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DISSERTATION

Changes in peripheral blood PD-1 receptor expression under anti-PD-1 Immune Checkpoint Therapy

Veränderungen der PD-1-Expression in peripherem Blut unter Anti-PD-1-Immun-Checkpoint-Therapie

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List of Abbreviations

PD-1	Programmed Death 1
HNSCC	Head and neck squamous cell carcinoma
RCC	Renal cell carcinoma
PBMCs	Peripheral blood mononuclear cells
IgG4	Immunoglobulin G4
irAEs	Immune-related adverse events
HPV	Human papilloma virus
mRNA	Messenger ribonucleic acid
CD	Cluster of differentiation
IgV	Immunoglobulin variable type
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
CTLA-4	Cytotoxic T-lymphocyte-associated Protein 4
PD-L1	Programmed Death Ligand 1
PD-L2	Programmed Death Ligand 2
DC	Dendritic cells
IL-12	Interleukin 12
IL-2	Interleukin 2
SHP-2	Src homology region 2 domain-containing phosphatase-2
ZAP-70	Zeta-chain-associated protein kinase 70
PKC-θ	Protein kinase C θ
TCR	T cell receptor
Syk	Spleen tyrosine kinase
PLCγ2	Phosphatidylinositol-specific phospholipase Cγ2
Erk	Extracellular-signal regulated kinases
BCR	B cell receptor
MHC	Major histocompatibility complex
AP-1	Activator protein 1
NFAT	Nuclear factor of T cells
NF-κB	Nuclear factor of κB
BATF	Basic leucine zipper transcriptional factor ATF-like
APC	Antigen presenting cell

NSCLC	Non-small-cell lung cancer
TNF-α	Tumor necrosis factor α
IFN-γ	Interferon γ
IFN-α	Interferon α
mTOR	Mammalian Target of Rapamycin
VEGF	Vascular Endothelial Growth Factor
FDA	Food and drug administration
TILs	Tumor-infiltrating lymphocytes
EBV	Epstein Barr Virus
NK cells	Natural killer cells
LDH	Lactate dehydrogenase
HLA	Human leukocyte antigen
TME	Tumor microenvironment
HLA-DR	Human leukocyte antigen – DR isotype
STD	Standard deviation
SD	Stable disease
PR	Partial response
PD	Progressive disease
Fc	fragment crystallizable
PBS	phosphate-buffered saline
BSA	bovine serum albumin
PE	Phycoerythrin
APC	Allophycocyanin
BV	Brilliant violet
FITC	Fluorescein
Cy	Cyanine
PerCP	Peridinin chlorophyll
BUV	Brilliant ultra violet
ELISA	Enzyme-linked immunosorbent assay
TMB	Tetramethylbenzidine
FSC	Forward scatter
SSC	Side scatter
WB	Whole blood
SEM	Standard error of the mean

Mono	Monocytes
Baso	Basophiles
Tregs	Regulatory T cells
p16	Protein 16
iRECIST	Response Evaluation Criteria in Solid Tumors (for immunotherapy)
MR	Mixed response
CR	Complete response
TGF-ß	Transforming growth factor β

1 Abstract

Immune checkpoint receptors are key regulators of the immune system, especially in autoimmunity and cancer. Programmed Death 1 (PD-1) is one of the most relevant immune checkpoints known and research around it as well as approval of anti-PD-1 monoclonal antibodies has led to a revolution in cancer therapy in the last decade. While trial results and remission rates have been impressive compared to therapies used before, not all patients benefit from immune checkpoint therapy. It is still largely unknown what distinguishes patients who respond to anti-PD-1 inhibition from those who do not, and reliable biomarkers predicting therapy outcomes are urgently needed. We set out to analyse PD-1 expression in peripheral blood immune cells of head and neck squamous cell carcinoma (HNSCC) and renal cell carcinoma (RCC) patients before and throughout treatment with anti-PD-1 monoclonal antibody Nivolumab to assess whether connections between PD-1 expression and therapy response can be drawn. As the basis for our longitudinal analysis, we established a protocol for whole blood preparation and antibody staining for flow cytometry. PD-1 blockade by Nivolumab poses a challenge in accurately detecting the receptor in flow cytometry. Secondary staining of PD-1 under these conditions has previously been successfully applied on peripheral blood mononuclear cells (PBMCs). We established a staining procedure utilizing a secondary IgG4 antibody and demonstrated that PD-1 staining under Nivolumab therapy can reliably be carried out on whole blood samples that were previously stabilized and frozen. We show that PD-1 expression is significantly higher on immune cells of HNSCC patients compared to RCC patients before and during Nivolumab therapy. We attributed higher PD-1 expression in HNSCC to patients with HPV-positive tumors. Higher PD-1 expression levels at the end of our trial period were associated with the occurrence of immune-related adverse events (irAEs), most distinctly in CD8+ CD38+ activated effector T cells. We compared PD-1 expression in responders and non-responders to Nivolumab therapy and observed elevated levels of PD-1 in T cells of patients with stable disease or partial response to therapy compared to patients with progressive disease. However, our data was non-significant in this regard and larger trials are needed to investigate such associations in RCC and HNSCC patients.

Immun-Checkpoint-Rezeptoren sind entscheidende Regulatoren des Immunsystems, insbesondere bei Autoimmun- und Krebserkrankungen. Programmed Death 1 (PD-1) ist einer der wichtigsten bekannten Immun-Checkpoints und seine Erforschung sowie die Zulassung von Anti-PD-1 monoklonalen Antikörpern hat zu einer Revolution der Krebstherapie im letzten Jahrzehnt geführt. Während Studienergebnisse und Remissionsraten im Vergleich zu vorher genutzten Therapien beeindruckend sind, profitieren nicht alle von Immun-Checkpoint-Therapien. Es ist immer noch weitgehend unbekannt, was Patient*innen, die auf PD-1-Inhibitoren ansprechen von denen unterscheidet, die nicht ansprechen. Es werden deshalb dringend zuverlässige Biomarker für die Voraussage des Ansprechens benötigt. Wir haben in dieser Arbeit die PD-1-Expression in Immunzellen des peripheren Blutes von Patient*innen mit Kopf-Hals-Tumoren (HNSCC, engl. head and neck squamous cell carcinoma) und Nierenzellkarzinomen (RCC, engl. renal cell carcinoma) vor sowie während der Therapie mit dem monoklonalen Anti-PD-1-Antikörper Nivolumab untersucht, um einzuschätzen, ob Verbindungen zwischen der PD-1-Expression und dem Therapieansprechen gezogen werden können. Als Grundlage für unsere longitudinale Analyse etablierten wir ein Protokoll für die Aufbereitung von Vollblut-Proben und Antikörper-Färbungen für durchflusszytometrische Messungen. Die PD-1-Blockade durch Nivolumab stellt eine Hürde in der genauen Detektion des Rezeptors mittels Durchflusszytometrie dar. Eine sekundäre Färbung von PD-1 ist bereits erfolgreich auf peripheren mononukleären Blutzellen (PBMCs) angewendet worden. Wir etablierten ein Färbeverfahren mit einem sekundären Anti-IgG4-Antikörper und konnten so demonstrieren, dass die Färbung von PD-1 unter Therapie mit Nivolumab zuverlässig auf vorher stabilisierten und gefrorenen Vollblut-Proben durchgeführt werden kann. Wir zeigen, dass die PD-1-Expression auf Immunzellen von HNSCC-Patient*innen vor und während der Therapie mit Nivolumab signifikant höher im Vergleich mit der von RCC-Patient*innen ist. Wir konnten die höhere PD-1-Expression HNSCC-Patient*innen mit HPV-positiven Tumoren zuordnen. Höhere PD-1-Expressionslevel am Ende unseres Studienzeitraumes waren zudem mit dem Auftreten von immunvermittelten Nebenwirkungen (irAEs, engl. immune-related adverse events) assoziiert, besonders in CD8+ CD38+ aktivierten Effektor-T-Zellen. Wir verglichen die PD-1-Expression von Respondern und Non-Respondern auf die Nivolumab-Therapie und beobachteten erhöhte PD-1-Werte in T-Zellen von Patient*innen mit Stable Disease oder Partial

Response unter Therapie verglichen mit Patient*innen mit Progressive Disease. Jedoch waren unsere Daten in diesem Zusammenhang nicht signifikant und größer angelegte Studien werden benötigt, um solche Zusammenhänge in RCC- und HNSCC-Patient*innen gezielt zu untersuchen.

2 Introduction

2.1 Programmed Death 1

PD-1 is an inhibitory receptor expressed primarily on T cells, though also found on a variety of leukocyte subsets. It was first described by Tasuku Honjo et al. at Kyoto University in 1992 as a member of the immunoglobulin gene superfamily (1). The group discovered PD-1 exploring cell death and apoptosis. They described PD-1 mRNA as highly upregulated upon induced programmed cell death and therefore hypothesized that PD-1 might be an inducer of programmed cell death, while not being its sole responsible factor. The authors had already reported on the membrane-spanning polypeptide encoded by the PD-1 gene, though they had only speculated about its molecular associations with possible ligands (1). Pursuing deeper understanding of the newly discovered receptor, the same group went on studying it in the following years and subsequently identified that PD-1 was induced on the surface of activated T and B lymphocytes by signalling through antigen receptors. In these studies, a self-manufactured anti-PD-1 monoclonal antibody was used (2). Later on, a connection to insufficient and dysregulated immune responses was drawn in several studies with PD-1-deficient transgenic mice (3, 6).

For his early and crucial work on PD-1, which would pave the way for a breakthrough in cancer medicine, Honjo – together with James P. Allison – received the Nobel Prize for physiology or medicine in 2018.

PD-1 is a type I transmembrane glycoprotein consisting of an immunoglobulin variable type (IgV) extracellular domain, a transmembrane domain and a tail in the cytoplasm functioning as an intracellular binding site for signalling molecules through an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) (7). It belongs to the B7/CD28/CTLA-4 receptor family and – contrary to most other members of that family – is monomeric in solution as well as when expressed on the cell surface (7). The front β sheet face in the extracellular variable type immunoglobulin domain of PD-1 serves as the binding site for the respective β sheets in the IgV domains of its ligands Programmed Death Ligand 1 (PDL-1) and Programmed Death Ligand 2 (PDL-2), enabling the complex formation of both respective IgV domains (7).

Glycosylation is an important posttranslational modification in immunoglobulin type proteins, in part determining their structure and, therefore, function. The common binding of N-Glycans to the asparagine side chains of a protein is called N-glycosylation. PD-1 has four potential N-linked glycosylation sites in its IgV domain (N49, N58, N74, and N116), with carbohydrates at these sites defining the domain's conformity (8).
In principle, PD-1 glycosylation was found to be non-essential for PD-1/PD-L1 interaction – which was likely due to the glycosylation sites being sufficiently far away from the region engaged in ligand binding (8). However, in vitro data suggests that N-glycan core fucosylation of PD-1 at N49 and N74 positively affects its cell surface expression and therefore cells' susceptibility for PD-1 ligation and its effects (9). Thus, reduced PD-1 cell surface expression through blocking PD-1 core fucosylation by genetic ablation or pharmacologic inhibition showed improved T cell anti-tumor effects (9). Furthermore, glycosylation of PD-1 is suggested to potentially alter the receptor's orientation on the cell surface, and thus might indirectly affect binding of ligands (7).
The glycosylation status of PD-L1 at its three potential N-linked glycosylation sites (N192, N200 and N219) is crucial for its T cell suppressive function in cancers, stabilizing the receptor and potentially sparing it from ubiquination (8).
On the other hand, PD-1 glycosylation was found to be non-essential to the binding of blocking anti-PD-1 monoclonal antibodies, with Nivolumab binding to a recently discovered N-terminal loop outside its IgV domain, which furthermore does not overlap with Pembrolizumab's binding site (10, 11).

PD-1's ligands PDL-1 and PDL-2 are also type I transmembrane glycoproteins. PDL-1 is physiologically expressed on a variety of cells, including hematopoietic cells such as T cells, B cells, dendritic cells (DC) and macrophages as well as on other tissue such as vascular or stromal cells (12). PDL-2 expression is limited to a narrower range of cells, including DC, macrophages and B cells. Both ligands have been associated with inflammation and they are both expressed by cancer cells, while PDL-1 is clinically more relevant due to its much more common expression (12).
Cell activation plays a key role in the expression and upregulation of PDL-1. It has been shown that IL-12 and interferons increase the receptor's expression (13, 14). Based on findings like that, the concept of 'adaptive resistance' in tumors has been discussed as an explanation for the upregulation of PDL-1 on tumor cells and its microenvironment.

The concept postulates that cytokines released by tumor-infiltrating T cells be key inducers of upregulated expression of PDL-1, resulting in a vicious circle maintained by the immune system trying to fight cancer cells while promoting resistance to itself (12).

2.1.1 PD-1 ligand interaction and signalling

As stated above, ligation of PD-1 to PDL-1 and PDL-2 is possible through the binding of the receptors' respective IgV domains. In PD-1-expressing cells, this leads to a cascade of intracellular signalling through the tyrosine motifs ITIM and ITSM in the receptor's cytoplasmic tail (15).

It has been shown in mutational studies that PD-1 conveys its inhibitory effects solely through ITSM: Mutated ITIM had no effect on the inhibitory function of PD-1 – characterized by inhibited production of T cell growth factor IL-2 and T cell expansion – whereas mutated ITSM did, with the authors concluding that factors binding to ITSM be the crucial determinants of inhibition (15, 16).

Through PD-1 engagement, both tyrosine motifs are phosphorylated. Upon ligation, ITSM mainly recruits the phosphatase SHP-2 (Src homology region 2 domain-containing phosphatase-2), leading to dephosphorylation of signaling molecules engaged in downstream pathways (Figure 1), e.g., CD3ζ, Zap70 and PKC-θ in T cell receptor (TCR) downstream signaling, and Igβ, Syk, PLCγ2 and Erk in B cell receptor (BCR) downstream signaling, resulting in inhibitory effects (16, 17). Phosphorylation of PD-1-specific ITSM (PD-1-Y248) is discussed as a marker for T cell inhibition. Serving as the key molecular mechanism of PD-1 enfolding its suppressive effects on the immune system, it might in fact be a more significant biomarker than PD-1 expression in assessing the pathway's inhibitory effects (18).

The TCR complex is responsible for recognizing antigens presented by major histocompatibility complex (MHC) molecules on antigen presenting cells. The cascade set in motion by this engagement is the crucial mechanism of T cell activation.

