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IDENTIFYING AND TARGETING VULNERABILITIES IN CANCERS THAT METASTASIZE TO THE BONE AND BONE MARROW

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Identifying and targeting vulnerabilities in cancers that metastasize to the bone and bone marrow

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By

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Till min kära familj.

To my beloved family.

إلى عائلتي العزيزة

À ma chère famille.

Ambition is the path to success.
Persistence is the vehicle you arrive in.
– Bill Bradley

POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer är och har alltid varit ett bestående faktum av livet. Förr viskades berättelser som passerade från generation till generation på grund av rädsla. Idag förs konversationer i större utsträckning öppet, och i samband med snabbt utvecklande teknologier upptäcks cancer tidigare, och kan därmed behandlas. Den enda föränderliga faktorn som är relaterad till cancer är just vetenskapen.

År 1889 beskrev en forskare vid namnet Steve Paget sin teori "*seed and soil*" om hur cancer sprids. Spridningen av cancer, även kallat metastas, påminner om ett frö (*seed*) som växer beroende av omgivningen (*soil*) det befinner sig i och näringen det får. Fröet ensamt resulterar inte i cancer. Däremot, med rätt omgivning och näring kan det både växa och överleva. På samma sätt betraktas cancerceller som individuella celler (frön), och när de är tillräckligt mogna blir de benägna att etablera sig och forma en tumör. Det är fortfarande oklart hur och varför spridning av en cancerform har en specifik destination. Exempelvis sprids bröstcancer mestadels till skelettet, huden, hjärnan och njurarna, medan tarmcancer sällan sprider sig till de nämnda organen, utan istället sprider sig tarmcancer till lungorna och levern. Två cancerformer som har tendens att sprida sig till skelettet, och även benmärgen, är klarcellig njurcancer hos vuxna och neuroblastom, som utvecklas i binjuren, hos små barn. Både njuren och binjuren är viktiga organ som reglerar kroppens näring och hormoner. I klarcellig njurcancer är behandlingsstrategierna framgångsrika när cancern fortfarande är lokal och befinner sig i njuren. När den spridits till skelettet eller benmärgen är sannolikheten för överlevnad mellan 10–12%. Överlevnaden för barn med neuroblastom har ökat drastiskt de senaste 30 åren med en överlevnad på över 80%. Likväl, när neuroblastom spridit sig till skelettet eller benmärgen svarar patienter sällan på terapi och överlevnaden är nedslående, mindre än hälften botas och vid ett återfall är överlevnaden mycket begränsad. Fortfarande föreligger enorma kunskapsbrister om mekanismerna bakom cancerspridning till skelettet och benmärgen.

Den omvälvande teknologiska utvecklingen, särskilt enkelcellsanalys, har hjälpt forskare där ofantliga mängder av data har genererats för bredare biologisk tolkning av cellulära förändringar, genuttryck och molekylära kommunikationskanaler mellan cancerceller. Således valde vi att använda enkelcellsanalys för en fördjupad insikt av hur cancer sprider sig till benmärg och skelettet. Arbetet i den här avhandlingen bygger på ett gediget internationellt samarbete mellan forskare som söker förståelse bakom orsaken till varför en andel av patienterna inte svarar på en viss behandling, medan andra patienter gör det. I **Delarbete I** och **II** används enkelcellsanalys på tumörvävnad från patienter som diagnostiserats med klarcellig njurcancer och benmetastaserad klarcellig njurcancer med tumörinfiltrering i benmärg. Arbetet demonstrerar en komplicerad mikromiljö med ett försvagat immunsystem. Vi upptäckte potentiella

kommunikationskanaler varvid en blockering kan främja ett minskat sjukdomsförlopp. Dessutom identifierades fyra gener som vid samtida uttryck är associerade med lägre överlevnad hos patienter med klarcellig njurcancer, samt en ökad sannolikhet för att cancern sprider sig till skelettet.

I **Delarbete III** föreslår vi ett behandlingsalternativ bestående av inhibering av ett enzym involverat i cancercellernas ämnesomsättning, dihydroorotate dehydrogenase (DHODH) i kombination med befintlig terapi, temozolomide. Tillsammans påvisar de kurativ kapacitet *in vivo* och *in vitro* för högriskneuroblastom. Vi föreslår därför att detta bör testas kliniskt för dessa barn.

Avsikten med våra studier är att bidra med ökad kunskap om de bakomliggande biologiska mekanismerna för att en cancerform sprider sig till ett specifikt organ men inte ett annat, samt hur vi kan behandla en aggressiv barntumör. Vi riktade in oss på två cancerformer som sprider sig till skelettet och benmärgen; klarcellig njurcancer och neuroblastom. Den avskalade karakteriseringen av mikromiljön avslöjade kommunikationskanaler som kan blockeras med läkemedel. Det återstår dock fler experimentella och farmakologiska studier innan våra fynd når den kliniska praktiken. Vår forskningsgrupp hoppas att insikterna om den dynamiska mikromiljön kan bistå likasinnade forskare för framtida experiment, fördjupad kunskap och utveckling av bättre och botande behandling.

ABSTRACT

Evolving technologies enable scientist to ask and seek answers to advanced questions, above and beyond. During the past decade single-cell RNA sequencing (scRNA-seq) has revolutionized our understanding of biology, in particular within cancer research. Contributing with its high-resolution has unraveled complicated biological circuits, along with a profound understanding of heterogeneity, cellular and molecular processes, including gene expression profiles and transcriptional states. Especially in the quest of understanding cancer metastasis, aggressive disease phenotype and treatment resistance.

In **Paper I** we utilize scRNA-seq on tissue material from untreated human primary clear cell renal cell carcinoma (ccRCC) patients. We dissect the tumor microenvironment (TME) and discover a metastatic gene signature that is upregulated in bone metastatic ccRCC patients that could serve as a predictive tool. We describe an immunosuppressive microenvironment with tumor associated macrophages (TAMs) with a M2 phenotype overexpressing *TREM2*, further associated with low survival outcome. Computational interactive receptor-ligand analysis reveals *CD70* (on tumor) and *CD27* (on cytotoxic T cells and regulatory T cells) as a potential pharmaceutical target.

In **Paper II**, a continuation of **Paper I**, we aimed to understand genetic alterations involved in metastatic disease from the primary site, namely bone metastatic ccRCC, including comparison of a normal bone marrow to malignant. Scrutinizing the complex TME we detect, as well here, an immunosuppressive microenvironment with transcriptionally different TAMs, exhausted T cell phenotype and a tumor associated mesenchymal stem/stromal cell (TA-MSC) population that upregulates bone remodeling genes. Concluding that the relevance of TA-MSC subset causes excessive bone resorption via *RANK/RANKL/OPG* signaling pathway.

Finally, in **Paper III**, we evaluate brequinar, a *DHODH* inhibitor, as a therapeutic agent in high-risk neuroblastoma (NB). We find *DHODH* as an independent risk factor in high-risk NB demonstrating prolonged survival in preclinical models when inhibited. In combination with temozolomide, already in clinical use for these children, a synergistic effect is achieved *in vivo*, proposing a promising approach to validate clinically.

Taken together, the presented thesis has provided comprehensive insights in the progression of ccRCC in a primary and a metastatic setting, including the identification of potential vulnerable targets. Additionally, a potential combination treatment *in vitro* and *in vivo* is presented as a promising treatment option in high-risk NB by inhibiting *DHODH* together with standard care of treatment agent temozolomide.

LIST OF SCIENTIFIC PAPERS

I. **Alchahin AM***, Mei S*, Tsea I, Hirz T, Kfoury Y, Dahl D, Wu CL, Subtelny AO, Wu S, Scadden DT, Shin JH, Saylor PJ, Sykes DB, Kharchenko PV, Baryawno N.
A transcriptional metastatic signature predicts survival in clear cell renal cell carcinoma.
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II. Mei S*, **Alchahin AM***, Tea I*, Kfoury Y, Hirz T, Sarkar H, Wu S, Subtelny AO, Zhang Y, Salari K, Wu CL, Randolph MA, Scadden DT, Dahl DM, Shin J, Kharchenko PV, Saylor PJ, Sykes DB, Baryawno N.
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III. Olsen TK*, Dyberg C*, Embaie BT, **Alchahin AM**, Milosevic J, Ding J, Otte J, Tümmler C, Hed Myrberg I, Westerhout EM, Koster J, Versteeg R, Ding HF, Kogner P, Johnsen JI, Sykes DB, Baryawno N.
DHODH is an independent prognostic marker and potent therapeutic target in neuroblastoma.
JCI Insight. 2022 Aug 9;7(17): e153836.

**Equal contribution.*

CONTENTS

LIST OF ABBREVIATIONS

α-SMA	Alpha smooth muscle actin
ANG-1	Angiopoietin 1
APOL1	Apolipoprotein 1
BM	Bone marrow
BMDC	Bone-marrow derived cell
BMP	Bone morphogenetic protein
CAF	Cancer associated fibroblast
ccRCC	Clear cell renal cell carcinoma
CSF	Colony-stimulating factor 1
CTC	Circulating tumor cell
CTL	Cytotoxic T cell lymphocyte
CTLA-4	Cytotoxic T lymphocyte associated antigen 4
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
DEG	Differentially expressed genes
DTC	Disseminated tumor cell
EC	Endothelial cell
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
eT^{reg}	Effector regulatory T cell
FAP	Fibroblast activation protein- alpha
FASL	Fas ligand
FOXP3	Forkhead box P3
HIF1α	Hypoxia-inducible factor-1 alpha
HSC	Hematopoietic stem cell
ICAM-1	Intracellular adhesion molecule-1
ICB	Immune checkpoint blockade
IFN- γ	Interferon gamma
LAG3	Lymphocyte activation gene-3

LEPR	Leptin receptor
M-CSF	Macrophage colony-stimulating factor
MAPK	Mitogen-activated protein kinases
MET	Tyrosine-protein kinase Met
MetSC	Metastatic stem cell
MSC	Mesenchymal stem/stromal cell
mTOR	Mammaliam target of rapamycin
NB	Neuroblastoma
NG2	Neuroglial 2 proteoglycan
NF-kB	Nuclear factor kappa B
NSCLC	Non-small celllung cancer
PCA	Principal component analysis
PD-1	Programmed cell death protein
PDGF	Platelet-derived growth factor
PDGFR β	Platelet-derived growth factor receptor beta
PMN	Pre-metastatic niche
RGS5	Regulator of G-protein signaling-5
RUNX2	Runt-related transcription factor 2
SAA1 and 2	Serum amyloid A1 and A2
SCF	Stem cell factor
scRNA-seq	Single cell RNA sequencing
SPP1	Osteopontin
SRE	Skeletal related events
STAT3	Signal transducer and activator of transcription 3
TAM	Tumor associated macrophage
TA-MSC	Tumor associated mesenchymal/stem stromal cell
TCR	T cell receptor
TIMP-1	Tissue inhibitor of metallopeptidase
TGFβ	Transforming growth factor beta
TKI	Tyrosine kinase inhibitor

TME	Tumor microenvironment
TNF α and β	Tumor necrosis factor alpha and beta
TRAIL	TNF-related apoptosis inducing ligand
TPO	Thrombopoietin
T^{reg}	Regulatory T cell
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor A
VHL	Von Hippel-Lindau tumor suppressor
WHO	World Health Organization
WNT	Wingless-related integration site

1 INTRODUCTION

1.1 Cancer and cancer metastasis

Cancer is the second leading cause of death estimated by the World Health Organization (WHO) in 2020. Approximately 1 in 6 deaths are due to cancer worldwide, totaling nearly 10 million deaths. The International Agency for Research in Cancer estimated that there are around 19.3 million new cancer cases every year worldwide, where roughly 400 000 are children[1]. Despite the devastating numbers, the general survival range is between 85-90% for patients with primary tumors. Survival is different for each cancer type, where lung and pancreatic cancer comprises with the lowest survival (18% and 11% respectively) and local breast cancer is the most curable type. Nevertheless, prevalent cancer types are lung, breast, colorectal, prostate, skin cancer and stomach[2]. Although, the most common types of cancers vary in every country[1,2]. Cancer normally develops through multiple processes occurring in normal cells leading to tumor development. The disease progression creates many complications and when a primary tumor is developed, it can obtain the capability to spread, resulting in metastasis.

Metastasis is the consequence when circulating cancer cells reinitiate a fully developed tumor at a distant site[3]. It has long been reported that over 90% of all cancer related deaths are associated with metastases[4-6]. As mentioned above, demographic implications may affect the percentage. For instance, a population-based study in Norway supports the notion, in addition they show retrospectively that at least 66.7% of solid cancer related deaths are linked to metastasis[7]. The lack of mechanism of action in metastatic spread and resistance to treatment of these tumors is the most challenging obstacle scientists and health care professionals face in their journey to defeat the spread of cancer. To understand how health care can meet this challenge one need the understanding of the characteristics and underlying driving forces that enable a cancer type to spread in a certain way to a certain site and not another. Reviving the "seed and soil" principle that Paget described in 1889, metastasis is postulated by the frequency of engagements that malignant cancer cells, the seed, have with their adjoining microenvironment, the soil. The seed *per se* will not flourish and grow. A hospitable soil will serve the purpose. Similarly, each cancer cell must be studied as an individual cell, proficient enough to develop in its own desired environment, a sophisticated niche. The crosstalk that happens between the cellular and molecular compartments has in recent years increased and nourished our knowledge. However, the gap of understanding the mediation of site-specific metastasis is needed to be investigated for the identification of new targeted therapies.

1.2 Primary mechanisms for metastatic spread

The mechanism of metastasis involves five major steps (Figure 1) enabling cancer cells to spread from the primary tumor into the blood vessel or lymphatic system to a distant site. Starting with *invasion* and *migration*, cancer cells remodel the extracellular matrix (ECM) mechanistically with metalloproteinases while they migrate and navigate through the microenvironment[8]. During the second step, including *angiogenesis* and *intravasation*, cancer cells form premature vasculature around itself enabling nutrients and oxygen to be transported[9]. The new vessels are vastly permeable due to absent basement membrane. Consequently, plasma protein leaks out, further promoting formation of new vessels, and tumor intravasation, to then, enter the blood stream. The angiogenic process aid metastatic progression by facilitating the path of tumor cells through the lymphatic and vascular system. Following, the third step, *survival* in the blood stream and *adhesion to the endothelium*, is succeeded by fairly few cancer cells[10]. The circulating tumor cells (CTCs) are trapped in vessels, due to a larger diameter than the microvasculature. During the adhesion process, collision of CTCs occurs with the endothelium via P-selectin or E-selectin, and they adhere through intracellular adhesion molecule -1 (*ICAM-1*) or vascular cell adhesion molecule-1 (*VCAM-1*). Lastly, cancer cells exit the blood vessel through *extravasation* and *colonize* the new site. This secondary site, metastatic site and (or) niche, retain ECM and cell types appropriate for tumor cell survival and growth[11]. This includes areas surrounding the blood capillaries where cancer cells can survive, seed and grow or settle into latency to later be reactivated and colonize, further generating a new macroscopic tumor[11].

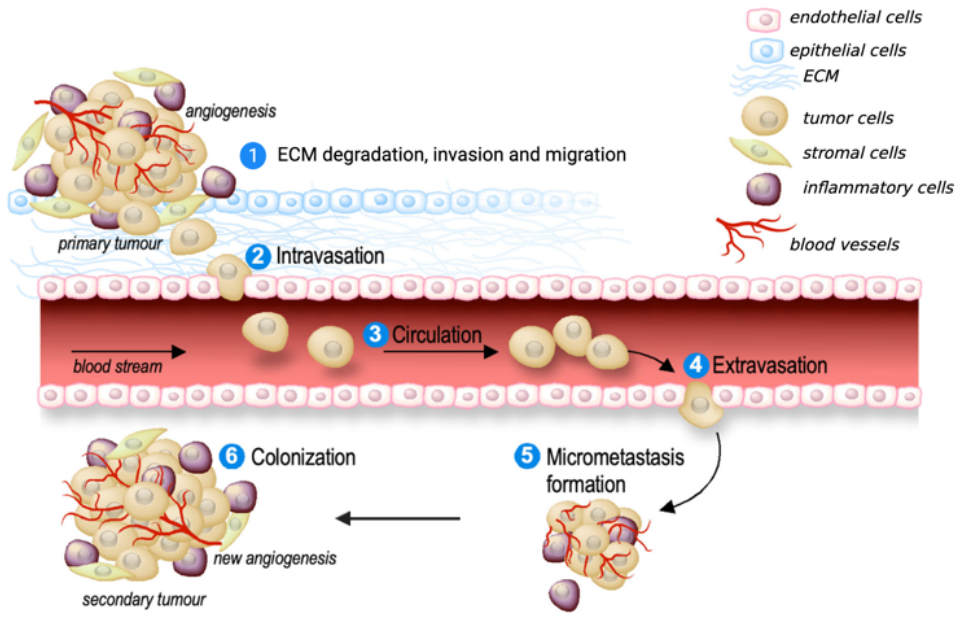

Figure 1: Metastatic process. Granted permission from author[12]. Adjusted in Biorender.com by A.M.A.

