Cellular and molecular cartography of muscle-invasive bladder cancer: Who benefits from neoadjuvant chemotherapy?

Wahlin, Sara

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SARA WAHLIN
DEPARTMENT OF CLINICAL SCIENCES LUND | FACULTY OF MEDICINE | LUND UNIVERSITY

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Who benefits from neoadjuvant chemotherapy?

Sara Wahlin

LUND UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 16th of May 2025 at 09.00 in the Belfrage Hall, Biomedical Center D15, Lund, Sweden

Faculty opponent
Professor Lars Dyrskjøt
Department of Clinical Medicine, Aarhus University, Aarhus, Denmark

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Title and subtitle: Cellular and molecular cartography of muscle-invasive bladder cancer: Who benefits from neoadjuvant chemotherapy?

Abstract:

Muscle-invasive bladder cancer (MIBC) is characterised by significant molecular heterogeneity, with the tumour composition being further sculptured by local microenvironmental influences. This inherent complexity contributes to the divergent disease trajectories and the variable therapeutic susceptibility observed among patients. Consequently, there is an evident need for prognostic and predictive biomarkers to guide clinical strategies based on the tumour's molecular fingerprint.

Standard treatment for MIBC consists of cisplatin-based neoadjuvant chemotherapy (NAC) followed by radical cystectomy. This thesis aimed to further explore tumour-cell intrinsic features and the local immune landscape to gain a deeper understanding of potential determinants of prognosis and NAC response. Tissue-based proteomic and genomic analyses on all (*n*=145) or selected (*n=15*) tumour specimens from a retrospective cohort of patients who underwent radical cystectomy with (*n*=65) or without (*n*=80) NAC were combined with *in vitro* experiments.

In *Paper I,* examination of the local immune repertoire revealed that an immune-enriched micro-environment at the time of cystectomy, characterised by elevated densities of T and B lymphocyte populations and high expression levels of the checkpoint molecules PD-1 and PD-L1, was independently associated with a reduced risk of recurrence. However, the immune contexture did not predict NAC response. *Paper II* investigated the clinical implications of the putative proto-oncogene RNA-binding motif protein 3 (RBM3). Elevated tumour-specific RBM3 expression was significantly associated with an improved clinical outcome in patients treated with NAC compared to untreated cases. The implicated link between RBM3 and chemosensitivity was corroborated by *in vitro* studies, which demonstrated decreased chemosensitivity of T24 cells following RBM3 silencing. Transcriptomic analyses provided a potential mechanistic explanation, suggesting involvement of RBM3 in cell cycle progression. *Paper III* sought to elucidate the impact of spatial intratumour heterogeneity (ITH) on therapeutic benefit. In-depth genomic, phenotypic, and immune profiling was performed across geographically separated tumour regions in 15 patients exhibiting divergent biological responses to NAC. Distinct patterns of ITH were identified, some of which correlated with NAC response. Opposing trends were observed between small-scale and chromosomal-level alterations, but with a significant proportion of optimal responses linked to DDR deficiency. Molecular subtype ITH was denoted among complete responders. Consistent with the findings of *Paper I*, the immune milieu provided limited predictive information.

In conclusion, this thesis contributes to the ongoing characterisation of the biological sum of tumour cell-intrinsic and extrinsic features, the clinical significance of which must be carefully delineated. This will become increasingly important with the advent of novel neoadjuvant treatment strategies.

Key words: Muscle-invasive bladder cancer, neoadjuvant chemotherapy, biomarkers, local immune microenvironment, RBM3, spatial intratumour heterogeneity

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Faculty of Medicine

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To all patients

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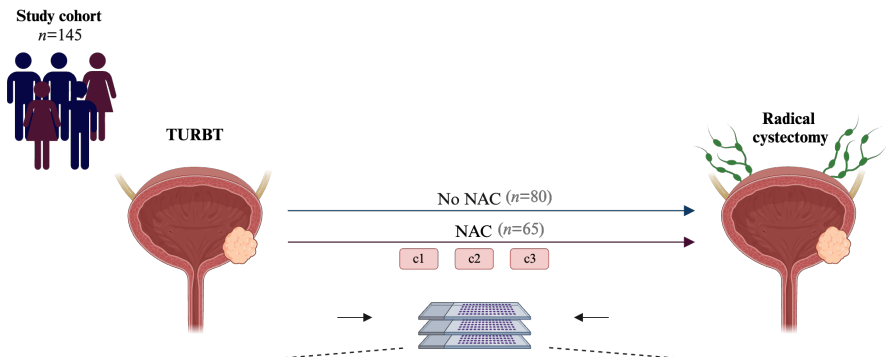
Thesis at a glance



Overarching aim

Exploration of tumour cell characteristics and the local immune landscape to delineate biological signatures of NAC response and clinical outcomes in MIBC

	Paper I	Paper II	Paper III
Objectives	Examine the clinical significance of tumour-infiltrating immune cells and the PD-1/PD-L1 axis.	Investigate the cellular properties of RBM3 and its influence on the disease course and NAC response.	Multilayered analyses of spatial intratumour heterogeneity and its implications for the therapeutic benefit of NAC.
Methodology	The densities and spatial localisation of $CD8^+$, $FoxP3^+$, $CD20^+$, $PD\text{-}1^+$, and $PD\text{-}L1^+$ cell populations evaluated in paired TURBT and cystectomy specimens using IHC.	IHC assessment of tumour-specific RBM3 expression in clinical specimens. *In vitro* functional characterisation and analyses of chemo-sensitivity.	Genomic, phenotypic, and immune profiling across spatially separated tumour domains (n=15 cases) using targeted deep sequencing, IHC, and mIF.
Conclusions	An immune-rich contexture independently correlated with a favourable prognosis but did not serve as a predictor of NAC efficacy.	Defines RBM3 as a candidate predictive biomarker of chemotherapy response, possibly through cell cycle regulation.	Spatial ITH manifests across multiple biological scales, with distinct patterns of alterations and ITH linked to NAC response.
	Published in *Oncoimmunology* (2019), 1st Author	Published in *BMC Cancer* (2022), 1st Author	Manuscript in preparation 1st Author

Abstract

Muscle-invasive bladder cancer (MIBC) is characterised by significant molecular heterogeneity, with the tumour composition being further sculptured by local micro-environmental influences. This inherent complexity contributes to the divergent disease trajectories and the variable therapeutic susceptibility observed among patients. Consequently, there is an evident need for prognostic and predictive biomarkers to guide clinical strategies based on the tumour's molecular fingerprint.

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In conclusion, this thesis contributes to the ongoing characterisation of the biological sum of tumour cell-intrinsic and extrinsic features, the clinical significance of which must be carefully delineated. This will become increasingly important with the advent of novel neoadjuvant treatment strategies.

Populärvetenskaplig sammanfattning

Urinblåsan fungerar inte bara som en tillfällig lagringsplats för urin utan utgör även en viktig skyddsbarriär som förhindrar att utsöndrade ämnen och bakterier når blodomloppet. Urinblåsans slemhinna utsätts under en livstid för långvarig exponering för substanser i urinen, varav en del kan vara skadliga. Tobaksrökens giftiga biprodukter samt yrkesmässig exponering för industrikemikalier är särskilt välkända för att kunna orsaka cellskador i urinblåsan och därigenom öka risken för cancerutveckling. Urinblåsecancer är en av de vanligast förekommande cancerformerna världen över, inklusive i Sverige, med 3 200 nya fall varje år. Antalet insjuknade har ökat över tid och denna utveckling förväntas fortsätta. Majoriteten av patienterna är vid diagnos över 70 års ålder, med en tydlig överrepresentation av män. Det första sjukdomstecknet är vanligen synligt blod i urinen vilket, med få undantag, skall föranleda snar medicinsk utredning.

Urinblåsecancer är inte *en* enhetlig sjukdom utan inrymmer ett brett spektrum av tillstånd med varierande prognos och behandlingsmöjligheter. För de flesta patienter (70-75%) upptäcks cancern i ett tidigt skede med utbredning begränsad till urinblåsans ytliga skikt. Dessa tumörer kan behandlas lokalt och prognosen är mycket god, även om det föreligger en viss risk för återfall och djupare tumörväxt framgent. Hos en fjärdedel av patienterna är sjukdomen dock avancerad redan vid diagnos, då cancern vuxit djupare genom blåsväggen in i det muskellager som omger slemhinnan och i vissa fall även spridit sig till närliggande vävnader eller andra delar av kroppen. Dessa så kallade muskelinvasiva tumörer har ett generellt aggressivt sjukdomsförlopp och kräver mer omfattande behandling, där bot oftast inte går att uppnå vid spridd sjukdom. Om muskelinvasiv cancer upptäcks i ett stadium med begränsad tumörväxt finns det dock möjlighet till botande kirurgi där urinblåsan och närliggande lymfkörtlar avlägsnas. Trots detta drabbas nära hälften av patienterna av återfall. Inför operation rekommenderas därför upprepade kurer med cellgifter, benämnd neoadjuvant behandling, i syfte att avdöda cancerceller som kan ha spridit sig utanför urinblåsan och senare ge upphov till recidiv. Neoadjuvant behandling med cellgifter har på gruppnivå visat sig förbättra överlevnaden, men är också förenad med risk för allvarliga biverkningar och endast en tredjedel av patienterna har nytta av behandlingen. Det föreligger således även inom gruppen muskelinvasiva tumörer en betydande variation vad gäller behandlingskänslighet och prognos. Detta beror främst på de distinkta biologiska egenskaper som cancerceller förvärvar över tid och som leder till att en tumör består av olika populationer av celler med varierande känslighet för cellgifter. De senaste årens metodutveckling har möjliggjort detaljerade analyser av gen- och protein-förändringar inom celler, vilket markant har ökat förståelsen för denna sjukdom. Dock är den molekylära bilden fortsatt ofullständigt kartlagd och det kvarstår ett

stort behov av att identifiera biologiska kännetecken – *biomarkörer* – av klinisk relevans som kan användas för att förutspå sjukdomsförlopp och möjliggöra en mer individanpassad behandling.

I ljuset av detta behov har syftet med denna avhandling varit att fördjupa kunskapen om både cancercellernas och immunförsvarets egenskaper vid muskelinvasiv urinblåsecancer, med särskilt fokus på betydelsen för prognos och känslighet för cellgifter. Avhandlingen omfattar tre delstudier, samtliga baserade på en retrospektiv kohort inkluderande 145 patienter som opererats vid Skånes Universitetssjukhus, varav 65 erhållit neoadjuvant behandling med cellgifter. Vävnadsanalyser har utförts på tumörprover insamlade vid diagnostillfället samt vid efterföljande operation och därefter kopplats till överlevnad och svar på behandling.

Kroppens immunsystem har en oumbärlig roll i att upptäcka och oskadliggöra sådant som är främmande, inklusive cancerceller. Detta immunsvar involverar flera olika typer av immunceller tillhörande både det medfödda och förvärvade immunförsvaret. Trots detta lyckas ibland skadade celler undkomma immunförsvarets kontroll varpå tumörer kan uppstå. Därtill har tumörceller förmågan att manipulera immunförsvaret för egen vinning och skapa en tumörfrämjande omgivande miljö. Muskelinvasiv urinblåsecancer är en av flertalet cancerformer där immunförsvaret har betydelse för prognosen och det kan även påverka tumörernas känslighet för cellgifter. I *delarbete I* undersöktes därför betydelsen av tumörinfiltration av olika typer av immunceller tillhörande det förvärvade immunförsvaret. Detta gjordes genom att färga in vävnaden med specifika antikroppar mot dessa celler, en metod kallad immunhistokemi, så att de sedan kan räknas i mikroskop. Resultaten visade att höga nivåer av samtliga analyserade immunceller var en gynnsam prognostisk faktor, med lägre risk för återfall efter kirurgi oberoende av andra kända prognostiska faktorer såsom ålder, kön, tumörstadium och neoadjuvant behandling. Vi utvärderade även om nivåerna av immunceller påverkade hur tumörerna svarade på cellgiftsbehandling, men såg ingen sådan koppling. Dessa resultat ger ytterligare stöd för att analys av immunceller kan bidra till en bättre bedömning av prognosen vid muskelinvasiv urinblåsecancer.

För att vidare undersöka potentiella biomarkörer för prognos och svar på cellgiftsbehandling fokuserade vi i *delarbete II* på ett protein – RNA-binding motif protein 3 (RBM3) – som har visats vara mer uttryckt i cancerceller jämfört med normala celler och som kopplats till ökad celldelning och cellöverlevnad. Trots det har höga nivåer av RBM3 i tumörceller tidigare främst förknippats med gynnsam tumörbiologi och prognos vid flera olika cancerformer. RBM3 har också föreslagits som en biomarkör för ökad känslighet för cellgifter, inklusive platinum som utgör basen i behandlingen av muskelinvasiv urinblåsecancer. I denna studie analyserades

RBM3-uttryck i tumörceller med immunohistokemi. Resultaten visade att patienter med tumörer med höga RBM3-nivåer som inte fått cellgiftsbehandling innan operation hade kortare tid till återfall och sämre överlevnad jämfört med patienter med höga RBM3-tumörnivåer som fått behandling. Det potentiella sambandet mellan RBM3 och känslighet för cellgifter undersöktes vidare genom cellodlingsexperiment, som visade att nedreglering av RBM3 resulterade i sämre behandlingseffekt. RBM3 visade sig dessutom vara involverat i celldelning även i urinblåsecancer vilket skulle kunna ge en funktionell förklaring till den ökade behandlingskänsligheten. Sammanfattningsvis ger resultaten stöd för att RBM3 har potential som en biomarkör för att förutspå svar på cellgiftsbehandling och framtida validerande studier är motiverade för att bekräfta detta fynd.

Som tidigare nämnts är tumörheterogenitet ett fenomen som kan påverka svar på cellgiftsbehandling, då olika populationer av cancerceller inom en tumör kan uppvisa en varierande grad av känslighet. En fördjupad förståelse för hur tumörheterogenitet tar sig uttryck, och vilka biologiska mekanismer som driver känslighet eller resistens mot behandling, är därför avgörande för att kunna optimera behandlingsstrategier. I *delarbete III* kartlades tumörheterogeniteten i 15 fall med varierande svar på neoadjuvant behandling, i syfte att identifiera mönster kopplade till behandlingseffekt. För detta ändamål utfördes detaljanalyser av flera geografiskt åtskilda tumöromården inom varje fall, med fokus på genetiska förändringar och immuninfiltration. Tumörområdena indelades även i så kallade molekylära subtyper, vilket är ett etablerat klassificeringssystem för urinblåsecancer som speglar underliggande tumörbiologi och har betydelse för prognosen. Även om antalet studerade fall var begränsat kunde vi identifiera distinkta mönster mellan behandlingskänsliga och resistenta tumörer. Behandlingskänsliga tumörer uppvisade ett högre antal mutationer, det vill säga förändringar i arvsmassan, varav flera var kopplade till det cellulära maskineri som ansvarar för att reparera uppkomna skador i arvsmassan, exempelvis sådana som orsakas av cellgiftsbehandling. Defekt skadereparation har i flertalet tidigare studier kopplats till ökad känslighet för cellgifter och resultaten från denna studie ger ytterligare stöd för detta, oavsett molekylär subtyp och grad av immuninfiltration. Vidare observerades en heterogen sammansättning av molekylära subtyper bland behandlingskänsliga tumörer, vilket bör beaktas i framtida studier. Förändringar i arvsmassan kan, utöver mutationer, även omfatta större genomiska avvikelser som påverkar flera gener, ibland tusentals. Vi fann att det totala antalet av sådana större förändringar inte skiljde sig åt mellan tumörerna, men att tumörer med sämre behandlingssvar tenderade att ha en mer heterogen förekomst av dessa. En detaljerad karaktärisering av den lokala immunmiljön, inkluderande såväl medfödda som förvärvade immuncellspopulationer, visade i likhet med *delarbete I* en begränsad förmåga att förutsäga svar på cellgifter.

Sammanfattningsvis har denna avhandling bidragit med ytterligare insikter till den komplexa molekylära bilden av muskelinvasiv urinblåsecancer, och belyser särskilt den lokala immunmikromiljön, tumöruttryck av RBM3 och tumörheterogenitet i relation till prognos och/eller behandlingskänslighet för neoadjuvant cellgifts-behandling. Resultaten understryker att flera faktorer kopplade till tumörerna och deras omgivning bör beaktas för att på bästa sätt kunna förutsäga behandlingseffektivitet och utfall, vilket kommer att bli ännu viktigare i och med införandet av nya neoadjuvanta behandlingsstrategier.

List of Papers

This thesis is based on the following papers:

Paper I

Wahlin S, Nodin B, Leandersson K, Boman K, Jirström K. Clinical impact of T cells, B cells, and the PD-1/PD-L1 pathway in muscle invasive bladder cancer. *Oncoimmunology* 2019;8(11):e1644108.

Paper II

Wahlin S, Boman K, Moran B, Nodin B, Gallagher WM, Karnevi E, Jirström K. Pre-clinical and clinical studies on the role of RBM3 in muscle-invasive bladder cancer: longitudinal expression, transcriptome-level effects and modulation of chemosensitivity. *BMC Cancer* 2022;22(1):131.

Paper III

Wahlin S, Petersson A, Svensson M, Nodin B, Lehn S, Boman K, Pietras K, Leandersson K, Sjödahl G, Jirström K. Attributes associated with response to neoadjuvant chemotherapy in the molecular terrain of muscle-invasive bladder cancer. *Manuscript in preparation.*

Papers not included in the thesis:

Andersson N, Ohlsson J, **Wahlin S**, Nodin B, Boman K, Lundgren S, Jirström K. Lymphocyte antigen 6 superfamily member D is a marker of urothelial and squamous differentiation: implications for risk stratification of bladder cancer. *Biomarker Research* 2020;8(1): 51.

Olsson Hau S, **Wahlin S**, Cervin S, Falk V, Nodin B, Elebro J, Eberhard J, Moran B, Gallagher W, Karnevi E, Jirström K. PRR11 unveiled as a top candidate biomarker within the RBM3-regulated transcriptome in pancreatic cancer. *The Journal of Pathology Clinical Research*. 2022;8:65-77.

Abbreviations

Ba/Sq	Basal/Squamous
BC	Bladder cancer
CI	Confidence interval
CIN	Chromosomal instability
CNA	Copy number alteration
CSS	Cancer-specific survival
CTLs	Cytotoxic T lymphocytes
Cx	Cystectomy
DCs	Dendritic cells
DDR	DNA damage response
FFPE	Formalin-fixed paraffin-embedded
GU	Genomically unstable
HRR	Homologous recombination repair
HR	Hazard ratio
mIF	Multiplex immunofluorescence
IHC	Immunohistochemistry
InDels	Insertions and deletions
ITH	Intratumour heterogeneity
MIBC	Muscle-invasive bladder cancer
MMR	Mismatch Repair
MVAC	Methotrexate, Vinblastine, Adriamycin, Cisplatin
NAC	Neoadjuvant chemotherapy
NGS	Next-generation sequencing
NMIBC	Non-muscle invasive bladder cancer
NER	Nucleotide excision repair
OS	Overall survival
PD-1	Programmed cell death-1
PD-L1	Programmed death-ligand 1
RBM3	RNA-binding motif protein 3
RBPs	RNA-binding proteins

RC	Radical cystectomy
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
siRNA	Silencing RNA
TCF	Tumour cell fraction
TDS	Targeted deep sequencing
TMA	Tissue microarray
TME	Tumour microenvironment
TNM	Tumour, node, metastasis
Tregs	Regulatory T cells
TTR	Time to recurrence
TURBT	Transurethral resection of bladder tumour
UC	Urothelial carcinoma
VAF	Variant allele frequency

Introduction

Omnis cellula e cellua
Virchow (1855)

Chapter I: Cancer – fundamental traits

All cells arise from pre-existing cells. Integral to the *cell theory* rooted in the mid 19th century[1] are the principles that cells represent the most basic unit of structure and function and that they propagate through division. This provides the foundation for understanding the processes that enable the continuity of life; from the evolutionary shift from unicellular cells to complex multicellular organisms, and the mechanisms through which new life emerges. These are sophisticated and meticulously controlled processes, occurring trillions of times during the human lifespan. Each time a cell divides, it must accurately replicate its entire genome, that is, six billion base pairs. Errors are inevitable. Multiple interconnected networks operate to repair or manage arising cellular damage, to maintain the essential state of equilibrium. However, errors that enable cells to circumvent such control mechanisms can culminate in the outgrowth of aberrant cells. Malignant cells then have the capacity to establish their own multicellular community, which interacts dynamically with the surrounding microenvironment to form an intricate tumour ecosystem.

This thesis seeks to further explore the local tumour composition of muscle-invasive bladder cancer. Tumour cell characteristics and the immune contexture are explored, with a particular focus on their relationship to patient outcomes and the susceptibility to oncological treatment. The following introduction aims to provide a context for these studies.

Carcinogenesis – the arise of malignant cells

Carcinogenesis, the neoplastic transformation of normal cells and the formation of malignant tumours, constitutes a multifaceted, multistep process. Numerous models of tumour evolution have been proposed, more or less cancer cell gene-centric,

reflecting several pathways to tumour formation[2]. A convergence of genomic, epigenetic, and phenotypic alterations, in conjunction with an adaptive permissive local microenvironment, is likely needed, though the relative contribution of these factors may vary[2,3]. Common acquired traits during the transformation process have been characterised as *Hallmarks of Cancer*[3], as delineated below. A critical aspect of the malignant transformation is the acquisition of alterations in genes that confer a proliferation advantage for the cell, collectively referred to as driver genes, encompassing both proto-oncogenes and tumour suppressor genes. Constitutive activation of proto-oncogenes or loss-of-function mutations or deletions in tumour suppressor genes can result in a near-chronic state of proliferation[4]. From an evolutionary perspective, carcinogenesis can be understood through the lens of natural selection, as theorised by Darwin[5]. In this framework, genomic alterations are accumulated, traditionally viewed as a random process in nature with regard to their functional consequences, although maybe not as random as thought[6]. These alterations can give rise to multiple cell subpopulations, known as subclones, through the preferential selection of alterations that confer an increased fitness and survival potential. The resulting subclonal landscape is shaped by a complex interplay of factors, including competitive or cooperative interactions, spatial constraints, and the selective pressure imposed by therapeutic interventions[7].

Tumour heterogeneity

The co-existence of distinct subclones, each defined by unique molecular alterations and phenotypic states, both arises from and fuels the microevolution observed within tumours. This mosaicism, termed tumour heterogeneity, manifests at varying degrees across multiple biological scales, ranging from being detectable through high-resolution molecular profiling to microscopically evident distinct morphological growth patterns. Tumour heterogeneity has been found to be present in nearly all cancer types[8].

Tumour heterogeneity can be described temporally, as dynamic changes in clonal composition over time, or spatially, as the clonal co-existence across different tumour areas at a given time point (Figure 1). Spatial heterogeneity can be further delineated into intratumour heterogeneity (ITH), which denotes the regional variability of cell subpopulations within a single tumour, and intertumoural heterogeneity, encompassing discrepancies between tumours originating from the same histology within the same patient (intrapatient) or between patients (inter-patient). There is additionally the concept of circulation heterogeneity, referring to discrepancies in tumour characteristics between tissue-derived and liquid biopsy analyses[9].

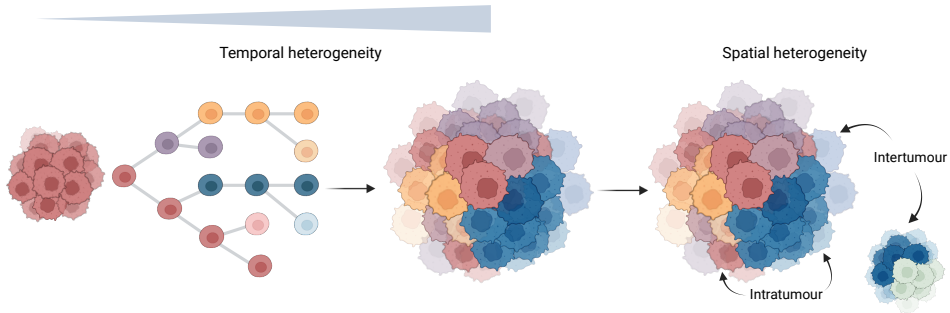

Figure 1. Temporal and spatial tumour heterogeneity. Subclones harbouring alterations that enable cells to disseminate from the primary tumour to distant sites give rise to both intratumour and intertumour heterogeneity.

Tumour heterogeneity has important consequences for both the therapeutic efficacy of anti-cancer therapies and for patient survival[10-12]. It can undermine standard linear treatment models, which predominantly targets proliferative cells, as evident by transient treatment responses and the emergence of resistant cell populations[13]. Consequently, novel treatment strategies that consider the evolutionary dynamics of tumours and leverage their inherent fragilities are currently being evaluated. Such strategies include the simultaneous or sequential administration of agents exerting opposing selective pressures to "trap" tumours[14], or adaptive treatment approaches designed to control, rather than eradicate, tumours to prevent the dominance of resistant clones in the metastatic setting[13].

Hallmarks of Cancer

Decades of research aimed at elucidating the molecular foundations of oncogenesis have compiled a rich and highly complex picture, though it remains fragmented. In 2000, *The Hallmarks of Cancer* were introduced with the objective of conceptualising this complexity into a generalisable framework of acquired, fundamental core alterations that determine cancer growth. These six hallmarks are considered to be ubiquitously present in cancer cells: (i) self-sufficiency in growth signals, (ii) insensitivity to growth-inhibitory signals, (iii) evading apoptosis, (iv) replicative immortality, (v) sustained angiogenesis, and (vi) tissue invasion and metastasis[4].

Subsequent advancements have expanded the original list, which to date enumerates fourteen traits. Genomic instability was incorporated as a key mechanism underlying hallmark capabilities[15]. Furthermore, mechanisms contributing to altered genomic profiles through means other than changes in the DNA sequence, i.e. non-mutational epigenetic reprogramming, was added[3]. In addition, the influence of the tumour microenvironment, in particular the impact of the immune system, were acknowledged[3].

Cell proliferation and cell cycle control

As delineated in the hallmarks, the inherent capacity of neoplastic cells to proliferate and divide is a fundamental driver of tumourigenesis. Under normal physiological conditions, cell proliferation is primarily driven by dynamic mitogenic signals and is carefully choreographed by branched intrinsic signalling pathways, linked to cell survival, energy metabolism, and the cell division cycle, specifically transit through the initial G_1-phase[15]. Many negative regulatory programs rely on tumour suppressors, e.g. the retinoblastoma protein (pRB) and p53, which serve as critical nodes controlling whether cells should proliferate, or, alternatively, undergo senescence – an irreversible arrest of the cell cycle – or apoptosis[4]. The duration and intensity of mitogenic signalling differ significantly between physiological and malignant states. In cancer cells, such fluctuations in growth stimuli are typically absent; rather, a defining characteristic is the persistent state of excessive cell division[4,16]. This can arise through various mechanisms: activating alterations in stimulatory pathways, oncogenic duplication, deletion of tumour suppressor genes, disruption of regulatory circuits, and epigenetically-driven dysregulation of gene expression[16].

The cell cycle consists of two distinct phases: interphase and M-phase (Figure 2). During the pre-replicative G_1-phase, regulatory networks orchestrate the critical decision of whether a cell will progress through the cell cycle or remain in G_1. Additionally, cells may enter into quiescence, a non-proliferative state (G_0), which represents the predominant condition for most cells. Upon receiving appropriate mitogenic signals, cells transition into the synthesis phase (S-phase), wherein DNA replication occurs. Following regulatory control in the G_2-phase, cells advance into M-phase, where the genetic material is condensed into chromosomes and centrally aligned. Filamentous structures known as the mitotic spindle attach to the centromeres on the chromosomes to ensure accurate separation of the duplicated DNA. The precise segregation of genomic, and subsequently, cytoplasmic components yield two genetically identical cells, after which they re-enter interphase[17].