As mentioned above, TCR downstream signalling is a central target of the phosphatases recruited upon PD-1 ligation. This eventually leads to a decrease in the activation of transcription factors, such as activator protein 1 (AP-1), nuclear factor of T cells (NFAT) and nuclear factor of κB (NF-κB) and through that to the inhibition of T cell activation as well as proliferation, survival and the cells' effector functions. Besides downregulation of transcription factors promoting activation, PD-1 can also upregulate

transcription factors that counteract T cell effector functions, such as basic leucine zipper transcriptional factor ATF-like (BATF). (12)

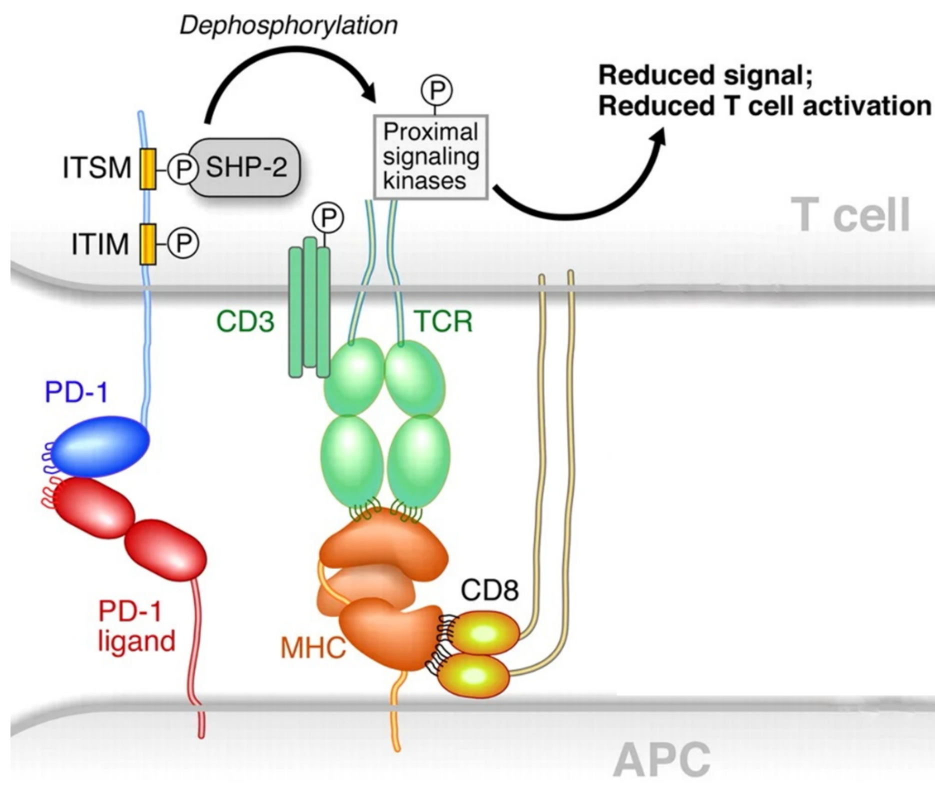

Figure 1: PD-1 downstream signalling in T cells.
Upon ligation of PD-1 on a T cell with PD-L1 expressed by an antigen presenting cell (APC), ITIM and ITSM are phosphorylated. ITISM recruits phosphatase SHP-2 which dephosphorylates downstream TCR-associated signalling kinases. This leads to reduced T cell activation.
Source: (19)

CD28 is a costimulatory receptor on T cells and plays a key role in T cell activation. Its ligands are CD80 (B7-1) and CD86 (B7-2), which are expressed by antigen presenting cells. Data published by a group in 2017 suggests that PD-1 ligand interaction majorly affects CD28 signalling in addition to TCR signalling, and that its downstream cascade might in fact be an equally or more important target in the pathway altogether (20). It was shown that CD28 is preferentially dephosphorylated compared to TCR upon PD-1 ligation with PDL-1, leading to inactivation of the pathway and the affected T cells, and that effective anti-PDL-1 therapy is reliant on CD28 expression of CD8+ T cells (20, 21). In trying to connect their findings from animal studies with clinical observations in

humans, one of the cited groups analysed T cells of NSCLC (Non-small-cell lung cancer) patients treated with anti-PD-1 therapy. CD8+ highly Ki-67-positive effector T cells that had been activated by checkpoint blockade were predominantly CD28-positive, indicating a significant role of CD28 in the effectiveness of anti-PD-1 therapy (21). Interestingly, this is in accordance with another paper of the same laboratory that identified the CD8+ PD-1+ T cell population responsible for the proliferative burst after anti-PD-1 checkpoint therapy. There too, the authors showed that amongst other markers, the population was characterised by high CD28 expression. (22)

PD-1/PDL-1 binding is commonly described as binding in trans, referring to the interaction of receptors on two different cells. In contrast, cis binding refers to the binding of receptors on the same cell. To date, trans binding is the most relevant aspect in models and research dealing with PD-1/PDL-1 interaction, and in understanding its role in cancer and autoimmunity. However, there is recent data showing cis binding of PD-1 and PDL-1 with high affinity on certain tumor-infiltrating antigen presenting cells (APCs) and cancer cells expressing both receptors, thus competing with their trans interaction (23). The authors suggest that cis binding might be a relevant regulatory mechanism in the PD-1/PDL-1 pathway (23), since blocking of tumor-expressed PD-1 by therapeutic monoclonal antibodies may release PDL-1 from a current interaction with its ligand, enabling or amplifying the axis' inhibitory effects on the immune system and, in conclusion, leading to an unwanted and paradox therapy effect (24).

2.1.2 PD-1 in T cell exhaustion

High PD-1 expression is a fundamental feature characterizing T cell exhaustion, either due to chronic infection or cancer (25). In 1998, exhausted T cells were first described as virus-specific activated CD8+ cells that – lacking the ability to produce antiviral cytokines – were unable to engage in antiviral activity in an experimental setting in chronically infected mice, resulting in viral immune evasion (26). Before loss of effector functions, T cells present distinct changes of partial impairment in advance to that. A group reported on the hierarchical loss of functions in CD8+ T cells upon chronic infection, starting with the disappearing ability of IL-2 production and targeting cell lysis in vitro, followed by compromised production of TNF-α and, finally, IFN-γ, marking the latter as most resistant to the state of functional exhaustion (27). In these experimental

settings, the final stage of exhaustion was defined by the deletion of virus-specific T cells (26, 27).
CD4+ T cells can also enter the state of exhaustion, characterized by a lack of effector cytokine production like IL-2 and TNF-α. However, less is known about the underlying mechanisms and consequences. (28-30)

It has become clear that T cell exhaustion plays a pivotal role in cancer, too (31). In animal studies, tumor infiltrating cells such as tumor-specific CD8+ T cells have been shown to exhibit an exhausted phenotype with downregulated effector functions and high expression of immune checkpoints like PD-1 (32, 33). This has also been confirmed to be the case for numerous cancers in humans (31, 34, 35).
In 2006, a group found PD-1-mRNA to be highly upregulated during chronic viral infection on virus-specific CD8+ T cells in a genome-wide analysis in mice (25). In this study, effector T cells also lost their ability to engage in antiviral activity – characterised by downregulated IFN-γ production upon stimulation – and exhibited absence of proliferation. The authors followed this by blocking the PD-1 pathway with an anti-PDL-1 antibody, which resulted in restored T cell effector functions, such as production of both IFN-γ and TNF-α and, as a consequence, reduction in viral load in the treated mice (25). Accompanying this discovery, similar studies showed that blocking the PD-1 pathway in cancer models led to comparable effects of restored effector functions, and thus anti-tumor effects could be achieved (31, 36, 37).
Based on the implications of such research results, the development of immune checkpoint therapy targeting the PD-1 pathway in the treatment of cancer was initiated.

2.2 Immune Checkpoint Therapy

The adaptive immune system's functionality relies on self-regulation. While its core functions lie in identifying and fighting pathogens and aberrant cells, recognising healthy cells as well as maintaining self-tolerance is just as crucial. Immune checkpoints such as the PD-1/PDL-1 pathway are important mechanisms of self-regulation of the adaptive immune system.
While intact, these pathways are responsible for preventing or limiting pathophysiological disorders like autoimmunity and cancer. However, cancers can alter the PD-1 pathway through upregulation of the receptor's ligand PDL-1 on cancer cells and other cells of the tumor microenvironment (TME). This results in the suppression of

PD-1-expressing immune cells, which prevents them from recognising and fighting malignant cells. (12)
Therefore, inhibiting the pathway by blocking either PD-1 or PDL-1 reinvigorates the immune system's suppressive effects on the growth of malignant cells.
Based on this idea, anti-CTLA-4 (cytotoxic T-Lymphocyte-associated antigen 4) antibody Ipilimumab was the first immune checkpoint inhibitor approved in the US and EU in 2011, significantly improving overall survival in the treatment of patients with metastatic melanoma (38, 39). While representing a breakthrough in the treatment of melanoma, it should also revolutionize the treatment of cancer with immunotherapy altogether and pave the way for the development of therapeutic antibodies targeting the PD-1 pathway.

2.2.1 Nivolumab

Nivolumab is a monoclonal anti-PD-1 antibody first approved for the treatment of melanoma in 2014 in the US (40). Alone, or in combination with Ipilimumab, it significantly improved overall survival and progression-free survival compared to Ipilimumab monotherapy: In 2015, trial results were published that showed a significantly improved progression-free survival in previously untreated melanoma patients administered with Nivolumab or Nivolumab combined with Ipilimumab compared to Ipilimumab monotherapy (41). Results of a trial that compared Ipilimumab and Nivolumab in advanced stage melanoma published in 2017 showed a significantly improved survival in patients treated with Nivolumab (42). And in 2018, the 4-year outcomes of a trial confirmed durable and sustained survival benefits in advanced stage melanoma patients treated with combination therapy of Ipilimumab and Nivolumab or Nivolumab alone compared to Ipilumumab monotherapy (43).
Following this breakthrough in the treatment of melanoma, Nivolumab was approved for several other types of cancer in the years after, starting with NSCLC in 2015. Today, it is furthermore approved for the treatment of advanced RCC, Hodgkin's lymphoma, HNSCC, urothelial carcinoma, malignant pleural mesothelioma, certain forms of colon cancer and squamous oesophageal cancer. (44) Trials for the treatment of additional forms of cancer are ongoing.

Compared to most drugs used for chemotherapy, adverse drug reactions to Nivolumab are generally milder, yet prevalent and possibly serious. The most common side effects

include fatigue, rash, diarrhoea, decreased appetite and nausea. irAEs pose a particular risk in this form of treatment, and their diagnosis and treatment should be given special attention due to their potentially serious nature. Among the most common irAEs in Nivolumab treatment are pneumonitis, as well as inflammation of the gastrointestinal tract like colitis, and of the endocrine glands like hypophysitis and thyroiditis as well as hepatitis. (45)

The pathophysiology of irAEs has not yet been finally understood. However, as PD-1 blockade hampers PD-1-mediated peripheral tolerance and cytotoxic T cells are reinvigorated, auto-immune events in the shape of irAEs might be the result.

Nivolumab is a human IgG4 monoclonal antibody with high affinity and specificity for PD-1. (46) Binding to PD-1, Nivolumab blocks the receptor from interacting with its two ligands PDL-1 and PDL-2. In addition to this direct receptor neutralizing drug effect, considerably strong induction of receptor internalization upon ligation has been speculated about as another level of modulation (47).

In a phase I trial, PD-1 mean peak receptor occupancy by Nivolumab was measured at 85% and mean plateau occupancy at 72%, while PD-1 occupancy seemed to be independent from drug dose, which was likely due to high drug-receptor affinity. Decay of occupancy was reached after 85 days. (46)

Animal studies showed considerable anti-tumor effects of anti-PD-1 antibodies. In mice treated with therapeutic antibodies, eradication of various tumors as well as improved survival could be shown (48, 49).

The mechanisms underlying these effects have been described in large parts as results of a decrease of immunosuppressive effects carried out through the PD-1 pathway following PD-1 blockade. This was characterised early on by the upregulation of markers of T cell activation such as Ki-67, IFN-γ and granzyme B (49, 50).

Besides Nivolumab, other PD-1-inhibiting monoclonal antibodies have been approved for cancer treatment, most notably Pembrolizumab (51).

2.2.2 Anti-PD-1 immunotherapy in renal cell carcinoma (RCC)

Representing the most common form of kidney cancer, RCC is among the 10 most diagnosed kinds of cancers in the population of the western world. Its prevalence rises with higher age, being diagnosed on average at 64 years. (52)

RCC has been described as highly immunogenic (53-55) and is resistant to conventional chemotherapy (56). Therefore, accompanying and following well-

established surgical therapy, there have been various approaches to systemic adjuvant treatment, beginning with IL-2- and IFN-α-based immune therapies in the 1980s and 90s, with antibody-based targeted therapies starting to be added to therapy regimens in the early 2000s (57). Today, among the standard therapies for irresectable RCC, or in a palliative situation, are multi kinase inhibitor Sorafenib, receptor tyrosine kinase inhibitors like Sunitinib, mTOR inhibitors like Everolimus, as well as VEGF-antibodies such as Bevacizumab, still accompanied by IL-2 and IFN-α under certain circumstances (58).
In 2015, anti-PD-1 checkpoint inhibitor Nivolumab was first FDA-approved for second line treatment of advanced RCC (59). In 2019, Nivolumab combined with Ipilimumab as well as Pembrolizumab in combination with tyrosine kinase inhibitor Axitinib were also approved for first line treatment of RCC, with both combinations significantly improving therapy outcomes compared to Sunitinib (60, 61). While survival rates have improved with anti-PD-1 checkpoint therapy, prognosis of RCC is still poor, especially in advanced stages (52).

Increased levels of tumor-infiltrating lymphocytes (TILs) have been shown in multiple analyses in the majority of RCC patients, while more substantial infiltration with immune cells has been associated with poor outcome (53-55). Immunosuppressive effects carried out by TILs might be responsible for tumor immune escape in RCC patients. In line with theories describing T cell exhaustion as a result of permanent antigen exposure, TILs in RCC have been characterized as expressing an exhausted phenotype. This suggests that high antigen exposure followed by immune cell exhaustion might be a mechanism responsible for immune evasion of renal cell carcinoma (62, 63).
Blocking the PD-1/PD-L1 pathway potentially enhances the compromised anti-tumor immunity in RCC patients.

While Nivolumab is clearly a more effective therapy choice compared to prior standard drugs in RCC therapy, it has turned out that – as in other types of cancer – there is only a relatively small fraction of patients showing a good response to anti-PD-1 therapy, while the responsible factors are still to be determined.
A trial investigating the role of PD-1 expression on TILs in RCC patients found that there was no link of receptor expression levels to therapy outcome, marking it inferior to

other established biomarkers, namely primary tumor stage and the pathohistological tool Fuhrman nuclear grade (63). More recently, another study proposed high baseline levels of soluble PD-1 as a biomarker predicting favourable therapy response in RCC patients treated with Nivolumab, as it showed a positive association to overall progression-free survival in two cohorts (64). Further research is necessary to evaluate whether markers immediately involved in the PD-1/PDL-1 pathway might be reliable tools in predicting anti-PD-1 therapy response in RCC.

