Diploma thesis

Correlation of Metastasis Location and Outcome in Patients Treated with Checkpoint Inhibitors with NSCLC, RCC and Melanoma

submitted by

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Graz, 15.11.2019

Affidavit

I hereby formally declare, that I have written the submitted thesis independently and without any illegitimate assistance from third parties. I confirm, that I used no other than the declared sources for the preparation of this academic work. All used sources have been indicated as such and acknowledged by means of complete references in the text.

Graz, 15th of November, 2019 Patrick Philipp Reimann eh

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Abbreviations

AFP	Alpha-fetoprotein
Alpine-TIR	Alpine Tumor Immunology Registry
APC	Antigen presenting cell
BCL10	B cell lymphoma 10
β_2m	β_2 microglobulin
CARMA1	Caspase recruitment domain containing membrane-associated guanylate kinase protein 1
CEA	Carcino-embryonic antigen
CI	Confidence interval
CR	Complete remission
CRAC	Calcium release-activated calcium
CT	Computer tomographie
cTEC	Cortical epithelial thymic cell
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
DAG	Diacylglycerol
DC	Dendritic cell
DN	Double negative
DP	Double positive
ER	Endoplasmic reticulum
ERAD	ER - associated protein degradation
ERK	Extracellular signal-regulated kinase
FDA	Food and Drug Administration
HBV	Hepatitis B virus

HCV	Hepatitis C virus
HER-2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HR	Hazard ratio
ICAM-1	Intercellular adhesion molecule 1
ICB	Immune checkpoint blockade
ICI	Immune checkpoint inhibitor
IFN-α	Interferon-α
Ii	Invariant chain
IKK	IκB kinase
IL	Interleukin
IP3	Inositol 1,4,5-trisphosphat
IP3R	IP3 receptor
irAE	Immune related adverse event
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
Lat	Linker for activation of T cells
Lck	Lymphocyte protein tyrosine kinase
LFA-1	Lymphocyte function-associated antigen 1
MALT1	Mucosa-associated lymphoid tissue 1
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex

MIIC	MHC class II compartment
MRI	Magnetic resonance imaging
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κB
NK	Natural Killer
NSCLC	Non-small cell lung cancer
ORR	Overall response rate
PD-1	Programmed death-1
PD-L1	Programmed death ligand-1
PD-L2	Programmed death ligand-2
PET	Positron emission tomography
PFS	Progression-free survival
PIP2	Phosphatidylinositol 4,5-bisphosphate
PLCγ1	Phospholipase Cγ1
PR	Partial response
PSA	Prostate-specific antigen
RCC	Renal cell carcinoma
SOCE	Store-operated calcium entry
TAA	Tumor-associated antigen
TAP	Transporter associated with antigen presentation
TGF-β	Transforming growth factor β
TNF	Tumor necrosis factor
TSA	Tumor-specific antigen
TCR	T cell receptor

VEGF	Vascular endothelial growth factor
ZAP-70	Zeta-chain-associated protein kinase of 70 kDa

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Abstract

Introduction: Checkpoint inhibitors play an increasingly important role in today's cancer therapy. The effect of checkpoint inhibitors depends on many factors such as presence of immune cells in tumor metastases and tumor intrinsic properties. We investigated the association between metastatic site and outcome in patients treated with immune checkpoint inhibitors.

Material and Methods: We retrospectively analyzed 103 patients from the Austrian cohort of the Alpine Tumor Immunology Registry (Alpine-TIR), with metastatic NSCLC (72 patients), metastatic RCC (16 patients) and metastatic melanoma (15 patients).

Results: Among those patients 24.3% had bone, 20.4% liver, 31.1% lung, 17.5% suprarenal gland and 7.8% brain metastasis, respectively. The median overall survival from diagnosis to death for all patients was 52.3 month. In the univariate cox analysis, lung metastases (hazard ratio: 2.384, 95% confidence interval: 1.160 - 4.899; p=0.018) correlated with a significantly shorter overall survival. With overall survival calculated from commencing checkpoint inhibitor treatment to death, also liver metastases (hazard ratio 2.374, 95% confidence interval: 1.114 – 5.062; p=0.025) showed a significantly poorer prognosis. Other metastasis location such as suprarenal gland, bone and lymph nodes did not show a correlation.

Conclusions: In conclusion, in the presented cohort, the efficacy of checkpoint inhibitors is dependent on the metastatic location. The existence of liver metastasis was associated with reduced overall survival from start of checkpoint inhibitor therapy, while lung metastasis showed a significantly poorer overall survival from diagnosis. This retrospective analysis demonstrated preliminary data for a predictive effect of location of metastasis in patients treated with immunotherapy.

Abstract - German

Einleitung: Die Entwicklung von Checkpoint-Inhibitoren hat die Tumortherapie in den letzten Jahren sehr stark beeinflusst. Der Einsatz dieser Substanzen führt zur Blockade von wichtigen Signalpfaden und ermöglicht dadurch eine bessere Bekämpfung der Tumorerkrankung durch das Immunsystem. Allerdings ist die Wirkung der Checkpoint-Inhibitoren von vielen unterschiedlichen Faktoren abhängig. Hierzu zählen unter anderem die Konzentration von Immunzellen in den Metastasen und spezifische Mutationen. Diese Arbeit untersucht den Zusammenhang zwischen Metastasenlokalisation und Effektivität der Checkpoint-Inhibitoren.

Material und Methoden: Hierfür wurden im Rahmen einer retrospektiven Analyse 103 PatientInnen aus der österreichischen Kohorte des Alpine Tumor Immunology Registry (Alpine-TIR) untersucht, welche an metastasiertem NSCLC (72 PatientInnen), metastasiertem RCC (16 PatientInnen) und metastasiertem Melanom (15 PatientInnen) litten.

Ergebnisse: Von den untersuchten PatientInnen wiesen zum Diagnosezeitpunkt 24,3% Knochenmetastasen, 20,4% Lebermetastasen, 31,1% Lungenmetastasen, 17,5% Nebennierenmetastasen und 7,8% Gehirnmetastasen auf. Das mediane Gesamtüberleben in der Kohorte lag bei 52,3 Monaten. In der univariaten Cox-Analyse zeigten PatientInnen mit Lungenmetastasen ein signifikant verringertes Gesamtüberleben im Vergleich zu PatientInnen ohne Lungenmetastasen (Hazard ratio: 2.384, 95% CI: 1.160 – 4.899; p=0.018). Das mediane Überleben von Beginn der Checkpoint-Inhibitor-Therapie zeigte sich bei PatientInnen mit Lebermetastasen, im Vergleich zu PatientInnen ohne Lebermetastasen, signifikant verringert (HR=2.374, 95% CI: 1.114 – 5.062; p=0.025). Andere Metastasenlokalisationen wie Knochen, Nebenniere, Lymphknoten und Gehirn zeigten keine statistisch signifikanten Veränderungen.

Diskussion: Zusammengefasst, zeigen die Ergebnisse dieser Kohorte, dass die Effektivität der Checkpoint-Inhibitoren auch von der Lokalisation der Fernmetastasen beeinflusst wird. So zeigte sich in der Studie die Existenz von Lebermetastasen verknüpft mit einem verringerten medianen Überleben. Dieses Ergebnis konnte auch in anderen Studien nachgewiesen werden. Insgesamt

zeigte diese Studie vorläufige Daten, welche einen prognostischen Einfluss der Metastasenlokalisation bei PatientInnen mit NSCLC, RCC und Melanom unter Immuntherapie demonstrieren.

1 Introduction

Cancer is expected to be the leading cause of death in the 21st century. The incidence as well as the mortality is continuously growing world-wide. The reasons are complex and include rapid population growth and aging. Bray and colleagues estimated that there were 18.1 million new cases of cancer and 9.6 million cancer deaths in 2018 throughout the world. They also stated that Europe accounts for 23.4% of all cases and 20.3% of global deaths, whilst representing only 9% of the world-wide population. In Europe in 2018, 2.05 million new cancer cases occurred in men and 1.85 million in women. The leading cancer types of the European population were female breast cancer (13.4%), colorectal cancer (12.8%), lung cancer (12.0%), and prostate cancer (11.5%). The estimated numbers of cancer deaths were 1.08 million in men and 850.000 in women.[1,2]

Lung cancer was still the most common cause of cancer related deaths in the year 2018 in Europe as well as world-wide. Especially in men lung cancer continued to be the most frequent cause of death from cancer (24.8%), while in woman lung cancer was in second place behind breast cancer (14.2% vs. 16.2%). Ferlay estimated 388.000 lung cancer caused deaths, which matches 20.1% of all cancer related deaths, in Europe in 2018. Incidence and mortality vary depending on sex, age, socioeconomic status and geography, with higher rates in North America and Europe where smoking was established earlier. 85% of the lung cancer patients are diagnosed with the subtype NSCLC and a majority of these already suffer from metastatic disease at time of diagnosis.[3] Although incidence and mortality rates are decreasing in most of the European and North American countries, they are increasing in many low- and middle-income countries.[1,2,4]

In Europe, melanoma of the skin was newly diagnosed in about 144.200 patients, accounting for 3.7% of all cancer cases in 2018. 71.200 cases (49%) occurred in men versus 73.000 (51%) in women. The number of melanoma related deaths was 15.200 (1.4%) in men and 11.900 (1.4%) in women. Renal cancer caused 54.700 deaths throughout Europe in 2018 and just over 136.500 new cases were diagnosed, which can be divided into 84.900 (62%) in men and 51.600 (38%) in women. The renal cell carcinoma (RCC) is the most common cancer of the kidney and its incidence is increasing world-wide, with the highest rates occurring in developed countries.[2,5]

1.1 Hallmarks of cancer

While the proliferation of normal cells is modulated through various control mechanisms, malignant cells are able to evade these restrictions. In 2000, Hanahan and Weinberg published the hallmarks of cancer, consisting of six alterations in cell physiology, which are shared by all cancer types an lead to uncontrolled tumor growth. These common traits include the self-sufficiency in growth, insensitivity to growth inhibitory signals and avoidance of apoptosis. Furthermore, cancer cells can establish their own blood supply, have a limitless replicative potential, are able to invade local tissues and develop metastasis.[6] In 2004, Schreiber and co-authors proposed the circumvention of immune-surveillance as another important trait of cancer.[7] This concept was included as seventh hallmark in an update of the study, published in 2011 by Hanahan and Weinberg **(figure 1.1)**.[8]

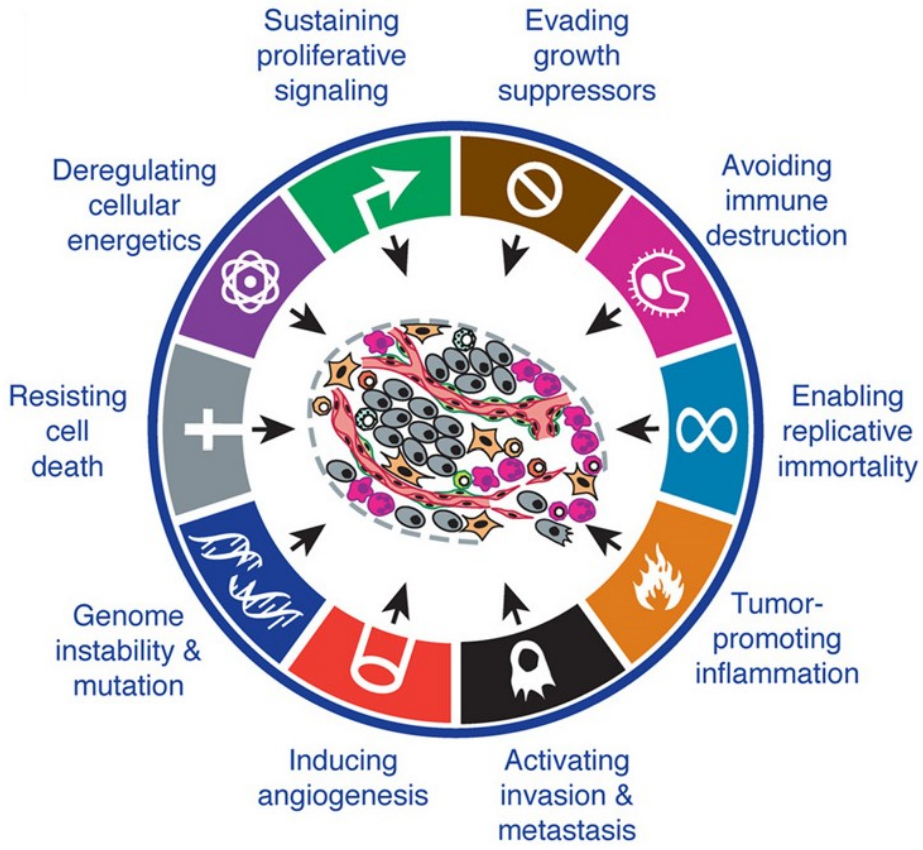

Figure 1.1: The hallmarks of cancer.

(Adapted from Hanahan D and Weinberg RA. 2011 [8])

1.2 Immune-surveillance and cancer immunoediting

Back in 1909, Paul Ehrlich stated, that the immune system plays a central role in the suppression of tumor growth. In mid-20th century tumor transplantation models demonstrated that tumors could be suppressed by the immune system. This evidence prepared the way for the immune-surveillance hypothesis, which was postulated in 1957 by Burnet and Thomas.[9] Immune-surveillance is a process whereby cancerous or precancerous cells are identified by the immune system and eliminated before they can form established tumors.[10] However, the fact that immunocompetent individuals still develop tumors led to the concept of cancer immunoediting.[11] This theory is based on the evidence that the immune system exerts both, host-protecting and tumor-sculpting effects.[11,12] In 2004, Dunn and colleagues stated that cancer immunoediting implies the three phases elimination, equilibrium and escape **(figure 1.2)**.[12]

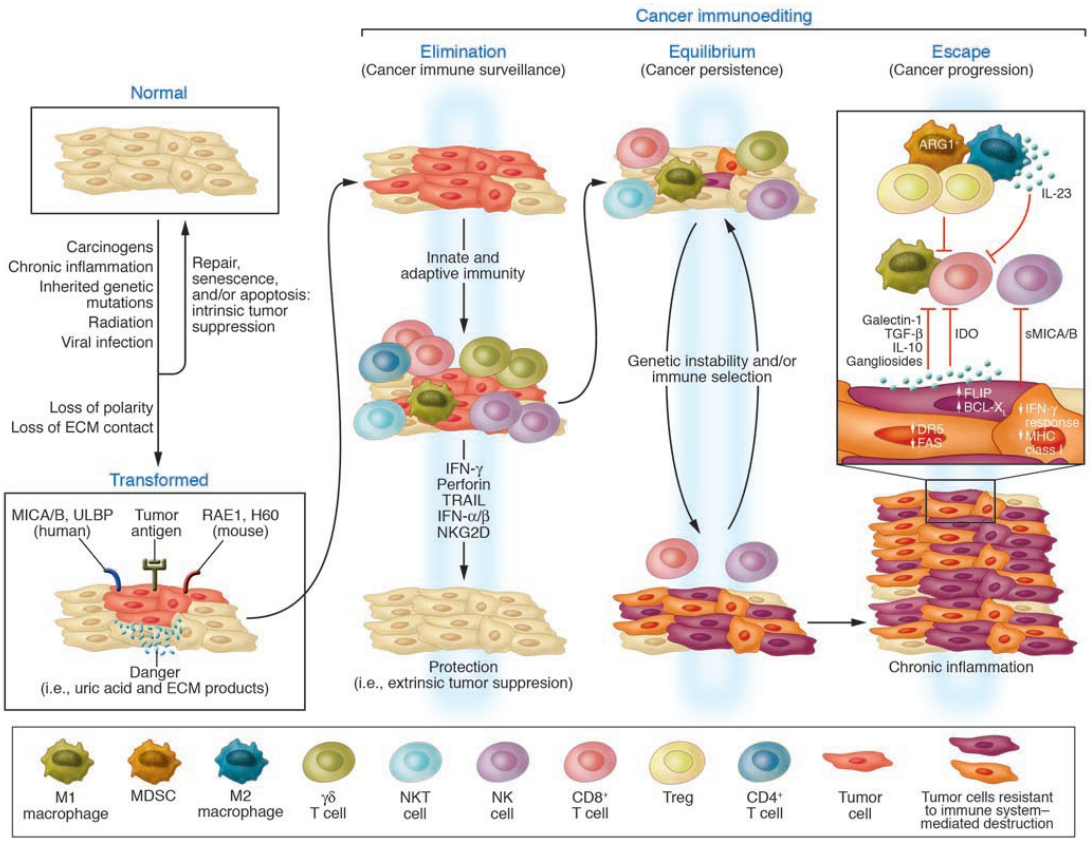

Figure 1.2: Cancer immunoediting.