As previously highlighted, cell cycle progression is stringently monitored by evolutionary conserved pathways for accurate cycling and genomic integrity[17]. The process is primarily governed by phase-specific oscillation of the activity of cyclin-dependent kinases (CDKs), which function in concert with cyclins to phosphorylate and thereby activate target proteins (Figure 2)[17]. The expression of CDKs and cyclins is intricately regulated through feedback loops, ensuring successive and unidirectional transition[18]. Furthermore, the tumour suppressor RB functions as a critical gatekeeper during G_1-phase, integrating both cell intrinsic and extrinsic cues to determine whether the cell should commit to a new cycle[15]. Additionally, several cell cycle checkpoints exist at critical time points to restrict proliferation of genomically aberrant cells[17]. Throughout interphase, the presence of DNA damage

activates checkpoint proteins, e.g. *ATM*, eliciting intracellular responses that halt cell cycle progression and activate the DNA repair machinery[17]. In the G_1-phase, p53 functions as a key regulator, halting cycle progression in response to DNA damage or cellular stress until normalisation. If the damage is irreparable, p53 induces either senescence or apoptosis[15,17]. Beyond G_1/S-transition, checkpoints are positioned to monitor DNA damage induced by replication stress during the S-phase, which is activated in response to the accumulation of single-stranded DNA, and during the M-phase, ensuring the correct attachment of chromosomes to the mitotic spindle[17]. If mitotic defects persist despite mitotic arrest, cells may exit the M-phase without proper chromosome segregation, resulting in a tetraploid (4N) state, which commonly triggers p53-mediated apoptosis[19].

A mounting number of studies indicate that the persistent cell division in cancer is primarily due to alterations preventing apoptosis and cell cycle exit, rather than by totally uncontrolled cell cycle propagation, as reviewed in[17]. The decision to exit the cell cycle is governed by the DNA damage checkpoint, which is accordingly frequently dysregulated across cancers, e.g. inactivation of *TP53*[17,20]. Cancer cells must however not only evade cell cycle exit, a successful entry into the S-phase is also needed. Accordingly, aberrations in key regulators of G_1/S-phase transition, e.g. *RB1*, is a recurrent trait[21], and may subsequently elicit replication stress and genomic instability[17]. In contrast, subsequent checkpoints are less frequently disrupted, indicating that cancer cells may depend on their functional integrity to cope with replication stress, avert mitotic catastrophe, and to evade apoptosis[17].

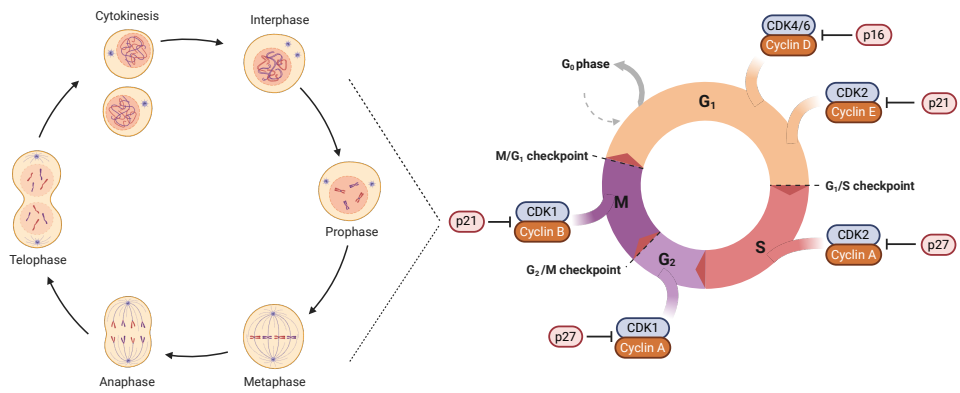

Figure 2. The cell division cycle. During interphase (left), the cell replicates its entire cellular content, corresponding to the G_1, S, and G_2-phases (right). The subsequent prophase marks the onset of the DNA and cell divison into two daughter cells, corresponding to the M-phase. The cell division cycle is sequentially orchestrated by cyclin-dependent kinases (CDKs) and cyclins, with phase-specific checkpoints at critical transition points.

Genomic alterations

Cancer is fundamentally a genetic disease, albeit not exclusively so[22]. DNA damage can arise from endogenous sources, including cell cycle-related alterations during replication and segregation, defective DNA repair pathways, reactive oxygen species, and inflammation-induced reactive species. Exogeneous sources encompass occupational and environmental carcinogens such as chemicals, ultraviolet and ionising radiation, and chronic virus infections[23,24]. The resulting DNA damage can range from small-scale to whole chromosomal-level alterations.

Small-scale alterations

These include base pair substitutions, referred to as single nucleotide variants (SNVs), which involve the replacement of a single nucleotide within the sequence, and base insertions and deletions (InDels), defined as the incorporation or removal of nucleotide bases, respectively (Figure 3A). The tumourigenic impact of such alterations is dependent on their genomic localisation and functional consequences on protein activity. Accordingly, somatic mutations can be classified as driver mutations, known to promote tumour progression and consequently being positively selected for during tumour evolution. By definition, driver mutations are located in cancer-associated genes. The remaining mutations are classified as passengers[25].

Somatic mutations are acquired alterations, in contrast to germline variants that are inherited and consequently present in all cells. Single nucleotide polymorphisms (SNPs) are the most prevalent form of germline variants. They involve single base pair substitutions and are typically observed in at least 1% of the population. SNPs have been implicated in modulating disease risk, including cancer susceptibility[26].

DNA damage repair mechanisms

The genome is continuously subjected to damage, which must be precisely repaired to preserve the integrity of the DNA sequence and ensure accurate reading during replication. The absolute majority of damage events go unnoticed, but, as mentioned, alterations affecting crucial cellular processes can have deleterious impacts[27]. DNA damage is counteracted by multiple repair pathways, operating with high efficiency. The DNA damage response (DDR) network senses DNA lesions, ranging from single-base mismatches to DNA double-strand breaks, and subsequently initiates intracellular signalling cascades to activate the appropriate repair mechanisms. These include: (i) base excision repair (BER), which addresses small, non-helix-distorting base lesions (ii) mismatch repair (MMR), which recognises base incorporation errors and base damage, (iii) nucleotide excision repair (NER), which removes bulky, helix-distorting DNA adducts, (iv) homologous recombination (HR), which repairs double-strand breaks by utilising a homologous template, typically the sister chromatid; and (v) non-homologous end joining (NHEJ), which directly ligates double-strand breaks of juxtaposed

sequences without the need for a homologous template, accordingly being a more error-prone repair process[28] (Figure 3B). However, in malignant cells, the capacity of these repair networks may become overwhelmed, and defects in any of the repair pathways can lead to genomic instability, thereby promoting tumourigenesis[29].

Chromosomal-level alterations

The human karyotype comprises 46 chromosomes, with a diploid (2N) chromosomal configuration in somatic cells. Copy number alterations (CNAs) refer to deviations in the number of copies of larger genomic regions in somatic cells (Figure 3C). These can manifest as copy number gains or losses. Losses may be hemizygous when affecting a single allele or homozygous when both maternal and paternal alleles are impacted. The loss of one allele is also referred to as loss of heterozygosity (LOH). The term aneuploidy denotes an abnormal number of chromosomes.

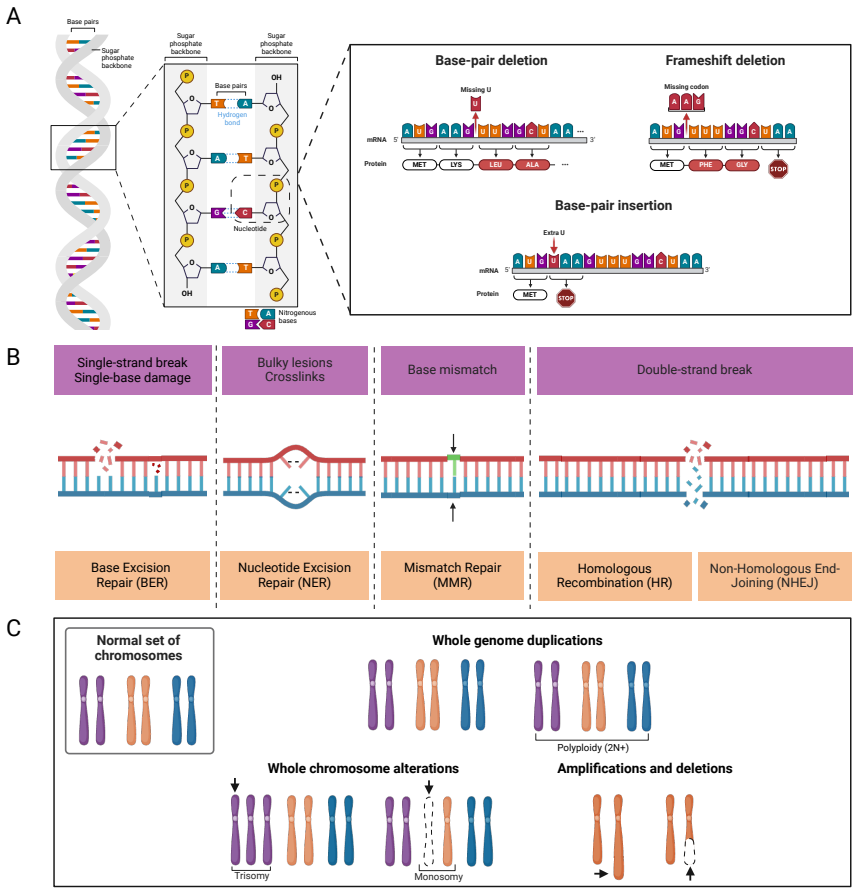

Figure 3. Genomic alterations. A) Examples of small-scale insertions and deletions, B) DNA damage repair mechanisms, C) Chromosomal-level alterations.

A gain or loss of a large chromosomal segment can affect numerous genes, including oncogenes and tumour suppressors, potentially leading to substantial impact on cellular function. Accumulation of chromosomal abnormalities and aneuploidy are frequent consequences of chromosomal instability (CIN). CIN refers to the sustained high rate of chromosomal aberrations resulting from errors in the accurate segregation of chromosomes during mitosis, yielding numerical and/or structural (rearrangement) alterations[17]. Various mechanisms can contribute to CIN, including premitotic replication stress, defects in sister chromatid cohesion, disruption of spindle assembly checkpoint signalling, the presence of multiple centromeres causing multipolar mitosis, and aberrations in kinetochore-microtubule dynamics[17,30]. Additionally, loss-of-function alterations in cell cycle regulation proteins, such as p53, RB, and BRCA1/2, as well as telomere dysfunction, are known to contribute to CIN[30].

Normally, aneuploidy is poorly tolerated and frequently results in cell cycle exit and/or apoptosis[17]. In the context of tumourigenesis, however, aneuploidy can paradoxically serve both as a tumour suppressor and tumour promoter. The resulting unbalanced gene expression disrupts cellular homeostasis, and aneuploidy has been shown to promote inflammation, proteotoxic stress, replication stress, and increased genomic instability. However, cancer cells can adapt to survive such changes, with aneuploidy *per se* driving adaptive responses[31]. Whole genome doubling (WGD), resulting in polyploid genomes (≥4N), has been shown to confer multiple advantages, including enhanced tolerance to mitotic errors[32]. Accordingly, consistent with being hallmarks of cancer, CIN and aneuploidy are frequently observed, with up to 80% of tumours exhibiting chromosomal abnormalities, particularly in more advanced stages[15,33]. CIN has been correlated to immune evasion, metastasis, therapy resistance, and a worse prognosis[31]. Moreover, chemotherapy-induced cellular stress has been implicated in the emergence of poly-aneuploid cancer cells (PACCs, ≥4N+). These cells exhibit an increased ability to endure stress by exiting the cell division cycle and transition into quiescence or reversible senescence[34]. These hibernating states enable cells to maintain their genomic integrity, as therapy-induced DNA damage could otherwise initiate apoptotic programs. Once the stress is alleviated, PACCs can re-enter the cell cycle, and undergoing depolyploidisation through asymmetric division, generating non-polyploid daughter cells. This adaptation mechanism, induced by selective pressure, serves as a pathway to therapeutic resistance[34].

The Central Dogma

Considering the plethora of multidirectional and complex regulatory biological networks, thankfully, there is at least one other fundamental linear principle beyond the cell cycle direction, namely the Central Dogma of Molecular Biology. This principle is integral to and positioned at the very heart of the process that enables

cellular life: the conversion of genomic information encoded in the DNA sequence, via RNA, into functional proteins. In 1957, Francis Crick theorised that, in light of the absence of supporting observations and biochemical processes, information encoded in proteins cannot reversibly be recoded into nucleic acids[35,36]. This does however not imply a strictly unidirectional flow from DNA to RNA to protein, rather, the dogma still allows for the transfer of information between nucleic acids, i.e. DNA and RNA. Indeed, shortly thereafter, reverse transcriptase was discovered in RNA retroviruses – an enzyme that enables RNA to be converted into DNA for subsequent integration into the host genome[37,38]. It has been reasoned that the restrictive principle reflected by the central dogma represents an evolutionary trade-off, wherein the inability of the intrinsic machinery to reverse proteins back into nucleic acids was outweighed by the ability to generate extensively greater diversity through the use of amino acids and subsequent protein folding[39].

During the initial phase of transcription within the cell nucleus, the DNA double helix is unwound and serves as a template for the synthesis of a complementary RNA strand. The RNA transcript is then released and undergoes post-transcriptional modifications: a 5' cap consisting of a modified guanine nucleotide is attached to the first nucleotide of the RNA strand, in addition to a poly(A) tail at the 3' end. These modifications are important for stabilisation, protection from exonucleolytic degradation, and for cytoplasmic transport. Additionally, the RNA molecule undergoes splicing to remove intronic sequences, producing a mature messenger mRNA (mRNA). In the subsequent phase of translation, protein synthesis occurs within cytoplasmic ribosomes. Here, the mRNA is read in sets of three nucleotides, known as codons, each of which specifies a particular amino acid. Amino acids are sequentially added, leading to the elongation of a polypeptide chain. This process continues until the ribosome encounters a stop codon in the mRNA sequence. The newly synthesised protein may then undergo further post-translational modifications to attain its final structure, functional activity, and subcellular localisation.

The cellular functions of RNA-bindings proteins

Proper post-transcriptional gene regulation, i.e. the control of gene expression after RNA synthesis until translation, is vital for subsequent protein synthesis and maintenance of cellular homeostasis[40]. RNA-binding proteins (RBPs), over 1500 being identified to date, reversibly bind to RNA via specific RNA-binding domains (RBDs), with RNA recognition motifs being among the most prevalent. These interactions orchestrate the full spectrum of RNA metabolism: transcription, maturation, splicing, modification, intracellular location, translation, and degradation[41] (Figure 4). As such, RBPs are highly evolutionary conserved across species and one of the most prominent classes of proteins within cells, generally ubiquitously expressed. RBPs are capable of recognising hundreds of transcripts, albeit with varying affinities and specificities, thereby forming complex regulatory

networks[42]. These networks are rigorously controlled through feedback and feedforward mechanisms, as any substantial changes can influence a broad range of transcripts and disrupt cellular equilibrium. Indeed, altered expression of certain RBPs has been correlated with several diseases, including cancer[41]. Although the expression pattern of RBPs mirrors that of housekeeping genes, with only 2% being tissue-specific, changes in their levels are often associated with tissue-specific dysfunction[40]. Possible explanations for this observation include differences in expression levels of RNA molecules bound by RBPs across tissues, as well as the dynamic properties of the networks they form, where disturbances may be differentially tolerated and managed across distinct cell types[40].

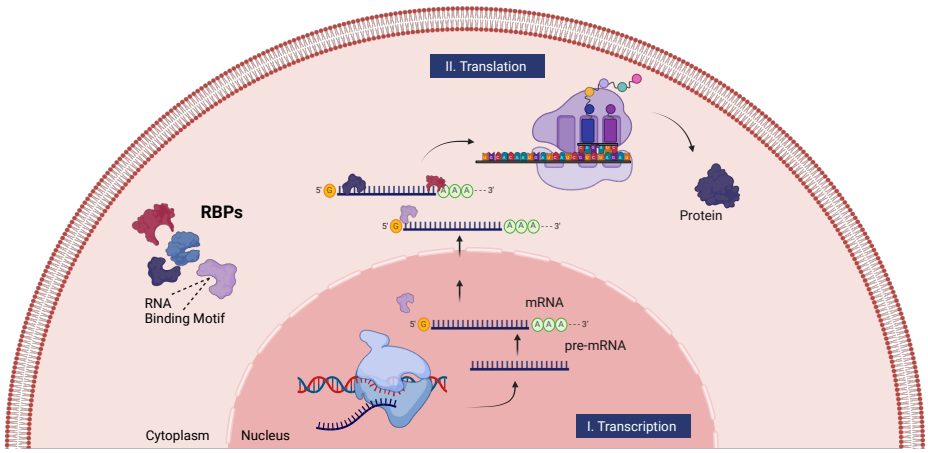

Figure 4. Post-transcriptional gene expression regulation by RNA-binding proteins. DNA is transcribed into RNA, which then undergoes processing, maturation, and transport into the cytoplasm, where it serves as a template for subsequent protein synthesis. All aspects of RNA metabolism, from post-transcription to translation, are under the influence of RBPs.

Amongst RBPs involved in cancer, the nuclear *RNA-binding motif protein 10* (*RBM10)* is one of the most frequently altered[40]. Somatic alterations in *RBM10* have been identified in several cancer types, including pancreatic[43] and bladder cancer[44]. In line with the implicated context-dependent roles of RBPs, RBM10 exhibits both tumour suppressive and oncogenic properties, potentially mediated by RBP-influenced alternative splicing of mRNAs and the aforementioned dynamics of the regulatory networks[42]. However, despite their essential and multifaceted roles during RNA processing, the mechanisms underlying the function of most RBPs remain to be deciphered[41].

RBM3 – a versatile regulator

In this thesis, the clinical implications of another RBP, namely *RNA-binding motif protein 3 (RBM3)*, was investigated in the context of MIBC. The *RBM3* gene is

located at the X chromosome, along with several other RBPs including *RBM10* and *RBMX*, and is mapped to the Xp11.23 region. Its encoded protein comprises approximately 157 amino acids including two RNA recognition motifs, with an estimated molecular weight of 17 kDa[45]. RBM3 is expressed in both the nuclear and cytosolic compartments and exhibits dual DNA- and RNA-binding capabilities[46]. Initially discovered as a cold-shock protein[47], subsequent investigations have illuminated a broad regulatory influence on the proteome. This includes stabilisation of mRNAs containing AU-rich elements[48], involvement in the posttranscriptional biogenesis of microRNAs[49], and in promoting global protein synthesis[50]. In addition to hypothermia, *RBM3* transcription is induced under various cellular stress conditions, including hypoxia, endoplasmatic reticulum stress, and serum deprivation, where it mediates cytoprotection and attenuates apoptosis[47,51-53]. *RBM3* has garnered interest within neurological research, where it has been identified as a regulator of cell proliferation and differentiation during early development stages[54], as well as a mediator of neuroprotective responses under induced hypothermia[55].

These characteristics are particularly relevant in the context of cancer, where RBM3 has been described as a putative proto-oncogene[48]. *In vitro* studies using cancer cell lines have demonstrated that RBM3 can induce stem-like characteristics via enhanced β-catenin signalling[56] and facilitate cell cycle progression by promoting G_2/M-phase transition, thereby mitigating mitotic catastrophe[48]. Protein-based analyses on patient-derived tumour specimens across several solid malignancies have shown RBM3 upregulation in preinvasive and cancerous tissues compared to normal tissue[53,57-59], as well as correlations between high tumour-specific RBM3 levels and favourable prognosis in colorectal[60], breast[57], prostate[61], urothelial bladder[62], and epithelial ovarian cancer[63]. However, similar to the dual function role observed for RBM10, high *RBM3* mRNA levels have been linked to worse survival in the TCGA pancreatic cancer cohort[64], with similar observations in prostate cancer[58]. Notably, high RBM3 expression has been associated with increased sensitivity to platinum-based treatments in metastatic testicular non-seminomatous germ cell[59], metastatic colorectal[65], and ovarian cancer[63]. In ovarian cancer, silencing *RBM3* expression *in vitro* resulted in decreased cisplatin sensitivity, which was attributed to its identified functional involvement in cell cycle regulation[63] and DNA damage response[66]. Hence, RBM3 may exert both pro-oncogenic and suppressive biological functions depending on the context, with a notable potential link to chemosensitivity.

Microenvironmental crosstalk sculpts tumour fate

Tumour cell-intrinsic alterations are fundamental for initial cell transformation. However, cancer is not a disease exclusive to cancer cells; genomic and phenotypic alterations are necessary, but insufficient on their own. Rather, tumours are dynamic ecosystems of myriads of non-cancerous cells, integrated into a fibrous and

vascularised scaffold known as the extracellular matrix (ECM), forming the tumour microenvironment (TME). The reciprocal interactions within the TME spans all phases of the tumour's evolutionary trajectory, from initiation and invasion to dissemination, and can further substantially impact the efficacy of antitumoural therapy[22].

The surrounding microenvironment of tumour cells can constrain tumour outgrowth through additional extrinsic suppressor mechanisms. The immune system has a crucial role in detecting aberrant cells, as will be further delineated. Another key suppressor mechanism involves cell-to-cell dependencies between adjacent cells, which are essential for maintaining tissue integrity by hindering inappropriate proliferation. In other words, cells can sense their transformed neighbours[67]. It was observed that normal cells in 2D cultures arrest proliferation upon reaching confluence[15], through contact inhibition mediated by cell surface molecules[68]. This suppressor mechanism is often circumvented by transformed cells, with downregulation of cell adhesion molecules, such as E-cadherin[15]. Furthermore, certain cell subsets are anchorage-dependent, meaning that they are normally attached to the ECM. This is necessary for obtaining the survival signals that suppress intrinsic apoptotic pathways, to prevent independent cell proliferation or migration. Extensive proliferation or aberrant cell morphologies causing stretching of focal adhesions can result in detachment from the ECM[67]. This results in activation of a programmed cell death pathway known as anoikis[69]. Cancer cells can however rapidly acquire resistance to these extrinsic suppressor pathways, thereby fostering a tolerant and supportive milieu[22]. Resistance to cell-to-cell or cell-to-ECM dependencies liberates cancer cells, allowing for cell polarisation into a mesenchymal state. This process, referred to as epithelial-to-mesenchymal transition (EMT), is characterised by increased motility, invasion, and dissemination[69].

A significant proportion of the cellular heterogeneity within tumours arises from non-cancerous cells, which may be either tissue-resident or recruited to the tumour site. The constellation of the TME may vary according to tumour origin and display considerable interpatient diversity[22]. The multilineage origin of cells encompasses innate and adaptive immune cell subsets, cancer-associated fibroblasts (CAFs), vascular or lymphatic endothelial cells, pericytes, adipocytes, and neurons. These cells possess substantial phenotypic plasticity, with both stimulatory and suppressive characteristics. The bidirectional signalling between cancer cells and their surrounding plays a crucial role in modulating these dynamics. Cancer cells strive to organise a tumour-supportive environment. By utilising cell-to-cell interactions and paracrine signalling, they can alter the vasculature and ECM, and recruit and reprogram non-cancerous cells to their advantage[22]. Additionally, cancer stem cell activity can be modulated by signals from the TME[70].

CAFs are dominant components of the tumour stroma harbouring both opposite pleiotropic and antagonistic phenotypes. Activation of CAFs by various signalling pathways can result in altered synthesis and remodelling of the ECM. A dense stromal network can obstruct intercellular signalling, impair immune cell recruitment, and hinder tumour access of therapeutic agents. CAFs can additionally modulate the immune response by direct cross-talk with immune cell subsets and impact angiogenesis[71]. Neovascularisation is essential during tumourigenesis, to enable sufficient supply of oxygen and nutrients. This initiates the angiogenic switch, wherein previously quiescent endothelial cells are activated, and new blood vessels are formed. However, these capillaries typically exhibit aberrant structural organisation and can compromise the recruitment and homing of immune cells[72].

Chapter II: Immunological perspectives on cancer

The multifaced immune response: Extrinsic barrier or facilitator

The immune system serves a pivotal role in surveying, recognising, and eliminating transformed cells – a process termed immunosurveillance[73]. The mechanistic basis of immune responses triggered by the presence of tumour cells shares many parallels with those elicited by other pathological insults: inflammation, wound healing, and the immune defence against exogenous pathogens[73]. While acute microbial invasion elicits a roboust inflammatory response, cancers initially provoke a more subtle inflammatory state, which may become increasingly more apparent as the tumour progresses[74]. Cancer is therefore linked to chronic inflammation, which contributes to immune exhaustion and the formation of a pro-tumourigenic state[75]. Chronic inflammation elicited by other factors such as viral infections can *per se* be conducive to tumour development[76]. However, acute inflammatory processes may also play a role, as exemplified by the use of the attenuated *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) vaccine, which evokes T cell immunity and serves as an efficient treatment for superficial bladder cancer[77]. A further challenge arises from the endogenous origin of tumour cells, requiring the immune system to recognise them as non-self despite often subtle antigenic alterations[78].

The renaissance of the principle of tumour-immune interaction roots back to the mid-19th century, when Virchow observed the colonisation of leukocytes in neoplastic tissue, hypothesising that cancer and inflammation are intricately related[79]. The concept that the immune system can prevent tumour progression was founded by Ehrlich in the early 20th century[80]. Indeed, Coley observed that live and inactivated microbes could induce tumour regression in sarcoma patients, leading to the idea that cancer could be eradicated by stimulating immunity, thus offering a potential therapeutic approach[81]. The notion of the immune system as a tumour recogniser and eliminator was later formalised through the cancer immuno-surveillance hypothesis[82]. A dual relationship between the immune system and tumours was subsequently described, with tumours being analogous to a chronic, non-healing wound[83]. Thus, the initial anti-tumour immune response can be suppressed by the influence of tumour cells. The central role of this process was solidified when tumour-promoting inflammation and the avoidance of immune destruction were integrated as *Hallmarks of Cancer*[15].

Innate and Adaptive immunity

The immune system consists of two arms: innate and adaptive immunity. The actions of these two arms are complementary in achieving a roboust and efficient short- and long-term immune response. *Innate immunity* serves as the first line of defence, enabling a rapid immune reaction. Innate immune cells are promptly recruited to the site of injury, directed to the chemokine and cytokine rich milieu. These cells respond in a non-specific manner, as their activation is not triggered by a particular antigen. In contrast, *adaptive immunity* refers to a highly specific immune response, which is acquired through the initial actions of the innate immunity. Presentation of antigens to the adaptive immune cells bridge these two immune arms, resulting in a maximal immune response. This is pivotal for immunosurveillance. Additionally, adaptive immune cells enable the establishment of long-term memory, allowing for an efficient response upon subsequent encounters with the same antigen[84].

The different innate and adaptive immune actors exhibit a diverse array of phenotypes and cellular functions. Adding to the complexity, the phenotypic polarisation of these cells is a dynamic process, modulated by microenvironmental cues. The following section summarises the function of these immune cell populations, with particular focus on those explored in this thesis.

Innate immune populations

A multitude of innate immune cell populations have been shown to be relevant in the context of cancer. These include dendritic cells (DCs), natural killer (NK) cells, invariant natural killer T (iNKT) cells, monocytes, macrophages, γδ T cells, neutrophils, mast cells, eosinophils, and myeloid-derived suppressor cells (MDSCs)[22]. While these cells exert distinct function in immunosurveillance, they share the intrinsic ability of innate sensing without previous priming[85].