2.2.3 Anti-PD-1 immunotherapy in head and neck squamous cell carcinoma (HNSCC)

HNSCC summarizes a group of cancers deriving from the mucosal epithelium of the mouth, pharynx and larynx (65). While malignancies in the oral cavity and larynx are often associated with tobacco use and alcohol consumption, as well as other geographically distinct forms of substance use like chewing betel quid, carcinomas of the pharynx are progressively linked to high-risk HPV infection (65). Globally, HNSCC is estimated to represent the sixth most common form of cancer, with incidences rising (65, 66). Notably, in India, Taiwan and parts of China, use of areca nut or betel quid is widely common and associated with substantially higher case numbers of HNSCC, i.e., making it the most common form of cancer in the Indian male population (65, 67, 68). The disease is typically diagnosed in older patients, while substance abuse derived forms are associated with later disease development than HNSCC cases linked to infection with HPV or EBV (65).

In 2016, Nivolumab was approved for treatment of recurrent or metastatic HNSCC previously treated with a platinum-based therapy, significantly improving overall survival compared to the previous therapies of choice, namely Methotrexate, Docetaxel or Cetuximab (69). Pembrolizumab was approved for treatment of metastatic or recurrent HNSCC in the same year (70). In addition, Pembrolizumab was also approved for first-line treatment in 2019 (71).

Over the last few decades, survival rates of HNSCC patients have been improving moderately due to new therapy forms, while a considerable part of that development is attributed to increasing incidences of HPV-positive HNSCC, which is typically diagnosed among a younger patient population with more favorable outcomes (72).

HNSCC lesions have been shown to be highly infiltrated by TILs, while proportions of the various immune cells involved vary between HPV-positive and negative tumors (73).

A group that investigated TIL patterns in HNSCC patients showed that HPV-associated tumors were infiltrated by significantly higher numbers of TILs compared to HPV-negative samples, a relevant part of which were CD8+ T cells with the ability to produce IFN-γ upon stimulation in vitro (73). Furthermore, the authors reported on significantly higher expression of PD-1 on HPV-positive tumor tissue as well as higher PDL-1 expression on tumor tissue compared to lymph nodes, regardless of HPV status (73). In line with these findings and in diametrical contrast to data on RCC, another group showed that PD-1 expression on tumor-infiltrating T cells in HNSCC was a favorable biomarker associated with improved overall survival in HPV-associated cases (74). Both groups proposed that positive PD-1 status of T cells might be a sign of prior anti-tumor activity during cancer formation, making the cells susceptible for reinvigoration through PD-1 blockade (73, 74).

2.3 Biomarkers for therapy response prediction

Anti-PD-1 immunotherapy has been in use for several years now, and the number of approvals for the treatment of more and more types of cancers is rising. As the use of drugs like Nivolumab and Pembrolizumab is becoming ubiquitous and represents potentially substantial improvements in survival and quality of life for a great number of patients, the demand for reliable biomarkers helping in the prediction of therapy benefit is high.

While checkpoint therapy has revolutionized cancer treatment and prolonged the life of many, it is still a challenge to confidently determine who will benefit from the treatment and who will not and would perhaps be better advised with a different kind of therapy.

2.3.1 PDL-1 expression

An obvious approach to predicting response to anti-PD-1 or anti-PDL-1 based immunotherapy is assessing PDL-1 expression on tumor tissue. Starting in the early clinical trials, PDL-1 expression has therefore been analysed and compared to patients' therapy response. In a trial including patients with melanoma, NSCLC and renal cell carcinoma receiving Nivolumab in 2012, PDL-1 positivity was defined at a 5% expression threshold, with 25 of the 42 patients included being positive for PDL-1 expression assessed by immunohistochemical analysis (45). 9 (36%) of the 25 PDL-1 positive patients had an objective response, compared to none in the group of patients with PDL-1 negative tumors (45). In a trial comparing Nivolumab to Docetaxel in the

treatment of NSCLC in 2015, the authors reported on a strong predictive association of

PDL-1 expression and therapy response throughout all defined efficacy endpoints, including progression-free survival and overall survival in the treatment with Nivolumab (75). Another group showed similar results, observing better efficacy in the treatment of NSCLC with Pembrolizumab in patients with a PDL-1 positivity of tumor cells of 50% or higher (76). Results like these were matched in a trial comparing Nivolumab to Ipilimumab and a combination of both in the treatment of melanoma: The group with positive PDL-1 status showed advantages in progression-free survival and objective response rate (41).

These data indeed suggest a connection of PDL-1 positivity and response to anti-PD-1 therapy. However, considerable response has been shown in patients with PDL-1-negative tumors as well. Most trials investigating correlation of PDL-1 status with therapy benefit have reported sizeable rates in overall response in patients with PDL-1 negative tumors, e.g., 41% to Nivolumab monotherapy and 54% to a combination of Nivolumab and Ipilimumab in the aforementioned melanoma trial (41, 77). Results like these have led to the realization that PDL-1 expression on tumor cells alone is not a sufficiently reliable biomarker in the prediction of response to anti-PD-1 therapy or even exclusion from treatment. Possible explanations for the described poor prediction reliability are inconsistent threshold values for PDL-1 positivity, different antibodies used for staining PDL-1 for immunohistochemistry as well as factors determining therapy response that are possibly independent from the PD-1 pathway (77).

Nevertheless, assessment of PDL-1 status plays an important role in characterizing tumors in the clinical setting. Interestingly, in a trial on NSCLC, results suggested that patients with negative PDL-1 status might indeed benefit to the same or even higher extent from chemotherapy or another immunotherapy compared to Pembrolizumab (76). Apart from this example, data on the predictive value of PDL-1 expression has been ambiguous and it is therefore not yet a standard tool in the consideration of different therapy choices in the majority of cases (77).

2.3.2 Tumor-infiltrating lymphocytes

Lymphocytes, most of all CD8+ effector T cells, are considered as the crucial intermediary in the effectiveness of immune checkpoint therapy targeting the PD-1 pathway. In order to be able to employ their regained potential impact on tumor cells,

immune cells need to be in close proximity to the malignant tissue or the tumor microenvironment.

In addition to CD8+ T cells, TILs are mainly comprised of CD4+ T cells, B cells and NK cells (78).

The significance of TILs in cancer survival and therapy response has been studied since before the introduction of immune checkpoint therapy, and it has been shown that high TIL levels correlate with better therapy response in various cancer types (79-84).

2.3.3 TCR clonality

Engagement of TCRs with MHC molecules is the key mechanism of antigen recognition through T cells and therefore fundamental to functionality of the adaptive immune system. TCRs are protein complexes that are highly variable within individuals and the diversity of a TCR repertoire results in the extent of efficacy to which T cells can recognize antigens (85).

In cancer patients, the TCR repertoire can evolve to become more clonal, i.e., continuous exposure to tumor-specific antigen might lead to selection and expansion of tumor infiltrating T cells that recognize that particular antigen. It has been shown that high intratumoral TCR clonality might be associated with clinical benefit in patients receiving immune checkpoint therapy (83, 86).

2.3.4 Markers in peripheral blood

In the clinical setting, finding predictive markers that can easily be determined in peripheral blood testing would be ideal. Cell counts and ratios of particular immune cells to one another have been under investigation as outcome predicting biomarkers for immune checkpoint therapy. For example, improved survival was correlated with baseline normal or low absolute neutrophil count and low neutrophil-to-lymphocyte ratio (87-91). Low baseline LDH has been reported as another favourable marker for immune checkpoint therapy response in various studies (92-98).

2.3.5 Genetic signatures

There have been studies focusing on genetic differences and changes in genetic expression patterns of cancer cells in biopsies from responders and non-responders to immune checkpoint therapy. For example, response to anti-PD-1 antibody Nivolumab

was associated with pre-existing IFN-γ-mediated gene signatures and increased HLA expression on tumor cells (99-102).

2.3.6 Neoantigen-specific T cells

Tumor-specific alterations in the DNA lead to the formation of so-called neoantigens. These protein sequences do not derive from the human genome but are the result of mutations. In virus-associated tumors such as HPV-derived HNSCC, open reading frames in the viral genome can lead to the formation of parts of the neoantigen landscape as well. (103) It has been shown that peripheral PD-1+ T cells in tumor patients are partially specific to these neoantigens (104, 105). This data hints at the possibility of observing neoantigen-specific T cell activity within tumors by using circulating T cells of the same pool as surrogates. (104-106)

2.3.7 PD-1 receptor expression

There are many factors potentially playing into the effective results of immune therapies. The role of PD-1 receptor expression level itself has been analysed for possible significance in predicting the response to anti-PD-1 immune checkpoint therapy. High PD-1 expression of CD8+ T cells in the TME has been associated with improved therapy response in cancer patients receiving anti-PD-1 checkpoint therapy (107). The authors of this study furthermore found that substantial reactivation of PD-1+ CD8+ effector T cells - and not PD-1+ Tregs - was crucial for anti-tumor effects. They concluded that the balance of PD-1+ CD8+ T cells and PD-1+ Tregs in the TME might be a promising biomarker in PD-1 checkpoint therapy response prediction. (107)
In a trial that longitudinally analysed peripheral blood samples of lung cancer patients treated with anti-PD-1 antibodies, the authors described CD8+ Ki-67+ effector T cells proliferating after treatment initiation to be mostly PD-1 positive, inferring that this expansion of cells were a direct result of blocking the PD-1 pathway (108). These cells were furthermore characterized by activation markers CD38+ and HLA-DR and had considerable cytotoxic potential assessed through intracellular granzyme B expression levels in most patients with a response to therapy (108). Additional examining of factors characterizing therapy response in peripheral blood samples showed that of patients with an early PD-1+ CD8+ T cell expansion, 57% achieved partial clinical response and 21.5% presented disease progression. Among patients without, with delayed or with PD-1 negative CD8+ T cell expansion in peripheral blood analysis, 53.8% showed

progressive disease and just 15.4% showed partial clinical response to therapy. Based on these findings, the authors suggest that early PD-1+ CD8+ T cell expansion in peripheral blood might be associated with better clinical outcomes in NSCLC patients treated with anti-PD-1 targeted therapies and that it could be utilized for monitoring therapy response. (108) These results were later matched by another study identifying high baseline circulation of the same PD-1+ CD8+ T cell population to be associated with improved therapy response in another group of NSCLC patients receiving Nivolumab (109).
Similar findings have recently been shown for gastric cancer patients treated with Pembrolizumab (110). The authors of this paper showed that high levels of baseline PD-1 in peripheral CD8+ T cells was associated with clinical therapy benefit. Additionally, they saw a significant increase in Ki-67 positivity among PD-1+ CD8+ T cells during therapy with Pembrolizumab which was correlated to sustained clinical benefit, suggesting that immune checkpoint blockade supports proliferation in PD-1+ cells (110).

A group that studied key checkpoint inhibitors on tumor-infiltrating T cells in multiple types of cancer found that TILs, amongst other receptors, expressed significantly higher levels of PD-1 compared to peripheral T cells; however, peripheral expression levels indeed positively correlated with their respective levels in TILs (111). Under these premises, characterization of peripheral blood T cells, including the assessment of PD-1 receptor status, may offer an informative insight into the potential of the anti-tumor activity that could be unleashed by anti-PD-1 checkpoint therapy.

3 Research question

Based on the cited data on NSCLC and gastric cancer patients, we hypothesized that a connection of PD-1 expression levels on T cells and therapy response might also be present in patients suffering from other types of cancers, too. We therefore recruited patients with RCC and HNSCC who had all been treated with anti-PD-1 monoclonal antibody Nivolumab for longitudinal peripheral blood sampling before and during therapy. Our research question was whether the extent as well as changes in PD-1 expression levels on immune cells stained in whole blood samples of tumor patients treated with Nivolumab could be associated with clinical outcome, specifically irAEs and therapy response. To date, PD-1 expression levels in cancer patients have mainly been studied on T cells; we wanted to assess whether observations on this subject could also be made in other immune cells. In addition, we wanted to evaluate whether PD-1 expression levels in peripheral blood are substantially different in RCC and HNSCC patients.

4 Material and methods

Table 1: Patient characteristics

Patients	N	17
Age	mean (STD) range	59,64 (12,34) 37-78
Gender	male female	14 3
Cancer type	HNSCC *p16+* *p16-* RCC	9 *5* *4* 8
irAEs	yes no	6 11
Type	*pneumonitis* *enterocolitis* *interstitial nephritis* *ulcerative colitis exacerbation*	3 1 1 1
irAE treatment	*glucocorticoids* *Vedolizumab*	5 1
Consequences of irAEs	*Nivolumab termination* *Nivolumab interruption*	4 3
Response	SD PR PD	1 6 10

STD: standard deviation, HNSCC: head and neck squamous cell carcinoma, RCC: renal cell carcinoma, irAEs: immune-related adverse events, SD: stable disease, PR: partial response, PD: progressive disease

4.1 Patients

We longitudinally collected whole blood samples of 17 patients treated with anti-PD-1 checkpoint inhibitor Nivolumab monotherapy. 9 patients suffered from HNSCC and 8 patients from RCC.

Whole blood samples of the patients were collected at four times throughout the Nivolumab treatment: Before the 1st, 2nd, 4th and once more before the 6th infusion with Nivolumab, i.e., respectively prior to therapy initiation, and about 2, 6 and 10 weeks after.

The study was approved by the Ethics Committee of Charité-Universitätsmedizin Berlin. Informed consent of all study participants was obtained regarding the prospective blood sampling and review of clinical data.

4.2 Preservation and preparation of whole blood samples

Whole blood samples from patients were treated with Smart Tube Proteomic Stabilizer (PROT1, Smart Tube Inc.) according to the company protocol:

500 µl whole blood were incubated with 700 µl Proteomic Stabilizer for 10 minutes at room temperature and then frozen at -80°C.

In bulk, frozen samples were fully thawed to 4°C in 30 minutes. Smart Tube 1000X thaw-lyse buffer concentrate was diluted to make a 1X working concentration. 1X thaw-lyse buffer was added at a ratio of 4:1 to the total volume of the stabilized samples (4.8 ml thaw-lyse buffer to a total sample volume of 1.2 ml). After incubation for 10 minutes, samples were centrifuged at 600x g for 5 minutes, supernatants were discarded and 12.5 ml thaw-lyse buffer was added to incubate for a further 10 minutes. After repeated centrifugation at 600x g for 5 minutes, the thawing was complete.

100 µl of the sample were transferred to a new tube, and to block Fc receptors before antibody staining, 10 µl Beriglobin were added and samples were stored 10 minutes in the dark for incubation. Afterwards, the samples were washed twice using phosphate-buffered saline with 0.2% bovine serum albumin (PBS/BSA 0.2%) to remove any unbound Beriglobin which could otherwise disturb the staining process.

First, secondary anti-IgG4-PE antibody (Biozol Mouse Anti-Human IgG4 Fc-PE, clone HP6025) was added to the sample at a 1:1,000 ratio. After 15 minutes incubation in

darkness, all other antibodies were added to the samples for another 15 minutes incubation (Table 2).

One sample was incubated with all antibodies but secondary anti-IgG4-PE to serve as a negative control sample for potential background signal in the channel of the secondary antibody. Another sample was incubated with a PD-L1 antibody instead of the PD-1 antibody (PD-L1 data was not included in this paper). Samples were washed twice with PBS/PBS 0.2%, centrifuged at 300x g for 5 minutes, then 500 µl PBS/BSA 0.2% were added and samples were transferred into tubes through filtering caps before analysis with a Beckman Coulter CytoFlex LX Flow Cytometer.

