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Significance of circulating Hsp70 levels and immunophenotyping as potential biomarkers for bladder cancer patients

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Contents

List of Abbreviations

ADCC ..... Antibody-dependent cellular cytotoxicity
CD ..... Cluster of differentiation
CLP ..... Common lymphoid progenitor
ELISA ..... Enzyme-linked immunosorbent assay
FACS ..... Fluorescence Activated Cell Sorting
FCS ..... Fetal calf serum
FSC ..... Forward Scatter
HCC ..... Hepatocellular carcinoma
HNSCC ..... Head and neck squamous cell carcinoma
HSC ..... Hematopoietic stem cell
Hsp70 ..... Heat shock protein 70
HSP70 ..... Heat shock protein group 70
ICI ..... Immune checkpoint inhibitor
ICL ..... Innate lymphoid cell
iNK ..... Immature NK cell
KIR ..... Killer cell immunoglobulin-like receptor
LMPP ..... Lymphoid-primed multipotential progenitor
MHC ..... Major histocompatibility complex
mHsp70 ..... Membrane-bound Hsp70
MIBC ..... Muscle invasive bladder cancer
mNK ..... Mature NK cell
NBD ..... Nucleotide binding domain
NCAM ..... Neural cell adhesion molecule
NCR ..... Natural cytotoxicity receptor
NK cells ..... Natural Killer cells
NKP ..... NK cell progenitor
NMIBC ..... Non-muscle-invasive bladder cancer
NSCLC ..... Non-small cell lung cancer
OS ..... Overall survival
PBL ..... Peripheral blood lymphocytes
PBS ..... Dulbecco's Phosphate Buffered Saline
PD-1 ..... Programmed cell death protein

PD-L1 ..... Programmed cell death ligand 1
PD-L2 ..... Programmed cell death ligand 2
RACE-IT Radiation therapy before radical Cystectomy combined with Immunotherapy
RCT ..... Radiochemotherapy
ROS..... Reactive oxygen species
RT ..... Radiotherapy
SBD ..... Substrate binding domain
SOP ..... Standard operating procedure
SSC ..... Side Scatter
TIL ..... Tumor-infiltrating lymphocyte
TME ..... Tumor microenvironment
TNF..... Tumor necrosis factor
Treg cells ..... Regulatory T cells
TURBT..... Transurethral resection of the bladder tumor
WBC ..... White blood cells

Abstract

The immune system plays an essential role in developing, defending, and combating cancer. It creates homeostasis with various counterparts, which on the one hand, enables immune tolerance against itself and, on the other hand, prevents the development of tumor cells. If this balance gets out of joint, tumors grow and progress, which in turn influences the composition of the immune system through the release of various mediators and cytokines. To improve the knowledge in this field of immuno-oncology, the immune phenotype of 16 patients with bladder cancer was determined, absolute numbers for lymphocytes and the percentages of B cells, T cells, and NK cells in all lymphocytes were measured both at diagnosis and their kinetics during therapy. Since the dynamics of the composition of the lymphocyte subpopulations and absolute lymphocyte counts depend on many factors, the biomarkers NLR and Hsp70 were also considered. An increasing NLR, as a marker for a systemic inflammatory response, can be associated with a compromised immune system and thus with a worse outcome. Hsp70 is a chaperone that typically supports proper folding and transport of proteins but is also upregulated upon exposure to stress, such as irradiation or increased cell division. In addition, membrane-bound Hsp70 serves as a tumor-specific recognition structure for NK cells. It can trigger a $CD8^+$ T cell response by presenting Hsp70 chaperoned peptides on MHC-I molecules.

Hsp70 can also influence NLR via the induction of inflammatory and anti-inflammatory responses. This study found increased mean values for Hsp70 and NLR in bladder cancer patients compared to a healthy control group. In addition, a decrease in Hsp70 concentration in the blood was observed in all Hsp70-positive patients after radioimmunotherapy, which either decreased or increased after tumor removal. In the patient population, reduced mean percentages of $CD19^+$ B cells and $NKG2D^+$ NK cells were observed compared to healthy individuals, while increased percentages of CD45+ and CD8+ T cells were noted. On the other hand, the rate of $CD4^+$ T cells decreased, suggesting an opposite development of $CD4^+$ and $CD8^+$ T cells.

Furthermore, two patients showed recurrence and metastasis associated with increasing Hsp70 levels. These findings provide supportive evidence for the use of Hsp70 as a biomarker for the early detection of cancer and monitoring of treatment response and prognosis. All the results were considered and evaluated in terms of their interdependence to advance the understanding of cancer as a holistic challenge.

1. Introduction

1.1. Bladder cancer

1.1.1. Epidemiology

In 2020, bladder cancer accounted for 573.000 new cases and 213.000 deaths worldwide, making it the 10th most frequently diagnosed cancer globally.
The disease affects men more often than women, with incidence and mortality rates for men of 9.5 and 3.3 per 100.000, which are approximately four times higher than those for women. Its prevalence is increasing, especially in developed nations which may be based on its most significant risk factor – tobacco smoking (Sung et al., 2021). Cancer of the bladder is a disease of the elderly since about 80% of the cases are diagnosed in patients aged 65 and older. In the US, the 5-year survival rate for bladder cancer is 77.1%, with a significant dependency on the stage of the disease. While the 5-year survival rate for cases of bladder carcinoma in situ is 95.8%, it drops to only 4.6% for metastatic disease (Saginala et al., 2020). Smoking tobacco is indeed the most significant risk factor for bladder cancer, contributing to approximately 50-65% of new cases yearly. It increases the risk of bladder cancer three to four times, making it a major concern for public health. Its relative risk for mortality from tobacco smoking is surpassed by lung cancer mortality, which is the leading cause of death related to smoking (Freedman et al., 2011). Other risk factors for developing bladder cancer include environmental and occupational exposure to carcinogens like aromatic amines, polycyclic aromatic hydrocarbons, or chlorinated hydrocarbons (Zeegers et al., 2001), red meat (Wang et al., 2012), obesity (Sun et al., 2015) and the protozoan schistosomiasis (Inobaya et al., 2014). According to a meta-analysis, approximately 81.8% of bladder cancer cases could be attributed to preventable risk factors, underlining the significant impact of modifiable risk factors on the development of this disease. In contrast, only about 7% of bladder cancer cases are attributed to genetic causes (Al-Zalabani et al., 2016).

1.1.2. Histology and pathogenesis

Histologically, bladder carcinoma can be divided into urothelial carcinoma, squamous cell carcinoma, adenocarcinoma, and other rare subtypes such as micropapillary

urothelial carcinoma or small cell carcinomas. Urothelial carcinoma accounts for over 90% of bladder cancers, whereas squamous cell carcinoma comprises only 5% (Mori et al., 2020). In addition, the bladder may be affected by invasive local growth of other cancer entities, such as prostate, testicular, ovarian, cervical, and endometrial cancers (Van Hemelrijck et al., 2014). Bladder cancer is staged by TNM classification, distinguishing between non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC), being particularly critical both prognostically and therapeutically. NMIBC is limited to extension into the mucosa or submucosa, while invasive muscle growth extends to the muscularis or serosa (Chehab et al., 2015).

1.1.3. Diagnostics

The most common initial symptom of a bladder tumor is painless macrohematuria. Therefore, the first diagnostic steps consist of a urine test strip and urine cytology. If atypical urothelial cells are detected in the cytologic analysis, sonography, cystoscopy, and CT urography are useful (Babjuk et al., 2017). After the initial cystoscopy, transurethral resection of the bladder tumor (TURBT) is usually attempted. In this way, information about tumor location, tumor size, the appearance of the tumor, and the entire bladder wall can be obtained, as well as a histological examination of the tumor tissue (Quarles et al., 2021). CT of the chest, abdomen, and pelvis should be performed as part of tumor staging (Alfred Witjes et al., 2017).

1.1.4. Therapy

1.1.4.1. Standard Treatment of MIBC

The standard treatment of MIBC from stage T2 currently consists of neoadjuvant cisplatin-based chemotherapy followed by radical cystectomy with lymph node dissection and urinary bladder reconstruction, depending on the patient's constitution. In addition, preoperative radiotherapy may be offered as it can downsize the tumor after 4 to 6 weeks (Alfred Witjes et al., 2017). Despite the option of radical cystectomy, patients with locally advanced bladder cancer at stage cT3/T4 cN0/N+ cM0 have a poor prognosis (Schmid et al., 2020). Even the addition of perioperative chemotherapy to surgery does not improve outcomes, leading to an overall survival (OS) rate of less than 35% (Hautmann et al., 2006).

1.1.4.2. Immunotherapy

For local immunomodulation, there is the option of intravesical injection of Bacillus Calmette-Guerin (BCG), an attenuated strain of Mycobacterium bovis. Investigated in 1974, intravesical therapy with BCG is nowadays part of the standard therapy for NMIBC (Wolacewicz et al., 2020). Treatment with BCG elicits an inflammatory response that attracts various immune cells attacking cancer. These cells include $CD4^+$ and $CD8^+$ T lymphocytes, NK cells, macrophages, granulocytes, and dendritic cells (DCs). BCG also kills some cancer cells directly (Zhang et al., 2020). Due to the described poor prognosis of advanced MIBC, research is concerned furthermore with possible new therapeutic options, such as recent advances in immunotherapy. Much immunotherapy research revolves around checkpoint inhibitor pathways, such as the PD-1/PD-L1 pathway (Havel et al., 2019). Programmed cell death protein (PD-1) and its ligands Programmed cell death ligand 1 (PD-L1) and 2 (PD-L2) are part of the immune system and provide immune tolerance in the tumor microenvironment (TME), which can be detrimental to patient survival in the presence of cancer (Wolacewicz et al., 2020). PD-1 inhibits the innate and adaptive immune system and is expressed on the surface of activated T cells, especially on tumor-specific T cells, B cells, NK cells, macrophages, dendritic cells, and monocytes (Ahmadzadeh et al., 2009).

On the one hand, PD-1 is necessary to prevent excessive immune responses, but it also contributes to carcinogenesis through its immunosuppressive effect (Salmaninejad et al., 2018). Through various transcription factors and binding sites, cancer can increase the transcription and expression of PD-1 and is therefore associated with an immune-suppressing tumor microenvironment (Han et al., 2020). Its ligand PD-L1 is expressed by macrophages, activated T cells, B cells, and other cells, especially under inflammatory conditions (Sharpe et al., 2007). In addition, PD-L1 is expressed on the surface of tumor cells to evade the anti-tumor immune response by binding various receptors and activating proliferative and survival pathways (Dong et al., 2018). The expression of PD-L1 is associated with the severity of bladder cancer. In addition, bladder tumors with high expression of PD-L1 are more malignant, showing a higher recurrence rate and lower OS (Nakanishi et al., 2007). Therefore, given the poor prognosis of locally advanced bladder cancer described above, immune checkpoint inhibition is also gaining importance for this cancer entity. In 2020, the immune checkpoint inhibitors (ICIs) Atezolizumab, Avelumab, Durvalumab, Nivolumab, Pembrolizumab, and Ipilimumab were already approved for the treatment

of bladder cancer, and further data is currently being 2collected to allow ICIs to be integrated into standard therapy. Nivolumab is, for example, a human IgG4 monoclonal antibody approved by the FDA in 2017 and the EUA for advanced bladder cancer. Overall, good results were achieved despite severe adverse events (Wolacewicz et al., 2020). Consequently, the phase II trial RACE-IT is currently being conducted at Klinikum rechts der Isar. All patients examined in this dissertation are part of this study, the design of which is discussed in further detail in Chapter 3.1.

1.2. Immune cells

Immune cells are essential components of the immune system, playing a crucial role in defending the body against pathogens and maintaining overall health. In the context of oncology, they also hold significant importance. Specifically, the focus of the next section will be on lymphocytes in relation to cancer, as they have been the subject of detailed study in the dissertation.

1.2.1. B cells

B cells, or B lymphocytes, can produce antibodies as mature plasma cells to fight off pathogens and thus form the basis for the specific humoral immune system. Up to 25% of all tumor-infiltrating lymphocytes (TILs) are B cells, making them the second most abundant immune cell in the tumor microenvironment after T cells. B cells can inhibit tumor progression by producing tumor-specific antibodies, support tumor killing via NK cells, support phagocytosis via macrophages, and promote tumor cell death by priming T cells. However, at the same time, B cells can promote tumor growth and progression by producing tumor growth factors and autoantibodies, including an inhibitory T cell response (Yuen et al., 2016).

1.2.2. T cells

T cells, like B cells, also belong to the lymphocytes and can be divided into $CD4^+$ and $CD8^+$ T cells based on their surface proteins. $CD4^+$ T cells, called T helper cells, develop into different subtypes depending on the cytokines acting on them. For example, a naive $CD4^+$ T cell can develop into Th1, Th2, Th17, TFH, and Treg cells (Zhu et al., 2008). Most T helper cells support other immune cells, such as B cells and

cytotoxic T cells, in their function and thus lead to increased immune response. In contrast, Treg cells, also known as regulatory T cells, regulate the remaining T cell subtypes by preventing excessive recruitment and autoimmunity (Wan et al., 2009). However, several studies have shown a correlation between high tumor-infiltrating Treg cell numbers and a good prognosis, such as colorectal cancer (Hu et al., 2017; Xu et al., 2017). The described paradox may be attributed to the substantial heterogeneity of Treg cells. Therefore, despite the rather pro-tumoral effect of Treg cells, it is essential to investigate their function precisely for the respective cancer entity. Cytotoxic $CD8^+$ T cells are necessary to kill pathogenic or altered cells directly. Naive $CD8^+$ T cells circulate in the body and develop into cytotoxic T cells upon antigen presentation. These then bind to the target and, in turn, lead to cell lysis via the release of cytokines. Several retrospective studies have found a strong positive correlation between tumor-infiltrating cytotoxic $CD8^+$ T cells and cancer survival (Gun et al., 2019).

