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TRANSLATIONAL STUDIES OF EPITHELIAL CANCER

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Translational studies of epithelial cancer

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Till min familj

“Det har jag aldrig provat förut, så det klarar jag säkert” – Pippi Långstrump

ABSTRACT

Tumors stemming from specialized epithelial cells cause the most common cancer types and are among the leading causes of death in the western world. Although great strides have been made in early cancer detection, defining prognostic factors, and improving survival with novel treatments, cancers such as colorectal cancer and prostate cancer are incurable in their advanced stages. In the work presented in this thesis, we aim to explore the biological underpinnings of tumor initiation and progression and determine novel pharmaceutical treatment strategies against epithelial cancer. We do so by combining preclinical methodologies of cell culture and animal models of disease with epidemiological studies of population and patient cohorts. EphB tyrosine kinase receptors promote intestinal tumor proliferation via the tyrosine-protein kinase Abl1 (Abl kinase) (Genander et al., 2009; Holmberg et al., 2006). In **paper I**, we find that the Abl kinase inhibitor imatinib blocks EphB receptor regulated tumor initiation and growth in mouse models of early-stage intestinal tumors, reduces proliferation in *ex vivo* human adenomas and prolongs survival of tumor bearing mice. We propose imatinib as a possible prevention and early treatment strategy for people prone to develop intestinal adenomas. In **paper II**, we explore the immune system and antiviral immunity as potential markers of prostate cancer prognosis. In prostate cancer patients, the Human Leukocyte Antigen (HLA) alleles HLA-A*02:01 and HLA-A*11 are associated with poor disease recurrence free survival after prostatectomy. Immunity to the human herpesvirus cytomegalovirus (CMV) in prostate tumors is associated with particularly poor disease recurrence free survival in HLA-A*02:01^{+} prostate cancer patients. In **paper III**, we find that CMV commonly chronically infects epithelium in the healthy and malignant prostate, prostate cancer metastases and prostate cancer cell lines. Experimental and therapeutic inhibition of CMV in *in vitro* and *in vivo* models of prostate cancer reveal that CMV promotes its viability and growth and propose that CMV targeting drugs can be repurposed against prostate cancer. In **paper IV,** we describe that CMV seropositivity is associated with high CMV abundance in healthy and malignant prostate. Studying a large prospective population cohort, we find that CMV seropositivity is not associated with prostate cancer incidence but is associated with increased risk of dying from prostate cancer after receiving a prostate cancer diagnosis.

LIST OF SCIENTIFIC PAPERS

I. Kundu P*, Genander M*, Strååt K, **Classon J**, Ridgway RA, Tan EH, Björk J, Martling A, van Es J, Sansom OJ, Clevers H, Pettersson S, Frisén J. *An EphB-Abl signaling pathway is associated with intestinal tumor initiation and growth.* Sci Transl Med. 2015 Apr 1;7(281):281ra44. doi: 10.1126/scitranslmed.3010567.
* These authors contributed equally to the study

II. **Classon J***, Zamboni M*, Engblom C, Alkass K, Mantovani G, Pou C, Nkulikiyimfura D, Brodin P, Druid H, Mold J, Frisén J. *Prostate cancer disease recurrence after radical prostatectomy is associated with HLA type and local cytomegalovirus immunity*. Mol Oncol. 2022 Oct;16(19):3452-3464. doi: 10.1002/1878-0261.13273. Epub 2022 Aug 31.
* These authors contributed equally to the study

III. **Classon J**, Stenudd M*, Zamboni M*, Alkass K, Eriksson CJ, Pedersen L, Schörling A, Thoss A, Bergh A, Wikström P, Adami HO, Toft Sörensen H, Druid H, Frisén J. *Cytomegalovirus promotes proliferation and survival of prostate cancer cells and constitutes a therapeutic target.* Manuscript.
* These authors contributed equally to the study

IV. **Classon J,** Britten A, Alkass K, Druid H, Gkrania-Klotsas, Frisén J. *Cytomegalovirus seropositivity is associated with increased prostate cancer mortality.* Manuscript.

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Reichenbach B*, **Classon J***, Aida T, Tanaka K, Genander M, Göritz C. *Glutamate transporter Slc1a3 mediates inter-niche stem cell activation during skin growth.* EMBO J. 2018 May 2;37(9). pii: e98280. doi: 10.15252/embj.201798280. Epub 2018 Apr 3.
* These authors contributed equally to the study

CONTENTS

LIST OF ABBREVIATIONS

Abl kinase	Tyrosine-protein kinase ABL1
AIDS	Acquired Immunodeficiency Syndrome
APC	Adenomatous Polyposis Coli
AR	Androgen Receptor
CI	Confidence Interval
CMV	Human Cytomegalovirus
CRPC	Castration Resistant Prostate Cancer
DNA	Deoxyribo Nucleic Acid
EBV	Epstein Barr Virus
EPIC	European Prospective Investigation of Cancer
FAP	Familial Adenomatous Polyposis
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPV	Human Papilloma Virus
HR	Hazard Ratio
IgG	Immunoglobulin G
KSHV	Kaposi's Sarcoma-associated Herpesvirus
MHC	Major Histocompatibility Complex
NHANES	National Health and Nutrition Examination Survey
PSMA	Prostate-Specific Membrane Antigen
pRB / *RB1*	Retinoblastoma protein
s-PSA	Serum Prostate Specific Antigen
TCR	T-cell Receptor

1 INTRODUCTION

Cancer is one of the most common causes of death in the western world. Many of these deaths are due to cancer originating in organs with specialized epithelial cells, such as the lung, intestine, breast, and prostate (Siegel et al., 2022). Cancers are comprised of abnormal cells that have gained functions that allow uncontrolled growth and invasion into its surroundings. This erratic behavior can e.g., occur due to mutations or by viruses that e.g., can integrate into the cells' genome (e.g., Human Papilloma Virus (HPV)-16 and HPV-18) or otherwise tip the balance from tumor suppressive cellular functions to oncogenesis (e.g., Epstein Barr Virus (EBV)). Most cancer types can also develop the capacity to spread to other areas of the body and produce metastases. Cancerous cells are often accompanied by an altered immune microenvironment, which sometimes leads to a more pro-tumorigenic milieu. To improve cancer survival, it is imperative to identify mechanisms behind the erratic behaviors of cancer cells and its altered microenvironment. This knowledge can be used to find novel therapies and biomarkers for early cancer detection and monitoring of disease progression. This thesis focuses on characterizing fundamentally different mechanisms that could play important roles for tumor development and cancer progression and find potential future cancer therapies.

Increased cancer prevention efforts, early tumor detection and intervention will decrease cancer mortality. In addition to broad cancer screening programs, specific tumor initiation pathways could be therapeutically targeted to reduce tumor burden, motivating further characterization of the molecular underpinnings of tumor formation. In intestinal adenomas and carcinomas, the gene adenomatous polyposis coli (*APC*) is commonly mutated and lose its tumor suppressing function. Carriers of *APC* defects, with a genetic syndrome called familial adenomatous polyposis (FAP), develop a substantial amount of benign intestinal tumors (polyps / adenomas) that are at risk of transforming into cancer. FAP patients undergo regular endoscopic examinations and detected tumors are surgically removed. *APC* defects sets a cascade of cellular events in motion, including upregulation of EphB tyrosine kinase receptors, which have broad and crucial functions in tumor development (Batlle et al., 2002; Caspi et al., 2021). For example, EphB tyrosine kinase receptors promote intestinal tumor proliferation via the Abl kinase (Genander et al., 2009; Holmberg et al., 2006). In **paper I,** we experimentally find that the Abl kinase inhibitor imatinib can be used as a targeted pharmacological therapy to prevent tumor formation in FAP patients, as an early intervention strategy.

In addition to early detection and intervention, it is also of great importance to correctly identify the tumors that will actually develop into lethal disease so that we do not give unnecessary treatments with severe side effects to those that do not benefit from them. In fact, most men

with a prostate tumor do not die from cancer (Zlotta et al., 2013). However, around 25% of patients diagnosed with prostate cancer will develop lethal, metastatic, disease. Despite extensive research efforts, it is unclear what tumor will stay indolent and what tumor will become deadly. Besides clinical and histological features, serum prostate specific antigen (s-PSA) and rare germline mutations in deoxyribo nucleic acid (DNA) repair genes, few prognostic biomarkers are known (Cooperberg et al., 2009; Eggener et al., 2020). Although classically viewed as immunologically inactive, prostate tumors are infiltrated by several types of immune cells (Sfanos et al., 2018). An increasing number of studies demonstrate a perturbed immune system in individuals with prostate cancer, making immune markers interesting candidates as new prognostic biomarkers.

A person's potential repertoire of adaptive immune cells is mainly determined by his or her combination of inherited human leukocyte antigen (HLA) gene alleles, that are responsible for presenting antigens from e.g., microbes to T-cells, a subset of adaptive immune cells. Not much is known about HLA-type and immunity to viruses in prostate cancer. In **paper II** we explore the role of HLA-type as biomarkers of prostate cancer outcomes. The role of the immune system in cancer is further highlighted be the fact that infectious agents contribute to around 15% of human cancers in the world (Plummer et al., 2016). But it is not certain that microbes cause the most common epithelial cancers. In addition to causing cancer, viruses can perturb functions of cancer cells once they are formed, as exemplified by HPV, whose presence in tumors is associated with superior prognosis in oropharyngeal cancer (Ang et al., 2010). Viruses may indirectly modulate cancer phenotypes by altering the immune microenvironment or by directly altering cellular processes in infected cancer cells (Virgin, 2014).

In the world, 83% have antibodies against the human herpesvirus CMV (Zuhair et al., 2019), a DNA virus that establishes life-long infection. CMV is a modulator of the immune system to which a large part of the adaptive immune system is focused on keeping in check (El Baba and Herbein, 2021). **In paper II,** we find that local immunity to CMV, but not to EBV or Influenza A, is associated with disease recurrence of prostate cancer after prostatectomy in HLA-A*02:01^{+} patients. CMV can potentially have direct cancer modulating properties (Herbein, 2018; Michaelis et al., 2009) and may chronically inhabit several epithelial organs and their corresponding cancer types, including the prostate (Boldogh et al., 1983; Gordon et al., 2017; Hendrix et al., 1997; Kuhn et al., 1995; Samanta et al., 2003; Shnayder et al., 2018).

Like other herpesviruses, CMV stays around for life in a chronic phase termed latency, characterized by little to no production of virus particles and long-term persistence of episomal, circular, viral genomes. Except for hematopoietic lineage cells (Bolovan-Fritts et al., 1999;

Sinclair and Sissons, 2006; Taylor-Wiedeman et al., 1991), whereabouts and characteristics of CMV infection is ambiguous. This is due to inconsistent detection methods for chronic CMV infection of which latency has been proven an elusive state to capture (Wick and Platten, 2014). In **paper III,** we improve detection methods of chronic CMV infection and with these determine CMV prevalence and infection characteristics in benign prostate and prostate cancer *in vivo* and *in vitro*. A central role for CMV as a regulator of cell viability and proliferation was uncovered in a series of experiments in prostate cancer cell lines. Preliminary data suggest that CMV seropositive males have higher risk of dying from prostate cancer than CMV seronegative men in a North American prospective cohort study (data not shown). In **paper IV,** we find that CMV seropositive men generally have more CMV in their prostates than CMV seronegative men, implying that CMV seropositivity may act as a surrogate marker for prostate CMV abundance. We find an association between CMV seropositivity and prostate cancer mortality in prostate cancer patients in a UK based prospective cohort with long-term follow up, implying that CMV is a risk factor for lethal prostate cancer and suggest that CMV may drive disease progression.