Table 2: Fluorochromes used for flow cytometry

Antibody	**Fluorochrome**	**Clone**	**Company**
CD25	APC	2A3	BD
CD16	APC-750	3G8	Biolegend
CD4	BV510	RPA-T4	Biolegend
CD8	BV785	RPA-T8	Biolegend
CD127	FITC	A019D5	Biolegend
HLA-DR	PerCP5.5	L243	Biolegend
CD45	BUV395	HI30	BD
CD3	A700	UCHT1	Biolegend
CD14	FITC	HCD14	Biolegend
CD56	Pe Dazzle	HCD56	Biolegend
CD123	Pe/Cy7	6H6	Biolegend
CD19	BV605	HIB19	Biolegend
CD38	BV650	HB-7	Biolegend
IgG4	PE	HP6025	Biozol
PD-L1	BV421	29E.2A3	Biolegend
PD-1	BV421	EH12.2H7	Biolegend

4.3 Establishment of PD-1 assessment under anti-PD-1 therapy

Directly monitoring PD-1 receptor status in patients being treated with anti-PD-1 monoclonal antibodies can only be done directly at baseline prior to therapy, since all available fluorochrome-conjugated antibodies bind to the same epitope region of PD-1 as Nivolumab and are therefore hindered from binding during therapy (112). It has been shown that attempted direct staining of PD-1 leads to highly inaccurate detection in flow cytometric analysis due to the relatively high saturation of PD-1 with Nivolumab. PD-1 expression can be indirectly measured by using a secondary anti-IgG4 antibody, as was successfully applied in a phase I study on the safety, clinical activity and pharmacodynamics of the antibody that would later become known as Nivolumab (46). Direct PD-1 staining has nevertheless been attempted, which presumably led to failed or insufficient detection of PD-1 in the presence of therapeutic anti-PD-1 antibody (46, 50, 113, 114). We conducted prior testing on T cells of a healthy individual to demonstrate the effect of Nivolumab incubation on PD-1 detectability by anti-PD-1 antibody as well as secondary staining of Nivolumab in flow cytometry (Figure 2).

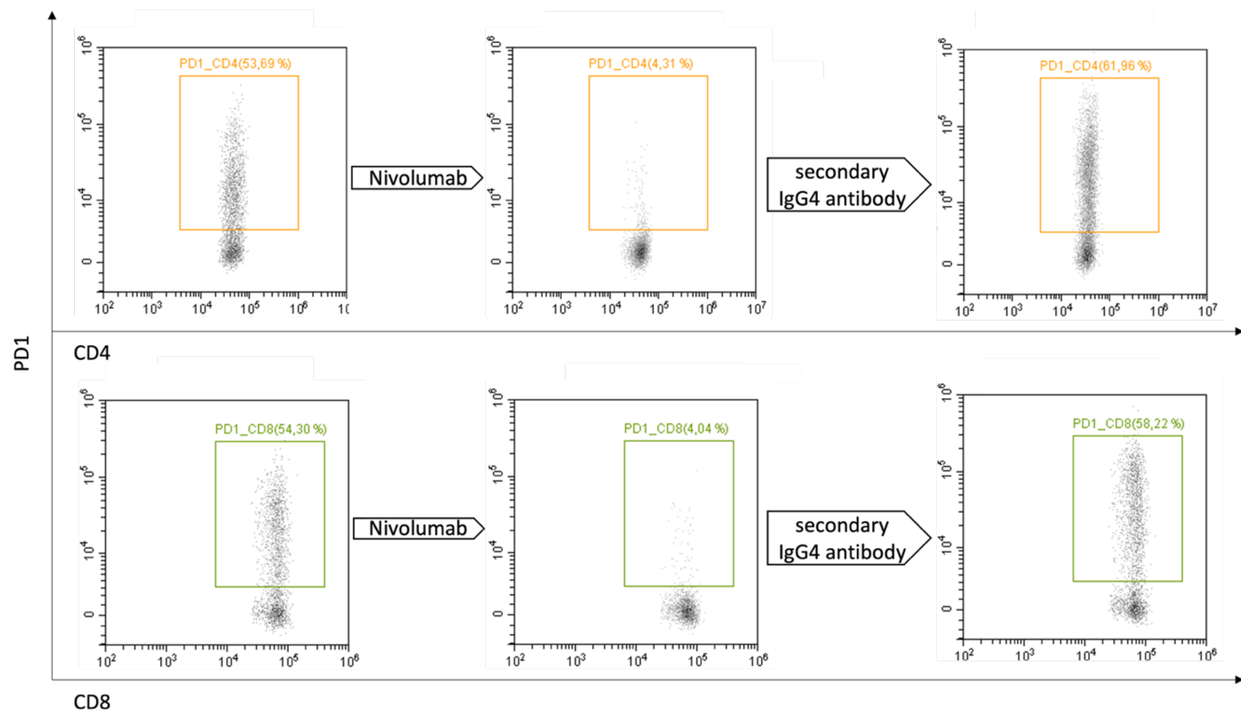

Figure 2: Effect of Nivolumab on PD-1 detection.

On the left, PD-1 expression of CD4+ and CD8+ T cells of a healthy individual stained with anti-PD-1-BV421 is shown. Incubation with 1 mg/ml anti-PD-1 monoclonal antibody Nivolumab led to considerably reduced PD-1 signals, as shown in the middle dot plots. On the right, secondary IgG4 antibody was used to stain Nivolumab-incubated samples.

Consequently, we established a staining process with an anti-IgG4 secondary antibody. An additional criteria to be considered in the process were was the reliable staining of whole blood samples previously treated with stabilizing buffer and frozen at -80°C for considerable amounts of time.

4.4 Nivolumab ELISA

For quality control of our flow cytometric panels, we carried out an enzyme-linked immunosorbent assay (ELISA) in patient serum samples from before and after therapy initiation to assess Nivolumab serum levels. Nivolumab ELISA was carried out using a ready-to-use kit (ImmunoGuide Nivolumab ELISA (mAB-based)) and the included instructions were followed:

100 µl of assay buffer was pipetted into each well of a 96-well plate. Five standard concentrates in a 10X concentration, A through E (concentration of 6,000, 2,000, 600, 200 and 0 ng/ml Nivolumab), were diluted at a ratio of 1:10, and patient's serum samples at a ratio of 1:200. 50 µl of diluted standards and samples were pipetted into a microtiter plate well. The plate was covered with adhesive seal and shaken carefully before incubation for 60 minutes at room temperature. Incubation solutions were decanted and washed five times with 350 µl diluted wash buffer per well. 100 µl of enzyme conjugate (horseradish peroxidase-anti human IgG) were pipetted into each well. The plate was covered and carefully tapped before incubation for another 30 minutes at room temperature. Wells were washed again five times with 350 µl washing buffer. 100 µl of TMB substrate solution (containing 3,3',5,5'-Tetramethylbenzidine) were pipetted into each well and the plate was incubated for another 15 minutes at room temperature in the dark. The substrate reaction was stopped by adding 100 µl of stop solution into each well and mixing contents by shaking the plate. Optical density was measured at 450 nm directly after on a Tecan plate reader.

4.5 Flow cytometry

Flow cytometry is a laser-based technology used for analysing cells according to characteristics such as size, granularity or expression of antigen pattern. Soluted cells are transported through a tube inside the flow cytometer in a way in which one cell at a time passes a laser, and the light is scattered and detected by sensors within the machine. Conclusions regarding characteristics of the cells can be drawn from these detections. Scatters of visible light are detected and measured in two directions, resulting in a forward scatter (FSC), characterizing a cell's size, and side scatter (SSC) which is indicative of cells' internal granularity (115). Further molecular properties of cells can be made visible in flow cytometry by using antibodies linked to fluorescents or fluorochromes that, for example, bind to particular cluster of differentiation (CD) molecules on the surface of immune cells, such as CD4 or CD8. The fluorochromes cover a color spectrum and each has its particular and characteristic wavelength of peak excitation and emission. The lasers used have particular wavelengths in order to induce excitation in the respective fluorochromes.

Flow cytometry was introduced in the 1960s and has been technically improved in the decades thereafter. Today, the method is widely used in research and diagnostics in immunology, haematology and other disciplines (116). Immunophenotyping is the most

common application of flow cytometry, as it allows for numerous markers - most typically CD molecules - to be stained with antibodies in a mix of immune cell populations. The application of this method is extended by the possibility to characterise the functionality of cells by also staining messenger substances like cytokines on the surface of cells as well as intracellularly. Through stimulating whole blood samples or isolated peripheral blood mononuclear cells and measuring their cytokine response, for example, evidence of the functionality of the immune system can be obtained.
Since each fluorochrome has a respective spectrum of fluorescence, spectra can and do overlap, especially when using different fluorochromes with similar emission spectra. In such situations, so called compensation must be carried out in order to mathematically correct for this fluorescence "spillover" between signal detectors. Basically, the signal of a fluorochrome falsely perceived by a particular detector is removed, leaving only the signal that was intended to be measured. (117)

In our study, we used a staining panel consisting of 16 fluorochrome antibodies (Table 2) and flow cytometric measurements were carried out on a Beckman Coulter CytoFlex LX Flow Cytometer. This flow cytometer houses six lasers and allows for the detection of up to 21 separate colour parameters.

4.6 Data analysis

Per patient and blood draw, we took measurements of two samples with anti-IgG4 secondary antibody, namely the samples including PD-1 or PD-L1 antibody respectively, and one control sample without anti-IgG4 antibody. In data analysis, we averaged both anti-IgG4 values and subtracted the channel value from the negative control sample lacking anti-IgG4 antibody. For overall PD-1 assessment, we added the value of the unbound PD-1 channel.
Flow cytometric data was analyzed and gated with Beckman Coulter CytExpert software. GraphPad Prism was used for statistical data analysis and graph creation. Changes in PD-1 expression levels over time and within patient groups were statistically assessed using a mixed effects model combined with the Tukey's multiple comparison test. The Mann-Whitney test was used to assess differences between subgroups. Exploratory p-values were obtained and statistical significance was defined as $p < 0.05$.

5 Results

We longitudinally analyzed whole blood samples of cancer patients in order to assess the significance of PD-1 expression levels on peripheral blood immune cells in patients undergoing anti PD-1 checkpoint therapy. The first blood draw of patients was carried out right before their first infusion with therapeutic monoclonal anti-PD-1 antibody Nivolumab. Further visits with blood draws were conducted before their second, fourth and sixth drug administration.
17 patients receiving Nivolumab monotherapy were included in our study. 9 suffered from HNSCC, and 8 from RCC.

Assessment of PD-1 expression levels under anti-PD-1 checkpoint therapy
Our goal in this study was to accurately detect PD-1 expression levels in cancer patients treated with anti-PD-1 checkpoint therapy in a longitudinal manner. However, therapeutic monoclonal antibody Nivolumab binds PD-1 and impedes direct staining with diagnostic antibodies for flow cytometric analysis. To overcome this hurdle in accurate PD-1 detection, we included both direct and secondary staining antibodies in our flow cytometry panel in order to capture unbound PD-1 as well as receptors bound with Nivolumab in patients' whole blood samples (Figure 3).
During data analysis, we added up both values respectively in order to assess the overall PD-1 receptor expression level.

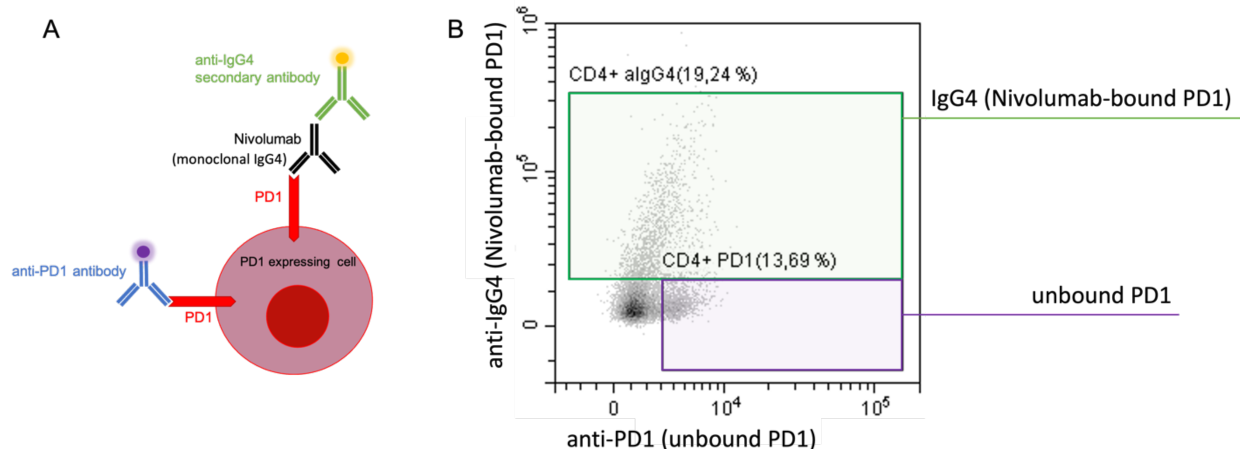

Figure 3: Staining of Nivolumab-bound PD-1 and unbound PD-1

A: PD-1 bound by Nivolumab was stained using anti-IgG4 secondary antibody, whereas unbound PD-1 was stained in the same samples through anti-PD1 antibody.

B: Staining signal of Nivolumab-bound PD-1 and unbound PD-1 on CD4 T cells.

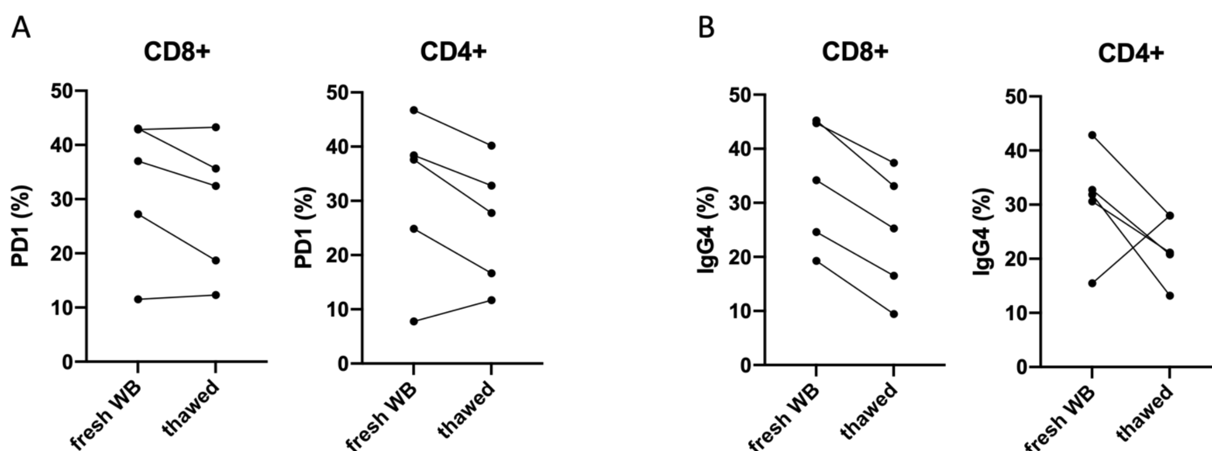

Figure 4: Assessment of the effect of stabilization and freezing of whole blood samples

A: Individual PD-1 detection levels on CD8+ and CD4+T cells in fresh whole blood samples of 5 healthy individuals, and after stabilization, freezing and thawing of the samples.