Dendritic cells

Central for mounting an optimal adaptive anti-tumour immune response is the potency of recruited APCs to internalise, process, and present antigens[67]. Amongst APCs, DCs are by far the most potent[86]. DCs can acquire antigens through mechanisms including phagocytosis of apoptotic tumour cells or debris and receptor-mediated endocytosis[87]. The diverse repertoire of genetic and epigenetic alterations within cells generates a diverse array of antigens, facilitating differentiation between self and tumour cells by the host immune system[88]. Following antigen uptake, DCs process the antigens intracellularly, load them onto major histocompatibility complexes (MHC), referred to as human leukocyte antigen (HLA) in humans, and present these complexes on the cell surface for recognition by the adaptive immune arm[89]. DCs can additionally initiate an immune response by sensing stressed or damaged cells via binding of damage associated molecular patterns (DAMPs) to their pattern recognition receptors (PRR)[90]. DCs represent a

phenotypically diverse cell population, displaying both pro- and anti-tumour phenotypes. Immunostimulatory qualities include, in addition to T and B cell activation, the secretion of cytokines that favour differentiation of T cells into effectors and interferon (IFN)-γ, which stimulates cytolytic cell function[91]. In contrast, immunosuppressive soluble molecules released from tumour cells can curtail DC maturation, resulting in the maintenance of an immature phenotype unable of T cell priming[91]. These factors can additionally induce the expression of inhibitory checkpoints, as described below.

Macrophages
Tissue-resident macrophages are primarily descendants of circulating monocytes. Similar to DCs, macrophages harbour APC properties, enabling antigen presentation via phagocytosis of damaged cells[67]. During the initial response, macrophages serve as a key source of pro-inflammatory cytokines, including tumour necrosis factor-alpha (TNFα) and interleukin-1 (IL-1), to promote the inflammatory process and the recruitment of additional immune cells. This response is subsequently regulated as macrophages can transition into a suppressing phenotype[92].

The functional spectrum of tumour-associated macrophages (TAMs) has been conceptualised into two states: the pro-inflammatory M1 phenotype and the anti-inflammatory M2 phenotype. M1 macrophages are characterised by their ability to present antigens, recruit cytotoxic immune cells, and secrete pro-inflammatory factors, e.g. IFN-γ, TNFα, and IL-6. The M2 phenotype is associated with inhibition of T-mediated cytotoxicity, the induction of immunosuppressive T cells, and angiogenesis[93]. Importantly, these states are not static, rather, highly contextualised and plastic[94].

NK and iNKT cells
These cytotoxic immune populations, integrating both innate and adaptive functions, are critical mediators of immunosurveillance. NK cells dynamically survey the microenvironment and can be activated in an antigen-independent manner through a finely regulated interplay of activating and inhibitory receptor signalling, enabling the distinction between normal and aberrant cells. As NK cells rely on inhibitory receptors that interact with MHC I molecules, tumour-associated MHC downregulation – a common immune evasion strategy – disrupts the inhibitory signal[95]. This activates cytotoxic pathways including granule exocytosis of perforin and granzymes and apoptosis initiation via Fas-FasL interactions[96]. iNKT cells are hybrids of NK and T cells characteristics, co-expressing NK surface receptors and invariable T-cell receptors (TCR) with limited diversity. Unlike conventional T cells, iNKT cells recognise glycolipid antigens presented by non-classical MHC molecules, including CD1a on APCs, contributing to their maturation[97]. In addition to its cytotoxic properties, iNKT cells can secrete

cytokines, including IFN-γ, which facilitates recruitment of innate immune subsets[95].

Adaptive immune populations

The two prototypic adaptive immune populations, T and B lymphocytes, predominantly reside in secondary lymphoid organs, including lymph nodes, until activation. In the presence of cancer, the most important functions of adaptive immunity are to distinguish the recognition of aberrant antigens from native antigens, evoke a roboust anti-tumour response upon antigen recognition, and establish immunological memory[98].

T lymphocytes

T lymphocytes possess a diverse repertoire of cell surface TCRs, generated through somatic recombination, which enable broad antigen recognition[99]. The primary T cell subsets are $CD8^+$ cytotoxic T lymphocytes (CTLs), $CD4^+$ T helper (T_H) cells, and $CD4^+$ $FoxP3^+$ regulatory T cells (Tregs). The activation of CTLs constitutes the backbone of the T cell response. Within the lymph node, APCs present antigens on MHC-I molecules to naïve $CD8^+$ T cells, initiating a three-step activation process essential for roboust T cell activation and cytotoxic properties (Figure 5)[100]. The activation of tumour-specific T cells induces a rapid clonal expansion of these cells. Similarly, APCs present antigens via MHC-II to prime naïve $CD4^+$ T_H cells, and a simultaneous cross-presentation to $CD4^+$ T_H cells and $CD8^+$ CTLs can further amplify the T cell response[67]. Activated T cells can subsequently specialise into memory T cells, establishing immunological memory.

Activated T cells home to the tumour site, where antigen recognition on tumour cells elicits a cytotoxic T cell response. The release of cytotoxic molecules, including perforin and granzymes, induces tumour cell apoptosis. Similar apoptosis-inducing mechanisms are observed for NK cells, although they employ distinct pathways to tumour recognition and harbour distinct cytolytic profiles[101]. $CD4^+$ T_H cells can enforce the cytotoxic response by secreting stimulatory signals, such as IFN-γ and IL-2[102]. However, T_H cells are not uniformly pro-inflammatory; polarisation into alternative phenotypes, such as T_H2, can promote tumour progression by inhibiting CTLs and stimulate M2 polarisation[102].

Tregs are T cells harbouring immunosuppressive functions, an important physiological mechanism to prevent autoimmunity. However, in the context of cancer, Tregs can attenuate the anti-tumour response through interactions with tumour cells as well as innate and adaptive immune cell lineages[103]. Tregs can impair antigen presentation by DCs[103], secrete inhibitory cytokines (IL-10 and TGF-β) or granzymes and perforins to inhibit or induce cytolysis of NK cells and CTLs[104]. Tregs can further reduce IL-2 levels, thereby depriving lymphocytes of essential proliferative signals[103].

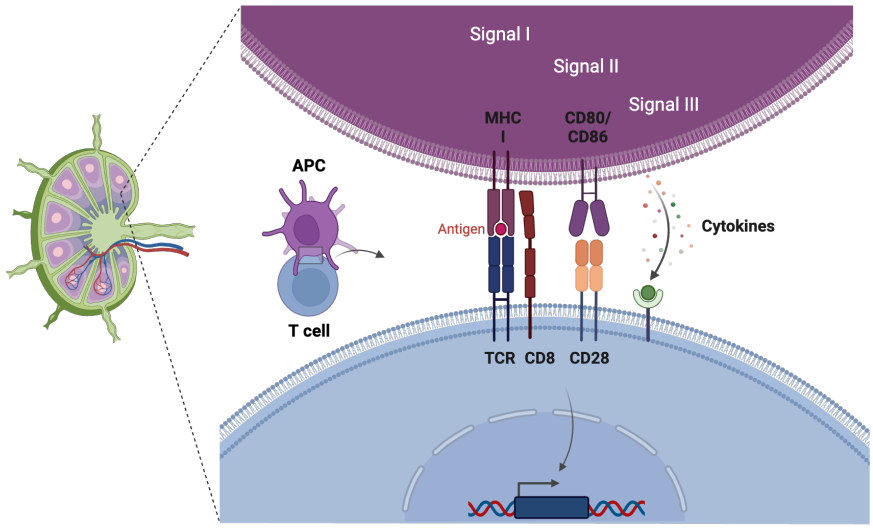

Figure 5. Mechanisms of antigen presentation. Antigen presentation by antigen-presenting cells (APCs) to naïve T cells, where full activation of T cells requires a three-step signalling process: 1) Antigen recognition: Antigenic peptides presented on MHC-I molecules are recognised by the T cell receptor (TCR), 2) Co-stimulatory activation: Simultaneous co-stimulatory signalling via CD80/CD86-CD28 interaction, and 3) Instructive cytokine release: Pro-inflammatory cytokines are released, directing T cell polarisation[100].

B lymphocytes

Compared to T lymphocytes, the role of B lymphocytes in the immunological response to cancer remains less untangled, although their dual pro- and anti-tumourigenic properties are well recognised[98]. The activation of the T and B cell lineage is integrated to mount a potent anti-tumour response, with B cells being responsible for the humoral, i.e. antibody, defence. Similar to T cell activation, tumour antigens are presented to naïve B cells within lymph nodes, facilitated by follicular helper (Tfh) cells. Antigen recognition by B cells leads to the formation of germinal centres, where activated B cells proliferate and undergo clonal selection, resulting in the expansion of B cells with the highest affinities for the target antigen. These expanded B cells subsequently specialise into effector B cells, antigen-secreting plasma cells (PCs), or memory B cells[98]. B cell activation may also occur peripherally near the tumour site, where dense networks of B and T cells form tertiary-lymphoid structures (TLSs)[105]. At the tumour site, antibodies secreted by tumour-infiltrating B cells can activate the complement system and mediate antibody-dependent cellular cytotoxicity (ADCC) or cellular phagocytosis (ADCP), where binding of antigen-antibody complexes to receptors of certain innate cells trigger cytotoxic killing or phagocytosis of tumour cells, respectively[98]. Antibody release can further amplify the innate response[98].

Akin to Tregs, B cells and PCs can adopt a regulatory immunosuppressive phenotype. Via secretion of anti-inflammatory molecules (IL-10, IL-35, or TGF-β), these regulatory B cells (Bregs) interfere with activation of APCs and effector T cells, while inducing Tregs[106]. The presence of tumour-infiltrating B cells as well as TLSs have been associated with favourable prognosis across multiple cancer types, whereas opposite correlations are denoted for Bregs[98].

Host recognition of cancer cells

The presence of tumour cells can alert the immune system through various mechanisms. Physical damage and tissue stress induced by invasive growth can *per se* signal the immune system to the location[67]. Neovascularisation, the release of extracellular matrix products, secretion of pro-inflammatory molecules – including IL-1, TNFα, and type I IFNs – during stromal remodelling or by tumour cells, as well as danger signals arising from tumour-cell damage, provides substrate for activation of APCs[67,107]. Innate immune cell – NK cells, NKT cells, and macrophages – can be rapidly recruited to the site, where they recognise stress-induced molecules, resulting in activation of cytotoxic effector mechanisms. The local secretion of interferons (IFN), such as IFN-γ, can suppress tumour proliferation as well as intensify the immune response. IFN-γ activated macrophages can contribute to tumour elimination. APCs, predominantly DCs, internalise tumour antigens, resulting in activation and phenotypic maturation. Influenced by chemo- and/or cytokines, DCs migrate to lymph nodes where they interact with the adaptive immune arm[89]. In the lymph node, APCs induce activation of naïve $CD4^+$ T_H cells, naïve $CD8^+$ CTLs, or cross-stimulation of these, resulting in an effective activation of the adaptive immune response[67]. During this process, B lymphocytes may also become activated for the generation of a humoral response. Activation of T cells leads to clonal expansion, after which tumour-specific T cells relocate to the tumour. Tumour-specific CTLs recognise the target antigen, leading to the induction of tumour cell death. $CD4^+$ T_H cell-induced IL-2 release helps sustain the CTL response. Furthermore, the secretion of IFN-γ by T cells can further augment tumour elimination[67].

Immune evasion: Strategies behind tumour escape

The intricate and dynamic interplay between the host immune system and the immunomodulatory effects initiated by cancer cells, which may occur concurrently or sequentially during tumour evolution, is inherently challenging to encapsulate due to its complexity and multidimensional nature. Additionally, significant aspects of these processes remain to be fully elucidated. Nevertheless, its essence has be categorised into three distinct phases, collectively referred to as immunoediting, which ultimately govern tumour fate[73]:

1. *Elimination*
   The proliferation of malignant cells that have bypassed the intrinsic tumour-suppressor regulation can trigger an innate immune response. resulting in immune-mediated elimination[73]. This process, known as immunosurveillance, constitutes an unceasing cyclic process in need of reactivation whenever novel tumour populations with distinct genomic profiles emerge[67]. The immune system can recognise these cells by different means, as outlined previously.

2. *Equilibrium*
   The elimination phase can effectively eradicate a substantial proportion of malignant cells; however, it may remain incomplete[67]. While a sustained immune pressure may be sufficient to constrain tumour growth, the emergence of tumour subclones harbouring novel resistant traits undermines the ability for complete eradication. This leads to a dynamic equilibrium phase, which in humans can persist for many years[73]. During this latency, tumour cells may ultimately be eradicated, maintained in equilibrium, or progress to the final phase of immunoediting – the escape[67].

3. *Escape*
   The continued interclonal evolution, driven by the positive selection of alterations conferring a fitness advantage, is considered the primary driving force of resistance to host immunity[73]. These alterations encompass genetic, epigenetic, and phenotypic modifications that can affect all aspects of the immune system, from initial innate immune sensing to execution of effector functions, leading to the avoidance of immunological recognition and the formation of a beneficial tolerant milieu[67]. Conceivably, both innate and adaptive immune responses must be outmanoeuvred, ultimately culminating in clinically detectable disease[67].

Thus, intratumour heterogeneity provides a simmering foundation for immune escape. Resistance can arise at multiple levels, and a conceptual framework categorising resistance mechanisms into globally, regionally, or locally acting has been proposed[75]. Clonally expressed resistance mechanisms can lead to a global reduction in immune pressure across the tumour bed as well as systemic immune tolerance, and these tumours are viewed as *immune cold*[75]. In contrast, regionally or locally expressed alterations, confined to specific tumour regions or discrete subclones, respectively, do not exert comparable macro-level effects[108]. Consequently, T cells can still infiltrate substantial portions or the tumour, and these tumours can be classified as *immune hot*[75].

The exploration of the molecular basis for how tumours can sculpture the immune response to their advantage has uncovered a complex array of pathways. Malignant cells can adopt a state of low immunogenicity, wherein downregulation of immunological signposts on the tumour cell membrane facilitates evasion. Reduced transcription of genes involved in antigen presentation, downregulation or loss of MHC proteins, low expression of co-stimulatory molecules, and decreased levels of NKG2D ligands allows tumour cells to avoid recognition by T and NK cells, or to be treated as self-antigens resulting in immune anergy[67]. Tumour cells can further develop insensitivity to IFN-γ signalling[109]. Release of tumour derived factors, including TGF-β, IL-10, and Indoleamine 2,3-Dioxygenaes (IDO), can directly suppress T cell activity. Moreover, tumours can induce a tumour-privileged tolerant milieu by skewing the distribution of immune cell populations from effector cells towards suppressors, such as Tregs, T_H2 helper cells, M2 macrophages, and MDSCs[67]. The architecture of the microenvironment can be further remodelled through vascular dysfunction, hypoxia and stromal changes, creating a physical barrier hindering immune cell recruitment and mobility, and hence, the mounting of an efficient response[75].

The PD-1/PD-L1 axis: A tumour ally and a therapeutic target

An additional route for immune escape is by leveraging host immunosuppressive pathways. As a critical mechanism for maintaining self-tolerance to prevent autoimmunity, as well as limiting tissue damage during immune reaction to pathogens, the immune system is equipped with intrinsic regulatory mechanisms[110]. The counteracting stimulatory and inhibitory signals, known as immune checkpoints, operate throughout each phase, from the activation of APCs to the final effector response, to fine-tune the immune reaction[88]. These signals are typically mediated by membrane-bound receptors and ligands. Among the immune checkpoints that regulate T cell inhibition, programmed-death 1 (PD-1) and its ligands, programmed-death ligand 1/2 (PD-L1/2), play pivotal roles. In response to prolonged immune activation, immune cells can upregulate PD-1 at the cell surface, which induces inhibitory downstream pathways leading to an exhausted immune state[111]. PD-1 is commonly expressed on activated T cells[111], but can also be present on DCs, NK cells, macrophages, and B cells[110]. Similarly, PD-L1 can be expressed by both innate and adaptive immune populations[112]. Additionally, PD-L1 can also be upregulated by the non-immune surrounding milieu, including by the blood vasculature[22].

Tumour cells can hijack this inhibitory pathway by expressing PD-L1/2, thereby suppressing T cell function. PD-L1 upregulation can be induced by oncogenic signalling or as an adaptive response to evade anti-tumour T cell immunity[88]. Exploiting this pathway has led to the development of antibodies targeting PD-1 or PD-L1, referred to as immune checkpoint inhibitors, which disrupt the inhibitory

signal to reinforce the anti-tumour response (Figure 6)[88]. These treatments have resulted in a paradigm shift in the management of many cancers.

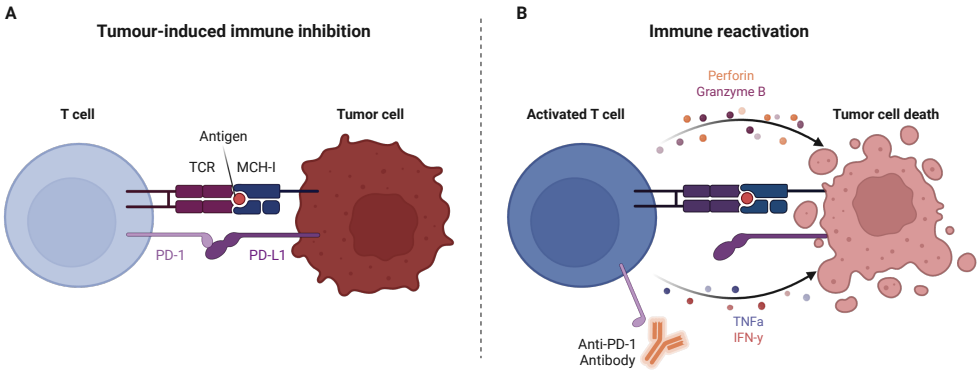

Figure 6. Regulation of immune responses via the PD-1/PD-L1 axis. A) Inhibition of the T cell-mediated anti-tumour response by PD-L1 expression on tumour cells. B) Blocking the PD-1/PD-L1 interaction using immune checkpoint inhibitors can restore T-cell functionality, resulting in tumour cell apoptosis.

Genomic instability and immunogenicity

Paradoxical correlations between highly aberrant genomes and immune responses have been observed, with loss of genomic integrity instigating both pro-inflammatory circuits[113,114] and facilitating immune evasion[115]. Various determinants of genomic instability have been highlighted in this context, including mismatch repair (MMR) and homologous recombination deficiency, *TP53* mutations, and chromosomal abnormalities[116,117].

Small-scale alterations

Accumulation of genomic alterations has a detrimental impact on cell survival, triggering inflammatory signalling pathways leading to rapid extinction of damaged cells[118]. Defective genomic maintenance in malignant cells can result in a high mutational burden, fuelling the translation of aberrant proteins, i.e. neoantigens, important for immune surveillance[115,117]. MHC-presented neoantigens are commonly detected by intratumoural CTLs[119] as well as $CD4^+$ T cells[120], and their presence has additionally been shown to influence the therapeutic efficacy of immune checkpoint blockade[121-123]. In non-small cell lung cancer, the number of non-synonymous mutations was more closely correlated to response to checkpoint inhibition than the total exonic mutational burden[124], with similar findings observed in melanoma[123], emphasising the need to consider the neoantigen potential of mutations[125]. In MMR-deficient tumours, the immunogenic state of the TME is predominantly attributed to the infiltration of activated $CD8^+$ and $CD4^+$ T cells[118], with the degree of neoantigen clonality serving as a key determinant in shaping the

T cell response[126]. The diluted expression of subclonal antigens increases the likelihood of being bypassed by APCs and thus, results in an inadequate T cell priming[75].

In addition to the influence of the T cell lineage, tumours harbouring higher MHC-I neoantigen loads have been associated with enhanced humoral immune responses[127]. Furthermore, B cells can co-localise with T cells within tumours, forming highly-organised ectopic lymphoid clusters known as tertiary lymphoid structures, which have been shown to be important for T cell phenotyping[128].

However, the high prevalence of genomic instability in clinically detectable cancers indicates the emergence of immune tolerance to genomic damage. Genomic instability serves as a substrate for Darwinian selection, conferring a fitness advantage to tumour populations capable of evading immune recognition. Tumour cells with reduced MHC expression may hence be preferentially selected for. In MMR deficient tumours, deleterious mutations in genes involved in antigen processing and MHC complex assembly, including *B2M* and *HLA,* are frequently observed. Furthermore, *TP53* loss has been implicated in immune evasion, leading to attenuated $CD4^+$ and $CD8^+$ T cell responses, accompanied by an accumulation of immune suppressive myeloid cells and Tregs[129]. Alongside the skewed predominance of exhausted effector cells over immunosuppressive phenotypes, elevated PD-1/PD-L1 expression within the TME has further been reported for genomically unstable tumours[118].

Chromosomal-level alterations

Similar to small-scale variants, the relationship between CIN and immune mechanisms is intricate and context-dependent. Chromosomal mis-segregation during mitosis can lead to the formation of cytoplasmic micronuclei, which, upon rupture, expose double-stranded DNA into the cytoplasm. Other nuclear processes, including replication stress and telomere dysfunction, can further cause accumulation of cytoplasmic DNA[130]. This alarms the cytosolic sensing cGAS-STING pathway, eliciting a downstream signalling cascade that induces a pleiotropic pro-inflammatory response, characterised by the production of type I interferons (IFN-I) and TNFα, alongside immune cell recruitment[115]. T cell and NK cells can be steered to the tumour site and activated through STING-induced chemokine gradients[131]. Cancer-derived DNA and cGAS/STING molecules have further been shown to stimulate innate immune sensing and maturation, with subsequent enhanced priming of $CD8^+$ T cells[132]. Additionally, the presence of karyotypic abnormalities can trigger intracellular stressors that promote the expression of immune-activating ligands on the tumour cell surface, including MHC molecules and NKG2D ligands, enforcing immune recognition by APCs, NK cells, and $CD8^+$ T cells[118]. Although genomic rearrangements can cause frameshift

alterations, it remains unclear whether CIN contributes to neoantigen-driven immune response[118].

While the acute induction of CIN and aneuploidy initially enhance immunogenicity, these responses can be attenuated over time, yielding a tolerant milieu permitting continued tumour progression and metastatic spread[133]. Persistent activation of the cGAS/STING pathway redirects the inflammatory signalling towards NF-κB activity while suppressing IFN-mediated effects, resulting in reduced MHC-I expression and resistance to apoptosis[118]. Specific chromosomal gains or losses have been associated with accumulation of Tregs, M2 macrophages, and the secretion of immunosuppressive soluble factors, thereby contributing to an exhausted T cell state[118]. For instance, one of the most recurrent homozygous deletions across various cancers affects the p-arm of chromosome 9[33], which has been linked to reduced TNFα and NF-κB signatures, and T-cell depletion[134]. Loss of heterozygosity at the HLA locus is a recurrent phenomenon that facilitates immune evasion[135].

Chemotherapy-induced immune alterations

Beyond tumour cell-induced modulation of immunogenicity, the administration of conventional chemotherapy can exert dual immunoregulatory impact on the immune system, with off-target effects modulating both innate and adaptive activity[136]. Chemotherapy as an immunological stimulus may appear paradoxical, as systemic chemotherapeutics is traditionally regarded as counteracting such processes by suppressing immune cell clonal expansion and inducing bone marrow suppression. This holds true, as high doses are correlated with leukopenia, and several agents are routinely used as immunosuppressants in the management of severe systemic autoimmune disorders[137]. However, for certain cytotoxic agents, the induced anti-tumour immune response contributes to agonistic therapeutic effects beyond direct tumour cell destruction, with postulated synergistic mechanisms spanning several cellular pathways across multiple cancer types[137].

Accumulation of DNA damage

The cell-intrinsic DNA damage response inflicted by genotoxic agents – including DDR-enzyme inhibitors (e.g. topoisomerase inhibitors and PARP inhibitors), direct DNA-damaging agents (alkylating agents and nucleoside analogues), or indirectly via antimitotic agents (e.g. microtubule targeting agents) – has been linked to the activation of the cGAS-STING pathway[138]. This results in an enhanced anti-tumour immunity, via mechanisms delineated in the previous section. The therapeutic advantage of combining standard-of-care chemotherapy with immune checkpoint inhibition has been observed in multiple clinical trials, indicating a synergistic therapeutic effect in certain cancers[137]. Although chemotherapy can enhance tumour antigenicity through increased mutation and neoantigen loads[139], since the DNA damage induced by cytotoxic agents occurs over relatively short timeframes, it is

hypothesised that the immunostimulatory effects potentiated by immunotherapy may not be primarily driven by enhanced mutagenesis. Rather, these effects are inclined to be mediated through other immune mechanisms, such as cGAS-STING activation[130].

Immunogenic cell death

The cellular consequences of exposure to chemotherapy treatments spans from inducing apoptosis or necrosis to senescence. The first two modalities represent cytotoxic effects resulting in cell death. Apoptosis is a cell self-generated and tightly-regulated sequential programme, reputedly immune tolerogenic. In contrast, necrosis is an accidental and unregulated form of cell death, leading to the uncontrolled release of inflammatory cellular content such as nucleic acids and heat shock proteins[140,141]. The cytostatic effects of treatment can induce a senescence state, where cells, in response to nonlethal stress, enter a stable state of cell cycle arrest. However, while dormant, these cells remain metabolically active, providing a means for evading therapy-induced eradication with a risk of later tumour relapse[142].

Chemotherapy is believed to exerts its predominant anti-tumour effect through apoptosis[143]. The disruption of cellular homeostasis induced by chemotherapeutics results in a state of cellular stress, activating a complex cascade of stress-responsive signalling pathways that attempt to restore equilibrium. If this cannot be achieved, apoptosis is initiated[144]. While apoptosis was initially considered immunologically inert, this perspective was revised following the findings of Casares *et al.*[145], who demonstrated that chemotherapy-triggered tumour cell apoptosis can activate a potent anti-tumour $CD8^+$ T cell response, functioning as a form of tumour vaccination providing subsequent protection against lethal inoculation with viable tumour cells. Intensive research aimed at deciphering the underlying basis for this observation has converged on the functional capacity of certain cytotoxic agents, such as anthracyclines and oxaliplatin, to elicit an *immunogenic tumour cell death.* This is preceded by endoplasmatic reticulum stress and the release of alarmins that mobilise the immune system[146]. These alarmins, or DAMPs, include calreticulin, a multifunctional protein primarily located in the endoplasmic reticulum. Exposure to anthracyclines can initiate a rapid translocation of calreticulin to the cell membrane, where its exposure functions as an *eat-me* signal that flags the cells for the innate system[144]. Calreticulin promotes efficient phagocytosis by immature DCs. Subsequent release of the nuclear high mobility group box 1 protein (HMGB1) from late-stage apoptotic cells interacts with DCs by binding to the TLR4 receptor, further stimulating antigen processing[143]. Additional release of intracellular molecules, such as exocytosis of ATP, operates as an extracellular *find-me* signal, recruiting innate immune cells to the location[146]. Collectively, these molecules serve as adjuvants for an innate immune activation and maturation, ultimately culminating in an anti-tumour T-cell response[146].

A significant advantage of this phenomenon is that chemotherapy can indirectly stimulate an immune attack towards cancer cells that are inherently treatment resistant[137]. However, if compared to the immune responses caused the presence of pathogens, treatment-induced immunogenic cell death likely results in considerably less effective immune responses[147].