Medications that suppress androgen signaling and chemotherapy are standard of care for metastatic prostate cancer. Although several new targeted therapies for select patients have been approved in the last decade, including PARP inhibitors and prostate-specific membrane antigen (PSMA) targeted therapies, metastatic prostate cancer is still not curable (Sandhu et al., 2021). Resistance to current drugs is inevitable and strategies to overcome or circumvent this issue are needed to improve prostate cancer survival. **In paper III**, we explore CMV as a therapeutic target in observational and experimental studies and propose repurposing of existing drugs as prostate cancer therapeutics.

2 TUMOR DEVELOPMENT AND PREVENTION

A tumor typically arises from one cell, the cell-of-origin, that acquires cancerous alterations and clonally expand. With time and selective pressure from e.g., anti-cancer drugs, additional alterations can occur that helps the cancer cells adapt and thrive in new environments such as metastatic niches. Although there are genes generally known as oncogenes and tumor suppressors, such as *TP53*, *APC* and *BRCA2*, loss-of-function of suppressors do not result in cancer all over the body, but rather result in risk of distinct tumor types. Risk of tumor formation at different sites in the body is suggested to be related to stem cell division rates in the particular tissue (Tomasetti et al., 2017; Tomasetti and Vogelstein, 2015). For example, skin and intestine are organs in which stem cells proliferate often and are also common sites of cancer. This exemplifies the complexity of tumor formation and that interactions with the microenvironment are crucial. The immune system is surveilling the body for aberrant cells (Burnet, 1970) and when the immune system is impaired, such as in AIDS patients, particular tumor types will arise (Grulich et al., 2007). In contrast, an overactive immune system is also linked to cancer, as chronic inflammation may be a pro-tumorigenic environment. Viruses have the potential to directly induce tumor formation by e.g., expression of oncogenes or indirectly by promoting long-term chronic inflammation (White et al., 2014).

Many cancers can be prevented by early detection and intervention, in which a precancerous tumor is removed before it becomes malignant. For certain tumor types, drugs can reduce cancer risk, including selective estrogen receptor modulators that reduce breast cancer risk (Cuzick et al., 2015). Individuals with increased risk of cancer, like patients with FAP, would benefit greatly from pharmaceutical cancer preventing treatments. Today, endoscopic excisions of polyps and larger surgical interventions are routinely used and can prevent early onset of intestinal cancer in patients with FAP but does not adequately prevent disease and are associated with severe morbidities. For heterogenous diseases such as prostate cancer, identifying all men with tumors and treating them may not be the primary goal, but rather it is important to pinpoint those who will develop lethal cancer and prevent advanced disease. Vaccinations against viruses that induce cancer have had tremendous success in lowering incidence of certain cancers. How mutations and viruses can give rise to cancer are well-studied phenomenon but are still not fully understood. Learning how these processes occur allows us to identify treatment targets and cancer prevention strategies with high specificity and few side effects.

2.1 COLORECTAL TUMOR FORMATION AND PREVENTION

Genetic changes play an essential role for tumor formation. Around 80% of human colorectal cancer have mutations that inactivate *APC* which results in hyperactivated Wnt signaling (Cancer Genome Atlas, 2012). Loss of APC and consequent Wnt hyperactivation severely alters cellular processes relevant for tumorigenesis (Caspi et al., 2021). Furthermore, around 5% of the patients with colorectal cancer have a cancer predisposing syndrome such as FAP, with germline mutations in *APC*. Cells with inactive Apc are unable to degrade the Wnt master regulator β-catenin, resulting in persistent Wnt activity (Caspi et al., 2021). An intestinal stem cell that loses *Apc* can become the cell-of-origin of an intestinal tumor (Barker et al., 2009). In contrast, Wnt pathway mutations are late events in prostate cancer, and often correlate with metastatic progression (Robinson et al., 2015). Restoring Apc activity in mouse models of intestinal tumors can induce tumor regression (Dow et al., 2015), equal to "oncogene addition" that has been described for e.g., the MYC oncogene in other tumor types (Dhanasekaran et al., 2021). Several ways of targeting Wnt signaling in colorectal cancer are currently explored but none have reached clinical use (Caspi et al., 2021). For example, restoring the β-catenin degradation complex with tankyrase inhibition (Schatoff et al., 2019), is actively investigated, but the important tissue homeostasis maintaining functions of Wnt signaling makes broad potential antagonists prone to unwanted side effects. The enzymes cyclooxygenase and ornithine decarboxylase are regulated by APC, have heightened expression upon *APC* loss in tumors and drugs that inhibit these enzymes have been clinically tested in patients with FAP. Unfortunately, drugs inhibiting cyclooxygenase and ornithine decarboxylase have not shown clear cancer preventing effects, even when administered in combination (Burke et al., 2020).

There are many different experimental models that can be used to study tumor development and potential therapies. For example, studying mice carrying specific intestinal tumor causing mutations are very informative. However, a single mouse model is often not able to capture all stages of colorectal cancer development and progression nor the heterogeneity of the disease between patients. $Apc^{multiple\ intestinal\ neoplasia\ (Min)/+}$ mice, that carry a heterozygous nonsense mutation in *Apc*, is a common mouse model for intestinal tumor formation and growth, that was described in the 1990s. After spontaneous loss of the remaining functioning allele, they frequently develop tumors in the small intestine but rarely in the colon (Levy et al., 1994). Although FAP patients commonly develop adenomas in the small intestine (Bulow et al., 2004), humans mainly develop intestinal cancer in the colon or rectum. Altering the genetic background of $Apc^{Min/+}$ mice or treating mice with inflammatory inducing agents can also confer higher frequency of colon adenomas (Burtin et al., 2020; Cooper et al., 2001). A more

recently developed mouse model e.g., uses conditional (time controlled) silencing of the *Apc* gene (shApc), in which tumors in the colon are more frequent (Dow et al., 2015). Taken together, the regulation of tumor positioning along the intestinal tract upon *Apc* perturbation may be complex and can potentially be relevant when one aims to translate knowledge from mouse models to human disease. Organoids derived from either mice or humans can also be used as models of tumor initiation or progression *in vitro* and *in vivo* and can be genetically and pharmacologically manipulated. Another method is *ex vivo* tissue slice cultures, used in **paper I**, in which short-term treatment responses can be assessed.

A group of tyrosine kinase receptors called EphB receptors are Wnt target genes in intestinal stem cells in which they regulate proliferation and cell positioning upon activation by ephrin ligands (Batlle et al., 2002; Holmberg et al., 2006). In tumors, EphB receptors promote proliferation through the Abl kinase but also inhibit progression from adenoma to invasive cancer, independent of Abl kinase activity (Batlle et al., 2005; Genander et al., 2009). General targeting of EphB functions, e.g., with receptor antagonists or monoclonal antibodies, potentially reduce proliferation and tumor suppressor functions (Batlle et al., 2005; Genander et al., 2009). The Abl kinase inhibitor imatinib reduced adenoma proliferation in a similar manner as inhibition of EphB activity (Genander et al., 2009). Described in two case-reports, imatinib treatment of chronic myelogenous leukemia in FAP patients was associated with regression of intestinal polyps (Itsukuma et al., 2007; Tobon et al., 2020). Although enticing, the mechanism and therapeutic effect of imatinib had not been explored. In **paper I,** we examine this therapeutic opportunity in model systems of FAP.

2.2 PROSTATE TUMORIGENESIS

As for colorectal cancer, genetic changes are also central for prostate cancer development. A large number of single nucleotide polymorphisms and a small number of mutations in genes, such as *BRCA2* and *HOXB13*, are associated with risk of prostate cancer (Shi et al., 2021). A recent survey identified 97 mutated genes in prostate cancer, with most of them being present in less than 3% of prostate tumors analyzed (Armenia et al., 2018). Two somatic mutations that have been extensively studied are point mutations in *SPOP* (found in 9% of primary prostate tumors) and common gene fusion events such as *ETS* transcription family fusions with *TMPRSS2* (found in 51% of primary prostate tumors) (Armenia et al., 2018). It is unclear if *TMPRSS2-ERG* fusions are oncogenic on their own, or if additional genomic alterations, such as *PTEN* loss, is needed to drive oncogenesis (King et al., 2009). *TMPRSS2-ERG* fusions can, with or without cooperation, initiate proposed pre-cancerous lesions called prostate

intraepithelial neoplasia. Furthermore, presence of *TMPRSS2-ERG* fusion transcripts can be detected in urine and be used as a biomarker of prostate cancer risk (Tomlins et al., 2011). In bioinformatically reconstructed cell lineage trees, *SPOP* mutations and *TMPRSS2-ERG* fusions appear early in tumors, whereas alterations in *PTEN* appear late (Baca et al., 2013). This model of sequential mutations increasing the total mutational burden favors the classical view of tumor development from benign to aggressive, as was originally described for colon cancer (Fearon and Vogelstein, 1990). But the opposite has also been described, in which massive DNA damage occur at once in an event called chromothripsis (Baca et al., 2013).

In addition to prostate intraepithelial neoplasia, a lesion that is associated with surrounding inflammation, called proliferative inflammatory atrophy, has also been suggested as a prostate cancer precursor state (Sfanos et al., 2018). A potential role of inflammation associated phenotypes in prostate cancer oncogenesis is supported by epidemiological studies that have found associations between non-steroidal anti-inflammatory drugs and lower cancer incidence (Doat et al., 2017). But an inverse association has also been observed between inflammation and prostate cancer in case-control studies (Langston et al., 2021). These results are suggested to depend on collider stratification bias when restricting the study population to clinically detected prostate cancer and excluding s-PSA guided biopsies (Langston et al., 2021). Chronic inflammation can play a prominent role in intestinal tumor formation by promoting DNA damage and epigenetic alterations (Schmitt and Greten, 2021).

Androgens, that are ligands for the androgen receptor (AR), play a central role in prostate cancer biology and may be associated with its occurrence (Morgentaler, 2007). Men treated with androgen reducing 5-alpha-reductase inhibitors, often used to treat benign prostate hyperplasia, may have lower incidence of prostate cancer (Chau and Figg, 2018) but it is not clear that addition of androgens promotes prostate cancer growth in steady state (Morgentaler, 2007) and there is no positive correlation between serum testosterone levels and prostate cancer incidence (Morgentaler, 2007). In a mouse model of *Ar* overexpression, pre-cancerous lesions developed in the prostate and a specific mutation in *Ar* can cause prostate cancer prone to metastasize in mice (Han et al., 2005; Zhou et al., 2015). Androgen signaling is altered in prostate cancer, in which it drives proliferation instead of differentiation. Several molecular events are involved in this switch, including altered binding capacities of AR to DNA as co-regulators and other transcription factors have distorted expression in prostate cancer (Zhou et al., 2015). Discovery of AR modulators are of particular importance, as androgen signaling is central to prostate cancer biology and is a central therapeutic target.

We can conclude that there may be several roads that lead to prostate cancer, but how genetic and environmental factors converge to cause cancer is less explored. Of note, it is curious that cancer does not arise in seminal vesicles, which are located in proximity to the prostate and also contains glandular epithelial cells. Understanding the biological differences between these two glands may be the key to decipher prostate oncogenesis.