1.2.3. Natural Killer cells

Natural Killer cells (NK cells) comprise 5-20% of peripheral blood lymphocytes, belong to the innate immune system, and have cytotoxic capabilities. Hence NK cells are responsible for the first line of defense against infectious diseases and cancer. Compared to T cells, NK cells do not have to be primed and activated by prior contact with the antigen (Gun et al., 2019). NK cells release T cells recruiting chemokines like IFN-γ and TNF-α. Furthermore, NK cell cytotoxicity is mediated via granzyme B/perforin, cell death receptor interactions, and antibody-dependent cellular cytotoxicity (ADCC) (Pockley et al., 2020). NK cells are also related to the chaperone Hsp70. The presence of Hsp70 has been linked to the activation of NK cells, suggesting that in cases of cancer with increased Hsp70 expression, an elevated immune response by NK cells may be observed. (Lobinger et al., 2021; Multhoff, 2009). Finally, some clinical studies have shown reduced NK cell activity in advanced cancer, and that low NK cell activity in tumor patients is often associated with poor survival outcomes (Gun et al., 2019). Since NK cells are also becoming attractive for therapeutic purposes, their unique properties will be discussed in more detail below.

1.2.3.1. Maturation and development

NK cells can develop in the bone marrow and secondary lymphoid organs such as the tonsils, spleen, and lymph nodes (Scoville et al., 2017). They belong to the innate lymphoid cell family (Shimasaki et al., 2020). On the way to becoming mature NK cells, hematopoietic stem cells (HSCs) differentiate into lymphoid-primed multipotential progenitors (LMPPs). Through the expression of various clusters of differentiation (CD) antigens, the LMPPs mature into common lymphoid progenitors (CLPs), which are progenitor cells for the Pro-B cells, Pre-T cells, NK cell progenitors (NKPs) and other innate lymphoid cells (ICLs). With the expression of CD122, the CLPs irreversibly form into cells of the NK cell lineage. The subsequent expression of the surface marker neural cell adhesion molecule (NCAM) or also called CD56, on the surface of immature NK cells (iNK), can then finally lead to the formation of mature NK cells (mNK) (Abel et al., 2018). Figure 1 concludes the schematic development from HSC to iNK. The expression of $CD56^{bright}$ and $CD56^{dim}$ is discussed in the following paragraph.

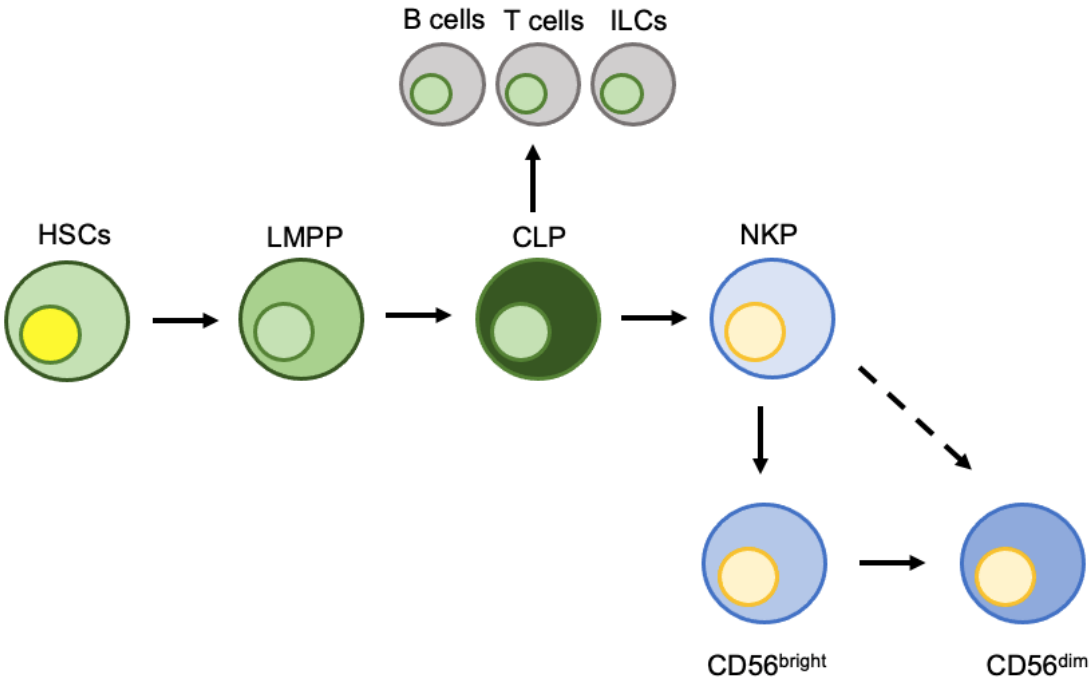

Figure 1 Development of NK cells

Adapted from Natural Killer Cells: Development, Maturation, and Clinical Utilization, Front Immunol 2018 Vol. 9

With the addition of different surface markers, NK cells can develop through six stages and thus express different characteristic CD antigens at each stage. For example,

activation receptors such as NKG2D, CD335 (Nkp46), and CD337 are expressed in reaching stage 3. In stage 4, high amounts of CD56 ($CD56^{bright}$) are expressed, then downregulated again in stage 5 ($CD56^{dim}$). Furthermore, in addition to downregulation, CD16 is supplemented in stage 5. This expression of CD56 represents the transition from iNK to mNK, with most NK cells circulating in the blood belonging to the $CD56^{dim}$ population. $CD56^{bright}$ NK cells are considered less mature and are more likely to remain in the secondary lymphoid organs. Downregulation of CD56 also contributes significantly to the anti-tumor cytotoxicity and cytolytic function of NK cells. $CD56^{bright}$ NK cells, on the other hand, are known to produce inflammatory cytokines. $CD56^{dim}$ cells have reached the final stage of maturation at the time of CD57 expression in stage six (Abel et al., 2018).

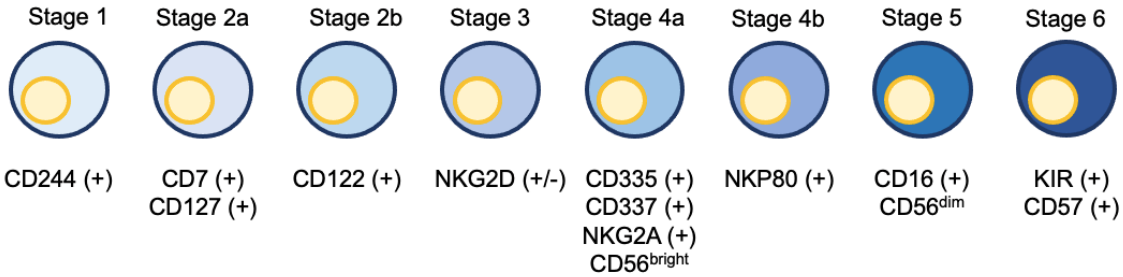

Figure 2 Stage-specific surface markers of NK cell development

Adapted from Natural Killer Cells: Development, Maturation, and Clinical Utilization, Front Immunol 2018 Vol. 9

Phenotypically, NK cells are defined by the expression of CD56 and the lack of expression of CD3, with $CD56^{bright}$ NK cells known as immunoregulatory NK cells and $CD56^{dim}$ NK cells known as cytotoxic NK cells (Farag et al., 2006). This process of differentiation and maturation is essential for the development of diverse immune cell populations and the establishment of an effective immune system.

1.2.3.2. Receptors and their ligands

The interaction of activating and inhibitory receptors mainly determines the function of NK cells. The most critical activating receptors include the group of natural cytotoxicity receptors (NCRs) and NKG2D. In contrast, the inhibitory receptors consist mainly of HLA-class-I specific inhibitory receptors such as killer cell immunoglobulin-like receptors (KIRs) and CD94/NKG2A. Ligands of the activating receptors are mainly

increased or completely newly formed during cell stress, viral infection, or tumor transformation. They are hardly expressed on the target cells of NK cells in a healthy state. On the one hand, the HLA-recognizing inhibitory receptors ensure recognition of the patient's healthy cells and, thus, self-tolerance. However, they can also lead to a decreasing inhibitory effect in the case of cell damage or cell transformation and a resulting loss of the HLA proteins as ligands. In addition, other receptors, such as PD-1, influence the function of NK cells because they ensure tolerance to T cells and a coordinated joint function of T and NK cells when tumor cells express the ligand PD-L1. The following figure shows all inhibitory and activating receptors with their respective ligands, with NKp46, NKp30, NKp44, and NKp80 belonging to the NCRs (Sivori et al., 2019).

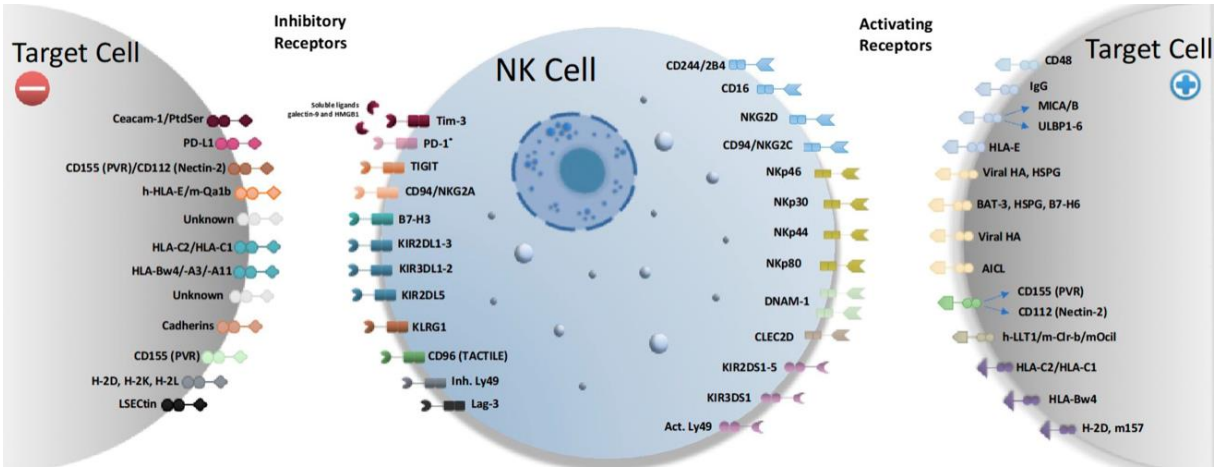

Figure 3 Inhibitory and activating Receptors and Ligands of NK cells

Cited from Approaches to Enhance Natural Killer Cell-Based Immunotherapy for Pediatric Solid Tumors, Cancers 2021 13 (11), 2796, CC BY license

In this section, only a selection of the most important and not all known receptors and ligands have been mentioned. However, it is also interesting that NK cells, in contrast to T cells, do not possess surface T cell receptors (TCRs) and, as a result, do not cause graft-versus-host disease (GVHD). This unique characteristic makes NK cells a promising therapy option for the targeted killing of cancer cells (Shimasaki et al., 2020).

1.2.4. Effector functions and killing mechanisms

NK cells use various mechanisms to eliminate tumor cells. As previously mentioned, NK cells can secrete vesicles with pro-apoptotic contents, such as the protease granzyme B and the pore-forming perforin, to induce apoptosis by binding to receptors on target cells (Talanian et al., 1997). Moreover, NK cells indirectly can activate the adaptive immune system with B cells and T cells by releasing pro-inflammatory interferon-γ and interferon-α and thus kill via cytokine-mediated pathways (Abel et al., 2018). Like T cells, NK cells can also induce apoptosis after being triggered by activating receptors with the help of Fas-Fas-ligand interactions and via TNF-death ligands (Bradley et al., 1998). In contrast to T cells, which require the presentation of a foreign antigen by antigen-presenting cells on MHC molecules for activation, NK cells are capable of killing target cells in an MHC-independent manner without prior stimulation. The high expression of MHC molecules serves to safeguard healthy body tissues from being targeted by NK cells. Furthermore, a reduction or absence of MHC expression, frequently observed on tumor metastases, triggers NK cell-mediated cytotoxicity. Consequently, NK cells have the ability to identify and eliminate cells with diminished MHC expression, thereby targeting cells that may evade T cell-mediated killing. (Narni-Mancinelli et al., 2011). Additionally, NK cells have the capability to induce apoptosis through antibody-dependent cellular cytotoxicity (ADCC). This mechanism is mediated by the low-affinity Fc gamma receptor CD16, which interacts with antibodies bound to the target cell. As CD16 is exclusively expressed on the surface of NK cells, they uniquely utilize this mechanism for inducing apoptosis in target cells. (Cooper et al., 2001; Pockley et al., 2020). In summary, while T cells express a single TCR, NK cells possess numerous activating and inhibitory receptors that collaborate in a balanced and intricate manner to effectively prevent and combat infections as well as cancerous cells.

1.3. Tumor microenvironment

The tumor microenvironment (TME) is the histological surrounding of a tumor. It consists of heterogeneous cellular and acellular components and regulates oncogenesis with its secreted factors. In addition to malignant cells, adipocytes, fibroblasts, tumor vasculature, lymphocytes, dendritic cells, myeloid-derived suppressor cells, and cancer-associated fibroblasts can be found in the TME, with

each of these cell types having different properties that are either tumor-suppressive or progressive (Arneth, 2019; Tesi, 2019). Looking at the TME, it becomes evident that malignant cells evade the immune system's mechanisms, allowing them to grow and spread uncontrollably. As described by Hanahan and Weinberg in 2000, several acquired capabilities of cancer cells contribute to their development: “Self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis” (Hanahan et al., 2000). These “hallmarks of cancer” were supplemented in 2011 by the points “reprogramming of energy metabolism and evading immune destruction” and confirmed in 2022 (Hanahan, 2022; Hanahan et al., 2011). For a better overview, the provided illustration, Figure 4, depicts the influencing factors for tumor genesis according to Hanahan & Weinberg.