(Adapted from Swann JB and Smyth MJ. 2007 [10])

In the elimination phase the immune system identifies malignant cells and eliminates them before they become clinically apparent.[10] This phase includes innate and adaptive immune responses, which occur due to upregulated danger signals from the transformed cells.[7,9] The persisting tumor cells enter the equilibrium a phase of tumor dormancy where the immune system keeps tumor growth in check.[7,10] During this phase a continuous sculpting of tumor cells leads to the selection of cell variants with increased resistance to immune attack.[9,12] These cells are able to evade the antitumor immune response and enter the escape phase. Now, the immune system is incapable to suppress tumor growth, resulting in the development of clinically detectable malignant tumors.[12,13]

1.3 The role of the immune system

The immune system consists of the innate and the adaptive immunity, whereby the adaptive arm can further be divided into humoral and cellular response. The innate immune system forms the first line of defense and consists of various cells (granulocytes, monocytes, natural killer cells, macrophages, mast cells and dendritic cells). It provides an unspecific inflammatory reaction and eliminates foreign as well as altered cells, whereas the adaptive arm leads to expansion and differentiation of B cells and T cells, which results in a more specific response to the pathogen.

To ensure a faultless functioning it is necessary, that our immune system can differentiate between self and non-self. One of the key processes to accomplish this task is the antigen presentation via TCR and MHC.[14,15]

1.3.1 Human leukocyte antigen (HLA) system

The HLA system, the major histocompatibility complex in humans, is encoded by two highly polymorphic gene families, located at chromosome six.[16] HLA molecules are membrane bound glycoproteins that are divided in two classes; Class I and Class II.[16] Class I molecules are expressed on platelets and almost all nucleated cells, whereas HLA class II molecules occur on the surface of

B lymphocytes, activated T lymphocytes and a restricted range of antigen presenting cells (APCs) including monocytes and dendritic cells (DCs).[16,17]

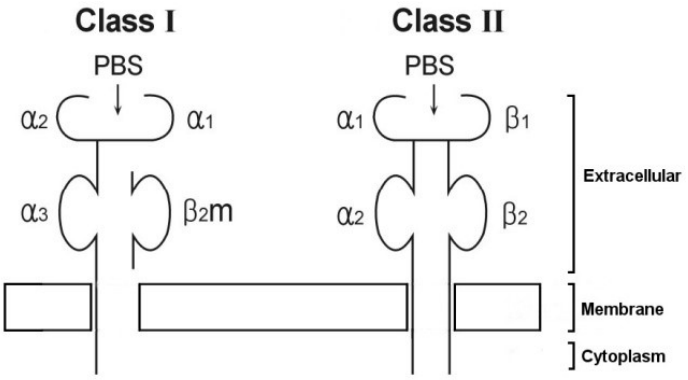

Figure 1.3: Schematic diagram of HLA class I and class II molecules.
PBS is peptide binding site.
(Adapted from Choo SY. 2007 [17])

HLA class I is formed by an alpha heavy chain and a light chain β_2-microglobulin (β_2m), whereas HLA class II consists of two non-covalently associated glycosylated chains; α and β **(figure 1.3)**.[17]

MHC class I molecules present protein fragments, from cytosolic and nuclear origin, at the cell surface to $CD8^+$ T cells **(figure 1.4a)**.[18,19] This mechanism starts with the degradation of the antigens by the proteasome. Then, the resulting peptides are carried into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP). In the ER the MHC class I molecules are assembled from an alpha heavy chain and a β_2m light chain. Now, a peptide inserts itself into the peptide-binding groove. This is necessary to stabilize the whole complex. Peptides which fail to bind on MHC class I molecules are removed from the ER via ERAD pathway. Peptide-MHC class I complex is transferred via Golgi to the cell surface for antigen presentation.[18]

MHC class II molecules present extracellular antigens to $CD4^+$ T cells **(figure 1.4b)**.[19] Extracellular antigens are taken up via phagocytosis, undergo

degradation in the endosomal compartment and are translocated to the MHC class II compartment (MIIC).[17-19] In the endoplasmic reticulum the α- and β-chains form a complex with the invariant chain (Ii). The resulting heterotrimer is transported via Golgi to the MHC class II compartment. This works either directly or via the plasma membrane. In the MIIC the invariant chain is degraded, leaving only the class II-associated Ii peptide (CLIP) in the peptide-binding groove of the MHC class II heterodimer. This fragment is exchanged for an antigenic peptide with the help of the dedicated chaperon HLA-DM. The whole complex is then transferred to the plasma membrane for antigen presentation.[18]

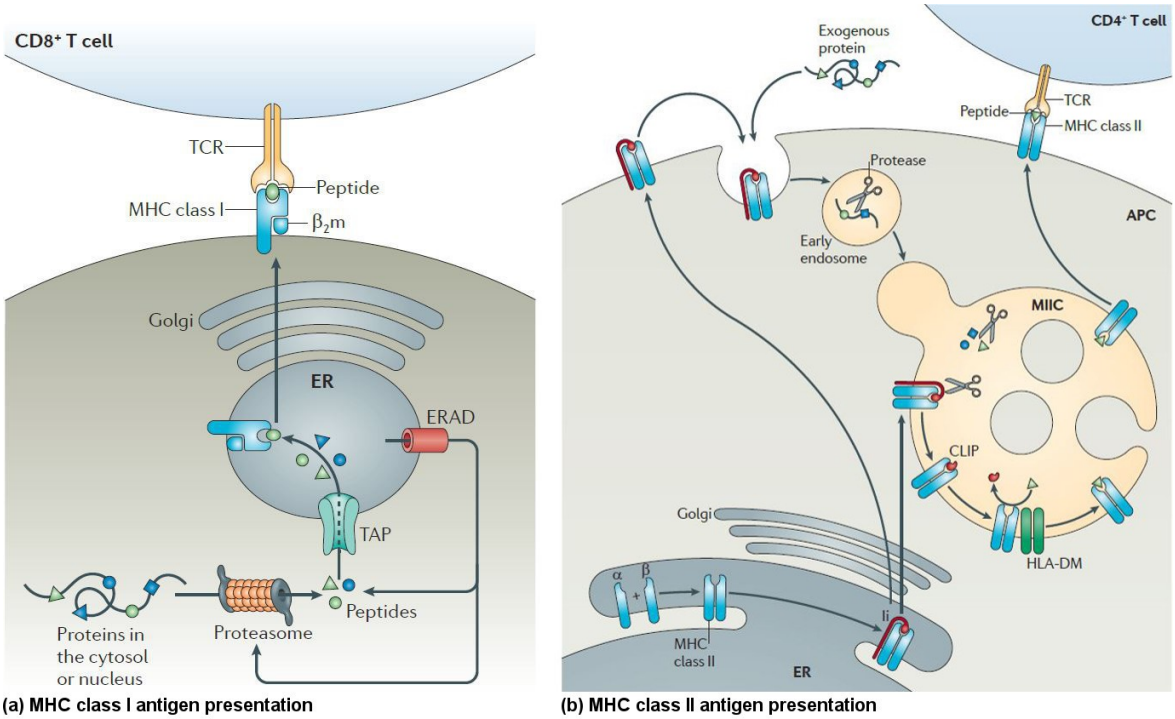

Figure 1.4: Antigen processing and presentation.

(Adapted from Neefjes et al. 2011 [18])

1.3.2 T cells

In general T cells are divided into $CD4^+$ T cells and $CD8^+$ T cells. $CD4^+$ T cells provide regulatory tasks, such as activating neighboring cells or recruiting of new immune cells, through secretion of cytokines and chemokines. In contrast,

$CD8^+$ T cells are able to destroy cells directly by secreting cytotoxic molecules and cytokines.

1.3.2.1 T cell development

The T cell development **(figure 1.5)** takes place in the thymus, which is divided into an outer cortex and an inner medulla.[20] The process starts with lymphoid progenitors which migrate from the bone marrow to the thymus. These early thymocytes lack the CD4 and CD8 co-receptors and are called double negative (DN).[21] They account for 5% of the thymocytes.[20] The double negative thymocytes can further be divided into four developmental subsets based on the expression of the CD25 (IL-2 receptor α), CD44 (Pgp-1) and CD117 receptors.[22,23] The cells of the first stage (DN1) are able to develop into other thymus-derived lineages such as DCs and NK cells. Therefore, they are not committed T cell precursors. DN1 thymocytes ($CD25^-$, $CD44^+$, $CD117^+$) start transition to the DN2 subset by expressing CD25. This transition also leads to TCRβ chain rearrangement **(figure 1.6A)** resulting in the DN2 ($CD25^+$, $CD44^+$ and $CD117^+$) subset. These cells contain joined D and J segments of TCRβ chain. Next, DN2 cells lose expression of CD44 and CD117. By joining of the V gene segment to the DJ segments the progress to the DN3 ($CD25^+$, $CD44^-$, $CD117^{low}$) subset is completed.[23] At this point the β-selection occurs, a checkpoint which verifies the generation of a functional TCRβ chain.[23,24] If no functional TCRβ chain is produced, the cell dies by apoptosis.[24] First step of the β-selection is the formation of the pre-TCR complex, which consists of a TCRβ chain covalently coupled to a pre-TCRα chain and is associated with CD3 molecules.[24,25] The expression of this pre-TCR complex leads to downregulation of CD25 and blocking of further TCRβ chain rearrangement (allelic exclusion).[23-25] This allows DN3 cells to continue to the DN4-stage ($CD25^-$, $CD44^-$ and $CD117^-$).[23] Pre-TCR signaling also results in proliferative expansion and initiates rearrangement at the TCRα locus.[24,25] Furthermore, expression of CD4 and CD8 is enabled and the thymocytes are now called double positive (DP).[23,25] To produce the mature TCRα chain random rearrangement of V and J gene segments at the TCRα locus is necessary **(figure 1.6B)**.[26] If the pairing of the new TCRα chain with the TCRβ chain is successful, the new TCRαβ-CD3 complex is expressed at the cell surface.[27]

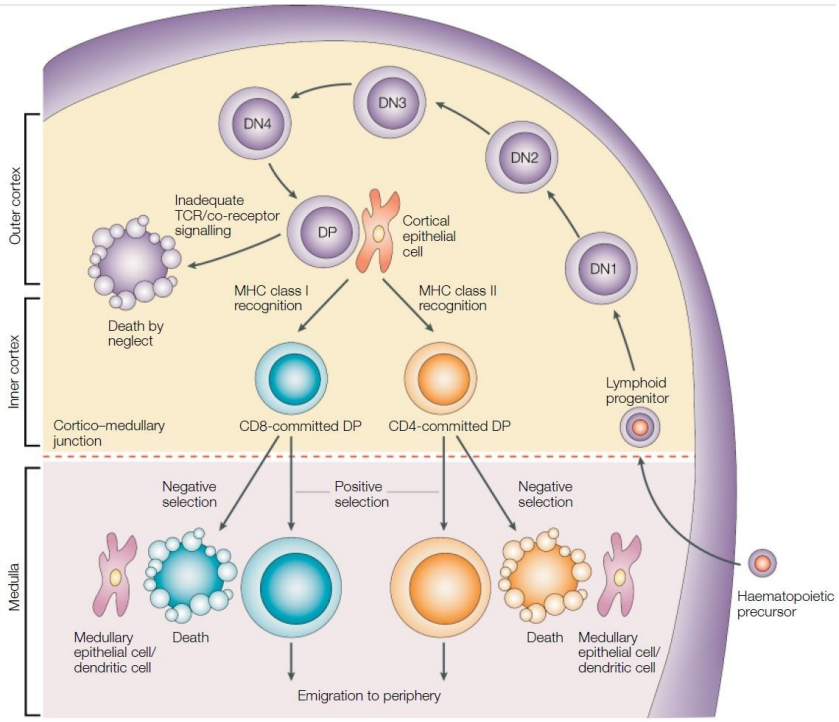

Figure 1.5: T cell development in thymus.