2.3 VIRAL CARCINOGENESIS

The immune system is designed to recognize and eliminate foreign objects, including viruses. Cells of the innate immune system, such as neutrophiles and macrophages act as the first broad defense, in which they recognize and neutralize the target while attracting other cells to the site. The adaptive immune system, with B- and T-cells as its major players, has specificity and can form memory and is therefore important for maintaining responses to encountered pathogens over time. Viruses are masters of escaping immune recognition and can establish chronic lifelong infections, of which several are linked to cancer.

Eight viruses are recognized as human carcinogens. These are EBV, hepatitis B virus, hepatitis C virus, human T-cell lymphotropic virus type 1, HPV, Human Immunodeficiency Virus (HIV), Kaposi's Sarcoma-associated Herpesvirus (KSHV), and Merkel Cell Polyomavirus. There is a tight link between the immune system and viral carcinogenesis. For example, tumor development caused by EBV, KSHV and Merkel Cell Polyomavirus is often linked to defects in the immune system, as they frequently appear in e.g., acquired immunodeficiency syndrome (AIDS) patients (Feng et al., 2008; Grulich et al., 2007), why HIV is considered carcinogenic. HPV associated cancer, such as epithelial cancer of the cervix, may also be increased upon immune suppression. On the other hand, chronic inflammation caused by an infection can induce cancer, exemplified by hepatitis viruses and liver cancer (Moore and Chang, 2017). Interestingly, immune suppression is not associated with increased risk of most common epithelial cancers (Grulich et al., 2007), generally suggesting that classical tumor viruses do not cause these malignancies.

Koch's postulates dictates that causality between a microbe and a disease can only be determined if all diseased cases harbor the microorganism and not healthy individuals (Evans, 1995). But most often, viruses are more frequent in the population than their associated tumors. Epidemiological observation studies, such as case-control and cohort studies, can be used to examine if carriers have an increased cancer risk. Using these methodologies, being immune to one of several DNA viruses (herpes simplex virus 2, CMV, HPV, KSHV) have not clearly been associated with increased prostate cancer incidence (Huang et al., 2008; Korodi et al.,

2005; Sutcliffe et al., 2007; Sutcliffe et al., 2012). CMV seropositivity and prostate cancer incidence have been studied in case-control studies, a study design that can capture potential associations but is prone to bias. A prospective cohort study, in which risk of prostate cancer can be determined over time in relation to CMV serostatus at start of follow up will be more conclusive. To my knowledge, CMV immunity has not been associated with development of other tumor types. On the other hand, men with varicella zoster virus reactivations that require care for shingles, have a slightly higher risk of being diagnosed with prostate cancer (Tsao et al., 2020), but patients with cancer also more commonly get shingles (Hansson et al., 2017). These associations may reflect a perturbed immune response in prostate cancer patients. Observational studies assume that viral immunity (e.g., defined as seropositivity) equals a chronic infection. But for example, studies finding CMV DNA in CMV seronegative individuals have repeatedly been reported since the 1980's (Hendrix et al., 1997; Kraat et al., 1992; Larsson et al., 1998; Mendelson et al., 1996; Roback et al., 2001; Stanier et al., 1989; Taylor-Wiedeman et al., 1991; Zhang et al., 1995), and anti-CMV T-cells and B-cells detected with experimental methods, can be identified in 46-100% of CMV Immunoglobulin G $(IgG)^-$ individuals (Ashokkumar et al., 2020; Litjens et al., 2017; Loeth et al., 2012; Terlutter et al., 2018). Interestingly, it was possible to re-activate CMV in a CMV IgG^- donor *ex vivo* (Soderberg-Naucler et al., 1997).

A large body of research is devoted to discerning if common viruses, including CMV, are present in tumors. Presence of viruses and their infection characteristics in common epithelial cancers, among others, is disputed as detection rate can differ from 0% to 100% of tumors depending on methodology (Wick and Platten, 2014). Several epithelial organs may perhaps be infected with chronic CMV (Gordon et al., 2017; Hendrix et al., 1997; Shnayder et al., 2018), including the benign and malignant prostate gland (Boldogh et al., 1983; Kuhn et al., 1995; Samanta et al., 2003). CMV is difficult to find by bulk RNA-sequencing and whole genome sequencing in prostate tissue (Shnayder et al., 2018; Tang et al., 2013; Zapatka et al., 2020). Acute CMV infection can easily be detected by a plethora of methods, but its chronic latent state is more elusive and may require optimized methods to capture, as exemplified in hematopoietic cells, well-characterized CMV reservoirs (Bolovan-Fritts et al., 1999; Cheng et al., 2017). It is likely that CMV infections in tissues are primarily latent. Like other herpes viruses, CMV can, with help of cellular and viral proteins, maintain its genomes as episomes that can be replicated during the cell cycle and be passaged to daughter cells (Bolovan-Fritts et al., 1999; Tarrant-Elorza et al., 2014). Latency has classically been viewed as a restricted infection state in which only a few viral genes were expressed, but the modern view of latency is defined as viral persistence in which no viral particles are being produced (Lieberman, 2016)

in which the virus actively perturb cell functions while suppressing immune surveillance (Shnayder et al., 2020).

CMV has been described as an inhabitant of precancerous epithelial cells and cancer cells in prostate biopsies, whereas CMV infection was rare in benign epithelium (Samanta et al., 2003). In this study, 22 patients were examined of whom CMV serostatus was not determined (Samanta et al., 2003). An association between a virus and a tumor is considered strong if all tumor cells are infected and if surrounding cells are uninfected. This argument is flawed since tumor development may take a long time, viral carcinogenesis may not be 100% efficient and other oncogenic mechanisms may co-exist. Furthermore, multiple cancer cells-of-origin may be present, arising through different mechanisms and with unique mutation profiles (Andreoiu and Cheng, 2010), perhaps resulting in both virus positive and virus negative hubs. It is also possible for tumors to be largely virus negative even though they were caused by one. For example, HPV may predispose development of squamous cell carcinoma through a 'hit and run' mechanism in which HPV impairs DNA damage response to ultraviolet light and cause oncogenic mutations (McBride, 2022) .

The CMV virion consists of a 235 000 bp large double stranded DNA helix in a nucleocapsid packaged in tegument proteins and an outer envelope. The virus enters cells via attachment of viral glycoprotein to cell surfaces and cooperation with receptors such as PDGFRα and EGFR (Soroceanu et al., 2008; Wang et al., 2003). Viral DNA is then deposited in cell nuclei. CMV, with around 700 putative open reading frames that can be expressed (Stern-Ginossar et al., 2012), will affect many cellular processes including those that promote cell survival and proliferation in order for viral replication to occur during an acute infection. It is noteworthy that only a fraction of CMV genes are needed for viral replication, implying that the rest may be used to perturb cellular functions that promote persistence (Hein and Weissman, 2022). The cellular state of an acute infection is temporary and ends once the cell dies, infection is cleared or, if latency ensues. During acute infection or when ectopically expressed, several CMV genes may promote oncogenic processes such as the ability to induce telomerase activity, impair DNA repair, dysregulate tumor suppressor genes and activate oncogenes (Cobbs, 2019; Costa et al., 2013; O'Dowd et al., 2012). The CMV kinase UL97 is capable of phosphorylating and inactivating retinoblastoma protein (pRB), a known tumor suppressor, resulting in activation of cell cycle processes (Hume et al., 2008). A genetically engineered mouse strain that expresses the CMV gene *US28* in intestinal epithelial cells show accumulation of β-catenin, adenoma and carcinoma formation (Bongers et al., 2010). Although these findings are

suggestive, it is unclear if they reflect the biology of *in vivo* CMV infection in tissues and tumors. Detailed studies of CMV in its proper cellular contexts are required.

Host and viral genetics and environmental factors could all play a role in defining cancer risk. For example, a particular strain of the common virus EBV (particular viral genetics) is associated to high risk of nasopharyngeal carcinoma in China (particular host genetics) (Xu et al., 2019). It could be possible that certain strains of CMV have the capacity to transform certain cell types in restricted conditions. In the 1970's it was reported that a strain of CMV isolated from a child's prostate could immortalize embryonal lung cells *in vitro* and tumors could be formed from these *in vivo* (Geder et al., 1976; Geder et al., 1977). These results have been difficult to reproduce and have been dismissed by the cancer research community. A recent study found that infection with a clinical CMV strain can transform breast epithelial cells (Kumar et al., 2018). This study raises several questions; Are these potentially oncogenic strains common? Does this strain infect epithelial cells *in vivo* and act in similar ways as *in vitro*? If so, these findings may have major implications on cancer prevention strategies.

In a study from the 1990's, patients with HIV had reduced risk of developing Kaposi's sarcoma when treated with the anti-viral drug foscarnet or ganciclovir (Mocroft et al., 1996) but was not effective in eradicating or reducing size of established tumors (Little et al., 2003). This indicates that tumor formation is dependent on actively replicating KSHV, but that latency with restricted viral gene expression ensues once the tumors are established. Koch's postulates state that the microorganism should be possible to isolate, propagate in culture, and cause disease again when re-introduced to the host (Evans, 1995). In general, it is often challenging to isolate and propagate viral particles in virus-associated tumors due to the non-virus producing nature of them (Moore and Chang, 2017). Anti-viral drugs such as ganciclovir and aciclovir are activated to inhibit viral DNA synthesis by specific viral kinases. For CMV, this viral kinase is UL97 and has classically been described to be expressed during acute infection (Littler et al., 1992; Talarico et al., 1999). Classically defined anti-viral drugs can also have cellular effects independent of viral DNA inhibition. For example, activated aciclovir is a weak acute inhibitor of cellular DNA polymerase (Furman et al., 1984). If activating viral kinases are expressed during latency, they can have broadened effects on cells, including cancer cells. Latent viruses can be functionally analyzed in experimental settings. For example, Merkel cell polyomavirus is considered an oncogenic virus partially due to Merkel cell carcinoma cells being addicted to expression of a viral oncogene *in vitro* (Houben et al., 2010). Similar to oncogenic viruses, cancers can also be dependent on bacteria. For example, gastric cancer can potentially be

prevented by *Helicobacter pylori* eradication and result in tumor regression (Nakagawa et al., 2019).

Vaccines against HPV and hepatitis B have remarkably reduced incidence of cervical cancer and liver cancer respectively. CMV vaccines are under development but none have yet showed clinical success (Nelson et al., 2018). Development of an efficient vaccine is complicated by the large number of viral strains (Gorzer et al., 2010; Renzette et al., 2015; Ross et al., 2010) and that viral-neutralizing antibody responses may be strain-specific (Martins et al., 2019).

3 CANCER PROGRESSION AND PROGNOSIS

Although colon cancer and prostate cancer both originate from specialized epithelial cells, these tumor types have tremendously different prognosis with colon cancer patients having around 65% five-year survival in contrast to 98% of prostate cancer patients being alive after five years (Siegel et al., 2022). Radical prostatectomy for localized prostate cancer increases prostate cancer survival in comparison to watchful waiting (Bill-Axelson et al., 2018), but around 35% of patients who undergo radical prostatectomy progress with recurrent disease within 10 years after surgery or local radiation with curative intent (Freedland et al., 2005). Patients with advanced prostate cancer, most often with distant metastases, only have a 30% 5-year survival rate (Siegel et al., 2022). Since prostate cancer patients often live for several years after their diagnosis of prostate cancer and initial treatment, surrogate markers of cancer survival are often needed in longitudinal studies. Increase in s-PSA, also called biochemical recurrence, is commonly used in studies, but it unfortunately has low sensitivity. The novel tool PSMA positron emission tomography imaging, that allows for visualization of small metastases in prostate cancer patients that are undetected with standard imaging, may be a potential complement to biochemical recurrence as surrogate markers of prostate cancer survival (Wang et al., 2022).