Chemotherapy-induced phenotypic enrichment

Chemotherapy can further modulate the composition of immune populations. Tumours favourably promote the myelopoiesis of MDSCs. Although MDSCs originate from the same progenitor lineage as DCs and macrophages, they develop into functionally distinct cells with immunosuppressive properties. The secretion of soluble molecules by MDSCs that inhibit the functionality of NK and T cells can have detrimental effects on the tumour immune response[148,149]. MDSCs have been shown to be sensitive to various chemotherapeutics, including alkylating agents, gemcitabine, and 5-fluorouracil[137]. Additionally, several agents have been demonstrated to promote macrophage differentiation towards a pro-inflammatory M1 phenotype[137].

Leukopenia

Leukopenia, a condition characterised by a deficiency in circulating white blood cells induced by lymphotoxic chemotherapy, is a commonly observed adverse effect causing increased susceptibility to infections. Transient leukopenia, however, may conversely foster an enhanced anti-tumour T cell response[143]. In addition to the previously mentioned antigen-driven clonal expansion, T cell proliferation is additionally sustained through homeostatic circuits. Disruption of this equilibrium triggers a compensatory secretion of promitotic growth factors, stimulating the proliferation of antigen-naïve and central memory T cells. This process can reshape the immune response through enhanced antigen recognition, differentiation into cytotoxic T cells[137], increased T cell homing to the tumour mass[143], and eradication of immunosuppressive phenotypes, such as Tregs[150].

Unfavourable antagonistic immune responses

In contrast, the administration of chemotherapeutics may also cause immune paralysis, curtailing the anti-tumour immune responses. The overall suppression of immune function induced by high-dose treatment regimens is likely attributable to the depletion of effector cells and/or stem-like T cells otherwise capable of replenishing the antigen-recognition capacity[137]. Furthermore, persistent activation of the DNA damage repair system, for example via continued treatment-induced genotoxicity, may lead to cGAS-STING induced immunosuppression[118] and has also been linked to the emergence of cellular senescence and a senescence-associated secretory phenotype (SASP)[130]. Senescence can be a favourable effect of chemotherapy as it impairs tumour cell proliferation and reduce tumour burden.

However, the SASP state, characterised by the release of a broad pro-inflammatory secretome of cytokines and chemokines, can have both pro- and anti-tumour effects. While it can increase the influx of cytotoxic cells for clearance of senescent cancer cells, it can also foster stemness features, epithelial-to-mesenchymal transition, and immunosuppression[151].

Future rationales

Although important immunological consequences of genomic instability as well as exposure to chemotherapy have been deciphered, several key questions remain unresolved. The majority of identified mechanisms stem from *in vitro* or murine models, needing further verification in patient-derived samples. A deeper understanding of these mechanisms will be particularly crucial for predicting responses to immunostimulatory treatments and for identifying potential avenues for therapeutic intervention as anti-tumour strategies[118].

While the implementation of chemoimmunotherapy is expanding across both early and late treatment settings, the mechanistical understanding of why combinatory treatment is favourable in certain situations but not universally so is still in its dawn. The prevailing approach involves the integration of checkpoint inhibitors into standard-of-care chemotherapy regimens, often given concurrently with minimal optimisation of treatment protocols[139]. Other agents beyond checkpoint blockade are also being explored[137]. Conceivably, the potential immunostimulatory effects of chemotherapy may be confined to a distinct therapeutic window, during which its immunogenic potential can be maximised[137]. As discussed above, prolonged exposure has been associated with a risk of inducing a tolerogenic immune environment. Given that many chemotherapy regimens are based on the maximum tolerated dose paradigm, it is plausible that reduced chemotherapy dosages during combinatorial treatments may be more favourable, although this requires careful empirical evaluation[137]. Thus, with this window of opportunity in mind, the evaluation of treatment schedules – including the selection and sequencing of agents, dosing strategies, and timing – is crucial for identifying optimal combinations that may enhance tumour-specific immunity while minimising unnecessary toxicity[137]. Such refinements could hopefully ultimately translate into an increased number of patients in the stable plateaus of Kaplan-Meier survival curves, representing the long-term survivors following chemoimmunotherapy.

Chapter III: Bladder Cancer

The concepts discussed are of significant relevance to bladder cancer (BC), a malignancy characterised by extensive genomic instability and a heterogeneous tumour landscape. This section delineates key biological and clinical attributes of BC, with particular emphasis on muscle-invasive tumours, and the major clinical challenge that motivated this thesis.

Normal bladder histology

The urinary bladder lining, termed urothelium, consists of specialised epithelial cells forming a protective barrier against the toxic constituents of urine and potential microbial invasion. It is structurally adapted to endure persistent mechanical tension, as well as osmotic and hydrostatic pressures[152]. The urothelium comprises three distinct cellular layers: superficial (umbrella) cells, intermediate cells, and basal cells (Figure 7, page 56). Located at the apical surface, umbrella cells form a critical high-resistance barrier. These fully differentiated, often multinucleated, cells are distinguished by the presence of intracellular vesicles, which facilitate the dynamic modulation of urothelial cell surface area via controlled endo- and exocytosis. The cell surfaces of umbrella cells are coated with glycocalyx, a dense, thin membrane of glycoproteins, glycolipids, and proteoglycans, which serve as a protective barrier. The intermediate cells form a multilayer structure and maintain connections to the basement membrane through long cytoplasmic extensions[153]. In contrast, basal cells, positioned at the basal lamina, represent the least differentiated cell population. These cells constitute the majority of the urothelium and can be subdivided into two distinct populations: K5-basal cells, which express cytokeratin 5 (Krt 5) and p63, and K14-basal cells, which express Krt14, Krt15, and p63. The latter population possesses progenitor capacity; however, it remains unclear whether these cells represent a distinct population or a phenotypic state induced by the need for urothelium renewal[153]. The mitotic index within the urothelium remains low (0.1%)[154] and the rate of cellular turnover is notably slow in comparison to other epithelia[155]. However, following injury or inflammation, cell proliferation is significantly upregulated, mediated by signalling pathways including Wnt, ELF3, and p63, resulting in the restoration of the urothelium within days to weeks[152]. Urothelial differentiation is regulated by the nuclear receptor PPARγ, which modulates the expression of several transcription factors, including GATA3 and

FOXA1, thereby promoting a luminal cell phenotype. In the absence of PPARγ, p63 preserves the undifferentiated basal phenotype by suppressing epithelial lineage genes[156]. Altered p63 function is implicated in BC oncogenesis[157].

Located beneath the basal membrane is the lamina propria, an elastic stromal layer including blood vessels and neural tissue. This is encircled by a smooth muscle layer known as the muscularis propria or musculus detrusor, which is further encased by perivesical adipose tissue.

Bladder carcinoma

Epidemiology

As the urothelium extends from the proximal urethra via the ureters to the renal pelvis, urothelial carcinoma can arise throughout the urinary tract. Among these malignancies, bladder cancer is by far the predominant diagnosis. Bladder cancer (BC) represents a significant public health concern. It ranks as the tenth most commonly diagnosed malignancy globally and the thirteenth leading cause of cancer-related mortality[158]. In 2020, approximately 570,000 newly diagnosed cases and 210,000 attributable deaths were reported[159]. The incidence of BC is increasing, and projections by the World Health Organisation indicate that it is expected to double over the next two decades[160]. The highest incidence rates are observed in high-income regions, including Europe, North America, and Western Asia, a pattern associated with differences in tobacco use and environmental and occupational toxin exposure, amongst other causes[160]. In Sweden, the annual incidence is approximately 3,200 cases, with 700 associated deaths. The mortality rate has not improved over the last decades[161]. Consistent with global trends, the incidence has been rising in recent years. The disease primarily affects older individuals, with a median age at diagnosis of 76 years, and occurrence before 50 years of age is rare. The incidence in men is three times higher than in women, irrespective of age[162].

Risk factors

Age and sex

As noted, age and sex are important epidemiological risk factors for BC initiation. In addition to discrepancies in incidence rates, women generally have a more aggressive disease course and subsequent higher mortality[163]. The observed sex dimorphism remains poorly understood but is likely the consequence of multiple factors. One potential explanation is divergent smoking patterns; however, the male-to-female ratio has been shown to remain even when known risk factors are accounted for, including cigarette use[164]. Delays between the onset of symptoms and diagnosis constitute another important factor, with women presenting with

haematuria initially being more likely to be diagnosed with and receive treatment for cystitis[165]. The influence of hormonal and non-hormonal biological sex differences in BC has been increasingly recognised. Androgens have been implicated in promoting BC tumourigenesis[164], whereas oestrogen signalling mediated via its nuclear receptors ERα and ERβ can exert contrasting effects[166]. ERα activation has been shown to suppress tumour initiation and invasion, with opposing results denoted upon ERβ activation[166]. Additionally, loss of chromosome Y is a recurrent trait in BC, observed in 10-40% of cases irrespective of tumour grade and stage. Chromosomal loss has been correlated with more aggressive tumour features and a worse prognosis, partially attributed to its induction of an immunosuppressive TME with high infiltration of TAMs, checkpoint inhibition expression, and exhausted CTLs[167].

Tobacco use

Cigarette smoking is the principal risk factor for BC carcinogenesis, accounting for over 50% of cases, with similar proportions observed in both men and women. Tobacco-related carcinogenesis includes genotoxic mechanisms, such as the formation of DNA adducts and epigenetic modifications; however, the precise molecular pathways remain incompletely characterised. Smokers exhibit a three-to fourfold increased risk of BC development relative to non-smokers, with the risk increasing proportionally to cumulative tobacco exposure[168]. Among former smokers, the risk declines over time, with a reduction exceeding 30% within the first 1-4 years, although it remains elevated for decades[168,169]. These observations underscore the critical importance of both primary and secondary interventions for smoking prevention and cessation.

Occupational exposure

Occupational exposure to aromatic amines, including benzidine, 4-aminobifenyl, and β-naphthylamine found in cigarettes as well as in the dye, chemical, and rubber industries, along with other organic compounds and combustion byproducts has been associated with an increased risk of BC[170]. Genetic polymorphism in enzymes involved in carcinogen detoxification, e.g. NAT2 and GSTM1, have been linked to bladder cancer risk[171].

Chronic inflammation

Chronic inflammation, induced by for example recurrent infections or calculi, may be associated with an elevated risk of BC. In North Africa, chronic inflammation resulting from *Schistosomiasis haeomatobium* infection represents a distinct risk factor for squamous cell carcinoma (SCC) but also urothelial carcinoma[172].

The disease spectrum

Bladder cancer can present as flat lesions, papillary growths into the bladder lumen, or solid tumours infiltrating the bladder wall, each with distinct genomic profiles and clinical implications. Bladder cancer is classified into non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC), the latter defined by tumour infiltration into the detrusor muscle or beyond. While early-stage disease is often treatable with minimal impact on survival, approximately 5-20% of non-invasive tumours progress into muscle-invasive disease[173]. At diagnosis, 25-30% of cases are muscle-invasive[174], characterised by an aggressive natural disease course and a high risk of metastasis, representing a lethal condition[175]. Given that this thesis focuses on MIBC, the following descriptions will primarily pertain to this disease.

Carcinogenesis

In-depth analysis of the normal urothelium has uncovered an intricate mutational landscape, marked by significant interindividual diversity, highlighting the influence of genetic factors and exposure to carcinogens. The clonal architecture of the normal urothelium is characterised by APOBEC-driven mutagenesis and extensive positive selection, with chromatin remodelling genes, such as *KMT2D, KDM6A, ARID1A,* and *RBM10,* being frequently altered. In contrast, larger chromosomal-level alterations are sparse[176].

Based on histopathological and molecular observations, a two-pathway model of bladder cancer carcinogenesis has been proposed, however, encompassing multiple subpathways. Originating from hyperplasia or dysplasia, the acquisition of LOH of chromosome 9 and mitogenic mutations in *FGFR3, PIK3CA,* and *STAG2* can give rise to low-grade papillary tumours. Additional loss of *CDKN2A* might provide a route for these tumours into invasive disease. Invasive tumours are thought to originate from flat dyplasia and carcinoma *in situ* (CIS), through acquisition of 9p/9q LOH, *RB1* and *TP53* alterations, as well as genomic instability[177]. Furthermore, the field cancerization hypothesis is pertinent to bladder cancer, wherein the epithelial field acquires pro-tumourigenic genomic alterations with subsequent increased susceptibility to further carcinogenic events[178]. The interactions between genetically altered cells and their microenvironment play a pivotal role in determining which subclones are preferentially selected. These selected subclones can proliferate, forming patches or fields that are predisposed to progression toward malignancy[178]. Thus, the presence of CIS might serve as an indicator of the genomic heterogeneity of the bladder lining. Comprehensive whole-organ analysis has revealed over 100 dysregulated pathways as initiators of carcinogenesis, a process that unfolds over more than a decade, beginning with a dormant phase followed by a progressive phase during the course of one to two

years. The mutations identified in this process commonly affected progenitor cells[179].

The genomic landscape

MIBC is characterised by a high mutational burden (>7 mutations per Mb), only exceeded by lung cancer and melanoma[180]. Early alterations commonly affect chromatin remodelling genes and the promoter region of the telomerase reverse transcriptase (*TERT*) gene, the latter being crucial for telomere maintenance and can also upregulate oncogenic signalling[181]. Alterations in the *TERT* gene have been reported in up to 70% of tumours[182]. While activating mutations in *FGFR3* are less frequent compared to NMIBC, upregulated expression is often observed. Deletion of chromosome 9, which encompasses key tumour suppressor genes such as *CDKN2A* and *TSC1*, occurs early in carcinogenesis, affecting the pRB and p53 pathways and mTOR signalling, respectively. Nearly all MIBC tumours exhibit loss of cell cycle checkpoints due to alterations in *TP53*, *RB1*, and *ATM*, or through aberrations in downstream effectors, such as *E2F3* (6p) and *MDM2* (12q) amplifications, as well as mutations in the *FBXW7* gene. DDR genes are often affected. The activation of RAS-MAPK and PI3K pathways is a recurrent feature, often driven by gain-of-function mutations or amplifications of upstream regulators, including *ERBB2* (17q), *ERBB3* (12q), and *EGFR* (7p)[183,184]. In addition, chromosomal-level alterations and aneuploidy are commonly observed, with whole genome-doubling denoted in approximately 50% of tumours[183]. In addition to previously mentioned copy number alterations, amplifications of PPARγ (3p) are commonly observed[184].

Molecular subtype classification

To deepen the understanding of the biological behaviour of BC and to identify molecular features that could refine prognostication and treatment prediction, significant research efforts have sought to compile a taxonomy system of molecular subtypes, each enriched with certain characteristics. These subtype classifications are based on gene expression profiles, offering the advantage of serving as a link between genomic aberrations and the phenotypic consequence of these. In MIBC, several classifiers have been proposed, each applying different rationales. The most fundamental classification is the stratification into luminal and basal phenotypes. In 2020, a consensus classification was published, integrating previous classification schemes, which resulted in the delineation of six molecular subtypes: (i) luminal papillary, (ii) luminal nonspecified, (iii), luminal unstable, (iv) stroma-rich, (v) basal/squamous (Ba/Sq,), and (vi) neuroendocrine-like. These subtypes exhibit distinct intrinsic oncogenic mechanisms, patterns of immune and stromal cell infiltration, and histological and clinical features. Additionally, these subtypes

confer prognostic implications, with Ba/Sq, and in particular, neuroendocrine-like, tumours being associated with worse outcomes. The consensus classifier employs a single-sampled predictor model to assign a molecular subtype to each tumour sample based on the predominant subtype signature. It additionally yields correlation values with the remaining subtypes as well as a separation score that informs the degree to which a sample aligns with its assigned class. These metrics can thus inform the potential presence of intratumoural subtype heterogeneity[185].

Histology

The molecular heterogeneity of BC extends to the morphological level, manifesting as distinct histological subtypes and divergent differentiation patterns, indicative of the inherent plasticity of progenitor cells. Over 95% of BC cases are of epithelial origin, with the vast majority (90-95%) being classified as urothelial carcinoma (UC). The morphological spectrum of UC includes squamous and glandular differentiation, as well as several histological subtypes, including micropapillary, sarcomatoid, plasmacytoid, or nested variants, some of which harbour unique molecular alterations. Non-urothelial histological subtypes comprise SCC, adenocarcinoma, and neuroendocrine carcinoma[9]. Of these, SCC is the most prevalent (3-5%), though its incidence is markedly higher in regions with endemic *S. haeomatobium* infections. Unlike UC, SCC exhibits a nearly equal male-to-female ratio[186]. Primary adenocarcinoma is a rare histological variant. While it can arise from the urachus – a remnant of the embryonic connection between the bladder and umbilicus – most cases originate from progressive glandular metaplasia of the urothelium or represent metastases from extravesical malignancies[187]. Small cell (neuroendocrine) carcinoma is an uncommon but aggressive tumour type, sharing clinical features with high-grade urothelial carcinoma[9].

The tumour microenvironment

In addition to cancer cell-intrinsic features, the cellular contexture of the bladder cancer TME holds both prognostic and predictive significance. As observed in numerous other malignancies, CAFs constitute the most abundant mesenchymal cell type within the BC TME. CAFs can adopt multiple functionally distinct phenotypes, several of which are only beginning to be characterised in BC, including the myofibroblastic and inflammatory subtypes[188,189]. These cells exert broad influence on the TME to facilitate BC progression, including the secretion of chemoattractants, growth factors such as FGF2 and EGFR, angiogenic factors, and metalloproteinases to promote degradation of the ECM[190]. Consistent with their predominantly tumour-promoting phenotypes, preclinical studies have shown that the presence of CAFs can enhance bladder cancer cell viability and induce cisplatin resistance, primarily through the activation of IGF-1/ERβ-signalling[191]. CAFs have

been further highlighted in the context of cisplatin-based neoadjuvant chemotherapy (NAC) treatment, where resistant cancer cell subpopulations were found to co-localise with TGFβ-activated CAFs[192]. Beyond their influence on cancer cells, CAFs also promote an immunosuppressive environment. In metastatic urothelial carcinoma, resistance to checkpoint inhibition has been linked to TGFβ signalling in fibroblasts, particularly in immune-excluded tumours with $CD8^+$ T cells confined to stromal compartments[193].

Normally, the bladder lumen is constantly exposed to microorganisms. As a result, the bladder is colonised by a plethora of tissue-resident immune populations, including macrophages, dendritic cells, neutrophiles, NK cells, and γδ T cells[190]. The immune system continues to exert a pivotal role during tumourigenesis. The anti-tumour immune response is generally more pronounced in MIBC than in NMIBC[194-196], with the Ba/Sq subtype exhibiting the highest degree of immune infiltration[185,194]. CTLs are the primary effector cells in this response, where the high mutational burden characteristic of BC, in particular MIBC, increases the likelihood of neoantigen recognition. Accordingly, high densities of $CD8^+$ T cells, as well as $CD3^+$ T cells, signify a favourable prognosis in BC[195-198], with their spatial distribution within the tumour bed recognised as a key determinant[196,198]. In contrast, tumour-infiltrating $FoxP3^+$ Tregs have been associated with both improved and worsened outcomes in MIBC[199,200], with similar opposing trends observed in NMIBC[196,201]. TAMs are important modulators in sculpturing an immuno-suppressive TME. High abundancies of M2 tumourigenic phenotypes have been linked to disease progression and metastasis in BC[202]. However, certain studies suggest that these cells may instead exert a protective role in MIBC[196,202]. In contrast to these immune populations, tumour-infiltrating B lymphocytes are less studied. In MIBC, high densities of $CD19^+$ B cells, interferon-stimulated B-cells, and TLS have been identified as prognostically favourable[203,204]. However, the presence of B cells has also been associated with the manifestation of more aggressive tumour features[205]. The dual correlations observed for multiple immune subpopulations underscore the intricate nature of the multicellular microenvironment, where the phenotypic signature of immune cells can be highly context-dependent, warranting further in-depth analyses. The immune contexture has additionally shown predictive value. In MIBC, high tumour fraction of infiltrating immune cell and low stromal signatures are associated with improved survival and response to checkpoint blockade[206]. The predictive significance of immune cells in relation to cisplatin-based chemotherapy will be emphasised in the subsequent Discussion section.

Clinical presentation and diagnostic procedures

Symptoms

The predominant initial symptom of urothelial carcinoma is visible blood in the

urine, macroscopic haematuria, which is observed in approximately 60% of diagnosed cases over 40 years of age[207]. Macroscopic haematuria is among the isolated alarm symptoms demonstrating the highest positive predictive value for malignancy[208]. In a prospective observational study encompassing 11,000 patients, 22.4% of patients presenting with macroscopic haematuria were diagnosed with BC. The incidence increased with advancing age, reaching 35% among patients aged ≥75 years[209]. Lower-risk symptoms include irritative urinary symptoms, dysuria, frequent urination or urge, pain, and recurrent urinary tract infections[209].

Diagnostic procedures

Accordingly, macroscopic haematuria requires early medical assessment, as delays in diagnosis and treatment correlate with reduced survival rates[210]. An exception, however, applies to women aged ≤40 years presenting with a first episode of classic haemorrhagic cystitis symptoms who become asymptomatic following treatment, as this is a common aetiology of macroscopic haematuria in these patients[211].

Diagnostic evaluation includes radiological imaging of the bladder and kidneys (CT urography) to exclude potential sources of haematuria in the upper urinary tract and to determine advanced stages of bladder cancer. Endoscopic examination of the bladder lumen (cystoscopy) is additionally performed, as well as microscopic analysis of the urine (cytology). Complementary imaging using nuclear medicine or radiological techniques is employed in cases of muscle-invasive disease where curative treatment is considered[211].

Transurethral resection of bladder tumour (TURBT)

TURBT under general anaesthesia is the gold standard diagnostic procedure for more precise staging and grading of BC, which is critical for guiding treatment decisions. If CT imaging confirms the presence of bladder cancer, TURBT can be performed directly without the need for prior cystoscopy. Following physical examination, a thorough endoscopic evaluation of the bladder lumen is performed, with subsequent resection of the exophytic tumour compartment as well as separate resections of the tumour base and the resection margins. In cases of NMBIC, TURBT may additionally serve a therapeutic purpose if complete tumour resection is achieved. The quality of the procedure is essential for ensuring diagnostic accuracy and, in the context of NMIBC, optimising therapeutic outcome. Several factors can influence the quality of the procedure, including the surgeon's technical expertise and experience, as well as anatomical and procedural challenges. Adequate representation of the detrusor muscle in TURBT tissue specimens is obligatory for determining invasion depth. In cases were the muscle is absent or inadequately represented, or when tumour stage 1 (Figure 7) is confirmed, a second-look resection is recommended to minimise the risk of understaging[211]. Restaging from NMIBC (T1-stage) to MIBC is common, reported in 20% of cases, and has significant consequences for the subsequent treatment decision[212].

Histopathological diagnosis

TURBT specimens are subsequently evaluated for histological grading into low-grade or high-grade, with the latter encompassing both NMIBC and MIBC. High-grade NMIBC is defined by a considerable risk of recurrence and progression to MIBC. In addition to tumour grade, the pathological tumour stage (pT) is determined based on the depth of invasion. The histological type, as well as the presence of divergent differentiation patterns and CIS, if applicable, is reported. Histopathological evaluation of TURBT samples presents inherent challenges due to sometimes limited tissue availability, as well as the presence of mechanically- and cautery-induced artefacts, haemorrhage, and necrosis, all of which can influence interpretability. Moreover, interobserver variability, particularly in the assessment of NMIBC, constitutes an important source of diagnostic inconsistency[213]. The clinical implementation of molecular-based tumour classifications for both NMIBC and MIBC holds future potential to complement and refine diagnostic precision as well as prognostication[185,214,215].

Diagnostic tissue-based assessments are complemented by urine cytology, offering high specificity but relatively lower sensitivity for the detection of bladder cancer, particularly high-grade (G3) urothelial carcinoma. The cell appearance is classified according to the Paris System for Reporting Urinary Cytology, which diagnoses high-grade BC with >90% accuracy[216]. To improve diagnostic sensitivity, especially for low-grade cancers, and enable early non-invasive detection, additional urine-based molecular diagnostic tests are under investigation. These include *TERT* promoter mutations[217], *FGFR3* alterations[218], as well as broader gene panels[219], however, further prospective validation and clinical benchmarking against cytology are needed[220].

Tumour staging

While the clinical tumour stage (cT) can be preliminarily evaluated through imaging, cystoscopy, and physical examination, the pathological tumour stage (pT), determined by histopathological examination provides the most definitive tumour classification. Tumour staging is reported according to the Union for International Cancer Control (UICC) Tumour, Node, and Metastasis (TNM) classification of malignant tumours (8th edition, Figure 7)[221]. NMIBC comprises Tis, Ta, and T1 stages, whereas MIBC corresponds to pT-stage ≥2. The T stage is closely linked to patient outcome and serves as the primary prognostic factor for guiding treatment selection. The following section details the current treatment rationale for organ-confined or locally advanced MIBC.

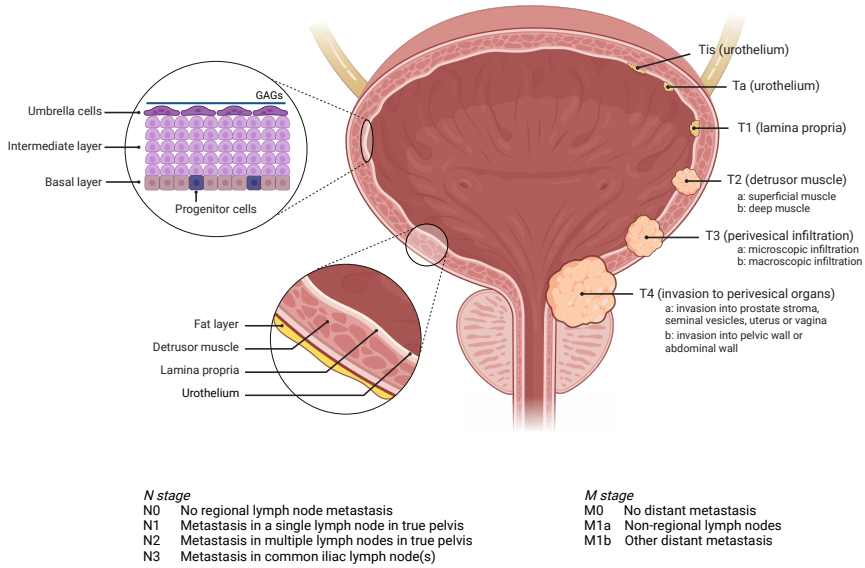

Figure 7. Bladder cancer staging. Illustration of the composition of the normal urothelium and bladder wall (right) and staging according to the TNM classification (left). Abbreviations: T: Tumour, N: Nodal, M: Metastasis.

Curative treatment strategies for muscle-invasive bladder cancer

Radical cystectomy

In 1886, the German surgeon dr. Bardenheuer wrote *I have always asked the question about bladder cancer if it would be possible to remove the whole bladder*. The following year, he performed the first cystectomy[222]. Over the course of the next century, significant advancements have been made. Today, the standard treatment for patients with MIBC staged T2-T4aN0M0 is radical cystectomy (RC) preceded by neoadjuvant chemotherapy. For patients with locally advanced disease, defined as T4b, N+, or solitary distant metastasis, induction chemotherapy with platinum-based combination regimens should be considered, with the aim of achieving remission and subsequent surgical treatment. Another indication for RC is BCG-unresponsive or high risk NMIBC[211].

Prior to surgery, the patient's overall health status, co-morbidities, and age are evaluated to assess the feasibility of surgery and to determine the extent of the procedure. RC involves cystectomy, pelvic lymph node dissection up to the common iliac bifurcation, and urinary diversion. In men, the prostate, seminal vesicles, and distal ureters are excised, while in women, the urethra, anterior vaginal wall, uterus, and distal ureters are additionally removed[223]. Post-surgery, histopathological evaluation is conducted as outlined for TURBT specimens. The presence of CIS, lymphovascular invasion, and lymph node metastases are reported, in addition to radicality[162].

