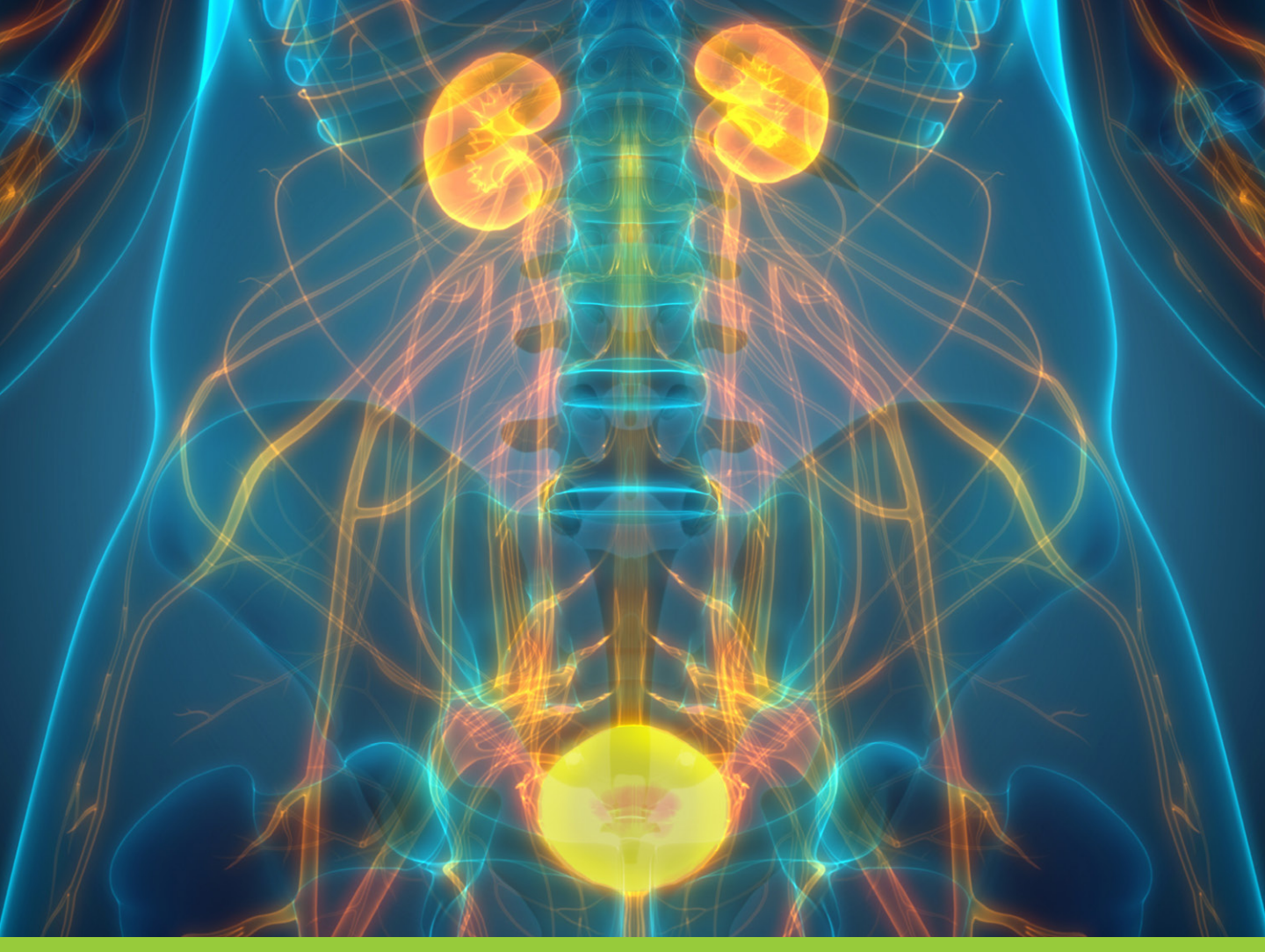

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Editors: Yair Lotan, Nathan Lawrentschuk, and Jack Schalken

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1st ICUD-WUOF International Consultation
October 2020

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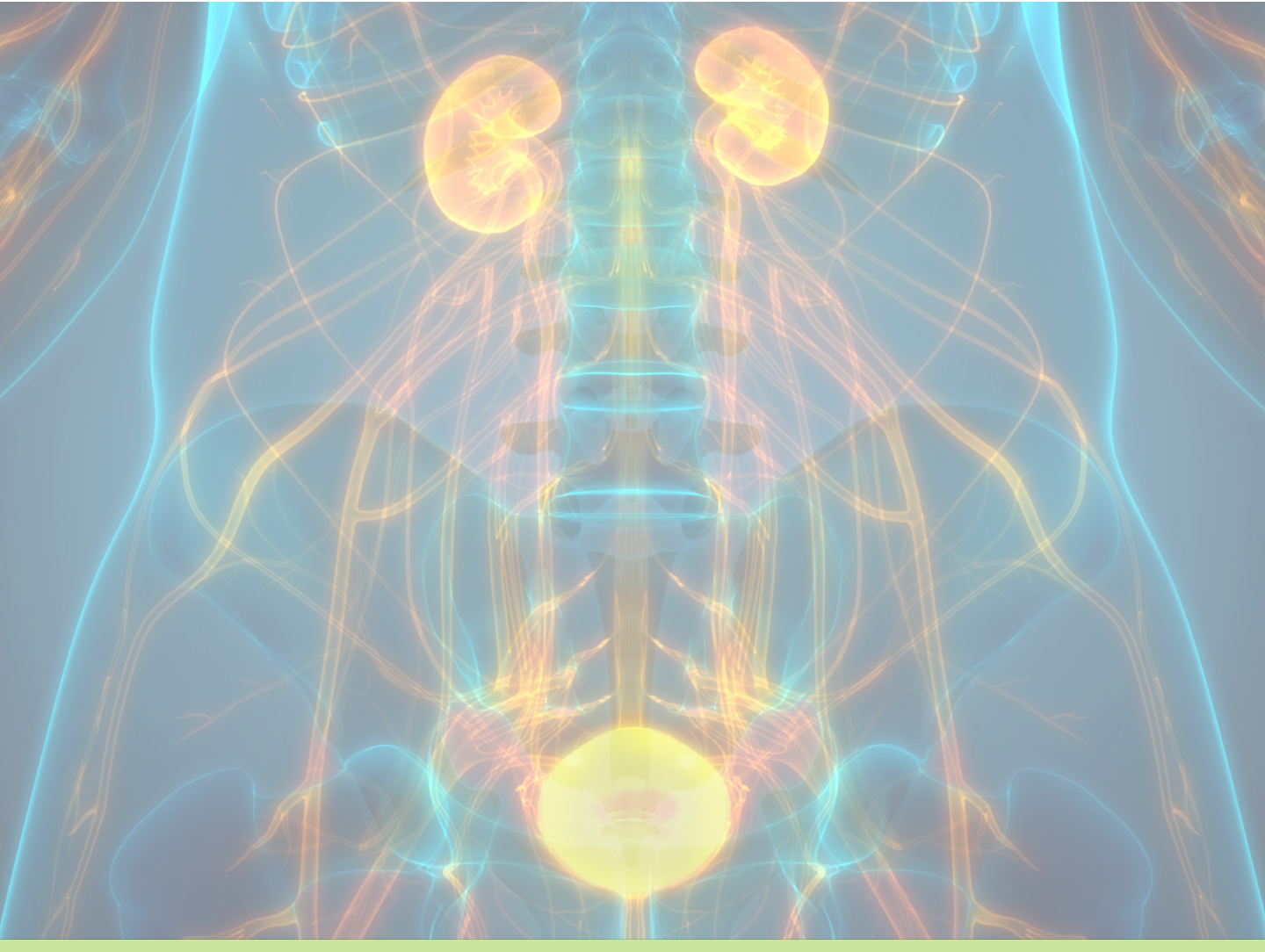

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Table of Contents

Preface

ICUD and the World Urologic Oncology Federation

Laurence Klotz,
CM, MD, FRCSC

The International Consultation on Urologic Diseases (ICUD) is a 40-year-old organization that produces book-length overviews of major topics in urology. These have typically defined the state of the art of the topic and serve as important internationally recognized references. They have been widely read and highly respected as sources for reliable information.

The chapters are prepared with input from diverse experts from around the world. The list of ICUDs is below. These texts have been major undertakings, involving scores or more of editors, chapter chairs, and writing committees.

The structure of the ICUD has evolved considerably over the past 4 decades. The initiative began in 1981 as a voluntary collaboration with support from international and national urological associations. The Consultations were supported, initially informally, by the World Health Organization (WHO) and the Union for International Cancer Control (UICC). The ICUD was formally established as a scientific, international non-profit NGO under Belgian law on June 28, 1994, to facilitate collaboration on an "organization to organization" basis with the WHO and UICC.

The principal aim of the ICUD has been consistent throughout its tenure: to promote improvement in the management of urological diseases around the world by producing evidence-based recommendations. This is achieved by bringing together experts in urology to produce recommendations based on a process that analyzes the available literature using an evidence-based approach. The recommendations must be suitable for adoption in all parts of the world, recognizing that resources and cultural influences differ widely between countries. The recommendations are not intended to be used as guidelines, although historically many ICUD recommendations have been incorporated into national guidelines.

The ICUD was for led many years by Saad Khoury (Paris, France), and subsequently by Paul Abrams (Bristol, UK). After many years the SIU became involved, initially as a collaborating partner. Eventually the SIU took over the management of the initiative. The World Urologic Oncology Federation (WUOF), an affiliate of the SIU, is the umbrella group for the 20 societies of Urologic Oncology around the world. It seemed like the perfect organizational partner for the Oncology ICUDs. Therefore, in 2018 the WUOF assumed the responsibility for the Oncology component of the ICUDs. This current edition, on Molecular Biomarkers in Urologic Oncology, has been conceived, organized, and managed by the WUOF.

The ICUD differs from national guidelines in important ways. Most obviously, it represents a uniquely international perspective, drawing input from virtually every region in the world. As such, the recommendations are less influenced by regional or national considerations.

The ICUD process has also evolved. Historically, groups of experts responsible for specific chapters would meet face to face, often on multiple occasions and for several days at a time, to hammer out consensus and resolve disagreements. While dedicated face-to-face meetings of the many individuals involved have become more challenging to implement (due mainly to resource limitations), the advent of social media and virtual meetings has facilitated the ability to collaborate across oceans and continents. This edition will be published in digital form only, which enables the development of a high-quality document using limited resources.

This important document comprehensively reviews progress in Molecular Biomarkers in Urologic Oncology. Industry sponsorship was critical to the success of this initiative. Many of our sponsors are small companies for whom the support for this project represented a significant investment. We thank them for their support. We are also grateful for the outstanding efforts of Ms. Patty Djan, who managed the ICUD sponsorship program.

A book like this requires a large team with diverse skills and talents. The production team, led by Areti Malapetsas of Medit Global, was superb. Ms. Malapetsas expertly managed the book production and was the senior medical copyeditor. The other members included Stephanie MacLean, Jodie Duffield, Baykent Tukeli, and Jeany Papadionissiou, chapter copyeditors; Christian Bello and Nicholas Floratos, proofreaders; and Falasteen Alfranji, graphic design. The quality of this book is a testament to their enthusiasm and expertise.

Laurence Klotz
Managing Editor, ICUD Oncology
Chairman, World Urologic Oncology Federation

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miR Scientific		

Past ICUD Consultations

2018 ICUD-SIU. Congenital Lifelong Urology: Caring for the Adolescent and Adult Patient with Congenital and Childhood GU Conditions
Seoul, South Korea
Chairs: Dan Wood and Hadley M. Wood

2017 ICUD-SIU. Bladder Cancer
Lisbon, Portugal
Chairs: Peter Black and Paulo Gontero

2016 ICUD-ISC. 6th International Consultation on Incontinence
Tokyo, Japan
Chairs: Paul Abrams, Linda Cardozo, Adrian Wagg, and Alan Wein

2016 ICUD-SIU. Urological Management of the Spinal Cord Injured Patient
Buenos Aires, Argentina
Chair: Shaun Elliott and Reynaldo Gomez

2015 ICUD-SIU. Image Guided Therapy in Urology
Melbourne, Australia
Chairs: Rafael Sánchez-Salas and Mihir Desai

2014 ICUD-EAU. Minimally Invasive Surgery in Urology
Stockholm, Sweden
Chairs: Walter Artibani and Jens Rassweiler

2014 ICUD. Men's Health (facilitated by AUA)
Orlando, United States
Chairs: Ajay Nehra, Ridwan Shabsigh, and Graeme Jackson

2014 ICUD-SIU. Stone Disease
Glasgow, Scotland
Chairs: John Denstedt and Jean de la Rosette

2014 ICUD-EAU. Medical Management of Urological Malignancy (MMUM)
Lisbon, Portugal
Chairs: Christian Stief and Christopher Evans

2013 ICUD-AUA. Topic Consultation on Anticoagulation in Urological Surgery
Chair: Stuart Wolf

2013 ICUD-SIU. Children's Congenital Anomalies
Vancouver, Canada
Chairs: Catherine de Vries and Rien Nijman

2013 ICUD-SIU. Upper Tract Transitional Cell Carcinoma
Vancouver, Canada
Chairs: Arnolf Stenzl, Shahrokh Shariat, and Serena Matin

2012 ICUD-EAU. 5th International Consultation on Incontinence
Paris, France
Chairs: Paul Abrams, Linda Cardozo, and Alan Wein

2012 ICUD-SIU. Male LUTS
Fukuoka, Japan
Chairs: Chris Chapple, Kevin McVary,

and Claus Roehrborn

2011 – 2nd International Consultation on Bladder Cancer
Vienna, Austria
Chairs: Mark Soloway and Henk van der Poel

2011 – 4th International Consultation on Prostate Cancer
Berlin, Germany
Chairs: Manfred Wirth and Gerald Andriole

2010 – 1st ICUD-EAU International Consultation on Renal Cell Cancer
Barcelona, Spain

2010 – 1st ICUD-SIU International Consultation on Urethral Stricture
Marrakesh, Morocco
Chairman: Gerry Jordan

2010 – 1st ICUD-SIU International Consultation on Obstetric Vesico-Vaginal Fistula
Marrakesh, Morocco
Chairmen: Dirk de Ridder and Sherif Mourad

2009 – 3rd International Consultation on Sexual Medicine
Paris, France

2009 – 1st International Consultation on Genito-Urinary Infections
Stockholm, Sweden

2009 – 1st ICUD-SIU International Consultation on Testis Cancer
Shanghai, China

2008 – 4th International Consultation on Incontinence
Paris, France

2008 – 1st International Consultation on Penile Cancer
Santiago, Chile

2007 – 2nd International Consultation on Stone Disease
Paris, France

2006 – 1st Consultation on Congenital Anomalies
Cape Town, South Africa

2005 – 6th International Consultation on New Developments in Prostate Cancer & Prostate Diseases
Paris, France

2004 – 1st International Consultation on Incontinence
Monte Carlo, Monaco

2004 – 1st International Consultation on Bladder Tumors
Honolulu, Hawaii

2003 – 2nd International Consultation on Erectile and Sexual Dysfunctions
Paris, France

2002 – Consultation on Genitourinary Trauma
Stockholm, Sweden

2002 – 3rd International Consultation on Prostate Cancer New Treatment Modalities
Paris, France

2001 – 2nd International Consultation on Incontinence

Paris, France

2001 – 1st International Consultation on Stone Diseases
Paris, France

2000 – 5th International Consultation on Benign Prostatic Hyperplasia
Paris, France

2000 – 1st International Consultation on Nosocomial Infections in Urology
Paris, France

1999 – 2nd International Consultation on Prostate Cancer
Paris, France

1999 – 1st International Consultation on Erectile Dysfunction
Paris, France

1998 – 1st International Consultation on Incontinence
Monte Carlo, Monaco

1997 – 4th International Consultation on Benign Prostatic Hyperplasia
Paris, France

1996 – 1st Consultation on Prostate Cancer
Monte Carlo, Monaco

1995 – 3rd International Consultation on Benign Prostatic Hyperplasia
Monte Carlo, Monaco

1994 – 4th International Symposium on Recent Advances in Urological Cancer Diagnosis & Treatment
Paris, France

1993 – 2nd International Consultation on Benign Prostatic Hyperplasia
Paris, France

1991 – 1st International Consultation on Benign Prostatic Hyperplasia
Paris, France

1990 – 3rd International Symposium on Progress Urinary Tumors
Paris, France

1989 – 2nd International Symposium on Progress Urinary Tumors
Paris, France

1987 – 1st International Symposium on Progress Urinary Tumors
Paris, France

1986 – Prostate Cancer
Paris, France

1985 – Bladder Tumors
Paris, France

1984 – 1st International Symposium on Testicular Cancer
Paris, France

1983 – Kidney Tumors
Paris, France

1981 – Prostate Cancer
Paris, France

Table of Contents of the Chapters

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

Chapter 8A

Chapter 9

Chapter 10

Chapter 11

Chapter 12

Chapter 13

Chapter 14

Chapter 15

Chapter 16

Chapter 17

Chapter 18

Abbreviations Used in the Text

Abbreviation	Full Term	Chapter #
AACR	American Academy of Cancer Research	15
ADT	androgen deprivation therapy	8
AFP	α-fetoprotein	16
AJCC	American Joint Committee on Cancer	12
ALCAM	activated leukocyte cell adhesion molecule	10
AMACR	α-methylacyl-CoA / alpha-methylacyl coenzyme A racemase	13, 15
AML	angiomyolipoma	14
APC	adenomatous polyposis coli	5
APOA1	Apolipoprotein A-I	10
APOA2	Apolipoprotein A-II	10
APOE	Apolipoprotein E	10
AQP1	aquaporin-1	14
AR	androgen receptor	6, 7
ARCC	Global Advanced Renal Cell Carcinoma	15
AR-V	androgen receptor splice variant	5
ASCO	American Society for Clinical Oncology	5, 11
asRNA	antisense RNA	4
ASSURE	Adjuvant Sorafenib or Sunitinib for Unfavorable Renal Carcinoma	15
ASTRO	American Society for Radiation Oncology	5
AUA	American Urologic Association	5, 9, 10, 16
AUC	area under the curve	3, 5, 9, 10, 16
AUROC	area under receiver operating curve	6
BAP1	BRCA1-associated protein 1	15
BCa	bladder cancer	11
BCG	bacillus Calmette-Guérin	10, 11, 12
BCL2	B-cell / CLL lymphoma 2	11
BCR	biochemical recurrence	6, 8, 9
BEST	Biomarkers, EndpointS and other Tools	4
bp	base pairs	14, 16
BPH	benign prostatic hyperplasia	6, 9
BRCA1	breast cancer susceptibility gene 1	11

BRCA2	breast cancer susceptibility gene 2	11
BTA	bladder tumour antigen	10
^{11}C-choline	carbon 11 choline / carbon-11 labelled choline	4, 8
CAGEKID	Cancer Genomics of the Kidney	15
CAIX	carbonic anhydrase IX	10, 13, 14, 15
CAIX FL	full-length isoform of carbonic anhydrase IX	10
CAPRA	Cancer of the Prostate Risk Assessment	3, 5
CBS	composite biomarker score	14
CCP	cell cycle progression	3, 5
ccRCC	clear cell renal cell carcinoma	13, 14, 15
CD10	cluster differential marker 10	13
CD31	cluster of differentiation 31	14
CDC	collecting duct carcinoma	13, 17
CDH	Cadherin-6	14
cDNA	complementary DNA	10
CEA	carcinoembryonic antigen	10
CEC	circulating endothelial cell	14
CE-MS	capillary electrophoresis mass spectrometry	10
CEPC	circulating endothelial progenitor cell	14
cfDNA	cell-free DNA	6, 7, 14, 15
CGI	cytosine-phosphate-guanine island	8A
CHIP	clonal hematopoiesis of indeterminate potential	7
chRCC	chromophobe RCC	13, 14
CIS	carcinoma in situ	10, 11
CISCA	cisplatin / doxorubicin / cyclophosphamide	9, 11
CK	cytokeratin	13, 14
CLIA	Clinical Laboratory Improvement Amendments	2
CMV	cisplatin, methotrexate, vinblastine	11
CNA	copy number alteration	10
COBRA	combined bisulphite restriction analysis	14
CONSORT	Consolidated Standards for the Reporting of Trials	2
CPCT	Centre for Personalized Cancer Treatment	5
CPE	concordance probability estimate	3
CpG	cytosine-phosphate-guanine	8A
cpRCC	clear cell papillary RCC	13
CR	complete response	11, 12
CRC	circulating rare cell	14

CRC-UMF	cells with uncertain malignant features	14
CRP	C-reactive protein	14, 15
CRPC	castrate-resistant prostate cancer	8
CSF1	colony-stimulating factor-1	14
CSM	cancer-specific mortality	11, 14
csPCa	clinically significant prostate cancer	9
CSS	cancer-specific survival	11, 14, 15
CT	computed tomography	8, 16
CTC	circulating tumour cell	7, 14, 15
ctDNA	circulating tumour DNA	7, 15, 17
CTLA-4	cytotoxic T-lymphocyte antigen 4 /cytotoxic T lymphocyte–associated protein 4	11, 14, 15
CTR1	high-affinity copper uptake protein 1	11
CUETO	Club Urológico Español de Tratamiento Oncológico	11
CXCR4	C-X-C chemokine receptor type 4	14
CyPRIT	Cytokine Panel for Response to Intravesical Therapy	10
2DICAL	2-dimensional image converted analysis of liquid chromatography mass spectrometry	14
DBS	dried blood spots	4
ddPCR	droplet digital polymerase chain reaction	10
DDR	DNA damage repair / response	5, 11
DFS	disease-free survival	14
DLX1	distal-less homeobox 1	9
DNase	deoxyribonuclease	4
DNMT1	DNA methyltransferase 1	8A
DRE	digital rectal examination	3, 6, 9
DRUP	Drug Rediscovery Protocol	5
4E-BP1	phosphorylated-4E-binding protein 1	14
EAU	European Association of Urology	5, 8, 10, 11, 16
EC	European Commission	2
ECM	extracellular matrix	10
ECOG	Eastern Cooperative Oncology Group	15
EGFR	epidermal growth factor receptor	10, 11
ELIZA	enzyme-linked immunosorbent assay	6, 10
ELOVL7	elongation of very long chain fatty acids protein 7	8
EMA	European Medicines Agency	4

EMT	epithelial-to-mesenchymal transition	11, 14
EN2	engrailed-2	6
ENO1	alpha-enolase	14
EORTC	European Organisation for Research and Treatment of Cancer	11, 14
EPCA	early prostate cancer antigen	3
EpCAM	epithelial cell adhesion molecule	7, 10
ER	estrogen receptor	11
ERBB2	erb-b2 receptor tyrosine kinase 2 (formerly HER2/*neu*)	11
ERCC1	excision repair cross-complementing 1	11
ERCC2	excision repair cross-complementing 2	11
ERG	erythroblastosis virus E26 oncogene homologues	9
ERSPC	European Randomized Study of Screening for Prostate Cancer	3, 6, 18
ERV	endogenous retrovirus	8A
ET	erythroblast transformation	9
EU	European Union	2
EV	extracellular vesicle	6, 14
^{18}F-choline	fluorine-18 labelled choline	8
%fPSA	% free PSA	9
FDA	US Food and Drug Administration	2, 6, 7, 9, 10, 14, 15
FDA-NIH	Food and Drug Administration and National Institutes of Health	4
FDG/^{18}F-FDG	fluorodeoxyglucose / ^{18}F-fluorodeoxyglucose / fluorine-18 labelled fluorodeoxyglucose	4, 8
FFPE	formalin-fixed, paraffin-embedded	4, 5, 8A
FGFR	fibroblast growth factor receptor	12
FGFR3	fibroblast growth factor receptor 3	10, 11
FH	fumarate hydratase	13
FINC	fibronectin-1	14
FISH	fluorescence in situ hybridization	10, 11, 13
FLT	ferritin light chain	14
fPSA	free PSA	9
FQP	Full Qualification Package	2
^{68}Ga-PSMA	^{68}Gallium prostate-specific membrane antigen / gallium-68 labelled PSMA	4, 8
G9a	histone H3 lysine 9 methyltransferase	8A

GAPDH	glyceraldehyde 3-phosphate dehydrogenase	14
GC	Genomic Classifier	5
GCNIS	germ cell neoplasia in situ	16
GCT	germ cell tumour	16
GLUT1	glucose transporter 1	8
GPS	Genomic Prostate Score	5
Gs	Gleason score	6
GSTM1	glutathione-S-transferase	4
GSTP1	glutathione-S-transferase P1	5, 6
GU	genomically unstable	11
β-hCG	beta–human chorionic gonadotropin	1, 2, 16
H3K9	histone 3 lysine 9	8A
HAI-1	hepatocyte growth factor activator inhibitor-1	10
HAVCR	hepatitis A virus cellular receptor	14
HD IL2	high-dose interleukin 2	15
HER2	human epidermal growth factor receptor 2	2, 11
HG	high grade	10
HGF	hepatocyte growth factor	14, 17
HIF	hypoxia-inducible factor	15
HIF-1α	hypoxia-inducible factor 1α	4, 11, 14, 15
HIF-2α	hypoxia-inducible factor 2 alpha subunit	14
hK2	kallikrein 2	9
hKIM-1	human kidney injury molecule-1	13
HLRCC	hereditary leiomyomatosis renal cell carcinoma–associated RCC	13
HNPCC	hereditary nonpolyposis colorectal cancer	17
hNSE	human neuron-specific enolase	14
HOXC6	homeobox C6	9
HP1	heterochromatin protein 1	8A
HR	hazard ratio	5, 6, 10, 15
HS	H-score	14
HSC71	heat shock cognate 71	14
Hsp	heat shock protein	11, 14
Hsp27	heat shock protein 27	14
HSPC	hormone-sensitive prostate cancer	8
HSPE1	heat shock protein family E member 1	14
IAP	inhibitor of apoptosis protein	15

IAP	immunosuppressive acidic protein	14
IBCN	International Bladder Cancer Network	10
ICAM1	intercellular adhesion molecule-1	14
ICB	immune checkpoint blockade	14, 15
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use	4
ICI	immune checkpoint inhibitor	14
ICOS	inducible T-cell co-stimulator	14
IDI	integrated discrimination improvement	3
IDLN-MSP	idiolocal normalization of real-time methylation-specific polymerase chain reaction	8A
IDO	indoleamine 2,3-dioxygenase	14
IDO-1	indoleamine 2,3-dioxygenase 1	14
IFN-α	interferon-α	15
IFN-γ	interferon-γ	11
IGCCCG	International Germ Cell Cancer Collaborative Group	16
IHC	immunohistochemistry	6, 11, 13, 14
IL2	interleukin 2	10, 15
IL8	interleukin 8	10, 11
IL6	interleukin 6	10, 14
IL8	interleukin 8	10, 14
IL10	interleukin 10	10, 14
IL-18BP	interleukin-18 binding protein	14
IMDC	International Metastatic RCC Database Consortium	15
IMP3	insulin-like growth factor 2 mRNA-binding protein	15
IMPACT	Identification of Men with a genetic predisposition to Prostate Cancer: Targeted	6
IMRCCD	International Metastatic Renal Cell Carcinoma Database Consortium	14
INR	international normalized ratio	2
ITGA3	integrin subunit alpha 3	6
ITGB1	integrin subunit beta 1	6
iTRAQ	isobaric tags for relative and absolute quantitation	14
4K	four kallikrein	9
KDM5C	lysine-specific demethylase 5C	15
KIM-1	kidney injury molecule-1	14, 15

KIRC	Kidney Renal Clear Cell Carcinoma	14
KLK3	kallikrein 3	9
Ksp-cadherin	kidney-specific cadherin	13
^{177}Lu	Lutetium-177	8
LASSO	least absolute shrinkage and selection operation	14
LC-MS	liquid chromatography–mass spectrometry	14
LCP1	L-plastin	15
LDH	lactate dehydrogenase	14, 15, 16
LDHA	L-lactate dehydrogenase A chain	14
LDL	low-density lipoprotein	2
LG	low grade	10
LH	luteinizing hormone	16
LHRH	luteinizing hormone-releasing hormone	8
LINE	long interspersed nuclear element	8A
LNCaP	human prostatic carcinoma cell line	8
lncRNA	long noncoding RNA	4, 14
LOI	Letter of Intent	2
LR	likelihood ratio	2
LS	Lynch syndrome	17
LTD	Laboratory Developed Test	2
LVI	lymphovascular invasion	11, 16
MAGE-A	melanoma-associated antigen A	17
MAH	macrohematuria	10
MATV	metabolically active tumour volume	8
MC	methotrexate / cisplatin	11
mCRPC	metastatic castrate-resistant prostate cancer	5, 7
MDR1	multidrug resistance mutation 1	11
MEC	methotrexate, epirubicin, cisplatin	11
MeDIP	methylated DNA immunoprecipitation	14
MIBC	muscle-invasive bladder cancer	10, 11, 12
MIH	microhematuria	10
MiPS	Michigan / Mi-Prostate score	6, 9
miRNA	microRNA	4, 6, 10, 11, 14, 15
MiT	microphthalmia-associated transcription	13
MLH1	MutL homologue 1	14
MLPA	multiplex ligation-dependent probe amplification	10

MMP	matrix metalloproteinase	10, 11, 15
MMP-7	matrix metalloproteinase-7	14
MMP-9	matrix metalloproteinase-9	14
MMR	mismatch repair	17
MMRd	mismatch repair deficiency	7
mpMRI	multiparametric magnetic resonance imaging	5, 6, 9
mRCC	mestastatic renal cell carcinoma	14, 15
mRNA	messenger RNA	4, 10
MRE11	meiotic recombination 11	11
MS	mass spectrometry	6, 14
MSI	microsatellite instability	17
MSKCC	Memorial Sloan-Kettering Cancer Center	15
MSP	methylation-specific polymerase chain reaction	10
mTOR	mammalian target of rapamycin	15
MTSCC	mucinous tubular and spindle cell carcinoma	13
MVAC	methotrexate, vinblastine, doxorubicin, and cisplatin	11
MVEC	methotrexate, vinblastine, epirubicin, cisplatin	11
NAC	neoadjuvant chemotherapy	11, 12
NAT2	*N*-acetyltransferase 2	4
NCCN	National Comprehensive Cancer Network	3, 5, 8
NCI	National Cancer Institute	15
NER	nucleotide excision repair	11, 17
NGAL	neutrophil gelatinase-associated lipocalin	14, 15
NGS	next-generation sequencing	5, 17
NLR	neutrophil / lymphocyte ratio	15
NLST	National Lung Screening Trial	12
NM23A	nonmetastatic cell 1 protein	14, 15
NMIBC	non-muscle invasive bladder cancer	10, 11, 12
NMP	nuclear matrix protein	14
NMP22	nuclear matrix protein 22	10, 14, 15
NMR	nuclear magnetic resonance	14
NNMT	nicotinamide N-methyltransferase	14, 15
NPV	negative predictive value	2, 9, 10, 12, 16
NRI	net reclassification improvement	3
NSGCT	nonseminomatous GCT	16
NuMA	nuclear mitotic apparatus protein	10

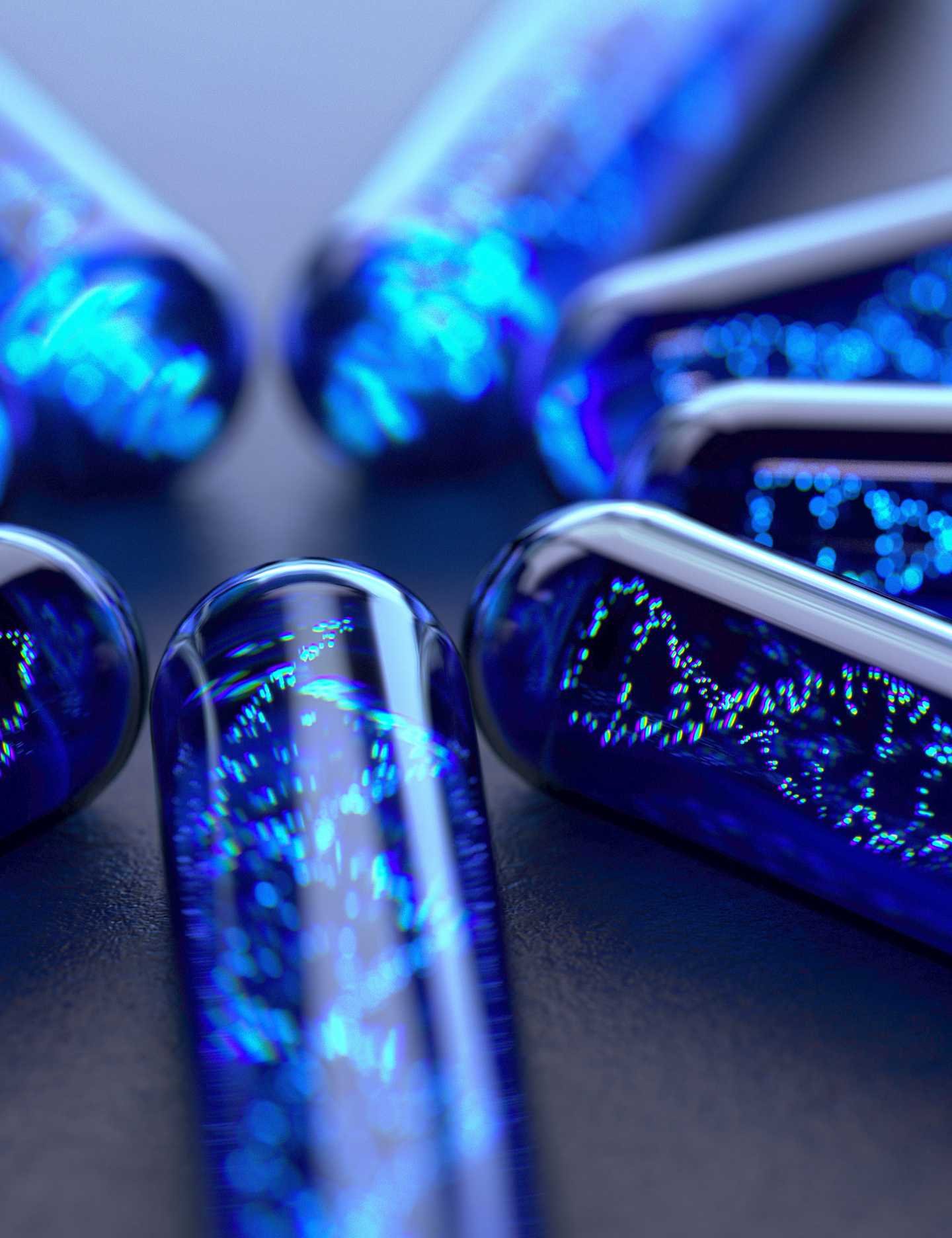
OPN	osteopontin	14
OR	odds ratio	5, 6, 9, 10
OS	overall survival	8, 11, 14, 15
p2PSA	[-2]proPSA	9
pAkt	phospho-Akt	14
PAP	prostatic acid phosphatase	9
PAR	protease-activated receptor	11
PARP	poly (ADP-ribose) polymerase	5, 7
PARP1	poly (ADP-ribose) polymerase 1	11
PBCG	Prostate Biopsy Collaborative Group	3
PBMC	peripheral blood mononuclear cell	14
PBRM1	polybromo 1	15
PCa	prostate cancer	6, 9, 10
PCA-1	prostate cancer antigen-1	6
PCA3	prostate cancer antigen-3	2, 3, 6, 9
PCPT	Prostate Cancer Prevention Trial	3
PCPT-RC	Prostate Cancer Prevention Trial risk calculator version 1.0 / 2.0	6 / 9
PCR	polymerase chain reaction	4, 5, 7, 10, 11
PC-RPLND	post-chemotherapy RPLND	16
PD-1	programmed cell death 1	2, 11, 14, 15, 18
PDGFB	platelet-derived growth factor beta polypeptide	14
PD-L1	programmed cell death 1 ligand 1	1, 2, 4, 11, 12, 14, 15, 18
PDX	patient-derived xenograft	17
PET	positron emission tomography	4, 8
PET/CT	positron emission tomography / computed tomography	5, 8, 15
PFS	progression-free survival	8, 14, 15
PHI	prostate health index	3, 9
PI3K	phosphatidylinositol-3-kinase	10
PI-RADS	Prostate Imaging Reporting and Data System	9
PK	pyruvate kinase	14
PKM2	pyruvate kinase-muscle-2	14
PLCO	Prostate, Lung, Colorectal and Ovarian	1, 12, 18
PLIN2	perilipin-2	14
PLND	pelvic lymph node dissection	11

POC	point of care	10
PPARγ	peroxisome proliferator-activated receptor γ	11
PPV	positive predictive value	2, 9, 10, 12
PR	partial response	12, 16
pRCC	papillary renal cell carcinoma	13, 14
ProtecT	Prostate Testing for Cancer and Treatment	9
PROTRACT	PROstate cancer TReatment optimization via Analysis of Circulating Tumour DNA	7
PRS	polygenic risk score	3
PSA	prostate-specific antigen	1, 2, 3, 5, 6, 7, 8, 9, 18
PSAD	PSA density	6, 9
PSADT	PSA doubling time	9
PDAV	PSA velocity	9
PSMA	prostate-specific membrane antigen	5, 8
PTGS2	prostaglandin-endoperoxidase synthase 2	14
PTEN	phosphatase and tensin homologue	5, 15
PUR	Prostate Urine Risk	6
PY	pack-years	12
QALY	quality-adjusted life years	9
QP	Qualification Plan	2
qPCR	quantitative polymerase chain reaction	10
qRT	quantitative reverse transcription	16
qRT-PCR	quantitative reverse transcription polymerase chain reaction	10
RASSF1	Ras association domain-containing protein 1	5
RASSF1A	Ras association domain family member 1A	14
RB1	retinoblastoma protein	11
RC	radical cystectomy	11, 12
RCC	renal cell carcinoma	10, 13, 14, 15, 17
RCCM	RCC marker	13
RECIST	Response Evaluation Criteria In Solid Tumors	4, 8, 11,
REDECT	REnal Masses: Pivotal Study to DETECT Clear Cell Renal Cell Carcinoma With Pre-Surgical PET/CT	15
REMARK	REcommendations for tumour MARKer prognostic studies	3,14
RFS	recurrence-free survival	14

RMB	renal mass biopsy	14
RNase	ribonuclease	4
RNase MRP	ribonuclease mitochondrial RNA processing	4
RNase P	ribonuclease P	4
RNF185	ring finger protein	14
RNU	radical nephroureterectomy	17
RO	renal oncocytoma	13
ROC	receiver operating characteristic	2, 14
RPLND	retroperitoneal lymph node dissection	16
RR	response rate	15
RRM1	ribonucleotide reductase catalytic subunit M1	11
rRNA	ribosomal RNA	4
RT	radiotherapy	11
RTC	randomized controlled trial	14
RTK	receptor tyrosine kinase	10
RT-PCR/rtPCR	reverse transcription polymerase chain reaction	6, 11
S2C	S-(2-succinyl cysteine)	13
S6RP	S6 ribosomal protein	14
S100A8	S100 calcium-binding protein A8	14
S100A9	S100 calcium-binding protein A9	14
SAA	serum amyloid A	14
SCC	squamous cell cancer	11
SCD1	stearoyl-CoA desaturase enzyme 1	8
SCGN	secretagogin	14
SCOTRRCC	Scottish Collaboration on Translational Research into Renal Cell Carcinoma	15
SDC1	syndecan 1	10
SDH	succinate dehydrogenase	13
SD-R	stable disease–response	14
SERPINA1	serpin family A member 1	10
SERPINE1	serpin family E member 1	10
SETD2	SET domain containing 2	14, 15
shRNA	short hairpin RNA	4
SINE	short interspersed nuclear element	8A
siRNA	small interfering RNA	4
snoRNA	small nucleolar RNA	4
snRNA	small nuclear RNA	4

SNP	single-nucleotide polymorphism	4, 11, 12, 15
SNV	single-nucleotide variant (formerly, SNP)	4, 11, 12, 15
SOC	standard of care	9
SOGUG	Spanish Oncology Genitourinary Group	15
SRM	small renal mass	14
SSIGN	Stage, Size, Grade, and Necrosis	14, 15
STARD	Standards for Reporting of Diagnostic accuracy studies	2
STM	serum tumour marker	16
S-TRAC	Sunitinib as Adjuvant Treatment for Patients at High Risk of Recurrence of Renal Cell Carcinoma Following Nephrectomy	15
SUO	Society of Urologic Oncology	5
SUV_{max}	maximum standardized uptake value	8
SWI/SNF	switch-sucrose nonfermentable	14
TARGET	Treatment Approaches in Renal Cancer Global Evaluation Trial	14, 15
TATI	tumour-associated trypsin inhibitor	14
TC	tumour cell	14
TCGA	The Cancer Genome Atlas	1, 11, 14, 17
TDO	tryptophan 2,3-dioxygenase	14
TGFβ	transforming growth factor β	11
TGFβ1	transforming growth factor β 1	13
TH1	type 1 helper T cell	10, 11, 14
TIC	tumour-infiltrating cell	14
TIL	tumour-infiltrating lymphocyte	4, 15
TIM-3	mucin-domain containing-3	14
TIMP-1	tissue inhibitor of metalloproteinase 1	14, 15
TK1	thymidine kinase 1	14
TKI	tyrosine kinase inhibitor	14, 15
TLDAs	TaqMan Low-density-Arrays	14
TMA	tissue microarray	11
TMPRSS2	transmembrane serine protease 2	5
TMT	trimodality therapy	11
TNF-α	tumour necrosis factor-α	14
TNFRII	tumour necrosis factor receptor 2	14
TNMS	tumour-node-metastasis–serum markers	16
tPSA	total PSA	9

TPx	thioredoxin peroxidase	14
TRAIL	tumour necrosis factor–related apoptosis-inducing ligand	14, 15
TPx	thioredoxin peroxidase	14
TRIPOD	Transparent Reporting of studies on prediction models for Individual Prognosis Or Diagnosis	6
tRNA	transfer RNA	4
TRUS	transrectal ultrasound	6, 9
Tu M2-PK	Tumour M2-PK	14
TUR	transurethral resection	10, 11, 12
TURBT	transurethral resection of bladder tumour	10, 12
TURP	transurethral resection of the prostate	5
UC	urothelial carcinoma	10
UCB	urothelial cancers of the bladder	17
UISS	University of California at Los Angeles Integrated Staging System	15
ULN	upper limit of normal	14
USA	United States of America	2
UTUC	upper tract urothelial carcinoma	10, 17
VEGF	vascular endothelial growth factor	11, 14, 15
VEGFA	vascular endothelial growth factor A	10, 15
VEGFC	vascular endothelial growth factor C	14
VEGFR1	vascular endothelial growth factor receptor-1	14
VEGFR2	vascular endothelial growth factor receptor-2	15
VEGFR3	vascular endothelial growth factor receptor-3	14
VHL	von Hippel-Lindau	14, 15
VIS	vasoactive intestinal peptide	6
WES	western blot	11
WHO	World Health Organization	10, 16
XRCC1	x-ray repair cross-complementing group 1	11
YST	yolk sac tumour	16



CHAPTER 1

Introduction: Personalized Medicine in Urologic Oncology

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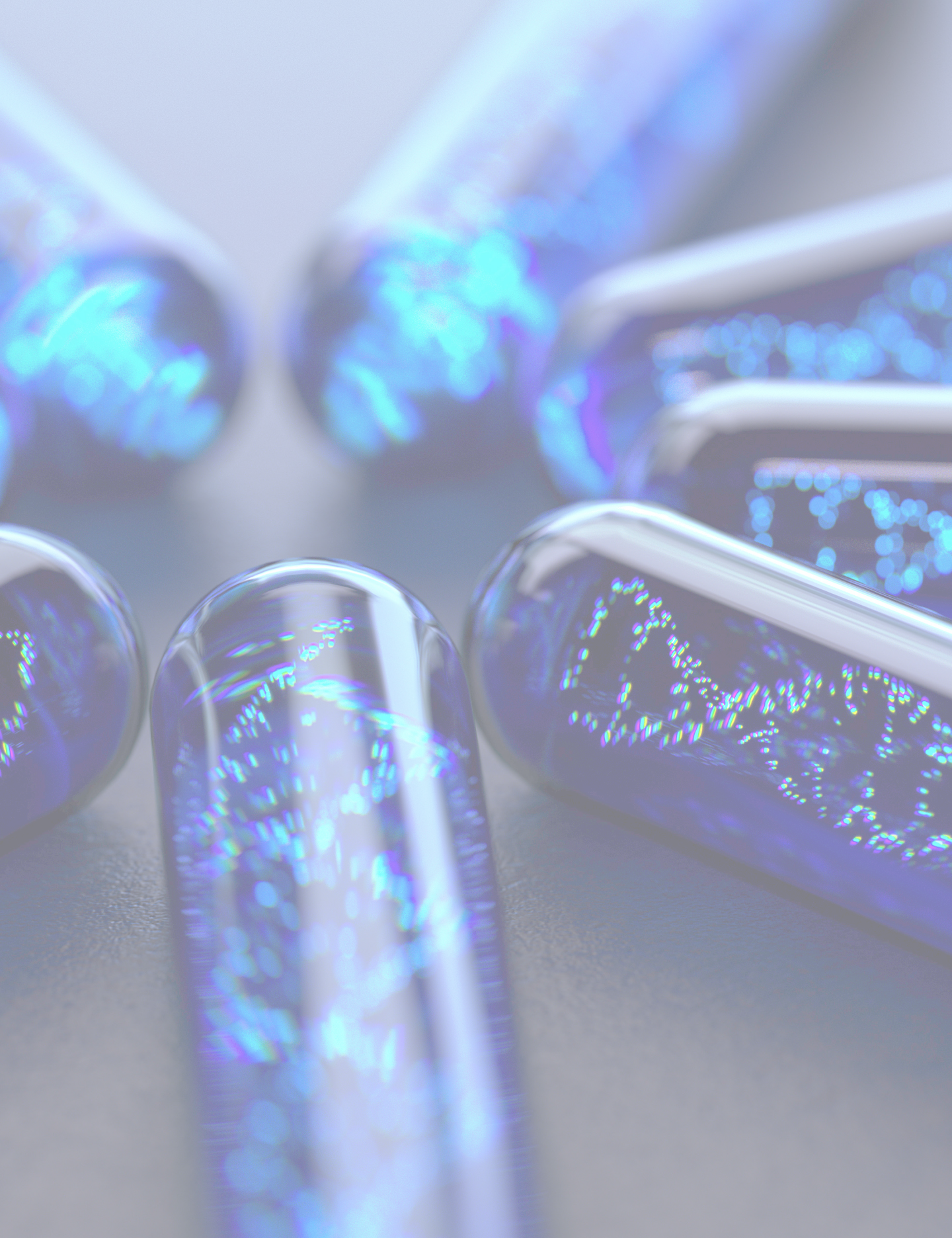
In an ideal world, the diagnosis and treatment of disease would be done in a noninvasive manner and with precision. Biologic testing would be performed using easily available samples such as blood, urine, sputum, and skin swab that would provide information regarding the cause of a disease as well as prognosis and optimal treatment. The current state of the science is that there is progress toward these goals in some areas while significant gaps are still present before molecular markers become incorporated into common practice. In this document, we will review the current state of affairs regarding molecular markers for the diagnosis and management of urologic malignancies and discuss their current role and research developments.

Incorporation of biomarkers into clinical practice thus far has met significant challenges. Some biomarkers have been adopted without sufficient evidence of effectiveness, resulting in controversy. An ideal marker would fulfill strict criteria demonstrating that it provides clinical benefit, information that is independent from other clinical factors, cost-effectiveness, reproducibility, and be comprehensively validated. The pathway to incorporate molecular markers should be standardized, similar to that required for approval of medications. A marker should pass through several phases, in which it is tested against the clinical question that it is supposed to answer and then validated in multicentre prospective cohorts. Many markers do not survive the initial phase of discovery and evaluation in small retrospective cohorts. This phase, while exciting, is subject to risk of bias and overfitting. If a marker is developed that shows promise, the next phase of evaluation should involve validation in independent cohorts. Finally, a test of clinical utility should be performed on a prospective cohort where the marker is evaluated for its ability to change clinical management to the benefit of the patient. Very few markers have been incorporated into clinical guidelines. Diagnosis and management of testis cancer, for example, includes the use of alpha fetal protein and beta–human chorionic gonadotropin (β-hCG). These markers help in differentiating histology and can guide the use of chemotherapy. Use of prostate-specific antigen (PSA), in contrast, was developed for surveillance of patients with prostate cancer but incorporated into screening programs. The use of PSA for screening has resulted in decades of controversy, as evaluation of the impact of prostate cancer screening on survival occurred many years after widespread use of the marker for this purpose. In the United States, this resulted in significant bias in studies such as the Prostate, Lung, Colorectal and Ovarian (PLCO). Inevitable contamination may render accurate assessment of the impact of PSA unfeasible. For bladder cancer, certain markers such as urine cytology have been incorporated into guidelines despite poor sensitivity for low-grade cancer and moderate sensitivity for high-grade disease. Other urine markers have not been adopted due to failure to fulfill stringent criteria of performance. The emphasis on a high standard of evidence prior to incorporating markers into clinical practice has merit. This is particularly important due to the risk associated with false-positive results, which often lead to unnecessary testing and anxiety. Furthermore, these tests may contribute significantly to the cost of health care, a worldwide concern.

The Cancer Genome Atlas (TCGA) is a landmark cancer genomics program that sequenced and molecularly characterized more than 11,000 cases of primary cancer. This major effort has provided important new information regarding urologic cancers. This data is now being evaluated for its role in characterizing tumour behaviour, prognosis, and response to therapy. This study, along with advances in next-generation sequencing, is providing novel information that will advance the goals of personalized medicine. There is a need for a significant effort to interpret this data using bioinformatics and to validate the utility of this data in clinical care. There are

multiple new clinical trials that are based on targeting specific mutations or enriching the patient population for the expression of immunological markers such as programmed death 1 ligand 1 (PD-L1), related to the use of checkpoint inhibitors. Understanding the significance of these molecular alterations and how to use them to guide therapy will likely take years; performing trials is critical. Patients with metastatic disease, especially urothelial cancer, have a short life expectancy. These patients don't have the luxury of being treated with multiple therapies in an attempt to find one that works. Thus, using molecular tests with predictive value to identify the treatment early on with the highest likelihood of response may make a major difference for these individuals.

This is an exciting time of transition with regard to molecular markers. Rapid advances in our understanding of cancer biology provide multiple opportunities to develop and validate molecular markers that will have a profound impact on patient management. This review will provide information on currently available and potential future markers. For patients, researchers, and providers seeking to incorporate markers into practice, it is important to evaluate how well the markers perform overall and whether they can provide a tool that improves patient care.



CHAPTER 2

Basics of Biomarker Development and Interpretation

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Table of Contents

2.1 Introduction and Definition

The "biomarker" term comes from "biological marker", which refers to a broad category of medical signs and characteristics that can be observed and measured.[1] A biomarker can be defined as a characteristic that indicates a normal biological process or a pathogenic one. It also can indicate biological responses to an intervention, including the therapeutic ones. A biomarker can be a physiological characteristic or a biological substance, including molecular, biochemical, and histologic ones, or even radiographic characteristics that can be measured. There are several definitions for biomarkers in the literature. In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*."[2]

By definition, the biomarkers must be objective and quantifiable characteristics of a biological process. They can be useful to provide indications of potential benefits and hazards associated with an intervention in the research context. In the clinical scenario, they can help diagnosis and risk stratification, and indicate responses to treatments. The biomarkers can be divided into categories according to their purposes, including susceptibility or risk biomarker, diagnostic biomarker, monitoring biomarker, prognostic biomarker, predictive biomarker, pharmacodynamic or response biomarker, and safety biomarker.

A biomarker does not access individuals' feelings, functions, or survival specifically. Those are clinical data, and clinical researches focus on clinical endpoints such as morbidity and mortality instead of biochemical features. However, some biomarkers can be used as surrogate endpoints. There is a subset of biomarkers with substantial scientific evidence that can consistently predict clinical outcomes accurately.[3] For many diseases, clinical endpoints such as mortality or disease recurrence may take a long time, while biomarkers may provide earlier data and allow for smaller and more efficient studies.

2.2 Types of Biomarkers

2.2.1 Diagnostic biomarkers

This category includes a broad set of molecular tests and image exams. The marker can confirm the presence of a disease or condition of interest.[4] Some examples are: sweat chloride that confirms cystic fibrosis; blood sugar and hemoglobin A1c that may be used as a diagnostic biomarker of type 2 diabetes mellitus; and the ejection fraction, as a diagnostic biomarker of heart failure.

2.2.2 Susceptibility or risk biomarkers

These biomarkers refer to people without clinically apparent disease. Many genetic tests are emerging, such as the BRCA1 and BRCA2 mutations tests and PCA3. The BRCA mutations indicate an increased risk for contralateral

breast cancer. The urinary prostate cancer antigen-3 (PCA3) analysis shows a higher risk for prostate cancer.[4,5] Susceptibility biomarkers help clinicians identify higher-risk patients who benefit from a closer follow-up with investigational image exams and biopsies.

2.2.3 Prognostic biomarkers

The prognostic markers are associated with clinical outcomes such as overall survival and recurrence-free survival, and they help in the identification of a more aggressive disease course.[6] They indicate an increased or decreased likelihood of a future clinical event, including disease recurrence, progression, and mortality. Those data are useful to stratify risk and guide the selection of treatments. Traditional prognostic oncological biomarkers are tumour size, number of positive nodes, and metastasis.

Some biochemical markers can be used for this purpose, such as the prostate-specific antigen (PSA) for prostate cancer, and human chorionic gonadotropin (HCG) and alpha-fetoprotein for some testicular cancers. [7,8] Also, molecular and genetic test usage has increased in the past decades.[5,9] Prognostic markers help patient selection in clinical trials, as eligible criteria. Selecting patients with a higher likelihood of experiencing an event enriches the study by increasing its statistical power.

2.2.4 Predictive biomarkers

Predictive markers can predict the activity of specific therapy and are used to help treatment decisions. They can indicate the likely benefit of a specific treatment to a specific patient.[10] The human epidermal growth factor receptor 2 (HER2) is one example of a predictive biomarker. Negative HER2 tumours do not respond to trastuzumab treatment for breast cancer.[11,12] Recently, new therapies have aimed to block immunotolerance by inhibition of programmed cell death 1 (PD-1).[13,14] The expression of PDL-1 indicates a subset of responder patients who benefit from the treatment.[15,16] Some predictive biomarkers are co-approved along with a specific drug to help patients' selection to treatment. Some authors may call those biomarkers as "companion biomarkers" in this particular scenario.[17–19]

2.2.5 Monitoring biomarkers

A monitoring biomarker is a biomarker that is measured serially to evaluate a disease's status or medical treatment effect. Each measure brings information; however, the monitoring biomarkers extract even more information from the repeated analysis. The curve of biomarker values along time can indicate the current status or a future condition. For example, the hepatitis C virus ribonucleic acid level may indicate treatment response; international normalized ratio (INR) is used to monitor the effect of anticoagulation when using warfarin.[20,21] PSA may be used to monitor prostate cancer response to treatment, and a progressive elevation after surgery indicates disease recurrence.[22]

Monitoring biomarkers help early detection and treatment of a medical condition before it can cause complications—the INR monitoring is essential to prevent gastrointestinal bleedings, hematuria, and even cranial bleeding.[23] In oncologic settings, monitoring biomarkers can indicate disease relapse before it can cause symptoms or even metastasis. The early detection of disease progression leads to early treatment and improves survival.[24]

2.2.6 Pharmacodynamic or response biomarkers

This kind of biomarker shows the biological response of an individual exposed to a medical product or environmental agent. It is a subset of monitoring biomarkers. These biomarkers do not necessarily reflect the effect of an intervention but indicate pharmacologic effects. So, pharmacodynamic biomarkers may not be accepted as surrogate endpoints. However, they provide useful information for patient management, helping make decisions such as dosing adjustments and treatment continuation or stoppage. One particular importance is to help drug development in the setting of new drug initial trials. Pharmacodynamic biomarkers are useful on a daily basis to estimate patients' responses and to adjust individual drug doses. For example, blood pressure measurements may be used as a response biomarker of antihypertensive treatments. Other examples are serum low-density lipoprotein (LDL) and hemoglobin A1c, which can evaluate response to treatments for diabetes mellitus and dyslipidemia.[25,26]

2.2.7 Safety biomarkers

Some biomarkers can provide risk assessment and allow mitigation of injuries. That is particularly important during initial studies about a drug. Monitoring biomarkers can indicate safety hallmarks by monitoring toxicity and collateral effects. They can point out safety issues and allow treatment modification or interruption before an injury consolidates. Examples include creatinine for potentially nephrotoxic drugs, transaminases and bilirubin for potentially hepatotoxic drugs, creatinine phosphokinase for drugs that may cause muscle damage; and QT interval to mitigate the potential risk of *torsades de pointes*.[27–29]

2.3 Biomarkers Basics

To use a biomarker in daily practice, one must understand its characteristics to be able to apply each biomarker in a suitable scenario. The primary characteristics include sensitivity, specificity, accuracy, and positive predictive and negative predictive values. The likelihood ratios and receiver operator curves bring essential information to interpret the biomarkers and help establish cutoff values.[30] In the next section, we explain these concepts and how their interpretation supports the application of the biomarkers. The sensitivity and specificity are the first fundamental measures of dichotomous tests. They affirm the biomarker's capacity to identify what it is supposed to do.

The **sensitivity** is the proportion of diseased individuals with a positive test; in other words, it is the proportion of really affected patients. On the other hand, **specificity** is the proportion of disease-free individuals with

a negative test result. They are the truly nonaffected patients who are correctly identified by a biomarker. To determine the true positive and true negative cases, one needs a "gold standard" method to compare. This test can be the pathologic findings from a biopsy or surgery or a widely accepted test. If one plots the true and false positives and negatives in a 2 x 2 table, the result will be the following.[31]

	Disease Present	Disease Absent
Biomarker +	A (true positives)	B (false positives)
Biomarker -	C (false negatives)	D (true negatives)

Considering the table above, the sensitivity and specificity can be defined as the following formulas.

Sensitivity = A / (A+C)
Specificity = D / (B+D)

A test's accuracy is defined as the proportion of test results (both positive and negative) that are concordant to actual health status. In other words, the accuracy gives the percentage value of correct answers provided by the test. The following formula gives the accuracy.

Accuracy = (A+D) / (A+B+C+D)

The previously presented measures are useful to assess test validity, but sometimes they are not so helpful for a determined clinical scenario. When the disease status is unknown, physicians use tests to understand its likelihood. If all tests were perfect, all patients with a positive result would always actually have the disease. However, such optimal performance is rare. Thus, the concepts of positive predictive value (PPV) and negative predictive value (NPV) are essential to help physicians and surgeons making their choices. Those measures help them to answer the question: "Given that this test is positive, what is the probability that this patient has the disease?". The ideal test would have both NPV and PPV of 100%.[32]

The **positive predictive value (PPV)** is defined as the proportion of individuals with positive tests who have the disease. On the other hand, the **negative predictive value (NPV)** is the proportion of individuals with a negative result who are disease free. The number of false-positive and false-negative results affect all the measures presented so far. However, the PPV and NPV are useful measures to help the interpretation of the biomarkers in daily practice. They are calculated using the following formulas.[33]

Positive Predictive Value (PPV) = A / (A+B)
Negative Predictive Value (NPV) = D / (C+D)

Looking at the formulas, one can understand that false-positive and false-negative results directly affect the predictive values. However, it is crucial to keep in mind that several other factors also influence the predictive values. The most important ones are the disease prevalence in the community, the study sample, and the specificity and sensitivity of a particular test.[32]

The specificity has an essential effect on the test's both predictive values, while higher sensitivity increases PPV. Beyond those intrinsic test measures, population characteristics such as disease prevalence also interfere with predictive values. As prevalence increases, the NPV decreases. Thus, a positive biomarker or a positive test for a common disease will have a high PPV. On the other hand, rare diseases have low PPV. However, in this scenario, negative tests or biomarkers have high NPV and are useful to rule out disease diagnosis.

Those features may sound complicated or much theoretical at first glance but are essential to support clinical practice. If one has a test with good sensitivity and specificity and has a positive result for a prevalent disease, the subject likely has the disease. However, considering the same conditions, the likelihood would not be so high for a rare disease.[33]

The **likelihood ratio (LR)** is another method to describe the performance of diagnostic tests. In general, it indicates how much a particular test result raises the probability of disease and provides an alternative way for PPV and NPV interpretation. When the LR is greater than 1, it indicates an increased likelihood of the disease and the other way around. Some authorities define likelihood ratios of ≥5 or ≤0.2 as moderate and ≥10 or ≤0.1 as massive shifts in probability. Thus, they have a great impact on clinical decision-making.[34,35]

$$\text{LR for positive test} = \frac{\text{probability +test among diseased individuals}}{\text{probability +test among disease-free individuals}}$$

LR for positive test = sensitivity / (1-specificity)

LR for a negative test = specificity / (1-sensitivity)

Several diagnostic tests and biomarkers return continuous values instead of positive or negative values. However, they are often dichotomized because the clinical outcome is generally binary. To use them in clinical practice, studies evaluate several cutoff values to define a threshold to separate positive and negative results.[36] Therefore, the sensitivity and specificity vary based on the adopted cutoff values. Lowering the cutoff point will increase sensitivity, while specificity usually decreases, and the other way around.

This relation is more evident when one analyzes a well-known biomarker: prostate-specific antigen (PSA). This is an important biomarker for prostate cancer and has significantly changed the prostate cancer diagnosis and follow-up. As PSA rises, so does the prostate cancer and lymph node disease risks. Patients with PSA above 10 ng/mL have a positive likelihood ratio of prostate cancer above 50%. It means that those patients have more than 50% of chances to have prostate cancer.[37]

When PSA levels are between 4 and 10 ng/mL, the prostate cancer risk is about 25%.[38,39] It is still a considerable risk compared to the 4% cancer risk of the general population above 50 years old. However, it shows how the lower cutoff decreases specificity. The cutoff value of 4.1 ng/mL has a 6.2% false-positive rate and detects only 20.5% of cancer cases (sensitivity). To improve sensitivity, lowering the cutoff to 1.1 ng/mL would identify 83% of cancer cases. However, this low threshold also increases false positives, and it would subject 61% of the men to an unnecessary prostate biopsy.[40,41]

For continuous tests, a high threshold leaves a high percentage of undetected patients; whereas a low threshold has a better detection but carries a risk of unnecessary morbidity. Therefore, the choice of the ideal cutoff depends on the characteristics of the disease. The most important ones are the relative significance of the disease, morbidity, cost, risk for progression, and availability of effective treatment.[30,42]

To better understand this relation, it is possible to plot the true-positive rates and the false-positive rates (1-specificity) for each cutoff. The resulting graphic is the **Receiver Operating Characteristic (ROC) curve**.

2.4 Receiver Operating Characteristic Curve

The ROC curve of a perfect test would fill the entire area of the ROC space. The closer the curve is from the left upper corner, the more accurate the test is. The upper left corner indicates a test that achieves a high true-positive rate (sensitivity) while maintaining a low false-positive rate (1-specificity). On the other hand, the curve that approaches the 45 degrees diagonal through the ROC space is a poor-performing test. The diagonal line indicates a test that produces false-positive and -negative results at the same rate of true results.[36]

The area under the curve is a precise measure of accuracy and very useful to compare different tests. An area of 1 indicates the perfect test, which fills the entire space, as mentioned before, and has no false results. As the test accuracy decreases, so does the area under the curve. An area of 0.5 indicates a 45-degree diagonal ROC curve and means a poor test.

The area under the ROC curve gives us a measure of a test's ability to correctly classify individuals with or without the disease.[43] The calculation is mathematically complex but easily performed by computer software. Both parametric (maximum likelihood technique) and non-parametric (trapezoid rule) tests are used to determine the area under the curve and its standard error. A modification of the Wilcoxon rank-sum test[7] is a frequent choice to identify the statistical significance of the difference between tests.

How to select the optimal cutoff? It is crucial to consider the test or biomarker's objectives and the impact and costs of both false-positive and false-negative results. These include the costs of unnecessary further diagnostic tests and psychological distress while, on the other hand, there are costs for patients' health by an undiagnosed disease. A common approach is to give equal importance to sensitivity and specificity. This approach points to the nearest top-left corner.[44] It assumes that the balance between false-positive and false-negative results is equal, but it is not always true, though. It is crucial to consider the clinical scenario where the test is used to identify each

one's importance. The first scenario has a high clinical risk of missing a particular diagnosis. It is usually much more critical to miss the diagnosis than to overdiagnose cancer. In this case, the high specificity is essential to a confirmatory test. Therefore, the clinical scenario and the test scope must be accessed to understand the most suitable balance between the two types of errors and select the most appropriate cutoff.[36] The screening tests are included in this scenario.[45] The second scenario is when the test confirms a specific condition, or clinically speaking, it can "rule-in" or "rule-out" a disease.

2.5 Screening Tests

Screening tests have a particular scope: to classify individuals regarding the probability of a disease, rather than establish a definitive diagnosis. The main objective is to identify people at risk of the disease and allow them to identify it at an early stage when effective therapy is possible. That's why the screening tests prioritize high sensitivity even if the specificity is relatively low.[32,46]

There are some critical features to establish the suitability of a screening test or biomarker:

1. the disease must be frequent and a significant health problem;
2. the natural history must be well known, and the disease must have a significant latent of presymptomatic phase;
3. there must be an effective treatment that may benefit patients in terms of mortality or morbidity when started at earlier phases;
4. the screening test must be acceptable for the majority of the population;
5. the cost should not be excessive relative to the costs of medical care; and
6. screening must be a continuous process.

The concept of a screening test for early diagnosis of relevant diseases is very welcomed. However, screening tests can bring some potential disadvantages, though. A screening test must have high sensitivity, and it usually comes with low specificity, resulting in a high percentage of false-positive results. Those individuals experience unnecessary anxiety and unnecessary complementary exams, which are sometimes more invasive, including biopsies. The rate of unnecessary invasive tests must be taken in account, along with their complications. Another concern is the ability to alter disease prognosis.

Earlier diagnosis will point at a higher survival period even if the mortality is not changed. It is called lead-time bias, in which the survival occurs only because of a shift in date of diagnosis. Screening program results over mortality must be compared with the natural history of the disease to access survival improvement correctly.[47–51] The absence of such data or controversies about the optimal treatment obscure the interpretation of screening programs. Finally, the relative economic and human resources should not be excessive considering the need to develop a population-based screening program.[32]

2.6 Evaluation of Biomarkers

The evaluation of a biomarker relies on an exploratory process of the biomarkers' characteristics and comparison to an ideal biomarker. Good biomarkers have more characteristics similar to an ideal marker. Ideally, the attributes of a biomarker should include the following:[52–54]

- The first one is clinical relevance. The biomarker must provide evidence that supports some rational basis for its use. The evidence reflects some measurements or changes in a physiologic or pathologic process over a relatively short time;
- High sensitivity and specificity to evaluate treatment effects;
- Reliability, the ability to measure the biomarker analytically. There's a need to detect changes in the biomarker with acceptable accuracy, precision, robustness, and reproducibility;
- Practicality, defined as noninvasiveness or only modest invasiveness to prevent inconvenience and discomfort to healthy volunteers or patients;
- Simplicity, means accessible equipment and low cost. Simplicity facilitates the widespread acceptance for use in drug development and clinical practice.

The biomarker development process consists of sequential phases: pre-analytical and analytical validation, clinical validation, regulatory approval, and demonstration of clinical utility.

The pre-analytical phase analyzes quality indicators, and standardizes indicators such as sample collection, process, and storage. The analytical phase illustrates several features of the test: sensitivity, specificity, linearity, precision, limit of detection, accuracy, reproducibility, repeatability, and robustness.

2.7 Types of Studies

Randomized clinical trial (RCT) testing the efficacy of new medical interventions uses specific endpoints to measure the clinical benefit.[55] RTCs provide the highest level of evidence for clinal benefit; however, the time and resources needed to demonstrate benefits at the endpoint are often substantial.

Required evidence includes a combination of a clear mechanistic rationale and, in most cases, data from multiple RCTs showing the effect on the surrogate endpoint. The surrogate endpoints predict the impact on the clinical outcome of interest. Observational studies can provide supportive data to surrogate endpoints; however, they cannot prove etiology, causation, or mechanism.[56] Therefore, they cannot validate a surrogate endpoint alone in most of the cases.

There are many studies in progress to validate biomarkers, which can illustrate these phases. Most of them are related to cancer, such as programmed cell death 1 ligand 1 (PD-L1) (bladder cancer and melanoma), Interferon Gama Release (cancer vaccines), and MHC class 1 epitope (melanoma).[57–60]

Well-controlled prospective clinical trials can properly evaluate the clinical utility of a predictive test. In contrast, retrospective analysis of collected data from previously completed trials may be used if adequately justified.

To improve diagnostic test reports' quality, including completeness of data and transparency, we recommend following the Standards for Reporting of Diagnostic accuracy studies (STARD). STARD provides a checklist of items that should be reported in any diagnostic accuracy study. It was inspired in the Consolidated Standards for the Reporting of Trials (CONSORT) statement that is used for randomized controlled trials. STARD proposed checklists as well[61,62] and was designed to apply to all types of medical tests, as the starting point for building more specific instruments to stimulate complete and transparent reporting. It was created in 2003 and updated in 2015.[63,64]

STARD, together with additional implementation initiatives, helps authors, editors, reviewers, readers, and decision makers collect, appraise, and apply the evidence needed to strengthen decisions and recommendations about medical tests.[65] The CONSORT Statement, most recently updated in March 2010, is an evidence-based minimum set of recommendations including a checklist and a flow diagram for reporting RCTs and is intended to facilitate the complete and transparent reporting of trials and aid their critical appraisal and interpretation. The CONSORT statement may beneficially influence the completeness of reporting trials published in medical journals.[66,67]

2.8 Typical Study Design

2.8.1 Discovery of biomarkers

The identification of biomarker candidates is the first step toward clinical implementation. At the discovery phase, there are two principal approaches. The first one is the knowledge-based approach. This consists of selecting biomarker candidates based on the existing molecular mechanisms underlying disease initiation or progression. The second is the unbiased approach, which involves the untargeted identification of differentially expressed proteins between the two analyzed groups.

2.8.2 Qualification

When using an unbiased strategy, after identifying a candidate biomarker, the next step is its qualification. The qualification phase can investigate different expressions of the biomarker using alternative methods. It is also essential to determine whether biomarker expression varies from diseased patients to healthy individuals, if this was not performed at the discovery phase. In both phases, the goal is to identify candidate biomarkers with high sensitivity. Thus, the emphasis is on documentation of a consistent association between the indicator and the disease of interest.[68,69]

2.8.3 Verification

The goal of biomarker verification is to determine whether the candidate biomarker has sufficient potential for success to warrant investment in time-consuming and expensive clinical validation studies. The analysis typically requires a large number of samples attempting to include a broad range of patients and controls. Although high sensitivity remains essential, the verification process is focused on the specificity. This is the case because it is crucial to understand whether the candidate biomarker can point toward a specific condition. So, the verification process assesses biomarker specificity by attempting to capture variations in the tested population.

2.8.4 Prioritization of the candidates

The daunting costs of clinical validation of biomarkers and the limited resources make it crucial to prioritize the biomarkers with the most promising indicators.

The prioritization considers the knowledge about the candidate biomarkers. The insights into the biology of the protein of interest and the understanding of the disease's pathophysiology can contribute to selecting biomarkers with a higher potential of usefulness in clinical practice.

2.9 Validation

Validation is an essential step in the quest to deliver high-quality research data. There are three principal criteria: the nature of the question that the biomarker is designed to address, the degree of certainty required for the desired answer, and the assumptions about the relationship between the biomarker and clinical endpoints.[70,71]

A validated biomarker is measured and approved by analytical systems with well-established performance characteristics. Moreover, it is supported by a widespread agreement in the scientific community, considering the physiologic, toxicologic, pharmacologic, and clinical significance.

Pilot studies performed during the verification phase, and careful prioritization usually eliminate most candidate biomarkers from further consideration. In real clinical practice, the few remaining indicators are usually validated.

The clinical validation process requires a systematic study of the impact of other clinical covariates. Yet some pathophysiological-related conditions may have an association between the putative biomarker and the disease state. Thus, biomarker validation is a lengthy and expensive process that requires enrollment of large numbers of patients with diversified characteristics and outcomes. Extensive and independent studies can demonstrate the value of the candidate biomarker. This robust clinical evidence is crucial for successful implementation in clinical practice.

Thus, the validation essentially informs all the potential uses of the biomarkers. It establishes whether the biomarkers and the tests used to assess them are fit-for-purpose.[72–74]

In other words, the validation can conclude if the test can be associated with a medical product or a development tool sufficiently to support its use. The validation also informs if the biomarker is appropriate for specific contexts of use and risk assessment. Validation is especially important when a biomarker is used as a surrogate endpoint for clinical outcome assessment, such as the safety and effectiveness of a specific medical product.[68,70]

A biomarker that fails to represent disease processes and therapeutic effect or fails to detect severe harms from a medical product may lead to erroneous decisions. A diagnostic product based on an analytically invalid test or non-informative biomarker is not safe or effective because it might lead to inappropriate clinical decisions.

Considering the importance of the validation to identify whether a biomarker is acceptable for its intended purpose, validation must rely on a process to establish the performance of a test, tool, or instrument. The elements of the validation process include but are not limited to the Analytical Validation and Clinical Validation.

2.9.1 Analytical validation

The analytical validation ensures specificity, accuracy, precision, and other characteristics of a biomarker test. It establishes if the performance biomarker's characteristics are acceptable in terms of sensitivity, specificity, accuracy, precision, and other relevant features using a specified technical protocol (which may include specimen collection, handling, and storage procedures). This validation process is used for tests, tools, and instruments; it evaluates the technical performance but not its usefulness. Therefore, analytical validation evaluates the performance characteristics.[65] In contrast, the clinical qualification or validation is a statistical process linking biologic and clinical endpoints to a biomarker.[75,76]

2.9.2 Clinical validation

Clinical validation ensures if the test or device performs as intended, establishing that the test, tool, or instrument acceptably identifies, measures, or predicts the concept of interest. In a regulatory context, the concept is the aspect of an individual's experience, a clinical, biological, physical, or functional state that the assessment is intended to capture (or reflect).

2.9.3 Fit-for-purpose validation

Generally, validation should demonstrate that a method is "reliable for the intended application". Accordingly, the rigor of biomarker method validation increases as the biomarker data are used for increasingly advanced clinical or otherwise business-critical decision-making. For biomarker assays, validation was proposed for the adoption of a continuous and evolving *fit-for-purpose* strategy.[77]

Fit-for-purpose method validation provides for efficient drug development by conserving resources in the exploratory stages of biomarker characterization. By definition, exploratory biomarker data would be used for less critical decisions than data describing a well-qualified biomarker. The former one requires an advanced assay validation to ensure the confidence of the measurements. This concept was provided by *The 2003 Biomarker Conference Report*, which offered a snapshot of trends in biomarker applications across the drug development continuum.[78,79]

2.9.4 Cross-validation

Cross-validation is a statistical method that helps to eliminate some spurious positive biomarkers before the validation stage. It has three types; among these, the "holdout" method is majorly used in biomarker validation. Generally, the analysis of multiple independent uni- or multivariate datasets shows each marker's prognostic potential.[80,81]

2.10 Importance of External Validation

External validation using data from a completely different study provides the highest irrefutable evidence that a tool validates. The more external validations the better, particularly when they come from more heterogeneous populations that put stress on the generalizability of the risk tool. Internal validation is more convenient and perhaps the only option for introducing the risk tool in a timely fashion, but it is no substitute for external validation.[77]

2.11 Concept of Biomarker "Qualification"

When considering a biomarker's qualification, the United States Food and Drug Administration (FDA) describes specific recommendations for regulatory submission and eligibility. However, it does not establish legal responsibility for the qualification. Therefore, this document reflects an agency's guidance and advice.

The qualification of a biomarker comes to promote reliability and add greater value to its results. The qualification request can be sent to the regulatory authorities if this biomarker helps, directly or indirectly, in decision-making.[81]

- Stage 1: Letter of Intent (LOI);
- Stage 2: Qualification Plan (QP);
- Stage 3: Full Qualification Package (FQP).[82]

Letter of Intent (LOI)	Indicates the qualification process of a biomarker for a proposed context of use (COU) in drug development
Qualification Plan (QP)	Defines the intended development to generate the necessary supportive data to qualify the biomarkers for the proposed COU
Full Qualification Package (FQP)	Contains all accumulated data to support the qualification of the biomarker for the proposed COU
Qualification Recommendation	Contains FDA's determination on whether the biomarker is qualified for the proposed COU based on a comprehensive review of the FQP

Source: US Food & Drug Administration. About Biomarkers and Qualification. Published June 28, 2019. Accessed August 13, 2020. https://www.fda.gov/drugs/cder-biomarker-qualification-program/about-biomarkers-and-qualification[82]

The availability of a biomarker for use in the desired context is released only after the biomarker has been specifically qualified for its use.[83]

2.12 Laboratory Developed Tests

The FDA classifies an *in vitro* test as a Laboratory Developed Test (LDT) when directed for clinical use, and designed and produced in a single laboratory. If the test is not designed or manufactured, even partially or entirely in the same laboratory, it cannot be considered an LDT.

From 1976 to the present, the scenario of laboratories that carry out LDTs has changed considerably, whether in the physical space or acquired technologies, with innovative software and devices. The current tendency to propose a more personalized medicine brought forward the importance of biomarkers and test devices. Laboratory developed tests improve clinical decisions and offer better results for high-risk conditions.[84]

Thus, laboratories can produce LDTs, with great advantage for those patients who do not have commercially available tests for a specific disease.[85] Due to this situation, the FDA considered that LDTs currently have an increased risk potential for patients, if there is not adequate supervision.[86]

At the same time, closer and stringent regulation can lead to better analysis and more reliable results of LDTs. In contrast, indiscriminate use can increase financial costs and delay innovations, making it difficult for new technologies to be more accessible. The LDTs have played a critical role at the forefront of diagnostic innovation, particularly in academic and university clinical laboratories.

Even though the FDA does not regularly inspect LDTs, this is still done at the federal level. Laboratories that perform tests in the United States must follow Clinical Laboratory Improvement Amendments (CLIA) standards, which are applied by Medicare and Medicaid Service Centers and not by the FDA.[87]

The FDA is responsible for determining the complexity of each test after the approval of new *in vitro* diagnostic products. Tests that do not go through the standard categorization process, such as LDTs, are considered highly complex.[88,89]

2.13 FDA Approval in the United States / EC Mark in Europe

Each country or group of countries has its regulations regarding the release of medication, medical devices, and biomarkers. The objective is to ensure a standard of quality, safety, efficacy, and agility in the investigation and release of these new measures. Two distinct signatories are currently confronted, the United States of America (USA) with the FDA, and the European Union (EU) with the European Commission (EC).[90]

Some parameters can be compared between both institutions: centralization of coordination, transparency, access to the population, resources, and data evaluation. The FDA evaluates data with the primary focus on safety and efficiency for approval, especially for high-risk devices. Performance is an essential principle in the USA, so the FDA requires evidence that the device performs the functions.[91]

Evidence states that the FDA deprives US citizens of devices and drugs for longer because of the slow approval process, while the European Union is considered more agile for clearance.[92] At the same time, this easiness and agility of EC can eventually be questionable and worrisome to the detriment of patient safety.[93]

Regarding transparency, the FDA is more concerned with the data released, being more transparent, issuing proven presale review reports, and possible adverse effects. On the other hand, the EC does not have that same concern, leaving specific data disclosure to competent authorities. However, as EC regulates more quickly, the European population has faster and easier access to medications and devices, in some cases, years earlier.[94,95]

The funding differs in that the FDA has 80% federal funding and 20% regarding user usage. On the other hand, the EC has variable financing made by the country members of the bloc and paid by sponsors.

In an analysis, the FDA may be influenced by the political climate while the EU depends on the funding sector. Both institutions have differences in several criteria due to cultural factors. However, both have a common goal: to provide their patients with reliable and safe medicines and medical devices.

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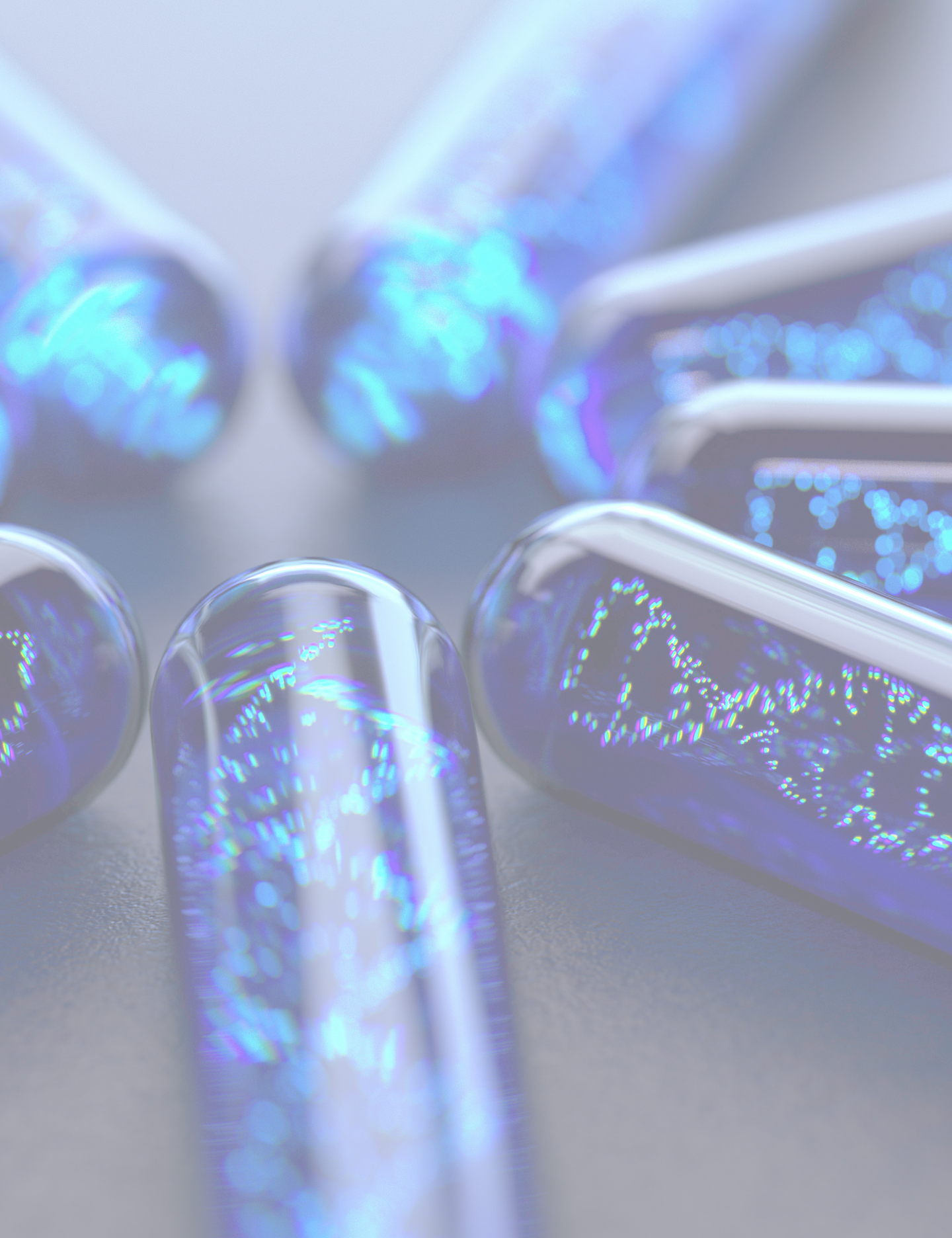

CHAPTER 3

How to Evaluate Biomarkers

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Table of Contents

3.1 Summary

In this chapter, we discuss 7 principles of biomarker development: 1) Biomarkers should predict risk rather than be categorized into being above or below a fixed cutpoint; risk prediction allows individualization of care. 2) Choose a clinically relevant outcome: many endpoints commonly used in biomarker studies, such as incident prostate cancer or advanced surgical pathology, are problematic. 3) Evaluate the biomarker on the patients to whom the biomarker would be applied in practice. 4) Follow the REMARK guidelines for the conduct and reporting of biomarker studies. 5) Biomarker research is comparative; the question is not whether a biomarker provides us with information, but whether it provides us better information than we already have, from clinical features or a currently used biomarker. 6) Report discrimination, calibration, and net benefit; a biomarker must be able to discriminate better than existing predictors, but risk predictions must be close to a patient's true risk; decision analysis is required to determine whether using the biomarker in clinical practice would change decisions and whether doing so would improve outcomes. 7) Conduct impact studies; evaluate how use of the biomarker in the real world affects outcomes.

3.2 Introduction

Biomarkers are used either to assess the risk for a current diagnostic state, such as having biopsy-detectable cancer, or predict the risk for a future event, such as prostate cancer death. In the former case, the biomarker gives the clinician information at less cost, risk, and inconvenience than the diagnostic test; in the latter case, it provides information that cannot be known, because it occurs in the future. Here, we will review methodologic considerations for serum prostate cancer biomarkers. We will neither discuss how biomarkers are discovered nor how best they can be measured accurately and reproducibly: we start from early-phase studies in humans evaluating the association between the biomarker and outcome and move to the later-phase trials (ie, impact studies) examining the effects of the biomarker when used in the clinic.

To be used most effectively, biomarkers need to be integrated with other information available to the clinician, such as the patient's age or stage of the tumour. This can be done informally by "clinical judgment," by using cutpoints and clinical rules, or by using a prediction model. In the case of prostate-specific antigen (PSA) for early detection of prostate cancer, an early approach was to use the clinical rule of "PSA > 4 or positive digital rectal examination (DRE)." This subsequently evolved to the more informal clinical judgment approach, where the urologist considers the age of the patient, the recent clinical history (such as symptoms of benign prostate disease) and the DRE, as well as the absolute level of PSA. In the past 10 to 15 years, there has been a move to statistical methods of risk prediction. Using statistical models such as the "PCPT risk calculator"[1] or the "PBCG model",[2] the urologist enters clinical data about the patient's age, race, DRE, family history, and history of prior negative biopsy, as well as the level of PSA, and obtains a percentage risk for high-grade cancer. The advantage of prediction models is that they give more accurate predictions than either informal clinical judgment, as numerous studies have demonstrated that computer models outperform clinicians,[3–5] or the risk groupings used for clinical prediction rules.[6–9] Moreover, use of prediction models allows for greater individualization of care. A man who is older, has comorbidities, or is averse to medical procedures, but has a PSA level just above 4 might reasonably ask

whether his PSA warrants a biopsy; comparably, a man anxious about prostate cancer who has a PSA just below 4 might want reassurance that his risk was indeed low. It is only by using predicted probabilities that urologists can have a rational conversation about risk that considers patient preferences and characteristics.

Statistical methods for building models are described at length in various publications and will not be further discussed here.[10] Instead, we will focus on approaches to assess the predictiveness of a biomarker in two different scenarios, when the biomarker is used independently and when it is incorporated into a prediction model.

3.3 Can the Biomarker Predict the Outcome of Interest?

3.3.1 Choose the right outcome

The appropriate clinical endpoint for a biomarker is sometimes more complex that it appears. Well-known studies such as the PRACTICAL collaboration have developed polygenic risk scores (PRS) for the endpoint of incident prostate cancer.[11] But incident prostate cancer is not synonymous with cancer-related mortality or morbidity. The central problem of prostate cancer early detection is overdiagnosis, reflecting that cancers are diagnosed that would never cause symptoms during the course of the patient's natural life. It is thus not as useful to know a man's risk for a prostate cancer diagnosis as it is to know the risk for prostate cancer metastasis or death: a man at high risk for prostate cancer death might want to consider screening to find a cancer early before it spreads; it is not at all clear what a man should do if he is at higher risk for prostate cancer. Biomarkers or models that predict the risk for any-grade cancer on prostate biopsy can be subject to a similar criticism: we want to find cancers that we would consider treating (eg, Grade 2 or higher disease); we do not need to know about the risk for all cancers, including Grade group 1 disease, the most appropriate management strategy for which is to order a second biopsy.

Naturally, the ideal endpoint for any biomarker is cancer-specific morbidity (ie, metastasis) or mortality. However, this is challenging given that such endpoints may occur 10 or 20 years after diagnosis, and their use has been attempted for only a handful of prostate cancer biomarkers, including the 4Kscore,[12,13] the DECIPHER score,[14] and of course, PSA.[15,16]

3.3.2 Does the biomarker distinguish between samples of clearly distinguishable patients?

Investigators can test whether biomarker levels differ in clearly distinguishable groups of people. These studies can be performed relatively quickly, as samples can be obtained from patients based on an outcome status already achieved as opposed to following a cohort of patients prospectively until the outcome of interest occurs or waiting to accrue patients undergoing a procedure such as biopsy. For example, in the notorious early prostate cancer antigen (EPCA) study,[17] levels of EPCA in men with prostate cancer were compared to healthy men, healthy women, and patients with other diseases, such as liver cancer or benign lung disease.

Diagnostic accuracy should instead be assessed using a sample representative of the population as shown in **Table 3–1**. In scenarios A and B, we have a biomarker with high sensitivity and specificity (both 90%) for advanced disease, but unable to distinguish localized disease (sensitivity and specificity of 50%). Scenario A represents a sample with an equal number of patients in each disease group, where the sensitivity and specificity in the entire population for detecting cancer is 70%. However, if the distribution of patients were more reflective of the population as in scenario B, the sensitivity and specificity drop to 58% and 63%, respectively.

TABLE 3–1 Two Hypothetical Studies That Sample Individuals Without Disease, with Benign Disease, with Localized Disease, or with Advanced Cancer*

	Cancer (Advanced or Localized)	No Cancer (No Disease or Benign Condition)
Scenario A		
Biomarker Positive	(90% x 250) + (50% x 250) = 350 (true positives)	(10% x 250) + (50% x 250) = 150 (false positives)
Biomarker Negative	(10% x 250) + (50% x 250) = 150 (false negatives)	(90% x 250) + (50% x 250) = 350 (true negatives)
Total	500	500
Scenario B		
Biomarker Positive	(90% x 50) + (50% x 200) = 145 (true positives)	(10% x 250) + (50% x 500) = 275 (false positives)
Biomarker Negative	(10% x 50) + (50% x 200) = 105 (false negatives)	(90% x 250) + (50% x 500) = 475 (true negatives)
Total	250	750

*The biomarker's sensitivity and specificity for advanced disease and those without disease are high (both 90%); however, the biomarker is unable to correctly identify patients with localized disease or those with a benign condition (sensitivity and specificity are 50%). Scenario A: Equal numbers of patients are included in each group (healthy, benign, local cancer, and advanced cancer). The overall sensitivity and specificity are both 70%. Scenario B: Sampling of patients reflects the prevalence of groups seen in the clinic. In this example, 25% of patients have no disease, 50% have benign condition, 20% of patients have localized cancer, and 5% have advanced cancer. The sensitivity is 58% and the specificity is 63%.

3.3.3 Is the biomarker associated with the outcome in the patients for whom the biomarker would be applied in practice?

Just as drugs are studied in the patients who would receive the drug were it shown to be effective, markers should be studied in the patients to whom the biomarker would be applied in practice. The development of free-to-total PSA ratio is a good example of a marker that moved from research on convenience samples to the target population of men considering biopsy. First, Christensson *et al.* demonstrated an association between the ratio of a PSA isoform, free PSA, and the total amount of PSA in serum (free-to-total PSA ratio) is significantly lower among men with prostate cancer than in men with benign prostate hyperplasia.[18] Catalona *et al.* then

assessed whether free-to-total PSA can enhance the specificity of prostate cancer screening by confirming that the association of free-to-total PSA and prostate cancer on biopsy remains significant among men with total PSA values of 4.1–10 ng/mL indicated for prostate biopsy in clinical practice.[19]

3.3.4 How well does the biomarker predict the outcome of interest compared to information available to the clinician?

A useful biomarker should provide additional information available to the clinician. In the Catalona *et al.* example above, measurement of free-to-total PSA ratio added information about prostate cancer risk over and above total PSA and DRE.[19] Assessments of discrimination or clinical utility (explained in detail below) can be used to compare the performance of a new biomarker to the performance of an existing model or existing biomarker.

Alternatively, a biomarker can be combined with other clinical factors by building a prediction model. When a biomarker is incorporated into a prediction model, the Wald test corresponding to the biomarker tests whether a biomarker adds predictive value in addition to the other clinical factors. For example, Klein *et al.* assess the added value of the Genomic Prostate Score by demonstrating that it is significantly associated with the risk for adverse pathology on multivariable logistic regression analysis when added to a model containing standard clinical predictors—age, PSA, clinical stage, and biopsy—either entered as separate variables or combined into a risk score such as the Cancer of the Prostate Risk Assessment (CAPRA) score.[20,21] If it is shown that the biomarker is independently predictive of outcome, the degree of added predictive value should be assessed.

3.4 Assessing Predictiveness

In the previous sections, we have outlined necessary steps to biomarker development. These culminate in establishing that the biomarker adds to established predictors in predicting a clinically relevant outcome in a group of patients to whom the biomarker would be applied in practice. In the next section, we will discuss metrics to assess the degree to which the biomarker predicts better than the established predictors.