B: Individual IgG4 detection levels on CD8+ and CD4+T cells in fresh whole blood samples of 5 healthy individuals, and after stabilization, freezing and thawing of the samples.

WB: whole blood

To validate the reliability of flow cytometric analysis after stabilizing, freezing and thawing samples, we tested the antibody detectability of PD-1 and anti-IgG4 secondary antibody on whole blood samples of 5 healthy individuals. The results showed that the biobanking process led to a diminished rate of PD-1 staining by an average of 4.5% compared to the fresh samples, while the loss in staining rates of anti-IgG4 antibody was 8.8% on average in the thawed samples compared to the fresh ones (Figure 4). However, we assessed the resulting binding rates to be sufficiently stable throughout all our establishing flow cytometric measurements.

T cell populations were characterized as CD3+ as well as CD4 and CD8 positivity respectively. Tregs were defined by a CD4+ CD25+ phenotype. Activated T cell populations were further characterized by their expression of CD38.
We demonstrated that levels of PD-1 on T cells bound by Nivolumab increased after the first infusion with the drug and stayed elevated steadily throughout all of the period studied. In turn, levels of unbound PD-1 decreased considerably after the first administration of Nivolumab (Figure 5 and 6).
These developments were shown throughout all analysed T cell populations, though most distinctly in CD8+ CD38+ activated effector T cells.
B cells were characterized by CD19 positivity, and NK cells by expression of CD56. On B cells and NK cells, the development described for T cells were also shown. However, differences between bound and unbound levels were markedly less distinct (Figure 6).
Before the sixth infusion with Nivolumab, we observed a slight and statistically non-significant increase in the expression of unbound PD-1 in all studied cell subsets, with the Nivolumab-bound PD-1 levels still being higher.

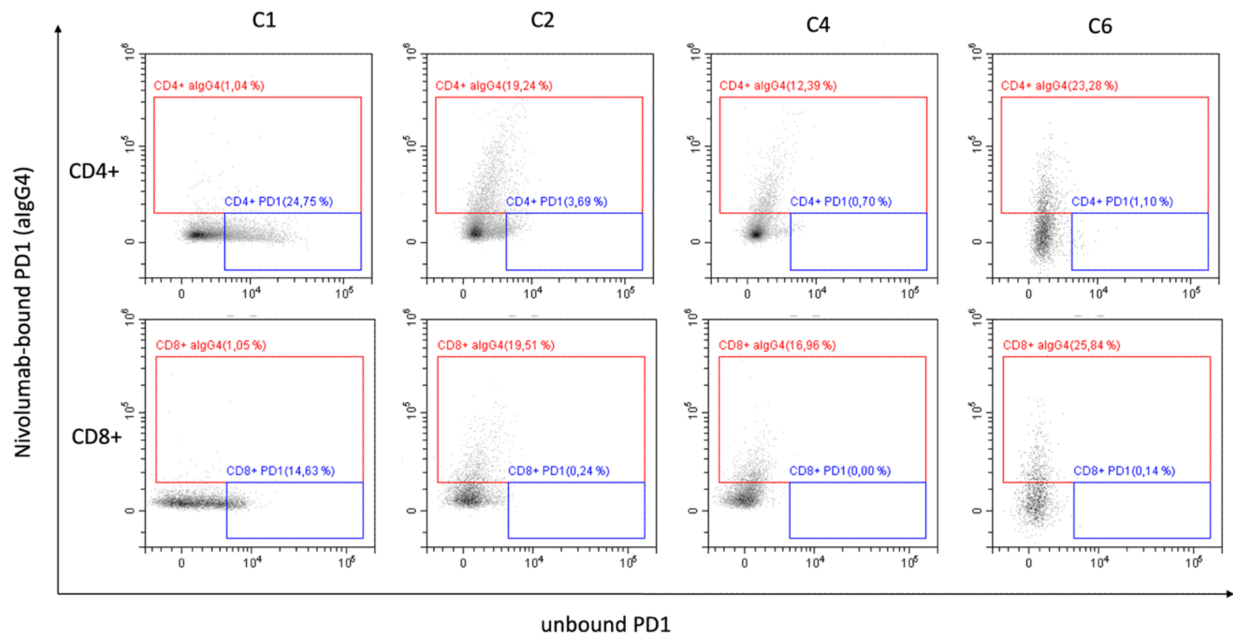

Figure 5: Detection of Nivolumab-bound and unbound PD-1 in the course of therapy
Representative CD4 and CD8 T cell PD-1 expression data of one patient. PD-1 analysis by flow cytometry was performed using anti-PD-1 antibody for staining unbound PD-1 and anti-IgG4 secondary antibody for detection of Nivolumab bound to PD-1. Analysis was carried out at four points throughout the trial (C1-C6).

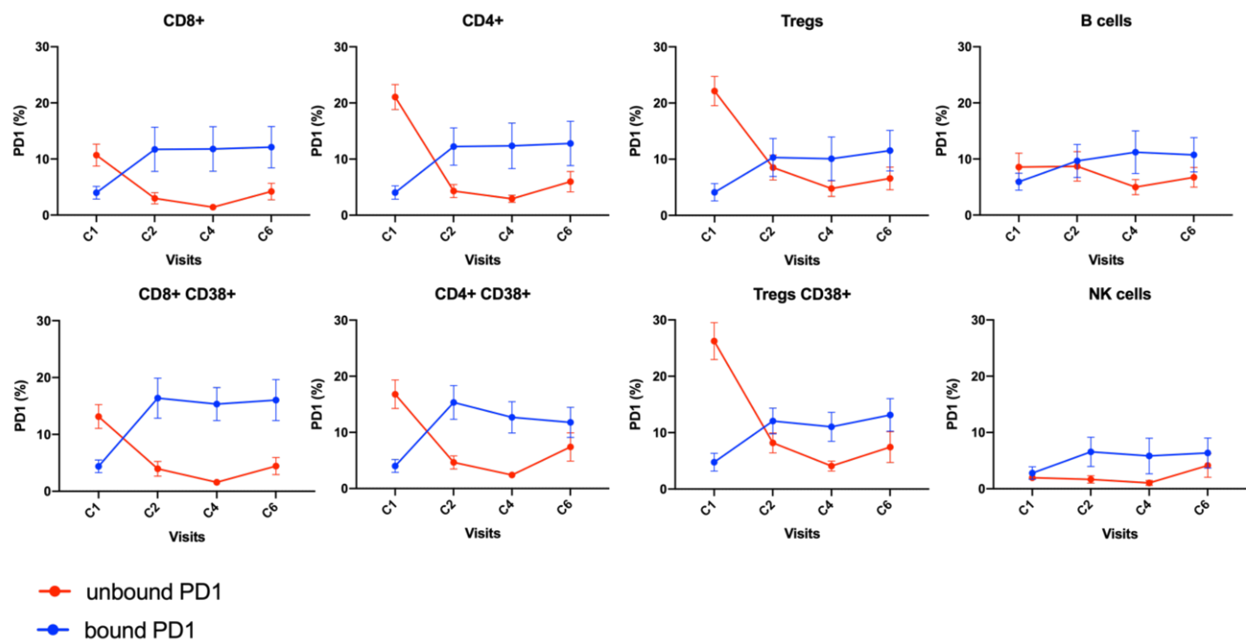

Figure 6: Unbound and Nivolumab-bound PD-1 expression levels before and after therapy initiation on immune cell populations and subpopulations

Unbound PD-1 and PD-1 bound by Nivolumab were assessed separately at baseline (C1) and over time after therapy initiation (C2-C6).

The graphs depict the mean plus SEM of data acquired of 17 patients.

PD-1 expression on leukocyte subsets in whole blood

We began our analysis by determining the overall PD-1 expression on leukocyte subsets without dividing patients into subgroups (Figure 7).

All analyzed T cell populations showed substantial levels of PD-1 before therapy initiation. Mean total PD-1 expression at baseline and over all patients was 14.26% on CD8+ T cells, 24.65% on CD4+ T cells and 25.6% on CD4+ CD25+ Tregs.

T cell activation plays an immanent role in the anti-cancer effects aimed at in immune checkpoint therapy. Recently activated T cells can be detected by their expression of CD38. Activated T cell subsets showed high mean overall PD-1 expression with CD8+ CD38+ T cells at 17.16%, CD4+ CD38+ T cells at 20.64% and CD38+ Tregs at 30.91%.

In comparison to T cell populations, B cells and NK cells showed slightly less total PD-1 expression. Mean expression levels of PD-1 were at 4.47% on NK cells and 14.09% on B cells.

Apart from T cells, B cells and NK cells, we assessed PD-1 expression levels of monocytes, DC and basophiles. Mean total PD-1 expression at baseline was 8.90% in monocytes, 17.27% in DC and 6.68% in basophiles. While we did measure considerable levels of PD-1, especially in DC, we did not observe major changes in expression over the time of our study in any of the above-mentioned leukocyte subsets. For further analysis, we excluded monocytes, DC and basophiles due to comparably low PD-1 expression levels.

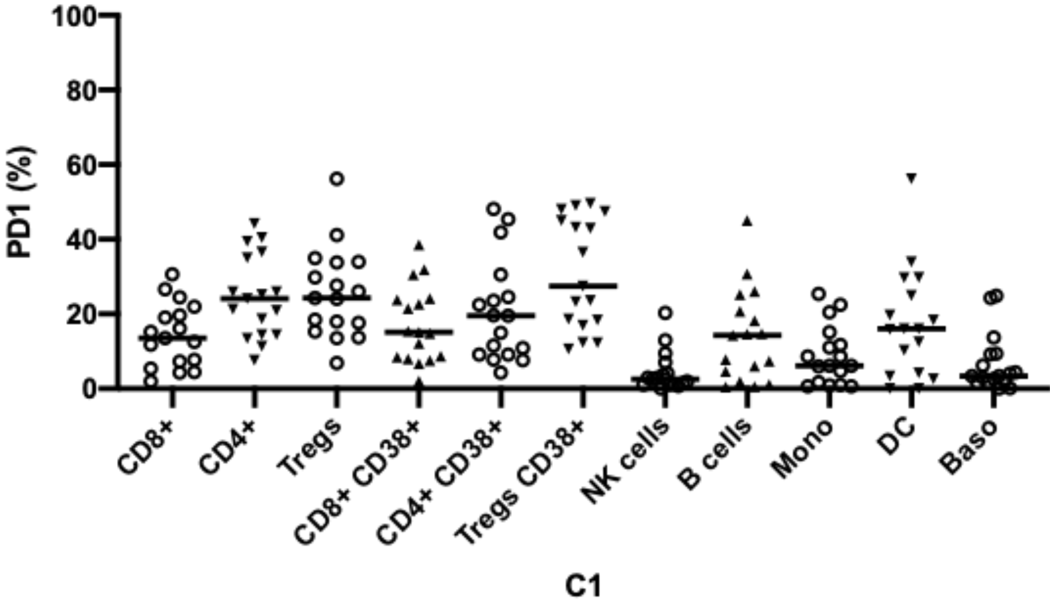

Figure 7: Baseline PD-1 expression on leukocyte subsets

PD-1 expression of leukocyte subsets at baseline (C1).

Horizontal lines represent means.

Mono: monocytes, Baso: basophiles

We longitudinally assessed PD-1 receptor levels over the study period (Figure 8). Total PD-1 expression on CD8+ effector T cells showed a non-significant decline over the course of our study. The decline in PD-1 on CD4+ helper T cells was significant (p=0.0121). The same effect was observed on CD4+ CD25+ regulatory T cells (Tregs) (p=0.0207) in an even more distinct manner. The described changes occurred from before the first to the fourth administration of Nivolumab. At the sixth administration, the levels rose again.
In contrast to CD8+ T cells, PD-1 levels on CD8+ CD38+ T cells showed a slight but non-significant rise over time. Total PD-1 levels of CD4+ CD38+ T cells and CD38+ Tregs significantly declined between C1 and C4, respectively (p=0.0491 and p=0.0004), followed again by a rise of levels at C6.
Mean total PD-1 expression levels of B cells and NK cells remained relatively stable throughout the trial period.

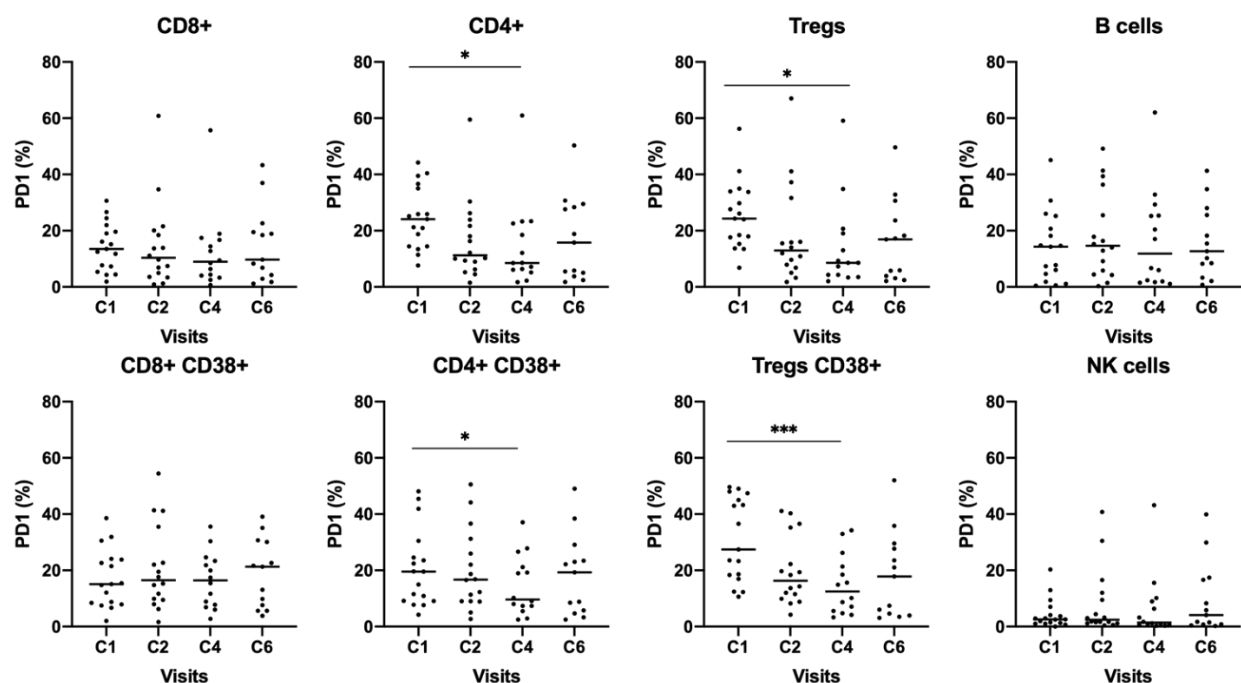

Figure 8: PD-1 expression before and throughout Nivolumab therapy
Scatter plot of PD-1 expression and means on leukocyte subsets throughout the trial period. PD-1 expression levels were analyzed before therapy initiation (C1) as well as before the 2nd, 4th and 6th infusion with Nivolumab (C2-C6).
$* p < 0.05$, $** p < 0.05$, $*** p < 0.001$

HNSCC patients show significantly higher PD-1 expression on T cells and other immune cells compared to RCC patients

The 17 patients included in our study suffered from different tumor entities: 9 patients from HNSCC and 8 patients from RCC. We compared the HNSCC to the RCC group. Throughout all visits (C1-C6), patients with HNSCC presented considerably higher levels of total PD-1 on T cell populations, NK cells and B cells, especially before therapy initiation at C1 (Figure 9).