Cancer cells that have migrated, managed to settle in a distant site are disseminated tumor cells (DTCs), and they give rise to metastasis. These diverse states can occur constantly until removal of the primary tumor. One of these states that is harder to detect is when cancer cells exhibit a quiescent phenotype (e.g., non-proliferative) and enter latent phase[11]. Though, cells that embrace the tumor-initiating ability form metastatic stem cells (MetSCs). When they exit their quiescent state, the MetSCs may recreate their lineage in the host organ and release metastatic progeny into the circulation to start secondary progression in the same or another organ. Eventually when they recur, they become clinical metastasis[13]. Generally, it is challenging to define their dormancy, however interpreting them as non-proliferative DTCs is considered dormant and it has not yet developed into a micro metastasis[14–16]. In addition, there are potential models for the behavior of dormant tumor cells as; very slow growth, proliferative, or non-replicative until being reactivated by the tumor microenvironment (through for instance immune surveillance or angiogenic switch)[17]. These might exist simultaneously in different niches. The whole process is considered to have existed already in a pre-established niche, so called, a pre-metastatic niche (PMN)[18].

The presence of a "pre-metastatic niche" reveals that there are organized chronological events occurring resulting in the metastatic process. This pre-metastatic niche contributes to the metastatic process in a series of separate phases:

- *Priming*

Initially, cells in the primary tumor become hypoxic and inflammatory by undergoing uncontrolled proliferation. This results in production of different tumor-derived secreted factors, extracellular vesicles and other molecules that could stimulate recruitment of bone-marrow derived cells (BMDCs), including regulatory/suppressive immune cells. They are recruited to the upcoming metastatic organ site, consequently initiating reprogramming of the stromal environment in the distant organ. The cancer cells undergo *intravasation* at the primary tumor site by invading the basement membrane into the blood or a lymphatic vessel. Further cells *migrate* through the adjacent lymph nodes or blood vessels, moving to other organs in the body[18].

- *Licensing*

In this step, the regulatory immune cells and BMDCs are in continuous mobilization into the secondary site as a response from the tumor-derived secreted factors and extracellular vesicles from tumor cells. This step could be the intermediate stage, the link between the pre-metastatic niche and the maturation towards initiating a tumor metastasis. The secondary site becomes prepared, "licensed" for seeding and colonization of tumor cells[18,19].

- *Initiation*

Now the licensed pre-metastatic niche is mature, and the primary-tumor educated cells are in action. They simultaneously contribute to initiating the metastatic process. By enabling *extravasation* of CTCs moving from the vasculature they promote the attraction of tumor cells to colonize in the newly formed niche. In addition, *latency* of tumor cells is also regulated by the pre-metastatic niche by pushing them into a quiescent state until the environment is fully appropriate for their metastatic growth. It has been shown that chemotherapy, surgery and anti-tumor immune response can push tumor cells into dormancy[19,20]. However, an established pre-metastatic niche in the appropriate environment will promote *colonization*, *survival*, *seeding* and *development* of metastatic tumor cells, resulting in the formation of micro metastases[18,19].

- *Progression*

Fully educated tumor cells from the primary tumor continue to aggressively colonize the metastatic organ with the help of the pre-metastatic niche. The components in the niche directly stimulate tumor growth and expansion, resulting in progression of *macro metastases*. The established metastatic lesion might in turn encourage tumor dissemination to expand into clinically detectable metastases. This stage also results in the formation of new blood vessels, creating blood supply allowing the tumor for continuous growth[18,19]. The recruitment of BMDCs and the formation of the pre-metastatic niche are essentials in forming the tumor microenvironment and the further development of metastasis.

1.3 Tumor microenvironment

When cancer cells inhabit a new environment (or niche), such as in a host organ/site, it stimulates a process where the tumor tissue, containing components of the prior organ where the tumor originated in, is generated. This response is a signature for the complicated heterotypic interactions between the host tissue and cancer cells, generating fibrous or connective tissue, called the tumor stroma or the TME[21]. Constituents of the TME involves cellular and non-cellular components, including capillaries, activated fibroblasts, immune cells, cancer associated fibroblasts (CAFs), basement membrane and ECM in the surrounding of the cancer cells[21,22]. The ECM function as a structural support for cells both in healthy organs and in tumors[21]. In normal organs it undergoes continuous remodeling, as well as in the TME. Here, proteases, such as MMPs and cathepsins, that degrade the ECM components, are secreted by CAFs and TAMs. Simultaneously new molecules are produced by CAFs[21]. In addition, in early stages of the tumor growth, the ECM have the capacity to suppress tumorigenesis. However, it has been demonstrated that it can also support the metastatic cascade and tumor growth, while simultaneously suppressing infiltration, activation and function of immune cells, further contributing to therapy resistance[23]. The interplay of different cell

populations becomes critical to defeat the tumor cells, favorably before the tumor cells manage to suppress the physiological defense, in other words, combatting the immune system.

1.3.1 Microenvironment in normal homeostasis

In normal adult tissue, stem cells exist in certain niches that contain the cellular and molecular factors maintaining and favoring their self-renewal, including access to differentiation signals. Niches that act as gate-keepers of the blood system comprises of mesenchymal stem/stromal cells (MSCs), hematopoietic stem cells (HSCs), and endothelial cells[21]. The formation and location of stem cell niches have been defined in different tissues, such as in the brain, bone marrow (BM), intestinal epithelium and epidermis[24-26]. Depending on the components in the niche of the host environment it will determine the survival and fitness of the metastatic-initiating DTCs. A key role in metastatic niches is the capacity of the DTCs to maintain their renewal capacity ("stemness"). The signaling pathways known for this purpose is Notch and wingless-related integration site (Wnt). They stimulate self-renewal within stem cell niches in the BM[27], the intestinal mucosa[24] and the brain[28]. These signaling pathways has been observed in cancers as colorectal cancer[29], glioblastoma[30] and pancreatic cancer[31].

The niche of HSCs mainly resides in the BM with a microenvironment that is highly regulated by balancing differentiation signals, stem cell self-renewal[32] and quiescence[27]. Within the niche, factors like angiopoietin 1 (*Ang-1*), stem cell factor (*SCF*), thrombopoietin (*TPO*), hypoxia-inducible factor 1-alpha (*HIF1α*), and transforming growth factor beta (*TGF-β*) maintain stem cell quiescence, whereas Notch and Wnt promote self-renewal and proliferation. Another essential key niche factor in the BM that is required for HSC retention and HSC maintenance, is C-X-C motif chemokine 12 (*CXCL12*)[33]. Absence or deletion of this niche factor leads to depletions of the HSCs from the BM[33]. It is also reported to act as a chemoattractant for immature and mature hematopoietic cells and their homing to the BM. MSCs are the source of niche factors such as *SCF* and *CXCL12* in the mouse bone marrow[34]. Nevertheless, MSCs are cell types that regulates HSCs for long-term self-renewal, preserving their multipotency of their ancestors and maintain the generation of all blood types throughout life[35]. On the contrary, MSCs that regulate HSCs and expresses the niche factor *CXCL12*, have shown to be involved in progression of bone metastasis of breast[36] and prostate cancer[37]. The capacity of HSCs to reconstruct the entire hematopoietic system in adults makes them precious for the treatment of several hematopoietic disorders. For this reason, BM transplantation is used in therapy of hematopoietic diseases, to help the body rebuild the hematopoietic system with the help of HSCs[38].

1.3.2 Hematopoietic cells of the TME

Shifting focus from normal homeostasis, close examination of the TME reveal that multiple blood cell types might play an important role for the development of cancer. Accumulation of early white blood cells such as the immune myeloid cells are often seen in cancers. In the early process they are monocytes or polymorphonuclear cells as eosinophils or basophils. Further in their maturation they mature into cell populations as macrophages, monocytes, dendritic cells and neutrophils. Lymphocytes are another subtype of white immune blood cells including the cell types: Natural Killer cells (NK), T cells and B cells. These cells make up the majority of the BM mononuclear cells. Diverse T cell populations exists in the TME, lymphoid organs, lymph nodes and the invasive area[39]. In cancer, the tumor infiltrating capacity of immune cells has been measurable by an "immunoscore" first recognized in colorectal cancer and has now become a technical tool to predict recurrence in patients with risk of metastasis[40,41]. The majority of the T cell compartment consists of cytotoxic T cells (CTL), helper T cells (Th-cells) and regulatory T cells (T^{reg}).

In this thesis the focus lies on certain immune cell populations that are discussed in the presented scientific work.

1.3.2.1 Macrophages

Signals from the TME recruit and activate macrophages for various effects on tumor progression and metastasis. Researchers emphasized that macrophages were critical components in the creation of PMNs. Tumor secreting factors such as vascular endothelial growth factor (*VEGF*), *TNF-β*, tissue inhibitor of metallopeptidase (*TIMP-1*), make macrophages react and mobilize to the bloodstream to later cluster in the pre-metastatic sites[42,43]. Nonetheless, macrophages are known for decades to display different phenotypes, M1 and M2, in response to environmental changes[44].

- *M1 and M2 macrophage phenotype*

Generally, M1 exhibit the classical macrophage activity by secreting pro-inflammatory cytokines such as interleukin 6 (*IL-6*), *IL-12*, *IL-23*, C-X-C motif chemokine ligand 10 (*CXCL10*)[45], interferon (*IFN*)-γ and tumor necrosis factor alfa (*TNF-α*). M1-type macrophages steer antigen-presenting, phagocytic and cytotoxic functions[46] and promote inflammatory responses towards invasive pathogens and tumor cells. The M2-type, on the other hand, secrete anti-inflammatory cytokines *IL-10* and *TGFβ*, *VEGF* and *MPPs*[45,47]. This phenotype is inclined to be immunosuppressive, supporting tissue repair and tumor development[48,49]. However, the M1 and M2 phenotypes are not distinctly described, rather existing in a polarized spectra depending on signals from their environment.

- *Tumor associated macrophages (TAMs)*

Macrophages inhabiting the TME of solid tumors are characterized as TAMs. The phenotype of TAMs is polarized, shifting between M1 and M2[44]. Majority of TAMs reside in avascular areas where a hypoxic environment facilitates further processes, as angiogenesis[50,51]. Therefore, TAMs are abundant in various cancer types and exert important regulatory functions supporting tumor angiogenesis, metastasis, invasion, immune regulation and chemoresistance[48,51,52]. Hence, tumor cells are not enough for metastatic progression, rather an extensive crosstalk exists in the TME. The association to metastasis, angiogenesis and immune regulation has been used to classify and identify TAMs, for instance with the proteins *MMP2/9*, signal transducer and activator of transcription 3 (*STAT-3*), *CD163* and *CD206*[52].

Recently, scientists discovered a unique cell surface marker expressed on macrophages, *TREM2*. Previously, the activity of *TREM2* has been known to initiate signaling pathways downstream of *mTOR* (mammalian target of rapamycin) and *MAPK* (mitogen-activated protein kinases), resulting in cell activation[53]. In a cancerous setting *TREM2* is described to be expressed on tumor infiltrating macrophages[54]. *TREM2* expressing macrophages demonstrate to be selective in human tumor macrophages, and correlate with poor survival in colorectal carcinoma and triple-negative breast cancer[55]. Hence, clinical observations are consistent with the concept that TAMs expressing *TREM2* in the TME are associated with worse disease outcome[48].

1.3.2.2 T cells

- *Regulatory T cells (T^{reg})*

A critical constituent in maintaining homeostasis of the immune system and immune tolerance in the body, are the T^{regs}. Their classification is based on the expression levels of *forkhead box P3* (*FOXP3*) (and/or *CD25*) and the naïve marker *CD45RA*. Naïve and resting T^{regs} lack expression of *CD25* and *FOXP3*, but are expressing *CD45RA*, on the contrary, effector/activated T^{regs} have high levels of *FOXP3* and *CD25* and rather an absence of *CD45RA*[56]. They originate from the thymus, and when naïve T^{regs} exit the thymus without being activated, they have poor suppressive activity. Upon stimulation by T cell receptors (TCR) they differentiate and proliferate into highly suppressive and terminally differentiated effector T^{regs} (eT^{reg}). These eT^{regs} can prevent maturation of antigen-presenting cells (APCs). This activity is achieved by certain inhibitory cytokines as *IL-10*, *TGF-β* and *IL-2*[56]. Another mechanism of which T^{regs} can suppress the immune response is through immune checkpoint molecules, including *lymphocyte activation gene-3* (*LAG-3*), MHC class II pathway and T^{reg} activation through programmed cell death (*PD)-1/PD*-ligand *(PD-L)1* pathways[57-59].

- *Cytotoxic T cells (CTLs)*

Cytotoxic T cells, expressing cell-surface CD8, are dominant effectors in the immune response against cancer and form the current cancer immunotherapeutic stamina. They kill cancer cells by stimulating death ligands in the cells such as TRAIL (TNF-related apoptosis inducing ligand)-FasL, with mediating perforin and granzyme pathways for the same purpose[60]. This subset of T cells, emerging in the thymus, are committed to identifying antigenic peptides presented by MHC class I molecules[60]. When CD8+ T cells respond to an antigen, they start their effector phase where they exert killing of their targeted cells by Fas ligand (FasL)-mediated apoptosis stimulation and granule exocytosis. *IFN-γ* and *TNFα* are released by CTLs to induce cytotoxicity in the cancer cells. Further, coinhibitory receptors such as *PD-L1* and cytotoxic T lymphocyte associated antigen 4 (*CTLA-4*)[59] are then expressed by the activated T cells.

- *T cell exhaustion*

Sustained overexpression of the coinhibitory receptors on CD8+ T cells could lead to dysfunction or exhaustion, resulting in their impaired potential for combatting cancer[59]. These coinhibitory receptors include the above-mentioned *PD-1* and *CTLA-4*, as well as *TIM-3*, *LAG-3* and *TIGIT*. The characteristic of exhaustion has been observed during infections and in cancers[61,62]. The discovery of this condition has led to emerging therapeutic targets and the development of checkpoint inhibitors, antibodies targeting checkpoint receptors and/or their ligands[63]. Nonetheless, the number of fatal cases remain. Instead, the debate concerns co-administration of *TGF-β* with *PD-1* inhibition to facilitate tumor killing[64]. Hence, aiming for precision medicine in metastatic cases will require more than the target drug, and rather a combination of a facilitator to pave the way for the armed drug.

1.3.3 Non-hematopoietic cells of the TME

Metastatic signs in primary tumors starts when cancer cells appear in the interfering area, that is the site where they are exposed to the stresses of the surrounding tissue as immune surveillance and hypoxia[13]. Several stromal cell types produce signals triggered by cancers cells that the cancer can use for their own self-renewal, migration and survival to created distant metastases[65]. Enrichment of signals and cells that resemble those of a particular distant tissue, informs cancer cell in the primary tumor to grow in that specific tissue. For instance, in breast cancer, the ability of the existing CAF rich stroma selects cancer cells that best act on the CAF-derived factor *CXCL12*, and are thereby prepared to survive in the *CXCL12* enriched environment of the bone marrow[65]. Consequently, for this to be enabled, different stroma subsets in the tumor microenvironment are involved.

1.3.3.1 Stromal cells

- *Endothelial*

The inner layer of the blood vessels consists of endothelial cells (ECs). It is proposed that their interaction with HSCs in the sinusoid ECs is important for HSC function[66]. Endothelial cells are heterogeneous in antigen composition, gene expression, morphology, function and distribution. They exhibit different phenotypes in different tissues, but also in-between the layers of the vasculature in the same tissue[67,68], as the sinusoidal and arteriolar ECs. They are different by their structure where the sinusoidal ECs are lined in a single layer of the endothelium, and arteriolar are thicker blood vessels[69]. The vasodilator *VEGF-A* (vascular endothelial growth factor A), regulates the vasculature. In a tumor environment, occurring events lead to changes in the barrier shape of ECs resulting in intercellular gaps that leaks blood and fibrin into the surrounding tissue. In these conditions the expression of *VEGF-A* is high in tumors[70]. Damaged blood flow, hypoxia and tumor growth are subsequent consequences. Along with *VEGF-A*, tumor cells also secrete angiopoietin (*Ang*) and platelet-derived growth factor (*PDGF*). *Ang-1* and *Ang-2* contribute to adhesion between ECs. In metastatic tumors the ECs show tumor-aggressive potential compared to non-metastatic tumors by exhibiting an increase of gelatinase-collagenase IV MMPs (*MMP-2* and *MMP-9*), resulting in ECM degradation[71].