3.4.1 Discrimination

The area under the receiver operating characteristics (AUC) curve, also referred to as the concordance statistic (or C-index), is commonly used to assess discrimination: the probability that given a randomly selected patient without the disease and a randomly selected patient with the disease, the patient with the disease has the higher predicted probability.[22–24] Extensions of the concordance statistics in the presence of censored data to assess the discrimination of Cox proportional hazards models have also been developed including Harrell's C-index and a concordance probability estimate (CPE) developed by Gönen and Heller.[25–28]

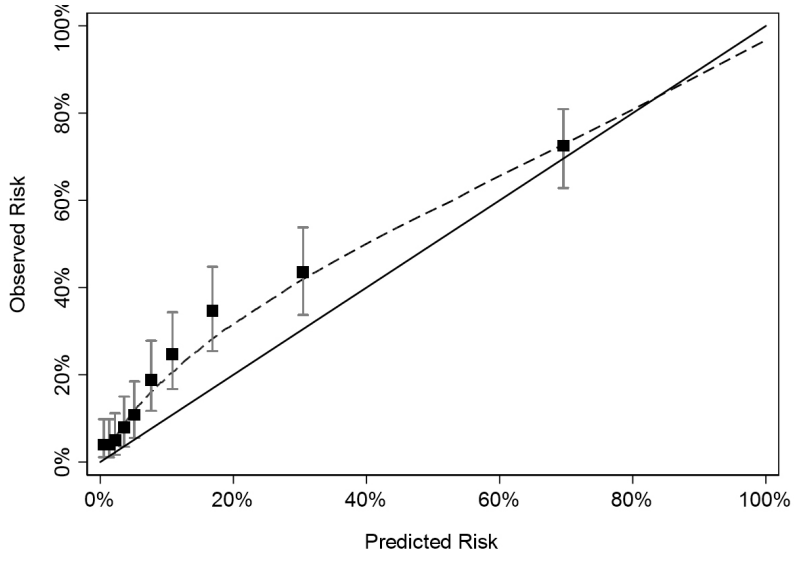
When comparing the discrimination of different models or biomarkers, investigators are encouraged to report the difference in discrimination along with 95% confidence intervals. Approaches to assess whether there is a significant difference in discrimination depend on the whether the models being compared are "nested." The classic example of a nested model is when a new biomarker is added to an existing model, for instance, when the two models are PSA, DRE, and age versus PSA, DRE, age, and free-to-total-ratio. A comparison of two existing models, such as the Prostate Cancer Prevention Trial (PCPT) versus the Prostate Biopsy Collaborative Group (PBCG) model, would be non-nested. In these cases, the DeLong test can be used to test for a difference in discrimination.[29] When models are nested, the *p*-value from the Wald test corresponding to the biomarker should be reported, the DeLong test being invalid.[30,31]

For example, Wei *et al.* assess the value of prostate cancer antigen-3 (PCA3) as a prognostic biomarker to predict high-grade prostate cancer on initial and repeat biopsy.[32] The authors demonstrate that PCA3 is significantly associated with high-grade cancer on initial and repeat biopsy after adjusting for the PCPT risk score (*p*-values≤0.003). They demonstrate moderate increases in discrimination for the detection of high-grade cancer with the addition of PCA3 after 10-fold cross-validation for initial and repeat biopsies of 0.74 to 0.78 and 0.74 to 0.79, respectively. Optimism and various strategies to adjust estimates of multivariable model performance to correct for optimism are highly encouraged and discussed in a later section.

3.4.2 Calibration

In order to be clinically useful, a prediction model must not only be able to discriminate between patients with and without the disease but also provide an accurate risk prediction. The degree to which predictions are in agreement with the observed outcomes is known as calibration.[33] A calibration plot visualizes the agreement between model predictions on the x-axis and the actual outcome on the y-axis. This is typically done by splitting the data into equally sized groups of increasing predicted probabilities (deciles) and plotting the mean of the observed outcome by the decile of prediction.[23] See **Figure 3–1** for an example of a calibration plot.

FIGURE 3–1 An example of a calibration plot for a model predicting the risk of high-grade prostate cancer on prostate biopsy. The dots show the average risk (along with 95% CI) of patients divided into 10 groups of increasing risk. The dots and dashed regression line fall above the 45-degree line for good calibration, demonstrating that patients had higher risk than that predicted by the model. This is particularly a problem for risks around 10%, the sort of risk at which a patient might opt for prostate biopsy. Such a calibration plot would raise questions about whether the model should be used to inform prostate biopsy decision-making.



A model with poor calibration in ranges of probabilities where treatment decisions reasonably can differ is likely to be of limited clinical value, even if discrimination is excellent: it is difficult to make a good decision if information about patient risk is wrong. Conversely, if a biomarker or model has poor discrimination, no adjustment or recalibration can improve its prognostic value use; however, if discrimination is good the biomarker or model can be recalibrated without sacrificing discrimination.[34] The calibration of a prediction model is nearly always excellent when assessed in the same dataset used to generate a multivariable prediction model. When a prespecified model is applied to an independent dataset, investigators are encouraged to assess calibration graphically with a calibration plot.

Some biomarkers such as the prostate health index (PHI)[35] and ExoDx Prostate IntelliScore[36] provide a score and decisions are made comparing to a proposed cutpoint, but these scores do not represent risk for disease. As such, it is not possible to assess calibration in the traditional sense. However, investigators can report the probability of the outcome above and below the previously proposed cutpoints to assess clinical value. For example, Donovan *et al.* present that the risk for high-grade disease on biopsy was 34% above versus 2.5% below a prespecified cutpoint for the ExoDx Prostate IntelliScore[36] and Jansen *et al.*[35] present the number of high-grade cancers missed and detected above a proposed cutpoint.

3.4.3 Clinical utility

A new biomarker is only of value if its use leads to improvement in patient outcome via a change in treatment decision patterns. A full assessment of the prognostic value of a biomarker or model must incorporate clinical consequences of the resulting decisions made. Imagine that we have data from a study of 1,000 men with elevated PSA levels and we calculate risk for cancer on biopsy based on a prediction model including a new biomarker represented in **Table 3–2**. This shows that 300 men had high-grade cancer and that among the 510 men with

a predicted risk of 10% or greater (our threshold to indicate biopsy), cancer was detected in 210 of these men (**Table 3–2**). Based on the clinical consequences illustrated in **Table 3–2**, we see that to determine whether it is better to biopsy all men or to use the statistical model and biopsy those with a 10% risk for high-grade cancer, we need to consider whether it is worth delaying the diagnosis of 90 high-grade cancers to avoid 490 biopsies.

TABLE 3–2 Hypothetical Results of a Biomarker Study for Prostate Biopsy Illustrating Clinical Consequences and Decision Analysis

Strategy	Biopsied	Biopsy Avoided	High-Grade Cancers Caught	High-Grade Cancers Missed	Unnecessary Biopsies	Net Benefit
Biopsy all men with elevated PSA	1,000	0	300	0	700	300 - (700 x (0.10 / 0.90)) = 222
Biopsy if risk ≥ 10% from statistical model based on molecular biomarker	510	490	210	90	300	210 - (300 x (0.10 / 0.90)) = 177

In some cases, the results will be fairly obvious, and we can rely on the reader's clinical judgment: if, for instance, there were only 10 high-grade cancers missed for a reduction of 490 biopsies, most would immediately see that the biomarker was of value. When results are not immediately clear, decision analysis can be of value. One of the simplest approaches, and the most widely used in the urologic literature, is "net benefit," which incorporates the consequences of clinical decisions of a prediction model or biomarker in the analysis.[37] Net benefit incorporates both discrimination (AUC) and calibration, making it an ideal statistic for comparing prognostic value.[38] A key aspect of net benefit is that the level of risk at which a patient opts to receive a biopsy is informative of how a patient weighs the relative harms of a false-positive (an unnecessary biopsy with risks of side effects including infectious complications and hospitalization) versus a false-negative (missing or delaying the detection of a high-grade cancer) result. This level of risk is termed threshold probability.[37] The threshold probability chosen in **Table 3–2** was 10%, corresponding to odds of 10:90, implying that missing a cancer is 9 times worse than doing an unnecessary biopsy.[39] A threshold of 10% corresponds to a "number-needed-to-test" of 1/10% = 10, meaning that 10 men need to be biopsied to find one cancer.[39,40] Applying this 9:1 ratio to the study results gives the findings in **Table 3–2**, where it can be seen that the biomarker is actually harmful. Even though the marker has reasonable sensitivity and specificity (~70% and ~60%), too many high-grade cancers are missed for the decrease in unnecessary biopsies achieved.[41] There are no significance tests (*p*-values) associated with comparing net benefits between strategies and it is rare that confidence intervals around the net benefit are reported. This is because the net benefit is a decision analytic statistic, that is to say, when choosing between different options, the most rational choice is, in general, that with the highest expected utility, irrespective of statistical significance.

One obvious issue is that the threshold can vary between patients or physicians: a patient worried about cancer might opt of a threshold of 6%, whereas one nervous about medical procedures might demand a 15% risk before considering biopsy. This variability in threshold probabilities underlines the value of a biomarker or prediction model to provide precise estimates of risk in order to make individualized treatment decisions that take into account patient preference. This is in distinction to dichotomized markers that place patients into high- or low-risk groups.

To account for this variation in preference, we can use decision curve analysis, in which the threshold probability is varied over a reasonable range and net benefit plotted against threshold probability.[37] By visualizing the decision curve, one can readily ascertain whether one strategy or model is optimal across the full range of threshold probabilities of interest. For more on decision curves, which are very widely used in urology research, a selection of further reading is available at www.decisioncurveanalysis.org.

3.4.4 Impact studies

Decision analytics techniques provide hypothetical assessments of clinical consequences, addressing questions such as "If urologists were to use a 10% risk from the model as a threshold for biopsy, what would be the results in terms of biopsies performed and cancers found?". Impact studies go one step further and assess the real-world consequences of a biomarker- or model-based strategy.

One example of an impact study would be to assess whether the results of the biomarker translated to changes in decisions. The cell cycle progression (CCP) score (marketed as the Prolaris test [Myriad Genetics, Salt Lake City, Utah, United States]), is a genomic test that combines levels of expression of various genes related to cell-cycle progression.[42] Crawford *et al.* investigated the impact of the CCP score by evaluating the change in treatment decisions before and after the physician received and discussed the patient's CCP test results.[43] Among those with a pre-CCP test recommendation of interventional therapy, there was a reduction of 37% in interventional therapy after the CCP test results were provided.[43] Similarly, Konety *et al.* reported a 65% reduction in prostate biopsies in men receiving the 4Kscore.[44] However, not all impact studies are consistent with clinical biomarker studies: for example, White *et al.* found that use of the PHI in practice led to a very large decrease in the capture of high-grade cancers, with an approximate 30% risk for high-grade cancer among men who avoided biopsy.[45]

A second reason to undertake impact studies is that some endpoints are not entirely predictable from clinical research. Early research on PSA certainly found that it found prostate cancer at an early stage, but it was unclear if prostate cancer screening regimens based on PSA would lead to reductions in mortality. The European Randomized Study of Screening for Prostate Cancer (ERSPC) is an example of an impact study that assesses whether a prostate cancer biopsy screening strategy based on total PSA biomarker can lead to a reduction in prostate cancer deaths.[46] Results from this trial illustrate the clinical benefit of a prostate cancer screening regimen based on total PSA, with an absolute risk reduction of death from prostate cancer being 1.8 per 1,000 men randomized.[47] Findings from the ERSPC illustrate the benefit in terms of prostate cancer deaths prevented as a result of this screening regimen while accounting for real-world deviations from the recommended screening regimen.

3.5 Study Design Issues

The REcommendations for tumour MARKer prognostic studies (REMARK) guidelines discuss study design considerations at length.[48] For instance, one key point is that assessors of the outcome should be blinded from the biomarker status. Another key concept is that of internal versus external validation. Interval validation is when a multivariable regression model is developed or a new cutpoint for a biomarker is selected and evaluated for performance on the same dataset. When a prediction model or biomarker cutpoint is developed and assessed on the same dataset, estimates of performance are over-optimistic, a phenomenon known as overfitting.[49,50] Harrell *et al.* describe methods for obtaining optimism-corrected internal assessments of performance including data splitting, cross-validation, and bootstrapping.[34] Optimism-correction techniques can also be applied to decision analysis when generating estimates of net benefit as outlined by Vickers *et al.*[51] Note when a model is generated in a separate independent cohort, optimism corrections are not necessary.

External validation not only solves the problem of over-optimism but also evaluates genuine differences between cohorts. A model predicting recurrence after radical prostatectomy, for instance, may be affected by surgeon skill—less-skilled surgeons having higher recurrence rates —or by differences in pathologic grading. An excellent practical example of external validation was a study showing that the risk of prostate cancer among Chinese men with a given PSA has been shown to be lower than for European men, the most likely explanation being that Chinese men have higher rates of benign disease. This true difference between cohorts will mean that prediction models using PSA will likely have poor properties when applied in China.[52]

3.6 Common but Flawed Approaches: Sensitivity / Specificity, Brier Score, NRI, and IDI

The reporting of sensitivity or specificity for a prediction model or continuous biomarker is discouraged, as these do not account for clinical consequences and should not be used to determine a cutpoint to justify a clinical decision. As a simple illustration, imagine that we have Test A with 90% sensitivity and 40% specificity and Test B with 40% sensitivity and 90% specificity, and our job is to decide which is superior. If the prevalence of disease were 2%, the positive predictive value of test A versus B would be 7.5% versus 3%; if the prevalence were 20%, positive predictive value would be 50% versus 27%. If 5% to 10% represented a reasonable range of threshold probabilities and the prevalence were 2%, then Test B would be superior; if 25% were a sensible threshold probability and the prevalence were 20%, then Test A would be superior.

In 2008, Pencina and colleagues introduced the net reclassification improvement (NRI) and integrated discrimination improvement (IDI), which they said to be more sensitive to the prognostic impact of a new predictor than the AUC.[53] There are, however, several major problems with these statistics, most fundamentally, a biomarker that is unassociated with the endpoint can lead to a statistically significant increase in NRI or IDI. Thus, the NRI and IDI are statistically invalid and should be avoided.[54,55]

To properly assess the value of a biomarker, we need some measure that incorporates both discrimination and calibration. The Brier score, the mean squared prediction error, which is sensitive to both discrimination and calibration, is an attractive alternative. However, the Brier score is inappropriately affected by prevalence.[56] Assel *et al.* demonstrate that due to this dependency, the Brier score may give undesirable results where clinical consequences are discordant with prevalence.[57] Consider a case where we have two binary biomarkers: one with a specificity of 95% and a sensitivity of 50% and the other has a sensitivity of 95% and specificity of 50%. In this scenario, the harms of missing disease far outweigh the harms of unnecessary treatment, so we would favour the test with a higher sensitivity; however, the Brier score favours the more specific test where disease prevalence is less than 50%.[57] As the Brier score does not assess clinical consequences, it should not be used to assess improvement in prognostic performance.

3.7 Against Cutpoints / for Risk Prediction

Categorizing a continuous biomarker or any other continuous variable in a prediction model is highly discouraged. As a simple example, imagine that instead of entering a PSA value into a model predicting biopsy result, we categorized PSA as greater or less than 10 ng/mL. This would mean that a man with a PSA of 9.9 ng/mL would be given the same risk as a man with a PSA of 0.5 ng/mL; patients with PSAs of 10.1 and 500 would similarly be given the same predicted risk. Categorizing markers throws out data and lessens our ability to predict.

A separate problem with categorization is when different investigators use different categories. For example, the CCP score has been categorized by different investigators as <-1, -1 to 0, 0 to 1, >1;[58] ≤0, 0 to 1, 1 to 2, >2;[59] and <0, 0 to 1, >1.[60]

Even more problematic is when investigators look at all potential cutpoints and use the cutpoint with the smallest *p*-value as opposed to a continuous term. An example is Chun *et al.*, who tested various levels of cutpoints of PCA3 to include in a nomogram.[61] This approach makes little sense because finding the cutpoint with the greatest strength of evidence against no association is not at all equivalent to finding a cutpoint with the greatest clinical utility. The authors demonstrate that the incremental increase in risk decreases at higher cutpoints of PCA3 (OR=3.24, 2.46, and 2.32 for cutpoints of 17, 24, and 35, respectively) indicating that there is likely non-linearity in the relationship between PCA3 and risk. The predictive accuracy for a continuous PCA3 along with non-linear terms would have likely resulted in a higher predictive accuracy. Another common example of risk categorization is defining a cutpoint of predicted risk based on the top left-hand corner of the receiver operating characteristics curve. This approach assumes that sensitivity and specificity are of equal importance, which is rarely if ever true in clinical practice: it is generally more harmful to miss a case of disease than to continue work-up unnecessarily. Bennette *et al.* discuss alternatives, such as including non-linear terms, to categorization of continuous variables.[62]

Categorization can also involve multiple biomarkers in the common, and questionable, practice of creating risk groups. The National Comprehensive Cancer Network (NCCN) proposes many such risk groupings.[21] Risk groups are often created by informal judgment. Alternatively, risk groups can be created by converting a multivariable model into a simplified scoring algorithm and those scores may also in turn be categorized into risk groups.

The argument is that risk grouping makes things easy for the physician. But there is little if any evidence that physicians are unable to interpret risk estimates, and only have the mental capacity to understand black-and-white risk groups. Risk grouping involves loss of information and it is hard to see how this could generally be in a patient's best interest.[63] Prediction modelling to generate a patient's individualized estimate of risk should be favoured when possible. This does not necessarily mean that a physician always uses the numerical risk estimate in the clinical consultation. The choice to do so will depend on a clinical evaluation of the patient's numeracy and decision style ("whatever you think is best, doctor" versus "I need all the information to make a decision") as well as the value of the risk estimate (eg, "I'm glad to tell you that your risk is very low, so no need to worry for the moment" versus "your risk is somewhere in the middle, so we need to discuss what to do next").

3.8 Conclusions

In this chapter, we have outlined the evaluation of prostate cancer biomarkers. Our key "takeaways" can be summarized as follows:

1. Biomarkers should provide a numerical risk prediction rather than be categorized into being above or below a fixed cutpoint; risk prediction allows for individualization of care.
2. Choose a clinically relevant outcome; many endpoints commonly used in biomarker studies, such as incident prostate cancer or advanced surgical pathology, are problematic.
3. Evaluate the biomarker on the patients to whom the biomarker would be applied in practice.
4. Follow the REMARK guidelines for the conduct and reporting of biomarker studies.
5. Biomarker research is comparative; the question is not whether a biomarker provides us with information, but whether it provides us better information than we already have, from clinical features or a currently used biomarker.
6. Report discrimination, calibration, and net benefit; a biomarker must be able to discriminate better than existing predictors, but risk predictions much be close to a patient's true risk; decision analysis is required to determine whether using the biomarker in clinical practice would change decisions and whether doing so would improve outcomes.
7. Conduct impact studies; evaluate how use of the biomarker in the real world affects outcomes.

It has often been noted that biomarker research has a poor track record of getting biomarkers into clinical practice. Following established principles of biomarker development increases the chances that a biomarker with good predictive properties would be incorporated into urologic decision-making and ultimately improve patient care.

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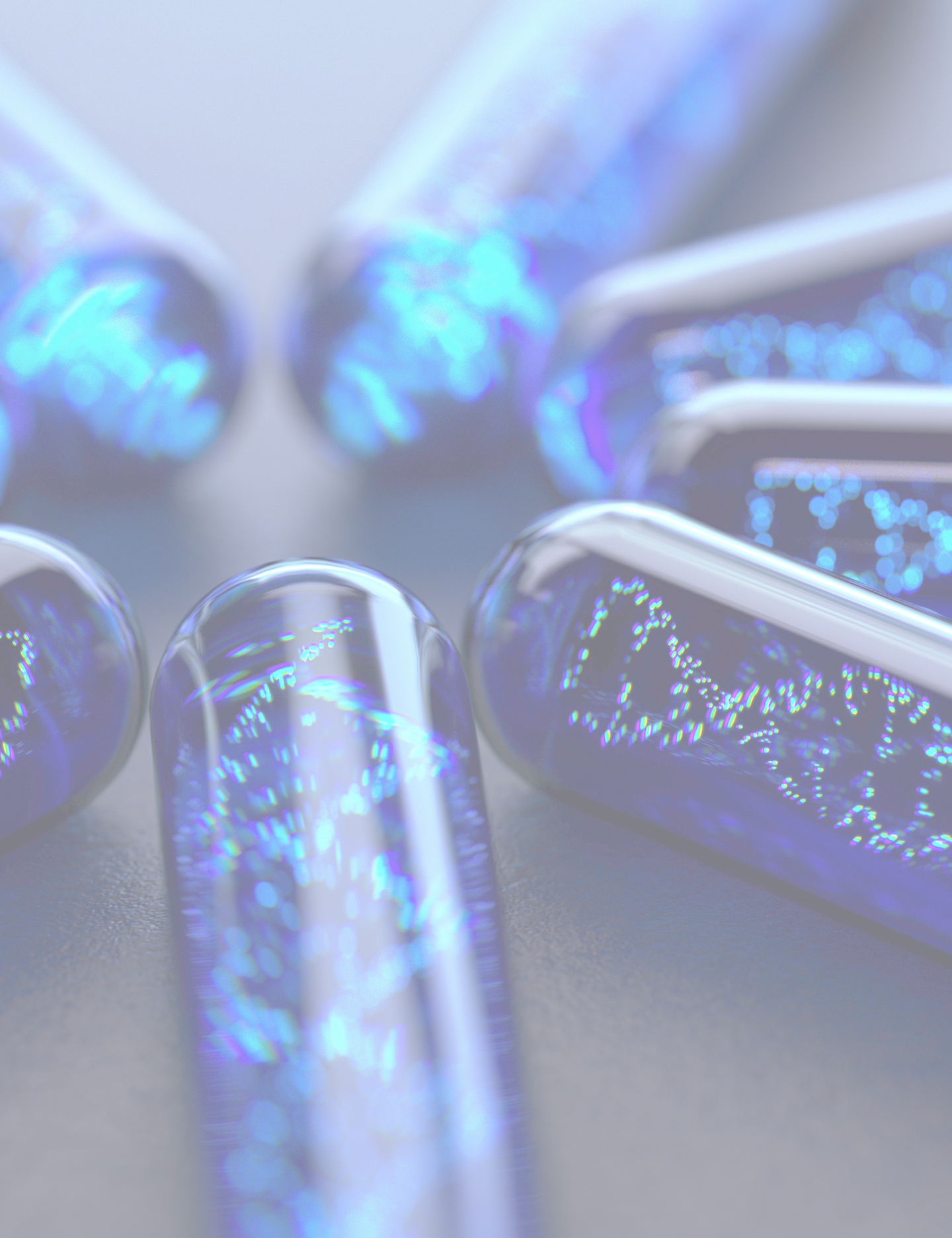

CHAPTER 4

Classification of Molecular Biomarkers

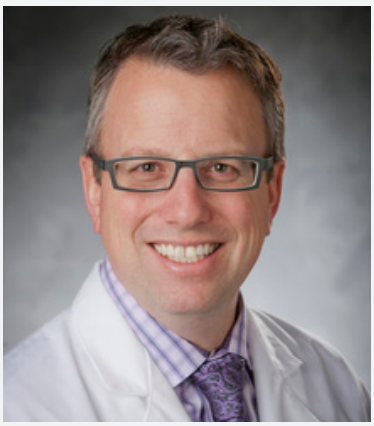

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Table of Contents

4.1 Introduction

A "biomarker" is any measurable characteristic that indicates the presence or absence of disease or the biological response to a stimulus, typically an exposure or intervention. The Food and Drug Administration and National Institutes of Health (FDA-NIH) Biomarker Working Group has produced a document called Biomarkers, EndpointS and other Tools (BEST), which defines seven categories of biomarkers based on their clinical usage: susceptibility and risk, diagnostic, monitoring, prognostic, predictive, pharmacodynamic and treatment response, and safety. In this chapter, we approach the classification of biomarkers in two additional ways: their bodily source and their measurement type.

4.2 Biomarkers by Source

Not all biomarkers can be classified by source (eg, imaging); although, all molecular biomarkers can. In this section, we discuss the sources from which most molecular biomarkers are obtained and the considerations that are associated with each.

4.2.1 Blood

Blood and its various components represent a valuable source for a wide variety of molecular biomarkers. Although direct sampling of tumour cells in solid tumours of urologic oncology is not accomplished with peripheral blood draws, circulating tumour cells, as well as cell-free circulating DNA, can be used for genomic biomarkers.[1,2] Proteomics, lipidomics, and metabolomics in the space of oncology, broadly, are growing fields that can be also applied to blood samples for additional biomarker evaluation.[3]

While blood is less invasive to obtain when compared to tissue or biofluids, particularly with repeat assessments, some level of invasiveness is required to obtain blood for analysis of many biomarkers. However, many patients with urologic malignancies are likely to undergo blood draws for other parts of their care and such samples can be leveraged for biomarker assessment. Blood is largely composed of water but contains additional components, including erythrocytes, leukocytes, platelets, fibrinogen and other clotting factors, proteins including albumins and globulins, glucose, and electrolytes. Importantly, any of these components may limit the assessment of a given analyte if the blood is not processed appropriately.[4,5] Also challenging to control is the variation of individual components that make up blood, which can occur in disease states such as dehydration, infection, or metastatic malignancy.[3,4,6]

In order to prevent degradation, blood and blood fractions have traditionally been cryopreserved in aliquots to limit the effects of thawing and re-freezing that could damage target analytes within the specimen. A major critique of this approach is that the cost associated with cryopreservation can be significant.[7,8] Alternative methods of storage have been evaluated in order to decrease storage costs tied to cryopreservation, including drying with newer methods such as lyophilization and isothermal vitrification; however, these methods are not yet standardized and data is emerging on their applicability to newer biomarkers and analytical methods.[9,10]

For low molecular-weight protein studies, drying on silica chips is an option but does not necessarily protect specimens at higher temperatures. Dried blood spots (DBS) using a paper system to evaporate water and contain blood components are useful in settings where access to cooling is limited for initial specimen handling. However, DBS requires controlled storage conditions for optimum protection of the specimen, and certain analytes are more susceptible to oxidative damage and are not good candidates for evaluation from DBS. Novel techniques for safeguarding blood components, with less cost, remain an area of exploration, and strategies such as silk-matrix stabilization may help overcome the need for cryopreservation in the future.[10]

Serum and plasma

Although whole blood has many uses for biomarker assessment, certain measurement modalities require sample refinement to optimize detection of a particular analyte and to minimize interference or contamination from other components. To this end, separating the cellular fraction out from the liquid portion of blood facilitates spectroscopy-based analysis with less interference from blood cells. The liquid fraction of blood can be isolated in the form of either serum or plasma. Plasma is distinct from serum in that it is stored in a way that prevents coagulation and clot formation. Consequently, various clotting factors, fibrinogen, and platelets are maintained in suspension in plasma. Serum, on the other hand, is allowed to clot over 30 minutes prior to use and can give a cleaner sample when interference from platelets and other contaminants is undesirable, while also potentially providing greater long-term stability with cryopreservation than plasma. There are trade-offs of the two forms,[4,11] although exceptions exist and the decision to use one liquid fraction over another should be individualized to the analyte of interest.[12]

Cellular fractions

Cellular components of blood, and characteristics therein, are also used in a variety of biomarkers. For example, ratios of neutrophils to lymphocytes have been found to be poor prognostic markers of systemic inflammation and correspond to worse outcomes in a variety of malignancies,[13,14] while anemia and thrombocytopenia have been used in risk stratification for renal cell carcinoma[15] and may broadly correlate with late-stage tumours.[16] Isolation of cellular fractions may be achieved by centrifugation and separation by size of cellular component or using advanced spectroscopy.[17,18] Cellular fractions are less subject to coagulation, as discussed above, when blood is stored as plasma. Consequently, reassessment of cellular biomarkers from blood samples may be facilitated with such specimens, although the anticoagulant or freezing technique used may affect the viability of cells.[19,20] Flow cytometry and other immunological techniques can be used to characterize the cellular components of blood to a high degree of precision using fluorescent antibody labelling. Using modern methods, dozens of markers can be evaluated on a single cell in one assay.[21]

4.2.2 Urine

In addition to being, perhaps, the least invasive liquid biomarker (with saliva), urine has the advantage of a simpler constituent matrix than many other biofluids. From a storage standpoint, urine is more thermodynamically stable than other biofluids and generally requires less processing for preservation. Also, in the case of urinary tract–facing malignancies, there is an opportunity to capture excreted tumour cells and their biochemical by-products. Urinary extracellular vesicles containing a wide variety of molecular biomarker classes have also been

discovered. As a consequence, a vast majority of the molecular biomarker classes are identifiable in urine. Of course, not all patients are able to supply urine for analysis, depending on their renal function or disease state. When urine can be provided, it is subject to variations in composition and pH, which can have varying effects on any given class of biomarker. Uniquely, urine is also subject to contamination by the urinary microbiome, which can make interpretation of the source of particular analytes challenging.[22–25]

4.2.3 Ejaculate and prostatic secretions

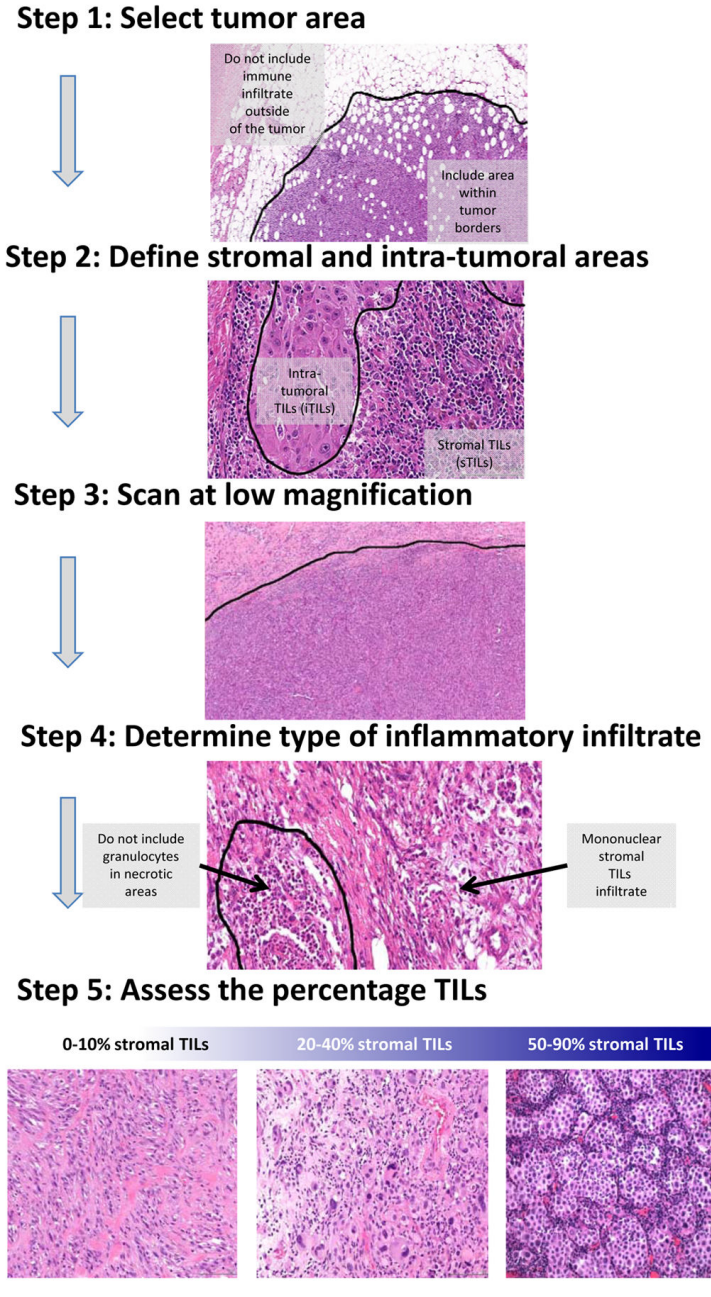
Of particular relevance to prostate cancer are prostatic biofluids, which capture analytes more effectively than other sources.[26] Of course, an intact prostate and ejaculatory pathway is required for procuring these specimens, which can somewhat limit the utility of such specimens. The post-prostatic massage urine is a proxy for capturing prostatic secretions, and so this particular biofluid is also subject to the constraints of urinary specimens noted above. There are also different social acceptability thresholds for semen and prostatic secretions, compared to other biofluids, making these secretions more procedurally intensive to collect. Recent efforts have shown the ability to collect RNA, DNA, proteins, and other molecular biomarkers from these biofluids.[26–30] Little data exists on storage considerations of prostatic secretions, although cryopreservation of seminal ejaculate is a standard practice in fertility scenarios.[2,27,30]

4.2.4 Tissue

Arguably, tissue is the most invasive specimen type to obtain, and using tissue has additional costs for procurement, processing, and storage. In the space of urologic oncology, though, tissue samples are often already obtained during routine clinical practice and may be used to identify biomarkers that guide treatment or provide prognostic information.[31,32] The full range of molecular biomarkers can be obtained from tissue samples, including more direct measurement of immune parameters at the tumour site (eg, tumour-infiltrating leukocytes), which influences both endogenous immune response to tumour as well as chemotherapy and immunotherapy efficacy.[33,34]

A major advantage of tissue specimens, compared to biofluid specimens, is the inherent ease with which one can optimize the signal-to-noise ratio in evaluating molecular biomarkers derived from tumours or tumour microenvironments. Depending on the biomarker of interest, a sample may be "enriched" prior to analysis to exclude normal tissue and prioritize the extraction of tumour tissue for analysis (eg, laser capture microdissection). Recently, efforts have been made to standardize the manner in which tissue samples for various types of tumours, including urologic tumours, are delineated from surrounding stroma on histopathologic analysis (**Figure 4–1**), with the intent of decreasing interobserver variability of certain biomarker assessments.[35]

FIGURE 4–1
A Representative Graphical Depiction of Tumour and Stroma Delineation During Measurement of Tumour-Infiltrating Lymphocytes, As Per the International Immuno-Oncology Biomarkers Working Group Guidelines[35]



Abbreviation: TIL, tumour-infiltrating lymphocyte.

Source: Reprinted with permission Wolters Kluwer Health, Inc., from: Hendry S, Salgado R, Gevaert T, et al. *Assessing tumor-infiltrating lymphocytes in solid tumors: a practical review for pathologists and proposal for a standardized method from the International Immuno-Oncology Biomarkers Working Group: Part 2: TILs in melanoma, gastrointestinal tract carcinomas, non-small cell lung carcinoma and mesothelioma, endometrial and ovarian carcinomas, squamous cell carcinoma of the head and neck, genitourinary carcinomas, and primary brain tumors.* Adv Anat Pathol. *2017;24(6):311–335. doi:10.1097/PAP.0000000000000161*

Similar to other sources of biomarkers, tissue-based biomarkers are subject to degradation and contamination. This is particularly true in fresh frozen tissue samples, where tissue will be subject to predictable ischemic changes in the *ex vivo* state, such as apoptosis and *in situ* coagulation until freezing occurs. The timeliness of such processing would affect the accuracy and quality of biomarker analysis across a range of analytes, including more sensitive proteins.[36]

Formalin-fixed, paraffin-embedded (FFPE) samples allow for storage of tissue at room temperature, and they increase the longevity of the specimen—regardless of storage temperature—due, in part, to the cross-linking of methylene bridges. However, depending on the analysis method for any given biomarker, residual paraffin (even after appropriate treatment) can contaminate the analysis of such a preserved sample.[36] There are trade-offs of additional processing considerations for FFPE samples obtained for clinical evaluation, and these may be associated with different potential contaminants or constraints in methodology for evaluation. For example, in the evaluation of DNA of FFPE samples, DNA fragmentation may occur to a greater extent than in fresh samples, and as a consequence, molecular assays and data-analysis approaches may require tailoring to account for specimen variability.[37,38] In the case of microRNA (miRNA) obtained from tissue, the expression of various miRNAs has even been shown to be affected by variations in fixation pH or solution (formalin ± ethanol).[39]

Despite the challenges associated with procuring, processing, storing, and interpreting the results of biomarker analysis from tissue samples, tissue samples remain an important component of biomarker discovery and validation.

4.3 Biomarkers by Type

4.3.1 Genomic biomarkers

The European Medicines Agency (EMA), in consort with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), has defined a genomic biomarker as "a measurable DNA and/or RNA characteristic that is an indicator of normal biologic processes, pathogenic processes, and/or response to therapeutic or other interventions."[40]

Factors affecting genomic biomarkers

In general, DNA and RNA are reliable biomarkers, though there are some commonly encountered situations in biospecimen collection that occur in routine clinical medicine that can affect nucleic acid quantity and quality and, hence, impact their accuracy as biomarkers. A few of these conditions are described here.

Prefixation time: Prefixation time is the duration of time between obtaining the biopsy or surgical specimen and its preservation. As the tissue samples removed are ischemic during this interval, several important biologic processes occur in the tissue that can affect nucleic acids. RNA, in particular, is susceptible to the effects of this "cold" (ie, <37 °C) ischemia. Changes that are seen during cold ischemia include increased expression (quantity)

of RNA molecules from hypoxia response genes (eg, hypoxia-inducible factor 1α [HIF-1α]); digestion and loss of RNA molecules with short half-lives; and broad RNA degradation and reduction in quality, starting at about 5 to 6 hours at room temperature.[41] In general, the shorter the time from patient to preservation (preservative or freezing), the better.

Formalin: Formalin fixation is a common method used to preserve biological tissue samples that have been obtained surgically or by biopsy. Formalin-fixed tissue samples are usually then paraffin-embedded to allow for the cutting of thin slices for histological examination. As FFPE samples are abundant and represent the standard method of clinical tissue preservation in most hospitals, FFPE samples are probably the second most important source of biomarker measurement, after blood. Formalin is an aqueous formaldehyde solution that is 37% by mass, usually buffered to pH 7, and mixed with 10% methanol as a stabilizer. Formalin has several effects on DNA that affect DNA quality, including DNA denaturation (breaks interstrand hydrogen bonds in AT-rich regions) and cross-linking with cytosine residues (creates artificial CT or GA mutations during polymerase chain reaction [PCR] reactions).[42] As a result of these and other effects on DNA, formalin induces artificial mutations at a rate of approximately 1 mutation per 500 base pairs (bp). RNA shares these formalin effects, but it is also affected by formalin in other ways, including electrophilic attacks of adenine residues, which affect the poly(A) tail of messenger RNA, impacting oligo(dT) primers, and methylol additions, which impede reverse transcription.[42,43] Factors that increase the formalin-induced artificial mutation rate include increasing formaldehyde concentration, increasing temperature, increasing duration of fixation, and decreasing pH.[42]

Tissue nucleases: Deoxyribonucleases (DNases) and ribonucleases (RNases) are tissue nucleases (enzymes) that digest DNA and RNA, respectively. RNA molecules are particularly susceptible to degradation by RNases, and for this reason, RNase inhibition is part of most RNA extraction protocols. DNase is felt to be an important contributor to DNA degradation in FFPE tissue samples.[44]

Storage conditions: The age of the FFPE sample and the storage temperature can have an impact on nucleic acid quality.[45] In general, storage at -20 °C is better than at room temperature, and a shorter storage duration is better.

DNA

DNA has many attributes that make it an excellent biomarker. First, DNA tends to be a very stable molecule—a biological requirement, as it directs the replication of all human cells—and is consequently affected less by environmental conditions than many other molecules. Second, many characteristics are measurable in DNA, including single-nucleotide variants (SNVs, formerly single-nucleotide polymorphisms [SNPs]), variability of short repeated segments (eg, microsatellites), epigenetic modifications (eg, methylation), haplotypes, deletion mutations, insertion mutations, copy number variations, and cytogenetic variations (eg, translocations, duplications, deletions, or inversions).

One important distinction with DNA is the difference between germline changes and somatic changes. Germline DNA is the complement of genes that an individual is born with and can pass on to future progeny. Generally, blood leukocytes are used as the source for germline DNA, but there are scenarios (eg, leukemia) where this is not ideal, and buccal swabs, saliva, or other normal tissue are used. Most evidence suggests that buccal swabs and saliva yield similar DNA quality to blood leukocytes, though quantity is usually less.[46,47] Germline DNA alterations can inform the presence of an inherited tumour syndrome (eg, von Hippel-Lindau disease), a susceptibility to exposures (eg, glutathione-S-transferase [GSTM1] null and *N*-acetyltransferase 2 [NAT2] slow acetylator increase the risk for bladder cancer), an ability to metabolize drugs, and a susceptibility to developing certain diseases or adverse events to treatment.

Somatic DNA refers to DNA collected from an affected tissue or organ, usually a tumour, and reflects a change that occurred in the DNA after conception. Somatic alterations are not passed on to children. Somatic alterations are useful for predicting responsiveness to treatment (eg, microsatellite instability and programmed death 1 ligand 1 [PD-L1] response), determining prognosis, and diagnosing the presence or absence of disease.

RNA

RNA is the transmitter of genetic information coded in the DNA and is therefore a significantly more dynamic molecule than DNA. RNA quantity and composition change significantly from tissue to tissue under normal physiologic conditions. Characteristics that are measured in RNA include sequences, splicing, expression levels, and subtype (eg, microRNA). As alluded to above, while RNA is a more responsive molecule and, perhaps, a better reflector of genetic activity within a particular tissue, it is also substantially less stable and is affected by a larger number of environmental conditions than DNA.

There are numerous types of RNA molecules and they are generally classified as the following: (a) those involved in protein synthesis; (b) those involved in RNA modification; and (c) those whose function is mainly regulatory.[48] A nonexhaustive summary of the main types of RNA is found in **Table 4–1**.

TABLE 4–1 Main Types of RNA

Protein synthesis	
Type	**Function**
Messenger (mRNA)	Transcription of the information contained in DNA exons (recipe for a protein) Subject to alternative splicing, which creates different protein isoforms
Ribosomal (rRNA)	Primary constituent of the ribosomes, where mRNA is translated into protein Most abundant RNA in cells (about 80%)
Transfer (tRNA)	Carries an amino acid matching the mRNA to the ribosome, required for translation
RNA modification	
Type	**Function**
Small nuclear (snRNA)	Processing and splicing of mRNA in the nuclear spliceosome
Small nucleolar (snoRNA)	Involved in methylation and pseudouridylation of rRNA and tRNA
Ribonuclease P (RNase P)	Riboenzyme (enzyme made of RNA) that cleaves RNA
Ribonuclease MRP (RNase MRP)	Riboenzyme that processes rRNA in the nucleus
Regulatory	
Type	**Function**
Micro (miRNA)	Single-stranded RNA, 22–bp length, interferes with other RNAs
Small interfering (siRNA)	Double-stranded RNA, 20–25 bp length, interferes with other RNAs
Long noncoding (lncRNA)	Single-stranded RNA, >200 bp length, interferes with other RNAs
Short hairpin (shRNA)	Artificial RNA molecule designed to inhibit other RNAs, has a tight hairpin turn structure
Antisense (asRNA)	Single-stranded RNA complementary to an mRNA to which it binds and inhibits

4.3.2 Protein

Proteins are the workhorses of the cell and are often highly dysregulated in diseased states. Proteins can be isolated from nearly all biofluids (eg, blood, urine, prostatic secretions) but, like all analytes, they are also subject to degradation and alteration. Human blood and urine contain proteases that cleave proteins into smaller peptides. These peptides, in turn, can be cleaved by peptidases into even smaller pieces.[49] Interestingly, the pattern of cleavage can be used as a signature to identify certain cancers.[50] Adding protease inhibitors to biospecimens can help reduce artifactual changes in proteins caused by enzymatic degradation, though these additions can also affect downstream applications.

Urine can be a particularly challenging source for protein biomarkers because of dramatic changes in pH (ranges from 4–8), the influence of hydration status on protein concentration, and proteolysis that occurs during storage in the bladder.[51] About 30% of urinary proteins are derived from glomerular filtration and 70% from the renal tubules and urothelium, so the urine protein pool is a mix of systemic and local-regional sources.[52]

Protein-based biomarkers have generally been focused on the quantification of a particular protein or isoform. However, assessment of post-translational modifications is also important. Examples of post-translational modifications that can be important to biomarkers include phosphorylation, methylation, glycosylation, ubiquitination, acetylation, and lipidation, among others.[53]

4.3.3 Glycans

The attachment of carbohydrates to molecules, such as proteins and lipids—a process known as glycosylation—is common and occurs in >50% of human proteins.[54] Several important glycoproteins have been found to be good biomarkers in urology, including α-fetoprotein, prostate-specific antigen, and human chorionic gonadotropin. There are different forms of protein glycosylation, including N-linked (glycan attached to the nitrogen of asparagine) and O-linked (glycan attached to the oxygen of threonine and serine). Tumours may show differences in the amount, size, and type of glycosylation when compared to normal tissue. For example, N-linked glycans tend to become larger and more branched, whereas O-linked glycans tend to be truncated and expose underlying peptide epitopes. Other glycans can be important biomarkers, too. For example, glycolipids (glycans bound to lipid molecules) and glycosaminoglycans (mucopolysaccharides) have been studied as biomarkers.

4.3.4 Lipids

Lipids are key molecules in cellular metabolism and are a critical structural component in the biological membranes that wrap all human cells. Lipids are different from other biomolecules in that they are soluble in organic solvents, which is an important processing step in lipid analysis and characterization.[55] Lipids are subdivided into eight classes, each of which has had some biological role described in cancer biology: fatty acyls, glycerophospholipids, glycerolipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides.[56] Mass spectroscopy and related techniques are the main tools used for profiling biological lipids.

4.3.5 Imaging

Perhaps nonintuitively, imaging also can serve as a biomarker.[57,58] Examples of widely available imaging-based biomarkers include basic radiological lesion characteristics (eg, size, shape, location), lesion density (computed tomography), lesion echogenicity (ultrasound), lesion signal intensity (magnetic resonance imaging), and contrast enhancement. The Response Evaluation Criteria In Solid Tumors (RECIST) criteria for evaluating tumour response to therapy is one example of a radiological biomarker that is commonly used in clinical trials.[59,60] More recently, functional molecular imaging has been developed, whereby specific molecular features are studied using novel radiological ligands. For example, in positron emission tomography (PET) imaging, a multitude of functional biomarkers are being explored to improve the detection of cancer, including, ^{18}F-fluorodeoxyglucose (^{18}F-FDG), carbon 11 choline (^{11}C-choline), ^{68}Gallium prostate-specific membrane antigen (^{68}Ga-PSMA), and numerous others. In other cases, theranostic imaging is being pursued where a molecular target is imaged in a patient *in vivo* prior to the administration of a targeted agent against that molecular target.[61] Magnetic resonance spectroscopy is yet another example of biomolecular imaging where tissue metabolism can be evaluated with imaging.[62]

4.3.6 Pathology

The histological evaluation of tissue samples (or blood smears) is not only a routine clinical component of cancer care but also an important source of clinical biomarkers. Many standard descriptors of tissue morphology can be quantified and used as biomarkers. Common examples in genitourinary oncology include tumour grade, presence of lymphovascular invasion, presence of mitoses, and histological tumour type and subtype, among many others. More recently, digital imaging has allowed for a new era of digital pathology, where pattern recognition and artificial intelligence software tools can be used to characterize tissue sections, with increasingly precise and reproducible methods.[63,64] It is highly likely that in the future, digital pathology tools will form the backbone for the analysis of most tissue sections.

4.4 Conclusion

Biomarkers can be obtained and characterized from a highly diverse set of biological sources of measurement. There is no clear optimal biomarker, and each has inherent strengths and flaws. The future will likely consist of a collation of large networks of biomarkers that are merged computationally to provide a consensus picture of the pathological process that is occurring in the patient. This will undoubtedly require new informatic tools and artificial intelligence in order to parse signals from noise but will probably also lead to a new era of precision medicine for the patient and the physician.

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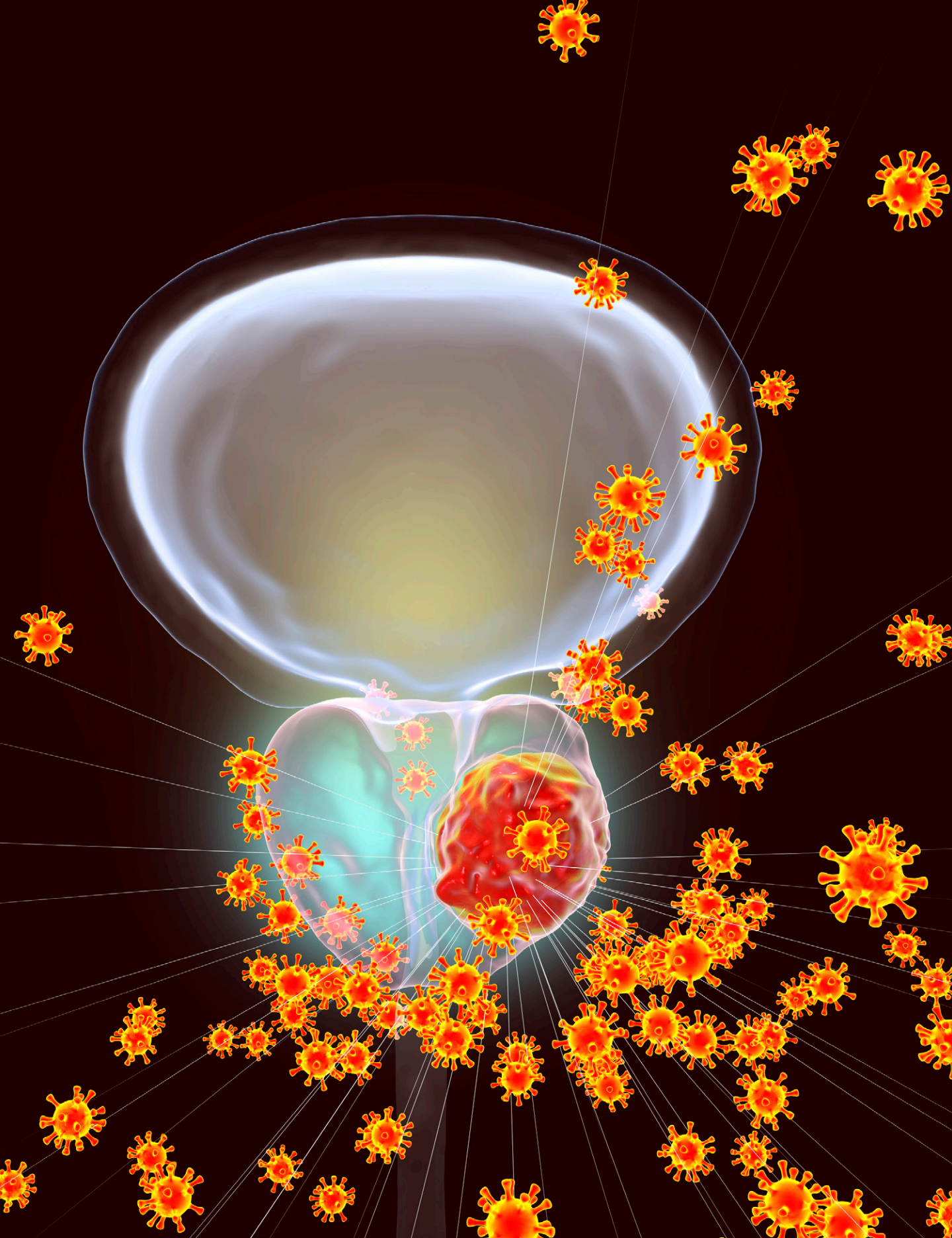

CHAPTER 5

Tissue-Based Biomarkers for Prostate Cancer

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Table of Contents

5.1 Introduction

Tissue-based biomarkers in prostate cancer is an exciting area of uro-oncology where much research and development is occurring. In the era of precision medicine, there is no better example than prostate cancer, which demonstrates the need for clinicians to tailor their management to every individual case. Tissue-based biomarkers provide us with an opportunity to try to predict the behaviour of subtypes of prostate cancer so that we can make an informed decision about the next step in treatment. These different biomarkers (**Table 5–1**) have the potential to allow clinicians to do this both at the stage of diagnosis and for prognosis.

TABLE 5–1 Biomarker Tests in Prostate Cancer

Clinical indication	Biomarker test	Stage of management	Description
Prediagnosis—considering repeat biopsy	ConfirmMDx[a,b]	Patients with negative first biopsy for consideration of repeat biopsy	Uses methylation-specific PCR to analyze status of epigenetic biomarkers: GSTP1, APC, RASSF1
	Prolaris[b,c]	Postbiopsy-confirmed, low to favourable intermediate-risk patients	Signature using 31 CCP genes standardized to form a CCP score from -3 to +3
	Oncotype Dx[a,b,c]	Postbiopsy-confirmed, low to favourable intermediate-risk patients	17-gene assay then converted to Genomic Prostate Score from 0 to 100
	ProMark[b,c]	Postbiopsy-confirmed, low to favourable intermediate-risk patients	Quantitative analysis of 12 proteins then converted to a score between 0 and 1
	Decipher (postbiopsy[b,c])	Postbiopsy-confirmed, low to favourable intermediate-risk patients	22-RNA marker panel used to produce a score from 0 to 1
Adjuvant vs salvage radiotherapy	Decipher (post–radical prostatectomy[b,c])	For patients post–radical prostatectomy who need risk stratification for biochemical recurrence	22-RNA marker panel used to produce a score from 0 to 1
Assess tumour aggressiveness	Ki-67	Has potential at biopsy stage or post–radical prostatectomy to predict cancer aggressiveness	Nuclear protein used as a proliferation marker that can be measured using immunohistochemistry
	PTEN	Has potential at biopsy stage or post–radical prostatectomy to predict cancer aggressiveness	A tumour suppressor gene located on chromosome 10
	TMPRSS2:ERG gene fusion	Has potential to predict cancer aggressiveness	Fusion of enzyme transmembrane protease (TMPRSS2) and ERG gene that can be detected in tissue or urine

Guidelines mentioning consideration of use: a, EAU; b, NCCN; c, ASCO.

Abbreviations: ASCO, American Society of Clinical Oncology; CCP, cell cycle progression; EAU, European Association of Urology; NCCN, National Comprehensive Cancer Network; PCR, polymerase chain reaction; PTEN, phosphatase and tensin homologue; TMPRSS2, transmembrane serine protease 2.

5.2 Prediagnosis—Considering Repeat Biopsy

5.2.1 ConfirmMDx

ConfirmMDx (MDx Health; Irvine, California, United States) uses methylation-specific polymerase chain reaction (PCR) to quantify in prostate tissue the methylation of known epigenetic markers of prostate cancer. This draws on the theory that epigenetic changes related to prostate cancer have a "field effect" and therefore will show changes in morphologically normal tissue (as seen by a pathologist under a microscope), if it is adjacent to significant prostate cancer. The assay was created to decide on the need for a repeat biopsy in patients with a previously negative biopsy. ConfirmMDx analyzes the methylation status of 3 epigenetic biomarkers: glutathione-S-transferase P1 (GSTP1), adenomatous polyposis coli (APC), and Ras association domain-containing protein 1 (RASSF1); all these markers have demonstrated promise as useful prostate cancer biomarkers.[1] ConfirmMDx uses formalin-fixed, paraffin-embedded (FFPE) prostate tissue from biopsy.

The multicentre DOCUMENT[2] trial from the United States and the MATLOC[3] study from Europe were the first validation studies of ConfirmMDx. A recent meta-analysis by Partin *et al.*[4] combined these two studies to form one set of 803 men. All men had a negative first core biopsy, of which all the cores were profiled using ConfirmMDx, then had a repeat biopsy within 30 months. It was found that ConfirmMDx had a negative predictive value of 89.2% (sensitivity and specificity of 64.8% and 63.8%, respectively). On multivariate analysis, high methylation intensity was found to be predictive of high-grade cancer (odds ratio [OR], 6.44; 95% CI, 2.57–16.13). Van Neste *et al.*[5] also combined the two study cohorts and found that DNA methylation was the most significant and best performing risk factor for identifying men with occult, high-grade prostate cancer, based on residual tissue of a prior biopsy negative for prostate cancer.

Clinically, Wojno *et al.*[6] showed that ConfirmMDx has the potential to decrease rates of repeat prostate biopsy by ten fold, and therefore concluded that a prospective, randomized, multicentre, clinical utility trial is needed to further analyze this.

Guidelines

Current European Association of Urology (EAU) guidelines state that more information can be gained through ConfirmMDx testing when considering re-biopsy after a previously negative biopsy.[7] But the guideline panel also mention that given the limited available data, no recommendation can be made regarding its routine application.[7] The National Comprehensive Cancer Network (NCCN) guidelines state that the use of ConfirmMDx should be considered in patients thought to be higher risk, despite a negative prostate biopsy.[8]

5.3 Active Surveillance Versus Treatment

5.3.1 Prolaris (Myriad Genetics Inc; Salt Lake City, Utah, United States)

A change in genes that control cell cycle progression (CCP) have been shown to have a significant impact on tumour cell biology.[9] These genes, called CCP genes, have been shown to have prognostic value in cancer such as breast cancer and now in prostate cancer.[10,11]

Cuzick *et al.* (2007)[11] used this idea to create a gene signature and score to predict aggressiveness of disease. This signature was developed through selecting 126 CCP genes and analyzing them on 96 commercially available prostate tissue samples. The result was a final signature consisting of 31 CCP genes thought to correlate with cell proliferation. A CCP score (from -3 to +3) was produced by normalizing the quantitative results with the results of 15 housekeeping genes. This signature was then retrospectively assessed in two groups: 366 patients who underwent radical prostatectomy, with the endpoint being biochemical recurrence (prostate-specific antigen [PSA] >0.3 ng/mL); and in 337 patients with clinically localized prostate cancer diagnosed through transurethral resection of the prostate (TURP), with the endpoint being biochemical recurrence and prostate cancer–specific mortality. It was found that on multivariate analysis, CCP score was a significant predictor of biochemical recurrence (hazard ratio [HR], 1.77; 95% CI, 1.40–2.22; $p<0.001$) in the radical prostatectomy cohort. It was also a significant predictor of prostate cancer–specific mortality (HR, 2.57; 95% CI, 1.93–3.43; $p<0.001$) in the TURP cohort, with a median follow-up of 9.8 years.[11] This ultimately indicated a more aggressive tumour profile.

The CCP score has since been validated by multiple groups.[12–15] Bishoff *et al.*[12] showed that in a 582-patient cohort from three different centres, CCP score was a strong predictor of biochemical recurrence (HR/score unit, 1.47; 95% CI, 1.23–1.76; $p=4.7 \times 10^{-5}$) on multivariate analysis. CCP score was also a strong predictor of metastatic disease (HR/score unit, 4.19; 95% CI, 2.08–8.45; $p=8.2 \times 10^{-6}$) in this study. Cuzick *et al.* (2015)[14] found that in 585 men diagnosed through prostate biopsy but managed conservatively, CCP score, combined with the widely used Cancer of the Prostate Risk Assessment (CAPRA) score, was highly predictive of prostate cancer death (HR, 2.17; 95% CI, 1.83– 2.57; $p=<10^{-20}$). Some 1,062 men who were definitively treated were also analyzed by Canter *et al.*[15] and again, CCP score was shown to be predictive of 10-year metastatic disease risk (HR/score unit, 2.21; 95% CI, 1.64–2.98; $p=1.9 \times 10^{-6}$).

The CCP score has also been shown to directly affect clinical decision-making. In an ongoing registry, Crawford *et al.*[16] found a reduction of invasive therapy recommendations of 37.2% after CCP testing led to a 49.5% reduction in surgical interventions and a 29.6% reduction in radiotherapy. Shore *et al.*[17] found that 32% of urologists indicated that the test results would definitely or potentially change treatment decisions. This shows the potential for CCP gene testing to impact on the pretreatment decision-making of clinicians.

Guidelines

Further evidence is required before the CCP score is put into routine use, likely due to study limitations inherent in all retrospective studies. Current EAU guidelines state that results of prospective multicentre trials need to be awaited before a final recommendation can be made.[7] In NCCN guidelines, the CCP score is mentioned as a potential tool in the postbiopsy setting in very low–risk and low-risk patients with prostate cancer who have a life expectancy of 10 years or more and are considering active surveillance as a preferred treatment option.[8] American Urological Association (AUA)/American Society for Radiation Oncology (ASTRO)/Society of Urologic Oncology (SUO) guidelines suggest that the CCP score has not yet been proven to have a substantial role in the selection of active surveillance candidates.[18] However, new American Society of Clinical Oncology (ASCO) guidelines state that CCP score may be offered in situations in which the assay result, when considered as a whole with routine clinical factors, is likely to affect management when identifying patients who would benefit from active surveillance.[19]

5.3.2 Oncotype Dx Genomic Prostate Score

The Oncotype Dx Genomic Prostate Score (GPS) (Genomic Health; Redwood City, California, United States) is a 17-gene assay (12 cancer-related genes and 5 reference genes) that has been used in breast cancer and colon cancer, and is now available for use in prostate cancer.[20] The technique used is a quantitative reverse-transcriptase PCR on prostate biopsy tissue. This is then converted to a score (0–100), which is used to predict adverse pathology, clinical recurrence, and prostate cancer.[21] It was designed to aid in the decision for active surveillance or active treatment in the low-risk to intermediate-risk prostate cancer group.[21,22] A strong point of this test is that only 5 ng of tumour RNA is required for valid test results.[23]

Oncotype Dx GPS was initially developed, then validated by Klein *et al.* in 2014[21] through a discovery prostatectomy study (n=441), a biopsy study (n=167), and a prospective, independent clinical validation study (n=395). All candidates were low to intermediate clinical risk who met the criteria for active surveillance, but decided to undergo radical prostatectomy. After first testing 732 candidate genes, the group chose 17 genes representing multiple biological pathways and combined them to make a GPS algorithm. This assay was run on the prospective group. GPS was shown to predict high grade (OR/20 GPS units, 2.3; 95% CI, 1.5–3.7; $p<0.001$) and high stage (OR/20 GPS units, 1.9; 95% CI, 1.3–3.0; $p=0.003$) on surgical pathology after adjusting for established clinical factors and CAPRA score.[21]

A recent prospective trial by Eggener *et al.*[25] enrolled 1,200 men with very low to favourable intermediate clinical risk cancer, 143 of whom elected for immediate radical prostatectomy. The authors confirmed, again, the ability of GPS to predict adverse pathology on prostatectomy specimen (OR/20 GPS units, 2.0; 95% CI, 1.1–3.7; $p=.024$). They also found that GPS decreased a decisional conflict scale score from 27 to 14 among their clinicians.

Conversely, Lin *et al.*[26] published a recent study analyzing the GPS of men at initial biopsy and its correlation with adverse pathology at surveillance biopsy or radical prostatectomy after a period of active surveillance. On univariable analysis, GPS was not found to be significantly associated with adverse pathology on radical prostatectomy; the only significant predictor was PSA density. On multivariable analysis adjusting for PSA

density and Gleason score, GPS also did not reach clinical significance (HR, 1.17; 95% CI, 1.00–1.43; p=0.066). GPS also did not show a significant association with upgrading of pathology at surveillance biopsy.

Cullen *et al.*[24] studied the value of GPS in prognostication. In a cohort of 402 men (20% African American), investigators found that GPS predicted time to biochemical recurrence post-prostatectomy (HR/20 GPS units, 2.7; p<0.001). Van Den Eeden *et al.*[27] looked at the predictive capability of GPS for prostate cancer death. They found that GPS was associated with time to prostate cancer death (HR/20 GPS units, 3.23; 95% CI, 1.84–5.65; p<0.001), but included low-, intermediate-, and high-risk patients in their study.

Guidelines

Guidelines by EAU and AUA state Oncotype Dx GPS is a potential tool that needs multicentre, prospective trial results before a final recommendation can be made.[7,18] The NCCN allows consideration of the use of Oncotype Dx post-biopsy for very low to favourable intermediate-risk patients with prostate cancer patients who have at least a 10-year life expectancy, who have not received treatment for prostate cancer, and who are candidates for active surveillance or definitive therapy.[8] ASCO guidelines state that Oncotype Dx GPS may be offered in situations in which the assay result, when considered as a whole with routine clinical factors, is likely to affect management when identifying patients who would benefit from active surveillance.[19]

5.3.3 ProMark

ProMark (Metamark; Cambridge, Massachusetts, United States) is a protein-based biomarker panel consisting of 12 proteins that are quantitatively analyzed through multiplex immunofluorescence assay of prostate tissue samples. It builds on the work of Shipitsin *et al.*,[28] who identified 12 biomarkers that predict prostate cancer aggressiveness. A risk score (0–1) is given based on the results of the assay.

A validation study by Blume-Jensen *et al.*[29] was performed on 381 patients with a refined 8-biomarker proteomic assay. Their endpoints were favourable pathology, defined as Gleason ≤3+4 and organ-confined disease (≤T2) and nonfavourable pathology, defined as Gleason ≥4+3 or non–organ-confined disease (T3a, T3b, N, or M). A second co-primary endpoint was also defined as a Gleason 6 or non-Gleason 6 pathology. A favourable risk score of ≤0.33 and a nonfavourable risk score of >0.8 was found. At a risk score of ≤0.33, the predictive value for favourable pathology in very low–risk and low-risk NCCN groups and low-risk D'Amico groups was 95%, 81.5%, and 87.2%, respectively, which was higher than the current risk classification groups themselves (80.3%, 63.8%, and 70.6%, respectively). At a risk score of >0.8, the predictive value for unfavourable pathology was 76.9% across all risk groups.

Guidelines

ProMark is mentioned as a potential tool in the postbiopsy setting in very low– and low-risk patients with prostate cancer who have a life expectancy of 10 years and more, and who are considering active surveillance as a preferred treatment option in NCCN guidelines, but ProMark is not mentioned in the EAU guidelines.[7,8] ASCO guidelines state that ProMark may be offered in situations in which the assay result, when considered as a whole with routine clinical factors, is likely to affect management when identifying patients who would benefit from active surveillance.[19]

5.3.4 Decipher in postprostate biopsy

The Decipher Genomic Classifier (GC) (Decipher Biosciences; San Diego, California, United States) is a 22-RNA marker panel that represents multiple biological pathways that are involved in aggressive prostate cancer, including cell proliferation, cell structure, immune system modulation, CCP, and androgen signalling.[30] It is scored from 0 to 1; a 0.1 increase in score represents a 10% risk in metastasis; a score of >0.6 is considered high risk for disease progression. The GC was first used on radical prostatectomy specimens from the Mayo Clinic, but the Decipher GC has also shown promise in predicting metastasis and prostate cancer–specific mortality (PCSM) from analysis of prostate biopsy tissue. Klein *et al.* (2016)[31] analyzed 57 prostate cancer biopsy specimens of patients who were treated with radical prostatectomy at the Cleveland Clinic. The study demonstrated that the GC remained a predictor of metastasis at 10 years after radical prostatectomy on multivariate analysis, when measured on prostate biopsy tissue (HR, 1.72/10% increase; 95% CI, 1.07–2.81; p=0.02). Decipher GC was also predictive of Gleason ≥4 pathology (area under the curve [AUC], 0.71; 95% CI, 0.56–0.86) and rapid metastasis (within 5 years) (AUC, 0.87; 95% CI, 0.76–0.97). When combined with NCCN risk groups, the GC increased the AUC for metastasis prediction from 0.72 to 0.88.

Nguyen *et al.*[32] studied the use of the Decipher GC on biopsy of 235 men treated with radical prostatectomy (n=105) or radiation therapy and androgen deprivation therapy (n=130). Investigators confirmed that the GC remained a predictor of metastasis (HR, 1.37/10% increase in score; 95% CI, 1.06–1.78, p=0.018), but also a predictor of PCSM (HR, 1.57/10% increase in score; 95% CI, 1.03–2.48, p=0.037).

Guidelines

The NCCN guidelines have also included the Decipher GC at the diagnosis stage. They believe that men with low or favourable intermediate disease may consider the use of the Decipher GC during initial risk stratification and also may be considered during workup for radical prostatectomy.[8] ASCO guidelines state that the Decipher GC may be offered in situations in which the assay result, when considered as a whole with routine clinical factors, is likely to affect management when identifying patients who would benefit from active surveillance.[19]

5.4 Adjuvant Versus Salvage Radiotherapy

5.4.1 Decipher in post–radical prostatectomy

To recap, the Decipher GC is a 22-RNA marker panel that represents multiple biological pathways that are involved in aggressive prostate cancer, including cell proliferation, cell structure, immune system modulation, CCP, and androgen signalling.[30] It is scored from 0 to 1; a 0.1 increase in score represents a 10% risk in metastasis; a score of >0.6 is considered high risk for disease progression. The GC was first used retrospectively on 545 radical prostatectomy specimens from the Mayo Clinic with a median follow-up of 16.9 years.[30] The patients with biochemical recurrence (n=192) were compared to a control cohort (n=353) who didn't have recurrence post-prostatectomy. GC had an AUC of 0.75 compared to clinical only, which had an AUC of 0.69 in predicting cases. When combined, the AUC was 0.74. On multivariate analysis, GC remained the only significant prognostic

variable for early metastasis (OR, 1.36; 95% CI, 1.16–1.60; $p<0.001$). This highlighted the potential for GC to improve the prediction of patients who would benefit from adjuvant radiotherapy and those who could be watched.

Currently, there is still uncertainty in offering patients adjuvant external beam radiation post–radical prostatectomy. Adjuvant external beam radiation was shown in three randomized control trials to be of benefit in progression-free and recurrence-free survival in patients with adverse pathology postsurgery;[33] however, other studies show that 50% of these patients were overtreated and would have remained cancer free.[34] This creates a niche for a biomarker to help determine which patients should go on to receive multimodal therapy for their prostate cancer. The Decipher GC shows promise in this regard.

A meta-analysis of five studies studied the performance of the Decipher GC on men post-prostatectomy.[35] The investigators showed on multivariate analysis that the GC remained a statistically significant predictor of metastasis (HR, 1.30; 95% CI, 1.14–1.47; $p<0.001$) per 0.1 unit. They concluded that it can independently improve prognostication of patients post-prostatectomy within nearly all clinicopathologic, demographic, and treatment subgroups.

A large prospective study has also been reported confirming the ability of the Decipher GC to predict aggressiveness of disease. Den *et al.*[36] reviewed 2,342 radical prostatectomy patients from different centres tested in 2015 and found that the GC had positive correlation with Gleason score and pathologic T stage. The main limitation here was lack of long-term follow-up.

Confidence has also been proven for the Decipher GC in clinical decision-making and patient anxiety. The prospective PRO-IMPACT study of 150 cases of patients considering adjuvant radiotherapy and 115 cases of patients considering salvage radiotherapy showed that decisional conflict score and prostate cancer–specific anxiety change after Decipher GC testing.[37] It found that the Decipher GC testing affected clinical decisions in offering both adjuvant and salvage radiotherapy (18% and 32% of cases, respectively).

Guidelines

Current NCCN guidelines state that the Decipher GC can be considered as part of counselling for risk stratification in patients with PSA resistance/recurrence after radical prostatectomy.[8] ASCO guidelines recommend the option of using the Decipher GC when considering adjuvant radiotherapy versus salvage radiotherapy in a post-prostatectomy setting.[19]

5.5 Molecular Pathology

5.5.1 Ki-67

Ki-67 is a nuclear protein that has been used as a proliferation marker of cancer. It is measured using the MIB-1 antibody in immunohistochemistry and is reported as the percentage of cells staining positively, which reflects the cell proliferation status. Theoretically, Ki-67 could be useful at multiple stages of clinical evaluation, at the biopsy stage, and post–radical prostatectomy.

At the biopsy stage, Tollefson *et al.*[38] showed that Ki-67 expression was associated with local or systemic progression, and with prostate cancer–specific death in a cohort of 451 men with a median follow-up for 12.9 years. Every 1% increase in Ki-67 expression resulted in a 12% increased risk of PCSM after radical prostatectomy. The investigators concluded that Ki-67 added predictive value to other existing prognostic algorithms and should be considered a standard, given its low cost and rapid evaluation time. Fisher *et al.*[39] confirmed that Ki-67 was a significant predictor of prostate cancer death (HR, 2.78; 95% CI, 1.42–5.46; p=0.008) in a cohort that was mostly treated with radical prostatectomy and had a mean 9.03 years of follow-up. However, the investigators did state that Ki-67 staining and interpretation was prone to variation among evaluators and future research needs to account for this variation.

Ki-67 evaluation in radical prostatectomy specimens have been studied as early as 1996 by Bettencourt *et al.*,[40] who found that men with higher Ki-67 levels had earlier progression and a lower 5-year, recurrence-free survival rate.

More recently, Tretiakova *et al.*[41] studied 1,004 prostate specimens from multiple institutions. They used Ki-67 as a continuous variable and found that it provided independent prognostic value for recurrence-free survival (per 1% increase; HR, 1.04; p=0.008), overall survival (per 1% increase; HR, 1.07; p=0.02), and disease-specific survival (per 1% increase; HR, 1.10; p=0.02). The investigators were unable to define an ideal cutoff point for Ki-67. However, this group did show that a cutoff point of >5%, which has been used empirically in many other studies, had an HR of 1.47, which was comparable to Gleason score (HR, 1.29–1.81), margin status (HR, 1.59), and log(PSA) increase by 1 unit (HR, 1.54), although slightly lower than seminal vesicle invasion (HR, 2.07). Further to this, Mathieu *et al.*[42] analyzed 3,123 patients post-prostatectomy and showed Ki-67 to be a strong predictor of biochemical recurrence (HR, 1.19; p=0.019), and could help with decision-making regarding adjuvant radiotherapy or optimization of follow-up scheduling.

Guidelines

Ki-67 remains one of the most widely studied biomarkers for prostate cancer and shows promise as a prognostic tool, but it is not yet recommended in NCCN guidelines and not mentioned in EAU guidelines.[7,8]

5.5.2 Phosphatase and tensin homologue

Phosphatase and tensin homologue (PTEN) is a tumour suppressor gene, located on chromosome 10, which is inactivated in many different tumours, including prostate cancer. It has been shown to be inactivated in up to two-thirds of prostate cancer cases[43] and shows promise as a prognostic marker for bad clinical outcomes. Florescence in situ hybridization is the gold standard assay to detect PTEN loss in tumour tissue, but recently, PTEN immunohistochemistry assay has been shown to have high specificity (91%) for 2 intact copies of the PTEN gene and high sensitivity (97%) for homozygous PTEN gene deletions.[44]

In a study by Murphy *et al.*,[45] PTEN loss was shown to be infrequent (2%) in clinically insignificant prostate cancer (defined as low-volume Gleason score 6), 13% in high-volume Gleason 6 prostate cancer, and 46% in Gleason ≥7 prostate cancers. Further to this, Lotan *et al.*[46] investigated whether PTEN loss could identify Gleason 6 cancer on biopsies that were upgraded after radical prostatectomy. They found PTEN loss in 18% of upgraded patients, compared to 7% of nonupgraded patients (p=0.02), concluding that PTEN loss could aid in Gleason score accuracy. As demonstrated by Guedes *et al.*,[47] in Gleason 3+4=7 disease, PTEN loss at biopsy was also shown to remain significantly associated with an increased risk for nonorgan-confined disease (HR, 2.46; 95% CI, 1.34–4.49; p=0.004).

PTEN loss has also been tested as a prognostic biomarker. In a study of 902 men with clinically localized prostate cancer who underwent radical prostatectomy,[48] men with markedly decreased PTEN staining had a higher risk for biochemical recurrence (OR, 1.67; 95% CI, 1.09–2.57; p=0.02). Murphy *et al.*[45] also showed that among Gleason score 7 or higher tumours, those with PTEN loss had a recurrence rate of 80%, compared to 55% in those with intact PTEN. Lahdensuo *et al.*[49] found PTEN loss increased risk for PCSM (HR, 2.156; 95% CI, 1.169–3.976; p=0.014) in a univariate analysis that compared those with a total loss of PTEN to those with partial or no loss of PTEN.

Guidelines

Like Ki-67, PTEN is not recommended in NCCN guidelines and not mentioned in EAU guidelines.[7,8]

5.5.3 TMPRSS2:ERG gene fusion

The fusion of the enzyme transmembrane protease serine 2 (TMPRSS2) and the *ERG* gene is a well-known phenomenon in prostate cancer that can be detected in up to 50% of cases. It has been demonstrated that this fusion has led to a decrease in 8-year overall survival (25% vs 90%; p<0.001) in a watchful-waiting cohort of 445 patients[50] and in a correlation with cancer-specific death rates (95% CI, 1.3–5.8; p<0.01).[51] However, controversy still surrounds its use, as the gene fusion status has not yielded any definitive results.

A meta-analysis of predictive significance by Song *et al.*[52] found that in a review of 76 articles between 2015 and 2017, the TMPRSS2:ERG gene fusion was associated with T stage and metastasis, but not with biochemical recurrence or PCSM. In a subsequent meta-analysis of 6,744 patients from 17 studies by Liu *et al.*,[53] the

investigators confirmed that there was no significant association between *ERG* rearrangement and cancer recurrence.

The gene fusion status can also be detected through urine. Leyten *et al.*[54] demonstrated that by adding TMPRSS2:ERG gene fusion, the prostate cancer antigen 3 (PCA3) test sensitivity rose from 68% to 76%; however, this could not be confirmed in validation studies.

5.6 Predictive Biomarkers

Predictive biomarkers play a role in men mainly with castrate-resistant prostate cancer. At present, in this cohort of men, there is no cure. However, these biomarkers help predict response of patients to certain drugs and therefore can aid clinicians in tailoring treatment to prolong life.

5.6.1 Androgen receptor splice variant 7

Advances have been made in recent years in understanding the mechanisms that drive advanced prostate cancer. One of these mechanisms studied is the androgen receptor. We now know that androgen receptor splice variants (AR-Vs), such as AR-V7, are associated with poorer outcomes and prognosis. Consequently, therapies have been targeted at androgen receptors, where drugs such as abiraterone and enzalutamide have been introduced into clinical practice, and have both shown improvements in survival benefits of patients. Despite this, most patients will still have disease progression. Therefore, it is imperative that we better understand that response of patients to these novel drugs to maximize their use.

The PROPHECY trial run by Armstrong *et al.*[55] was a multicentre prospective trial of circulating tumour cell AR-V7 detection in men receiving abiraterone or enzalutamide. The investigators enrolled 118 men with metastatic castrate-resistant prostate cancer (mCRPC) and found that men with AR-V7 had lower progression-free survival compared to AR-V7–negative patients. They validated AR-V7 as a predictive marker of short progression-free survival and overall survival. It has been pointed out that this trial did include patients with multiple poor prognosis factors and therefore should not be validated for "all-comers."[56]

5.6.2 DNA damage response genes

DNA damage response (DDR) is an integral pathway in the survival of both normal and malignant cells. The repair of damaged DNA allows the cell to remain viable for proliferation and function. Genes that are known to be involved in this pathway, such as BRCA1, BRCA2, ATM, and PALB2, have been shown to be mutated in prostate cancer, especially in advanced stages of disease. A systematic review of DDR gene mutations found that prevalence for DDR germline and/or somatic mutations was 27% in general prostate cancer and 22.67% in mCRPC.[57]

Poly (ADP-ribose) polymerase (PARP) inhibitors target the DDR pathway in malignant cells and make them more susceptible to cell death.[58] Recently, the PROfound group studied the effects of the PARP inhibitor olaparib

in men with mCRPC and known homologous recombination repair gene alterations.[59] Initial results show that olaparib performed well against enzalutamide/abiraterone. It showed a significant benefit for progression-free survival and superior overall response rate (OR, 20.86; $p<0.0001$) and time to pain progression (HR, 0.44; $p=0.0192$).

5.7 Future Directions

There remains an optimism and an urgency for the integration of biomarkers into routine clinical practice. As can be seen, biomarkers hold much potential in improving the accuracy of diagnosis and prognosis, and in predicting treatment response, not only as sole entities, but also through the merging of biomarkers with existing algorithms and new imaging modalities. Notwithstanding our exponentially increasing amount of knowledge and research, there is still a long way to go in understanding this complex disease.

As compared to new imaging modalities, namely, multiparametric magnetic resonance imaging (mpMRI), and prostate-specific membrane antigen positron emission tomography/computed tomography (PSMA PET/CT), tissue-based biomarkers have a somewhat slower rate of advancement. Currently, the evidence for their use is limited because most studies are retrospective in nature, and may not encompass a wide enough variety of prostate cancer patients or ethnicities. Multicentre, prospective, long-term trials are needed to study the true efficacy of the biomarkers. Cost-effectiveness and impact on clinical decision-making also need to be assessed before biomarkers are integrated into regular clinical practice.

There is new cutting-edge technology continually being developed or refined that will make a large impact on the clinical landscape. One such technology that is gaining momentum is next-generation sequencing (NGS). NGS allows for large-scale (millions to billions) analysis of DNA nucleotides simultaneously, which is a phenomenal improvement from the original Sanger's method developed in 1977. The potential benefits of NGS are its sensitivity, faster turnaround, and lower cost. Although a relatively new technology, NGS instruments have improved over the past 10 years.

In prostate cancer specifically, NGS has particularly shown promise in advanced phase of disease. Beltran *et al.*[60] showed that NGS could be used in detecting genomic alterations in formalin-fixed paraffin-embedded (FFPE) prostate cancer tissue. Building on this development, the Drug Rediscovery Protocol (DRUP) group from The Netherlands categorized more than 10 subtypes of prostate cancer based on genomic alterations.[61] Similarly, the Centre for Personalized Cancer Treatment (CPCT) group in The Netherlands is currently recruiting prostate cancer patients and obtaining tumour biopsies, which will be analyzed using NGS platforms.[62] The group's aim is to form a bank of tumour-related genetic mutations to develop a realistic view of the genomic complexity of prostate cancer. The results of study are eagerly awaited and is another step toward providing more targeted, personalized care.

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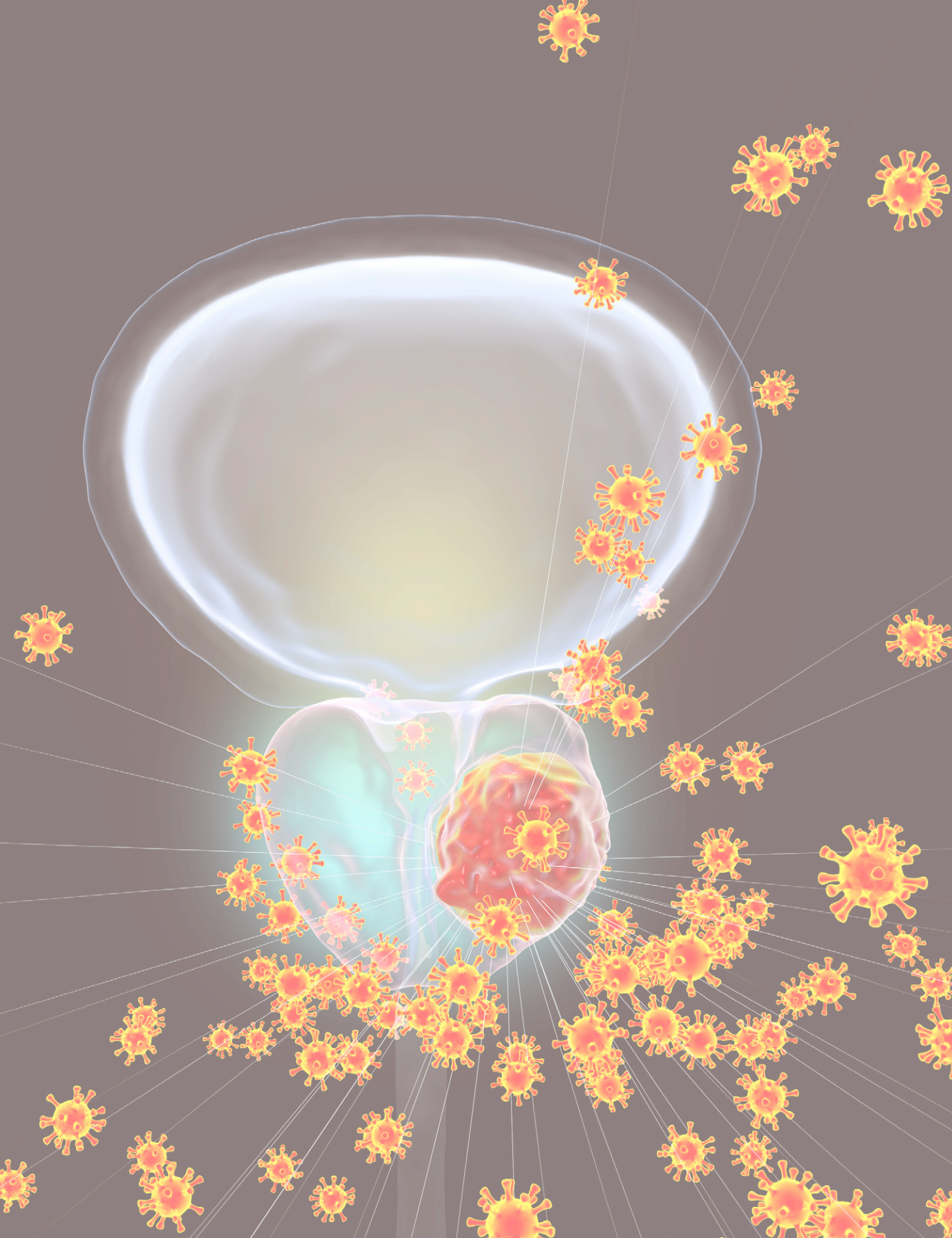

CHAPTER 6

Urine-Based Biomarkers for Prostate Cancer

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Table of Contents

6.1 Introduction: Urine As a Source of Prostate Cancer Biomarkers

Prostate cancers (PCa) are usually multifocal[1,2] and can be highly heterogeneous.[2,3] Currently the most thorough way to assess the volume, grade, and stage of prostate cancer is to examine an excised prostate. With the prostate *in situ* it's not so simple. Biopsy sampling is variable, with significant amounts of both upgrading and downgrading on prostatectomy analysis.[4,5] Multiparametric magnetic resonance imaging (mpMRI) has improved enormously in the past 10 years but still misses significant cancers (Gleason >4), has a high false-positive rate of around 50%,[6] and there are interoperator inconsistencies.[7] Is there a better way to sample the whole prostate without surgical removal? Is urine the answer?

The prostate is a secretory organ that lies just below the bladder. It makes prostatic fluid, which is a complex secretion product of epithelial cells. Prostatic secretions make up 30% of the volume of semen and their composition can reflect preneoplastic or malignant changes.[8] The prostate is continually secreting and these secretions come from all areas of the prostate where PCa is found.[9,10] These secretions flow into the urethra from where they are flushed out of the body on urination.[9] If a cancer is present, then tiny bits of tumour (cells, extracellular vesicles, and molecules) are also carried with the secretions and these can be detected in urine.[11,12] Prostate cancer cells in prostatic secretions and urine have been investigated for more than 70 years.[11–13] The advantages of a urine "liquid biopsy" are that urine can be collected at low cost, is completely noninvasive, and has the potential to sample all secretory areas of the prostate in one go. The prostate resides next to the rectal wall, and most urine studies have examined urine samples collected after a digital rectal examination (DRE), in which a finger is inserted into the rectum and the prostate is stroked on its posterior side to push prostatic secretions into the urethra. A DRE is used to boost the levels of prostatic secretions collected in the urine.[14,15] Prostatic secretions are washed out in the first 10 to 15 mL of urine, which can be collected for biomarker analysis.

Whole and fractionated urine samples have been analyzed for cells, DNA, RNA, proteins, and metabolites, which could act as cancer biomarkers. The current state and future directions of urine analysis for prostate cancer diagnosis and prognosis are described herein.

6.2 Analysis of Whole Unfractionated Urine

There have been a number of investigations examining the utility of biomarkers in whole unfractionated urine for PCa diagnosis. As early as 1979, Grayhack *et al.* found that C3, C4, and transferrin proteins were elevated in prostatic fluid from PCa patients[8] and in 1982 prostate cancer antigen-1 (PCA-1) protein was detected in urine from PCa patients but not in age-matched non-PCa men.[13] However, it wasn't until 2003, when levels of prostate cancer antigen-3 (PCA3) transcripts in urine were found to be linked to PCa, that the potential for urine molecular diagnostics in clinical urologic practice took off.[16]

6.2.1 RNA in whole urine

In 2012 the US Food and Drug Administration (FDA) approved the first PCa urine test called the Progensa PCA3 test, a post-DRE whole urine–based biomarker test for predicting the likelihood of a positive result for PCa on repeat biopsy.[17,18] PCA3[19] is a prostate-specific long noncoding RNA that is found to be overexpressed in more than 95% of prostate cancer tissues.[16] PCA3 was first investigated as a urinary PCa marker by de Kok *et al.* in 2002.[20] Then in a multicentre validation study[21] it was shown to predict Gleason score (Gs) ≥7 cancer with a negative predictive value of 80%[22] and had improved diagnostic use over prostate-specific antigen (PSA).[17] Investigations into a direct relationship between the PCA3 test and PCa volume or Gleason pattern have been unclear and have yielded opposing results in different studies.[20,23,24]

The PCA3 score is calculated as PCA3 mRNA/KLK3 mRNA x 1000.[17] A PCA3 score threshold of 35 delivered a sensitivity and specificity of 58% and 72%, respectively, for presence of significant cancer on rebiopsy.[25,26] Metanalysis by Luo *et al.* (2014)[27] suggested that reducing the cutoff to 20 could reduce the rate of false-negative results. Luo *et al.* also found heterogeneity among published data sets with PCA3 test sensitivity ranging from 47% to 82%, the reasons for which were unknown, and which may underlie the poor uptake of the PCA3 test in the clinic.

TMPRSS2:ERG is a fusion gene found in ~50% of prostate tumour foci; however, due to the multifocal and heterogeneous nature of PCa, tumour foci with and without a TMPRSS2:ERG can be found in individual prostates,[28] and it may be present in ~70% of PCa-radical prostatectomies,[29] making its detection more useful than was originally apparent. Young *et al.* found that urine transcript levels of TMPRSS2:ERG aided PCA3 in prediction of PCa and correlated with ERG expression in PCa tissue.[30] Tomlins *et al.* combined detection of PCA3 and TMPRSS2:ERG fusion transcripts with either serum PSA levels or the multivariate Prostate Cancer Prevention Trial risk calculator version 1.0 (PCPT-RC). This combined analysis they called Mi-Prostate score (MiPS).[31] When compared to PSA or PCPT-RC alone, MiPS had a significantly improved area under receiver operating curve (AUROC) for the detection of any PCa and Gs ≥7 on biopsy.

Further gene transcripts have been investigated for additional improvements and Van Neste *et al.* combined reverse transcription polymerase chain reaction (RT-PCR) data from HOXC4, HOXC6, TDRD1, DLX1, KLK3, and PCA3 with clinical information from two independent multicentre prospective collections (n=906).[32] The final model built by the authors did not require PCA3, TMPRSS2:ERG, age, family history of PCa, or PSA but included a combination of PSA density (PSAD), DRE result, HOXC6, and DLX1, using KLK3 as a reference for relative biomarker quantitation. The authors' analyses indicated a strong net benefit and potential for a further reduction in unnecessary biopsies over the PCA3 test, PSA, and the PCPT-RC, with a validation cohort AUROC of 0.9 for detection of Gs ≥7 cancer. This combination of markers has been developed as SelectMDX, which has been reported to be able to reduce diagnostic costs in a study covering five European countries, the degree of benefit varying with the amount of overtreatment in each country's clinical procedures.[33]

6.2.2 MS-based urinary proteomics for detection of PCa

Shotgun mass spectrometry (MS) is a useful tool that enables a global assessment of the proteome for disease-associated biomarkers. In most studies, MS candidate biomarkers are then verified by immune-based assays such as an enzyme-linked immunosorbent assay (ELISA) and Western blot.[34] The above methods have revealed a number of PCa protein biomarkers in urine, though as cohort numbers are frequently small and verification methodologies are variable, there is often little overlap between findings or subsequent validation publications.[35,36] However, some protein biomarkers have been confirmed in follow-up studies. Sequeiros *et al.* examined 64 proteins suggested from in-house proteomics investigations and the literature in urinary extracellular vesicles (EVs) in 107 patients (53 PCa patients; 54 patients with benign diseases, eg, BPH, HGPIN).[37] The significant differential abundance of 14 proteins between benign disease and PCa was confirmed. The best performance for PCa detection was a combination of transglutaminase-4 and adseverin (AUROC, 0.65). In agreement with urinary data, the proteins were found to have differential expression between PCa tissue (n=136) and benign samples (n=98), tested using immunohistochemistry (IHC).

MS proteomics has been applied to investigate naturally occurring urinary peptide metabolites as biomarkers to guide prostate biopsy[38] and identify clinically significant PCa (GS ≥7).[39] In a study by Frantzi *et al.* of 823 PCa patients with PSA <15 mg/mL, the investigators identified 19 biomarkers, including peptides from alpha-1 collagen of types (I), (XI), (XVII), and (XXI) and alpha-2 type (I), (V), and (IX), protein phosphatase 1 regulatory subunit 3A, chemokine (C-X3-C motif) ligand 1, and Semaphorin-7A.[39] These were integrated in a support vector machine model, which, upon independent validation in 280 PCa patients, resulted in an AUROC of 0.81, outperforming PSA (AUROC, 0.58) and the European Randomized Study of Screening for Prostate Cancer (ERSPC) risk calculator (AUROC, 0.69) for significant PCa.

Studies by Geisler and Jia have examined urine for the presence of protein biomarkers originally found in tissue. Geisler *et al.* investigated tissue proteomes by MS in men with and without biochemical relapse (BCR) up to 5 years post-surgery.[40] Tissue vinculin was found to be significantly upregulated in PCa tissue and this was also found in Western blot analysis of urine from PCa patients. The authors noted a trend for vinculin to be higher in urine from men with relapse (n=15) than in those without relapse (n=133), with a detection accuracy for BCR of 40% sensitivity and 61% specificity. Jia *et al.* examined the presence of PCa-detected N-linked glycopeptides in both urine and serum using MS.[41] Approximately 40% of the tissue glycopeptides were also detected in urine, while the overlap with serum was lower (13%).[41] A significant decrease of inactive tyrosine-protein kinase 7, ICOS ligand, zinc alpha-2 glycoprotein, fibrillin-1, and Golgi apparatus protein 1 was reported in urine from patients with high grade PCa (n=10).

MS has been used to tentatively reveal proteins in urine that may be of use in PCa diagnosis and prognosis. However, the majority of the studies have limitations, such as being underpowered, comparing advanced disease with healthy individuals (inappropriate targeted population), or having missed comparisons with current clinical standards of care. Further research will be required to develop this field to fruition.