In CD8+ T cells, significantly higher levels of PD-1 in HNSCC patients were found at C1 (p=0.0111), with PD-1 expression levels in HNSCC and RCC patients remaining relatively stable over the study period. CD4+ T cells in the HNSCC group showed higher expression of PD-1 at C1 (p=0.0025), C4 (p=0.0426) and C6 (p=0.0295). Both groups showed declining levels of PD-1 on CD4+ T cells over time, with this alteration being significant in the RCC group (C1-C4 p=0.0104). CD4+ CD25+ Tregs exhibited a very similar course. Here, differences between the compared groups were significant at C1 (p=0.0360), C6 (p=0.0451), and also the RCC group, showing declining receptor levels over time (C1-C4 p=0.0054, C1-C6 p=0.0427).

Analysis of only activated T cells (CD38+) showed comparable results. Significant disparities in PD-1 expression between the HNSCC and RCC group were observed in CD8+ CD38+ T cells at C1 (p=0.0206), in CD4+ CD38+ T cells at C1 through C6 (p=0.0006, p=0.0418, p=0.0463, p=0.0186), and in CD4+ CD25+ Tregs at C1 (p=0.0079). In addition, CD38+ Tregs displayed significantly declining PD-1 levels over the course of the study period in both the HNSCC group (C1-C2 p=0.0205, C1-C4 p=0.0084) and the RCC group (C1-C4 p=0.0239, C1-C6 p=0.0074).

In B cells, the differences in PD-1 expression between the tumor entities were observable before therapy initiation (p=0.0464). However, differences in the expression levels between the groups were far less distinct and robust over time compared to all T cell populations analyzed. NK cells did not show significant differences in PD-1 expression between tumor entities, though mean expression levels were also consistently higher in the HNSCC group here, with receptor levels rising slightly over the study period.

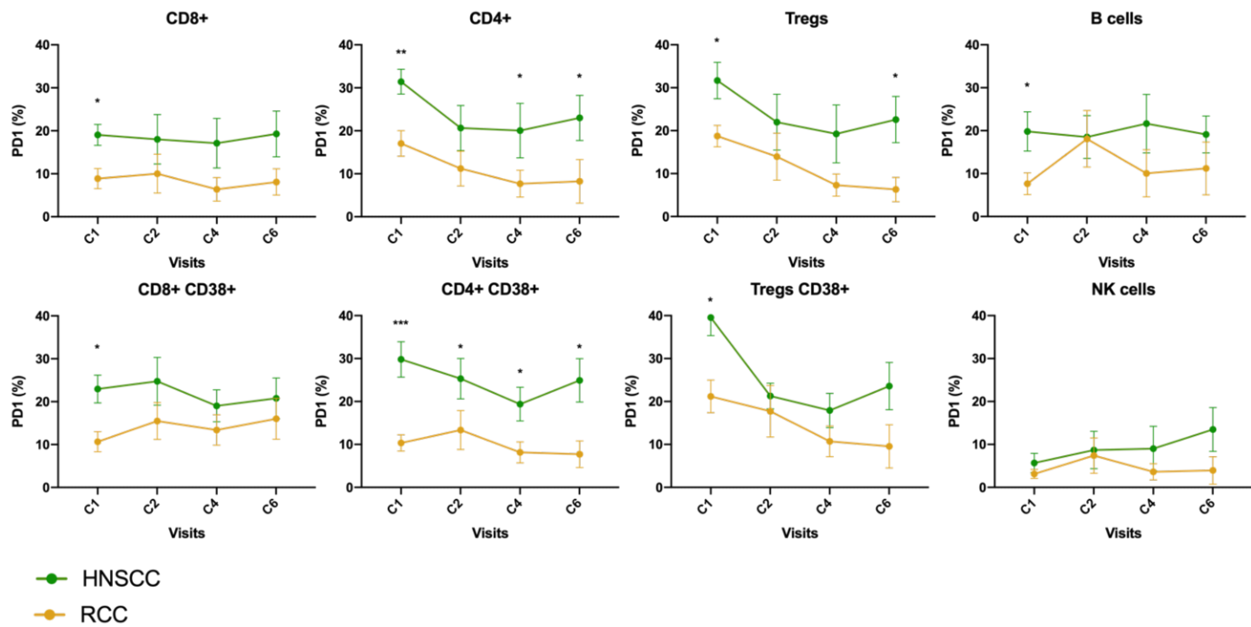

Figure 9: PD-1 expression in HNSCC and RCC patients

PD-1 expression on leukocyte subsets was assessed at baseline (C1) and before the respective cycles of Nivolumab therapy (C2-C6). Expression levels were compared between HNSCC and RCC patients.

The graphs depict the mean plus SEM of data acquired of 17 patients.

** $p < 0.05$, ** $p < 0.05$, *** $p < 0.001$*

HPV status plays an important role in HNSCC formation and immunogenicity. We furthermore stratified HNSCC patients according to tumor suppressor p16 status, which can be used as a surrogate marker for HPV positivity, and compared p16+ to p16- patients. Among the 9 HNSCC patients, 5 were categorized as p16+ by immunohistochemistry carried out as part of their clinical diagnostic procedure.
We showed that on all analyzed leukocyte subsets, PD-1 expression was higher in p16+ patients from C1 to C4. At C6, PD-1 levels slightly were slightly elevated in the p16- group in CD8+ T cells, CD8+ CD38+ T cells, CD38+ Tregs, B cells and NK cells compared to the p16+ group.
In Tregs, PD-1 expression was significantly higher in the p16+ group at C1. All other differences in PD-1 levels between groups and changes within groups were non-significant (Figure 10).

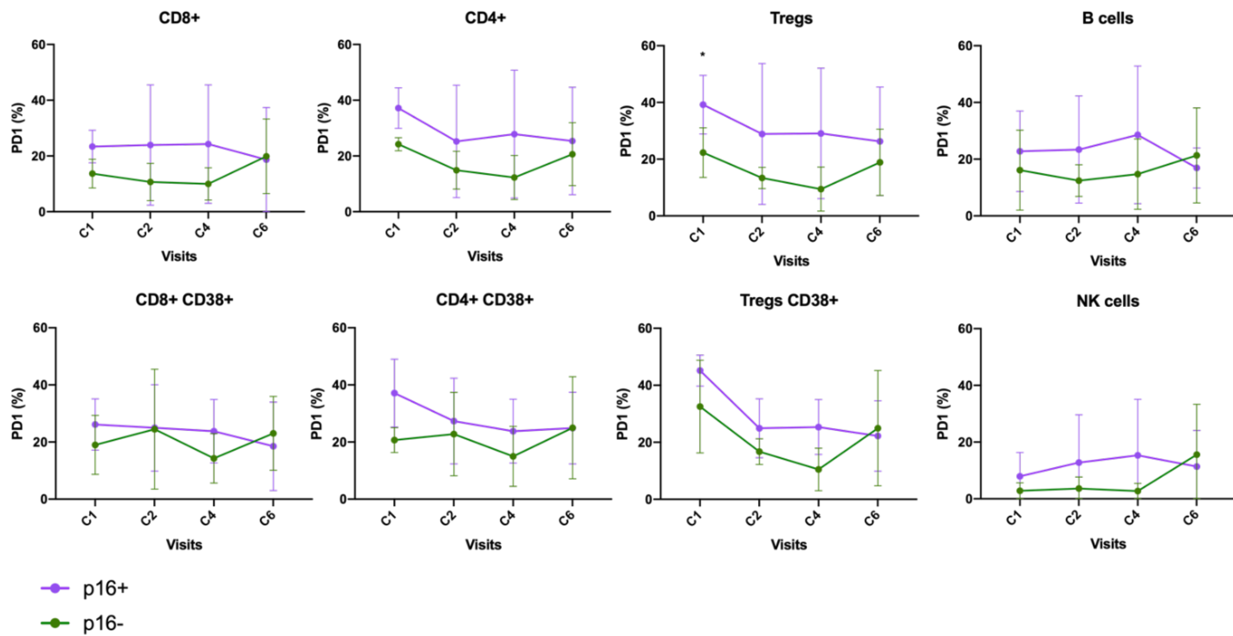

Figure 10: PD-1 expression in p16+ and p16- HNSCC patients

PD-1 expression levels were compared between p16+ and p16- HNSCC patients before therapy initiation (C1) and throughout the trial period (C2-C6).

The graphs depict the mean plus SEM of data acquired of 9 HNSCC patients.

** p < 0.05, ** p < 0.05, *** p < 0.001*

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irAEs correlate with PD-1 expression in activated T cells

Since the PD-1/PDL-1 pathway contributes majorly to maintaining peripheral tolerance of the adaptive immune system, its therapeutic interruption predisposes to provoke unwanted therapy effects, and especially those linked to dysregulated (auto-) immunity. Such irAEs are a common and serious problem in patients receiving anti-PD-1 checkpoint therapy and were also prevalent amongst patients included in our study. Out of 17 patients, six experienced such adverse events, which necessitated immunosuppressive treatment with glucocorticoids and, in one case, anti-integrin monoclonal antibody Vedolizumab. The events treated were cases of pneumonitis, enterocolitis, severe exanthema, interstitial nephritis and an exacerbation of ulcerative colitis. In four cases, irAEs with subsequent treatment led to termination of Nivolumab therapy. Three patients temporarily interrupted their treatment regimen. These events all took place after the respective completion of blood draws for our trial.

We divided the patients into an irAE and non-irAE group and compared the PD-1 expression levels between them (Figure 11).

In the T cell populations of patients in the non-irAE group there was a trend of declining levels of PD-1 over time, while in CD8+ T cells this effect was non-significant, and in CD4+ T cells it was apparent and significant (C1-C4 p=0.0020, C1-C6 p=0.0244). CD4+ CD25+ Tregs showed corresponding effects of decline (C1-C4 p=0.0042). Among CD38+ activated T cells, activated CD38+ Tregs were the only population with a significant decline of PD-1 (C1-C4 p=0.0114, C1-C6 p=0.0119) in the group of patients without irAEs, whereas in the irAE patients, PD-1 levels in CD4+ CD38+ and CD8+ CD38+ activated T cells were relatively stable over time, with just slight decreases respectively.

In the irAE group, we observed rising levels of PD-1 in all T cell subpopulations when looking at the entire study period, though none of these effects proved to be statistically significant, being probably due to high variation in the group. As in the overall T cell analysis (Figure 8), there seemed to be a tendency of dropping PD-1 levels until the fourth infusion with Nivolumab (C4), before a considerable rise till the sixth (C6).

Comparing the two groups, the most striking effect – after the relatively similar levels until C4 – was therefore the difference in PD-1 expression on T cell populations at C6. While it was observable in all subpopulations, this effect was significant only in CD8+ CD38+ activated T cells (p=0.0056).

The occurrence of irAEs seems to be the key factor responsible for this increase. While it is observable in our data before the division into irAE and non-irAE subgroups, it became considerably more distinct after.

In B cells and NK cells, levels of PD-1 at C6 were higher in the group with irAEs as well, though again non-significantly.

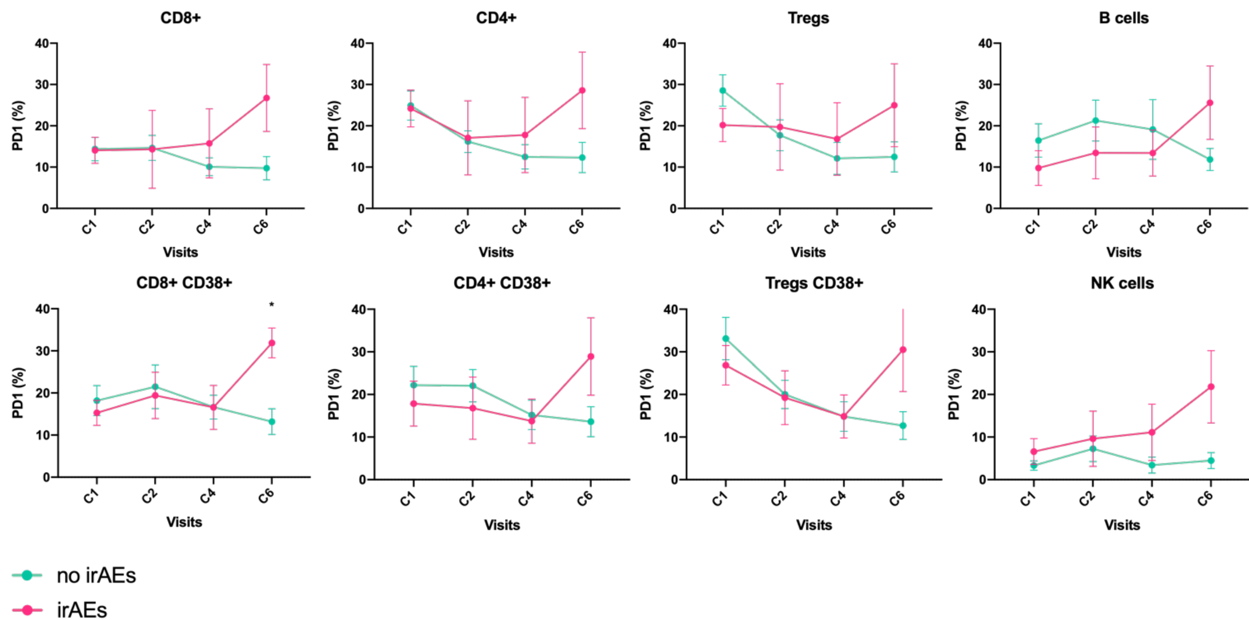

Figure 11: PD-1 expression in patients with and without immune-related adverse events (irAEs)

Comparison of PD-1 expression on leukocyte subsets between irAE and non-irAE patients under Nivolumab therapy throughout the trial period (C1-C6).