(Adapted from Germain RN. 2002 [21])

The next step in the T cell development is the selection of thymocytes **(figure 1.7)**. DP cells that cannot bind self-peptide-MHC at the surface of cortical thymic epithelial cells (cTEC) undergo apoptotic death (known as positive selection).[21,23,27] Also thymocytes with TCR with high affinity for self-MHC die by programmed cell death, because of their high autoreactive potential. This process is known as negative selection. Only cells that express a receptor with sufficiently low affinity for self-peptide-MHC are rescued from apoptosis.[27,28] Yates reported, that only 5% or less survive both positive and negative selection.[29]

Depending on the MHC molecule, which is recognized by the TCR, the cell commits to either the CD4 or the CD8 lineage. While recognition of MHC class I leads to the generation of a $CD8^+$ cytotoxic T cell, recognition of class II results in a $CD4^+$ helper T cell **(figure 1.5)**.[21,27,30] The fact that CD4/CD8 lineage choice is determined by the MHC-restriction specificity of the TCR was revealed first in 1988.[31] Since then, the mechanisms underlying lineage choice have been subject of many analyses and abstract models. Although these mechanisms have

been clarified significantly in the last years, many aspects continue to be debated.[30]

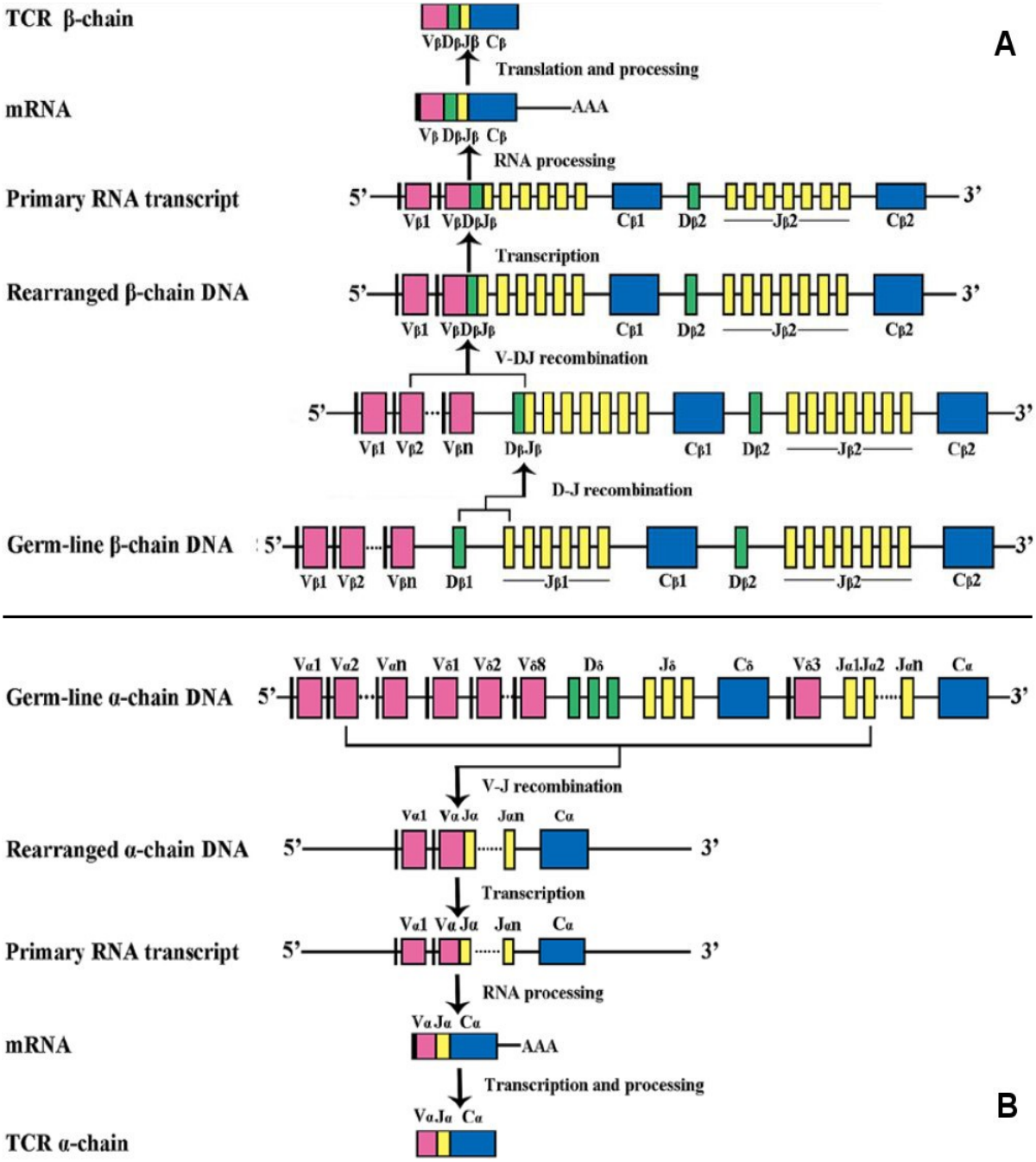

Figure 1.6: Gene rearrangement during T cell development.

A) TCRβ chain rearrangement.

B) Rearrangement of the TCRα chain.

(Adapted from Wang et al. 2016 [26])

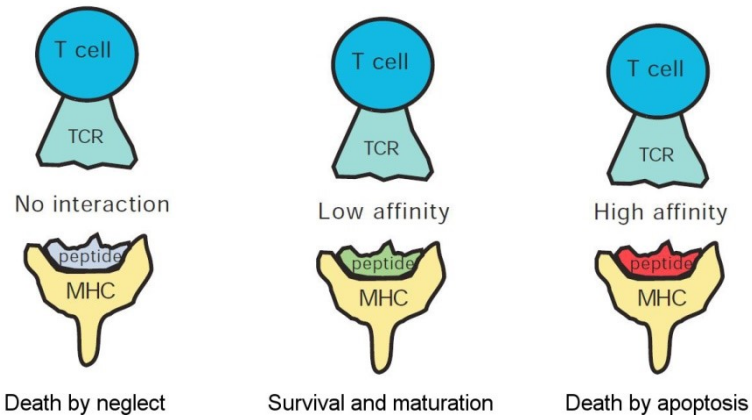

Figure 1.7: Consequences of TCR interaction with self-peptide-MHC.

(Adapted from Goldrath et al. 1999 [27])

In the last step of their development, the mature naïve T cells now leave the thymus and circulate throughout the secondary lymphoid organs. They keep patrolling in the periphery until they either encounter their specific antigen or die.[27]

1.3.2.2 T cell activation

The activation of T cells is a complex process, which takes place in the secondary lymphoid organs. Antigenic peptides bound to major histocompatibility complex molecules form a complex at the surface of antigen-presenting cells, which is recognized by the TCR-CD3 complex **(figure 1.8)**.

The T cell receptor consists of a α and a β chain, whereby the carboxy-terminal constant domains are associated with four intracellular polypeptide chains; gamma (γ), delta (δ), epsilon (ε) and zeta (ζ).[32] These chains form three subunits (εγ, εδ, ζζ), together known as CD3. The cytoplasmic tails of these three dimers contain the immunoreceptor tyrosine-based activation motifs (ITAMs).[32,33]

A stable interaction also requires either CD4 or CD8 as a co-receptor, which binds to the MHC molecule. The cytoplasmic segment of the co-receptor is connected to the lymphocyte protein tyrosine kinase (Lck).[28]

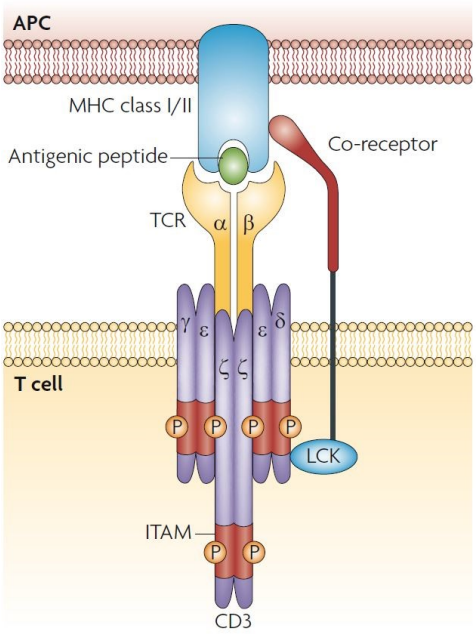

Figure 1.8: Model of the TCR-CD3 complex.

(Adapted from Gascoigne NRJ. 2008 [33])

Due to selection in the thymus, the TCRs of mature naïve T cells interact weakly with self-peptide-MHC.[34] This chronic interactions support the survival of T cells in the peripheral lymphoid tissues and are involved in their homeostatic proliferation.[35,36] Furthermore, the ability to recognize self-peptide-MHC complexes is required for full responsiveness to foreign antigens.[37,38] As a result, even the interaction of a single foreign peptide presented via an APC can lead to full-fledged activation of T cells.[28]

The activation of T cells is largely based on DCs, a specific type of APCs.[39,40] This cells act as sentinels in peripheral tissues and lymphoid organs.[40] Immature DCs express many uptake receptors (for example FcγR) and are also rich in MHC class II molecules, as well as other molecules, like invariant chain.[39] Antigen presentation by immature DCs leads to tolerance induction.[41] Maturation is provided by inflammatory cytokines, recognition of characteristic patterns of pathogens and necrotic cells.[42] This maturation stimulus results in upregulation of B7-1 (CD80), B7-2 (CD86), CD40 and production of interleukin-12 (IL-12) and other cytokines and chemokines.[39,41] Antigen presentation by this activated matured dendritic cells leads to activation of T cells.[41]

The activated DCs travel to the lymph nodes and other lymphoid tissues, where they face the naïve T cells, which are continuously sampling the APCs. If the TCR of a T cell binds to a peptide-MHC complex on the surface of an activated DC, the activation is started. This process last for several hours and requires a stable connection between the two cells.[43,44] Therefore, rapid cytoskeletal rearrangements lead to accumulation of TCRs, CD28 and lymphocyte function-associated antigen 1 (LFA-1) molecules at the surface of the T cell. Together with peptide-MHC complex, B7 and intercellular adhesion molecule 1 (ICAM-1), which are expressed by the DC, they form the immunological synapse (IS).[32,45-48] While TCR-peptide MHC complexes congregate at the center of the IS, ICAM-1-LFA-1 complexes accumulate at the periphery.[45,47,49]

The TCR-peptide MHC interaction starts the activation process of a T cell by initiating several intracellular signaling pathways **(figure 1.9)**. First, TCR stimulation, assisted by the co-receptor, leads to recruitment of Lck resulting in the phosphorylation of the CD3 ITAMs.[28,50] Now, Zeta-chain-associated protein kinase 70 (ZAP-70) is activated. This cytosolic tyrosine kinase in turn phosphorylates the scaffolding molecule Lat (linker for activation of T cells).[28,50,51] This protein is important for recruitment of different signaling proteins, resulting in activation of phospholipase Cγ1 (PLCγ1).[52] PLCγ1 generates the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) out of phosphatidylinositol 4,5-bisphosphate (PIP2) by hydrolysis.[53]

IP3 binds to IP3 receptors (IP3Rs), which are located at the endoplasmic reticulum membrane, leading to release of calcium from the ER.[50,53,54] This Ca^{2+} release from the ER leads to decrease of the Ca^{2+} concentration in the ER, which triggers the store-operated calcium entry (SOCE) pathway, resulting in opening of calcium release-activated calcium (CRAC) channels in the plasma membrane. Now, influx of extracellular calcium into the cytosol elevates the intracellular Ca^{2+} concentration, which in turn activates Ca^{2+}-dependent enzymes, such as calcineurin.[53,54] Calcineurin dephosphorylates NFAT (nuclear factor of activated T cells) thereby enabling the import of NFAT into the nucleus.[54]

Not only the NFAT but also the NF-κB (nuclear factor κB) pathway requires the activity of PLCγ1. NF-κB dimers are located in the cytosol of resting T cells.[55] After TCR triggering, the protein kinase C-theta (PKCθ) is activated via DAG. This leads to the formation of the CBM complex, consisting of CARMA1 (caspase recruitment domain-containing membrane-associated guanylate kinase protein-1), BCL10 (B cell lymphoma 10) and MALT1 (mucosa-associated lymphoid tissue 1), which results in the activation of the trimeric IKK (IκB kinase) complex and thereby allowing translocation of NF-κB to the nucleus.[50,55,56]

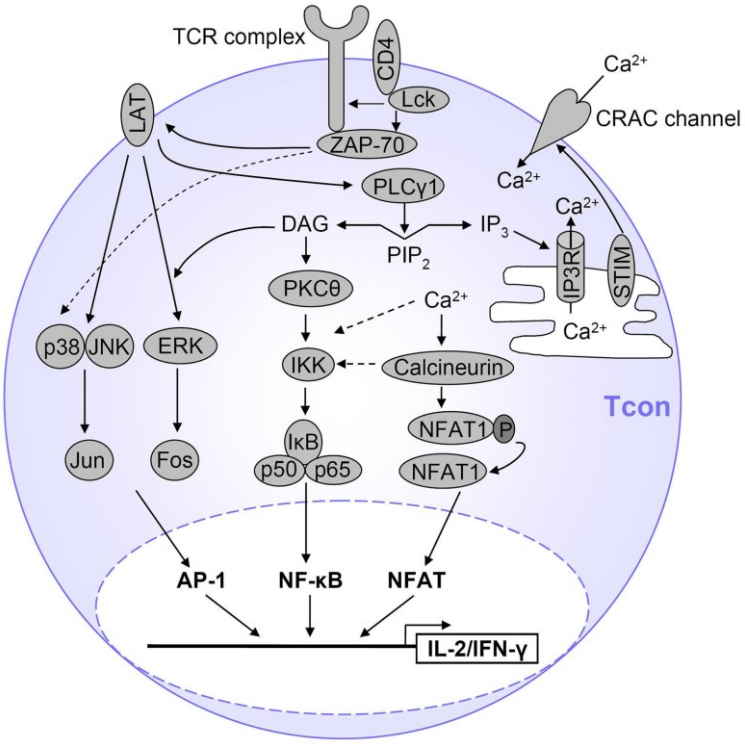

Figure 1.9: Intracellular signaling pathways in T cells.

(Adapted from Schmidt et al. 2012 [50])

The activator protein 1 (AP-1) pathway is the third main signaling pathway and largely independent of PLCγ1.[50] A mitogen-activated protein kinase (MAPK) cascade leads to activation of ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38 isoforms.[57] These MAPKs promote expression and activation of the transcription factors Fos and Jun, which together form the transcription factor AP-1.[58]

In the nucleus the three activated transcription factors NFAT, NF-κB and AP-1 play a key role in activation and proliferation of T cells as well as production of IL-2 and IFN-γ.[50]

TCR-peptide MHC interaction is not enough to activate T cells. There are three main signals required for successful activation: 1) recognition of the antigen; 2) co-stimulation through engagement of various surface molecules, including CD28, CD27 and CD40; 3) stimulation of the T cell through IL-12 or interferon-α (IFN-α) produced by the DC.[28,41,48,59]

1.3.3 Tumor antigens

To destroy malignant cells the immune system must be able to recognize them. Therefore, the so-called tumor antigens can be used. In general, tumor antigens are divided into tumor-specific antigens (TSA) and tumor-associated antigens (TAA). TSAs are unique tumor antigens expressed by the malignant cells and do not exist in normal tissue, whereas TAAs are overexpressed in cancer cells but can also exist in normal tissue. Study of these tumor antigens may allow the further development of safe and effective immunotherapies like adoptive cell therapy and cancer vaccines. A small selection of the wide range of tumor antigens known today is shown in **table 1.1**.[26,60-62]

Table 1.1: Selection of explored tumor antigens.