6.3 Analysis of Urine Cell Sediment

6.3.1 PCa cells in urine

It has been estimated that up to 80% of the cells found in urine originated in the prostate.[42,43] Prostate cancer cells were first detected in urine samples by microscopy in 1947;[12] they are frequently present in large cell clusters[44] and most often are detected in urine from men with higher risk and advanced cancers.[42] Urine can contain many different cell types including bladder urothelial cells, squamous cells, seminal vesical cells, prostate cells, red blood cells, and white blood cells.[44] Multiple biomarkers are required therefore to fully distinguish cell types. The relative proportions of these different cell types in urine can vary post-DRE[42,45] and according to prostate disease state.[46] For example, high levels of white blood cells and bacteria are observed in men with prostatitis;[47] and sperm, bacteria, blood cells, kidney renal tubular cells, corpora amylacea, plus prostate cancer cells are seen in men with PCa or prostate/urinary tract problems.[44,48]

Several studies have investigated PCa cells in urine cell sediment using molecular or protein biomarkers. Staining for AMACR, Nkx3.1, and nucleolin in combination was able to enhance detection of prostate cancer cells in urine sediment but lacked sensitivity.[48] Detection of ERG overexpression in urine cells by immunocytochemistry was associated with raised PSA levels and advanced tumour grade and stage,[49] but again lacked sensitivity to detect all cancer cells. Nickens *et al.* used a multiplex set of four antibodies to detect ERG, AMACR, PSA, and prostein proteins in cells collected post-DRE; however, sensitivity and specificity for PCa were <70% compared with biopsy.[50]

Improved detection of PCa in post-DRE urine samples has been achieved with better probes and better harvesting of cells. Two fluorescent approaches have shown promise; OligoFISH® probes had an 80% specificity compared to biopsy data using a four chromosome (7, 16, 18 and 20) FISH panel for aneuploidy analysis.[43] Sensitivity and specificity of >98% to detect PCa in urine, compared to biopsy, was reported using a fluorescent peptide probe to detect VPAC receptors, which bind vasoactive intestinal peptide (VIP), a neuropeptide linked to development, growth, immune system, and cancer.[51]

6.3.2 Cell RNA

Measuring for the presence of TMPRSS2:ERG in urinary sediment has been shown to have a low sensitivity for PCa detection, 37%, compared to 62% for PCA3;[52] however, combining both markers increased sensitivity to 73%.[52,53] Leyten and colleagues (n=433, including 196 PCa) assessed urinary TMPRSS2:ERG and PCA3 in addition to the ERSPC risk calculator.[54] They noted a stepwise improvement in AUROC for detection of cancer from 0.79 (ERSPC alone) to 0.83 (EPSRC + PCA3) to 0.84 (ERSPC + PCA3 + TMPRSS2:ERG). They also reported that TMPRSS2:ERG added significant predictive value to the ERSPC calculator to predict biopsy Gleason, whereas PCA3 did not. In contrast, TMPRSS2:ERG provided negligible benefit when TMPRSS2:ERG and PCA3 were added to the PCPT-RC.[55] TMPRSS2:ERG has been reported to be less common in Chinese populations[56]

and detection of TTTY15:USP9Y gene fusion transcripts, which is found in 35% of Chinese men with PCa,[56] has improved PCa detection in urine sediments (*n*=226, AUROC 0.83).[57]

Combining additional markers with PCA3 has improved PCa detection in cell sediment, for example, combinations of: i) AMACR, TRPM8, and MSMB;[58] ii) TMPRSS2:ERG, GOLPH, and SPINK1,[59] and iii) HIST1H2B, SPP1, and ELF3.[60] In contrast, Leyten *et al.* found that PCA3 was unnecessary when HOXC6, DLX1, and TDRD1 were used for PCa detection,[61] TDRD1 being a direct target of ERG and coexpressed with ERG in PCa.[62] Other probe combinations without PCA3 include a panel of 6 genes overexpressed in PCa tissue (CCND1, LMTK2, FN1, GSTP1, HPN, and MYO6) that was used in the analysis of 156 urine sediments from patients with PCa (*n*=67), showing a sensitivity of 80.6% and specificity of 62.9% for PCa detection (AUROC, 0.80).[63]

6.3.3 Cell miRNA

microRNAs (miRNAs) are short (18–24 nucleotides) noncoding RNAs involved in regulating gene expression. miRNA dysregulation is frequently observed in cancer,[64] and miRNAs are relatively durable in biofluids as they are stabilized by RNA-binding proteins.

A number of diagnostically useful miRNAs detected in urine have been found: miR-21 expression is controlled by the androgen receptor (AR) and it has been implicated in progression to castration resistance,[65] increased cell motility, and apoptotic resistance.[66] miR-125b is also regulated by AR and overexpressed in PCa. It induces tumour growth by targeting proapoptotic genes such as Puma, BAK1,[67] and TP53.[68] miR-205 is a tumour-suppressor miRNA, with strong evidence connecting its loss to early stages of PCa development.[69] miR-205 can reduce tumour migration and invasion by targeting ERB3 and promote apoptosis by targeting BCL2 and MAPK.[69] Despite miR-205 being downregulated in PCa, it is a constituent of several miRNA urinary biomarker panels. AUROCs vary from 0.6 to 0.85 for detection of PCa using multiple combinations of miRNAs,[68,70] and AUROC is 0.74 for distinguishing low-risk from high-risk disease.[71]

The small size and low concentration of miRNAs in biofluids make them challenging to work with. As new technologies such as next-generation sequencing mature, it may become easier to overcome these difficulties.

6.3.4 Cell DNA methylation

Epigenetic alterations are defined as heritable changes in gene expression with no change to the DNA code. In cancer, DNA hypermethylation silences tumour suppressors and other important regulatory genes.[72] It is easily detectable by PCR and it occurs early in tumourigenesis, making it an ideal biomarker for early detection as well as disease progression and risk stratification of patients.[73,74]

Pioneering work in the detection of PCa and significant PCa (Gs ≥7) was performed by Cairns *et al.* in 2001, who showed that methylation of the glutathione S-transferase P1 (GSTP1) gene was detectable in urine of men with PCa (sensitivity, 27%).[75] GSTP1 is underexpressed due to hypermethylation in >90% of PCa,[75] a change that is relatively PCa specific, typically being overexpressed in most other cancers. For these reasons, it is a stalwart

of PCa-methylation analysis. Follow-up studies improved performance by examining gene panels that included APC, RARbeta, RASSF1A, PTGS2, and ABCB1, with aberrant methylation detectable in >85% of cases.[76] Notable examples include "epiCaPture", a 6-gene DNA methylation panel (GSTP1, SFRP2, IGFBP3, IGFBP7, APC, and PTGS2) that can detect 85% of aggressive PCa (Gs ≥8) with a 70% improvement in the specificity of PSA[77] and ProCUrE, a two-gene DNA methylation panel (HOXD3 and GSTP1) with a sensitivity of 57.1% and a specificity of 97% for significant PCa detection.[78]

Accurate assessment of disease state and prognosis is critical to timely treatment intervention. Zhao *et al.* established a 4-gene panel (APC, CRIP3, GSTP1, and HOXD8) with some ability to predict cancer progression in patients on active surveillance (odds ratio [OR], 2.559; 95% CI, 1.257–5.212) from post-DRE urine.[79] They subsequently incorporated microRNAs and reported that miR-24, miR-30c, and CRIP3 methylation could predict reclassification of patients on active surveillance (AS).[80]

There are currently no commercially available DNA methylation–based urine tests for PCa.[76] A number of factors centered around the lack of consensus on standard methodologies prevent clinical uptake.[81] Firstly, urine storage: methylated DNA is stable for up to 28 days in urine stored at -20 ºC/-80 ºC, but at room temperature, a preservative is required.[82] Secondly, assay sensitivity: most urine DNA methylation assays use bisulfite conversion of unmethylated cytosines into uracil, leaving hypermethylated cytosines preserved for downstream analysis. However, this process degrades >90% of the DNA,[83] which reduces detection sensitivity.[84] False-positive results can arise when unmethylated cytosines are not converted. A study assessing 12 different bisulfite kits discovered that recovery and conversion varied greatly between them.[85] PCR analysis of bisulfite-treated DNA can vary depending on DNA input and the storage of the less stable single-stranded bisulfite-converted DNA.[86] Thirdly, target choice: DNA hypermethylation typically occurs at multiple sites in CpG islands up to 2 kb in length. Thus, the choice of CpG sites to assess in a biomarker assay is of prime importance. Proximity to the transcription start site and transcription factor–binding motifs, and DNase hypersensitivity are all factors that can affect sensitivity and specificity.[74] Large, multicentre, standardized urine collections and clinical follow-up are needed to reduce the unknowns and bring PCa methylation biomarkers to fruition.

6.4 Urine Supernatant

6.4.1 RNA in urine extracellular vesicles

Large numbers of extracellular vesicles (EVs) can be found in urine.[14] EVs are produced by a wide range of mammalian cell types and were originally described as "cell dust" before their initial characterization as lipid-bound vesicles in 1983.[87] EVs function as intercellular messengers that can bind to and influence the phenotype of cells they come in contact with (see reviews for more detailed information on EV biogenesis and function).[88,89] Cancer cells also produce EVs, which can enhance vasculature,[90] increase metastasis,[91] influence the immune system,[92] and can contain PCa-specific mRNAs such as TMPRSS2:ERG fusion gene transcripts.[52] EVs contain lipids, RNA, DNA, and proteins, including membrane receptors.[88,93,94] The majority of EVs in first-catch adult

male urine originate from the prostate,[14,95] but EVs also come from the kidney,[96] bladder, and blood cells.[95] An advantage EVs have over urine cell sediment is that the cell transcriptome is likely to alter on becoming detached and coming in contact with urine.[97,98] EV membranes protect the nucleic acids contained therein, resisting RNase and DNase digestion.[96]

The majority of publications refer only to analysis of small numbers of gene transcripts in EVs, namely PCA3, ERG, TMPRSS2:ERG, and KLK3, which have been found to be useful in PCa diagnosis and detection of Gleason ≥4 cancer.[14,99–103] Additional genes with diagnostic potential have been also been found: AGR2 splice variants,[104] Birc5,[100] and decreased expression of CDH3, reflecting data from PCa tissue.[105] In contrast, Connell *et al.* used a NanoString panel of 167 gene probes, the majority of which were selected due to published evidence of overexpression in PCa tissue.[95] Analysis of expression data in 535 urine EV samples resulted in the construction of the Prostate Urine Risk (PUR) signatures from 39 gene probes. In contrast to all other urine analyses, instead of a single cancer signature, the investigators constructed four PUR signatures, which were built around samples categorized as non-cancer (PUR-1), plus the three D'Amico risk groups for cancer aggression, namely Low-risk (PUR-2), Intermediate-risk (PUR-3) and High-risk (PUR-4). Each sample could have representation from all four signatures and the sum of the four PUR signatures in each sample was "1". Connell *et al.* found that PUR-4 could predict the presence of Intermediate- or High-risk cancer on transrectal ultrasound (TRUS) biopsy (AUROC, 0.77).[95] On examination of an active surveillance cohort (n=87), PUR-4 had a significant association with time to disease progression (interquartile range hazard ratio [HR], 2.86) and could divide patients into two groups with rates of progression to treatment intervention of 10% and 60% up to 5 years after urine collection (HR, 8.23). A strong PUR-1 signature correlated with stability of low-grade disease, which did not progress in the 5-year follow-up. The PUR-2 and PUR-3 signatures had less utility but were hypothesized as integral to the creation of a clearer signature for higher-grade Gleason cancer detectable by PUR-4.

A few studies have attempted to compare PCa mRNA transcripts contained in both cell and EV urine fractions. Levels of prostatic transcripts appear to be higher in the EV fraction,[14,15,99] but may have better diagnostic utility in the cell sediment. Dijkstra *et al.* examined PCA3 and TMPRSS2:ERG in 30 men scheduled for biopsy and observed that the cell sediment had better diagnostic utility,[106] with a caveat that 10% of cell sediments were unusable due to the formation of crystals during centrifugation while none of the EV samples were lost. Hendriks *et al.* observed that KLK3, PCA3, and ERG expression was highest in whole urine, followed by EV, and lowest in the cell sediments.[15] The authors reported that PCA3 transcripts were expressed significantly higher in PCa patients compared to non-PCa in both the whole-urine and cell-sediment fractions but not in the EV fraction. Webb *et al.* compared RNA yields from cell sediment and EVs in 200 patients and found them to be highly variable, with no apparent correlation.[99] This observation suggests that examination of RNA biomarkers in whole urine could be obfuscated by the unknown relative contribution of transcripts from the different urine fractions and may suggest that independent analysis of the two separate fractions should be recommended.

6.4.2 CfDNA

Cell-free urine DNA (cfDNA) has been reported to be both present inside EVs and bound to the outside of EV membranes.[96] The source of the DNA has been hypothesized to be apoptotic cells[96] and mitochondria.[107]

cfDNA yields from EVs have been reported to be low (18 pg/mL urine);[108] however, cfDNA has been used in detection of methylated GSTP1 found in men with PCa, which was not present in urine from men with benign prostatic hyperplasia (BPH).[94] Casadio *et al.* used copy number analysis of c-Myc, BCAS1, and HER2 by qRTPCR to distinguish PCa from non-PCa, with an AUROC of 0.8, while copy number gains of AR, presence of TMPRSS2:ERG, loss of PTEN, and other chromosomal regions have been detected in a small cohort of castrate-resistant men (n=10).[109]

6.4.3 Supernatant proteins

Thousands of proteins encapsulated on and within EVs have been identified by mass spectrometry analysis of urine from PCa patients, with some such as integrin subunit alpha 3 (ITGA3) and integrin subunit beta 1 (ITGB1) being linked to metastasis.[110] For a thorough review see recent papers by Pang *et al.*[111] and Wu *et al.*[112]

Probably the most thoroughly investigated urine protein is engrailed-2 (EN2), a transcriptional repressor member of the homeobox family.[113] EN2 can be secreted from normal and PCa cells[114] , which is unusual for a transcription factor, and then can be internalized by other cells to effect transcriptional changes in, for example, stroma.[115] The main role of EN2 is to regulate neural development and embryonic axonal guidance. Rather than remaining silenced after birth, EN2 is re-expressed in a range of cancers including bladder and prostate cancer, where it may regulate androgen-receptor activity in androgen-sensitive prostate cancer cells.[116,117] The first formal clinical evaluation of EN2 as a diagnostic marker in urine compared 82 PCa patients and 102 control patient samples.[118] EN2 was identified in the urine of 66% of biopsy-proven PCa patients, some of whom had undetectable levels of serum PSA. This was in contrast to <15% positivity in control groups (some of whom would have been expected to harbour occult prostate cancer), giving a specificity for the test of 88.2% (AUROC, 0.8; p<0.001). Men with prostate cancer had a 10-fold higher level of EN2 in their urine versus non-cancer controls, indicating a high diagnostic potential for EN2.

The potential use of EN2 as a marker of "significant disease" was investigated in two studies that compared pre-prostatectomy urinary EN2 levels with tumour stage and volume in prostatectomy specimens.[119,120] Using an EN2 level >42 ng/mL, 88 men (70%) were found to be EN2 positive. A strong relationship was demonstrated with PCa volume (p=0.006; linear regression) but not prostate volume. Higher EN2 levels correlated with advancing tumour stage, for example, pT3a versus pT2b (p=0.027) and positive margins (p=0.008). In a second prospective study of 57 men that used tumour volume cutoff levels of 0.5 mL, 1.3 mL, and 2.5 mL to define "significant disease", markedly higher levels of urinary EN2 were seen for each level (p<0.0001).[120]

Urinary EN2 was also evaluated in men at high risk of developing prostate cancer participating in the IMPACT study (Identification of Men with a genetic predisposition to Prostate Cancer: Targeted). EN2 levels in urine collected prior to the diagnosis of cancer in 267 BRCA1/2 mutation carriers and 140 controls correlated with prostate cancer diagnosis (AUROC, 0.816; sensitivity, 66.7%; specificity, 89.3%).[121,122]

There have however been no large-scale EN2 trials due to the lack of a robust commercially available test for EN2 protein in urine, which may be due to the very high net charge of the protein causing attachment to some

plastic surfaces. Indeed, a recent study looking at commercially available ELISA kits for EN2 found no significant diagnostic value for urinary EN2 in prostate cancer patients.[123] Novel approaches are in development such as a graphene-based biosensor[124] and examining urine cfRNA EN2 transcripts.[95]

6.5 Multiplex Biomarker Analysis

In general, two experimental approaches are used in biomarker development: those with a few biologically relevant candidates such as the PCA3 test[19] and data-driven high-dimensionality experiments with thousands of candidates.[125] Recent evidence suggests the latter will be the future direction of PCa-urine analysis.[95,126] There is a unique challenge for statistical analyses when the number of variables far outweigh the number of observations, and overfit is all but guaranteed, making feature selection an important task. While selection of "useful" variables may seem trivial, it is unfortunately not the case, and must be carefully considered to avoid both discarding useful information and producing overoptimistic results.

Current best practice for feature selection would be to use a train/validation data set split,[127] but this can be undesirable due to the cost of data acquisition. Instead, resampling and simulation-based approaches can be used to produce robust internally validated predictive models that can be interrogated for clinical utility or updated in further data sets.[126,128] These methods can maximally use all available data and reduce overfitting, which can destine a model for failed external validation. A recent example of this is the development of a multimodal model for biopsy prediction by Connell *et al.* (2020), which combined urine cfRNA and DNA methylation data and used a bootstrap resampling approach to feature selection based on Random Forests.[126] Designed to learn nonlinear patterns, Random Forests also implement bootstrap aggregation, which can be used to generate out-of-bag predictions that are unbiased, and so can be used to internally validate models without the need for an external data set or refitting the model in future studies.[129,130] The reported "ExoMeth" Random Forest model was therefore inherently internally validated, and showed good clinical utility for ruling out a cancer finding in biopsy-naïve patients (AUROC, 0.91; 95% CI, 0.87–0.95) or for the detection of Gleason ≥3 + 4 disease (AUROC, 0.89; 95% CI, 0.84–0.93). "ExoMeth" will not require retraining to be applied to new data sets, bringing it one step closer to external validation and clinical realization.

A key shortfall of using TRUS biopsy Gleason data as the outcome variable is the small number of tumour cores taken and the underlying assumption that each Gleason pattern is discrete when, realistically, it is often composed of complex mixtures. Connell *et al.* considered this and binned patient biopsy results into categories; no cancer found on biopsy, majority Gleason = 3, and majority Gleason ≥4.[126] Significantly, these labels were then treated continuously, recognizing that disease proportions within labels are not discrete and allowing for more nuanced error metrics to be used. Whether this approach more accurately reflected the underlying PCa biology remains to be validated using template biopsy or prostatectomy data sets.

The Transparent Reporting of studies on prediction models for Individual Prognosis Or Diagnosis (TRIPOD) guidelines provide a good framework for the development of multiplex biomarker models and describe what is reasonably achievable with different scenarios for the development, internal validation, and external validation

of multivariable prognostic or diagnostic models.[128] The evidence-level hierarchy supplied within the guidelines is useful in exploring what is possible with the data available, or what can be possible with careful study design incorporating robust validation strategies such as external centres, or temporally distanced cohorts. Clear study design will greatly improve the likelihood of promising results being realized, and not destined for an interminable loop of repeated validation studies and evidence generation.

6.6 Urine Biomarkers and the DRE

A large problem with the use of urine in diagnosis is the inconsistency in the amounts of prostatic material between samples. The DRE is one source of this variation. When men turn up at a hospital, nerves would very often mean that they would urinate before seeing the physician and flush out all the prostatic secretions from the urethra. To replenish the prostatic biomarkers in the urethra, urine is collected after a digital rectal examination of the prostate in the clinic whereby the physician would stroke the prostate with a finger pushing prostate secretions into the urethra shortly before urination. However, Webb *et al.* found that urine cfRNA yields correlated with the clinician who performed the DRE, with median differences of more than 10 fold between clinicians, which were hypothesized as being linked to the clinician's DRE technique.[99]

A number of studies have confirmed that RNA yields from urine collected in the clinic without a DRE are less than a tenth of the samples collected with a DRE[14,99] and levels of prostate markers such as KLK3 were also much lower.[15] However, studies by Donovan *et al.* and McKiernan *et al.* using non-DRE urine found AUROCs of 0.8 and 0.77, respectively, for detection of Gs >7 using PCA3 and ERG combined with clinical parameters,[101,102,131] strongly suggesting that non-DRE urine could be of use. A more recent paper by Webb *et al.* took this one step further.[99] Their hypothesis centred around the finding by Huggins in 1945 that the prostate was constantly secreting.[9] Webb *et al.* therefore asked men to collect a urine sample at home from their first urination of the day and found that RNA yields were comparable to those collected post-DRE from the same patients a week earlier.[99] Significantly, detection of PCA3 and TMPRSS2:ERG by RT-PCR proved to be much more sensitive in these "morning" samples compared to the post-DRE samples. While this study was limited by the low number of samples (n=14), it does suggest that urine collections could be performed at home and sent to the lab for analysis by post without the man having to visit the hospital, which would make disease monitoring in, for example, active surveillance cohorts much cheaper and simpler. Webb *et al.* also suggested that consistency between samples could be further improved by collecting a second urine sample one hour later.[99] While median PCa transcript levels were not as high as the "morning" samples, they were still better than the post-DRE samples, and using a fixed collection time period may prove to add the extra level of consistency required to make urine biomarkers a viable option. Augustus *et al.* have investigated the best time points and fractions for collecting the greatest DNA yields for DNA-methylation analysis, and they determined that the second and third collections of the day yield more DNA than first-void morning urine.[132]

6.7 Conclusions

The secretory nature of the prostate, the prostate's internal luminal structure, and interconnectivity with the urinary system all make urine a valuable non-invasive source of prostate biomarkers. Urine has shown utility in predicting biopsy outcome and monitoring disease progression, and in the future could lead to the development of a PCa screening test. However, the translation of potential biomarkers from research into clinical practice is a slow process, with many factors to consider.[84] Biomarker sensitivity and specificity often vary greatly between studies, and it is difficult to inter-compare data from cohorts with different ranges of disease severity. In addition, the impact of many variables on urine biomarker performance is still unclear; these variables include tumour size and location, method of sample collection and storage, variability in biomarker extraction, and analysis. However, this is a developing area and the field is evolving toward multi-omics integration of biomarkers for increased diagnostic performance. We suggest that a strong collaboration with clinicians is necessary at every step of the development process to produce a successful urine biomarker that has impact in the clinic to improve patients' lives.

6.8 References

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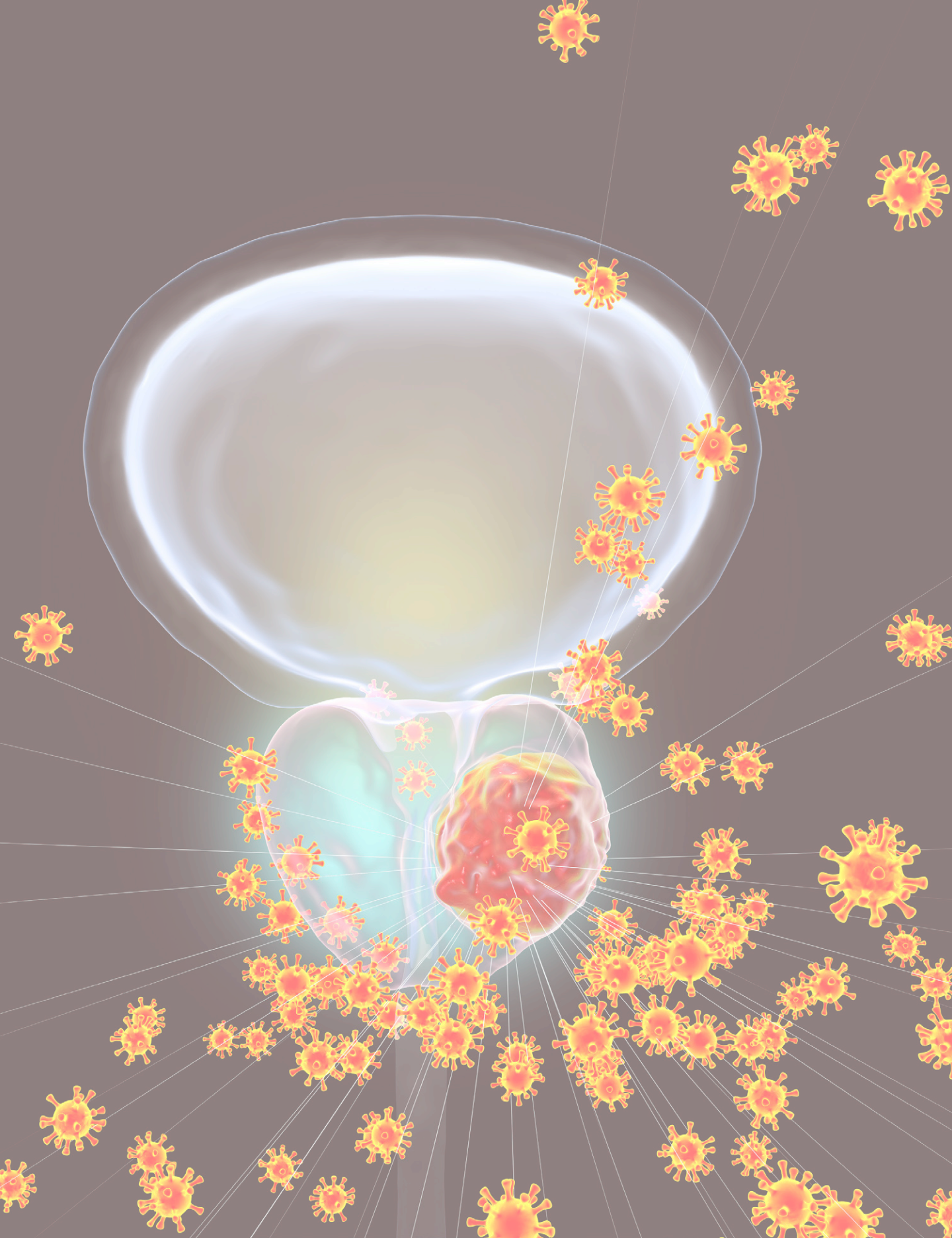

CHAPTER 7

Circulating Tumour DNA and Circulating Tumour Cells as Biomarker Sources in Prostate Cancer

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Table of Contents

7.1 Etiology of Cell-Free DNA

When cells die, genomic DNA can escape and diffuse into surrounding tissue. Consequently, cell-free DNA (cfDNA) is present in many bodily fluids. The most familiar fluid is peripheral blood, but cfDNA has been purified from urine, sputum, cerebrospinal fluid, and ascites. In individuals without known disease, the cfDNA in each fluid appears to be predominantly derived from cell types that comprise the immediate neighbourhood. Therefore, in plasma partitioned from peripheral whole blood, most cfDNA originates from cells of the hematopoietic lineage.[1,2] With conventional nucleic acid extraction methodology, blood from healthy individuals will yield about 5 ng of cfDNA (approximately 750 diploid genomes) per 1 mL of double-spun plasma.[3] Urine cfDNA (typically extracted from supernatant, rather than cell pellet or sediment DNA) is present in lower concentrations than plasma cfDNA, but this can be compensated by larger volume collections.

CfDNA that is purified from plasma has a periodic fragment pattern consistent with apoptotic processing, representing the intervals at which caspase-activated DNase cleaves DNA. As caspase-activated DNase preferentially cleaves unprotected DNA falling between chromatosomes, the most common cfDNA fragment length is 167 base pairs.[4] In urine, cfDNA is also highly fragmented, with some studies suggesting a periodic size even smaller than in plasma.[5,6] Notably, the presence of high molecular weight DNA purified from plasma can be indicative of a technical failure post-blood collection. For example, whole blood samples that were incorrectly stored prior to processing into plasma can contain large genomic DNA fragments originating from leukocyte lysis. For this reason, serum is not an alternative to plasma for cfDNA profiling.

Injury and disease can alter the etiology of cfDNA.[1,7,8] In people with cancer, genomes from tumour cells undergoing apoptosis can be shed into body fluids. These tumour-derived cfDNA fragments are termed circulating tumour DNA (ctDNA), and they can be detected against a backdrop of cfDNA from benign cells using assays to identify somatic alterations or epigenetic marks. As such, there is much excitement about the minimally invasive diagnostic potential of cfDNA analysis. The half-life of cfDNA is typically measured in hours but varies by the enzymatic activity in each body fluid.[9] Moreover, the kidneys, liver, and spleen all contribute to clearing cfDNA fragments in circulating blood. This rapid turnover means that detection of ctDNA in blood represents a real-time cancer biopsy.

7.2 Approaches for Cell-Free Circulating Tumour DNA Analysis

In people with cancer, the proportion of cfDNA that is tumour derived (ie, the ctDNA fraction) is highly variable.[10] This variability cannot be easily estimated prior to the profiling of purified cfDNA, and it represents the major technical challenge for ctDNA detection and characterization. Assays must therefore be highly sensitive to detect the possibility of rare ctDNA fragments, in the order of one in hundreds to thousands of normal cfDNA fragments.[11] Importantly, tumour fragments can be so rare in the bloodstream that sampling probability means that they may not be present in a few millilitres of peripheral blood.[12] As such, with modern approaches it is

often the collection volume and total number of cfDNA genomes present that imposes the theoretical limits of detection, rather than the technology itself.

Prostate cancer has a relatively low mutation rate and is driven in part by genomic structural rearrangements and copy number changes.[13] Optimal ctDNA assays for detection or characterization of prostate cancer must therefore be able to identify somatic alterations beyond just exonic mutations. The simplest type of assay that can detect somatic alterations in ctDNA is polymerase chain reaction (PCR) amplification of a few target regions. PCR-based approaches rely on either prior knowledge of tumour genotype (eg, from whole-exome sequencing of the matched primary tumour) or the plausible presence of recurrent hotspot mutations with clinical import. A well-known example is the *EGFR*-activating mutation L858R in non-small cell lung cancer, which can be detected with rapid and cheap PCR-based plasma cfDNA assays;[14] patients who test positive may benefit from tyrosine kinase inhibitors. Unfortunately, with the possible exceptions of the androgen receptor (*AR*) and *SPOP* genes, prostate cancer does not harbour highly recurrent hotspot mutations with obvious clinical relevance,[15,16] so broader approaches are required.

At present, most clinical research using ctDNA in prostate cancer has applied targeted next-generation sequencing approaches that capture a limited number of exons (and occasionally introns) for a set of known cancer-related genes.[17] Due to the lower cost, targeted sequencing assays are preferable to conventional whole-exome or -genome approaches. For tumour tissue–based analysis, sequencing coverage of 30X to 100X is adequate to characterize the somatic prostate cancer genome.[13,18] However, ideal sequencing depths for ctDNA are typically above 1000X and often considerably higher.[11] This can be expensive, and hence the selection of genes or regions for cfDNA sequencing is a delicate balance between cost, genome coverage, and desired detection sensitivity. Most commercial targeted sequencing assays contain several ubiquitous cancer genes that are relevant for prostate cancer, such as *TP53*, *MYC*, and *BRCA2*. However, although commercial assays are typically marketed as "pan-cancer", their designs are skewed toward mutation-driven cancers such as lung, skin, and colorectal. Important prostate cancer genes that are not always present in historical pan-cancer approaches include *SPOP*, *FOXA1*, and *CDK12*.[19] The selection of specific regions to sequence within each gene must also be carefully considered; inclusion of intronic and untranslated regions can enable identification of structural rearrangements and gene deletions. In prostate cancer, *PTEN*, *RB1*, *MSH2, FOXA1,* and *AR* are often disrupted by structural rearrangements affecting introns.[13,20–23] Some of these genes can also be perturbed by partial or entire locus deletions. From an assay design perspective, comprehensive detection of structural rearrangement breakpoints generally requires probes tiled across introns. As introns can span thousands of base pairs, their inclusion in targeted sequencing assays can significantly increase the cost of ctDNA profiling.

The ctDNA fraction of a sample determines the type of somatic alterations that can be detected. Advances in library preparation techniques (eg, duplex unique molecular identifiers) and bioinformatic approaches (eg, digital error suppression) mean that somatic mutations can be identified at ~0.1% frequency.[24,25] However, there are mathematical limits on the detection of copy number changes that cannot be simply overcome by technological improvements. For example, even the detection of entire chromosome arm level deletions requires that a cfDNA sample have a ctDNA fraction of at least 5% (an attribute that is rare outside of progressing metastatic disease).[26]

CtDNA purity must be even higher to enable detection of focal deletions. In prostate cancer, several focal copy number changes have clear clinical relevance (eg, deletions affecting *PTEN*, *MSH2*, *BRCA2*).[27] Therefore, cfDNA assays should report ctDNA fraction and discriminate between a true negative result (ie, tumour wild-type status) versus the inability to detect a change due to low tumour-DNA purity. End users must be aware that limits of detection vary by the type of alteration and even the gene in question.

In research settings, selected prostate cancer cfDNA samples have been subjected to whole-exome or -genome sequencing.[21,26,28,29] Whole-exome sequencing is generally only cost-justifiable in samples with above 20% to 40% ctDNA fraction, but in such scenarios can provide a sensitive snapshot of somatic mutations and copy number changes. Standard whole-genome sequencing of cfDNA is not feasible outside of bespoke analyses, but so-called low-pass whole-genome sequencing is a more cost-effective alternative that has shown promise for wide uptake.[30] With this method, the entire genome is sequenced at a shallow depth, normally less than 1X. Low-pass whole-genome sequencing can provide an estimate of ctDNA fraction (although not below 3–5%) and yields a low resolution genome-wide copy number profile. As no targeted capture step is required, the technique is cheap and quick to perform, and software packages for data analysis are publicly available.[30] However, low-pass whole-genome sequencing does not inform on somatic mutations, complex structural rearrangements, or focal copy number changes. Furthermore, the continual improvement of modular capture assays and targeted designs incorporating genome-wide targets means that it is possible to incorporate a backbone for whole-genome copy number profiling (eg, leveraging germline single nucleotide polymorphisms) into modern targeted sequencing assays.

Ideally, deep sequencing of plasma cfDNA is accompanied by similarly deep sequencing of matched leukocytes (ie, the buffy coat layer that forms during whole blood centrifugation). Leukocyte sequencing helps identify pathogenic germline alterations affecting genes such as *BRCA2* and *TP53*.[31] However, it also allows resolution of somatic mutations in cfDNA that are related to clonal hematopoiesis of indeterminate potential (CHIP) rather than cancer.[20,32] In an elderly population such as that with prostate cancer, somatic expansions in the hematopoietic lineage are common.[33] CHIP is linked with an increased risk for leukemia and also cardiac events or strokes,[34] and while CHIP-related outcomes in the context of cancer patients are unknown, they may be adverse.[35] Importantly, cells of the hematopoietic lineage comprise the bulk of normal cfDNA, so any somatic mutations associated with CHIP are simultaneously present in plasma cfDNA.[32] These mutations may be falsely interpreted as originating from cancer cells if matched leukocyte sequencing is not performed. In CHIP, most mutations fall in genes associated with hematopoietic cancers (eg, *DNMT3A*, *TET2*, *ASXL1*), but some prostate cancer genes such as *TP53* and *ATM* can be recurrently mutated.[36] Not all commercial ctDNA assays include leukocyte sequencing, and therefore cannot easily resolve somatic variants linked to CHIP.[19,37,38]

Ultimately, there is no single ctDNA testing approach that can inform on all possible scenarios in prostate cancer. As such, the choice of assay should be governed by the scientific or clinical question of each investigator.

7.3 Circulating Tumour Cells: Background and Clinical Impact

Circulating tumour cells (CTCs) are cancerous cells that have detached from a solid tumour and entered the bloodstream in a process known as intravasation. A tiny subset of CTCs seed new metastatic lesions in distant sites after extravasation.[39,40] CTCs are also presumed to facilitate "cross-seeding" between existing metastatic deposits.[41] In some rare contexts, CTCs cluster together forming circulating tumour microemboli, which can also include supporting non-tumour cells. As circulating tumour microemboli retain a semblance of the original tumour microenvironment, they may have increased metastasis-initiating capacity compared to single CTCs.[42,43] CTCs are rare in individuals diagnosed with prostate cancer. Only half of patients with confirmed metastatic disease have more than four CTCs per 7.5 mL of blood,[44] and this proportion is considerably lower in earlier-stage and localized disease. CTCs are undetectable in most localized prostate cancer patients 24 hours after surgery, indicating a short half-life that parallels ctDNA.[45] In breast cancer, the half-life of a single CTC is less than 3 hours.[46]

The major challenge of CTC analysis is to isolate the tumour cell signal from the denominator of billions of white and red blood cells. CTC isolation techniques fall into two broad categories. Firstly, use of cell surface markers that are relatively specific to cancer cells compared to leukocytes. The most recognized cell surface marker is epithelial cell adhesion molecule (EpCAM), which is expressed by most epithelial cells (but not endothelial cells or leukocytes). Prostate-specific membrane antigen is a promising disease-specific example.[47,48] It is also possible to enrich CTCs by using hematopoietic cell–specific markers to deplete leukocytes from the melange of blood-borne cells. The second broad CTC isolation approach is to use distinctive physical attributes, such as CTC size and density, typically through the use of microfluidic devices.[49]

CellSearch is the only Food and Drug Administration (FDA)-approved CTC isolation system. However, CellSearch relies on EpCAM expression, and therefore cannot capture CTCs that do not express the marker, such as cells that have undergone epithelial-to-mesenchymal transition. Regardless, enumeration of CTCs by the CellSearch system is linked to prostate cancer patient prognosis in the advanced-disease setting.[50–52] A decline in the number of detected CTCs may even be a surrogate biomarker for patient response to therapy in the castration-resistant setting, while an increase can predict poor outcomes.[44,53–58] However, CTC counts can be highly variable, and a large subset of patients are CTC negative by CellSearch, even in the metastatic setting. Despite the development of numerous CTC isolation techniques beyond CellSearch, including marker-independent methods and even apheresis,[59] to date no others have been validated for use in a clinical setting.[60]

7.4 Circulating Tumour DNA Abundance as a Prognostic Biomarker

Similar to CTC count, the abundance of ctDNA is a potentially clinically impactful variable, even without further characterization of prostate cancer molecular subtype. In metastatic castration-resistant prostate cancer

(mCRPC), plasma ctDNA abundance is associated with clinical measures of disease burden such as lactate dehydrogenase and prostate-specific antigen (PSA) levels, and the presence of visceral metastatic lesions.[26,29,61–63] Accordingly, high ctDNA fractions (as a proportion of total cfDNA) are associated with poor overall survival and short progression-free survival in mCRPC patients treated with standard of care.[26,61,64–66] The converse is also true, where low or undetectable ctDNA appears to be a marker of good prognosis.[26] Importantly, ctDNA fraction in mCRPC appears to provide independent prognostic information to standard clinical factors, suggesting that simple assays of ctDNA abundance could become part of prognostic models.[26,66]

As ctDNA abundance is closely related to the volume of proliferative disease, effective therapy has a rapid impact.[20,66,67] In metastatic castration-sensitive prostate cancer, one week of androgen deprivation therapy can reduce ctDNA fractions by ten-fold.[67] In mCRPC, declines in ctDNA are associated with PSA responses to abiraterone or enzalutamide, while patients with a rising ctDNA fraction while on treatment are at greater risk for progression.[29,68–70] Importantly, PSA can be unreliable as a response biomarker in late-stage disease due to reduced tumour reliance on AR signalling.[71] As such, the detection of changes in ctDNA fraction during treatment is a potential surrogate biomarker of response and should be explored in prospective biomarker trials.

To date, there are few ctDNA studies in localized prostate cancer. Detection of cancer-associated mutations in cfDNA (and therefore, detection of ctDNA) has offered opportunities for early diagnosis in other cancers.[25] For example, in urothelial carcinoma, detection of *TERT* promoter or *FGFR3* hotspot mutations in urine cfDNA may be an alternative to cytology for diagnosis.[72,73] Similarly, in bladder cancer patients undergoing surveillance, detection of *FGFR3* and *PIK3CA* hotspot mutations in plasma or urine cfDNA may be indicative of disease progression.[74] However, in localized prostate cancer, the availability of serum PSA screening (and the continued problem of overdiagnosis) has minimized the demand for new tools that simply detect early-stage cancer. Regardless, plasma ctDNA fragments appear to be extremely rare in patients with localized prostate cancer.[75] It is likely that plasma is suboptimal for cell-free tumour-derived nucleic acid analysis in this setting, especially compared to urine. Urine-based detection of *TMPRSS2-ERG* fusion transcripts and the long noncoding RNA *PCA3* has shown considerable promise for augmenting standard PSA-based screening.[76,77] In other cancers, persistent detection of plasma or urine ctDNA after curative therapy can indicate the presence of residual disease and therefore predict clinical recurrence.[78–81] However, the high sensitivity of PSA testing to infer residual disease after radical prostatectomy or radiation therapy also means that ctDNA-detection assays are less warranted in localized prostate cancer compared to some other malignancies.

It is of critical importance to recognize the prognostic impact of CTC and ctDNA abundance when considering the relevance of molecular alterations detected in liquid biopsies. For example, detection of the truncated *AR* splice variant *ARv7* in CTCs is associated with AR-targeted therapy resistance in mCRPC.[82,83] As there appears to be no association between *ARv7* detection and response to taxane-based chemotherapy, *ARv7* is a potential predictive biomarker.[84,85] However, the probability of detecting *ARv7*-positive CTCs is related to the number of tested CTCs, meaning that the theoretical utility of *ARv7* as a predictive biomarker is considerably diminished when controlling for CTC count.[86] Similarly, it is a constant challenge to resolve the biomarker potential of individual genomic alterations detected in ctDNA from the prognostic weight conferred by the mere presence of ctDNA itself.

7.5 Relationship of Circulating Tumour DNA and Circulating Tumour Cells to Tumour Tissue Biopsy

Tissue is the gold standard for derivation of tumour molecular features. As such, results from liquid biopsies are typically expected to align with those from tissue-based analyses. The rapid uptake in cfDNA sequencing was facilitated by data that suggested mutation and copy number profiles from plasma ctDNA were similar to those obtained via invasive biopsy of metastatic lesions. In an early study of 45 patients with mCRPC, deep targeted sequencing of same-day metastatic tissue biopsies and plasma cfDNA collections demonstrated high concordance for typical prostate cancer driver gene alterations such as *TP53* mutation, *AR* amplification, *SPOP* mutation, and *PTEN* deletion.[87] In a parallel study, copy number profiles were highly concordant when applying low-pass whole-genome sequencing to mCRPC patient-matched tissue and ctDNA.[88] More recently, high tissue-ctDNA concordance for driver gene alterations has been reported in patients at diagnosis with *de novo* metastatic castrate-sensitive prostate cancer,[67] and even among genomically or pathologically distinct patient subsets such as those with somatic mismatch repair defects or neuroendocrine features.[21,28] Collectively, the similarity between patient-matched tissue and ctDNA is consistent with the findings from rapid-autopsy studies where the vast majority of truncal driver alterations were conserved across metastatic sites.[41,89] Nevertheless, subclonal or late-arising alterations associated with acquired treatment resistance (eg, *AR* amplification or mutation), and neutral passenger mutations, are likely to vary between metastatic lesions and therefore between a single biopsy site and total ctDNA. It is important to note that, while analysis of ctDNA may theoretically capture intra-patient heterogeneity (ie, via sequencing of DNA shed from more than one tumour site), in practice it is challenging to resolve distinct populations from bulk ctDNA.

In theory, genomic analysis of multiple individual CTCs prevents potential "homogenization" of intra-patient mCRPC heterogeneity. However, comparison of CTCs to patient-matched tissue has been complicated by low CTC counts in mCRPC and the noise inherent when amplifying DNA from single cells. Several studies have overcome the latter challenge by deriving low-resolution whole-genome copy number profiles from individual cells.[59,90,91] By examining large tracts of chromosomal DNA, technical noise can be reduced, and it is possible to derive robust prostate cancer copy number profiles that appear similar to those obtained via tumour tissue.[91] Somatic mutations can also be identified, although technical false positives are to be expected when amplifying a single nuclear genome.[90,92,93] In general, concordance between tissue and CTCs is reasonable for truncal alterations (those that are shared across the majority of CTCs and/or tissue foci sampled), suggesting that CTCs can be a tumour tissue surrogate.[92,94] Interrogation of biomarkers such as *SLFN11* mRNA expression in platinum-resistant prostate cancer, and *PTEN* deletion in mCRPC, supports the concept of CTCs as a tissue proxy, as both these candidate biomarkers have shown high consistency between patient-matched tissue and CTCs.[94,95] Indeed, the ability to inform upon non-genomic features (such as transcriptional output) is a key potential advantage of CTCs over ctDNA analysis.

Ultimately, somatic profiles derived from liquid biopsies will never be identical to those from tissue biopsy. This is partly due to biological variability, such as intervening lines of therapy between a diagnostic tissue sample and a late-stage blood collection, or differential rates of ctDNA/CTC shedding between metastatic lesions. Indeed,

there is some evidence to suggest that the site of metastasis influences availability of ctDNA in blood collections, as brain lesions appear particularly difficult to sample for plasma cfDNA.[96] However, technical issues can also negatively influence concordance,[97] as can incidental detection of other somatic clones such as those related to clonal hematopoiesis or subclinical cancers.[35]

7.6 DNA Damage Repair Defects as Prognostic and Predictive Biomarkers

DNA damage repair defects are common in prostate cancer, particularly in metastatic disease.[98,99] In general, DNA repair defects are linked to poor prognosis across the disease spectrum,[100] but their precise prognostic relevance is contingent on a number of factors. For example, there are several distinct DNA repair pathways and hundreds of individual genes with different degrees of involvement. Alterations in each pathway and even gene can have drastically different downstream genomic and clinical effects, in addition to the specific class of alteration observed.

The most commonly affected DNA repair gene in prostate cancer is *BRCA2*, which is altered at the germline and/or somatic level in ~10% of metastatic patients.[13,101,102] Biallelic *BRCA2* defects result in a compromised ability to repair double-strand DNA breaks and reliance on alternative repair pathways that are more error-prone in this context.[103] These errors mount across the genome, meaning that across cancer types, *BRCA1/2* defects are associated with a distinctive mutational "scarring" pattern that can be identified via whole-genome or -exome sequencing.[104–106] Typically, only biallelic *BRCA2* loss is linked to genomic scarring.[103,107] As monoallelic *BRCA2* deletion is common in prostate cancer, allelic status is an important variable to capture when searching for pathogenic DNA repair defects. In mCRPC with high ctDNA fractions, loss of heterozygosity across deleterious germline *BRCA2* mutations is evident in ctDNA, suggesting that cfDNA sequencing could help identify patients with functional *BRCA2* loss.[108] It is plausible that broad cfDNA sequencing will also be able to identify genomic signatures of defective homologous recombination repair, as has been demonstrated by tumour tissue sequencing.[107,109] However, copy number profiles and loss of heterozygosity metrics are integral to current models of "BRCAness", so such an approach would currently be appropriate only in patients with sufficiently high ctDNA fractions.

Prospective and retrospective studies have suggested that *BRCA2* defects detected via leukocyte and cfDNA sequencing are linked to poor mCRPC patient outcomes in the context of AR-targeted therapy.[26,66,108,110] An association between plasma ctDNA DNA repair defects and poor outcomes has also been observed in metastatic castration-sensitive disease.[66] However, these associations appear variable across patient cohorts and were not confirmed in some retrospective studies using tumour tissue.[111,112] Regardless, mCRPC with biallelic *BRCA2* defects is vulnerable to therapies that exploit defective homologous recombination repair, such as platinum-based chemotherapy or poly (ADP-ribose) polymerase (PARP) inhibitors.[113–115] In recent clinical trials of PARP inhibitors in mCRPC with DNA repair defects, benefit is most overt in the subset with *BRCA2* mutations or deep deletions.[58,116,117]

BRCA2 is not the only DNA repair gene that is recurrently altered in prostate cancer. Other established homologous recombination repair genes such as *BRCA1*, *PALB2*, and *RAD51* are mutated or deleted at frequencies below 1%.[27,101,118] In other cancers, loss of these genes is associated with genome-wide signatures of defective homologous recombination repair,[105] so it is plausible that affected mCRPC tumours are vulnerable to PARP inhibitors. However, to date no clinical trials have been powered to address this question. Conversely, *ATM* and *CDK12* mutations are in aggregate as prevalent as *BRCA2* defects in mCRPC, but their association with homologous recombination repair is tenuous and the implications for patient prognosis and PARP inhibitor response appear to be quite different. Studies using cfDNA and/or tumour tissue sequencing have demonstrated that *CDK12* mutations are linked to a distinct tandem duplicator phenotype and poor clinical prognosis with standard of care treatments.[119–123] In mCRPC patients harbouring *CDK12*-mutant tumours, PARP inhibitor response rates have been disappointing regardless of patient selection via liquid or tissue biopsy. There is some suggestion that the frequent tandem duplications in *CDK12*-mutant tumours result in an elevated neoantigen burden and sensitivity to immune checkpoint blockade, but this hypothesis is untested in clinical trials.[121,122] Conversely, *ATM* mutations have not been linked to a genomic phenotype. The general prognosis of mCRPC with *ATM* mutations is unclear, but similar to *CDK12*, response rates to PARP inhibitors also appear to be reduced in comparison to *BRCA2*.[117] Preclinical work suggests *ATM* loss may sensitize prostate tumours to ATR inhibitors.[124] Currently, prospective plasma ctDNA sequencing is under evaluation in several phase 2/3 clinical trials of PARP inhibitors in mCRPC, and upon regulatory approval is likely to be key for patient biomarker screening. The largest hurdle to be overcome for reliance on plasma ctDNA screening is the detection of *BRCA2* biallelic deletions, which (unlike *ATM* and *CDK12*) are recurrent in mCRPC. Patients with somatic biallelic deletion of *BRCA2* can have exceptional responses to platinum chemotherapy or PARP inhibitors, and they are not at risk of developing resistance through reversion mutations;[125] screening approaches leveraging tissue or mutation signature analyses may be required to capture these cases.

DNA mismatch repair defects are present in 3% to 5% of mCRPC,[21,27] but are less common in the context of localized disease.[126] In prostate cancer, *MSH2* and *MSH6* alterations predominate and can take the form of complex structural rearrangements, thus complicating detection strategies.[127] Similar to homologous recombination repair–deficient tumours, those with mismatch repair deficiency (MMRd) display distinctive mutational signatures, including hypermutation (C>T transitions, particularly in the NCG trinucleotide context) and microsatellite instability. MMRd signatures can be detected in plasma ctDNA from patients with mCRPC.[20,21,128] Although high tumour mutational burden is not exclusive to MMRd etiology, in prostate cancer there are no other common causes of hypermutation, and assays that merely assess tumour mutational burden in ctDNA can be used.[21] Targeted sequencing panels that cover ~1.1 Mb of the coding genome (ie, most commercial approaches) are equivalent to whole-exome sequencing for tumour mutational burden estimation.[129,130] Patients with MMRd mCRPC may respond to immune checkpoint inhibitors,[131] so there is a need to test ctDNA as a prospective biomarker.

A key strength of liquid biopsy is the opportunity for frequent collections. In the context of DNA repair defects and PARP inhibitors, this is relevant for identifying and detecting resistance mechanisms and predicting responses to further lines of therapies aimed at DNA repair–defective cancers (eg, platinum chemotherapy[132]).

BRCA2 reversion mutations can be detected in plasma ctDNA at clinical progression on platinum chemotherapy or PARP inhibitors.[19,69,133–137] Importantly, plasma cfDNA sequencing identifies a greater diversity of *BRCA2* reversion mutations than biopsy of a single metastatic site.[133] It is plausible that regular plasma cfDNA screening could detect emergence of *BRCA2* reversion mutations prior to clinical progression, offering opportunities for earlier interventions.

7.7 *AR* Mutations, Amplifications, and Genomic Structural Rearrangements

Missense mutations in the *AR* ligand-binding domain can alter ligand affinity and drive therapy resistance and/or indicate potential vulnerabilities. Overall, *AR* mutations are found in approximately 10% of ctDNA-positive mCRPC, but only a few point mutations are widely recurrent, principally L702H, W742L/C, H875Y, and T878A.[26,61,62,68,138,139] As exonic point mutations are straightforward to detect via plasma cfDNA sequencing, the biomarker implications of *AR* missense mutations are relatively well understood. *AR* W742L/C mutations are a resistance mechanism to bicalutamide and are frequently identified in the plasma ctDNA of patients who have been exposed to this drug. Next-generation AR-targeted therapies such as enzalutamide and abiraterone have activity against *AR* W742L/C, meaning that detection of this mutation via liquid biopsy may predict durable responses to such agents.[26,68] *AR* T878A and L702H tend to arise after later lines of therapy, permitting agonism of the AR by progesterones and glucocorticoids, respectively.[140,141] While detection of these alterations in plasma cfDNA is linked to poor outcomes, switching to different AR-targeted therapies or steroid regimens may be effective in some scenarios.[142] Overall, after controlling for ctDNA fraction, the detection of *AR* mutations in plasma cfDNA appears to indicate better patient prognosis compared to other types of *AR* alterations such as *AR* copy number gain.[26]

AR copy number gain is the most frequent category of *AR* gene alteration in mCRPC, enabling prostate tumours to adapt to low androgen levels during treatment.[13] Like *AR* mutations, *AR* copy number changes are not present in untreated prostate cancer and arise primarily after androgen deprivation therapy. As such, archival primary tissue is not appropriate for assessment of *AR* gene status in mCRPC patients, and only a fresh tumour tissue biopsy or liquid biopsy is informative. *AR* copy gain in plasma cfDNA has been associated with shorter progression-free survival and overall survival in mCRPC patients treated with AR-targeted therapy.[61,66,68,143–146] However, measuring *AR* copy gain in plasma cfDNA is complicated by the variability of ctDNA fractions between patients with mCRPC. Resolution of a single extra gene copy on the X chromosome (where the *AR* gene is located) requires a ctDNA fraction of approximately 20%, whereas the signal from eight *AR* copies can be detected in samples with a ctDNA fraction of only 5%.[26] In a prospective study that corrected for ctDNA fraction, detection of *AR* gain was no longer significant as a predictor of progression-free survival on abiraterone or enzalutamide in a multivariate model with standard clinical prognostic factors.[26] In reality, *AR* gain is not a binary variable, rather a continuous spectrum of increasing *AR* copies. It is plausible that there is an *AR* copy dose-effect relationship with patient prognosis in the advanced-disease setting.[26] An alternative approach is to accept that detection of *AR* gain in plasma cfDNA is a composite biomarker of high ctDNA fraction and *AR* copy number amplification. Indeed,

in a recent analysis of several clinical trial cohorts, an absolute "plasma *AR* copy number" of 1.92 identified a small subset of mCRPC patients with highly aggressive disease in the context of abiraterone or enzalutamide treatment.[147] Interestingly, plasma *AR* copy gain does not associate with poor outcomes in mCRPC patients treated with taxane-based chemotherapy, suggesting an opportunity for a predictive biomarker.[148]

The acquisition of *AR* copy gain in a tumour cell requires a series of structural rearrangements affecting the *AR* gene locus. Genomic breakpoints can fall within the chromosomal region coding for the *AR* gene body itself, and they are termed *AR*-GSRs (genomic structural rearrangements). Some *AR*-GSRs can result in a transcript coding for a truncated ligand-binding domain, similar in concept to the splice variant *ARv7* but usually distinct in nucleic acid sequence.[149,150] While the downstream consequences are challenging to predict from DNA level breakpoints alone, *in vitro* studies have suggested that select *AR*-GSRs give rise to constitutively active AR proteins and drive therapy-resistant phenotypes. *AR*-GSRs can be detected via ctDNA sequencing of *AR* introns, and are linked to primary resistance to AR-targeted therapies.[26,151] The presence of *AR*-GSRs is positively correlated with *AR* copy number,[149,152] and *AR*-GSRs appear to be more abundant in patients with late-stage disease compared to initial mCRPC progression.[20] This enrichment may suggest a positive selective pressure for tumours to acquire ligand-binding domain truncating *AR*-GSRs over the course of AR-targeted therapy, but to date no study has examined this hypothesis in serial plasma cfDNA samples from the same patients.

CTC-based interrogation of the *AR* has largely been limited to splice variants.[86] However, a method for single sample *ARv7* and *AR* ligand-binding domain mutation detection has recently been described.[153] Moreover, *AR* amplification status inferred via fluorescence in situ hybridization is correlated between paired metastatic tissue and CTCs, indicating potential for cytogenetic analyses to complement sequencing approaches.[154] Finally, it is important to note that a combination of *AR* intron sequencing in plasma cfDNA and RNA sequencing of CTCs can be used to identify both *AR*-GSRs and splice variants.[151]

7.8 Other Common Genomic Alterations as Potential Biomarkers

There are several other frequently altered genes in advanced prostate cancer beyond the *AR* and those involved in DNA repair. The tumour suppressor *TP53* is altered in approximately 10% of localized tumours,[15] but in more than 50% of mCRPC.[16,101] For mCRPC patients, detection of *TP53* alterations in plasma cfDNA is linked to worse overall survival, and poor response to AR-targeted therapy.[26,142,152] This relationship appears to be independent of ctDNA fraction and clinical prognostic factors.[26] Prostate tumours that have lost the tumour suppressor triumvirate of *TP53*, *RB1*, and *PTEN* tend to be clinically aggressive, and are primed for lineage plasticity and rapid adaptation to therapy-induced bottlenecks.[155,156] Detection of ctDNA-based alterations in *TP53*, *RB1*, and *PTEN* is a key opportunity to identify potentially aggressive disease variants at an early stage.[66] Single-CTC sequencing can also identify deletions of these tumour suppressors.[91] Future studies will need to examine less common prostate cancer tumour suppressors such as *CHD1*, whose deletion was recently shown to confer AR-targeted therapy resistance *in vivo*.[157] There is some suggestion that *CHD1* deletions detected in ctDNA associate

with shorter overall survival in mCRPC,[66] however, *CHD1* deletions co-associate with *SPOP* mutations, a known good prognostic factor when identified in either ctDNA or tissue.[26,158]

PTEN deletion is the most common PI3K pathway alteration in mCRPC patients.[101] Other alterations affect this pathway, including activating missense mutations in *AKT1* and *PIK3CA*, which are found in 6% of cases.[159] Tumours with PI3K alterations may be reliant on PI3K signalling for survival and therefore represent a therapeutic vulnerability. *PTEN* deletion appears to be a biomarker for selecting patients most likely to respond to PI3K pathway inhibition.[160] Consequently, the pan-Akt inhibitor ipatasertib is under evaluation in a phase 3 clinical trial in mCRPC patients with *PTEN* defects (NCT03072238). It is unclear whether other PI3K signalling pathway alterations will be relevant for ipatasertib response if the drug is approved. However, a recent ctDNA-based study suggested that patients with somatic truncal hotspot mutations in *AKT1* or *PIK3CA* are particularly reliant on the pathway and may have strong responses to ipatasertib.[159] Clearly, prospective evaluation of various PI3K pathway defects as predictive biomarkers is imperative to determine if these patients represent a molecular subtype of mCRPC poised to derive benefit from Akt inhibitors. It is possible that ctDNA or CTC assays focused on hotspot regions in *AKT1* and *PIK3CA*, in conjunction with assessment of *PTEN* copy number, would be sufficient to identify the majority of vulnerable tumours. This is in contrast to the DNA repair alterations discussed previously, where assays must be broad to capture complex events and pan-genome signatures.

7.9 Non-Genomic Information Available in Cell-Free DNA

CtDNA fragments carry additional information beyond genomic alteration status, meaning that ctDNA profiling can inform on aspects of the epigenome. Nucleosomes protect cfDNA from degradation by circulating nucleases, and their positioning can be inferred from whole-genome mapping of cfDNA fragments. Patterns of nucleosome spacing can indicate tissue of origin and have been crucial in demonstrating that plasma cfDNA is largely derived from hematopoietic cells.[1] In addition, cfDNA fragmentation patterns have been shown to vary between plasma from cancer patients versus healthy individuals, indicating diagnostic potential.[2] Lastly, the non-random fragmentation pattern of ctDNA means that even transcription factor activity and gene expression can be inferred from whole-genome cfDNA sequencing data.[161,162]

Epigenetic marks, such as cytosine methylation, are tissue- and cancer-specific features present on cfDNA/ctDNA fragments.[163,164] As such, the tissue of origin can be predicted through methylation profiling of cfDNA.[8,165] Importantly, the number of cell type–specific methylation marks in a tumour cell vastly outnumber the somatic mutation count. Therefore, plasma cfDNA methylation assays have potential for greater ctDNA detection sensitivity than assays reliant on capturing somatic mutations, especially in the context of early cancer diagnosis.[163,164] Detection of prostate lineage methylation marks on cfDNA can provide an accurate measure of ctDNA fraction and even resolve patients with *AR* copy gain.[166] It is also possible that cfDNA methylation profiles could help distinguish mCRPC that is abiraterone sensitive versus resistant.[167] Finally, a recent study demonstrated that in a subset of mCRPC patients with high ctDNA fractions, simultaneous whole-exome sequencing and whole-genome bisulfite sequencing can identify neuroendocrine prostate cancer.[28]

7.10 Future Directions

True clinical translation of recent ctDNA correlative research will require prospective clinical trials. In the context of DNA repair and PARP inhibition, cfDNA analysis is part of patient screening protocols in several ongoing phase 2 or 3 clinical trials.[116] The innovative ProBio clinical trial (NCT03903835) is an outcome-adaptive, multi-arm, platform trial testing the utility of liquid biopsies to tailor treatment decisions for men with mCRPC.[168] The initial arms in this trial will test prognostic and predictive biomarkers for many of the current standard of care therapies such as abiraterone and cabazitaxel. PC-BETS (NCT03385655; also known as IND234) is another multi-arm umbrella trial, but set in a later stage than ProBio, testing investigative agents such as adavosertib, darolutamide, palbociclib, and ipatasertib. CtDNA fraction as a potential biomarker is also being prospectively tested in the phase 2 PROTRACT (PROstate cancer TReatment optimization via Analysis of Circulating Tumour DNA) clinical trial (NCT04015622). In PROTRACT, mCRPC patients who have progressed on abiraterone are randomized to physician's choice of enzalutamide or docetaxel, or a biomarker-driven stratification based on pre-treatment ctDNA fraction. The results from these and similar trials testing potential CTC biomarkers will be crucial for moving liquid biopsy profiling toward routine clinical use in metastatic prostate cancer.

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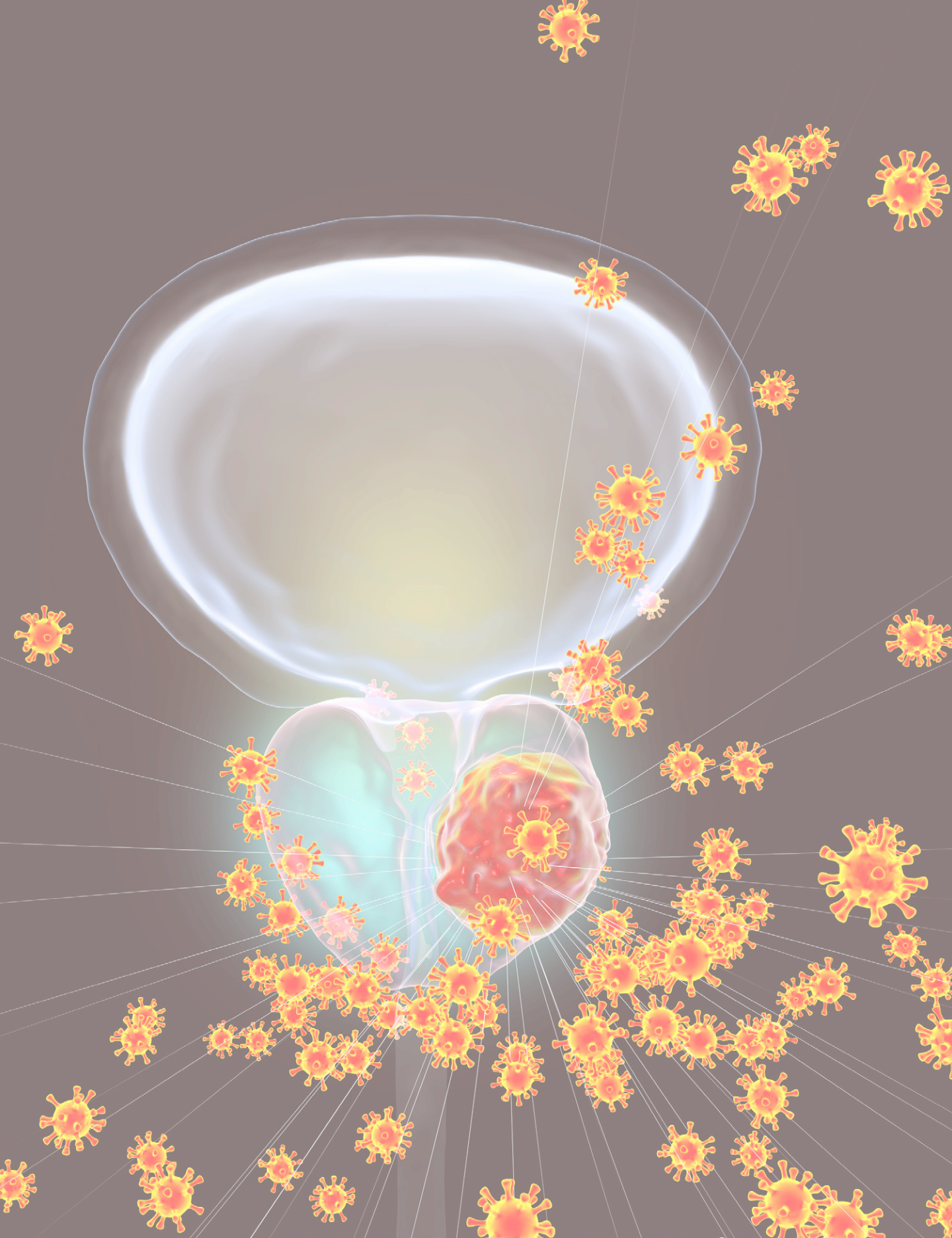

CHAPTER 8

Molecular Imaging of Urologic Cancers as a Biomarker

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Table of Contents

Molecular imaging with positron emission tomography/computed tomography (PET/CT) has established a clear role in diagnosis and staging of urologic cancers, and it provides valuable information about disease prognosis. In recent times, extensive progress has been made in the development of radiotracers that bind to biologically relevant compounds and provide personalized and precise imaging of disease. An example is the application of gallium-68 (^{68}Ga)-radiolabelled prostate-specific membrane antigen (PSMA) for PET/CT imaging of prostate cancer.

This chapter focuses on the role of various molecular imaging modalities as biomarkers in the prognosis of urologic cancers.

8.1 Prostate Cancer

The natural history of prostate cancer is evolution from a clinically localized tumour to metastatic hormone-sensitive disease and finally to a metastatic castrate-resistant state. Imaging plays a crucial role in various clinical phases of prostate cancer, from diagnosis and staging to restaging of biochemically recurrent disease.

Heterogeneity of prostate cancer likely accounts for the variability in therapeutic responses and presents a significant challenge when defining parameters to predict outcomes.

Biochemical markers such as prostate-specific antigen (PSA) have long provided the foundation to assess prognosis in metastatic prostate cancer. In hormone-sensitive metastatic disease, PSA can be used as a prognostic marker, with levels after seven months of androgen deprivation therapy (ADT) inversely correlated with median survival.[1,2] With volume of metastatic disease on conventional imaging now established as a prognostic indicator following the CHAARTED study,[3] and PSA and its various kinetics known to lack accuracy to predict response to treatment and subsequent prognosis in metastatic castrate-resistant disease,[4,5] the opportunity to explore molecular biomarkers as a prognostic tool in prostate cancer has arisen.

Large volumes of clinical and translational research focused on the biology of metastatic prostate cancer, both hormone-sensitive and castrate-resistant, have resulted in the introduction of an array of novel hormonal agents such as enzalutamide and abiraterone, and the development of the Prostate Cancer Clinical Trials Working Group.[6] This committee has emphasized the importance of biological tumour profiling to better define predictive and prognostic markers to facilitate optimal sequencing of targeted therapies, with molecular agents potentially able to play a key role as disease biomarkers.[7]

The diagnostic utility of various novel radiotracers in prostate cancer have been thoroughly explored in the literature, allowing assessment of their prognostic value as molecular biomarkers.

8.1.1 Fluorodeoxyglucose

Metabolic imaging in oncology began by capitalizing on the increased glycolysis of malignant cells, using the radiolabelled glucose analogue ^{18}F-fluorodeoxyglucose (FDG) to demonstrate sites of disease.[8]

The accumulation of FDG in malignant tissue relies upon the Warburg effect, whereby a metabolic change occurs in cancer cells to increase glucose uptake and convert it to lactate even in the presence of normal oxygen levels.[9] This "switch" to aerobic glycolysis facilitates the accelerated proliferation of malignant cells.[10] It has been shown to be related to overexpression of cellular membrane glucose transporters (predominantly glucose transporter 1 [GLUT1]) and increased hexokinase enzymatic activity.[11,12]

In contrast with the majority of solid neoplasms, prostate cancer demonstrates marked heterogeneity. Its cells generally do not have this "glycolytic switch", and without increased glycolysis its cells do not consistently exhibit FDG uptake on PET/CT.[8] However, FDG uptake in prostate cancer does provide valuable information regarding treatment response, assessment, and prognosis.

^{18}F-FDG uptake associated with poorly differentiated prostate cancer

Preclinical studies have shown GLUT1 expression to be significantly higher in poorly differentiated prostate cancer cell lines compared to the well-differentiated hormone-sensitive lines.[13] Clinically, this correlates to aggressive, poorly differentiated prostate cancer displaying higher FDG uptake on PET/CT.[14]

The presence of metabolically active prostate cancer on ^{18}F-FDG PET/CT is thought to be a poor prognostic indicator, given that ^{18}F-FDG uptake is associated with more aggressive, poorly differentiated disease.[14] Two studies have demonstrated that intra-prostatic ^{18}F-FDG uptake has a poorer median cancer-free survival, and the intensity of activity measured by maximum standardized uptake value (SUV_{max}) is inversely associated with prognosis (**Table 8–1**).

TABLE 8–1 Studies Demonstrating Increased ^{18}F-FDG Uptake Is associated with Poorly Differentiated Prostate Cancer and Poorer Prognosis

Study	Year	Cohort	Findings
Beauregard *et al.*[15]	2015	44 men with untreated high-risk prostate cancer (Gleason ≥8)	^{18}F-FDG avid foci of disease were demonstrated within the prostate gland in 44%, in lymph nodes in 13%, and in bone in 6%. Absence of intra-prostatic ^{18}F-FDG uptake was associated with a median cancer-free survival probability of 70.2%, compared with 26.9% when there was ^{18}F-FDG uptake within the prostate.
Oyama *et al.*[16]	2002	42 men with varying grades of untreated prostate cancer	Higher primary tumour SUV_{max} was associated with a significantly poorer prognosis measured by relapse-free survival than those with lower SUV_{max}.

Abbreviations: ^{18}F-FDG, fluorine-18 labelled fluorodeoxyglucose; SUV_{max}, maximum standardized uptake value.

Neuroendocrine differentiation in prostate cancer is androgen-independent and potentially represents a key mechanism in treatment and castration resistance.[17] Subsequently, disease progression and poor prognosis are associated with this neuroendocrine variant. A case series of 23 patients with metastatic neuroendocrine prostate cancer who underwent ^{18}F-FDG PET/CT demonstrated those with median survival of <2.2 years had more avid bony and soft-tissue metastatic deposits and higher average SUV_{max} of the involved lesions, compared to those with a median survival of ≥2.2 years.[18]

^{18}F-FDG uptake associated with poor prognosis in castrate-resistant prostate cancer

FDG uptake in androgen-sensitive prostate cancers has been shown in preclinical studies to decrease with androgen withdrawal. The positive impact of androgen on GLUT1 and hexokinase expression was seen in androgen-sensitive, androgen receptor–positive prostate cell lines, compared to no effect in androgen-independent, androgen receptor–negative lines.[14,19,20] Clinically, this translates to hormone-sensitive prostate cancer (HSPC) demonstrating lower FDG uptake than castrate-resistant prostate cancer (CRPC).

The metabolic burden of disease demonstrated on ^{18}F-FDG PET/CT in CRPC is thought to be predictive of prognosis.[5,21] Studies have shown that independent factors that predict survival in CRPC are the intensity of ^{18}F-FDG uptake on PET/CT, as measured by SUV_{max}, and the number of metabolically active lesions (**Table 8–2**).

In conjunction with appropriate multi-disciplinary discussion and consideration of other clinical parameters such as PSA, high-volume disease on ^{18}F-FDG PET/CT may prompt a change in management given the associated poor outcomes. Even in the HSPC group, ^{18}F-FDG uptake can provide valuable prognostic information about time to treatment failure and survival probability.

TABLE 8–2 Studies Demonstrating Intensity and Number of ^{18}F-FDG Avid Lesions Predict Survival in CRPC

Study	Year	Cohort	Findings
Meirelles *et al.*[22]	2010	43 men with CRPC bony metastases	OS was inversely associated with SUV_{max} of ^{18}F-FDG-avid lesions. Prognosis was dependent on an SUV_{max} threshold of 6.10. Median survival 14.4 months when SUV_{max} >6.10 and 32.8 months when SUV_{max} <6.10.
Jadvar *et al.*[23]	2013	87 men with metastatic CRPC	Sum of the SUV_{max} of up to 25 ^{18}F-FDG-avid nodal, bony, or soft-tissue metastases demonstrated a hazard ratio of 1.01 for predicting OS. Marked increased chance of death when the sum of the SUV_{max} of metabolically active lesions exceeded 20.
Vargas *et al.*[24]	2014	38 men with metastatic CRPC	Number of metabolically active metastases on ^{18}F-FDG PET/CT was inversely associated with OS.
Jadvar *et al.*[25]	2019	76 men with metastatic HSPC	Both sum of SUV_{max} and number of lesions were associated with time to hormonal treatment failure on univariate analysis. When sum of SUV_{max} were grouped into quartiles, survival probability for patients in the fourth quartile group was significantly worse, with a hazard ratio of 6.2.

Abbreviations: CRPC, castrate-resistant prostate cancer; SUV_{max}, maximum standardized uptake value; ^{18}F-FDG, fluorine-18 labelled fluorodeoxyglucose; OS, overall survival; PET/CT, positron emission tomography/computed tomography; HSPC, hormone-sensitive prostate cancer.

Guidelines

The European Association of Urology (EAU) does not recommend ^{18}F-FDG PET/CT to evaluate disease at any stage of prostate cancer management. PET/CT altogether is described as having no routine role in the assessment of treatment response in metastatic hormone-sensitive and castrate-resistant disease, although there is a strong recommendation to adapt follow-up when progression is suspected.[1]

The National Comprehensive Cancer Network (NCCN) guidelines similarly do not recommend routine ^{18}F-FDG PET/CT in men with prostate cancer, and particularly not in initial staging of newly diagnosed disease. However, ^{18}F-FDG is one of only four FDA-approved radiotracers that can potentially assess prostate cancer with PET/CT.[26]

Take-home messages

- Presence and degree of ^{18}F-FDG uptake on PET/CT is associated with more aggressive disease and poorer prognosis.
- CRPC generally exhibits higher ^{18}F-FDG uptake on PET/CT compared to HSPC; thus, ^{18}F-FDG PET/CT can be used to monitor development of castrate resistance.
- In CRPC, the number of FDG-avid lesions and the degree of ^{18}F-FDG uptake (SUV_{max}) on PET/CT are independent prognostic factors.
- Degree of ^{18}F-FDG uptake on PET/CT can be used to predict time to treatment failure and survival probability in HSPC.

8.1.2 Choline

Molecular imaging can interrogate tumour biology by capitalizing on altered cellular metabolic pathways, including increased glycolysis and altered lipid metabolism. Lipid precursors such as acetate and choline can be used as alternative PET radiotracers when labelled with fluorine-18 or carbon-11.[8]

Increased lipid synthesis is directly coupled to deregulation of oncogenes and oncosuppressors that occurs early in prostate cancer carcinogenesis, while upregulation and increased activity of lipid metabolizing enzymes such as fatty acid synthase and choline kinase occurs at all stages.[8]

Choline kinase is required for the synthesis of essential cellular membrane phospholipids and demonstrates oncogenic activity when overexpressed by cancer cells.[27] Radiolabelled choline PET/CT capitalizes on the increased expression and activity of lipid metabolizing enzymes in prostate cancer, and conveys valuable diagnostic information, particularly in restaging of biochemically recurrent disease where it has demonstrated a respectable pooled sensitivity of 85% to detect nodal and distant disease.[28] It follows that uptake of ^{11}C-choline or ^{18}F-choline by prostate cancer cells would provide useful information about treatment response and prognosis.

Radiolabelled choline uptake associated with disease progression in biochemically recurrent disease

Overexpression of fatty acid synthase and choline kinase play a key early role in lipid-mediated oncogenicity in prostate cancer, and have both been associated with poor prognosis and reduced disease-free survival in preclinical studies.[29,30] The increased expression of these lipid metabolizing enzymes is exploited by radiolabelled lipid precursor tracers such as choline, translating to avid disease on PET/CT.

The presence of ^{11}C-choline or ^{18}F-choline uptake on PET/CT can be used as a molecular biomarker, as it is thought to be predictive of prognosis.[5,31] Various studies have demonstrated that prostate cancer with ^{11}C-choline or ^{18}F-choline avidity on PET/CT is associated with earlier disease progression, earlier treatment failure, and poorer prostate cancer–specific survival in the setting of biochemical recurrence after radical therapy (**Table 8–3**).

TABLE 8–3 Studies Demonstrating ^{11}C-Choline or ^{18}F-Choline Avid Disease on PET/CT Is Associated with Poorer Prognosis in Biochemical Recurrence After Radical Treatment

Study	Year	Cohort	Findings
Gacci *et al.*[32]	2014	103 men with BCR following curative intent treatment	Three PSA kinetic parameters had sensitivity 86% to predict disease progression on 6-month follow-up ^{18}F-choline PET/CT: • Increase in PSA from baseline >5 ng/mL • Decrease in PSA doubling time <6 months • Increase in PSA velocity >6 ng/mL/month
Reske *et al.*[33]	2012	27 men with BCR following RP	^{11}C-choline avid disease on PET/CT associated with treatment failure and less freedom from biochemical failure after salvage radiotherapy, compared to patients with negative PET/CT.
Breeuwsma *et al.*[34]	2012	64 men with BCR following RP	Disease-specific survival with median follow-up of 50 months was significantly higher in patients with negative PET/CT, compared to those with ^{11}C-choline avid disease.
Giovacchini *et al.*[35]	2014	195 men on ADT with BCR following RP	Median prostate cancer–specific survival in patients on ADT significantly poorer in those with ^{11}C-choline avid disease (11.2 years), compared to those with negative PET/CT (16.4 years).
Giovacchini *et al.*[36]	2015	302 men with BCR following RP	15-year prostate cancer–specific survival probability in hormone-naïve patients significantly poorer in those with ^{11}C-choline avid disease (42.4%), compared to those with negative PET/CT (95.5%).

Abbreviations: ADT, androgen deprivation therapy; BCR, biochemical recurrence; ^{11}C-choline, carbon-11 labelled choline; CRPC, castrate-resistant prostate cancer; ^{18}F-choline, fluorine-18 labelled choline; PET/CT, positron emission tomography/computed tomography; PSA, prostate-specific antigen; RP, radical prostatectomy.

Radiolabelled choline uptake associated with poor prognosis and disease progression in castrate-resistant prostate cancer

Preclinical studies have demonstrated specific lipid metabolizing enzymes to be regulated by hormone-dependent pathways, such as the elongation enzyme ELOVL7 (elongation of very long chain fatty acids protein 7), which is required in the metabolism of long-chain fatty acids and is overexpressed in higher-grade prostate cancer[37]. The fatty acid desaturation enzyme SCD1 (stearoyl-CoA desaturase enzyme 1) is overexpressed in both hormone-sensitive and -resistant cell lines, but in CRPC the increased expression correlates to a higher Gleason grade.[38] These enzymes are potentially examples of how radiolabelled choline can reflect prostate cancer progression and prognosis as it evolves from the hormone-sensitive to the castrate-resistant stage.

The prognostic significance of ^{18}F-choline avid metastatic disease burden on PET/CT was examined in a prospective study of 30 men with CRPC.[39] Three PET-based factors were found to be significantly associated with poorer overall survival (OS) in the cohort, reflecting the value of molecular imaging as a predictor of prognosis. Higher metabolically active tumour volume (MATV), as derived by SUV_{max} and lesion volume, higher total lesion activity, as derived by mean SUV_{max} and MATV, and highest lesion SUV_{max} were all factors that predicted poorer overall survival with hazard ratios of 2.02, 1.93, and 1.26, respectively.

Radiolabelled choline can be used as a marker of treatment response in castrate-resistant prostate cancer

PET/CT imaging using radiolabelled choline has shown promising results to predict response to varying treatment modalities, including conventional ADT, chemotherapy, and other novel hormonal agents for CRPC.[7,31] The previously discussed study by Giovacchini *et al*. demonstrated the value of ^{11}C-choline to assess response to ADT in men with BCR, with median prostate cancer–specific survival significantly poorer in those with ^{11}C-choline avid disease.[35]

Radiolabelled choline PET/CT has been investigated as a biomarker for response to treatment modalities for CRPC, including the first-line chemotherapy agent docetaxel and the novel hormonal agents enzalutamide and abiraterone (**Table 8–4**). Intensity of uptake and burden of metastatic disease on baseline choline PET/CT are both promising prognostic parameters to predict disease progression and progression-free survival. Interestingly, treatment response on choline PET/CT varied as a predictor of survival, with some studies demonstrating significance, and other studies showing no correlation.

TABLE 8–4 Studies Demonstrating ^{11}C-Choline or ^{18}F-Choline Avid Disease on PET/CT Is Associated with Poorer Prognosis in Metastatic CRPC

Study	Year	Cohort	Therapy	Findings
Ceci *et al.*[40]	2016	61 men with metastatic CRPC	Docetaxel	Higher tumour burden (>10 avid bone lesions) on baseline ^{11}C-choline PET/CT significantly associated with increased probability of disease progression. Progressive disease on ^{11}C-choline PET/CT observed in 44% of patients with PSA response post-chemotherapy.
Schwarzenböck *et al.*[41]	2016	32 men with metastatic CRPC	Docetaxel	No significant correlation between changes in ^{11}C-choline uptake on PET/CT and conventional therapy response assessment (using lesions on CT and PSA levels), at early and late docetaxel cycles.
De Giorgi *et al.*[42]	2014	43 men with metastatic CRPC	Abiraterone	Treatment response on ^{18}F-choline PET/CT and PSA decline were both significant predictors of PFS and OS on univariate analysis. Only treatment response on ^{18}F-choline PET/CT remained a significant predictor of PFS and OS on multivariate analysis.
De Giorgi *et al.*[43]	2015	36 men with metastatic CRPC	Enzalutamide	Treatment response on ^{18}F-choline PET/CT and PSA decline were both significant predictors of PFS. Only PSA decline was a significant predictor of OS.
Maines *et al.*[44]	2016	30 men with metastatic CRPC	Enzalutamide	^{18}F-choline uptake measured by SUV_{max} on baseline PET/CT only, was a significant prognostic factor for PFS and OS. No significant correlation between biochemical and treatment response on ^{18}F-choline PET/CT was demonstrated.

Abbreviations: ^{11}C-choline, carbon-11 labelled choline; CRPC, castrate-resistant prostate cancer; ^{18}F-choline, fluorine-18 labelled choline; OS, overall survival; PET/CT, positron emission tomography/computed tomography; PFS, progression-free survival; PSA, prostate-specific antigen; SUV_{max}, maximum standardized uptake value.

Guidelines

In 2019, the EAU updated their guidelines to recommend choline PET/CT for restaging in the BCR setting after radical prostatectomy, where PSA is >1 ng/mL, PSMA PET/CT is unavailable, and the results will significantly influence subsequent treatment.[1] Stronger evidence is stated for the use of choline PET/CT in PSA recurrence following curative intent radiotherapy, although it is only recommended if curative intent salvage treatment is appropriate. Alongside these recommendations, an emphasis is placed on the sensitivity of choline PET/CT to detect disease, being strongly dependent on serum PSA level and kinetics.[45]

The EAU guidelines do not recommend choline PET/CT to assess progression of CRPC, stating the value is unclear but likely to not be as beneficial as for patients with BCR or HSPC.[1] It is recommended that treatment response and management of CRPC be based upon PSA progression, radiographic progression on conventional imaging with CT and bone scan, and clinical deterioration.

The NCCN guidelines recommend that ^{11}C-choline PET/CT is considered to detect sites of metastatic disease in the BCR setting following radical prostatectomy or curative radiotherapy, with ^{11}C-choline being one of the four FDA-approved PET radiotracers.[26] Specifically, the recommendation is to consider ^{11}C-choline PET/CT following bone scan for evaluation of equivocal findings.

Take-home messages

- Presence of radiolabelled choline uptake on PET/CT is associated with higher rates of treatment failure, disease progression, and poorer prostate cancer–specific survival in BCR.
- Higher degree of metastatic disease burden on choline PET/CT is associated with poorer overall survival in CRPC.
- Radiolabelled choline PET/CT imaging has shown potential as a valid biomarker to predict response to chemotherapy and novel hormonal agents in CRPC.

8.1.3 Prostate-specific membrane antigen

Prostate-specific membrane antigen (PSMA) was first detected on a human prostatic carcinoma cell line (LNCaP) as an integral membrane glycoprotein.[46] Unlike PSA, which is a secreted protein that acts as a serum tumour marker, PSMA is a transmembrane protein expressed by epithelial cells and has folate hydrolase activity.[47] PSMA demonstrates limited expression in benign prostate tissue but is overexpressed 100 to 1,000 fold in prostate cancer cells, with further overexpression in metastatic and castration-resistant disease.[48]

In benign prostate tissue, PSMA expression is seen in the cytoplasm and apical side of the epithelium surrounding prostatic ducts. This cytoplasmic PSMA is called PSM' and demonstrates no folate hydrolase activity. In comparison, malignant transformation of prostate cells results in PSMA being transferred to the luminal surface of the prostatic ducts where it develops folate hydrolase activity and expression is increased.[49]

PSMA is an attractive target for molecular imaging with PET/CT given these appealing biological characteristics. Of the available tracers and ligands able to image PSMA-expressing tumours, gallium 68 radiolabelled PSMA (^{68}Ga-PSMA-11) and second-generation fluorinated PSMA agents (^{18}F-DCFPyL, ^{18}F-PSMA-1007) are the most frequently used to demonstrate PSMA avid disease.[7]

The value of PSMA PET/CT in diagnostic and staging contexts has been well described in recent years. Compared to conventional imaging, the superior sensitivity and specificity of ^{68}Ga-PSMA PET/CT to detect nodal and distant metastatic disease in biochemically recurrent prostate cancer was demonstrated in a large meta-analysis.[50] The results of a large prospective study to assess the value of ^{68}Ga-PSMA PET/CT to stage primary high-risk prostate cancer are imminent,[51] but the large volume of retrospective literature certainly supports PSMA PET/CT in this context. The improved sensitivity to detect metastatic disease, along with favourable biological characteristics that demonstrate increased PSMA uptake in higher-grade and androgen-independent tumours,[7,49] makes PSMA PET/CT a very valuable imaging biomarker to assess prognosis in prostate cancer.

PSMA as an imaging biomarker to assess response to systemic therapy

PSMA expression levels have been shown to increase with increasing stage and grade of prostate cancer.[52–54] PSMA overexpression is also associated with high serum PSA levels and probability of recurrence.[55] Of clinical importance is the fact that PSMA is upregulated when tumours become androgen independent, and when ADT is introduced.[56]

There is insufficient clinical data to directly correlate PSMA expression in primary prostate tumours with prognosis, but it's certainly an area that will expand now that PSMA PET/CT is becoming a more accepted modality to stage high-risk and restage biochemically recurrent disease.

PSMA PET/CT potentially fills the void of a reliable imaging modality to assess response to systemic therapies in metastatic prostate cancer.[57] Currently, Response Evaluation Criteria In Solid Tumors (RECIST) criteria remains the standard to define metastatic burden.[58] However, bone metastases are not measurable using this convention, which is clearly an issue given the predilection of prostate cancer to metastasize to bone. Emerging data suggests PSMA PET/CT is more sensitive than bone scan to detect metastases,[59] and potentially more specific, assessing prostate cancer itself rather than osteoblastic activity within the bone.[60]

Preliminary data on the role of PSMA PET/CT in assessment of response to non-hormonal systemic therapy in metastatic HSPC is limited but promising (**Table 8–5**). The role of PSMA uptake on PET/CT to predict prognosis is yet to be addressed in the published literature.

TABLE 8–5 Studies Using PSMA PET/CT to Assess Response to Non-Hormonal Systemic Therapy in Metastatic Prostate Cancer

Study	Year	Cohort	Therapy	Findings
Anton *et al.*[61]	2018	28 men with metastatic HSPC	Docetaxel	Post-docetaxel PSMA PET/CT demonstrated reduced disease burden in all patients. Complete response was seen in 36% and reduction to oligometastatic disease (<5 metastatic sites) was seen in a further 43%. Complete response correlated with PSA ≤0.2 ng/mL, an established marker of good prognosis.
Seitz *et al.*[62]	2018	7 men with metastatic HSPC	Docetaxel	^{68}Ga-PSMA PET/CT showed superior concordance with biochemical response to conventional CT in the metastatic HSPC group (86% vs 50%).
		16 men with metastatic CRPC	Docetaxel	Outcome prediction less concordant in the metastatic CRPC group, 56% concordance with ^{68}Ga-PSMA PET/CT versus 33% with conventional CT.

Abbreviations: CRPC, castrate-resistant prostate cancer; ^{68}Ga-PSMA, gallium-68 labelled PSMA; HSPC, hormone-sensitive prostate cancer; PET/CT, positron emission tomography/computed tomography; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen.

Based on preliminary studies and anecdotal data, PSMA PET/CT appears to be a promising new modality to assess response to systemic therapy, particularly in metastatic hormone-sensitive prostate cancer, before clonal evolution occurs. However, more studies are clearly required before this will become the standard of care, in order to validate its prognostic ability and define parameters for disease progression and disease response.[57]

PSMA uptake significantly affected by androgen receptor pathway

Preclinical work has shown the androgen receptor suppresses PSMA expression, and thus androgen receptor pathway blocking treatments, such as ADT, upregulate PSMA. This was then replicated in human metastatic CRPC, where PSMA PET/CT demonstrated a 7-fold increase in PSMA uptake following initiation of ADT, signifying the potential for flares of PSMA activity on PET/CT when ADT is commenced.[63]

Further preclinical work has demonstrated that both ADT and the novel antiandrogen agent abiraterone can result in increased PSMA expression, with the effect more pronounced with abiraterone.[64]

The effect of systemic androgen deprivation on PSMA uptake on PET/CT in both metastatic hormone-sensitive and castrate-resistant prostate cancer has been explored in two prospective studies (**Table 8–6**). Both consolidate the preclinical findings that androgen deprivation or blockade induces change in PSMA expression, but also demonstrate the difference this effect can have on PSMA uptake on PET/CT between hormone-sensitive and

castrate-resistant disease. This difference is clearly critical when considering how to interpret PSMA expression on PET/CT as both a prognostic indicator and in assessing disease response to treatment.

TABLE 8–6 Studies Demonstrating the Effect of Androgen Deprivation on PSMA Uptake on PET/CT Between Metastatic Hormone-Sensitive and Castrate-Resistant Prostate Cancer

Study	Year	Cohort	Therapy	Findings
Aggarwal *et al.*[65]	2018	4 men with metastatic HSPC	LHRH agonist + short-term bicalutamide	49% of all metastatic lesions between both groups showed an initial increase in SUV_{max} on ^{68}Ga-PSMA PET/CT consistent with flare effect rather than disease progression. 68% of metastatic HSPC lesions showed an initial increase in SUV_{max} on ^{68}Ga-PSMA PET/CT, compared to 41% in metastatic CRPC group.
		4 men with metastatic CRPC	Enzalutamide	
Emmett *et al.*[66]	2019	8 men with metastatic HSPC	LHRH agonist +/- short-term bicalutamide	Median 30% reduction in SUV_{max} on ^{68}Ga-PSMA PET/CT by day 9 following initiation of ADT. Patterns of PSMA uptake heterogeneous after day 9, with persistently high SUV_{max} in 37.5% and marked reduction in 62.5%.
		7 men with metastatic CRPC	Abiraterone OR enzalutamide	Median 45% increase in SUV_{max} on ^{68}Ga-PSMA PET/CT by day 9 following initiation of novel antiandrogen. Patterns of PSMA uptake more homogeneous after day 9. All SUV_{max} increases plateaued by day 28.

Abbreviations: CRPC, castrate-resistant prostate cancer; ^{68}Ga-PSMA, gallium-68 labelled PSMA; HSPC, hormone-sensitive prostate cancer; LHRH, luteinizing hormone-releasing hormone; PET/CT, positron emission tomography/computed tomography; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; SUV_{max}, maximum standardized uptake value.

Preliminary evidence suggests PSMA expression may be dynamic and changes with introduction of systemic therapies. Early restaging with PSMA PET/CT following commencement of androgen deprivation may facilitate identification of those at risk for early progression to castrate-resistant disease, thus rendering PSMA uptake on PET/CT a powerful prognostic molecular biomarker.[57]

Lack of PSMA uptake may represent more advanced disease

Not all prostate cancer overexpresses PSMA, with up to 10% exhibiting low PSMA expression and thus low or minimal uptake on PSMA PET/CT.[67,68] The effect on prognosis of high-risk localized prostate cancer with minimal or no PSMA expression is yet to be addressed, however, it appears in the metastatic setting, so this confers a poor prognosis.