What determines cancer prognosis is to a large extent unknown, but some environmental, hereditary and tumor specific alterations have been identified. General or systemic factors can be defined as environmental factors, such as smoking, viral immunity and diet, and hereditary factors with germline mutations. These factors may impact the body as a whole, but also exert local functions in primary tumors and metastases and determine cancer outcomes. For example, smoking can induce systemic inflammation that could result in tumor-promoting microenvironments. Cancer cell intrinsic or extrinsic tumor specific features, such as tumor differentiation grade, somatic mutations and the local tumor microenvironment all have an impact on tumor outcomes. Identified risk factors for prostate cancer mortality include obesity, smoking, carrying germline mutations in e.g., *BRCA2* (Rawla, 2019) and the tumor specific factors Gleason score and T-stage, but current models are insufficient and do not accurately predict prostate cancer prognosis.

3.1 CANCER PROGRESSION AND PROGNOSIS: TUMOR CELL ALTERATIONS

In chapter 2, I discussed how tumors arise. In the intestinal tract, APC loss of function is very often an early event, and experimental evidence shows that its perturbation causes formation

of intestinal adenomas. Here I introduce how a benign adenoma turns into an invasive carcinoma. There is a clear-cut switch when an intestinal adenoma transitions into a malignant carcinoma. Loss of APC function and Wnt hyperactivation is not sufficient to cause tumor invasion and metastases, for which additional insults are required. It has been estimated that three to six mutations that can be considered as disease drivers are present in each colorectal carcinoma, with mutations in *KRAS* and *TP53* being found in around half of cases (Cancer Genome Atlas, 2012). An extended mutational landscape is accompanied by changes in gene expression, of which particular genes are crucial for cancer progression. For example, EphB receptor expression is often lost in carcinomas, and can promote E-cadherin dependent invasive cancer growth in $Apc^{Min/+}$ mice (Batlle et al., 2005; Cortina et al., 2007). The ability for cells to lose contact with their neighbors, allowing them to detach and migrate, is a hallmark of the core cancer associated concept epithelial-to-mesenchymal transition. The extent of lost EphB receptor expression is associated with colorectal cancer prognosis (Jang et al., 2020). Why EphB expression is downregulated is unknown, as Wnt signaling is still active in carcinomas (Batlle et al., 2005). EphB receptors are expressed in prostate cancer but their role in tumor formation and progression is less characterized then in colorectal cancer.

In general, metastases from patients with CRPC contain larger numbers of mutations compared to primary tumors (van Dessel et al., 2019), suggesting that these mutations may promote formation of metastases. Alterations in *AR* is common in metastases, along with mutations in *TP53*, *PTEN*, *Retinoblastoma protein* (*RB1)*, *MYC*, *APC* and DNA repair genes (van Dessel et al., 2019). Although the first metastasis is formed from the primary tumor, formation of additional metastases is a dynamic process in which subclones from one or more metastases can form another, resulting in intermingling and polyclonal metastases (Gundem et al., 2015). Due to difficulties in material acquisition, most studies on prostate cancer metastases have been performed in patients with CRPC and are therefore biased toward also identifying treatment induced alterations in the genome, since inhibition of androgen signaling e.g., can induce DNA damage, as AR promotes DNA repair (Polkinghorn et al., 2013). Targeted sequencing of circulating tumor DNA in patients with *de novo* metastatic cancer show a similar mutational profile as CRPC, but without *AR* alterations (Vandekerkhove et al., 2019). Mutations in *AR* are also uncommon in tumor tissue from patients with castration sensitivity disease but are strongly associated with swift development of CRPC (Stopsack et al., 2020). Loss of any of *RB1*, *TP53* and *PTEN* are more common in metastatic CRPC (92%) compared to metastatic (63%) and localized (39%) castration sensitive prostate cancer (Hamid et al., 2019). In summary, genetic mutations may promote a metastasis friendly phenotype in tumor cells but can only partly predict poor prostate cancer prognosis.

In prostate tumors, its phenotype is of importance when determining prognosis. Of all prostate cancers, the majority are adenocarcinomas with AR and *KLK3*/prostate specific antigen (an AR target gene) expression, but 2% have a neuroendocrine phenotype accompanied with poor prognosis (Wang et al., 2021). In addition to Gleason score, cribriform architecture and intraductal growth patterns of tumors are histological features associated with worse outcome (Kweldam et al., 2016). Patients with a large number of structural anomalies in the tumor genome or multiple subclones formed from a common cancer cell-of-origin are more likely to have aggressive disease features (Espiritu et al., 2018; Lalonde et al., 2014). Using a combination of expression of selected genes, e.g., with the Decipher test, it is possible to improve prognostic estimations compared to using standard clinical variables (Jairath et al., 2021). Integrating measures of genomic instability with presence of hypoxia in tumors increased the precision of predicting disease recurrence after local treatment (Lalonde et al., 2014). Combining tumor genomics, epigenomics and other measures to understand prostate cancer biology and prognosis hold great promise for development of assays to determine prognosis.

3.2 CANCER PROGRESSION AND PROGNOSIS: THE IMMUNE SYSTEM AND VIRUSES

Although mutational accumulation can drives colon cancer development, genomic heterogeneity of these tumors cannot be used to predict prognosis (Schmitt and Greten, 2021). In contrast, components of the tumor microenvironment, including the composition of immune cells and their function, is tightly linked to colorectal cancer mortality and response to therapies (Schmitt and Greten, 2021). Innate immune cells, and adaptive immune cells, including cells of the B- and T-cell lineages, can both be present within tumors and surround them. Cells of the B-cell lineage produce a variety of antibodies that e.g., can neutralize viruses and act as regulators of T-cells. T-cells ($CD3^+$) can broadly be classified into cytotoxic ($CD8^+$) T-cells and helper ($CD4^+$) T-cells of which several subtypes exist including memory T-cells ($CD8^+$ and $CD4^+$) and regulatory T-cells (mostly $CD4^+$). An ‘immunoscore’, measuring the density and location of $CD3^+$ and $CD8^+$ T-cells can predict colorectal cancer prognosis. In most solid tumor types, including colorectal cancer, presence and magnitude of T cell infiltration is associated with favorable prognosis (Fridman et al., 2017), suggesting that they have the capacity to identify cancer as foreign and fight it. Adaptive immune cells in tumors may specifically detect tumor epitopes such as tumor neo-antigens but may also be bystander cells that respond to non-tumor epitopes, such as viruses (Rosato et al., 2019).

For prostate cancer, an association between T-cell infiltration and prognosis is unclear, with conflicting data showing associations with both worse (Ness et al., 2014; Petitprez et al., 2019; Zhao et al., 2019) and improved prognosis (Yang et al., 2021). Specifically, regulatory helper T-cells ($CD4^+FOXP3^+$) in prostate cancer is associated with adverse outcome (Davidsson et al., 2013). Through the use of single-cell RNA-sequencing techniques, the immune composition of prostate cancer is emerging (Chen et al., 2021; Tuong et al., 2021) and support a fairly "suppressed" immune state (Tuong et al., 2021). This idea is also corroborated by prostate tumors having low expression of genes encoding for granzyme B and perforin, two proteins that are used by active $CD8^+$ T-cells (Rooney et al., 2015).

Naïve T-cells, cells that have the capability of encountering and responding to antigens, develop in the thymus. Here, a complex process ensues involving positive selection of well-functioning T-cells and negative selection of autoreactive T-cells. Every naïve T-cell have a unique TCR, of which the majority are composed of alpha (α) and beta (β) chains. Part of TCRα and TCRβ are complementary determining regions, that determine antigen specificity. When a naïve T-cell encounters an antigen presented by a cell, it does so in the context of HLA-type and will only proliferate, clonally expand, and differentiate in the presence of a particular HLA-type and epitope. The highly polymorphic HLA genes are responsible for the large diversity in immune responses among individuals and populations. Classical HLA genes are divided into major histocompatibility complex (MHC) I (*HLA-A*, *HLA-B*, *HLA-C*) and MHC II genes (including *HLA-DPB1*, *HLA-DQA1*, *HLA-DQB1*, *HLA-DRB1*) of which two alleles are present in all individuals.

$CD8^+$ T-cells recognize antigens presented by MHC class I, which can be found on all cell types with nuclei. Upon receiving proper co-stimulatory signals, $CD8^+$ T-cells can kill a cell containing foreign antigen. $CD4^+$ T-cells recognize antigens presented by MHC class II, which are typically expressed by subsets of immune cells, but can e.g., also be aberrantly expressed by cancer cells. When $CD4^+$ T-cells recognize a foreign antigen, they can produce signals such as cytokines to communicate with other immune cells. Particular HLA alleles are associated with development of autoimmune diseases and can also be associated with increased or decreased risk of certain cancers (Dendrou et al., 2018). In general, HLA-type is associated with the specific mutations found in a tumor (Marty et al., 2017b) and allow mutations to be visible or not for T-cells as tumor mutation epitopes are visualized in the context of MHC. As a mechanism of immune escape, prostate cancer cells can reduce HLA protein expression, which is associated with poor prognosis (Ylitalo et al., 2017).

HLA and viral immunity are tightly linked. For example, T-cells can only respond to the CMV pp65 epitope NLVPMVATV in carriers of HLA-A*02:01. Variability in HLA-type is associated with differential susceptibility for infectious diseases and autoimmune diseases, implying that HLA-type can determine functional outcomes of anti-viral immunity (Blackwell et al., 2009). It has been estimated that 5-30% of all memory T cells in blood, which is an enormous amount, are specific for CMV in CMV seropositive individuals (Sylwester et al., 2005). The size of the antiviral T-cell response and titers of CMV targeted IgG antibodies increase with age (Komatsu et al., 2003; Parry et al., 2016), which is proposed to be dependent on increased frequency of subclinical micro-reactivation events from e.g. monocytes in blood (Parry et al., 2016). On the other hand, CMV directly modulate immune pathways including type I interferon signaling and antigen presentation so that the virus is not eliminated (Elder et al., 2019).

CMV is a major determinant of immune functions throughout life (Brodin et al., 2015; Looney et al., 1999). An active anti-CMV immune response may boost the immune system in young people (Furman et al., 2015). Young CMV seropositive people have a greater response to seasonal influenza vaccination and a bacterial toxin than young CMV seronegative people (Furman et al., 2015; Pera et al., 2014). In contrast, an active anti-CMV immune response has been associated to poor health and increased mortality in the elderly, in particular in patients with cardiovascular disease (Olsson et al., 2000; Savva et al., 2013; Simanek et al., 2011; Strandberg et al., 2009; Wang et al., 2010). As humans grow old the immune system weakens, which has been suggested to be CMV dependent, although recent evidence questions this paradigm (den Elzen et al., 2011; Furman et al., 2015; Marandu et al., 2015; Moss, 2019; Smithey et al., 2018). These proposed systemic effects of CMV could also have profound effects on tumor microenvironments and cancer progression. T-cells specific to pathogens such as viruses can not only be detected in circulation, but also in tissues and cancer (Masopust et al., 2001; Rosato et al., 2019). Resident memory T-cells expand upon local reactivation of herpes simplex virus-2, to counteract virus production (Wakim et al., 2008). CMV specific T-cells are present in tissues, but their role in the fight against viral re-activation events (Gordon et al., 2017) and potential tumor altering effects are unclear.