Despite curative-intent surgery, approximately 50% of patients will eventually die from metastatic disease. In a large cohort of MIBC cases (n=507), nodal metastases were identified in 24% of patients despite negative preoperative staging, underscoring the metastatic propensity of MIBC. The 5-year overall survival (OS) for patients was limited to 62% for patients with organ-confined disease and 26% for patients with nodal involvement. Distant metastases developed in 51% of patients with initial nodal spread, compared to 25% of those with organ-confined tumours[224]. The most significant histopathological prognostic variables after surgery are the pT and pN-stages. Additional risk factors for disease recurrence include lymphovascular invasion and tumour location near the bladder neck or trigone[223].

Neoadjuvant treatment

These high recurrence rates reflect the presence of micrometastases at the time of surgery, emphasising the need for systemic treatment. Neoadjuvant treatment is beneficial as it is administered at the earliest time-point when the micrometastatic burden is conceivably low and the tolerability generally better than in the post-surgical setting. This improves the likelihood of tolerating dose intense regimens, and, in addition to eradication of micrometastases, enables a favourable downstaging of the primary tumour. In MIBC, there is strong evidence for recommending cisplatin-based combination therapy to eligible patients[223]. The administration of NAC has not been demonstrated to adversely affect surgical morbidity[223]. The efficacy of chemotherapy in MIBC has been recognised for decades, with effect of the MVAC regimen (methotrexate, vinblastine, doxorubicin, and cisplatin) published in 1985[225], followed by a landmark study in 2003 showing that neoadjuvant MVAC improves survival in locally advanced BC[226]. Meta-analyses have demonstrated an overall 5-8% improvement of 5-year OS[227,228], and an increased 10-year survival from 30% to 36%[229] with NAC compared to surgery only, which is clinically meaningful. For responders, particularly in patients with complete remission (pT0N0), NAC can have a major positive impact on OS[230]. NAC should be administered exclusively as cisplatin-based combination chemotherapy, as alternative regimens or monotherapy have demonstrated inferior efficacy in metastatic BC and have therefore not been comprehensively evaluated in the neoadjuvant setting[223]. The most frequently used regimens are dose dense (dd)-MVAC and GC (gemcitabine-cisplatin), given 3-4 cycles prior to surgery. The optimal form – MVAC or GC – is under debate, but dd-MVAC has shown higher degree of complete response (ypT0N0) compared to GC (41.3% vs. 24.5%)[231], as well as an improved survival[228,232]. For tumours exhibiting small cell differentiation, cisplatin-etoposide is recommended[162,223]. Side effects of treatment include myelosuppression, anorexia, vomiting, alopecia, and, particularly with cisplatin, renal dysfunction, neuropathy and ototoxicity, all of which require careful monitoring during treatment.

Unfortunately, response rates are limited to 30-50% of patients[232,233], and the inability to prospectively identify responders leads to considerable overtreatment of low-risk patients and non-responders. As a result, these patients are subjected to unnecessary chemotherapy-related toxicity and potential delays in curative surgery. This presents a significant clinical challenge, as will be further highlighted below.

In addition to NAC, studies exploring novel neoadjuvant treatment combinations are ongoing. Recently, results from the awaited NIAGARA trial were reported, showing an improved OS with the addition of perioperative checkpoint blockade (Durvalumab) to conventional cisplatin-based NAC (85% *vs.* 75% at 24 months), and comparable treatment-related adverse events[234]. In cisplatin-ineligible patients, administration of single-agent ICI (Atezolizumab) conferred a clinical benefit, with the expression of $CD8^+$ T cells correlating with therapeutic outcomes[235].

Radiotherapy and trimodality treatment

Curatively intended radiotherapy serves as an alternative to RC for well-informed patients who are ineligible for or decline surgery, preferably with concurrent radiosensitising chemotherapy (chemoradiotherapy), which has shown improved locoregional control and overall survival compared to radiotherapy alone[236]. Trimodality treatment, comprising maximal radical TURBT followed by chemoradiotherapy, constitutes a bladder-sparing approach and has shown comparable oncological outcomes to RC, however proper patient selection is warranted[223,237]. Subsequent surveillance is essential for detection of local bladder recurrences, where salvage surgery may be required[184].

Adjuvant treatment

Chemotherapy

Given the evidence supporting NAC, the proportion of chemotherapy-naïve patients eligible for adjuvant chemotherapy is relatively low. One theoretical advantage of adjuvant over neoadjuvant administrated treatment lies in the ability to base treatment decisions on more accurate tumour classification through histo-pathological evaluation of cystectomy specimens compared to TURBT specimens, thereby minimising overtreatment. Additionally, adjuvant treatment does not introduce delays to surgical intervention for patients with insufficient response to NAC. However, a notable disadvantage is the potential delay in chemotherapy treatment to patients with micrometastases, and chemotherapy may be difficult to initiate post-surgery due to insufficient postoperative recovery or complications.

The administration of adjuvant platinum-based chemotherapy for patients with high-risk disease (pT3/4 and/or pN+) is a subject of ongoing debate. A recent systematic review and meta-analysis of ten RCTs, involving 1183 participants treated with adjuvant cisplatin-based chemotherapy after radical cystectomy or

radiotherapy for MIBC, concluded a significant OS benefit with adjuvant chemotherapy (HR 0.82, 95% CI 0.70-0.96), corresponding to an absolute improvement in survival of 6% from 50% to 56% at 5-year follow-up. Significantly prolonged RFS and metastasis-free survival were additionally denoted (11% and 8%, respectively)[238]. The EAU guidelines, as well as the national Swedish recommendations, currently advocate offering adjuvant cisplatin-based combination chemotherapy to eligible patients within this high-risk group if no prior NAC has been given[211,223].

Immunotherapy

Adjuvant immunotherapy has been assessed in several phase III RCTs, in which patients were assigned to treatment with checkpoint inhibitors for 12 months versus obseration or placebo. The CheckMate 274 trial (Nivolumab) demonstrated a significant improvement in DFS, with similar results in the interim analysis of the AMBASSADOR trial (Pembrolizumab)[239,240]. In contrast, Imvigor010 (Atezolizumab) did not meet its primary endpoint of DFS[241]. TMB and PD-L1 expression as predictive biomarkers have been assessed with conflicting results[223]. The use of circulating tumour DNA (ctDNA) to stratify patients for adjuvant immunotherapy has shown promising potential[242]. For patients with high-risk disease following radical cystectomy and tumour-cell PD-L1 expression ≥1%, adjuvant immunotherapy may be considered, including for patients previously treated with NAC[162].

Who benefits from NAC?

The heterogeneous biological responses to NAC have spurred research efforts to elucidate the underlying mechanisms of therapeutic susceptibility.

Cisplatin constitutes the cornerstone of NAC regimens[223]. From a molecular standpoint, cisplatin exemplifies how even minor modifications in chemical structures can markedly influence cells' biological activity. Upon cellular uptake, cisplatin is activated through the displacement of chloride ions by water molecules, resulting in the formation of highly reactive electrophilic species. Cisplatin causes cellular damage by inducing reactive oxygen species, disrupting calcium homeostasis, and by eliciting endoplasmic reticulum stress. However, its principal target molecule is the DNA where it reacts with nucleic acids, primarily purine residues. This results in the formation of 1,2-intrastrand crosslinks between purine bases, although interstrand crosslinks have also been identified to contribute to cisplatin's toxicity[243]. The DNA damage activates the DNA repair machinery, leading to cell cycle arrest or the initiation of apoptotic programs, as previously described. The key signaling cascade connecting cisplatin-induced DNA damage to apoptosis involves the sequential activation of *ATM* and *ATR*, with subsequent activation of p53. However, p53-deficient cells can still respond to DNA damage,

underscoring the involvement of alternative pathways[243]. The genotoxic effects are not confined to malignant cells but also extend to healthy cells, contributing to the side effects of chemotherapy. Nevertheless, cancer cells are reliant on persistent cell division and become increasingly dependent on cell cycle control mechanisms. Consequently, excessive DNA damage is more likely to precipitate catastrophic genomic instability with fatal consequences for these cells[17]. In other words, impaired DNA repair constitutes an Achilles' heel of tumours and can subsequently hypersensitise cancer cells to cisplatin[243].

Indeed, defects spanning multiple DDR-related pathways have been implicated for NAC response in MIBC, including *ATM, RB1, FANCC, ERCC2,* and *BRCA*1/2[244-247]. In a cohort of cisplatin-treated patients with locally advanced or metastatic MIBC (*n*=100), stratified based on the presence or absence of mutations in DDR genes, patients harbouring mutations in ≥1 genes exhibited significantly improved OS. The presence of DDR mutations correlated with a higher overall mutational and CNA burden[248,249]. The clinical utility of stratifying patients based on DDR status has been evaluated in a single-arm, phase II non-inferiority trial (NCT02710734). In this study, sequencing of pre-NAC TURBT specimens was performed to identify somatic mutations in *ATM, RB1, FANCC,* and *ERCC2*. Patients with tumours harbouring ≥1 DDR alteration, and no clinical evidence of residual disease following dd-MVAC, were assigned to active surveillance, whereas the remaining patients proceeded to radical cystectomy. In the active surveillance cohort, recurrence was denoted in 68% (17/25) of cases; however, 48% avoided cystectomy without developing metastatic disease. Further long-term follow-up is warranted, but these findings suggest that additional refinement of patient selection criteria may be necessary[250]. Moreover, the presence of DDR genes may additionally be predictive of ICI response[251].

Beyond DDR mutations, additional genomic features, including the degree of genomic instability, have been associated with cisplatin response[247]. The predictive value of molecular subtypes remains uncertain. While some studies suggest preferential selection of the basal phenotype[252,253], others have reported inferior response rates to NAC in these tumours relative to luminal subtypes[254,255]. Moreover, transcriptomic profiling of NAC treated MIBC cases revealed that high stromal infiltration correlated with impaired outcome[256].

Resistance to therapy can arise from the selective expansion of pre-existing resistant clones or arise *de novo* as a consequence of chemotherapy-driven adaptation. Common resistance mechanisms include but are not restricted to: (i) blocked DNA access, for example downregulation of membrane transporter for drug uptake or increased drug efflux, (ii) impaired recognition and repair of DNA lesions, (iii) downregulation of apoptotic signalling, and (iv) altered drug metabolism. Often, multiple resistance mechanisms are simultaneously activated[257]. Additionally, the

underlying assumption of chemotherapy – that all cells are rapidly proliferating – is unlikely, posing a challenge to treatment efficacy.

The quest for biomarkers

Prediction is very difficult, especially about the future

Niels Bohr

The rich molecular variability inherent to MIBC is intrinsically linked to the divergent clinical disease trajectories observed in these patients, underscoring the need for reliable prognostic and predictive tools that could better anticipate the disease course and inform optimal treatment strategies. Although significant efforts have outlined important determinants, no molecular tools have yet been integrated into routine clinical practice. In the neoadjuvant setting, the standard practice of performing a TURBT procedure offers a valuable opportunity to examine biological tissue signatures, or *biomarkers.*

Present investigation

Seek, and ye shall find
Matthew 7:7

Aim

The overarching aim of this thesis was to deepen the understanding of the determinants of prognosis and response to neoadjuvant treatment in patients with muscle-invasive bladder cancer, through the exploration of tumour cell-intrinsic and extrinsic signatures within the local tumour microenvironment.

Specific aims

Paper I

To examine the prognostic significance of the local immune composition, including tumour-infiltrating T lymphocytes, B lymphocytes, and the PD-1/PD-L1 pathway, and its potential to predict NAC efficacy.

Paper II

To investigate the prognostic and predictive value of tumour cell RMB3 expression and elucidate cellular mechanisms potentially influencing chemosensitivity.

Paper III

To decipher the impact of spatial genomic, phenotypic, and immunogenic intratumour heterogeneity on therapeutic benefit and molecular alterations contributing to therapy resistance.

Study cohort

In *Papers I-II*, the study cohort comprised a retrospective, consecutive series of 145 patients diagnosed with MIBC, all of whom underwent diagnostic TURBT followed by radical cystectomy and pelvic lymph node dissection with curative intent at Skåne University Hospital, Sweden, between January 1st 2011 and December 31st 2014. Of these, 115 patients were diagnosed with *de novo* MIBC, while 13 had previously received intravesical BCG treatment. Histological subtypes were identified in 27 of 135 evaluable cases. The demographic characteristics, including age at diagnosis and sex distribution, were representative of the broader Swedish patient population[258]. A total of 65 patients received cisplatin-based NAC prior to definitive surgery, using the four-drug MVAC regimen, and 12 patients received adjuvant chemotherapy. Follow-up commenced at diagnosis and continued until death or August 31st 2018, with a mean duration of 4.3 years. Disease recurrence was identified in 51 cases, of whom 17 had received NAC, and MIBC was the recorded cause of death in 50 cases. Tumour specimens procured during TURBT were collected for all cases, with paired tumour tissue from radical cystectomy and regional lymph node metastases available for 135 and 27 cases, respectively.

Non-randomised, retrospective patient enrolment is inherently associated with a risk of introducing bias and may further constrain the ability to adequately control for confounding variables. In these studies, all patients who underwent treatment for MIBC within the defined four-year period were included. Given that approximately half of the patients received NAC, comparative analyses between the treated and untreated cohorts were feasible, providing insights into the putative prognostic and predictive value of investigated biomarkers despite the studies' retrospective design. Notably, NAC-treated patients were significantly younger than untreated patients, which may also impact outcomes. To address this potential bias, prognostic analyses considered not only overall survival (OS), but also time to recurrence (TTR) and cancer-specific survival (CSS). Clinicopathological annotations were obtained from medical records, in which a significant proportion of relevant parameters are routinely reported and thus available for all cases.

In *Paper III,* 15 cases from the aforementioned retrospective cohort exhibiting divergent biological responses to NAC were selected. Subsequent comprehensive molecular exploration of genomic, phenotypic, and immune profiles of chemo-naïve and matched residual tumours were conducted. To enable detailed analyses of NAC-induced tumoural responses, cases were included based on the following criteria: availability of sufficient viable tumour material for multiregional assessment, no prior history of intravesical therapy, and completion of a minimum of two cycles of NAC. Clinical data were updated at the end of April 2023.

Ethical considerations

Ethical approval for all studies was obtained from the Ethics Committee at Lund University, Sweden (reference number 445-2007 with amendments: 35-2008, 611-2007, and 748-2014), with permission granted for opt-out consent. The studies further comply with relevant guidelines and legislation, including the EU Council Convention on Human Rights and Biomedicine, and the Declaration of Helsinki Ethical Principles for Medical Research Involving Human Participants.

These retrospective studies were conducted independently of clinical management and did not influence treatment decisions. All tissue-based analyses were performed on archival tumour specimens procured during routine diagnostic or surgical interventions, whereby no additional harm was caused. Although the analysed tissue material originates from the years 2011 to 2014, it continues to serve as a valuable resource for ongoing and future research. Accordingly, careful planning of the sampling strategy was undertaken to ensure that the studies' objectives could be addressed while minimising any unnecessary impact on the biological material. In this context, the use of tissue microarrays (TMAs) represents a highly suitable approach, enabling the concurrent analysis of multiple spatially distinct tumour regions while preserving the integrity of the remaining tissue for further research.

The collected data, comprising clinical annotations extracted from medical charts and genetic information obtained through DNA sequencing, including germline samples, are inherently sensitive and require stringent measures to ensure privacy and confidentiality. All data have been de-identified through pseudonymisation and securely stored. Additionally, particular care has been taken during data processing and presentation to prevent the disclosure of any characteristics that could inadvertently lead to patient re-identification.

Furthermore, transparency constitutes one of the fundamental principles of research, essential for ensuring credibility, reproducibility, and scientific progress. In biomarker research, the Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) guidelines provide a framework for standardised, high-quality reporting of tumour biomarker studies[259], facilitating the evaluation of analytical validity and potential clinical relevance. The corresponding checklist for reporting[259] has been adhered to where applicable throughout these studies.

Methodological considerations

A true adventure begins when imagination collides with reality
Karel Capek

This section outlines the principal methodologies utilised in this thesis, discussing their strengths, limitations, and the rationale behind their selection.

Tissue-based analyses

Tissue preparation

The use of FFPE tissue specimens: Opportunities and hurdles

All tissue-based analyses were conducted using Formalin-Fixed Paraffin-Embedded (FFPE) tissue specimens. The formaldehyde fixation process induces inter- and intra-molecular cross-links between protein and nucleic acids, thereby stabilising cellular structures and preventing enzymatic degradation[260,261]. Subsequent paraffin embedding provides a solid matrix supporting tissue integrity. Thereby, FFPE enables long-term tissue storage at ambient temperatures, while maintaining tissue architecture[262]. FFPE is the gold standard for tissue preservation in clinical routine, where thin sections of FFPE blocks can be cut for histological evaluation and biomarker analyses. In clinical practice, archival FFPE specimens may be invaluable for re-assessing diagnoses and conducting supplementary molecular analyses to inform treatment decisions. For research purposes, FFPE samples serve as an indispensable resource, providing extensive accessibility to biomaterial for genomic, transcriptomic, and proteomic explorations in both retrospective and prospective studies, while permitting routine histopathological diagnostics.

However, the FFPE procedure introduces several challenges for molecular analyses and downstream data interpretation. Variability in fixation and storage conditions can significantly affect analytical outcomes. At the protein level, fixation-induced alterations include conformational changes, post-translational modifications, and cross-linking of proteins, which may obscure the immunoreactivity of epitopes[263-265]. To recover epitope structure, i.e. unmask the antigen, antigen retrieval techniques are employed to reverse covalent cross-links, with heat-induced epitope retrieval (HIER) being the most widely used method[264]. In genomic analyses, FFPE-induced degradation and modification of DNA and RNA, along with the chemical effects of extraction agents, can result in low yields, fragmented material, and sequencing artefacts, including increased deamination of cytosine to thymine and

guanine to adenine, yielding C:G > T:A miscalls[266,267]. An alternative to FFPE, offering improved nucleic acid quantity and quality, is the use of fresh frozen (FF) tissue. However, FF tissues require preservation at ultralow temperatures, and histomorphological assessment becomes more challenging[266]. While FF is generally regarded as the optimal substrate for sequencing, strong correlations have been reported for targeted deep sequencing (TDS) and whole-exome sequencing (WES) of FFPE- and FF-derived DNA[268-270]. Within the 100,000 Genome Project, whole-genome sequencing (WGS) was performed on paired FFPE and FF samples, yielding a 71% concordance in the detection of SNVs, whereas CNA calling demonstrated greater noise in the FFPE dataset. Importantly, 98% of clinically actionable alterations were detected using FFPE[271]. Advancements in sequencing technologies and analytical frameworks, including *in situ* transcriptomic profiling[272], have further strengthened the position of FFPE as a valuable resource in genome medicine[266,271].

Tissue Microarrays – a foundation for biomarker research

Throughout this thesis, tissue microarrays (TMAs) have been utilised for candidate biomarker evaluation and exploration of spatial ITH. Since their initial introduction by Kononen *et al.* in 1998[273], TMAs have become integral to biomarker research, facilitating the simultaneous assessment of multiple tumours while saving both tissue material and reagents compared to whole-tissue section evaluation, thereby enabling high-throughput biomarker screening[274]. The TMA technology is compatible with an armamentarium of *in situ*-technologies, including immunohistochemistry (IHC) and immunofluorescence (IF). During TMA construction, cylindrical tissue biopsies, usually 0.6-2 mm in diameter (herein, 1 mm) are extracted from multiple donor FFPE blocks, derived from one or several tumours. These cores are then systematically organised into an array within a recipient paraffin block, which is subsequently sectioned (typically 3-5 μm) and mounted onto glass slides for further analysis, as illustrated in Figure 8 (page 69).

Choosing a core sampling strategy: Impact of spatial intratumoural heterogeneity

A major consideration requiring attention in all biomarker studies is the extent to which the analysed tumour material accurately portrays the tumour's biological characteristics, as this directly influences interpretability. Specifically, this pertains to the degree of spatial ITH, reflecting the mosaicism of coexisting tumour cell populations which potentially harbour distinct molecular features of prognostic and predictive impact[275]. This complex issue remains a focal point of intense research. There are numerous factors that can influence the degree of detected ITH, including the cancer type being studied, timing of tissue sampling relative to tumour evolution and administered treatments, the level of molecular analyses (DNA, RNA, or protein), sampling coverage in relation to overall tumour dimensions, and the spatial expression pattern of the markers under investigation.

Hence, a recurrent concern is whether TMAs provide a sufficiently accurate representation of the donor tumour, given the small diameter of the tissue cores sampled, or whether whole tissue sections are preferred. This question has been extensively examined across multiple cancer types, with findings generally demonstrating a high concordance between the molecular heterogeneity detected using TMAs and that observed in whole tissue sections[273,276], including bladder cancer[277]. However, the assumption that whole tissue sections, with a thickness of 3-5 μm, reliably represent the entire tumour is not universally applicable. It has been reported that sampling of two or three distinct regions yields more comprehensive and representative data than a conventional single-sample[278,279]. Accordingly, in clinical research, the primary focus may not be whether TMAs reflect whole tissue sections, but rather the extent to which they correspond to clinically relevant associations, as discussed by Simon *et al.*[274].

While the optimal tissue sampling strategy remains an area of ongoing investigation, various approaches may be considered. These are contingent upon the specific requirements of downstream analytical objectives. In genomic studies, achieving sufficient tumour purity is essential, as admixture with non-malignant cells can dilute tumour-specific signals, thereby increasing the likelihood of obtaining false-negative results. Consequently, a commonly adopted strategy when using macro-dissection is to target tumour-enriched regions, identified through histopathological evaluation using haematoxylin and eosin (H&E) staining. To improve precision, laser capture microdissection (LCM) can be utilised to selectively isolate regions of interest, thereby enriching the tumour cell fraction. The integration of LCM with low-input sequencing has, for instance, enabled in-depth analyses of microbiopsies across the bladder tissue landscape, yielding crucial insights into the evolutionary trajectories of bladder cancer[176]. For tissue-based proteomic analyses, visual detection of target proteins with single-cell resolution mitigates sensitivity to tumour purity. Thus, sampling from regions of low cellular density is feasible. Areas adjacent to necrotic or haemorrhagic regions, however, are typically avoided due to the risk of protein detection interference and signal contamination. Examples of sampling strategies that can be employed include collecting a predefined number of regions from representative tumour areas, systematic sampling from equally spaced areas or grid-based approaches, and/or targeted focus on capturing micro- or macroscopically heterogeneous tumour domains.

In *Papers I-II,* triplicate tissue cores were macrodissected from representative tumour areas for each specimen type and case, using different donor blocks where available. In *Paper III*, a more comprehensive tissue sampling approach was implemented. All available tumour blocks underwent thorough H&E assessment prior to TMA construction, with regions intentionally selected to capture spatially distinct tumour domains, i.e. superficial and invasive core and border regions, as well as morphological heterogeneity. For tumour-enriched regions designated for

DNA sequencing, an adjacent tissue core within a defined radius of 0.5 cm was marked for parallel immune and molecular subtype phenotyping. In the case of immune profiling, additional regions from tumour-immune interfaces and adjacent immune-enriched stromal areas, were included. Although the regions of interest were carefully macrodissected, some may consequently not have been fully captured. The utilisation of LCM could have further refined sampling accuracy.

Spatial protein-based analyses

The use of tissue-based proteomic analysis constitutes a powerful means of examining tumour-specific protein expression, detailing the microenvironmental tumour landscape, and mapping cell-cell interactions. These techniques offer single-cell, and in some instances, subcellular resolution, while preserving the spatial context and morphology of the tissue. In this thesis, single-plex IHC and multiplex immunofluorescence (mIF) staining of TMAs were performed (Figure 8).

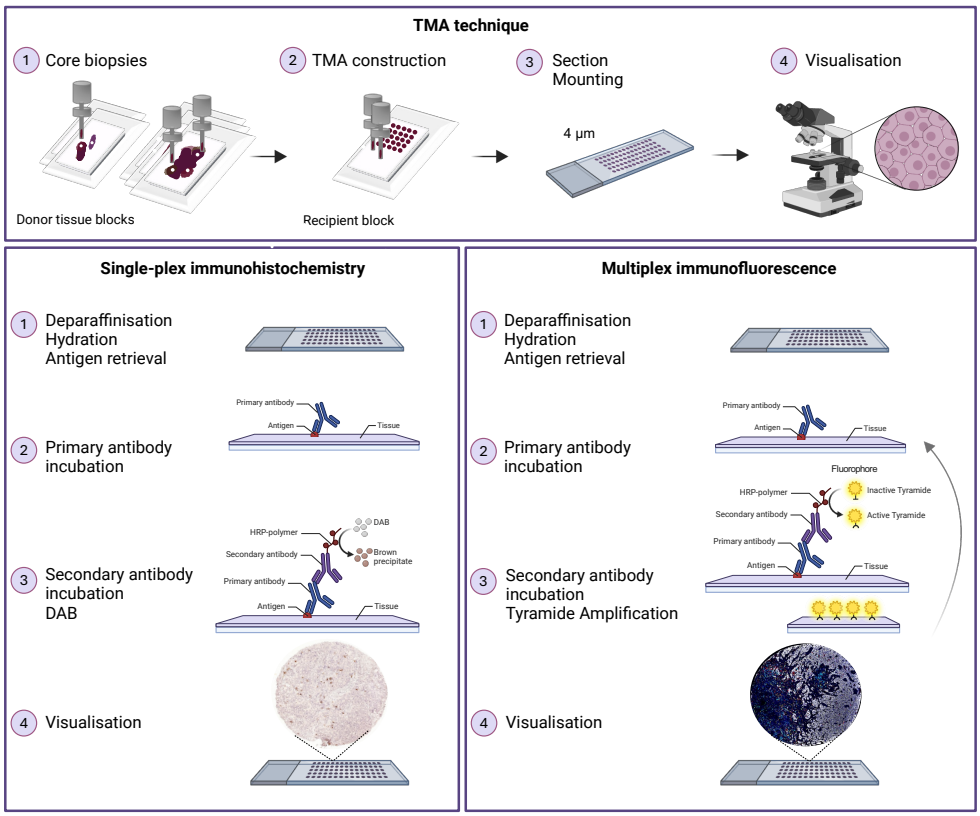

Figure 8. Protein-based tissue analyses. Schematic illustration of the construction of tissue microarrays (TMAs) and the subsequent application of single-plex IHC and and multiplex IF workflows.

Target detection: Selecting appropriate antibodies

Prior to staining, selecting antibodies with validated selectivity and specificity for the target antigen is essential to ensure reliability and reproducibility[280]. *A priori* knowledge of the target antigen's expression profile and cellular localisation, i.e. cell surface membrane, cytoplasm, or nucleus, is therefore important. In this regard, the open-access database *Human Protein Atlas*[281] serves as a valuable resource by providing extensive information on protein expression across a wide array of human tissues and cancer types, in addition to antibody validation.