A study of 16 patients with metastatic castrate-resistant disease and either low PSMA expression or discordant ^{18}F-FDG avid disease demonstrated median overall survival of 2.5 months.[69] This likely reflects tumour heterogeneity following several lines of therapy, but also demonstrates the utility of ^{18}F-FDG PET/CT in patients with known or suspected metastatic prostate cancer, but negative PSMA PET/CT. Sites of low PSMA expression frequently exhibit high metabolic activity reflected by ^{18}F-FDG uptake, and thus imaging with both tracers may provide a more comprehensive assessment of disease burden, tumour heterogeneity, and prognosis.[57]

Prostate cancer with neuroendocrine differentiation, whether primary[70] or as a result of androgen deprivation induced clonal evolution,[71,72] does not reliably express PSMA, and thus disease progression in this subgroup is not dependably reflected by PSMA PET/CT.

PSMA-based radioligand therapies in metastatic castrate-resistant prostate cancer

PSMA targeted therapies using radionuclides, or theranostics, have revolutionized metastatic castrate-resistant prostate cancer unresponsive to conventional therapies. Lutetium-177 (^{177}Lu) is a β-emitter labelled to the same small PSMA molecules used for PSMA PET/CT imaging, and it enables effective delivery of radiation to sites with high PSMA expression.[73] Initial trials suggest variable treatment responses, with PSA decline ≥50% seen in 35% to 64% across the three studies.[73–75]

Given that a key concept of ^{177}Lu-PSMA treatment is "see-what-you-treat", the prospective phase 2 trial enhanced patient selection by performing both ^{68}Ga-PSMA PET/CT and ^{18}F-FDG PET/CT to elucidate PSMA-negative tumours that were ineligible for ^{177}Lu-PSMA targeted therapy.[57,73] It was this subgroup of 16 patients with low PSMA expression and/or ^{18}F-FDG discordant disease that demonstrated very poor prognosis.[69]

It has been postulated that the flare effect of androgen receptor blockade resulting in increased PSMA uptake could potentially be used to improve the therapeutic activity of PSMA-targeted radioligand therapies such as ^{177}Lu-PSMA.[65]

Several prognostic biomarkers in men with metastatic castrate-resistant prostate cancer receiving ^{177}Lu-PSMA targeted therapy have recently been identified.[76] High ^{18}F-FDG avid molecular tumour volume of more than 207 mL, high ^{68}Ga-PSMA uptake with SUV_{mean} greater than 10.55, serum lactate dehydrogenase more than 240 U/L, serum alkaline phosphatase more than 126.5 U/L, and bone scan index of more than 5.3% all conferred a poor prognosis. Interestingly, neither the intensity of ^{18}F-FDG uptake nor the ^{68}Ga-PSMA avid tumour volume was predictive of prognosis.[76]

Guidelines

In 2019, the EAU updated their guidelines to recommend PSMA PET/CT for restaging in the BCR setting post–radical prostatectomy where PSA is > 0.2 ng/mL and the results will significantly influence subsequent treatment.[1] They also recommend PSMA PET/CT in PSA recurrence following radical radiotherapy where patients are fit for curative salvage treatment. Currently, PSMA PET/CT is not recommended in the primary staging or diagnosis settings, but an emphasis is placed on the promising evidence, particularly in staging of newly diagnosed high-risk disease.

The EAU guidelines do not recommend PSMA PET/CT to assess progression of CRPC, stating the value is unclear but likely to not be as beneficial as for patients with BCR or HSPC.[1] It is recommended that treatment response and management of CRPC be based upon PSA progression, radiographic progression on conventional imaging with CT and bone scan, and clinical deterioration.

The NCCN guidelines do not recommend PSMA PET/CT at any stage in the management of prostate cancer, as currently there are no FDA-approved PET radiotracers.[26]

Take-home messages

- Increased PSMA expression demonstrated on PET/CT is associated with higher grade and stage of prostate cancer, and it is associated with higher probability of recurrence.
- ADT-upregulated PSMA expression results in a 'flare' effect on PET/CT following commencement of hormonal therapy.
- Poor PSMA expression may represent more advanced disease particularly in CRPC, and concurrent imaging with ^{18}F-FDG PET/CT in those with poor PSMA uptake can facilitate more accurate staging.
- Degree of PSMA uptake is a marker of prognosis in men with CRPC receiving ^{177}Lu-PSMA targeted radioligand therapy.

FIGURE 8–1 Patient with recurrent prostate cancer underwent serial ^{18}F-fluorocholine PET/CT. Lower panel (2a-e) demonstrates initial staging with ^{18}F-fluorocholine PET/CT with intense uptake right prostate (2b) and no evidence of nodal or distant disease, where presenting PSA was 7.0 ng/mL. Upper panel (1a-e) demonstrates the same patient 12 months later following external beam radiotherapy and ADT where ^{18}F-fluorocholine PET/CT demonstrates focal residual uptake in right prostate (1b) and moderately avid bilateral external and internal iliac nodes (1c, 1d, 1e). The patient went on to develop metastatic CRPC. Red arrows (1d) indicate tracer uptake within the ureters in relation to the avid nodes.

Source: Caption and images are courtesy of Dr. Nathan Lawrentschuk.

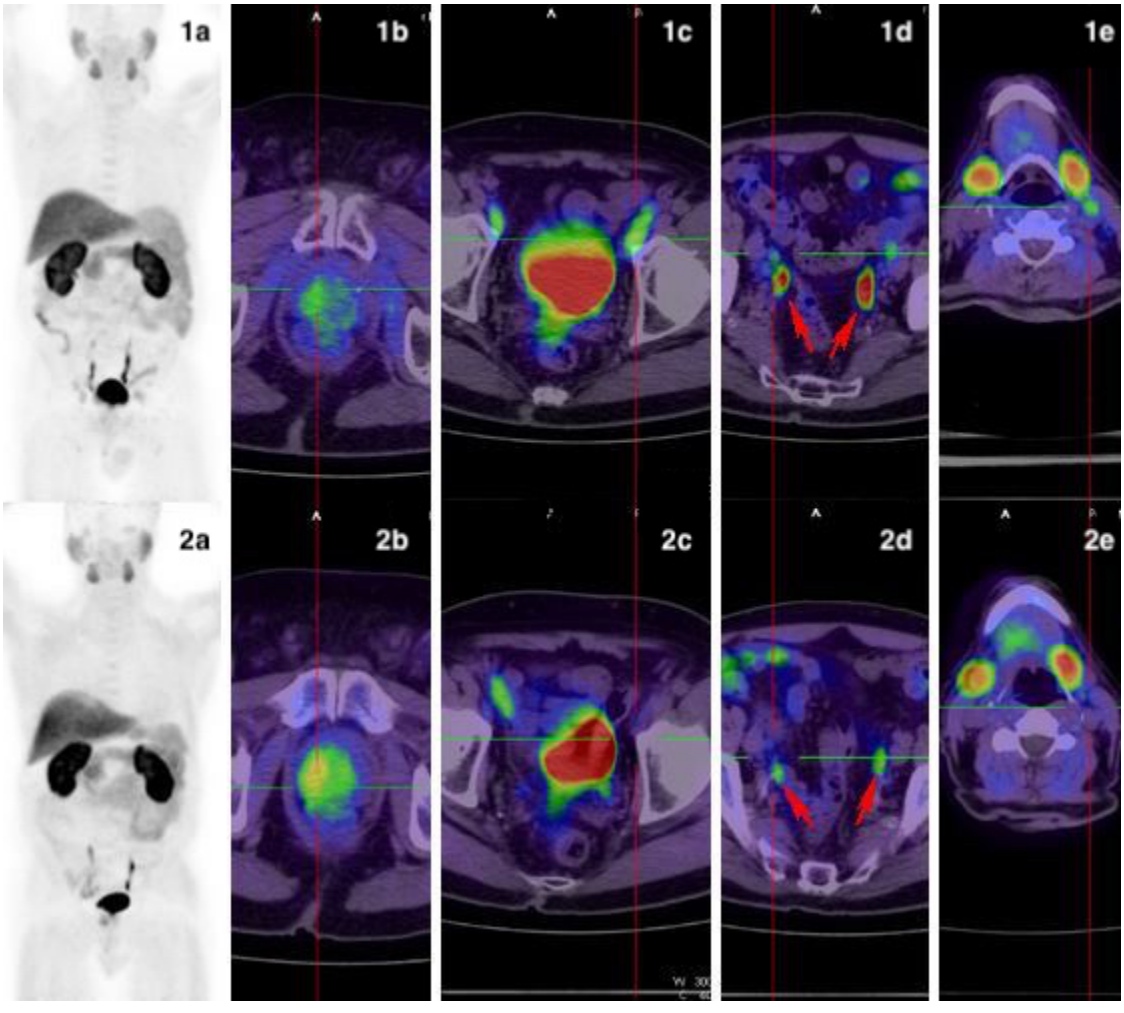


FIGURE 8–2 Patient with metastatic hormone-sensitive prostate cancer with bony metastases treated with up-front docetaxel underwent serial ^{68}Ga-PSMA PET/CT to assess disease response. Pretreatment ^{68}Ga-PSMA PET/CT demonstrates extensive uptake within the prostate most marked left posterior base extending to left seminal vesicle (1a) and two foci of mildly PSMA avid bony lesions in the right pubic ramus (1b) and right posterior ilium (1c). Following treatment, complete resolution of all PSMA-avid primary and metastatic disease was seen (2a-c).

Source: Caption and images are courtesy of Dr. Nathan Lawrentschuk.

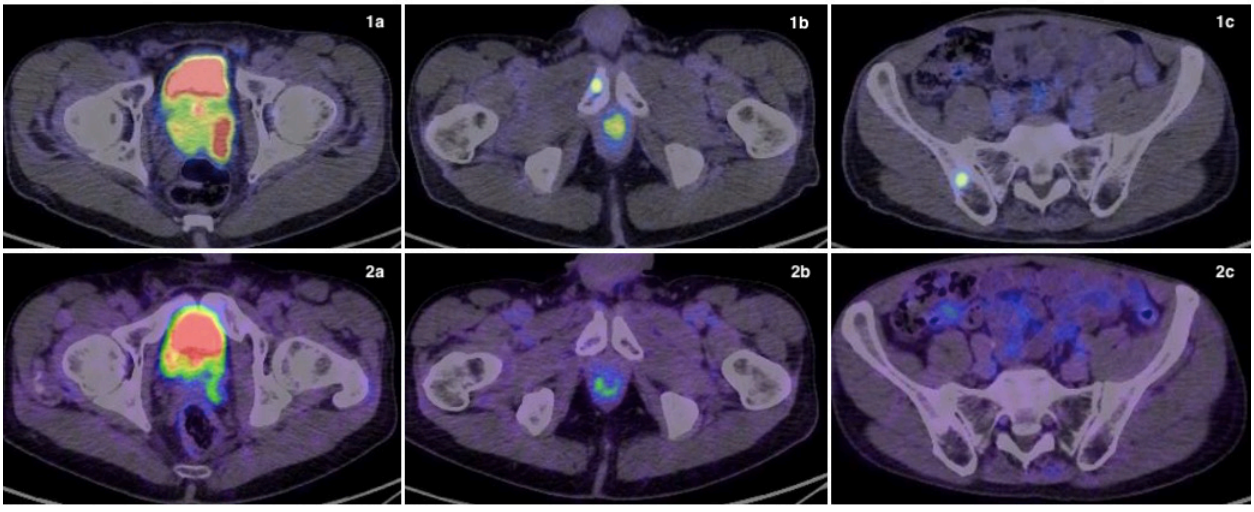


FIGURE 8–3 Patient with metastatic CRPC and extensive bony disease initially treated with ADT and docetaxel. PSA then increased to 31.4 ng/mL on enzalutamide, and patient underwent both ^{18}F-FDG PET/CT (1a-b) and ^{68}Ga-PSMA PET/CT (2a-b). Extensive osseous metastases largely demonstrating concordant FDG and PSMA uptake, except for the lesion in the left acetabulum (red arrow 1a and 1b), which is FDG-avid but shows no PSMA uptake. Consistent with presence of ^{18}F-FDG discordant disease, the prognosis was poor.

Source: Caption and images are courtesy of Dr. Nathan Lawrentschuk.

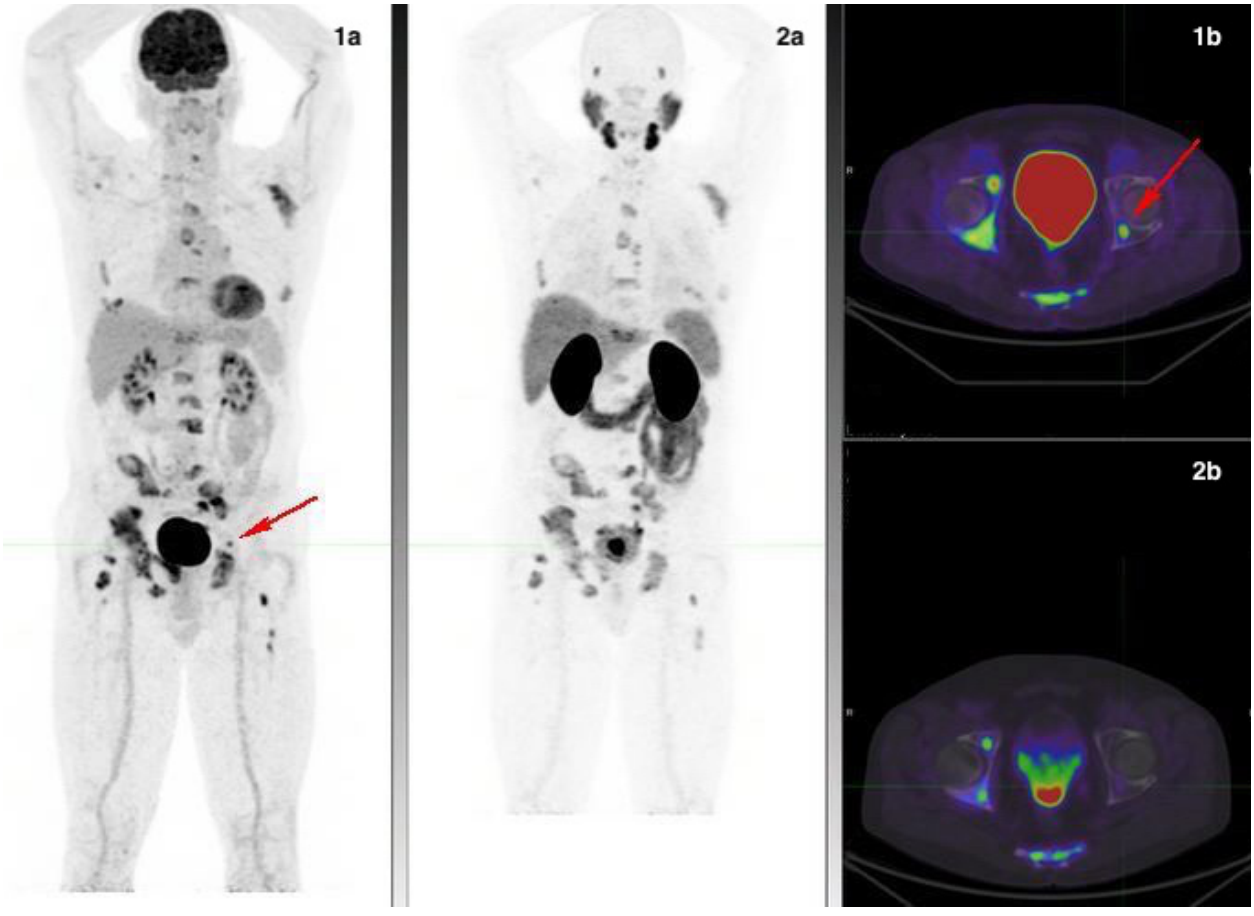
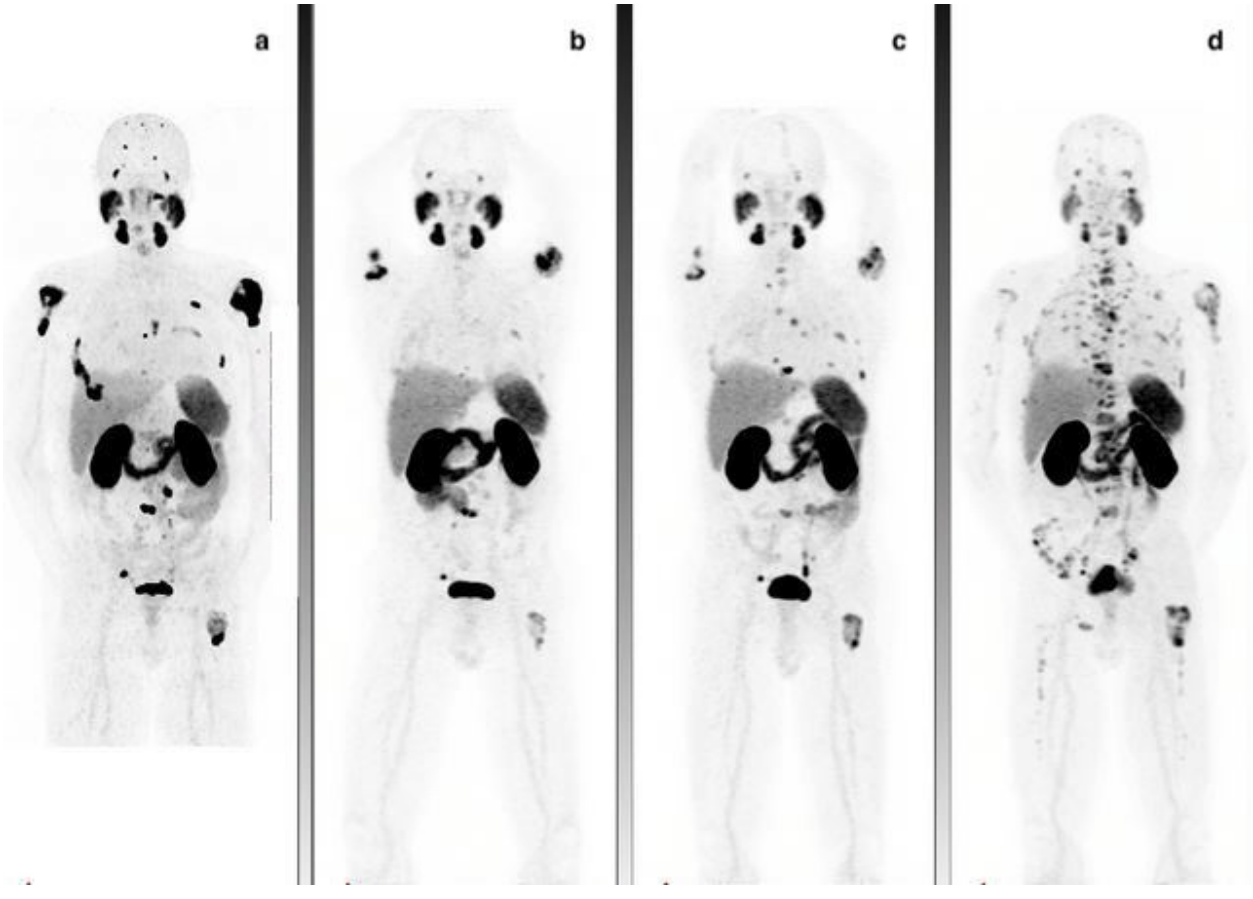


FIGURE 8–4 Patient with metastatic CRPC and extensive nodal, bony and visceral disease initially treated with ADT and docetaxel, followed by enzalutamide. Given ongoing PSMA-avid disease, patient enrolled in trial for ^{177}Lu-PSMA targeted therapy with baseline scan demonstrating extensive disease (a). Initially partial PSMA response is seen to ^{177}Lu-PSMA (b) before progressive disease is demonstrated 3 months later (c) and at 6 months after five cycles of treatment (d).

Source: Caption and images are courtesy of Dr. Nathan Lawrentschuk.



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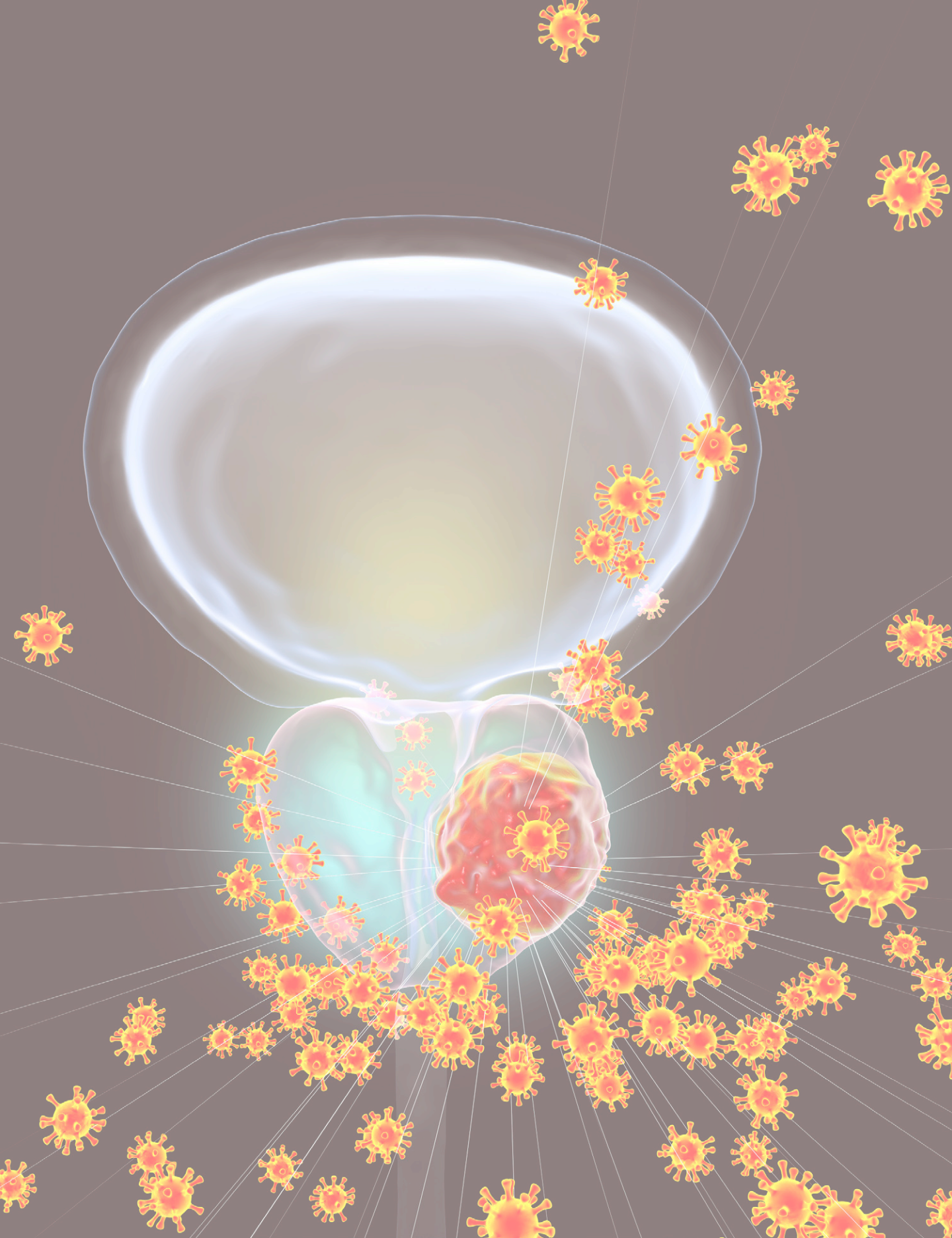

CHAPTER 8A

Epigenetics to Identify and Stratify Prostate Cancer: A Perspective of Prostate Cancer Diagnosis and Prognosis on the Basis of Epigenetics

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Table of Contents

8A.1 Introduction: History of Epigenetics

The ancient Greek philosopher Aristotle (384–322 BC) first developed the concept of epigenetics. He introduced epigenesis (ἐπιγένησις) as the process of how an individual organic form comes into being from the unformed. In modern times, Conrad Hal Waddington (1905–1975) contemplated the rapid, plastic, and complex processes of human ontogenesis, and coined the term "epigenetics" to describe the mechanisms by which the genes of the genotype bring about phenotypic effects and the causes behind them.[1] From the modern-day epigeneticist's point of view, accumulating evidence underlies the notion that the cellular phenotype is not a direct consequence of the genome, but rather an epigenetic annotating system that dictates how the genome will be used to manifest the intended cell type and function. Figuratively speaking, it is like a full-body corset whose intentionally positioned gaps determine that only the desirable parts of the body are featured, so that only the person's intended shape becomes visible. Furthermore, environmental factors interact with this epigenetic layer on the genes to determine their effects on cellular morphology and function. We consider genes, their regulating epigenetic mechanisms, the exogenous noxae that affect them, and the resulting cellular phenotype as co-evolving, steadily interacting parts of one unit. Disturbances of external influencing stimuli lead to adaptive changes of the epigenetic mechanisms, which may even lead to drastic morphological and functional changes, once a certain stimulus threshold is exceeded. Therefore, the gene-centered approach to biology of recent times, mainly owing to the groundbreaking findings of Gregor Mendel, must become considerably extended in an effort to achieve significant progress in cancer research.

8A.2 Epigenetics in Cancer

Unimpaired gene expression is a fundamental prerequisite for the integrity of a differentiated and functional cell. Ideally, all genes necessary for the constitution of a functional cell are qualitatively and quantitatively expressed in a cell type–specific manner. Epigenetic mechanisms decisively contribute to this process. Unnecessary genes and numerous intergenic sequences with transcriptional and interfering potential are epigenetically silenced. Among these repetitive element families are long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), and numerous fragments of viral origin, such as endogenous retroviruses (ERVs), which have evolutionarily accumulated in high numbers within the genome.[2] By this epigenetic silencing, these elements remain dormant, and do not interfere with the genetic material and the cell type–specific transcriptome. Hence, it is guaranteed that cell type–specific gene expression remains unaffected and contributes to a highly specialized and functional phenotype.

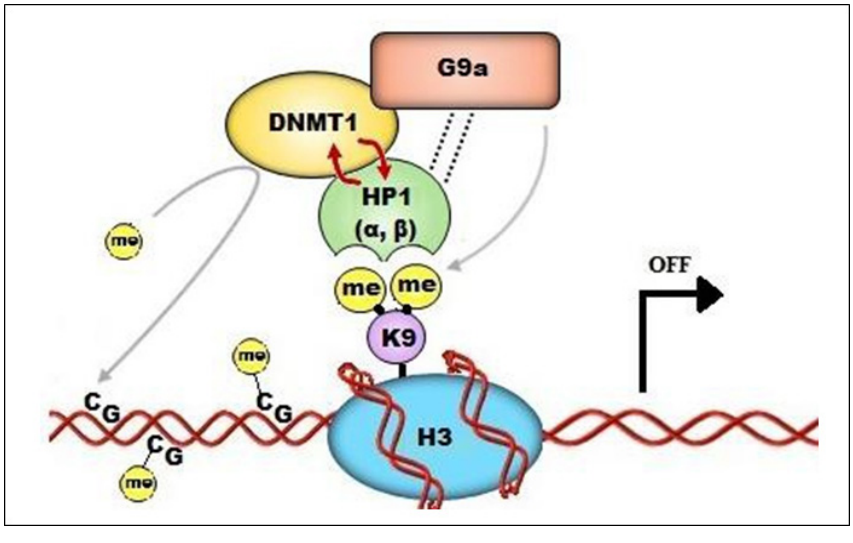
Key epigenetic mechanisms responsible for these gene expression levels and primarily for a wide variety of distinct cellular phenotypes generated from the same genome are histone modifications, chromatin remodelling, and DNA methylation.

DNA methylation is the most prominent and most widely investigated epigenetic mechanism, which is involved mainly in gene regulation and silencing of repetitive sequences.[3] DNA methylation occurs only in the context of cytosine-phosphate-guanine (CpG) dinucleotides. Arrangements of CpG dinucleotides in approximately 1,000 to

1,500 base pair–long CpG islands (CGIs), with an elevated CG content, are associated with repetitive elements, such as LINE 1, and with the promoter regions of 60% of all human genes. Normally, methylation of these CpGs occurs with heterochromatization and correlates with gene repression.

It is important to highlight that the currently proposed timeline of the epigenetic gene silencing process begins with the interference of the transcription of a gene. This may be caused by environmentally harmful factors. For instance, these factors may be aromatic amines in cigarette smoke, a main risk factor for bladder cancer, which cause mutations in key cancer-related genes by forming DNA adducts.[4] Recently, Erichsen and colleagues suggested that this may be also the case for key genes of the methyl group metabolism itself with severe consequences for the methylome, which includes all methylated CpG dinucleotides of the genome shaping its use.[5,6] Subsequently, due to heretofore unknown mechanisms, such a mutation-driven aberrant gene silencing event leads to attraction of histone methyltransferase G9a to the 5' region of the regulatory gene. This enzyme transfers methyl groups to the histone 3 lysine 9 (H3K9) residue and hence establishes a H3K9 dimethyl (diMe) or H3K9 trimethyl (triMe) repressive chromatin mark. This epigenetic modification allows for binding of the heterochromatin protein 1 (HP1) repressor, which subsequently recruits DNA methyltransferase 1 (DNMT1). This enzyme confers methyl groups onto DNA. In this way, DNA methylation leads to constitutive heterochromatic gene silencing.[7] (**Figure 8A–1**).

FIGURE 8A–1 Model of Synergistic Interplay Between Enzymes and Repressors During Epigenetic Gene Silencing and Heterochromatin Formation (*Adapted from Smallwood A, Estève P-O, Pradhan S, Carey M. Functional cooperation between HP1 and DNMT1 mediates gene silencing*. Genes Dev. *2007;21(10):1169–1178. doi:10.1101/gad.1536807)*



Abbreviations: G9a**,** histone H3 lysine 9 methyltransferase; DNMT1, DNA methyltransferase 1; H3, histone 3; HP1, heterochromatin protein 1; K9, lysine 9; me, methyl group.

On the other hand, loss of DNA methylation is associated with gain of transcriptional competence. In other words, the affected transcription unit can be expressed, as long as all other essential requirements for its expression are met, such as the availability of required transcription factors. For instance, following demethylation, intragenic LINE 1 elements coding for a potent endonuclease can be reactivated, and the endonuclease can interfere with the integrity of the genetic material and the transcription of corresponding protein coding genes.[8]

Thus, we see that DNA methylation is a key player in cell type–specific genome organization and control, preventing gene activation, much like a locking system providing for the long-term memory of gene repression decisions,[9] mediated by the main mechanism of heterochromatin formation.

However, in cancer, drastic aberrations of these epigenetic mechanisms and the epigenetic profiles on the genome take place.

One example is the well-known observation of promoter CGI hypermethylation, which silences known tumour-suppressor genes, such as BRCA1, CDKN2A, VHL, and RB1, in a broad spectrum of tumour entities, including breast, pancreas, colon, bladder, and prostate, among others. Of note, in some cases, this is thought to be of causal relevance and some of these epigenetic alterations are considered essential for cancer cell survival, with important implications for therapy.[2] Another example is global DNA hypomethylation, a genome-wide decrease in methylcytosine affecting ERVs and retrotransposons (SINEs and LINEs) in particular, which has been broadly documented to occur in many cancer types. Specifically, substantial global LINE 1 DNA hypomethylation is seen in chronic lymphocytic leukemia and in colon, lung, prostate, urothelial, ovarian, hepatocellular, gastric, and breast cancers.[10,11]

Interestingly, more than 90% of early urothelial carcinomas adopt hypomethylation of LINE 1 retrotransposons at an early stage of the carcinogenesis process, whereas more than 80% of advanced prostate cancers, which are thought to be particular life-threatening, exhibit this same epigenetic alteration. In prostate carcinogenesis, hypomethylation is associated with tumour progression.[12–14] Here, hypomethylation increases with tumour grade and stage, where particularly pronounced hypomethylation has been seen in node-positive tumours.[12] Analyses on mortality stratified by Gleason score revealed that the association between LINE 1 hypomethylation and mortality from prostate cancer was present only in patients with a Gleason score of at least 8. These data suggest that increased mortality from prostate cancer is associated with lower levels of LINE 1 methylation in the tumour tissue. DNA hypomethylation arises later in prostate cancer progression than CGI hypermethylation and contributes to metastatic tumour heterogeneity.[15]

It is mainly the 5' CpG-rich regulatory promoter region that is aberrantly affected by this reduced methylation. Notably, in both cancer types (urothelial carcinoma and prostate cancer), this feature has been linked to LINE 1 expression and mobilization, and to genomic instability, which is thought to be at least in part caused by LINE 1 encoded endonuclease activity. Of note, this epigenetic aberration is thought to be involved in cancer development and progression. The LINE 1 promoter consists of a CpG-rich methylated and heterochromatic promoter region in healthy tissues, whereas the same region is largely unmethylated and accessible for expression, and therefore

euchromatic in the aforementioned tumour entities. Interestingly, LINE 1 is one of the most abundant class I retrotransposable elements. It comprises 17% of the human genome, with approximately 500,000 copies, including the rarer class of full-length (~6 kb) LINE 1 retrotransposon sequences with intact internal promoters, of which there are approximately 7,000 copies in the human genome.[16] It is clear that the feature of LINE 1 hypomethylation provides a prominent starting point with major technical advantages for the efficient application of molecular detection techniques.

Thus, LINE 1 hypomethylation is considered useful as an early detector of cancer and also as a prognostic indicator.[10] However, it remains unknown whether LINE 1 demethylation is one cause for cancer initiation, cancer progression, or both. Recently, Erichsen *et al.* developed an *in vitro* model by which they were able to induce rapid and efficient LINE 1 demethylation and transcription initiation at will, such as in primary uroepithelial cells, and based on this new evidence that study is poised to answer this major question.[6]

Today, we can identify a plethora of disturbances of the normal DNA methylation patterns in cancer cells. A major focus of current research worldwide seeks to take advantage of biomarkers in the search for differentially methylated CGIs, as these latter elements are associated with gene promoter regions particularly of tumour suppressor genes. Such disturbances denote the fundamental functional importance of DNA methylation in cancer development and consequently bear relevance for therapy and prognosis. But it must be noted that unfortunately this notion pushes forward an obtrusive bias.

8A.3 Use of DNA Methylation Changes for Diagnosis and Prognosis of Prostate Cancer and Other Cancer Entities

Based on our own cancer epigenetic studies over the past 20 years, we currently believe that the cancer methylome changes to be largely what, at first glance, we would like to term as “pleiotropic.” That is, a DNA methylation disturbance does not impart one specific functionality feature on a cancer cell, and as such, its effects appear to be inconsistent in different patients with the same tumour entity. In particular, we observe a variety of such DNA methylation aberrations in cancer on CpG dinucleotide positions of genes that normally exhibit cell type–specific expression and partake in highly specialized functions, such as olfactory receptors, killer cell immunoglobulin-like receptors, crystallines, etc. Consequently, we assume that the loss of the proper epigenetic control of such specialized cell type–specific genes may be well tolerated and therefore appear at the first instance of cancer. On the other hand, we extracted a smaller core part of consistently differentially methylated CpGs—a valuable specific methylation signature present in every single prostate or bladder cancer patient. This core comprises CpG positions within the regulatory elements of genes known to have functional relevance for cancer.

Over the past few years, we carried out many genome-wide DNA methylation screening analyses in tissue from patients with urothelial carcinoma, prostate cancer, and other tumour entities. Histological hematoxylin and eosin–stained sections from formalin-fixed, paraffin-embedded (FFPE) tissue specimens (tumour tissue and

healthy tissue) were reviewed for tumour (stage and grade); healthy tissue content and the target area were marked by a trained pathologist for DNA isolation. Methylated DNA as an epigenetic mark has the major advantage of stability and it most easily survives various forms of sample processing, including FFPE.[17] We interrogated the entire methylome by performing immunoprecipitation with a specifically methylated cytosine binding antibody, analytical DNA methylation array technology, and a bioinformatic analysis packet developed in-house. Our notion is that whenever possible, we should always assess all 28 million CpGs of the genome, as these may be differentially methylated in cancer. A fundamental goal in this approach is to find out all differentially methylated single CpG dinucleotides in optimally pathologically reviewed, homogeneous samples compared to corresponding healthy tissue. Furthermore, we seek to determine whether single CpG dinucleotides are consistently differentially methylated in a statistically significant number of best-characterized prostate cancer specimens in comparison to healthy tissue. Indeed, sophisticated bioinformatic analyses uncovered a plethora of differentially methylated CpG positions occurring in all cancer samples, but not in the controls (**Figure 8A–2**). This method allows for the identification of the differential CpG methylation signatures of any pathologically defined and properly excised tumour tissue of a given stage and grade or of tumour-surrounding fluids. These differences will then be used by idiolocal normalization of real-time methylation-specific polymerase chain reaction (IDLN-MSP) for fast, cost-effective, and reliable diagnosis and prognosis, after thorough validation studies. IDLN-MSP is a method developed for reliably and accurately measuring DNA methylation in cancer samples with diverse genetic abnormalities.[18] For instance, this will help reliably detect prostate cancer in pathologically cryptic biopsies. Furthermore, comparisons of these genome-wide differentially methylated CpGs seem to allow for epigenetic classifications that may be valuable, once their relation to pathological and/or clinical classification has been understood and verified (**Figure 8A–3**).

We are currently identifying markers for a crucial target in urological cancer, specifically for the stratification of indolent and aggressive prostate cancer. To that end, we are exploring differentially methylated CpGs that solely and consistently arise in prostate cancer specimens from patients who have left active surveillance, as these patients have been clearly identified to exhibit progression by the gold standard clinical criteria; these CpGs do not occur in the samples from patients with indolent prostate cancer.

In our experience and knowledge, cancer-specific DNA methylation differences are the only known biological features so far that consistently occur in a specific tumour type of a certain stage and grade. Furthermore, we see that some of these DNA methylation patterns consistently and simultaneously appear in many different tumour entities at once. These observations open a new perspective on developing new potent “pan-cancer” biomarkers. An example is shown in **Figure 8A–4**: Initial experiments show LINE 1 hypomethylation detection in urinary cell-free and cellular DNA from urothelial cancer patients with a sensitivity of 94% and a specificity of 95%, indicating that detection of LINE 1 hypomethylation may be used for early cancer detection as well as for monitoring purposes.

FIGURE 8A–2 Short Section of a Heatmap of: Localized, Consistently Differentially Methylated Cytosine-Phosphate-Guanine–Rich Probes in 20 Prostate Cancer Samples (T) and Eight Prostate Cancer Adjacent Healthy Tissue Samples (C). The higher the value and the brighter the red colour, the higher the methylation of the corresponding 60 nucleotide-long, CpG-rich probe (numbered casket). Blue colour indicates hypomethylation.

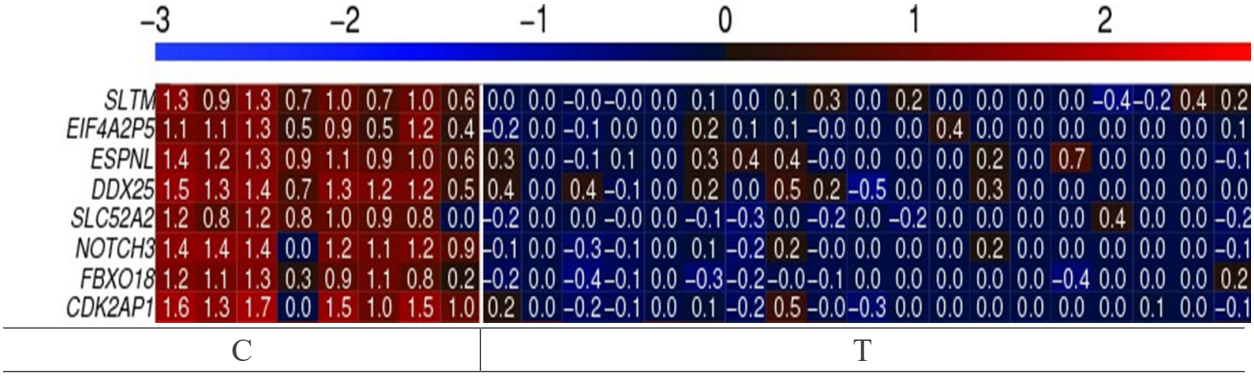
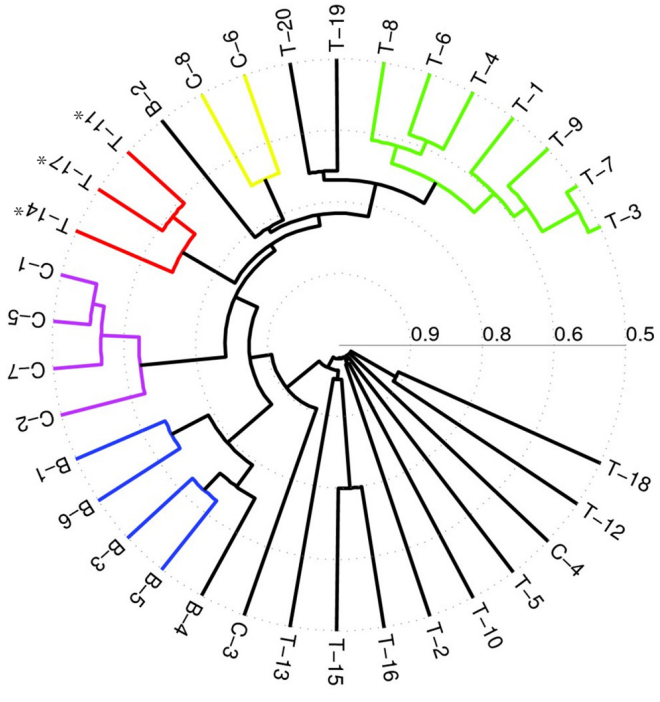
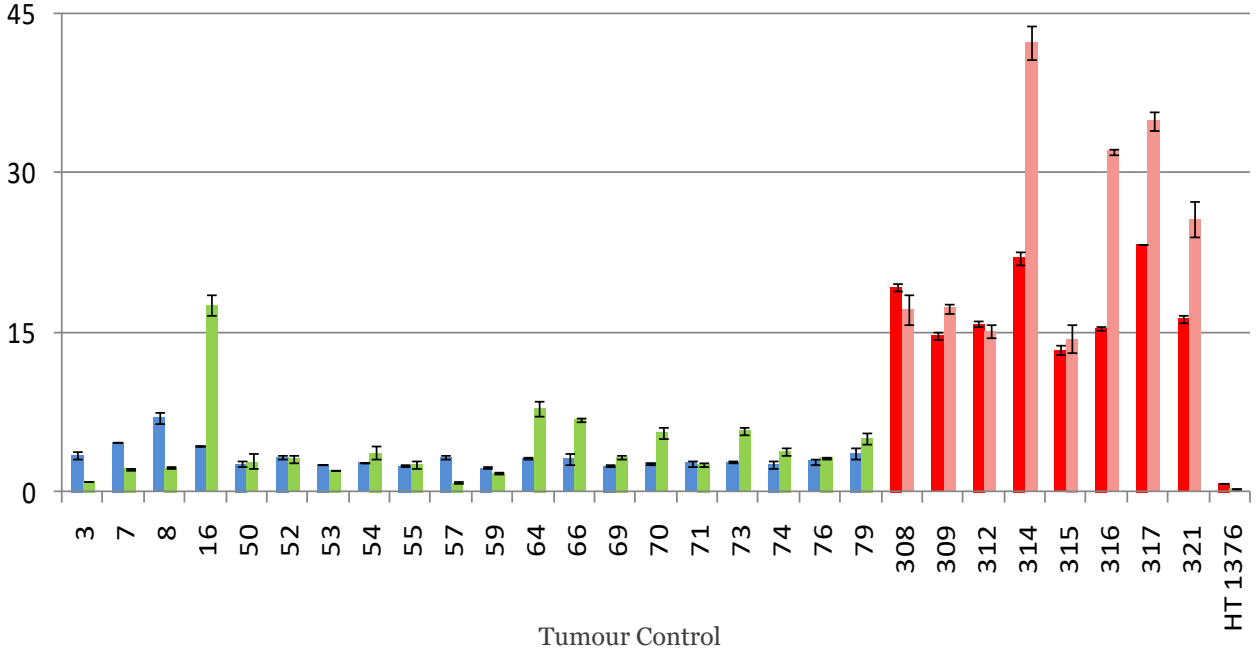


FIGURE 8A–3 Hierarchical Clustering of: Prostate Cancer Samples of Various Stages and Grades (T), Benign Prostatic Hyperplasia Samples (B), and Prostate Cancer–Adjacent Tissue Samples Reviewed as Pathological or Healthy (C). This classification has been performed by the correlation metric and the average linkage method, based on all similarities and dissimilarities of all differential methylated CpG dinucleotides of the genome.



*Death due to prostate cancer.

FIGURE 8A–4 LINE 1 Real-time IDLN-MSP PCR from Urinary Cellular and Cell-free DNA. The LINE 1 DNA methylation status was relatively quantified from urinary cellular DNA (blue and red bars) and cell-free DNA (green and light red bars) from 20 bladder cancer patients (tumour) and eight healthy individuals (control), and DNA from the bladder cancer cell line HT 1376.



Abbreviations: IDLN-MSP, idiolocal normalization of real-time methylation-specific polymerase chain reaction; LINE 1, long interspersed nuclear element.

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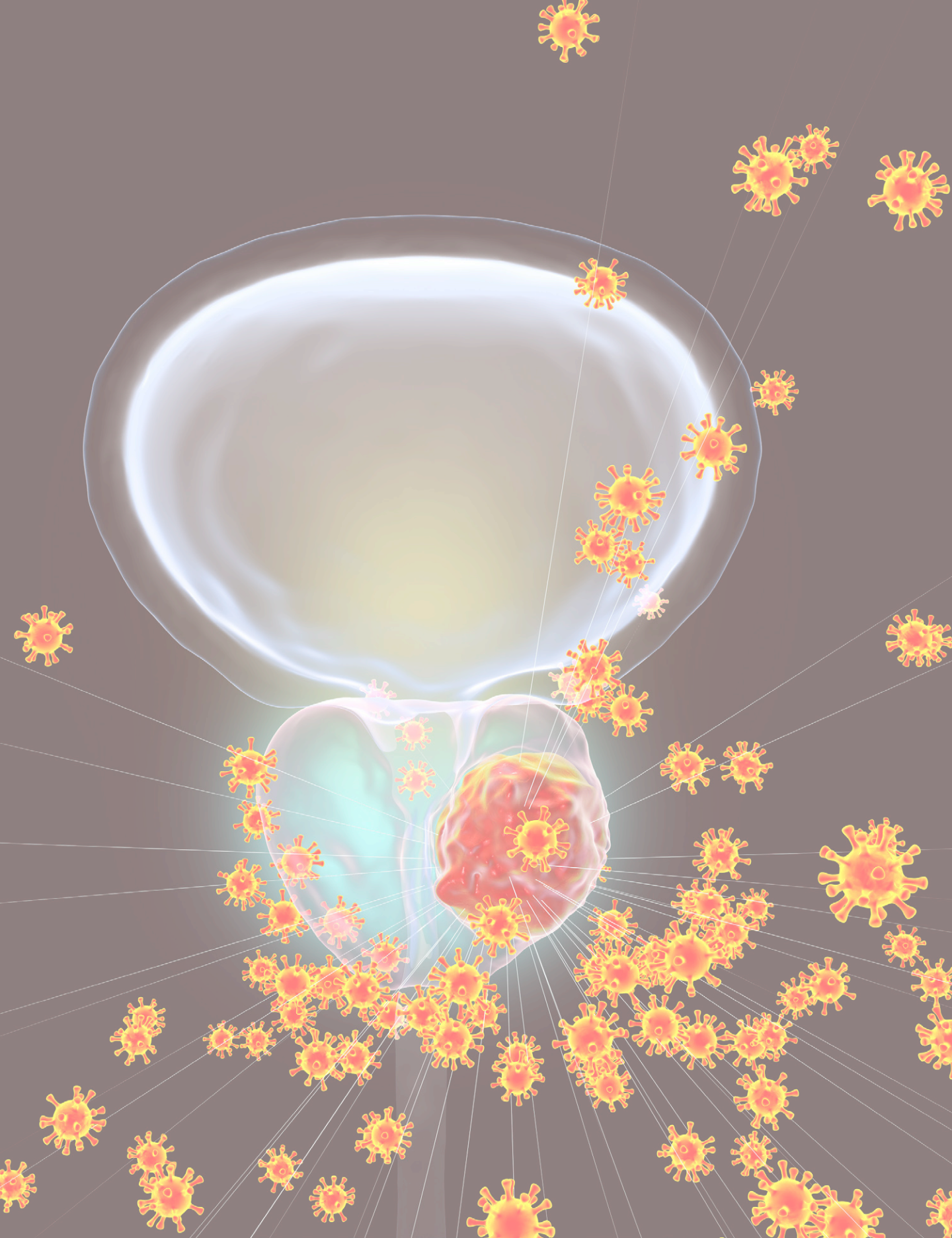

CHAPTER 9

The Clinical Applications of Serum and Urinary Biomarkers in Prostate Cancer

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Table of Contents

9.1 Introduction

Prostate cancer (PCa) is a heterogeneous disease with a highly variable clinical course and behaviour. Traditional risk stratification of PCa has been based on standard clinical parameters such as prostate-specific antigen (PSA), clinical stage, and biopsy Gleason scores. After the introduction of widespread PSA testing, the pendulum swung toward overdiagnosis of clinically insignificant PCa and the subsequent overtreatment with its associated morbidity.[1] On the other hand, traditional nomograms, while allowing risk stratification of patients to a certain degree, may not allow accurate prediction of outcomes for an individual patient with PCa, leading to potential under-treatment of high-risk disease.[2,3] Due to these limitations, leading to discordant care, many areas of uro-oncology have recognized the need for the development of reliable biomarkers to aid decision-making in various challenging clinical contexts. Finding the ideal biomarker that can accurately predict a patient's individual risk and improve outcomes has become an important focus in clinical cancer research. This is particularly true for prostate cancer.

At every stage of the PCa journey, from screening and diagnosis to advanced disease, patients and clinicians face dilemmas and decisions that can impact long-term outcomes. The first challenge in PCa management is knowing which patient to biopsy when presented with an elevated PSA. Once PCa is diagnosed, patients and families wish to understand the aggressiveness of the disease, its lethality, and whether treatment is required and in what form. When considering undergoing definitive treatment for PCa, patients are concerned about how successful that treatment will be and what the risk for disease recurrence is. In the postoperative setting, clinicians seek to predict which patients are likely to require adjuvant treatment.

In recent years several commercially available blood, urine, and tissue assays have entered clinical practice. Novel biomarkers may help clinical decision-making in the setting of an elevated PSA, refine patient selection for prostate biopsy, or assist further intervention decisions after an initial negative biopsy such as when to undergo a repeat biopsy. After the diagnosis of PCa, tissue biomarkers may help to confirm the decision to manage a patient with active surveillance or proceed to intervention. Biomarkers have also been validated to predict the risk for disease recurrence, progression, development of metastases, and death.[4–8] The use of biomarkers may also help to determine the intensity of ongoing surveillance.[9]

Although there is evidence to support the use of biomarkers to guide management decisions, the optimal scenario in which to use them, how to interpret the results, and how to incorporate those results into clinical decision-making can be confusing. Many biomarkers are expensive, limiting their widespread use in clinical practice. The general principle that the value of a test is dependent on the test's ability to effect true clinical change is valid here. An investigation should be undertaken only if it is expected to change management or improve clinical outcomes. Therefore, just because a biomarker can be used in a certain situation does not automatically mean that it should be used. However, in the era of personalized and precision medicine, it is important for clinicians to be aware of what tests are available, what clinical questions they seek to answer, and the limitations they have.

In this chapter, we discuss the various noninvasive serum and urinary biomarkers that show promise in the management of prostate cancer.

9.2 Classification of Biomarkers in Prostate Cancer

Biomarkers can be classified in many ways—according to the source (blood or urine—**Table 9–1**) or the clinical decision juncture at which they are used (**Figure 9–1**).

TABLE 9–1 Commercially Available Biomarkers That Are US Food and Drug Administration (FDA) and Clinical Laboratory Improvements Amendments (CLIA) Approved

Biomarker	Molecular Markers Tested
Serum	
Prostate-Specific Antigen (PSA)*	PSA
Prostate Health Index (PHI)* Beckman Coulter Inc, Brea, California, United States	tPSA, %fPSA, p2PSA
4Kscore OPKO Lab, Miami, Florida, United States	tPSA, fPSA, intact PSA, hK2
Urine	
Progensa Prostate Cancer Antigen-3 (PCA3)* Hologic, Marlborough, Massachusetts, United States	PCA3
ExoDX Prostate (Intelliscore) Exosome Diagnostics Inc, Waltham, Massachusetts, United States	PCA3, ERG
Michigan Prostate Score (MiPS) Detroit, Michigan, United States	TMPRSS2-ERG mRNA, PCA3
SelectMDX MDx Health, Irvine, California, United States	HOXC6, DLX1

*FDA-approved biomarkers.
Abbreviations: hK2, kallikrein 2; fPSA, free PSA; %fPSA, % free PSA; p2PSA, [-2]proPSA; tPSA, total PSA.

Figure 9–1 Serum and Urinary Biomarkers According to Their Clinical Applications

SCREENING / PRE–1st BIOPSY	PREVIOUS NEGATIVE BIOPSY / PRE–2nd BIOPSY
PSA	4Kscore
4Kscore	PHI
PHI	PCA3
PCA3	
SelectMDx	
MiPS	
ExoDx	

The ideal biomarker has high sensitivity and specificity, is cost-effective, and uses an easily acquired sample source such as blood and urine. It must also be independently reproducible in populations that are representative of those in whom the marker would be clinically applied.

It is important to differentiate between diagnostic, prognostic, and predictive markers.[10] Diagnostic markers indicate the presence of a disease but may not provide further information on the characteristics of the disease.[11] Diagnostic assays incorporating PSA isoforms such as prostate health index (PHI) and 4Kscore are examples of diagnostic biomarkers.

Prognostic markers provide information related to a prespecified clinical endpoint and are correlated with a time-to-event outcome such as biochemical recurrence, or cancer-specific or overall survival. Prognostic markers are useful in the clinical management of patients, as they provide information about the aggressiveness of the disease. They can help determine potential candidacy of various treatment strategies, for example, active surveillance in patients with indolent disease or multimodal therapy for patients with virulent and aggressive disease.

Predictive markers indicate the likely benefit a specific patient may have from a specific treatment and may be used to make more specific choices between treatment modalities. For example, a patient expressing a certain predictive marker may benefit more from a certain treatment than a patient not expressing that marker. Examples of predictive makers include tissue-based gene expression assays that may predict which patient is likely to benefit from adjuvant radiotherapy after radical prostatectomy.[11]

Therefore, prognostic markers reflect the effects of the disease and patient characteristics on outcome, whereas predictive markers reflect the effects of treatment on outcome.

9.3 Serum Biomarkers

9.3.1 Prostate-specific antigen (PSA)

Measurement of serum PSA is the most widely used test to aid in the detection of early prostate cancer, despite its well-known limitations including false-positive rates, poor specificity for prostate cancer, and overdiagnosis.

PSA is a member of the serine protease kallikrein family and is encoded by the kallikrein 3 (KLK3) gene. It is secreted by prostatic tissue into blood, urine, and semen. However, it is not tumour specific, as it is secreted by both benign and malignant prostatic tissue. Therefore, apart from PCa, many benign processes such as inflammation, benign prostatic hyperplasia (BPH), and trauma may lead to increased PSA levels. Human PSA was first purified in 1979 and introduced as a biomarker in prostate cancer in the 1980s after its homogeneity to prostatic tissue and usefulness as an indicator of prostate cancer were demonstrated.[12,13] It gained FDA approval for PCa screening in 1994 after it was shown to have higher sensitivity than prostatic acid phosphatase (PAP) in the detection of prostate cancer.[13]

Historically, a serum PSA level above 4 ng/mL was the accepted cutoff to predict the potential presence of prostate cancer.[14] However, it has since been recognized that 20% of patients diagnosed with prostate cancer have a PSA lower than 4 ng/mL.[15,16] The positive predictive value (PPV) of a PSA level less than 4 ng/mL is not clearly defined.[16] Furthermore, it has been reported that when using a PSA cutoff of 4 ng/mL, the specificity of PSA in detecting PCa is only 12.8%, leading to high false-positive rates and unnecessary biopsies.[17] As a strategy to improve the diagnostic capacity of PSA at low levels and to enable better discrimination between PCa and BPH, the measurement of the ratio of free to total PSA was proposed, and many studies have demonstrated its efficacy.[18–21] In a large, prospective, multicentre clinical trial of 773 patients with PSA levels between 2 and 10 ng/mL, it was shown that a % free PSA (%fPSA) cutoff of 25 detected 95% of cancers and avoided 20% of unnecessary biopsies with minimal loss in sensitivity.[22] The cancers associated with a higher %fPSA tended to be in older populations and less aggressive in tumour grade and volume. Catalona *et al.*'s multivariable analysis showed %fPSA to be an independent predictor of prostate cancer (odds ratio [OR], 3.2; 95% CI, 2.5–4.1; $p<0.001$) and more significant than total PSA (tPSA). Generally, the lower the %fPSA, the higher the risk for cancer. However, prostate volume has been found to have an influence on %fPSA. Stephan *et al.* reported diagnostic value in determining %fPSA in patients with prostate volume <40 cm^3, but they advised caution in using %fPSA in those with larger glands.[23]

Other PSA metrics have also been employed to enhance the diagnostic and predictive capacity of PSA. These include PSA density (PSAD), PSA doubling time (PSADT), and PSA velocity (PSAV). PSA density is a quotient of serum PSA and prostate volume and may be a means of distinguishing between BPH and PCa.[24] A higher PSAD may not only indicate the presence of PCa, but also reflect the aggressiveness of cancer in a gland. Studies have shown a correlation between a higher PSAD and adverse PCa prognostic features.[25] A high PSAV and rapid PSADT, especially in the setting of post-treatment biochemical recurrence (BCR), is associated with an increased risk for castration resistance, metastases, as well as cancer-specific and overall mortality.[26]

9.3.2 Prostate health index (PHI)

Prostate health index (PHI; Beckman Coulter Inc, Brea, California, United States) combines three quantitative kallikrein immunoassays—tPSA, %fPSA, and [-2]proPSA (p2PSA)—via a mathematical algorithm into a single score. It received FDA approval in 2012 and is indicated as an adjunct to tPSA in the distinction of PCa from benign prostatic conditions in men aged over 50 years with tPSA between 4 and 10 ng/mL and nonsuspicious digital rectal examination (DRE) findings. It seeks to enhance the specificity of detecting overall and high-grade PCa. Numerous validation studies have addressed the clinical utility of PHI. A prospective, multi-institutional trial enrolled 892 men with no history of PCa, normal DRE, and tPSA of 2–19 ng/mL and found that within this range of PSA, at a sensitivity of 80–95%, the specificity and area under the curve (AUC) of PHI exceeded those of PSA and %fPSA.[27] Similarly, Stephan *et al.*'s evaluation of 1,362 patients with tPSA of 1.6–8.0 ng/mL who underwent systematic biopsy with ≥10 cores, showed that PHI (AUC, 0.74) outperformed %p2PSA (AUC, 0.72), p2PSA (AUC, 0.63), %fPSA (AUC, 0.61), and tPSA (AUC, 0.56) in predicting PCa.[28] Furthermore, these large series have demonstrated the advantage of PHI over %fPSA in distinguishing between high-grade disease (Gleason score ≥4+3) and low-grade disease or negative biopsies.[27,28] Catalona *et al.* showed that an increasing PHI was associated with a 4.7-fold increased risk for PCa and a 1.6-fold increase in higher-grade disease.[27] Other prospective studies have indicated the ability of PHI to detect aggressive (Gleason score ≥7) with more specificity than tPSA and %fPSA in biopsy-naïve men, reducing the need for unnecessary biopsies.[29]

The utility of PHI in detecting clinically significant prostate cancer (csPCa) was explored by Tosoian *et al.*, who analyzed the median PHI density (based on the prostate volume) of 118 patients with PSA >2 and negative DRE who underwent prostate biopsy. The median PHI density was higher for those with csPCa (1.21) compared with those with clinically insignificant PCa (0.70) and negative biopsies (0.53), with $p<0.001$. The authors used a threshold of 0.43 and found that PHI density was 97.9% sensitive and 38% specific for csPCa and 100% sensitive for Gleason ≥7 disease.[30] They concluded that discriminative ability of PHI density (AUC, 0.84) for csPCa was higher than PHI, PSA, PSAD, %fPSA, and prostate volume (AUC, 0.52–0.79). Additionally, up to 38% of unnecessary biopsies could be avoided while missing only 2% of csPCa.[30] Another advantage of PHI over %fPSA is the lack of influence of patient age and prostate volume.[27]

A recent study has evaluated the impact of PHI on clinical decision-making. White *et al.*'s observational study prospectively enrolled more than 500 men who had a PHI test and compared them with 683 men as a historical control group. The authors found that men who received a PHI test had a significantly lower biopsy rate compared with the control group (36.4% vs 60.3%, $p<0.0001$). Physician questionnaire responses also showed that the PHI test impacted the physicians' management plans including the decision to defer biopsies when PHI score was low and to perform biopsies when the PHI score was intermediate or high.[31] Similarly, Tosoian *et al.*'s prospective comparative analysis of 345 men receiving a PHI test demonstrated that PHI testing reduced the rate of biopsy procedures while the detection of higher-grade cancers remained unchanged.[32]

Another potential application of the PHI score is the integration into a multivariable model with multiparametric magnetic resonance imaging (mpMRI). This may be particularly useful in a patient with a negative prostate biopsy for whom a repeat biopsy is being considered. Gnanapragasam *et al.* prospectively evaluated the combined value

of PHI and mpMRI in men suspected of having PCa and needing a repeat biopsy.[33] The authors found that adding PHI to mpMRI improved the prediction of overall and csPCa (AUC, 0.71 and 0.75, respectively) compared with mpMRI or PSA alone (AUC, 0.64 and 0.69, respectively). A PHI threshold of 35 achieved a negative predictive value of 97%. In determining whether to re-biopsy men with a negative mpMRI, PHI performed better than PSA and PSAD in identifying csPCa. With a PHI threshold of >35, 42% of men avoided biopsy while only one of 21 significant cancers were missed.[33]

9.3.3 4Kscore

The 4Kscore (OPKO lab, Miami, Florida, United States) comprises a panel of four kallikrein (4K) markers—tPSA, %fPSA, intact PSA, and human kallikrein 2 (hK2)—and combines clinical variables (age, DRE findings, and previous biopsy status) into a model to predict the likelihood of having csPCa (Gleason score ≥7) on prostate biopsy. It may have value in reducing unnecessary prostate biopsies.[34] The merit of the 4Kscore in the pre-biopsy setting has been extensively evaluated, especially in Europe and the United States.[6,35–43] The algorithm was developed in accordance with data derived from the European Randomized Study of Screening for Prostate Cancer (ERSPC) and the Prostate Testing for Cancer and Treatment (ProtecT) trials.

Vickers *et al.* reported on 2,914 men in the ERSPC study who underwent prostate biopsy for PSA ≥3 ng/mL, where PCa was diagnosed in 28% of men.[40] Incorporating the 4Kscore with PSA and age significantly improved predictive accuracy with (AUC, 0.78 vs 0.70) and without (AUC, 0.76 vs 0.64) DRE results ($p<0.001$). The authors also concluded that for every 1,000 men, the addition of the 4Kscore would reduce 513 unnecessary biopsies, albeit with the trade-off that 12% of csPCa would be missed.[40] A smaller series of 262 men showed similar results, with the 4Kscore showing improved diagnostic accuracy than "base" clinical models incorporating PSA, age, and DRE findings only.[35] This series reported AUC increases from 0.63 to 0.78 in PCa prediction and 0.77 to 0.87 for prediction of high-grade PCa. Parekh *et al.* prospectively evaluated the diagnostic performance of the 4Kscore in 1,012 men undergoing prostate biopsy regardless of PSA or clinical findings where csPCa (Gleason ≥7) was found in 23% of patients.[6] Compared to the Prostate Cancer Prevention Trial Risk Calculator (PCPT-RC) 2.0 model, the 4Kscore increased the AUC (0.74 vs 0.82, $p<0.0001$) in detecting csPCa. The authors concluded that along with a higher predictive capacity for csPCa with the inclusion of the 4Kscore, up to 58% of biopsies could be avoided, with a delayed diagnosis in 1.3% to 4.7% of csPCa.

Further validation of the 4Kscore was undertaken by Stattin *et al.* in a large, representative cohort study from Sweden of 40,379 men, with 12,500 men followed for more than 15 years. PSA and kallikrein markers were measured in cryopreserved blood and the 4Kscore was studied for the predictive ability to detect the risk for distant metastases during long-term follow-up. The authors proposed a risk-stratification approach to prostate biopsy using PSA and 4Kscore thresholds. For instance, in patients with PSA ≥ 3 ng/mL, if a 4Kscore of 7.5% is used as a cutoff, the risk for distant metastases at 5, 10, 15, and 20 years would be much greater for those patients with 4K ≥7.5%, (high-risk group—2.4%, 5.6%, 9.9%, and 16.4%), compared to those patients with 4K < 7.5% (low-risk group—0%, 0.2%, 1%, and 1.8%).[41] Furthermore, the authors concluded that men in their 50s with a modest PSA elevation and low 4K risk could be exempt from biopsy with the reassurance of a low long-term risk of developing distant metastases.

The 4Kscore has been evaluated in men with previously negative prostate biopsy but persistently elevated PSA and found to have superior predictive capacity for high-grade cancers while minimizing unnecessary biopsies.[43] In a study cohort of 925 men, the 4Kscore was found to have higher discriminatory accuracy than PSA and DRE alone (AUC, 0.68 vs 0.58, $p<0.001$) in detecting PCa on repeat biopsy. For the prediction of high-grade (Gleason ≥7) PCa, the 4Kscore outperformed clinical factors alone (AUC, 0.87 vs 0.76. $p=0.003$). Furthermore, if a 4K risk threshold of 15% was used, 712 repeat biopsies would potentially be avoided in every 1,000 men, with only 3 of the 53 cancer diagnoses that were missed being Gleason ≥7.[43]

A comparative analysis of the 4Kscore and PHI was conducted in a population-based cohort study of 531 men with PSA 3 to 15 ng/mL undergoing initial prostate biopsies.[42] The predictive value of the tests were similar for predicting any PCa (AUC, 69 for 4K; AUC, 70.4 for PHI) as well as high-grade PCa (AUC, 71.8 for 4K; AUC, 71.1 for PHI).[42] Compared to a base model of age and PSA, both 4K and PHI had higher AUC ($p<0.0001$). Using high-grade PCa risk thresholds of 10% for 4K and 39 for PHI, 29% of biopsies could potentially be spared with the caveat that 10% of high-grade cancers could be missed.[42] Using simple serum tests such 4K and PHI in risk stratification during screening could be one option to reduce harm associated with unnecessary biopsies, but this must be used with caution due to the risk for delayed or missed diagnosis of a proportion of high-grade cancers.

Although there is a low level of evidence to support their use as primary screening test in the detection of PCa, both PHI and 4K scores are mentioned by the European Association of Urology (EAU), the American Association of Urology (AUA), and the National Comprehensive Cancer Network (NCCN) as potential marker tests that may be used to risk-stratify patients in the early detection of PCa. Their benefits of predicting the risk for high-grade PCa and reducing unnecessary biopsies in men with PSA ranging from 2 to 10 ng/mL is recognized, as is their superior performance over %fPSA based on prospective multicentre studies.[44] However, as no validated cutoff points or thresholds have been identified for either test, PHI and 4K must be used judiciously.

9.4 Urinary Biomarkers

As the recipient of prostatic secretions and cancer cells as well as a convenient, easy-to-obtain biological specimen, urine is a precious commodity when it comes to the discovery of biomarkers in prostate cancer. Although many potential urinary biomarkers have been identified, only PCA3 has received FDA approval for clinical use. In this section, we discuss the commercially available, FDA- and CLIA-approved urinary biomarkers in the diagnosis and risk stratification of PCa.

9.4.1 PCA3

Progensa prostate cancer antigen-3 (PCA3; Hologic, Marlborough, Massachusetts, United States) is a noncoding large-chain RNA that is highly overexpressed in the majority of malignant prostate tissue compared to benign prostate tissue.[45,46] PCA3 is detectable in the urine of men with prostate cancer and the Progensa PCA3 gene assay measures PCA3 mRNA concentrations in the first void urine collected after DRE.[47] PCA3 is thought to be independent of prostate size and serum PSA levels.[48,49] In 2012, the FDA approved the use of PCA3 to facilitate the

decision-making process to re-biopsy men with a previous negative biopsy. Although the exact function of PCA3 is largely unknown, multiple studies have evaluated the clinical utility of the PCA3 assay in the early detection of PCa and as a prognostic marker in the active surveillance of patients with low-risk PCa.[50–52]

A key European, multicentre, prospective study by Haese *et al.* enrolled 463 men who had undergone previous negative biopsies and were scheduled for a repeat biopsy.[49] Their post-DRE, first-void urine samples were tested for PCA3 mRNA, and the PCA3 assay scores were correlated with repeat biopsy outcomes. The study demonstrated that a higher PCA3 score was associated with a greater probability of a positive repeat biopsy. Men who had a PCA3 score ≥35 had a 39% chance of having a positive biopsy compared with 22% in men with a PCA3 score < 35 ($p<0.0001$). The mean PCA3 score was significantly higher in men with a positive biopsy than those with a negative biopsy (63.8 vs 35.5, $p<0.0001$). In univariate analyses, PCA3 was found to be a statistically significant independent predictor of PCa on repeat biopsy and to significantly improve the predictive accuracy in multivariate analyses. Furthermore, a PCA3 threshold of 35 provided greater diagnostic accuracy than a comparable %fPSA cutoff of 25%. PCA3 was found to be independent of tPSA, prostate volume, and patient age.[49]

Further supporting the utility of PCA3 in patients with a prior negative biopsy, Wu *et al.* conducted a single-centre retrospective analysis of 103 men and found that using a PCA3 score threshold of 25 yielded a sensitivity and a specificity of 0.67 and 0.64, respectively.[53] In their multivariate analysis, although PCA3 was found to be independently associated with PCa with an AUC of 0.64, the highest diagnostic accuracy was derived from a multivariable model comprising PCA3, PSAD, PSA, DRE, and transrectal ultrasound (TRUS) findings (AUC, 0.82).[53]

A prospective validation trial by Wei *et al.* sought to assess the diagnostic accuracy of PCA3 in the early diagnosis of PCa in 859 PSA-screened patients undergoing initial biopsy and a repeat biopsy after prior negative biopsy.[52] For the detection of any PCa, the authors demonstrated a positive predictive value (PPV) of 80% for PCA3 > 60 at initial biopsy and a negative predictive value (NPV) of 88% for PCA3 < 20 at repeat biopsy. They highlighted the value of adding PCA3 to risk estimation models to improve the stratification of PCa. Their findings supported the use of PCA3 in reducing unnecessary biopsies in men with a prior negative biopsy and concluded that for initial biopsy, a PCA3 > 60 significantly increases the probability of cancer detection.[52]

In another European, prospective, multicentre study, de la Taille *et al.* evaluated 516 men with PSA ranging from 2.5 to 10 ng/mL, scheduled for initial biopsy, comparing the diagnostic accuracy of PCA3 to total PSA, PSAD, and %fPSA. The authors observed that the mean PCA3 score was higher in a positive versus a negative biopsy (69.6 vs 31.0, $p<0.0001$). PCA3 was also higher in men with csPCa (Gleason ≥7, >33% positive cores, and significant vs indolent cancer). PCA3 scores > 35 had the highest diagnostic accuracy, with a sensitivity of 64% and a specificity of 76%. The authors also highlighted that the PCA3 score was independent of age, total PSA, and prostate volume, and outperformed total PSA, PSAD and %fPSA.[54]

The ability of PCA3 to predict tumour volume and assist in selecting low-risk patients for active surveillance has been studied in Ploussard *et al.*'s prospective evaluation of 106 low-risk PCa patients prior to radical

prostatectomy. The authors reported that PCA3 scores strongly correlated with tumour volume.[55] A PCA3 score >25 was associated with a threefold increase in the risk for csPCa. On multivariate analysis, a PCA3 score >25 was a predictive factor for tumour volume >0.5cm^3 (OR, 5.4; p=0.01) and for significant cancer (OR, 12.7; p=0.003). Using their predictive cutoff of 25, the authors concluded that PCA3 is indicative of tumour volume and the presence of significant cancer and can be a useful tool in selecting better candidates for active surveillance.[55] Nakanishi *et al.* also found a significant association between PCA3 and tumour volume as well as Gleason grade in prostatectomy specimens, with PCA3 being the best predictor of tumour volume on multivariate analysis.[48] Additionally, PCA3 had the ability to detect low tumour volume of <0.5cc (AUC, 0.757), making it a potentially useful tool in the selection of patients with low-risk PCa.

As PCA3 is the only urinary biomarker with FDA approval, the various guidelines do mention the use of urinary PCA3 to risk-stratify patients after a previous negative biopsy and to determine the need for a repeat biopsy. American Urologic Association (AUA) guidelines do not recommend PCA3 as a primary screening tool, but it may be used in conjunction with other markers to determine the need for prostate biopsy. However, guidelines have not defined a threshold for PCA3 score to guide decision-making. And as no direct correlation has been made between PCA3 scores and malignant tumour characteristics, PCA3 is not currently recommended as a tool in monitoring or guiding active surveillance decisions.

9.4.2 ExoDx Prostate Intelliscore

The ExoDx Prostate Intelliscore (EPI; Exosome diagnostics, Waltham, Massachusetts, United States) is a newer, novel urine exosome gene expression assay used to predict the risk for PCa on biopsy. Exosomes are one of two types of microvesicles found in prostate secretions.[56] Exosomes may be secreted by normal and malignant tissues, but elevated exosome secretions have been found in malignant biofluids such as the urine of patients with PCa. Exosomes encompass a portion of the parent cell cytoplasm containing proteins and RNA that closely resemble the cell of origin.[57] They lack ribosomal RNA but are rich in mRNA, which acts as a unique footprint of specific tumour cells and may give information about the specific tumour genotype that underlies the varying phenotypes that are seen in a heterogeneous disease such as PCa. Exosomes isolated from post-DRE urine contain PCA3 and ERG (erythroblastosis virus E26 oncogene homologues).[57] The EPI test has the advantage of not requiring a pre-collection DRE, making it noninvasive and convenient for patients and clinicians. The test uses an algorithm independent of clinical variables to provide a risk score, with 15.6 being the threshold between high-grade and low-grade PCa.[58]

McKiernan *et al.* compared the performance of the EPI assay with biopsy outcomes in men with PSA ranging from 2 to 20 ng/mL, then went on to validate the prognostic score.[59] They found that incorporating EPI into the standard of care (SOC) improved the discrimination of high-grade disease from low-grade or benign disease. Validation in 519 patients showed the superior performance of EPI plus SOC (AUC, 0.73) over SOC alone (AUC, 0.63) in predicting high-grade disease (p<0.001). Using an EPI threshold score of 15.6, they concluded that 27% of biopsies could have been avoided, missing 5% of high-risk cancers.[59]

A recent prospective, randomized, clinical utility study by Tutrone *et al.* examined the clinical utility and the impact of the EPI score on decision-making in men presenting for initial prostate biopsy with PSA of 2–10 ng/mL.[58] A total of 1,094 patients and 72 urologists were involved in the study. Of the urologists, 68% were purportedly influenced by the EPI score in their decision to recommend or defer a biopsy, the main reason for noncompliance with EPI results being a rising PSA. Of the patients, 87% with a positive EPI score were recommended to proceed to biopsy, leading to the detection of 30% more high-grade PCa than the control arm. On the other hand, 63% of patients with a negative EPI score were recommended to defer prostate biopsy, and the authors estimated that 49% fewer high-grade cancers were missed due to biopsy deferral compared to SOC.[58]

The latest NCCN guidelines do mention EPI as a potential investigative marker, but it is not currently in mainstream practice.

9.4.3 MiPS

The Mi-Prostate Score (MiPS; Detroit, Michigan, United States) assay was developed at the University of Michigan Rogel Cancer Center. It is a urine multiplex analysis that combines PSA with two PCa-specific biomarkers—PCA3 and an RNA marker that is found only when TMPRSS2 and ERG abnormally fuse. ERG is an erythroblast transformation (ET) transcription factor that is overexpressed in 57% of prostate cancers.[60] In more than 90% of cases that overexpressed ERG, there was found to be fusion with TMPRSS2, with the fusion possibly leading to the overexpression. TMPRSS2-ERG (T2-ERG) fusion is thought to occur in 50% of PCa cases and is a strong indicator of PCa.

In a trial of 48 patients undergoing prostate biopsy, Salami *et al.* found an association between the presence of T2-ERG in post-DRE urine and PCa (OR, 12.02; $p<0.001$).[61] Although PCA3 had higher sensitivity (93%), T2-ERG had the highest specificity (87%) in predicting PCa. T2-ERG had better discriminative ability (AUC, 0.77) compared with PCA3 (AUC, 0.65) and serum PSA (AUC, 0.72). Combining all three factors into a multivariable algorithm improved the AUC for cancer prediction to 0.88, with a specificity of 90% and a sensitivity of 80%, and the combination was superior to any individual marker alone.[61] Earlier trials have compared T2-ERG alone with the combination of T2-ERG and PCA3. While individual sensitivities range from 32% (T2-ERG) to 62% (PCA3), the combination of the markers improved the sensitivity to 73%.[62] T2-ERG had a PPV of 94% in predicting PCa in those with a history of negative biopsies and a persistently elevated PSA, making it a potential tool in the decision to re-biopsy a patient.[62] Laxman *et al.* showed that compared to PCA3 alone (AUC, 0.662), T2-ERG in combination with PCA3 and a multiplex panel of urinary transcripts (AUC, 0.758) improved the early detection of PCa.[63]

A large validation trial by Tomlins *et al.* in 1,225 patients assessed the ability of the MiPS multivariable model incorporating PSA, PCA3, and T2-ERG in predicting PCa and high-grade PCa on biopsy.[64] The authors showed that models incorporating T2-ERG had higher AUC than PSA in predicting any PCa (0.693 vs 0.585) and high-grade PCa (0.729 vs 0.651). The MiPS model integrating T2-ERG outperformed other models that included only PCA3 and PSA in the detection of PCa ($p<0.001$).[64]

The association between ERG overexpression and biochemical recurrence has been studied in a cohort of 1,180 men treated with radical prostatectomy.[65] Pettersson *et al.* showed that while T2-ERG or ERG overexpression was associated with tumour stage at diagnosis, there is no significant association with biochemical recurrence or lethal disease, suggesting that MiPS is not useful in predicting recurrence or mortality in men who have undergone radical prostatectomy.[65]

The Mi-Prostate Score is currently an investigational tool according to NCCN guidelines and not routinely used in mainstream practice.

9.4.4 SelectMDx

SelectMDx (MDx Health, Irvine, California, United States) is a post-DRE urine-based gene assay risk score that aims to predict a patient's risk for high-grade PCa. The algorithm measures the mRNA signatures of two genes implicated in prostate carcinogenesis consisting of homeobox C6 (HOXC6) and distal-less homeobox 1 (DLX1)—and combines these with clinical factors such as age, family history, previous negative biopsies, and DRE findings. Leyten *et al.* described a 3-gene urinary panel including HOXC6 and DLX1 that was shown to have additional value over serum PSA and PCA3 in the detection of PCa and high-grade PCa and reducing the risk for overtreatment.[66]

Van Neste *et al.* developed a multivariable model incorporating HOXC6 and DLX1 on a cohort of 519 patients and subsequently validated the risk score in an independent cohort of 386 patients in two prospective multicentre studies.[7] The mRNA levels were measured by reverse transcriptase PCR of post-DRE urine prior to prostate biopsy. The authors identified the mRNA signature risk score in combination with PSAD and previous negative biopsies to be the most significant factors, with an overall AUC approaching 0.90 (95% CI, 0.85–0.95). Another model adding DRE as a risk factor was also tested, with an AUC of 0.86. When comparing this model to the Prostate Cancer Prevention Trial Risk Calculator (PCPT-RC) and PCA3, the authors found that adopting this model could reduce biopsies by 42% and reduce unnecessary biopsies by 53%, with a NPV of 98% for Gleason ≥7 disease.[7]

Correlating SelectMDx with mpMRI results, Hendriks *et al.*'s retrospective observational study of 172 patients reported a positive association between the risk score and the final Prostate Imaging Reporting and Data System (PI-RADS) grade.[67] Median SelectMDx scores were higher in patients with an MRI suspicious of a significant lesion compared with those with a negative mpMRI ($p<0.01$). SelectMDx was also shown to have some value in predicting the mpMRI result, with an AUC of 0.83 compared to PSA (AUC, 0.66) and PCA3 (AUC, 0.65). The authors concluded that SelectMDx would be a useful adjunct in identifying patients at risk for high-grade PCa and selecting patients for radiological diagnostics.[67]

The cost-effectiveness of SelectMDx compared to SOC was evaluated by Dijkstra *et al.*, who concluded that the judicious use of SelectMDx to reduce the overdiagnosis and overtreatment of PCa in men with PSA > 3 ng/mL may lead to reduction in costs and gains in quality-adjusted life years (QALY).[68]

9.5 Future Challenges and Directions

The past decade has witnessed an ever-growing array of prostate cancer biomarkers. Many are clinically validated to improve on clinical parameters alone by more accurately determining prognosis and specific oncologic outcomes. However, whether a genomic test may be predictive of a clinically significant response to a particular management strategy is a more complex question. Retrospective data indicates that genomic classifiers may improve risk stratification; however, their clinical utility in decision-making has not been validated prospectively or in clinically meaningful outcomes such as decreasing morbidity from treatment and/or disease.

Genomic tests must add value beyond existing multivariable models used in clinical practice in order to be of benefit to the management of men with prostate cancer. For example, genomic tests used in a prediagnostic setting should yield improvements not only on PSA but also on existing validated models that incorporate clinical parameters such as age, PSA, clinical stage, biopsy Gleason score, family and medical history, and ethnicity. Similarly, genomic tests used after treatment must improve on readily available multivariable models that incorporate known pathological risk parameters. What constitutes incremental improvement in risk determination remains unknown, and changes in AUC with the incorporation of genomic tests often yield very modest improvements. Given the costs associated with many of the genomic tests used in clinical practice, merely providing reassurance and reinforcing a clinical decision already supported by current evidence-based guidelines is insufficient and may not be cost-effective.

TABLE 9–2 Modified Consensus Statements on the Use of Biomarkers in PCa. *(Adapted from Cooperberg MR, Carroll PR, Dall'Era MA,* et al. *The state of the science on prostate cancer biomarkers: The San Francisco Consensus Statement*. Eur Urol. *2019;76(3):268–272. doi:10.1016/j.eururo.2019.05.013).*[9]

1.	Potential biomarkers must improve on existing validated multivariable models that incorporate all available clinical information in order to be clinically useful.
2.	Biomarker study outcomes should be tailored to patients' disease states and capture information that can impact on a patient's quantity and quality of life.
3.	The results of biomarker studies must be extrapolated with caution in populations that are different from the study population.
4.	Randomized controlled trials of biomarkers in diverse populations of men are necessary to fill the gaps in knowledge regarding the utility of biomarkers in the various stages of prostate cancer.
5.	Clinicians should consider the cost-effectiveness of the various biomarkers and ensure that the costs are balanced with the benefits for the management of men with prostate cancer.
6.	Clinicians ordering biomarker studies must be able to have an open discussion with the patient regarding the application of results in the context of the patient's individual situation.

Biomarkers, as with any other type of investigation, need to lead to improved clinical outcomes by driving changes in treatment decisions including timing and intensity of treatment as specified by that individual patient's risk. As outlined in this chapter, the available serum and urinary biomarkers have been tested for numerous endpoints but none one of them are perfect, and clinicians must be judicious in their use and application. Cooperberg *et al.* have summarized their recommendations regarding the use of PCa biomarkers in the San Francisco consensus statements (**Table 9–2**).[9]

Biomarkers offer great potential to optimize the care for men with prostate cancer. However, clearly defining the optimal genomic test for the right patient under the right circumstances such that it meaningfully impacts outcome warrants further study.

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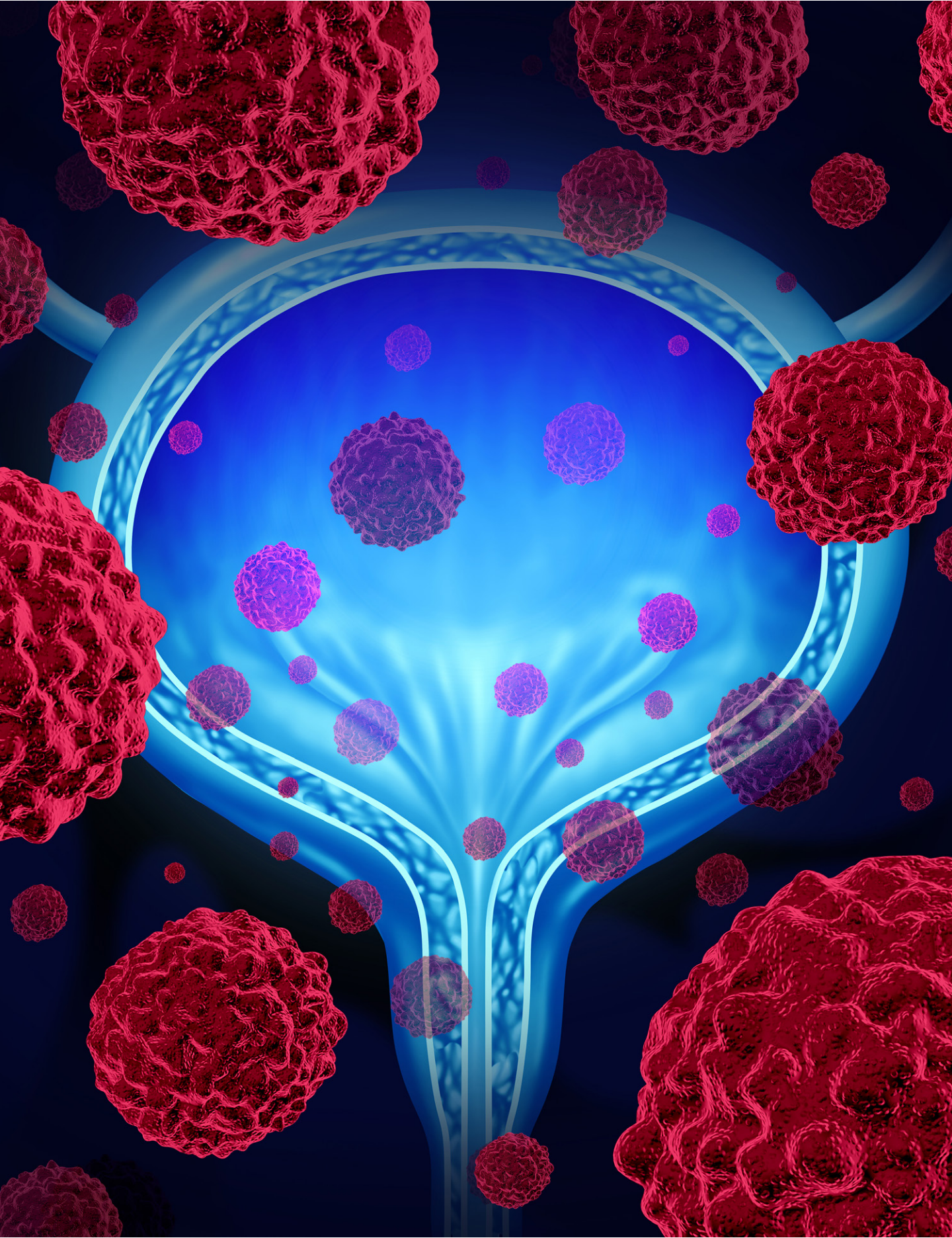

CHAPTER 10

Urinary-Based Markers for Bladder Cancer Detection

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Table of Contents

10.1 Introduction

Cystoscopy still represents the gold standard for diagnosis of bladder cancer. However, cystoscopy represents a procedure that can be associated with discomfort for the patient. Moreover, nonpapillary lesions (eg, carcinoma in situ [CIS]) may be missed by white light cystoscopy, leading to controversies about its value as a gold standard diagnostic procedure. Therefore, significant efforts have been made in the past two decades to improve the sensitivity of cystoscopy (eg, blue light cystoscopy) and develop diagnostic tests that may replace cystoscopy in the future. Some of these tests have been approved by the US Food and Drug Administration (FDA); however, cytology remains the only test with a clear recommendation by current guidelines (European Association of Urology [EAU], American Urological Association [AUA]). Continuous efforts are made to develop markers not only for diagnostic purposes but also for prediction of therapy response and monitoring of disease. These efforts include the use of high-throughput technologies such as next-generation sequencing. In this chapter, several approaches for urine-based detection or analysis of bladder cancer and their current roles in clinical management of patients with bladder cancer will be discussed. These approaches include cell-based detection systems, DNA- and RNA-based approaches, and platforms for protein analysis.

10.2 DNA-Based Detection of Bladder Cancer

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Bladder cancer is known to have a high mutational burden. In other words, most tumour cells in bladder cancer harbour many different mutations.[1,2] Various types of DNA alterations can occur in bladder cancer, such as mutations, copy number alterations (CNAs, deletions or gains of parts of a gene), and genomic rearrangements (coupling of two unrelated genes). Until now, none of the DNA-based urine assays that are based on detection of mutations have been FDA approved for use in the clinic. Prospective validation of most urine assays is still awaited.

10.2.1 Urine DNA

Urine contains mostly water (91–96%).[3] Besides water, urine contains several chemical components, such as nitrogen (eg, in the form of urea), phosphorus, calcium, potassium, and creatinine. Lastly, urine contains organic components, for instance inflammatory cells, urothelial cells, and sometimes bacteria.[3] As the largest component of urine is water, the variation in concentration of all other components is highly dependent on the patient's fluid intake. Therefore, the concentration of DNA in the urine depends highly on the fluid intake of the patient. The minimum volume of urine needed to sufficiently analyze for DNA biomarkers is thus different in every patient. In most studies, between 10 and 100 mL of urine is used.[4–8]

Urine can be analyzed as whole urine or it can be spun down to separate the cell pellet from the supernatant. The cell pellet can be used to extract cellular DNA, and the supernatant can be used to extract cell-free DNA. Overall, DNA is very stable and is therefore relatively easy to process.

When using the urine's cell pellet to extract the DNA, one must realize that inflammatory cells also contain a nucleus and could therefore potentially influence the detection of oncogenic DNA alterations, due to a larger amount of normal background DNA.

10.2.2 DNA mutations

FGFR3

When a specific growth factor (for example, a cytokine or hormone) binds to the extracellular domain of a receptor tyrosine kinase (RTK), the intracellular domain is activated. This activation results in the stimulation of a downstream cascade that can affect various processes in the cell, such as cell proliferation and growth, eventually resulting in cell division. The RTK-RAS-MAPK signalling pathway and the RTK-PI3K-Akt signalling pathway are some of the most frequently affected signalling pathways in cancer.[9] Fibroblast growth factor receptor 3 (*FGFR3*) is a type of RTK that is especially important in bladder cancer. Approximately two-thirds of non-muscle invasive bladder cancers (NMIBCs) have activating *FGFR3* mutations. The number of activating mutations is much lower in muscle-invasive bladder cancer (MIBC),[10] with only <15% of tumours harbouring *FGFR3* mutations; however; >40% of MIBCs overexpress *FGFR3*.[11] Tumours with an *FGFR3* mutation grow slowly and are less likely to progress to MIBC than wild-type *FGFR3* tumours.[11–14] Several hotspot mutations in the *FGFR3* gene have been identified as oncogenic.

RAS

There are three known *RAS* genes, which code for *KRAS*, *NRAS*, and *HRAS*. RAS proteins are GTPases and can be in an inactivated state when bound to guanosine diphosphate, or they can be activated when bound to guanosine triphosphate.[15] Activation leads to downstream signalling and oncogenic mutations may result in a constant state of activation. Mutations in the *KRAS* gene are most frequent in cancer. For instance, *KRAS* is mutated in 90% of pancreatic cancers and 45% of colorectal cancers.[16] In bladder cancer, *HRAS* is the most commonly mutated *RAS* gene; *HRAS* mutations are present in approximately 5% of bladder tumours.[1]

PIK3CA

Phosphatidylinositol-3-kinase (PI3K) consists of a regulatory subunit and a catalytic subunit.[17] The *PIK3CA* gene encodes for the catalytic subunit: phosphatidylinositol-4,5-bisphosphate-3-kinase catalytic subunit α. Approximately 20% of bladder cancer tumours harbour a mutation in the *PIK3CA* gene.[1] PI3K can be activated by RTKs or via crosstalk via the RTK-RAS-MAPK pathway.

TERT

Mutations in the telomerase reverse transcriptase (*TERT*) gene are frequent in bladder cancer, with >70% of bladder tumours harbouring a *TERT* promotor mutation.[18] Telomeres are protective repetitive nucleotide sequences at the end of a chromosome and shorten at every cell cycle. Telomerase increases the telomere length, thereby increasing the amount of possible cell divisions a single cell can make. The presence of a *TERT* mutation was found to be more frequent in tumours that also harboured *FGFR3* mutations; however, it was not associated

with the stage or grade of the tumours.[18] Overall, significant overlap between different mutations occurs, albeit not mutually exclusive.

10.2.3 Gene hypermethylation

Genome-wide methylation analyses have identified several genes that are significantly hypermethylated in bladder cancer cells versus normal urothelial cells.[19] Methylation of several genes was found to be useful for the diagnosis of bladder cancer, with some markers being highly specific for bladder cancer. Gene hypermethylation has also been proposed in predicting disease progression.[20,21]

10.2.4 DNA-based urinary biomarker assays

In the literature, various DNA-based urinary markers have been developed. For most of these, prospective validation is still lacking. The urine-based markers suggested in the literature can be subdivided into markers used for detection of primary tumours (eg, in a patient with hematuria) or markers used for detection of recurrent tumours (eg, in a patient previously treated for bladder cancer). Furthermore, as mentioned previously, markers based on cell pellet DNA exist, as well as cell-free DNA-based markers.