Associations between CMV immunity and cancer outcomes can be examined in epidemiological studies. In general, parts of the world with relatively low CMV seroprevalence such as Australia, Europe and North America, have a lower number of prostate cancer deaths per diagnosed prostate cancer case than parts of the world in which CMV seroprevalence is higher, such as in Africa, Asia and South America (Wong et al., 2016; Zuhair et al., 2019).

Surely, this association may be explained by a large number of factors including heterogenous availability to health care and methods of diagnosing cancer, treatment options and genetic differences. Interestingly, CMV seropositivity is associated with increased cancer mortality in men, but not women, in the North American large population cohort study National Health and Nutrition Examination Survey (NHANES) III (Okedele et al., 2020). Preliminary results from a collaborative unpublished study indicate that CMV seropositive men have higher risk of dying from prostate cancer than CMV seronegative men, even after adjusting for age and potential confounding factors such as smoking and surrogate variables of socioeconomical status. These observations suggest that there could be an association between CMV and prostate cancer mortality, a connection that is intriguing to explore further.

Several attempts have been made to examine direct roles of CMV for cancer phenotypes. The CMV viral load found in gastric tumors is positively correlated to development of lymphatic metastases (Zhang et al., 2017). In mice, latent murine cytomegalovirus infection can increase the number and size of lung metastases seeded from primary breast tumors (Yang et al., 2019). Super-infecting the prostate cancer cell line PC3 have been proposed to promote expression of the proliferation marker ki67, the pro-survival protein Bcl-2 (Samanta et al., 2003) and promote cell invasion in *in vitro* experiments (Blaheta et al., 2006). High abundance of CMV IE protein expression in glioblastoma is proposed as a marker of poor prognosis (Rahbar et al., 2013). Overexpression assays with IE1, pp71, US28 or gB in glioblastoma cells have indicated that these proteins have properties that can promote proliferation or cancer cell invasion (Rahman et al., 2019). Several CMV genes have known anti-apoptotic properties during acute infection *in vitro*, including UL36, UL37 and UL38 (Hein and Weissman, 2022). To understand if these findings are biologically meaningful, a comprehensive view of presence, characteristics and function is needed since evidence in isolation are difficult to interpret.

4 TREATMENT OF ADVANCED CANCER

The five pillars of cancer treatment are surgery, radiation, chemotherapy, immunotherapy, and targeted therapy. Surgery and local radiation of metastases have proven to be valuable and effective treatment strategies for advanced epithelial cancers. Pharmacological treatment options of advanced epithelial cancer have significantly increased the last decades. Fifteen years ago, the options were few for patients with advanced prostate cancer, with androgen deprivation therapy including the anti-androgen bicalutamide as clinical practice. Today, there are multiple options. Chemotherapeutic agents, second-generation anti-androgens, PSMA-targeted therapy, and PARP inhibitors all have proven effects on prostate cancer outcome (Yamada and Beltran, 2021). Although these new agents have increased prostate cancer survival, drug resistance is inevitable and advanced prostate cancer is still incurable. Finding drugs that can overcome resistance will be important to further improve prostate cancer survival and perhaps find a cure.

Virus-infected tumors have been proposed to respond well to immunotherapy, since viral proteins expressed in cancer cells could be recognized by the immune system and allow cell killing (Nghiem et al., 2016). Unfortunately, targeting T cells with immunotherapy with immune checkpoint blockade has not yet shown clear benefits in prostate cancer except for very rare super responders (Yamada and Beltran, 2021), nor in brain tumors (Preusser et al., 2015), both tumor types potentially associated with CMV. Novel types of immunotherapies, including CAR T-cell therapy, are under development and in early phase clinical trials of prostate cancer. T cell targeted immunotherapy has been very successful for other tumor types and somehow their potential to fight prostate cancer might be awakened.

4.1 TARGETED THERAPIES IN PRECISION ONCOLOGY

Precision oncology entails defining the molecular profile of tumors and including this information when deciding on treatment for a patient. Most biomarkers that define subsets of cancer patients in general responding to targeted therapies are alterations in the genome, including *BRCA2* mutated tumors responding to PARP inhibitors and tumors with defects in mismatch repair responding to immune checkpoint blockade with pembrolizumab (Ku et al., 2019; Yamada and Beltran, 2021). Some alterations and drug responses, such as these two examples described, are common to several tumor types. Some tumor alterations are rare, so to study drug responses, patients across tumor types are grouped together and studied in e.g., so called basket trials (Mateo et al., 2022), under the naïve assumption that the tumor alterations are functionally similar across cancers. For lung cancer patients, a large number

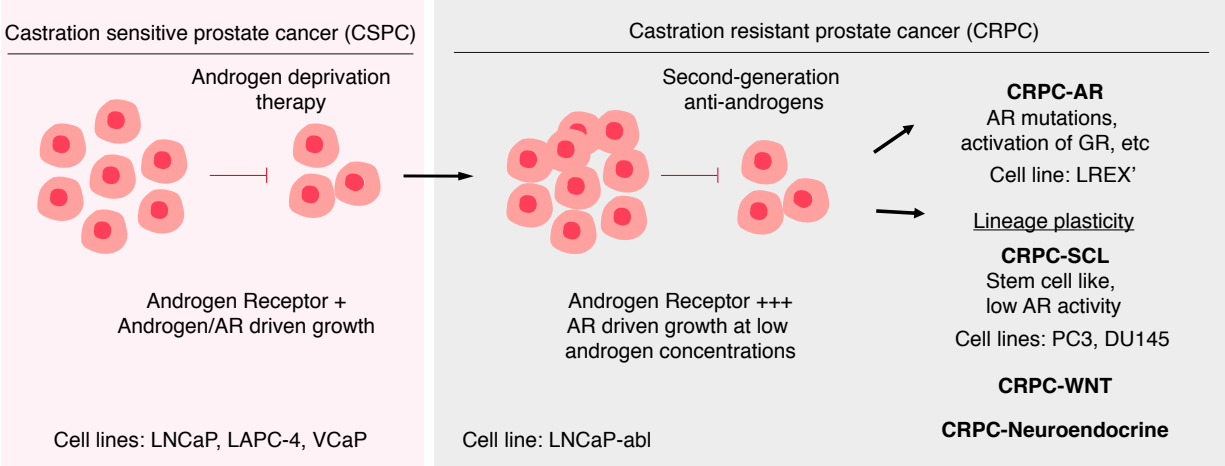

Figure 1. *Prostate cancer cell line phenotypes and resistance mechanisms to anti-androgen therapy. AR: Androgen Receptor. GR: Glucocorticoid Receptor. SCL: Stem Cell Like.*

of genomic alterations, including alterations in *ALK*, *ROS1* and *EGFR*, can be tested and used as guidance for selecting a targeted therapy (Mateo et al., 2022). In prostate cancer, known actionable disease drivers are much fewer. Other types of biomarkers than genomic alterations, including those involving changes in the transcriptome, proteome, microbiome, or particular profiles of the immune system, can potentially be used. In **paper III** and **paper IV,** we study the abundance of CMV and CMV seropositivity as markers of patients that could potentially respond to anti-viral therapies. Efficiency of targeted therapy relies on the biological impact of the therapeutic target and how well the drug works. Heterogeneity of tumor alterations within a cancer patient can limit their detection and drug efficiency. In prostate cancer patients, analyzing molecular features of metastases, which are usually located to bone, is difficult. Surrogate markers of metastases, such as cell-free DNA in liquid biopsies, are being developed to simplify molecular profile analyses of advanced prostate cancer (Mizuno and Beltran, 2022). As for patients with a particular mutational profile, not all patients with virus infected tumor cells will respond to anti-viral treatment, and developing tools to further pinpoint treatment benefactors, for example in liquid biopsies, will be important.

In most patient with treatment-naïve advanced prostate cancer, primary and metastatic tumor cells express AR. Drugs that target and inhibit androgen signaling, i.e., medical castration, are given to all patients with advanced prostate cancer. These include classical GnRH agonists (such as leuprorelin) or novel GnRH antagonists (degarelix, relugolix), with the goal of lowering systemic testosterone levels (androgen deprivation therapy), in combination with second-generation anti-androgens (e.g., enzalutamide or abitarone acetate) (Yamada and Beltran, 2021). The first-generation anti-androgen bicalutamide was used for several decades but has now been replaced. Anti-androgen therapy can with clinical benefit be given in

combination with the chemotherapeutic drug docetaxel (Yamada and Beltran, 2021). AR targeted therapies prolong life of prostate cancer patients, but castration resistance, defined as either s-PSA relapse and/or metastatic outgrowth, inevitably occurs after around 10-15 months (Yamada and Beltran, 2021). Therefore, an understanding of how treatment resistance occurs is needed and it is urgent to find new cancer drugs.

4.2 RESISTANCE TO TARGETED THERAPIES

Cancer cells can bypass anti-androgen therapy via multiple mechanisms, resulting in castration resistant clones that survive and thrive. Resistance can either be primary, with all or a fraction of cells having these properties at treatment initiation, or acquired, with all or a fraction of cells gaining resistance during treatment. Some genetic alterations in castration sensitive prostate cancer are associated with early resistance to castration, including *AR* alterations in rare patients, *MYC* and *TP53* mutations and alterations in *NOTCH* genes, suggestive of primary resistance (Stopsack et al., 2020). Acquired genomic alterations of *AR* are very common, including amplification of *AR*, amplification of an *AR* enhancer, point mutations in the *AR* gene (Ku et al., 2019). Other adaptations to maintain AR activity include increased intratumoral androgen production, increased expression of the glucocorticoid receptor, or increased AR expression (Ku et al., 2019). In addition, detection of the constitutively active *AR* splice variant *AR-V7* in circulating tumor cells, is associated with early resistance to second-generation anti-androgens in patients with CRPC (Antonarakis et al., 2014; Armstrong et al., 2019). Creative ways to inhibit sustained AR signaling in CRPC are being explored, including blocking RORγ, a transcriptional regulator of AR (Wang et al., 2016) and utilizing novel technology to induce specific degradation of AR protein (Han et al., 2019).

Some patients with metastatic CRPC develop phenotypes that are AR signaling independent, have poor prognosis, and are challenging to treat. These tumors have altered their cellular phenotype from a luminal cell like adenocarcinoma, in a paradigm called lineage plasticity. Non-AR driven CRPC metastases are e.g., associated with increased proliferation, DNA repair and inflammation (Thysell et al., 2022) and can be divided into neuroendocrine, Wnt dependent, or stem cell-like subtypes (Tang et al., 2022). After AR active/dependent CRPC, representing around half or CRPC cases, stem cell-like CRPC is the next most common subtype with up to 30% displaying this phenotype (Tang et al., 2022). Neuroendocrine CRPC, reminiscent of small-cell lung cancer, is a well-studied phenotype that can clearly be distinguished histologically by expression of specific markers such as synaptophysin and chromogranin A and most often exhibit low AR signaling activity (Ku et al., 2019). The

plethora of possible resistance phenotypes, that can theoretically all co-exist in a patient, makes this disease state particularly difficult to treat. Intense research efforts are directed towards understanding resistance mechanisms and finding novel treatment options for this deadly disease state (Ku et al., 2019).