Tumor antigen	Cancer histology	Description	Reference
Carcino-embryonic antigen (CEA)	Colorectal carcinoma, lung cancer, etc.	Produced by colorectal tissue and increased in tumors of the gastrointestinal tract	[60,63,64]
HER-2/neu	Breast cancer, ovarian cancer, etc.	Plays a critical role in cell proliferation and is overexpressed in various cancers	[65-67]
Prostate-specific antigen (PSA)	Prostate cancer	Secreted by epithelial prostate cells and overproduced in malignant situations	[60,68]
Alpha-fetoprotein (AFP)	HCC, gastric cancer, etc.	Typically expressed in fetal liver cells and cancerous cells	[60,69,70]

1.4 Immune checkpoints in tumor escape

1.4.1 Escape strategies

To provide tumor growth the cancerous cells must evade recognition by the immune system of the host. The fact that most of the tumor antigens are 'self-proteins' is very important, because they are tolerated by the immune system.[71,72] In the last years further escape mechanisms were explored although not all of them are completely understood right now. Some tumors can modulate tumor cell surface antigens by transferring them from the cell surface to the cytoplasm and thereby making themselves immunologically invisible.[72] For example, downregulation of MHC class I molecules leads to reduced antitumor activity of cytotoxic T cells.[73] Malignant cells can also disturb the interaction of tumor cells with cytolytic cells by changing the expression of cell adhesion molecules.[74] Another mechanism, used by tumors to block immune response is the secretion of cytokines, which results in inhibition of immune cell development and function. Among others, these immunosuppressive factors include IL-10, tumor necrosis factor (TNF), tumor growth factor β (TGF-β) and vascular endothelial growth factor (VEGF).[71,72] Further, there is some evidence that cancer cells can express death receptor ligands (e.g. FasL), which, in case of interaction with their receptors, trigger signaling pathways leading to apoptotic cell death.[71,75] One of the main escape strategies is the activation of negative immunological checkpoints leading to unresponsiveness and functional exhaustion of tumor specific T cells. CTLA-4 and PD-1 belong to the best studied among these checkpoints.[71,72,76,77]

1.4.2 T cell exhaustion

During acute infections, naïve T cells are activated, leading to proliferation and generation of effector T cells. After clearing of antigen and/or inflammation, some of the remaining $CD8^+$ T cells differentiate into self-renewing memory T cells, which are capable to reactivate effector functions rapidly in the case of secondary infections. On the other hand, persisting T cell stimulation during chronic infections or cancer leads to functionally silenced effector T cells **(figure 1.10)**. This state is called 'exhaustion'.[78]

The first exhausted T cells have been observed in mice suffering from infection with the lymphocytic choriomeningitis virus. This mouse model was also used to demonstrate the reversibility of T cell exhaustion.[79] In humans, T cell exhaustion has been noted in cancer and various virus infections like human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV). In tumor patients, exhaustion of tumor specific T cells seems to promote tumor immune escape.[80] Among other things, exhaustion results in an upregulated expression of multiple inhibitory receptors, loss of effector cytokine secretion, transformed cell metabolism. These inhibitory receptors include PD-1 and CTLA-4. Blocking these pathways, which are overexpressed in exhaustion, can reverse this state of T cell dysfunction and possibly recover anti-cancer immunity.[78,80]

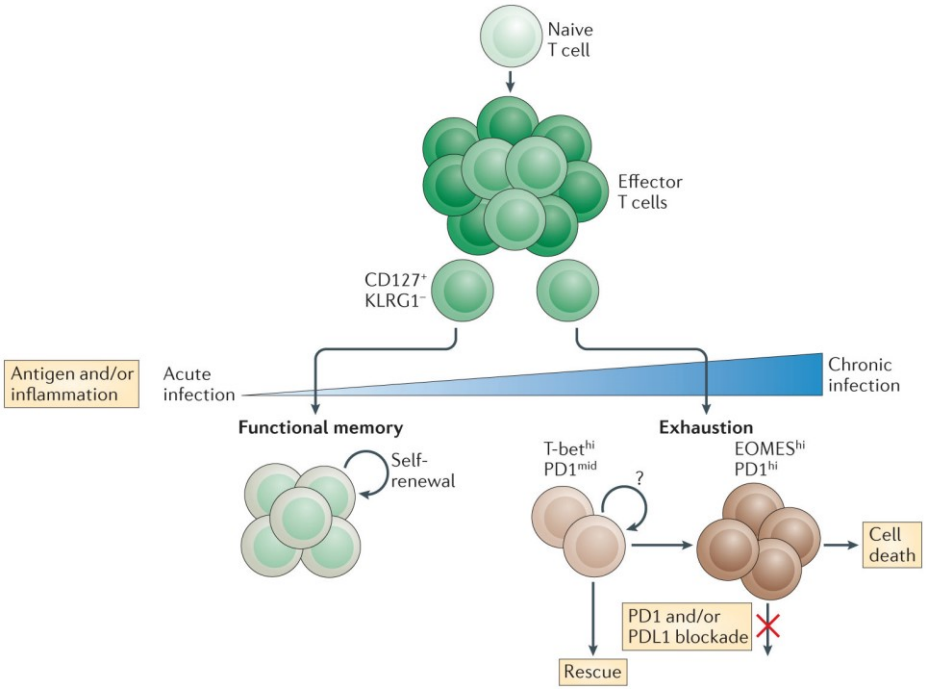

Figure 1.10: Development of T cell exhaustion.

(Adapted from Wherry et al. 2015 [78])

1.4.3 CTLA-4 pathway

Brunet and colleagues discovered the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) in 1987 as a new member of the immunoglobulin superfamily.[81] Further experimental examination showed that CTLA-4 is a

negative regulator of immune function. CTLA-4 is a CD28 homolog, but has a much higher binding affinity to B7. In contrast to CD28, binding of CTLA-4 to B7 does not produce a stimulatory signal.[15,82] But this competitive binding alone only accounts for a part of the inhibitory effect. In fact, some evidence suggests that CTLA-4:B7 binding leads to inhibition of cellular proliferation and reduced production of IL-2 via tyrosine phosphatases.[82] Because of these mechanisms full activation of T cells via APCs is prevented **(figure 1.11)**.[15]

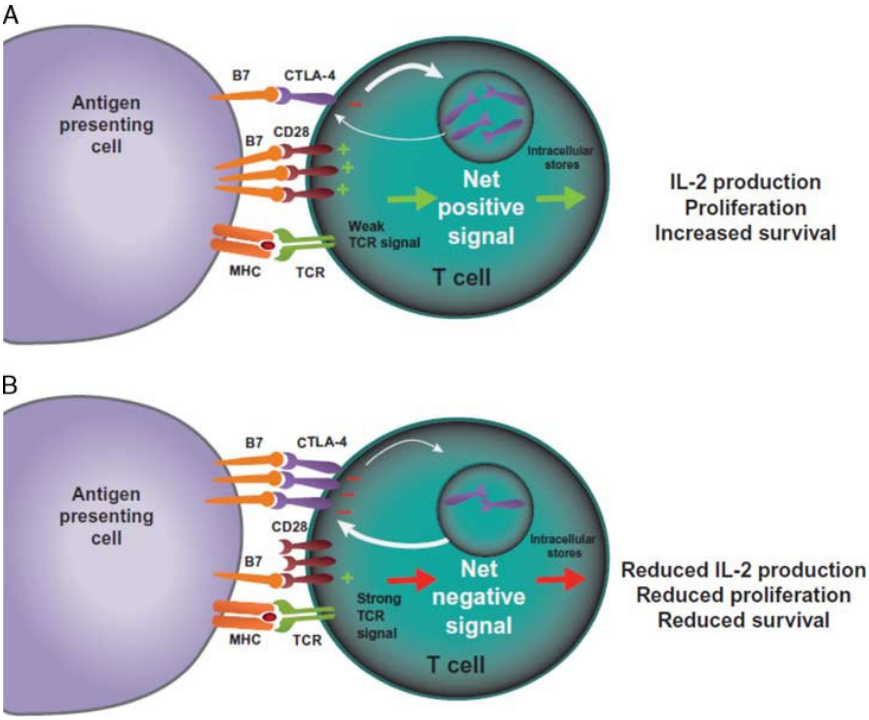

Figure 1.11: Effect of CTLA-4:B7 binding on T cells.

A) CD28:B7 binding predominates, which leads to a positive stimulatory signal.

B) CTLA-4:B7 binding prevails, resulting in a negative stimulatory signal.

(Adapted from Buchbinder et al. 2016 [15])

Preclinical studies demonstrated that inhibition of the CTLA-4 pathway leads to improved survival, decreased tumor growth and rejection of preestablished tumors providing the rationale for development of antibodies targeting this immune checkpoint.[83] The CTLA-4 blockade supports activation and proliferation of effector T cells independent of TCR specificity, resulting in an increased peripheral T cell diversity.[84,85] The first clinically successful inhibitor of CTLA-4 was a fully

human monoclonal antibody called ipilimumab. It was first approved in 2011 for the treatment of metastatic melanoma. In addition to the prolonged overall survival, ipilimumab also showed a better durability of responses.[86]

1.4.4 PD-1 pathway

Programmed death-1 (PD-1) is a monomeric protein located at the cell surface and a member of the immunoglobulin superfamily. It was discovered by Honjo and colleagues in 1992.[87,88] Today, there are two known ligands: programmed death ligand-1 (PD-L1) and programmed death ligand-2 (PD-L2). While PD-L1 is expressed on various cells including leukocytes, non-hematopoietic cells and non-lymphoid tissues such as heart and lung, PD-L2 is located only on the surface of monocytes and dendritic cells.[84,89,90] Expression of PD-L1 also occurs on different tumor types and can be induced on malignant cells by inflammatory cytokines and various tumorigenic signaling pathways.[15]

Similar to the CTLA-4 pathway, PD-1:PD-L1 signaling has negative effects on T cell activity. The binding of PD-1 results in reduced interferon-γ, TNF-α and IL-2 production and inhibition of T cell proliferation **(figure 1.12)**. It also reduces T cell survival. In contrast to CTLA-4, PD-1 functions during the effector phase of T cell activation, mainly in peripheral tissues. Programmed death-1 is broadly expressed on activated T cells. Prolonged TCR stimulation, which occurs during cancer, can cause an upregulated expression of PD-1 on the T cell surface and results in suboptimal control of tumors.[15] Higher expression levels of PD-1 are associated with poorer tumor prognosis.[84]

Blocking PD-1 pathway with antibodies has shown an improved survival as well as prolonged progression-free survival (PFS) in patients with different tumor types, such as non-small cell lung cancer (NSCLC), renal cell carcinoma and metastatic melanoma. The first approved antibodies targeting PD-1 were pembrolizumab and nivolumab. The clinical use of these drugs led to new challenges like pseudoprogression and the handling of immune related adverse events (irAEs).[15,82] These irAEs include rash, pruritus, diarrhea, pneumonitis, hypophysitis, arthritis, myositis etc. The term pseudoprogression describes an

initial increase in tumor volume as a result of inflammation and accumulation of immune cells at the tumor site.

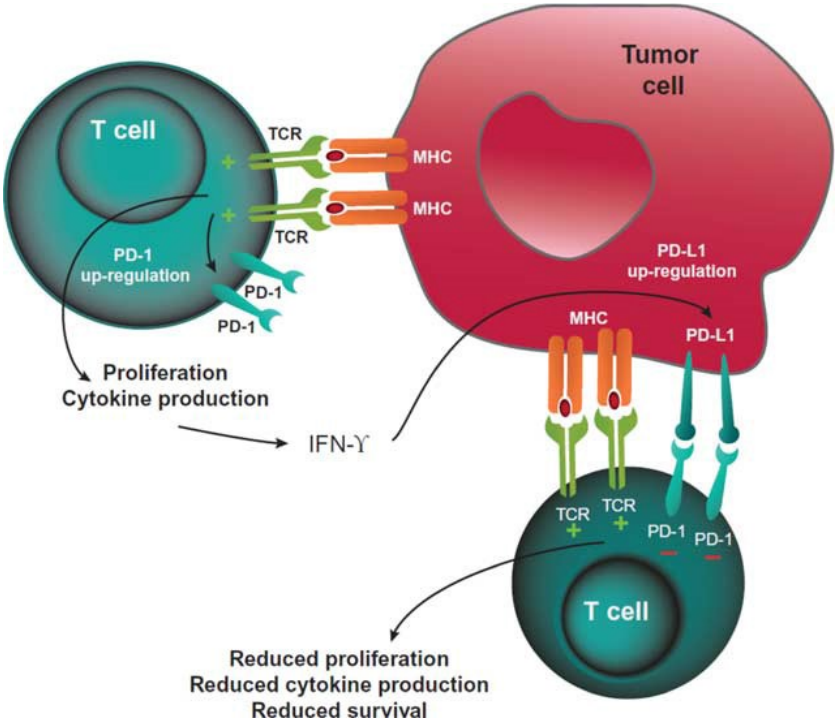

Figure 1.12: Inhibition of T cells via PD-1 signaling.

(Adapted from Buchbinder et al. 2016 [15])

1.5 Immune checkpoint inhibitors in cancer therapy

Checkpoint inhibitors play an increasingly important role in today's cancer therapy. The success of cancer immunotherapy has been driven by years of basic research in the fields of tumor immunology and cell biology. The fact, that T cell activation requires co-stimulation and the identification of negative signaling pathways, which downregulate T cell activation, provided key insights into tumor immune escape mechanisms. The blockade of these immune checkpoints, which normally act as a failsafe mechanism of the immune system, has turned out to be an effective treatment for many tumor types. Today, immune checkpoint inhibitors (ICIs) are standard of care for the treatment of various malignancies **(table 1.2)**.

Following a study of Hodi and colleagues, ipilimumab (Yervoy®) became the very first approved immune checkpoint inhibitor for cancer therapy by the FDA in 2011.

Hodi et al investigated 676 patients with unresectable stage III or IV melanoma, who suffered from progressive disease while receiving therapy. They were randomly assigned to three different treatment arms, receiving ipilimumab, ipilimumab plus gp100 or gp100 alone. The median overall survival was 10.1 month among patients receiving ipilimumab alone, whereas patients who received gp100 alone had a median overall survival of 6.4 month (hazard ratio for death 0.66, P=0.003). There was no difference in overall survival between ipilimumab alone and ipilimumab plus gp100.[91] Ipilimumab is also investigated as treatment for other cancer types, including RCC, NSCLC, prostate cancer, and others. Beside ipilimumab, another anti-CTLA-4 antibody called tremelimumab is currently being investigated in various clinical trials.[92]

Evidence that PD-1 plays a role in suppression of effector T cells in peripheral tissues led to the development of immune checkpoint inhibitors targeting PD-1 in cancer patients.[92] The first approved PD-1-targeting immune checkpoint blockade (ICB) therapy was nivolumab (Opdivo®) a fully human IgG4 monoclonal antibody produced by Bristol-Myrs Squibb. This drug showed improved objective response rates versus chemotherapy in patients with unresectable or metastatic melanoma who suffered from progressive disease following therapy with ipilimumab +- a BRAF inhibitor.[93] Another phase III trial demonstrated improved objective response rate (40% vs. 13.9%), progression-free survival (5.1 vs. 2.2 months) and overall survival (72.9% vs. 42.1% at 1 year) for nivolumab compared to dacarbazine in patients with previously untreated melanoma without BRAF mutation.[94] Recently, nivolumab was also approved as treatment for NSCLC following a study from Brahmer et al.[95] In addition, nivolumab showed significant benefits in the treatment of several other cancer entities like advanced renal cell carcinoma and advanced urothelial carcinoma.[96,97] Moreover, an improved objective response rate in patients with relapsed or refractory Hodgkin's lymphoma was demonstrated.[98] This led to the approval of nivolumab as first immune checkpoint inhibitor for treatment of a hematological cancer by the FDA.[92]

Another humanized IgG4 monoclonal antibody targeting PD-1 is pembrolizumab (Keytruda®). Following some trials that showed improved overall and progression-free survival in PD-L1 positive metastatic NSCLC compared to

docetaxel or platinum-based chemotherapy, pembrolizumab was granted approval as therapy for advanced NSCLC.[99,100] Further, it is used for treatment of unresectable or metastatic melanoma.[101,102] Various trials also led to pembrolizumab's approval for treatment of urothelial carcinoma, Hodgkin's lymphoma and colorectal cancer.[92,103,104]

A different approach to eliminate signaling through the PD-1 pathway is based on blocking of PD-L1. Therefore, various monoclonal antibodies are available, including atezolizumab (Tecentriq®), avelumab (Bavencio®) and durvalumab (Imfinzi®). In 2016, Atezolizumab became the first approved anti-PD-L1 antibody, receiving authorization for treatment of certain indications of metastatic urothelial carcinoma and NSCLC.[92] Later on, avelumab was granted marketing approval based on significant benefits in objective response in patients suffering from platinum-refractory metastatic urothelial carcinoma.[105] Also, it received approval for treatment of patients with metastatic Merkel cell carcinoma.[92] As third PD-L1 inhibitor, durvalumab received approval for progressive metastatic urothelial carcinoma. Furthermore, it showed improved progression-free survival in NSCLC patients in the PACIFIC trial. This led to its authorization for therapy of stage III NSCLC in patients, who did not have a progress after chemoradiotherapy.[92,106]

Table 1.2: Selection of approved immune checkpoint inhibitors.