Mutation marker assays

In 2010, Zuiverloon *et al.* described that *FGFR3* mutations in the urine of patients treated for NMIBC increase the risk for recurrence by 3.8 fold over a 3.5-year follow-up period. Only 11% of *FGFR3*-negative urine samples were associated with a recurrence of bladder cancer.[22] In this study, the recurrence was not always directly detected at the concomitant cystoscopy; however, it did occur over time. In another study that included 191 patients, 74 (39%) had a positive analysis before transurethral resection (TUR) (*FGFR3* mutation group), and *FGFR3* mutation analysis showed a sensitivity of 0.73 and a specificity of 0.87.[23] The presence of an *FGFR3* mutation at the time of diagnosis was associated with a shorter time to recurrence (p=0.02). Patients with a positive urine test had 70% of recurrences during the 2-year follow-up.[23] Christensen *et al.* analyzed multiple urine supernatants from several NMIBC patients and patients undergoing radical cystectomy for the presence of *FGFR3* mutations and *PIK3CA* mutations. High levels of tumour DNA in urine supernatants of patients with NMIBC were associated with disease progression.[24] High levels of tumour DNA in urine supernatants of patients who had radical cystectomy was associated with a worse recurrence-free survival.

Since 2013, several studies have been conducted with single or combined use of *TERT* mutation analysis in urine samples.[5,6,18,25] A study by Allory *et al.* demonstrated the feasibility of the detection of *TERT* mutations in urine by SNaPshot® analysis. Sensitivity of detection of bladder cancer was higher in patients with primary tumours (62%) than in patients with recurrent tumours (42%). Interestingly, *TERT* mutations were also detected in patients without a bladder tumour (specificity of 73%). Combining *TERT* mutation analysis with *FGFR3* mutation analysis improved sensitivity of the assay in patients with primary tumours to 70% and in patients with recurrent tumours to 50%.[18] Combining *TERT* mutation detection with *FGFR3* mutation detection was also attempted by Critelli *et al.*[5] In fact, a panel of mutations was analyzed combining *TERT*, *FGFR3*, *PIK3CA*, and *RAS* gene mutations. The

combination of *TERT* and *FGFR3* mutations resulted in a sensitivity of 67%; adding *RAS* and *PIK3CA* mutations increased the sensitivity by another 2%. The authors therefore concluded that a combination of *TERT* and *FGFR3* mutations would be most interesting.[5] Another panel of mutations was tested by Ward *et al.* using multiplex polymerase chain reaction (PCR) and next-generation sequencing.[25] This technique was preferred by the authors due to low DNA input requirements. The mutation panel included the following genes: *TERT*, *FGFR3*, *TP53*, *HRAS*, *KDM6A*, and *RXRA*. DNA extracted from cell pellets was analyzed and resulted in an overall sensitivity of 70% and a specificity of 97% (see **Table 10.2–1** for mutation marker assays).

TABLE 10.2–1 Mutation Marker Assays

Reference	Year	Gene	Technique	Population	Sens, %	Spec, %
Zuiverloon *et al.*[22]	2010	*FGFR3*	SNaPshot analysis	Surveillance	58	86
Couffignal *et al.*[23]	2015	*FGFR3*	PCR	Surveillance	73	87
Christensen *et al.*[24]	2017	*FGFR3*, *PIK3CA*	ddPCR	NMIBC MIBC	36 11	NR NR
Descotes *et al.*[6]	2017	*TERT*	PCR	Surveillance	81	90
Allory *et al.*[18]	2014	*TERT* FGFR3 *FGFR3*, *TERT*	SNaPshot analysis	Hematuria Surveillance Hematuria Surveillance Hematuria Surveillance	62 42 36 19 70 50	90 73 NR 90 NR 71
Critelli *et al.*[5]	2016	*TERT*, *FGFR3*, *PIK3CA*, *RAS*	SNaPshot analysis	NMIBC (primary) - All genes - *TERT* - *FGFR3* - *TERT* + *FGFR3* - *PIK3CA* - *RAS* MIBC (primary) - *TERT* - *FGFR3* - *PIK3CA* - *RAS*	 69 52 42 67 13 5 80 17 4 0	 NR NR NR NR NR NR NR NR NR NR
Ward *et al.*[25]	2016	*TERT*, *FGFR3*, *TP53*, *HRAS*, *KDM6A*, *RXRA*	Multiplex PCR, next-generation sequencing	Primary/recurrent	70	97

Abbreviations: ddPCR, droplet digital polymerase chain reaction; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer; NR, not reported; PCR, polymerase chain reaction; Sens, sensitivity; Spec, specificity.

Methylation marker assays

One of the first to report a urine-based biomarker assay considering the methylation status of genes was Renard *et al*. A two-gene panel containing the methylation status of the *TWIST1* and *NID2* genes demonstrated high sensitivity (90%) and also high specificity (93%) for the presence of bladder cancer.[26] Methylation status in this study was determined via methylation-specific polymerase chain reaction (MSP). An attempt to externally validate these results did not yield the same level of performance, with a sensitivity of 79% and a specificity of 63%. However, these markers were still considered potentially useful in future assay development.[27]

In 2014, Su *et al*. suggested a three-gene methylation panel analysis via pyrosequencing, containing the *SOX1*, *IRAK3*, and *L1-MET* genes.[28] After internal validation, the assay resulted in a predictive capacity of 95%, as represented by the area under the curve (AUC) in the receiver operating characteristics analysis. The authors concluded that this urinary-based biomarker assay may reduce invasive cystoscopies in patients and identify patients at higher risk for disease recurrence. In another pilot study, quantitative methylation–specific PCR was employed to evaluate an eight-gene panel on the tumour tissues of patients in follow-up after treatment for NMIBC.[29] The genes included in this assay were: *ARF*, *TIMP3*, *RAR-β2*, *NID2*, *CCNA1*, *AIM1*, *CALCA*, and *CCND2*. Only the methylation status of *NID2*, *CCNA1*, and *CCND2* was significantly associated with recurrence of NMIBC. Three genes were also analyzed in urine from patients under surveillance. Interestingly, one of the three suggested markers was positive in 83% of cytology-negative bladder cancer cases. Prospective validation is still awaited.

Finally, a large 150 CpG loci biomarker panel called the UroMark assay was recently presented.[30] This next-generation bisulphite sequencing assay was evaluated in two independent case-control cohorts and resulted in an AUC of 97%, with a sensitivity of 98% and a specificity of 97%. This assay awaits prospective validation (see **Table 10.2–2** for methylation marker assays).

TABLE 10.2–2 Methylation Marker Assays

Reference	Year	Gene	Technique	Population	Sens, %	Spec, %
Renard *et al.*[26]	2010	*TWIST1*, *NID2*	MSP	Hematuria	90	93
Abern *et al.*[27]	2014	*TWIST1*, *NID2*	MSP	All (Renard threshold validation) All (new threshold) - Hematuria - Surveillance	79 75 38 58	63 71 86 66
Su *et al.*[28]	2014	*SOX1*, *IRAK3*, *L1-MET*	Pyrosequencing	Surveillance - Test - Validation	 86 89	 80 97
Maldonado *et al.*[29]	2014	*CCNA1*, *CALCA*, *CCND2*	Quantitative MSP	Surveillance	73	70
Feber *et al.*[30]	2017	150 CpG loci (UroMark)	Next-generation bisulphite sequencing	Hematuria	98	97

Abbreviations: MSP, methylation-specific polymerase chain reaction; Sens, sensitivity; Spec, specificity.

Combined marker assays

Several studies have assessed the use of various combinations of DNA-based biomarkers (eg, combined mutation and methylation assays). *FGFR3*, *PIK3CA*, and *RAS* mutation analysis was combined with microsatellite analyses and methylation analysis in 136 patients in follow-up after treatment for NMIBC.[31] Sensitivity of the assay varied between 66% and 68% after patient stratification based on tumour DNA analysis. Kandimalla *et al*. combined the methylation analysis of three genes (*OTX1*, *ONECUT2*, and *OSR1*) with *FGFR3* mutation analysis.[32] Sensitivity increased from 74% to 79% when *FGFR3* mutation analysis was included in the assay. Another study combined methylation status of *HS3ST2*, *SLIT2*, and *SEPTIN9* genes with the mutation status of *FGFR3* and the age and smoking status clinical parameters.[33] Roperch *et al*. found a sensitivity of 97% in a primary diagnostic setting versus a sensitivity of 90% in a surveillance setting.

Mutations in *TERT* and *FGFR3* were combined with the methylation status of *SALL3*, *ONECUT2*, *CCNA1*, *BCL2*, *EOMES*, and *VIM* in a study by Dahmcke *et al.*[34] In 475 patients, 99 bladder cancer cases and 376 benign cases, the assay showed an AUC of 94% in patients presenting with gross hematuria. In a large international study of patients in follow-up after treatment for NMIBC, almost 2,500 urine samples from 977 patients were analyzed for mutation status of *FGFR3* and *TERT* and mutation status of *OTX1*.[35] Sensitivity was 57% in patients with primary low-grade (LG) tumours and increased to 72% for patients with primary high-grade (HG) tumours. In 2016, a study was published describing a urine-based assay including the mutation status of *FGFR3*, *TERT*, and *HRAS* combined with the methylation status of *OTX1*, *ONECUT2*, and *TWIST1* combined with the age of the patients.[8] The assay resulted in an optimism-corrected AUC of 92%, with a sensitivity of 97% and a specificity of 83%. The assay was externally validated in an independent patient cohort and proved robust with an AUC of 96%.[36] Finally,

Springer *et al*. describe the UroSEEK assay, which combines mutations in 11 genes and copy number changes on chromosome 39.[37] Sensitivity was 83% in patients presenting with hematuria and 68% in patients in follow-up after treatment for NMIBC. Combining the assay with cytology increased sensitivity to 95% in the hematuria patients and to 71% in the surveillance cohort (see **Table 10.2–3** for combined marker assays).

TABLE 10.2–3 Combined Marker Assays

Reference	Year	Gene	Technique	Population	Sens, %	Spec, %
Zuiverloon *et al*.[31]	2013	Mutation: *FGFR3*, *PIK3CA*, *RAS* Methylation: 41 CpG islands in 23 genes Microsatellite: 12 different primers	SNaPshot analysis Methylation-specific MLPA	Surveillance - *FGFR3* - Cytology - *FGFR3* + cytology - *FGFR3* + microsatellite - *FGFR3* + methylation Surveillance, patients stratified by inclusion tumour - *FGFR3* - *FGFR3* + *PIK3CA* + *RAS* - Microsatellite - *FGFR3* + microsatellite - Methylation - *FGFR3* + methylation	 49 56 76 71 75 66 71 67 82 68 75	 66 57 42 44 22 50 63 40 82 42 22
Kandimalla *et al*.[32]	2013	Mutation: *FGFR3* Methylation: *OTX1*, *ONECUT2*, *OSR1*	SNaPshot analysis	Surveillance - Methylation only - Combined	 74 79	 90 77
Roperch *et al*.[33]	2016	Mutation: *FGFR3* Methylation: *HS3ST2*, *SLIT2*, *SEPTIN9* Clinical parameters: age, smoking status	Allele-specific PCR Quantitative multiplex-MSP	Hematuria Surveillance	97 90	84 65
Dahmcke *et al*.[34]	2016	Mutation: *TERT*, *FGFR3* Methylation: *SALL3*, *ONECUT2*, *CCNA1*, *BCL2*, *EOMES*, *VIM*	ddPCR	Hematuria - All - *TERT* - *FGFR3* - *SALL3* - *ONECUT2* - *CCNA1* - *BCL2* - *EOMES* - *VIM* - *TERT*, *FGFR3*, *ONECUT2*, *CCNA1*	 97 82 41 68 78 67 63 46 76 97	 77 84 98 97 94 97 98 97 96 80

TABLE 10.2–3 Combined Marker Assays *(Cont'd)*

Reference	Year	Gene	Technique	Population	Sens, %	Spec, %
Beukers *et al.*[35]	2017	Mutation: *FGFR3*, *TERT* Methylation: *OTX1*	SNaPshot analysis	Surveillance - Previous LG - Previous HG	 57 72	 59 55
van Kessel *et al.*[8]	2016	Mutation: *FGFR3*, *TERT*, *HRAS* Methylation: *OTX1*, *ONECUT2*, *TWIST1* Clinical parameter: age	SNaPshot analysis MSP	Hematuria	97	83
van Kessel *et al.*[36]	2017	Mutation: *FGFR3*, *TERT*, *HRAS* Methylation: *OTX1*, *ONECUT2*, *TWIST1* Clinical parameter: age	SNaPshot analysis MSP	Hematuria	93	86
Springer *et al.*[37]	2018	Mutations in 11 genes Copy number changes on chromosome 39 Cytology	Multiplex PCR Singleplex PCR Aneuploidy assays	Primary - 10 genes combined - *TERT* - Aneuploidy - UroSEEK (assays combined) - Cytology - UroSEEK + cytology UTUC - 10 genes combined - *TERT* - Aneuploidy - UroSEEK (assays combined) Surveillance - 10 genes combined - *TERT* - Aneuploidy - UroSEEK (assays combined) - Cytology - UroSEEK + cytology	 68 57 46 83 43 95 64 29 39 75 52 57 28 68 25 71	 NR NR NR 93 100 93 NR NR NR NR NR NR NR 80 100 82

Abbreviations: ddPCR, droplet digital polymerase chain reaction; HG, high grade; LG, low grade; MLPA, multiplex ligation-dependent probe amplification; MSP, methylation-specific polymerase chain reaction; NR, not reported; PCR, polymerase chain reaction; Sens, sensitivity; Spec, specificity; UTUC, upper tract urothelial carcinoma.

Other marker assays

Urinary cell-free DNA integrity was proposed as a urinary biomarker by Casadio *et al.*, with an AUC of 83% in a primary diagnostic setting. DNA integrity was defined as the sum of the sequences longer than 250 bp of three different oncogenes combined: *c-Myc*, *BCAS1*, and *HER2*.[4] Brisuda *et al.* used urinary cell-free DNA quantification as a urine-based biomarker. In this study, DNA was quantified using real-time PCR of the *GAPDH* gene. The total amount of cell-free DNA in a urine sample had a discriminatory capacity of 73% as represented by the AUC.[38] (see **Table 10.2–4** for other marker assays)

TABLE 10.2–4 Other Marker Assays

Reference	Year	Gene	Technique	Population	Sens, %	Spec, %
Casadio *et al.*[4]	2013	*c-Myc*, *BCAS1*, *HER2*	Real-time PCR	Primary/hematuria	73	84
Brisuda *et al.*[38]	2016	*GAPDH*	Real-time PCR	Not specified	42	91

Abbreviations: PCR, polymerase chain reaction; Sens, sensitivity; Spec, specificity.

10.3 Conclusions

Overall, many different approaches to DNA-based urine biomarkers have been undertaken and numerous different combinations of assays have been attempted. A common denominator remains the lack of prospective validation. Still, several DNA-based biomarkers show great potential for use in clinic. Most promising is the use of DNA-based urine biomarkers in primary diagnostics of bladder cancer and the use of DNA-based biomarkers in the follow-up of patients treated for low-risk NMIBC. Even though it is still too early to tell, it is likely that some of these biomarkers will be included in future guidelines.

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10.5 RNA-Based Urinary Markers

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Various urinary cellular and protein-based biomarkers have shown a higher sensitivity than urinary cytology, but at the expense of a lower specificity. They are thus unable to replace cystoscopy in bladder cancer screening, detection, and surveillance in the near future.[1,2] At the moment, a high number of RNA-based urinary gene panels are being developed and validated, with the aim of improving diagnostic accuracy in bladder cancer without decreasing specificity.[1] In the following chapter, we provide an overview of new messenger RNA (mRNA)- and microRNA (miRNA)-based urinary biomarkers that are currently being tested in clinical trials for bladder cancer detection, screening, or surveillance.

10.5.1 Messenger RNA–related urinary biomarkers

1. Cxbladder™

The Cxbladder assay (Pacific Edge Diagnostics, United States) evaluates five mRNAs related to bladder cancer, including *MDK* (affects migration and angiogenesis in cancer cells), *HOXA13* (affects cell differentiation), *CDC2/CDK1* (essential to mitotic cell cycle and cell proliferation), *IGFBP5* (inhibits cell apoptosis), and *CXCR2* (marker of inflammation that is used to reduce false-positive results by identifying patients with "nonmalignant" inflammatory conditions) (**Table 10.5–1**). The Cxbladder assay consists of three bladder cancer tests with different indications: Cxbladder Triage, Cxbladder Detect, and Cxbladder Monitor.

Cxbladder Triage has been designed to identify patients with low risk of having bladder cancer in the clinical setting of gross hematuria without history of bladder cancer. The test incorporates clinical characteristics such as age, sex, smoking history, and hematuria history, as well as the results from mRNA expression analysis. The test aims to identify with a high negative predictive value (NPV) those patients who may not require cystoscopy. Thus, an individual risk profile was created based on clinical factors, along with the mRNA analysis.[3] Of the 695 patients with macrohematuria (MAH) registered across three cohorts (cohort of the study by O'Sullivan,[4] 517 patients from 9 urologic departments in New Zealand and Australia, 178 patients from two additional centres in New Zealand), samples from 587 patients were available for modelling comprising 72 urothelial carcinoma (UC)-positive and 515 UC-negative samples. Of the 45 samples from patients with microhematuria (MIH) provided, 40 were suitable for analysis. All 45 patients had received a full urologic work-up, and clinical truth was confirmed as UC-negative in all patients.

The genotypic-phenotypic model (G + P INDEX) combining urinary expression of *IGFBP5*, *HOXA13*, *MDK*, *CDK1*, and *CXCR2* genes (genotypic) with age, gender, frequency of MAH, and smoking history (phenotypic) resulted in a 100% detection rate of high-grade (HG) tumours in 587 patients with MAH. Moreover, the sensitivity, NPV, and diagnostic accuracy was 95%, 98%, and 86%, respectively. In MIH, 80% of patients without evidence of bladder cancer were correctly triaged out using the G + P INDEX, thus not requiring a full urologic work-up.[3]

Cxbladder Detect was created for patients with a higher risk for bladder cancer. In this case, physicians can use Cxbladder Detect, which can inform about a bladder cancer diagnosis when combined with other tests in a urologic work-up. O'Sullivan *et al.*[4] analyzed Cxbladder Detect in a marker-comparison trial including 485 patients presenting with MAH but without history of bladder cancer. Urinary samples for mRNA analysis were collected prior to cystoscopy. HG and pT1 tumours were detected in 97% and 100%, respectively, with an overall sensitivity and specificity of 82% and 85%, respectively. In addition, the sensitivity was significantly higher for low-grade (LG) tumours as compared to cytology (pTa LG: 91% vs 68%).

A survey of urologists was used to evaluate the potential utility of the Cxbladder Triage and Cxbladder Detect tests in reducing the overall number of invasive and diagnostic procedures used in the diagnostic work-up of patients presenting with MIH.[5] Interestingly, the projected total number of diagnostic procedures was reduced by 5% and 25% and the number of invasive procedures by 11% and 31%, following disclosure of results in Triage (data from genomic markers and clinical variables such as age, gender, frequency of MAH, and smoking history) and Triage and Detect (expression of five genomic markers), respectively.

TABLE 10.5–1 Overview of Diagnostic Characteristics Polymerase Chain Reaction Assays for Detection/ Surveillance of Bladder Cancer

Marker/Test	Manufacturer	Description	Target genes
Cxbladder	Pacific Edge	PCR assay for detection of mRNA expression of 5 genes	*CDC2*, *HOXA13*, *MDK*, *IGFBP5*, *CXCR2*
Xpert Bladder Cancer	Cepheid	PCR assay for mRNA expression analysis of 5-gene panel	*CRH*, *IGF2*, *UPK1B*, *ANXA10*, *ABL1*
TaqMan Array (12 + 2 panel)	Thermo Fisher	PCR assay of a 12 + 2 gene-set panel	*ANXA10*, *AHNAK2*, *CTSE*, *CRH*, *IGF2*, *KLF9*, *KRT20*, *MAGEA3*, *POSTN*, *PPP1R14D*, *SLC1A6*, and *TERT* + *ASAM* and *MCM10*

Abbreviations: mRNA, messenger RNA; PCR, polymerase chain reaction.

The trial by Kavalieris *et al.*[6] evaluated the **Cxbladder Monitor** test and collected 1,036 urine samples from 763 patients undergoing routine surveillance for recurrent bladder cancer. Remarkably, the Cxbladder Monitor offered a high sensitivity and NPV of 93% and 97% for detection of bladder cancer. In a similar group of patients (bladder cancer surveillance), Lotan *et al.*[7] confirmed a high sensitivity and NPV of the test in 803 patients. The overall sensitivity and NPV was 91% and 96%, outperforming current FDA-approved urinary biomarkers such as cytology (sensitivity: 22%), NMP22 enzyme-linked immunosorbent assay (ELISA) (26%), and NMP22 BladderChek (11%). A summary of published trials presenting an overview of the diagnostic performances of Cxbladder assays is shown in **Table 10.5–2**.

Summary on Cxbladder test

The five published studies on different clinical scenarios (detection of bladder cancer in at-risk population and surveillance for recurrent bladder cancer) consistently showed promising diagnostic performance with a high sensitivity (even in LG tumours on surveillance) and NPV, but at the expense of a lower specificity compared to cytology. For bladder cancer screening and detection, Cxbladder has the potential to reduce the frequency of diagnostic and invasive procedures in patients presenting with hematuria; however, further data are necessary to confirm. At the moment, no clear evidence exists confirming that the application of Cxbladder, especially in a screening setting, has an effect on cancer-specific mortality.

2. Xpert Bladder Cancer

The Xpert Bladder Cancer Monitor (Cepheid, United States) is an mRNA-based urinary marker test developed for bladder cancer surveillance that measures the levels of five target mRNAs (CRH, IGF2, UPK1B, ANXA10, and ABL1) from a voided urine sample by real-time RT-PCR (**Table 10.5–1**). All five mRNAs are based on cell proliferation and survival (IGF2), cell growth and signal transduction (ANXA10), epigenetic dysregulation (UPK1B), and response to neuroendocrine stress, immunity, and inflammation (CRH).[8–10] A schematic overview of published trials concerning the diagnostic performances of Xpert Bladder Cancer are presented in **Table 10.5–2**. In detail, Wallace *et al.*[11] reported on the first data of the Xpert Bladder Cancer test. After initial evaluation of the 10 best biomarkers (UPK1B, IGF2, CRH, ANXA10, ABL1, KRT20, AR, PIK3CA, UPK2, and MGEA5) within a training set of 483 urine specimens, regression analysis was performed to identify a five-mRNA model to predict bladder cancer status in an independent test cohort of 450 participants. Xpert Bladder Cancer confirmed a diagnostic accuracy of 87%, with an overall sensitivity of 73%. It has to be considered that the specificity was 90% and 77% in the hematuria (no history of bladder cancer) and surveillance populations, respectively.[11] The trial by van Valenberg *et al.*[12] included bladder cancer patients on surveillance, as well as a control group (healthy volunteers or patients without a history or clinical evidence of bladder cancer who were referred for urologic consultation). The overall sensitivity and NPV was 74% and 93%, respectively, and 83% and 98% for HG tumours on bladder cancer surveillance, respectively. The overall specificity was 80%. In contrast, the specificity was much higher in the nonsurveillance cohort (control group), with 95% overall (urinary incontinence, 100%; benign prostatic hyperplasia, 95%; prostate cancer [PCa], 96%; and kidney stones, 83%; respectively).

Focusing on bladder cancer surveillance, the marker-comparison study by Pichler *et al.*[13] compared the diagnostic performance of the Xpert Bladder Cancer with voided urine and bladder washing cytology in 140 patients with non-muscle invasive bladder cancer (NMIBC). The overall sensitivity and NPV of the Xpert Bladder Cancer were significantly superior to those of bladder washing cytology (84% vs 33% and 93% vs 76%), also achieving a high sensitivity in the subgroup of patients with low-risk bladder cancer (LG 77%; pTa 82%). Moreover, the specificity was similarly high compared to bladder washing cytology (91% vs 94%). D'Elia *et al.*[14] also evaluated Xpert Bladder Cancer in patients undergoing bladder cancer surveillance. The overall sensitivity (46.2%) was higher for Xpert Bladder Cancer compared to cytology (11.5%). LG and HG tumours were detected by Xpert Bladder Cancer with a sensitivity of 40% and 85.7%, respectively. In contrast to the results published by Pichler *et al.*,[13] the specificity (77%) for Xpert Bladder Cancer was lower compared to cytology (92.7%).

Summary on Xpert Bladder Cancer test

Four trials were published in the literature, focusing on both bladder cancer detection in patients without history of bladder cancer and surveillance populations with previous history of bladder cancer. In bladder cancer surveillance, the sensitivity was excellent for HG tumours (83–100%) and between 40% and 77% for LG tumours. The overall sensitivity ranged between 46.2% and 84%. While the specificity in the bladder cancer detection population was very high (90–95%), nonhomogeneous data concerning specificity were confirmed for bladder cancer surveillance (77–91%). These problems can be overcome by using balanced cohorts (patients with bladder cancer recurrence to nonrecurrence), as well as by using independent validation cohorts in further trials. At the moment, no clear evidence exists confirming that the application of the Xpert Bladder Cancer, especially in a detection setting, has an effect on cancer-specific mortality.

3. TaqMan Array (12 + 2 gene panel)

Mengual *et al.*[15] identified a 12 + 2 gene expression signature for bladder cancer diagnosis and prediction of tumour aggressiveness on voided urine samples using a TaqMan PCR array (based on quantitative reverse transcription polymerase chain reaction [qRT-PCR] platform). The target genes are displayed in detail in **Table 10.5–1**. Phase 1 included 365 urinary samples, 244 bladder washings from histologically confirmed bladder cancer patients, and 121 voided urine sample controls. These samples were consecutively analyzed by qRT-PCR using a TaqMan array, containing a final group of 48 genes (for 291 samples). In the second phase of the study, 211 voided urine samples were included, and analyzed by the TaqMan array that contained the described 48 genes. Due to the fact that the effect of RNA degradation is much more apparent in voided urine than bladder washings,[16] a complementary DNA (cDNA) preamplification step was applied in the second phase of this trial. Interestingly, there was a decrease in overall sensitivity in detecting bladder cancer in voided urine (70%) compared to bladder washings from phase 1 (98%), while specificity remained stable (96% for voided urine vs 99% for bladder washings). The addition of two other genes (*ASAM* and *MCM10*) to the 12-gene set panel resulted in an overall sensitivity and specificity of 79% and 92%, respectively, in discriminating between LG and HG tumours in bladder cancer patients (tumour aggressiveness) (**Table 10.5–2**).

Subsequently, the validation study by Mengual *et al.*[16] was performed and evaluated the diagnostic performance of the presented 12 + 2 gene expression panel using an independent cohort of 207 urinary samples. The overall sensitivity and specificity were 80% and 86%, respectively, in discriminating between bladder cancer and control samples, and 75% and 75%, respectively, in discriminating between LG and HG tumours (**Table 10.5–2**). A four-gene signature was designed and evaluated in a prospective, blinded, multicentre trial including 789 patients. The signature of two genes (GS_D2) had the best diagnostic performance, being equal to or better than cytology (sensitivity, 81.48%; specificity, 91.26%).[17]

Summary on TaqMan Array (12 + 2 gene panel)

The training and validation study analyzing the diagnostic accuracy of a specific 12 + 2 gene set panel on bladder washings and voided urine samples confirmed comparable sensitivities and specificities in bladder cancer detection (sensitivity: 70%, 80%, and 98%; specificity: 86%, 96%, and 99%) and in discriminating between LG and HG tumours (sensitivity: 75–79%; specificity: 75–92%). These findings were confirmed in a prospective, blinded multicentre trial.[17] Nevertheless, it should be kept in mind the difficulty in obtaining a sufficient quantity of high-quality RNA from voided urine compared to bladder washings, as presented by Mengual *et al.*[15,16]

TABLE 10.5–2 Diagnostic Performance Characteristics of Messenger RNA–Based Urine Markers for Detection/Surveillance of Bladder Cancer

	Reference	Study design	Indication	N of included patients	Sens	Sens for HG tumours	Spec	NPV
Cxbladder	O'Sullivan *et al.*[4]	Marker-comparison study, prospective	MIH	485	82%	97%	85%	-
	Lotan *et al.*[7]	Marker-comparison study, prospective	Bladder cancer surveillance	803	91%	97%	-	96%
G + P INDEX	Kavalieris *et al.*[3]	Cohort, prospective	MIH and MAH	695 (MAH)/45 (MIH)	95%	-	45%	98%
	Kavalieris *et al.*[6]	Cohort, prospective	Bladder cancer surveillance	763	93%	97%	-	97%
Xpert Bladder Cancer	van Valenberg *et al.*[12]	Marker-comparison study, prospective	Bladder cancer detection + surveillance	239 (surveillance)/508 (detection)	74%	83%	80% (surveillance) 95% (detection)	93%
	Wallace *et al.*[11]	Cohort, prospective	Bladder cancer detection, surveillance	484 (training cohort) + 450 (validation cohort)	73%	-	90% (hematuria) 77% (surveillance)	-
	Pichler *et al.*[13]	Marker-comparison study, prospective	Bladder cancer surveillance	140	84%	100%	91%	93%
	D'Elia *et al.*[14]	Marker-comparison study, prospective	Bladder cancer surveillance	230	46.2%	85.7%	77%	83%

TABLE 10.5–2 Diagnostic Performance Characteristics of Messenger RNA–Based Urine Markers for Detection/Surveillance of Bladder Cancer *(Cont'd)*

	Reference	Study design	Indication	N of included patients	Sens	Sens for HG tumours	Spec	NPV
TaqMan Array (12 + 2 gene panel)	Mengual *et al.*[15]	Cohort, prospective	Bladder cancer detection + bladder cancer aggressiveness	341/235 (control)	98%	100%	99%	95%
	Mengual *et al.*[16]	Case-control study	Bladder cancer detection + bladder cancer aggressiveness	207 (independent set)/404 (training set)	80%	-	86%	-
	Ribal *et al.*[17]	Cohort, prospective	Bladder cancer detection	525	81.5%	-	91.3%	-
CAIX (full-length isoform relative percentage, cutoff of 10%)	Malentacchi *et al.*[23]	Cohort (bladder cancer, RCC, and PCa)	Bladder cancer, RCC, and PCa detection	138/89 (control)	80%	-	72%	82%
	de Martino *et al.*[24]	Marker-comparison study, prospective	Bladder cancer detection	196/123 (control)	86.2%	88.4%	95.1%	81.2%
Survivin	Shariat *et al.*[26]	Marker-comparison study, prospective	Bladder cancer detection	117/92 (control)	64%	-	93%	67%
	Weikert *et al.*[27]	Marker-comparison study, prospective	Bladder cancer detection	35/33 (control)	68.6%	-	100%	-
	Horstmann *et al.*[28]	Pilot study, prospective	Bladder cancer detection	32/17 (control)	53%	83%	88%	-

Abbreviations: CAIX, carbonic anhydrase IX; HG, high grade; MAH, macrohematuria; MIH, microhematuria; NPV, negative predictive value; PCa, prostate cancer; RCC, renal cell carcinoma; Sens, sensitivity; Spec, specificity.

10.5.2 Other urinary messenger RNA expression targets in bladder cancer

1. Carbonic anhydrase IX

Carbonic anhydrase IX (CAIX) is known to be an enzyme of the carbonic anhydrase family that regulates the pH value as a response to hypoxia.[18] It is upregulated in hypoxic conditions, resulting in cell proliferation and tumour progression. Thus, CAIX has been intensively investigated as a prognostic and predictive biomarker in renal cell carcinoma (RCC).[19,20] In bladder cancer tissue samples, previous studies have shown that more than 70% of samples show expression of CAIX, whereas normal urothelial tissue shows no significant expression of CAIX. Moreover, CAIX expression within the tumour has been shown to be higher in Ta tumours compared to T1 to T4, and higher in LG than HG tumours.[21]

Malentacchi *et al.*[22] evaluated the role of CAIX as a urinary biomarker. They confirmed that the CAIX full-length isoform plays an important role in bladder carcinogenesis and, in addition, the relative percentage of CAIX full-length isoform seems to be associated with the presence of bladder cancer. Another trial by Malentacchi *et al.*[23] was designed to perform an in-depth validation of urinary CAIX mRNA expression in samples from various urologic oncological cancer entities (renal, bladder, prostate), including 93 patients with bladder cancer and a healthy control group of 89 subjects, measuring the full-length isoform (CAIX FL) and the total CAIX mRNA concentration. As the evaluation of the total CAIX mRNA concentration may result in misleading data due to the variable relative expression of CAIX FL, the FL relative percentage (FL%) was calculated. The urinary FL% levels were significantly higher in cancer patients (median: bladder cancer, 58.8%; PCa, 71.4%; and RCC, 84.9%) compared to healthy subjects (median: 2.6%). Receiver operating characteristic (ROC) curves indicated the best diagnostic performance for FL% with a sensitivity and specificity of 90% and 72%, respectively, and positive predictive value (PPV) and NPV of 83% and 82%, respectively. The highest AUC value was achieved for bladder cancer (AUC, 0.896), compared to RCC (AUC, 0.848) and PCa (AUC, 0.717) (**Table 10.5–2**).

A further trial by de Martino *et al.*[24] evaluated the diagnostic performance of urinary CAIX expression by quantitative polymerase chain reaction (qPCR) using the TaqMan Gene Expression Assay in a bladder cancer cohort (n=195) and 123 controls with hematuria (n=123) defined as the training cohort. Data were also validated in an independent cohort of 155 subjects who were prospectively accrued in 2014. Importantly, the presence of CAIX within the tumour was significantly associated with the detection of CAIX in the urine. The sensitivity and specificity was 86.2% and 95.1%, respectively, compared to cytology, which was 43.5% and 100%. In addition, the diagnostic accuracy was higher than cytology (90.5% vs 71.7%), especially in LG tumours (90% vs 61.8%). The high accuracy could be confirmed in the validation cohort of the study (AUC, 88.3%). On the contrary, CAIX expression decreased with higher tumour grades and tumour stages (sensitivity for HG tumours: 88.4% for CAIX vs 70.5% for cytology) (**Table 10.5–2**).

Summary on urinary CAIX expression

CAIX expression has been confirmed in both bladder cancer tissue and urine samples of bladder cancer patients. Its tumoural expression seems to be increased in LG tumours and Ta cancers, whereas normal urothelial tissue remained CAIX negative. Concerning CAIX as a urinary bladder cancer biomarker, the relative percentage of the full-length CAIX isoform seems to be most predictive when detecting bladder cancer on ROC (AUC, 0.896). CAIX expression decreased with higher tumour grade and stage. Thus, CAIX could be an innovative option in the detection and follow-up of LG tumours. Nevertheless, due to the few published studies with limited data, CAIX must be validated in further prospective marker-comparison trials with other urinary mRNA targets.

2. Survivin

Survivin is a 16.5 kDa member of the inhibitor of apoptosis protein family that is overexpressed in many malignancies but rarely detected in normal differentiated adult tissues. A meta-analysis by Ku *et al.*[25] included 14 studies about the predictive role of survivin in bladder cancer detection, with a pooled sensitivity and specificity of 77.2% and 91.8%, respectively. However, only three of these trials were prospectively designed.[26–28] Moreover, discrepancies in the diagnostic performances of urinary survivin mRNA were confirmed across the literature, with sensitivity ranging from 53% to 68.6%, and specificity ranging from 88% to 100% (**Table 10.5–3**).[26–28] Nevertheless, sensitivities were superior to those of cytology, and without reducing specificity. Most trials included only a limited number of patients and control groups.

Summary on Survivin expression

At the moment, no clear statement and recommendation about urinary survivin as a predictive biomarker can be made due to the low levels of evidence provided.

TABLE 10.5–3 Performance Characteristics of microRNA Urinary Signature Panels for Detection/Surveillance of Bladder Cancer

Reference	Target genes	Indication	N of included patients	Sens	Accuracy	Spec	NPV
Sapre *et al.*[30]	miR16, miR200c, miR205, miR21, miR221, and miR34a	Bladder cancer surveillance	131	88%	74%	48%	75%
Yun *et al.*[38]	miR-145 and miR-200a	Bladder cancer detection and surveillance	207	77.8% (miR-145) 54.4% (miR-200a)	73%	61.1% (miR-145) 65.7% (miR-200a)	-
Wang *et al.*[36]	miR-200, miR-205, miR-192, miR-155, and miR-146a	Bladder cancer detection	75	-	-	-	-
Hanke *et al.*[29]	Ratio of miR-126:miR-152	Bladder cancer detection	47	72%	77%	82%	-
Miah *et al.*[37]	miR-135b/15b/1224-3p	Bladder cancer detection	121	94.1%	-	51%	-
Puerta-Gil *et al.*[39]	miR-143, miR-222, and miR-452	Bladder cancer screening for MIH and detection	94	-	85% (miR-452) 77% (miR-222)	-	-

Abbreviations: MIH, microhematuria; NPV, negative predictive value; Sens, sensitivity; Spec, specificity.

10.5.3 Urinary microRNA targets

A novel class of biomarkers that has recently been introduced and investigated is microRNAs (miRNAs). Their expression in body fluids (circulating miRNAs) such as blood and especially urine is relatively stable and can be quantified reliably by qRT-PCR.[29–31] miRNAs are a class of single-stranded non-coding RNAs that play an essential role in the negative regulation of gene expression at the posttranscriptional level. Recently, their potential role as a mediator for intercellular communication with a hormone-like mechanism in cancer has also been established.[32] Whereas single miRNAs are mostly not cancer-specific, many miRNA signatures have been validated and may be able to serve as biomarkers for uro-oncological cancer entities such as bladder cancer.[31,33] miRNAs are dysregulated in human cancers and can work as tumour suppressors or oncogenes. In bladder cancer and other tumour entities, members of the miRNA-200 family are involved in the tumour cell adhesion, cell migration, invasion, and metastasis, controlling the epithelial-to-mesenchymal–transition process and sensitivity to epidermal growth factor receptor (EGFR) therapy.[34,35] Interestingly, different urinary miRNA signatures are found to be up- or downregulated, making them potential biomarkers for bladder cancer detection.[29,36–39] The diagnostic performances of those miRNA signatures for bladder cancer detection are shown in **Table 10.5–3**, with heterogeneous results concerning sensitivity (54.4–94.1%) and specificity (51–82%). A possible explanation

for the high variability in the results is the inclusion of heterogeneous control groups (including patients with hematuria, urinary inflammation, and benign urologic conditions), and that only a few of these trials validated their miRNA signatures with independent cohorts.[30]

Only two studies evaluated the predictive role of urinary miRNA signatures in bladder cancer surveillance, comparing miRNA expression levels of urine from disease-free patients with a previous history of bladder cancer versus patients with verified recurrence at time of urine collection. Yun *et al.*[38] published the first data about the prognostic value of miRNA-200a in predicting recurrence on bladder cancer surveillance (odds ratio [OR], 0.449). In this case-control study, 207 patients with diagnosed primary bladder cancer and a healthy control cohort (n=144) were included. Moreover, miRNA-145 expression was able to detect NMIBC with a sensitivity and specificity of 77.8% and 61.1%, respectively. Nevertheless, an independent cohort was not included to corroborate these findings. Using a case-control design, Sapre *et al.*[30] identified a urinary miRNA signature including 6 miRNAs (miR16, miR200c, miR205, miR21, miR221, and miR3) as the best predictor in discriminating between disease-free patients and bladder cancer recurrence on surveillance (AUC, 0.85). These findings were also successfully confirmed in an independent cohort (AUC, 0.74), resulting in a high sensitivity of 88%, but a low specificity of 48% (**Table 10.5–3**).

Summary on urinary miRNA target signatures

The miRNAs may represent potential disease biomarkers in bladder cancer detection and surveillance in the future. Nevertheless, identified miRNA signatures were heterogeneous, depending on different published studies, with few trials confirming their results by independent validation cohorts. This resulted in a low degree of reproducibility in the clinical practice. Moreover, diagnostic performances of miRNA signatures were very broad ranging. Most trials included only a small number of patients (between 47 and 207 patients); thus, larger prospective studies, especially in the surveillance cohort, are urgently needed prior to drawing any final conclusion. Another open question is the implementation and application of different analytical platforms and bioinformatics in clinical practice.[31]

10.5.4 Advantages and limitations of RNA-based urinary markers

The most important advantages of RNA-based urinary markers are their automation, easy handling, brief hands-on sample preparation time, technical instrument systems that automate and integrate all complex PCR processes, and high-quality standards, including in-sample quality controls. In addition, RT-PCR–based urinary tests have the advantage of being reliable, easy to perform, and objective in contrast to cytology.[13]

One of the major limitations of the application of RNA-based urinary techniques is the difficulty in obtaining a sufficient quantity of high-quality RNA from voided urine compared to bladder washings, resulting in artificial research conditions with optimized specimen collection and handling.[15,40] It has been shown that bladder washing samples yielded higher amounts of better-quality RNA than voided urine samples.[15] This is important to know, as both the Cxbladder and Xpert Bladder Cancer test were analyzed on fresh midstream voided urine samples. A multiplex cDNA preamplification technique, as used by the TaqMan Array, may be helpful for overcoming this limitation. In addition, a positive ABL1 signal, indicating that the urine sample contains sufficient human cells and human RNA, is required for a valid test result when using Xpert Bladder Cancer, thus improving the consistency of the test results.[13]

Another limitation is the fact that there is a wide variability in the cost of RNA-based urine tests. For widespread use in the future, these tests should be available at reasonable costs. The high costs of novel RNA-based urinary markers necessitate further assessments of their additional value and a comparison with cytology, DNA, and protein-based urinary markers. Thus, further marker-comparison trials are urgently needed to estimate the cost-effectiveness of RNA-based urinary markers, developing, for example, a Markov model to compare the cost and effectiveness of RNA-related urinary markers. Another challenge of RNA-based urinary markers in pre-analytics is the mRNA instability, resulting in an advantage for commercial test systems (working with RNA-stabilizing tubes) compared to single urinary mRNA targets (CAIX or survivin). Standardized processes are indispensable for RNA analysis when promoting those tests to the general public.

Ongoing clinical trials of mRNA-based urinary biomarkers concerning bladder cancer surveillance are summarized in **Table 10.5–4**.

TABLE 10.5–4 Ongoing Clinical Trials of Messenger RNA–Based Urine Markers for Surveillance of Bladder Cancer

	Study name	N of patients	Indication	Primary endpoint	Status*	NCT number
Cxbladder Detect	-	300	Bladder cancer surveillance	Performance characteristics	Not yet recruiting	NCT03673202
	Cxbladder Hematuria Clinical Utility Study	600	Subjects presenting with hematuria randomized based on clinical risk and marker status	Reduction rate in cystoscopy procedure count	Not yet recruiting	NCT03988309
Xpert Bladder Cancer	ANTICIPATE X	1,100	Bladder cancer surveillance for low-and intermediate NMIBC	Bladder cancer recurrence	Recruiting	NCT03664258
	-	100	Spinal cord individuals with symptoms or clinical findings suspicious for bladder cancer	Urine tumour mRNA concentration	Recruiting	NCT02538809
	-	530	Bladder cancer surveillance	Bladder cancer recurrence	Recruiting	NCT03125460
	-	24	Bladder cancer surveillance of patients treated with BCG	Rate of negative Xpert tests in patients with no recurrence at first follow-up cystoscopy	Unknown	NCT02895620
	-	200	Bladder cancer surveillance	Bladder cancer recurrence	Recruiting	NCT03715660

*Status according to https://clinicaltrials.gov/, last accessed August 6, 2019.

Abbreviations: BCG, bacillus Calmette-Guérin; mRNA, messenger RNA; NMIBC, non-muscle invasive bladder cancer.

10.6 International Recommendations on RNA-Based Urinary Markers

According to the current guidelines of the European Association of Urology (EAU), RNA-based (molecular) urinary biomarkers are currently not recommended for the screening and detection of bladder cancer in patients with microscopic hematuria or for bladder cancer surveillance. At the moment, cystoscopy cannot be replaced by any RNA-based urinary marker test due to the low levels of evidence provided.[41] Additionally, the American Urological Association (AUA) guidelines also do not routinely recommend any urinary biomarkers in surveillance of NMIBC.[42]

10.7 References

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10.8 Protein Markers

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The urinary protein components have been (and still are) under intensive study for their use as biomarkers in bladder cancer management. Technical difficulties in protein analysis relate to the extensive complexity and variability of protein physicochemical properties, as well as the large dynamic range of protein concentrations (spanning at least six orders of magnitude in the case of urine), which collectively underscore the need for application of multiple different methodologies to allow for protein detection and quantification at high resolution.[1,2] Despite these difficulties, assays evaluating urinary protein markers (nuclear matrix protein 22 [NMP22], complement factor H and complement factor H–related protein, and bladder cancer–associated mucins) have been approved by the FDA for use in bladder cancer detection and/or surveillance, and many more have been reported and await proper validation. A brief overview is provided below, aiming to highlight representative studies in each case (summarized in **Tables 10.8–1 and 10.8–2**). For more exhaustive recent systematic reviews on protein markers, the interested reader may refer to Frantzi and Vlahou, Zuiverloon *et al.*, D'Costa *et al.*, Frantzi *et al.*, Latosinska *et al.*, and Sathianathen *et al.*[3–8]

TABLE 10.8–1 Urinary Proteins Associated With Bladder Cancer*

Protein marker	Type of assays	Main protein function	Study cohort	Sensitivity	Specificity	Risk	Reference
NMP22	-POC qualitative (Bladder Check) -Quantitative immunoassay (ELISA)	Nuclear matrix protein	-Meta-analysis Primary diagnosis (n=1,313 for ELISA; n=1,816 for POC)	67% (ELISA) 47% (POC)	84% (ELISA) 93% (POC)		Chou *et al.*[15]
			-Meta-analysis Primary diagnosis and follow-up (n=10,119 both tests)	68% (overall)	79% (overall)	-	Mowatt *et al.*[13]
			-Review (follow-up; n=2,041, ELISA)	71% (median; range: 47–100)	73% (median; range: 55–98)		van Rhijn *et al.*[25]

TABLE 10.8–1 Urinary Proteins Associated With Bladder Cancer* *(Cont'd)*

Protein marker	Type of assays	Main protein function	Study cohort	Sensitivity	Specificity	Risk	Reference
BTA (complement factor H–related protein and complement factor H)	-POC (BTA stat®) -ELISA (BTA TRAK)	Complement regulators	-Meta-analysis Primary diagnosis (*n*=829 for ELISA; *n*=1,160 for POC)	75% (ELISA) 70% (POC)	65% (ELISA) 75% (POC)	-	Glas *et al.*[28]
			-Meta analysis Primary diagnosis (*n*=1,021)	76% (POC)	78% (POC)		Chou *et al.*[15]
			-Meta analysis (*n*=3,175)	67% (POC)	75% (POC)		Guo *et al.*[29]
			-Review (Follow-up; *n*=3,461 for ELISA: *n*=555 for POC	*58% (POC; range: 29–74) *71% (ELISA; range: 60–83)	*73% (POC; range: 56–86) *66% (ELISA; range: 60–79)		van Rhijn *et al.*[25]
CK8 and CK18 fragments	-POC (UBC® *Rapid*)	Structural proteins	Meta-analysis Primary and follow-up: *n*=623	59.3%	86.1%	-	Schmitz-Dräger *et al.*[14]
CK19 fragments	Quantitative immunoassay (CYFRA 21-1)	Structural protein	Meta-analysis Primary and follow-up (*n*=1,495)	82%	80%	-	Huang *et al.*[36]
MDM5	Quantitative immunoassay (ADXBLADDER)	DNA replication	Primary prospective (*n*=856)	73%	69%	-	Dudderidge *et al.*[43]
EpCAM	Quantitative immunoassay	Cell adhesion	NMIBC progression (*n*=683)	-	-	2.04 higher risk for diease-specific death	Snell *et al.*[42]
HA-1	Quantitative immunoassay	HA-1	NMIBC progression (*n*=683)	-	-	2.14 higher risk for diease-specific death	Snell *et al.*[42]

TABLE 10.8–1 Urinary Proteins Associated With Bladder Cancer* *(Cont'd)*

Protein marker	Type of assays	Main protein function	Study cohort	Sensitivity	Specificity	Risk	Reference
Cytokine panel (9 cytokines)	Quantitative immunoassay (CyPRIT)	Immune response	Intermediate- and high-risk NMIBC (*n*=130)	80% (prediction of recurrence)	77.4% (prediction of recurrence)	-	Kamat *et al.*[49]
IL-6/IL-10 ratio	ELISA	Immune response	Intermediate-risk NMIBC and controls (*n*=65)	83% (prediction of recurrence after BCG)	76% (prediction of recurrence after BCG)	-	Cai *et al.*[48]
10-protein panel	Quantitative immunoassay	MMPs, ECM, inflammatory and plasma proteins	Surveillance (*n*=125)	79%	88%	-	Rosser *et al.*[51]
10-protein panel	Multiplex immunoassay	MMPs, ECM, inflammatory and plasma proteins	Primary diagnosis (*n*=200)	85%	81%	-	Shimizu *et al.*[52]
Peptide panel	Mass spectrometry (CE-MS)	Panel of 116 (primary) and 106 (surveillance) peptide fragments from ECM and plasma proteins	Primary (*n*=425 training, *n*=270 test sets) Surveillance (*n*=425 training, *n*=211 test)	91% 87%	68% 51%	-	Frantzi *et al.*[32]
Peptide panel	Mass spectrometry (CE-MS)	Panel of 36 peptides, mainly of ECM and plasma proteins	Surveillance (*n*=98, including *n*=50 used as test set)	-	-	HR, 5.76 for prediction of disease relapse	Krochmal *et al.*[56]

*Highlighted are meta-analyses, as available, and studies with employed cohorts reflecting a clinically relevant context of use. **In bold**: Markers have been approved by the FDA; when available, the commercial name of the respective assay is provided.

Abbreviations: BCG, bacillus Calmette-Guérin; BTA, bladder tumour antigen; CE-MS, capillary electrophoresis mass spectrometry; CyPRIT, Cytokine Panel for Response to Intravesical Therapy; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; EpCAM, epithelial cell adhesion molecule; HA-1, hepatocyte growth factor activator inhibitor-1; HR, hazard ratio; IL, interleukin; MMP, matrix metalloproteinase; NMIBC, non-muscle invasive bladder cancer; POC, point of care.

TABLE 10.8–2 Sensitivities per tumour stage, grade, or risk groups for the FDA-Approved markers, based on the most recent large meta-analysis.*

Protein marker	Sensitivity (%) - tumour stage	Sensitivity (%) - tumour grade	Reference
NMP22 (qualitative-POC)	Ta: 39 (30–49) T1: 53 (40–66) ≥T2: 69 (51–83) CIS: 57 (22–86)	G1: 36 (23–51) G2: 42 (29–56) G3: 65 (52–77)	Chou *et al.*[15] (number of included studies: 2–3)
NMP22 (quantitative-ELISA)	Ta: 48 (36–60) T1: 72 (60–81) ≥T2: 82 (70–89) CIS: 66 (38–86)	G1: 44 (32–57) G2: 58 (47–69) G3: 75 (65–83)	Chou *et al.*[15] (number of included studies: 6–12)
BTA (qualitative-POC)	Ta: 49 (41–56) T1: 74 (66–80) T2: 89 (83–93) CIS: 68 (52–81)	G1: 39 (30–48) G2: 63 (54–71) G3: 81 (75–87)	Chou *et al.*[15] (number of included studies: 10–19)
BTA (quantitative-ELISA)	Ta: 53 (39–66) T1: 82 (67–91) T2: 88 (72–96) CIS: 58 (23–86)	G1: 51 (36–67) G2: 63 (47–76) G3: 86 (73–93)	Chou *et al.*[15] (number of included studies: 2–4)

*The median sensitivities and respective ranges, as well as the number of considered studies in each case (ranges are provided), are shown.

Abbreviations: BTA, bladder tumour antigen; CIS, carcinoma in situ; ELISA, enzyme-linked immunosorbent assay; FDA, US Food and Drug Administration; POC, point of care.

10.8.1 FDA-approved protein markers

Nuclear matrix protein 22

NMP22, or nuclear mitotic apparatus protein (NuMA), is a component of the nuclear supporting scaffold, detected at increased levels in the urine of bladder cancer patients, as a result of the tumour-associated increased cell turnover.[9] The two commercially available tests for detecting urinary NMP22 (the point of care [POC] NMP22 BladderChek and the NMP22 ELISA immunoassay [Alere/Abbott, United States]), have received approval by the FDA for use as adjuncts to cystoscopy in bladder cancer surveillance (both tests) and detection of the disease in high-risk or symptomatic populations (for the NMP22 BladderChek test only).[8,10–12] The reported sensitivity and specificity rates of the assays for cancer detection differ among different studies (ranging from 47% to more than 90%).[13–15] Nevertheless, there is an emerging consensus that the provided sensitivity is, in general, superior to that of cytology for the detection of low-grade (LG) disease[10,14,15] and also that important confounders in the measurements are hematuria, presence of stones, infections, or instrumentation.[16,17]

In brief, the marker has been evaluated for application in multiple different contexts of use, including detection of tumour in screening of general or high-risk populations, including patients with hematuria, or as predictors of tumour aggressiveness at primary or surveillance settings. As prominent examples of the former, NMP22 was implemented in a prospective trial of a more than 6-year duration, including workers exposed to occupational risk factors in Germany.[18] The very low incidence rate (14 positive cancer cases out of 1,722 enrolled patients) limited the statistical power of the study. Along the same lines, when NMP22 was implemented in the screening of 1,502 high-risk asymptomatic individuals, positive testing was received for 85 (5.7%) subjects.[19] Collectively, these results reflect the difficulties in biomarker implementation for bladder cancer screening associated with the low disease prevalence, even in the case of high-risk patients, a result that is supported by further large-scale studies.[20] A higher potential for the marker has been suggested in predicting tumour aggressiveness during surveillance, prior to transurethral resection of the bladder tumour. As an example, the analysis of 302 patients under surveillance suggested an increased risk for invasive tumour (>33-fold) or grade 3 tumour (21-fold) in the presence of a positive NMP22 and cytology tests.[21]

As in the case of all individual biomarkers (also described below), the combination of NMP22 with clinicopathological factors or other molecular assays increases accuracy rates in disease detection: in a study by Lotan *et al.*, a nomogram incorporating NMP22 together with demographic factors (age, gender, ethnicity), risk factors (smoking), type of hematuria, and cytology results provided an accuracy of 80% in predicting bladder cancer in patients with hematuria (23 patients out of 381).[22] The combination of additional molecular markers (such as fluorescence in situ hybridization [FISH] or immunocytology) has been shown to provide increased accuracy rates in detecting primary cancer in a group of 808 patients in comparison to each individual marker alone.[23]

Complement factor H and complement factor H–related protein

The POC BTA stat (Polymedco, United States) and sandwich immunoassay-based BTA TRAK tests detect the complement factor H and complement factor H–related protein, which reflect cancer-associated abnormalities of the complement cascade and immune evasion.[24] Similarly to the NMP22 assay, varying accuracy rates have been reported ranging from 57% to 83% (sensitivities) and 60% to 92% (specificities), depending on tumour stage, grade, and context of use (primary vs surveillance settings), with higher sensitivities, in general, being observed for primary, advanced stage, and advanced grade tumours.[25–27] As supported by a respective representative meta-analysis,[28] BTA stat and BTA TRAK could detect newly diagnosed cancer cases with sensitivities of 70% (BTA stat) or 75% (BTA TRAK) and specificity of 75% (BTA stat) and 65% (for BTA TRAK; number of analyzed samples for BTA stat=1,160; for BTA TRAK=829). In comparison, cytology detected the disease with a sensitivity of 55% and a specificity of 94% (number of analyzed samples=3,444). Similar overall performance rates for BTA stat were also received in two more meta-analyses.[15,29] The results collectively reflect the existence of confounders (hematuria, infection, renal calculi, stones, presence of stents, all increasing the levels of complement factor H), which limit the assay specificity, and consequently its widespread use as a stand-alone diagnostic test. As previously mentioned, these factors have been also shown to significantly affect the results of other urine markers such as NMP22.

10.8.2 Non-FDA-approved, exploratory protein markers

Besides the FDA-approved markers, several additional protein biomarkers have been reported in the literature for the initial detection or surveillance of bladder cancer and/or monitoring of treatment response. As recently reviewed,[3,5,7] of the most commonly reported are (among others) proteins of the extracellular but also nuclear matrix, apolipoproteins (Apolipoprotein A-I [APOA1], Apolipoprotein A-II APOA2], Apolipoprotein E [APOE]), and other plasma proteins (α1 antitrypsin, heparin cofactor II), including angiogenic factors (such as angiogenin, vascular endothelial growth factor A [VEGFA]), as well as inflammatory factors (such as interleukins [ILs] 2, 6, 8, and 10, and tumour necrosis factor [TNF]-α). Lately, these proteins are being combined in biomarker panels or multiparametric classifiers that appear to better reflect disease heterogeneity and tolerate instabilities and inconsistencies in comparison to individual markers. For these more repeatedly reported markers and protein panels, a brief introduction with representative studies from the existing literature is provided below.

Fibrinogen (A and B), as well as cytokeratin fragments (mainly of cytokeratins 8 and 18), have been reported in association to bladder cancer in multiple independent studies, as individual markers or components of marker profiles.[30–33] For the measurement of cytokeratins, a commercially available quantitative ELISA-based test as well as a POC assay have been developed (UBC *Rapid*, IDL Biotech AB, Sweden). A meta-analysis suggested an overall sensitivity and specificity of 59.3% and 86.1%, respectively, for the POC test (n=623).[14] A recent study involving approximately 500 individuals was conducted for the validation of the UBC *Rapid* test, particularly for the detection of low-risk non-muscle invasive bladder cancer (NMIBC).[34] The cohort included patients at primary diagnosis or under surveillance, mainly with low-grade (LG) NMIBC (n=134), but also high-grade (HG) NMIBC (n=48) or HG muscle-invasive bladder cancer (MIBC) (n=60), as well as individuals with other diseases of the urinary tract and healthy subjects (n=226). The overall sensitivity was 53.3%, with sensitivities for the low- and high-risk subgroups of 38.8% and 68.3%, respectively, at a specificity of 93.8% for healthy controls. Similar results were also obtained in a recent prospective phase 2 study from Sweden[35] involving close to 300 individuals, corresponding to newly diagnosed (n=94), or under surveillance (n=75) cases, patients with benign diseases of the urinary tract (n=51) and healthy controls (n=50). Increased detection rates in the primary versus surveillance settings and for HG in comparison to LG disease (sensitivity of 79.2% vs 60%, respectively), with a specificity of 61.4% were observed.[35]

In analogy to the UBC test, the CYFRA 21-1 test has been established for the measurement of cytokeratin 19 fragments in urine. The overall reported sensitivities and specificities from the different studies, as estimated in a recent meta-analysis, are 82% and 80%, respectively;[36] nevertheless, validation of these findings, especially in proper control populations (such as patients with hematuria) is pending.

Of the different apolipoproteins reported in association to bladder cancer, Apolipoprotein A-I has been highlighted in multiple independent studies following application of different proteomic techniques (including gel[37] methodologies or those based on high-resolution liquid chromatography/tandem mass spectrometry[31,38]). Reported AUCs ranged from 0.83 to 0.98 in the different studies (with sensitivities and specificities >80%). Nevertheless, these rates represented comparisons of multi-stage or grade bladder cancer cases to healthy or hernia controls, thus calling into question the clinical applicability of the findings. Recently, Apolipoprotein

A-I was used as a component of multiparametric classifiers for the investigation of clinically relevant primary or surveillance cohorts, with promising results[32] (described below). Similar restrictions with use of suboptimal controls apply for additional proteins repeatedly reported in association to bladder cancer (such as α1 antitrypsin;[30,31,39,40] heparin cofactor II[31,38]), reflecting the need for proper validation studies.

Cell membrane receptors and urinary fragments, as well as further nuclear proteins, are emerging biomarkers for bladder cancer. Recently, the shed ectodomains of epithelial cell adhesion molecule (EpCAM) and hepatocyte growth factor activator inhibitor-1 (HAI-1)[41] were quantified by ELISA in a large cohort of NMIBC (n=683).[42] The fragments were found to have a prognostic value with reported 2.14 times higher risk for disease-specific death for HAI-1 and 2.04 times for EpCAM in the presence of elevated protein levels. Validation of these results is pending. In addition, the mini-chromosome maintenance-MCM5 protein involved in DNA replication, as quantified by ELISA (ADXBLADDER, Arquer Diagnostics Ltd, Sunderland, United Kingdom), was recently evaluated in a prospective multicentric trial for its association to bladder cancer in urine. The assay was implemented for the quantification of the protein in urine sediments of 856 patients with hematuria. Cancer was detected in 74 cases, of which 54 were predicted by MDM5, corresponding to an overall marker sensitivity of 73% (100% in the case of MIBC), superior to that of cytology (48% in a subset of n=173 patients), with a specificity of 69%.[43]

Multiple studies on cytokines and combinations thereof have been conducted as a means to monitor response to bacillus Calmette-Guérin (BCG) in patients with high-risk NMIBC (reviewed by Kamat *et al*).[44] Of the most extensively studied is IL-2, secreted by CD4+ T cells, whose increased levels following treatment administration have been associated with outcome of BCG treatment.[45] Nevertheless, the complex interplay of different cytokines has limited the use of IL-2 or other tested individual cytokines (such as IL-12, TRAIL, TNF-α, etc) as predictive markers; instead, the predictive value of cytokine ratios (such as IL-6/IL-10 as markers of type 1 helper T cell [Th1] and Th2 response, respectively)[46] or cytokine combinations (such as IL-8 and IL-18 marking the early response of polymorphonuclear cells)[47] have been under investigation. As an example, the IL-6/IL-10 ratio was found to predict recurrence following BCG treatment with a sensitivity of 83% and a specificity of 76%,[48] also validated in a subsequent cohort of high-risk NMIBC (n=72).[46] Recently, Kamat *et al.*, described a panel of 9 cytokines (Cytokine Panel for Response to Intravesical Therapy [CyPRIT]; including IL-1, IL-2, IL-6, IL-8, IL-18, TRAIL, interferon [IFN]-γ, IL-12, and TNF-α) as having potential predictive power to monitor response to intravesical therapy.[44,49] In this study, involving 125 patients with intermediate or high-risk NMIBC, cytokine levels were measured at different time points (baseline, immediately before and after BCG instillation at 6 weeks, and at first maintenance course). A nomogram constructed based on the changes in abundance of these cytokines prior to and after the sixth instillation of BCG had superior accuracy (85.5%) in predicting BCG response in comparison to any of the individual cytokines.[49] In a more recent study, and using serial urine samples (baseline, week 7, week 13, week 28, and end of treatment) from a phase 2 study investigating intravesical BCG complemented (or not) with intradermal HS-410 therapy, changes in 105 cytokines were monitored.[50] Modelling of the measurements supported predictive values for the percent change between week 13 and baseline, particularly for IL-18 binding protein-a (hazard ratio [HR], 1.995), and IL-8 (HR, 0.27).[50] Collectively, these results suggest a predictive value of cytokine panels for monitoring BCG response; nevertheless, large-scale validation of the presented models is required to rule out potential data overfitting, frequently occurring during such multiparametric approaches.

Besides the simultaneous evaluation of multiple cytokines, further combinations of different protein markers in the form of panels or multiparametric classifiers have emerged the past few years with promising results. Individual (considered in combination) or multiplex ELISA-based assays have been applied involving the quantification of matrix metalloproteases in combination with plasma proteins (such as various apolipoproteins), and angiogenic and inflammatory markers for the detection of primary or recurrent bladder cancer (reviewed by Frantzi and Vlahou).[3] In a retrospective study, Rosser *et al*. analyzed urine samples from a total of 125 patients under surveillance (*n*=53 recurrences; *n*=72 negative samples for tumour relapse) for a panel of 10 proteins (IL-8, MMP9, MMP10, serpin family A member 1 [SERPINA1], VEGFA, angiogenin, CAIX, APOE, serpin family E member 1 [SERPINE1], and syndecan 1 [SDC1]). A sensitivity of 79% with a specificity of 88% for recurrence detection was observed.[51] Analysis of a similar 10-marker panel (replacing SERPINA1 with α1 antitrypsin) in the form of a multiplex assay provided a sensitivity of 85%, with a specificity of 81% in the detection of bladder cancer in samples received prior to cystoscopy (n total =200).[52] Combination of the marker panel to demographics provided an AUC of 0.891, when applied in a multicentric study of 686 subjects (bladder cancer cases and benign controls).[53] Collectively, these studies showed the added value of combining different markers over analyzing them individually, a conclusion that was further supported by a recent meta-analysis of all existing studies on the aforementioned panels.[54]

Besides the use of multiplex ELISA, mass spectrometry-based platforms are increasingly being used for the simultaneous measurement of multiple markers. The use of a capillary electrophoresis-based platform for profiling the urinary peptidome (smaller proteins of <15 kD), peptides mainly originating from extracellular matrix proteins (collagens, fibrinogen), but also plasma proteins (apolipoprotein A, histidine-rich glycoprotein, and others) in association with bladder cancer have been described.[32,33,55] In a recent multi-institutional study involving samples from a total of 1,357 individuals under primary diagnosis (*n*=721) or surveillance (*n*=636) and separated into discovery and test sets, classifiers composed of 116 (primary) or 106 (surveillance) peptides were generated using support vector machines. When applied to the test sets (*n*=270 for the primary and *n*=211 for recurrences), these panels detected cancer with AUC of 0.87 (primary) or 0.75 (surveillance).[32] A predictive value (HR, 2.24) of the 106-classifier for bladder cancer relapse was supported in a subsequent study,[56] using a longitudinal cohort of 98 patients (mainly of NMIBC). In the same study, the prognostic value of individual peptides was also evaluated, based upon which a 36-peptide panel was constructed and predicted disease relapse with an HR of 5.76 in a test set of *n*=50 samples.[56]

10.9 Conclusions

Despite the large number of existing studies and reported associations with bladder cancer, a limited number of protein markers has progressed in the biomarker implementation pipeline. Among the attributed reasons are the frequent lack of proper study design (use of healthy subjects as controls, use of small sample sizes that collectively do not represent a clinically relevant context of use), and also the impact of urine as a biological matrix, including hematuria as a prominent confounder complicating the establishment of robust clinically applicable ELISA assays.[57] Nevertheless, as previously mentioned, frequently reported associations of specific proteins with disease phenotypes exist; in the form of marker panels or nomograms in combination to clinicopathological information, these can increase accuracy in disease detection and/or prognosis of disease evolution. Of note, clear evidence now proves the direct links of urinary proteins to tissue events; this is exemplified in the recent study by Egloff *et al*.[58] investigating the expression of activated leukocyte cell adhesion molecule (ALCAM) in both bladder tissue and urine and at both mRNA and protein levels. While no changes at the mRNA level could be observed, a decrease in the tissue protein levels was supported by immunofluorescence with cancer stage progression, which was related to the cleavage of the protein ectodomain and its shedding in urine. The latter urinary shed protein fragment was found to associate with bladder cancer tumour stage and overall survival.[58] Collectively, the obvious functional links, in combination with technological advancements allowing the simultaneous detection of marker panels, provide a solid basis for further validation of urine protein markers. Ideally, the field should rapidly advance toward prospective validation trials where, side by side, the different protein/proteomic panels (and potentially markers from other molecular levels) will be evaluated and their clinical utility in relevant bladder cancer contexts defined.

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10.11 Cell-Based Urinary Markers

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10.11.1 Introduction

More than five decades ago, Dr. George Papanicolaou hypothesized that microscopic evaluation of exfoliated cells in the urine was a potentially useful method to detect urinary tract malignancies. His idea took advantage of access to multiple samples of urine specimens that potentially contain cells from the whole surface of the urinary tract, as upper tract, bladder, and urethra continuously defoliate and eliminate their cells in the urine stream, giving, if collected properly, a valuable material to be analyzed.

However, until now cytology is the only marker that has been broadly implemented as a decision tool in daily clinical practice. Among the different markers studied, none have been able to meet all the requirements of the ideal marker as described by the International Bladder Cancer Network (IBCN).[1] Very often, after an initial laboratory and clinical evaluation, many of the bladder cancer markers have been abandoned in favour of the search for new markers with potentially improved performance.

In this review, we will discuss the potential of urinary cytology and other cell-based bladder cancer marker tests approved by the FDA, such as ImmunoCyt/uCyt+ (Scimedx; Denville, New Jersey, United States) and fluorescence in situ hybridization (FISH) UroVysion (Abbott Molecular; des Plaines, Illinois, United States). We will talk also about the more recently released cell-based bladder cancer marker CellDetect (Zetiq Technologies Ltd.,; Tel Aviv, Israel) **(Table 10.11–1)**. Regarding ImmunoCyt/uCyt+, we want to note that Scimedx has, at the moment, stopped production.

TABLE 10.11–1 Marker Test and Manufacturer

Marker/Test	Manufacturer	Description	FDA status
ImmunoCyt/ uCyt+	Scimedx, United States*	Immunocytochemical assay for detection of expression of CEA and bladder cancer–associated mucins	Approved for follow-up; not on the market at the moment
UroVysion	Abbott Molecular, United States	Multicolour FISH assay for detection of numerical aberrations of chromosomes 3, 7, 17, and locus 9p21	Approved for diagnosis and follow-up
CellDetect	Zetiq Technologies Ltd., Israel	Platform technology comprising a proprietary plant extract and 3 dyes that enables colour discrimination between malignant (red) and benign (green) cells based on specific metabolic alterations exclusive to the tumour	Not approved

*No longer produced.

Abbreviations: CEA, carcinoembryonic antigen; FDA, US Food and Drug Administration; FISH, fluorescence in situ hybridization.

10.11.2 Cell-based bladder cancer markers

All cell-based diagnostic tests require laboratory equipment and trained and dedicated personnel. Nearly 10 % of the assays[2] are not informative because of an insufficient number of cells. Collecting methods differ. Voided urine (50–100 mL) is the most convenient and easily obtained specimen. However, since urine normally has an acidic pH, shed urothelial cells are damaged. Moreover, as it is not a direct sampling method, contamination from external genitalia and vagina can be seen. Strong hydration could dilute cells so that a larger amount of urine is needed. Cellularity is higher in the first morning urine; however, in these samples degenerative changes that can damage cells are observed. Therefore, the second morning urine is preferred, when possible.[3]

To improve the cellularity of the samples and reduce the contamination from non-urothelial cells, *catheterized urine* and *urinary bladder washing/barbotage could be used*. Bladder washings can be obtained by placing a catheter into the bladder and vigorously irrigating with saline (ie, barbotage). Bladder wash cytology yields more tumour cells in the sample and is more sensitive in identifying cancer, especially for high-grade (HG) tumours, but it also yields a higher false-positive rate than voided urine cytology, reducing specificity[4] and also increasing invasiveness and patient discomfort.[3] Therefore, the urine sampling technique and clinical history should be always specified on the cytology requisition form, since inflammatory disease, intravesical therapy (BCG, mitomycin), chemotherapy (cyclophosphamide), drug abuse (ketamine), the presence of stones, radiotherapy, or other malignancies, and manipulation (cystoscopy or catheterization) could modify cell membrane cytoplasm and nuclear morphology of urothelial cells.[5]

10.11.3 Urine cytology

Urine cytology remains the gold standard and the only urine marker with a clear recommendation by the European Association of Urology (EAU) and the American Association of Urology (AUA)[6,7] for the diagnosis and surveillance of high-grade (HG) bladder cancer, but always in combination with cystoscopy.

Many studies have evaluated the accuracy of urine cytology in the detection of bladder cancer. Overall, the reported sensitivity ranges from 20% to 97.3%; specificity ranges from 74% to 99.5%.[4,8,9]

Urine cytology has an excellent specificity and negative predictive value (NPV), with few false-positive cases for HG urothelial carcinoma (UC) and *carcinoma in situ (CIS)* (specificity, 83–99%).[6,10,11] Sensitivity for HG tumours has been historically reported as high as 80% to 90%, but with a poor sensitivity for LG tumours (sensitivity, 10–30%). More contemporary series, however, reported a lower sensitivity for high-risk patients also. Freifeld *et al.*[12] evaluated the sensitivity of cytology using several prospective studies that were designed to evaluate blue-light cystoscopy with hexaminolevulinate (Photocure; Olso, Norway) and the commercially available Cxbladder tests (Pacific Edge Ltd,; Dunedin, New Zealand) that measure the mRNA of five gene expression markers, for the detection of recurrent UC. The specificity and sensitivity of cytology for the overall cohort were calculated on a total of 1,487 urine samples. The pooled sensitivity and specificity for cytology was 40.8% and 92.8%, respectively, and 54.3% for HG/World Health Organization (WHO) grade 3 disease. In a multicentric prospective validation study, van Valenberg *et al.*[13] compared cytology and UroVysion to a newly developed urine assay,

Xpert Bladder Cancer Monitor ([Xpert, CE-IVD] Cepheid; Sunnyvale, California, United States), which measures five mRNA targets that are frequently overexpressed in bladder cancer, in patients previously diagnosed with NMIBC. Of the eligible patients, 239 with a history of bladder cancer had results for all assays. Forty-three cases of recurrences occurred. Xpert had overall sensitivity of 74% and 83% for HG tumours. Cytology showed 30% sensitivity for the all the grades, with 50% sensitivity for HG tumours.

Sensitivity and specificity depend on the threshold used to consider a cytological result as positive, and this depends mostly on how different studies considered atypical cytological diagnosis.[11]

The definition of *atypical urothelial cells* causes the greatest controversy among pathologists. Some have defined atypia as cells that are reminiscent of, but not diagnostic of, HG bladder cancer. Others define it as clusters of urothelial cells, suspicious for LG bladder cancer, and yet others believe damaged urothelial cells should be reported as atypical.[3,6,11,14,15] In recognition of the need to correct this situation and reduce the ambiguous category of atypia, an international panel of cytopathologists and urologists convened in Paris in May 2013. The goal of the Paris system was to define morphological criteria for the various categories in urinary tract cytopathology and also to standardize the reporting system in order to be universally acceptable and globally used.[16] The published diagnostic categories are shown in **Table 10.11–2**. Atypia is still strongly dependent on the pathologist's interpretation. As a result, there is a wide interobserver and intra-observer variability, which is the reason why the rates of atypia vary from 1.9% to 23.2% among institutions.[11,17] In a small survey sent to a voluntary group of US laboratories, the reported percentages of their atypia categories ranged from 0.8% to 22% (mean, 12.9%).[17] A similar survey sent to 20 international groups including France, Canada, and Japan showed similar results, with atypia ranging from 1.8% to 23.7% (mean, 13.75%).[16]

TABLE 10.11–2 Diagnostic Categories for the Paris System for Reporting Urinary Cytology

1. Nondiagnostic/unsatisfactory 2. Negative for high-grade urothelial carcinoma (HG UC)
3. Atypical urothelial cells 4. Suspicious for high-grade urothelial carcinoma (HG UC) 5. High-grade urothelial carcinoma (HG UC) 6. Low-grade urothelial carcinoma (LG UC)
7. Other: primary and secondary malignancies and miscellaneous lesions

A further problem for urologists is: What to do with atypia? In a retrospective analysis on more than 4,000 urine samples, Kapur *et al*. reported a diagnosis of atypia in 6.9% of the voided urine specimens and in 7.9% of instrumented specimens.[18] Subsequently, they detected a tumour in 32.7% of cases in the group of patients who underwent instrumented urine sampling and in 46.6% of cases in the voided urine samples. In patients with a previous diagnosis of bladder cancer, there is a higher likelihood of a subsequent malignant diagnosis after an atypical specimen.[17] In such cases, follow-up should be adapted to the specific situation. Some authors and AUA guidelines suggest but do not recommend the possibility of using tumour markers such as ImmunoCyt/uCyt+ or FISH UroVysion, as a reflex test to investigate atypia categories.[19–22]

The category *suspicious for urothelial carcinoma*, in contrast to atypia, means that the pathologist sees very rare cells with features that are compatible with UC, or that there are only some features of malignancy. The *Urothelial carcinoma* report means that the presence of tumour cells is undeniable, fitting the criteria for UC as outlined.[23,24] In that case, there is the need to evaluate the entire urothelium. While the most common finding in cases of positive cytology is HG solid tumours or CIS in the bladder, we should not forget that during follow-up of high-risk non-muscle invasive bladder cancer (NMIBC), a "non-CIS" recurrence could occur.[10] In patients with *divergent results* (ie, positive cytology and negative cystoscopy), patients should be re-evaluated carefully with a subsequent cystoscopy and multiple random biopsies of the bladder. Blue light cystoscopy is recommended in order to identify suspicious areas and avoid uninformative biopsies.[25] The upper urinary tract as well as the urethra (prostatic and penile) should be evaluated in cases of negative bladder pathology and persistent positive cytology. Retrograde pyelography of the upper urinary tract and selective cytology from the ureters and ureterorenoscopy should be performed.[10]

Controversial results in the literature are available for the *prognostic value of cytology*. Todenhöfer *et al.* showed that patients with positive cytology, uCyt+, FISH UroVysion, or NMP22 test at time of negative cystoscopy exhibit a shorter time to disease recurrence and progression. Positive cytology was independently associated with an early recurrence (HR, 2.8 [1.2–7.0]; p=0.02).[26] Furthermore, it has been reported that positive urine cytology before second transurethral resection of the bladder (TURB) (OR, 6.8; 95% CI, 2.3–19.9; p<0.01) is an independent prognostic factor of residual tumour and thereby increases the risk for the subsequent need of a radical cystectomy.[27] In a prospective study of 91 patients, however, Bell *et al.* did not confirm the prognostic value of cytology for recurrence and progression.[28]

10.11.4 ImmunoCyt/uCyt+

The ImmunoCyt/uCyt+ test is based on an immunofluorescence technique that uses three monoclonal antibodies to recognize antigens that exhibit increased expression on LG UC cells. The test consists of a cocktail of antibodies labelled with fluorescent markers: M344 and LDQ10 bind to surface glycoprotein mucins and 19A211 binds to a glycosylated form of carcinoembryonic antigen (CEA). This test was initially designed as an adjunct test for cytology using the same urine sample. The test can be performed in 2 hours and its result can be influenced by urinary infections or by the effect of intravesical therapies.[29]

The global sensitivity of immunocytology ranges between 78% and 90% and is higher than that of cytology, especially for LG cancers, whereas its specificity has been reported between 68% to 87% and tends to be lower than that of cytology (**Table 10.11–3**).[2,30,31] Generally, the rate of samples that are not evaluable is reported to be between 6% and 10%.[2,29] Most studies reported mixed cohorts composed of patients with symptoms suggestive of bladder cancer, such as microscopic or gross hematuria, and patients followed after bladder cancer resection (**Table 10.11–4).**[32–42] Prevalence of bladder cancer varies from 4% for studies in cohorts of patients with microscopic hematuria[32] to 70% in cohort studies with surveillance or with suspicion of bladder cancer.[33] Positive predictive value (PPV), negative predictive value (NPV), and specificity vary according to bladder cancer prevalence. As for other bladder cancer urinary markers test, hematuria was reported to reduce the PPV of ImmunoCyt/uCyt+.[38]

TABLE 10.11–3 Performance Characteristics of Cellular-Based Urine Markers for Bladder Cancer Detection

	Reference	No. of patients	No. of studies included	Context	Sensitivity	Specificity	No. of patients with tumour
Immunocytology meta-analyses	Chou *et al.*[30]	1,876	7	Primary diagnosis	85% (78–90%)	83% (77–87%)	401
	Mowatt *et al.*[31]	4,199	10	Mixed	84 % (77–91%)	75% (68–83%)	NA
	Schmitz-Dräger *et al.*[2]	4,899	20	Mixed	81% (median)	75% (median)	1,252
UroVysion meta-analyses	Chou *et al.*[30]	651	2	Primary diagnosis	73 (50–88%)	95% (87–98%)	144
	Mowatt *et al.*[31]	3,321	14	Mixed	76% (65–84%)	85% (78–92%)	NA
	Schmitz-Dräger *et al.*[2]	2,852	21	Mixed	72% (median)	80% (median)	792
CellDetect	Davis *et al.*[62]	217	1	Surveillance	84%	84%	96

Abbreviation: NA, not available.

TABLE 10.11–4 Sensibility, Specificity, Positive Predictive Value, and Negative Predictive Value for Urine Cytology, Immunocyt/uCyt, and UroVysion Fluorescence In Situ Hybridization*

Reference	No. of patients	Suspicious of cancer or Surveillance	Cancer detected, %	Sensitivity, % (cytology) ImmunoCyt/ uCyt	Specificity, % (cytology) Immu-noCyt/ uCyt	PPV, % (cytology) Immu-noCyt/ uCyt	NPV, % (cytology) Immu-noCyt/ uCyt	Additional comments
Schmitz-Dräger *et al.*[32]	189	189 -	4	87	91	-	-	All MIH
Schmitz-Dräger *et al.*[33]	61	61 -	28	(47) 88	(95) 79	(80) 62	(82) 94	All gross hematuria
Lodde *et al.*[34]	235	98 **173**	43,4	G1 (5) 85 G2 (55.5) 100 G3 (85.6) 92 **G1 (4.3) 78.2** **G2 (20) 70** **G3 (94) 94.4**	(95) 79.2 **(93.9) 84.7**	(95.6) 75 **(86.2) 63.8**	(56.7) 82.4 **(71.8) 53.2**	Mixed
Pfister *et al.*[35]	694	236 **458**	27.9 **22.9**	G1 (19.9) 60.7 G2 (46.3) 756 G3 (63.8) 76.8	(95.5) 71.7 **96.1**	76.8	81.9	Mixed Multicentric
Toma *et al.*[36]	126	47 **79**		G1 (42.9) 85.7 G2 (87) 739 G3 (75) 83.3	(-) 73.8	(75) 63.2	(88) 8.5	Mixed Compared to UroVysion
Sullivan *et al.*[37]	100	0 **100**	24	**LG (21) 61** **HG (27) 91**	**(97) 63**	**(71) 43**	**(78) 88**	Surveillance Compared to UroVysion
Horstmann *et al.*[38]	221	0 **221**	51.1	**G1(57) 62** **G2 (92) 82** **G3 (96) 72**	**(62) 72**	**(69) 72**	**(79) 74**	Surveillance Compared to UroVysion
Messing *et al.*[39]	341	0 **221**	15.4	**(23) 81**	**(93) 81**	**(-) 38**	**(-) 95**	Surveillance
Vriesema *et al.*[40]	104	0 **104**	25.6	**50**	**73.4**	**39.3**	**80**	Surveillance

TABLE 10.11–4 Sensibility, Specificity, Positive Predictive Value, and Negative Predictive Value for Urine Cytology, Immunocyt/uCyt, and UroVysion Fluorescence In Situ Hybridization *(Cont'd)**

Reference	No. of patients	Suspicious of cancer or Surveillance	Cancer detected, %	Sensitivity, % (cytology) ImmunoCyt/ uCyt	Specificity, % (cytology) Immu-noCyt/ uCyt	PPV, % (cytology) Immu-noCyt/ uCyt	NPV, % (cytology) Immu-noCyt/ uCyt	Additional comments
Comploj *et al.*[41]	2,217	30% **70%**	63	G2 (8) 57 G2 (36) 75 G3 (73) 79	(97.9) 72.3	(65.2) 22	(92.2) 95.2	Mixed 7,422 tests
Todenhöfer *et al.*[42]	808	808 0	14.2	G1–2 (57.7) 69.2 G3–CIS (89) 83.3	(87.5) 76.6	(43.3) 34.3	(94.3) 94.5	Suspicious of bladder cancer Compared to UroVysion
UroVysion				**Sensitivity, %**	**Specificity, %**	**PPV, %**	**NPV, %**	
Bubendorf *et al.*[46]	153			G1 71 G2 76 G3 94				
Sarosdy *et al.*[45]	497	497 0	10.1	G1 48 G2 70 G3 88	77.7	21	98.5	Gross hematuria
Todenhöfer *et al.*[42]	808	808 0	14.2	G1–2 61.5 G3–CIS 94.4	76.6	34.4	94.7	Suspicious of bladder cancer
Toma *et al.*[36]	126	47 **79**		G1 51.1 G2 63 G3 83.3	73.8	83.8	77.8	Mixed Compared to UroVysion
Horstmann *et al.*[38]	221	0 **221**	51.1	**G1 54** **G2 83** **G3 88**	**63**	**68**	**71**	Surveillance Compared to UroVysion

*Bolded numbers show results of surveillance cohorts.

Abbreviations: CIS, carcinoma in situ; HG, high grade; LG, low grade; MIH, microhematuria; NPV, negative predictive value; PPV, positive predictive value.

In a cohort of patients under surveillance, Messing *et al.*,[39] Têtu *et al.*,[43] and Mian *et al.*[44] also reported a good sensitivity of immunocytology for CIS (nearly 100%).

ImmunoCyt/uCyt+, despite being an operator-dependent exam, seems to have a lower interobserver variability than conventional cytology. In a large French prospective multicentre study including 694 patients, 458 undergoing surveillance for a previous bladder cancer,[35] the sensitivity for G1 and G2 patients was 17% and 47% for cytology and 60% and 75.6% for ImmunoCyt/uCyt+. For G3 patients, sensitivity was 63% for cytology and 76.8% for ImmunoCyt/uCyt+. In particular, the analysis showed that the sensitivity of urinary cytology in the 10 centres was much more variable (sensitivity between 27.3% and 68%), compared to ImmunoCyt/uCyt+ (sensitivity between 42.9% and 83.3%).

The ImmunoCyt/uCyt+ test has been discussed as a potential aid to prolonging intervals between cystoscopies in patients who are under surveillance. A retrospective study performed by Lodde *et al.* reported that in 216 patients under surveillance, during 26 months of follow-up, nearly 70% of control cystoscopies were negative. Particularly, in the low-risk group defined according to the EAU guidelines, 84 patients underwent 131 cystoscopies with the diagnosis of only 30 pTaG1 tumours and without any progression. The authors proposed a reduction of the surveillance scheme with annual cystoscopies and ImmunoCyt/uCyt+ and cytology tests every 6 months. The additional cost of the immunocytological test would in these cases be compensated by the reduction in the number of cystoscopies.[34] Nevertheless, no data from large prospective studies are presently available and surveillance based exclusively on urinary markers is not recommended by AUA and EAU guidelines.[6,7]

Recently, Odisho *et al.*[19] proposed using ImmunoCyt/uCyt+ in patients whose cytology was atypical but not clearly positive, so-called reflex ImmunoCyt/uCyt+. This approach was performed retrospectively on 506 urine samples from 324 patients who presented with hematuria or who were under surveillance. The reference to define true and false-positive tests was the 90-day abnormal cystoscopy. ImmunoCyt/uCyt+ sensitivity was 73%, specificity 49%, and PPV 36.4%. The authors proposed that reflex ImmunoCyt/uCyt+ testing could be used in patients with atypical cytology, with a NPV of 83.7% and that cystoscopy, in patients with LG cancer, could be avoided. However, ImmunoCyt/uCyt as a reflex test is not recommended by current guidelines but considered optional in this setting.[6]

Todenhöfer and colleagues assessed the diagnostic accuracy of different markers alone and when using them in combination in a cohort of 808 patients without previous diagnosis of bladder cancer. The overall sensitivity and specificity of ImmunoCyt/uCyt+ was 73.9% and 76.6% when used as single marker with an area under the curve (AUC) of 0.75. Cytology and fluorescence in situ hybridization (FISH) were found to be the single tests with the best overall performance (AUC, 0.78/0.79). Combinations of 2, 3, and 4 markers were found to increase the AUC to various extents compared with the use of single markers, but also consistently increased the cost.[42]

10.11.5 Fluorescence in situ hybridization (Multi-target multicolor FISH - UroVysion™)

The multitarget cytogenetic FISH UroVysion™ test (Abbott Molecular; des Plaines, Illinois, United States) consists of 4 probes of different colours to identify numerical alterations of chromosomes 3, 7, and 17 and the loss of the specific locus 9p21, present on the short arm of chromosome 9.[30] To consider a test positive, according to the manufacturer, 25 cells should be morphologically altered with 4 or more of these cells showing a gain of 2 or more of chromosomes 3, 7, or 17, or when 12 or more cells show a loss of the two copies of locus 9p21. Although this test was initially approved for the surveillance of patients with bladder cancer in conjunction with cystoscopy, it was then also approved as a diagnostic aid in patients with hematuria.[45]

The urine FISH diagnostic test has been shown to be more sensitive than cytology in detecting bladder cancer at the cost of a lower specificity. Its sensitivity ranges from 50% to 88% and its specificity from 78% to 92% (**Table 10.11–3**).[2,30,31] Bubendorf *et al.* reported that FISH has a sensitivity twice as high as cytology in pTa and pT1 tumours (50.9% vs 29.8%). In invasive tumours (pT1 and pT2–4), the test showed a sensitivity of 88% compared to 68% for cytology.[46]

In subsequent publications, using modified criteria for the definition of FISH-positive samples, Bubendorf himself[47] reported a sensitivity of FISH that was triple that of cytology in pTa tumour (73% vs 24%). For invasive tumours, he reported a sensitivity of 100% versus 58% for cytology. Notably, the scoring algorithm of the manufacturer was developed for voided urine, whereas Bubendorf *et al.*[47] and Zellweger *et al.*[48] progressed from voided urine to a fine-tuned algorithm for bladder washings. According to Bubendorf, non-tetrasomic cells are regarded as abnormal by FISH if they show 3 or more copies of any of the signals for chromosomes 3, 7, and 17 and the 9p21 locus, or if there is a heterozygous or homozygous loss of 9p21 (one copy or both copies lost). Since tetrasomic cells were not counted, they could lower the cutoff of the number of aneusomic cells to define a FISH-positive specimen and still retain a high specificity. A tetraploid pattern of the FISH signals is a common finding in reactive umbrella cells in bladder washings from patients with benign irritative bladder pathology. It is likely that a higher fraction of umbrella cells is obtained by the bladder washing procedure than by spontaneous shedding of umbrella cells in voided urine.[48] Since a tetraploid pattern represents a simultaneous gain of all chromosomes, it can lead to a positive FISH result as defined by the manufacturer's criteria. Therefore, the scoring system from Zellweger *et al.* considered FISH positivity by considering rare cells (≤10) with a tetraploid pattern as FISH negative. In addition, those authors showed that the presence of 9p21 deletion by FISH could also predict a high risk for recurrence at the time of a negative follow-up cystoscopy.

Mischinger *et al.*, in a retrospective cross-section study, compared scoring algorithms from the manufacturer to that defined by Bubendorf and Zwellinger.[49] The method used by Zellweger *et al.* showed highest total accuracy of all FISH assessments (76.5%), which was the result of a high specificity (78.8%). Additionally, the highest total PPV (47.3%) suggested more power in indicating a need for random biopsies in patients with negative cystoscopy and positive FISH. Tetraploid cells could neither be associated with bladder cancer development nor recurrence or progression. The algorithm proposed by Zellweger *et al.* had the lowest total

sensitivity (68.4%) for all FISH methods, whereas the method developed by Bubendorf *et al.* surpassed the manufacturer's algorithm in total sensitivity (76.8% vs 71.9%) and NPV (90.7% vs 89.9%). Due to the highest sensitivity and NPV, the interpretation algorithms suggested by Bubendorf *et al.* and the manufacturer are preferable for patients under surveillance, whereas the application of the algorithm proposed by Zellweger *et al.* is preferable in the primary diagnostic setting requiring high specificity and PPV.[49] Other studies confirmed the high sensitivity of FISH (96.4%) and the lower specificity compared to cytology (**Table 10.11–4**).[36,38,42]

The issue of positive results in the setting of normal cystoscopy has been followed in various studies, and the existence of anticipatory positive tests has been discussed.[50] The term "anticipatory positive" refers to patients who have a positive test but negative cystoscopy at time of testing and will develop a tumour during follow-up. The concern is that a positive result might be due to either a false-positive result or the presence of cancer that is too small to visualize with cystoscopy. In these patients, it has been discussed that the test can highlight molecular changes some months before cystoscopy or cytology. Yoder and colleagues,[51] for example, reported that two-thirds of patients who were FISH positive but had negative cystoscopy (27% of the entire cohort) developed cystoscopically visible cancer in the following 29 months. Seideman *et al.*[52] also reported in a retrospective multicentre study from 664 patients that, in patients who were FISH positive, mean time to recurrence was 12.6 months, compared to 17.9 months in FISH-negative patients (p=0.03). In multivariate analysis, only pathologic stage, cystoscopic findings, and cytology but not UroVysion were independently associated with recurrence. To the contrary, progression to ≥T2 bladder cancer occurred in 34 (5.1%) patients in this same cohort and, in multivariate analysis, only initial T stage and FISH result were found to be independent predictors of progression (p<0.05).[50] Currently, however, there are no clear recommendations for different surveillance for patients who have a negative cystoscopy and cytology but positive FISH test.