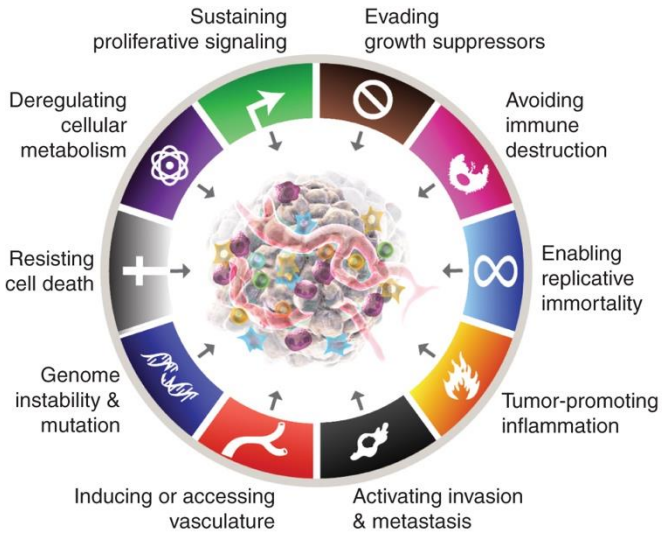

Figure 4 Hallmarks of Cancer – Reprinted from Hallmarks of Cancer

New Dimensions, Cancer Discov. 2022 Vol. 12 Issue 1 Page 31-46, with permission from AACR

As previously mentioned, the specified characteristics underwent multiple revisions and additions from 2000 to 2022, resulting in the current inclusion of eight hallmark capabilities and two enabling characteristics within the "hallmarks of cancer." The two enabling characteristics are “tumor-promoting inflammation” and “genome instability and mutation”, which contribute to tumor development and growth by activating the capability functions. These diverse capabilities across various dimensions enable the

invasive and destructive growth of cancer, necessitating the development of mechanisms to control it. Important cells in the TME for tumor control are the $CD8^+$ and $CD4^+$ T cells, B cells, and NK cells described above, as they can all contribute in both directions to cancer development. Also crucial for tumor progression are cells and messengers that increase tumor cell stemness, promote angiogenesis and migration, create drug resistance, and generally suppress the immune system (Arneth, 2019). As mentioned previously, the PD-1/PD-L1 pathway and its role in promoting cancer progression are also present within the TME. It provides an example of drugs that do not target the tumor directly but rather the cellular functions and their interaction in the TME. Also, treating tumor cells with radiotherapy can lead to changes in the TME. It is a well-established understanding that ionizing radiation induces cell death by causing DNA double-strand breaks, making it a viable option for cancer therapy. (Daguenet et al., 2020). However, the abscopal effect, for example, can also lead to a tumor mass reduction of other tumor parts than the irradiated ones (Reynders et al., 2015). This effect is a systematic immunological reaction of the body against the tumor triggered by irradiation. In this way, even non-irradiated metastases can shrink by altering the immune response. In addition, the bystander effect can result in enhanced local tumor control through the localized communication of irradiated cells with neighboring cells or those located just a few millimeters away (Daguenet et al., 2020). In both effects, releasing cytokines and chemokines, for example, stimulates NK and T cells, which in turn can induce an immune response against the tumor, locally or distantly (Garnett et al., 2004). These systemic immunological responses do not occur with every irradiation, and it is difficult to predict when an effect will occur. However, it is interesting to note that it seems to be an increased response that appears with combination therapies, such as the use of an additional immune checkpoint therapy (Y. Liu et al., 2018). Therefore, it is evident that cancer research encompasses not only the tumor itself, but also the surrounding factors and the interactions of its collaborators or antagonists.

1.4. Heat shock protein 70 (Hsp70)

1.4.1. Overview

Another essential protein of the TME is the Heat shock protein 70, also called Hsp70, HSPA1A, or Hsp70.1 (Balogi et al., 2019). Hsp70 is a chaperone that typically supports

the proper folding of proteins, promotes the transport of newly formed polypeptide chains, and helps assemble multiprotein complexes (Radons et al., 2005). The human HSP70 family includes 13 distinct members that differ in expression, localization, and amino acid sequences (Radons, 2016). The expression of HSP70 can be caused by, as the name describes, heat stress but also by other forms of stress such as ischemia, food deprivation, inflammation and infection, chemotherapy, and radiotherapy leading to the formation of reactive oxygen species (ROS) and also during cell proliferation and differentiation (Lindquist et al., 1988; Multhoff et al., 2015). Hsp70, the major stress-inducible member of the HSP70 group, occurs intracellular and extracellular and on the cell surface of tumor cells. In contrast, it is not found on the surface of healthy cells (Radons et al., 2005). Depending on the expression site, the Hsp70 proteins perform different tasks and operate differently. In contrast to healthy cells, tumor cells have higher Hsp70 contents inside the cell due to their higher proliferation rate. The anti-apoptotic effect of Hsp70 thus results in better protection against cell death in tumor cells. Resistance to therapeutic agents can also be caused by the increased intracellular Hsp70 level (Vargas-Roig et al., 1998).

Extracellular Hsp70 can be released from dead and inflammatory cells as a free protein or vesiculary bound to exosomes of active, living cells (Radons, 2016). It has been found that membrane-bound Hsp70 (mHsp70) is primarily present on tumor cells and not on normal cells (Radons et al., 2005). As Hsp70 does not possess a conventional transmembrane domain, it is hypothesized that these proteins depend on the lipid rafts of tumor cells to be transported externally and reach the outer membrane surface (Vega et al., 2008). Exosomal Hsp70 is predominantly released by actively proliferating tumor cells through lipid rafts, and as a result, it can serve as an indicator of the viable tumor size in the bloodstream of cancer patients (Gunther et al., 2015; Werner et al., 2021). In conclusion, HSPs have many functions that need to be fully understood despite their discovery in 1962. The HSP family member Hsp70 will be discussed in more detail in the following section.

1.4.2. Structure and function

Many other vital functions complement the classical function of Hsp70 as a molecular chaperone. Hsp70 protein consists of an N-terminal nucleotide-binding domain (NBD), which binds and hydrolyzes ATP, and a C-terminal substrate binding domain (SBD),

which binds unfolded polypeptides (Flaherty et al., 1990). Thus, intracellularly, Hsp70 binds ATP-dependently to polypeptides, prevents aggregation of unfolded peptides, transports proteins, and regulates intracellular signaling (Multhoff et al., 2015). Moreover, Hsp70 mediates antigen-cross presentation by presenting Hsp70 chaperoned peptides on MHC-I molecules, eliciting a $CD8^+$ T cell immune response (Radons et al., 2005).

Without HSP-chaperoned antigens, membrane-bound Hsp70 can also serve as a tumor-specific recognition structure for activated NK cells (Gross et al., 2003). After binding to Hsp70, NK cells mediate tumor cell killing, which is mediated by granzyme B. Thus, NK cells recognize Hsp70 without a presented antigen, whereas $CD8^+$ T cells only recognize the presented antigen but not the actual Hsp70 (cross-presentation). In addition, extracellular and lipid-bound Hsp70 in the extracellular milieu can induce inflammatory immune responses (Multhoff et al., 2015).

1.4.3. Role of Hsp70 in tumor cells

Hsp70 maintains the cellular balance of the proteome by supporting a proper folding of denatured proteins. Therefore, it is not surprising that Hsp70 is upregulated during conditions of homeostatic imbalance, like cancer (Balogi et al., 2019). The cells in the TME encounter harmful factors like hypoxia, acidosis, free radicals, and nutrient deficiency. The rapid and undifferentiated growth of tumor cells and the resulting misfolded proteins make the environment in the TME stressful. Due to this, the levels of Hsp70 increase in cancer cells, which leads to improved growth, decreased senescence, and resistance to stress-induced apoptosis (Xie et al., 2003). Thus, upregulated Hsp70 levels protect tumor cells from apoptotic cell death (Beere, 2005) and promote cell survival and tumor progression (Gabai et al., 2009). Since Hsp70 is a recognition structure for activatory C-type lectin NK cell receptors (Gross et al., 2003), it may also provide an anti-tumor immune response and tumor regression. It has been shown that incubation of NK cells with Hsp70 or an Hsp70-derived peptide (TKD) together with low-dose interleukin-2 (Multhoff et al., 2001) can increase the proliferation rate and migratory ability of NK cells towards highly aggressive, Hsp70-positive tumor cells (Gastpar et al., 2005; Multhoff et al., 1999). In addition, it has been shown that the killing mechanism by activated NK cells is mediated by an increased release of the pro-apoptotic granzyme B which results in tumor cell apoptosis (Multhoff

et al., 2015). In summary, elevated Hsp70 plasma and serum levels may correlate with increased living tumor mass. Additionally, Hsp70 may influence the peripheral blood lymphocyte composition. This has been demonstrated, for example, in patients with non-small cell lung cancer (NSCLC) (Gunther et al., 2015) and patients with hepatocellular carcinomas (HCC). The HCC study showed that patients with chronic liver cirrhosis who subsequently developed HCC had higher Hsp70 serum levels than healthy individuals. However, patients with HCC had the highest serum Hsp70 levels (Gehrmann et al., 2014). In addition, another study conclusively showed that patients with pancreatic cancer had higher Hsp70 serum levels than healthy controls or patients with chronic pancreatitis (Dutta et al., 2012). These findings indicate that Hsp70 serum levels are elevated in patients with inflammatory diseases but reach the highest levels in cancer patients. Consequently, as published by Multhoff and colleagues in 2015, it can be proposed that “serum Hsp70 levels will provide a useful biomarker and a minimally invasive approach for predicting the presence of tumors and monitoring the outcome of a therapy shortly” (Multhoff et al., 2015).

1.4.4. Therapeutic relevance

As mentioned before, Hsp70 is present only on the membrane of cancerous cells and not on healthy tissues. Exposure to stressors such as radiation and chemotherapy can elevate the expression of Hsp70. This makes membrane-bound Hsp70 an important biomarker and a potential target for cancer treatment as it creates more opportunities for further therapy. Studies have shown that high levels of HSPs can help tumor cells survive and resist radiation therapy. However, when human lung and breast cancer cells were treated with the heat shock response inhibitor NZ28, they showed a significantly better response to radiation therapy. NZ28 works by reducing Hsp70 levels, inhibiting the activation of its transcription factor HSF1, and making the cells more sensitive to radiation. Researchers believe that combining NZ28 with Hsp90 inhibitors and radiation therapy could be a promising new approach to treating cancer (Schilling et al., 2015). New therapies utilizing activated NK cells that can detect membrane-bound Hsp70 are on the horizon, including those for NSCLC patients with mHsp70-positive tumors. A recent phase II clinical trial showed promising results when autologous NK cells, stimulated with ex vivo TKD/IL-2 after radiochemotherapy (RCT), were administered to these patients. The intention-to-treat group showed increased

progression-free survival compared to those treated with standard RCT. This therapy shows potential for use in other cancer entities, as Hsp70 is expressed on the surface of various tumor cells (Multhoff et al., 2020).

1.5. Aim of the Dissertation

Numerous factors contribute to the onset, development, and treatment of cancer, and scientists worldwide are exploring diverse avenues of research in this field. As such, this thesis delves into the intricacies of a specific cancer type, its treatment, and the implications for both its tumor microenvironment (TME) and the overall physiology. The study investigates the kinetics of absolute lymphocyte counts, compositions of lymphocyte subpopulations, and potential prognostic factors such as the neutrophil-to-lymphocyte ratio and the potential biomarker Hsp70 to gain a comprehensive understanding of 16 bladder cancer patients and their immuno-oncology. Data is being gathered not only about the patient cohort as a whole, but also about individual patients, in order to demonstrate the significance of cancer research for both the broader population and individual patients, as various patient-specific factors can impact the onset, prognosis, and outcome of cancer.

2. Material

2.1. Chemicals and Reagents

Table 1: Chemicals and Reagents

Chemicals and Reagents
Ethanol
Na_2Co_3
$NaHCO_3$
$2NH_2SO_4$ Stop Solution

2.2. Solutions and Buffers

Table 2: Solutions and Buffers

Solutions and Buffers	Company
BioFX TMB Super Sensitive One Component HRP Microwell Substrate	Surmodics, Minnesota, USA
CELLCLEAN Auto	Sysmex Corporation, Kobe, Japan
CELLPACK DCL	Sysmex Corporation, Kobe, Japan
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma life science, Sigma-Aldrich, St. Louis, USA
FACS Lysing Solution	BD Biosciences, San Jose, USA
FACSClean	BD Biosciences, San Jose, USA
FACSFlow	BD Biosciences, San Jose, USA
FACSRinse	BD Biosciences, San Jose, USA

Material

Fetal calf serum (FCS)/Fetal Bovine Serum	Sigma life science, Sigma-Aldrich, St. Louis, USA
Fluorocell WDF	Sysmex Corporation, Kobe, Japan
FoxP3 Buffer Set A	BD Biosciences, San Jose, USA
FoxP3 Buffer Set B	BD Biosciences, San Jose, USA
HRP Protector	Candor Bioscience, Wangen, Germany
Liquid Plate Sealer	Candor Bioscience, Wangen, Germany
StabilZyme Select Stabilizer	Surmodics, Minnesota, USA
Tween	Calbiochem, Merck, Darmstadt, Germany

2.3. Proteins and Antibodies

Table 3: Proteins and Antibodies

Antibody	Conjugate	Clone	Volume	Company
CD16	PE	Mouse	10 µL	BD Biosciences, San Jose, USA
CD19	PE	Mouse	20 µL	BD Biosciences, San Jose, USA
CD25	APC	Mouse	5 µL	BD Biosciences, San Jose, USA
CD3	PerCP	Mouse	10 µL	BD Biosciences, San Jose, USA
CD335 (NKp46)	PE	Mouse	10 µL	Beckman Coulter, Brea, USA
CD337 (NKp30)	PE	Mouse	10 µL	Beckman Coulter, Brea, USA

Material

CD4	FITC	Mouse	20 µL	BD Biosciences, San Jose, USA
CD45	APC	Mouse	1 µL	Beckman Coulter, Brea, USA
CD56	FITC	Mouse	5 µL	BD Biosciences, San Jose, USA
CD56	PE	Mouse	5 µL	BD Biosciences, San Jose, USA
CD56	APC	Mouse	10 µL	BD Biosciences, San Jose, USA
CD69	APC	Mouse	5 µL	BD Biosciences, San Jose, USA
CD8	FITC	Mouse	20 µL	BD Biosciences, San Jose, USA
CD8	PE	Mouse	20 µL	BD Biosciences, San Jose, USA
CD94	FITC	Mouse	5 µL	BD Biosciences, San Jose, USA
cmHsp70.1	FITC	Mouse	200ng/mL	Multimmune GmbH, Munich, Germany
cmHsp70.2	FITC	Mouse	1 µg/mL	Multimmune GmbH, Munich, Germany
FoxP3	PE	Mouse	20 µL	BD Biosciences, San Jose, USA
IgG1	FITC	Mouse	5 µL	BD Biosciences, San Jose, USA
IgG1	PE	Mouse	5 µL	BD Biosciences, San Jose, USA
IgG1	PerCP	Mouse	5 µL	BD Biosciences, San Jose, USA

Material

IgG1	APC	Mouse	1 µL	Life Technologies Corporation, Frederick, USA
NKG2D	PE	Mouse	10 µL	R&D Systems, Minneapolis, USA
Streptavidin			1 µL	Senova GmbH, Weimar, Germany