Drug name	Target	Certain indications (selection)	Initial approval (FDA)
Ipilimumab	CTLA-4	Unresectable or metastatic melanoma	2011
Nivolumab	PD-1	Metastatic NSCLC, unresectable or metastatic melanoma, advanced RCC and urothelial carcinoma, classical Hodgkin's lymphoma	2014
Pembrolizumab	PD-1	Metastatic NSCLC, classical Hodgkin's lymphoma, urothelial carcinoma	2017
Atezolizumab	PD-L1	Urothelial carcinoma, metastatic NSCLC	2016
Avelumab	PD-L1	Metastatic Merkel cell carcinoma, advanced urothelial carcinoma	2017
Durvalumab	PD-L1	Urothelial carcinoma, NSCLC	2017

1.6 Metastases and outcome in cancer therapy

Metastatic spread is a key feature of malignant tumors and the reason for most cancer deaths.[6,107] About 40% of the patients suffering from non-small cell lung cancer present with metastatic lesions at diagnosis.[108] The most common metastatic sites are liver, bone, CNS, adrenal glands and the respiratory system.[109] The metastatic pattern differs not only between cancer types but also between histological subtypes.[107,108] In patients with adenocarcinoma, the most common metastatic site is bone, while in patients with squamous cell carcinoma it is lung. In many studies, patients with a single metastatic lesion showed better overall survival compared to patients suffering from multi-site metastatic disease.[108,109] However, Liao and colleagues reported no difference in overall survival between patients with three and four metastatic sites. In lung cancer, several reports showed a worse overall survival in patients with liver metastasis.[108,109] By contrast, patients with lung metastasis had an increased prognosis. In patients with only a single brain, bone and lung metastasis, surgery on distant lesions led to an improved outcome compared to patients who received no surgery. Patients with a single liver metastatic site did not benefit from metastasectomy.[109]

In patients suffering from renal cell carcinoma, the most common metastasis sites were lung (45,2%), bone (29,5%), lymph nodes (21,8%), liver (20,3%), adrenal (8,9%) and brain (8,1%).[110] Bianchi et al found, that a younger age is associated with an increased rate of multiple metastatic sites. The overall survival of patients suffering from renal cell carcinoma differs according to the metastasis location.[110] Abdel-Rahman showed, that patients with isolated liver metastases have worse overall survival compared to other single organ metastatic lesions especially bone ($p < 0.0001$), lung ($p < 0.0001$) and distant nodal metastases ($p = 0.003$). He also reported worse overall survival in patients with single organ brain ($p = 0.015$) and lung ($p = 0.011$) metastasis compared to bone metastasis. Further on, patients with distant metastasis except distant lymph nodes benefit from surgery.[111]

Patients with metastatic cutaneous melanoma have poor five year survival rates (16%) and median survival (< 1 year).[112] Especially patients with multiple metastatic lesions have a poor prognosis.[112,113] The most common metastatic

sites are lung, liver, distant lymph node, skin, bone and brain. Patients with isolated bone, brain and liver metastasis showed worse overall survival and melanoma-specific survival (time from diagnosis to death due to cutaneous melanoma) compared to patients suffering from skin and distant lymph node metastasis. Surgery of the metastatic lesion showed benefit for patients with lung metastasis ($p < 0.0001$), whereas surgery of other metastatic sites did not show any benefit.[112]

1.7 Aims and outline of this thesis

The use of checkpoint inhibitors like PD-, PD-L1 and CTLA-4 antibodies has changed the therapy of several cancer entities significantly during the last years and emerged as a standard treatment option in patients suffering from metastatic melanoma, advanced non-small cell lung cancer and renal cell carcinoma. The effect of checkpoint inhibitors depends on many factors such as presence of immune cells in tumor metastases and tumor intrinsic properties. However, only a few predictive biomarkers, like PD-L1 expression, for the benefit of checkpoint inhibitor therapy are currently available. Previous reports showed that response to immune checkpoint inhibitors also depends on the tumor microenvironment, which differs according to the metastasis location and on the presence of T cells in tumor metastases.[114,115] Therefore, the efficacy and outcome of checkpoint inhibitors may depend on the metastatic site. Knowledge of these variations may help to identify patients who benefit from additive local therapy.

Within the Alpine Tumor Immunology Registry (Alpine-TIR) we investigated the correlation of metastatic site and outcome in a cohort of patients treated with checkpoint inhibitors with NSCLC, RCC and Melanoma.

The following aims were specified:

- To estimate the outcome and prognosis of patients treated with either CTLA-4 or PD-1 antibodies depending on the metastasis location.
- To identify metastatic sites, which affect the efficacy of checkpoint inhibitors to identify patients who benefit from additive local therapy.

2 Material and Methods

2.1 Population and design

We performed a retrospective study on all incident cases of metastatic non-small cell lung cancer, metastatic renal cell carcinoma and metastatic melanoma, which were treated with checkpoint inhibitors at the Academic Teaching Hospital Feldkirch (Feldkirch, Austria), Landeskrankenhaus Bludenz (Bludenz, Austria), Landeskrankenhaus Bregenz (Bregenz, Austria), or the Landeskrankenhaus Hohenems (Hohenems, Austria). All patients were recruited between August 2017 and December 2018.

The diagnosis was confirmed with pathological or cytological specimens in all patients. Prior to the start of therapy, the patients were staged using computer tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET). Only patients with at least one distant metastasis were included. Patients suffering from two or more distant metastases were scored on each metastasis location. Staging was performed according to the 7th edition of the Union for International Cancer Control TNM classification. The two main endpoints were overall survival calculated from start of checkpoint inhibitor treatment to death as well as diagnosis to death.

The study received admission from the local ethics committee.

2.2 Data collection

Data were collected in a standardized fashion and recorded in the Alpine Tumor Immunology Registry (Alpine-TIR). The recorded socio-demographic and clinical variables were extracted from the electronic patient record system. A selection of the collected variables is shown in **table 2.1**.

Table 2.1: Selection of the recorded parameters.

Parameter	Value
Tumor entity	Melanoma, NSCLC, RCC
Gender	Male, Female
Date of birth	Date
Nationality	Austria, Switzerland, etc.
Smoking status	smoker, nonsmoker, ex-smoker
Basis of diagnosis	histology, cytology, MRI, CT, PET
Death certificate	Date
Date of incidence	Date
Topography	head, thorax, abdomen, upper-, lower extremities
Morphology	adenocarcinoma, squamous cell carcinoma, etc.
Location of metastases	lung, liver, bone, CNS, kidney, lymph node, etc.
PD-L1 expression status (tumor cell)	% positive
Last patient examination	Date
Month of follow-up	Number
History of atopy	N/A, yes
Autoimmune disorders	rheumatoid arthritis, autoimmune thyreoiditis, etc.
Infectious diseases	Hepatitis B, Hepatitis C, HIV, etc.
cTNM	clinical TNM staging (version 7)
pTNM	pathological TNM staging (version 7)
UICC	UICC-Stage
Radiotherapy	Yes (including start and stop date, dose, location, schedule) or No
Surgery	Yes (+ date, location) or No
Result of surgery	RX, R0, R1, R2
Adjuvant therapy	Substance, Date
Maintenance therapy	Substance
Recurrence	Yes (date, location), No
Received doses of checkpoint inhibitor	> 2 or <= 2

Parameters collected at each examination	Value
Administered substances	checkpoint inhibitors, chemotherapeutics, etc.
Single dose for each substance	Dosage in mg
Cumulative dose for each substance	Dosage in g
Adverse events	Pneumonitis, Hepatitis, Colitis, Dermatitis, Diarrhea, Nausea, Pruritus, etc.
Grad of adverse events	Grad 1 – 4 according to the Immunotherapy toxicity guidelines from Kumar and Harris approved in October 2016
ECOG	ECOG-Score from 0 - 5
Laboratory values collected at each examination	**Value**
TSH	mU/L
ACTH	pg/mL
Sodium	mmol/L
Potassium	mmol/L
Blood glucose	mg/dL
Creatinine	mg/dL
Bilirubin	mg/dL
ASAT	U/L
ALAT	U/L
CRP	mg/dL
Hemoglobin	g/dL
Leukocytes	G/L
Monocytes	%
Neutrophils	%
Thrombocytes	G/L
Lymphocytes	%
Eosinophils	%

2.3 Statistical analysis

Median overall survival was calculated from the start of treatment with checkpoint inhibitors to death as well as diagnosis to death. Patients who were lost to follow-up or patients without a death certificate were censored at the date of the last examination. We defined the overall response rate (ORR) as the percentage of patients who had complete remission (CR) or at least partial remission (PR) as the best overall response. Survival analysis was performed employing Kaplan Meier survival curves and statistical significance was calculated using log-rank test. Hazard ratios were calculated using univariate cox regression analysis. A p-value less than 0.05 was considered statistically significant. All the statistical analyses were performed using SAS software, version 9.4 (SAS Institute Inc., Cary, NC, US).

3 Results

3.1 Patient characteristics

Among the 198 patients in the Austrian cohort of the Alpine Tumor Immunology Registry, 103 patients had confirmed metastatic non-small cell lung cancer (72 patients), metastatic renal cell carcinoma (16 patients) or metastatic melanoma (15 patients). The detailed patient characteristics are shown in **table 3.1**. There were 40 (38.8%) women and 63 men in the analyzed cohort. The median age at diagnosis was 62.5 years (range, 32.6-86.8), while the median age at start of first checkpoint inhibitor was 63.7 years (range, 32.8-86.9) **(table 3.2)**. The majority of patients were active (35%) or former (19.4%) smokers, while only 11.7% were never smokers. Most patients received checkpoint inhibition as first line (30.1%), second line (43.7%) or third line (23.3%) treatment, whereas only a minority received immune checkpoint inhibitors later. The majority of the patients received either nivolumab (53.4%) or pembrolizumab (32%). Only 4 patients (3.9%), all of them with metastatic melanoma, were treated with a combination of nivolumab and ipilimumab. Overall, 81 (78.6%) patients received more than two doses of immunotherapy, while 22 patients received 2 or less.

Table 3.1: Detailed patient characteristics.

Variable	Melanoma (N=15) n (%)	Non-small cell lung cancer (N=72) n (%)	Renal cell carcinoma (N=16) n (%)	Total (N=103) n (%)
First checkpoint inhibitor				
Atezolizumab		2 (2.8%)		2 (1.9%)
Durvalumab		9 (12.5%)		9 (8.7%)
Nivolumab	2 (13.3%)	37 (51.4%)	16 (100.0%)	55 (53.4%)
Nivolumab + Ipilimumab	4 (26.7%)			4 (3.9%)
Pembrolizumab	9 (60.0%)	24 (33.3%)		33 (32.0%)

Variable	Melanoma (N=15)	Non-small cell lung cancer (N=72)	Renal cell carcinoma (N=16)	Total (N=103)
	n (%)	n (%)	n (%)	n (%)
Line of first checkpoint inhibitor				
1	13 (86.7%)	17 (23.6%)	1 (6.3%)	31 (30.1%)
2	2 (13.3%)	32 (44.4%)	11 (68.8%)	45 (43.7%)
3		20 (27.8%)	4 (25.0%)	24 (23.3%)
4		2 (2.8%)		2 (1.9%)
6		1 (1.4%)		1 (1.0%)
Immunotherapy - Received > 2 doses				
No	4 (26.7%)	17 (23.6%)	1 (6.3%)	22 (21.4%)
Yes	11 (73.3%)	55 (76.4%)	15 (93.8%)	81 (78.6%)
Gender				
Female	6 (40.0%)	28 (38.9%)	6 (37.5%)	40 (38.8%)
Male	9 (60.0%)	44 (61.1%)	10 (62.5%)	63 (61.2%)
Smoking Status				
Ex-Smoker		20 (27.8%)		20 (19.4%)
N/A	8 (53.3%)	17 (23.6%)	8 (50.0%)	33 (32.0%)
Nonsmoker	5 (33.3%)	3 (4.2%)	4 (25.0%)	12 (11.7%)
Smoker	2 (13.3%)	31 (43.1%)	3 (18.8%)	36 (35.0%)
Missing		1 (1.4%)	1 (6.3%)	2 (1.9%)
Metastases				
Yes	15 (100.0%)	72 (100.0%)	16 (100.0%)	103 (100.0%)
Metastasis location (more than one applicable)				
Bone	3 (20.0%)	19 (26.4%)	3 (18.8%)	25 (24.3%)
CNS	4 (26.7%)	4 (5.6%)		8 (7.8%)
Duodenum	1 (6.7%)			1 (1.0%)
Gaster		2 (2.8%)		2 (1.9%)
Ileum	2 (13.3%)			2 (1.9%)
Liver	4 (26.7%)	11 (15.3%)	6 (37.5%)	21 (20.4%)

Variable	Melanoma (N=15)	Non-small cell lung cancer (N=72)	Renal cell carcinoma (N=16)	Total (N=103)
	n (%)	n (%)	n (%)	n (%)
Lung	6 (40.0%)	18 (25.0%)	8 (50.0%)	32 (31.1%)
Lymph node	8 (53.3%)	59 (81.9%)	10 (62.5%)	77 (74.8%)
Muscle	1 (6.7%)			1 (1.0%)
Peritoneum			1 (6.3%)	1 (1.0%)
Pleura		5 (6.9%)		5 (4.9%)
Soft tissue	2 (13.3%)	3 (4.2%)	2 (12.5%)	7 (6.8%)
Suprarenal gland	2 (13.3%)	13 (18.1%)	3 (18.8%)	18 (17.5%)

Table 3.2: Median age of the patients.

Variable	Overall (N=103)			Melanoma (N=15)			Lung cancer (N=72)			Renal cell carcinoma (N=16)		
	n	median	(min, max)	n	median	(min, max)	n	median	(min, max)	n	median	(min, max)
Age at diagnosis (years)	103	62.5	(32.6, 86.8)	15	71.3	(32.6, 86.8)	72	62.5	(38.1, 83.9)	16	56.5	(44.2, 76.9)
Age at start of first checkpoint inhibitor (years)	103	63.7	(32.8, 86.9)	15	71.3	(32.8, 86.9)	72	63.9	(41.4, 84.0)	16	58.1	(47.3, 77.6)

3.2 Metastatic patterns

The distribution of metastasis locations for all patients is shown in **table 3.1**, whereby more than one metastatic site was applicable. In patients with metastatic melanoma the most common locations were lymph node (53.3%), lung (40%), liver (26.7%), CNS (26.7%) and bone (20%). Among patients with RCC the most frequent metastatic sites were lymph node (62.5%), lung (50%), liver (37.5%), bone (18.8%) and suprarenal gland (18.8%). Patients with NSCLC most commonly suffered from metastasis in lymph node (81.9%), bone (26.4%), lung (25%), suprarenal gland (18.1%) and liver (15.3%).