The role of UroVysion as a reflex test for patients with atypical cytology was addressed in 2 studies, Lotan *et al.* and Schlomer *et al.*[20,22] The researchers prospectively evaluated a population comprising 50 patients with no history of cancer and 70 who underwent cystoscopy for cancer surveillance and thereafter 108 patients with no history of cancer and 108 who underwent cystoscopy for cancer surveillance. In patients with cystoscopically visualized lesions, UroVysion had a PPV of 100%, but there were false-negative results. In patients with equivocal cystoscopy and a history of cancer, all HG tumours were detected and there were no false-negative findings. In patients with equivocal cystoscopy and no prior cancer, the PPV was 100% and there were no false-negative results. In patients with negative cystoscopy, the UroVysion test detected all cancers, but the PPV was 10% and 29% in patients with and without a history of cancer, respectively. Schlomer *et al.* concluded that in patients with atypical cytology and obvious tumour on cystoscopy, the reflex assay is unnecessary but may help avoid unnecessary evaluation while identifying those who would need further evaluation in patients with atypical cytology and indeterminate cystoscopic findings.[20]

Virk *et al.* reported on the role of FISH as a reflex test for atypical cells in urine. In 377 patients with atypical cells in voided urine, the authors found that 16.45% were diagnosed with UC within 12 months. FISH analyses for patients with atypical cytology were not evaluable in 11.94%, positive in 16.71%, and negative in 71.35%. UroVysion sensitivity, specificity, PPV, NPV, and accuracy were 44.64%, 81.82%, 47.17%, 80.25%, and 71.91%,

respectively. FISH showed a high false-positive rate of 52.83% at the time of urine analyses, which remained high even after extended follow-up, arguing again against "anticipatory positive" results.[53]

Results from UroVysion have shown an association with outcome in patients with NMIBC. In an average period of 14.2 months, a study comprising FISH results of urine from 51 patients showed that those with negative FISH relapsed or progressed in 4.3% of cases; in the case of a loss in the p16 locus with or without an aneusomy of chromosome 3, the recurrence and/or progression rate was 15.0% and 60% in the case of aneuploidy of chromosome 7 and/or 17. The authors proposed to stratify patients according to UroVysion results into patients with low and high risk for recurrence.[54] Maffezzini and collaborators have shown similar results in their study including 126 patients.[55] Kawauchi *et al.*[56] (using bladder washing urine) and Krüger *et al.*[57] (on paraffin tissue) have shown in two independent studies that the loss of 9p21 predicts relapse but not progression.

Kamat *et al.* first reported a prospective clinical trial to determine whether FISH results during BCG immunotherapy can predict therapy failure following TURB for high-risk NMIBC.[58] FISH was performed before bacillus Calmette-Guérin (BCG) and at 6 weeks, 3 months, and 6 months during BCG treatment. Cox proportional hazards regression was used to assess the relationship between FISH results and tumour recurrence or progression. A total of 126 patients participated in the study. At a median follow-up of 24 months, 31% of patients had recurrent tumours and 14% experienced disease progression. Patients who had positive FISH results were 3 to 5 times more likely than those with negative FISH results to experience recurrent tumours and 5 to 13 times more likely to have disease progression ($p<0.01$).

Recently, two multicentric prospective studies confirmed the predictive value of FISH in detecting recurrence and progression in HG bladder cancer treated with BCG.[59,60] Liem *et al.*[59] collected three bladder washouts at different time points during treatment (t_0= week 0; pre-BCG; t_1= 6 weeks following TURB; t_2= 3 months following TURB) in a multicentre, prospective study including patients with bladder cancer treated with BCG. Of 114 patients, 31.6% developed a recurrence after a median time of 6 months. A positive t_2 FISH test was associated with a higher risk for recurrence ($p=0.001$). Patients with a positive FISH test 3 months following TURB had a 4.0 to 4.6 times greater risk of developing a recurrence compared to patients with a negative FISH. The authors concluded that FISH could therefore be a useful additional tool for physicians when determining a treatment strategy.[59] Lotan *et al.*[60] also recruited a total of 150 patients with bladder cancer treated with BCG. At 9 months of follow-up, there were 46 events, including 37 recurrences and 9 progressions. For events with positive FISH, the HR was 2.59 (95% CI, 1.42–4.73) for the baseline FISH test, 1.94 (95% CI, 1.04–3.59) for the 6-week test, and 3.22 (95% CI, 1.65–6.27) at 3 months. Patients with positive results at baseline, 6 weeks, and 3 months had events 55% of the time and patients with negative results at each time point had no event 76% of the time.[60] However, using the test to change management decisions is limited due to the discordance between results and outcomes, as well as the variance of tests results with time.

10.11.6 CellDetect

CellDetect technology is a novel cell staining method based on a proprietary plant extract that enables colour discrimination between benign and malignant cells while preserving critical features of cell morphology. The discriminative capacity of the stain is related to specific metabolic alterations and increased metabolic activity observed in neoplastic cells. Preclinical studies and clinical trials demonstrated the applicability of this technology in many cell culture lines and various cancers (**Table 10.11–3**).[61,62]

Two studies from the same group have been published. The first consists of an open label, two-step study at tertiary medical centres.[61] The study enrolled patients with newly diagnosed or a history of UC. Step 1 involved staining archived biopsies. The slides were evaluated by two independent pathologists who determined the concordance of the new staining technology with the hematoxylin and eosin–based diagnosis. Step 2 included staining urine specimens with the new method and comparing findings to the patient final diagnosis and the results of standard urine cytology. A total of 58 archived biopsies were collected. The concordance of staining using the new platform technology with the hematoxylin and eosin–based diagnosis was 100%. The new method applied to 44 urine smears showed 94% sensitivity and 89% specificity to detect UC. Compared to standard urine cytology, the new technology had overall superior sensitivity (94% vs 46%), particularly for LG tumours (88% vs 17%, each $p<0.005$). There was no significant difference in specificity between the two staining techniques.[61]

Recently, the same group reported the results of a multicentre, prospective, blinded study conducted in nine hospitals that included patients with a documented history of bladder cancer and monitored for UC or scheduled for bladder cancer surgery.[62] Cystoscopy and/or biopsy was used as a reference standard to determine sensitivity and specificity. Smears were stained by CellDetect and interpreted by two cytologists blinded to the patient's final diagnosis. The findings were compared with those of standard urine cytology and BTA stat. A total of 217 voided urine specimens were included: 44% were positive by histology and 56% were negative by either cystoscopy or histology. The overall sensitivity of CellDetect was 84%. Notably, the sensitivity for detecting LG NMIBC tumours was greater than that of BTA stat (78% vs 54%) and more than two-fold higher compared with standard cytology (33%). The specificity was 84% in patients undergoing routine surveillance by cystoscopy. At a median follow-up of 9 months, 21% of the patients with positive CellDetect and negative cystoscopy developed urothelial cancer, which was significantly higher compared with the 5% of the true negative cases. The test is promising but further studies with samples from patients with other urologic malignancies and with bladder washing urine sampling are needed. Moreover, hematuria and strong inflammation seem to interfere with the reading of the slides because of the high number of superimposed cells.[62]

10.12 Conclusion

Cytology has been the standard of care cell-based urinary marker for the detection of bladder cancer for decades. It is an important component in the diagnosis and follow-up of patients with bladder cancer, especially for HG tumours. However, the test shows unsatisfactory sensitivity in LG tumours. To address this important limitation, cell-based markers that provide improved sensitivity have been introduced.

ImmunoCyt/uCyt+ and UroVysion are superior compared to cytology in the diagnosis of LG bladder cancer and comparable for the detection of HG tumours. Nevertheless, the specificity is lower than that of cytology and PPV is low. The immunocytological test ImmunoCyt/uCyt+ has been removed from the market, although several studies suggested that the test might be a good adjunct to cytology in order to detect LG tumours in urine samples. A potential but untested use could be to use a marker to reduce the number of cystoscopy procedures in the surveillance setting of low- and intermediate-risk bladder cancer with ImmunoCyt/uCyt+, but no large prospective and cost-benefit analyses are available at the moment justifying such an approach.

There is a current role for use of cell-based markers in adjudicating atypical cytology or cystoscopy. It is in this area that the guidelines support utilization of UroVysion and ImmunoCyt.

In addition to detection of recurrences during surveillance, cell-based markers represent potential tools for early detection of BCG failure. Several studies suggest that a positive UroVysion test after BCG induction predicts poor response to BCG, with higher likelihood for recurrence and progression.

CellDetect technology has been recently introduced on the market with promising results that should be confirmed by larger clinical trials.

To promote the use of cell-based urinary markers in daily clinical practice, prospective trials including panels of these markers are urgently needed. Otherwise, the likelihood that markers other than cytology will have a role for clinical decision-making is low.

10.13 References

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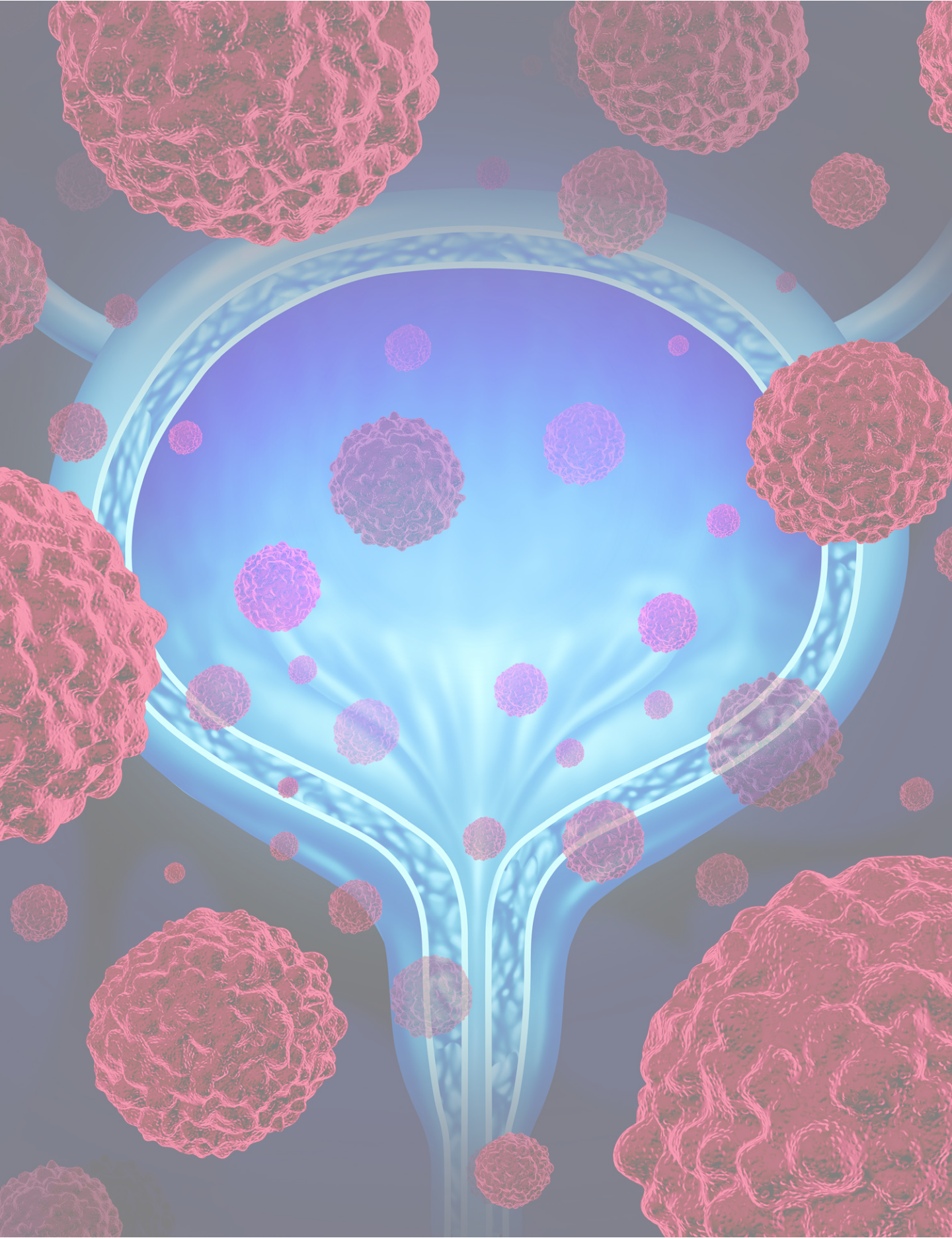

CHAPTER 11

Bladder Cancer Tissue-Based Biomarkers

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Table of Contents

11.1 Introduction

There have been significant innovations in the treatment of bladder cancer (BCa). While most treatments are standardized, we are transitioning from the era of "one size fits all" to the era of "precision medicine," where treatments are personalized and tailored to patients' tumour characteristics. Biomarkers play an undeniable role in this setting, allowing for patient risk stratification, predicting response to treatments, and paving the way for targeted therapies. In this section, the technical aspects of tissue-based biomarkers as well as their current role in terms of clinical utility, both in non-muscle invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC), will be reviewed.

11.2 Technical Aspects of Tissue-Based Biomarkers in Bladder Cancer

Several technical aspects should be considered when working with tissue specimens:
First of all, there are important differences between using fresh, frozen, or formalin-fixed material. The use of fresh or fresh-frozen tissue enriches the quality of RNA-derived material as compared to formalin-fixed tissue.[1] However, current commercial kits have improved the amount and quality of RNA material that can be extracted. DNA is usually more stable and unless long nucleic acids are required, DNA of sufficient quality for analysis can be obtained for the majority of the polymerase chain reaction (PCR)-based methods in fresh, fresh-frozen, or formalin-fixed material.

Second, it is critical to consider that bladder tumours are extremely heterogeneous. When working with non-muscle invasive disease, this heterogeneity might be less relevant than with advanced disease and tumour cell types might be more homogeneous. Thus, analyses performed using different paraffin blocks could provide similar results. However, when working with muscle-invasive disease, tissue heterogeneity might be extremely high, with differential ratios between tumour and stromal cells among paraffin blocks leading to very different results if microdissection is not performed. In gene profiling, a study demonstrated that differential results can be obtained in non-muscle and muscle-invasive tumours comparing fresh-frozen material, frozen-microdissected tissue, and laser-microdissected frozen tissue.[1] Using immunohistochemistry to evaluate tissue heterogeneity may lead to significant differences when staining sections from different blocks. There is a trend toward using tissue microarrays because of the possibility of measuring hundreds of tumours in one single slide. However, in muscle-invasive tumours, it is advisable to evaluate protein patterns of expression in single slides because of the risk for heterogeneity in a high number of patients.

Third, sample handling is very important to preserve the quality of the specimen, whether it is for nucleic acid extractions or protein patterns measurements by immunohistochemistry. In frozen material, it is important that freezing is performed within 30 minutes from the time the specimen is removed. It is advisable to have freezers on-site in the operating room. When working with paraffin-embedded tumours, it is important that specimens are maintained no longer than 24 hours in formalin for fixation and that blocks are well orientated to be cut

within that time. This may also affect nucleic acid quality for PCR-based measurements, and differences among specimens from different hospitals can be observed due to sample handling of paraffin-embedded material.[2,3]

Fourth, the use of controls is mandatory. Comparison with normal urothelium should always be performed in any experiment at the DNA, RNA, or protein level.[4] Matching normal urothelium might not always be available, and the presence of epigenetic or genetic alterations in "adjacent normal" might affect interpretation of results.[5] Access to normal urothelium obtained from patients without BCa is recommended. When working with immunohistochemistry protein patterns, it is important to use technical controls that can prove that each step has been correctly performed. These should include analyses on slides without adding primary and secondary antibodies, blocking nonspecific binding of antibodies, or using different tissue type controls.

Finally, several considerations should be made in the use of tissue microarrays (TMAs) in immunohistochemical patterns of expression. Indeed, these arrays represent an efficient way to measure hundreds of cases in a single experiment while minimizing cost and use of the primary antibody. It is important to account for tissue heterogeneity, which may be particularly important for invasive tumours. Using duplicate specimens can help validate TMA findings. These tissue arrays should include different tissue types surrounding the spots containing bladder tumours, which may serve as negative and positive controls for any tested antibody so that each staining is internally controlled.[4]

11.3 Tissue-Based Biomarkers in Non-Muscle Invasive Bladder Cancer

In general, prognostic information for patients with NMIBC is highly desirable, as guidance for further treatment and follow-up is urgently needed. So far, this information relies exclusively on clinicopathologic parameters.[6–9] However, additional information reflecting the biology of a given tumour could greatly impact patient management. Therefore, tremendous research efforts have been made throughout the past decades to identify and characterize molecular markers related to tumour prognosis. Numerous different markers related to different pathways of cell growth and differentiation and of various molecular origins have been explored. This chapter aims at the definition of the challenges and summarizes the current status of tissue-based molecular markers in NMIBC. To improve the readability and clinical utility, we restricted this review to more widely studied markers, aiming at covering more recent achievements, and focusing on suggestions about how to implement molecular markers in clinical practice.

As previously discussed, to include a given marker in clinical decision-making, several steps must be taken, including technical aspects and questions considering thorough clinical validation. The necessity of validating the prognostic role of a specific marker is greatly underestimated. Confirming the findings by internal and external validation is still rare in the current literature, especially in NMIBC. Another shortcoming in many studies is the lack of considering established prognostic clinical and pathological information.[8,9] Finally, inclusion of a marker in clinical decision-making requires confirmation of its relevance in a prospective trial. As this aspect has been

largely neglected in the past, current guidelines do not consider tissue markers in patient management.[6] The p53 trial results triggering a decision for or against chemotherapy in advanced BCa has remained a rare exception.[10] It is only today, in the age of personalized medicine, that molecular markers are included in randomized trials, although again, this is still a domain of MIBC. Key prognostic biomarkers for disease recurrence and progression in NMIBC are listed in **Table 11-1** and briefly described below.

11.3.1 Prognostic markers for disease recurrence

There are two general classes of markers: prognostic markers and predictive markers. A prognostic biomarker provides information about a patient's overall outcome (cancer recurrence/survival/overall mortality) regardless of therapy, while a predictive biomarker gives information about the effect of a therapeutic intervention.

Numerous biomarkers have been investigated to determine prognostic risk of tumour recurrence in NMIBC.[11–13]

The p53 tumour suppressor gene is probably the first molecular alteration that has been extensively studied, but there have been conflicting results regarding its association with prognosis. Several groups report a worse outcome,[14,15] but this was not confirmed by other investigators.[16,17] Similarly, Shariat *et al.*[18] found a mutant p53 genotype more frequently in recurrent tumours, while another group[19] observed an increased recurrence rate in patients with wild-type tumours.

Using p53 as a marker has issues due to multiple cell cycle regulators that may have overlapping roles. Considering the biological diversity of NMIBC, along with its intratumoural heterogeneity, the research focus on tissue marker research has shifted from investigation of single alterations to consideration of combined alterations, including gene classifiers for distinguishing between recurrent and nonrecurrent NMIBC, with promising results.[18,20–22] Nevertheless, results from a large, international retrospective validation study of 404 patients investigating a 26-gene signature found no association with tumour recurrence.[23] Fibroblast growth factor receptor 3 (FGFR3) mutations have been correlated with the prognosis of patients with NMIBC. A retrospective multicentre study investigated the prognostic potential of FGFR3 status and three molecular markers (MIB-1, p53, and P27kip1) in 286 patients with NMIBC, with a mean follow-up of 5.5 years. While p53 and P27kip1 expression yielded no additional information, the combination of FGFR3 and MIB-1 was found to be an independent predictor of disease recurrence rate.[17] More recently, dysregulation of several microRNAs (eg, miR-138, miR-26b-5p, and miR-221/222 cluster expression) has been suggested to predict tumour recurrence;[24–26] however, validation and prospective assessment are lacking.

In summary, the role of molecular markers in the prognostication of NMIBC disease recurrence seems limited, not only due to technical reasons but also because clinical parameters (eg, multiplicity, tumour size, incomplete transurethral resection [TUR]) importantly affect this event and mitigate the impact of biomarkers.[17]

TABLE 11-1 Prognostic Biomarkers in Non-Muscle Invasive Bladder Cancer

Molecular pathway	Bio-marker	Method	n	Results	Reference
Cell cycle regulation and proliferation	**RB**	IHC	74	No association with progression	Têtu *et al.*[40]
	P21	IHC	207	No association with progression	Liukkonen *et al.*[41]
			244	No association with recurrence	Pfister *et al.*[42]
	P27	IHC	61	No association with recurrence and progression	Park *et al.*[43]
	Ki-67	IHC	61	No association with recurrence and progression	Park *et al.*[43]
Cell death pathways	**p53**	IHC	69	Overexpression predicts disease progression	Serth *et al.*[14]
		IHC	104	Overexpression predicts disease recurrence	Vorreuther *et al.*[15]
		IHC	286	Overexpression alone predicts disease progression, but not in combination with FGFR3 mutation	van Rhijn *et al.*[17]
		IHC	83	Overexpression predicts disease recurrence and progression	Shariat *et al.*[18]
		rtPCR	105	No prognostic value	Moonen *et al.*[19]
	BCL2	IHC	100	No association with recurrence	Tzai *et al.*[44]
		IHC	93	No association with recurrence	Wu *et al.*[45]
Cell growth signalling	**FGFR3**	rtPCR	286	Mutation associated with higher recurrence-free and progression-free survival	van Rhijn *et al.*[17]
	ERBB2 (HER2)	IHC	88	Association with recurrence and progression	Hegazy *et al.*[46]
		rtPCR	141	Association with recurrence and progression	Lim *et al.*[47]
		rtPCR	34	Association with progression	Breyer *et al.* (2017)[48]
	Survivin	IHC	233	Association with progression	Breyer *et al.* (2016)[49]
			115	Association with recurrence and progression	Senol *et al.*[50]
			283	Association with progression and survival	Fristrup *et al.*[51]
Angiogenesis markers	**VEGF**	IHC	185	No association with recurrence	Chow *et al.*[52]
			140	No association with recurrence and progression	Theodoropoulos *et al.*[53]
	HIF-1α	IHC	140	No association with recurrence and progression	Theodoropoulos *et al.*[53]
Immune markers	**PD-L1**	rtPCR	296	Association with recurrence, progression, and survival	Breyer *et al.* (2018)[38]

Abbreviations: BCL2, B-cell/CLL lymphoma 2; FGFR3, fibroblast growth factor receptor 3; HIF-1α, hypoxia-inducible factor 1α; IHC, immunohistochemistry; PD-L1, programmed cell death 1 ligand 1; RB, retinoblastoma; rtPCR, reverse transcription polymerase chain reaction; VEGF, vascular endothelial growth factor.

11.3.2 Prognostic markers for disease progression

A plethora of different markers has been tested for the prognostication of tumour progression in NMIBC. The main concern with progression of disease is from NMIBC to MIBC, especially in patients with T1 disease. There are only a few biomarkers found to be clinically relevant and deserving of discussion.

p53 alteration is one of the first and certainly most frequently studied markers in this context. Most of these studies, including a combined analysis of 23 studies,[27] reported a correlation between p53 overexpression or p53 mutation and tumour progression. However, as p53 alterations are closely related to tumour grade, stage, and other molecular changes, an independent prognostic value of this parameter remains a matter of controversy.[16–18] Immunohistochemical p53 overexpression has also been tested in combination with other alterations, frequently related to cell cycle regulation. Shariat *et al.*[18] in analyzing p53, pRB, P21, and P27 expression in a large retrospective analysis, concluded that the combination of altered markers rather than a single marker alteration was associated with tumour progression on multivariable analysis. These findings were supported by the work of Chan and his group, who examined the expression of angiogenin, matrix metalloproteinase-2, p53, RB, and PAI-1 in NMIBC.[22] However, prospective validation of these findings is lacking. There was one prospective study, where every patient with high-grade NMIBC treated with TUR of the bladder underwent immunohistochemical staining for 5 biomarkers (p21, p27, p53, Ki-67, and cyclin E1). Results indicated that there were no differences in survival based on the number of altered markers, nor was biomarker status a significant predictor of recurrence or progression. While marker alterations have marginally improved discrimination of European Organisation for Research and Treatment of Cancer (EORTC) and Club Urológico Español de Tratamiento Oncológico (CUETO) models, their models were found to have only moderate predictive ability.[28]

A molecular grading based on the combination of FGFR3 mutation and MIB-1 expression has been shown to be significantly associated with disease progression. The molecular grading system based on this observation was validated in a retrospective analysis with a mean 8.8 years follow-up interval.[29] A more recent investigation, using a more detailed molecular grading system, confirmed the earlier findings.[30] Results from another large prospective study of 1,239 patients from the same group demonstrated that molecular grading based on FGFR3 mutational status and methylation of GATA2 was able to improve the European Association of Urology (EAU) NMIBC risk score in predicting tumour progression.[31] The prognostic relevance of the FGFR3 mutational status was further corroborated by prospective investigations.[32,33]

Development of gene classifiers and subtyping using microarrays is another option for combining molecular information.[34,35] In a large, prospective Scandinavian-based trial of 1,224 patients, Dyrskjøt *et al.* demonstrated that the results of a 12-gene, real-time qualitative PCR assay yielded independent prognostic information on tumour progression.[36] Nevertheless, with a 66% sensitivity and specificity to predict tumour progression as a stand-alone assay, it becomes obvious that, at this stage, information obtained by molecular makers is not sufficient and needs to be integrated with established clinicopathologic variables.

11.3.3 Predictive markers of response to intravesical therapy

Regarding tumour recurrence and progression, various tissue-based biomarkers have been evaluated for prediction of response in patients with NMIBC undergoing bacillus Calmette-Guérin (BCG) therapy. To date, the best available evidence comes from a validation study based on two Nordic multicentre trials comparing treatment with BCG with other intravesical adjuvant therapies.[37] In this report, ezrin, CK20, and Ki-67 have been analyzed in a TMA. In BCG-treated patients, at multivariable analysis, none of the variables correlated with disease recurrence and only tumour multifocality was associated with disease progression.

Following the advent of immunotherapy with checkpoint inhibitors, also in the NMIBC setting (recently, the use of pembrolizumab in patients with BCG-unresponsive carcinoma in situ [CIS] has been approved by the FDA), the role of potential immune markers to predict response to BCG has been investigated with promising results. However, it should be noted that these findings need to be externally validated before they could be considered for clinical practice. The prognostic role of programmed cell death 1 receptor (PD-1) and programmed cell death 1 ligand 1 (PD-L1) mRNA expression has been recently assessed:[38] high mRNA expression of PD-L1 was able to independently predict improved recurrence-free, progression-free, and cancer-specific survival (CSS) in T1 NMIBC patients treated with BCG. Moreover, the involvement of certain classes of lymphocytes is of major significance in the immunotherapy response as a reflection of the immune system activation. It has been shown that BCG generates a Th1 response and, therefore, an increased Th1 activity has been observed in patients responding to intravesical immunotherapy.[39] Conversely, the presence of a preexisting Th1 immunologic environment within the tumour was associated with BCG failure. Consequently, the CD4+/CD8+ lymphocyte ratio in the primary specimen was investigated as a biomarker to predict BCG response.

11.4 Tissue-Based Biomarkers in Muscle-Invasive Bladder Cancer

11.4.1 Prediction of oncological outcomes

The standard treatment for patients with MIBC is radical cystectomy (RC) with neoadjuvant chemotherapy (NAC). However, despite the administration of adequate therapy and the recent development of new treatment strategies, such as trimodal therapy or targeted therapies, MIBC remains an aggressive disease characterized by a generally unfavourable prognosis,[54] high rates of recurrence,[55] and progression to metastatic disease. There are significant challenges in accurately staging the disease and insufficient ability using clinical/pathological factors alone to predict recurrence and progression, and importantly, an inability to predict response to systemic therapies (chemotherapy and immunotherapy) and radiotherapy. The consequence is that many patients are undertreated, overtreated, or given therapies which are unlikely to benefit them.

Some clinical preoperative features, such as clinical stage, presence of concomitant CIS at the time of TUR, tumour grade, presence of variant histology at TUR, presence of lymphovascular invasion (LVI) at TUR, and presence of preoperative hydronephrosis have been found to be associated with worse oncologic outcomes after RC.[56] Although several nomograms and prognostic models aiming to predict long-term prognosis of patients treated with RC[57] and stratify patients for selection for NAC, based on these clinical and pathological parameters, have been proposed, none of them have proved sufficiently accurate to entered in clinical practice.[58,59] Understanding the molecular pathology and biology of BCa could be useful to improve patient stratification and clinical decision-making. Recently, several reports have focused on molecular biomarkers as diagnostic and prognostic tools in MIBC, although their application in clinical practice remains unclear to date. In this section, the role of tissue biomarkers in the prediction of prognosis after RC, and in the response to neoadjuvant and adjuvant therapies will be reviewed.

11.4.2 Prediction of disease stage at radical cystectomy

An accurate prediction of disease stage at RC is of fundamental importance to risk stratification and to patient selection for neoadjuvant systemic therapies. Despite international guidelines recommending NAC in cT2–T4a N0M0 patients, compliance with this recommendation remains low, especially in cT2 disease. This is probably due to the fact that data from two prospective randomized clinical trials (SWOG 8710 and BA06 30894) showed a greater survival benefit for patients with ≥cT3 disease compared to those with cT2 (survival gain of 42 vs 19 months, respectively).[60] However, a critical point is represented by the poor accuracy of clinical staging and, consequently, by the high rate of upstaging at final pathology. Therefore, an accurate preoperative staging of the disease is essential for decision-making regarding preoperative therapies. Several tissue-based biomarkers have been investigated for this purpose and have been integrated in predictive models. Mitra *et al*. firstly developed a pre-cystectomy decision model to predict pathological upstaging and oncological outcomes in cT2 patients undergoing RC.[61] This model was based on clinicopathologic variables such as the preoperative presence of hydronephrosis, evidence of deep muscularis propria invasion, and LVI as well as tumour growth pattern and count. Subsequently, Shariat *et al*. tested the accuracy of a preoperative panel of tissue-based biomarkers (p53, p21, p27, Ki67, and cyclin E1); the number of altered biomarkers was categorized as favourable (≤2 altered markers) or unfavourable (>2).[62] An unfavourable biomarker score was able to predict T-stage upstaging, but not T- or/r N-stage upstaging; moreover, the overall accuracy of the model in the prediction of the T-stage upstaging was low (62%). Recently, a genomic subtyping classifier was used to evaluate pathological upstaging in a multi-institutional cohort of patients with cT1–T2 urothelial carcinoma treated with radical cystectomy.[63] Luminal tumours, characterized by a strong expression of markers typically associated with the luminal subtype (ie, peroxisome proliferator-activated receptor γ and KRT20) and lower expression of basal-associated, epithelial–mesenchymal transition (EMT)-associated, immune-associated, and stromal-associated markers, showed a lower rate of upstaging to nonorgan-confined disease compared to nonluminal tumours (34% vs 51%). Pending external validation, molecular characterization promises to transform the paradigm of BCa risk stratification, thus paving the way to an even more personalized approach.

11.4.3 Prediction of oncological outcomes after radical cystectomy alone

There has been an interest in predicting likelihood of recurrence in patients who have undergone cystectomy alone. These patients may benefit from adjuvant therapies such as chemotherapy[64] and there are multiple trials evaluating the value of adjuvant checkpoint inhibitors. Key biomarkers evaluated for prediction of oncological outcomes are listed in **Table 11-2**.

TABLE 11-2 Prognostic Biomarkers After Radical Cystectomy Alone in Muscle-invasive Bladder Cancer

Molecular pathway	Biomarker(s)	Method	n	Results	Reference
Cell cycle regulation	**RB**	IHC	38	Association with cancer-specific mortality	Cordon-Cardo *et al.*[73]
	P21	IHC	692	Association with disease recurrence and cancer-specific mortality	Shariat *et al.* (2010)[82]
Cell death pathways	**p53**	IHC	692	Association with disease recurrence and cancer-specific mortality	Shariat *et al.* (2010)[66]
		IHC	243	Association with disease recurrence and cancer-specific mortality	Esrig *et al.*[65]
	ERBB2 (HER2)	IHC	354	No association with oncological outcomes	Soria *et al.*[70]
		IHC	198	Association with disease recurrence and cancer-specific mortality	Bolenz *et al.*[71]
	Survivin	IHC	222	Association with disease recurrence and overall mortality	Shariat *et al.* (2007)[75]
Angiogenesis markers	**VEGF**	IHC	286	Association with cancer-specific mortality	Herrmann *et al.*[83]
Markers of tumour cell invasion	**E-cadherin**	IHC	25	Association with survival	Bringuier *et al.*[84]
	MMPs	IHC	54	Association with disease recurrence	Vasala *et al.*[85]

Abbreviations: IHC, immunohistochemistry; MMP, matrix metalloproteinase; RB, retinoblastoma; VEGF, vascular endothelial growth factor.

Currently, *p53* is the most widely studied prognostic biomarker in patients treated with RC, with conflicting results reported in the literature. In patients with BCa confined to the bladder, p53 has been associated with progression and survival, independent of tumour grade, stage, and lymph-node status.[65] Shariat *et al.*[66] evaluated data from 692 patients with advanced BCa (pT3–pT4, with or without N+) treated with RC and pelvic lymph node dissection (PLND): at multivariable analysis, p53 expression was independently associated with recurrence and cancer-specific mortality (CSM). These findings were partially confirmed in a meta-analysis by Malats *et al.*[67]: a prognostic value of p53 overexpression for disease recurrence was observed in 9 of 34 studies (27%).

Human epidermal growth factor receptor 2 (HER2) is a tyrosine kinase transmembrane receptor involved in cell cycle regulation and cell proliferation. HER2 overexpression has been extensively studied in breast and gastric cancers, and it has been found to be associated with malignant transformation and oncogenesis.[68,69] HER2 status has been tested also as a prognostic marker in patients with MIBC treated with RC, with or without perioperative systemic therapies. Soria *et al.*[70] found that HER2 overexpression was associated with adverse pathological features at RC, such as the presence of lymph node metastasis. However, HER2 overexpression did not translate into worse oncological outcomes. Conversely, Bolenz *et al.*[71] reported that patients with HER2 positivity had a significantly increased risk for both disease recurrence (hazard ratio, 1.95; p=0.003) and CSM (hazard ratio, 2.06; p=0.004), compared to those with HER2 negativity.

The *retinoblastoma protein (RB1)* is a tumour suppressor gene, which acts as a negative regulator of cell cycle progression and has been proved to be dysregulated in several cancers. The loss of RB1 expression is an adverse prognostic biomarker in MIBC.[72,73] Inactivating RB1 mutation translates into a lower expression of FGFR3 levels and is associated with significantly worse CSS.[74]

Survivin, an inhibitor of apoptosis, was found to be associated with disease recurrence (p=0.04), CSM (p=0.03), and all-cause mortality (p=0.04) in 222 consecutive patients treated with RC and PLND.[75] These results have been externally validated and survivin status has been incorporated into a nomogram for the prediction of outcomes in patients with pT1-3N0M0 disease. The addition of survivin has improved the accuracy of the model over standard clinicopathologic features for prediction of disease recurrence and CSS.

However, even if tissue-based markers such as p53, RB1, and survivin have shown to be associated with long-term outcomes in patients with MIBC treated with RC, their role in clinical practice as single markers remains limited, mainly due to their insufficient accuracy. Models based on the assessment of multiple markers (p53, p21, RB, cyclin E1, and p27) have shown higher predictive accuracy compared to those based on single markers.[76–78]

A prospective study of 216 patients treated with RC and lymphadenectomy, and who underwent immunohistochemical staining for p53, p21, p27, cyclin E1, and Ki-67, found that in a multivariable model adjusting for pathologic stage, margins, lymph node involvement, and adjuvant chemotherapy, only LVI and a number of altered biomarkers were independent predictors of recurrence and CSS.[79] At present, molecular markers have not been used in routine care, as clinical trials demonstrating a clinical utility have not been performed.

As discussed, BCa molecular characterization is acquiring increasing importance in the prediction of prognosis in patients with MIBC. In 2014, the Cancer Genome Atlas Research Network initially identified four clusters of high-grade MIBC according to their prognosis.[80] The "cluster I" tumours present a papillary-like morphology, with overexpression of FGFR3, decreased expression of miR-99a and miR-100, and high expression of luminal breast differentiation markers, including GATA3 and FOXA1. Similar characteristics in breast differentiation markers are shared with "cluster II" tumours, which also present overexpression of uroplakins, E-cadherin, and members of the miR-200 family. Moreover, overexpression of ERBB2 (erb-b2 receptor tyrosine kinase 2, formerly HER2/*neu*]) and estrogen receptor-β by these tumours suggests a potential beneficial role of hormonal therapies. "Cluster III tumours," defined as basal/squamous-like for their similarities with basal-like breast

cancers and squamous cell cancers of the head, neck, and lung, are characterized by overexpression of epithelial lineage genes. Since 2014, several different molecular classifications have been proposed, and only recently, an international consensus on MIBC molecular subtypes has been achieved.[81] Six different classes of MIBC have been identified: luminal papillary (24%), luminal nonspecified (8%), luminal unstable (15%), stroma-rich (15%), basal/squamous (35%), and neuroendocrine-like (3%). These classes differ regarding the underlying oncogenic mechanisms, the infiltration by immune and stromal cells, and the histological and clinical characteristics, including outcomes. Specifically, compared to luminal papillary tumours that were taken as reference, luminal nonspecified and stroma-rich tumours showed similar outcomes, while luminal unstable, basal/squamous, and neuroendocrine-like subtypes were associated with worse survival, the latter representing the class with the worst prognosis (hazard ratio, 2.18; $p<0.05$). Moreover, this consensus classification, besides providing a promising tool for risk stratification, suggests possible therapeutic implications such as those related to targeted therapies, thereby representing a milestone for MIBC classification.

11.4.4 Prediction of response to neoadjuvant chemotherapy

Several potential tissue biomarkers have been recently evaluated as molecular predictors of response to cisplatin-based NAC, including DNA repair genes, regulators of apoptosis, receptor tyrosine kinases, genes involved in cellular efflux, and molecular subtypes. Key biomarkers investigated for prediction of response to NAC are listed in **Table 11-3**.

TABLE 11-3 Biomarkers Associated With Response to Neoadjuvant Chemotherapy

Molecular pathway	Biomarker(s)	Method	Drug(s)	N	Results	Reference
Cellular efflux	**CTR1**	IHC	Cis	47	Association with pathologic response	Kilari *et al.*[102]
Cell death pathways	**p53**	IHC	MVAC	111	Association with survival	Sarkis *et al.*[100]
		IHC	MVAC	44	Does not predict pathologic response	Plimack *et al.*[96]
	BCL2	IHC	Cis + radiotherapy	51	Low levels associated with better prognosis	Cooke *et al.*[101]
DNA repair	**BRCA1**	rtPCR	Cis	57	Low/intermediate levels predict pathologic response	Font *et al.*[86]
	ERCC1	IHC	Cis	38	Does not predict pathologic response but predicts survival	Ozcan *et al.*[91]
	ERCC2	rtPCR	Cis	50	Predicts pathologic response	Van Allen *et al.*[93]

Abbreviations: BCL2, B-cell/CLL lymphoma 2; BRCA1, breast cancer susceptibility gene 1; Cis, cisplatin-based; CTR1, high-affinity copper uptake protein 1; ERCC1, excision repair cross-complementing 1; ERCC2, excision repair cross-complementing 2; IHC, immunohistochemistry; MVAC, methotrexate, vinblastine, doxorubicin, cisplatin; rtPCR, reverse transcription polymerase chain reaction.

DNA repair genes

Cisplatin acts as an alkylating agent and interferes with DNA replication and gene transcription, thereby promoting cell death. The DNA damage caused by cisplatin is repaired by two pathways: the homologous recombination (HR) pathway, which includes the BRCA1, BRCA2, and RADS51 genes; and the nucleotide excision repair (NER) pathway, which includes several genes, such as ERCC15, CDK7, DDB1-2, and XPA. Alteration in NER and/or HR genes has been suggested as affecting the clinical response to cisplatin-based chemotherapy.

The *breast cancer susceptibility gene 1 (BRCA1)* gene encodes a nuclear protein that is recruited to the site of DNA breaks and modulates chemoresistance, responding to DNA damage with several different mechanisms. In breast cancer cell lines, ovarian cancer cell lines, and non–small cell lung cancer cell lines, a decrease in BRCA1 expression increases the sensitivity to cisplatin and is associated with a better survival after cisplatin-based chemotherapy. Font *et al.*[86] evaluated BRCA1 mRNA levels on TUR specimens of 57 patients treated with cisplatin-based NAC before RC. Intratumoural expression levels were assessed by real-time quantitative PCR. Patients with low to intermediate BRCA1 levels were found to have a significantly higher pathological response (defined as pT0-1 at RC specimen) compared to patients with high BRCA1 levels (66% vs 22%, p=0.01). Moreover, patients with low to intermediate levels had a significant improvement in median overall survival (OS) compared to patients with high BRCA1 levels (168 vs 34 months, p=0.02). BRCA1 levels were found to be an independent prognostic factor for OS also at multivariable analysis. However, these results have not been yet externally validated.

The *excision repair cross-complementing 1* (*ERCC1*) encodes a protein that is involved in DNA repair and DNA recombination. It has been associated with cisplatin resistance in ovarian, gastric, cervical, colon, and non–small cell lung cancers in several studies,[87–89] whereas its role in BCa remains debated. Choueiri *et al.*[90] found no association between ERCC1 expression and response to cisplatin-based NAC, whereas Hemdan *et al.*, who evaluated ERCC1 expression in paraffin-embedded bladder tumour samples of patients treated with NAC, found that patients negative for ERCC1 had an improved OS compared to patients with ERCC1 positivity.[176] Similar results were reported by Ozcan *et al.*,[91] who reported similar rates of pathological complete response after NAC between patients with low and high ERRC1 expression, but high levels of ERCC1 were found to be significantly associated with worse disease-free and OS.

The *excision repair cross-complementing 2* (*ERCC2*) gene encodes an NER helicase whose loss of function has been reported to be associated with cisplatin sensitivity in preclinical models.[92] Initially, Van Allen *et al.*[93] prospectively evaluated the whole-exome sequencing on pre-chemotherapy tumours and germline DNA of 25 nonresponder patients and 25 responder patients with MIBC who received NAC and RC. The authors found that the ERCC2 gene was the only significantly mutated gene enriched in responder patients. Similar results were then replicated in a validation study.[94]

Genomic alterations in the DNA repair-associated genes *ATM*, *RB1*, and *FANCC* were found to be predictors of response (87% sensitivity, 100% specificity) and improved OS (p=0.007) after methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) chemotherapy for MIBC.[95] These results were validated in an external cohort

of patients treated with gemcitabine/cisplatin. Another study published by Plimack *et al.*[96] evaluated all coding exons of 287 cancer-related genes in patients treated with dose-dense MVAC and found that mutations of ATM, RB1, or FANCC were associated with pathological complete response and improved OS.

Finally, Iyer *et al.*[97] evaluated the role of a DNA damage repair (DDR) gene set. Results indicated that the most frequently mutated DDR genes were BRCA1 (12%) and ATR (12%), and the presence of a deleterious DDR gene alteration was associated with chemosensitivity (positive predictive value for <pT2N0, 89%).

Tyrosine kinase receptors

The ERBB family of proteins is composed of four classes of tyrosine kinase growth factor receptors, including the epidermal growth factor receptor (EGFR), and the ERBB2, ERBB3, and ERBB4 receptors. The ERBB2 is an orphan receptor and its specific ligand remains unknown. The overexpression of ERBB2 has been demonstrated in several tumours. Recently, Groenendijk *et al.*[98] evaluated the expression of ERBB2 in pre-chemotherapy tumour tissue of 38 responder patients and 33 nonresponder patients with MIBC. Results indicated that 9 of 38 patients with complete response had a missense mutation of ERBB2, whereas none of the 33 nonresponder patients had this kind of mutation.

Regulators of apoptosis

The *p53* gene encodes for a nuclear phosphoprotein that is involved in transcriptional regulation; it acts as a tumoural suppressor gene that is able to respond to DNA damage and induce apoptosis. The p53 mutation is the most frequent genetic alteration found in human tumours, and it usually results in mutated protein accumulation in the nucleus and in the loss of its apoptotic activity. Although it has been demonstrated that p53 mutations confer sensitivity to cisplatin in BCa cells *in vitro*,[99] conflicting results regarding their role in response to NAC have been reported. Sarkis *et al.*[100] evaluated data of 90 patients treated with MVAC and RC, and they found that p53 nuclear overexpression was an independent prognostic factor for survival (p=0.001). Conversely, in a phase 2 trial by Plimack *et al.*,[96] p53 mutation was not associated with pathologic response in patients treated with MVAC.

The *B-cell/CLL lymphoma 2 (BCL2)* gene belongs to the BCL2 family of regulator proteins involved in the regulation of apoptosis. Cooke *et al.*[101] evaluated the expression of BCL2 and p53 genes in 51 patients with MIBC, who were randomized to receive either radiotherapy alone or radiotherapy plus cisplatin-based NAC. No difference was found in BCL2 positivity when the whole cohort was considered, but BCL2-negative patients receiving NAC had a significantly better prognosis, with a median survival of 72 months compared to 17 months in BCL2-positive patients. The authors concluded that quantification of BCL2 in patients undergoing radiotherapy for advanced BCa may be able to identify those who could benefit from NAC.

Genes involved in cellular efflux

High-affinity copper uptake protein 1 (CTR1) encodes a protein involved in cellular efflux and plays a role in the influx of platinum. Kilari *et al.*[102] evaluated CTR1 expression in 47 patients with MIBC treated with NAC and found that CTR1 expression was significantly associated with pathological response.

Gene expression profiling and molecular subtypes of bladder cancer

Recent studies evaluated the role of molecular profiles for decision-making and counselling of patients treated with NAC and RC. Choi *et al.*[103] identified three molecular subtypes of MIBC on fresh-frozen tissue obtained from TUR: basal, luminal, and p53-like. The basal subtype is characterized by p53 activation, positive CK 5/6, high levels of EGFR, absence of cytokeratin 20, and squamous/sarcomatoid differentiation. This type of tumour was associated with worse survival and recurrence, but responded to NAC, suggesting that early management with NAC could be the best option to improve survival. The luminal subtype is characterized by the peroxisome proliferator-activated receptor γ (PPARγ) pathway, estrogen receptor (ER) transcription, and FGFR3 mutations; some of these tumours responded to NAC, although no specific biomarker was determined. The p53-like tumours were characterized by wild-type p53 gene expression. In the study, none of the patients with this subtype responded to NAC. More recently, Seiler *et al.*[104] evaluated the entire transcriptome profiling of 343 pre-NAC TUR specimens, developing a genomic subtyping classifier able to predict consensus subtypes (claudin-low, basal, luminal-infiltrated, and luminal). The claudin-low tumours were associated with poor OS, independently of the NAC regimens administered. Patients with basal tumours had the most improvement in OS with NAC compared to surgery alone, whereas patients with luminal tumours experienced the best OS, with or without NAC. A consequent cluster evaluation of RC specimens[105] found an inconsistency with clusters of pre-NAC TUR specimens. In the recently developed international consensus on molecular classification of MIBC,[81] no significant association was found between the consensus classed and oncologic outcomes in patients treated with NAC. In conclusion, although tissue markers seem to play a role in patient selection for NAC before RC, their use in clinical practice remains controversial. Validation studies and prospective, well-conducted trials are needed to confirm the reported preliminary results.

The initial results of a randomized phase 2 trial investigating the role of a gene expression model (COXEN) as a predictive biomarker in patients undergoing NAC and RC were presented at the 2019 American Society for Clinical Oncology (ASCO) meeting.[106] Patients were randomized to receive either NAC with dose-dense MVAC or gemcitabine/cisplatin, based on their personal gene expression profile. The primary endpoint of the trial was to assess whether the prespecified, treatment-specific gene expression profile was prognostic for pathologic response to NAC. Some 167 patients were included in the study and the COXEN was found to be a significant predictor for downstaging in the pooled analysis, but not in individual arms. These findings will also be used to validate DNA repair genes and subtyping signatures.

11.4.5 Prediction of response to systemic chemotherapy

This section will review molecular biomarkers that have been shown to predict response to chemotherapeutics in the adjuvant setting. Such response may be evidenced by prolongation of survival (usually measured by progression-free survival, disease-free survival, or OS). In the adjuvant setting, such endpoints may be more suitable for trial designs compared to traditional criteria (ie, Response Evaluation Criteria In Solid Tumors [RECIST]), as the primary tumour and organ involved is surgically removed.[107] Herein, "chemotherapy" refers to any drug therapeutic modality, targeted or otherwise, that is not primarily an immunotherapeutic. While NAC response was discussed in the preceding section, we will herein discuss determinants of response to systemically

administered chemotherapy in the adjuvant setting for muscle-invasive disease and for metastatic cancer. Key biomarkers predictive of response to systemic chemotherapy that have been evaluated for BCa are listed in **Table 11-4** and are briefly described below.

Cell cycle and proliferation

Homeostatic regulation of the urothelium is tightly controlled, and regulators of the cell cycle are crucial for maintaining this equilibrium.[108] The genesis and progression of BCa is centrally characterized by dysregulation of the cell cycle, which results in sustained proliferation signalling.[109] While many molecules have a role in controlling the cell cycle, the main players are cyclins and cyclin-dependent kinases.[110] Cell cycle regulators and markers of proliferation are known to be important prognostic markers in BCa,[111] and several have been evaluated as predictors of chemotherapy response.[112,113] MicroRNAs are small, single-stranded noncoding RNAs that can exert their function through post-transcriptional gene expression regulation and cell cycle control. A combination of such regulatory RNAs and transcription factors has also been shown to be predictive in patients with metastatic BCa treated with cisplatin-based therapy.[114] However, there is yet to be a clinically validated role for them as predictive markers.

Cell death pathways

The series of cell death events that occur throughout normal development and in response to a variety of initiation stimuli is referred to as apoptosis. This process is effected by molecules called caspases, which may be directly or indirectly modulated in cancer cells, thereby allowing them to escape apoptosis.[115] In turn, caspases are regulated by several molecules involved in the detection of DNA or mitochondrial damage, including p53 and BCL2.[116] The prognostic and predictive value of p53 has been extensively evaluated in BCa, as previously reported.[117] While initial retrospective studies were promising,[65] subsequent studies have reported conflicting conclusions regarding the predictive role of p53.[112,118,119] A phase 3, randomized controlled trial was unable to support the chemotherapeutic predictive role for p53 in invasive disease.[10] BCL2 is an important anti-apoptotic member of the intrinsic pathway that inhibits caspase activation. Expression of this protein in primary tumours has been correlated with response to platinum-based BCa therapies.[101,119]

DNA damage repair

Many chemotherapeutic agents act by causing DNA damage, thereby impeding the replicative potential of cancer cells, if their DNA integrity is sufficiently disrupted.[120] Several chemotherapy agents used in BCa cause DNA damage, including alkylating agents that crosslink DNA strands (cisplatin); antimetabolites that mimic normal pyrimidine bases (gemcitabine, 5-fluorouracil) or that inhibit DNA synthesis (methotrexate); and anthracyclines that intercalate DNA base pairs and inhibit the topoisomerases that uncoil DNA for replication (doxorubicin, epirubicin). DNA repair, such as nucleotide excision repair, HR recombination, or mismatch-repair mechanisms can correct such chemotherapy-induced defects. Cancer cells that have deficient DNA damage repair mechanisms are unable to rectify such damage induced by these chemotherapy agents and are therefore more susceptible to being killed by the agents. Proteins involved in DNA damage detection and repair that play a role in BCa chemotherapy response include BRCA1, BRCA2, RAD51, PAR, PARP1, ERCC1, ERCC2, and RRM1. This has been the most promising class of predictive markers thus far for chemotherapeutic response. In the neoadjuvant setting, a study showed that 38% of patients in the combined discovery and validation cohorts who underwent platinum-based chemotherapy had an alteration in one or more of three DNA repair–associated genes; 91%

of these patients had pathologic stage T1 or less at the time of RC.[95] Similarly, a validation study found an *ERCC2* mutation in 21% of patients with muscle-invasive disease, and 80% of the marker-positive patients had pathologic stage T1 or less at the time of RC following NAC.[121] In a study of patients with advanced or metastatic urothelial cancer receiving platinum-based palliative chemotherapy employing next-generation sequencing, 341 genes, including 34 DNA DDR-associated genes, were evaluated.[122] The investigators identified 47% patients who harboured at least one DDR mutation. Patients with DDR gene alterations had significantly longer progression-free survival and OS compared with patients with wild-type DDR genes. In the setting of advanced urothelial carcinoma, overexpression of ERCC1, RAD51, and PAR has been correlated with worse survival for patients treated with first-line platinum combination chemotherapy.[123,124]

Drug transport

For most chemotherapies to work, they must enter the cancer cell and disrupt a biological process. This implies that proteins that affect drug transport into the cancer cell or that alter drug metabolism could have important effects on chemotherapy efficacy. The *MDR1* gene encodes P-glycoprotein, a membrane-associated protein member of the superfamily of ATP-binding cassette transporters involved in transporting various molecules across extracellular and intracellular membranes. It has been studied in the context of chemotherapeutic response in BCa, with conflicting reports regarding its predictive value.[112,125]

Cell growth signalling

Several peptide growth factors and their associated tyrosine kinase receptors are responsible for modulating growth signals from external cues and transmitting them via signal transduction pathways into the nuclei of urothelial cells. Aberrations in these growth factor receptors and/or the signals transmitted by them can result in an abnormal increase in the rate of transduction of growth signals, thereby leading to uncontrolled cellular proliferation and tumour formation. Such kinases are the targets of several new systemic therapies in oncology. Several tyrosine kinase inhibitors have been tried in BCa, including lapatinib (inhibits EGFR and HER2/*neu* pathways) and pazopanib (inhibits FGF, PDGF, and VEGF pathways). While these drugs appear to have limited activity in BCa, the possibility of biomarker enrichment for response has been assessed with mixed results.[126–130]

DNA mutations and alterations

Both germline (patient) and somatic (tumour) DNA have been assessed for alterations that might predict response to chemotherapy agents, a field known as pharmacogenomics.[131] In BCa, germline single-nucleotide variants (SNVs, formerly single-nucleotide polymorphisms [SNPs]; single base-pair changes that occur in a patient's genes) have been tested as predictors of chemotherapy response.[132,133]

Immunologic factors and other molecules

Several other factors have been assessed for their ability to predict chemotherapy response, including immunological markers.[134] In some cases, these factors have been identified using a logical discovery process, such as proteomics, while in other cases, the rationale behind biomarker selection is less clear. Several of these factors are summarized in **Table 11-4**.

TABLE 11-4 Biomarkers Associated With Systemic Chemotherapy Response

Molecular pathway	Biomarker(s)	Method	Drug(s)	n	Results	Reference
Cell cycle and proliferation	**cyclin D1**	IHC	Cis	63	Overexpression predicts better chemo response	Seiler *et al.*[113]
	CCND1*	FISH	Cis	63	Does not predict chemo response	Seiler *et al.*[113]
	Ki-67	IHC	CMV, MVAC	99	Does not predict chemo response	Siu *et al.*[112]
	miRNAs	rtPCR	MVAC, GC	83	Increased miR-21, miR-372, and E2F1 associated with chemo response and survival	Bellmunt *et al.*[114]
Cell death pathways	**p53**	IHC	MC, MEC CMV, MVAC	83	Overexpression predicts improved survival in chemoresistant patients	Qureshi *et al.*[118]
		IHC	CMV, MVAC	99	Does not predict chemo response	Siu *et al.*[112]
		IHC	CISCA, MVAC	25	Overexpression associated with worse response	Kong *et al.*[119]
		IHC	MVAC	114	Does not predict chemo response (phase 3 RCT)	Stadler *et al.*[10]
	BCL2	IHC	Cis	51	Low expression predicts better response to chemoradiation	Cooke *et al.*[101]
		IHC	CISCA, MVAC	25	Overexpression associated with worse response	Kong *et al.*[119]
DNA repair	**BRCA1**	IHC	Cis	104	Does not predict chemo response	Mullane *et al.*[123]
		rtPCR	GC, GCT	57	Does not predict chemo response	Bellmunt *et al.*[124]
	BRCA2	IHC	Cis	104	Does not predict chemo response	Mullane *et al.*[123]
	RAD51	IHC	Cis	104	Overexpression associated with worse survival	Mullane *et al.*[123]
	PAR	IHC	Cis	104	Overexpression associated with worse survival	Mullane *et al.*[123]
	PARP1	IHC	Cis	104	Does not predict chemo response	Mullane *et al.*[123]
	ERCC1	IHC	Cis	104	Overexpression associated with worse survival	Mullane *et al.*[123]
		rtPCR	GC, GCT	57	Overexpression associated with worse survival	Bellmunt *et al.* (2007)[124]
	RRM1	rtPCR	GC, GCT	57	Does not predict chemo response	Bellmunt *et al.* (2007)[124]

TABLE 11-4 Biomarkers Associated With Systemic Chemotherapy Response *(Cont'd)*

Molecular pathway	Biomarker(s)	Method	Drug(s)	n	Results	Reference
Drug resistance	**MDR1**	rtPCR	MVEC	108	Overexpression associated with inferior outcome	Hoffmann *et al.*[125]
	P-glycoprotein (MDR1)	IHC	CMV, MVAC	99	Does not predict chemo response	Siu *et al.*[112]
	Caveolin-1	rtPCR	GC, GCT	57	Does not predict chemo response	Bellmunt *et al.* (2007)[124]
Cell growth signalling	**FGFR3**	WES	Pazopanib	3	Mutation associated with partial response	Pinciroli *et al.*[126]
	ERBB2 (HER2)	WES	Pazopanib	3	Mutation associated with improved response	Pinciroli *et al.*[126]
		IHC	Lapatinib	116	Does not predict chemo response	Powles *et al.*[127]
		IHC	Lapatinib	34	Does not predict chemo response	Novara *et al.*[128]
	HER1	IHC	Lapatinib	116	Does not predict chemo response	Powles *et al.*[127]
	EGFR	IHC	Lapatinib	34	Overexpression associated with response	Novara *et al.*[128]
	VEGF	Serum	Sunitinib	26	Does not predict chemo response	Grivas *et al.*[129]
		Serum, IHC	Pazopanib	18	Does not predict chemo response	Pili *et al.*[130]
	HIF-1α	IHC	Pazopanib	18	Does not predict chemo response	Pili *et al.*[130]
DNA markers	**Germline SNVs**	Microarray	Cabazitaxel	45	SNVs predicted chemo response and toxicity	Duran *et al.*[132]
		Microarray	Cis	210	SNVs predicted chemo response	Gallagher *et al.*[133]
Immune markers	**IL-8**	Luminex xMAP	Sunitinib	38	Underexpression associated with longer time to progression	Bellmunt *et al.* (2011)[134]
Other	**Metallothionein**	IHC	CMV, MVAC	99	Overexpression associated with worse survival	Siu *et al.*[112]

**CCND1*, formerly *Bcl-1*.

Abbreviations: BCL2, B-cell/CLL lymphoma 2; BRCA1, breast cancer susceptibility gene 1; BRCA2, breast cancer susceptibility gene 2; Cis, cisplatin; CISCA, cisplatin/doxorubicin/cyclophosphamide; CMV, cisplatin, methotrexate, vinblastine; EGFR, epidermal growth factor receptor; ERCC1, excision repair cross-complementing 1; FGFR3, fibroblast growth factor receptor 3; FISH, fluorescence in situ hybridization; GC, gemcitabine/cisplatin; GCT, gemcitabine/cisplatin/paclitaxel; HIF-1α, hypoxia-inducible factor 1α; IHC, immunohistochemistry; IL-8, interleukin-8; MC, methotrexate/cisplatin; miRNAs, microRNAs; MDR1, multidrug resistance mutation 1; MEC, methotrexate, epirubicin, cisplatin; MVAC; methotrexate, vinblastine, doxorubicin, cisplatin; MVEC, methotrexate, vinblastine, epirubicin, cisplatin; PAR, protease-activated receptor; PARP1, poly (ADP-ribose) polymerase 1; RRM1, ribonucleotide reductase catalytic subunit M1; SNVs, single-nucleotide variants (formerly, single-nucleotide polymorphisms [SNPs]; VEGF, vascular endothelial growth factor, WES, western blot.

11.4.6 Prediction of response to systemic immunotherapy

The advent of systemic immunotherapy in the management of advanced BCa represents a quantum leap over the past few years, especially for patients who have not responded to cisplatin-based therapies. Several immune checkpoint inhibitors have shown promising activity in this space, including agents targeting the programmed cell death 1 (PD-1) receptor, its ligand PD-L1, and cytotoxic T-lymphocyte antigen 4 (CTLA-4).

Two classes of drugs have recently been approved by the FDA for treatment of urothelial cancer. Atezolizumab,[135] a PD-L1–targeting agent, as well as nivolumab[136] and pembrolizumab,[137] two PD-1–blocking monoclonal antibodies, have demonstrated activity as second-line agents, while atezolizumab[138] and pembrolizumab[139] have also been approved as first-line treatment for cisplatin-ineligible patients. Durvalumab[140] and avelumab[141] are two other drugs that have been granted breakthrough therapy designation by the FDA for patients with locally advanced or metastatic urothelial cancer.

While these developments are promising, a majority of patients still do not respond to treatment,[135–138,140–143] thereby resulting in a significant financial burden and potential treatment-related side effects. This highlights the need for appropriate biomarkers to aid in selecting patients who are most likely to benefit from checkpoint targeting therapy. While several biomarkers have been explored in the context of these clinical trials, no pretreatment recommendations can be definitively made at this point based on any molecular predictors, as a significant proportion of patients do respond to treatment despite testing negative for a biomarker. Indeed, current FDA approvals for checkpoint inhibitors in urothelial cancer are independent of biomarker status. Nevertheless, biomarker-based selection for immunotherapy remains an area of active interest that is likely to grow in the years to come.

PD-L1

PD-L1 expression in urothelial cancer has been associated with higher tumour grade, worse outcomes, and decreased postoperative survival.[144,145] Detection of PD-L1 on tumour samples with immunohistochemistry has been used in several clinical trials to evaluate the feasibility of PD-L1 as a predictive biomarker with mixed results. In the IMvigor210 trial, a higher PD-L1 expression score was associated with a higher response rate.[135] In contrast, the CheckMate 275 trial showed meaningful responses to nivolumab, irrespective of PD-L1 expression levels.[136] Lack of standardized testing and evaluation of PD-L1 may be partially responsible for these discrepancies. For example, there are several variations in antibodies (SP142,[135,138], 28-8,[136,142] 22C3,[137,146] SP263, and 73-10[141]) and staining platforms used in assays employed in prior trials. Additionally, PD-L1 expression has been variously assessed: on tumour-infiltrating immune cells in the IMvigor210 trial,[135] on tumour cells in the CheckMate 275 trial,[136] and on both tumour cells and immune cells in the durvalumab trial.[143] Furthermore, there are variations in percentage cutoffs used to define high and low expression. Finally, PD-L1 expression is dynamic, and a single biopsy is unlikely to provide a complete assessment of status for the entire duration of disease. Therefore, evaluation of the predictive value of PD-L1 positivity is difficult and correlations with response to treatment or survival vary between trials.

The Cancer Genome Atlas subtypes

Molecular subtypes of MIBC have recently been categorized according to gene expression. The Cancer Genome Atlas (TCGA) described four subtypes of BCa based on cluster analysis of messenger RNA.[147] Tumour samples from recent clinical trials have been analyzed based on these subtypes and correlated to treatment response.

Exploratory analyses from the cisplatin pretreated arm of the IMvigor210 trial showed TCGA subtypes to be independently predictive of response to atezolizumab treatment.[135] PD-L1 immune cell prevalence was highly enriched in the basal subtype versus the luminal subtype (60% vs 23%; $p<0.0001$), with expression of 15% in papillary-like luminal cluster I, 34% in cluster II, 68% in squamous-like basal cluster III, and 50% in basal cluster IV. Elevated PD-L1 tumour cell expression was almost exclusively seen in the basal subtype (39% in basal vs 4% in luminal; $p<0.0001$), and did not correlate with objective response rate. Response to atezolizumab occurred in all TCGA subtypes, but was significantly higher in luminal cluster II than in other subtypes, which demonstrated an objective response rate of 34% ($p=0.0017$).[135] For cisplatin-ineligible patients, responses were seen across all subtypes and were more frequent with the luminal II subtype.[138] In CheckMate 275, all four urothelial carcinoma molecular subtypes were represented; basal 1 subtype had the highest proportion of responders.[136] These discrepancies may be partially attributable to the fact that both trials allowed biopsy specimens from the primary tumour, lymph nodes, or metastatic lesions for TCGA subtyping, which may lead to inaccurate tumour classification. Additionally, the criteria for molecular subtyping differed in each study, highlighting a challenge in standardizing TCGA classification. Until further details emerge, TCGA subtype may not be a reproducible predictive biomarker for immunotherapy at this time.

Mutational load

High mutational load may be associated with improved response to immunotherapy, particularly for checkpoint inhibitors. In the IMvigor210 trial, cisplatin-ineligible patients in cohort 1 with the highest mutational load had significantly longer OS. In cohort 2 (cisplatin-pretreated patients), the median mutational load was significantly increased in responders compared with nonresponders. The relationship between mutational load and response was unrelated to TCGA subtype.[135] However, there is currently no standardized definition of mutational burden relative to the depth of sequencing performed. Targeted sequencing panels may also not adequately cover gene fusions, truncations, and translocations. Furthermore, germline variants may not be silenced by informatics techniques that filter common germline single-nucleotide variants. These challenges currently limit the use of tumour mutational burden as a predictive biomarker for immunotherapy.

Transforming growth factor β

Tumour microenvironment is the primary location for interaction between tumour cells and the host immune system. Transforming growth factor β (TGFβ) plays a complex role in regulating the tumour microenvironment. High preoperative TGFβ1 plasma levels are associated with poor outcomes in patients with urothelial carcinoma.[148] In the IMvigor210 trial, tumours analyzed by RNA sequencing showed that TGFβ attenuated tumour response to PD-L1 blockade by contributing to the exclusion of T cells.[149]

Interferon γ gene signature

CheckMate 275 findings revealed that higher values of a 25-gene interferon-γ (IFN-γ) signature were associated with a greater proportion of responders to nivolumab and higher PD-L1 expression, while patients with a high IFN-γ signature were more likely to respond to nivolumab than were those with low IFN-γ signature.[136] The strongest IFN-γ signature was noted in patients with basal 1 subtype. These patients were more likely to have a high IFN-γ signature score than patients with the other subtypes. Genomic defects in IFN-γ pathway genes have been described in patients who do not respond to anti–CTLA-4 treatment.[150]

Chemokines and CD8+ T-cell infiltration

Different immune cell subsets are recruited into the tumour microenvironment. Complex interactions occur between chemokines and their receptors, and these populations have distinct effects on tumour progression and therapeutic outcomes.[151]

CheckMate 275 findings indicated that the highest CXCL9 or CXCL10 expression was observed in nivolumab responders, in the basal 1 subtype, and in the subgroup of patients with PD-L1 expression of ≥5%, with CXCL9 and CXCL10 expression at least three times higher than in other subgroups. Additionally, a 12-chemokine signature was highly enriched in tumours from nivolumab responders. The highest CD8 expression was associated with nivolumab responders and basal 1 subtype.[136] In the IMvigor210 cohort of cisplatin-pretreated patients (cohort 2), responses to atezolizumab were most closely associated with high expression of two IFN-γ-inducible, type 1 helper T cell (TH1)-type chemokines, CXCL9 and CXCL10.[135] Consistent with increased T-cell trafficking chemokine expression, tumour centre CD8+ T-cell infiltration was also associated with both PD-L1 expression on immune cells and response to atezolizumab. Consistent with PD-L1 immune cell expression, CD8 T-effector gene expression was elevated in luminal cluster II and basal clusters III and IV, and not in luminal cluster I.

11.4.7 Prediction of response to radiotherapy

Today, radiotherapy is generally administered in the context of organ preservation therapy in BCa. With careful patient selection, maximal transurethral tumour resection, chemotherapy, and radiation (trimodality therapy [TMT]) yield oncological outcomes and quality of life comparable to RC in muscle-invasive disease.[152–154] Given the lack of randomized controlled trials, TMT has often been used as an alternative in patients with muscle-invasive disease who are medically unfit for RC. Between 50% and 90% of patients with muscle-invasive disease treated with TMT can experience complete response.[155] While those who do not achieve complete response may undergo salvage cystectomy, such patients often have unfavourable CSS due to metastasis.[155,156] It is therefore imperative to carefully select patients who may be the most optimal candidates for TMT. Several studies have looked at biomarkers that can predict response to TMT; the logic for evaluation of these biomarkers is generally based on their ability to predict response to the radiotherapy aspect of TMT. Important biomarkers that are predictive of response to radiotherapy in the context of TMT that have been evaluated for MIBC are listed in **Table 11-5** and are briefly described below. The chemotherapy aspect in these regimens generally employs radiosensitizers such as cisplatin, fluorouracil, and mitomycin-C; other studies have also evaluated the activity of gemcitabine and paclitaxel as radiosensitizers.[157]

TABLE 11-5 Biomarkers Associated With Radiotherapy Response

Molecular pathway	Biomarker(s)	Chemoradiation regimen	n	Results	Reference
Cell proliferation	**Ki-67**	RT 59.4 Gy + cisplatin	70	Higher Ki-67 associated with higher CR	Rödel *et al.*[159]
		RT 40 Gy + cisplatin	94	Higher Ki-67 associated with higher CR	Tanabe *et al.*[160]
		RT 40.5 Gy (median) + cisplatin	62	No association with response	Matsumoto *et al.*[161]
Cell death pathways	**Apoptotic index**	RT 59.4 Gy + cisplatin	70	Higher index associated with higher CR	Rödel *et al.*[159]
	Bax/BCL2 ratio	RT 40.5 Gy (median) + cisplatin	62	Higher ratio associated with higher CR	Matsumoto *et al.*[161]
DNA repair	**ERCC1**	RT 40–66 Gy + cisplatin or nedaplatin	22	Expression loss associated with higher CR	Kawashima *et al.*[164]
	ERCC1, XRCC1	RT 48.6 Gy (median) + cisplatin	157	Positive expression associated with improved survival	Sakano *et al.*[165]
	MRE11	RT 55 Gy	179	High expression associated with improved survival	Choudhury *et al.*[166]
	DDR alterations	RT or chemoradiation	48	Presence of alterations associated with trend to improved recurrence-free survival	Desai *et al.*[167]
Cell growth signalling	**ERBB2**	RT 40 Gy + cisplatin + other agents	55	Positivity associated with lower CR	Chakravarti *et al.*[168]
		RT 40 Gy + cisplatin	119	Positivity associated with lower CR	Inoue *et al.*[169]
		RT 64.8 Gy + paclitaxel with trastuzumab (group 1, ERBB2+) or without trastuzumab (group 2, ERBB2-)	66	CR rates, 72% for group 1 and 68% for group 2	Michaelson *et al.*[171]
Other	**Molecular subtype**	RT 40 Gy + cisplatin	118	CR rates, 52%/45%/15% for GU/SCC-like/urobasal	Tanaka *et al.*[174]
	Hsp60	RT 40 Gy + cisplatin	54	Positivity associated with better response	Urushibara *et al.*[175]

Abbreviations: CR, complete response; DDR, DNA damage response; ERCC1, excision repair cross-complementing 1; ERCC2, excision repair cross-complementing 2; GU, genomically unstable; Hsp60, heat shock protein 60; MRE11, meiotic recombination 11; RT, radiotherapy; SCC, squamous cell cancer; XRCC1, x-ray repair cross-complementing group 1.

Cell proliferation

Ki-67 is an established marker of cellular proliferation that reflects the biological aggressiveness of malignancies. High Ki-67 labelling index has been shown to be an independent risk factor for recurrence and cancer-related death in patients with BCa undergoing RC.[158] Two retrospective studies have reported that higher Ki-67 labelling index was significantly associated with an improved CR rate and improved CSS among patients receiving chemoradiation for BCa.[159,160] However, another study found no association between Ki-67 labelling index and chemoradiation response; higher labelling index was also associated with worse CSS.[161] In the latter study, the total radiation dose (median, 40.5 Gy) and CR rate (34%) were lower than those of the study by Rödel *et al.* (59.4 Gy and 71%, respectively).[159,161] In the study by Tanabe *et al.*, patients received chemoradiation at 40 Gy; 73% eventually underwent partial cystectomy of the original tumour site or salvage cystectomy to completely eradicate possible residual cancer cells.[160] These conflicting results may be attributable to differences in therapeutic intensity and the possibility that the residual cancer cells with high Ki-67 labelling index that survived relatively low-dose chemoradiation may proliferate and be associated with more aggressive disease.

Cell death pathways

Cell death is quantified by the apoptotic index, the ratio of apoptotic cells to the total number of tumour cells, and has been correlated to tumour progression as well as to recurrence-free survival and OS in BCa.[162] Higher apoptotic index has been associated with a higher CR rate in patients with invasive BCa treated with chemoradiation.[159] However, no significant association with chemoradiation response was observed for immunohistochemical expression of p53 or BCL2.

The BCL2 family of proteins plays a crucial role in the intrinsic apoptotic pathway; it includes anti-apoptotic members such as BCL2, as well as pro-apoptotic members such as Bax and Bad. A study evaluating immunohistochemical expressions of p53, BCL2, Bax, and apoptotic index in patients with invasive bladder tumours receiving chemoradiation found no significant associations between each biomarker and treatment response.[161] However, a higher Bax/BCL2 ratio was significantly associated with higher CR rates.

DNA damage repair

Ionizing radiation induces cell death primarily by introducing double-strand DNA breaks. After exposure to ionizing radiation, such damage is detected by the meiotic recombination 11 (MRE11)-RAD50-NBS1 complex, resulting in activation of the DDR pathway.[163] Failure to repair this DNA damage results in tumour cell death. Loss of ERCC1 expression has been associated with improved CR rates in patients with MIBC undergoing chemoradiation.[164] The investigators of this study also demonstrated that *ERCC1* suppression removed BCa cell resistance to ionizing radiation *in vitro*. Another group reported that positive expression of ERCC1 or x-ray repair cross-complementing group 1 (XRCC1) was significantly associated with improved CSS among patients with MIBC undergoing cisplatin-based chemoradiation.[165] As chemoradiation sensitivity may be a surrogate for prognosis among patients with BCa with invasive disease treated with TMT, findings of the above studies appear to be conflicting.

Higher MRE11 expression has been associated with improved CSS in patients with BCa with invasive disease treated with definitive radiotherapy.[166] A trend toward an inverse prognostic effect of MRE11 overexpression was

noted among patients undergoing RC, and CSS was significantly improved for those treated with radiotherapy than RC among patients with MRE11-overexpressing tumours. This suggests that MRE11 expression may allow patient selection for radiotherapy versus RC.

As with systemic chemotherapy, the prognostic impact of DDR gene alterations has been analyzed by next-generation sequencing in patients with BCa with invasive disease undergoing chemoradiation or radiotherapy.[167] The presence of DDR gene alterations, most commonly identified in *ERCC2*, showed a trend for improved bladder or metastatic recurrence-free survival.

Cell growth signalling

Studies have evaluated the association of ERBB2 (formerly HER2/*neu*) and EGFR with chemoradiation response and prognosis following TMT. Positive ERBB2 has been associated with lower CR rates and positive EGFR has been associated with improved CSS.[168] However, reasons for the positive association between EGFR expression and improved CSS remain to be elucidated. The association between positive ERBB2 expression and lower CR rates has been validated by a subsequent study, which also demonstrated poor CSS corresponding to ERBB2 overexpression in patients with invasive BCa receiving chemoradiation.[169]

These data suggest that ERBB2 inhibitors such as trastuzumab, an anti-ERBB2 monoclonal antibody, can potentially improve outcomes of patients with BCa with ERBB2-overexpressing tumours undergoing chemoradiation.[170] This was the basis for a biomarker-driven phase 1/2 clinical trial designed to overcome chemoradiation resistance in patients with ERBB2-overexpressing BCa.[171] The study indicated possible improvement of chemoradiation response with ERBB2-targeted therapy; 66 evaluable patients were treated with radiation and either paclitaxel and trastuzumab (group 1) or paclitaxel alone (group 2), according to the presence (group 1) or absence (group 2) of ERBB2 overexpression in tumour tissues. The CR rate at 1 year for group 1 was nearly equivalent to that of group 2 (72% vs 68%, respectively), with comparable toxicity profiles between the two groups.

Other biomarkers

Whole genome expression profiling techniques have been used to define novel molecular subtypes of urothelial carcinoma.[172] Using an initial 20-gene panel, one study characterized three such subtypes, based on immunohistochemical expression patterns of cyclin B1 and keratin 5: genomically unstable; squamous cell cancer–like; and urobasal exhibiting prognostic differences.[173] These molecular subtypes may be used to predict chemoradiation response in muscle-invasive disease.[174] CR rates were 52%, 45%, and 15% for genomically unstable, squamous cell cancer–like, and urobasal subtypes, respectively.

Heat shock proteins (Hsps) are produced by cells in response to exposure to stressful stimuli, and they may be involved in therapeutic resistance and tumour aggressiveness. Members of this protein family, including Hsp27, Hsp60, Hsp70, and Hsp90, have been interrogated in patients with BCa with invasive disease treated with chemoradiation.[175] This study noted that positive Hsp60 expression was independently associated with favourable responses.

11.5 Conclusions

The literature review of the utility of tissue-based biomarkers in BCa through the past two decades reveals that research has moved its focus away from immunohistochemical analysis and tumour-related phenotypic changes to the analysis of genetic alterations. Furthermore, as previous research has demonstrated that a single marker has insufficient potential to predict the course of disease and response to therapy, a trend toward marker combinations and genetic classifiers, mostly combining these findings with clinical parameters, is observed.

In summary, literature information about the prediction of disease recurrence in NMIBC is inconclusive, and study quality is insufficient to warrant clinical use of tissue biomarkers for this purpose. Even less data is available for prediction of response to intravesical BCG therapy. With regard to disease progression, external prospective validation studies suggest that mutational FGFR3 status and gene signatures may improve models based on clinicopathologic information.

In MIBC, tissue-based biomarkers are increasing in importance, as they may predict patient response to systemic chemotherapy and immunotherapy. The advent of molecular characterization holds the promise of revolutionizing the paradigm of decision-making in the treatment of MIBC, especially in the years characterized by the advent of systemic immunotherapy.

Prospective studies in well-defined patient cohorts and with clinically meaningful endpoints are needed for drawing definitive conclusions about the utility of tissue-based biomarkers in BCa. Until then, despite their promising value, the role of tissue-based biomarkers should be limited to the experimental setting.

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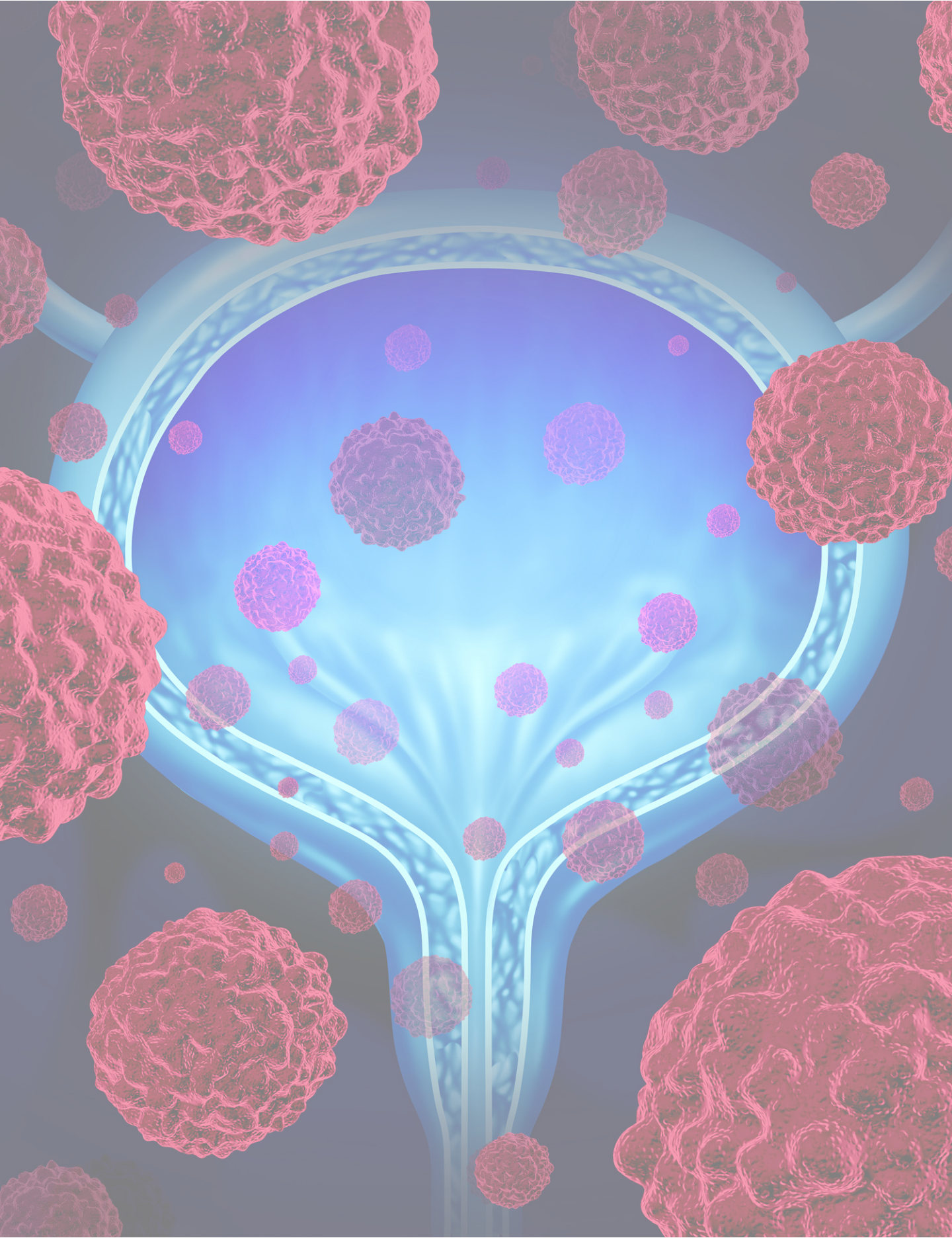

CHAPTER 12

Clinical Utility of Bladder Cancer Biomarkers

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Table of Contents

12.1 Introduction

Bladder cancer is estimated to be responsible for about 550,000 new diagnoses and almost 200,000 deaths worldwide in 2018.[1] The need for frequent follow-up including invasive procedures such as cystoscopies, repetitive procedures such as transurethral resection of bladder tumour (TURBT) and intravesical instillation therapy in non-muscle invasive stages as well as systemic treatment with or without radical local treatment in advanced stages makes bladder cancer one of the most expensive cancers to treat.[2] Prognostic and predictive biomarkers have the potential to fundamentally change bladder cancer treatment algorithms, which may both result in improved patient comfort and oncological outcomes as well as decrease the socioeconomic burden of the disease. Intense research activities in this field have recently resulted in the approval of the first agent for this disease targeting a specific mutation (fibroblast growth factor receptor [FGFR]) by the FDA.[3] Yet, many areas of bladder cancer diagnosis and treatment remain unchanged for decades, and this is only in part due to the therapeutic success of the status quo. In order to integrate biomarkers into clinical practice patterns, specific considerations for the different disease stages and settings should be kept in mind.

12.2 Considerations for Biomarkers for Bladder Cancer Screening

Due to the rather low incidence of bladder cancer in the general population and even in high-risk populations, there is currently no recommendation for bladder cancer screening.[4] In general, for screening purposes in daily routine, a point-of-care test on a dichotomous basis could be performed easily in the ambulatory setting and should provide an initial risk assessment that could be used to tailor the need for further clinical investigation. Urine is obviously the most promising resource for such a test. On the one hand, a urine-based biomarker test in such a setting requires a high sensitivity and negative predictive value (NPV) in order to avoid missing people at risk. On the other hand, given the low incidence of bladder cancer, a high specificity and positive predictive value (PPV) is also needed to avoid unnecessary (invasive) evaluation due to false-positive results. In addition, given the application of a urine marker as a screening tool, the costs for test analysis should be low and not exceed the usual costs for standard diagnostic work-up of bladder cancer by a large margin. Finally, an earlier detection after test analysis should ideally demonstrate a significant improvement in oncological outcomes. As it seems to be difficult to demonstrate an overall survival benefit in the general population or even high-risk populations, a reduction of rates of invasive disease may serve as a surrogate parameter. Most current urine markers have a PPV that is too low to justify their use in screening, as the number of unnecessary evaluations will far exceed the finding of cancer. Targeted screening of very high–risk populations may result in a high enough cancer incidence, but prospective studies will be necessary to demonstrate a survival benefit or at least a reduction of muscle-invasive disease. It is known that male sex, higher age, and duration and intensity of smoking are associated with higher risk for bladder cancer. A study evaluating these factors and incidence of bladder cancer in the Prostate, Lung, Colorectal and Ovarian (PLCO) trial and the National Lung Screening Trial (NLST) found that in men older than 70 years with smoking exposure of 30 pack-years (PY) and more, incidence rates were as high as 11.92 (PLCO) and 5.23 (NLST) (per 1,000 person-years).[5] There is not a strong association of family

history and bladder cancer. However, in the presence of distinct single-nucleotide variants (SNVs, formerly single-nucleotide polymorphisms [SNPs]) that are associated with a significant risk for bladder cancer, screening for bladder cancer may be justified. Upper tract urothelial carcinoma develops in up to 28% of patients with known Lynch syndrome. While no ideal screening test exists for this disease, it is not unreasonable to use routine urine analysis and evaluate patients using the American Urological Association guideline of 3 or more red blood cells per high-power field.[6] Patients with Lynch syndrome who develop upper tract disease usually present at a younger age, with a higher female preponderance and a predisposition to bilaterality.

12.3 Considerations for Biomarkers for Bladder Cancer Diagnosis in the Setting of Hematuria

Patients with hematuria have a markedly increased risk of having bladder cancer (gross 10–40% depending on other risk factors or microscopic 2–5%) and therefore need further clinical work-up.[7,8] This work-up consists of cystoscopy with cytology as well as contrast-enhanced imaging of the upper urinary tract.[4] These procedures are costly, invasive, or uncomfortable. Therefore, it would be desirable to replace them with a biomarker test in order to spare full evaluation from patients who do not need it. Again, urine seems to be the most promising medium for a biomarker test in such a scenario. Large efforts have been undertaken to risk stratify patients with hematuria based on demographic and clinical factors as well as various genetic and protein markers.[4] Dichotomous or semiquantitative tests indicating the individual risk for the presence of bladder cancer seem to be suitable to guide clinicians toward or against further testing. Similar to the screening setting, the combination of a high sensitivity and NPV is needed to avoiding missing a tumour. A high specificity and PPV are also desirable to avoid unnecessary evaluations for false-positive test results but are less important than in the screening setting, given the lower number of these “quasi”-prescreened patients and related costs.

As the rate of cancer in patients with hematuria exceeds screening cohorts by far, identifying high-risk patients who always need evaluation is important. Several studies have found that the cancer yield increases with gross hematuria, male gender, and increasing age.[9,10] Unfortunately, many patients with high-risk disease are not adequately evaluated.[11–13] The goal for markers could be to not only improve risk stratification of patients so that higher-risk patients get evaluation but also identify which lower-risk patients need to be evaluated and which patients may be safely monitored without invasive testing.[14,15]

12.4 Considerations for Biomarkers in the Surveillance of Patients with Non-Muscle Invasive Bladder Cancer

When patients are diagnosed with low- to intermediate-risk non-muscle invasive bladder cancer (NMIBC), the follow-up course is usually associated with frequent cystoscopies to rule out recurrent or progressive disease, especially in those undergoing intravesical instillation therapy. These examinations are cumbersome to many

patients and are associated with significant costs. Therefore, investigators have focused on biomarkers to accurately detect the presence or absence of a recurrence as an alternative to invasive diagnostic procedures. Given the low risk for progression in low- and low-grade intermediate-risk NMIBC (<15% at 5 years), but high risk for recurrence, one needs to consider the goals for a biomarker. A biomarker with a high specificity will reduce the number of cystoscopies performed due to false-positive findings, but if sensitivity is not high, it may miss recurrences.[16] This may be acceptable in order to reduce the number of cystoscopies, as low-grade recurrences are unlikely to progress. As an alternative, some investigators have proposed alternating cystoscopy with a marker. This is even more important, as most markers have a better sensitivity for high-grade disease and would therefore be more likely to catch the rare case of a low-grade tumour that progresses.

On the other hand, patients with a history of high-risk bladder cancer have the highest risk for recurrence (50% at 5 years).[16] Therefore, a useful biomarker test must have a high sensitivity and NPV in order not to miss any high-grade recurrence that may result in progression to muscle-invasive stages. This could even come at the cost of lower test specificity.

Another area where molecular markers could also aid in clinical decision-making for patients with NMIBC would be in predicting response to intravesical therapy. Adding a biomarker test to a standard work-up in this setting may identify patients who are unlikely to benefit from bacillus Calmette-Guérin (BCG) and require early radical treatment. Nonetheless, this issue is difficult to be addressed in clinical trials, as bladder cancer is a heterogeneous disease and the intravesical therapies used are non-specific to a distinct molecular target for which one could test. Therefore, predicting response to intravesical therapy will remain challenging in the future. This circumstance is also complicated by the fact that immunotherapy with BCG and also chemoinstillation induce inflammatory changes, which can sometimes impair the diagnosis of a tumour recurrence or influence test results. However, maybe adding a biomarker test to a standard work-up in this space could identify patients unlikely to benefit from BCG, who might require more radical treatment, early.[17] Furthermore, markers may be able to select populations at higher risk for enrollment in clinical trials.[18]

12.5 Considerations for Biomarkers in the Muscle-Invasive Setting

In muscle-invasive bladder cancer (MIBC), there are several distinct needs that could benefit from biomarkers. Patients with American Joint Committee on Cancer (AJCC) stage 2 or 3 disease have variable rates for recurrence and progression. Furthermore, there is a problem with understaging approximately 40% of patients.[19] There is level 1 evidence for use of neoadjuvant chemotherapy (NAC), but NAC is underused due to concerns about toxicity and a relatively small survival benefit.[20] There is a greater benefit to NAC in patients who have non-organ–confined disease, so identifying which patient is likely to have micrometastatic disease would be valuable. As there is also variable response to NAC, a marker to identify likely responders would be important to avoid treatments that are toxic and unlikely to benefit the patient. Furthermore, while cystectomy is the main treatment for MIBC, trimodality therapy is a reasonable alternative in some patients, and predicting responsiveness to this type of treatment would be valuable.

In patients who do not get NAC, there is a role for adjuvant therapy in patients with non-organ–confined disease.[21]

In addition, proponents of an adjuvant approach argue that there is a considerable risk for overtreatment with NAC, as histopathological risk factors determined in radical cystectomy (RC) specimens correlate much stronger to survival compared with histological parameters obtained by TURBT.[22,23] Pathological complete or partial response (downstaging to non-muscle invasive tumour stages) after NAC is reported in 40% to 50% of the patients and associated with excellent survival.[24] On the contrary, the majority of patients will exhibit persistent muscle-invasive disease (≥ypT2) after RC, which is associated with poor outcomes.[24]

A predictive biomarker in this setting should be capable of identifying patients in need for systemic treatment and also give information about whose tumours are sensitive to systemic treatment—maybe in the future even to which treatment. Given the high degree of intratumoural heterogeneity of MIBC,[25] biomarker expression levels should be homogeneous within the tumour lesion to reliably predict response. Ideally, a biomarker assessed in the transurethral resection (TUR) specimen should have a rather high sensitivity than specificity in order to avoid undertreatment of patients with MIBC. By contrast, a high specificity but low sensitivity may result in the underuse of neoadjuvant treatment, but this may be alleviated in the case an effective adjuvant treatment is available and safely applicable even in patients with comorbidities, ie, impaired renal function. For the decision-making on the necessity of adjuvant treatment in the case of lack of a downstaging effect after neoadjuvant therapy, the currently available histopathological parameters (ie, tumour and nodal stage) can be considered accurate enough to determine clinical need; however, a biomarker might help select between different treatments in the future.[26] Prediction of response in the adjuvant setting after failure of a neoadjuvant approach requires ideally a biomarker that exhibits an even higher specificity compared to the (primary) neoadjuvant setting, as the group of ≥ypT2 patients after RC exhibits a very dismal prognosis.[24]

Patients who have a locally advanced tumour on cystectomy and have not received previous neoadjuvant therapy might benefit from a prognostic biomarker informing on who will experience recurrence and who will not as well as a predictive biomarker of which treatment might be associated with the best response. The prognostic biomarker should again display a high sensitivity to avoid undertreatment in this scenario.

12.6 Considerations for Biomarkers in the Metastatic Setting

In the metastatic setting, predictive biomarkers are urgently needed to determine which tumour will likely respond to which treatment, to prevent patients from receiving ineffective therapies in this very aggressive disease state. These biomarkers might likely be blood or tissue based. Ideally, biomarkers should be assessable sequentially via blood draw so the patient doesn't need to be biopsied multiple times and the biomarker does validly represent the status quo as tumours might evolve over time. Groundwork on these fronts has been done by genomic characterization of bladder tumours; however, validated biomarkers are largely lacking.

Biomarkers to predict response to therapy are critical. There is already an approved targeted therapy for bladder cancer in the metastatic setting targeting FGFR mutations.[27] The role for biomarkers such as programmed cell death 1 ligand 1 (PD-L1) to predict response to checkpoint inhibitors is controversial but necessary in certain cases. In first-line, cisplatin-ineligible patients, the use of PD-L1 inhibitors is nowadays only approved after PD-L1 testing, whereas in the second-line setting, data from a randomized trial support the use of, for example, pembrolizumab in an unscreened population of platinum-pretreated patients.[28,29] Given the low response rates in both settings (~25% complete response [CR] + partial response [PR]), it will be important to combine PD-L1 inhibitors with other targeting agents to improve response. Nonetheless, in the future, it can be expected that the decision to use single- or multiagent targeted therapy in any line of systemic therapy will be based on marker expression. Given the high mutational burden and heterogeneity of response to treatment in metastasized tumours, the critical question is whether the tissue obtained at primary diagnosis can accurately reflect the tumour biology after multiples lines of systemic treatment. Therefore, the implementation of robust biomarkers in the different metastatic settings will require first a better understanding of the biological processes during progression of metastatic disease. This will obviously require well-designed biopsy studies to systematically assess alterations in tumour biology during the process of metastasis formation.

12.7 Conclusion

There are many areas in bladder cancer where biomarkers can have an important role in improving clinical decision-making. The current information from stage and grade of disease is insufficient to adequately stage or predict outcomes for most patients. Biomarkers have the potential to shed light into the clinical behaviour of tumours to allow for a personalized approach to care. There is also a potential for improved understanding of the biology of the disease in order to determine which patients need more intensive therapy and which therapies to use.