Several prostate cancer models exist to study its biology, resistance mechanisms and treatment strategies, including cell lines, organoid cell lines and rarely used patient derived xenograft models. The cell lines LNCaP, VCaP and LAPC-4 are derived from CRPC, but exhibit AR dependent growth *in vitro* and as xenografts *in vivo* as they respond to anti-androgen therapy. LNCaP kept in androgen deprived conditions become resistant to initial growth inhibition and start to grow again accompanied by altered properties such as increased AR expression (established cell line called LNCaP-abl) (Culig et al., 1999). Increased AR expression drives resistance to first-generation anti-androgen therapy (Chen et al., 2004). Further selective pressure from second-generation anti-androgen therapy *in vivo* can result in resistance driven by GR, that can take over features of AR signaling (established cell line called LREX') (Arora et al., 2013) or lineage plasticity upon additional genetic insults in e.g., *TP53* and *RB1* (Mu et al., 2017). The cell lines PC3 and DU145 are AR low/negative cell lines classified as stem-cell like CRPC (Tang et al., 2022). Organoids can be used to study all CRPC subtypes, including the more uncommon neuroendocrine and Wnt associated states. Studying cell lines that represent different disease and treatment resistance states allows us to gain insights of broad biological functions of CMV in prostate cancer, as studied in **paper III**, and can get clues to how many patients and pinpoint who will respond to antiviral therapy.

4.3 ANTI-VIRAL DRUGS AS CANCER THERAPY

Aciclovir and ganciclovir are widely used anti-viral drugs that are activated by viral kinases, including the CMV kinase UL97. Activated aciclovir and ganciclovir are nucleoside analogues that inhibit viral DNA synthesis with distinct mechanisms. Once activated through phosphorylation, aciclovir can have anti-cancer effects, even in uninfected cells (Yao et al., 2013). Aciclovir-triphosphate can, with low affinity, inhibit human DNA polymerase, resulting in DNA chain termination, DNA damage and finally cell death (Furman et al., 1984). The ganciclovir pro-drug valganciclovir quickly impaired growth in xenografts of a medulloblastoma cell line that tested positive for CMV (Baryawno et al., 2011), findings that have been questioned (Hortal et al., 2017). The authors suggest that CMV actively replicates in xenografts and that foci with active replication are reduced in number with valganciclovir

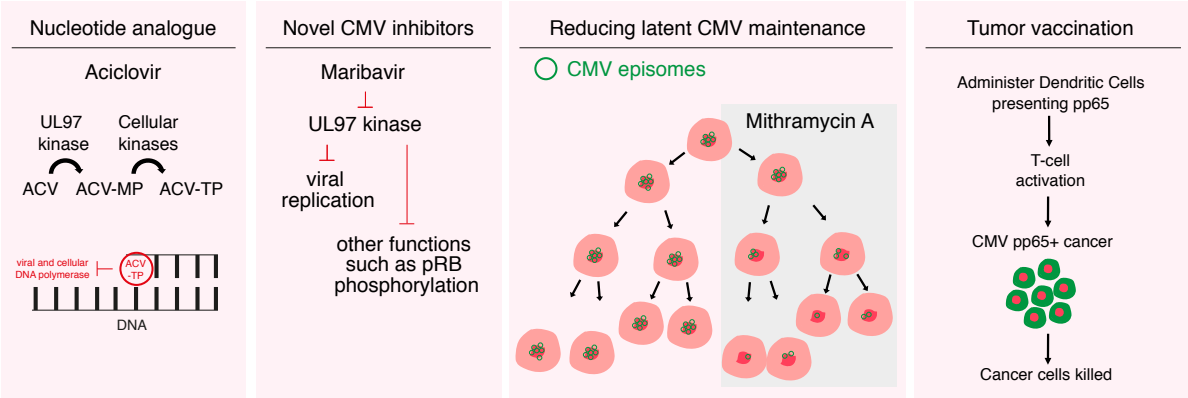

Figure 2. *Examples of therapies targeting CMV that could potentially be used as anti-cancer therapies. ACV: Aciclovir. ACV-MP: Aciclovir-monophosphate. ACV-TP: Aciclovir-triphosphate. pRB: Retinoblastoma protein.*

treatment. One would think that active viral replication would attract immune cells and induce tissue damage, acting an "oncolytic virus", but these phenotypes are not reported.

Injection of murine cytomegalovirus epitopes together with an adjuvant into implanted tumors in mice with persistent cytomegalovirus infection resulted in activation of murine cytomegalovirus specific T-cells, broad immune activation, and remarkable tumor control (Cuburu et al., 2022). Viral peptide treatment of human tumors in a mouse model improved responses to the immunotherapy regimen PD-L1 checkpoint blockade (Rosato et al., 2019). A vaccine containing the CMV immunodominant protein pp65 could provide a similar effect *in vivo*, kickstarting an anti-viral immune response that could be efficient against tumor cells (Batich et al., 2017; Mitchell et al., 2015).

The ganciclovir pro-drug valganciclovir has been clinically tested in a phase II clinical trial for glioblastoma (Stragliotto et al., 2013). The trial did not meet the primary endpoint of alterations in tumor volume after 6 months (Stragliotto et al., 2013), but an additional analysis of survival over a longer period of time found a clear improvement for patients receiving valganciclovir (Soderberg-Naucler et al., 2013). These results have been questioned due to difficulties in study design, selection bias and pit-falls in survival analyses (Wick and Platten, 2014). It is possible that effects of ganciclovir on survival in glioblastoma patients (Soderberg-Naucler et al., 2013) are due to DNA damage and apoptosis of rare CMV infected cancer stem cells (Soroceanu et al., 2015).

Several treatments for tumors infected with CMV are plausible. A toxin bound to CMV-US28 can be internalized by US28 expressing cells and have cytotoxic functions (Krishna et al., 2017; Spiess et al., 2015). CMV-UL138 can downregulate a drug transporter that results in entrapment of the chemotherapeutic drug vincristine within cells (Weekes et al., 2013). Although, vincristine has only shown modest effects on advanced prostate cancer combined

with other agents (Daliani et al., 2003), exemplifying the need for studying CMV related outcomes in specific cellular contexts. Latent CMV is maintained by Topoisomerase IIβ and Specificity Factor 1 in hematopoietic cells and targeting these proteins with the chemotherapeutic drugs ellipticine and mithramycin A respectively, can reduce the ability of CMV to replicate its episomal genome (Tarrant-Elorza et al., 2014). During acute infection, UL56 and UL97 are important for assembling viral particles after viral DNA replication. The CMV-UL56 inhibitor letermovir and the CMV-UL97 kinase inhibitor maribavir are two novel well-tolerated drugs with documented anti-CMV effects in phase III clinical trials (Maertens et al., 2019; Marty et al., 2017a). Depending on expression and function of UL56 and UL97 in cancer, letermovir and maribavir may be explored and repurposed as cancer drugs. Furthermore, effects of emerging vaccines against CMV on cancer risk and cancer mortality will be exciting to explore in the future.

5 RESEARCH AIMS

Paper I To examine if the Abl kinase inhibitor Imatinib is effective in animal models of intestinal cancer and human adenomas *ex vivo*.

Paper II To examine if HLA-type is associated with prostate cancer recurrence after prostatectomy and to decipher its mechanism.

Paper III To examine presence, characteristics, function, and role as a therapeutic target of CMV in the benign prostate and prostate cancer.

Paper IV To examine if CMV serostatus is associated with CMV prostate abundance, prostate cancer incidence and prostate cancer mortality in a prospective cohort study.

6 PRESENT INVESTIGATION

6.1 PAPER I

In **paper I,** we use mouse models of intestinal cancer and *ex vivo* slice cultures of human intestinal adenomas to examine the role of EphB receptor-Abl kinase signaling and the Abl kinase inhibitor Imatinib as a therapeutic for tumor prevention and early intervention. EphB receptors promote proliferation of intestinal epithelial cells through Abl kinase (Genander et al., 2009). Imatinib reduced the number of proliferating cells per adenoma and decreased the number of visible adenomas in $Apc^{Min/+}$ mice. Treatment with ephrin-B2-fc, which acts as an EphB antagonist, reduced the number of small adenomas detected by β-catenin staining, and did not have additive effects to imatinib. This implies that imatinib acts strictly through EphB receptors, and no other group of tyrosine kinase receptors, to inhibit proliferation. Adenomas in $Apc^{Min/+}$ mice do not transform to carcinomas, but the mice have reduced lifespan due to bleedings from large intestinal tumors. Long-term imatinib treatment increased the lifespan of $Apc^{Min/+}$ mice. Short-term imatinib treatment of *ex vivo* slice cultures of adenomas from FAP patients reduced phosphorylation of Abl kinase, as expected, and reduced markers of proliferation. These data suggest that imatinib has translational potential to reduce adenoma size and/or adenoma burden.

$Apc^{Min/+}$ mice sporadically develop adenomas. To determine if imatinib could inhibit tumor initiation in a more controlled setting, transgenic animals in which the *Apc* gene was conditionally deleted in intestinal stem cells (Lgr5-GFP-$CreER^{T2}$/$Apc^{fl/fl}$) were used. In this CreER-LoxP system, tamoxifen administration allows for Cre to relocate to the cell nucleus from the cytoplasm in Lgr5 expressing cells. Here, Cre acts as a recombinase to remove exon 14 from the *Apc* gene, resulting in a frame shift mutation and inactive Apc protein (Shibata et al., 1997). In this model, in contrast to $Apc^{Min/+}$ mice but similar to mice in which *Apc* gene expression is knocked down using short hairpins (Dow et al., 2015), mice develop tumors in the colon in addition to small intestine. Adenomas in the colon, but not small intestine, of imatinib treated animals were smaller and fewer.

EphB receptors promote adenoma proliferation but also suppress the transition to carcinoma by regulating E-cadherin and subsequent tumor compartmentalization (Cortina et al., 2007). Loss of EphB receptor expression results in carcinoma development and invasive growth (Batlle et al., 2005). When targeting EphB receptors with a drug, it is crucial that the tumor

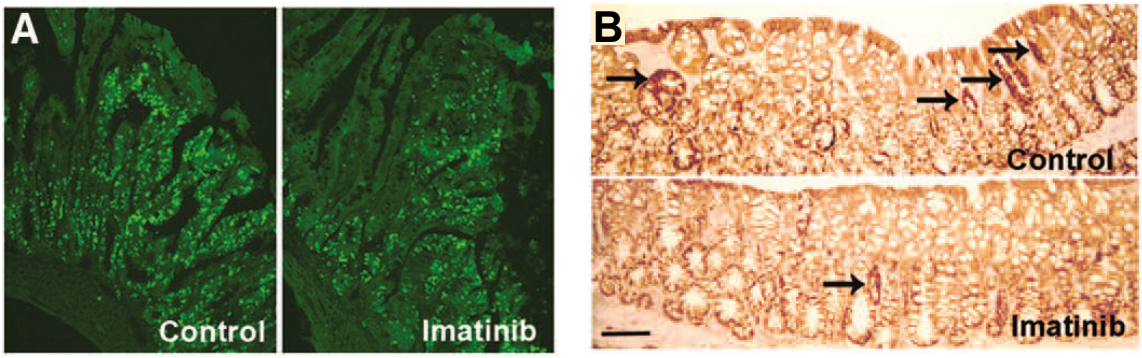

Figure 3. A. *Imatinib treatment reduces the number of BrdU-incorporating cells in small intestinal adenomas in* $APC^{Min/+}$ *mice.* ***B.*** *Imatinib reduces the number of adenomas in Lgr5-GFP-*$CreER^{T2}$*/*$Apc^{fl/fl}$ *mice. Adapted from (Kundu et al., 2015). Reprinted with permission from AAAS.*

suppressor function is maintained. Imatinib increased EphB receptor expression in adenomas of $Apc^{Min/+}$ mice and did not alter pathways associated with cytoskeletal regulation in a microarray gene expression screen. These findings suggest that EphB's tumor suppressive functions are not perturbed by imatinib.