- *Pericytes*

Pericytes are cell types that are found in the walls of small blood vessels in tissues and are involved in maintaining structural integrity of the vessels, regulating blood flow and assists in formation of new blood vessels. In the BM there are at least two types of pericytes identified according to their location in the blood vessels: sinusoidal and arteriolar[72]. They can be distinguished by known markers where pericytes in the arteriolar express neuroglial 2 (*NG2*) proteoglycan, and are negative for the leptin receptor (*LEPR*). Whereas the sinusoidal pericytes are negative for *NG2* but express the *LEPR*[72]. Pericytes are otherwise identified depending on their anatomical location and along with several markers such as platelet-derived growth factor receptor β (*PDGFRβ*), *NG2*, α smooth muscle actin (*αSMA*) and *Nestin*[73]. Tumor pericytes present pro-angiogenic functions and their activated phenotype are characterized by high expression of *αSMA* and regulator of G-protein signaling-5 (*RGS5*) compared with pericytes in normal tissue[74]. In hepatocellular carcinoma (HCC) and non-small cell lung cancer (NSCLC) a subset of pericytes with induced glycolysis have demonstrated disrupted support for blood vessel function, further correlated with lower survival[75]. Furthermore, pericytes have shown to have an important role, particularly in brain metastasis of breast cancer[76], where tumor cells demonstrate pericyte mimicry and show pro-metastatic behavior[77]. By blocking the neo vasculature dynamics *in vitro*, it

was possible to reduce metastasis in uveal melanoma[77] or radiation induced glioma recurrence[78].

- *Fibroblasts*

Fibroblasts produce the connective tissue in the ECM during normal development, and their physiology and function changes with age[79]. Most fibroblasts originate from the primitive mesenchyme which they share with other mesenchymal lineages as adipocytes, chondrocytes and osteoblasts[80]. There are markers for subtypes of fibroblasts as *αSMA* (also called *ACTA2*) and fibroblast activation protein-α (*FAP*). In normal adult tissues *FAP* cannot be found and is mainly associated with stroma of primary and metastatic tumors[81,82]. Fibroblasts are also involved in tissue repair and is activated when tissue is damaged. Otherwise, they are quiescent until injury, though it is still unclear how these resting fibroblasts become active. It is suggested there are two states; reversible and irreversible determination by epigenetic regulations[83,84].

Cancer associated fibroblasts (CAFs)

CAFs are universal components of tumor stroma and are found in several solid tumors[85]. These are identified by cell surface expression of FAP, a serine peptidase[86]. In solid tumors, their overexpression has been linked with metastases and worse survival[81,82]. On the primary site CAFs might promote metastasis by releasing growth factors and cytokines into the circulation to promote the progression of the cancer cells at a distant site[87]. CAFs might impact the rigidity of the ECM in primary tumors and therefore enhance tumor cell invasion and generate pathways to guide for cancer cell penetration[88].

1.3.4 Cells specific to the bone marrow TME

1.3.4.1 Mesenchymal stem/stromal cells (MSCs)

MSCs are multipotent stromal cells recruited from the BM and have the ability to differentiate into adipocytes, osteoblasts and chondrocytes[89]. The MSCs can attract tumor cells to the BM via secreted chemokines and therefore support the process of epithelial-to-mesenchymal transition (EMT), a process critical for tumor cell migration, and also promote bone metastasis. Furthermore, they can be recruited to the TME where they could mediate immune suppression[90,91].

1.3.4.2 Osteoblasts

Osteoblasts, cells of osteogenic origin, remodel the bone. Osteoblasts produce several cytokines and growth factors important for HSC maturation[92,93] and it is suggested to have a role in the regulation of the HSC in the BM[92]. A protein secreted by the osteoblasts, osteopontin (*SPP1*), is involved in locating HSC and inhibit their proliferation[94,95]. On the contrary, a receptor that is expressed by osteoblasts, Jagged1

(notch receptor ligand) instead enhance the number of HSCs[96]. High osteoblastic containing lesions are detected when there is an accelerated bone growth, a mineralized tissue with malignant cells, leading to cancer growth. This is often seen in patients with bone fractures and pain[97]. For instance, in breast cancer bone metastasis, it has been observed that the DTCs are found within the bone areas where there is an enrichment of osteoblasts[98]. This is clarified as osteoblast expressing nuclear factor kappa B (*NF-kB*) (RANK ligand, RANKL) *RANKL* and *SDF1* (or *CXCL12*) in bone. These are cytokines in breast cancer cells, which leads to tumor growth when they bind to their respective receptors *RANK* and *CXCR4* on the cancer cells[16].

1.3.4.3 Osteoclasts

Together with osteoblast, osteoclasts regulate bone resorption and formation, where a premature state of osteoclasts can decrease the number of osteoblasts[99], reducing their capacity to enter the marrow cavity. Their ability of regulating bone resorption is important for the renewal of skeleton in the human body and also allowing passage of the HSCs. In cancer, an increase of osteoclast function, resulting in bone resorption, induce a release of numerous growth factors from the bone matrix such as *TGFβ*, which also affects HSCs[100]. In turn these components support tumor growth and more activation of the osteoclasts[101].

In 1990 it was observed that the formation of osteoclasts, osteoclastogenesis, can be achieved by the monocyte-macrophages lineage, hence not only from immature cell populations[102]. It was not until the beginning of the 21st century that it became clear that two essential molecules are expressed to stimulate osteoclastogenesis: macrophage colony-stimulating factor (M-CSF) and receptor for activation of *RANKL*[103]. Also known as OPGL. When M-CSF, important for the maturation of macrophages, binds to its receptor (colony stimulating factor 1; CSF-1, c-Fms) on premature osteoclast precursors, they provide signals that are necessary for their proliferation and survival[102]. Upon stimulation, osteoclasts are therefore believed to be involved in orchestrating PMN formation[103]. Together with immune cells of the hematopoietic lineage and the stromal cells, these host cells are closely distributed within the BM, near the microenvironment and where the stem cell niches are found. These niches represent important interdependence between mesenchymal progenitor cells and HSC differentiation and the link of bone formation with bone resorption. When tumor cells arrive and reside in the BM they remodel the bone and the BM into a tolerant environment where they can be comfortable and grow[104].

1.4 Bone metastasis

The location of the established pre-metastatic niche is thought to not be manifested by chance. A pattern of site-specific metastasis has been observed among most cancers.

For instance, breast carcinomas often form metastases in the bone, skin, brain and kidneys. On the contrary, colon carcinomas rarely metastasize to those sites, and normally form metastasis in the lung and liver. Similar to breast cancer, prostate cancer form metastases in the bone and only in rare cases to the lung and liver. Nevertheless, the most common sites that cancers metastasize to are to the bone, liver, lung and the lymph nodes[105]. Bone metastasis is the foremost cause of morbidity with a varying survival rate highly depending on the primary tumor. A retrospective population-based study showed that patients presenting with bone metastasis at diagnoses and the median survival in months vary among different cancer types, with the shortest being pancreatic bone metastasis (ranging between 1-7 months, with a median value of 3 months) and the longest in breast cancer (ranging between 8-57 months with a median of 27 months)[106]. Among solid tumors, breast cancer bone metastasis was the only one present a five-year overall survival of 10%, while the three-year survival of lung bone metastasis was 2%[107]. The most common cancers that are prone to spread to bone are primary tumors of the breast, prostate, and lung. Though many other cancers can metastasize to bone, including: thyroid, kidney, melanoma, lymphoma, sarcoma, uterine and gastrointestinal[106-108].

Bone metastasis is characterized by reduced mobility, pathological fractures, spinal cord compression, severe pain, hypercalcemia, bone marrow aplasia and skeletal related events (SREs)[105]. Usual sites for bone metastasis are: spine, ribs, hips, sternum and skull. Cancer cells can either spread to one or several bones. In general, bones are under constant change and new bone tissue is generated, while old bone tissue break into minerals circulating in the blood. Cancer cells tend to disturb the normal bone remodeling process which leads to weakening of the bones, further causing bones to become frail or thick depending on the affected type of bone cells. Bone metastasis is therefore driven by either if there are many new bone cells forming (often in metastasized prostate cancer, driven by osteoblasts), *osteoblastic*. Or if too much bone is destroyed (seen with metastasized breast cancer, often driven by osteoclasts), *osteolytic*[109]. Destruction of the balance often lead to tumors being able to form bone malignancies in the bone and bone marrow[110,111].

1.4.1 Normal BM microenvironment

The BM is found within the bones and the preferred site for homing and regulation of HSCs is in the trabecular regions of the metaphysis, closer to the surface of the bone[112] (Figure 2). Here, it has been proposed that HSCs show a more self-renewal capacity compared to being located in the central marrow[113]. Nevertheless, other studies have suggested that HSCs might be randomly distributed in the BM[114]. In addition, some studies have observed that the location is age-dependent where the “older” the HSCs are, the more likely they are distributed to one site and not to the bone surface as when being “young”[115].

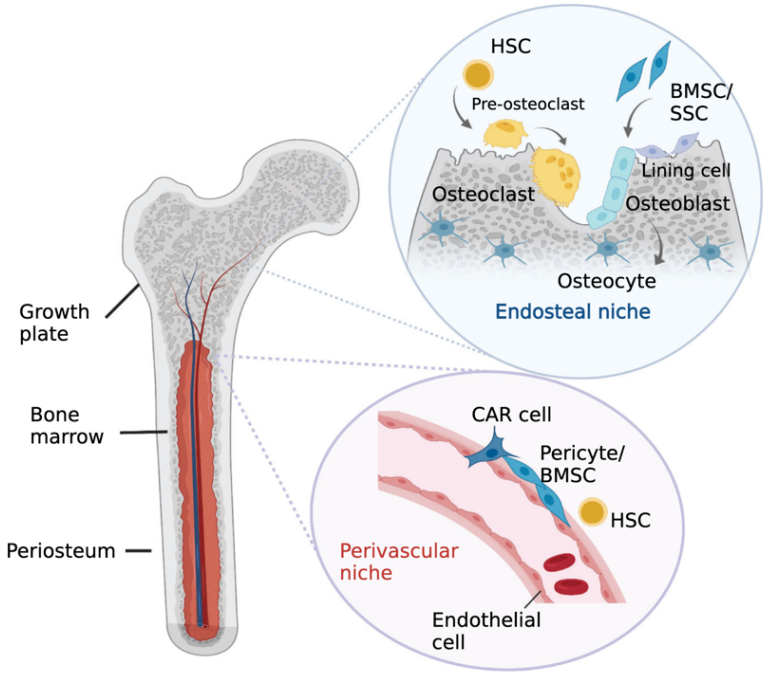

Figure 2: Normal bone marrow niche. Granted template permission by the author[116]. Adjusted in Biorender.com by A.M.A.

A variety of cells reside in the bone since it is a tissue that regulate osteogenesis and hematopoiesis. The inner surface, cortical and trabecular parts, of the bone is covered by a thin vascular membrane of connective tissue where MSCs, osteoblasts and osteoclasts are found, forming the *endosteal niche*. Numerous of blood vessels builds up a network of supplies to both the bone and the BM, nurturing the BM via nutrient canals[117]. Inside the bone, along with the sinusoidal space, arteries host thin arterioles that makes it possible for them to come into the central marrow. By forming a permeable passage system, the sinusoid allows for mature blood cells to enter the circulation. Following the branches of arterioles are networks of nerves that subdivide towards the smooth muscles cells or are latent in the hematopoietic tissue with hematopoietic cells[118]. The BM arteriolar and sinusoidal endothelial cells are both surrounded by perivascular cells[119], forming the *perivascular niche*, which is known to be the environment supporting the HSCs and lies close to the blood vessels[72]. A study show that inactive (quiescent) HSCs exists closer to arterioles than to sinusoids, indicating that there are individual and spatially different perivascular niches for quiescent and proliferating HSCs in the BM[72].

1.4.2 Bone metastatic microenvironment

The total vigorous interactions govern the microenvironment of the BM, and control the formation of blood and bone[27]. The BM is also the root of self-renewal and developmental pathways such as Wnt, Notch, Hedgehog, *TGF-β* family and *CXCL12/SDF-1*, facilitating colonization and outgrowth of tumor cells in the bone[120]. This extensive

communication accelerates their journey and passage into distant sites. After arriving in the bone marrow, tumor cells proliferate and interact with mesenchymal and hematopoietic precursors at various stages of differentiation[121]. These connections provide essential signals for both tumor cells and host bone and bone marrow cells during tumor colonization of bone. The interplay of the niches dictates the metastatic progression[97,122].

1.4.2.1 The role of the perivascular niche in bone metastasis

Endothelial cells, pericytes and BMSCs, constituents of the perivascular niche (Figure 3), are typically close to the sinusoids, demonstrating an important role for maintaining full potential and survival of HSCs[35,123]. The BMSCs, endothelial cells, osteoprogenitors and osteoblasts tend to express *CXCL12* in the perivascular niche[124]. The receptor of *CXCL12*, CXC chemokine receptor type 4 (*CXCR4*), is a common molecular nominator shared by HSCs and DTCs, that often inhabit the same space in the bone compartment[66]. Throughout dissemination and homing to a secondary organ, less than 0.1% of DTCs survive, however several perivascular niche cells can assist DTC homing to the bone through the production of *CXCL12* (mainly by BMSCs). These "new" bone inhabitants tend to have high expression of *CXCR4* in contrast to the primary tumor[125].

1.4.2.2 The role of the endosteal niche in bone metastasis

This niche predominantly involves different key bone cells produced from mesenchymal stroma cells such as osteoblasts, osteocytes and bone-lining cells, and from hematopoietic progenitors, the osteoclasts[35]. The *endosteal niche* is critical in modulating osteolytic and osteoblastic bone metastasis. When the bone microenvironment has been colonized by tumor cells, the bone matrix signifies a congenial "soil" to support the metastatic growth of cancer cells comprising diverse

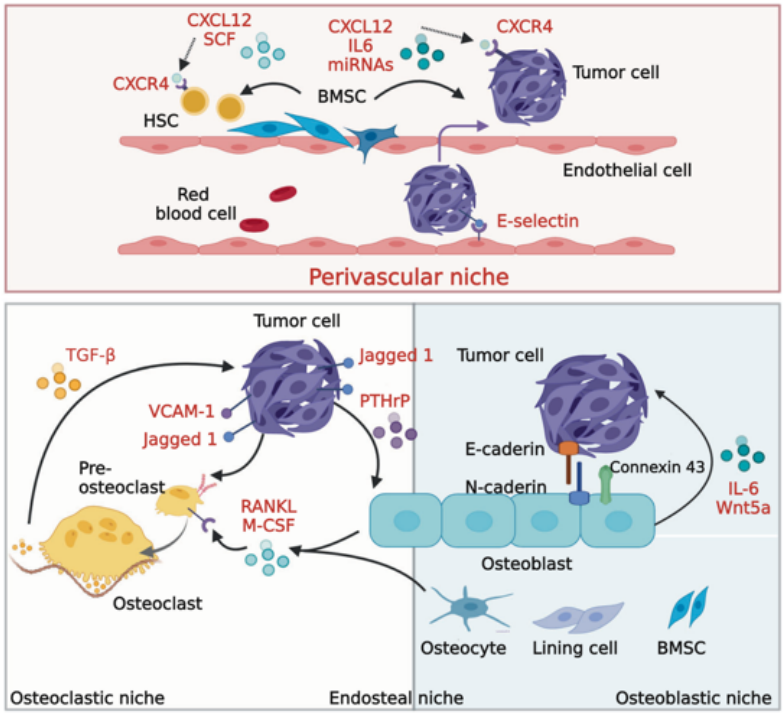

Figure 3: Illustration of the perivascular niche and the endosteal niche. Granted template permission from the author[116]. Adjusted with Biorender.com by A.M.A.

minerals, sufficient nutrients and growth factors. A phenomenon called the "*vicious cycle*" is a mechanism of osteolytic bone metastasis, where activation and recruitment of osteoclasts are associated with tumor invasion. By activating the maturation of osteoclasts, degradation of the bone matrix, results in release of growth factors, such as *TGF-β*, further triggering the vicious cycle. Cancer cells take advantage of the bone remodeling process through the vicious cycle. In cancers that are osteoblastic, as prostate cancer bone metastasis, the vicious cycle additionally comprises of a formation of too many bone cells. Tumor cells produces osteoblast activating factors as *TGF-β*, bone morphogenetic proteins (BMPs), *EGF* and *PDGF*. These growth factors promote the formation of new bone. Furthermore, the activated osteoblasts produce *IL-6*, promoting cancer cell colonization and growth in the bone microenvironment[126].

The remodeling process is regulated by signaling pathways and transcriptions factors such as osterix (*SP7*) and *RUNX2* (runt-related transcription factor 2). Both are important transcription factors responsible to ensure osteoprogenitors differentiation to mature osteoblasts. Consequently, bone formation is initiated by secreted proteins from the bone matrix[126] contributing to bone turnover.

As the seed and soil hypothesis, the fondness for the bone as a proclaimed site of metastasis is reliant on the specific characteristics of the tumor, and the receiving bone microenvironment.