The graphs depict the mean plus SEM of data acquired of 17 patients.

** $p < 0.05$, ** $p < 0.05$, *** $p < 0.001$*

Response to therapy does not significantly correlate with PD-1 expression in leukocyte subsets

Initial response to Nivolumab therapy was evaluated according to iRECIST criteria (118) and clinical assessment by week 16 after therapy initiation. The categories established for therapy response were progressive disease (PD), partial response (PR), mixed response (MR), stable disease (SD) and complete response (CR). While none of the patients included in our study reached complete response, 10 showed disease progression, 6 had partial response to therapy, and 1 patient showed disease stability at the time of staging.

We divided our cohort into patients with progressive disease and with partial response or stable disease (Figure 12).

In general, we did not observe significant differences in the compared groups. There was a tendency towards higher levels of PD-1 in the SD/PR group in the T cell populations. Expression levels were relatively stable in CD4+ and CD8+ T cells as well as in their activated CD38+ subsets in both groups. However, CD4+ CD25+

Tregs showed a significant decline in PD-1 expression over the study period in the PD group (C1-C2 p=0.0421, C1-C4 p=0.0077), but not in SD/PR group. In activated CD38+ Tregs, declining levels were prevalent in the PD group (C1-C4 p=0.0419) as well as in the SD/PR group (C1-C4 p=0.0214).

In B cells and NK cells, we observed relatively similar and stable PD-1 expression levels in patients with PD and SD/PR as well as over the period studied.

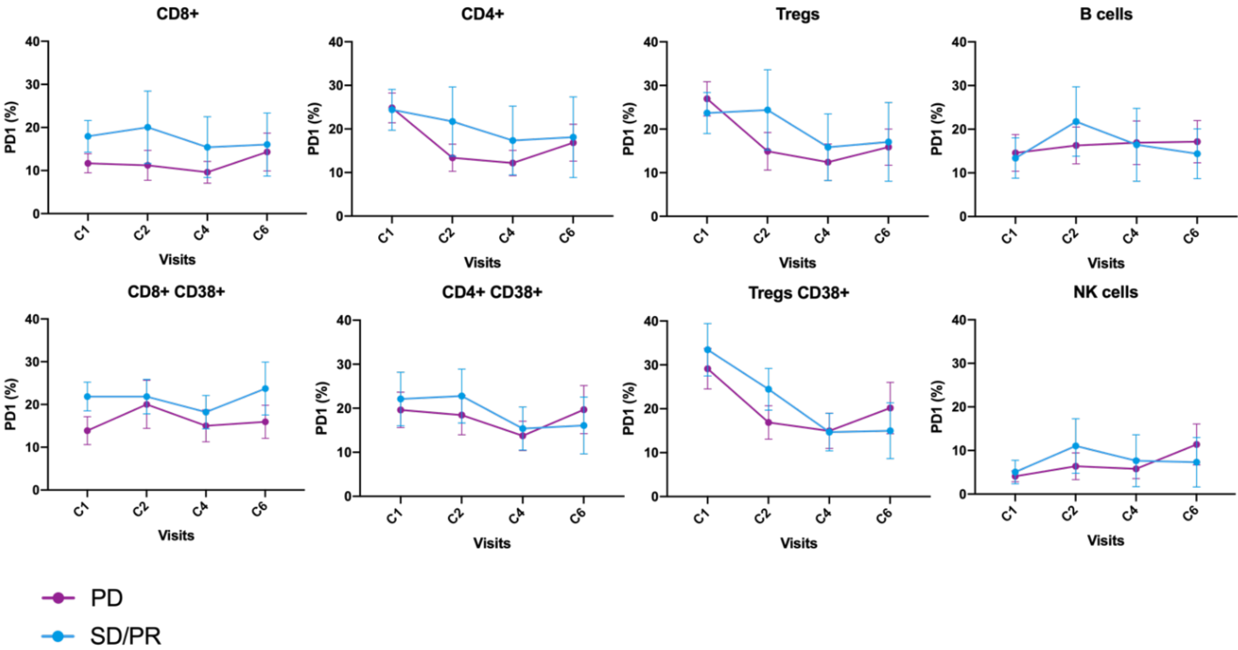

Figure 12: PD-1 expression in patients with progressive disease and stable disease or partial response under Nivolumab therapy

PD-1 expression in leukocyte subsets in responders and non-responders to Nivolumab therapy over the trial period (C1-C6).

The graphs depict the mean plus SEM of data acquired of 17 patients.

** p < 0.05, ** p < 0.05, *** p < 0.001*

Nivolumab ELISA

Since treatment with Nivolumab started after the first time of drawing patients' blood (C1), data at that point should not have shown any binding of anti-IgG4 antibody in flow cytometry. However, in working with antibodies and flow cytometry, unspecific binding behaviour may occur at times. To distinguish minor unspecific binding signal in flow cytometry from errors being made in the trial setting (e.g., patients being administered with Nivolumab first and blood samples taken after), we carried out a Nivolumab ELISA analysis of patients' serum samples taken at the first and second time of blood drawing (C1 and C2) to evaluate the presence of Nivolumab.

Our analysis showed that patient ICB005 had apparently been administered with Nivolumab before blood drawing at C1, and therefore high anti-IgG4 binding behaviour in flow cytometry was plausible in that case. Data of this patient was subsequently excluded from analysis. All other patients showed no sign of Nivolumab at C1 with the measured absorbance levels outside the established standard curve and considerable Nivolumab serum levels at C2 (Figure 13).

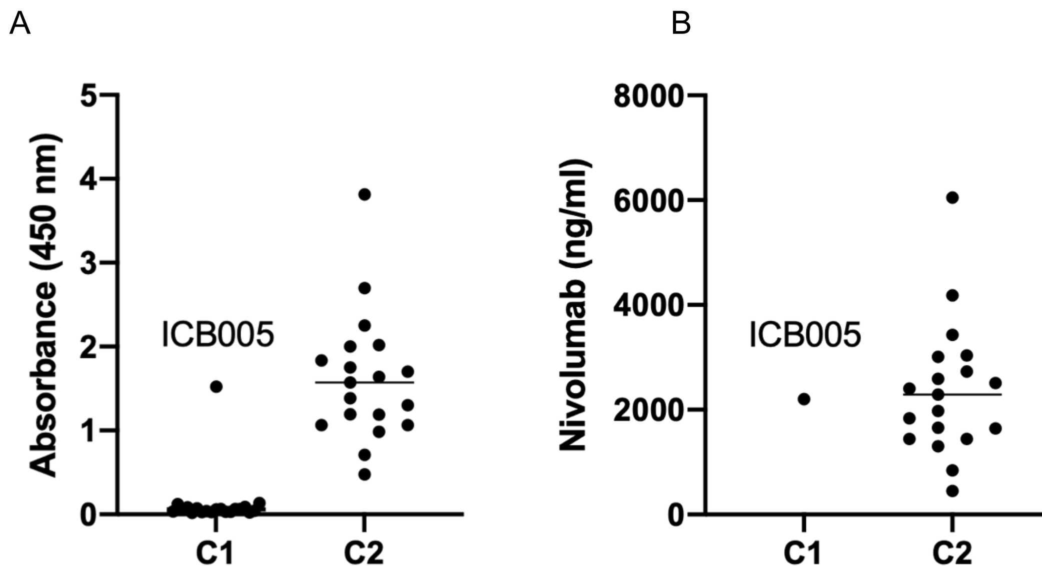

Figure 13: Quantification of Nivolumab serum levels in patients before (C1) and after (C2) therapy initiation

A: Measured absorbance of patient samples at 450 nm in Tecan Plate Reader.

B: Nivolumab serum levels determined by the established standard curve.

6 Discussion

The introduction of immune checkpoint therapies has led to a revolution in the treatment of cancer. While the results on overall response to drugs like the anti-PD-1 inhibitors Nivolumab or Pembrolizumab have been remarkable and have led to approval for treatment of various types of cancer, only subgroups of patients show substantial response to the drugs in most trials (41-43, 59, 69, 70). Biomarkers reliably predicting therapy response and characterizing patient populations susceptible for and benefitting from immune checkpoint therapy remain to be established.

HNSCC and RCC are among the cancer entities for which the anti-PD-1 inhibitors Nivolumab and Pembrolizumab have recently been approved, and biomarkers predicting therapy response are urgently needed (60, 61, 69, 70).

Kamphorst et al. described the expansion of CD8+ Ki67+ PD-1+ effector T cells to characterize immune response to PD-1 blockade therapy via peripheral blood sampling in NSCLC patients treated with anti-PD-1 inhibitor Nivolumab (108). Early expansion of this T cell subset was associated with better therapy response in the patient cohort, and the authors proposed that peripheral blood sampling including PD-1 receptor assessment might be a promising tool in predicting and monitoring responses to anti-PD-1 immune checkpoint therapy (108).

Both HNSCC and RCC have been described to be highly immunogenic and infiltrated with large numbers of TILs, while high infiltration levels have been associated with poor outcome in RCC and, in contrast, improved outcome in HNSCC (53-55, 73, 74). The role of PD-1 expression on immune cells and its potential implications for the response to immune checkpoint therapy in these tumour entities remains to be unravelled. We recruited patients with either HNSCC or RCC upon initiation of monotherapy with anti-PD-1 inhibitor Nivolumab for longitudinal peripheral blood sampling before and during treatment. PD-1 receptor status was assessed on immune cell subsets from whole blood samples at four time points through flow cytometry.

Faced with the difficulty in detecting bound PD-1, i.e., receptors blocked by therapeutic monoclonal antibody Nivolumab, we established a staining process utilizing an anti-IgG4 secondary antibody for the assessment of overall as well as differential bound and unbound PD-1 expression levels in whole blood samples through flow cytometry. We emulated our biobanking procedure on whole blood

samples of healthy donors incubated with Nivolumab and subsequently frozen at -80°C. Samples of 5 healthy individuals showed the reliability of the process through corresponding results in antibody staining behavior in fresh and thawed samples in flow cytometric analysis. However, the minor loss of PD-1 antibody detection as well as secondary IgG4 antibody detection were observed.

As a basis for our further results, we tested our method of Nivolumab-bound PD-1 detection in patient samples by comparing levels of bound and unbound PD-1 before and after therapy initiation. We showed that unbound PD-1 in patient samples suddenly declined after therapy initiation, whereas Nivolumab-bound PD-1 detection rates increased, which demonstrated that Nivolumab binds the majority of PD-1 on the studied cells. This is in line with early data on Nivolumab pharmacodynamics showing strong drug affinity to PD-1, resulting in high, dose-independent and sustained receptor occupancy upon antibody administration (45, 46). The cited papers demonstrated that even several weeks after drug administration, Nivolumab binding rates remain high (45, 46) and therefore secondary staining of the monoclonal antibody can be used as a reliable method to assess PD-1 expression levels in longitudinal studies.

T cells are considered the main carriers of PD-1 and are therefore the key targets of anti-PD-1 checkpoint therapy. Our data showed diverging levels of bound and unbound PD-1 throughout the trial period most distinctly in all T cell subsets studied. Among T cells, CD8+ CD38+ activated T cells showed the largest gap between unbound and bound receptor levels after therapy initiation. Since T cells relatively express the highest levels of PD-1, the large gap between bound and unbound levels seems plausible. The longevity of this observation throughout our trial period could hint at a low turnover of the PD-1 receptor on T cells.
While the differences between bound and unbound levels were less distinct in B cells and NK cells, they were still clearly observable. The fact that PD-1 is expressed by other immune cells – especially under conditions of chronic infection and cancer – has been well described (2, 12, 119). However, the unravelling of the role of immune cells other than T cells in anti-PD-1 checkpoint therapy has only just started.
We observed that Nivolumab binds PD-1 expressed by T cells, B cells, NK cells, DC, monocytes and basophiles. Binding on cells other than T cells has previously been

shown in PBMC samples of a small cohort of cancer patients under anti-PD-1 checkpoint therapy (120).
At baseline, we showed that all studied immune cell subsets express PD-1 to a certain extent. Expression levels were highest in CD4+ T cells and CD4+ CD25+ Tregs, and higher in CD38+ activated T cells compared to their respective populations of reference. We showed that CD4+ T cells express high levels of PD-1 (in fact, on average, higher levels than CD8+ T cells), although the role and mode of action of CD4+ T cells in this regard remains to be elucidated.
While we observed that PD-1 is expressed on dendritic cells, monocytes and basophiles, their population numbers as well as PD-1 expression levels were relatively low, which made it difficult to obtain robust PD-1 expression values by secondary antibody staining during immune checkpoint therapy. Unspecific background staining signal of the secondary antibody was increased in our flow cytometric data in these populations as well. Thus, we decided to exclude DC, monocytes and basophiles from further analysis.
PD-1 expression was relatively low on NK cells as well. However, staining behaviour seemed far more consistent and plausible in flow cytometric data, being likely due to higher numbers of NK cells analyzed in the respective samples. It has been shown that anti-tumor effects of anti-PD-1 checkpoint therapy in part seem to rely on NK cell activity and that PD-1 expression in NK cells marks an activated rather than an exhausted phenotype in these cells (121). Considering the interesting role of NK cells in anti-PD-1 checkpoint therapy, we decided to include them in further data analysis. The fact that we did not observe significant changes in overall NK cell PD-1 expression throughout our trial period could be due to relatively low baseline levels of PD-1.

PBMCs are a crucial tool in studying biological processes in and around the immune system and the default sampling method for analyses in this field in many cases. However, there has been a discussion evolving that challenges the dominance of PBMCs used for analyses in immunological studies (122, 123). Data suggests that PBMCs might not represent in vivo conditions as accurately as was assumed. It has been shown that various immune cells might be affected by the process (122, 123). One group showed that PBMC separation using Ficoll resulted in altered distribution of T cell sub-populations compared to whole blood samples, most notably in CD8+ T

cells as well as in altered T cell response upon antigen stimulation (123). However, the authors underline the importance of PMBCs, especially in cryopreservation for later use, and suggest the advantages of whole blood sampling being primarily in fresh probes that are analyzed right away (122, 123). Another study examined potential difficulties in stabilizing whole blood samples for biobanking through freezing and, in particular, observed changes in antibody-based detection of cell surface markers upon thawing the samples (124). However, none of the surface markers we used in our antibody panel was among the ones that were found to be problematic by this group. Considering the possible limitations of both whole blood and PBMC sampling, we concluded that stabilizing and freezing whole blood samples to thaw and process in bulk later was an appropriate way to minimize inter-day variance, e.g., caused by changes in environmental or laboratory conditions such as reagent batch variance and that the process might result in the improved ex vivo condition of samples obtained.