2.4. Devices

Table 4: Devices

Devices	Company
Sysmex XN-350	Sysmex Corporation, Kobe, Japan
FACSCalibur	BD Biosciences, San Jose, USA
2030 Multilabel Reader VICTOR X4	PerkinElmer LAS, Rodgau, Germany
Heraeus FRESCO21 Centrifuge	Thermo Fisher Scientific, Waltham, USA
Heraeus MEGAFUGE 16R Centrifuge	Thermo Fisher Scientific, Waltham, USA
Heraeus LABOFUGE 400R Centrifuge	Thermo Fisher Scientific, Waltham, USA
Combi-Spin PCV-2400	Grant Instruments, Camebridgeshire, UK
Pioneer PX224	OHAUS Corporation, Parsippany, USA
pH 3110	Xylem Analytics, WTW Weilheim, Germany

2.5. Software

Table 5: Software

Software	Company	Version
Cell Quest Pro	BD, Franklin Lakes, USA	6.0
Endnote	Clarivite Analytics, Philadelphia, USA	9.3.3
Excel	Microsoft, Redmond, USA	16.54
GraphPad Prism	GraphPad Software, La Jolla, USA	8.2.1
PerkinElmer2030	PerkinElmer LAS, Rodgau, Germany	4.0.0
PowerPoint	Microsoft, Redmond, USA	16.54
Word	Microsoft, Redmond, USA	16.54

3. Methods

3.1. Study Collective and Standard operating procedure

This dissertation includes sixteen patients who participated in the RACE-IT (Radiation therapy before radical Cystectomy combined with Immunotherapy) trial and received treatment according to the specified protocol. All these patients were diagnosed with stage T3/4 N0/N1 M0 urothelial bladder carcinoma.
More detailed patient characteristics can be taken from the following Figure 5:

Characteristics		N	%
age (years)	<50	1	6.25
	50-59	3	18.75
	60-69	3	18.75
	70-79	9	56.25
gender			
	female	7	43.75
	male	9	56.25
pTNM			
	T3	11	68.75
	T4	5	31.25
	N0	10	62.50
	N1	4	25.00
	N2	2	12.50
	M0	15	93.75
	M1	1	6.25

Figure 5 Patient characteristics

Only patients who had not received prior chemotherapy or radiotherapy and were ineligible for cisplatin treatment were included in the RACE-IT trial. Blood samples of included bladder cancer patients were provided from the urology department of the Klinikum rechts der Isar, Technical University of Munich, at three different time points. Time point *t0* describes sample collection before radioimmunotherapy, *t1* after radioimmunotherapy, and *t2* 12 weeks after radical cystectomy. After blood samples were received, flow cytometry was used to measure peripheral blood lymphocyte subpopulations, an automated hematology analyzer to obtain a complete blood count,

and Hsp70 compELISA to measure Hsp70 plasma levels performed for each time point. A complete timeline of patient treatment and SOP is shown below in Figure 6.

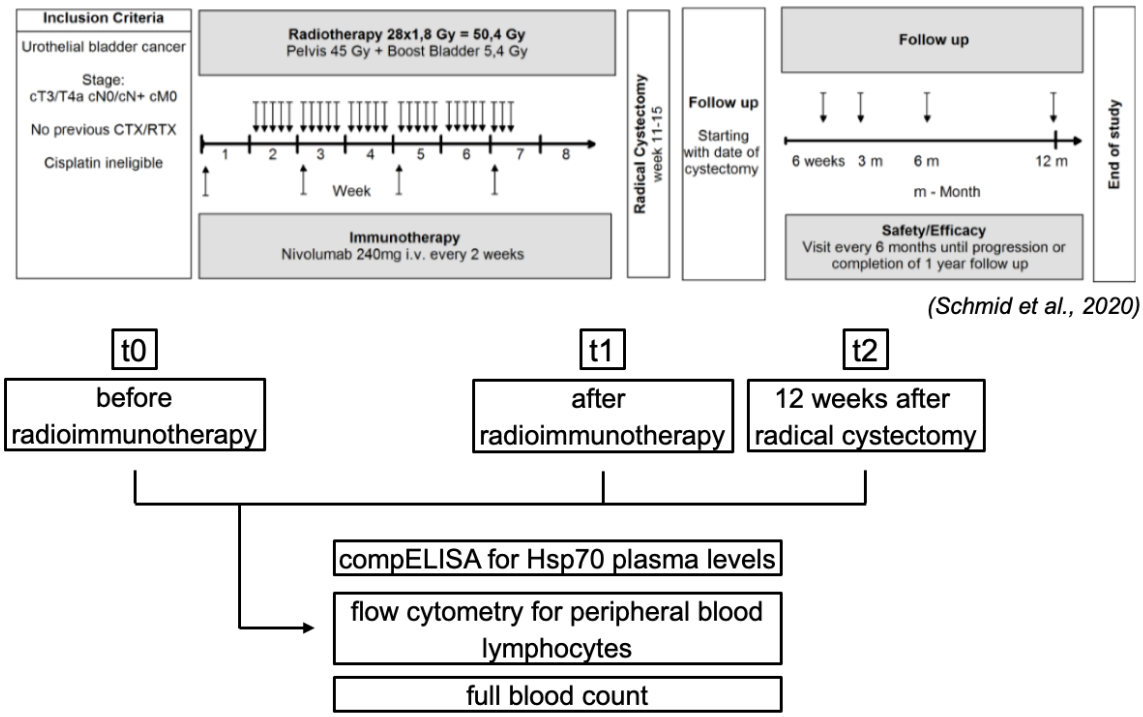

Figure 6 Timeline patient treatment and SOP

Healthy donors were recruited for the study, which was approved by the ethics committee of the medical faculty of TUM.

3.2. Flow cytometry

Flow cytometry or FACS (Fluorescence Activated Cell Sorting) analysis allows the counting and analysis of cells in a fluid stream. One of the main applications is to use fluorescent dye-labeled samples, e.g., antibodies or receptors, to describe and measure specific properties of cells or cell populations at the single-cell level. In flow cytometry, the cells to be examined flow one after the other through a narrow measuring chamber called a flow cell due to hydrodynamic focusing. A precise laser illuminates the cells labeled with fluorescence dyes. When the monochromatic laser beam energizes the electrons of the fluorescence dye, they are raised to a higher energy level. After the pulse of the laser, the electrons fall back to their original energy level while emitting photons. The number of photons emitted measured by a photodetector is proportional to the number of bound fluorescence-labeled antibodies

per cell. In addition, information such as cell size and internal structure of the cells can be determined by light diffraction and scattering, where the Forward Scatter (FSC) represents the cell size and the Side Scatter (SSC) the granularity of cells. Since the different fluorescence dyes are excited at a standard wavelength but have characteristic emission spectra for each dye, multiple dyes and, thus, multiple cell types and different cell properties can be studied in one FACS analysis. The FACS analyses aimed to separate the patient's peripheral blood lymphocytes (PBL) into their lymphocyte subpopulations and display these in percentages.

EDTA blood from bladder cancer patients was used for the FACS analyses performed. Twenty antibodies were added to 14 tubes, each filled with 100 µL blood, and incubated for 15 minutes at room temperature. After a wash step with FACS Buffer (1:10 Dilution of fetal calf serum (FCS) in Dulbecco's Phosphate Buffered Saline (PBS)), erythrocytes were lysed by adding FACS Lysing Solution and incubating for 15 minutes at room temperature. After another washing step, the staining process for T, B, and NK cells was completed. The marker FoxP3 used for staining Treg cells is a transcriptional repression factor of the forkhead or winged-helix family of transcription factors (Fontenot et al., 2005). Therefore, cell lysis is required for staining with the FoxP3-PE antibody. Thus, FoxP3 buffer sets A and B were added to the remaining tubes to release the intracellular and intranuclear proteins. Subsequently, after two more washing steps, the fluorescently labeled antibodies for the isotype IgG1-PE and FoxP3-PE were added and incubated for 30 minutes at room temperature to identify the FoxP3 positive regulatory T cells. After two final washing steps, the stained cells were ready for analysis with FACSCalibur.

3.2.1. Analysis

The CellQuestPro software from BD was used to analyze the generated data. The measured data was plotted in dot plots, representing each measured event as a dot. One antibody signal each is represented by X and Y axis. Thus, it can be read in the coordinate system and calculated which relative cell numbers are positive for one, both, and none of the antibodies. As described above, a further staining step was added to represent the regulatory T cells, so a combination of three antibodies results here. To define which cells are classified as antibody positive and negative, the four isotypes IgG1-FITC, IgG1-PE, IgG1-PerCP, and IgG1-APC were stained, and gates

for the following evaluation were defined according to them. An evaluation example is shown the following Figure 7.

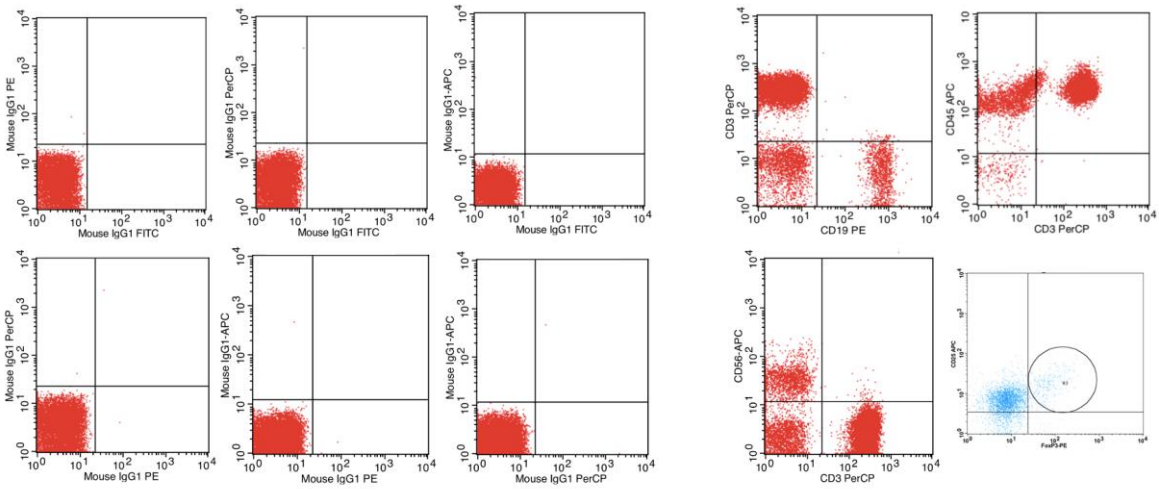

Figure 7 Flow cytometry analysis

3.3. Automated hematology analyzer

With the automated hematology analyzer of Sysmex, full blood counts of each patient were created. EDTA blood samples were aspirated with an aspiration volume of 25 µL and put into different analyzation channels in the device. White blood cells (WBC) were detected using fluorescence flow cytometry, and absolute counts of their subpopulations were measured. Due to this technology, absolute lymphocyte numbers and neutrophil-lymphocyte ratio could be calculated.

3.4. ELISA

3.4.1. Aliquots

Plasma was produced by centrifugation of EDTA blood at 1500*g* for 15 minutes at 4 º Celsius. For the preparation of serum aliquots, blood was centrifuged for 10 minutes with 800*g* at room temperature after clotting for 30 minutes in a serum separator tube that contained a clotting activator. Serum and plasma were aliquoted to 180 µL parts and stored in the - 80 º Celsius freezer. Due to better availability and more consistent storage at -80 º Celsius, plasma, rather than serum, was used for the ELISA in this study.

3.4.2. Operating principle ELISA

In an Enzyme-linked immunosorbent assay (ELISA), various proteins such as antibodies, hormones, viruses, or Hsp70 can be detected and quantified on a plate. The ELISA used for this series of experiments functions according to the principle of sandwich ELISA. Here, two antibodies bind to two different, non-overlapping epitopes of the protein to be analyzed and are thus bound from both sides as in a sandwich. To measure Hsp70 plasma levels of bladder cancer patients, 96-well plates were coated overnight with 1 µg/mL cmHsp70.2 in a sodium-carbonate buffer. The next day, the plate was washed with PBS and Tween-20 at a concentration of 0.05%. Afterwards, the formation of nonspecific bindings was blocked with liquid plate sealer for 30 minutes. After another wash step, the plasma samples diluted in StabilZyme Select Stabilizer were applied to the 96-well plates and incubated for 30 minutes at room temperature. An eight-point concentration standard curve of Hsp70 was inserted into each assay with concentrations of 100ng/mL, 50ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25ng/mL, 3.125ng/mL, 1.5625ng/mL, and 0 ng/mL. After another wash, the wells were incubated with 200 ng/mL biotinylated cmHsp70.1 in HRP-Protector for another 30 minutes at room temperature. After a final wash, 57 ng/mL HRP-conjugated streptavidin dissolved in HRP-Protector was applied to the wells at room temperature for 30 minutes. Colorimetric analysis was performed with the addition of substrate reagent (BioFX TMB Super Sensitive One Component HRP Microwell Substrate) at room temperature. After 15 minutes, the color change was stopped by applying 2NH2SO4. The absorbance was measured at a wavelength of 450 nm with a correction from the absorbance at a wavelength of 560 nm in an Elmer microplate reader. The performed and described Elisa was developed and published in the working group of Prof. Multhoff (Werner et al., 2021). To better identify possible errors in the experimental process, two adjacent wells were incubated with plasma from one aliquot as duplicates for each patient sample measured. The standard curve was also plotted in pairs of two per concentration. To generate controls between each 96-well plate, at least two equal plasma samples from 2 healthy donors were measured on each plate. An example of the plate distribution is shown below in Figure 8, where the numbering 1 to 38 exemplifies different patient samples in measured ELISA.