1.4.3 Therapeutic approaches

Currently the treatment for bone metastasis is individualized. The treatment plan depends on the type of primary cancer, the stage, which bones that are involved, prior cancer treatment and overall health. Radiation therapy slows metastatic growth and reduce pain. Chemotherapy reduces the tumor cells and if necessary, surgery is performed to fix broken bones and pain, then some physical therapy to strengthen muscles to help with mobility[97]. To date, there are no curative therapeutics in bone metastatic tumors. Most treatments are palliative where the purpose is to alleviate the pain induced by the disease. Agents that were first discovered, which retrospective plus follow-up studies are based on, and that are currently still in use for targeting bone are:

1.4.3.1 Bisphosphonates

Bone-building drugs similar to those used in osteoporosis; these strengthen bones and reduce the pain of metastases. In the late 90´s bisphosphonates were discovered to tumor-induced hypercalcemia and therapy of bone metastasis[127]. Osteoclast inhibition by bisphosphonates causes a reduction in the number of HSCs and delays hematopoietic recovery[128]. Bisphosphonates, such as commercially available alendronate, is used as preventive care in solid tumors that have metastasized to the bone. In breast cancer bone metastasis, a duration of 2-year treatment with bisphosphonate prolonged survival to a median of approximately 50 months[129].

1.4.3.2 Denosumab

A human monoclonal antibody inhibiting RANKL and preventing the development of osteoclasts[130]. Denosumab has demonstrated similar efficiency as bisphosphonates in preventing SRE by 8.2 months before first event, and reduced the risk of first SRE by 17%[131]. In non-squamous non-small cell lung cancer bone metastasis patients denosumab prolonged the median overall survival by 21.4 months compared to 10.5 months in untreated patients[132].

1.4.3.3 Radioactive elements (radiopharmaceuticals)

Normally they are injected into a vein and find, and kill, cancer cells in the bones. In prostate bone metastasis, radium-223 has shown to be beneficial effects with an overall survival of 14.9 months compared to 11.3 months in the placebo group[133,134].

Despite current treatment approaches, bone metastasis remains a challenging and fatal disease. The recent decade has provided more biological insights into novel therapeutic approaches. In some cases, a combination is favorable, as for instance immune checkpoint blockade (ICB) together with palliative radiotherapy in breast cancer bone metastasis[135], resulting in a median progression-free survival of 4.1 months. For other solid bone metastatic tumors, one drug agent that acts both as tyrosine kinase inhibitors (TKIs) and VEGFR inhibitor, showed that half of the patients had progression

free survival after 3.5 months[136]. In bone metastatic RCC median time to deterioration was 5.6 months with cabozantinib (TKI and VEGFR inhibitor) compared to 1.9 months with everolimus (mTOR inhibitor) separately. In NSCLC bone metastases it was shown that ICB together with bone targeted therapy increased overall survival from 15.8 to 21.8 months[137], indicating a synergistic response. However, some of these new therapeutic approaches will require future retrospective analysis to evaluate their outcome and impact on survival rates. Approved investigation studies for immunotherapies in melanoma, prostate, breast and lung cancer patients that reports on bone metastasis has been estimated by others to roughly 1%[138].

Overall, bone metastasis carries a depressingly low future for patients. Two independent population based studies show the five-year incidence and survival of bone metastases by tumor type presented in table 1[139] with a low overall 5-year survival[106-108]. At time of diagnosis of solid tumors, the rate of bone metastasis is more than 20% in renal and lung cancer patients. Yet, prostate and breast cancers most commonly metastasize to the bone. And the shortest survival time among patients with bone metastasis are patients that initially manifests with cancer in the gastrointestinal tract[106].

Type of cancer	Percent of cases that metastasize after 5 years	5-year survival rate after metastasis
Prostate	24,5%	6%
Lung	12,4%	1%
Renal	8,4%	5%
Breast	6,0%	13%
GI	3,2%	3%

Table 1: Cancer types; ratio that metastasize and 5-year survival.

This thesis will look further into two forms of cancer that are prone to metastasize to the bone and have bone marrow involvement: RCC in adults and NB in children.

1.5 Renal cell carcinoma (RCC)

1.5.1 Kidney physiology

In the human body, the kidney plays an important role in maintaining homeostasis of solutes and fluids, as well as handling the secretion of components from the blood that the body does not want, as waste and acids, to therefore keep a correct water balance. The functional unit of the kidney is the nephron, constituting of the glomerulus, the place for blood filtration, and the collecting duct system draining urine towards the lower urinary tract. The resorptive activity in the kidney is mainly in the proximal tubule, predominantly contributing to the overall cellular arrangements in the kidney[140]. Proximal tubular cells are susceptible to toxic and ischemic injury; thus, their role is important during disease progression. They can acquire the capability to dedifferentiate, to further proliferate and substitute impaired epithelial cells. However, some cells do not succeed to redifferentiate, resulting in nonstop production of TGF- β, consequently promoting cancer or fibrosis[141].

1.5.2 RCC etiology

RCC is a group of malignancies arising from the epithelium of the proximal part in the renal tubules, where the tumor normally develops[142]. The most common histological subtype of RCC is clear cell RCC (ccRCC) and accounts to 75-80% of all RCC cases. The other renal cancers are papillary RCC (pRCC; 10-15%) and chromophobe RCC (5-10%)[140]. Approximately one-third of RCC patients have developed bone metastasis[143] of which ccRCC is most common. The 5-year survival of ccRCC bone metastasis is less than 10% compared to non-metastatic ccRCC with 75%[144].

1.5.3 Clear cell renal cell carcinoma (ccRCC)

Loss of chromosome 3p and second-hit loss-of-function mutation in *VHL* (Von Hippel-Lindau tumor suppressor; located in chromosome 3) often characterizes ccRCC, along with chromosomal loss of 14q and chromosomal gain of 5q[145]. *VHL* is responsible for degrading the transcription factor hypoxia-inducible factor (*HIF*), resulting in transcription of hypoxia-inducible genes as VEGF[146]. Hence, an increase of VEGF in the TME of ccRCC is a consequence of *VHL* protein inactivation. Receptors on tumors, immune, vascular endothelial and stromal cells interacting with VEGF, lead to multiple downstream effects as cell proliferation, tumor angiogenesis, cell migration and metabolism[42]. Other predominantly deactivated genes in ccRCC are *PBRM1* (polybromo 1), *BAP1* (BRCA-associated protein 1), *SETD2* (SET Domain Containing 2), and histone modifiers such as *UTX* (Ubiquitously transcribed tetratricopeptide repeat, X chromosome) and *ARID1a* (AT-Rich Interaction Domain 1A) and *KDM5a* (Lysine Demethylase 5A)[147]. Due to the varied genomic profile and complex chromosomal alterations along with somatic copy number alterations (SCNAs), patients with ccRCC

show different developmental outcomes[148]. A study of ccRCC reported that within distinct tumor subclones parallel development of mutations of the same pathways, or genes, occur. For instance, the same tumor could have mutations of *BAP1* and *SETD2*, but they could not coexist in the same tumor clone. Nevertheless, *VHL*-driven tumors at early ccRCC tumor progression, did not show developmental divergence compared with a single clone, implying that *VHL* may be the ancestor for the subsequent aberrations through disease advancement[149].

1.5.4 Treatment approaches in ccRCC and metastatic ccRCC

Partial or radical nephrectomy for patients with no signs of distant metastasis is still the standard procedure with curative intentions for localized ccRCC[150]. If evidence of concurrent establishment of micro metastases from the primary tumor, the surgical approach is considered to be effective for targeting initial steps of potential metastatic cascade as intravasation of tumor cells[150]. The treatment of metastatic ccRCC is not involving surgery, rather it includes various targeted therapeutics, including tyrosin kinases, mTOR signaling and checkpoint inhibitors.

1.5.4.1 Inhibitor of tyrosine kinases

The treatment of metastatic ccRCC have changed over the past decade. The spontaneous discovery of somatic *VHL* mutation in ccRCC[151], lead to development of agents aiming to prevent tumor angiogenesis by targeting circulating *VEGF* and its receptors. The principal target of *VEGFR*-targeting tyrosine kinase inhibitor (TKI) used in RCC is *VEGFR2*[152]. The *VEGF-VEGFR2* interaction on endothelial cells will stimulate a cascade of multiple downstream pathways, eventually resulting in tumor growth[153]. Early discovered inhibitor sunitinib, (inhibitor of both *VEGF* and *PDGFR*) showed significant progression free survival in metastatic ccRCC with 11 months compared to 5 months with the previously widely used interferon alfa[154].

1.5.4.2 Mammalian target of rapamycin inhibitor (mTOR)

There is a particular interest in using mTOR inhibitors in renal carcinomas due to its modulatory effects on the production of *HIF2α*, as well as controlling several signaling cascades involved in cell proliferation and survival. The upregulation of hypoxia-inducible genes, such as *VEGF* and erythropoietin, mediated by the transcription factor *HIF2α*, yields an important role of *HIF2α* in tumor survival and cell proliferation in hypoxic condition[155]. In normal conditions, the protein complex containing *VHL* degrades *HIF2α*, however, in almost 90% of ccRCC cases the *VHL* genes is lost[145]. The subsequent accumulation of *HIF1α* and *HIF2α* is due to inactive *VHL* further promoting RCC pathogenesis. The mTOR inhibitor everolimus was recently evaluated[156] showing prolonged progression-free survival (14.7 months) in combination with levantinib (*VEGFR* inhibitor).

1.5.4.3 Immune checkpoint inhibitors

Evolving understanding of immune infiltration in tumors has prompted research to investigate the status in metastatic ccRCC. In particular, T cells are highly infiltrating ccRCC tumors[157], hence therapeutic approaches have been modified by the addition of ICB as an option with antiangiogenic agents. Combinations were considerably effective with response rates between 42-71%[156,158-161]. Nevertheless, in the past decade treatments including immunotherapy with PD-1 monotherapy and combination of CTLA-4 and PD-1 inhibition have yielded efficient treatment results in metastatic ccRCC and non-metastatic ccRCC[158,162,163] with a median overall survival of 24 months. The development of immunotherapy shows significantly improved responses. In addition, recent trials points to a significantly improved progression free survival with dual use of antiangiogenic drugs with immunotherapy, highlighting the role of the TME and new strategical approaches in treating metastatic ccRCC[159,161,164].

Target	**Therapeutic agent**
Programmed cell death 1 protein (PD-1)	*Nivolumab[162], pembrolizumab[165]*
Programmed cell death ligand 1 (PD-L1)	*Avelumab[166], atezolizumab[164]*
Anti-cytotoxic T lymphocyte associated protein 4 (CTLA-4)	*Ipilimumab[158]*
Vascular endothelial growth factor (VEGF) inhibitor TKI	*Axitinib[167], sunitinib[154], pazopanib[168], cabozantinib[169], Lenvatinib[165], bevacizumab[170]*
Mammalian (mechanistic) target of rapamycin (mTOR) inhibitor	*Everolimus[171]*
Multi protein kinase; VEGFR, PDGFR	*Sorafenib[172]*

Table 2: Therapeutic agents in RCC.

With emerging technologies crucial gaps of knowledge are unraveled in ccRCC, as for instance the cell-of-origin, reprogramming of immune cells after treatment, and immune characterizations of untreated RCC patients[157,173,174]. By colonizing in multiple sites, through lymphatic and hematogenous paths, we know that ccRCC forms metastasis in the bone, brain, liver, lung, pancreas, muscle and skin[175]. Metastatic ccRCC is therefore a

promising disease model for studying metastatic development, besides understanding the biological and cellular mechanisms beyond chromosomal and genetic implications.

1.6 Neuroblastoma

NB is a pediatric solid tumor deriving from cells within the neural crest. The majority of NB cases are diagnosed during infancy, as approximately 90% of all cases are detected within the first 5 years of life. The median age at diagnosis is 18 months[176]. The disease frequently metastasizes to secondary sites. Usual sites of NB metastasis include the BM, bone, lymph nodes, skin and the liver. Patients with metastasis account for 50% of all NB patients at diagnosis where 70% have BM involvement[177].

1.6.1 Etiology

Neuroblastoma is known for the amplification (detected as multiple gene copies in unfavorable tumor cells) and increased expression of *N-myc* (*MYCN; v-myc myelocytomatosis viral related oncogene, neuroblastoma derived*) oncogene. In the early 80's oncogene amplification was discovered, and subsequently the preeminent transcriptional regulator of cellular differentiation, growth and metabolism in human NB[178,179]. Experimental investigations have relied enormously on *MYCN* when using a mouse model that overexpresses human *MYCN* in murine neural crest cells, driven by rat tyrosine hydroxylase (*TH*) promoter. This model was engineered with spontaneous recapitulation of the human disease for research purposes[180], resembling human NB characteristics regarding genetic modifications[181,182], pathology[183] and microRNA expression profile[184]. Currently this transgenic mouse model (*tgTh: MYCN*) is the most widely used preclinical *in vivo* model to study NB disease manifestation, biology and pharmacological approaches[180,185].

The *MYC* family, of which *MYCN* belongs to, comprises transcriptional factors that are crucial for cell proliferation and cell growth. In cancer, oncogenic *MYC* tend to be a central conductor of metabolic processes, and consequent gene expressions generate increased levels of adenosine 5′-triphosphate (ATP, human body's energy supplier) and key cellular compartments, supporting unrestrained cell proliferation[186]. In NB, an amplification of the *MYCN* oncogene indicates a poor clinical prognosis, detected in approximately 20% of NB cases, a group identified with aggressive high-risk NB. Alongside, segmental chromosomal aberrations as 1p-or 11q-deletion and 17q-gain are also presented in high-risk NB patients[187-189]. These heterogenous disease characteristics[190,191] challenges the therapeutic approaches in high-risk NB and consequently, treatment resistance occurs. In theory, arising treatment resistance evolves where subsets of tumor cells evade treatment, or mechanisms unrelated to genetics, namely transcriptional reprogramming[192].

1.6.2 Transcriptional heterogeneity in NB

NB is thought to be initiated during early embryonal development in neural crest cells, whose major derivatives are the sympathoadrenal cells. These precursor cells differentiate into, for instance, mesenchymal cells, Schwann cells, chromaffin cells, sympathetic neurons or fibroblasts. Some argue that along the differentiation route, cells become abnormal, resulting in unsuccessful differentiation and further development of NB. Nevertheless, the ancestor cell in NB is yet a riddle.

During the past decade novel single cell transcriptomic technologies has uncovered the complexity of the disease, arguing for transdifferentiation among cells of the sympathetic lineage[193,194]. Unraveling two neuronal phenotypes, the lineage committed adrenergic cells (ADR) and undifferentiated mesenchymal cells (MES), assists to link exclusive patterns of gene expression associated with epigenetic programs. Cells with ADR phenotype tend to differentiate along the sympathetic noradrenergic lineage, whilst MES cells are undifferentiated MES-like cells or similar to the neural crest cells. Both states cohabit in patient tumors and are consistently observed in established cell lines, the difference being that the undifferentiated MES state show resistance to therapy[195-201]. Nonetheless, there are remaining conflicts regarding existence of MES cells in patients and experimental models since it has been puzzling to demonstrate that MES cells are in fact involved in NB treatment resistance, beyond *in vitro* cell culture studies[196,202]. Identifying the correct mechanisms underlying treatment resistance in high-risk NB, due to heterogeneity across and within patients, remains to be elucidated.

1.6.3 NB staging

NB is a highly heterogenous disease, both in clinical and biological aspects and it has for a long time been understood that clinical stage is important for prognosis, in particular metastatic spread beyond the primary tumor[203]. The International Neuroblastoma Risk Group (INRG) created a clinico-biological risk classification system of NB to promote a common language to simplify treatment options and comparisons. For that purpose, a new staging system was defined categorizing the disease into four different stages based on primary disease features (L1 and L2, Table 3) and metastatic spread (M and MS, Table 3)[187,204].

Stage	Disease pattern
L1	Tumor is solely in the local area and no image defined risk factors for surgical removal are detected.
L2	Tumor is local and close to nearby tissues; some image defined risk factors are detected
M	Tumor has spread to other parts of the body except of areas mentioned in MS below.
MS	Tumor has spread only to skin, liver and/or bone marrow (less than 10 % bone marrow involvement) in patients younger than 18 months.

Table 3: Staging of NB.

The gradings in the INRG risk classification system are based on clinical stage (Table 3), grading of tumor differentiation and biological characteristics of *MYCN*- status, histology, 11q abnormalities and ploidy[187]. Lately, the INRG classification was revised adding alteration in chromosome 1p as a biological risk marker and improved the risk factor arrangements in the previously established groups; Low risk, Intermediate risk and High risk. Finally, the modified risk classifier accounts for further age spectrum of patients younger than five years old and older than five years[205]. Despite increasing survival in NB, the total mortality remains between 20-35% for advanced disease. Low-and-intermediate risk NB has a median overall survival of 95%, whereas metastatic high-risk NB has only 40-60% overall survival. *MYCN*-amplified NB are generally more aggressive than non-*MYCN* amplified NB.