We then examined anti-tumor effects of imatinib in two mouse models of intestinal carcinoma. In the first model, *Apc* loss, *Trp53* (encoding p53) and *Kras* mutations were conditionally induced in cells expressing Villin, which includes intestinal epithelial cells (Villin $Cre^{ER}Apc^{fl/+};p53^{fl/R172H};KRas^{G12D/+}$). Imatinib did not increase lifespan of these mice, a crude measure of its effects. In intestinal organoids from Villin $Cre^{ER}Apc^{fl/fl};p53^{fl/fl}$ mice (Sato et al., 2009) transplanted into nude mice, imatinib reduced tumor growth, but did not show crude effects on invasion, determined histologically. Although these results rule out striking effects of imatinib in models of colorectal cancer, it will be important to determine what cellular processes are altered, e.g., if imatinib can restore EphB receptor expression in carcinomas and promote a more adenoma-like phenotype. Since carcinomas contain more alterations than adenomas, it is possible that imatinib may have EphB independent effects, if any. To further explore effects of imatinib as treatment of human adenomas and carcinomas, it would be possible to establish long-term organoid cultures or patient-derived xenografts from different stages of tumor development and thoroughly examine drug-induced phenotypes molecularly as well as histologically *in vitro* and transplanted to mice *in vivo*. In these models, the genetic and epigenetic heterogeneity of tumors are possible to study, making results more generalizable than using genetically engineered mouse models.

Imatinib is a well-tolerated drug that is most often safe to use long-term, but severe adverse effects such as cardiotoxicity can sometimes develop (Kalmanti et al., 2015). The human equivalent dose given to mice in **paper I** was lower than the standard dose given to patients with regular intake (300mg/day instead of 400mg/day) but was still effective (Kundu et al., 2015), suggesting that a lower dosage with potentially reduced risk of severe side effects could be used for FAP patients. Short term treatment may also be efficient in reducing tumor burden. Further understanding of underlying biological mechanisms of tumor growth and EphB signaling can guide in the search of more specific and less toxic targeted therapies. For example, EphB receptors cooperates with EphA4 receptors to promote proliferation, and EphA4 can be inhibited with a selective antagonistic peptide (Jurek et al., 2016).

6.2 PAPER II

In **paper II,** we use next-generation sequencing data, bioinformatic tools and viral immunity screening methods to find markers and mechanisms of prostate cancer progression. The prostate gland and prostate cancer are inhabited by a multitude of immune cells, including cells of the T- and B-cell lineage (Sfanos et al., 2018). HLA complexes present specific antigens, including viral antigens, on cell surfaces to T-cells with matching T-cell receptors. In patients diagnosed with *de novo* metastatic prostate cancer, the two HLA alleles HLA-A*02:01 and HLA-A*24:02 are associated with prognosis (Stokidis et al., 2020). Other than that, HLA-type is unchartered territory in prostate cancer research.

HLA-genes are highly polymorphic, and identification of specific HLA-alleles require specialized methods. Classically, HLA-type has been determined by complex series of Polymerase Chain Reaction, but other methods including HLA-targeted sequencing are now available and used to determine immune compatibility in stem cell transplant donors and recipients. Bioinformatical tools can retrieve HLA-type from genome wide association studies, exome sequencing, whole genome sequencing and RNA-sequencing data with varying specificity. The tools used in this study, ArcasHLA (Orenbuch et al., 2020) for RNA-sequencing and HLA*LA (Dilthey et al., 2019) for whole genome sequencing data have high specificity and the two HLA-typing methods showed good HLA-typing overlap in 13 studied patients.

We analyzed the next generation sequenced prostatectomy prostate cancer cohorts CPC-GENE and TCGA-PRAD. The HLA-alleles HLA-A*02:01 and HLA-A*11 were associated with disease recurrence after prostatectomy in low-intermediate risk (CPC-GENE; adjusted

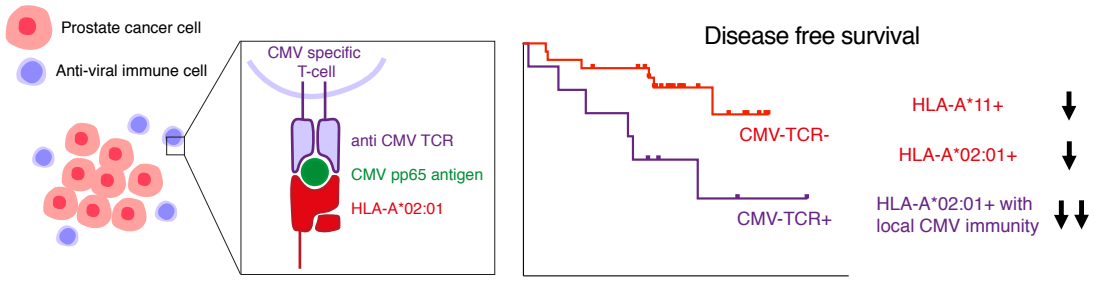

Figure 4. *Summary of study and results in paper II. Adapted from Molecular Oncology.*

hazard ratio (HR) 2.1 95% Confidence Interval (CI) 1.2-3.6, p=0.011) and high-risk (TCGA-PRAD; adjusted HR 5.5 95% CI 3.0-10.1, p<0.001) prostate cancer respectively in Cox proportional hazard models adjusted for Gleason score, s-PSA, T- and N-stage. These results imply that HLA-A*02:01 is indeed associated with prognosis in prostate cancer, as previously reported in patients with *de novo* metastatic disease (Stokidis et al., 2020). Interestingly, HLA-A*02 alleles may also be associated with prognosis of other tumor types, including ovarian cancer (Gamzatova et al., 2006). After validation in independent cohorts, we hope that HLA-type, e.g., determined using a blood test, can be incorporated in models to decipher risk of aggressive prostate cancer.

We found no evidence for a mutation dependent mechanism of cancer progression in HLA-A*02:01 carriers. We examined if adaptive immunity to viruses could be detected in tumors and explain poor prognosis. B-cell mediated anti-viral immunity was detected in prostate glands with the IgG antibody screening method VirScan (Xu et al., 2015) and Enzyme-Linked Immunosorbent Assay. Software such as MiXCR (Bolotin et al., 2017) can be used to infer TCRs and B-cell receptors from next generation sequencing data. The number of TCRs detected per prostate tumor was low and reflect the sparsity of their gene expression in T-cells. The full repertoire of TCRs in prostate cancer will therefore be underestimated with bulk RNA-sequencing. Sequencing single T-cells sorted from prostate cancer and using specific TCR amplification protocols, or screening T-cell binding capacity to viral epitopes would result in higher accuracy and information on phenotypes, that was not possible in this study. TCR sequences with known epitope binding specificity are compiled in the VDJ database (Bagaev et al., 2020; Shugay et al., 2018). This database naturally contains more information for the most studied viruses, epitopes and HLA-types than rarely studied ones, biasing our results. We detected local T-cell immunity towards CMV, EBV and Influenza A in around 20% of tumors respectively in HLA-matched donors.

There was no association between detection of a local T-cell response to Influenza A nor EBV immune response and disease-free survival, but notably, patients with a local T-cell response to CMV had particularly poor disease-free survival. In other types of tumors, the seemingly opposite has been described, whereby antiviral T-cell activation can promote anti-tumor responses (Rosato et al., 2019; Strickley et al., 2019). The phenotypic state of anti-CMV T-cells in prostate cancer is unknown, but anti-CMV $CD8^+$ T-cells in other sites of the body are mainly effector memory and terminally differentiated effector T-cells (Gordon et al., 2017). They may act to perturb the tumor microenvironment, as they may do systemically (Moss, 2019), and promote a pro-tumor milieu that allows cancer cells to escape the prostate gland and form metastases. Bystander tumor infiltrating $CD8^+$ T-cells that respond to e.g., EBV or CMV in colorectal and lung cancer can have a distinct phenotype to neoantigen specific T-cells that implies that they are not actively stimulated by antigens (Simoni et al., 2018). Nevertheless, CMV could potentially be present in the prostate and exert direct effects on prostate cancer cells (Samanta et al., 2003). I thoroughly explore this scenario in **paper III.** Anti-CMV immune response as a risk factor of prostate cancer prognosis is further examined in **paper IV.**

This study is limited by a small sample size and to analysis of surrogate markers of prostate cancer outcomes, e.g., biochemical recurrence in the absence of detectable metastases by standard imaging. Antiviral T-cell responses in HLA-A*11 carriers are not well studied, and it was therefore not possible to investigate these with the current study design.

6.3 PAPER III

Paper III is a comprehensive study of the presence, characteristics, and function of CMV in prostate cells and its role as a prostate cancer therapeutic target *in vitro* and *in vivo*. To characterize CMV infection in prostate tissue, we collected prostates from post-mortem donors, prostatectomy samples from prostate cancer patients and prostate cancer bone metastases. As for latent CMV in hematopoietic cells (Cheng et al., 2017), CMV is difficult to find by bulk RNA-sequencing and whole genome sequencing in prostate tissue (Shnayder et al., 2018; Tang et al., 2013; Zapatka et al., 2020). In contrast, some quantitative polymerase chain reaction assays specifically identified CMV DNA and CMV proteins were detected by immunoblot. CMV DNA^+ cells in prostate epithelial cells *in situ* expressed CMV protein and several CMV proteins were detected with immunohistochemistry with concordant expression patterns. These findings indicate that latent/chronic CMV can inhabit the prostate gland, in which CMV proteins are expressed and virion production is rare. Abundance of CMV in prostates was heterogenous, with a mean 46% of epithelial areas being infected (n=41). Primary prostate

tumors and bone metastases are also often, but not always, infected with CMV. Whole areas of glands or patches of cells could be infected. Our findings indicate that the prevalence of CMV in prostate cancer is overestimated in (Samanta et al., 2003) who describe CMV-IE1 protein detection in 20 of 20 biopsies containing prostate intraepithelial neoplasia and/or cancer.

Endogenous CMV was detected in cell lines derived from advanced prostate cancer. Presence of CMV in cell lines was validated by reduced CMV protein expression and function after gene specific knock down with small interfering RNA. CMV persists through interactions between cellular proteins and the core CMV gene locus *UL122-UL123* (Collins-McMillen et al., 2018; Tarrant-Elorza et al., 2014). Knock down of *UL122-UL123* reduced CMV abundance in prostate cancer cell lines, allowing us to study functional outcomes of CMV loss. In seven of nine cell lines, CMV loss induced apoptosis, reminiscent of oncogene addiction (Dhanasekaran et al., 2021) and an observed phenotype upon *UL122-UL123* knock down in endogenously CMV infected glioblastoma stem cells (Soroceanu et al., 2015). From these data, it is difficult to establish that CMV would be a cancer-causing virus since the viral dependency may have arisen late in tumor progression. Up until recently it has not been possible to grow cell lines as e.g., organoids from primary prostate cancer (Gao et al., 2014; Karkampouna et al., 2021). In such models we could explore this hypothesis further. CMV positively regulated *AR* and *AR-V7* expression and promoted AR driven proliferation. Reduction of CMV-UL97 protein partly mimicked CMV loss and reduced cell viability in cell lines with CRPC phenotypes. It is therefore possible that CMV can promote resistance phenotypes (Watson et al., 2015).