Pooled for NSCLC, RCC and melanoma, the most common metastasis locations were lymph node (74.8%), lung (31.1%), bone (24.3%), liver (20.4%) and suprarenal gland (17.5%).

3.3 Overall response

In the melanoma cohort overall response rate was 13.3% and 20% reached stable disease as best overall response. The ORR for patients with NSCLC was 26.4%, while another 26.4% reached stable disease. The RCC cohort showed an ORR of 18.8% and stable disease in 43.8% of the patients. In the pooled cohort overall response rate was 23.3% and 28.2% showed stable disease, while 35.9% of the patients showed progressive disease.

Table 3.3: Responses among the different tumor entities.

Variable	Melanoma (N=15) n (%)	Lung cancer (N=72) n (%)	Renal cell carcinoma (N=16) n (%)	Total (N=103) n (%)
Best response				
CR			2 (12.5%)	2 (1.9%)
PR	2 (13.3%)	19 (26.4%)	1 (6.3%)	22 (21.4%)
SD	3 (20.0%)	19 (26.4%)	7 (43.8%)	29 (28.2%)
Missing	10 (66.7%)	34 (47.2%)	6 (37.5%)	50 (48.5%)
PD	5 (33.3%)	26 (36.1%)	6 (37.5%)	37 (35.9%)

3.4 Overall survival

In the cohort pooled for NSCLC, RCC and melanoma, the median overall survival was calculated from checkpoint inhibitor start to death as well as diagnosis to death. Median overall survival from diagnosis was 52.3 month, while median overall survival from checkpoint inhibitor start was not reached **(figure 3.1)**.

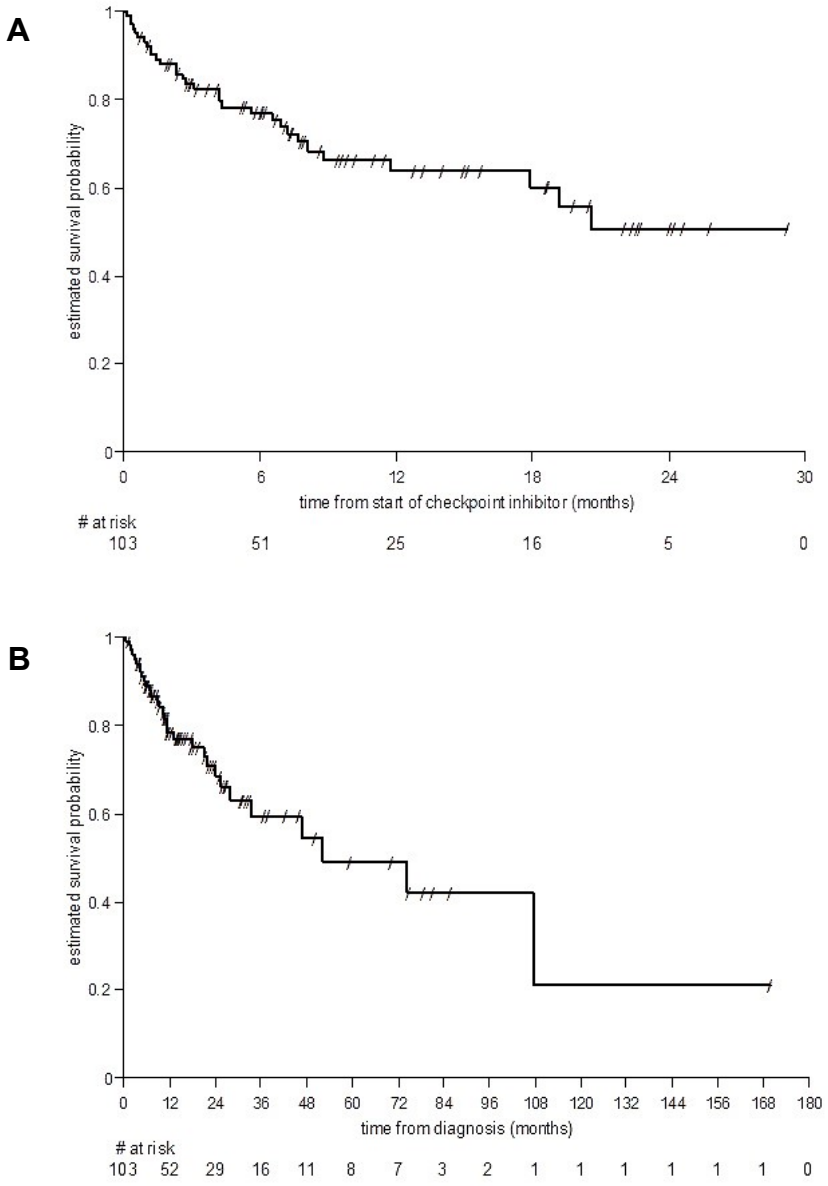

Figure 3.1: Kaplan-Meier curve of median overall survival for pooled cohort.

A) OS calculated from checkpoint inhibitor start to death.

B) OS calculated from diagnosis to death.

Calculated for each tumor entity, median OS from checkpoint inhibitor start was 19.2 months in patients with NSCLC, while it was not reached in the RCC and melanoma cohort. Median OS from diagnosis was 46.7 months in the non-small cell lung cancer cohort and not reached in patients with renal cell carcinoma and Melanoma **(figure 3.2)**.

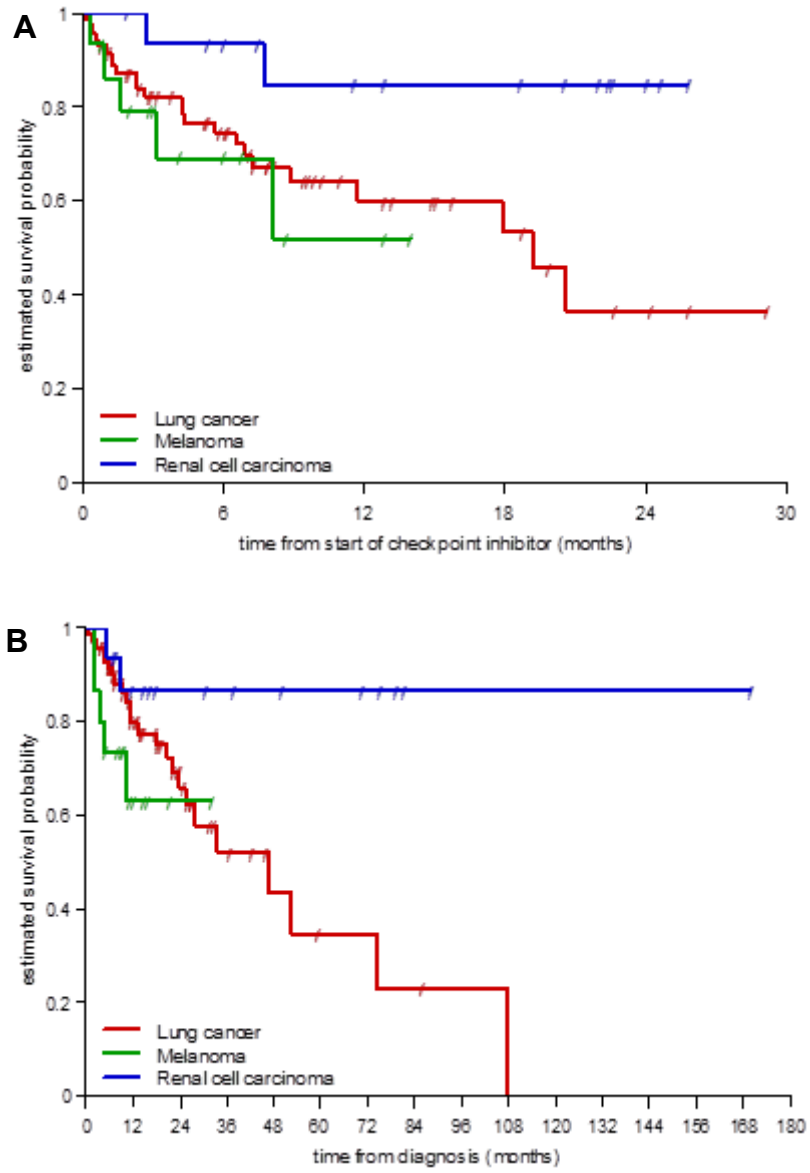

Figure 3.2: Kaplan-Meier curve of OS for each tumor entity.

A) OS from start of checkpoint inhibitor.

B) OS from diagnosis.

3.5 Overall survival for different metastatic sites (pooled cohort)

In the pooled cohort of this study, containing patients with metastatic non-small cell lung cancer, metastatic renal cell carcinoma and metastatic melanoma, 21 patients suffered from liver metastasis, while 82 patients did not. Among the patients with liver metastasis the median overall survival, calculated from start of checkpoint inhibitor treatment to death, was 6.5 month, whereas not reached in patients without liver metastasis. Therefore, median overall survival was significantly worse in patients with liver metastasis, with a calculated hazard ratio of 2.374 (95% CI: 1.114 – 5.062) and a P-value of 0.025 **(table 3.4)**.

However, calculated from diagnosis, the median OS was 11.3 months in patients with liver metastasis compared to 52.3 months in patients without them. Therefore, with HR=2.068 (95% CI: 0.952 – 4.489) and P=0.066 **(table 3.5)**, no statistically significant difference was detected between these two patient cohorts. The Kaplan-Meier curves of OS regarding the existence of liver metastasis are shown in **figure 3.3**.

The analysis of this study also included 32 patients with lung metastasis and 71 patients without lung metastasis. The median overall survival from diagnosis was 20.8 months for patients who had developed lung metastasis, while patients without lung metastasis showed a median overall survival of 74.4 months. In the univariate cox regression we calculated a hazard ratio of 2.384 (95% CI: 1.160 – 4.899) and a P-value of 0.018 **(table 3.5)**. Thus, the development of lung metastasis was associated with a statistically significant worse overall survival in our study.

However, calculated from start of immunotherapy to death, the median overall survival in patients with lung metastasis was 8.8 months versus not reached in patients without metastasis of the lung. The calculated HR was 1.879 (95% CI: 0.926 – 3.815) and the P-value was 0.081 **(table 3.4)**. Therefore, this difference was not statistically significant. The related Kaplan-Meier curves are shown in **figure 3.4**.

The univariate cox regression analyses, regarding the different metastatic locations of the pooled patient cohort in our study, are shown in **table 3.4** and **table 3.5**, subdivided into OS calculated from diagnosis to death and OS calculated from start of immunotherapy to death.

Table 3.4: Univariate cox regression with OS from start of checkpoint inhibitor.

Metastatic site	HR (95% CI)	P-value
Lung (Yes vs. No)	1.879 (0.926 - 3.815)	0.081
Liver (Yes vs. No)	2.374 (1.114 - 5.062)	0.025
CNS (Yes vs. No)	2.034 (0.705 - 5.871)	0.189
Lymph nodes (Yes vs. No)	0.582 (0.278 - 1.221)	0.152
Bone (Yes vs. No)	1.565 (0.718 - 3.409)	0.26
Suprarenal gland (Yes vs. No)	1.095 (0.448 - 2.677)	0.842
Pleura (Yes vs. No)	2.556 (0.770 - 8.486)	0.125
Soft tissue (Yes vs. No)	0.000 (0.000 - Inf)	0.997

Note: for soft tissue there was no event in the group with metastases, therefore the results are not meaningful

Table 3.5: Univariate cox regression with OS from diagnosis.

Metastatic site	HR (95% CI)	P-value
Lung (Yes vs. No)	2.384 (1.160 - 4.899)	0.018
Liver (Yes vs. No)	2.068 (0.952 - 4.489)	0.066
CNS (Yes vs. No)	2.881 (0.982 - 8.455)	0.054
Lymph nodes (Yes vs. No)	0.574 (0.272 - 1.211)	0.145
Bone (Yes vs. No)	1.781 (0.808 - 3.923)	0.152
Suprarenal gland (Yes vs. No)	1.444 (0.588 - 3.547)	0.422
Pleura (Yes vs. No)	1.322 (0.379 - 4.615)	0.662
Soft tissue (Yes vs. No)	0.000 (0.000 - Inf)	0.997

Note: for soft tissue there was no event in the group with metastases, therefore the results are not meaningful

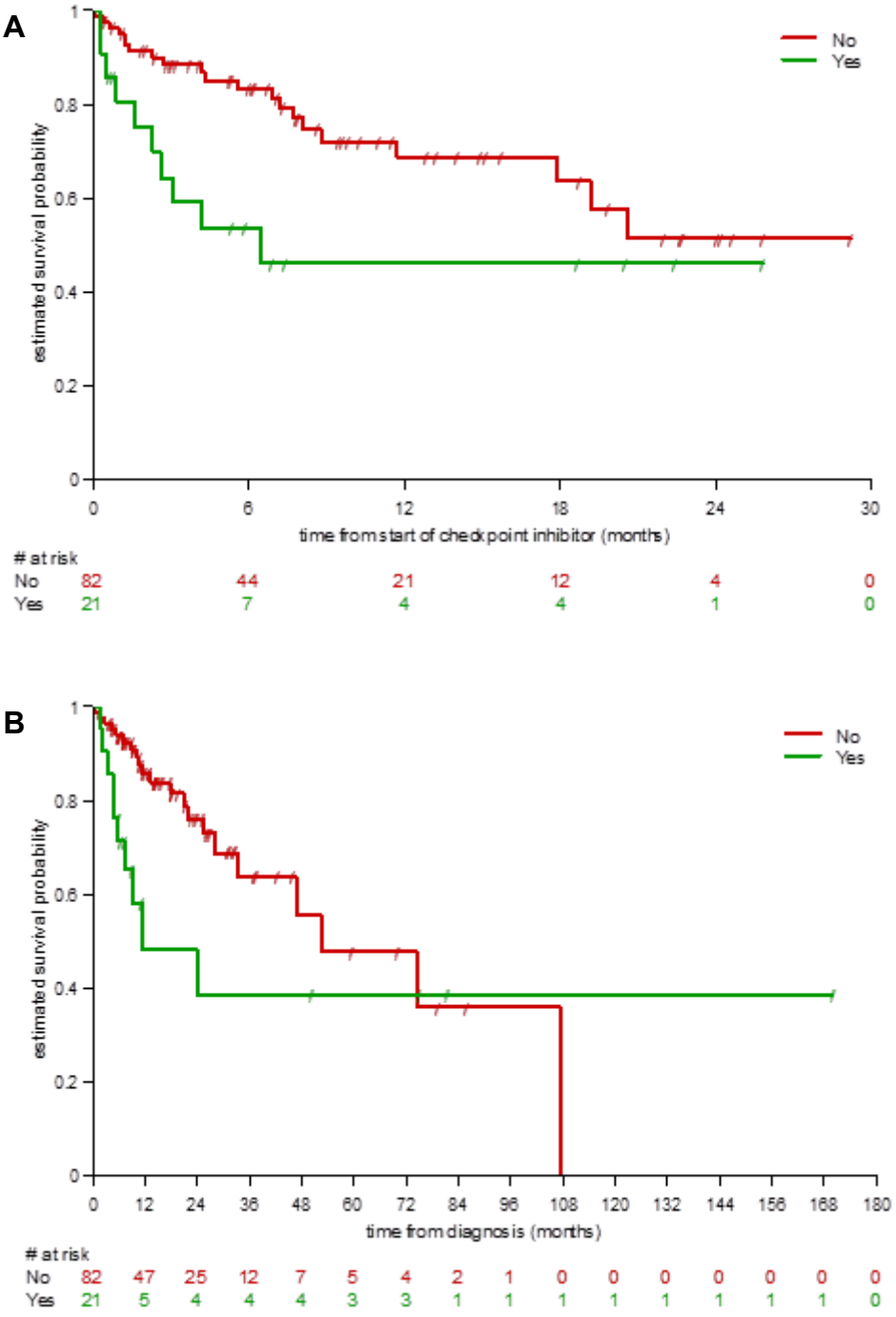

Figure 3.3: Kaplan-Meier curve of OS for liver metastasis (pooled cohort).