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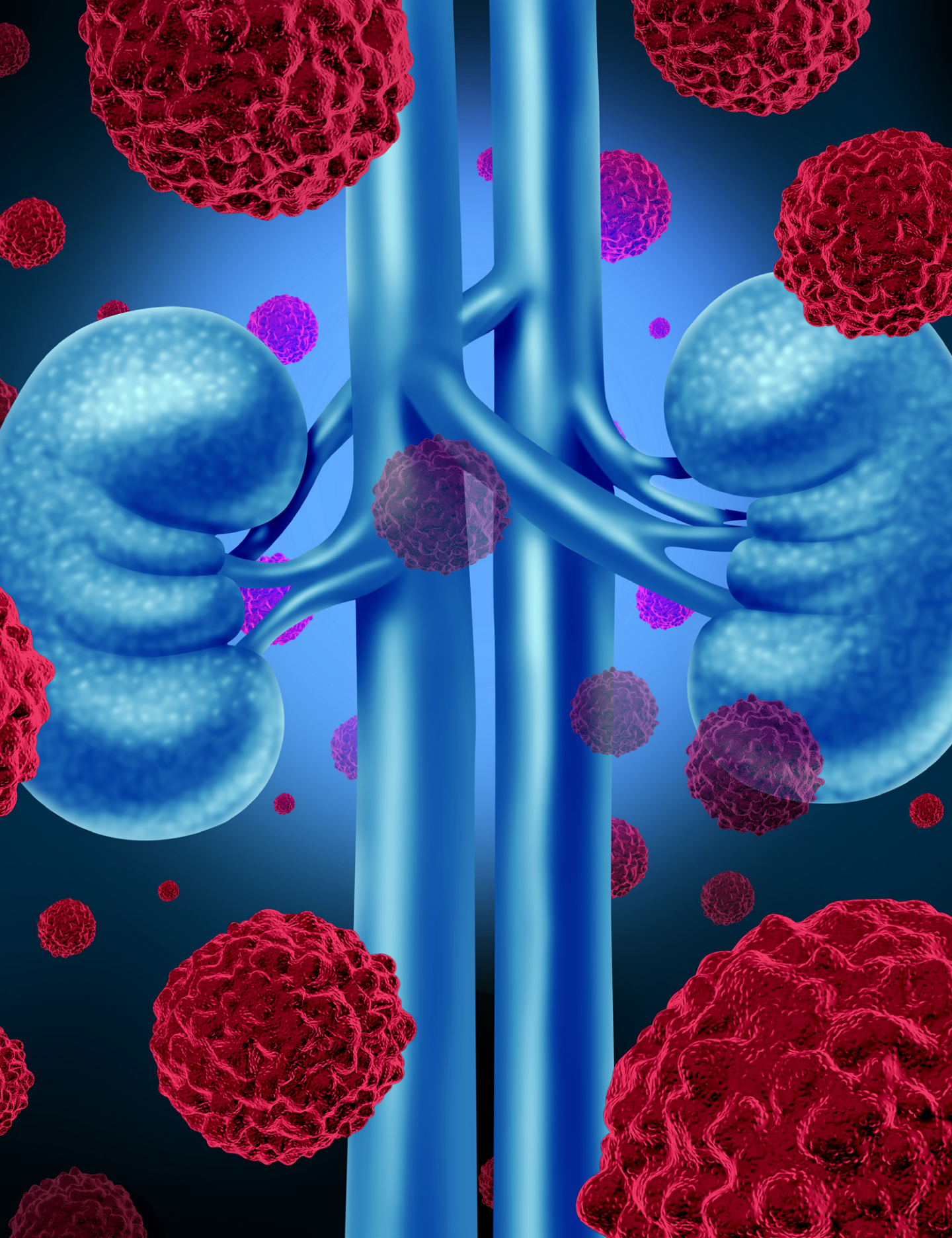

CHAPTER 13

Tissue-Based Markers for Renal Cell Carcinoma

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Table of Contents

13.1 Introduction

With an increase in renal cell carcinoma (RCC) subtypes, the role of classification now relies on a range of techniques and features for distinguishing between tumours, involving the analysis of anatomical, morphological, immunohistochemical, and molecular characteristics. There has been however more attention recently drawn to the role of immunohistochemistry (IHC) staining. The use of tissue-based IHC markers serves multiple purposes, where it is especially important for aiding histological subtyping, differentiating from non-renal neoplasms or other metastatic disease, and using for prognostication as well as guidance of treatment selection.[1] Furthermore, the classification of subtypes itself conveys important prognostic information.[2] IHC staining provides not only information on presence or absence of a marker but also quantitative data including extent of staining within a specimen.

Although many advances have been made in IHC staining for RCCs, challenges still arise in this constantly evolving landscape. Substantial tissue heterogeneity exists across the tumour subtypes, and even within subtypes themselves.[3] Small volume samples are increasingly required to be analyzed as diagnostic biopsies occur more frequently. Furthermore, the interpretability of IHC staining can be significantly impacted by variations in tissue acquisition, processing, and analysis.[3] This, in combination with the need to use as few markers for staining as possible to reduce costs while obtaining an accurate diagnosis, leaves this field in ongoing development.[4] Because of these various challenges, IHC staining will continue its current role as a supportive adjunct to the classification of RCCs.

This chapter will outline the current landscape of tissue-based markers that have been reported for the various RCC subtypes, with a primary focus on the most common subtypes (**Table 13-1**). A detailed overview of markers has been explored for clear cell RCC, papillary RCC, chromophobe RCC, and renal oncocytoma. Validated and novel tissue-based markers will also be outlined that may help distinguish the overlap between chromophobe RCC and renal oncocytomas. Tissue-based markers will also be concisely outlined for other RCC subtypes. Markers for diagnosis extend beyond distinguishing between subtypes, and they must also aid in distinguishing from non-renal tumours such as urothelial carcinoma. Other useful IHC markers will also be examined.

13.2 Clear Cell Renal Cell Carcinoma

Clear cell RCC (ccRCC) makes up more than 75% of diagnosed RCCs.[5] These tumours are typically characterized by their clear cytoplasm, nests of cells arranged among alveolar architecture, accompanied by thin-walled sinusoid-like vessels.[6] ccRCCs are more likely to present as tumours of larger sizes compared to other subtypes.[6]

The IHC staining profile of ccRCC has been widely characterized. Carbonic anhydrase 9 (CAIX) is a transmembrane carbonic anhydrase protein that is involved with CO_2 transfer. Its function has been implicated in the mediation of cell proliferation and tumour progression. CAIX displays widespread membranous staining in non-necrotic areas, with high diagnostic specificity in combination with other markers.[6,7] Typically, all ccRCC specimens would stain either moderately or strongly positive for CAIX.[6] Vimentin, a mesenchymal marker, is also highly

expressed in ccRCC, with up to 87% staining of specimens reported in the literature.[8,9] Expression for epithelial membrane antigen (EMA) can be found in up to 85% of specimens, where the pattern of staining in ccRCC is mostly membranous.[10] The pattern of staining for EMA may provide insight into disease-free survival and the development of metastatic disease.[11]

Variable cytokeratin (CK) staining patterns may be found. Broad spectrum CK (pan-cytokeratin) can be detected in ccRCC using AE1/AE3 antibody. The co-expression of CAIX and pan-cytokeratin is typical of ccRCC.[12] In addition, the CAM 5.2 antibody may also be used, achieving roughly a 60% positivity for detecting CK8 expression in ccRCC specimens.[13] Although CK7 is commonly detectable in other RCCs, ccRCC usually stains negative for CK7.[6,8] Immunoreactivity for CK19 may be observed infrequently, in fewer than 20% of specimens.[8,14] ccRCCs are not immunoreactive to 34βE12, an antibody for high molecular weight CK.[14]

Alpha-methylacyl coenzyme A racemase (AMACR), a mitochondrial enzyme for fatty acid oxidation, is not typically present on staining in ccRCC. However, some weak positivity for AMACR may be seen in up to 18% of cases.[6] The expression of the cell adhesion molecule E-cadherin is also typically negative, although it may be detectable in tumours of higher grade.[10,11,15] Kidney-specific cadherin (Ksp-cadherin), detectable in the distal convoluted tubules, can be detectable in moderate intensity in some specimens (30%).[16] Parvalbumin, claudin 7 and 8, and CD117 are some other markers that may be also tested. In ccRCC, there is typically no expression of parvalbumin, a calcium-binding protein involved in intracellular calcium regulation, in primary or metastatic ccRCC specimens.[17] However, up to 23% detection has been reported in smaller collections of cases.[9] Claudin 7 and 8, two protein components found in intercellular tight junctions, are also typically not expressed in ccRCC specimens.[18–20] CD117 (or c-Kit), a receptor tyrosine kinase, is typically negative in ccRCC specimens, with immunoreactivity reported in up to 3% of specimens.[21,22]

Human kidney injury molecule-1 (hKIM-1), a type 1 transmembrane glycoprotein present in injured proximal tubules, can be detected in ccRCCs, in up to 74% of specimens.[23] Diffuse expression of hKIM-1 is more commonly detected in patients with higher-grade ccRCC disease.[23] However, hKIM-1 expression can also be found in clear cell carcinomas of the ovary, uterus, and invasive adenocarcinomas of the colon.[23] Among lectins, galectin-1 and galectin-3 have been implicated in roles for tumour progression and angiogenesis. In a study of IHC staining for galectin-1 and galectin-3, staining was present in normal renal tissue localized to the renal tubules, with galectin-1 additionally being detectable in glomeruli.[24] However, in ccRCC specimens, high expression was present for galectin-1 (51%) and galectin 3 (78%).[24]

A number of sensitive but nonspecific markers may also be used for confirming RCCs. RCC marker (RCCM), an antibody directed at the brush border of the proximal tubule, is present in approximately 85% of specimens.[25] Its degree of staining is more consistently positive for lower-grade tumours, identifiable as immunoreactivity in the surface membrane, compared to higher-grade tumours.[26] However, RCCM is also reported to be expressed in other non-renal tumours, up to 27% in those with clear cell morphology.[27] Cluster differential marker 10 (CD10), is also expressed widely in ccRCC specimens, in up to 94% of specimens, primarily in a cell surface staining pattern; however, it may also be detectable in non-renal tumours.[26]

The use of IHC markers may aid in distinguishing between RCC subtypes with clear and eosinophilic morphology; for instance, ccRCC may appear similar to chromophobe RCC (chRCC). A suitable shortlist panel of IHC markers to assist with diagnosis may include vimentin, RCCM, CAIX, Ksp-cadherin, CD117, and parvalbumin.

13.3 Papillary Renal Cell Carcinoma

Papillary RCC (pRCC) is the second most common RCC subtype, accounting for up to 15% of renal cell tumours.[5] These tumours characteristically display prominent papillary features, with small cells with less cytoplasm, as well as the presence of psammoma bodies.[6] There is diffuse immunoreactivity to AE1/AE3 antibody and CAM 5.2 antibody, but no expression of high molecular weight CKs. There can be strong membranous staining for CK7, especially for type 1 pRCC, where more than 80% staining can be achieved.[8,28] Up to 90% of specimens have immunoreactivity to CK19 staining, with greater likelihood of detection in type 1 tumours.[8,14]

AMACR staining demonstrates strong granular positivity within the cytoplasm, for both type 1 and 2 pRCC, in up to 96% of specimens.[29] EMA staining is often reported as immunoreactive in pRCC in the literature. In one study, 100% of pRCC specimens were reported to express EMA, with different staining patterns across pRCC types. Type 1 pRCCs had predominantly membranous staining, whereas type 2 pRCCs displayed both membranous and cytoplasmic staining.[30] Other studies have suggested that roughly 65% of pRCC specimens may stain positive for EMA.[10] pRCCs express E-cadherin variably depending on grade and type of pRCC. Although overall expression is low (15%), specimens were more likely to express E-cadherin if they were higher grade, or if they were type 2 pRCCs.[11] pRCCs consistently do not express Ksp-cadherin.[16] There can be strong staining for vimentin in pRCCs. Previously reported studies of pRCC specimens have demonstrated 78% to 100% of specimens staining positive for vimentin.[8,10]

pRCC specimens often stain negative for CAIX, though some (13%) may demonstrate weak positivity near necrotic areas.[6] RCCM stains positive in a strong surface staining pattern in pRCC.[25,26] Similarly, CD10 also shows positive staining in up to 93% of specimens with a surface staining pattern.[26] hKIM-1 can be identified in pRCCs. In one study, 28 of 30 pRCC specimens were immunoreactive to hKIM-1, with 50% of them displaying diffuse hKIM-1 staining.[23] Reports show expression of parvalbumin in pRCC as variable, with detection reported in 0% to 71% of specimens.[9,17] pRCC typically does not express CD117, with reports of immunoreactivity in approximately 5%.[22,31] There is variable reported data for membranous staining of claudin 7 in a range of 28% to 78% of pRCCs.[19,20] Claudin 8 does not stain well in pRCCs, seen in as few as 14% of specimens.[20] In pRCC, it has been reported that there are up to 83% of specimens with >50% immunoreactivity to galectin-1. In contrast, fewer than 6% of pRCC specimens demonstrated >50% expression of galectin-3.[24]

For a tumour with papillary growth morphology, a helpful IHC panel would include CK7, AMACR, CD10, RCCM, TFE3, and CD57.[4,32]

13.4 Chromophobe Renal Cell Carcinoma

Chromophobe RCC (chRCC) represents 6% to 11% of diagnosed RCCs.[7] It is typically characterized by large pale cells with prominent cell membranes. Upon using routine dyes, these tumours are found to characteristically lack cytoplasmic colouring. chRCCs produce immunoreactivity to CAM 5.2 antibody in the majority of specimens (59%).[14] chRCC specimens also demonstrate up to 73% overall immunoreactivity to AE1/AE3 antibody, with a mixed staining pattern of membranous staining and diffuse cytoplasmic staining of smaller cells.[14] There is up to 73% immunoreactivity reported for CK7 in chRCC.[7,14] chRCC does not usually express CK19, although immunoreactivity has been reported in some studies, in up to 23% of specimens.[8,14] chRCC typically does not stain for high molecular weight CK with 34βE12 antibody.[14]

chRCC is reported to consistently stain for EMA, where the pattern of staining is diffusely cytoplasmic, although some specimens may also additionally stain with a membranous pattern.[10,11] Typically, chRCCs stain positive for E-cadherin, where up to 95% of specimens may be immunoreactive. Ksp-cadherin may also be expressed, located on the basolateral cell membrane surface of distal tubules and collecting ducts, and it may be detected in up to 86% of specimens.[16,33]

chRCCs stain positive for the intracellular calcium-binding protein parvalbumin in up to 100% of specimens.[9,17] Staining pattern is most commonly a strong cytoplasmic as well as nuclear immunoreactivity in most cells.[17] chRCCs may also demonstrate immunoreactivity for tight junction proteins, claudin 7 (91%) and less frequently claudin 8 (27%).[18,20] Claudin 7 and claudin 8 are reported to be expressed in a membranous distribution in chRCC if the tumour is immunoreactive.[18] Up to 96% of chRCCs stain positive for receptor tyrosine kinase CD117 using conventional sections.[22] chRCC also demonstrates high expression of both galectin-1 (100%) and galectin-3 (63%).[24]

These tumours typically stain negative for vimentin, although some reactivity has been reported in specimens with sarcomatoid features.[7,8,34] When stained for hKIM-1 marker, there is consistent negative expression in chRCC specimens.[23] chRCC specimens typically are not immunoreactive to CAIX, CD10, RCCM, or AMACR.[26,29] To differentiate chRCCs from oncocytomas, Hale's colloidal iron stain may be used, where staining may demonstrate diffuse, granular cytoplasmic staining in up to 87% of chRCCs.[10]

13.5 Renal Oncocytoma

Renal oncocytomas (ROs) are similar to chRCC; they have overlapping morphology and IHC features. Typically, ROs consist of oncocytes, in a nest-like or alveolar arrangement among a fibrous stroma.[34] Immunoreactivity to AE1/AE3 pan-cytokeratin is variable, with up to 48.5% positivity amid oncocytoma specimens.[14] Limited immunoreactivity in roughly 40% of specimens may be observed for CK19, while there is no expression of high molecular weight CK.[14] ROs are widely immunoreactive to CAM 5.2 antibody.[35] CK7 may rarely be detected in ROs, with small scattered focal staining in up to 10% of specimens.[8]

ROs typically stain positive for E-cadherin; however, they may have some cytoplasmic expression of EMA (52%) and can express Ksp-cadherin in up to 76% of oncocytomas.[10,16,33] Parvalbumin is detectable in all RO specimens.[9] Reports of CD117 staining have demonstrated 100% staining in conventional sections of RO specimens.[22] There can be membranous staining for claudin 7 in up to 55% and mixed pattern expression of claudin 8 in up to 92% of RO specimens.[18–20] Oncocytomas are typically negative for vimentin, AMACR, CAIX, CD10, and RCCM.[4,10,26,29,34,36] Only limited immunoreactivity has been reported for hKIM-1, with staining found in less than 10% of specimens.[23]

A number of IHC markers may assist in distinguishing between oncocytomas and chRCC. In comparison to the strong granular positive staining in chRCC specimens, ROs typically stain negative for Hale's colloidal iron stain; however, variability in processing and technique has affected the interpretability and reproducibility of the findings.[10] CK7 IHC demonstrates only focal staining compared to the strong and diffuse staining in chRCCs.[34] In preliminary studies, S100A1 protein has been reported to be widely expressed in oncocytomas (100%), with either a strong diffuse cytoplasmic pattern, or more commonly, focal staining. This is in comparison to chRCCs, where only 30% demonstrated immunoreactivity to S100A1, with staining confined to the cytoplasm as well as the membrane.[37] S100A1 is also immunoreactive in ccRCC and pRCC specimens.[37] Caveolin-1, a scaffolding protein, was originally reported to be well expressed in chRCC and not expressed in ROs; however, this has been inconsistently reported and patterns of expression (membranous versus cytoplasmic) can vary across cancer subtypes.[34,38–40]

More recently studied markers that may help distinguish oncocytoma from chRCC have been analyzed for their utility.[41] Amylase α1A, a salivary-type digestive enzyme, was investigated in a large sample (n=129). The amylase α1A stain was able to produce 100% staining in RO specimens, compared to only 13% of chRCCs.[42] Another marker, Wnt-5a, involved in tumour development, similarly was able to produce 100% staining in RO specimens while only 16% of chRCCs stained positive.[43] FXYD2 is a marker that codes for a subunit of a distal tubule Na/K ATPase, with staining found in 17% of ROs, compared to 96% in chRCCs.[44] Ankyrin-repeated protein with a proline-rich region, a muscle protein, was present in 86% of ROs, compared to 0% in chRCC specimens.[45] CD63 is a glycoprotein that has been investigated for differential staining patterns in renal tumours. CD63 staining produces apical/polar staining in 94% of ROs, compared to the diffuse staining pattern observed in 96% of chRCCs.[46] Transforming growth factor β 1 (TGFβ1), a cytokine, has also been studied for its differential staining patterns. ROs demonstrated predominantly cytoplasmic staining, compared to the membranous staining in chRCC specimens.[47]

A range of novel markers have been explored in more recent years and are still under investigation. FOXI1, a transcription factor present in intercalated cells, demonstrates positive nuclear staining for oncocytomas, compared to chRCCs (96% vs 3%).[48] ELA, a ligand of apelin receptor, has also been studied. Apelin receptor is expressed in stem cells as well as kidney tissue, with roles in embryonic development.[49] Preliminary data have shown a significant increase in staining scores in RO compared to chRCC (1.28 ± 0.63 vs 0.21 ± 0.13). IHC staining of caspase 3, a protease involved in cellular apoptosis, was able to produce significantly lower staining for ROs compared to chRCCs (25% vs 86%).[50] Increased nuclear expression of leptin has also been reported in

RO compared to eosinophilic chRCCs.[51] Loss of RB1 protein on IHC staining may also be observed in up to 64% of chRCC specimens, while no loss in RB1 was identified in any RO specimen.[52] Analysis of the receptor tyrosine kinase ERBB4 demonstrated that ERBB4 may produce differing staining patterns, with less nuclear staining in ROs compared to chRCCs (12% vs 75%).[52] Further validation of these markers may lead to their adoption in routine histopathological classification.

TABLE 13-1 Summary of IHC Markers for Common RCC Subtypes

IHC Marker	ccRCC	pRCC	chRCC	Renal Oncocytoma
Pan-cytokeratin (AE1/AE3)	+	+	+	variable
CK7	-	Type 1: + Type 2: -	+	-
CK8/CK18 (CAM 5.2)	+	+	+	+
CK19	-	+	-	-
HMWCK (34βE12)	-	-	-	-
EMA	+	+	+	+
E-cadherin	-	variable	+	+
Ksp-cadherin	-	-	+	+
CAIX	+	-	-	-
AMACR	-	+	-	-
Vimentin	+	+	-	-
Parvalbumin	-	variable	+	+
c-Kit/CD117	-	-	+	+
CD10	+	+	-	-
RCCM	+	+	-	-
hKIM-1	+	+	-	-
Caveolin-1	+	+	+	-
S100A1	+	+	-	+

Abbreviations: +, ≥50% staining; -, < 50% staining.
AMACR, alpha-methylacyl coenzyme A racemase; CAIX, carbonic anhydrase 9; ccRCC, clear cell renal cell carcinoma; chRCC, chromophobe renal cell carcinoma; CK, cytokeratin; EMA, epithelial membrane antigen; IHC, immunohistochemistry; hKIM-1, human kidney injury molecule-1; HMWCK, high molecular weight cytokeratin; Ksp-cadherin, kidney-specific cadherin; pRCC, papillary renal cell carcinoma; RCCM, RCC marker.

13.6 Other Renal Cell Carcinoma Subtypes

13.6.1 Clear cell papillary RCC

Clear cell papillary RCC (cpRCC) tends to present as a histological mix of both clear cysts and papillary features. cpRCC tends to display fewer features of tumour aggressiveness such as extracapsular or vascular invasion, mitotic figures, or tumour necrosis, and as a result, these tumours are often diagnosed at an earlier stage.[6] In this particular subtype of RCC, there is a strong, diffuse positive cytoplasmic staining for CK7. Staining for AMACR is negative in cpRCC. There is diffuse membranous staining of CAIX, similar to ccRCC, but there is a unique "cup-like" staining pattern on the basolateral surface of tumour cells.[6] There may be some patchy or diffusely positive staining for 34βE12 high molecular weight CK.[7] Unlike ccRCC, this subtype is negative for CD10 and positive for CK7. pRCC may be differentiated by its positivity for CD10 and AMACR.

13.6.2 Microphthalmia-associated transcription family translocation carcinoma

Although several features may be shared with ccRCC and pRCC, microphthalmia-associated transcription (MiT) family translocation carcinomas should be distinguished due to differences in outcome, prognosis, and management strategy.[53] These tumours often consist of large, clear cells, arranged in nests, alveoli, or papillae.[6]

MiT family translocation RCCs typically do not express CKs or EMA, but they do express CD10 and RCCM. Although the primary diagnostic modality is a fluorescence in situ hybridization (FISH) assay, IHC markers may be used to distinguish MiT family translocation RCCs.[54] Translocations involving chromosome Xp11.2 are negative for CK7 and CAIX, but positive for AMACR.[29] A specific IHC marker, TFE3, may be used to identify this transcription factor that is overexpressed in this particular translocation.[6] A similar translocation-related tissue marker, TFEB, may be used to distinguish cells affected by a translocation at chromosome 6p21.[55] In relation to both chromosomal loci, an additional marker, cathepsin-K, may also be overexpressed and detectable in both Xp11.2 and 6p21 translocation tumours. Cathepsin-K has been reported to be expressed in all TFEB translocation RCCs, and in up to 60% of TFE3 RCCs.[56,57] Nevertheless, these IHC markers serve as a supportive aid to diagnosis only, due to the occurrence of false-positive and -negative results.[58]

13.6.3 Acquired cystic disease–associated RCC

Acquired cystic kidney disease may develop in patients with end-stage kidney disease, including those on dialysis.[59] Acquired cystic disease–associated RCC may result—these indolent tumours arise within these acquired cysts and can be multifocal or bilateral.[12,59] Their morphological features enable them to be diagnosed without heavy reliance on IHC. These tumours typically possess eosinophilic granular cytoplasm, a characteristic "sieve-like" intracytoplasmic bridging, along with large nuclei with prominent nucleoli.[59] They typically stain positive for AMACR, vimentin, CD10, RCCM, and CAM 5.2 and AE1/AE3 antibodies. They have variable expression of CK7 and CAIX, and they stain negative for E-cadherin and CD117.[59,60]

13.6.4 Tubulocystic RCC

This tumour subtype may appear similar to pRCCs, but remains separate with different immunophenotyping.[12] There is a characteristic "bubble wrap" appearance of cystically dilated tubules separated by fibrous stroma, and tumour cells have been described to have a "hobnail" appearance, in addition to possessing abundant eosinophilic cytoplasm and large nuclei.[61] There is usually diffuse positivity of AMACR.[29] They may also stain positive for CK7, but this can be weak. In a study of three tubulocystic RCC specimens, all three stained positive for CK19, as well as vimentin, while being negative for high molecular weight CK.[8] These tumours may also stain positive for CD10, PAX2, and PAX8.

13.6.5 Succinate dehydrogenase–deficient RCC

Loss of the SDHB subunit for succinate dehydrogenase (SDH) mitochondrial complex 2 predisposes individuals to developing succinate dehydrogenase–deficient RCC. These tumour cells typically present in solid nest or tubular arrangements, with cyst-like changes and pale eosinophilic fluid.[62] Characteristically, cytoplasmic inclusions may be observed using electron microscopy, signifying the enlarged mitochondria. Succinate dehydrogenase–deficient RCCs display variable CK expression, and as many as 68% may be negative.[62] CD10 staining may also be variable in this tumour subtype. These tumours may be positive for PAX8, EMA, as well as Ksp-cadherin. Typically, they stain negative for vimentin, CD117, RCCM, and CAIX.[63,64] Notably, due to the loss of the SDHB gene coding for a subunit for succinate dehydrogenase enzyme, these tumours will demonstrate universally negative immunostaining for SDHB.[63]

13.6.6 Collecting duct carcinoma

Collecting duct carcinomas (CDCs) present as focal tubulocystic cells that may colonize into the pelvic urothelium. Their aggressive nature enables them to differentiate into a urothelial-like appearance, meaning that care must be taken to exclude urothelial carcinoma as a diagnosis. CDCs display positivity for CK5/CK6, CK7, CK8, CK19, as well as high molecular weight CK (using 34βE12).[8] These tumours are positive for vimentin, PAX2, and PAX8.[8,10] They are however negative for CD10. A useful panel to differentiate CDCs from urothelial carcinomas may consist of: PAX8, p63, and GATA3. PAX8 staining has been reported to demonstrate positive staining in up to 90% of CDCs and in as little as 8.8% of urothelial carcinomas.[65] Staining for p63 may lead to immunoreactivity in 14% of CDCs and in 97% of urothelial carcinomas.[65]

13.6.7 Renal medullary carcinoma

Renal medullary carcinoma is closely related to collecting duct carcinomas, and typically identified in individuals with sickle cell trait or anemia.[66,67] Renal medullary carcinoma specimens demonstrate positive staining for CK7 and AE1/AE3 antibody, but negative for high molecular weight CK.[8] There is variable EMA staining. These tumours stain positive for PAX2 and PAX8. They may also stain positive for vimentin.[8] Stain for nuclear transcriptional regulator SMARCB1 (INI1) can be used to differentiate from other subtypes such as collecting duct carcinomas.[68] Use of OCT3/4 stain can also be used to distinguish from urothelial or collecting duct carcinomas.[69]

13.6.8 Multilocular cystic renal cell neoplasm of low malignant potential

Multilocular cystic renal cell neoplasms of low malignant potential may display identical immunoprofiling with classic low-grade ccRCC. Microscopically, they consist of multiloculated cysts with fibrous septae. It is important to histologically differentiate them from ccRCC with cystic change, based on the absence of higher-grade features such as tumour necrosis or hemorrhage. They may be distinguished from cystic ccpRCC by their clear cytoplasm and low-grade nuclei. These tumours are reactive to CAM 5.2, EMA, CK7, CAIX, PAX2, and PAX8.[70,71] There may exhibit patchy immunoreactivity for high molecular weight CK.[70] These tumours have been reported to have variable expression of CD10.[70,71]

13.6.9 Hereditary leiomyomatosis renal cell carcinoma–associated RCC

Histologically, hereditary leiomyomatosis renal cell carcinoma–associated RCC (HLRCC) historically was reported as being similar to type 2 pRCC or collecting duct carcinomas. These tumours produced varied architectural patterns including: papillary, tubular, tubo-papillary, and solid patterns.[72] Characteristically, these tumours display prominent nucleoli with perinucleolar halos.[73] They have positive epithelial staining for CK7, CAM 5.2 antibody, and CD10. The stroma is negative for CD117 and CKs. Loss of fumarate hydratase (FH), a Krebs cycle enzyme, is specific for HLRCC.[72] Due to FH deficiency, there is detectable immunoexpression of S-(2-succinyl cysteine) (S2C) from the accumulated fumarate, leading to strong and consistent nuclear and cytoplasmic expression in up to 100% of specimens. S2C staining was not detected in any ccRCC specimens, but it could be detected in up to 22% of type 2 pRCCs; however, the staining was predominantly cytoplasmic.[72]

13.6.10 Mucinous tubular and spindle cell carcinoma

Mucinous tubular and spindle cell carcinoma (MTSCC), although a distinct subtype, displays several morphologic and IHC similarities to pRCC, despite being genetically different. Features shared between the two subtypes include compact tubular architecture, papillae, mucin production, as well as presence of foam cells.[74] MTSCC typically consists of cuboidal cells and spindle cells that contain low-grade nuclei. Similar to pRCCs, these tumours stain positive for AMACR, CK7, PAX2, E-cadherin, and EMA, while staining negative/variably for RCCM, high molecular weight CKs (using 34βE12 antibody), and CD117.[8,74,75] There can be variable staining for vimentin.[8,74,75] However, MTSCC differs by having a lower frequency of expression for CD10 compared to pRCCs (15% vs 100%).[74]

13.6.11 Sarcomatoid morphology

A number of RCC subtypes may possess sarcomatoid features on microscopic examination. These typically indicate worse prognosis. Recent reports of ccRCC or chRCC with sarcomatoid features indicate variable staining patterns, where IHC stain immunoreactivity may not reflect the typical staining pattern of the subtype. A study of 42 cases with sarcomatoid differentiation demonstrated widespread positivity for vimentin in sarcomatoid

cells, and variable expression of markers CK, EMA, CK7, and CK18.[76] Most tumours expressed CAIX, CD10, and PAX8 (76%, 76%, and 69%, respectively).[76,77] In other studies, specimens with sarcomatoid components lacked E-cadherin immunoreactivity.[11] Patterns of immunoreactivity in sarcomatoid cells were indistinguishable between ccRCC or chRCC origin, for stains CAIX, CD10, vimentin, CK7, and CD117.[76]

13.7 Other Useful IHC Stains

Various other markers are useful for determining RCC origin versus other non–renal cell neoplasms. PAX2 and PAX8 are transcription factors for the development of kidney, Müllerian ducts, and other organs.[4,78] These markers are diffusely expressed in normal kidney tissue. They are also expressed in most renal neoplasms and serve as a highly sensitive markers, being present in up to 90% of histological subtypes. This, in addition to not being expressed in urothelial carcinoma, allows them to be beneficial in diagnostic workups.[65] However, their detection in non-renal tumours with clear cell morphology can be present, with up to 24% for PAX2 and 39% for PAX8.[27] In Müllerian-derived tumours of the ovary, vagina, and cervix, PAX2 and PAX8 detection has been reported to be in up to 69% and 100% of cases, respectively.[27] Some differences exist between the two—PAX2 is often not expressed in oncocytoma or chRCC while PAX8 may be detectable.

GATA3, an endothelial cell transcription factor, has also been used widely for its ability to distinguish RCC from urothelial origin. Several studies have demonstrated consistently negative staining for GATA3 in RCC specimens, whereas up to 71% staining in invasive urothelial carcinoma specimens may be achieved.[79] Even in RCC with sarcomatoid features, GATA3 staining demonstrated no immunoreactivity, making GATA3 a useful negative marker for determining renal tumour origin.[77]

CA-125, a marker classically associated with ovarian cancer, may also be used as a negative staining tissue-based marker for RCCs. It may be useful in distinguishing ccRCC from cases of Müllerian-derived tumours as well as other tumours with clear cell morphology. ccRCC does not stain for CA-125, whereas up to 88% of clear cell morphology tumours may be immunoreactive, including cancers of the ovary, vagina, lung adenocarcinoma, bladder, and skin.[27]

Another marker for ruling out RCC origin is CK20.[4] Notably, most RCC subtypes are negative for CK20, except for eosinophilic solid cystic RCCs.[80] This marker may assist in distinguishing RCCs from other CK20-positive tumours. CK20 may be detected in urothelial carcinoma specimens.[8] CK5 and CK6 may also be used as negative markers, where they are non-immunoreactive for most RCC subtypes except for collecting duct carcinomas. Other markers discussed in previous sections that may assist in non-renal neoplasm workup, include RCCM, CD10, vimentin, and CK.

13.8 Conclusion

Updates to the classification of RCC subtypes have led to greater demand for biomarkers. Some markers have been widely characterized in the RCC literature, allowing for adequate differentiation between some RCC subtypes and for distinguishing RCC from non-renal tumours. However, ongoing investigation into novel tissue-based markers is still underway, and these may help to further characterize this complex disease. The use of IHC tissue biomarkers will ultimately help indicate diagnosis, guide treatment, and inform prognosis.

Take-home messages

- RCC tissue-based markers allow for differentiation between RCC subtypes and for distinguishing RCC from non-renal tumors.
- There is ongoing investigation into novel tissue-based markers, and these may help to further characterize this complex disease.
- There is ongoing literature in the investigation for tissue-based markers that may effectively distinguish chromophobe RCC from renal oncocytomas.
- The use of IHC tissue biomarkers will ultimately help indicate diagnosis, guide treatment, and inform prognosis.

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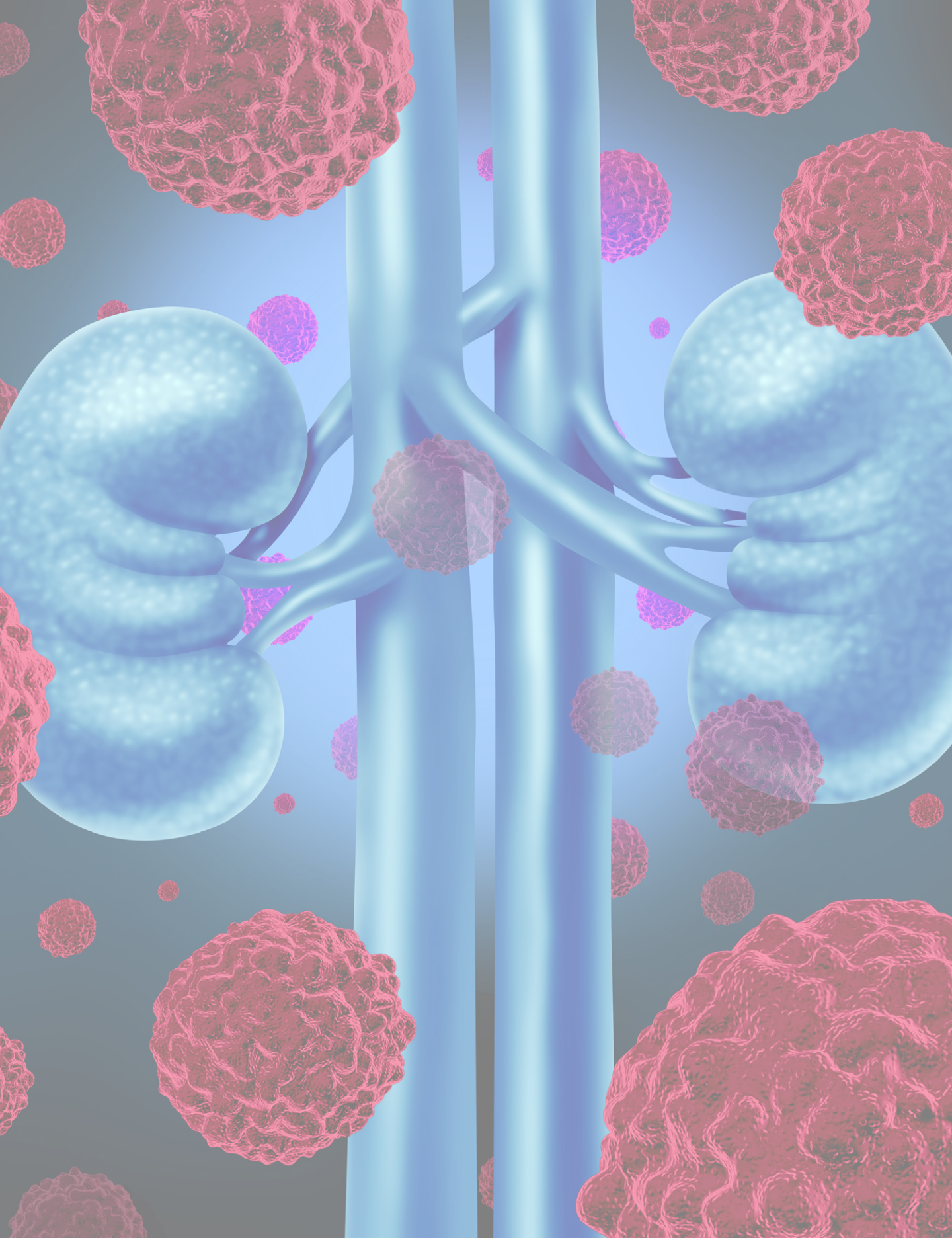

CHAPTER 14

Novel Expanding Renal Cell Carcinoma Biomarkers

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Table of Contents

14.1 Summary

Identification of reliable molecular biomarkers that can complement clinical practice represents a fascinating challenge in any cancer field. Patients with renal tumours are usually asymptomatic and these tumours are incidentally identified during imaging studies for unrelated reasons. However, in 25% to 30% of patients, the first diagnosis is accompanied by symptoms and associated with distant metastasis. Thus, early diagnosis may improve disease progression and avoid the side effects of inadequate treatment. Moreover, the ability to categorize patients' risk for recurrence after radical treatment or even predict benefit from a target therapy represents a compelling challenge. Here we reviewed the current state of the art on RCC biomarkers, focusing on the new approaches of genomics, liquid biopsy, proteomics, and metabolomics.

14.2 Biomarkers in Early Detection and Diagnosis

14.2.1 Introduction

Rena cell carcinoma (RCC) represents the third most common urological cancer in the United States, with an estimated 44,120 new cases in 2019.[1] Clear cell renal cell carcinoma (ccRCC) is the most frequent subtype, accounting for approximately 75% to 80% of RCC tumours and responsible for the majority of cancer deaths from RCC.[2] Recent advances in diagnostic techniques have increased early ccRCC detection; however, mortality rates remain steady.[3] Imaging studies are still unable to differentiate histology, and renal mass biopsy (RMB) presents a 10% to 20% non-diagnostic rate.[4] Therefore, it is highly desirable to have novel and reliable biomarkers suitable for RCC screening and early detection, particularly exploiting the benefits of new technologies.

14.2.2 Liquid biopsy

Circulating cell-free DNA

Liquid biopsy, such as circulating tumour cells (CTCs) or circulating cell-free DNA (cfDNA), constitutes a promising and less-invasive technique that can be a useful tool to overcome the limits of conventional diagnostic methods.[5] Most of cfDNA is double-stranded molecules that circulate as nucleoprotein complexes in blood.[6] DNA released from tumour cells by different molecular processes such as cell apoptosis, immune response-related necrosis, micrometastasis, and secretion has been considered to be an important cancer biomarker.[7]

Hauser *et al.* evaluated cfDNA using quantitative reverse-transcription polymerase chain reaction (RT-PCR). ACTB-106 detects fragmented cfDNA due to apoptosis and ACTB-384 detects long DNA fragments due to necrosis. In this analysis, both DNA fragments were increased in patients with RCC compared with healthy controls.[8]

Lu *et al.* evaluated cfDNA in healthy people and in M0 and M1 RCC patients. The 67 base pairs (bp) and 180 bp genomic cfDNA fragments did not differ between the three study groups while the 306 bp fragment was lower in RCC patients than in controls. As cfDNA fragment size is an indicator of the integrity of cfDNA molecules, the

authors showed that while in the control group there were no significantly different DNA concentrations for all genomic and mitochondrial fragments, the mitochondrial cfDNA was significantly higher in the metastatic group compared with the other groups. Furthermore, the cfDNA integrity index (ratio of longer to shorter fragments) was significantly better in the M0 than in the M1 subgroup.[9]

Some promising results have been published by Yamamoto *et al.* These investigators showed that median levels of cfDNA and median size of fragments from RCC patients were significantly higher than those from controls. In order to evaluate the diagnostic capacity of cfDNA, a receiver operating characteristic (ROC) curve analysis was performed and revealed a sensitivity and a specificity of 63.0% and 78.1%, respectively, with an optimal cutoff value of 2,876 copies/mL.[10]

The abundance and relative fragmentation of cfDNA in blood can be a universal marker for RCC,[8] yet the precise cfDNA metrics that are most clinically relevant remain controversial, possibly because of the heterogeneity of the available studies in terms of clinical stage, tumour pathology, blood sample management, and cfDNA measurements.

Circulating tumour cells

Circulating tumour cells are cells that have shed from a primary tumour into the vasculature or lymphatics. The detection and analysis of CTCs can assist with determining patient prognosis, personalized treatments, as well as initial diagnostic and monitoring procedures. Moreover, CTCs are particularly suited to interrogate functional heterogeneity by combining genetic and transcriptomic assessment of single CTCs[11] or by transcriptome and epigenome analysis.[12]

Diagnostic value of CTCs is often impaired by different methods of CTC collection and identification.[13] The different techniques include epithelial marker– or non-epithelial marker–dependent isolation, reverse transcription PCR–based methods, and morphological and cell size–based methods.[14] Even in the metastatic subgroup, the number of CTCs identified by an epithelial marker–dependent method is usually low. Moreover, RCC cells are inclined to lose their epithelial antigens through a process named "epithelial-to-mesenchymal transition" (EMT), in which morphological transformation leads to acquisition of mesenchymal features.[15]

Adding a new set of cell surface markers including carbonic anhydrase IX (CAIX) and CD147 to the conventional detection of CTCs through epithelial markers, such as the epithelial cell adhesion molecule (EpCAM), showed better results, but there was lack of specificity due to EpCAM expression in normal tissue, hypoxic or necrotic tissues, and in benign lesions.[16]

The RT-PCR approach has three main targets: CAIX, VHL, and Cadherin-6 (CDH-6). Considering the VHL mutations, Ashida *et al.* examined blood samples from 29 RCC patients with VHL gene mutations. Somatic mutations were detected in 69.0% of patients with sporadic ccRCC, while VHL gene mutations were detected in the CTCs of 75% of patients.[17] Otherwise, CDH-6 gene expression has been observed in only 45% of ccRCC patients and in none of the controls.[18]

Broncy *et al.* studied circulating rare cells (CRCs) in 30 RCC patients. These CRCs included CTCs and cells with uncertain malignant features (CRC-UMFs) according to cytomorphology. The authors performed a single-cell genetic analysis after isolation by size of tumour/trophoblastic cells and targeted VHL mutations in order to evaluate the specificity and sensitivity of cytopathology. A total of 29 of 30 patients harboured CRCs (20 had CTCs and 29 had CRC-UMFs) and 25 of 29 patients carried VHL somatic mutations in the tumour tissue. In total, 205 single CRCs consisting of 64 CTCs and 141 CRC-UMFs provided genetic data. Interestingly, 100% of CTCs and 83.2% of CRC-UMFs from the 25 patients with VHL-mutated tumour carried the same VHL mutation detected in the tumour. Thus, the specificity of the cytopathological approach was 100% while the sensitivity of cytopathology alone was low at 35%, compared with 72% achieved with genetically based identification alone.[19]

The role of microRNAs

MicroRNAs (miRs) are small 22-nucleotide noncoding RNA molecules that can modulate gene expression. They are implicated in the regulation of different process such as proliferation, migration, invasion, and apoptosis, and many of them can be easily found in tissues and bodily fluids.[20]

Wulfken *et al.* reported 109 miRs circulating at higher levels in the serum of RCC patients. Seven potential candidate miRs were selected and compared. The level of miR1233 was significantly increased in patients with RCC compared with healthy controls. ROC analysis determined a sensitivity of 90.9% and a specificity of 50% (area under the curve [AUC], 0.67). Thus, miR-1233 levels were validated in an independent cohort, confirming a higher mean value in RCC patients.[21]

After screening 30 miRs, Redova and colleagues further evaluated miR-378, miR-451, and miR-150, and successfully validated the first two molecules. Whereas miR-451 levels were decreased, levels of miR-378 were increased in the serum of RCC patients compared with controls. Combination of those miRs enables detection of RCC with a sensitivity of 81% and a specificity of 83% (AUC, 0.86).[22]

Zhao *et al.* found that miR-210 levels were higher in primary RCC tissues than in normal tissue. Furthermore, the serum level of miR-210 was significantly decreased in patients 7 days after nephrectomy and, consequently, a potential combined role in early detection and monitoring after radical treatment could be proposed.[23] Furthermore, Iwamoto *et al.* confirmed at the serum level that the expression of miR-210 was higher in RCC patients compared with healthy controls.[24] In addition, a meta-analysis conducted by Chen *et al.* including seven studies showed pooled sensitivity, specificity, diagnostic odds ratio, and AUC to predict RCC patients of 74%, 76%, 8.81, and 0.81, respectively.[25]

Chen *et al.* evaluated the expression levels of miR-129-3p and miR-129-5p at the tissue level. The investigators showed that miR-129-3p, but not miR-129-5p, was widely attenuated in human ccRCC and chromophobe renal cell carcinoma (chRCC), yielding a 73.5% accuracy in discriminating ccRCCs from normal tissues. The relative miR-129-3p expression levels significantly differed between malignant and benign kidney tumours.[26]

In a prospective cohort of 30 patients with proven ccRCC in comparison with healthy controls, Yadav *et al.* found that serum miR-34a, miR-141, and miR-1233 were able to diagnose ccRCC with a sensitivity of 80.76%, 75%, and

93.33%, and a specificity of 80%, 73.33%, and 100%, respectively, as compared to pathology specimens. Moreover, a combined approach using a panel of two serum miRNAs (miR-141 and miR-1233) allowed a diagnosis of ccRCC with a sensitivity of 100% and a specificity of 73.3%.[27]

Recently, Zhang and colleagues investigated whether miRNAs in serum exosomes could serve as biomarkers in ccRCC. Their findings showed that the expression levels of exosomal miR-210 and miR-1233 were significantly higher in RCC patients than in healthy individuals (both $p<0.01$). ROC analysis demonstrated that exosomal expression levels distinguished RCC patients from healthy individuals, with 70% sensitivity and 62.2% specificity for miR-210, and 81% sensitivity and 76% specificity for miR-1233.[28]

14.2.3 Metabolites as novel biomarkers of RCC

Metabolomic approaches have shown promising results in oncology, with the recognition of metabolic reprogramming as a central hallmark of cancer. Globally, RCC metabolic signature of the tumour microenvironment is characterized by alterations in metabolites associated with energy metabolism, especially those involved in glycolysis, amino acid metabolism, and fatty acid catabolism pathways, which are essential for cell growth and proliferation.[29]

Kim *et al.* first evaluated the utility of urine metabolomics analysis for metabolomic profiling. The authors identified a total of 212 molecules that are able to differentiate the presence of RCC. The rate of correct classification was 88.3%. Moreover, the authors found a small difference in the urinary metabolomic profile in a cohort of pre- and post-treatment RCC patients. Altogether, these results suggest a potential role for metabolic changes associated with disease monitoring after local treatment.[30]

Ganti *et al.* used a metabolomics approach to evaluate urine sample compounds appearing in RCC patients with chromatography coupled to mass spectrometry in two independent laboratories. They showed differential urinary concentrations of several acylcarnitines as a function of both RCC status and grade, with most acylcarnitines being increased in the urine of RCC patients and in those with high-grade disease. Many overlapping metabolites were detected by the two laboratories but two acylcarnitines (isobutyrylcarnitine and suberoylcarnitine) could significantly differentiate the presence of RCC from healthy subjects. Furthermore, urinary acylcarnitines were increased in a grade-dependent fashion. Acylcarnitines have both cytotoxicity and immunomodulatory properties, and thus they may play a role in decreasing the inflammatory response and providing a mechanism by which these cells are able to evade immune surveillance.[31]

In the same field, Niziol *et al.* showed that hydroxybutyrylcarnitine, decanoylcarnitine, propanoylcarnitine, carnitine, dodecanoylcarnitine, and norepinephrine sulfate were found in higher concentrations in both RCC tissues and the urine of patients with cancer.[32] The main differences between these studies are related to the analysis methods, which require some validation steps, management, and collection of urine samples. By combining nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), Smolinska *et al.* showed that the ideal manner forward is to employ the strengths of both modalities.[33]

14.2.4 Biomarkers from proteomics analysis

Proteomics offers a great platform to study the complex molecular events of tumourigenesis. Tissue proteomics has the advantage of large availability of relevant proteins.[34] On the other hand, less-invasive methods are required to obtain samples, thus serum and urine proteomes constitute another important source of research.[35] In RCC, upregulation in the glycolytic flux manifests through the increase of some glycolysis-relevant metabolites, including glucose, pyruvate, and lactate.[36]

Using isobaric tags for relative and absolute quantitation (iTRAQ) labelling and liquid chromatography–mass spectrometry (LC-MS), White *et al.* identified 55 proteins that are significantly dysregulated in RCC specimens. Dysregulation of alpha-enolase (ENO1), L-lactate dehydrogenase A chain (LDHA), heat shock protein beta-1 known as (heat shock protein 27 [Hsp27]), and 10 kDa heat shock protein, mitochondrial (known as heat shock protein family E (Hsp10) member 1 [HSPE1]) was confirmed in two independent sets of patients by western blot and immunohistochemistry (IHC). The expressions of AHNAK, ENO1, and Hsp27 were found to be significantly elevated in ccRCC compared with matched normal tissues, whereas HSPE1 was significantly downregulated in RCC patients.[34]

Kim *et al.* evaluated frozen specimens of RCC and adjacent normal tissue. The proteins upregulated in RCC were nicotinamide N-methyltransferase (NNMT), secretagogin (SCGN), L-plastin, human neuron-specific enolase (hNSE), nonmetastatic cell 1 protein (NM23A), ferritin light chain (FTL), and thioredoxin peroxidase (TPx). NNMT was the most commonly upregulated protein in all types of RCC compared with normal tissues. SCGN was elevated in ccRCC samples but not in papillary, chromophobe, or normal tissue while NM23A showed the same behaviour although the magnitude of changes was smaller than for the first two molecules. Compared to the diagnostic capabilities of individual markers, the triple combination of NNMT, FTL, and hNSE constitutes a potential diagnostic panel for RCC.[37]

Zhang *et al.* reported a quantitative proteomic analysis applying iTRAQ labelling and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis on serum samples from ccRCC patients and a control group. The investigators found that 16 proteins were significantly upregulated and 14 significantly downregulated in early-stage RCC. Serum heat shock cognate 71 (HSC71) was highly elevated compared with the control group.[38] Using a similar approach, another work group identified 27 differentially expressed proteins in early-stage RCC by iTRAQ, with the expression of C1QC, C1QB, S100A8, S100A9, ceruplasmin, and lumican found to be associated with tumour stage and/or grade.[39] Studying the expression of small Ca^{2+}-binding proteins belonging to the S100 family, Zhang *et al.* found that S100A8 and S100A9 serum expression levels were significantly upregulated in RCC patients.[40]

Yokomizo *et al.* studied plasma proteins digested by trypsin with resulting peptides analyzed by 2-dimensional image converted analysis of liquid chromatography mass spectrometry (2DICAL), and observed an increased expression of fibronectin-1 (FINC) in the plasma of ccRCC patients, especially in the early stage.[41]

Gbormittah *et al.* compared the proteome, glycoproteome, and N-glycome. They found a panel of 13 candidate glycoproteins and considering the removal of renal tumour, they reported a significant increase of two specific clusterin glycoforms (FA2G2S2 and A2G2S2) as a potential reliable biomarker for diagnosis and disease monitoring after curative treatment.[42] (See **Table 14–1** for list of studies of novel potential candidate biomarkers in diagnosis and early detection of RCC.)

14.3 Prognostic Biomarkers

14.3.1 Introduction

While most biomarkers for early detection and diagnosis remain at an early stage of the development process, more advances have been made for prognostic biomarkers for RCC. Many molecules have been proposed as potential prognostic markers for RCC. To date, few biomarkers have been taken beyond single studies, thus none are yet ready for routine clinical practice. Furthermore, emerging and promising approaches can serve as new platform in which novel potential biomarkers can be found. For any type of surgical treatment for renal tumours, there is a need for risk stratification aimed at personalized outcome prediction. The major endpoints evaluated and predicted using prognostic biomarkers across the following studies are disease/progression/recurrence-free survival (D/P/RFS), overall and cancer-specific survival (OS, CSS), and correlations with clinicopathologic features that might influence the prognosis among these patients.[43]

14.3.2 Liquid biopsy

CTC identification

Liquid biopsy is a potential tool to assist clinicians on risk stratification after treatment for curative intent.[13] Bluemke *et al.* collected data at different time points to evaluate CTC expression. A significant correlation between the detection of CTCs and positive lymph node status and the presence of synchronous metastases at the time of primary surgery was found. With such prerogatives, the presence of positive cytokeratin expression (CK+) CTCs with epithelial signature was independently correlated with worse OS at multivariate analysis.[44] Liu *et al.* showed a significant association of CTC numbers as well as the CTC expression status of Vimentin with disease progression.[16] Recently, Wang *et al.* investigated the relationship of dynamic changes of CTCs and Beclin-1 expression of CTCs and RCC prognosis in a cohort of patients receiving radical treatment. All patients received multiple CTC tests at three different time points. For the metastatic group, the number of mixed CTCs at 12 months was significantly higher than the number of mixed CTCs preoperatively and at 6 months. Of note, in this group, the number of preoperative Beclin-1–positive CTCs was significantly higher than the number of preoperative Beclin-1–negative CTCs. Thus, a variation trend of CTCs and Beclin-1–expressing CTCs was significantly associated with the onset of metastatic disease.[45]

cfDNA

One of the most promising uses of liquid biopsy is to determine the risk for recurrence after nephrectomy and to serve as a surveillance biomarker during the follow-up after primary treatment. In a study including 30 pre-nephrectomy patients, Al-Qassab *et al.* sought to develop whole exome sequencing of cfDNA with next-generation sequencing (NGS) to interrogate 14 commonly mutated genes: VHL, PBRM1, SETD2, BAP1, KDM5C, KIT, NFE2L2, MET, TP53, CDKN2A, FGFR3, PIK3CA, BRAF, MUC4. Of the preoperative RCC patients, 67.0% had detectable somatic mutations, resulting in nonsynonymous, frameshift, stop-gain, or splice site mutations, compared with 3.1% of controls. By demonstrating the feasibility of gene-specific whole exome sequencing of cfDNA, the investigators suggest that even the low tumour burden sheds detectable quantities of cfDNA, thus the role of cfDNA as a monitoring biomarker was proposed.[46] Wan *et al.* measured plasma levels of cfDNA before and after surgery for localized disease. Mean preoperative level of plasma cfDNA in patients who developed recurrence disease was significantly higher than in those with localized disease or controls.[47] Analyzing cfDNA extracted from healthy people and in M0 and M1 RCC patients, Lu *et al.* developed two different models that incorporated clinicopathologic features with specific expression pattern among cfDNA fragments. Particularly, APP genes (APP-1, APP-2, APP-3), Alu sequences (SINE-1 and SINE-2), and mitochondrial DNA fragments (Mito-1 and Mito-2) showed significant correlation in terms of OS and RFS.[9] As a quantitative experience, Yamamoto *et al.* divided their cohort into two subgroups based on the length of cfDNA fragments (cutoff value of 166 bp). Whereas cfDNA fragmentation correlated with poorer PFS, cfDNA plasma levels were not associated with any survival outcomes.[10]

Lastly, an initial analysis by de Martino *et al.* evaluated the putative significance of total cfDNA and CpG island methylation among samples collected from 200 consecutive patients with localized disease and benign lesions. Total cfDNA levels were higher in patients with mRCC and increased necrotic component. Those cfDNA levels were associated with worse DSS. Of note, in multivariate analysis adjusted for tumour stage, size, grade, and necrosis, categorized total cfDNA levels (cutoff value, 2,400 GE/mL) were independent prognostic factors.[48]

miRNAs

In a comprehensive meta-analysis of 29 published studies, Tang *et al.* identified a robust panel of miRNA signature expression profiles to determine candidate miRNAs as prognostic biomarkers. Using a vote-counting strategy, the investigators reported that high expression of miR-21 and miR-210 and low expression of miR-141, miR-200c, and miR-429 were associated with worse CSS following RCC resection.[49] In a systematic review and meta-analysis across 27 different studies and including a total of 2,578 subjects, Gu *et al.* found that elevated expression of miR-21, miR-1260b, miR-210, miR-100, miR-125b, miR-221, miR-630, and miR-497 was associated with a poor prognosis in RCC patients. Conversely, decreased expression of miR-106b, miR-99a, miR-1826, miR-215, miR-217, miR-187, miR-129-3p, miR-23b, miR-27b, and miR-126 was associated with worse prognosis. Importantly, results from meta-analysis revealed that elevated miR-21 expression was associated with shorter OS, CSS, and DFS, whereas decreased expression of miR-126 was associated with shorter CSS, OS, and DFS.[50]

Also regarding specific circulating long noncoding RNAs (lncRNAs), defined as RNA transcripts longer than 200 nucleotides that are not transcribed into a protein, results were promisingly. The expression profile of six lncRNA transcripts with potential prognostic interest were next validated. A significant increase of lnc-ZNF180-2

expression in advanced RCC tissue compared with localized RCC was observed. Furthermore, lnc-ZNF180-2 expression levels were an independent predictor of PFS, CSS, and OS.[51]

In the same attempt as above, Qu *et al.* sought to establish an lncRNA signature to improve postoperative risk stratification after radical treatment. The investigators built a classifier named RCClnc4 based on four lncRNAs. RCClnc4 significantly stratified patients into high-risk versus low-risk groups in terms of clinical outcomes and remained an independent prognostic factor in multivariate analyses after adjusting for clinical and pathologic factors. More importantly, RCClnc4 signature achieved a higher accuracy than clinical staging systems such as TNM and tumor Stage, Size, Grade, and Necrosis (SSIGN) score.[52]

14.3.3 Genetic and DNA methylation biomarkers

It is becoming increasingly clear that epigenetic variations, such as promoter methylation, play an important role in renal carcinogenesis, biological aggressiveness, and progression. DNA methylation is defined as a covalent addition of a methyl group to cytosines that precede a guanosine (the CpG dinucleotide), which are clustered mainly as CpG islands in the promoter region of genes bringing functional silencing.[53] Furthermore, DNA methylation alterations are often shown to be associated with clinicopathologic features and RCC patient survival or both.[54] CpG island methylation markers reflect tumour biology, allowing the identification of patients with "high epigenetic risk" who can benefit from tailored management in order to improve survival outcomes.

In a recent systematic review across 49 studies, for nine genes (SFRP1, BNC1, GREM1, RASSF1A, PCDH8, SCUBE3, GATA5, LAD1, and NEFH), promoter methylation was found to be associated with patient survival, and the prognostic value of these genes was independently validated in other studies.

If the analysis of the single methylations had sometimes led to conflicting results, the validity of these biomarkers was then also evaluated for biomarker combinations. To develop a 5-CpG–based assay for ccRCC prognosis using formalin-fixed paraffin-embedded specimens, a panel composed by methylation of PITX1, FOXE3, TWF2, RIN1, and EHBP1L was validated in three independent sets from China, United States, and The Cancer Genome Atlas (TCGA) data set. Stratifying patients into two groups from this 5-CpG panel, the investigators defined low- and high-risk categories. Preliminary results showed an important correlation between the high-risk group and poorer OS.[55] Recently, with the same endpoint, Chen *et al.* identified seven specific prognosis subgroups based on the DNA methylation spectrum of RCC from the TCGA database. The specific DNA methylation patterns reflected differentially in the clinical index, including TNM classification, pathological grade, clinical stage, and age. In addition, 437 CpGs corresponding to 477 genes from 151 samples were identified as specific hyper/hypomethylation sites for each specific subgroup. Next, the investigators constructed a Bayesian classifier to determine the function of the prognosis prediction model, with 437 specific CpG sites as characters (AUC, 0.95).[56]

14.3.4 Prognostic value of metabolomic approaches

Analyzing tumours and their matched tissue, Gao *et al.* studied the metabolomic RCC profile. Creatine, glutamate, and glutamine were found at higher concentrations in tissues of tumours at the most advanced

stages.[57] The glycolysis-relevant metabolites (glucose, glucose-6-phosphate, and fructose-6-phosphate) were found to be significantly increased in high-grade disease, suggesting that glucose metabolism is more prominent with increasing tumour grade. Consequently, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), enolase 2, and pyruvate kinase-muscle-2 (PKM2) were found to be increased in tumour compared with normal tissues. L-lactate follows the same tendency in a grade-dependent manner. Also levels of carnitine, acylcarnitines, and acetylcarnitines were associated with grade hinting at how the combination of these metabolites can predict the biological aggressiveness of RCC and thus influence its prognosis.[58] Furthermore, increased levels of glutathione were also grade and stage dependent.[59] Thus, the upregulation of antioxidant capacity in adaptation to intrinsic oxidative stress is indeed a common event in RCC, especially in the advanced stages.[60]

14.3.5 Other biomarkers

Serum concentrations of tumour type M2 pyruvate kinase (Tu M2-PK) and thymidine kinase 1 (TK1) have been reported as predictors of RFS at multivariate analysis adjusted for stage, grade, and tumour necrosis.[61] Among a prospective series of patients who underwent surgical/ablative therapy for RCC, elevated preoperative levels of Tu M2-PK were significantly correlated with adverse pathological features such as tumour size and advanced grade, which might influence the prognosis of these patients.[62] High urinary levels of cathepsin D were significantly associated with poorer OS on univariate analysis and approached significance at the multivariate level.[63]

A retrospective series of tissue microarrays showed that hepatitis A virus cellular receptor (HAVCR)/kidney injury molecule-1 (KIM-1) expression promoted invasive phenotype *in vitro* and more aggressive tumours *in vivo*, which might influence the prognosis of these patients.[64]

An *et al.* showed that the overexpression of S100A8 was significantly increased in cores of patients with higher T stage and higher Fuhrman grade. Multivariate analysis confirmed that high expression of S100A8 was significantly correlated with poor DFS.[65] (See **Table 14–2** for list of studies of novel potential candidate biomarkers in prognosis of RCC.)

14.4 Conclusions

Cancer biomarkers have shifted treatment and management of patients in many cancer types. Although, "personalized" medicine is becoming more common in our daily practice, none of the mentioned RCC biomarkers are in routine clinical use. Metabolomics and proteomics studies have shown excellent potential in terms of diagnostic accuracy; however, research in these areas still appears to be hypothesis generating. Most of publications mentioned in this chapter are aimed at understanding tumour biology due to the high heterogenicity of RCC.

Circulating biomarkers have attracted a lot of interest; however, the high diversity of techniques precludes any further conclusions. Surely due to the accessibility of tumour tissue, the growing use of liquid biopsy, accompanied by the increasing standardization methods of analysis and quantification of CTCs, cfDNAs, and miRNAs, is

providing promising results. Particularly, NGS of cfDNA is a novel technology that can complement tumour tissue. It has demonstrated its potential role across the diagnostic and prognostic fields of both localized and mRCC. Single molecule validations are being replaced by multipanel biomarkers to provide improved validation results. The use of multipanel biomarkers also reflects the role of molecular biology in current clinical nomograms as a transition tool from bench to bedside.

TABLE 14–1 Novel Potential Candidate Biomarkers in Diagnosis and Early Detection of Renal Cell Carcinoma

Biomarker	Source	Trend	Correlation/use	Reference
cfDNA	P S	↑	RCC and mRCC detection, association with histotype, monitoring after curative surgery	Hauser *et al.* (2010)[8] de Martino *et al.* (2012)[48] Lu *et al.* (2016)[9] Yamamoto *et al.* (2018)[10]
CTCs	B	↑	RCC detection and monitoring	McKiernan *et al.* (1999)[66] Ashida *et al.* (2000)[17] Allard *et al.* (2004)[67] Li *et al.* (2005)[18] Liu *et al.* (2016)[16] Broncy *et al.* (2018)[19]
miRNA				
miR-1233	S	↑	RCC detection	Wulfken *et al.* (2011)[21] Zhang *et al.* (2018)[28] Yadav *et al.* (2017)[27]
miR-451	S	↓	RCC detection	Redova *et al.* (2012)[22]
miR-378	S	↑	RCC detection	Redova *et al.* (2012)[22]
miR-21	T	↑	RCC detection, differential diagnosis between ccRCC, pRCC, and chRCC and oncocytoma	Faragalla *et al.* (2012)[68]
miR-15a	T U	↑	RCC detection, differential diagnosis between malignant and benign renal tumours	von Brandestain *et al.* (2012)[69]
miR-210	T U S	↑ ↑ ↑	RCC detection and disease monitoring after local treatment	Zhao *et al.* (2013)[23] Iwamoto *et al.* (2014)[24] Zhang *et al.* (2018)[28] Chen *et al.* (2018)[25]
miR-129-3p	T	↓	RCC detection, differential diagnosis between malignant and benign renal tumours	Chen *et al.* (2014)[26]
miR-34a	S	↓	RCC detection	Yadav *et al.* (2017)[27]
miR-141	S	↓	RCC detection	Yadav *et al.* (2017)[27]

TABLE 14–1 Novel Potential Candidate Biomarkers in Diagnosis and Early Detection of Renal Cell Carcinoma *(Cont'd)*

Biomarker	Source	Trend	Correlation/use	Reference
Metabolomics and Proteomics				
Acetylcarnitines	U T	↑	RCC detection, grade-dependent behaviour	Ganti *et al.* (2012)[31] Niziol *et al.* (2018)[32]
Tu M2-PK	P	↑	RCC and mRCC detection	Roigas *et al.* (2001)[70] Weinberger *et al.* (2007)[71]
AHNAK	T	↑	RCC detection	White *et al.* (2014)[34]
ENO1	T	↑	RCC detection	White *et al.* (2014)[34]
HSPE1	T	↓	RCC detection	White *et al.* (2014)[34]
NNMT	T	↑	RCC detection	Kim *et al.* (2010)[37]
HSC71	S	↑	RCC detection	Zhang Y *et al.* (2015)[38]
S100A8	S	↑	RCC detection	Zhang L *et al.* (2015)[40] Zhang L *et al.* (2016)[39]
S100A9	S	↑	RCC detection	Zhang L *et al.* (2015)[40] Zhang L *et al.* (2016)[39]
TATI	S	↑	RCC detection, clinical stage and grade	Lukkonen *et al.* (1999)[72] Paju *et al.* (2001)[73]
FINC	P	↑	RCC detection	Yokomizo *et al.* (2011)[41]

Abbreviations: cfDNA, cell-free DNA; ccRCC, clear cell renal cell carcinoma; chRCC, chromophobe renal cell carcinoma; CTCs, circulating tumour cells; ENO1, alpha-enolase; FINC, fibronectin-1; HSC71, heat shock cognate 71; HSPE1, heat shock protein family E (Hsp10) member 1; miRNA, microRNA; mRCC, mestastatic renal cell carcinoma; NNMT, nicotinamide N-methyltransferase; pRCC, papillary renal cell carcinoma; RCC, renal cell carcinoma; S100A8, S100 calcium-binding protein A8; S100A9, S100 calcium-binding protein A9; TATI, tumour-associated trypsin inhibitor; Tu M2-PK, tumour M2-PK; P, plasma; S, serum; B, blood; T, tissue; U, urine.

TABLE 14–2 Novel Potential Candidate Biomarkers in Prognosis of Renal Cell Carcinoma

Biomarker	Source	Outcomes Correlated	Reference
CTCs	B	RFS, OS	Bluemke *et al.* (2009)[44] Liu *et al.* (2016)[16] Wang *et al.* (2019)[45]
Beclin-1–positive CTCs	B	RFS	Wang *et al.* (2019)[45]
cfDNA	P	RFS, OS	de Martino *et al.* (2011)[48] Wan *et al.* (2013)[47] Lu *et al.* (2016)[9] Yamamoto *et al.* (2018)[10]
miR-378	S	DFS, clinical stage	Fedorko *et al.* (2015)[74]
miR-221	S	OS, CSM, lymphovascular invasion	Teixeira *et al.* (2014)[75] Vergo *et al.* (2014)[76]
miR-150	S	DSS, clinical stage	Chanudet *et al.* (2017)[77]
miR-451	S	Clinical stage	Redova *et al.* (2012)[78]
miR-21	T	CSS, OS, DFS, clinical stage, tumour grade, tumour size	Faragalla *et al.* (2012)[68] Tang *et al.* (2015)[49] Vergho *et al.* (2014)[79] Vergho *et al.* (2014)[76]
miR-126	T	DFS, CSS, OS	Vergho *et al.* (2014)[79] Vergho *et al.* (2014)[76] Khella *et al.* (2015)[80]
miR-106b	T	PFS	Slaby *et al.* (2010)[81]
miR-27a-3p	T	PFS	Nakata *et al.* (2015)[82]
miR-210	T	CSS	Tang *et al.* (2015)[49]
miR-141	T	CSS	Tang *et al.* (2015)[49]
miR-200c	T	CSS	Tang *et al.* (2015)[49]
miR-429	T	CSS	Tang *et al.* (2015)[49]
miR-486	T	CSM, clinical stage	Goto *et al.* (2013)[83]
miR-23b	T	OS	Ishihara *et al.* (2014)[84]
miR-27b	T	OS	Ishihara *et al.* (2014)[84]
lnc-ZNF180-2	T	PFS, CSS, OS, clinical stage	Ellinger *et al.* (2015)[51]
lnc-NBAT-1	T	OS	Xue *et al.* (2015)[85]
Metabolomics			
Creatine	T	Advanced tumour stages (T3-4)	Gato *et al.* (2012)[57]
Glutamate	T	Advanced tumour stages (T3-4)	Gato *et al.* (2012)[57]
Glutamine	T	Advanced tumour stages (T3-4)	Gato *et al.* (2012)[57]

TABLE 14–2 Novel Potential Candidate Biomarkers in Prognosis of Renal Cell Carcinoma *(Cont'd)*

Biomarker	Source	Outcomes Correlated	Reference
GAPDH	T	High grade	Wettersten *et al.* (2015)[58]
Enolase-2	T	High grade	Wettersten *et al.* (2015)[58]
PKM2	T	High grade	Wettersten *et al.* (2015)[58]
L-lactate	T	High grade	Wettersten *et al.* (2015)[58]
Glutathione	T	Advanced stages	Hakimi *et al.* (2016)[59]
Tu M2-PK	S	RFS	Nisman *et al.* (2010)[61] Gayed *et al.* (2015)[62]
TK1	S	RFS	Nisman *et al.* (2010)[61]
cathepsin D	U	OS	Vasudev *et al.* (2009)[63]
S100A8	T	DFS, tumour grade, stage	An *et al.* (2019)[65]

Abbreviations: cfDNA, cell-free DNA; CSM, cancer-specific mortality; CSS, cancer-specific survival; CTCs, circulating tumour cells; DFS, disease-free survival; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OS, overall survival; PFS, progression-free survival; PKM2, pyruvate kinase-muscle-2; RCC, renal cell carcinoma; RFS, recurrence-free survival; S100A8, S100 calcium-binding protein A8; TK1, thymidine kinase 1; Tu M2-PK, tumour M2-PK; P, plasma; S, serum; B, blood; T, tissue; U, urine.

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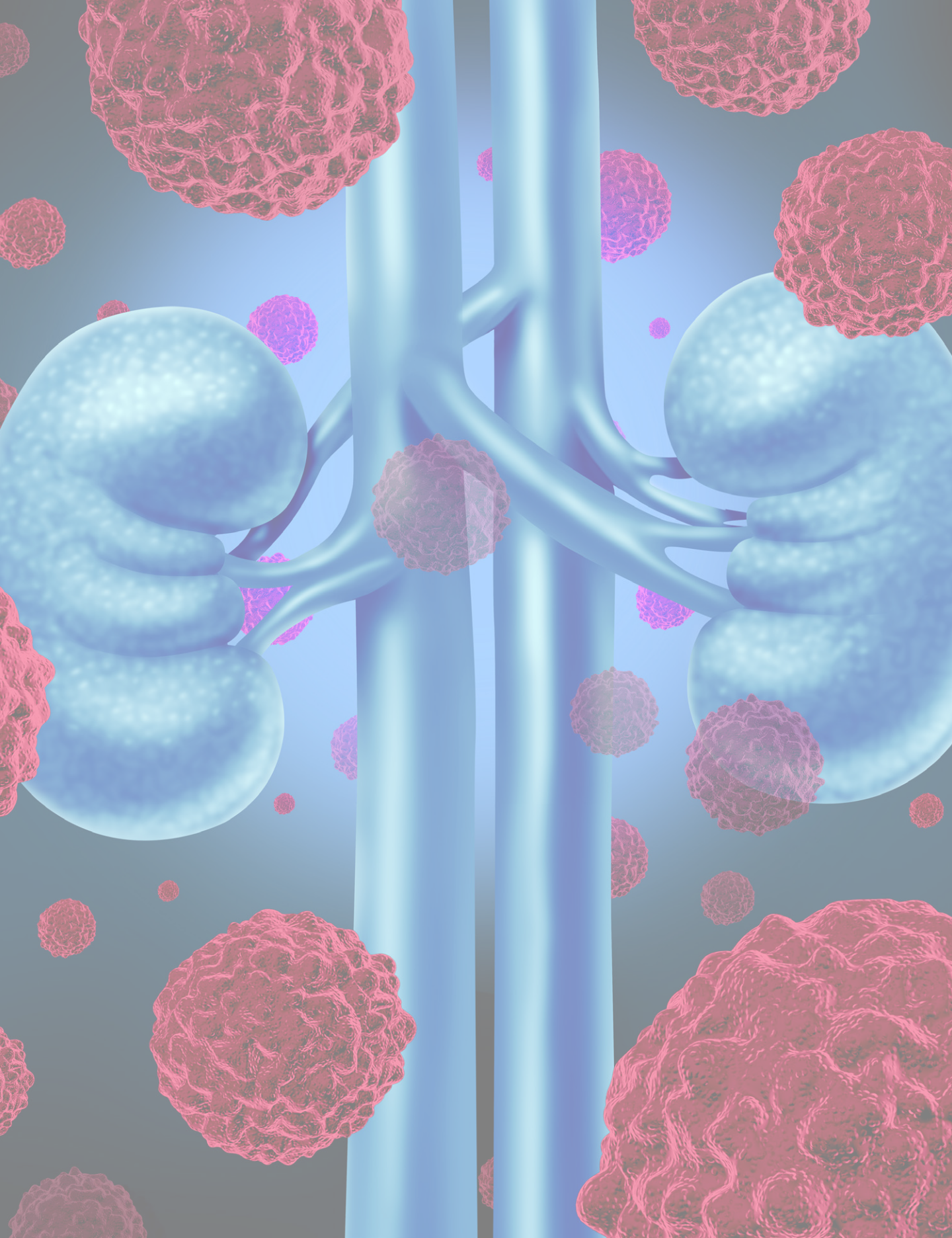

CHAPTER 15

Clinical Utility of Biomarkers in Renal Cell Carcinoma

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Table of Contents

15.1 Introduction

Renal cell carcinoma (RCC) is the sixth most common cancer in men and eighth most common cancer in women, accounting for 5% and 3% of all new cancer diagnoses, respectively.[1] Widespread use of cross-sectional imaging has resulted in increased detection of incidental small renal masses over the past three decades. Metastatic disease continues to comprise 17% of the diagnoses.[2]

While surgery remains the mainstay of treatment for localized RCC, a challenge remains in identifying the subset of patients with indolent or benign lesions who may be able to forego invasive intervention. This is of particular importance as we continue to treat an older and more complex population. At the other end of the spectrum, identifying aggressive tumours to institute curative treatment in a timely manner, as well as appropriate surveillance strategies, is also paramount. Additionally, the changing landscape of systemic therapies for metastatic renal cell carcinoma (mRCC) has unveiled a void in clinical tools to guide treatment decisions regarding this heterogeneous disease.

There is an imperative need to identify and validate molecular and genomic biomarkers to assist with the diagnosis and prognostication of RCC, as well as deliver on the promise of personalized medicine. An increasing understanding of the pathophysiology of RCC has allowed significant progress to be made in this field.

15.2 Renal Cancer Biology

Renal tumours demonstrate significant heterogeneity, with at least 16 known subtypes.[3] Understanding the biological pathways that lead to the development of these tumours is pertinent in identifying clinically useful biomarkers. Most of our current understanding of these processes comes from analyzing clear cell renal cell carcinoma (ccRCC), which accounts for 70% to 80% of all cases.

15.2.1 Molecular genetics

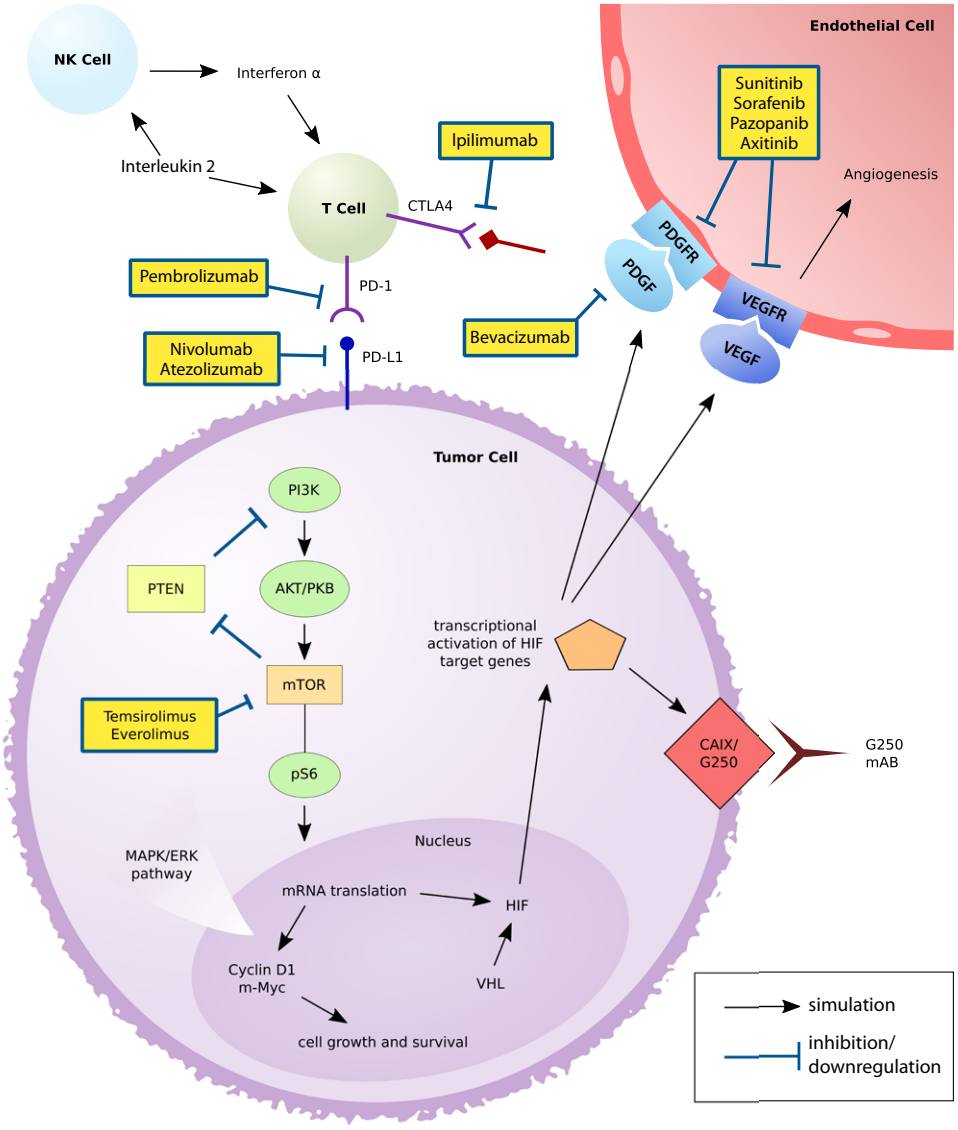
The study of hereditary RCC syndromes has led to a greater understanding of the underlying genetic and molecular basis of RCC. The von Hippel-Lindau (VHL) tumour suppressor gene is important in this regard and has been implicated in >80% of sporadic ccRCC.[4] Its best understood function relates to its role in proteosomal degradation of the hypoxia-inducible factor (HIF) family, particularly HIF-1α.[5,6] Inactivation of the VHL gene, due to genetic mutations or epigenetic alterations such as methylation, results in constitutive activation of HIF-1α. This leads to downstream overexpression of the hypoxia pathway in non-hypoxic conditions, such as modulation of angiogenesis via vascular endothelial growth factor (VEGF) (**Figure 15–1**).

Knowledge of this pathway led to the advent of VEGF-targeted tyrosine kinase inhibitors (TKIs) and monoclonal antibodies in the early 2000s. Further understanding of other intracellular signalling pathways, such as the oncogenic PI3K/Akt pathway and tumour suppressor gene phosphatase and tensin homolog (PTEN), has been extrapolated from other tumour streams. This also led to a paradigm shift in the treatment of mRCC with the introduction of mammalian target of rapamycin (mTOR) inhibitors in 2007.

Large-scale genomic sequencing studies have identified other genes involved in the pathogenesis of RCC, and may have prognostic utility. Polybromo 1 (PBRM1) is the second most commonly mutated gene in ccRCC, affecting approximately 45%,[7] followed by BRCA1-associated protein 1 (BAP1) in 15%.[8] Both of these genes have also been localized to chromosome 3p, like the VHL gene, and are associated with different pathologic features and outcomes. Several studies have now demonstrated that BAP1-mutant tumours tend to be of higher grade and stage, and have a significantly worse overall survival (hazard ratio [HR], 2.5–3).[9–11] Other genetic alterations implicated in the pathogenesis of ccRCC involve the SET domain containing 2 (SETD2) and lysine-specific demethylase 5C (KDM5C) genes.[7,12–14]

Though the exact pathways of carcinogenesis due to these mutations are yet to be established, the noted differences in tumour biology and outcomes certainly warrant a consideration for molecular classification of RCC, as opposed to the current morphological classification. Genetic arrays of RCC that correlate with varied biology and clinical outcomes are being developed and are likely to have a greater role in the future.[15]

FIGURE 15–1 Biologic Pathways and Markers in Renal Cell Carcinoma



Abbreviations: AKT/PKB, Akt/protein kinase B (gene); CAIX, carbonic anhydrase IX; CTLA-4, cytotoxic T lymphocyte–associated protein 4; G250 mAb, monoclonal antibody G250; HIF, hypoxia-inducible factor; MAPK/ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin; PDGF, platelet-derived growth factor; PD-1, programmed cell death 1; PD-L1, programmed cell death 1 ligand 1; PDGFR, platelet-derived growth factor receptor; PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homolog; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VHL, von Hippel-Lindau.

15.2.2 Immunology

The interplay between the immune system and cancer has been widely postulated for more than a century. Both innate and adaptive immunity play critical roles in recognizing and destroying malignant cells. Passive immunotherapy, in the form of interleukin 2 (IL2) or interferon, was used on this basis, though with modest benefits. Since then, considerable advances have been made in deciphering one of the hallmarks of cancer: evasion of immune recognition. Modulating immune checkpoint receptors and ligands that underpin this immunological escape is the basis of modern immuno-oncology (IO).[16]

Such immune checkpoint pathways include programmed cell death protein 1 (PD-1), programmed cell death 1 ligand 1 (PD-L1), and cytotoxic T lymphocyte–associated protein 4 (CTLA-4). Ordinarily, these proteins downregulate cytotoxic T-cell activation to maintain peripheral tolerance and prevent autoimmunity; however, cancer cells also elude the immune system by stimulating this pathway.[17] The idea of suppressing the natural brakes of the immune system to recognize and destroy cancerous cells led to the discovery of monoclonal antibodies directed against these immune checkpoint targets. In mRCC, checkpoint inhibitors are now considered "the new backbone in first-line treatment", and are recommended as a standard of care in treatment-naïve patients.[18]

15.3 Utility of Biomarkers in Renal Cell Carcinoma

Biological markers (or biomarkers) are defined as objectively measurable indicators of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic intervention.[19] They have long been anticipated to deliver on the promise of personalized or precision medicine, and thus lead to better patient care and lower healthcare costs.[20]

Diagnostic biomarkers are relevant throughout the course of a disease, such as in early diagnosis and surveillance after treatment. In urologic oncology, prostate-specific antigen has fulfilled these roles, and established itself as one of the most commonly used and sensitive biomarkers since its introduction more than 40 years ago.[21] There are several clinical challenges in early diagnosis of RCC that could be addressed with appropriate diagnostic markers. The management of small renal masses (<4 cm) is often a point of contention, as 20% of these lesions are benign. A diagnostic biomarker would reduce the number of invasive interventions performed for benign lesions. Another use for such a marker is for surveillance following resection, as about one-third of patients develop recurrent disease, and there is no consensus on a standardized follow-up strategy.[22]

Prognostic biomarkers can inform clinicians about the natural course of an individual cancer and guide their decision of how aggressively to treat. This is particularly useful in selecting adjuvant treatment in intermediate- and high-risk patients. A notable example is breast cancer multigene-expression assay Oncotype Dx, which has been clinically validated and shown to significantly change decisions regarding adjuvant chemotherapy.[23] While at this time, four of the five randomized controlled trials on adjuvant TKIs in RCC did not demonstrate a benefit in either progression-free survival (PFS) or overall survival (OS), there are ongoing trials for checkpoint inhibitors in this setting.[24–27] The Sunitinib as Adjuvant Treatment for Patients at High Risk of Recurrence of Renal Cell

Carcinoma Following Nephrectomy (S-TRAC) study was the only adjuvant trial that did demonstrate a PFS advantage, and the discrepancy could partly be explained by how patients were risk-stratified, demonstrating the need for identifying more precise prognostic biomarkers.[28]

Predictive biomarkers assess the probability of a patient benefiting from a particular treatment. This is relevant for the increasing repertoire of IO drugs in RCC, given the variability in response, high cost, and potentially devastating toxicities. The predictive value of KRAS oncogene mutational status in colorectal cancer is highly informative in understanding the immense utility of such markers. Some 30% to 50% of patients with metastatic colorectal cancer are known to have this mutation and it is now established that these patients do not respond to the commonly used anti-epidermal growth factor monoclonal antibodies. Routine testing for this gene spares these patients from toxicities of futile treatment, in addition to the estimated cost-saving of USD$600 million per year for unnecessary treatment.[29]

Despite significant advances in our knowledge of RCC at the molecular level, there are no validated biomarkers that can guide our management of this disease. High-throughput technologies in genomics, proteomics, and metabolomics have contributed to more than 150,000 papers on biomarkers in the literature. However, fewer than 100 biomarkers are validated for clinical practice.[29] In 2007, the American Academy of Cancer Research (AACR), in partnership with the US Food and Drug Administration (FDA) and the National Cancer Institute (NCI), formed the AACR-FDA-NCI Cancer Biomarkers Collaborative to accelerate the translation of cancer therapeutics by streamlining effective development of validated biomarkers and their use in clinical trials.[30]

This has been followed by a number of large-scale biomarker initiatives in RCC,[31] including:

- Cancer Genomics of the Kidney (CAGEKID) consortium, funded by the European Union;
- Biomarker pipeline and Tumour Cancer Genome Atlas (TCGA), funded by the National Institutes of Health;
- EuroTARGET (TArgeted therapy in Renal cell cancer: GEnetic and Tumour related biomarkers for response and toxicity); and
- Scottish Collaboration on Translational Research into Renal Cell Carcinoma (SCOTRRCC).

In 2017, the Kidney Cancer Research Network of Canada sponsored a consensus-based, priority-setting partnership to identify the top 10 research priorities in the management of kidney cancer. Identification and validation of biomarkers was still unanimously considered a top priority as the search for reliable markers continues.[32]

15.4 Renal Cancer Biomarkers

An array of serum-, urine-, and tissue-based biomarkers have been described, but each has its own practical limitations. Profiling complex fluids, such as serum and urine, requires awareness of the effect of other circulating proteases and nucleases on marker signals, as well as the need for pre-fractionation strategies, given the vast difference in orders of magnitude in protein concentrations (eg, albumin 40 g/L, compared to cytokines

1 pg/mL–10 pg/mL).[33] On the other hand, tumour heterogeneity may limit the utility of tissue-based markers.[34] The following section summarizes the current status of the most widely studied biomarkers in RCC.

15.4.1 Diagnostic biomarkers

Improved characterization of small renal masses is required to avoid surgical intervention in patients with benign or indolent lesions, and treat those with high metastatic potential in a timely manner. Given the high level of discordance in pathological subtyping as seen in non-ccRCC cases[35,36] or biopsy specimens,[37] a diagnostic biomarker would be particularly useful. Additionally, there is a role for such markers in ongoing surveillance, given the paucity of data to support the current follow-up strategies.

Carbonic anhydrase IX

Carbonic anhydrase IX (CAIX) is a downstream effector of HIF-1α and is thought to play a role in regulating intracellular and extracellular pH in tumour cells. It is highly expressed in 95% of ccRCC, compared to minimal expression in oncocytomas, or chromophobe and papillary RCC.[38–40] CAIX is also expressed in other tumours, including carcinomas of lung, breast, uterus, esophagus, and brain, as well as in normal gastric mucosa.[41] While this somewhat limits the use of CAIX as a diagnostic marker for metastatic disease, there is ongoing enthusiasm for its utility in characterization of the small renal mass. Several clinical trials have demonstrated the possibility of improving the performance of positron emission tomography/computed tomography (PET/CT) by using radiolabelled girentuximab, a chimeric monoclonal antibody against CAIX. REnal Masses: Pivotal Study to DETECT Clear Cell Renal Cell Carcinoma With Pre-Surgical PET/CT (REDECT), a phase 3, open-label, multicentre trial, assessed the diagnostic accuracy of ^{124}I-girentuximab PET/CT in 195 patients undergoing nephrectomy; it reported a sensitivity of 86.2% and specificity of 85.9%.[42] It would be interesting to see whether the results improve in the ongoing confirmatory phase 3 trial, ZIRCON, with use of ^{89}Zr-girentuximab, given the theoretical benefits in tumour uptake and spatial resolution with zirconium-89 over other isotopes.[43]

Circulating tumour cells and nucleic acids

Liquid biopsy has been a focus of interest in multiple cancer streams for more than a decade, as a tool to diagnose and monitor malignancy with minimal invasiveness. Furthermore, sampling blood or other bodily fluids has the theoretical benefit of a more complete representation of tumour burden and biology, as opposed to the sampling constraints of a tissue biopsy in heterogeneous tumours such as RCC.[44] Such diagnostic targets include circulating tumour cells, which are viable cells shed by the tumour into the circulation, and cell-free nucleic acids, which are thought to be released from apoptosis of tumour cells (**Table 15–1**). Analysis of these variables have been shown to mirror the genetic and epigenetic mutations from traditional tissue biopsies.[45] While there is no established clinical validity for these markers, their potential to overcome intratumoural heterogeneity and optimize early diagnosis of primary and recurrent disease remains promising.[46–55]

TABLE 15–1 Circulating Tumour Cells and Nucleic Acids as Diagnostic Biomarkers

Diagnostic biomarker	Value as biomarker	Reference
Circulating tumour cells (CTCs)	Limited success compared to breast or colorectal cancer due to reduced expression of epithelial markers (EpCAM) commonly used in CTC detection platforms, eg, CellSearch	Maertens *et al.*[52] Broncy *et al.*[54]
Cell-free nucleic acids	More stable and easier to isolate than CTC in RCC	de Martino *et al.*[49]
Cell-free DNA (cfDNA)	Higher levels reported in patients with RCC	Smith *et al.*[55]
Circulating tumour DNA (ctDNA)	More likely to be found in larger and locally advanced tumours, with highest fractions in metastatic RCC	Bergerot *et al.*[53]
Microribonucleic acids (miRNAs)	• Significantly higher levels in RCC tissue and serum of patients • Short noncoding nucleotides that regulate post-transcriptional gene expression, eg, miR-210, miR-378, miR-451, and miR-1233 • 97%–100% sensitivity of miR-based signatures to distinguish between histological subtypes of RCC	Wulfken *et al.*[46] Youssef *et al.*[47] Redova *et al.*[48] Zhao *et al.*[50] Ellinger *et al.*[51]

Gene expression profiling

Gene expression arrays have been created to differentiate between RCC subtypes and identify the aggressiveness and metastatic potential of tumours. Multiple studies have correlated the genetic expression profile of different types and grades of RCC with their morphological classification.[56–58] Analysis of these signatures from early-stage ccRCC has also informed us of additional pathways in tumourigenesis, including the downregulation of transcription factors required for normal renal development, such as GATA3, TFCP2L1, TFAP2B, and DMRT2. Other studies have identified a panel of up to 34 genes that is predictive of tumour aggression, and may function as a biomarker in the future.[59]

Urinary biomarkers

Urine is an easily accessed source for biomarkers. Profiling studies have identified two promising proteins originating from the proximal tubule, aquaporin 1 and adipophilin, that may be shed in urine and have diagnostic potential. Initial results indicate that both proteins are significantly elevated in urine from patients with RCC compared to healthy controls, declining to control levels following nephrectomy.[60,61] Nuclear matrix protein 22 (NMP22), an accepted urothelial cancer marker, was also found to be significantly elevated in urine samples from patients with RCC in a few studies more than 15 years ago; however, there have been no further reports since.[62–64] Other markers, such as kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and matrix metalloproteinases (MMPs), have been inadequate in differentiating renal malignancy.[65,66]

Tissue biomarkers

Diagnosis and classification of primary RCC can usually be established on morphological appraisal alone. However, immunohistochemistry is required in cases of benign mimics, or classification of tumour type on limited tissue such as from core biopsy of metastases. There is a wide panel of antibodies that are currently used for diagnostic purposes, including CK7, CD10, Pax 2, Pax 8, vimentin, and α-methylacyl-CoA (AMACR).[67]

Due to the particular difficulty in differentiating between benign and malignant eosinophilic tumours, a number of additional biomarkers have been studied to better characterize chromophobe RCC and oncocytoma, such as Hale's colloidal iron, several cadherins, and BCA2.[68] Additional analysis of distinct chromosomal aberrations, such as TFE3 and TFEB, is now established for translocation-associated RCCs.