The anti-herpes virus drug aciclovir had modest anti-cancer effects in pre-clinical models of prostate cancer, but we found no striking association between aciclovir usage and reduced prostate cancer mortality in a large Danish population study (HR: 0.95 (CI95% 0.90-1.01), p=0.09). This population study may be biased by confounding by indication, as e.g., varicella zoster reactivation, a common indication for aciclovir use, may itself alter prostate cancer outcomes. Men receiving care for shingles have a slightly higher risk of being diagnosed with prostate cancer (Tsao et al., 2020). The chemotherapeutic DNA-intercalating drugs ellipticine and mithramycin A, which had previously been used to study CMV persistence in hematopoietic cells (Tarrant-Elorza et al., 2014), reduced CMV abundance in models of prostate cancer and promoted CMV dependent anti-cancer effects on cell survival and proliferation. These drugs may be highly toxic, limiting their potential clinical use. In contrast, the novel anti-CMV drugs letermovir (inhibiting UL56) and maribavir (inhibiting UL97 kinase), developed to reduce active CMV replication, are well tolerated drugs (Maertens et al.,

2019; Marty et al., 2017a). Maribavir reduced cell viability in a larger number of cell lines than letermovir, largely mimicked effects of UL97 kinase knock down and showed anti-cancer effects in an *in vivo* model of prostate cancer.

6.4 PAPER IV

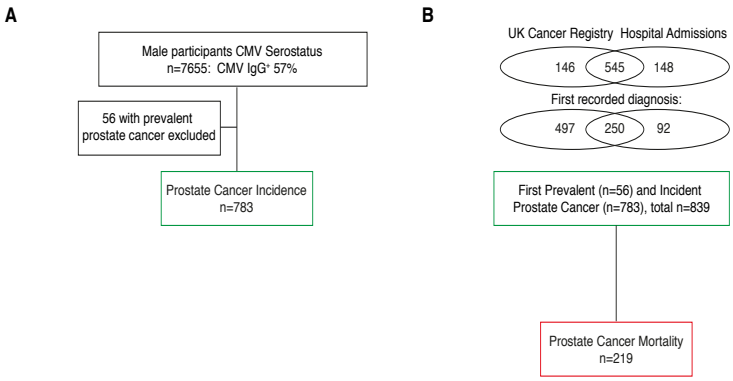

Figure 5. *Flow chart of study design in male CMV cohort nested in EPIC-Norfolk. A) All study participants followed until prostate cancer incidence. B) All patients with prostate cancer followed to prostate cancer mortality.*

In **paper IV**, we explored if CMV abundance in prostate tissue was associated with CMV serostatus and if CMV IgG seropositivity was associated with prostate cancer outcomes. CMV was present in a larger portion of prostate epithelium in CMV seropositive (n=24) than CMV seronegative (n=17) post-mortem donors. Primary prostate tumors in prostatectomy specimens were homogenously infected in CMV seropositive patients (8/10 tumors >90% CMV abundance) and CMV was often less abundant in CMV seronegative patients. CMV in seronegative individuals is well-documented (Hendrix et al., 1997; Kraat et al., 1992; Larsson et al., 1998; Mendelson et al., 1996; Roback et al., 2001; Stanier et al., 1989; Taylor-Wiedeman et al., 1991; Zhang et al., 1995), and may reflect an altered infection state with very few reactivation events that triggers a low-level CMV immune response (Ashokkumar et al., 2020; Litjens et al., 2017; Loeth et al., 2012; Terlutter et al., 2018). Due to the heterogenous pattern of CMV infection in prostate cancer, a large area of tissue may be required to accurately determine its abundance. CMV serostatus therefore provided an easily evaluated potential surrogate marker for CMV abundance in prostate.

We determined if CMV seropositivity was associated with risk of prostate cancer incidence and prostate cancer mortality in the European Prospective Investigation of Cancer (EPIC)–Norfolk cohort study. Of 7655 men aged 40-81 years old, 57% were CMV seropositive at baseline and were followed for a mean of 18 years (Gkrania-Klotsas et al., 2012, 2013). Date of prostate cancer diagnosis was identified through the UK cancer registry or by hospital admissions and prostate cancer as underlying cause of death was determined from death certificates. All time to event analyses were performed in men with two years or more follow

up time, to reduce bias from technical errors of delayed registration of prostate cancer diagnoses close to time of death.

In cox proportional hazard regression models adjusted for age and potential confounders, CMV seropositive men did not have higher risk of being diagnosed with prostate cancer (adjusted HR 1.03, 95% CI 0.89-1.19, p=0.687). This finding supports previous research that find no association between DNA virus immunity and prostate cancer occurrence (Huang et al., 2008; Korodi et al., 2005; Sutcliffe et al., 2007; Sutcliffe et al., 2012). Although we cannot exclude that CMV is oncogenic in specific settings, our findings here suggest that CMV in not a main cause of prostate cancer. CMV seropositivity was associated with increased risk of dying from prostate cancer in patients diagnosed with prostate cancer (adjusted HR 2.20, CI 95% 1.04-4.64, p=0.039). These findings suggest that CMV seropositivity is a biomarker of poor prostate cancer prognosis.

Strengths of this study include long follow-up time, no loss to follow-up other than death and high data completeness. In this prospective cohort it is possible to postulate causality, as CMV serostatus is determined prior to prostate cancer diagnosis or death, in contrast to a case-control study (Sutcliffe et al., 2012). Prostate cancer diagnosis data is valuable and information that e.g., is not available in the north American prospective population cohort NHANES III, which contains information of CMV serostatus (Okedele et al., 2020). Limitations include a homogenous study population preventing generalizability to other ethnicities than white males in the UK. Timing of CMV serology testing is a limitation, as it in most cases preceded prostate cancer diagnosis with several years. A fraction of men has surely seroconverted at time of prostate cancer and are therefore falsely allocated as CMV seronegative. It is unclear how many were diagnosed with prostate cancer via s-PSA testing or by showing clinical prostate cancer signs. One can speculate that CMV IgG^- men are more likely to get s-PSA testing as they in general are of higher socioeconomic status than CMV IgG^+ men. But the proportion of patients diagnosed with advanced prostate cancer was not higher in CMV IgG^+ men and adjusting for socioeconomic factors did not reduce the association between CMV IgG seropositivity and prostate cancer mortality, together suggesting that the association is independent of these factors.

The findings in **paper IV** are suggestive of CMV as a potential biomarker for prostate cancer mortality. Perhaps, patients with a particular HLA-type (e.g., HLA-A*02:01) and infiltration of local CMV immunity in prostate tumors, as found in **paper II,** are at highest risk for poor prostate cancer prognosis.

7 ETHICAL CONSIDERATIONS

This thesis includes several types of experiments and analyses of tissues and data from humans. As models of tumors and cancer we studied mouse models, cancer cell lines and cultured tumor tissues. In these models, we performed experiments to decipher biological mechanisms of tumor development and progression and evaluated responses to pharmacological treatments. Blood and tissues from post-mortem donors and cancer patients were analyzed to characterize biological processes. We also used data already synthesized from research study participants, including next generation sequencing data from prostate cancer patients and men with known CMV serostatus in a population cohort, to study prostate cancer prognostic factors.

Human prostate cancer cell lines are derived from prostate cancer patients. To establish human prostate cancer cell lines, they are typically passaged in immune deficient mice, after which they are all cultured in media containing serum from calves. It has proven difficult to eliminate the use of animals in establishing and maintaining cell cultures, even though the research is executed on cells from humans. Experiments were also performed in mouse models of cancer. Animal research can be reduced and refined by careful planning of experiments and using appropriate mouse models for the research hypothesis. However, redundancy and flexibility are needed to avoid unnecessary repetition of experiments. Replacement of animal research to other methods such as advanced *in vitro* systems, including organoids, is a sympathetic goal, but would require both basic scientists and drug agencies to reconsider the research process and requirements for i.e. drug approval. The animal experiments in this study have been planned taking the '3Rs' into consideration. We consider the experiments conducted to be ethically justified due to the lethal nature of colorectal and prostate cancer and the need for research that can result in new treatment options. Animals in experiments are under regular observation and humane endpoints are in place to reduce unnecessary suffering.

For studies on human tissue samples, next-generation sequencing data and population cohorts, careful risk-benefit analyses were performed and all necessary ethical permits for our studies were received. Informed consent was gathered from all study participants. Sharing of data, including next-generation sequencing data, allow us to perform efficient and cost-effective research. Strategies to keep genomic data from which an individual could be identified secure are employed but may need to be updated in the future.

8 CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, we find that HLA-type and CMV immunity are potential biomarkers of prostate cancer prognosis (**paper II and paper IV)**. We describe therapeutic strategies for prevention and treatment of epithelial cancer in its early (imatinib targeting EphB-Abl in intestinal cancer; **paper I**) and advanced stages (CMV-targeted therapies in prostate cancer; **paper III**).

Together with the case studies on imatinib-associated effects on adenomas in FAP patients (Itsukuma et al., 2007; Tobon et al., 2020), our preclinical data on effects of imatinib on early stage intestinal tumor formation and growth (Kundu et al., 2015) suggests that complementing surgery with drug therapy may delay cancer development.

In **paper II,** we find that carrying particular HLA alleles is associated with prostate cancer disease recurrence after prostatectomy. Validation of HLA-A*02:01 and HLA-A*11 as prognostic biomarkers in cohorts with long-term follow up will be important. If validated, HLA-type could be determined in blood tests and potentially be incorporated into prostate cancer risk stratification models.

Our results indicate that CMV does not cause prostate cancer and that we will not be able to prevent its occurrence with anti-viral therapies or by vaccination. On the other hand, we show both observational and experimental evidence that CMV seropositivity is associated with lethal prostate cancer and that CMV infection may directly modulate critical tumor behaviors. An anti-CMV immune response may promote an inflammatory, environment that encourage cancer cells to spread and grow at distant sites. Since CMV seropositive prostate cancer patients have a higher tumor CMV burden, it is also likely that CMV infected cancer cells have more aggressive features than uninfected cells. We have not explored if CMV infection in cancer cells promotes metastatic spread and growth but removing CMV from infected cancer cells results in programmed cell death and growth inhibition, which suggests that CMV promotes general pro-cancer features.

CMVs presence in bone metastases with diverse phenotypes suggest that anti-CMV therapy may have additive effects to current therapies and prolong life in patients that do not respond to treatments given to date. CMV could be targeted in a preventative setting to reduce risk of lethal cancer or in advanced prostate cancer to prolong life. The four tested anti-viral treatment strategies included nucleoside analogues (aciclovir, ganciclovir), chemotherapy (mithramycin A, ellipticine), UL56 inhibitor letermovir and the UL97 kinase inhibitor maribavir all had anti-cancer effects in prostate cancer models. Maribavir, recently approved for clinical use in the US, may be the most suitable candidate for a prostate cancer clinical trial since aciclovir does

not show striking clinical benefits in a large cohort study, chemotherapy can cause severe side effects and letermovir did not show broad efficiency across CMV infected cell lines.

I envision CMV as a precision oncology target. In addition to CMV serostatus, identification of factors that are associated with tumor CMV abundance and the functional heterogeneity of CMV to ultimately predict the response to anti-CMV therapies, will be important. The development of assays for detection of latent CMV in liquid biopsies from cancer patients could be useful to predict treatment response. Characterization of CMV in other tissues and tumor types than the prostate is also on the horizon, for example using pan-cancer tissue microarrays. The examination of CMV associated tumor types will help us understand if CMV has a general role in cancers and as a cancer treatment target.

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