A) Overall survival calculated from the start of immunotherapy to death.

(HR=2.374, 95% CI: 1.114 – 5.062, P=0.025)

B) Overall survival calculated from diagnosis to death.

(HR=2.068, 95% CI: 0.952 – 4.489, P=0.066)

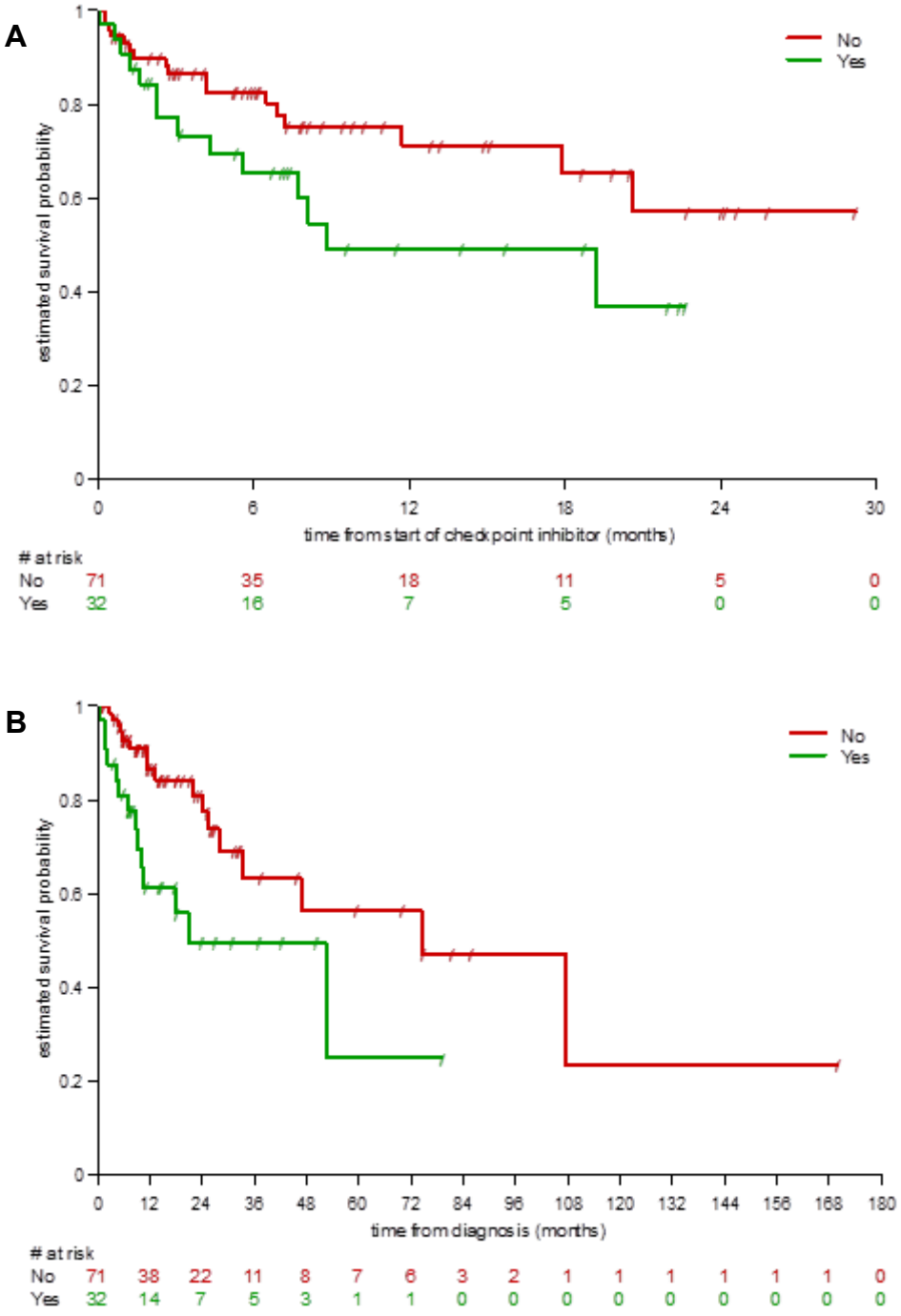

Figure 3.4: Kaplan-Meier curve of OS for lung metastasis (pooled cohort).

A) Overall survival calculated from start of checkpoint inhibitor treatment to death.

(HR=1.879, 95% CI: 0.926 – 3.815, P=0.081)

B) OS from diagnosis to death.

(HR=2.384, 95% CI: 1.160 – 4.899, P=0.018).

Calculated from checkpoint inhibitor start to death as well as diagnosis to death, the univariate cox regression showed no statistically significant difference in overall survival of the 77 patients suffering from lymph node metastasis compared to the 26 patients without lymph node metastasis **(figure 3.5)**. The hazard ratio was 0.582 (95% confidence interval: 0.278 – 1.221, P=0.152) from checkpoint inhibitor start **(table 3.4)** and 0.574 (95% CI: 0.272 – 1.211, P=0.145) from diagnosis **(table 3.5)**.

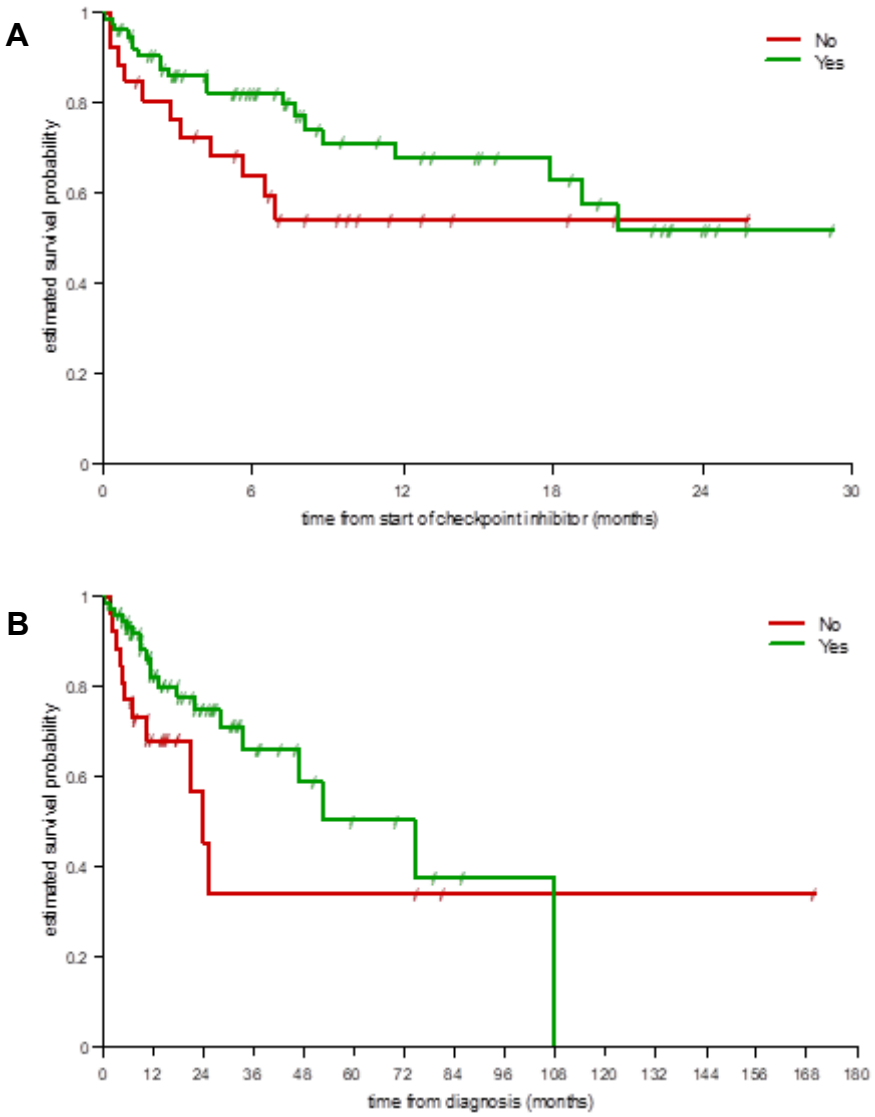

Figure 3.5: Kaplan-Meier curve of OS for lymph node metastasis (pooled cohort).

A) OS from start of checkpoint inhibitor (HR=0.582, 95% CI: 0.278 – 1.221, P=0.152).

B) OS from diagnosis (HR=0.574, 95% CI: 0.272 – 1.211, P=0.145).

The 25 patients with bone metastasis also showed no statistically significant difference in both main endpoints compared to those without this metastasis location **(figure 3.6)**. HR was 1.781 (95% CI 0.808 – 3.923, P=0.152) from diagnosis **(table 3.5)** and 1.565 (95% CI 0.718 – 3.409, P=0.26) from start of immunotherapy **(table 3.4)**.

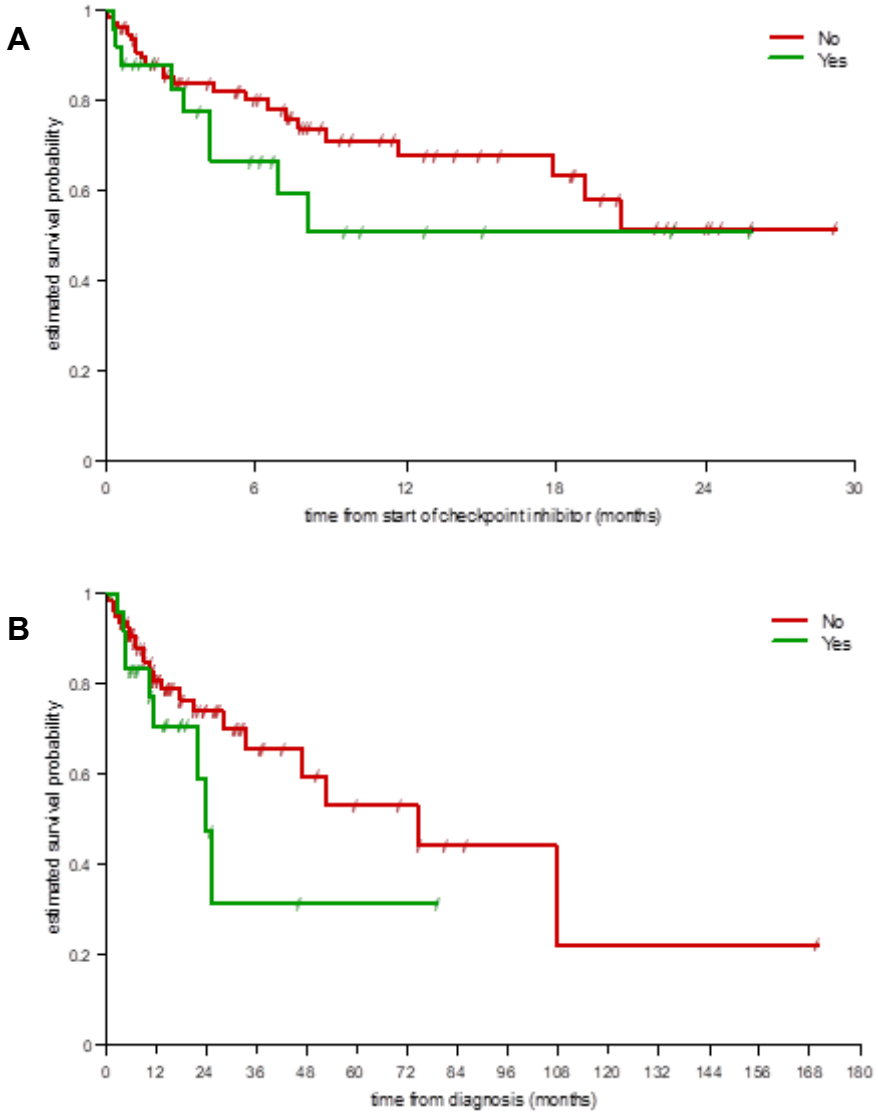

Figure 3.6: Kaplan-Meier curve of OS for bone metastasis (pooled cohort).

A) OS from immunotherapy start (HR=1.565, 95% CI: 0.718 – 3.409, P=0.26).

B) OS from diagnosis (HR=1.781, 95% CI: 0.808 – 3.923, P=0.152).

In the patients with suprarenal gland metastasis, there was no significant difference in overall survival compared to those without metastasis of the suprarenal gland **(figure 3.7)**. The hazard ratio was 1.095 (95% CI 0.448 – 2.677, P=0.842) from start of immunotherapy **(table 3.4)** and 1.444 (95% CI: 0.588 – 3.547, P=0.422) from diagnosis **(table 3.5)**.