1.6.4 Therapeutic approaches in high-risk NB

Treatment for patients with high-risk NB incorporates a strong multimodal schedule encompassing intense induction chemotherapy, surgery, radiation, high dose chemotherapy, stem cell transplant and in some cases immunotherapy and targeted therapies. The International Society of Pediatric Oncology – European Neuroblastoma group (SIOPEN) established a treatment program for high-risk NB patients, including rapid COJEC that is an induction therapy including high doses of five chemotherapeutic drugs (carboplatin, cisplatin, etoposide, cyclophosphamide and vincristine)[206]. Yet, resistance occur, and unfortunately patients that primarily respond to treatment may relapse due to therapy resistant disease. The overall survival for these resistant/relapsing patients is much lower than 50%[205].

1.6.5 Differentiation therapy

In general, cancers driven by *MYC* and *MYCN* with uncontrolled growth often relies on metabolic pathways. For instance, biosynthetic pathways activated by cancer cells involves purine and pyrimidine, as part of the *de novo pyrimidine synthesis*, to yield nucleotides needed for the fast-proliferating cells[207]. Metabolites of this pathway has been observed in NB, for example the purine byproduct hypoxanthine and the pyrimidines 2-deoxycytidine and cytidine[208]. Various approaches have intended to investigate differentiation in NB since it is considered to be the reason behind disease progression and resistance to therapy[209].

It was by the end of 1970s that multiple agents and signaling molecules, including cytokines, retinoic acid (RA), sodium butyrate and cAMP (cyclic adenosine monophosphate) showed evidence of terminal differentiation in embryonic carcinomas, acute myeloid leukemia (AML) and NB. The successes of differentiation therapy are harbored by the early discovery when treating acute promyelocityc leukemia (APL) with RA. It is a subgroup of AML accounting for 10-15% of the cases[210]. Retinoic acid had long been contemplated as the breakthrough of differentiation therapy. The movement from mortality to vitality on the differentiation avenue had finally reached a milestone by curing patients. Thereafter setting the successful hallmark of differentiation therapy[211], where in APL the 2-year event free survival of 97% when all trans retinoic acid (ATRA) was combined with arsenic oxide compared when ATRA was combine with chemotherapy (86%)[212]. In AML 52% had complete remission compared to without ATRA (39%) in elderly patients[213]. These results pioneered the idea of inducing terminal differentiation in cancer cells for clinical significance.

1.6.5.1 Dihydroorotate dehydrogenase (DHODH)

It was as early as in the 1980s that brequinar, a *DHODH* inhibitor, was first introduced as an agent against cancer[214]. The enzyme *DHODH* is positioned in the mitochondrial inner membrane (Figure 4). It is a universal enzyme and deprivation of *DHODH* activity would result in dysfunctional metabolism. Yet it is not understood if it can undergo mutations or overexpression in cancer patients. The following decade of the 1980s, several phase II clinical trials investigated the possibilities to target *DHODH* in solid tumor with a potent specific inhibitor, brequinar[215–219]. Unfortunately, results were disappointing. Today, a *DHODH* inhibitor, leflunomide, is approved to treat rheumatoid arthritis.

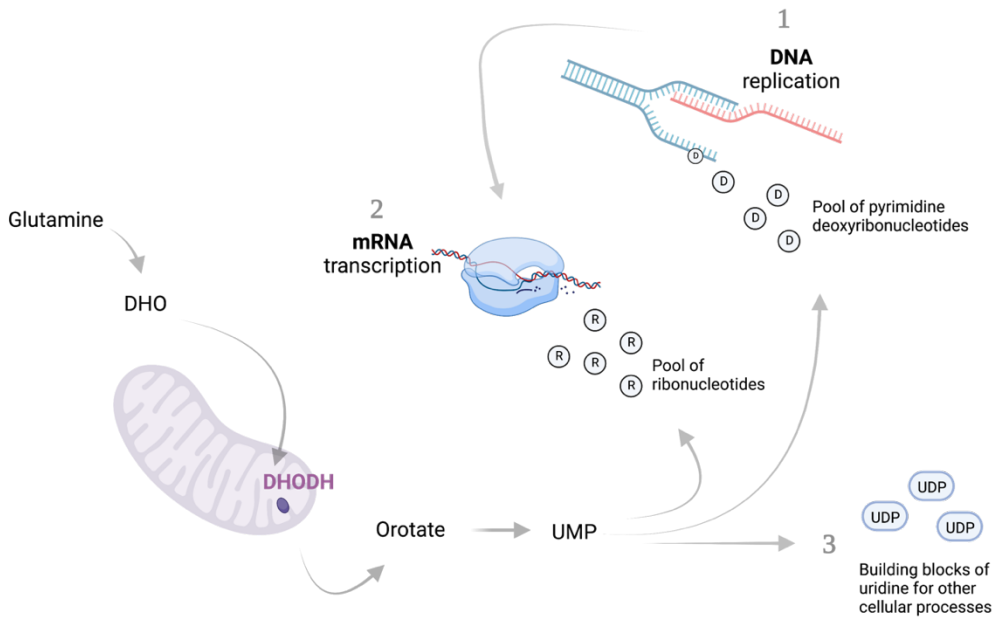

Figure 4: Illustration of parts of the *de novo* pyrimidine synthesis where DHODH action is critical for the formation of deoxyribonucleotides, ribonucleotides and other important components involved in metabolic pathways. Created by A.M.A in Biorender.com. Inspirational source[220].

Nevertheless, *DHODH* inhibition had demonstrated neural crest developmental blockade and reduced melanoma progression *in vivo*[221], and treatment of multiple myeloma[222]. This is specifically interesting since NB and melanoma are derived from neural crests. Recently, the concept of differentiation therapy in AML was revisited. With a large high-throughput chemical screening, *DHODH* inhibition induced differentiation in AML models *in vivo* and *in vitro* without harming normal cells[223]. This study showed similar effects as RA treatments, in addition, a consistency with early studies observing that DNA synthesis inhibition support differentiation in leukemia cell lines[224]. Nevertheless, there are precautions for using RA in therapy due to its toxicity on healthy cells increasing diverse side effects. Long-term usage may cause liver damage and other serious side effects.

In solid tumors the notion of differentiation is more challenging in contrast to leukemia due to multiple complex oncogenic circuits. Insights of RA in NB taught us that RA induces functional (acetylcholine esterase activity) and morphological changes as formation of vesicles, dendrite growth and growth arrest in both primary NB patient cells and cell lines[225,226]. As *MYCN* is, in many cases, amplified in NB, showed decreased expression when RA was administrated. The agent aided cells to progress from a stem-cell state to a mature neuronal state obtaining classical neuronal characteristics[227]. Today, in high-risk NB, RA is mainly used after high-dose chemotherapy and stem cell transplant, due to its efficiency in targeting minimal residual disease. High-risk NB patients receiving RA had a better 3-year event free survival of 46% compared to 29% for non-recipients. Similarly, when RA was used as continuation therapy the 3-year

event free survival was 55% compared with 18% without[228,229]. Therefore, the interest in reintroducing differentiation therapy in high-risk NB with a specific *DHODH* inhibitor, brequinar, is up for debate.

1.7 Single cell RNA sequencing

The saga of single-cell transcriptomics relates back to the early 90's primitive methods, when firstly used on small gene quantities[230,231]. Complementary DNA (cDNA) of a single cell was further expanded with amplification strategies such as *in vitro* transcription and PCR[232-235], to adapt to the single-cell RNA-sequencing (scRNA-seq) approaches. Plotting genetic structures to phenotype has been a lasting biological hurdle in medicine, and to uncover this, is by transcriptome analysis. It was in 2009 the first *bona fide* experiment of single cell transcriptomics was performed. Built on next-generation sequencing platform it was possible to characterize cells from early development[236]. This breakthrough brought science into the new era of discoveries. Researchers were prompted to pursue universal high-resolution analyses of single cell heterogeneity to uncover human biological paradoxes, especially in cancer. Critical assessment of the diversity of gene expressions among individual cells were prioritized. Finding and characterizing cells within a population would advance our understanding of drug resistance and relapse in cancer treatment[237]. Certainly, it did! The technology has provided revolutionary understanding in cancer as finding the origin of ccRCC[173], unraveling potential vulnerabilities to target in prostate bone metastasis[238] or elucidating transcriptional states in NB[196]. Parallel profiling of a large number of cells has been the driving force of the single cell transcriptomics revolution.

The revolution progressed fast during the decade following the discovery of Tang and colleagues[236]. Compared to bulk RNA sequencing technologies, scRNA-seq allows sequencing for the profiling of gene expression at the single cell level, and a much higher resolution than bulk RNA-sequencing[239]. Protocols were developed such as SMART-seq/SMART-seq2, differing in their amplification technology, sequencing length and transcript handling, as well as automatization of liquid handling in plates[240]. Compared to Smart-seq, the basis of many scRNA-seq techniques builds on inDrop/Drop-seq strategy that enable profiling of gene expression of thousands individual cells. The idea builds on using a microfluidic device to encapsulate single cells through oil droplet formation and simultaneously barcode the transcript. After the consecutive steps of cDNA synthesis, amplification and sequencing, the resulting reads can be assigned back to their originating cells based on the barcode[239]. The possibility to sample tens of thousands of cells at the same time, detecting rare transcripts, favors scRNA-seq over Smart-seq, including a much more reduced cost. The commercial *10X Single-Cell Chromium Solution* used in this thesis is based on the inDrop/Drop-seq technology.

The scRNA-seq technologies is still transforming the biological field and offers opportunities to study cell populations and individual cells existing in a tissue, in particular a tumor tissue. The processing of scRNA-seq experiments comprises of four steps[241]:

1) Isolation of single cells, often through enzymatic dissociation of tissue into a cell suspension, that are then captured as single cells. Often either through FACS sorting into plates or through encapsulation through oil emulsion droplets,
2) Barcoding and reverse transcription; single cells are lysed after isolation, where RNA inside each individual cell is converted to cDNA via reverse transcription,
3) cDNA amplification; where the cDNA is copied into multiple 10-20 extra copies through PCR
4) Finally, library construction and sequencing

Thus, there is an increased sensitivity and accuracy of scRNA-seq, in particular due to the reverse transcription and cDNA amplification. To prevent bias from PCR, the barcoded unique molecular identifier (UMI) of 6-8 base-pair is introduced barcoding each individual mRNA molecule within a cell during transcription. This effective reduction of PCR bias enables the same UMI to be tagged on PCR-duplicates arising from sequencing reads[242].

1.7.1 Managing the magnum of data

To integrate the large genomic data readouts produced from different molecules (RNA, DNA and protein)[243], computational methods improved rapidly alongside the first single cell transcriptomic dataset. This included low-level processing, quality control and data interpretation[244]. By dissecting the complex regulatory and cell-to-cell communication networks influencing cell identity, it has uncovered marker genes and identified developmental trajectories that relate to individual cells. The innovation of multi-omics catalyzes new computational systems simultaneously[243]. The joint pursuit of mapping cells in human tissue, building a "Google map" of the human body, aims to generate a global fundamental knowledge of each human tissue studied. Alongside, computational methodologies that have enabled researchers to approach the complex data is via pathway and gene set overdispersion analysis (PAGODA)[245], and conos[246] to overcome overlapping heterogeneities when multiple samples are combined. By further combining data with other aspects of single-cell gene expression dynamics, as RNA velocity[247], it enables to fully explore and exploit the granularity that usually exist in single-cell level expression measurements.

1.7.2 Continuous single cell transcriptomic method development

Nevertheless, to advance flexible and widespread sequencing technologies, single cell transcriptomic is integrated in many fields. Other technological combinations aim to

perform dual sequencing of the whole genome and mRNA in single cells, so called direct nuclear tagmentation and RNA sequencing (DNTR-seq). By using nuclease-free protein-DNA complexes to fragment the genome, RNA-seq is being used to sequence the transcriptome. Hence, it captures and sequences both transcriptomic and epigenomic information from single cells, providing a comprehensive view of gene expression and epigenetic regulation in a single experiment. Moreover, allowing for identification of changes in gene expression between cell states and novel regulatory mechanisms[248]. Novel strategies as cellular indexing of transcriptomes and epitopes (CITE-seq) uses measurements of gene expression and cell surface protein abundance simultaneously in one single cells. It combines the power of scRNA-seq with antibody-based proteomics, allowing researchers to analyze both the transcriptome and proteome of individual cells in a single experiment. In addition to UMI, oligonucleotide-labeled antibodies are used to bond with cell surface protein antibodies, further determining the gene expression levels of cell proteins[249]. In contrary, by adding a layer of spatial information to single cell technology, it enables in-depth understanding of the TME with slide-seq. Slide-seq and high-definition spatial transcriptomics (HDST) allow for more than 10 000 measurements of high-complexity, single cell and sub cellular detection[250,251]. This provides a comprehensive and quantitative measurement of the spatial distribution of gene expression in an entire tissue or organ. By transferring RNA from tissue fragments into a surface covered in DNA barcoded beads with known positions, allows the sites of the RNA to be inferred by sequencing.

During the rise of single cell transcriptomics, cancer researchers focused mainly on genetic alterations and cellular heterogeneity. It became evident that the influence of inherited epigenetic modifications also contributed to tumor development, a critical point for heterogeneity. Epigenetic changes in chromatin tissue in an individual cell was clearly revealed by linking it to scRNA-seq[252,253] with single cell assays for transposase-accessible chromatin with sequencing (scATAC-seq). This method is used to analyze open chromatin landscape at single-cell resolution, by combining high-throughput single cell sequencing with Tn5 transposase to capture open chromatin regions, granting researchers to study the epigenetic landscape at a cellular level. The method is a powerful tool for profiling chromatin accessibility at single cell resolution. It can detect subtle changes including coding and non-coding regions of the genome. It can also be used to identify regulatory elements and can be used to study transcriptional regulation and cell fate decisions[252].

The methodological advancements and refinements in single cell transcriptomics have assisted researchers to answer many challenging questions and consistently moves science forward. Particularly in cancer research. A plethora of new information is being added and shared worldwide. Prevalent findings in scRNA-seq unravel alterations of stromal and immune dynamics, particularly an immunosuppressive and pro-tumoral

microenvironment[238,254-256]. An excess of transcriptomic analyses offers knowledge about metastasis-associated genes, that can possibly serve as diagnostic biomarkers, to further predict patient prognosis. These possibilities untangle the complexities in cancer progression and how it is a crucial tool to excel the development of cures[257-259].

2 RESEARCH AIMS

The thesis focuses on identifying and targeting vulnerabilities in cancers that metastasize to the bone and the bone marrow. The overall aims are to provide insights of the molecular and cellular processes that are enhancing the processes of which a primary tumor spread to distant sites, and how to target an advanced primary tumor that is resistant to treatment.

The project specific aims are:

Paper I: Elucidating specific communication patterns in the tumor microenvironment of primary ccRCC to expand our understanding of cancer progression and carcinogenesis.

Paper II: Aiming to characterize tumor tissue of bone metastatic ccRCC patients, and untangle the metastatic microenvironment by including the comparison of malignant samples with immune and stroma cells from normal bone marrow.

Paper III: By targeting and inhibiting DHODH in a combinatory setting with the standard care therapeutic temozolomide, we aim to investigate their combined curative potential for treatment of high-risk NB. Here we use a transgenic NB mouse model.

3 MATERIAL AND METHODS

The intention of this section is to lay out a synopsis reflecting over materials and methods used in this thesis, and provide ethical considerations regarding studies involving animal and human material. Precise methodological details are elaborated in respective publications and manuscript. Hence, I chose to emphasize important research methods that were pivotal for the general research aims in this thesis, and reflect about strengths and limitations with the strategies applied.

3.1 Ethical considerations

In **Paper I** and **II**, we received tumor tissue from kidney, normal adjacent kidney tissue, bone tissues and bone marrow aspirates from patients diagnosed with primary ccRCC and bone metastatic ccRCC. Bone marrow aspirates were also received from patients that had undergone hip replacement surgeries to serve as control. Each study comprising human materials was permitted by the accountable institutional board, including informed consent from patients. Furthermore, to collect and work with patient samples, ethical permits were necessitated. We were fully responsible to guarantee that the appropriate investigational work was conducted on the samples, and ensuring respect to the patient's autonomy during the handling of human material. Similarly, we respect the value of the animal, and refine experiments to reduce number of mice used to yield enough data to answer the research questions pursued in **Paper III**. Despite the advancement of *in vitro* modeling for cancer research, resembling the real multicellular compartments comprising human physiology remain fatigue. Therefore, they do not yet optimize our understanding of pharmacodynamic and pharmacokinetic events of potential drugs. Hence, the use of animal model portrays a sustainable approach towards drug development, for now. Furthermore, animal work is justified with ethical permits, and progression of our work with animals is heavily regulated with guidelines provided from the institutions and national laws to certify animal health.

Our research purpose is to seal the gap of knowledge with comprehensive understandings, robust clinical relevance and ultimately significant evidence for new hypotheses in diagnosing and treating cancer patients. Lastly, by publishing our work in scientific journals with open access enables the research community to take part of and benefit from our findings.