	Samples				HSP70 Standard curve		Samples					
	1	2	3	4	5	6	7	8	9	10	11	12
A	1		9		c =100 ng/mL		17		25		33	
B	2		10		c = 50 ng/mL		18		26		34	
C	3		11		c = 25 ng/mL		19		27		35	
D	4		12		c = 12.5 ng/mL		20		28		36	
E	5		13		c = 6.625 ng/mL		21		29		37	
F	6		14		c = 3.125 ng/mL		22		30		38	
G	7		15		c = 1.5625 ng/mL		23		31		healthy control 1	
H	8		16		c = 0 ng/mL		24		32		healthy control 2	

Figure 8 ELISA Pipetting scheme

3.4.3. compHsp70 sandwich ELISA

In all described experiments, the compHsp70 Sandwich Elisa developed in the group of Prof. Multhoff was used. The central feature of this ELISA is that, unlike commercially available ELISA KITS, it can detect both free and vesicular Hsp70 (Werner et al., 2021). Usually, vesicular Hsp70 is prevented from being detected by the ELISA due to interactions with lipids such as PS, Gb3, or sphingolipids (Smulders et al., 2020). The newly developed compHsp70 ELISA detects free and vesicular Hsp70 using two antibodies (cmHsp70.1 and cmHsp70.2) that recognize its conserved epitopes in the C-terminal substrate binding domain. (Multhoff et al., 2011; Stangl et al., 2011). Since the compHsp70 ELISA detects both forms of Hsp70, it is impossible to distinguish precisely which part comes from the living tumor mass and which part from dead cells. However, the majority is exosomal Hsp70, so although elevated Hsp70 levels are also measured in the blood in the case of inflammation, even higher levels can be measured in patients with tumor diseases (Gehrmann et al., 2014; Werner et al., 2021)

3.4.4. Analysis

Excel was used for the evaluation of the measured data. With the help of measured values of the eight-point concentration standard curve of Hsp70, a function could be created with which the mean values of the determined wavelength minus blank could be converted into concentrations of Hsp70.

3.5. Statistical analysis

For flow cytometry and automated hematology analyzer, each sample was measured once as fresh primary material was required. For ELISA, each sample was measured four times in at least two independent experiments, and an average of these four values was calculated for the following evaluations. Differences between two data sets were calculated using the unpaired t-test, and differences between two paired data sets were calculated using the paired t-test when data were normally distributed. The Mann-Whitney-U rank sum test was performed when a Gaussian distribution could not be proven with the Normality test. When more than two data sets were compared and not normally distributed, the Kruskal-Wallis test was applied. Differences in *p-values < 0,05* were considered statistically significant, as shown in Table 6 below. A ROC curve (receiver operating characteristics) defined a threshold value for Hsp70. The software used for statistical analysis was GraphPad Prism 8.2.1.

Table 6 Significance levels

Significance level	Presentation
$p \leq 0.05$	* *significant*
$p \leq 0.01$	** *very significant*
$p \leq 0.001$	*** *highly significant*
$p \leq 0.0001$	**** *very highly significant*

4. Results

As mentioned earlier, the thesis encompasses the inclusion and evaluation of results from 16 patients. However, a total of 18 patients were assessed, with two being excluded due to non-compliance with therapy or withdrawal from the study, as depicted in Figure 9. The upcoming presentation of results will address the subdivision into Hsp70 positive and negative patients.

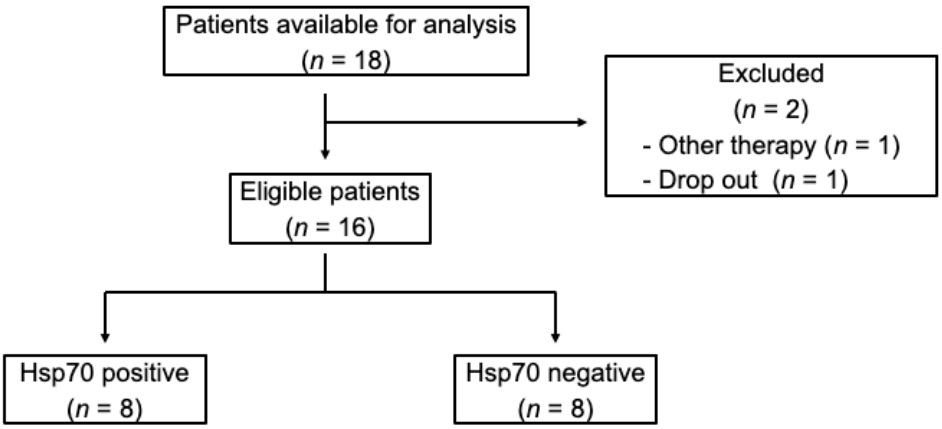

Figure 9 Patients included

4.1. Absolute lymphocyte counts

Using the automated hematology analyzer, absolute numbers of lymphocytes were measured. Since this measurement was performed with a new method and was established only during the ongoing patient recruitment, the absolute lymphocyte values could only be determined for up to 10 patients, offering insight into only a portion of the patient population. The Sysmex-XE 5000 device's company specifies a reference value for absolute lymphocyte counts of 1.26-3.35 G/l for healthy individuals. Bladder cancer patients under examination exhibited an average lymphocyte count of 1.29 G/l at the time of diagnosis (t0), indicating that they were still within the lower normal range. After radioimmunotherapy (t1), however, as shown in Figure 10, a significant decrease in the absolute lymphocyte count to an average value of 0.61 G/l could be observed (t0 vs. t1: 1.29 ± 0.70 G/l vs. 0.61 ± 0.32 G/l, *$p \leq 0.05$), which increased again to 0.71 G/l 12 weeks after radial cystectomy (t2) but was still

significantly below the initial value at t0 (t0 vs. t2: 1.29 ± 0.70 G/l vs. 0.71 ± 0.31 G/l, *p ≤ 0.05).

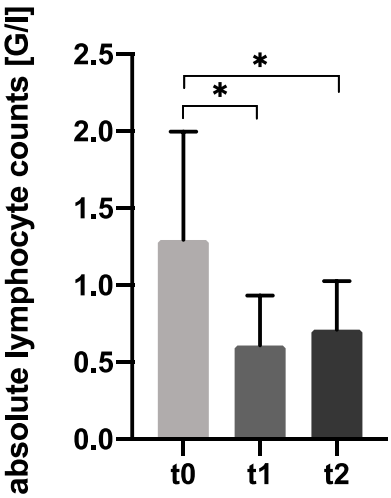

Figure 10 Absolute number of lymphocytes in G/l of patients

4.2. Composition of lymphocyte subpopulations

The following section presents the mean values of the percentages of lymphocyte subpopulations of all included patients. For comparison, a healthy collective with a similar average age was included. Sixteen healthy individuals, 15 bladder cancer patients at time points t0 and t1, and 13 bladder cancer patients at time point t2 were examined.

4.2.1. $CD19^{+}/CD3^{-}$ B cells

Figure 11 shows a significant decrease in the percentage of $CD19^{+/}CD3^{-}$ B lymphocytes of bladder cancer patients compared to a healthy collective. Before the start of therapy (t0), the percentage of the B cell subpopulation was 7.48 %, 4.24 percentage points lower than the 11.72 % in healthy patients (healthy vs. t0: 11.72 ± 2.87 % vs. 7.48 ± 3.81 %, **p ≤ 0.01). Following radioimmunotherapy, the B cell population initially dropped to 2.90 % (healthy vs. t1: 11.72 ± 2.87 % vs. 2.90 ± 2.29 %, ****p ≤ 0.0001). Subsequently, 12 weeks after radical cystectomy, the B cell population remained low at 4.44 % (healthy vs. t2: 11.72 ± 2.87 % vs. 4.44 ± 5.02 %, ****p ≤ 0.0001). Overall, a significant decrease in the percentage of B lymphocytes in bladder cancer patients compared to healthy patients could be shown, with the most

significant difference after radioimmunotherapy (t1). Comparing the development of the B cell percentages within the course of therapy of the patients, a decrease from 7.48 % to 2.90 % of B cells could be observed between the start of and the completion of radioimmunotherapy (t0 vs. t1: 11.72 ± 2.87 % vs. 2.90 ± 2.29 %, **p ≤ 0.01). Twelve weeks after radical cystectomy, the percentages of B cells increased slightly and not significantly to 4.44 % but remained below the pre-therapy level (t1 vs. t2: 2.90 ± 2.29 % vs. 4.44 ± 5.02 %, t0 vs. t2: 11.72 ± 2.87 % vs. 4.44 ± 5.02 %).

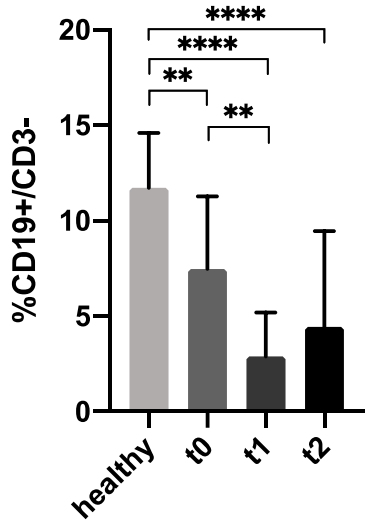

Figure 11 CD19⁺/CD3⁻ B cells, healthy vs. patients

4.2.2. CD3⁺/CD45⁺ T cells

As shown in Figure 12, healthy patients had an average T cell percentage of 67.00 %, whereas bladder cancer patients before the start of therapy (t0) showed a percentage of 78.34 % (healthy vs. t0: 67.00 ± 6.00 % vs. 78.34 ± 6.19 %, ****p ≤ 0.0001). After radioimmunotherapy and radical cystectomy, the values remained almost the same at 75.39 % (t1) and 75.40 % (t2) (healthy vs. t1: 67.00 ± 6.00 % vs. 75.39 ± 11.93 %, **p ≤ 0.01, healthy vs. t2: 67.00 ± 6.00 % vs. 75.40 ± 7.52 %, **p ≤ 0.01). Looking at the entire course of therapy from t0 to t2, it can be said that no relevant differences could be observed between the individual time points (t0 vs. t1 vs. t2: 78.34 ± 6.19 % vs. 75.39 ± 11.93 % vs. 75.40 ± 7.52 %). Thus, it can be stated that the percentage of CD3+/CD45+ T cells was higher in bladder cancer patients than in the healthy collective and that this increase was consistent throughout the therapy process.

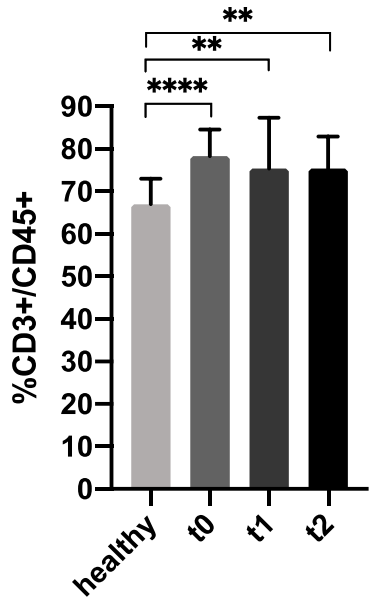

Figure 12 CD3⁺/CD45⁺ T cells, healthy vs. patients

4.2.3. CD3⁺/CD4⁺ T cells

Figure 13 shows the development of CD3⁺/CD4⁺ during therapy of bladder cancer patients compared to healthy persons. Compared with the healthy control group, a trend of decreased percentage of CD3⁺/CD4⁺ T cells could be measured, which was significant only after radioimmunotherapy (t1) and 12 weeks after radical cystectomy (t2) (healthy vs. t1: 46.96 ± 47.27 % vs. 38.58 ± 16.36 %, *p ≤ 0.05; healthy vs. t2: 46.96 ± 47.27 % vs. 37.08 ± 13.58 %, **p ≤ 0.01). This downward trend was also evident during therapy as a statistically significant drop in the CD3⁺/CD4⁺ proportion of T cells could be demonstrated. The mean value dropped from 47.27 % to 38.58 % to 37.08 % (t0 vs. t1 vs. t2: 47.27 ± 13.65 % vs. 38.58 ± 16.36 % vs. 37.08 ± 13.58 %, *p ≤ 0.05). Despite the negative trend, no statistically significant difference could be demonstrated between t1 and t2 since the percentage level remained almost at the same low level.

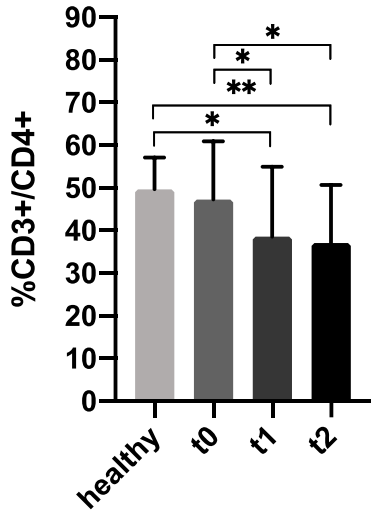

Figure 13 CD3⁺/CD4⁺ T cells patients

4.2.4. CD3⁺/CD8⁺ T cells

When examining the CD3⁺/CD8⁺ T cells in Figure 14, a contrasting trend to the progression of the CD3⁺/CD4⁺ T cells depicted in Figure 13 becomes apparent. A trend towards an increasing percentage of CD3⁺/CD8⁺ T cells with the course of therapy could be observed, although this was not statistically significant. Despite high standard deviations, the percentage from t0 via t1 to t2 increased from 20.58% via 25.02% to 28.33% (t0 vs. t1 vs. t2: 20.58 ± 7.99 % vs. 25.02 ± 17.26 % vs. 28.33 ± 17.11 %). Comparing the increased trend with a group of healthy individuals, a significantly high percentage of CD3⁺/CD8⁺ T cells of patients with bladder cancer was found after radical cystectomy (healthy vs. t2: 18.19 ± 5.20 % vs. 28.33 ± 17.11 %, *$p \leq 0.05$). This finding further reinforces the rising trend of CD3⁺/CD8⁺ T cell percentages with progressing therapy.

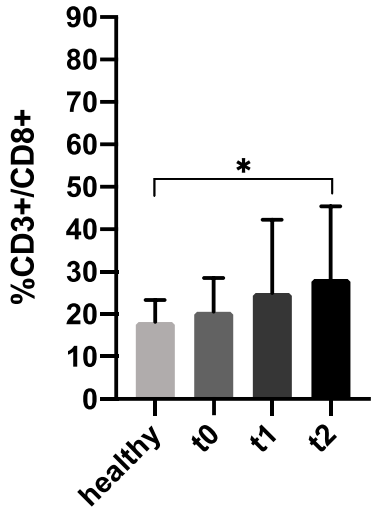

Figure 14 CD3⁺/CD8⁺ T cells patients

4.2.5. CD4⁺/FoxP3⁺/CD25⁺ regulatory T cells

Observing Figure 15, it becomes evident that the percentages of regulatory T cells in the overall lymphocyte population were lower in healthy individuals compared to the examined bladder cancer patients. The average percentage of Treg cells was 3.28 % in healthy individuals and 7.77 % in bladder cancer patients before radioimmunotherapy (healthy vs. t0: 3.28 ± 1.06 % vs. 7.77 ± 2.80 %, ****p ≤ 0.0001). Also, after radioimmunotherapy, the percentage remained significantly higher than in healthy individuals (healthy vs. t1: 3.28 ± 1.06 % vs. 9.21 ± 3.72 %, ****p ≤ 0.0001), as well as 12 weeks after radical cystectomy (healthy vs. t2: 3.28 ± 1.06 % vs. 8.75 ± 4.35 %, ****p ≤ 0.0001). Comparing the individual time points during therapy, it becomes apparent that the proportions of regulatory T cells remained consistently high between the start of radioimmunotherapy, after radioimmunotherapy, and until 12 weeks after radical cystectomy (t0 vs. t1 vs. t2: 7.77 ± 2.80 % vs. 9.21 ± 3.72 % vs. 8.75 ± 4.35 %).