We analysed PD-1 expression levels on leukocyte populations before therapy initiation and prior to the patients' second, fourth and sixth infusion with Nivolumab, respectively. Most notably, CD4+ T cells showed a decrease in PD-1 expression over the trial period, with the most distinct effects observed in CD4+ CD25+ Tregs, CD4+ CD38+ activated T cells and CD38+ activated Tregs. This decline was steady until the fourth Nivolumab administration, before a slight increase in expression levels before the sixth infusion.
It has been shown in various studies that regulatory T cells are able to suppress anti-tumor effects of the immune system (125). In a paper published in 1999, it was demonstrated that depleting the immune system of Tregs led to strong anti-tumor responses and tumor regression through cytotoxic T cell activity in mice (126). Other animal studies have confirmed this data and similar associations have been suggested in humans (125, 127).
High expression levels of PD-1 on Tregs as well as significant changes throughout our study period indicate the potential engagement of Tregs upon anti-PD-1 checkpoint inhibition in RCC and HNSCC. irAEs in our patient cohort all occurred after completion of blood draws. Changes in immunologic regulation in affected patients had perhaps already begun towards the end of our trial period. It has been implied that irAEs are partially result from the loss of Treg homeostasis (128), and the upregulation of PD-1 in

Tregs in our trial at C6 mirrored the counter effects to restore regulatory functions. Fittingly, rising PD-1 levels at C6 were only observed in the irAE group. However, this effect was observed in all studied immune cell subsets.

Both the PD-1/PDL-1 pathway and Tregs are known to play major roles in immune regulation, while Tregs themselves often also express high levels of PD-1. In fact, it has been shown that PDL-1 is a key inducer of Treg evolution (129, 130). The authors of the cited paper showed that development of naïve T cells into inducible Tregs depended on the presence of TGF-ß upon PD-1/PDL-1 ligation, while its absence led to differentiation into effector T cells (129). Promoting Treg development through PDL-1 expression is another possible immune escape mechanism of cancer cells, since Tregs negatively regulate effector T cells that could potentially target malignant tissue.
Accordingly, a group that studied TILs and circulating T cells in colorectal cancer patients found large numbers of potentially immunosuppressive Tregs that were likely derived from the TME and which expressed high levels of immune checkpoint receptors including PD-1 (131). Other trials have confirmed PD-1 as highly upregulated on Tregs in the TME of various types of cancers (132-134).

While it is the consensus that the effects of anti-PD-1 immune checkpoint therapy mostly rely on reinvigorating cytotoxic CD8+ T cells, it has furthermore been suggested that PD-1 blockade also leads to the limitation of the suppressive effects of Tregs on effector T cells and that this mechanism might unfold crucial additional therapy effects (134-136). Thus, assessing the PD-1 expression in Tregs of cancer patients treated with anti-PD-1 therapy is of utmost interest.

In CD8+ T cells, B cells and NK cells, PD-1 expression levels were relatively stable throughout the study period. Both a slight decrease of receptor expression on CD8+ T cells and an increase on CD8+ CD38+ T cells were non-significant.
We further analysed our data by grouping patients according to occurrence of irAEs and therapy response.

We compared PD-1 expression levels on leukocyte subsets in samples from HNSCC and RCC patients. Interestingly, we found that PD-1 expression was consistently

higher in HNSCC patients throughout the entire period studied. This circumstance was most significant at baseline on all T cell populations and B cells. In both cancer entities, significant upregulation of PD-1 on immune cells in peripheral blood has been described (137, 138). Yet, to our knowledge, PD-1 expression levels of HNSCC and RCC in peripheral blood have not been directly compared before. However, both tumor types have been described to be highly immunogenic and therefore infiltrated by large numbers of TILs, suggesting a critical role of immune checkpoints in tumor formation and immune evasion (55, 73).
HPV infection plays an outstanding role in tumor formation of HNSCC, and it has been shown that HPV positivity correlates with PDL-1 expression levels in oropharyngeal squamous cell carcinoma (139). The authors of the cited paper also reported that in their study, HPV-positive cancers were more likely to show increased TIL infiltration (139). Another group studied the role of the PD-1/PDL-1 pathway in immune resistance of HNSCC tumors and observed high levels of PDL-1 on tumor tissue and of PD-1 on CD8+ TILs respectively in HPV-positive HNSCC (140).
p16 is a tumor suppressor and highly associated with HPV positivity (141-143). While p16 expression is not strictly limited to HPV-positive tumors, it can be used as a surrogate marker for HPV positivity when cut-off values are applied (69, 70).
Among HNSCC cases included in our study, 5 out of 9 tumors had elevated p16 expression levels and were categorized as p16+ by immunohistochemistry. Since HPV infection can trigger a cascade that leads to induction and upregulation of p16 resulting in tumor formation (65), it can be assumed that HPV infection played a considerable role in HNSCC formation among HPV+ patients in our cohort and essentially influenced immunogenicity and the pronounced expression of immune checkpoint receptors such as PD-1 in immune cells.
Fittingly, PD-1 expression levels were higher in p16+ HNSCC patients across all analysed leukocyte subsets from C1 to C4. However, all but CD4+ T cells including CD4+ CD38+ activated T cells and Tregs showed higher PD-1 levels in the p16- group at C6. Differences between the groups were non-significant except for significantly higher PD-1 in Tregs in the p16+ group at C1. Nevertheless, our data suggests that higher levels of PD-1 in HNSCC peripheral immune cells can predominantly be attributed to HPV-positive tumors.

Additionally, we found that PD-1 levels of CD4+ T cells, CD4+ CD25+ Tregs and CD4+ CD25+ CD38+ activated Tregs in the RCC group significantly declined over the study period. Interpreting PD-1 expression as result of tumor antigen exposure, one could argue that such a decline might be a sign of effective therapy and tumor eradication, resulting in a decrease of antigen exposure, though patient numbers were too small to be further statistically stratified into subgroups of responders and non-responders in a meaningful manner.

IrAEs are potentially serious and represent a considerable obstacle in anti-PD-1 checkpoint therapy, since therapy disruption or discontinuation upon manifestation of irAEs is common. While the exact physiological mechanisms underlying the occurrence of irAEs are yet to be better understood, they are generally considered as inflammatory or auto-immune side effects of anti-PD-1 checkpoint therapy (144). Ever since therapies targeting immune checkpoints were established, irAE development upon treatment has been observed in clinical trials. Early on, various groups reported on a favourable connection between irAEs and the response to anti-CTLA-4 therapy in melanoma patients (145, 146). These findings were confirmed by similar data on melanoma patients treated with anti-PD-1 checkpoint inhibitors (147, 148).

In line with these results, irAEs have also been shown to correlate with better therapy response in RCC and HNSCC patients under anti-PD-1 or anti-PD-L1 therapy (149-151).

In our study, we compared PD-1 expression on peripheral blood immune cells of patients that developed irAEs and those who did not. Generally, we observed decreasing PD-1 levels in patients without irAEs, and increasing levels in patients with irAEs. The decrease of PD-1 in the non-irAE group was significant in CD4+ T cells, Tregs and CD38+ Tregs. As mentioned above, the occurrence of irAEs in our study seems to result in the upregulation of PD-1 in Tregs, perhaps as a mechanism to re-establish their regulatory function. Furthermore, our data suggests that lack of irAEs leads to downregulation or degradation of PD-1 under anti-PD-1 therapy. Without the trigger of auto-immune events, PD-1 upregulation might not be initiated as pronounced, or not at all.

IrAE development has been associated with the upregulation of interferon-γ secretion (152-154), a pro-inflammatory cytokine that is secreted by T cells and NK cells.

Furthermore, interferon γ has been shown to induce PD-1 expression through upregulation of its ligands (155-157). Higher levels of PD-1 in the irAE group in our trial as well downregulation of PD-1 in non-irAE patients are therefore plausible.

When comparing the PD-1 levels of the irAE to the non-irAE group throughout the trial period, the results were non-significant, with one exception: CD8+ CD38+ activated T cells showed significantly higher PD-1 expression levels in the irAE group at C6 compared to the group without irAEs. Activated effector T cells have been considered responsible for irAE development and they have been detected at the sites of several affected organs respectively (152, 158-161). Furthermore, it was previously shown that irAE onset correlates with higher PD-1 levels in peripheral CD4+ and CD8+ T cells in RCC patients (161). Simultaneous occurrence of PD-1 upregulation in recently activated CD38+ effector T cells and irAEs in our patient cohort might reflect that T cells activated by PD-1 blockade are in part responsible for irAE development. In a mouse model, it was observed that thyreoditis under anti-PD-1 therapy could be prevented completely by depletion of CD4+ T cells and partially by depletion of CD8+ T cells (152). While the results of higher PD-1 in irAE patients in our study were only significant in CD8+ CD38+ T cells, they were also observable in CD8+, CD4+ and CD4+ CD38+ T cells, and thus fit the cited data.
These findings have to be validated by carrying out analyses in larger clinical trials. However, our data hints at the occurrence of irAEs being associated with higher expression levels of PD-1 across various leukocyte subsets, most distinctly in T cells in HNSCC and RCC patients.

We sought to study the effects of PD-1 status on therapy response and divided our patients into a group with progressive disease and another one of patients with stable disease or partial response to therapy with Nivolumab, so as to compare PD-1 expression levels in peripheral blood leukocytes. Overall, expression levels were relatively stable over the study period and we did not observe significant differences between the two groups. However, PD-1 expression tended to be higher in the SD/PR group on the T cell subsets studied, most notably in CD8+ T cells and CD8+ CD38+ activated T cells.
While our results hint at higher levels of PD-1 in T cells being associated with improved therapy response, our data was non-significant in this regard, which again

necessitates further clinical trials with larger patient cohorts that might lead to more distinct results.
We furthermore observed a significant decrease in PD-1 expression on CD4+ T cells, CD4+ CD25+ Tregs and CD38+ activated Tregs in the PD group. The latter showed a similar decrease in the SD/PR group. As stated before, Tregs seem to play a major role in the formation of various kinds of cancer as well as in the effects of anti-PD-1 checkpoint therapy.
Since PD-1 receptors on Tregs are a potential target of anti-PD-1 checkpoint therapy, the decay and downregulation of PD-1 expression might coincide with the decline of therapy response. Hence, the therapeutic antibody might no longer be able to suppress the negative regulatory functions of Tregs on anti-tumor immunity.

While the role of T cells in anti-PD-1 checkpoint therapy has been studied extensively and the underlying mechanisms have been becoming more clear, the role of B cells in this regard is still largely unknown. It has been shown that B cells express considerable levels of PD-1; however, its function in the cells and role in cancer have remained mostly unclear (2, 12). Recent data suggest that peripheral PD-1+ B cells possess pronounced regulatory functions by suppressing CD4+ and CD8+ T cell proliferation in in vitro experiments (162). In line with this, it has been reported that elimination of immunosuppressive B cells led to significant improvement in treating prostate cancer with chemotherapeutic agent oxaliplatin in murine models (163). However, depletion of B cells does not seem to have effects on the anti-tumor activity of PD-1 inhibitors, as a study on murine melanoma and colon cancer models investigated (164).
It has also previously been shown that NK cells express PD-1 and that the PD-1 pathway might be a key regulator of NK cell exhaustion in various tumor entities (165-167). While the function of the inhibitory receptor in the cells and the possible implications for anti-PD-1 checkpoint therapy are still under investigation, recent data showed that poorer anti-tumor activity is associated with pronounced PD-1 expression in NK cells (166, 167). Furthermore, it was shown that PD-1 inhibition carries out its therapeutical effects not only through T cells but also NK cells (121). Cytotoxic T cells can only target cells that express HLA-I, whereas NK cells recognize cells with abnormal or absent HLA-I expression (165). Therefore, NK cells can additionally contribute to therapeutic PD-1 blockade effects.

Generally, we observed relatively low levels of PD-1 on NK cells compared to T cells. However, in our study, T cells, B cells and NK cells showed similar dynamics in PD-1 expression changes over the trial period, e.g., higher PD-1 levels in HNSCC patients compared to RCC patients and differences in patients with and without irAEs. This could hint at distinct similarities in the underlying regulatory mechanisms and signalling pathways of PD-1 expression in B cells, NK cells and T cells.

7 Limitations

The results of our study have to be considered with the caveat that there was a relatively small cohort of 17 patients. Broadening the inclusion criteria, e.g., including patients under treatment with Pembrolizumab or with further tumor entities could allow recruitment of a larger patient cohort without considerably extending the recruitment period. Furthermore, we chose an exploratory approach to our research questions and compared baseline PD-1 values to receptor expression under Nivolumab therapy as well as subgroups of patients according to properties like irAEs and tumor type. Including a control cohort with healthy individuals could lead to further interesting results in subsequent studies.

8 Summary

We showed that in stabilized and frozen peripheral whole blood samples of HNSCC and RCC patients treated with the anti-PD-1 monoclonal antibody Nivolumab, PD-1 could reliably be stained with antibodies for flow cytometric analysis. We stained unbound PD-1 directly and PD-1 bound by Nivolumab indirectly using a secondary IgG4-antibody. Following therapy initiation, we demonstrated that unbound PD-1 levels decreased and Nivolumab-bound levels increased on all leukocyte subsets included in our analysis and that receptor binding status remained relatively stable throughout our trial.

In comparing tumor entities, we showed that all studied leukocyte populations expressed higher levels of PD-1 in HNSCC patients compared to RCC patients throughout the entire study period, significantly so, in all T cell subsets, as well as B cells before the start of immune checkpoint therapy. By stratifying HNSCC patients according to tumor HPV status, we demonstrated that higher PD-1 expression in HNSCC patients could be attributed to HPV+ tumors.

In addition, we showed that immune cell PD-1 expression levels of patients with irAEs tend to be higher compared to those without irAEs, especially towards the end of the immune monitoring period in our trial. This observation was most distinct and only significant in CD8+ CD38+ activated T cells before the sixth administration of Nivolumab therapy.

We furthermore observed that PD-1 expression levels were more pronounced in patients with stable disease or partial response to therapy compared to patients with progressive disease. These results, however, were non-significant and larger trials are needed to investigate associations regarding therapy response.

We observed considerable expression of PD-1 in B cells and NK cells. While we witnessed changes in expression levels similar to those in T cells throughout the trial, our data did not show any significant associations to the occurrence of adverse events or therapy response.

In conclusion, while being limited by a relatively small patient cohort, the insights of our data provide a promising contribution on the path to finding reliable biomarkers for predicting the response to anti-PD-1 checkpoint therapy in RCC and HNSCC patients.

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Eidesstattliche Versicherung

„Ich, Theodor Schmidt, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Changes in peripheral blood PD-1 receptor expression under anti-PD-1 Immune Checkpoint Therapy" bzw. "Veränderungen der PD-1-Expression in peripherem Blut unter Anti-PD-1-Immun-Checkpoint-Therapie"selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst."

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Datum Unterschrift

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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Bescheinigung

Hiermit bescheinige ich, dass Herr Theodor Schmidt innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgende Beratungstermine wurden wahrgenommen:

- Termin 1: *04.12.2020*

Folgende wesentliche Ratschläge hinsichtlich einer sinnvollen Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- Beachtung der Abhängigkeit von Daten bei statistischen Tests
- Voraussetzungen und Vorteile eines linearen gemischten Modells
- Hypothesengenerierende Interpretation der p-Werte (explorativ)

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das Institut für Biometrie und klinische Epidemiologie übernimmt hierfür keine Haftung.

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