For the perspective of both human and animal, ethical and moral decisions made within research, are based on the general principle applied, that it is for the benefit of our future patients.

3.2 Sequencing of material

3.2.1 Tissue processing

In **Paper I** and **II** we used scRNA-seq, a powerful tool for profiling gene expression at the single cell level, offering great insight into the diversity of cell types and their states within a tissue or organism. However, it requires tissue to be in purified liquidous single cells before the subsequent transcription and amplifications phases. Tissue and DNA structures can easily be affected and destructed. In both theory and in practice, the processing of tissue is a process where risk of pure quality and quantity of cells decreases at every step. From the mechanical handling of the tissue, transferring the tissue, dissociating, lysing, sorting by flow cytometry. The risk of decreased number of cells can affect the end result. In both projects, for the bone tissue, kidney tumor and normal kidney tissue we use media together with collagenases I-IV to digest the connective components in tissue samples to liberate individual cells, and assistance with dispase in order to assist the dissociation. We also pre-warm the media in order to liberate the cells with minimal damage. Nevertheless, before sending the samples for sequencing and after completed experiment, we performed quality control of the final library to ensure input for sequencing.

3.2.2 Single cell RNA sequencing

Allowing profiling of gene expression at the single cell level, with a much higher resolution than bulk RNA-sequencing, scRNA-seq provides a way to study the heterogeneity of gene expression between cells, which can reveal important insights into the complexity of cellular biology. It enables identification of rare cell populations that are not easily detected in bulk RNA sequencing experiments. It is highly reproducible and enables detection of subtle gene expression differences, studying cell-cell interactions, and elucidate the underlying regulatory networks that control gene expression. In **Paper I** and **II** we map out the transcriptomes of individual cells, providing insights into the roles of different cell types in complex biological processes. Despite its excellence to answer the research questions for identification of cell types, depending on the end-goal of the research question, there are some limitations in scRNA-seq that are important to acknowledge when designing projects. Compared with bulk RNA-sequencing, sc-RNA-seq limitations includes low sequencing depth, hence limiting the accuracy of gene expression measurements, making it difficult to detect low abundance transcripts. There is a higher risk of technical noise, amplification bias and confounding effects from the environment. It is also very costly and demand experienced personal and special equipment. Despite, research goals, tissue analyzed or experimental setup, using scRNA-seq comes with a lot of challenges that are all rooted in data science.

3.2.3 Data science: Computational integration and analysis of magnum data

3.2.3.1 Quality control

When we pool samples before sequencing and when we combine the large datasets, a common error is the introduction of batch effects and noise. We want to ensure reliable results and it begins with confirming that only one cell exist in each oil droplet. There is a risk for doublets, multiplets, empty cells or dead/destroyed cells. To minimize the effect on the results one needs to filter them out. We look at number of expressed genes in a cell, number of transcripts in a cell and percentage of mitochondrial transcripts to filter out data. To detect them we looked at the gene counts that are larger than a set threshold. Usually if there are more than one cell in a droplet, we see a larger gene count. If the droplet is empty, no cells, then the gene count is very low. And if the cell is broken or dead in the droplet, we would see a lot of mitochondrial transcripts, since they have a different structure. Usually, the mitochondrial transcript proportion would be larger than 5% to interfere with the results. Furthermore, low quality cells can contain a large amount of noise or incorrect data that can bias downstream analytical results, and later incorrect conclusions. Removing them is important for reducing the chance of obtaining inaccurate or misleading results from single cell data analysis.

3.2.3.2 Data integration

To create a comprehensive picture of gene expression across cell types and conditions, multiple scRNA-seq experimental data are combined. To be able to identify subcellular populations in a large set of data, principal component analysis (PCA) was used along with conos[246]. PCA is a statistical technique used to reduce the dimensionality of data by transforming a large set of variables into a smaller set or uncorrelated variables. It helps to identify patterns in data, detect outliers and perform predictive analysis to identify the main factors explaining the underlying variance in a dataset. To further be able to integrate such huge and heterogenous datasets, we also use conos. It provides a platform to integrate scRNA-seq data from different sequencing platforms, allowing for the consolidation of data across different platforms. This helps to reduce the need for additional data processing and analysis. At the same time, it enables identification of recurrent cellular subpopulations by relying on multiple possible inter-sample mappings to generate a global graph linking all measured cells. Conos also allow users to easily explore the transcriptome of individual cells, identify cell types and subtypes, discover gene expression patterns and identify genes associated with patterns of expression.

3.2.3.3 Differential expression analysis

Previously, statistical methods have measured gene expression by looking at the mean difference, hence the expression of a gene is consistent through all populations. Or by variability, which presume that genes in one population are very similar, whereas expression levels vary a lot in another population ranging high to low. Nevertheless,

solely single cell measurements can find and assume the differences between populations. In both **Paper I** and **II** we find differentially expressed genes (DEG) and transcripts among cell populations. By looking at the gene expression differences between normal and disease states, we find genes as *TREM2* in macrophages and *FAP* in fibroblasts expressed in malignant states and not in normal conditions. To avoid batch effects on DEG, we perform pseudo-bulk DE analysis for the comparison between sample fractions (for example Tumor macrophages vs. Normal macrophages). Each sample will form a batch which will limit the potential bias. Recently Jordan *et. al* benchmarked different single cell DE methods and demonstrated Pseudobulk methods outperform common and specialized single-cell DE methods[260]. Pseudo bulk involves pooling together cells from different sources and then running a single experiment on the pooled sample. This technique is useful when studying gene expression and other cellular activities, as it provides a more accurate representation of the overall makeup of the sample than analyzing each cell type or population separately. By looking at the gene expression differences across pseudotime, it enables to measure continuous alteration of expression in a cell population. The goal with the pseudotime approach is to infer the underlying dynamics of data and uncover the order of events that lead to the observed data. However, using pseudotime approach has limiting abilities as well, as it cannot measure the exact dynamics of cell-to-cell transitions, and it is still difficult to identify the exact biological processes that are driving the changes in pseudotime. In order to fully explore and exploit the granularity that usually exist in single cell level expression measurements, we use RNA velocity[247] to understand the single cell gene expression dynamics.

3.2.3.4 Alignment to reference datasets

Categorizing cells into cell types or states is crucial for diverse secondary analyses, which involves unsupervised strategies including manual annotation of the clusters by referencing to existing literature. Subsequently two main caveats are important to bear in mind: (1) Manual annotation is a time-consuming process, which in turn (2) limit the reproducibility of the results. The accessibility of reference atlases has resulted in consistent development of methods that one can manipulate through supervised classification of cell states and types for contextual understanding. Despite the active development of several atlases, the computational challenges are parallel paradoxes.

3.2.3.5 The Cancer Genome Atlas (TCGA)

The TCGA is a publicly available database comprising of 33 clinically diagnosed cancer types that are profiled with genomic and proteomic data. It is a collaborative effort of the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI). TCGA has played a crucial role in recent years with its easy availability to assist both clinicians for enhanced patient stratification, including researchers in their journey

for new biomarkers that may serve as prognostic markers for improved patient survival. The TCGA can be used to validate findings from scRNA-seq by comparing gene expression patterns from the scRNA-seq data to the gene expression patterns from TCGA. This comparison can help to validate the findings from scRNA-seq and ensure that the results are accurate and reliable. Additionally, TCGA can be used to identify potential biomarkers for a given disease or condition that may have been overlooked in the scRNA-seq data. This can help to further refine the results from the scRNA-seq data and provide more reliable insights about the underlying biology. We used TCGA to validate gene expression in disease stage and preformed survival analysis. Survival analysis can be used to validate findings from scRNA-seq by looking at the expression data of genes associated with survival outcomes and comparing it to the survival data from TCGA. However, mainly primary tumors that are untreated are registered in the TCGA database, and oftentimes patients receive neoadjuvant treatments, further limiting accessible sample size for eventual transcriptomic analysis. This is important to acknowledge due to bias that may interfere in the interpretations of prognostic information.

3.2.4 Animal studies

3.2.4.1 Transgenic TH-MYCN mouse model

The engineered transgenic mouse model that recapitulates the human disease, it is the closest animal model to clinical manifestation of NB. Resembling the human disease in its spontaneous development in the adrenal gland and its fully developed immune system prosper pharmacological investigations. It is an ideal *in vivo* platform for studying the role of *MYCN* in NB pathogenesis, and allows for studies of *MYCN* induced changes in gene expression, cell signaling and tumorigenesis. Weiss and colleagues incorporated the human *MYCN* in the genome, hence all mice that are homozygous for the gene, establishes a tumor at around five-six weeks of age. However, around half of the mice that are hemizygous has tumor manifestation raging between seven to twenty weeks of age. Nevertheless, this mouse model is limited in its ability to accurately represent the heterogeneity among human tumors, the complexity of human *MYCN* oncogene and associated phenotypes.

3.2.4.2 Xenograft mouse model

In comparison to the transgenic model, a fairly easier *in vivo* model is a xenograft model. With the advantage of selecting appropriately modified, or certain characteristic, cell lines of interest are subcutaneously injected in the flank of immunodeficient host species, further presenting a tumor in the flank. Due to the absence of an effective adaptive immune system, it will allow for extraneous species to grow locally in the flank facilitating the surveillance of tumor development for future measurements and analysis. The ability to control the experimental progression and choose characteristics of the

cell lines injected, allow better strategy and possibly reduced cohort size for the intended experiment. Further, permitting a better prediction on investigational time compared to the transgenic mouse model where sufficient number of mice might differ from time to time due to breeding issues arising. Nonetheless, xenograft models do not accurately reflect the complexity of the human body and the lack of immune system in the host organism, subsequently lead to incomplete results due to the lack of natural niches in the tumor microenvironment. Further developed *in vivo* versions of xenografts are the orthotopic xenograft model or patient-derived xenograft models (PDX) implanted closer to the anatomical tumor location[261].

3.2.5 Protein validation

Validating certain populations and proteins has been crucial in **Paper I–III**.

3.2.5.1 Flow cytometry

For **Paper I** and **II** we use flow cytometry to sort certain cell populations or a bulk of cell populations for later scRNA-seq analysis. It is a technology with high sensitivity, accuracy and reproducibility. It performs analysis on particles or single cells as they flow past one or multiple lasers while suspended in a, usually salt based, buffer solution, or a suitable media. In both projects cells are labeled with a fluorophore conjugated with an antibody of interest to bind to the cells. Further it can be analyzed for a visible light scatter and for single or multiple parameters. The detectable light scatter is measured in two directions of which one is at 90° (Side Scatter or SSC), indicating inner complexity or granularity of the cell. And the forward direction (Forward Scatter, or FSC) signifying the size of the cell. The light scatter is independent of fluorescence.

In both **Paper I** and **II** we used flow cytometry early in the project to purify our single cells from red blood cells and non-viable cells. However, in **Paper I** we further validated *TREM2* expressing macrophages and *PDCD1* expressing T cells in the tumors compared to normal, and in **Paper II** we isolated mesenchymal cells in the considered normal bone marrow to compare the bone marrow niche in healthy and malignant circumstances. Usually, we sorted for a minimum of 100 000 cells with flow cytometry to account for eventual losses of cells during sample preparation until sequencing. The method allows for high throughput analyses allowing for rapid analyses of large number of samples with automation. However, it has a hard time to detect low concentrations, challenging us during the validation of the T cell populations in **Paper I**.

3.2.5.2 Western blot

Since the mid 70's, western blot, also known as immune blotting, has had an immense impact on research progression. It has become one of the most standardized methods in laboratories and is suitable for detecting proteins in samples, in addition to protein quantification. The method relies on protein sizes that are separated through gel

electrophoresis for subsequent transfer to nitrocellulose or polyvinylidene difluoride membranes. Further enabling protein binding to a primary antibody that is selective for the protein, followed by visualization via a chemiluminescent, or fluorescent antibody tag, to quantify the protein. An advantage of this method is that it is very easy, cheap, detects low concentrations and can be easily taught to be independently performed. It can detect post-translational modifications such as phosphorylation, glycosylation, acetylation etc. yielding information about the active profile of a protein. This further facilitates detection and quantification of different co-existing proteins of two independent samples, compared to for instance immunohistochemistry or immunofluorescence. Due to its size-based separation, a range of proteins can be detected, hence altered isoforms of a protein can also be measured. Nevertheless, comprising of several steps before actual analysis errors are likely to occur. A limiting aspect is the risk of cross-reactivity, due to the use of antibodies, may result in misleading positive result. In the case of **Paper III**, mass spectrometry might have been a more suitable approach due to its high sensitivity. However, it is highly expensive and require qualified personnel to use the machine.

3.2.6 *In vitro* studies

Colossal reliance on cancer cell lines in research investigations led to several crucial findings for further assessment. The simplistic use by reproducibility of drug testing, changes and growth pattern detection when gene expression is manipulated, enables cheap and reliable analyses. Usually, they are derived from biopsy samples or surgically resected tumor material, and inherent a relative level of resistance to treatment. However, with emerging technologies and understandings of the complex tumor microenvironment the use of cell lines has more or less reached a *cul-de-sac*. Contaminations and infections are common, they can become genetically unstable over time, and the risk of losing physiological characteristics or developing new characteristics are feasible. Cell lines, normally a two-dimensional culture, risk to lose their properties when adhering to plastic, and their growth in flasks lack the tumor microenvironment. Additionally, they may not accurately represent the *in vivo* phenotype and may therefore lack the heterogeneity of the clinical outcome. It is still fundamental at early stages, however, to open the door and embrace the complex tumor biology, challenges and improved treatment development, sophisticated *in vitro* models are needed.

4 RESULTS & DISCUSSION

4.1 PAPER I

4.1.1 The metastatic signature

Similarities in transcription profile of tumor cells and normal nephron cells were visualized in the PT2 subpopulation. When validated in the first study published on single cell transcriptomic data of ccRCC revealing the cell of origin, our annotated PT1 and PT2 aligned with the study[173], hence sustaining consistency with our results. Moreover, the CNV alterations of the tumor samples, showed that one cluster (C4) was enriched in patients with advanced ccRCC and metastasis on multiple sites. This cluster also displayed the most CNV aberrations. Intriguingly, the transcriptional profile of C4 reveal an upregulation of four genes; *SAA1* (serum amyloid a1), *SAA2* (serum amyloid a2), *APOL1* (apolipoprotein 1) and *MET* (tyrosine-protein kinase Met) which were associated with poor prognosis in two independent RCC cohorts. Applying this signature expression pattern on our analysis of seven ccRCC bone metastasis cases in **Paper II**, demonstrated an upregulation when compared with primary tumor cells, further validating a reliable tumor metastatic signature. Together these genes are involved in multiple processes responsible for events in the metastatic cascade. For instance, *SAA1* has shown to predict overall survival in lung cancer[262] and correlated with advanced stage in colorectal cancer with increased migration and invasion[263,264], further negatively associated with survival[265]. More importantly, we show that *SAA1* and *APOL1* independently are associated with advances disease stage of ccRCC. On the contrary, *MET* has shown to be involved in cell survival, migration and proliferation[266] and is currently targetable with cabozantinib[267]. In progressed renal carcinoma, after *VEGFR* treatment, cabozantinib showed prolonged progression free survival compared with mTOR inhibitor. Nevertheless, the possibility to target the metastatic signature only by targeting *MET*, may not be the sole solution due to the multiple concomitant events in the TME. Ongoing trials are keener on multitargeting in untreated advanced ccRCC patients, such as recently a triple combination utilizing nivolumab (*PD-L1*), ipilimumab (*CTLA-4*) and cabozantinib (*MET* and *VEGFR2*), showing promising effects[268]. Conclusively, this distinct tumor cell cluster comprising of a transcriptomic program is associated with metastatic potential and poor survival[265].

4.1.2 ccRCC portrays a complex immunosuppressive microenvironment

Investigating the T and myeloid cells separately, we identify the cytotoxic lymphocyte subpopulation (CTL-1) that expresses exhaustion markers as *PDCD1*, *TOX*, *HAVCR2*, *LAG3* and *CTLA-4*, and T^{reg} immunosuppressive function. Along with a M2-phenotypic TAM population overexpressing *TREM2*. More studies are pointing to different subsets of T cells infiltrating ccRCC tumors with increased exhaustion profile as the disease

progresses[157,269]. Immunotherapies targeting *CTLA-4* and *PD-1* are approved for patients with advanced ccRCC with encouraging results of overall survival[158,160,162].

Nevertheless, our results with the overexpression of *TREM2* in the TAM phenotypic macrophages in the tumor (Macro-2) has been shown to be upregulated in malignant tumors, including ccRCC[270]. It is associated with resistance to immune therapy and demonstrating poorer survival outcomes[55]. However, targeting macrophages in cancer is difficult due to their plasticity and dynamic nature, as they are constantly changing in response to the TME[44,51]. Additionally, macrophages are often found in close proximity to other cells, making it difficult to accurately target them with traditional therapies. Inhibiting *TREM2* on tumor infiltrating macrophages suppressed tumor growth and enhanced checkpoint blocking therapy in preclinical studies[55]. The overexpression we see in our ccRCC data suggests that it could be a potential prognostic marker and as a biomarker to aid clinicians to detect patient in need of checkpoint blockade immunotherapy.