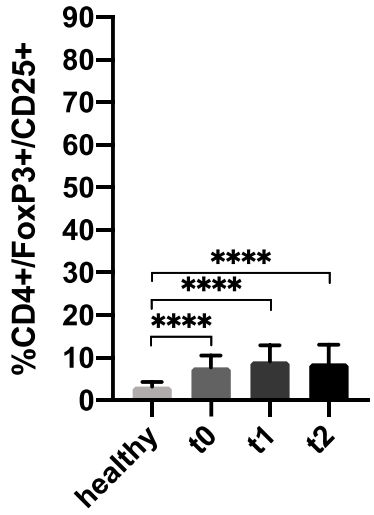

Figure 15 $CD4^+/FoxP3^+/CD25^+$ Treg cells, healthy vs. patients

4.2.6. $CD56^+/CD3^-$ Natural Killer cells

Over the recorded period, no significant difference in the percentage of $CD56^+/CD3^-$ NK cells between patients with bladder cancer and healthy individuals could be shown. The healthy patients shown in Figure 16 reached a percentage of $CD56^+/CD3^-$ NK cells in all lymphocytes of 10.97 %. Thus, in patients before and after radioimmunotherapy, an NK cell percentage of 10.18 % and 10.34 % was measured, and a percentage of 12.04 % was measured after radical cystectomy (healthy vs. t0 vs. t1 vs. t2: 10.97 ± 4.00 % vs. 10.18 ± 6.70 % vs. 10.34 ± 6.72 % vs. 12.04 ± 6.78 %). It could be observed that before and after radioimmunotherapy (t0 vs. t1), almost the same mean and standard deviation values were calculated.

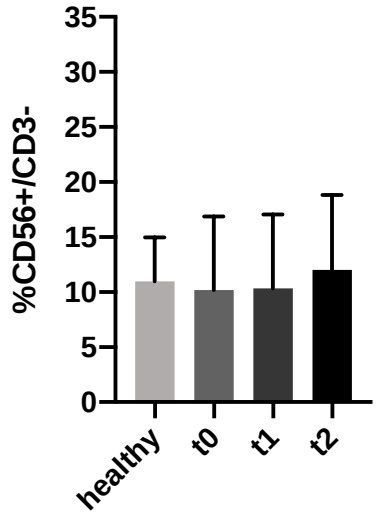

Figure 16 $CD56^+/CD3^-$ NK cells, healthy vs. patients

4.2.7. Activation marker-stained Natural Killer cells

As shown in Figure 17, in healthy individuals, an average of 10.19 % of all peripheral blood lymphocytes were NKG2D^{+}/CD3^{-} NK cells. Compared to patients with bladder cancer who have not received any therapy so far, a decreasing but not significant trend of percentage of NKG2D^{+}/CD3^{-} NK cells could be shown. Under progressive therapy, a further decrease in NKG2D^{+}/CD3^{-} NK cells was measured and became a significant difference after radical cystectomy (healthy vs. t2: 10.19 ± 3.75 % vs. 6.81 ± 2.99 %, *p ≤ 0.05).

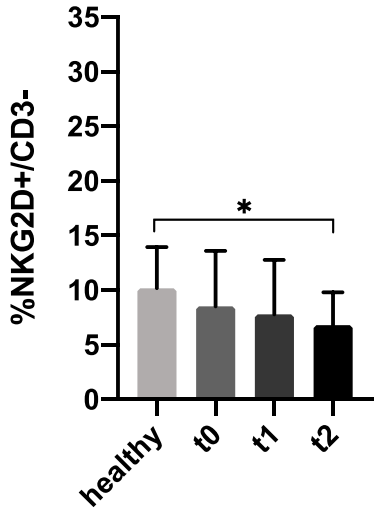

Figure 17 NKG2D^{+}/CD3^{-} NK cells, healthy vs. patients

While the staining with the activation marker NKG2D showed a decreasing trend with a significant drop at t2, the NK cells expressing NKp30, NKp46, and CD94 on their surface showed neither significant differences between healthy and diseased persons nor an apparent kinetic change during therapy. It should be noted that no drop in NK cell percentage could be found in all three cases examined; see Figure 18, Figure 19 and Figure 20.

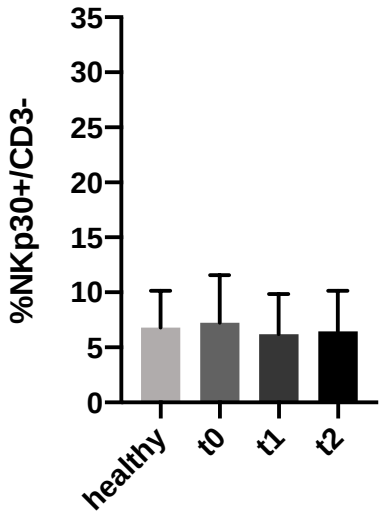

Figure 18 NKp30⁺/CD3⁻ NK cells, healthy vs. patients

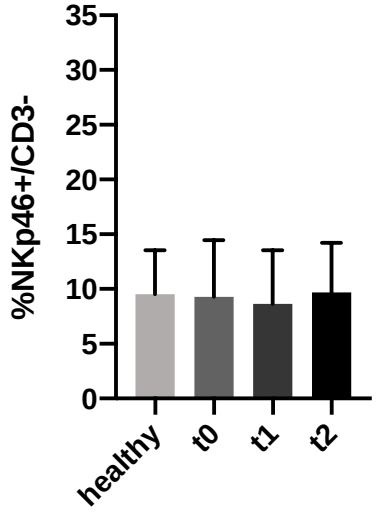

Figure 19 NKp46⁺/CD3⁻ NK cells, healthy vs. patients

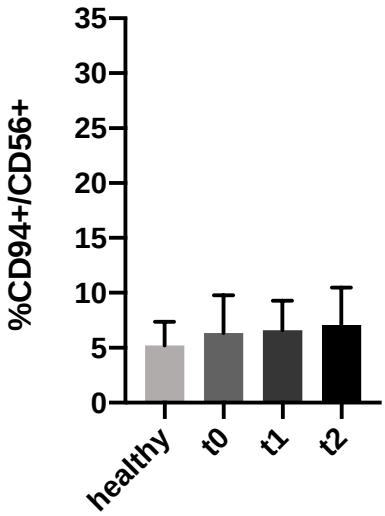

Figure 20 CD94⁺/CD56⁺ NK cells, healthy vs. patients

4.3. Neutrophil-Lymphocyte Ratio

The automated hematology analyzer measured the absolute values for neutrophils and lymphocytes. When the quotient of the absolute number of lymphocytes and the absolute number of neutrophils is determined, the neutrophil-lymphocyte ratio (NLR) is calculated. To differentiate between a high and low NLR, a 2018 meta-analysis was consulted. A limitation of the studies on NLR is that cut-offs for NLR are not defined equally high in each study. The meta-analysis by Hu et al. in 2019 included a total of 11945 patients with primary bladder cancer undergoing radical cystectomy from 18 published studies.

Figure 21 summarizes the cut-offs for high NLR, including only the studies with patients undergoing radical cystectomy (RC) without neoadjuvant chemotherapy (n=8). A cut-off of 2.1 (Kang et al., 2016; Zhang et al., 2015) and 2.5 (Gondo et al., 2012; Ozcan et al., 2015) was used twice each, and four times 2.7 (D'Andrea et al., 2017; Lucca et al., 2016; Tan et al., 2017; Viers et al., 2014). In the patient population studied in this dissertation, some NLR data were collected at all three stages of therapy. Due to logistical problems, however, taking measurements from every patient at every time was impossible, so the database is more limited here. Ten patients were measurable at time t0 and t1, whereas, at time t2, only eight could be measured. Although different patients were measured at different time points, the total data consists of more than ten patients.

As shown in

Figure 21, an average NLR of 4.47 was measured at the time before radioimmunotherapy (t0). A significant increase after radioimmunotherapy (t1) by 2.72 percentage points to 7.19 was presented (t0 vs. t1: 4.47 $\pm$ 3.84 % vs. 7.19 $\pm$ 2.88 %, *$p \leq 0.05$). At 12 weeks after radical cystectomy, the mean NLR of 7.41 was determined with an increasing standard deviation of 5.87.

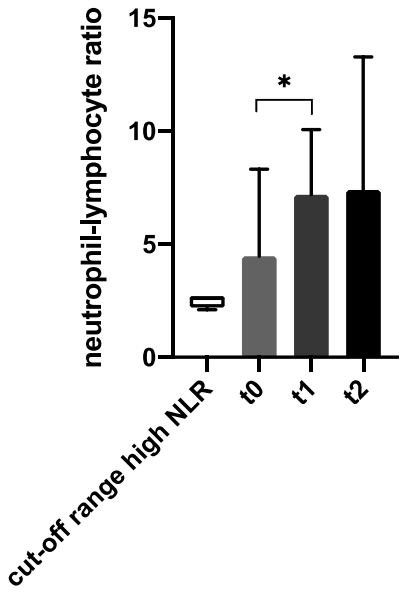

Figure 21 Neutrophil-Lymphocyte Ratio (Hu et al., 2019)

4.4. Hsp70 plasma levels

The measured values for Hsp70 plasma levels were first summarized to mean values for time points t0, t1, and t2 and compared with the Hsp70 plasma values of a healthy collective consisting of 108 patients. For healthy subjects, a mean Hsp70 value of 35.06 ng/mL with a standard deviation of 41.62 ng/mL could be observed, with the lowest measured value being 0.099 ng/mL and the highest measured value for Hsp70 being 158.2 ng/mL. For the measured patient values, 16 patients could be included at time t0, 12 at time t1, and 11 at time t2. As shown in Figure 22, a significant increase in Hsp70 plasma content could be found after therapy inclusion but before radioimmunotherapy compared to healthy individuals. This Hsp70 mean value between healthy individuals and patients with bladder cancer was increased 13.7-fold (healthy vs. t0: 35.06 ± 41.62 ng/mL vs. 480.9 ± 563.2 ng/mL, *$p \leq 0.05$). At the time point after radioimmunotherapy, a mean Hsp70 value of 401.7 ng/mL and 12 weeks after radical cystectomy of 514.4 ng/mL could be determined. No other significant trends could be detected compared with the healthy collective and among the individual time points (t0 vs. t1 vs. t2: 480.9 ± 563.2 ng/mL vs. 401.7 ± 495.1 ng/mL vs. 514.4 ± 606.9 ng/mL). From the perspective of Hsp70 means, trends exhibited complexity due to individual patients moving in different directions. This complexity contributes to the relatively high standard deviations, a topic that will be further explored in the following chapter.

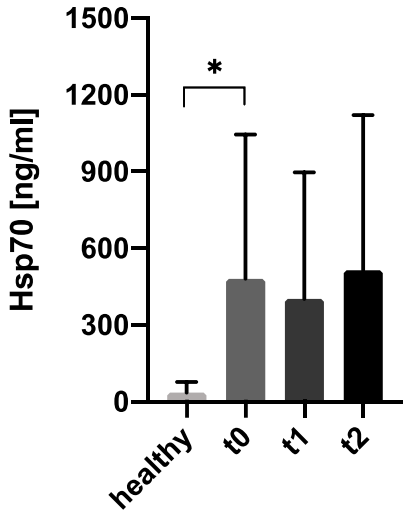

Figure 22 Hsp70 plasma levels of all patients

A cut-off of 121 ng/mL was established to differentiate between low and high plasma Hsp70 levels in bladder cancer patients, marking the transition from Hsp70 negative to Hsp70 positive patients. This Threshold was determined using a ROC curve (healthy vs. t0), see Figure 23, and has a specificity of 94.4% and a sensitivity of 50%. The area under the curve (AUC) (CI 95 %) is 0.68, and the p-value is 0.021 (*p ≤ 0.05).

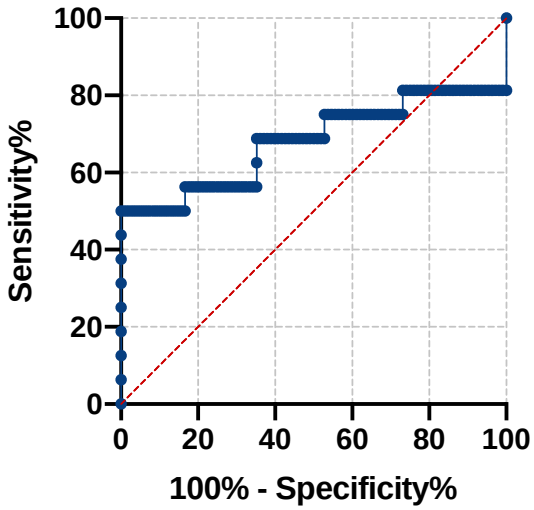

Figure 23 ROC Curve using Hsp70 values of healthy vs. t0 shown in Figure 22

Using this cut-off for Hsp70, an Hsp70 positive and an Hsp70 negative group could be formed. Individual patients were permanently assigned to one of the two groups for all

therapy time points. Eight patients could be assigned to each of the two groups. Thus, 50% each could be classified as Hsp70 positive and 50% as Hsp70 negative. Figure 24 plots the mean values for Hsp70 plasma levels for each time point in the group comparing Hsp70 positive vs. Hsp70 negative patients. With an average Hsp70 value of 940.5 ng/mL, the Hsp70-positive patients had the highest plasma Hsp70 within the group before the start of radioimmunotherapy (t0). After radioimmunotherapy (t1), an average Hsp70 value of 674.4 ng/mL and 12 weeks after radical cystectomy (t2), an Hsp70 of 790.0 ng/mL could be detected in plasma. Comparing the Hsp70 positive and Hsp70 negative groups at a time point, a significant difference in Hsp70 plasma content was noticeable. The Hsp70 value of the positive group at t0 was 43.9 times higher than that of the Hsp70 negative group (t0Hsp70+ vs. t0Hsp70-: 940.50 ± 442.67 ng/mL vs. 21.40 ± 311.4 ng/mL, ***p ≤ 0.001). After radioimmunotherapy at time t1, a 40.4-fold increase was shown, and 12 weeks after radical cystectomy (t2), a 30.9-fold increase could be observed (t1Hsp70+ vs. t1Hsp70-: 674.37 ± 489.91 ng/mL vs. 16.65 ± 38.48 ng/mL, *p ≤ 0.05, t2Hsp70+ vs. t2Hsp70-: 790.03 ± 607.94 ng/mL vs. 25.54 ± 29.59 ng/mL, *p ≤ 0.05).