Composite biomarkers

The optimal future biomarker will likely be a panel of biomarkers using the strengths of those mentioned above. One such biomarker is the composite 3-marker panel of nicotinamide N-methyltransferase (NNMT), L-plastin (LCP1), and nonmetastatic cell 1 protein (NM23A). This composite biomarker panel was evaluated in a cohort study of 189 patients and further validated in 100 patients. Plasma levels of NNMT, LCP1, and NM23A were significantly elevated in patients with kidney cancer. This composite assay had a positive predictive value of 87.2% and a negative predictive value of 97%.[69] The results are promising; however, they are yet to be externally validated.

15.4.2 Prognostic biomarkers

Accurate prognostic markers are the cornerstone of cancer management, and are indispensable in patient counselling, determining need for adjuvant therapies, and developing appropriate surveillance strategies. In RCC, several prognostic algorithms based on clinical and pathological variables have been developed and validated since Elson *et al.*[70] first pioneered multivariate modelling to predict cancer-specific outcomes for metastatic disease in 1988.

Currently, at least eight prognostic nomograms are frequently used for predicting the risk of relapse and survival in localized disease, and they have been shown to outperform TNM staging or Fuhrman grade alone.[71–75] These models were all developed from retrospective data, but some, such as the University of California at Los Angeles Integrated Staging System (UISS), Leibovich score, and Stage, Size, Grade, and Necrosis (SSIGN) score, have been central to the design of the recent RCC adjuvant trials.

However, recent prospective validation of these recurrence prediction models, using data from the Adjuvant Sorafenib or Sunitinib for Unfavorable Renal Carcinoma (ASSURE) trial, showed a substantial decrease in each of their prognostic abilities compared to previously reported results of up to 90% from retrospective external validation studies.[76] Most models only marginally outperformed standard staging and all had a C-index below 0.7.[77] As such, the search for more precise prognostic markers continues.

Routine blood markers

Biomarkers are not constrained to patented or expensive proteins overexpressed by RCCs. Everyday blood tests can be extremely useful in providing prognostic information about renal cancer. Serum lactate dehydrogenase (LDH), calcium, and hemoglobin have been widely reported to have independent prognostic significance in metastatic disease and are included in several nomograms, including the International Metastatic RCC Database Consortium (IMDC) risk model and Memorial Sloan-Kettering Cancer Center (MSKCC) score.[78,79] Hypercalcemia,

anemia, and elevated erythrocyte sedimentation rate have also been reported to be independent prognostic factors for cancer-specific survival (CSS) in localized ccRCC in a study of more than 1,700 patients.[80]

Several studies have reported that C-reactive protein (CRP) is a strong predictor of metastasis and overall mortality after nephrectomy for localized RCC. It also improved the predictive accuracy of other clinical and pathological predictors by up to 10%, though it did not have an additive benefit in all nomograms.[81–85] Findings from a study of 1,161 patients with localized and metastatic RCC concluded that CRP is a significant independent predictor of CSS and OS, when stratified to three subgroups of ≤4 mg/L, 4 mg/L to 10 mg/L, or ≥10 mg/L.[86] Immunohistochemical studies have also demonstrated significant intratumoural production of CRP, which can be correlated with survival outcomes.[87,88] Interestingly, after adjusting for tumour staining, preoperative serum CRP was not a significant predictor of OS (p=0.741) in this study.[88]

Thrombocytosis, another marker of the inflammatory milieu, is also an adverse prognostic factor in many cancers, including RCC.[89–91] However, in a predictive model comprising TNM stage, age, Fuhrman grade, histological subtype, and preoperative hemoglobin, thrombocytosis did not add any meaningful value, with a predictive accuracy gain of only 0.3%.[92]

Many investigators have demonstrated that increased peripheral blood or intratumoural neutrophils are associated with poor survival, especially in the context of IL-2 therapy.[93–97] Furthermore, a number of studies, including a systematic meta-analysis, have demonstrated that a higher blood neutrophil/lymphocyte ratio (NLR) portends a poorer prognosis.[98–100] NLR is emerging as a prognostic factor in several other cancers, and is thought to represent an impaired cell-mediated immune response due to systemic inflammation.[101]

Changes in coagulation pathways are also well recognized in malignancy. Cohort studies have reported significantly higher concentrations of plasma fibrinogen and D-dimer in patients with metastatic disease, and identified independent association with reduced CSS and OS.[102–104]

The VHL, HIF, and VEGF axis

Mutation of the VHL gene has been associated with a longer PFS and CSS in ccRCC in some studies.[105,106] However, this was not reproduced in other analyses.[107–109] Similarly, analysis of elevated HIF-1α levels and survival has varied, with some studies demonstrating favourable prognosis and others associating higher expression with worse outcomes.[110,111]

Increased VEGF concentration correlates with VHL gene inactivation, but in contrast, has consistently been associated with worse tumour stage, grade, necrosis, microvessel invasion, and CSS.[112,113] The phase 3 Treatment Approaches in Renal Cancer Global Evaluation Trial (TARGET) evaluated the role of VEGF as a prognostic biomarker in 712 patients with mRCC. Patients with a higher MSKCC score or Eastern Cooperative Oncology Group (ECOG) performance status had significantly higher baseline plasma VEGF levels. On multivariate analysis, VEGF level was an independent prognostic factor in patients on placebo, but not in those treated with sorafenib.[114] However, given that VEGF is contained within platelets and released on clotting, falsely elevated

results due to contamination of plasma with platelets or coagulation due to delays in processing the sample can occur, compromising its clinical applicability.[115]

CAIX is one of the HIF target genes and is associated with tumour growth, aggressive phenotype, and poor prognosis in most cancers.[116–118] In contrast, high CAIX expression is associated with a better prognosis in RCC in several studies.[119–122] In a larger study, however, CAIX expression was not an independent prognostic factor, after adjusting for the effect of nuclear grade, sarcomatoid differentiation, and tumour necrosis.[41] These findings were further validated at the 5-year follow-up of this study.[123]

Immunologic biomarkers

The B7 family of immune regulatory ligands produce co-stimulatory or co-inhibitory T cell signals, and therefore have been identified as promising prognostic biomarkers. B7-H1 functions as a negative regulator of immunity and its overexpression is independently associated with significantly increased progression to metastatic disease (relative risk [RR], 3.46; $p<0.001$), cancer-specific mortality (RR, 3.92; $p<0.001$), and overall mortality (RR, 2.37; $p<0.001$).[124,125] B7-H4—and to a lesser extent, B7-H3—have also been implicated as adverse prognostic factors.[126,127] Noninvasive immunoassays for the soluble forms of the B7 family are being developed with promising early results.[128]

Given the immunogenic nature of RCC, pathologic specimens harbour a high number of tumour-infiltrating lymphocytes (TILs). The prognostic significance of TILs is not established due to inconsistent findings on various multivariate analyses to date.[129–131]

Markers of cell proliferation and apoptosis

Various nuclear proteins that regulate cell growth, proliferation, and apoptosis are established as prognostic markers in other cancers. Some of these are also very promising in RCC, as summarized in **Table 15–2**.[132–146]

There is a plethora of other cell growth and survival markers currently under investigation, including MMP, vimentin, fascin, PTEN, ribosomal protein S6, caveolin, protein kinase B, serum amyloid A, NGAL, osteopontin, and cathepsin D, to name a few.[33,76]

TABLE 15–2 Markers of Cell Proliferation and Apoptosis as Prognostic Biomarkers

Prognostic biomarker	Role	Supporting literature	Reference
IMP3	Oncofetal RNA-binding protein; cell proliferation and invasion	Expression associated with significantly worse outcomes, with >1,400 patients total in 3 major studies: • 5-year OS: 27% vs 82%, $p<0.0001$ • OS HR: 1.42, $p=0.024$; risk of distant metastases: HR, 4.71, $p<0.001$ • Risk of metastases specifically in chromophobe or papillary RCC: HR, 13.45, $p<0.001$	Jiang *et al.* (2006)[136] Hoffmann *et al.*[141] Jiang *et al.* (2008)[142] Burdelski *et al.*[146]
Ki-67	Cell proliferation marker	• Poor prognosis in many cancers, including RCC • HR, 2.18 and 2.50 for CSS in 741 tumours with and without necrosis, respectively • Expression correlates with increasing stage and grade • Only independent predictor of RCC recurrence in a study of several markers (CAIX, CRP, HIF) in 216 patients; HR, 3.73	Bui *et al.*[133] Dudderidge *et al.*[135] Tollefson *et al.*[140] Inwald *et al.*[144] Abel *et al.*[145]
Survivin	Member of the IAP family	• Overexpressed in almost all human cancers, including RCC • Worse CSS, HR 2.4, $p<0.001$; and PFS, HR 1.9, $p=0.02$ • Confirmed in several retrospective studies	Altieri.[132] Parker *et al.*[137] Byun *et al.*[138] Krambeck *et al.*[139] Zamparese *et al.*[143]
p53	Induces apoptosis when DNA is damaged	• Overexpression is noted in 70% of papillary tumours, in 27% of chromophobe tumours, and in 12% of clear cell tumours • Prognostic significance not yet established	Zigeuner *et al.*[134]

Abbreviations: CAIX, carbonic anhydrase IX; CRP, C-reactive protein; CSS, cancer-specific survival; HR, hazard ratio; IAP, inhibitor of apoptosis protein; IMP3, insulin-like growth factor 2 mRNA-binding protein 3; OS, overall survival; PFS, progression-free survival; RCC, renal cell carcinoma.

Utility of biomarkers in prognostic models

Incorporation of molecular markers into existing prognostic models, as well as combining markers to create molecular signatures of the disease, will certainly be of greater utility than any single marker. A prognostic model using p53, CAIX, gelsolin, and vimentin, combined with metastatic status, T stage, and ECOG performance status, was 79% accurate in a cohort of 318 patients.[147] In another study of 634 patients, the integration of BioScore (which is based on expression of Ki-67), survivin, and B7-H1 to the UCLA Integrated Staging System (UISS) and Mayo Clinic Stage, Size, Grade, and Necrosis (SSIGN) models improved the prognostic accuracy of the models by 4.5% and 1.6%, respectively. Furthermore, patients with high BioScores were noted to be 5 times more likely to die from RCC (HR, 5.03; $p<0.001$).[148]

Lastly, the prognostic value of multigene assays, such as ClearCode-34 and 16-gene signature, has been reported to outperform the established predictive models and has now been validated in at least one prospective cohort. There are certainly caveats around tumour heterogeneity and misclassification due to sample bias; however, the results so far have been encouraging.[149–152]

15.4.3 Predictive biomarkers

The therapeutic landscape in metastatic RCC has transformed in the past decade with the introduction of targeted and IO treatments. Identifying markers that can reliably predict the response to specific treatments is essential to optimize patient management.

The only predictive biomarker validated in a phase 3 trial is the IMDC risk model. Data from the CheckMate 214 trial demonstrated that this model can effectively dichotomize mRCC patients into two distinct groups that benefit from different first-line treatment strategies.[153] The OS and objective response rates (RRs) were significantly higher with nivolumab plus ipilimumab (ipi/nivo) than with sunitinib among intermediate- and poor-risk patients,[154] resulting in a change of standard-of-care recommendations worldwide.[18,155,156]

The following section summarizes other predictive biomarkers under investigation. While the markers discussed aim to discern the efficacy of different treatments, markers that can predict the likelihood of severe toxicity in individual patients are equally important, given that severe adverse effects are reported in more than 20% of patients undergoing modern IO therapies.[157]

Immune checkpoint inhibitors

Although immune checkpoint blockade (ICB) is highly efficacious in treatment of patients with mRCC, there is significant variability in outcomes, highlighting the need for accurate predictive markers. Almost a quarter of patients in CheckMate 214 were classified as IMDC favourable risk and had significantly better outcomes on sunitinib alone compared to ipi/nivo (PFS, 25.1 months vs 15.3 months).[154] However, findings from the KEYNOTE-426 study demonstrated improved PFS and OS favouring pembrolizumab plus axitinib, irrespective of the IMDC risk group.[158]

Immunohistochemical expression of PD-L1 is the most studied marker in this realm. Studies to date in mRCC have not established its independent predictive value. In all prospective ICB trials, PD-L1 expression has been associated with worse prognosis, but not with response to checkpoint inhibitors.[154,158,159] Biologic and logistic challenges in using PD-L1 as a biomarker have been well described, including intratumoural heterogeneity, discordant expression in primary and secondary sites, dynamic expression, and variation in immunohistochemical assays.[160]

Results of a biomarker analysis from the JAVELIN Renal 101 trial were recently published, and included a 26-gene expression signature, and mutations and polymorphisms based on whole-exome sequencing, in addition to PD-L1 expression.[161] PD-L1 expression was not predictive of response to avelumab. Similar to previous studies, tumour mutational burden did not demonstrate any significant predictive value.[162] Several genetic mutations and the 26-gene signature were implicated in predicting treatment response; however, these findings need to be further validated.

Likewise, the phase 2 IMmotion-150 trial demonstrated the utility of gene expression signatures, reflecting angiogenesis and effector T-cell response, in predicting response to atezolizumab. A high angiogenic signature was associated with improved response rate and PFS in patients treated with sunitinib, and patients with high effector T-cell signature had better responses to ICB. These findings were subsequently confirmed by the phase 3 IMmotion-151 trial.[163]

Other predictive markers under investigation include TILs, mutation signatures and microsatellite instability, HLA classification, transforming growth factor β expression, PD-L2, CTLA-4, mutational or neoantigen burden, and commensal gut microbiome.[164,165] Ongoing integration of biomarker evaluation and validation in prospective studies is required to identify clinically significant markers.

VEGF-related therapies

A number of treatment-related toxicities have been shown to independently predict better treatment response to VEGF-related TKIs. In a pooled analysis of 544 patients treated with sunitinib, patients who developed treatment-related hypertension had significantly better outcomes compared with those who did not: objective RR, 54.8% versus 8.7%; PFS, 12.5 versus 2.5 months; and OS, 30.9 versus 7.2 months.[166] Other markers reported to have predictive value are neutropenia, hand-foot syndrome, and hypothyroidism.[167–169]

Several genomic and molecular markers have also been investigated. VHL mutation status failed to show any predictive value.[170,171] However, downstream effectors of angiogenesis have shown some promise. High IL-6 concentration is associated with improved PFS benefit from pazopanib compared with placebo, as well as improved OS benefit from bevacizumab plus interferon-α (IFN-α) compared with IFN-α alone.[172,173] The results for baseline levels of vascular endothelial growth factor A (VEGFA), VEGF receptor 2 (VEGFR2), VEGFR3, HIF-1α, HIF-2α, and CAIX have been variable and inconsistent. Similar limitations were seen in analysis of other markers such as osteopontin, MMP, tissue inhibitor of metalloproteinase 1 (TIMP-1), tumour necrosis factor–related apoptosis-inducing ligand (TRAIL), and NLR.[174,175]

The role of PD-L1 status was evaluated in two recent trials comparing the efficacy of cabozantinib to everolimus (METEOR) and sunitinib (CABOSUN). Though PD-L1 expression was associated with shorter PFS and OS in both studies, it was not predictive for response to either treatments.[176,177] Post-hoc analysis in patients from the COMPAARZ trial also confirmed the prognostic significance of PD-L1; however, results did not demonstrate any predictive value.[178]

Single-nucleotide variants (SNVs, known previously as single-nucleotide polymorphisms [SNPs]) in key genes encoding proteins related to the pharmacokinetics (CYP3A5), pharmacodynamics (VEGFR1, VEGFR3), and angiogenic pathways (FGFR2), as well as genetic mutations in key genes such as PBRM1 and BAP1, were associated with improved outcomes after sunitinib treatment in some pharmacogenomic studies.[179,180] Multigene assays and molecular subtyping have also shown predictive significance in small retrospective cohorts, and warrant further investigation in a prospective setting.[181,182]

mTOR inhibitors

In a retrospective analysis of the Global Advanced Renal Cell Carcinoma (ARCC) trial more than 10 years ago, comparing temsirolimus with IFN-α, survival benefit with temsirolimus was only observed in patients with high pretreatment LDH, supporting its role as a predictive marker. PTEN and HIF-1α levels did not predict differential treatment response.[183,184]

In 2018, the Spanish Oncology Genitourinary Group (SOGUG) prospectively analyzed immunohistochemical markers such as p-S6, p-AKT, p21, and PBRM1, as well as next-generation sequencing for mutational analysis on key genes of the mTOR pathway in 77 patients. On multivariate analysis, only negative immunohistochemical expression for BAP1 and PBRM1 was predictive of better response to mTOR inhibitors (OR 4.0, p=0.011, and OR 3.9, p=0.025, respectively).[185]

Interleukin 2

High-dose interleukin 2 (HD IL2) can induce durable and complete response in 6% to 10% of patients, and overall RRs of up to 25%.[186,187] It remains the only drug to date that can cure a small percentage of mRCC patients and thus, despite its significant toxicity and the emergence of better-tolerated agents, should not be entirely overlooked.

Stringent patient selection is critical, given the toxicity associated with HD IL2, and some attempts have been made to identify markers that predict treatment response. Following several retrospective studies, the Cytokine Working Group conducted a prospective Select trial to validate some proposed predictive markers of response to HD IL2. Response rate was not associated with CAIX, plasma VEGF, or fibronectin levels, as previously reported. Interestingly, RR and durable remission (PFS >3 years) was positively associated with PD-L1 expression.[188]

15.5 Conclusion

Our knowledge of RCC biology at the molecular level has increased greatly over recent decades, catalyzing the discovery of targeted therapies. However, there is need for progress in the development of clinical tools to guide our decision-making for management of this heterogeneous disease. The potential for biomarkers to improve diagnosis and treatment and to reduce healthcare costs is greater than any other area of current medical research. Volumes of literature have been published on numerous promising diagnostic, prognostic, and predictive RCC biomarkers, but none of these have yet been established for routine clinical use.

A major barrier to translating research findings to clinically applicable tools has been a lack of standardization in study methodologies and small sample sizes lacking statistical power to demonstrate any meaningful correlation. The recent shift to the collaborative efforts of large research networks involving industry and scientific experts in prospective trials, instead of the traditional model of small laboratory-based retrospective studies, is hoped to yield higher-quality data, and provide us with an exciting and unprecedented opportunity for the discovery and large-scale validation of reliable, precise, and cost-effective RCC biomarkers.

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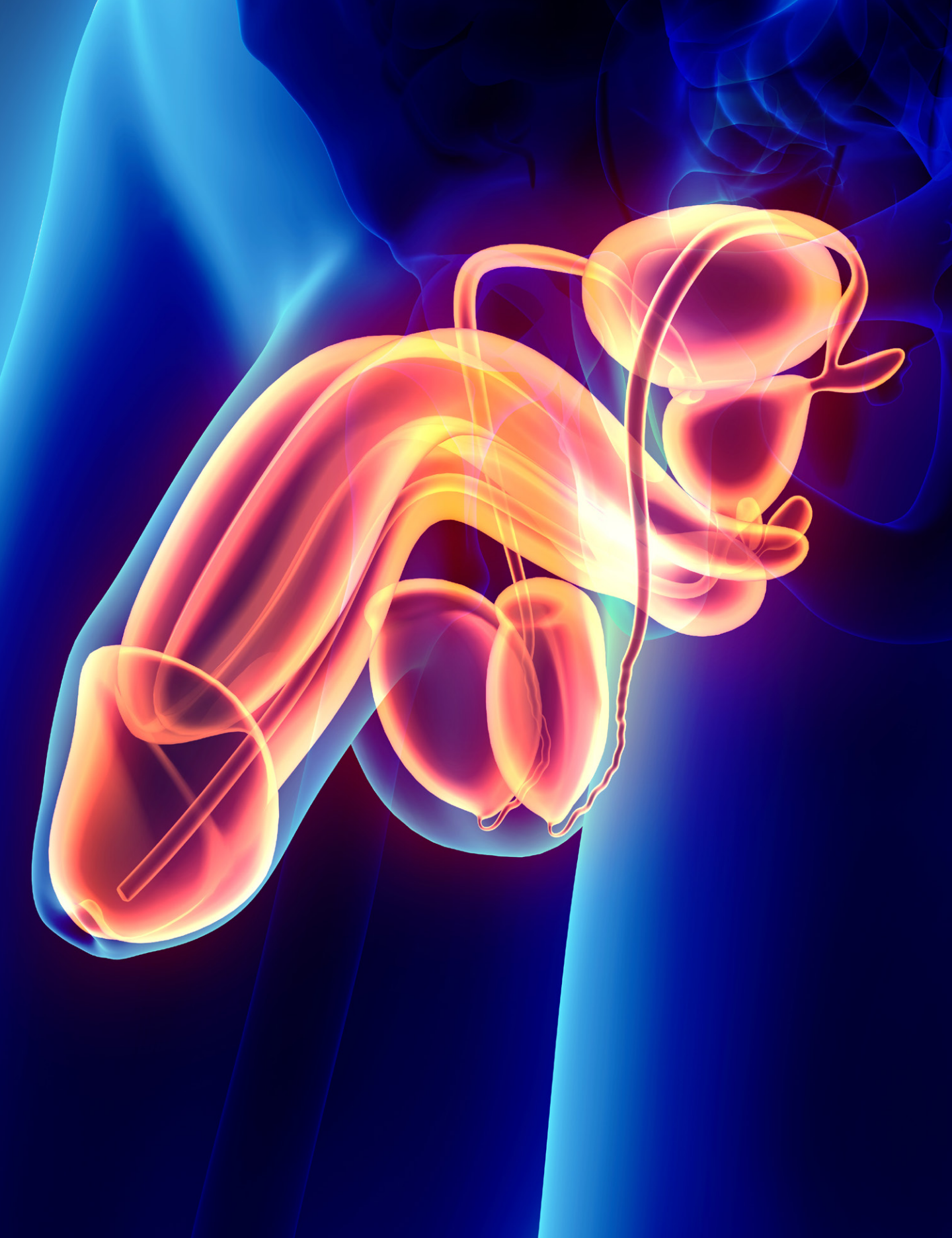

CHAPTER 16

Biomarkers in Testicular Germ Cell Tumours

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Table of Contents

16.1 Introduction

Testicular germ cell tumour (GCT) is the most common malignancy in men between the ages of 18 to 40 years.[1] Testicular germ cell tumours are histologically divided into seminoma and nonseminomatous GCT (NSGCT). While seminomatous tumours retain pluripotency and resemble primordial germ cells, NSGCT are undifferentiated (embryonal carcinoma), or differentiated toward embryonic germ layers (teratoma) or extraembryonic elements (choriocarcinoma or yolk sac tumour [YST]).[2] There is evidence that in the testis, both seminoma and NSGCT emerge through a common pathogenesis called germ cell neoplasia in situ (GCNIS), and approximately 15% of NSGCT contain both seminomatous and nonseminomatous GCT histologies.[3]

In some cases of GCT, substances called serum tumour markers (STMs) are secreted into the blood and are found at abnormally high levels. Circulating levels of these proteins are determined by the histologic composition and overall burden of the tumour. Lactate dehydrogenase (LDH), α- fetoprotein (AFP), and the beta subunit of human chorionic gonadotropin (β-hCG) are the three serum tumour markers that have an established role in GCT diagnosis, staging, and management. Only 10% to 15% of seminomas have elevated tumour markers and by definition never secrete AFP, while 60% to 85% of non-seminomas (NSGCT) secrete tumour markers (**Table 16–1**).[4]

TABLE 16–1 Histology-specific Serum AFP and β-hCG Levels[14]

Histologic subtype	AFP	β-hCG
Seminoma	–	±
Embryonal carcinoma	±	±
Choriocarcinoma	–	++
Yolk sac tumour	++	–
Teratoma	±	–

Abbreviations: ++, strongly positive levels, –, negative levels; ±, marker can be negative or moderately positive. AFP, α-fetoprotein; β-hCG, β–human chorionic gonadotropin.
Source: Adapted with permission from Springer Nature, from Murray MJ, Huddart RA, Coleman N. The present and future of serum diagnostic tests for testicular germ cell tumours. Nat Rev Urol. *2016;13(12):715–725. doi:10.1038/nrurol.2016.170*[14]

The current staging system for GCTs reflects the prognostic value of these biomarkers by including an S-stage classification.[5] Furthermore, in cases of disseminated disease, STM levels are associated with prognosis and have an important role in determining systemic therapy regimens.[6] However, the clinical utility of these markers is tempered by limited sensitivity and specificity. Issues with these serum markers include the fact that they are expressed in only a subset of GCTs, and they may be normal in the setting of early recurrence or low tumour burden (marker-negative, radiographically occult metastases). They also may be nonspecifically elevated by processes other than GCT, and they might not necessarily correlate with treatment response or post-treatment histology.[4,7] Nevertheless, STMs have an integral role in diagnosis and monitoring of patients with GCTs.

When managing patients with GCTs, there are multiple clinical scenarios where current tumour markers, whether tissue based or serum based, are inadequate for precise and individualized decision-making. For patients with a radiographically equivocal, STM-negative testicular mass, the diagnosis of GCT is not straightforward. The American Urologic Association (AUA) and European Association of Urology (EAU) recommend short-interval imaging or enucleation.[8,9] In the majority of men who present with clinical stage I disease (any T, N0M0S0), normal post-orchiectomy STMs, and no radiographic evidence of metastases, 10% to 50% of patients will have occult disease.[10] Diagnostic tools in this clinical setting are inadequate to inform personalized treatment decisions, with lymphovascular invasion (LVI) and the presence of embryonal carcinoma stratifying risk in NSGCT patients and size or rete testis invasion dictating risk in patients with seminomatous tumours.[10] In patients with stage I NSGCT who undergo primary retroperitoneal lymph node dissection (RPLND) and are found to have viable GCT elements, the decision to administer or withhold adjuvant therapy is only informed by the pathologic N stage, which is again a rudimentary classifier.

Among patients with clinical stage IIA (T any N1M0S0-1) disease, up to 30% will ultimately be found to have pathologic N0 disease, and there is no accurate way to identify these patients at the point of care.[11] For patients presenting with disseminated disease, we currently rely on STM decline, radiographic response, and symptom profile to assign treatment response or resistance. While certainly useful, there are significant limitations.[12] For patients with metastatic disease who have completed induction chemotherapy, our ability to predict the presence of fibrosis or necrosis, viable GCT elements, or teratoma is limited at best, with current prognostic models considered insufficient to make accurate decisions.[13] Finally, surveillance of patients with testicular cancer is costly and associated with repeated radiation exposure due to multiple computed tomography (CT) scans. Therefore there is certainly room for improvement.

In this chapter, we will discuss the features of established biomarkers in GCT diagnosis and management, as well as the promise of the next generation of biomarkers to fill critical gaps for patients with testicular cancer. Despite an immense and commendable body of literature suggesting various novel biomarkers for predictive and prognostic purposes in GCTs, the current chapter will be focused on clinically relevant potential biomarkers that show promise to enter the clinical arena. Specifically, we will focus on microRNAs (miRNAs, miR) that are poised to radically change the way patients with GCTs are managed.[10]

16.2 Conventional Serum Tumour Markers

16.2.1 Lactate dehydrogenase

Lactate dehydrogenase (LDH) is the least GCT-specific STM, but it has several important features. LDH, elevated in 40% to 60% of GCTs, may be the only elevated STM in patients presenting with pure seminoma.[12] LDH converts lactate to pyruvate, and it is found universally throughout cells of the body.[12] LDH is associated with cell turnover, making it nonspecific for GCTs. Elevated LDH levels are identified in other malignant processes, including lymphoma, renal cell carcinoma, bone tumours, and a variety of other cancers.[12]

Noncancerous situations where there is cell lysis or injury, including myocardial infarction, other muscular disease, and rheumatologic disorders, can also have elevated LDH levels.[12] LDH is often considered a surrogate for tumour bulk. Though it is less specific and sensitive than AFP or β-hCG for NSGCTs, it is incorporated into the International Germ Cell Cancer Collaborative Group (IGCCCG) risk criteria.[6] Circulating LDH levels are measured by an enzymatic assay that varies considerably from one laboratory to the next. A consistently reported half-life of LDH is not known, but the scale is on the order of days, not hours.[14]

16.2.2 α-Fetoprotein

α-Fetoprotein (AFP) is a protein containing a carbohydrate moiety and a ~600 amino acid α-globulin molecule.[14] AFP is normally produced by the fetal yolk sac, but primarily by the liver and to a lesser extent other gastrointestinal organs.[12] Albumin replaces AFP in the third trimester, but the long half-life of AFP means that it is elevated at birth and can remain elevated until two years of age physiologically.

As such, postpubertal AFP levels are generally low, as measured against World Health Organization (WHO) international standards, with normal values being <12 ng/mL, although some individuals will have slightly higher levels in the absence of disease.[14,15] AFP levels that are clinically quantitated with a chemiluminescent sandwich enzyme assay can have a different upper limit of normal parameters, depending on the institution.[14]

16.2.3 α-Fetoprotein in seminoma

By definition, elevated AFP levels preclude a diagnosis of a seminomatous GCT (See **Table 16–1**). Significantly elevated AFP levels are diagnostic for NSGCT, even when the orchiectomy specimen reveals seminoma only. Importantly, false or borderline elevations, which are discussed below, must be taken into account when deciding whether to classify a pure seminoma orchiectomy tumour as a seminoma or NSGCT, or when making a determination on the presence or absence of occult metastases based on STMs only. In some cases of seminoma with hepatic metastases where liver regeneration is taking place, AFP levels may be elevated.[16] Multiple cases of pure seminoma with marginally elevated AFP (typically <20 ng/mL) are reported in the literature, emphasizing the importance of familiarity with STM interpretation in GCT management.[17]

16.2.4 α-Fetoprotein in nonseminomatous germ cell tumour

α-Fetoprotein is produced in the majority of yolk sac tumours, also known as endodermal sinus tumours, and it can also be measured in the serum of patients with teratomatous or embryonal carcinoma elements (See **Table 16–1**). In pure immature teratoma specimens, AFP might be elevated due to gastrointestinal or liver elements within the tumour.[14] AFP level elevation and degree of elevation is related to clinical stage, with an elevated AFP in 10% to 20% of stage 1 tumours and in up to 40% to 60% of patients with metastatic disease.[12]

16.2.5 False-positive α-fetoprotein levels

Certain liver conditions, including hepatocellular carcinoma, chronic liver disease, history of gastric or hepatic surgery, and hereditary ataxia telangiectasia, can be associated with elevated AFP levels.[18] Patients with systemic

therapy may have damage to the lung or liver that can lead to elevated AFP levels.[19] Other relatively uncommon scenarios including gastrointestinal cancers, such as pancreatic, stomach, and colon cancers, along with lung cancer, have also been associated with elevated AFP levels.[12] Mildly elevated and stable AFP, when not accompanied by any other indication of testicular cancer, should be managed by ongoing surveillance.[15]

16.2.6 Half-life of α-fetoprotein

The half-life of AFP is approximately 5 to 7 days. Knowledge of half-lives and appropriate STM decline is mandatory for establishing correct clinical stage after orchiectomy and response to chemotherapy in cases of metastatic disease.[12]

16.3 Beta Subunit of Human Chorionic Gonadotropin

β–Human chorionic gonadotropin is the most commonly elevated STM in GCTs in adult patients, and it is found in both seminomatous and nonseminomatous histologies. For GCTs, hCG measurements are typically of both the free β subunit (monomer), and the α-β dimer (intact hCG), summed into the total hCG (β-hCG).[14] β-hCG is typically measured with a double antibody immunometric assay.[12] Normal β-hCG levels are <2 IU/L.

16.3.1 β–Human chorionic gonadotropin in seminoma

Pure seminoma containing syncytiotrophoblast cells can produce β-hCG, which occurs in 15% to 20% of seminoma cases.[12] Elevated β-hCG are generally associated with higher tumour bulk in seminomas, yet there is no association with metastatic potential in patients with stage I disease who normalize β-hCG levels after orchiectomy, nor is β-hCG incorporated into the IGCCCG risk stratification for patients with pure seminoma.[6]

16.3.2 β–Human chorionic gonadotropin in nonseminomatous germ cell tumour

Elevations in β-hCG are most striking in patients with significant choriocarcinoma elements yet can also be present in patients with embryonal carcinoma histologies (See **Table 16–1**). Overall, 10% to 20% of patients with stage I NSGCT and up to 40% of patients with disseminated disease will have elevations in β-hCG.[12] β-hCG in NSGCT is an important prognostic factor in the IGCCCG risk criteria.[6]

16.3.3 False-positive β–human chorionic gonadotropin levels

Elevated β-hCG levels, particularly mildly elevated levels, should be approached cautiously because a multitude of confounding factors can cause elevated β-hCG. Hypogonadism leads to increased production of luteinizing hormone (LH), which can cross-react with the assay to measure β-hCG. Furthermore, hypogonadism directly leads to compensatory increases in hCG production from the pituitary gland. This can be misleading, as hypogonadism can be seen following orchiectomy.[12] Marijuana use, tumour lysis following induction chemotherapy, and the presence of heterophile antibodies are all associated with elevated β-hCG levels that must be interpreted

with caution.[20,21] Though very high (> 10,000 IU/L) β-hCG levels are typically only seen in GCT and rarely in trophoblastically differentiated adenocarcinomas and pregnancy states, β-hCG can be elevated in a variety of other cancer types including lymphoma, leukemia, and neuroendocrine tumours across disease sites.[20]

16.3.4 Half-life of β–human chorionic gonadotropin

The half-life of β-hCG is usually 12 to 36 hours. This relatively quick decay is faster than AFP, and it can be helpful for clinical monitoring. In patients with elevated pre-orchiectomy β-hCG levels and no radiographic evidence of metastases, rapid normalization of β-hCG levels may indicate that orchiectomy was curative, while persistently elevated hCG levels (clinical stage IS disease) suggest occult metastatic deposits.

16.4 Clinical Applications of Serum Tumour Markers

Germ cell tumour guidelines uniformly recommend obtaining STMs prior to orchiectomy. This allows for confirmation of appropriate STM decline following orchiectomy, and the potential presence of NSGCT elements in patients with elevated AFP and seminoma on orchiectomy specimens.[8,22] As noted previously, normal STMs are insufficient to rule out a diagnosis of GCT. In patients with widely metastatic disease, elevated STMs and high clinical suspicion can be sufficient to initiate GCT-directed systemic therapy without histologic confirmation. Similarly, significantly elevated STMs in the setting of uncharacteristic histopathologic findings for confident GCT diagnosis may also lead to GCT-specific management.[8,22]

Following orchiectomy, nadir STM values are used for tumour-node-metastasis–serum markers (TNMS) or composite staging, dictating subsequent steps in management.[8,22] Among patients with disseminated disease, post-orchiectomy serum tumour markers are incorporated into the IGCCCG risk criteria, along with primary site and presence of non-pulmonary visceral metastases, dictating treatment regimen and serving as independent predictors of clinical outcomes.[6]

Taking half-life decay into account, serial measurement of STMs during treatment of metastatic disease indicates appropriate response to treatment or presence of chemotherapy-resistant disease. Following definitive therapy—orchiectomy with or without adjuvant treatment for stage I disease, and systemic therapy and/or locoregional therapy for stage II/III disease—STMs are measured at routine surveillance intervals.[6]

16.4.1 Serum microRNAs as biomarkers in testicular germ cell tumours

While conventional STMs have a critical role in the diagnosis, treatment, and monitoring of patients with GCTs, they remain hampered by lack of sensitivity and specificity as well as histology-specific considerations.[10] Over the past decade, an ever-growing body of work indicates that measurement of GCT-specific miRNAs is poised to radically change the way germ cell tumour patients are diagnosed, managed, and surveyed, ushering in an era of

personalized medicine. For the purposes of this review, we will focus on the role of serum miRNAs as potential biomarkers and will not discuss technical details of the assay or implications with respect to GCT pathogenesis.

MicroRNAs are short, noncoding RNAs that are involved in epigenetic regulation of gene expression.[10] In a landmark study, Voorhoeve and colleagues identified overexpression of the miR-371~373 cluster in germ cell tissues and cell lines, suggesting that this cluster functioned as oncogenes by virtue of suppression of the LATS2 tumour suppressor gene.[23] Subsequently, a body of work described the overexpression of 8 GCT-associated microRNAs (miR-371~373 cluster and the miR-302-367 cluster) regardless of histology (except teratoma), anatomic site of origin (gonadal vs extragonadal), gender (male vs female), and age (pediatric vs adult). These data suggested the first universal molecular abnormality of this heterogeneous disease.[24,25]

The next major advancement in GCT-related miRNA research occurred when Murray and colleagues described a quantitative reverse transcription (qRT) PCR methodology with a preamplification step whereby elevated levels of GCT-associated miRNAs were identified in the serum of a patient with extragonadal GCT compared with pooled normal controls.[26] Furthermore, the circulating levels of the miRNAs were informative with respect to treatment response and disease status on routine follow-up.[26] This seminal study formed the basis for an explosion of investigations into the performance characteristics and potential roles for these serum miRNAs in the diagnosis and management of patients with GCTs.

We will now review pivotal studies that evaluate the utility of these microRNAs across the clinical spectrum of GCT diagnosis and management.

16.4.2 Screening

To date, no large-scale studies have been conducted to assess whether GCT-associated miRNAs have utility in screening patients for the development of invasive GCT. Though purely speculative, there might be a role based on work done surrounding serum miRNAs and GCNIS.

Germ cell neoplasia in situ is considered to be the common precursor lesions for adult-type seminomas and NSGCT. Novotny and colleagues described the overexpression of GCT-associated miRNAs in tissue samples of patients with GCNIS compared with controls.[27] While initial reports of the ability of serum miRNAs to identify GCNIS were negative,[28] more recent studies indicate that up to 50% of patients with GCNIS-only tumours can be identified with serum GCT-associated miRNA tests.[29] These findings were reported in small numbers of patients and have not been validated.

Until definitive studies identifying patients with GCNIS-only tumours and distinguishing them from patients with invasive GCTs are available, it is imperative that patients with clinical GCNIS are not mistaken for patients with invasive GCNIS and overtreated. Nevertheless, it is conceivable that with more sensitive tests, patients with the GCNIS precursor lesion may be identified prior to development of invasive tumours.

16.4.3 Pre-orchiectomy

A solid testicular mass on physical exam or sonography is considered a malignancy until proven otherwise. However, there are non-malignant causes that are difficult to distinguish with a clinical exam, sonography, or both.[22] Conventional STMs might not be informative, as seminomas have elevated markers in only 10% to 15% of cases, while NSGCTs have elevated markers in about 60% to 85% of cases.[4]

Multiple groups have evaluated the performance characteristics of miRNAs prior to orchiectomy, uniformly demonstrating greater sensitivity and specificity than conventional STMs (**Table 16–2**). In 2013, using data from The Netherlands, Germany, and the United Kingdom, Gillis and colleagues described assessment of GCT-associated miRNAs in 80 patients with GCTs, non-cancer controls, matched pre/post orchiectomy specimens, and patients with non-GCT testicular masses.[7] This study indicated that a four-panel serum miRNA test (miRNA miR-367-3p from the miR-302/367 cluster, miR-371a-3p, miR-372-3p, and miR-373-3p) clearly distinguished GCT patients from controls. The test was normal in non-GCT testicular masses, outperformed conventional STMs, returned to normal following orchiectomy in patients with stage I disease, and trended toward higher levels in patients with metastatic disease.[7]

Overall sensitivity of the four-panel miRNA test was 98% with an acceptable specificity. Importantly, the four-panel miRNA set (miRNA 367, miRNA 371-373) outperformed the previously described eight-panel set that included the miRNA 302/367 cluster, and it established the four-panel set as the basis for future studies.

van Agthoven and Looijenga evaluated a larger cohort of 250 GCT patients compared with 60 non-GCT patients and 104 healthy controls to further investigate the discriminative ability of GCT-associated miRNA in the primary diagnosis of GCTs.[28] The GCT-associated panel accurately detected both seminomas and NSGCT at the time of diagnosis, with a 90% sensitivity and 91% specificity. Notably, the inability of the miRNA test to detect pure teratoma or GCNIS was highlighted in this study. While this may initially be perceived as a limitation of miRNA tests, it does suggest a possible utility of the test to discriminate teratoma from viable non-teratomatous GCT elements, distinguishing patients who might require surgical resection of teratoma.

Similarly, delineating GCNIS from invasive cancer has important management implications. Dieckmann and colleagues recently reported on a cohort of 616 GCT patients, 522 of whom provided preoperative samples, and 258 controls.[30] Importantly, only miR-371a-3p was assessed in this study, and it had a sensitivity of 90.1% and a specificity of 94% for the diagnosis of GCT regardless of histology using pre-orchiectomy specimens.

TABLE 16–2 Performance Characteristics of Serum Pre-Orchiectomy GCT Associated MicroRNAs for Prediction of GCT on Final Orchiectomy Histopathology

Reference	Sensitivity, %	Specificity, %	PPV, %	NPV, %	AUC
Gillis *et al.*[7]	98	48.3	NR	NR	0.96
van Agthoven and Looijenga[28]	90	91	94	7	0.962
Dieckmann *et al.*[30]	90.1	94	97.2	82.7	0.966

Abbreviations: AUC, area under the curve; GCT, germ cell tumour; NPV, negative predictive value; PPV, positive predictive value.

As expected, the performance characteristics of the serum test for diagnosis of teratoma was poor in this study and remains an area of future research. At the time of publication, a large and growing body of work indicates that serum miRNAs have excellent specificity and sensitivity for accurately identifying patients with malignant or viable GCT prior to orchiectomy.

16.4.4 Identification of occult metastases in patients with early-stage (I/II) disease

The majority of patients with GCT present with clinical stage I disease (any T, N0M0S0), normal post-orchiectomy STMs, and no radiographic evidence of metastases, yet 10% to 50% of patients will have occult disease and ultimately relapse.[10] Discriminatory tools in this clinical setting are limited, with LVI and the presence of embryonal carcinoma stratifying risk in NSGCT patients, and size or rete testis invasion dictating risk in seminoma patients.[10] For stage IA NSGCT, there is a consensus that surveillance is the preferred strategy in the absence of malignant somatic transformed elements, to avoid overtreatment in the 85% to 90% of patients who are cured with orchiectomy alone.[31]

Nevertheless, the minority of patients with occult disease not detectable by conventional imaging or STMs that are destined to recur would be well served to receive primary RPLND or adjuvant BEPx1, because both are associated with excellent recurrence-free survival rates and decreased toxicity compared with full-dose induction chemotherapy.[32] For patients with stage IB NSGCT, higher recurrence rates (45–50%) make adjuvant strategies more attractive for management in this setting, though we only have very rudimentary histopathologic information to guide the decision, such as the presence of LVI.[32] Similarly, accurate identification of patients with occult metastatic seminoma could lead to earlier, less intensive treatment.

Tumour bulk is reproducibly associated with circulating miRNA levels, and an important clinical question is whether radiographically occult metastases comprise a critical tumour mass to produce measurable serum miRNA levels.[33] Gillis and colleagues reported on 11 patients with both pre- and post-orchiectomy serum samples. Serum miRNA levels normalized following orchiectomy, but clinical follow-up was not reported, and it is unclear if subclinical metastases were actually present.[7]

Decrease in miR-371a-3p following orchiectomy was confirmed in a large multi-institutional study, and the miR-371a-3p test was associated with 83% sensitivity and 96% specificity for identifying relapses.[30] Notably, the disease burden at microRNA assessment and characteristics of clinical relapse are unclear, though these patients were presumably identified radiographically or serologically to have relapsed GCT.

Nappi and colleagues reported on 25 patients with low-risk stage I seminoma or NSGCT using plasma instead of serum, and the miR-371a-3p test correctly identified all patients that ultimately recurred (1 of 25) and those that did not.[34] Recently, Lafin and colleagues conducted a study of 24 patients with stage I/II GCT who had miRNA analysis done prior to chemotherapy-naïve RPLND. This study was the first to pathologically confirm the presence or absence of viable germ cell tumour elements in stage I or stage II disease. Benign histology was reported in 10 patients, teratoma in 3 patients, and viable GCT in 11 patients. On ROC analysis, miR-371a-3p had an AUC of 0.965, with a sensitivity and a specificity of 100% and 92%, respectively.[51]

If validated, this data has far reaching implications with respect to avoiding overtreatment or undertreatment in patients with stage I disease, while also avoiding overtreatment of the 20% to 30% of patients with marker negative, clinical stage IIA disease that are ultimately pN0 on final histology.[11]

Over the past decade, multiple reports indicate that normalization of serum miRNAs, or normal post-orchiectomy serum miRNA levels, are associated with low risk of relapse, whereby persistently elevated levels portend clinical relapse. These data suggest that miRNAs can serve as personalized biomarkers capable of detecting early recurrence or occult metastases with high specificity and negative predictive value. This would fulfill the need to individualize management strategies for patients with low-stage GCT. Ostensibly, these same principles apply for patients who receive primary RPLND with confirmed viable GCT on final pathology and are being considered for observation versus adjuvant chemotherapy. Specifically, a post-RPLND miRNA test may inform whether residual cancer is likely to be present or absent, and whether further therapy should be administered or withheld.

16.4.5 Response to treatment in patients with disseminated disease

Conventional STM decline carries important prognostic information when treating patients with metastatic disease as a marker of chemotherapy sensitivity or resistance, yet the information has significant limitations.[35] Dieckmann and colleagues elegantly demonstrated that in patients with stage II and III disease treated with chemotherapy, miRNA levels track treatment response and also suggest treatment resistance. Of the 118 patients with systemic disease, 70 had decreases in miR-371a-3p levels after the first cycle of chemotherapy, with stage II patients having no further reduction after the first cycle, and stage III patients having no further reduction after the second cycle. Despite very limited follow-up data overall, two patients who ultimately died of disease progression were known to have corresponding rises in miRNA levels to very elevated values.[30]

Mego and colleagues conducted a detailed analysis of the prognostic value of plasma miR-371a-3p levels in patients with metastatic disease. Among their cohort of 180 patients treated with systemic therapy, miRNA levels

were associated with IGCCCG risk group and response to chemotherapy.[36] These preliminary results suggest that miRNAs levels are associated with tumour bulk and can have prognostic and predictive implications with respect to treatment response.

16.4.6 Prediction of post-chemotherapy residual mass histology

Following induction chemotherapy for patients with primary metastatic or relapsed GCT, treatment response is evaluated with radiographic response and STM levels. For many patients with radiographic incomplete response, particularly with NSGCT histology, the next step is post-chemotherapy RPLND (PC-RPLND).[36] PC-RPLND is the only way to accurately differentiate whether residual masses harbour necrosis/fibrosis only, teratoma, or viable GCT elements.

In addition to being diagnostic, PC-RPLND is the only way to treat chemo-resistant teratoma and may be therapeutic in patients with viable chemotherapy-resistant GCT elements.[13,37] Importantly, up to 50% of patients will have fibrosis/necrosis only, 40% teratoma, and 10% viable GCT elements at PC-RPLND histology. In other words, 50% of patients will undergo an unnecessary, complicated operation.

Leão *et al.* recently reported on the performance characteristics of miR-371a-3p, miR-373-3p, and miR-367-3p levels in a cohort of 82 NSGCT patients undergoing PC-RPLND, 12 of whom (14%) had viable GCT on post-chemotherapy histology.[38] These investigators found that miR-371a-3p had the highest discriminative capacity to detect residual viable GCT after chemotherapy (AUC, 0.874) and declined predictably in response to treatment. Leão *et al.* grouped teratoma and benign processes (fibrosis/necrosis) separately from viable GCT, given the poor discrimination for teratoma.[38]

Nonetheless, their findings are encouraging and warrant prospective validation. Based on recent data from the TCGA suggesting that teratomatous elements express miR-375,[39] Lafin and colleagues sought to determine if this miRNA was secreted into the serum, and did not find this to be the case in three patients with teratoma-only on RPLND.[51] To date, there is no reliable serum marker to predict the presence or absence of teratoma in the primary or post-chemotherapy setting.

16.4.7 Novel therapeutic targets for chemotherapy-resistant disease

Chemotherapy resistance is a difficult clinical situation associated with a 50% risk for cancer-specific mortality despite availability of fairly effective salvage chemotherapy regimens.[40] miRNAs are potentially attractive therapeutic targets due to effects on multiple mRNA molecules among many different cellular pathways, and they can decrease drug resistance. Broadly, strategies for miRNA-based treatment include inhibition of overexpressed miRNAs with miRNA inhibitors or replenishment of underexpressed miRNA with miRNA mimics.[41] *In vitro* data suggest that this may be a viable strategy in GCTs as well.[42]

16.4.8 Surveillance

Serum miRNA testing offers the promise of radically changing the way all patients with seminomatous GCTs are surveilled, where teratoma is an exceedingly rare occurrence. Specifically, miRNAs hold the promise to supplant axial imaging and traditional blood tests for the monitoring of recurrence. In patients with NSGCT, miRNA examination could limit the number of axial scans required for surveillance, where evaluation for teratoma and the rare occurrences of malignant transformation and growing teratoma syndrome must be considered.[10]

16.5 Conclusions

Conventional STMs are critical and established biomarkers for the diagnosis, monitoring, and treatment of patients with GCTs. However, they are significantly limited in their ability to inform management decisions across the continuum of the disease from diagnosis to treatment of advanced-stage disease to post-treatment surveillance. There is a growing body of literature implicating the clinical utility of serum miRNAs in diagnosing and monitoring GCT patients.[26,28,43–48] Furthermore, two large clinical trials [AGCT1531 (NCT03067181), open and accruing; and SWOG-S1823, expected to open soon] further study the role of miRNAs in patients with GCTs. While the utility of miRNAs in identifying viable GCT has been supported by several studies, their application in identifying pure teratoma remains elusive.

The ability to differentiate teratoma from viable GCT has beneficial implications with respect to recommending surgery or chemotherapy. Hence, identification of additional markers is needed to better distinguish teratoma—a classically chemo-resistant tumour—from benign processes. Prior to implementation of GCT-associated miRNAs into routine clinical practice, methods for collecting and processing blood samples must be standardized, quantification and cutoff values established, normalization optimized, and large-scale validation must occur.[10,49,50]

Notwithstanding, serum miRNAs have the potentially to usher in an era of precision medicine for patients with GCTs across the disease spectrum.

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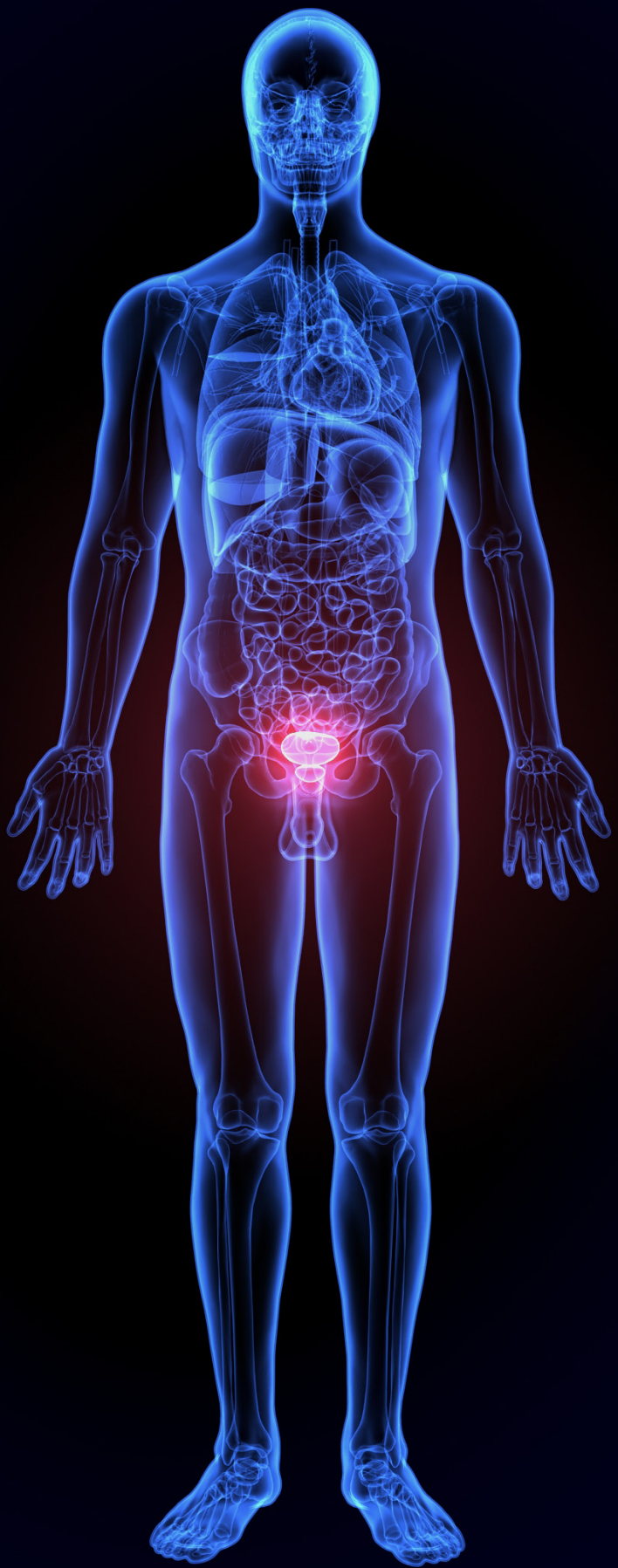

CHAPTER 17

Upper Tract Urothelial Carcinoma

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Table of Contents

17.1 Introduction

Upper tract urothelial carcinoma (UTUC) is a rare disease with a significantly lower prevalence than bladder urothelial carcinoma, resulting in an annual incidence of approximately 2 cases per 100,000 person-years in western countries.[1,2] In advanced-disease stages, UTUC has a poor prognosis, with a 5-year cancer-related survival of <50% for pT2/pT3 tumours and only <10% for pT4 tumours.[3] UTUC shares various common risk factors with bladder cancer (BCa), such as tobacco smoking or use of the analgesic phenacetin, albeit with a 6-fold higher risk for UTUC than for bladder cancer.[4,5] There are, however, specific etiologies that are unique to UTUC, such as exposure to aristolochic acid—which is caused by either oral intake of Chinese herbal medication or contaminated wheat, that is, in Balkan-endemic nephropathy—characterized by degenerative interstitial nephropathy, with an approximately 200-fold increased risk for UTUC compared to bladder cancer.[6,7] There is also strong evidence for differences in a genetic predisposition to the diseases, primarily observed in patients with Lynch syndrome. Lynch syndrome is caused by germline mutations in genes related to the DNA mismatch repair system, which leads to increased microsatellite instability (MSI). While MSI is rarely encountered in bladder cancer, it has been shown to occur in up to 30% of UTUC cases.[8–10] These differences support the view that UTUC should be considered a separate morphologic and pathologic entity with a partly shared but partly also diverging molecular biology compared to bladder cancer.

Regarding molecular characterization studies, initial molecular profiling reports have—due to low UTUC incidence—mainly focused on the molecular pathways and carcinogenesis present in bladder cancer. Data from The Cancer Genome Atlas (TCGA) in high-grade and invasive urothelial cancers of the bladder (UCB) has served as a framework for comparative evaluation of cases with isolated primary upper tract disease.[11,12] However, high-throughput molecular sequencing techniques and proteomic studies conducted in the past few years have included UTUC tumours and expanded our understanding of UTUC pathobiology and its relationship to bladder cancer.[13–15] A deeper understanding of the similar and divergent molecular pathways involved in the two entities could potentially help tailor disease-specific and patient-specific targeted precision therapies. Thus, whereas previous studies mainly focused on selected genes or biomarkers in UTUC, the past few years have provided a more comprehensive understanding of the molecular processes present in the disease, which now makes it possible to compare bladder cancer and UTUC on a molecular level.

A point worth emphasizing in this regard is the importance of case-mix and selection in these series, particularly the use of high-grade, predominantly invasive cancers in the published UCB TCGA studies with missing data on preceding or concomitant history of upper tract disease in these cohorts.[16] This factor underscores the issue of possible misrepresentation in some series from the possible spread of tumours originating in the upper tract that had seeded into the bladder and *vice versa*—where tumours from the bladder may be seeded into the upper tract by instrumentation or migration into the lower ureter. These distinctions may have implications for understanding the pathogenesis of these cancers and have to be considered when evaluating differences in genomic alteration between tumours arising *de novo* at these sites.

In this chapter, we will review some of the key UC molecular data specific to upper tract urothelial cancers and describe potential applications in clinical management.

17.2 Mutational Profiling Studies

Mutational profiling studies performed so far comprise sequencing studies of selected cancer-associated genes as well as comprehensive whole-exome sequencing (WES) of primary and matched metastatic tissue.[13–15,17–21] A similar set of genomic alterations observed for UTUC has been reported across these studies, suggesting a subtle but important difference in biology associated with these cancers. The alterations detected comprised *FGFR3*, *TP53/MDM2*, chromatin remodellers such as *KMT2D* or *KDM6A*, and other tumour suppressors/oncogenes such as *CDKN2A* or *RAS*. Noteworthy, the prevalence rates of certain mutations, such as *FGFR3* and *TP53/MDM2,* were associated with tumour grade.[13–15] Using a custom next-generation sequencing (NGS) assay interrogating the mutational status of 300 genes, Sfakianos *et al.* reported a higher prevalence of activating *FGFR3* alterations in low-grade compared with high-grade tumours (92% vs 35.6%).[15] This finding was confirmed by a WES study of primary tissue by Moss *et al.* and a targeted sequencing study interrogating 468 genes and including the largest sample size of UTUC tumours by Audenet *et al.*[13,14] WES data of the 31 samples by Moss *et al.* reported *FGFR3* mutations in 92% of low-grade and 60% of high-grade tumours and *TP53* mutations in 22.2% of tumours. Additionally, *FGFR3* and *TP53/MDM2* mutations tended to be mutually exclusive, with the latter being enriched in high-grade tumours and circulating tumour DNA (ctDNA) from metastatic UTUC patients.[13,18] The presence of *TP53/MDM2* mutations correlated with a higher degree of genomic instability, a higher frequency of copy number alterations, and worse clinical outcomes. In contrast, mutations in *FGFR3* were associated with a lower frequency in ctDNA from metastatic patients than in primary tissue. They were associated with better outcomes compared to tumours with mutations in *TP53/MDM2*.[18,22] Other genes with a difference in mutation frequencies between low- and high-grade tumours comprised *HRAS* (13.6% vs 1.0%), *CDKN2B* (15.3% vs 3.9%), *RB1* (0% vs 18.6%), and *ARID1A* (13.6% vs 27.5%). Alterations in chromatin remodellers did not seem to correlate with low- or high-grade disease.[15] Interestingly, *FGFR3* mutations among high-grade tumours were also associated with lower-stage (pTa/pT1/pT2) disease, whereas *TP53*, *CCND1*, *ERBB2*, *ERBB3*, and *KRAS* mutations were more common in >pT2 cancers.

Assessing the genomic profiles of UTUC tumours of patients with BCa recurrences, Audenet *et al.* reported a higher incidence of recurrent BCa in the case of *FGFR3*, *KDM6A*, and *CCND1* mutations. In contrast, the presence of *TP53* mutations in UTUC was associated with a lower incidence. Also, BCa recurrences and previous UTUC tumours within the same patient were clonally related to each other, strongly supporting a drop-down implantation pathway of recurrence.[14]

The genomic profiles in UTUC have also been shown to impact cancer-specific survival. In a multivariate analysis of 83 UTUC cases managed at a single institution, alterations in *TP53/MDM2* and *CCND1* were both independently associated with metastatic progression and disease-specific mortality. In contrast, mutations in *FGFR3* were associated with a lower risk for progression and death.[22] These data were used to generate a simplified model, stratifying patients into three risk categories based on mutational profile: low risk including *FGFR3* mutations, high risk including *TP53/MDM2* alterations, and intermediate risk including all other cases. This model predicted the risk for metastatic progression and disease-specific survival.

Studies comparing mutational profiles of UTUC and BCa reported a similar mutational landscape, with a difference only in a small number of genes.[14,15,21] Among the top mutated genes in both entities were *TP53*, *FGFR3*, *APC*, and chromatin remodellers. In the largest number of UTUC and BCa cases (195 UTUC and 454 BCa), Audenet *et al.* found lower alteration frequencies in *TP53*, *RB1*, and *ERBB2* in UTUC (26% vs 46%, 3% vs 20%, 8% vs 19%), whereas alterations in *FGFR3* and *HRAS* were more frequent in UTUC compared to BCa (40% vs 26%, 12% vs 4%).[14] In particular, high-grade UTUC samples harboured a higher frequency of *FGFR3* alterations than high-grade BCa samples (31% vs 21%), a finding consistent with several other studies addressing high-grade UTUC and high-grade BCa.[14,19] Regarding gene fusions, recurrent in-frame activating *FGFR3-TACC3* fusions were found in UTUC and BCa, whereas the *SH3KBP1-CNTNAP5* fusion was reported only in UTUC.[13,15,18] Noteworthy, tumour mutational burden was lower in high-grade UTUC than in BCa samples, which was also observed in metastatic BCa compared to metastatic UTUC samples obtained by rapid autopsy.[19,20] WES studies confirmed the APOBEC mutational signature, C>T transitions at CpG dinucleotides, and *ERCC2*/nucleotide excision repair (NER) and mismatch repair (MMR) signatures related to defective nucleotide excision repair as the most relevant mutational processes in UTUC.[13,19,20] Furthermore, based on an integrated analysis of tumour mutational burden, MSIsensor score, and mutational signature, only 7.2% of UTUC tumours were classified as MSI high/MMR deficient.[14]

17.3 RNA Profiling Studies, Molecular Subtypes, and Immune Environment

In recent years, large-scale genomic expression profiling identified several molecular subtypes in BCa. To date, four major classifiers have been used to group the BCa patients, including the i) University of Lund, ii) University of North Carolina (UNC), and iii) MD Anderson Cancer Center (MDACC) groups, and iv) TCGA classification using cDNA microarray or RNAseq.[23,24] The two defining features of these classifications are luminal and basal components that are analogous to the intrinsic breast subtypes. However, those studies mostly did not include UTUC. Another limitation regarding gene expression studies in UTUC is that most of the RNAseq studies focusing on UTUC are limited by sample size, with only three studies reporting data on more than 25 patients.[13,19,25] Based on these studies, several approaches were adopted to investigate the molecular features of UTUC related to gene expression profiling. The first approach used unsupervised consensus clustering of RNAseq expression data to segregate patients based on clinical variables such as grade, stage, survival outcomes. Applying this approach to RNAseq data from 28 patients, Moss *et al.* showed that UTUC is segregated into four molecular subtypes.[13] Cluster 1 had no *PIK3CA* mutations with enrichment of high-grade non-muscle invasive UTUC. Cluster 2 had 100% *FGFR3* mutations and was associated with a more favourable phenotype (low-grade, non-muscle invasive UTUC, no bladder recurrences). Cluster 3 also had 100% *FGFR3* mutations and was enriched for non-muscle invasive UTUC but with a high rate of bladder recurrences. The distinct feature of cluster 3 was 71% of mutations and no *TP53* mutations. Cluster 4 had the most aggressive phenotype (high-grade, muscle-invasive UTUC, shorter survival), with half of the patients harbouring mutations in *KMT2D*, *FGFR3*, and *TP53*.[13]

Another study used gene expression profiling to distinguish UTUC from its anatomic counterpart, renal cell carcinoma (RCC). Comparing the gene expression profile of 17 patients with collecting duct carcinoma (CDC) to 9 patients with muscle-invasive UTUC, Malouf *et al.* showed that CDC had a basal-like gene signature compared to UTUC, which had a luminal-like signature.[26] The study confirmed that GATA3, P63, and UPK are useful markers to differentiate UTUC from CDC.

The third study aimed to delineate UTUC from its biological counterpart, BCa. Robinson *et al.* compiled an RNAseq meta-dataset from 32 high-grade UTUC tumours and the TCGA BCa.[19] Using the (UNC) 47-gene signature (BASE47) classifier, most of UTUC clustered to luminal subtype compared to BCa (84.3% vs 64.1%). Similar results were obtained using the MDACC and TCGA classifiers. Furthermore, the authors characterized the immune contexture of UTUC through applying a 170-gene classifier comprising essential immune genes. Most of UTUC tumours were T-cell depleted (87.5%), with downregulated T-cell–related and interferon-γ (IFN-γ) signalling genes. Meanwhile, the BCa tumours were evenly distributed into T-cell–inflamed, and T-cell–depleted subtypes. Interestingly, *FGFR3* was found to be highly expressed in T-cell–depleted UTUC tumours. Based on mechanistic studies in cell lines, the authors showed that FGFR3 signalling shapes the immune-depleted phenotype in UTUC, and its inhibition could reverse this phenotype. This data supported the rationale for commencing a phase 3, placebo-controlled clinical trial, PROOF302, testing infigratinib in the adjuvant setting for patients with UTUC harbouring *FGFR3* genomic alterations (**NCT04197986**).

Other orthogonal gene expression analyses comparing UTUC and BCa confirmed that the luminal-papillary component is the defining hallmark of UTUC.[25,27] The enrichment of the luminal subtype in UTUC was further confirmed in a study analyzing 80 UTUC samples; 70 (87.5%) of these 80 tumours had a luminal phenotype, and only 10 (12.5%) could be assigned to the basal phenotype. The consensus classifier developed by the Bladder Cancer Molecular Taxonomy Group16 for muscle-invasive BCa also assigned 66 tumours (82.5%) to the luminal-papillary subtype, and 7 tumours (8.75%) to the luminal-unstable, 1 tumour (1.25%) to the luminal-nonspecific, 1 tumour (1.25%) to the stromal-like, and 5 tumours (6.25%) to the basal/squamous types.[25]

In a study by Sanford *et al.* on 22 UTUC and 19 BCa tumours, there was evidence of differential clustering among pT3 tumours and significant gene expression differences in 81 genes. Pathway analysis revealed differences in hepatocyte growth factor (HGF) and TNF signalling pathways. Again, this study reported that UTUC tended to have a high expression of genes associated with the luminal subtype.[27] Taken together, these studies confirm the enrichment of the luminal subtype in UTUC as a defining feature of the biology of UTUC.

Gene expression profiling was also used as a tool to identify targetable drugs in several other reports. The above-mentioned study compared the gene expression profile of 12 UTUC and 20 BCa tumours and showed that UTUC tumours expressed an increased number of genes belonging to the luminal subtype, and one of these genes, *SLITRK6*, was used as a drug target for the antibody-drug conjugate AGS15.[27,28] It is worth noting that an early study that compared UTUC to BCa in a small cohort (14 vs 10 patients) failed to show any significant gene expression differences between UTUC and BCa using unsupervised clustering, suggesting a sample size limitation rather than an inherent biologic attribute.[29] Also, another study failed to show distinct gene expression

patterns between 22 Lynch syndrome–associated UTUC and 19 BCa samples.[30] This study used unsupervised hierarchical clustering to group 41 Lynch syndrome–associated tumours into three clusters. Most UTUC tumours (65%) grouped in cluster 1.

Another advantage of gene expression profiling is to identify differentially expressed genes as potential diagnostic biomarkers. Over- or underexpressed genes can be immunohistochemically analyzed at the protein level, and several studies identified promising markers, such as FXYD3, after prior gene expression screening.[31] Some of these biomarkers had a prognostic value; for instance, the low protein expression of ALDH2 and high expression of CCNE1/SMAD3 were associated with shorter survival in a cohort of 103 patients with UTUC.[32] Similarly, low CK5/6 and high CK20 expression were associated with shorter survival in 15 patients with non-muscle–invasive papillary high-grade UTUC.[33] Further biomarkers are reviewed by Lie *et al.*[34] Another aspect that is significantly understudied is linking gene expression patterns to the tumour microenvironment. One study showed that expression of melanoma-associated antigen A (MAGE-A), a promising target for vaccine-based immunotherapy, is associated with infiltration of CD3-, CD8-, and CD45RO-positive T lymphocytes.[35]

17.4 Relationship with Other Diseases

17.4.1 Lynch syndrome and UTUC

Familial risk factors include Lynch syndrome (LS), also called hereditary nonpolyposis colorectal cancer (HNPCC), which predisposes patients, typically at a younger age, to developing synchronous and metachronous cancers in several organ systems. These include gastrointestinal, gynecologic (endometrium and ovaries), brain (glioblastoma), skin, and urothelial cancers, especially UTUC. UTUC is the third most common form of malignancy in LS patients and a significant cause for morbidity related to functional renal loss secondary to the often necessary surgery.[10,36] LS is characterized by inactivating germline mutations involving DNA mismatch repair (MMR) genes. Commonly identified alterations involve *MLH1*, *MSH2*, *MSH6*, or *PMS2*, as well as *EPCAM*, which affects methylation and silencing of *MSH2*. Aberrations in MMR gene function impairs DNA repair, resulting in accumulated DNA errors typically demonstrated by hypermutated profiles of LS cancers and MSI.[37] The strong association of these features with LS has led to recommendations for reflex testing of MMR protein by immunohistochemistry or evaluation for MSI in all LS-associated tumours in younger patients to identify affected individuals in order to provide genetic counselling and appropriate disease screening. While helpful, MMR immunohistochemistry and MSI scoring are not definitive, and additional germline testing may be appropriate, as some LS-affected individuals can be missed and Lynch-like cases may be misinterpreted.[8,38] In Lynch-like disorders, patients demonstrate the LS phenotype including MSI-high tumours, yet germline testing is negative. While not completely characterized, a number of processes may explain these findings including somatic mutations in MMR genes, heritable mutations impacting epigenetic gene silencing, germline mosaicism, or other undiscovered MMR-like alterations.[39] These patients, and potentially related kindreds, may be at risk for LS malignancies and stand to benefit from LS management strategies. However, it should be pointed out that studies reporting somatic MMR deficiency in UTUC without germline testing should be viewed critically, given

that a study by Robinson *et al.* reported that low mRNA and protein levels of MMR genes detected in sporadic UTUC without germline mutations were not associated with the presence of MSI and that somatic downregulation of MMR expression does not induce a hypermutated phenotype in non-Lynch UTUC patients.[19]

A study of UTUC tumours from patients with known LS assessed genomic mutations of 341 genes in 17 patients with confirmed LS and compared these mutations to those detected in sporadic UTUC.[15,40] Patients with LS-UTUC were significantly younger, had had less exposure to tobacco, and presented more frequently with a ureteral primary site compared with patients with sporadic UTUC. The median number of somatically altered genes per case was 47 (range, 9–204) with a high MSIsensor score (median, 25; range, 6–38) and thus significantly higher in LS-UTUC tumours than in tumours from the sporadic cohort. Although the genetic landscape was comparable between the two cohorts, certain differences were observed. For example, alterations in *KMT2D*, *CREBBP*, or *ARID1A* or in DNA damage response and repair genes were present at a significantly higher frequency in LS-UTUC (94% vs 23%). Notably, 5 of 11 amplification events involved *CDKN1B*, and *CIC*, *NOTCH1*, *NOTCH3*, *RB1*, and *CDKN1B* alterations were almost exclusive to LS-UTUC. Furthermore, LS-UTUC had a similar frequency of *FGFR3* mutations as sporadic UTUC, but it demonstrated a high rate of the specific hotspot point mutation at *R248C* (84%), which was not seen in sporadic cases. This particular mutation in UTUC appears to be highly specific to LS-affected individuals and could serve as a potential biomarker for the disease.

17.4.2 Balkan endemic nephropathy and aristolochic acid–induced UTUC

One of the most well-described endemic factors predisposing to UTUC is exposure to the phytochemical aristolochic acid, common in parts of Asia (also known as Chinese Herb Nephropathy) through use of herbal supplements such as birthwort or by contamination of wheat crops by the plant *Aristolochia clematitis* in the case of Balkan endemic nephropathy. Cumulative exposure to aristolochic acid results in a tubulointerstitial disease causing eventually renal dysfunction and often UTUC.[41,42] The disease is much more common in the Balkans and Asia, particularly Taiwan, and rare in western countries. The causative component, aristolochic acid, is a nephrotoxic carcinogen, which interacts with DNA to form dA-aristolactam, and dG-aristolactam adducts. These adducts accumulate in renal tissue and produce typical A:T→T:A transversions, leading to a distinct mutational pattern compared to sporadic UTUC.[43–45] Additionally, sequencing studies of AA-induced UTUC samples showed a higher number of mutations per sample than in sporadic cases.[43] Also, a signature related to age and a signature related to APOBEC enzymes could be identified.[45] The most frequently mutated gene was *TP53*, whereas *FGFR3* mutations were detected to a lower degree and were not A>T transversions.[43–45]

17.5 Translating Research Studies into Biomarkers for Clinical Trials

Translating genetic information into clinical decision-making is slowly being integrated as risk-based strategies are being developed. For this, reliability in obtaining sufficient material from endoscopic biopsies for representative sequencing from UTUC is necessary. This has been demonstrated in a recent study that tested the feasibility of performing NGS on biopsies taken during diagnostic ureteroscopy. The study confirmed that matched biopsy and radical nephroureterectomy (RNU) specimens were comparable regarding the presence and prevalence of genomic alterations, which would provide means for adopting routine genomic testing, along with histology, for developing prognostic and predictive data.[17,46] A recent development that enables testing the actionability of mutational events is the use of patient-derived xenograft (PDX) models in nude mice, which have recently been established from UTUC patients and can serve as models to study chemosensitivity and associated molecular alterations. Using these models, the authors tested the sensitivity of a UTUC-PDX with a hotspot mutation in *ERBB2* (*HER2 S310F*) to neratinib, a HER2 kinase inhibitor, and to a HER2 antibody topoisomerase I inhibitor drug conjugate. They were able to show that the antibody-drug conjugate was more effective than the selective HER kinase inhibitor at inhibition of UTUC-PDX growth25.

Other important targets in UTUC include *FGFR3*, which appears to play a major role in tumour pathogenesis in both low- and high-grade cancers. Clinical trials with FGFR3 inhibitors such as erdafitinib or pemigatinib are just now starting to include UTUC cancers in their eligibility criteria.[47,48] It will also be interesting to test whether inhibition of activated FGFR3 signalling will affect the immune system, given that *in vitro* studies have shown that inhibition of FGFR3 signalling can lead to immune cell activation and reverse the T-cell–depleted phenotype.[19] On the other hand, tumours with *TP53/MDM2* mutations often display a mutator phenotype and higher genomic instability, which may make them more responsive to chemotherapy or immunotherapy. Additional genomic biomarkers include *ERCC2* or other genes involved in DNA damage response, which have been shown to predict platinum sensitivity in UC.[49] In concordance with findings in MSI-high colon cancer, a distinct UTUC subtype that may show promising responses to immunotherapy is MSI-high tumours in patients with LS. In UTUC tumours due to exposure to aristolochic acid, however, the main mutational processes involve *TP53* mutations and are associated with a higher mutational load, which could also indicate a higher benefit from chemotherapy or checkpoint inhibitor therapy. In all of these cases, the application of consensus classifiers to determine UTUC mRNA expression subtypes could be used for understanding patterns of gene expression and for stratification before determining therapeutic approaches.[25,50]

In the past few years, we have learned much about the biology underlying the carcinogenesis and clinical behaviour of UTUC, the molecular drivers shaping UTUC expression patterns, and the different aspects of extrinsic and intrinsic factors contributing to disease development and progression. Translating these biological insights into therapeutic approaches will advance the field toward true precision medicine for UTUC patients.

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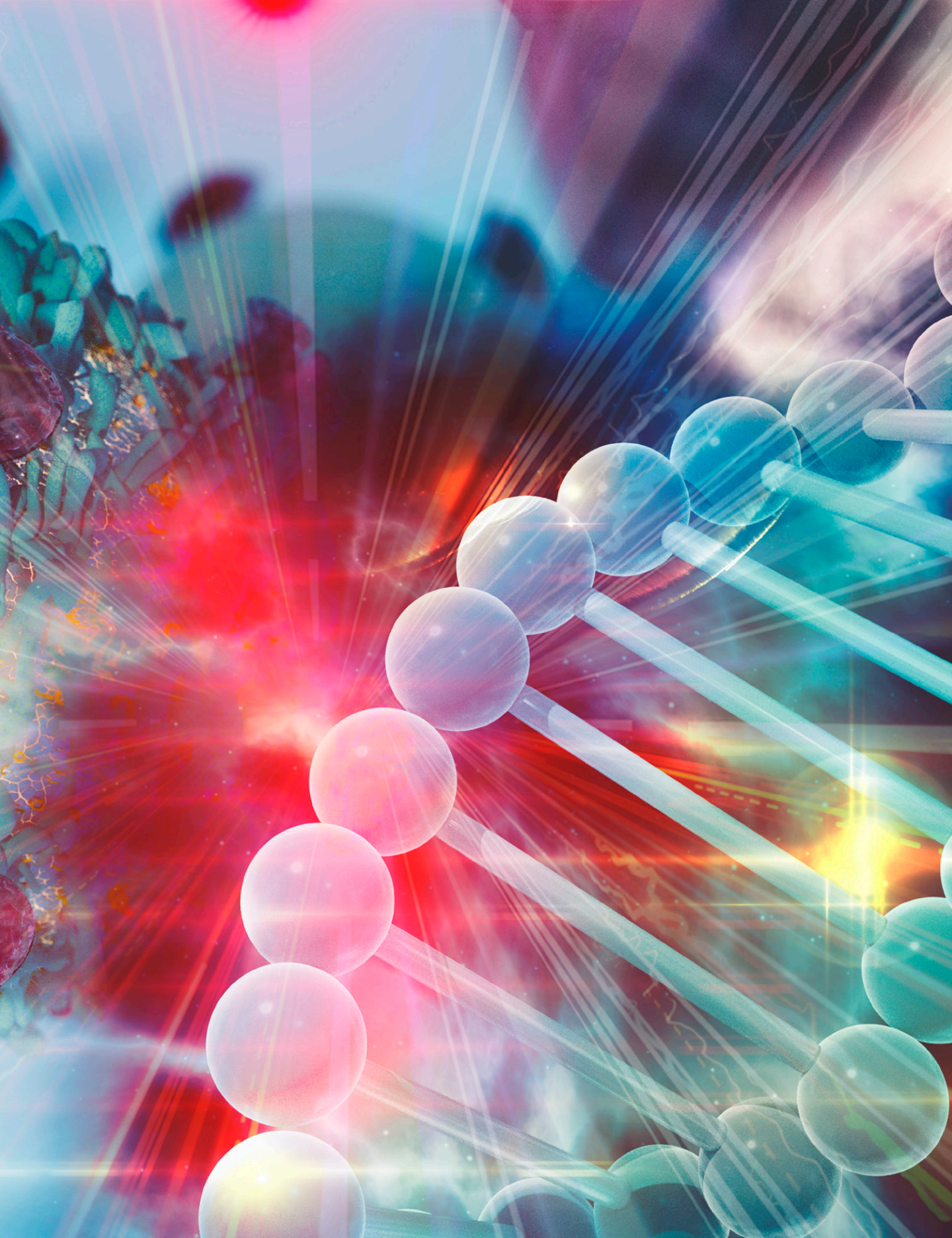

CHAPTER 18

The Future of Biomarkers in Urologic Oncology

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Table of Contents

18.1 The Future of Biomarkers in Urologic Oncology

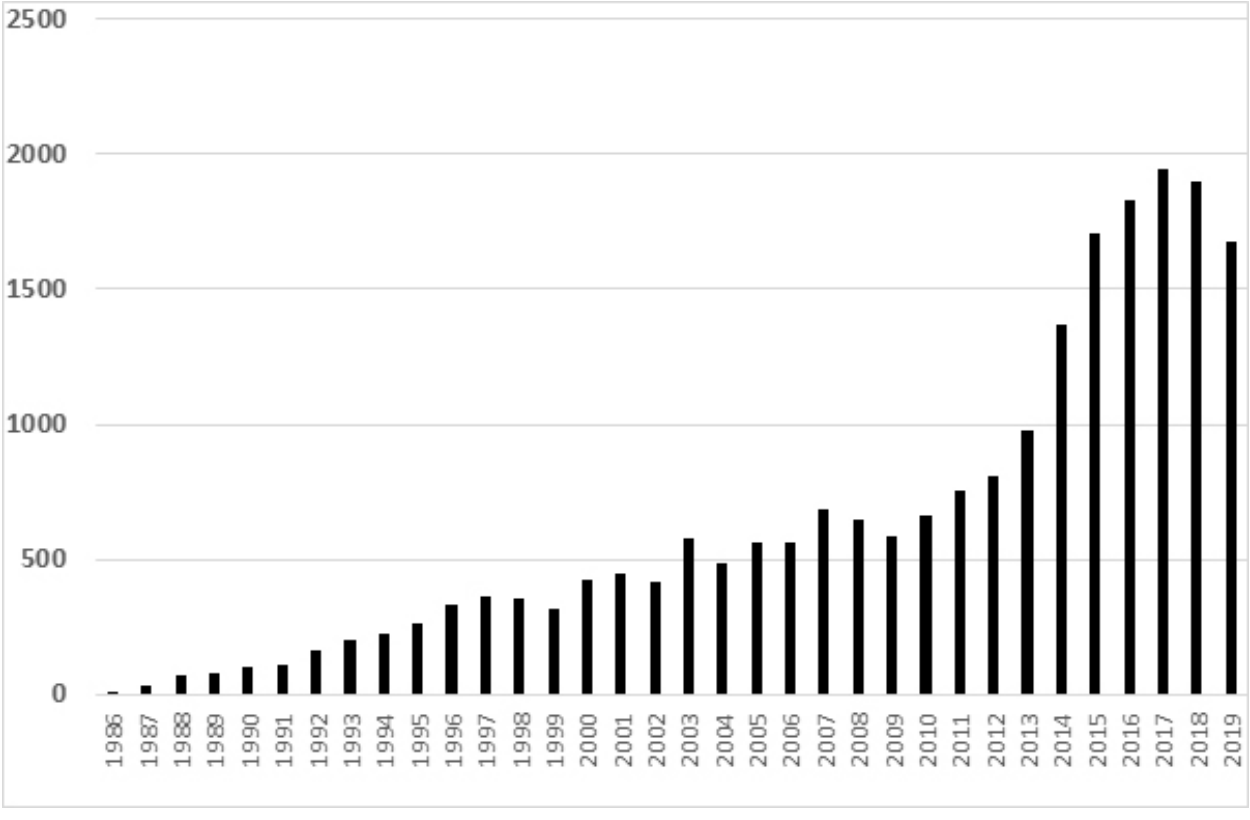
Officially, a biomarker refers to a quantifiable biological parameter that is measured and evaluated as an indicator of normal biological, pathogenic, or pharmacologic responses to a therapeutic intervention, as defined by the National Institutes of Health.[1] Accordingly, glomerular filtration rate, repeat blood pressure readings, hemoglobin A_{1C}, and gene expression profiling are all examples of "biomarkers." When used in translational research discussions, the term itself often alludes to a marker used to accelerate or aid in diagnosis or monitoring and provide insight into "personalized" medicine.[2]

In past decades, biomarkers traditionally were the domain of complex research studies seemingly struggling to make the transition from the laboratory to the clinic. Many contended that translational research was at its best—yet with apparent limited impact. While there was hope, there were few tangible benefits to a patient in your clinic who you would be seeing the following week after a conference where another candidate biomarker appeared promising to enter practice.[3]

Researchers were not deterred. Over a 25-year period from 1986 to 2009 almost 30,000 grants were awarded to studies funded by the National Institutes of Health in the United States with the title "biomarker" in them.[4] Fast forward to 2020 and this ICUD book demonstrates how far we have come in urologic oncology and where we could realistically be going with biomarkers. The breadth of publication chapters and clinical applications currently available is certainly complex and evolving. Journals have now been dedicated to biomarkers such as *Biomarkers in Medicine* as part of the *Future Medicine* group. Biomarkers are considered by many to be the cornerstones of a preventive and personalized medicine of the future.

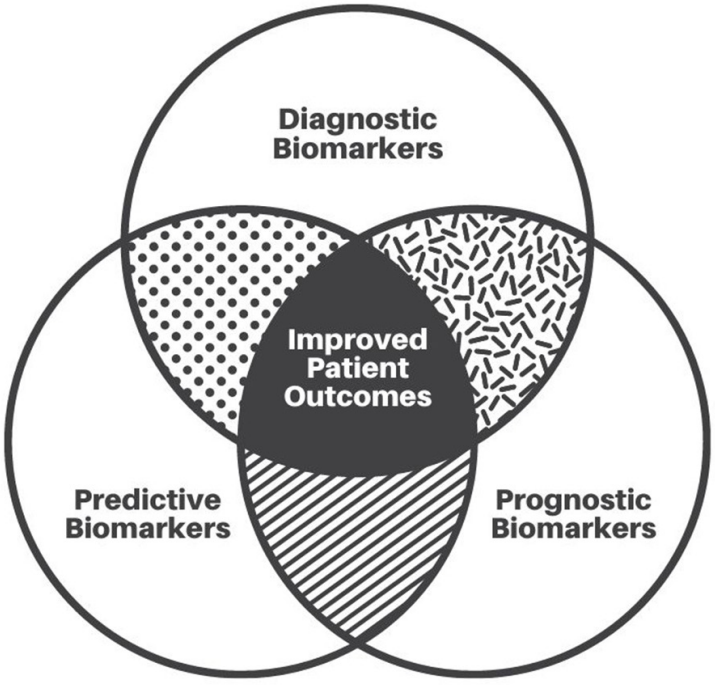
Understandably, the presentations and publications have followed. Urologic oncology has been particularly prolific (**Figure 18–1**). These studies have paved at least part of the way for individualizing cancer patient therapy based on molecular profiles. When and how to use testing and other biomarker-generating tools in the clinic is at present an evolving and unsolved issue.[5]

FIGURE 18–1 Publications in PubMed including the terms Biomarker, Urology, and Oncology from 1986 to 2019



Put simply, biomarkers may be summarized in three broad groups: Diagnostic, Predictive, and Prognostic. As outlined in **Figure 18–2**, there is overlap among all three subtypes, but a common goal is to improve outcomes in all of our patients.

FIGURE 18–2 The Interface Between Diagnostic, Predictive, and Prognostic Biomarkers (*Adapted from Kisluk J, Ciborowski M, Niemira M,* et al. *Proteomics biomarkers for non-small cell lung cancer*. J Pharm Biomed Anal. *2014;101:40–49. doi:10.1016/j.jpba.2014.07.038*[6])



Diagnostic biomarkers	-Directly correlate with disease presence -Should be the most specific and sensitive with early-stage disease -Developed form large cohorts of clinical examples
Predictive Biomarkers	-Useful to indicate treatment response -For predicting positive or negative responses to new treatments
Prognostic Biomarkers	-Should correlate with disease recurrence and survival data -Easy to determine; may be repeated at different time points

18.2 Diagnostic Biomarkers

It may be difficult to accept in 2020 but the best-known cancer biomarker that remains in widespread clinical use in oncology is prostate-specific antigen (PSA). PSA has been used by clinicians to detect early disease. Somewhat controversially, serum PSA has been widely used in screening for prostate cancer in the past decade, and has brought about a dramatic increase in early detection of the disease.[7] In the early days of its use, the upper limit of a normal PSA level was considered to be 4 ng/mL.[7,8] This was not age-adjusted and failed to take into account PSA velocity, PSA density, and other parameters such as the free : total PSA ratio. While debate still rages as to the utility of measuring PSA in asymptomatic men, it has brought about modest declines in mortality yet extremely significant reductions in rates of men presenting with metastatic disease.[9]

To bolster PSA as a screening tool we now have imaging as well as other biomarkers presented in this book. Despite progress, the very premise of PSA screening remains challenging with conflicting randomized trials to blame. For example, the European Randomized Study of Screening for Prostate Cancer (ERSPC) trial showed a survival benefit while the Prostate, Lung, Colorectal and Ovarian (PLCO) trial did not; but the PLCO was plagued by contamination issues that were blamed for its failure. Of course, we are still searching for reliable diagnostic biomarkers for urothelial cancer and have made some progress with renal tumours particularly via imaging and biopsy. Testicular and penile cancer have relied on self-examination and early presentation, which has not changed greatly in the biomarker era.

Likely, for diagnosis there will be a transition for some urologic cancers to the "liquid biopsy," which is the use of anything extracted from a bodily fluid, that could add significant clinical value. This noninvasive, or minimally invasive, biomarker testing could allow for rapid, economical, and repeat evaluation.[10] This repeat sampling feature would thus allow for the patient with high potential for a particular disease to self-sample urine and saliva or for clinical sampling of serum/plasma or whole blood.[2] To date, most of the liquid biopsy research has focused on the rare circulating tumour cells, but as this book has demonstrated, there are many more targets—from proteins to antibodies and RNA fragments.

Imaging will move to a yet more important element of diagnosis and directing who should get further interrogation. Screening with imaging becomes a real possibility.

18.3 Predictive Biomarkers

Predictive markers have taken on a greater importance, as investigations into biomarkers are aligned with providing personalized medicine. The concept seems easy—test tumour for biomarker, biomarker represents a target that can be attached by a matching agent, therefore yielding better outcomes. This is the opposite of a broader systemic therapies approach. But as programmed cell death 1 / programmed cell death 1 ligand 1 (PD-1 / PDL1) inhibitors have taught us, the expression and responses to biomarkers are not always linear.

Hence, translation of these findings into improved patient treatments will require clinical validation of potential biomarkers within well-defined patient cohorts and subsequent biomarker measurement within large patient groups to ensure that developed biomarker tests are robust. High-throughput methods of biomarker quantification and validation, including targeted mass spectrometry, are beginning to bridge this gap. There is thus considerable potential for molecular analysis methods to improve patient treatment outcomes in the future.[11]

Clinical research is evolving rapidly, from basket and umbrella trials to adaptive design precision oncology clinical studies, and genomic and molecular data often displace the classical clinical validation procedures of biomarkers. In this context, physicians must be aware of the clinical evidence behind these new biomarkers and "next-generation sequencing" tests available, in order to use these predictive tools in the right moment, and with a critical point of view.[5]

18.4 Prognostic Biomarkers

Although prognostic markers may seem to be the least important group compared to diagnostic and predictive markers, in many ways they will shape a patient's cancer journey. There are important decisions regarding use of neoadjuvant and adjuvant therapies that are made based on likelihood for micrometastatic disease. Clinical staging is often inadequate to assess "true" disease stage or outcomes. Biomarkers may improve identification of which patient might benefit from additional or multimodal treatment approaches and may spare some patients from toxic therapy. If first- and second-line therapies are now failing, then a patient should return to be tested for newer predictive markers with matched therapies and hopefully the cycle of effective treatment can then continue—to turn malignancies that cannot be cured into chronic diseases.

18.5 The Future

Predicting the future is always a gamble in medicine. Will imaging spearhead biomarkers and be the complete answer for diagnosis, prognosis, and prediction? Likely not—it will be combinations of modalities from the least invasive such as liquid biopsy through to targeted biopsies and intraoperative imaging that will all play a role. Pathway-based tests rather than single-marker diagnostics will be the wave of the future, enabling the delivery of the right drug to the right patient at the right time. An expansion of technologies capable of providing information on multiple markers that are altered in cancer prior to or after therapy will have the greatest impact.[12]

New technology and access to huge patient databases are providing new biomarker options and the focus is shifting to combinations of several or multiple biomarkers rather than the single markers that research has concentrated on in the past. Biomarkers will increasingly be used as part of routine clinical practice in the future, complementing clinical examination and physician expertise to provide accurate disease diagnosis, prediction of complications, personalized treatment guidance, and prognosis.[13]

Funding of exploratory markers will be inextricably linked to therapies that can exploit patients suitable for those treatments. Undoubtedly the private sector will continue to be also attracted by biomarkers where they are regarded as a possible solution to the paucity of research and development productivity in the pharmaceutical sector. Many biotech companies are already pursuing their use or offer specialized screening services.[4] Only then will we truly have the personalized medicine we desire for better patient outcomes.

In summary, clinicians ignore biomarkers at their peril but instead must embrace them and be at the forefront of trials to ascertain the utility of each biomarker in diagnosis, prediction, and prognosis. The sheer weight of numbers of candidate biomarkers is the only barrier, and resources that can distil biomarkers into their rightful position such as this ICUD book become documents of reference for anyone involved in urologic oncology—accepting that the field will move and change but many of the principles will remain sound.

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1st ICUD-WUOF International Consultation: Molecular Biomarkers in Urologic Oncology

Optimal diagnosis and management of urologic malignancies necessitates the use of noninvasive tests and methodologies. The ready availability of blood, urine, and tissue-based samples from patients has tremendous potential to enhance our ability to predict risk and natural history and improve the management of urologic cancers.

This international consultation on Molecular Biomarkers in Urologic Oncology represents the first effort of the World Urologic Oncology Federation (WUOF). The publication, prepared in the format of an e-book, reviews the current state of the art of molecular biomarkers for the diagnosis and management of urologic malignancies. It reviews the literature on the current role of these biomarkers in the identification and prognostication of urologic cancers and on research efforts with the potential for future developments. For patients, researchers, and providers seeking to incorporate biomarkers into clinical practice, it is important to evaluate how well the biomarkers perform overall and how they can and should be incorporated into improved patient care.

The 1st ICUD-WUOF International Consultation on Molecular Biomarkers in Urologic Oncology is the first publication of its kind on the role of biomarkers for urologic oncology prognosis and management, presenting an invaluable resource for healthcare providers to patients with urologic malignancies. We hope you enjoy reading this book and find it useful and relevant to your practice.

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