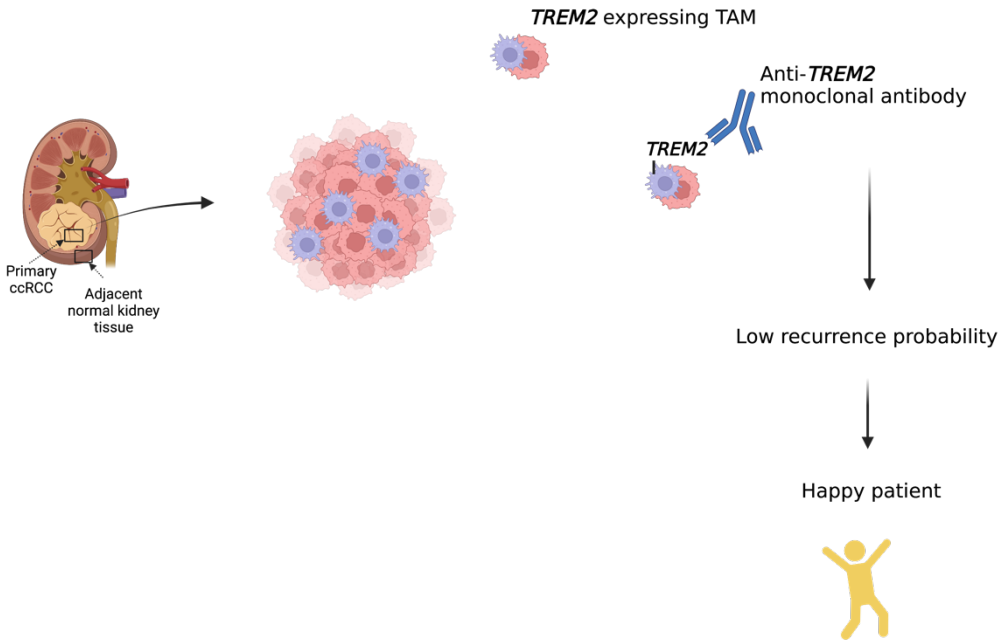

Figure 5: Suggested potential target of *TREM2*. Created by A.M.A with Biorender.com.

Furthermore, our ligand-receptor interaction analysis revealed that *TREM2* immunosuppressive macrophages (Macro-2) impacts T cell exhaustion via *C-X-C motif chemokine ligand 9/10 (CXCL9/CXCL10)* and *CXC-chemokine receptor 3 (CXCR3)* signaling[55], and vice versa. Indicating that if M2 macrophages are induced it would impair the responses of CTLs in the TME[271]. We show that this is the case in ccRCC as well, demonstrated by the increase of the exhaustion score of CTL-1 and the Marco-2 population.

Further ligand-receptor interaction analysis pinpoints certain cell types expressing particular genes and whom they communicate with. For instance, we found the axis of *CD70-CD27* highly interesting, since *CD70* (the ligand) is oftentimes overexpressed in solid tumors, predominantly in renal cancers (87%), although also in glioblastoma (42%), ovarian cancer (15%) and lung cancer (10%)[272]. Normally, *CD70* is found in T^{regs} and CD8+ T cells, and usually expressed after immune activation. Its receptor, *CD27*, is upregulated and circulated in the blood upon T cell stimulation, further serving as a diagnostic marker for T cell activation[273]. Provided that *CD27* is mainly found on exhausted T cells (CTL-1), and *CD70* in tumor tissue, with a correlation with T cell exhaustion, presumes that an upregulation of *CD70-CD27* interaction consequently led to T cell exhaustion. Currently clinical trials are recruiting patients to test *CD70*-targeted chimeric antigen receptor (CAR)-T cell therapy in solid tumors including RCC (NCT05420519, NCT05468190)[274]. Hence, it is yet an emerging therapeutic approach with limited proof of concept. In acute myeloid leukemia (AML) an engineered construct with *CD70* targeting CAR T showed antitumor effects both *in vivo* and *in vitro* without affecting the normal hematopoiesis, arguing for a possible therapeutic approach[275].

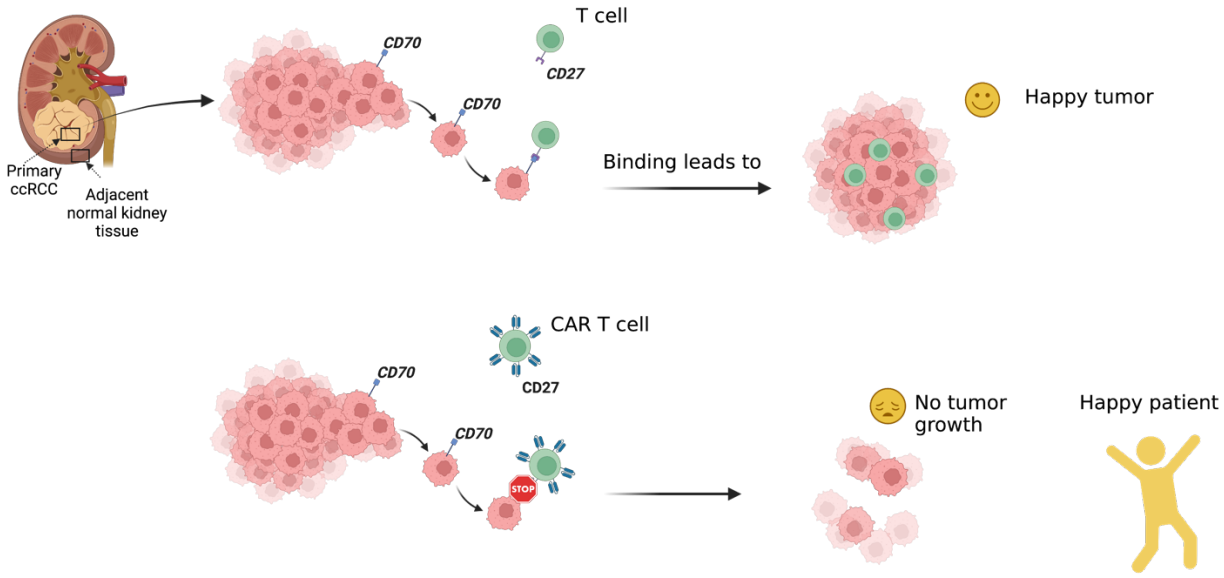

Figure 6: Suggested inhibition of *CD70-CD27* axis in primary ccRCC. Created by A.M.A with Biorender.com

4.1.3 Vasculature remodeling implicated in ccRCC progression

Deciphering the different stromal cell populations comparing normal kidney tissue and the malignant ccRCC tissue, we found endothelial, pericytes and fibroblasts. In turn, both endothelial and the pericytes displayed subpopulations, of which endo-1 displayed a phenotype that has been described as tumor associated endothelial cells (TECs). Genes that were upregulated in the TEC population was *PLVAP, CA2, SPARC, INSR, IGFBP7*[276]. With spatial validation we also observe that these genes in particular are enriched in tumor regions. Pericyte-1 was also enriched in the tumor, displaying a pro-inflammatory

capillary phenotype, compared to pericyte-2, that resembled vascular smooth muscles cells with less infiltration in the tumor region. Along with spatial validation of *RGS5, PDGFRB, THY1, ABCC9, AXL* in pericyte-1 we hypothesized that this pericyte population is an indication of an ongoing pro-inflammatory vascular remodeling process during ccRCC progression[277], moreover permitting invasion of tumor cells[6].

The mechanism of *VEGFR* TKI inhibitors in cancer are accepted for the idea that they target angiogenesis. The complete mechanism of the inhibition is not fully understood, but sunitinib showed to prevent capillary sprouting stimulated by *VEGF* and basic fibroblast growth factor in a vascular endothelium spheroid model[278,279]. Suggesting that tumor angiogenesis is disrupted by *VEGF* TKI inhibitor by blocking the capillary sprouting. Similar phenotype as we discovered in the pericyte-1, capillary pericytes. Named after their wrapping around capillaries enabling vessel sprouting via stimulation of endothelial tip cell formation, further promoting tumor angiogenesis and potential spread of tumor cells in the blood[277]. The Endo-1, displaying a TEC phenotype, called endothelial tip cells organize the sprouting of capillaries from pre-existing vessels and create new access points for tumor cells into the blood stream[276]. According to Folkman's theory; in the beginning the primary tumor and metastasis are avascular, then, when the tumor volume reaches over 1 mm^3 (10^5–10^6 cells) angiogenesis occurs supporting tumor growth[280]. Early micro metastases grow and migrate together with the pre-existing vasculature[281-283], suggesting that events involved in micro metastasis may not depend on angiogenesis. Evidence therefore suggests that *VEGFR* targeting TKIs would shatter the metastatic cascade, although monotherapy fails due to the consequent immune infiltration, as seen in hepatocellular carcinoma[284]. Further pointing to the hope of combination treatment.

Moreover, the microvasculature has shown to vary between organs. *In vitro* studies of the lung endothelium showed that mature vasculature was inhibited by *VEGFR2*, while in the liver endothelium by *VEGFR1*, further implying that angiogenesis in micro metastasis possibly varies depending on the tissue that the receptor lies in[285]. As mentioned earlier, the majority of the TKIs used in RCC therapy targets *VEGFR2*, although to a certain extend also *VEGFR1* and *VEGFR3*, and therefore the inhibition efficiency in a metastatic setting differs depending on the tissue.

In conclusion, it seems that there is a huge interactive signaling circuit in progressive ccRCC with multiple events occurring simultaneously. This may be the fundamental reason for the treatment challenges and how they vary from patient to patient.

4.2 PAPER II

As a continuation of **Paper I**, we were curious to understand the microenvironment in metastatic ccRCC to the bone and bone marrow. Studies at single cell resolution has unraveled profound insights of the tumor and immune microenvironment of primary and

advanced ccRCC[157,286]. However, single cell studies on bone metastatic ccRCC are lacking.

4.2.1 Immunosuppressive transcriptional states in ccRCC bone metastatic patients

Similar to primary ccRCC, in a bone metastatic setting, we found an immunosuppressive TME comprising of an enrichment of a M2-like phenotypic TAM macrophages expressing *TREM2* (macro-2) and an exhausted T cell profile. In advanced ccRCC *TREM2+* macrophages have been identified and has been linked with T cell exhaustion and anti-PD-1 resistance[55]. It has been shown that disruption or removal of TAMs reduces bone metastatic progression in prostate and breast cancer[238,287]. Compared to the TAM macrophages identified in **Paper I**, the composition of macrophages in bone metastasis also constituted with high levels of genes, such as *SPP1*, *CCL18* and *CXCL5*, that are involved in metastatic progression, tumor differentiation, bone formation and attachment of osteoclasts to the bone remodeling matrix[288-292]. This subset of macrophages was also associated with worse outcome.

Moreover, ligand-receptor interaction analysis implied a communication between *CCL18*, specific to macro-2, and *CCR8*, particularly expressed in T^{reg}, both upregulated in tumor. Within the tumor *CCR8+* T^{regs} has shown to have suppressive features[293]. In combination with the correlation of *CCL18* with CTL-3 exhaustion, we believe that the binding of *CCL18-CCR8* have a role in suppressing the immune responses in ccRCC bone metastasis. For instance, in breast cancer, *CCL18* has shown to promote metastasis[290]. Currently the first clinical trial aims to investigate anti-*CCR8* antibody given alone or in combination with pembrolizumab (*PD-1*) in patients with advanced solid tumors (NCT05537740). Pembrolizumab recently showed significantly longer overall survival, 79.2%, when combined with *VEGFs* inhibitor, levantinib, in advanced RCC compared to combination with levantinib plus everolimus (inhibiting mTOR; 66.1%) or only sunitinib (inhibiting *PDGFRs*, *VEGFRs*; 70.4%) after 24 months[156].

Considering a metastatic cascade and eventual growth of micro metastases, it remains unclear how immunotherapy affect intravasation or extravasation in the blood circulation, or how it influences tumor cell migration. In a metastatic melanoma mouse model, disseminated tumor cells were dormant, but could proceed with metastatic growth upon CD8+ T cell exhaustion[294]. Similarly, not much is known about the metastasis promoting function, although increased disease stages in a mouse model in colon cancer showed TAMs with elevated *PD1* expression levels[295]. Besides, in an osteosarcoma xenograft mouse model inhibition of *PD1* reprogrammed tumor infiltrating macrophages to a pro-inflammatory phenotype rather than immunosuppressive, resulting in regressed metastases in the lung[296]. Taken together, this suggest that inhibition of *PD1* could prevent metastatic niche formation caused by macrophages.

4.2.2 Bone marrow stroma from tumor samples is featured with tumor associated mesenchymal phenotypes (TA-MSC)

Similar to **Paper I**, we observe that malignant cells and proximal tubule cells are comparable. Both the primary and the metastatic disease show alterations in the transcriptional profile with significantly upregulated genes of ccRCC signature as *VEGFA*, *NDUFA4L2* and *PDK4*[286]. Indeed, the inferred CNV analysis on tumor cells from the bone metastatic patients show chromosomal losses with Chr3 in combination with loss of Chr9 and Chr14[148]. The observation of the high patient-to-patient variability, in particular in the metastatic patients, might explain the extensive transcriptional heterogeneity and complexity of metastatic tumors, furthermore explaining the challenges in targeting bone metastatic ccRCC.

A consistent EMT activation was observed[297,298], and we reveal that a certain MSC population acquire an EMT cell-state shift involved in promoting bone remodeling activity. MSCs are crucial in modulating the TME, and MSC-derived factors has shown to affect disease development in metastatic breast cancer[299], as well as prostate bone metastasis[300]. We first observed a stromal population induced by the tumor in the bone metastatic fraction characterizing the MSC subset, which was surprising, since it contrasted to previous findings of prostate bone metastases, where stromal cells were limited[238]. Our subset comprised of gene expression of *LEPR*, *NT5E*, *THY1* (*CD90*), *VCAM1* and *CXCL12*, constituents of the hematopoietic stem cell niche. Interestingly MSC-2 was augmented in bone metastasis with high EMT marker expression of *HTRA1*, *ITGA5* and *INHBA*[301,302] compared to MCS-1 that was seen in the normal BM stroma fraction. Strikingly, MSC-2 but not MSC-1, had a sustained expression of classical MSC markers *NT5E* and *THY1*, with a reduction of *VCAM1*, *LEPR* and *CXCL12*. A similar downregulation has been observed in bone marrow derived *Lepr+* MSCs in a leukemia mouse model[34]. In comparison with other stromal population and primary tumor cells, MSC-2 displayed the highest EMT signature score and EMT programs, as extracellular matrix and actin cytoskeleton organization, in bone met fractions. Hence, there is an elevated tumor invasion, motility, cell state plasticity and metastasis, which are part of the hallmarks of metastases[303,304].

This particular MSC-2 subset demonstrated disrupted cell adhesion capacity by high expression of *SPARC*[305], active ECM remodeling through expression of several collagen-associated genes as *COL6A2*, *COL3A1*, *COL4A1*, *COL4A2* together with promoted processes as tube morphogenesis, cell adhesion, collagen fibril organization and matrix organization[74]. These alterations proposes that this subpopulation, MSC-2, in ccRCC bone metastases resembles CAFs observed in other cancers[306]. Besides, the bone metastatic fraction constituted of a predominant expression of CAF markers *FAP*, *CD44* and *FN1*[307]. Previous studies in cancer have shown that CAFs can promote cancer cell proliferation and metastasis[308] by secreting *IL6*, *IL8* and *TGFB1*. Finally, MSC-2 also

showed an association with poor progression free and overall survival, suggesting that the CAF phenotype of MSCs is exclusive in metastatic BM, since they are not found in normal BM[309]. Thus, the alteration from MCS-1 to MSC-2 is complemented with EMT-like and CAF-like gene expression regulation correlated with survival. Therefore, we propose and name MCS-2 as tumor-associated mesenchymal stromal cells (TA-MSCs) with a possible tumor promoting capacity.

4.2.3 TA-MSC regulate bone remodeling in ccRCC bone metastasis

We found an extensive crosstalk among the different stromal populations. We also observed significant interactions between stromal and myeloid subsets comprising of interactions involved in bone remodeling as *RANKL–RANK*, *OSM–OSMR* and *VEGF–KDR*[310,311]. In the bone metastatic fraction, *RANKL* and *RANK* expression was upregulated in osteoclasts presenting enhanced gene expression associated with activation and differentiation (*CA2, TCIRG1, CLCN7, OSTM1 and ANXA2*)[312,313] . On the other hand, osteoblasts had a decreased expression of genes linked with osteoblast mineralization, proliferation and fibrous tissue integrity (*BMP4, ALPL, LRP5, BGLAP*), implying a disrupted osteoblast-mediated bone formation and induced bone resorption in bone metastatic ccRCC.