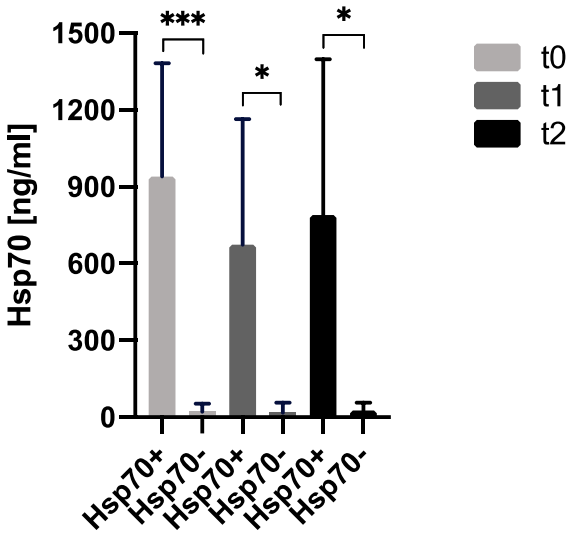

Figure 24 Hsp70 plasma levels of Hsp70 positive vs. Hsp70 negative patients (Threshold Hsp70 = 121 ng/mL)

4.4.1. Hsp70 negative patients

All patients with Hsp70 levels less than 121 ng/mL in all three measurements were assigned to the Hsp70 negative group. The highest measured value for Hsp70 in an

Hsp70 negative patient was 95.1 ng/mL, the lowest values for Hsp70 were not measurable and were thus assigned a value of 0 ng/mL. As shown in Figure 24, the mean values for Hsp70 in the Hsp70 negative group were 21.40 ng/mL before radioimmunotherapy, 16.65 ng/mL after radioimmunotherapy, and 25.54 ng/mL 12 weeks after radical cystectomy (t0Hsp70- vs. t1Hsp70- vs. t2Hsp70-: 21.40 ± 31.14 ng/mL vs. 16.65 ± 38.48 ng/mL vs. 25.54 ± 29.59 ng/mL).

4.4.2. Hsp70 positive patients

Examining the progression of Hsp70 plasma levels in Hsp70 positive patients revealed a decrease in all Hsp70 values from the beginning to the conclusion of radioimmunotherapy (t0 and t1). However, the development of these values differed between the end of radioimmunotherapy and the 12-week follow-up after radical cystectomy (t1 and t2). In Figure 25, the upper row illustrates patients whose Hsp70 levels decreased entirely. Conversely, the lower row depicts patients whose Hsp70 levels decreased between t0 and t1 but then increased again between t1 and t2. Regrettably, complete data for patients 2 and 13 was not available. However, based on their declining Hsp70 trends, they were categorized in the upper row.

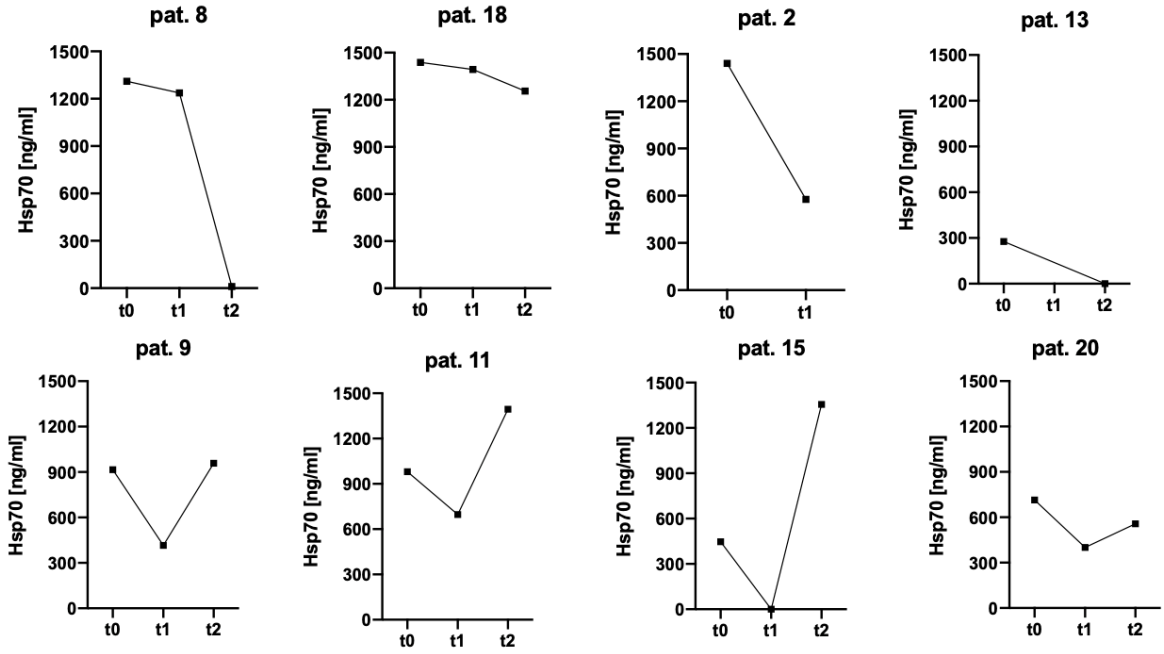

Figure 25 Hsp70 plasma level progression of all Hsp70 positive patients

To show the decrease of the Hsp70 plasma level again for all Hsp70 positive patients, the bar chart in Figure 26 was created. It shows a statistically significant decrease in Hsp70 between time points t0 and t1. As described in Chapter 4.4 and Figure 22, Hsp70 levels dropped from a mean of 940.5 ng/mL before radioimmunotherapy to a mean of 674.4 ng/mL after radioimmunotherapy (t0Hsp70+ vs. t1Hsp70+: 940.50 ± 442.67 ng/mL vs. 674.37 ± 489.91 ng/mL, *p ≤ 0.05). During the progression from t1 to t2, no significant trend could be identified, as the individual Hsp70 values of the patients exhibited diverse developments, as depicted in Figure 24 (t1Hsp70+ vs. t2Hsp70+: 940,50 ± 442,67 ng/mL vs. 790,03 ± 607,94 ng/mL).

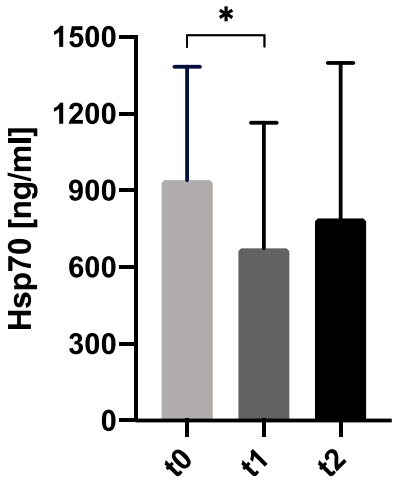

Figure 26 Hsp70 mean values of all Hsp70 positive patients

5. Discussion

5.1. Immunophenotyping – Composition and kinetics of plasma lymphocyte levels in tumor patients

In the context of immuno-oncology, immunophenotyping involves analyzing the percentage of different lymphocyte subpopulations in all lymphocytes and their development before, during, and after cancer. This analysis allows for insights into the interaction of the immune system and cancer, as well as the characteristics of the tumor itself and the prognosis of the disease.

The introduction of BCG as an immunological therapy option for bladder cancer in 1970 brought the term "immunosurveillance" into greater prominence, signifying the potential for immunological elimination of pathologically altered cells. This period also witnessed the gathering of significant new findings in urothelial carcinoma and immuno-oncology research. Since immune checkpoint inhibitors have recently started playing an increasing role in urothelial carcinoma, interest in bladder cancer immunology has grown strongly (Joseph et al., 2019).

To improve the knowledge of the immune system and its composition in bladder cancer patients, absolute lymphocyte counts, different lymphocyte subpopulations, and their behavior during disease and therapy were analyzed using an automated hematology analyzer and flow cytometry. Whereas the automated hematology analyzer can measure absolute values for lymphocytes, only compositions of lymphocyte subpopulation can be determined in flow cytometry. Thus, in the following discussion, only percentages and no absolute counts are described for single lymphocyte types.

All the cell types discussed below belong to the lymphocyte family, and their number and composition play an important role in tumorigenesis, progression, and healing. Since sufficient numbers and function of lymphocytes are essential for anti-tumor immunity, it seems reasonable that an association of absolute lymphocyte counts with outcome and prognosis could be found in different tumor entities, like breast cancer, ovarian cancer, lung cancer, and bladder cancer (Afghahi et al., 2018; Joseph et al., 2016; Karantanos et al., 2019; Milne et al., 2012). In patients with metastatic breast carcinoma, a decrease in absolute lymphocyte counts was associated with disease progression (Jimbo et al., 2022). This decrease was also observed in our patient population, which showed a drop in lymphocyte counts from an initial 1.29 G/l to 0.61

G/l and 0.71 G/l during the disease. However, at this time, no predictions can be made about any association with a worsened prognosis. It must also be considered that the patients were already undergoing therapy, and no differentiation can be made between disease progression and therapy effect.

Furthermore, a significantly lower number of lymphocytes was shown in patients with glioblastoma (Lobinger et al., 2021), especially in those who relapsed. In NSCLC patients treated with Nivolumab, absolute lymphocyte counts predicted overall survival (Karantanos et al., 2019). In patients with MIBC and advanced bladder cancer, low counts of absolute lymphocytes before treatment showed to be an adverse prognostic factor (Joseph et al., 2016). Since a significant decrease in the number of lymphocytes was found after radiotherapy in our study, it can be assumed that radioimmunotherapy downsizes the lymphocyte population and thus hurts anti-tumor immunity. The increasing trend after tumor removal is not significant but could be explained by the fact that the TME releases fewer immunosuppressive cytokines after tumor removal. This association was interpreted as possible tumor-induced immunosuppression that may lead to tumor progression (Joseph et al., 2016). These findings may underline the importance of lymphocytes and a functional immune system in the development and progression of tumors. To gather more information in this area, the composition of the lymphocyte subpopulations was examined in more detail.

As already described, B cells are the second most common cell in the TME. They are therefore exposed to a large dose of ionizing radiation during tumor irradiation which, in the best case, leads to DNA double-strand breaks in the tumor but also causes damage to healthy cells. The study performed here showed that circulating $CD19^+$ B cells of the examined bladder cancer patients were significantly lower than circulating B cells of the healthy collective during all three therapy time points. Moreover, a notable reduction was observed between pre-radioimmunotherapy and post-radioimmunotherapy, and a minor, non-significant increase was observed between post-radioimmunotherapy and 12 weeks after radical cystectomy, albeit remaining below the baseline value prior to treatment initiation. Similar observations were made in a breast cancer study in 2015, which showed severe depletion of B cells after radiation and recovery six weeks after the end of radiation (Sage et al., 2016). Also, in prostate patients, a B cell decrease directly after radiotherapy (RT), and a recovery one year after RT could be observed (Sage et al., 2017). A study performed by Belka

et al. also showed that of all lymphocytes, B cells are the most sensitive to local RT (Belka et al., 1999).

It should be noted, however, that in our study, bladder cancer patients received radioimmunotherapy and not RT; thus, the results are not precisely comparable. Compared to the healthy patient population, this study found a reduced number of circulating B cells even before the start of therapy in bladder cancer patients. It is unclear whether this deficit is due to an absolute drop in B cells or whether increased B cells migrate from the blood into the TME. Studies also demonstrated this correlation with patients suffering from head and neck squamous cell carcinoma (HNSCC). However, an increased percentage of $CD19^+$ B cells expressing the activation marker HLA-DR was shown simultaneously. This suggests that tumor production of growth factors and B cell chemoatractives lead to an increased migration of B cells into the tumor area, and thus a decreased percentage of B cells can be measured in the blood (Andrade et al., 2013).

Contrary to HNSCC patients, there was no significant difference in the percentages of $CD19^+$ B cells between glioma patients and healthy controls (Lobinger et al., 2021). This could be caused by a limited migration ability of lymphocytes across the blood-brain barrier (Dunn et al., 2007). Further evidence of B cell immigration into the TME of urothelial carcinoma was noted by Zirakzadeh and colleagues in 2013 with the description of clonal B cell expansion and increased immigration into tumor tissue of urinary bladder cancer patients, probably due to a T helper cell-dependent response to tumor antigens (Zirakzadeh et al., 2013). Furthermore, since B cells can present antigens and thus recruit T cells, a following study by Zirakzadeh et al. demonstrated the proximity of $CD20^+$ B cells and $CD3^+$ T cells in follicle-like structures near the tumor. Interestingly, B cells in follicle-like structures were found mainly in patients with an improved prognosis (Zirakzadeh et al., 2020).

T cells are essential in anti-tumor immunity in the follicle-like structures and the entire body. To identify T cells with flow cytometry, they were stained with antibodies for CD3 and CD45. The CD45 antigen is also known as a common leukocyte antigen and is a membrane glycoprotein expressed on almost all hematopoietic cells except mature erythrocytes. The antigen is known to have a general functional role in the differentiation and activation of hematopoietic cells (Nakano et al., 1990). Recent studies have shown that CD45, as a protein tyrosine phosphatase, phosphorylates and

activates the TCR to recognize and respond to antigens. It has been shown that CD45 controls T cell activation and, through a filtering property, recognizes weak, possibly even counterproductive signals, thus simultaneously preventing an unnecessary T cell-mediated immune response and enabling a regulated immune action of T cells (Courtney et al., 2019). The CD3 antigen, also described as the CD-3 receptor, forms the TCR-CD3 complex with the TCR, which is present on almost 95% of all human T cells (Chetty et al., 1994). As described by Call et al. in 2002, the TCR-CD3 complex plays „a critical role in the differentiation, survival, and function of T cells, and receptor triggering elicits a complex set of biological responses that serve to protect the organism from infectious agents“ (Call et al., 2002). Since CD45 as a common leukocyte antigen is expressed in T cells and other hematopoietic cells, but CD3 is only found on the membrane of T cells, only T cells, in general, are detected with $CD3^+/CD45^+$ staining and cytotoxic $CD8^+$ T cells with $CD3^+/CD8^+$ staining. CD3-negative cells, such as NK cells, are not detected despite their expression of CD8. $CD3^+/CD4^+$ staining detects T helper cells. In the bladder cancer patients examined, a significantly higher percentage of $CD3^+/CD45^+$ T cells could be measured at all measurement times compared to the healthy collective. However, no significant differences were found in the patient group during the disease; the percentage of T cells remained similar before the start of therapy, after radioimmunotherapy, and after radical cystectomy.