Most antibodies utilised for IHC belong to the IgG class, where the fragment antigen-binding (Fab) region mediates antigen binding. Antibodies are categorised as monoclonal or polyclonal, originating from a single B cell clone targeting a unique epitope or multiple B cell clones recognising distinct antigen epitopes, respectively. Monoclonal antibodies provide high specificity with minimal cross-reactivity and background staining. However, their binding capacity may be compromised by FFPE-induced epitope masking[282]. These antibodies are conventionally produced using the hybridoma technology, wherein B cells are fused with immortalised cancerous cells to generate hybridoma cell lines, from which antibodies are isolated and purified, yielding batch-to-batch consistency[283]. However, hybridomas are susceptible to genetic drift over time, which may compromise antibody performance[284]. To further enhance long-term consistency and reproducibility, recombinant systems have been developed. These systems allow the isolation of the nucleotide sequence encoding the monoclonal antibody for expression in mammalian cell lines. It is also possible to use entirely synthetic gene constructs[285]. In contrast, polyclonal antibodies offer broader binding capacity and sensitivity, but carry an increased risk of cross-reactivity and non-specific staining. As these are generated through animal immunisation, they are inherently associated with lot-to-lot variability[282].

All antibodies used in this thesis have been previously validated, with monoclonal, in some cases recombinant, antibodies employed for IHC. Particular emphasis has been placed on ensuring that, whenever feasible, all tissue regions from a single case were incorporated into the same TMA to mitigate the risk of artefactual heterogeneity arising from potential technical errors.

Single-plex immunohistochemistry

Since its inception in the 1940s[286], IHC has remained an indispensable antigen detection technique in clinical pathology, where the use of complementary staining enhances diagnostic precision and serves as a means for companion diagnostics. It is also a pivotal method in biomarker research[287]. In the present investigation, IHC was employed to examine the immune composition of the local tumour micro-environment as well as the tumour-specific expression of molecular subtype markers and RBM3.

IHC staining of FFPE specimens follows a stepwise procedure, where each phase can be optimised to maximise signal detection. Following deparaffinisation and rehydration, antigen retrieval is performed to unmask epitopes, as described above. In this thesis, HIER was used. Given the use of a peroxidase-dependent antigen detection system, naturally occurring peroxidase activity in the tissue is blocked by endogenous peroxidase inhibitors, preventing high background staining. A protein-blocking step may also be included to reduce non-specific binding to proteins mimicking the target epitope. Subsequent epitope detection is mediated by antibody binding, either through direct methods – utilising detection-labeled conjugated primary antibodies – or indirect methods, as employed in these studies. The tissue is incubated with the primary antibody for the optimised duration, followed by incubation with the secondary antibody conjugated to horseradish peroxidase (HRP), which binds to the primary antibody. Addition of a chromogenic substrate, such as 3,3′-diaminobenzidine (DAB), triggers an HRP-catalysed reaction, forming a brown precipitate at the antigen localisation site. Finally, tissue sections are counterstained to visualise cell structures, such as cell nuclei using haematoxylin, followed by visual inspection (Figure 8).

Multiplex immunofluorescence and digital image analysis

Analyses enabling the simultaneous assessment of markers allow for a more concise delineation of distinct cell populations, e.g. the co-expression of CD4 and FoxP3 to identify Tregs[288]. While multiplex conventional IHC allows for the concurrent detection of 2-3 antigens, they are limited by antibody species interference as well as weak and overlapping chromogen signals[289]. Thus, alternative approaches that offer an expanded dynamic signalling range are needed. Therefore, for spatial immune phenotyping in *Paper III*, mIF was utilised for the detection of six markers: CD4, CD8, CD20, FoxP3, CD68, and Pancytokeratin (PanCK) (Solid Tumour Immunology Kit, Akoya Bioscience). The methodological principles are analogous to conventional IHC, but rather than using chromogenic substrates for signal detection, fluorogenic substrates and tyramide signal amplification are used. The HRP-conjugated secondary antibody catalyses the conversion of fluorescently labelled tyramide molecules into free radicals, which covalently bind to tissue tyrosine residues in proximity to the antigen localisation site. Sequential staining cycles are then performed until all epitopes are fluorescently labelled. Between cycles, HIER is repeated to remove previously bound antibodies, thereby preventing cross-reactivity (Figure 8). After staining, 4′,6-diamidino-2-phenylindole (DAPI) is applied for nuclear visualisation. The stained tissue slides are then subjected to multispectral scanning to acquire high-resolution images. Each fluorophore emits light at a specific wavelength upon excitation, resulting in distinct emission spectra. The imaging system captures multiple images of the tissue at different wavelengths to record the complete emission signature of each fluorophore. Spectral deconvolution is then applied to separate overlapping signals, allowing high-precision localisation and quantification of each marker[289]. Autofluorescence

arising from FFPE preservation can be isolated from the tissue and excluded from analysis, thereby improving detection accuracy.

For the subsequent evaluation of acquired pixel data, various digital analysis pipelines can be implemented for cell identification and phenotyping. Herein, all digital analyses, summarised in Figure 9 (page 73), were conducted using the open-source software QuPath[290]. Unlike manual assessment of conventional IHC staining, where necrosis, staining artefacts, and tissue folds can be visually accounted for, digital analysis is inherently dependent on the quality of the input data. Therefore, systematic tissue curation is necessary prior to analysis to exclude such regions. Tissue curation is followed by cell segmentation, i.e. the assignment of pixels to individually identified cells. This step is crucial for accurate cell assignment. Several challenges can affect the performance of cell segmentation algorithms when analysing two-dimensional data derived from complex three-dimensional tissues. These challenges include the presence of cell-dense regions, morphologically aberrant cell structures deviating from spherical shapes, and variations in the spatial resolution of acquired images[291]. As many of these algorithms are trained on publicly available datasets, they may not be fully adapted to the specific tissue under investigation. One commonly used approach involves the initial identification of cell nuclei based on a defined threshold for the nuclear counterstaining signal (e.g. DAPI), combined with size and shape parameters. Thereafter, cell boundaries are determined by specifying the distance over which the cell should expand from the nucleus, unless it encounters the boundaries of neighbouring cells first. This method was exploited, resulting in effective cell detection. However, its relatively static nature renders it sensitive to cell shape variability. Consequently, an alternative approach using *Instanseg* was applied, enabling simultaneous nuclei and cell segmentation, with enhanced accuracy as the number of input channels increases[292]. It is built upon a U-Net architecture framework[293], and has been benchmarked against other widely implemented methods, such as CellPose and StarDist, with similar or superior performance[294]. Using its pretrained fluorescence model, the delineated cell boundaries were found to more closely conform to the natural contours of the cells and were hence utilised for further analysis.

Cell segmentation was followed by the development of a classifier for each marker. This can be done by visually determining a threshold for each channel, above which cells are classified as marker-positive. In this study, we employed a supervised machine learning approach based on a random trees classifier[295], integrated into QuPath. This hierarchical decision tree algorithm iteratively partitions the data based on cell feature values to construct a predictive model. At each branching node, the algorithm selects the optimal feature and threshold to split the data, continuing the process until a predefined stopping criterion – such as a maximum number of splits or the formation of homogeneous subgroups – is reached. The model was

trained using manually assigned cell calls from multiple cores representing all included TMAs and was subsequently applied across the dataset (Figure 9).

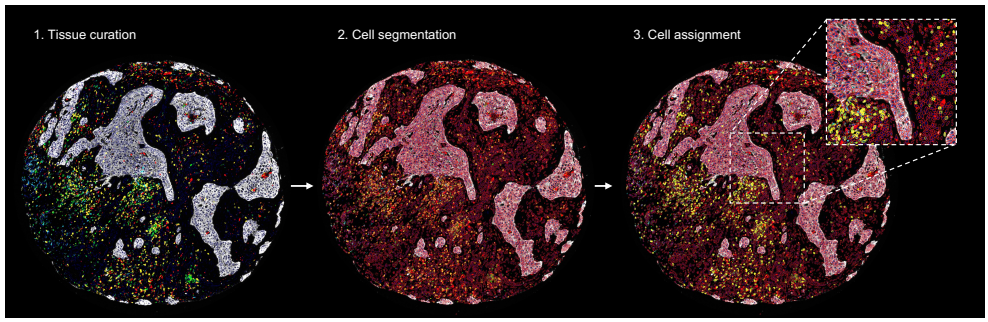

Figure 9. Digital image analysis of mIF data. Tissue cores were subjected to cell and nuclei segmentation using *Instanseg*, followed by cell phenotyping. A supervised machine learning approach was applied to train a classifier for each marker, as exemplified by CD8 (yellow).

Semiquantitative or automated tissue annotation: Advantages and disadvantages

Single-plex IHC staining was evaluated manually. Visual scoring remains widely utilised, allowing for a nuanced interpretation of staining patterns and cellular morphology, attributable to the pathologist's expertise and pattern recognition. However, as a semiquantitative method, it is inherently susceptible to intra- and inter-observer variability[287]. For biomarkers not implemented in clinical practice, the absence of standardised scoring methods and predefined cut-off values complicates reproducibility and comparability across studies. Consequently, a detailed assessment of staining patterns and cell distributions is essential to determine an appropriate evaluation strategy.

For certain biomarkers, such as PD-L1, which may be expressed on the membranes of both tumour and immune cells, distinguishing between these cell populations may be crucial, as explored in this thesis. Morphological examination facilitates this distinction unless counterstaining for specific cell populations has been performed. Scoring approaches can integrate both cell abundance and staining intensity, with the relevance of the latter varying depending on the marker. In *Paper I,* the percentage of marker-positive immune cells relative to the total immune cell count was quantified on a continuous scale (0-100%), with PD-L1 expression denoted separately for immune and tumour cells. In *Paper III*, IHC-derived immune cell counts were instead stratified into categories, adjusted to account for variations in cell densities. For mIF data, cell counts were normalised to the total cell count per core to enable comparison across cores and cases. The marker analysed in *Paper II,* RBM3, is expressed in both the nuclear and cytoplasmic compartments. However, given the previously shown stronger prognostic association with nuclear

localisation[57], analysis was restricted to nuclear frequency and intensity, in alignment with earlier studies[57,62].

Other critical considerations include blinding the observer to outcome data whenever possible to mitigate the risk of unintentional bias. Additionally, awareness of *diagnostic drift*, a gradual shift in scoring values when assessing larger materials or over prolonged periods, is essential[296]. Hence, IHC scoring was independently performed by two observers, with discrepancies resolved through consensus discussion.

As mentioned, manual assessment offers greater flexibility and readily adapts to variations in staining quality, whereas automated quantification is more susceptible to artefacts. However, automated analysis, despite its initially labour-intensive setup, enables objective, reproducible large-scale analyses and integration with computational pathology tools. A limitation is the inherent inability to interpret tissue architecture without proper training[297]. For immunofluorescence analyses, including a consecutively sectioned H&E-stained slide can aid morphological assessment, as used in *Paper III.*

Spatial genomic analysis

To delineate the extent of genomic spatial ITH and its potential relevance for chemotherapy response, targeted deep sequencing (TDS) was performed on DNA extracted from FFPE tumour specimens, along with matched normal tissue samples, in *Paper III*. Compared to whole-genome (WGS) or whole-exome (WES) sequencing, which analyse the entire genome or protein-encoding regions, respectively, TDS focuses on a selected subset of coding and non-coding regions. This method provides a cost-effective means of achieving high sequencing depth, thereby partially addressing the challenges induced by the FFPE process[269]. To this end, a pan-cancer panel (total size: 2.4 Mb), encompassing over 300 genes and complemented by a SNP backbone, was used for the assessment of small-scale and chromosomal-level alterations, sequenced on the Illumina NovaSeq 6000 platform.

DNA isolation

Nucleic acids exhibit a high selective binding affinity to silica-based membranes[298], providing the rationale for silica-based extraction methods. The stepwise procedure involves cell lysis, removal of contaminants, and reversal of formalin-induced cross-links by serine proteases. This is followed by the binding of nucleic acids to the silica membrane of the spin column through electrostatic interactions. After thorough washing, purified DNA is eluted from the silica into buffer for subsequent sequencing[299] (Figure 10A, page 76).

Next-generation sequencing

The first generation of genomic sequencing, known as *Sanger* sequencing, was introduced in the 1970s[300], marking the onset of a revolutionary era that has laid the foundation for a deeper understanding of biology. Subsequent advancements paved the way for the initiation of the *Human Genome Project,* which, after 13 years of research, successfully decoded the approximately three billion base pairs constituting the human genome by 2003[301], with the final gaps assembled in 2022[302]. The current standard, next-generation sequencing (NGS), enables high-throughput analysis through massive parallel sequencing of DNA and RNA[303].

Sequencing aims to determine the precise order of nucleotides, using *sequencing by synthesis*. The process, outlined in Figure 10B, consists of four key stages: (1) *Library preparation*, where DNA is fragmented and ligated to oligonucleotide adapters. To enable multiplex sequencing – the simultaneous analysis of multiple samples – unique adapters are assigned to each sample before pooling. For non-WGS approaches, molecular capture probes complementary to the sequences of interest are introduced to selectively enrich for these regions. (2) *Cluster generation,* where adapter-ligated DNA fragments bind to the flow cell surface of the sequencing instrument, followed by bridge amplification, forming clonal clusters that increase the local concentration of DNA. (3) *Library sequencing,* in which primers anneal to the DNA strands, and fluorescently labelled nucleotides – each corresponding to one of the four bases – are sequentially incorporated during DNA synthesis. A laser excites the fluorophores, generating emission signals that are recorded by the instrument. Each nucleotide generates a unique emission spectrum, enabling precise determination of the DNA sequence at the individual base level. (4) *Alignment and data analysis*, as further outlined in the following section.

In *Paper III*, paired-end sequencing (2 x 150 bp) was performed, where sequencing in both directions facilitates the identification of true variants. To mitigate technical noise and sequencing biases, unique molecular identifiers (UMIs) were incorporated. UMIs are short oligonucleotides that label individual DNA molecules, serving as molecular barcodes[304]. During library preparation, UMIs are co-amplified with the targeted sequences, and following sequencing, all reads sharing the same UMIs originate from a single template DNA molecule. Reads associated with a given UMI can subsequently be collapsed into a final consensus read during bioinformatic processing. This approach enables the detection of amplification-induced errors or technical artefacts in the nucleotide sequence that could otherwise be misinterpreted as false variants, thus improving variant calling specificity[305]. In *Paper III*, duplex UMIs (i.e. UMI-tagging of each strand of the double-stranded DNA molecule) were used to generate consensus reads where applicable, in terms of adequate sequencing depth.

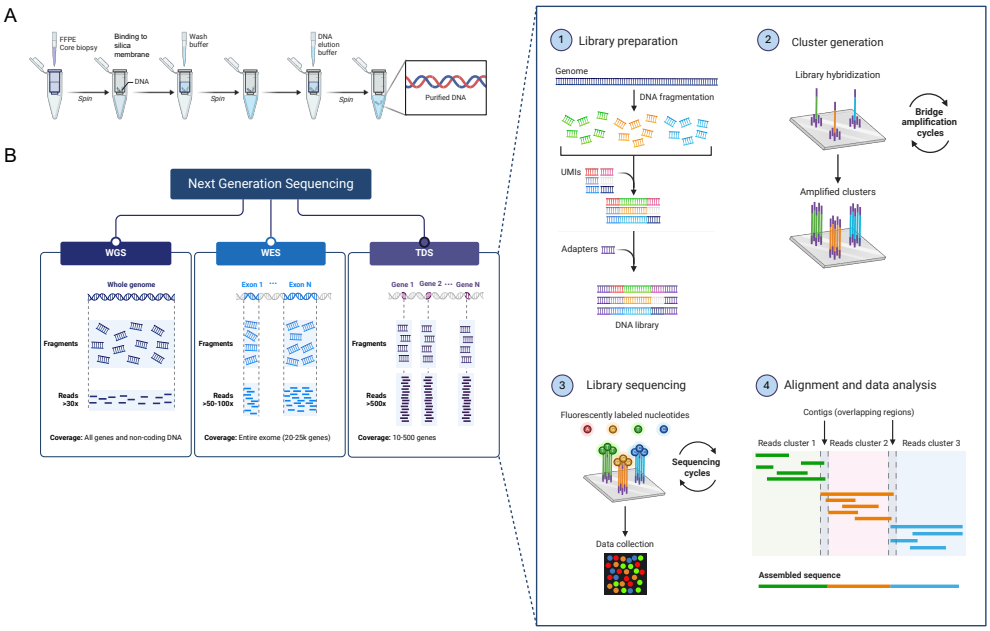

Figure 10. Principles of next-generation sequencing. A) DNA extraction from FFPE tumour core biopsies. B) The three distinct NGS approaches, characterised by different genome coverage and average read depth, of which a targeted approach was employed for the studies in *Paper III.* Abbreviations: UMIs: Unique molecular identifiers; WGS: Whole-genome sequencing; WES: Whole-exome sequencing; TDS: Targeted deep sequencing.

Bioinformatic processing

Bioinformatic processing refers to the computational analysis and interpretation of comprehensive complex datasets, including NGS data. The raw sequencing data are initially converted into FASTQ files, which contain information on the read name, called sequence, and base call quality scores for each nucleotide. These are subjected to quality control, where low-quality reads are excluded. The remaining reads are then aligned to the reference genome, in this case hg19/GRCh37. Short sequencing reads, due to DNA fragmentation or insertion-deletions, may inaccurately align to multiple genomic regions. However, by leveraging paired-end reads, the accuracy of alignment can be improved. The alignment information is stored in Sequence Alignment Map files (SAM files), which are compressed into Binary Alignment Map (BAM) files to facilitate downstream analyses. Herein, two distinct BAM files were generated for each sample: consensus BAMs, which include all reads (even singletons) but with consensus reads generated where applicable, and filtered BAMs, containing only high-quality duplex consensus reads. The latter were then primarily used to provide additional support for variants called from the consensus BAMs during variant calling. Technical artefacts arising during library construction may result in duplicate reads, which can be identified and removed prior to variant calling. The read depth, i.e. sequencing depth, at each

genomic locus is quantified as the number of reads aligned to the specific position. Variant calling is then performed to identify variants that deviate from the reference genome, with the inclusion of matched normal samples facilitating the distinction between somatic and germline variants. Typically, multiple variant callers are used, with FreeBayes[306] and VarDict[307] being employed in this study. Identified variants are then annotated to determine their significance. This process involves removing variants, e.g. SNPs, commonly found in the general population and identified through reference databases such as the genome aggregation database (gnomAD)[308], as well as variants estimated to have low functional impact on the resulting gene product, by using tools such as Variant Effect Predictor (VEP)[309]. In *Paper III*, only variants with predicted moderate or high impact were included. While truncating alterations, such as InDels or nonsense mutations, and alterations influencing RNA splicing are generally considered deleterious, the pathogenicity of non-synonymous single-base substitutions (missense mutations) may vary. Factors influencing the pathogenicity of these include their location, such as within critical regions of the protein-encoding sequence, the physicochemical properties of the amino acids involved, and the structural integrity of the protein[310]. In general, missense mutations are more likely to be deleterious when involving highly evolutionarily conserved residues, typically critical for the protein's function[311]. Although the potential functional consequences of missense mutations can be assessed through experimental functional assays, the most commonly used method is *in silico* prediction using computational tools such as PolyPhen-2[312] or SIFT[313].

For each genetic variant, the variant allele frequency (VAF) is calculated, representing the proportion of sequencing reads supporting the variant in relation to the total number of reads aligned to the locus. This metric is essential for subsequent interpretation of small-scale variants.

Chromosomal-level alterations were inferred from TDS data, by integrating sequencing depth and allele-specific information obtained from genome-wide SNPs. The analytic process begins with calculating read depth for each target region, followed by normalisation to mitigate technical biases introduced during sequencing, including amplification artefacts, variations in hybridisation efficiency, GC-content biases, and uneven sequencing coverage. A reference panel, in this case, a pooled set of matched normal samples, is utilised to establish a baseline for somatic copy number estimation. When using a TDS panel, genomic gaps between targeted regions may pose challenges for DNA detection. To address this, genome-wide SNPs can be incorporated into the panel and there are additionally algorithms developed to leverage information from off-target regions. As non-targeted sequencing reads will be present after sequencing, these can be used to generate large-scale CNA profiles, albeit with low coverage[314].

Next, copy number ratios are determined by comparing the read depth of the tumour samples with that of the reference panel for each given genomic region, yielding

log_2 ratios (Log_2R). Log_2R is a quantitative measure of the copy number configuration. A Log_2R of 0 corresponds to the same copy number as the reference, in normal cells a diploid state, whereas log2R >0 and <0 indicates copy number gains and losses, respectively. These calculations are supplemented with information on the allelic composition, i.e. deviations from the expected diploid state (2N, 1+1) of normal cells. To quantify these, SNPs identified in matched normal samples are analysed to determine the B-allele frequency (BAF), which represents the proportion of the non-reference allele (B allele) to the total number of alleles (B allele/(A+B alleles)) at a given locus, providing a measure of relative allelic abundance. In diploid regions, heterozygous SNPs (A+B) exhibit a BAF of 0.5, while homozygous SNPs display values of either 0 (B+B) or 1 (A+A). Deviations from these expected values indicate the presence of CNAs, but may also arise from other genomic events, such as loss of heterozygosity (LOH) or the presence of subclonal tumour cell populations. Thereafter, the software segments the genome into regions with similar copy number profiles, applying a threshold to differentiate alterations from background noise. In this thesis, CNVkit[314] was primarily used for CNA calling. The resulting segment files were exported for manual visualisation and careful curation of CNAs and allelic imbalances. To support this evaluation, CNAs were additionally identified using the Jumble bioinformatics algorithm[315]. The generated CNA profiles and scatter plots were utilised for complementary assessment of tumour cell ploidy, clonality, and allelic composition (Figure 11).

Calling CNAs from FFPE samples presents inherent challenges due to variations in sequencing depth and tumour cell purity[271]. Although TDS yields lower resolution compared to alternative detection methods, such as the molecular inversion probe (MIP) technology[316], the integration of data from two independent callers, combined with rigorous manual curation, was considered suitable for the purpose of this study.

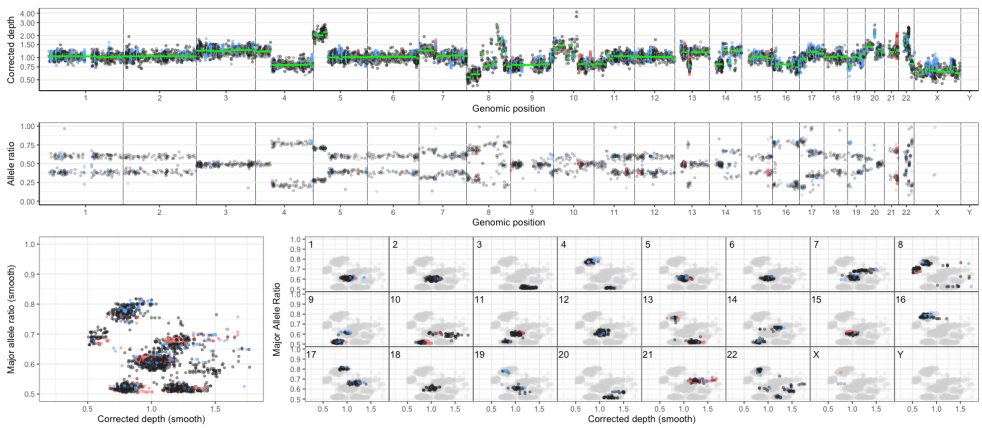

Figure 11. Genome-wide copy number alteration profile. Overview of called somatic CNAs for one tumour sample, with corresponding log_2 ratio and B-allele frequency values, derived from the Jumble copy number anlaysis algorithm[315].

Curation of genomic alterations in relation to intratumour heterogeneity

Regardless of the chosen NGS approach – WGS, WES, or TDS – low-frequency variants of uncertain significance are inevitably detected. Distinguishing true subclonal alterations from fixation- or sequencing-induced artefacts is challenging, especially in the context of tumour heterogeneity analyses. One approach for improving variant classification involves quality assessment using tools such as the Integrative Genomic Viewer (IGV)[317]. In this study, all detected variants underwent inspection in IGV to verify accurate read alignment and bidirectional sequencing support. To minimise the risk of introducing false heterogeneity, variants not present across all samples from a given case were additionally evaluated. Variants below the predefined VAF detection threshold were included if a corresponding tumour sample from the same patient exhibited the same variant at a higher VAF. Differences in tumour purity further complicate interpretation, as low-purity samples may obscure the detection of subclonal variants. Therefore, samples with insufficient tumour purity were excluded, as the absence of a subclonal variant in such cases could not be reliably attributed to true biological heterogeneity.

A similar strategy was applied to CNA analysis, with a particular focus on the precise delineation of breakpoints. To mitigate the risk of false inter-sample variability, biologically recognised stepwise evolutionary processes and back mutations were incorporated, where applicable, as previously described[318].

In addition to comparison of genomic profiles, curated SNVs/InDels and CNAs were utilised for clonal deconvolution and phylogenetic modelling. Tumour purity and the fraction of tumour cells exhibiting distinct genomic alterations were estimated using VAF values and allelic composition for small-scale variants, and Log_2R and BAF values for CNAs. Clonal deconvolution was then applied to delineate the subclonal architecture of the samples, whose ancestral relationships, i.e. the chronological order of events, were inferred using phylogenetic reconstruction. For this purpose, the DEVOLUTION algorithm[319] was employed, adapted to bulk sequencing data, in conjunction with the maximum parsimony algorithm[320].

In vitro-modelling

Cell lines

In *Paper II,* RMB-related biological processes were examined using an *in vitro* model of two bladder carcinoma cell lines: RT4 and T24. These cell lines are widely employed in bladder cancer research, providing a simplified representation of distinct disease states, and have previously demonstrated sensitivity to the chemotherapy agents applied in this study[321]. The RT4 cell line, established in 1970, originates from a human low-grade (G1) T1 transitional cell carcinoma[322]. It is

characterised by a non-invasive phenotype and commonly used as a model of superficial disease[323,324]. Molecular profiling has identified luminal subtype features[325] and *TP53* wildtype status[326]. The T24 cell line, established in 1973, derives from a high-grade (G3) invasive transitional cell carcinoma[327]. It exhibits low expression of luminal and basal markers[325], with instead a mesenchymal morphology[328], and is *HRAS* and *TP53* mutant[326]. In comparison to RT4 cells, T24 cells have shown increased invasive capacity *in vitro* and elevated levels of the cancer stemness marker CD44[329].

RBM3 silencing

Modulating the expression levels of a target gene facilitates the investigation of gene function. This can be achieved through overexpression and/or silencing, with the latter approach being employed in this study. Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)-Cas-based technologies have emerged as powerful tools for precise DNA editing, enabling effective and stable gene knockouts with minimal off-target effects[330,331], and their applications have been further expanded to include RNA editing[332,333]. While CRISPR/Cas-based tools are being increasingly utilised, RNA interference (RNAi), including the use of silencing RNA (siRNA), remains a widely adopted and cost-effective method for gene silencing. RNAi induces transient reductions in mRNA levels, i.e. gene knockdown, but is inherently associated with an increased risk of both sequence-related and unrelated off-target effects[334]. In *Paper II*, siRNA-mediated knockdown was used. siRNA exploits the endogenous microRNA-mediated silencing machinery present in all cells (Figure 12). siRNA consists of double-stranded RNA, which is introduced into the cytoplasm via various transfection methods, and in this study, a cationic liposome-based reagent was used[335]. Upon cell entry via endocytosis, siRNA is loaded into the pre-RNA-induced silencing complex (RISC), where the passenger strand is removed and degraded. The antisense strand remains bound to Argonaute 2 (Ago2) forming the mature RISC complex, where it provides the template for complementary base pairing with the target mRNA. Once bound to RISC, the mRNA is cleaved by Ago2 and released from the complex. The antisense strand remains associated with RISC, allowing for repeated cycles of binding and degradation of target mRNAs, thereby inhibiting subsequent protein translation[336]. The persistence of *in vitro* gene silencing is contingent upon the cell proliferation rate, with a duration of 4-7 days in rapidly dividing cells[337], during which thousands of target mRNA molecules can undergo degradation[338].

siRNA-mediated silencing of *RBM3* was subsequently confirmed at both the mRNA and protein levels, using qRT-PCR or Western blot and immunocytochemistry, respectively. In all experiments, non-targeting siRNA served as a control to assess off-target effects of siRNA delivery and as a reference for evaluating target-specific siRNA effects.