Furthermore, *OPG* (a decoy receptor antagonizing *RANK*) was decreased in the malignant TA-MSC compared to the benign fraction (Figure 7). This suggests that within the tumor there is an activation of the *RANKL–RANK* axis[310]. Our study points to the TA-MSC subpopulation as the source for *RANKL* and not the tumor itself. We further validated that *RANKL* expression does not exist in tumor cells from primary and metastatic ccRCC patients in **Paper I** and from Bi and colleagues[157]. Conclusively, the TA-MSC population acts as a main regulator of the bone remodeling detected in bone metastatic ccRCC patients via *RANKL–RANK/OPG* pathway, which is the major pathway promoting osteoclast-mediated bone resorption via osteoclast differentiation and maturation[314]

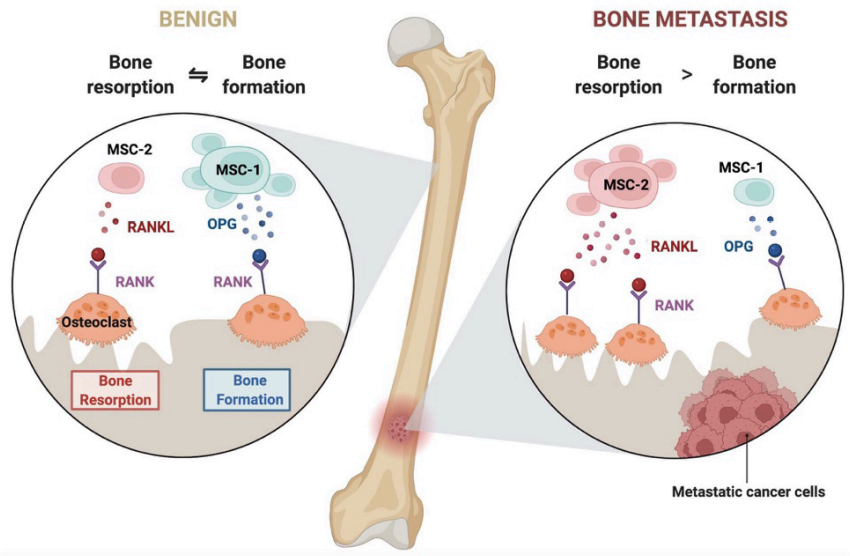

Figure 7: Bone remodeling in normal (left) and bone metastasis (right). Adopted from Paper II.

Another factor that independently can stimulate *RANKL* expression is Oncostatin M (*OSM*). When *OSM* is secreted by monocyte-derived macrophages, binding to its receptor *OSMR*, *RANKL* production is elevated[315]. We observed that *OSM* was significantly increased in Macro-2, but reduced in Macro-1 and Macro-3, while *OSMR* was expressed by TA-MSCs and tumor cells. This indicates that the existence of the *OSM-OSMR* interaction in bone metastatic ccRCC could possibly be an autonomous *RANKL*-stimulating pathway, further supporting an osteolytic microenvironment by abnormal osteoclast differentiation and formation in bone metastatic ccRCC. This is characteristic to osteolytic bone metastases[316]. It seems that cancer cells can manipulate some parts of the bone microenvironment for their growth, maintenance and homing.

Modulating immune cells, as previously mentioned with immune checkpoint blockade, has confirmed to be valuable in ccRCC[157]. Though our study also suggests that the targeting of stromal cells might also be an effective approach in ccRCC bone metastases.

4.3 PAPER III

4.3.1 NB cells with *DHODH* dependency result in worse outcome

With translational analysis on publicly available panel of cancer cell lines, we revealed three genes translating to the enzymes carbamoyl-phosphate synthetase 2 (*CAD*), *DHODH* and uridine monophosphate synthetase (*UMPS*) with strong correlation to each other. This indicated that cancer cell lines that were reliant on one enzyme of the *de novo pyrimidine* synthesis, was also relying on the other two, suggesting that cancer cells were counting on *UMP* production and pyrimidines. This included NB cell lines.

Compared to other solid tumor cell lines, *DHODH* was highly expressed in NB and rhabdoid tumors. Furthermore, in 2 independent cohorts of human NB, increased *DHODH* expression was significantly linked with poor survival across all disease stages. However, the expression levels of *DHODH* were utmost in high-risk and *MYCN*-amplified tumors. Additionally, the *DHODH* expression increased with the INSS staging system indicating its prevalence in aggressive NB. When analyzing publicly available NB patient cohorts by adjusting for known risk factors as age, disease stage, risk group and *MYCN* status, *DHODH* sustained as an independent risk factor correlating with worse overall survival. In particular, a subgroup of high-risk, non-*MYCN* amplified tumors with high *DHODH* expression had poor prognoses.

4.3.2 *MYCN* and *DHODH* dependency correlate with *DHODH* inhibition in preclinical NB models

When utilizing NB cell lines, *MYCN* amplified (SK-N-BE (2)C) and non-*MYCN*-amplified (SK-N-AS), a knockdown of *DHODH* and therapeutic inhibition of *DHODH* by the *DHODH* inhibitor brequinar, showed induced apoptosis. Similarly, treatment of brequinar *in vivo* induced caspase-3 activation in SK-N-BE (2)C xenograft tumors. The inhibition resulted in reduced intracellular nucleotide quantity, particularly in SK-N-BE (2)C cells, indicating a dependency on *de novo* synthesis. The pharmacological evaluation of brequinar *in vivo* in the xenograft mice injected with SK-N-BE (2)C or SK-N-AS cell lines showed considerably suppressed tumor growth in SK-N-BE (2)C xenografts and less noticeable effect in the SK-N-AS. After treatment termination the SK-N-BE (2)C xenograft model relapsed suggesting that the malignant cells were not completely eliminated or terminally differentiated. Similarly, tumor recurrence was observed after treatment discontinuation of brequinar in homozygous *TH-MYCN* NB mice. *In vivo*, brequinar reduced *MYCN/MYC* expression and *MYC* targets shown from GSEA of RNA-seq data from transgenic *TH-MYCN* and xenografts. Hence, *MYCN*-driven NB correlate with *DHODH* expression and are suggested to be less capable in rescuing pyrimidine, and rely more on *de novo pyrimidine* biosynthesis through *DHODH*.

4.3.3 ADRN cell state in NB is in particular targeted during DHODH inhibition

After the discoveries of the two malignant cell states that NB cell lines can exist in, we created an ADRN and MES score using the expression signatures described[195,196]. We observed that cell lines with high MES score displayed lower *DHODH* dependency. *In vitro*, SK-N-AS and SH-EP were also the most resistant to brequinar treatment. SH-EP is known to display MES phenotype and showed a similar sensitivity as non-malignant stromal MRC5 cell line. On the contrary, cell lines that were most dependent on *DHODH* had low MES score and high ADRN score. In SK-N-AS cells with high MES score, treatment with brequinar plateaued with increased dose without decreased cell viability, proposing that NB cells with MES phenotype are resistant to *DHODH* inhibition. Interestingly, SK-N-BE (2)C with the strongest ADRN phenotype was the most sensitive,

and the dependency of *MYCN* and *DHODH* correlated[195,196]. The sensitivity of ADRN tumor cells to *DHODH* inhibition may be the cause to the resulted imbalance of transcriptional states in the tumor tissue. Nevertheless, relapsed tumors showed increase of tumor cells expressing *VIM*, further implying on the increased MES phenotype, similar to previous studies[200]. An increase of transitioning cells was particularly observed in relapsed cases, which hypothetically could be due to brequinar effective killing of "pure" ADRN cells, subsequently a larger number of residual MES NB cells was presented.

4.3.4 Combining *DHODH* inhibition and temozolomide establishes therapeutic potential in *TH-MYCN* mouse model

Nevertheless, monotherapy of *DHODH* inhibition did not show curative potential *in vivo* in xenografts or in the transgenic *TH-MYCN* mouse model of NB. A combination of brequinar and temozolomide, a DNA alkylating agent frequently used in the treatment of NB[317], demonstrated a synergistic inhibitory effect *in vitro*. *In vivo* combinational treatment in tumor-bearing *TH-MYCN* mice with one dose of temozolomide and/or brequinar showed after 24 hours decreased proliferation and an increased apoptosis in the tumor tissue. The combination of temozolomide and brequinar resulted in significantly prolonged survival in 5 of 7 mice, even after 190 days of treatment discontinuation. Since temozolomide is already an approved agent and is clinically used for relapsed patient cases, the combination with *DHODH* provides a foundation for implementation in treatment strategies. Previously, pyrimidine reduction has been revealed to induce double-stranded breaks and DNA damage[318]. Therefore, the biological explanation for the synergistic effect observed could be pyrimidine reduction on DNA breakage and damage together with alkylation. *MYCN* appear to transcriptionally upregulate *DHODH* and enzymes of the pyrimidine synthesis by stimulating the production of nucleotides. Thus, the relationship between *MYC/MYCN* and *DHODH* appear to be bidirectional, where they all have a role in regulation of cell growth and proliferation. *DHODH* helps to regulate essential energy molecule ATP. *MYC* and *MYCN* are transcription factors that help regulate gene expression. *MYC* and *MYCN* directly activates the expression of genes involved in cell growth and proliferation, and *DHODH* helps to regulate the production of ATP needed for those processes. In turn, *MYC* can also indirectly regulate *DHODH* activity by affecting the expression of genes that control *DHODH* activity. Recently *MYC*-driven medulloblastoma has also highlighted the potential of *DHODH* inhibition as therapeutic strategy[319], possibly as monotherapy[320].

Finally, targeting and inhibiting *DHODH* in combination with temozolomide, an alkylating agent, demonstrate high efficiency in preclinical NB models. Therefore, high-risk NB patients may benefit by utilizing their metabolic dependencies by targeting *DHODH*. Conclusively, our data reveal that NB cell growth is mediated by *DHODH* with therapeutic impact, and serves as a potential candidate for clinical testing.

5 CONCLUSIONS

The presented thesis work elucidated vulnerable targets in primary ccRCC, ccRCC bone metastasis and NB. When their respective primary tumor advances it has the capability to metastasize to the bone and the bone marrow. These critical insights lay a solid foundation, in both diseases, for future researchers to exploit. We intended to complement and provide essential knowledge for adjusted or innovative therapeutic understanding and approach. Conclusively:

In **Paper I**, with scRNA-seq we dissect the TME of human ccRCC comprising of matched normal kidney tissue and tumor tissue. We unravel an utterly complex microenvironment with potential exploitation of a TAM population with M2 phenotype overexpressing *TREM2*, *CD70-CD27* axis and a metastatic signature constituting of four genes that could serve as predictive tool.

In **Paper II**, we built upon **Paper I** with comparative analysis of bone metastatic ccRCC compared with primary ccRCC and normal bone marrow. Similarly, we scrutinize the complicated TME and observe immunosuppressive characteristics including a distinct transcriptional TAM population and an increased exhaustion among T cells. A distinctive bone marrow stroma population from the tumor sample presented tumor associated mesenchymal phenotype (TA-MSC) supportive of EMT and a CAF phenotype. This particular stromal subpopulation, dysregulate *RANK/RANKL/OPG* signaling pathway consequently causing bone resorption.

Finally, in **Paper III**, we show that *DHODH* is an independent risk factor in high-risk NB and that *in vivo* inhibition of *DHODH* resulted in prolonged survival. When combined with an alkylating agent used in standard care of therapy in NB, it demonstrated curative potential.

6 FUTURE PERSPECTIVES

Metastasis is orchestrated through a number of complicated interactions involving malignant cells (the "seed") and the microenvironment (the "soil"). Targeting metastatic cascade is far more challenging than the ongoing debate regarding precision medicine, for now at least.

6.1 Identification of vulnerabilities in ccRCC and metastatic ccRCC

Looking at primary ccRCC and bone metastatic ccRCC, multiple concomitant biological events occur during disease progression, hence requiring multimodal treatment approaches. For instance, a recent study suggests a triple combination treatment in untreated advanced RCC by targeting *PD-1*, *CTLA-4*, *VEGFR2* and *MET*[268] with clinical effect. Principally, targeting four major aspects that are involved in the disease progression, as we also show in **Paper I** and **Paper II**; vascular remodeling, immunosuppression and MET overexpression.

When our immune system is working properly, it wants to kill cancer cells. That is their function. However, in the cases of primary ccRCC and bone metastatic ccRCC, the T cells are exhausted, allowing tumor cells to evade immune response. We suggest that in primary ccRCC *CD70-CD27* axis could be a potential target, possibly by the engineering of CAR-T cell therapy. With this approach, T cells are introduced with genetic information and are reprogrammed to produce special receptors, in this case it would be *CD27*, to bind to *CD70* on the ccRCC tumors. This is currently under investigation in clinical trials[274] (NCT05420519, NCT05468190).

Nevertheless, recent guidelines provided by the European Association of Urology[150], imply that partial nephrectomy still yields better quality of life when the disease is localized. However, metastatic RCC, including bone metastatic ccRCC, surgical approaches are not recommended since sunitinib (TKI blocking *VEGFR* and *PDGFR*) did not show poorer results compared to nephrectomy[321]. Due to the fact that bone metastasis harbors a depressing clinical outcome, treatment approaches are individualized, although multimodal. Because of the positive results of immune checkpoint blockade in combination with TKIs, there is an emerging ongoing debate regarding the use of them as adjuvant therapy in metastatic ccRCC to prevent the formation, survival and maturation of micro metastases[322]. Nevertheless, retrospective results of ongoing clinical trials are pending, and according to recent guidelines[150] adjuvant therapy is not recommended in metastatic ccRCC.

In bone metastatic ccRCC, or bone metastasis in general, it appears that RANK/RANKL/OPG balance remain the intended target. With this work we now understand that the TA-MSC cell population adjust and acquire the potential of the normal bone remodeling process, and overstimulate it, leading to, in this case of bone

metastatic ccRCC, to bone destruction. Bone is in general hard to treat due to its very dense tissue, and blood vessels, and other cells have difficulty in penetrating it. However, we highlight that *CCL18–CCR8* axis to also be involved in the metastatic progression. Interestingly there is a clinical trial that started their recruitment for investigating a combination therapy with an anti-*CCR8* antibody against advanced solid tumors (NCT05537740). Most innovative ongoing clinical trials focus on radiation therapy on bone metastases with modified emissions[135,323].

6.2 Reviving differentiation therapy in NB

Currently there are no ongoing clinical trials investigating *DHODH* inhibition in solid tumors. And yet, there are no clear answers for why the agent previously failed clinical trials. However, with the benefit of the emerging technologies and multiomic analysis *DHODH* is revealed and reintroduced as a strong candidate target[319,320,324-326].

Diseases driven by *MYC* or *MYCN* are metabolically reprogrammed as an integrated part of their growth path. The discovery of enhanced pyrimidine nucleotide production by *MYCN*, leading to upregulation of *DHODH*, is shedding light on *DHODH* inhibition to prevent *MYC/MYCN* driven tumor growth. In parallel studies of NB, studies in medulloblastoma and gliomas are illuminating the potential therapeutic outcome in preclinical models using a *DHODH* inhibiting agents alone, or in combination, including our work in **Paper III**[319,320,324-327]. Already a decade ago leflunomide tested *in vitro* in NB demonstrated apoptosis and was suggested as potential target[328]. The lingering question is, why has it not yet been implicated in clinical testing? There may be a simple answer. That is, that the enzyme is expressed in all our cells in our bodies for RNA, DNA and nucleotide synthesis, lack of proof showing its mutation or overexpression in cancer, and clinical trials has been unsuccessful to date, driving an uncertainty. It is reasonable to draw these logical conclusions. Although, the accumulating and evolving preclinical evidence demonstrating reduction in pyrimidine nucleotides could effectively slow proliferation of cancer cells by inhibiting *DHODH*, will eventually change the course of investigations for *DHODH* inhibiting agents.

6.3 Yet a puzzle

Since Paget's theory, in 1889, about "the seed and soil" evolved, the research community has gained an appreciable understanding of the diverse crossroads that tumor cells ought to pass, their cellular modifications and molecular interactions. The question is: What dictates what organs shall suffer when encountering a circulating cancer cell? It is a puzzle inside a puzzle. It is the same puzzle that Paget tried to solved when he was surprised that women suffering from breast cancer died because of liver metastasis. He thought, a more logical metastatic site from the breast would have been the spleen due to its larger artery compared to the artery of the liver.

This thesis work has been able to provide some answers, significantly and gratefully thanks to other scientists that dedicated their research time to develop sophisticated technologies. The question is, how would Paget have reacted if he would have had access to the information or technology we have today, back then? And where would we have been now in the pursuit for cancer cure?

7 ACKNOWLEDGEMENTS

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عمتي **سحر الشاهين,** مافي منك والله يخليلنا ياكي

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