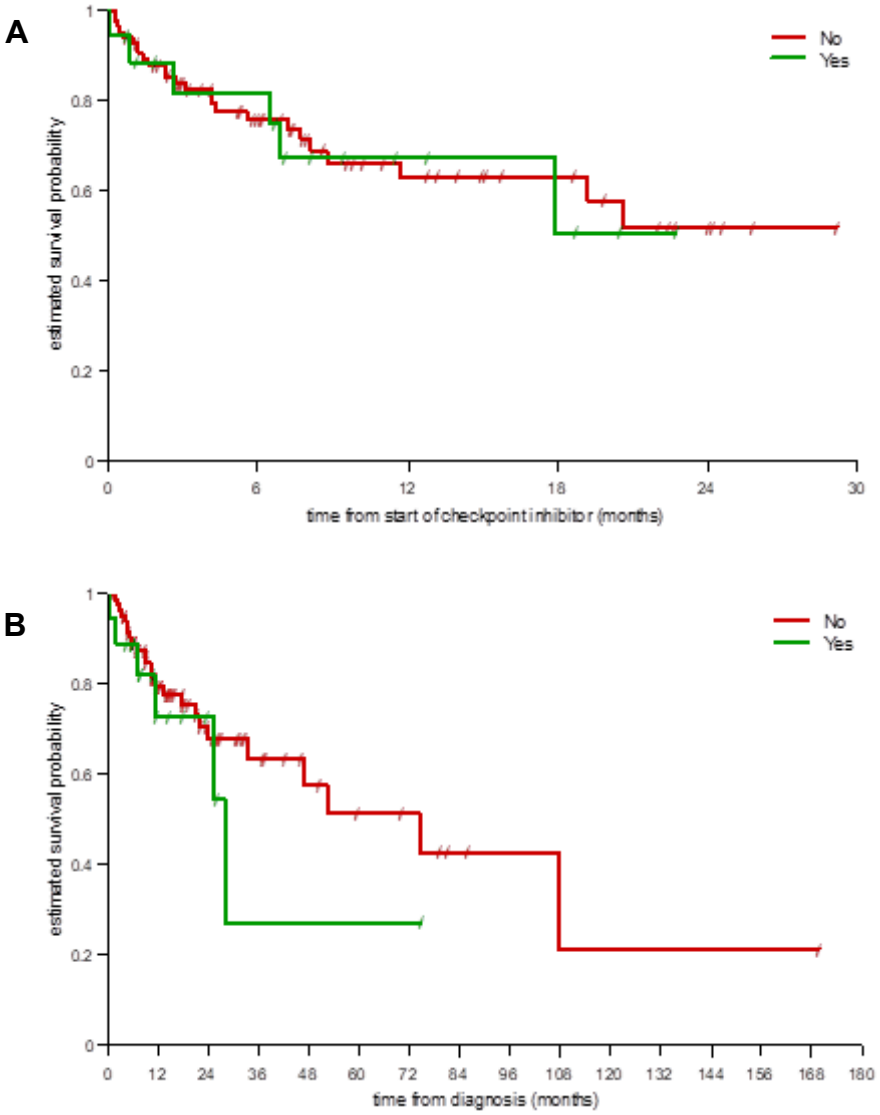

Figure 3.7: Kaplan-Meier curve of OS for suprarenal gland metastasis (pooled cohort).

A) OS from checkpoint inhibitor start (HR=1.095, 95% CI: 0.448 – 2.677, P=0.842).

B) OS from diagnosis (HR=1.444, 95% CI: 0.588 – 3.547, P=0.422).

In the univariate cox analysis there was no statistically significant difference in patients with pleura metastasis compared to patients without them **(figure 3.8)**. The calculated HR was 2.556 (95% CI: 0.770 – 8.486, P=0.125) from treatment start with checkpoint inhibitors **(table 3.4)** and 1.322 (95% CI: 0.379 – 4.615, P=0.662) from time of diagnosis **(table 3.5)**.

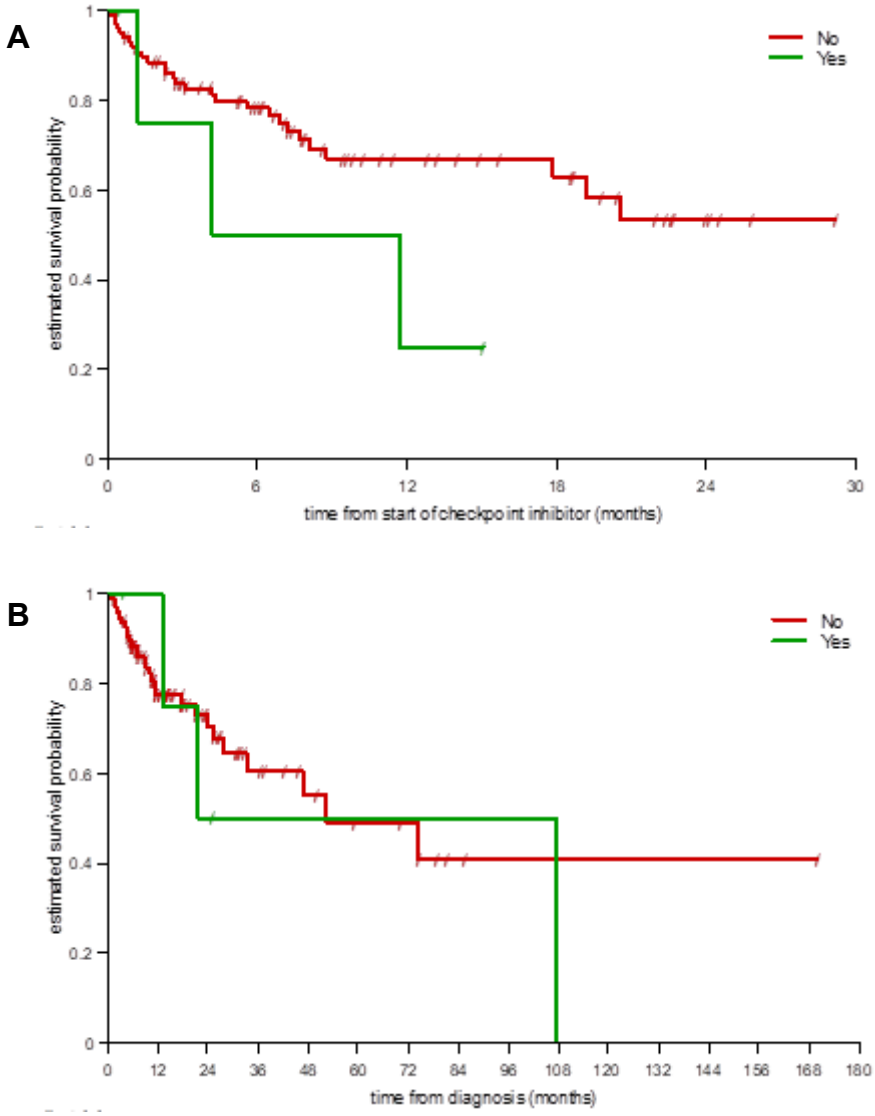

Figure 3.8: Kaplan-Meier curve of OS for pleura metastasis (pooled cohort).

A) OS from start of checkpoint inhibitors (HR=2.556, 95% CI: 0.770 – 8.486, P=0.125).

B) OS from time of diagnosis (HR=1.322, 95% CI: 0.379 – 4.615, P=0.662).

4 Discussion

The results of this study showed, that the overall survival differs according to the metastatic site in patients of the pooled cohort with NSCLC, RCC and Melanoma treated with ICI. Liver metastasis and lung metastasis were associated with a worse prognosis compared to the other metastasis locations. Since the current knowledge on tumor response to checkpoint inhibitors is limited, these findings may be useful in upcoming treatment decisions such as combination treatment.

In this study only NSCLC patients who presented with at least one metastatic site at initial diagnosis were included. 43% presented with a single metastasis location and 33.3% of the patients presented with two metastatic sites. Another 16.7% had three and the remaining 7% had four different metastatic locations at diagnosis. This is largely consistent with a study from Tamiya and colleagues, who reported 24.6% of the patients with one metastatic site, 36.6% with two different metastasis locations, 24% with three and 10% with four as well as 4.8% with more than four metastatic sites at diagnosis.[116] The most common metastasis locations in this analysis were lymph node (81.9%), bone (26.4%), lung (25%), suprarenal gland (18.8%), liver (15.3%) and CNS (5.6%). Several other reports demonstrated lymph node (56%), lung (20.1% - 57.2%), bone (32.8% – 36.2%), liver (13.4% – 25%), brain (2% - 36.9%) and adrenal gland (7.8% - 16.7%) as the most common metastasis locations in patients with non-small cell lung cancer.[107,115-117] The observed differences might be caused by the small number of included patients.

The 72 unselected patients with metastatic NSCLC, which were reviewed in this study, had a median overall survival of 19.2 months from start of checkpoint inhibitor therapy to death. This is significantly longer, than the OS reported in previously accomplished studies investigating patients with advanced non-small cell lung cancer.[95,99,118] This may be due to the fact, that we did not differentiate between distant lymph node metastasis and regional lymph node metastasis.

In the cohort of 15 patients with metastatic melanoma lymph node (53.3%), lung (40%), liver (26.7%), CNS (26.7%), bone (20%), suprarenal gland (13.3%) and soft tissue (13.3%) were the most common metastatic locations. A previous study of Abdel-Rahman from 2018 reported lung (50%), brain (33.6%), liver (24.3%),

distant lymph node (21.9%) and bone (21.3%) as the most common metastatic sites at diagnosis, while another report from Hao et al showed regional lymph node metastasis (45.7%), liver metastasis (24.4%) and subcutaneous metastasis as the most frequent locations.[112,113] Hence, the results of this study are consistent with previous reports.

Within the 16 patients suffering from metastatic renal cell carcinoma, 62.5% showed lymph node metastasis, 50% lung, 37.5% liver, 18.8% bone, 18.8% suprarenal gland and 12.5% soft tissue metastasis. In comparison, Bianchi and colleagues reported lung (45.2%), bone (29.5%), distant lymph node (21.8%), liver (20.3%), adrenal (8.9%) and brain (8.1%) as most common sites of distant metastasis in their cohort of 11157 patients with renal cell carcinoma.[110]

Regarding the metastatic site as a prognostic factor in advanced cancer, the existing studies show a mixed picture. The most reports available so far correspond in the assumption that a higher number of different metastatic locations is associated with a statistically significant poorer median overall survival in patients with NSCLC, RCC and Melanoma.[108,109,111,113,116,119] Therefore, we analyzed the effect of different metastatic locations on the median overall survival. In particular, our knowledge of the effect of metastatic sites on the outcome of patients treated with immune checkpoint inhibitors is currently limited. Thus, we also explored if the median overall survival from start of checkpoint inhibitor treatment to death varies depending on the location of distant metastasis.

In this study, the median overall survival of the pooled cohort including patients with NSCLC, RCC and Melanoma, was significantly lower in patients with lung metastasis (P=0.018) compared to other metastatic sites. This, however, contradicts various other surveys that did not report lung metastasis as unfavorable prognostic factor.[107,109,111,117] Liver and CNS metastasis were not unfavorable prognostic factors in this analysis, however the results were boarderline significant (P=0.066 for liver and P=0.054 for CNS metastasis), which might be due to the small number of included patients. Most of the available studies reported liver metastasis showing a significantly shorter survival compared to patients without liver metastasis, which is contrary to our results.[3,107,111,117,119,120] However, CNS metastasis were discussed

controversially throughout the existing literature. Sorensen et al demonstrated in 1988 that brain metastasis in patients suffering from NSCLC led to a shorter survival compared to those without brain metastasis.[121] Another study from Paesmans and colleagues reported the absence of brain metastasis as a significant factor related to longer survival.[122] However, other surveys did not show a statistically significant shortening of the survival in patients with CNS metastasis compared to those without CNS metastasis.[3,117,120] Furthermore, also bone metastasis showed diverging effects on the survival compared to patients without them. Finkelstein et al reported that bone metastases were associated with poorer prognosis in patients with NSCLC.[123] Also Bauml and colleagues identified bone metastasis as a negative prognostic factor in NSCLC, while other studies demonstrated contrary results.[119,120] In patients with renal cell carcinoma, Abdel-Rahman showed, that the existence of bone metastasis was not associated with worse survival compared to other metastatic sites.[111] This study reported no significant difference in the median overall survival regarding the existence of bone metastasis in the pooled cohort (P=0.152). Also patients with pleura metastasis (P=0.662) showed no significantly poorer OS in this thesis. This matches the results of previous trials.[3,117] Patients with adrenal gland metastasis were examined in a few previous studies and showed a significant poorer median OS compared to patients without adrenal gland metastasis.[3,117] However, Riihimäki et al reported no difference in OS whether patients suffer from suprarenal gland metastasis or not, which is consistent with the results of this study (P=0.422). Furthermore, this thesis also showed no statistically significant difference in median OS whether patients had lymph node metastasis or not (P=0.145). Other trials reported a poorer survival of patients with extrathoracic and abdominal lymph node metastasis.[3,117] This discrepancy is most likely due to the fact, that this study did not differentiate between distant and regional lymph node metastasis.

In this study we also calculated the median overall survival from start of immune checkpoint inhibitor treatment to death to investigate the influence of distant metastatic sites on the effect of checkpoint inhibitors. Therefore, we used the univariate cox regression analysis.

The presence of liver metastasis was associated with statistically significant worse median OS compared to patients without liver metastasis (P=0.025). Thus, presence of liver metastasis appears to affect the efficacy of checkpoint inhibitors in patients with non-small cell lung cancer, renal cell carcinoma and Melanoma. This result matches various previously published studies. For example, in a cohort of 201 patients with NSCLC treated with nivolumab, Tamiya et al reported a median progression-free survival of 1.15 month in the 24 patients with liver metastasis and a PFS of 3.25 month in 172 patients without liver metastasis, which is statistically significant (P=0.0008).[116] In another survey patients treated with pembrolizumab with melanoma and NSCLC suffering from liver metastasis showed a significantly poorer PFS compared to patients without liver metastasis.[124] In 2018 Schmid and colleagues postulated lower efficacy of immunotherapy in a cohort of patients with NSCLC with liver metastasis compared to patients without liver metastasis.[115] Lung (P=0.081), CNS (P=0.189), lymph node (P=0.152), suprarenal gland (P=0.842) and pleura metastasis (P=0.125) were not unfavorable prognostic parameters in this study. Regarding lymph node metastasis the known literature showed concordant results.[115,116,125] Adrenal gland lesions were more responsive to checkpoint inhibitors in the study of Nishino et al, while the cohort of Schmid et al showed a lower activity of ICI treatment in patients with suprarenal gland metastasis.[115,125] Regarding to Tamiya et al, brain (P=0.411) and bone (P=0.192) metastasis did not show a significantly poorer PFS in patients with advanced NSCLC. While Nishino and colleagues reported no difference in response rate of patients with lung metastasis compared to patients without lung metastasis in a cohort of patients with advanced non-small cell lung cancer treated with PD-1 inhibitors, Tamiya et al postulated a significantly worse median progression-free survival (P=0.006) in patients with lung lesions (median PFS of 2.27 months) compared to patients without them (median PFS of 3.52 months).[116,125]

This study has several limitations. Firstly, there is potential of bias due to their retrospective nature. Secondly, because of the low number of patients, the results need to be confirmed in larger trials. And thirdly, due to the fact that a pooled cohort was used for the analysis, there may be divergent results for each single tumor entity.

In conclusion, in the pooled cohort, the effect of immune checkpoint inhibitors is dependent on the metastatic site. In patients with liver metastasis the median OS, calculated from start of ICI therapy to death, is significantly worse compared to patients without liver metastasis, which is consistent with several other reports. Thus, this study showed no difference in median OS in patients with lung, brain, bone, lymph node, pleura and adrenal gland metastasis compared to patients without them. While the published literature is in line regarding lymph node metastasis, the other metastatic sites are discussed controversially. This study also showed that the overall outcome of patients with NSCLC, RCC and Melanoma is dependent on the metastatic location. Various studies confirm that the existence of lung metastasis is associated with poorer overall survival, while reporting divergent results regarding liver metastasis. The effect of brain and bone metastasis varies throughout the literature, while pleura metastasis did not show a significant influence. In summary, our preliminary data suggest a predictive effect of location of metastasis in patients treated with immunotherapy and may help in treatment selection such as combination treatment. However, these data needs to be validated in upcoming prospective clinical trials.

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