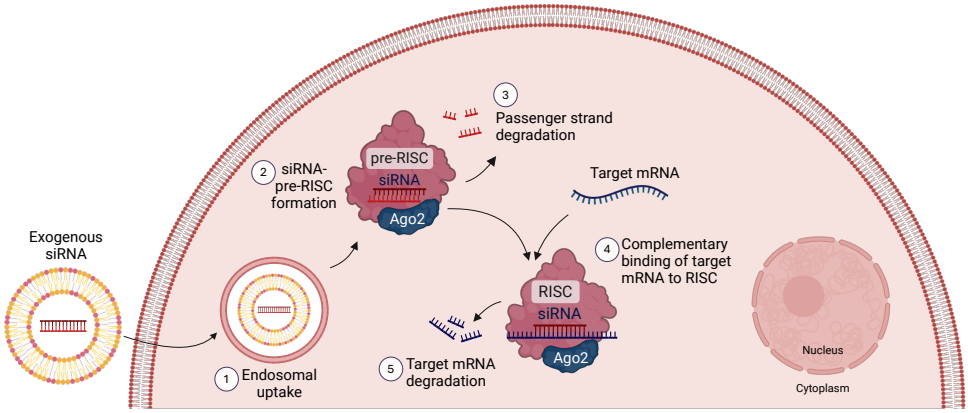

Figure 12. The mechanistic basis for siRNA-mediated gene silencing of target molecules. Abbreviations: Ago2: Argonaute 2; mRNA: Messenger RNA; RISC: RNA-induced silencing complex; siRNA: Silencing RNA.

Cell viability assay

Cell viability following siRNA-mediated silencing and exposure to cisplatin and gemcitabine was examined using a colorimetric Water-Soluble Tetrazolium (WST)-1 assay. In metabolically active cells, added tetrazolium salts are reduced to formazan dye by mitochondrial dehydrogenases, which can be quantified spectrophotometrically at specific wavelengths. Due to its negative net charge, WST-1 is primarily cell-impermeable, meaning the reduction process occurs extracellularly. This requires the addition of intermediate electron acceptors for transmembrane electron transport, with mitochondrial-derived NADH serving as the cellular reductant[339]. The measured dye reaction is assumed to be proportional to the number of viable cells[340].

Although the metabolic state of cells is often used as a measure of cell viability, it is important to note that a decrease in metabolic activity can reflect both cell stasis and actual cell death. Chemotherapeutic agents can exert cytostatic, cytotoxic, or combinatorial effects depending on the context and dosage. For instance, cisplatin-induced DNA lesions may result in transient cell cycle arrest, allowing DNA repair mechanisms to operate, or if the damage is irreparable, it may cause permanent proliferation arrest, i.e. cellular senescence, or apoptosis[341]. While a marked reduction in WST-1 signal indicates cell death, additional methodologies, such as flow cytometry-based Annexin V assays, caspase activity measurements, or Western blotting for apoptosis markers, are needed to accurately differentiate between cytostatic and cytotoxic effects[342].

Transcriptome-level effects of RBM3 and validation

To characterise cellular mechanisms associated with RBM3 in muscle-invasive disease, bulk RNA-sequencing was performed on siRBM3-treated and control T24

cells. RNA-seq is an NGS-based technology that enables comprehensive transcriptomic profiling, facilitating the identification of differentially expressed genes (DEGs) between experimental conditions and their associated biological processes. The core principles of RNA-seq are similar to those of DNA-seq but incorporate an additional reverse transcription step, wherein RNA is enzymatically converted into complementary DNA (cDNA), a more stable intermediate that enables subsequent processing using DNA-based NGS platforms. Sequencing can be conducted in either a single-end or paired-end configuration. In *Paper II*, a single-end strategy (75 bp, Illumina NextSeq 500 platform) was implemented, generating unidirectional reads. While single-end sequencing constitutes a cost-efficient approach, continuous advancements in sequencing technologies have rendered shorter paired-end reads a viable alternative[343]. Post-sequencing, the generated FASTQ files were processed to trim sequencing adaptors and filter out low-quality base calls, followed by alignment to the human reference genome (hg19/GRCh37). Read quantification and statistical analyses were performed to identify DEGs between siRBM3-treated and control T24 cells. The identified DEGs were then subjected to gene ontology (GO) enrichment analysis[344] to gain insight into molecular pathways associated with RBM3 modulation.

The obtained results were validated using quantitative PCR (qPCR) and flow cytometry-based cell cycle analysis. For qPCR, similar to RNA-seq, RNA was isolated, purified and reverse-transcribed into cDNA. To quantify the specific genes of interest, in this case, cell cycle regulators, fluorescently labeled oligonucleotide probes complementary to the target gene sequences were added. This was followed by amplification through repeated cycles of denaturation, primer annealing, DNA extension, and probe cleavage, resulting in detectable fluorescence emission. The fluorescence is proportional to the amount of dsDNA, reflecting gene amplification.

The principle of the employed cell cycle analysis is based on the quantification of Propidium Iodide (PI), a fluorogenic compound that intercalates into double-stranded nucleic acids. The PI fluorescence intensity provides a direct measure of cellular DNA content[345]. Samples were analysed using flow cytometry, wherein cells in suspension undergo hydrodynamic focusing towards a laser detection point, generating forward and side scatter signals from which cell metrics are inferred[346]. A gating strategy was applied to exclude debris and doublets. The constructed DNA histograms, with fluorescence intensity on the x-axis and cell counts on the y-axis, allowed for the discrimination of discrete cell cycle phases, using the Watson Pragmatic algorithm[347]:

1. G_1-phase (diploid, 2N): Low fluorescence intensity.
2. S-phase: Intermediate fluorescence intensity due to partial DNA replication.
3. G_2/M-phase (tetraploid, 4N): High fluorescence intensity.

Statistical considerations

Statistical models were predominantly applied to evaluate differences between patient groups and to extrapolate population-level patterns from the studied cohorts, i.e., statistical inference. The employed analyses are specified in each original paper.

In statistical hypothesis testing, the alternative hypothesis (H_1), which posits a relationship between variables, is tested against the null hypothesis (H_0), assuming statistical independence between variables. The *p*-value represents the probability of obtaining the observed, or more extreme, distribution under the null hypothesis, providing an indication of whether the result reflects a true effect or arises from random variation. The obtained *p*-value is then compared to the pre-defined significance alpha (α) level. If $p < \alpha$, H_0 can be rejected in favour of H_1. The significance level is commonly set at 0.05, which was applied consistently throughout this thesis. In hypothesis testing, two types of errors may occur: Type I error, where H_0 is incorrectly rejected, leading to false positives, and Type II error, where H_0 is erroneously accepted, resulting in false negatives. Type I errors are determined by the chosen significance level, whereas type II errors are influenced by additional factors, including statistical power. Statistical power is further shaped by various elements beyond the significance level, including sample size, effect size, and the extent of variability within the data. In retrospective studies, the sample size is often constrained by the availability of cases and/or biospecimens for analysis.

These concepts are central to the interpretation of the statistical analyses presented in this thesis. It is essential to emphasise that a *p*-value should not serve as an arbitrary threshold for scientific significance, rather, it should be regarded as the strength of evidence against H_0 and the accepted degree of uncertainty. Moreover, statistical significance does not inherently equate to clinical relevance; the magnitude of the observed effect and the clinical framework must be carefully considered. The restricted sample sizes in the subgroup analyses in *Papers I-II*, and the cohort encompassing fifteen patients in *Paper III*, increased the susceptibility to type II errors. Thus, *p*-values indicating a potential trend of association were transparently reported and interpreted within their respective context, while emphasising the need for careful consideration. In *Paper III*, certain statistical comparisons were deemed non-informative; therefore, case-specific observations were instead reported. Given that the primary objective of the study was to examine the extent of ITH, this approach was considered methodologically appropriate in this context.

On the contrary, conducting multiple statistical comparisons or tests on the same dataset increases the probability of incorrectly rejecting H_0 and consequently, inflated type I error rates. Mathematically, if twenty tests are performed with a significance level of 0.05, assuming H_0 holds for all tests, there is a 5% chance of

false-positive inference, which increases with the number of tests being performed. To address the issue of spurious associations, correction for multiple testing can be implemented through various methods, each with its distinct strengths and limitations. For clinical data, the Bonferroni and Holm-Bonferroni corrections were employed. The Bonferroni method is widely utilised to control the family-wise error rate (FWER), adjusting the target α-value as α/m, where m represents the number of tests performed. However, this approach can be overly stringent, potentially reducing statistical power. Consequently, for certain more extensive analyses, Holm-Bonferroni was applied as a less conservative alternative, wherein *p*-values are ranked and compared against progressively higher thresholds, given by $\alpha/(m - k + 1)$, with k representing the rank. For high-dimensional RNA-seq data, the Benjamini-Hochberg procedure for controlling the false discovery rate (FDR) was utilised, regulating the expected proportion of false positives among the set of rejected H_0. Given the explorative nature of the included immune analyses, multiple testing was not corrected for, acknowledging the potential for false positives while prioritising the minimisation of false negatives. In hindsight, in *Paper I,* adopting a less conservative approach to controlling the FWER could have been a viable alternative.

The choice of appropriate methods for group-wise comparison of distributions is contingent upon the nature of the data and the underlying assumptions. When the data adhere to a normal distribution, or approximate normality in larger sample sizes, and demonstrate homoscedasticity, parametric tests are preferred. However, for smaller sizes, as was the case throughout this thesis, non-parametric tests are recommended. Although these tests tend to be less powerful and provide less detailed information due to their reliance on rank-based analyses, they offer greater robustness to outliers and broader applicability.

Outcome analyses

In biomarker research, comparing survival or other clinical endpoints between groups is fundamental. In *Papers I-II,* the impact of biomarker levels on time to recurrence (TTR) was analysed, with additional evaluation of cancer-specific survival (CSS) and overall survival (OS) in *Paper II*. The four-year inclusion period mitigated the risk of systematic bias in outcome probability and thorough follow-up promoted data consistency. Nevertheless, inherent uncertainties in the accuracy of cause-of-death reporting pose a recognised limitation. For subsequent analyses, biomarker expression was dichotomised based on the median or using Classification and Regression Tree (CRT) analysis. The latter constitutes a binary tree algorithm that iteratively partitions the data to maximise differences between groups while maintaining relative homogeneity within each subset, thereby identifying an optimal cut-off for stratification based on outcome.

The Kaplan-Meier method, in conjunction with the log-rank test, is a well-established approach for time-to-event analyses, enabling the statistical comparison of outcome probabilities, most commonly survival, between distinct groups. Applied in *Papers I-II*, this non-parametric analysis estimates the probability of an event occurring at successive time points, accounting for censored data, and presents these estimates as time-dependent outcome curves. The log-rank test compares the observed event frequencies to those expected given that H_0 is true. While censored data generally does not alter the estimation of outcome probabilities, the relative influence of each event may become more pronounced as the number of individuals at risk decreases. To facilitate interpretation, the results were supplemented with life tables, detailing the number of individuals at risk at each specified time point. These methods offer valuable insights into outcome data, they do however not provide a measure of effect size, and due to its univariate nature, cannot adjust for potential confounding variables. Subsequently, additional analyses were performed using Cox proportional hazards regression. This method predicts a model for time-to-event data, evaluating the relationship between the time to a specified outcome and one or more covariates, thereby estimating the impact of these covariates on the timing of the event. The model assumes that the hazard, or event rate, at a given time point for an individual is proportional to the hazard for another individual, with potential variations arising from the covariates. It further posits that these hazard ratios (HRs) remain constant over time, i.e., the proportional hazards assumption. Cox regression does not require the explicit specification of a baseline hazard function, providing a flexible approach for outcome analysis. The results are presented as HRs, which represent the instantaneous risk of an event occurring in one stratum relative to another. However, for practical reasons, these can be interpreted similarly to incidence rate ratios. Since HRs are point estimates, 95% confidence intervals (CIs) are included to indicate the precision of these estimates. Both univariable and multivariable models were employed to assess hazard ratios for disease recurrence, cancer-specific mortality, and all-cause mortality, with multivariable models adjusting for potential confounders. Cox regression additionally accounts for potential dependency between covariates and allows for the examination of interaction between these, as utilised in both papers to assess potential interactions between biomarker expression and NAC treatment.

Results

A summary of the main results from *Paper I-III* is presented below, with additional details available in the original papers.

Paper I

Clinical impact of T cells, B cells, and the PD-1/PD-L1 pathway in muscle-invasive bladder cancer

Study outline

MIBC is characterised by an immunogenic local microenvironment, encompassing both pro-tumour and anti-tumour responses, the context of which has shown prognostic implications[195-197,348]. However, the influence of spatiotemporal heterogeneity remains insufficiently explored. This raises the question of whether immune infiltrative patterns in primary tumours obtained at TURBT and radical cystectomy, or in regional lymph node metastases, yield different prognostic information. To address this, TMAs were constructed from TURBT specimens from 145 cases, with paired tumour specimens from RC available for 135 cases, 65 of whom received cisplatin-based NAC. The quantities and spatial organisation of $CD8^+$ cytotoxic T lymphocytes (CTLs), $FoxP3^+$ regulatory cells (Tregs), $CD20^+$ B cells, PD-1^+ immune cells, and PD-$L1^+$ immune (PD-$L1^{IC}$) and tumour (PD-$L1^{TC}$) cells, were assessed using single-plex IHC (Figure 13).

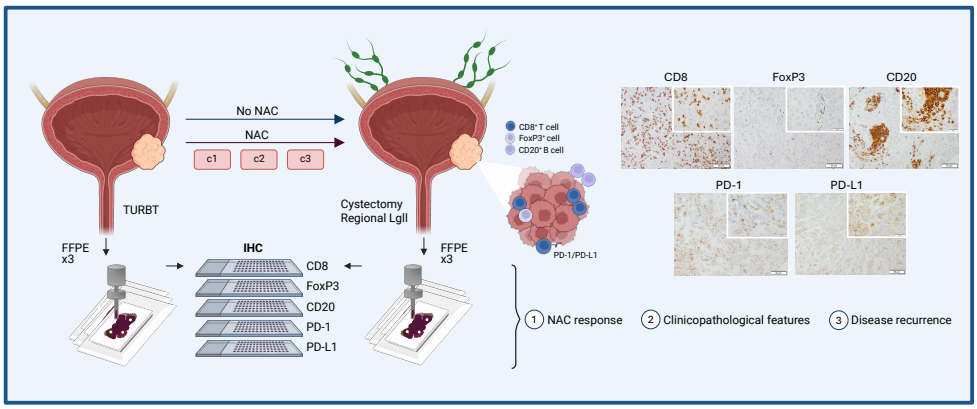

Figure 13. Schematic overview of *Paper I*. Tissue microarrays were constructed of matched primary tumours and regional lymph node metastases, with each specimen type represented by triplicate core biopsies. The expression levels of defined cell populations were analysed in relation to NAC response, clinicopathological features, and disease recurrence. Representative images of immune infiltration are presented, adapted from Wahlin *et al.*[349] licensed under CC BY-NC-ND 4.0 (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Longitudinal comparison reveals divergent immune infiltrative profiles

Comparison of tumour specimens demonstrated varying immune patterns, with significantly higher densities of CTLs, B cells, and PD-1^+ immune cells in cystectomy specimens and synchronous lymph node metastases compared to TURBT specimens. Except for higher quantities of B cells in lymph nodes, the densities of cell subsets were consistent between lymph nodes and cystectomy specimens. Stratification by NAC treatment showed higher densities of PD-1^+ immune cells in post-NAC residual tumours compared to pre-NAC TURBT. The same pattern was observed in NAC untreated cases. Additionally, NAC untreated cystectomy specimens harboured higher densities of CTLs and B cells compared to TURBT, while lymph node metastases exhibited higher quantities of CTLs, B cells, PD-1^+, and PD-$L1^+$ immune cells compared to TURBT. For all examined cell populations except B cells, immune infiltration into the tumour nest – defined as immune cells being juxtaposed to tumour cells – was evident in the majority of cases. No differences were observed in the spatial localisation of immune cells or the presence of lymphoid B cell aggregates before and after cystectomy. Correlation analyses demonstrated generally moderate to strong intercorrelations between all investigated immune subsets, and PD-$L1^{TC}$.

The predictive value of the pre-NAC immune composition

To examine whether divergent biological responses to NAC could be mirrored by distinct immune infiltrative patterns, the immune profiles of chemotherapy-naïve TURBT specimens were compared between responders (ypT0/Ta/CIS) and non-responders (≥pT1). No significant differences in immune infiltration or PD-$L1^{TC}$ expression were observed between response categories.

CTL and PD-$L1^{TC}$ infiltration differs according to tumour stage

The assessment of cell abundances in relation to established clinicopathological characteristics revealed a trend towards lower infiltration of CTLs in TURBT specimens and higher postoperative T-stages. In cystectomy specimens, the highest density of CTLs was denoted in pT2 tumours, with significantly lower expression in CIS/Ta lesions, and a gradual decline in pT3 and pT4 tumours. A similar trend was denoted for PD-$L1^{TC}$ expression in cystectomy specimens. High PD-$L1^{TC}$ infiltration in TURBT specimens further correlated with lower pN-stage.

Immune cell abundances correlate with disease outcome, but with specimen-type-dependent patterns

For subsequent analyses of the potential impact of immune infiltrates on TTR, cell densities were dichotomised into high and low categories based on median- and CRT-derived cut-off values. Kaplan-Meier estimates revealed that, overall, NAC-treated cases with immune-rich tumours had a significantly longer TTR compared to NAC-untreated cases with low immune infiltration. Interestingly, although a

potentially additive beneficial effect of NAC was observed for both low and high $CD8^+$ infiltrative tumours, no difference in TTR could be seen for NAC-untreated cases with high CTLs and NAC-treated cases with low CTLs.

The prognostic significance of immune cell subsets and $PD\text{-}L1^{TC}$ was further evaluated using Cox regression analysis. Using median cut-off values, high levels of CTLs and $PD\text{-}1^+$ immune cells in TURBT specimens were significantly associated with prolonged TTR; however, these associations did not remain significant in multivariable models, adjusted for established prognostic factors including age, postoperative T-stage, N-stage, and neoadjuvant and adjuvant chemotherapy. In contrast, when applying CRT-derived cut-offs, high densities of CTLs remained independently associated with a reduced risk of recurrence. In TURBT specimens, the presence of CTLs within the tumour nest *per se* was identified as an independent prognostic factor, with a similar non-significant trend observed in cystectomy specimens.

In cystectomy specimens, high densities of CTLs, $FoxP3^+$, $PD\text{-}1^+$, and $PD\text{-}L1^+$ immune cells, as defined by median cut-offs, were independently associated with prolonged TTR. For CRT-based cut-offs, these correlations remained significant, with the additional identification of high B cell infiltration as a favourable prognostic marker. No significant interaction between NAC and the density of any investigated immune subset was detected in relation to TTR.

Paper II

Pre-clinical and clinical studies on the role of RBM3 in muscle-invasive bladder cancer: longitudinal expression, transcriptome-level effects and modulation of chemosensitivity

Study outline

RBM3 has emerged as a putative proto-oncogene, exerting cytoprotective functions, including the prevention of mitotic catastrophe, facilitation of cell cycle progression, and attenuation of apoptosis[48,63]. Its potential prognostic significance has been highlighted in several solid malignancies, albeit with varying correlations[350], in addition to being linked to chemosensitivity, including to cisplatin[63,64]. These observations spurred the investigation of the potential prognostic and predictive value of RBM3 in MIBC. Tumour cell expression of RBM3 was evaluated by immunohistochemistry using the previously constructed TMAs (*Paper I*), and its correlation with NAC response, as well as recurrence and survival following radical cystectomy, was examined. Cellular processes associated with RBM3 were further explored *in vitro* (Figure 14).

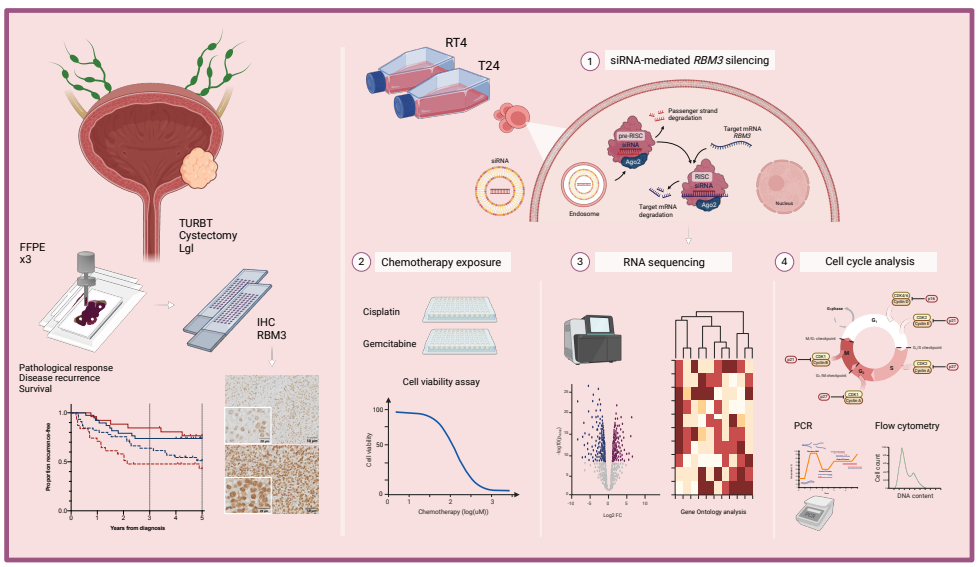

Figure 14. Graphical outline of *Paper II*. Tissue microarrays with samples from primary tumours and regional lymph node metastases (described in *Paper I*) were immunohistochemically stained for RBM3. RBM3 nuclear scores were determined by combining the fraction of RBM3 positive tumour cell nuclei and staining intensity, and subsequently correlated with NAC response and clinical outcomes. Functional studies examining cellular mechanisms linked to RBM3 were performed using T4 and T24 bladder cancer cell lines. Following siRNA-mediated *RBM3* silencing, cells were subjected to chemotherapy exposure, RNA sequencing, and cell cycle analyses.

RBM3 protein levels in relation to NAC response and clinical outcome

RBM3 was frequently expressed across the cohort, detected in TURBT specimens in 82% (116/141) of cases. Longitudinal comparison of paired primary tumour specimens from TURBT and RC revealed reduced RBM3 expression in cystectomy specimens, irrespective of NAC treatment, while expression levels remained consistent between primary tumours and regional lymph node metastases.

For subsequent analyses, RBM3 expression was dichotomised into low and high categories based on median nuclear scores across TMA cores for each case. No significant associations were observed in relation to clinicopathological characteristics. As the main objective was to investigate the prognostic and predictive value of RBM3, the analyses were centred on TURBT specimens. A trend towards increased NAC-induced pathological downstaging of the primary tumour (ypT0/Ta/CIS) was noted in tumours with high RBM3 expression compared to low expression (16/26 and 17/39 cases, respectively), although this did not reach statistical significance. This trend was less evident when nodal status (ypT0/Ta/CIS,N0) was considered. Given that local and distant recurrences predominantly occur within three years after treatment[351], analyses of 3-year and 5-year TTR were conducted. Kaplan-Meier estimates demonstrated that NAC treatment was the principal factor influencing recurrence risk. The highest proportion of recurrences was seen for NAC-untreated cases with high tumour-specific RBM3 expression. Conversely, cases with high tumour RBM3 expression who received NAC had a significantly lower recurrence rate, with the greatest difference in risk observed within the first three years following diagnosis. These associations were further examined using univariable and multivariable Cox regression models, with the latter adjusted for age at diagnosis, pT-stage and N-stage at RC, NAC, and adjuvant chemotherapy – variables previously identified as prognostic in this cohort[349]. While univariable analyses did not reveal any significant associations, multivariable analyses indicated an increased risk of recurrence in cases with high RBM3 expression at both 3-year and 5-year follow-up. A significant treatment interaction between NAC treatment and RBM3 expression was observed during the first three years. Additional Kaplan-Meier analyses showed that NAC-untreated cases with high tumoural RBM3 expression had significantly worse 5-year OS and CSS compared to those who received NAC. No significant differences in outcomes were observed between high and low RBM3 expression in the NAC-untreated cohort. While RBM3 was not prognostic in subsequent univariable Cox regression analysis, multivariable analysis identified high RBM3 expression as an independent factor of reduced 5-year OS, with a similar, though non-significant, trend observed for CSS.

RBM3 suppression increases cell viability following chemotherapy exposure

These findings, indicating a potential link between RBM3 expression and NAC benefit, prompted further exploration into the biological properties of RBM3. Two bladder carcinoma cell lines, RT4 and T24, were utilised, of which the latter, representing invasive disease[327], displayed higher RBM3 levels at baseline. Following siRNA transfection, cells were treated with cisplatin or gemcitabine, and cell viability was assessed using a WST-1 assay. In RT4 cells, RBM3 suppression resulted in only a modest shift towards reduced sensitivity to cisplatin. However, in T24 cells, RBM3 knockdown led to a significant increase in the number of viable cells after chemotherapy. A comparison of proliferation rates of non-chemotherapy-treated cells showed no differences after siRBM3 treatment, further supporting that the observed alterations in cell viability could be attributed to changes in chemo-sensitivity.

Transcriptomic analysis positions RBM3 at the G_1 to S-phase transition

RNA sequencing and gene ontology analysis uncovered that biological processes associated with RBM3-related genes primarily converged on cell cycle processes and regulation, with downregulated genes further specified to G_1/S-phase transition and the initiation of DNA replication. Cellular component analysis indicated connections to nuclear chromosome parts and the mini-chromosome maintenance (MCM) protein complex. To validate these findings, flow cytometry-based cell cycle analysis was performed. In T24 cells, RBM3 silencing resulted in a trend towards increased cell accumulation in the G_1-phase, accompanied by a marked decrease in the proportion of cells in the S-phase. G_1-phase progression and the G_1/S-phase transition are sequentially governed by several cyclins and CDKs, including cyclin D (1, 2, and 3)/CDK4/6 in mid-G_1 and cyclin E/CDK2 in late G_1, whose activity is further modulated by CDK inhibitors, including p16, p18, and p27[352]. After siRBM3 transfection, reduced expression of *CCND1*, *CDK2* and *CDK4,* and increased levels of *CCND3* and *CDKN1B* ($p27^{Kip1}$), were demonstrated using qRT-PCR, thus, confirming the results from the RNA sequencing. In conclusion, these analyses suggest a functional role for RBM3 in facilitating cell cycle progression by promoting G1/S-phase transition (Manuscript Figure 7) and initiation of DNA replication, providing a plausible mechanism for the observed increase in chemosensitivity in MIBC.

Paper III

Attributes associated with response to neoadjuvant chemotherapy in the molecular terrain of muscle-invasive bladder cancer

Study outline

Spatial intratumour heterogeneity poses a significant challenge to therapeutic efficacy. We reasoned that predictive biomarkers for NAC response need to be evaluated within this context to identify the combinations of molecular signatures and heterogeneity patterns that are conducive to a therapeutic benefit. Additionally, we hypothesised that intratumour variability *per se* could serve as an important predictive factor, as highly heterogeneous tumours are more likely to harbour treatment-resistant niches. To investigate this, paired primary tumours from TURBT and RC, as well as regional lymph node metastases where present, were collected from 15 cases exhibiting divergent biological responses to NAC, i.e., complete (n=5), partial (n=5), or non-responders (n=5). In total, 78 tumour regions (54 pre-NAC and 24-post-NAC) from spatially separated tissue compartments were subjected to TDS, along with matched normal samples, for mutational and copy number profiling. Genomic data were integrated with extensive molecular subtype and immune phenotyping across 316 tumour areas (231 pre-NAC and 85 post-NAC) using single-plex IHC and mIF (Figure 15).

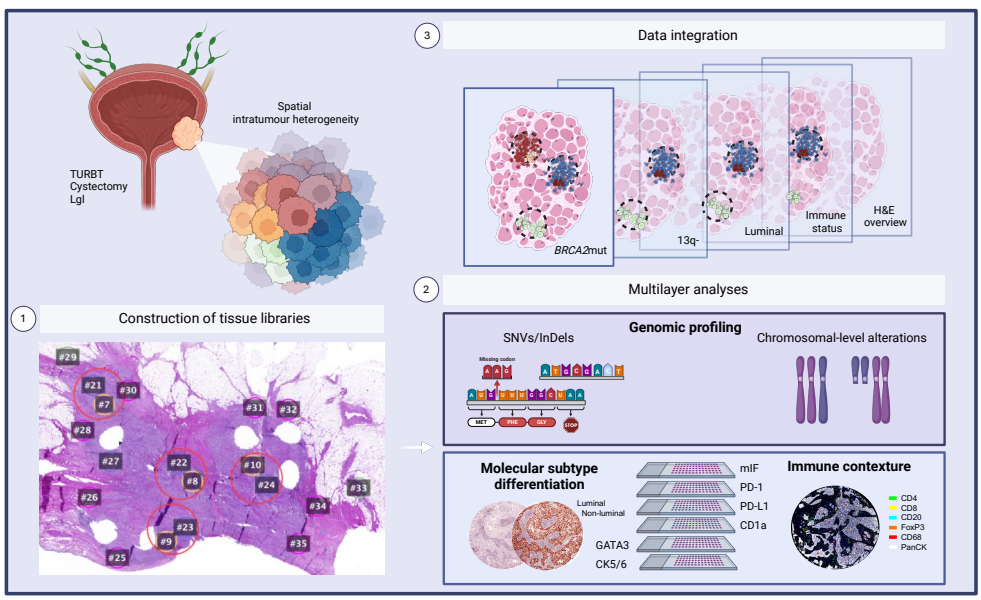

Figure 15. Study design of *Paper III*. Detailed case-specific tumour tissue libraries were constructed for 15 patients to examine the impact of spatial intratumoural heterogeneity on NAC response, focusing on small-scale and chromosomal-level structural alterations, complemented by parallel molecular subtyping and immune phenotyping.

Divergent patterns of intratumoural mutational and copy number heterogeneity across response groups

Analysis of the pre-NAC tissue landscape revealed diverse mutational profiles with 48% (141/294) of detected somatic mutations shared across intratumoural regions. Similar to the evolutionary paths described by Heide *et al*.[353], divergent mutational trajectories were evident. Some tumours exhibited early mutational stasis with few or no emergent mutations during progression to invasive disease, whereas other tumours acquired additional alterations in known driver genes with subsequent clonal co-existence. Complete responders were characterised by a significantly higher mutational burden compared to non-responders, however, the degree of subclonality did not appear to be associate with NAC response. In contrast, an opposite trend was observed for CNAs. While there was no difference in the absolute number of events or the percentage of the genome altered by CNAs, complete responders exhibited a higher prevalence of shared CNAs. Notably, all five cases dominated by extensive private or regional CNAs (<25% shared alterations) were non-complete responders.

Genomic responders identified by the presence DDR deficiency

We next sought to delineate the extent to which NAC responses could be attributed to a *genomic response,* defined by the presence of high-confidence functional alterations in MIBC therapy-relevant DDR pathways (NER, HRR, and MMR). Within this context, all five complete responses could be explained by the virtue of DDR deficiency, despite exhibiting phenotypic heterogeneity. The detected alterations encompassed all three pathways, with 12/13 alterations being either regional or shared across all intratumoural areas. The increased mutational burden observed in these cases may, at least in part, be attributed to the presence of deleterious DDR alterations, as the number of DDR mutations demonstrated a positive correlation with mutational burden, aligning with previous findings[248]. In contrast, DDR mutations were absent in 2/5 partial and 2/5 non-responders.

The influence of NAC on the genomic landscape

Comparison of pre-NAC and post-NAC mutational profiles in cases with residual disease demonstrated both patterns of consistent mutational profiles throughout treatment, implying inherent chemoresistance, whereas other tumours displayed early branching evolution, characterised by the loss of certain pre-NAC alterations and the extensive selection of resistant subclones. The latter is illustrated by the phylogenetic reconstruction of case 1145, a partial responder, with indications of NAC-induced clonal replacement (Manuscript Figure 3). When mapped back to the histological overview of the tumour, this branching heterogeneity extended to the morphological level, with the surviving tumour clone exhibiting a distinct growth pattern.

Notably, truncating clonal mutations in *ARID1A* were identified in four of five non-responders. These alterations persisted throughout NAC, being detected in all paired post-NAC samples, including lymph node metastases. Similar comparative CNA analyses demonstrated pronounced heterogeneity in several cases, suggesting NAC-induced emergence.

Intratumour phenotypic diversity within complete responders

Molecular subtype classification was inferred from GATA3 and CK5/6 protein levels. Unexpectedly, complete responders exhibited marked molecular subtype ITH, with two out of four cases demonstrating combinations of luminal, Ba/Sq, and double-negative staining patterns, alongside evidence of intracore heterogeneity in two cases. One complete responder primarily exhibited a double-negative staining pattern, consistent with its classification as a small cell neuroendocrine carcinoma. In contrast, cases with residual disease exhibited more stable tumour phenotypes, predominantly of the luminal subtype. Notably, no significant shifts in molecular subtypes were observed following NAC. Integration of molecular subtype classifications with genomic profiles revealed contrasting trends, including both the enrichment of non-shared and shared alterations among heterogeneous cases.

The predictive value of the local immune repertoire

For parallel immune assessment, additional tumour areas were sampled to represent tumour-immune border compartments and tumour-adjacent immune-enriched stromal areas, resulting in a total of 256 pre-NAC TURBT tumour cores. The presence of $CD20^+$ B cells, $CD8^+$ CTLs, $CD4^+$ Th-cells, $CD4^+FoxP3^+$ Tregs, $FoxP3^+$ cells, and $CD68^+$ macrophages were quantified using mIF. Serial tissue sections were stained for $CD1a^+$ DCs, immune cells expressing PD-1^+ and PD-$L1^+$ (PD-$L1^{IC}$), and PD-$L1^+$ tumour cells (PD-$L1^{TC}$) using single-plex IHC. Significant intra- and intertumour heterogeneity of the defined cell populations was observed. However, the degree of variability was marker-specific, with certain populations, i.e. $CD1a^+$ DCs and PD-$L1^+$ TCs, exhibiting greater spatial heterogeneity. No systematic differences in heterogeneity were apparent between the response groups. While the overall associations with NAC response were limited, non-complete responders displayed higher quantities of $CD1a^+$ DCs. Contrary, stratification by genomic instability showed that highly genomically unstable tumours harboured elevated PD-$L1^{TC}$ expression, increased $FoxP3^+$ cell infiltration, and a tendency towards higher CTL levels. Infiltration of DCs were not significantly associated with genomic instability.

Discussion

The immunogenic microenvironment

The multifaceted immune environment imposes constraints on tumour progression while also possessing pro-tumourigenic potential. Central to the anti-tumour host response are effector T cells[22]. Expanding upon the accumulating evidence of the prognostic significance of $CD8^+$ cytotoxic T cells in MIBC[195-197,354], high infiltration of these cells in both TURBT and cystectomy tumour specimens was independently associated with a reduced risk of recurrence. In line with previous findings, the spatial localisation of CTLs relative to tumour proximity constitutes a critical factor and should accordingly be considered in prognostic assessments[196]. Notably, the highest densities of CTLs, as well as PD-$L1^+$ tumour cells, were observed in T2 tumours, with a gradual decline in more advanced stages. This tumour stage-dependent immune activity, which has been shown to occur synergistically with other immune populations[198], suggests a state of immune exhaustion or evasion as the disease progresses. Moreover, genomic instability is well recognised to alert an immune response. In this context, a high mutational burden increases the likelihood of neoantigen presentation, providing a prerequisite for an augmented T cell effector phenotype[118]. The highly genomically unstable tumours in this study were characterised by increased infiltration of CTLs, with a compensatory upregulation of PD-L1 expression on tumour cells. The correlations between CTLs, tumour stage, and genomic instability may have additional predictive implications, as response to checkpoint blockade has been associated with the $CD8^+$ CTL phenotype in MIBC[193]. Nevertheless, the addition of immune checkpoint inhibitors to NAC was shown to confer improved overall survival in patients with advanced tumour stages[234], suggesting that reactivation of host immunity remains a feasible approach despite the observed immune exhaustion signals.

Chemotherapy can induce a range of multiple immunomodulatory effects, with certain agents demonstrating more potent immunogenic properties than others. For instance, doxorubicin, a key component of the MVAC regimen, has been shown to promote immunogenic cell death, whereas cisplatin does not appear to elicit this response[137]. The impact of chemotherapy on the tumour immune microenvironment remains an underexplored area. In *Paper I*, temporal heterogeneity between matched pre-NAC and post-NAC tumours of non-complete responders was denoted solely for PD-1^+ immune cells. A similar observation was noted in NAC-untreated cases, which also displayed higher levels of CTLs and B cells in Cx samples compared to TURBT. A potential explanation for this discrepancy could be the presence of spatial ITH; however, considering the systematically observed differences, another plausible explanation is that, in NAC-treated cases, the immune response induced by the TURBT-related tissue damage may have diminished by the

time of RC. Alternatively, this could reflect NAC-induced immunosuppression, which may hinder the immune response triggered by the radical cystectomy procedure. This may, in turn, be unfavourable, as higher immune cell densities at the time of definitive surgery were independently associated with a lower risk of recurrence. In contrast to these findings, Chelushkin *et al*. reported an increase in the percentage of intratumoural $CD8^+$ T cells, PD-$L1^+$ immune cells, and $CD68^+$ macrophages following MVAC[355]. Contrasting responses were also observed, including the upregulation of TGF-β signalling in CAFs and increased spatial separation between immune cells and adjacent cancer cells in post-NAC specimens[355]. Further studies are highly warranted to elucidate chemotherapy-induced microenvironmental alterations, particularly in light of the advent of neoadjuvant chemoimmunotherapy in MIBC.

In this thesis, no strong evidence was found to support the predictive value of the pre-NAC immune composition, although a potential association with the presence of $CD1a^+$ DCs was suggested. DC activation and maturation serve as the critical link to an effective anti-tumoural adaptive immune response, however, tumour-derived cytokines such TGF-β can promote an immature, tolerogenic phenotype[356]. Therefore, further functional characterisation of DCs within the local microenvironment of MIBC, using additional phenotypic surface markers, would be interesting. Moreover, given that other studies have highlighted a potential predictive value of the local immune environment – specifically increased B cell activation and intratumoural $CD3^+$ T cell enrichment in complete responders, alongside signs of immunosuppression in non-responders[357] – this warrants further investigation.

RBM3 as a candidate biomarker of chemotherapy response

RBM3 was found to be ubiquitously expressed in MIBC tumours, consistent with the well-established role of RBPs in RNA metabolism and cellular homeostasis[42]. The observation that NAC-treated patients with tumours exhibiting high RBM3 protein levels experienced significantly improved outcomes, compared to those not receiving NAC, suggests a potential predictive role for RBM3. This finding prompted further *in vitro* investigations, which demonstrated that RBM3 downregulation reduced chemosensitivity to both cisplatin and gemcitabine. Mechanistically, this effect is thought to be mediated by the promotion of cell cycle progression, particularly through the facilitation of G_1/S-phase transition and the initiation of DNA replication.

The observed adverse prognostic impact of high RBM3 levels in MIBC contrasts with findings from several studies on other solid tumours[350]. However, similar observations have been reported in pancreatic[64] and prostate cancer[58]. These discrepancies may reflect the extensive post-transcriptional regulatory functions of

RBPs, wherein distinct cell lineages may exhibit variable capacities to compensate for disruptions within these regulatory networks[42]. This context-dependent functional plasticity of RBPs is exemplified by *RBM10*, which has been implicated in exerting both oncogenic and tumour-suppressive functions across different tumour types[42].

The aberrant proliferative potential that hallmark malignant cells has primarily been attributed to their ability to evade cell cycle exit, necessitating an efficient transition into the S-phase to sustain continuous proliferation[17]. The upregulation of RBM3 expression may conceivably facilitate this process, consistent with observations of elevated RBM3 levels in malignant cells compared to normal counterparts, including premalignant lesions[53,57-59]. In BC, RBM3 has been found to be upregulated in CIS compared to normal urothelium[358]. This suggests that altered RBM3 expression may constitute an early oncogenic event in certain tumour types. Additionally, RBM3 has been shown to confer protection against mitotic catastrophe during cell division[48]. This may be particularly pertinent to MIBC, given the generally pronounced genomic instability that characterises these tumours. While RBM3 may exert cytoprotective functions, the same mechanisms could also increase the vulnerability of cells to DNA damage-inducing chemotherapeutics. RBM3 has previously been linked to cisplatin sensitivity in ovarian cancer cells *in vitro*, primarily attributed to cell cycle alterations, particularly G_2/M-arrest[66], as well as to gemcitabine sensitivity in pancreatic cancer cells[64]. If prospectively validated, these findings could have broad clinical implications in MIBC, extending beyond the context of NAC. In all chemotherapy settings, but particularly in palliative situations, patient frailty, comorbidities, and disease-related symptoms complicate treatment decisions, necessitating careful balancing of treatment efficacy with the potential impact of toxicity on quality of life. In metastatic UC, gemcitabine may be administered as monotherapy to frail patients[211] and predictive biomarkers are hence of significant value.

The potential clinical significance of RBM3 has been investigated in previous studies spanning both NMBIC and MIBC stages. These investigations have generally shown that RBM3 expression is associated with less aggressive tumour characteristics[62,358,359]. However, the prognostic value remains inconsistent, with some studies reporting improved survival outcomes[62,359], while others found no prognostic association[358]. Notably, in the latter study, RBM3 expression was found to be significantly upregulated in metastatic lesions compared to primary tumours[358]. Several factors may contribute to these inconsistencies, including differences in IHC assessment strategies, the interpretation of staining intensity, varying thresholds for biomarker positivity, tumour molecular profiles, and the potential intratumour heterogeneity of RBM3 expression. While high tumour RBM3 levels correlated with impaired OS, with similar non-significant trends observed for CSS and TTR, the results from *Paper II* suggest that the most

substantial influence of RBM3 is observed in the context of chemotherapy. Therefore, although the prognostic value of RBM3 in MIBC remains to be fully elucidated, future studies exploring RBM3 expression in relation to chemotherapy response are strongly warranted.

The influence of spatial intratumour heterogeneity

The central consideration regarding spatial ITH is its correlation with the evolutionary propensity of tumours, alongside its impact on tumour susceptibility to therapeutic interventions and subsequent clinical outcomes. Thus, the question is not only the extent of spatial ITH *per se*, but also *whether all heterogeneity matter?*

The extent and clinical impact of spatial ITH in MIBC are increasingly recognised. Post-NAC tumour heterogeneity has been demonstrated to predict impaired overall survival[360]. With regard to molecular subtypes, single-cell transcriptomic profiling has revealed significant intratumoural subtype heterogeneity[361]. Notably, pre-NAC mixed molecular subtype contextures – defined by the coexistence of at least two distinct consensus molecular subtypes – have been associated with adverse outcomes following NAC in the VESPER trial, despite comparable initial response rates[254]. A high prevalence of subtype ITH within Ba/Sq tumours has been reported in several other studies[362,363]. Unexpectedly, in *Paper III*, we observed significant ITH among complete responders, including mixed Ba/Sq phenotypic features. While such tumour characteristics are generally associated with more aggressive disease courses, these findings underscore that they may also display optimal biological responses to NAC.

The genomic tumour profiles of the 15 cases, incorporating both small-scale variants and larger structural alterations, demonstrated distinct evolutionary trajectories, however, these were not confined to any specific NAC response category. While complete responders were characterised by a higher somatic mutational burden, their overall subclonal distributions did not correlate with therapeutic benefit. In contrast, an opposite trend was observed for CNAs. All five cases harbouring extensively branching CNA profiles were non-complete responders. Phylogenetic reconstruction of one of these cases showed that CNAs provided greater resolution of the tumour's evolutionary history, with SNVs and CNAs contributing at different phases of the trajectory. It has been proposed that CNAs, rather than SNVs, exert a more prominent role in the development and maintenance of ITH[364].

Regarding distinct alterations, truncating *ARID1A* alterations were frequently observed in non-complete responders and persisted throughout chemotherapy treatment, being additionally detected in regional lymph node metastases. *ARID1A* deficiency has previously been described as being enriched in high-grade invasive

and metastatic tumours[365] and has been highlighted as a potential mechanism behind BCG resistance[181]. Thus, *ARID1A* is an area of interest for future research, particularly given that *ARID1A* mutations have been implicated in response to pharmacological inhibition of EZH2 methyltransferase[366]. Moreover, the identification of complete responders as genomic responders, based on the presence of DDR deficiency, aligns with current perspectives in the field[245,248,250]. The majority of DDR alterations within genomic responders were shared across intratumoural regions, suggesting that genetic homogeneity with regard to DDR deficiency may be a determining factor. This is of utmost importance to delineate, as various clinical scenarios may arise. For instance, a sequencing report may reveal a clonal deleterious DDR alteration in a therapy-relevant context of MIBC, providing a clear indication of the therapeutic benefit of NAC. Based on the results of *Paper III*, irrespectively of molecular subtype differentiation and immune status. Alternatively, the detected alteration may be subclonal, potentially accompanied by other negative predictive tumour characteristics. Thus, composite predictive models or hierarchical frameworks are likely to be required in clinical practice.

Overarching and study-specific strengths and limitations

The results of these studies are derived from a single retrospective cohort of MIBC patients. Although the cohort constitutes a consecutive, well-annotated series of cases with demographics reflecting the broader patient population, the inherent risks of confounding variables and bias associated with a retrospective design must be acknowledged, as further emphasised in the *Study cohort* section. Some of the observations in this study confirm earlier findings and/or align with subsequent literature, whereas others, such as the potential role of RBM3 in NAC response, are consistent with observations in other tumour types but have not been previously characterised in MIBC. Regardless, further validation, preferably in a prospective setting, is warranted.

A subset of NAC-treated patients received only one or two cycles of treatment, primarily due to intolerable toxicity. Retrospectively, the inclusion of these patients in the stratified analyses of NAC response could potentially have influenced the results. Nevertheless, the number of such cases was limited (5/65 and 8/65 cases, respectively), and their inclusion can be argued to reflect the real-world clinical context. In *Paper III*, only patients having completed a minimum of two cycles of NAC were included. Additionally, the requirement for sufficient tumour tissue for multiregional analysis, while potentially favouring more advanced tumour stages, could further strengthen that the observed responses were indeed induced by NAC.

A common challenge in biomarker research is the lack of harmonisation in the use of antibodies, scoring systems, and cut-off values, which complicates cross-study comparability. In these studies, only well-validated antibodies were utilised. Binary

classification of biomarker expression, as applied here, is frequently utilised in outcome analyses. Compared to continuous modelling, dichotomous approaches may obscure biologically relevant nuances, introduce misclassification bias, and influence reproducibility due to dataset-specific thresholds. However, dichotomisation offers several practical advantages, including the simplification of outcome analyses and the accommodation of skewed data distributions, potentially facilitating both clinical interpretability and clinical implementation. While the optimal prognostic thresholds remain to be established, the immune cell subsets in *Paper I* were assessed and reported on a continuous scale, subsequently dichotomised using two distinct methods. However, in *Paper III,* various degrees of ITH were observed. Addressing its potential implications for clinical outcome in larger cohorts would be of significant value. In *Paper II,* RBM3 expression was assessed using a scoring method widely adopted in previous studies; however, it should similarly be additionally evaluated in the context of potential ITH.

The *in vitro* investigations of RBM3 employed a model system of two human bladder cancer cell lines. While cell cultures represent simplified models that do not recapitulate the potential influence of the complex tumour microenvironment, they remain valuable for gaining initial mechanistic insights into markers of interest. Given the mesenchymal phenotype of T24 cells[328], further preclinical investigations incorporating additional (epithelial) muscle-invasive cell lines would be of considerable interest to broaden the understanding of the role of RBM3 across different cellular contexts, particularly in relation to molecular subtypes or the presence of DDR alterations.

Conclusions

In summary, the findings presented in this thesis highlight that:

- An immune-rich tumour microenvironment independently signifies a favourable clinical outcome, thus holds potential as a prognostic signature. However, the pre-NAC immune landscape may provide limited predictive value for NAC efficacy.
- Tumour-specific RBM3 expression emerges as a candidate beneficial predictive biomarker for NAC response in MIBC, mechanistically linked to the promotion of cell cycle progression.
- Spatial intratumour heterogeneity manifests across multiple biological scales, with some patterns associated with NAC response. This underscores the importance of integrative predictive models, although certain tumour characteristics may independently confer a therapeutic benefit from NAC.

Future perspectives

Doctors [...] prescribe medicines of which they know little, to cure diseases of which they know less, in human beings of whom they know nothing
Voltaire (1665)

As of today, 360 years later, this statement remains partially valid. While significant advancements have been made and important efforts are ongoing, prognostic and predictive tools have yet to be integrated into clinical practice for MIBC patients. This underscores the continued challenge of discerning the core(s) within the complex cellular architecture of MIBC and translating these insights into a robust, high-sensitivity, high-specificity biomarker suitable for clinical implementation. Evidently, there is no universal Achilles' heel in these tumours; thus, a composite signature of key traits must be carefully delineated. Central to this challenge is the influence of spatiotemporal tumour heterogeneity and treatment-induced dynamics. Nevertheless, opportunities to explore these aspects are expanding, notably through single-cell analysis or spatial *in situ* profiling, with additional methods available for studying the cellular effects of cisplatin *in vivo* using FFPE material[367].

The neoadjuvant treatment setting offers a critical window of opportunity in these patients. Not only for determining therapeutic efficacy but, most importantly, in terms of potential bladder-sparing approaches and improving patient outcomes. With the anticipated avenue of new orthogonal regimens, such as the combination of immunotherapy with chemotherapy[234], or targeted approaches[368], the ability to effectively stratify patients for personalised treatment strategies will become increasingly important, as will the early identification of non-responders. Currently, comparative analyses of pre- and post-NAC tumour tissue specimens – routinely obtained during diagnostic and definitive surgical procedures – serve as a valuable resource and has been used throughout this thesis. However, the tumour and host dynamics occurring during the interim period remain largely a black box, particularly in complete responders. In this regard, ctDNA holds significant potential in providing insights into tumour evolution, as well as in detecting circulating tumour heterogeneity[369].

Given the high degree of genomic instability in MIBC and the multifaceted tumour-host immune interactions that may result, further investigation into these dynamics is essential. This is particularly pertinent to the diverse immunological effects

induced by chemotherapy and how these could be harnessed to achieve the optimal synergistic effects of chemoimmunotherapy. These studies did not provide robust evidence supporting the predictive value of the immune repertoire for NAC response, although potentially indicating a role for tumour-infiltrating DCs. While further investigations are needed, including the exploration of phenotypic states as well as other immune cell subsets, the findings highlight the importance of considering circulating immune responses to better understand and monitor potential stimulatory or inhibitory immune effects of NAC. Furthermore, independently of NAC, the positive prognostic association of the immune contexture motivates further studies. Various immune populations have been proposed in this context, yet, as previously noted, the results remain inconsistent. The use of different methods, alongside the well-known context-dependent functional plasticity of immune cells, presents complicating factors that must be addressed in order to delineate the prognostic immune determinants. However, the consistent significance of tumour-infiltrating CTLs raises the question of whether the existing tumour classification system should be revised to reflect advancements in tumour immunology.

The indication of RBM3 as a potential predictive biomarker, with links to both cisplatin and gemcitabine, is a noteworthy finding. If prospectively validated, it could have implications in the neoadjuvant to the palliative setting. In this study, tumour-specific RBM3 expression was evaluated at the protein-level using FFPE samples, a method that could be feasibly translated into routine pathology. However, in addition to prospective validation, further assessment is needed to establish the optimal threshold for biomarker positivity, as the cut-off employed in this study may be highly dataset-dependent. Given the previously noted concordance between RBM3 mRNA and protein levels[63], transcriptomic profiling could present a viable alternative, provided similar findings are observed in MIBC. This approach could facilitate integration with the molecular subtype classification; however, its applicability may be complicated by the fact that it is primarily the nuclear expression of RBM3 that appears to hold significance, at least for prognostication purposes[62,359].

Lastly, based on the multilayer analyses conducted in *Paper III*, it can be concluded that candidate prognostic and predictive biomarkers should be evaluated through the lens of the potential influence of ITH. While intratumoural heterogeneity within consensus molecular subtypes has been associated with worse outcomes following NAC[254], this study revealed considerable subtype ITH among complete responders, emphasising the need for further research into its clinical implications. Furthermore, genomic responders to NAC were identified by the presence of DDR deficiency, independent of molecular subtype or local immune patterns, again emphasising the importance of integrating multiple molecular aspects into predictive models.

We are currently at the dawn of molecular pathology, where the integration of the fundamentals of histopathology with multiplex assays and high-resolution molecular technologies is posed to significantly advance patient management and clinical outcomes. Nevertheless, several critical questions remain to be elucidated regarding the optimal application of these tools and the key biological factors that must be considered. It is my hope that the work presented in this thesis has added at least one pixel to the intricate cartography of muscle-invasive bladder cancer. As Souza et al.[370] aptly noted, *we envision increasingly colourful and exciting times ahead.*

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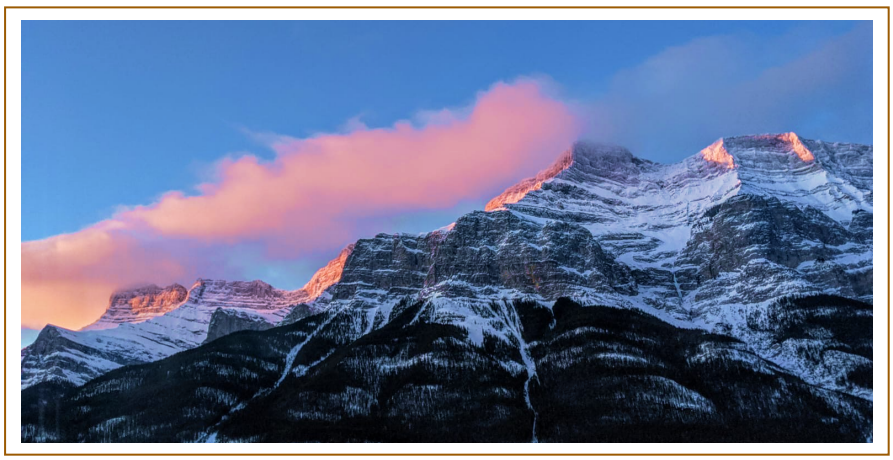

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About the thesis

Curative treatment for muscle-invasive bladder cancer remains a significant challenge due to the heterogeneity of the disease. Through exploration of the local tumour microenvironment, this thesis aimed to provide insights into prognostic and predictive molecular signatures. The doctoral studies have evolved in parallel with continued clinical practice – from medical school to the oncology residency programme – and as these paths have converged, also a sense that it has now come together.

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