Considering the different T cell subpopulations, a significantly relevant percentage decrease in $CD3^+/CD4^+$ T cells could be observed after radioimmunotherapy and after radical cystectomy. The $CD3^+/CD4^+$ T cells decreased by approximately 10 percentage points after therapy and remained at a similar level after radioimmunotherapy and cystectomy. A significant decrease in the percentage after radioimmunotherapy and 12 weeks after radical cystectomy could also be shown compared to the healthy control group. Overall, it can be stated that the percentage of the $CD3^+/CD4^+$ subpopulation was higher in healthy persons than in sick patients and that it also decreased in the treatment course up to significant differences. Regarding the $CD3^+/CD8^+$ T cell proportion, a trend towards increasing percentages from before the start of therapy, after radioimmunotherapy, and after cystectomy could be shown. The lowest percentages of the $CD3^+/CD8^+$ T cell group are found in healthy individuals. Higher values, which continued to increase during therapy, were determined for bladder

cancer patients. Although a positive trend was observed, no significance could be determined during ongoing treatment, with significance only evident when comparing healthy individuals and patients 12 weeks after radical cystectomy. Leaving aside the lack of significance in the $CD8^+$ T cell collective, it can be assumed that $CD4^+$ and $CD8^+$ T cells developed in opposite directions in this study - $CD8^+$ T cells increased while $CD4^+$ T cells decreased. A study collective of breast cancer patients demonstrated a significant increase in CD4+ T cells during radiotherapy. This is inconsistent with the $CD4^+$ T cell trends measured in this study. However, it should be noted that patients in this study received radioimmunotherapy and not radiotherapy alone. The radiation dose was 60 Gy for breast cancer patients and 50.4 Gy for bladder cancer patients (Sage et al., 2016). It must also be considered that this increase in breast cancer patients or decrease in bladder cancer patients can also be partly driven by an opposite development of regulatory T cells but cannot be proven with the available data.

Regulatory T cells can be described as a $CD4^+$ T cell subpopulation expressing the interleukin 2 (IL- 2) alpha receptor CD25 and the transcription factor FoxP3 and were stained as $CD4^+CD25^+FoxP3^+$ T cells. In the study collective presented here, a significant increase of approximately 2.4-fold in the percentage of regulatory T cells was measured between healthy persons and bladder cancer patients before the start of therapy. However, the percentage remained similarly high between the start of therapy and 12 weeks after radical cystectomy. It would, therefore, not explain the dynamics of the $CD4^+$ T cell decrease during this period by their kinetics. A generally high baseline level of regulatory T cells may lead to the suppression of $CD4^+$ T cells. The relatively consistent level of regulatory T cells in bladder cancer patients is consistent with an observation by Qu et al., who demonstrated an increase in regulatory T cells after radiotherapy and postulated that regulatory T cells might be less radiosensitive than other declining lymphocyte subpopulations or that regulatory T cells are increasingly being produced due to the destruction of other tumor cells during radiation therapy (Qu et al., 2010). However, while this assumption would not explain the high increase compared with healthy patients, it would explain the lack of decrease after radioimmunotherapy. In line with our findings, in various tumor entities, such as head and neck cancer, lung cancer, liver cancer, gastrointestinal cancer, pancreas cancer, breast cancer, and ovary cancer, a high percentage of regulatory T cells could also be detected, which contributes to an immunosuppressive, abnormal

phenotype in the peripheral blood but also in the TME itself (Nishikawa et al., 2010). The breast cancer cohort mentioned earlier showed an increase in the percentage of regulatory T cells following radiotherapy (Sage et al., 2016). Even though regulatory T cells are considered to have pro-tumoral properties, a good prognosis with a high rate of tumor-infiltrating regulatory T cells in patients with colon cancer has been demonstrated, as already mentioned in the introduction (Hu et al., 2017; Xu et al., 2017). Various functions of regulatory T cells are not yet fully understood, and it remains questionable whether these functions are anti-tumoral or pro-tumoral. Although there are many signs of an anti-tumoral immune dominating effect of regulatory T cells, there are also indications of a better outcome in patients with an increased percentage of regulatory T cells (Joseph et al., 2019).

Another essential point to consider is the use of checkpoint inhibitors as a part of the therapy of bladder cancer patients. Since PD-L1 is aberrantly expressed in bladder cancer cells, a regulatory function of tumorigenic PD-L1 in anti-tumor immunity can be hypothesized (Nakanishi et al., 2007). Therefore, manipulating the PD-1/PD-L1 pathway is also gaining importance for bladder cancer patients. During radiotherapy, patients were given four times each 240mg i.v. of Nivolumab, a PD-1 checkpoint inhibitor (Schmid et al., 2020). As described earlier, PD-1 is expressed on the surface of T cells, B cells, and NK cells, among others, and can suppress the anti-tumor response by binding its ligand PD-L1 on the membrane of tumor cells (Ahmadzadeh et al., 2009; Dong et al., 2018). If the ICI Nivolumab prevents this binding, this will have an impact on anti-tumor immunity and cellular balance in the TME and peripherally. Therefore, the T, B, and NK cells considered here should be less suppressed from the start of radioimmunotherapy, and anti-tumor immunity should benefit from using the drug. As there are no comparative data for the study cohort that received solely radiotherapy without radioimmunotherapy, it is not possible to determine the extent to which the dynamics of the lymphocyte subpopulations can be attributed to either therapy. Looking again at the B cells, a drop in percentage after radioimmunotherapy is evident. However, it is not possible to say whether this drop would have been more pronounced without the administration of ICI. At 12 weeks after radical cystectomy, an increasing but insignificant trend could be shown.

Furthermore, the question arises whether the cells are only reactivated using ICI or whether a difference in recruitment and proliferation is also shown. If cells only are

reactivated, there will be no increase in the percentage of cell numbers in FACS. Conversely, an increase in cell recruitment into the TME would likely result in a decrease. As described by Freeman et al., the binding of PD-1 and PD-L1 inhibits TCR-mediated proliferation and cytokine production (Freeman et al., 2000). Therefore, if ICIs prevent the binding, it is reasonable to assume that increased proliferation and growth of the T cell lymphocyte subpopulation should be possible. However, in our study, flow cytometry could not show a significant increase in $CD19^+$ B cell, $CD3^+$ T cell, $CD4^+$ T cell, nor $CD8^+$ T cell subpopulation after radioimmunotherapy. Only for $CD8^+$ T cells, in the absence of significance, an increasing trend in the percentage of their population could be assumed after radioimmunotherapy. Further studies with a control group are needed to assess whether the observed kinetics can be explained by either radiotherapy, immunotherapy, or both.

In general, it can be stated that bladder tumors drive overcoming immunosurveillance in several ways. On the one hand, it could be shown that both the frequency and the level of PD-1 expression on $CD4^+$ and $CD8^+$ T cells infiltrating bladder tumors is significantly higher compared to healthy tissue and the peripheral blood of patients and healthy individuals (Ahmadzadeh et al., 2009; Hartana et al., 2018; Wang et al., 2019). $CD8^+$ and PD-1 expression-strong cells infiltrating the tumor were shown to be functionally impaired (Ahmadzadeh et al., 2009), which may be explained by the chronic antigen presentation typical for PD-1 and the resulting exhausted phenotype. Through the phenomenon of the exhausted phenotype, it has been demonstrated that tumor-produced mediators can lead to suppressed perforin expression in cytotoxic T cells in the tumor vicinity.
In addition, $CD8^+$ mediated T cell response is directly blocked by tumor-dependent downregulation of IFN-γ and IL-12 and counter-directed production of immunosuppressive cytokines (Joseph et al., 2019). On the other hand, as already described, the immunological balance can shift toward the tumor due to an increased regulatory T cell proportion. Moreover, bladder tumors can down-regulate MHC I and II protein expression as a form of immunoediting and thus inhibit antigen presentation to T cells and provide reduced anti-tumor immunity (Cathro et al., 2010; Joseph et al., 2019; Nouri et al., 1990).

Since NK cells can also recognize antigens on MHC proteins and even detect downregulation of MHC molecules, they are also affected by this pathologic change in MHC expression (Narni-Mancinelli et al., 2011). Furthermore, NK cells can be activated without MHC-mediated antigen presentation (Smyth et al., 2006). As part of innate immunity, NK cells are one of the first activated cells of the anti-tumor response. They occupy a special position in immunosurveillance due to their multiple functions. Since all mature subpopulations of NK cells carry the surface marker CD56 on their surface (Abel et al., 2018), $CD56^+/CD3^-$ NK cells were analyzed first in our study collective. No significant differences were found between healthy and diseased persons in this subpopulation. Bladder cancer patients tended to have lower percentages of $CD56^+$ NK cells than healthy individuals, with almost the same mean values before and after radioimmunotherapy. At 12 weeks after radical cystectomy, a slight increase was measured, which, however, was then higher than the value for healthy individuals. The peripheral immune phenotype of $CD56^+$ NK cells of prostate cancer patients also did not change during radiotherapy (Sage et al., 2017). In contrast, a collective of breast cancer patients showed a significant drop in percentages of the NK cell subpopulation, which, however, returned to the initial level 6 months after the end of radiotherapy (Sage et al., 2016). Thus, the unchanging $CD56^+$ phenotype of the prostate cancer patients and the recovered $CD56^+$ baseline level of the breast cancer patients after radiotherapy are consistent with our observations. Consistent with the significant decrease in the percentage of $CD56^+$ NK cells during radiotherapy of breast cancer patients, a study by Belka et al. in 1999 also measured a decrease in the absolute NK cell count during radiotherapy and observed a recovery of the level before radiotherapy (Belka et al., 1999). In addition, to account for the important role of NK cells in anti-tumor immunity and to study the kinetics of their subtypes in more detail, lymphocytes were stained with the activation markers NKG2D, NKp30, NKp40, and CD94. In the $NKp30^+/CD3^-$, $NKp46^+/CD3^-$, and $CD94^+/CD3^-$ subpopulations, no significant differences between healthy and diseased individuals could be found. There were also no relevant percentage changes during and after radioimmunotherapy, and 12 weeks after radical cystectomy, neither a decrease nor an increase in NK cell percentage could be found. All three subpopulations thus behaved like the entire NK collective of $CD56^+/CD3^-$ cells. In experiments blocking NKG2D, reduced cytotoxicity of NK cells against bladder cancer cells was shown (Garcia-Cuesta et al., 2015). This demonstrates the importance of the receptor NKG2D, which binds overexpressed

stress ligands on tumor cells. However, for the NKG2D^{+}/CD3^{-} NK cell subpopulation, a significant difference was found between healthy and diseased individuals 12 weeks after radioimmunotherapy. At t2, the percentage was almost two percentage points lower than the value for healthy individuals. A lower but insignificant percentage was also measured during the disease without therapy. This trend continued after radioimmunotherapy and reached a significantly low value 12 weeks after cystectomy. The absence of a drop in the CD56^{+}, NKp30^{+}, NKp46^{+}, and CD94^{+} subpopulations and the relatively small decrease in the NKG2D^{+} subpopulation compared with B cells and the trend towards regeneration 12 weeks after radical cystectomy may be related to a higher intracellular level of glutathione in NK cells. Increased use of the antioxidant allows NK cells to maintain better cell homeostasis and thus better compensate for cell stress during irradiation. This mechanism explains the reduced radiosensitivity of NK cells (Multhoff et al., 1995). Why NK cells behave differently in different cancer entities is not clear. However, there are also different prognosis statements for bladder cancer depending on NK cell counts compared to other cancers. While in glioblastoma patients, higher NK cell counts show a trend towards better progressive free survival (Lobinger et al., 2021), and a study with gastric cancer patients has demonstrated that low NK cell activity leads to high tumor occurrence and metastasis and its extent correlates with its severity (Takeuchi et al., 2001), a retrospective study of patients with NMIBC showed that patients who relapsed two years later had higher baseline NK cell infiltration than disease-free patients. In addition, an association between tumor size and increased NK cell infiltration was determined (Krpina et al., 2014). These correlations may indicate reduced efficiency, poorer cytotoxicity, or even a negative effect of NK cells in bladder cancer (Joseph et al., 2019). A recent study examining fresh tissue from NMIBC and MIBC by flow cytometry demonstrated that improved survival might be related to CD56^{bright} NK cells in the TME. However, cytokine-producing CD56^{bright} NK cells are present in a much lower number near the tumor than CD56^{dim} NK cells (Mukherjee et al., 2018). This dependence on more active CD56^{bright} NK cells producing more cytokines than CD56^{dim} NK cells could explain the study's results by Krpina et al., as this study did not distinguish between the two subtypes (Joseph et al., 2019). It must be considered that only peripheral lymphocytes were determined in our performed flow cytometry, and therefore, it is not easy to predict their behavior in TME. As already described for B cell kinetic, an increased migration of NK cells into tumor tissue could be associated with a decreased number in the blood, but

this has not been proven. To prove these correlations, simultaneous flow cytometry of peripheral blood and TME must be performed. In addition, a separate analysis of $CD56^{bright}$ and $CD56^{dim}$ NK cells would be interesting. Finally, the kinetics of the percentage of NK cells should be considered in the context of the radioimmunotherapy received by the patients. In many studies, the immunophenotype is investigated before and after radiotherapy. However, in our collective, immunotherapy has a major impact on the patient, his immune system, and his outcome. As mentioned previously concerning T cells, inhibition of the PD-1/PD-L1 pathway can reactivate TCR-mediated proliferation and cytokine production by T cells (Freeman et al., 2000). Since NK cells can also carry the receptor PD-1 on their surface, this thus allows coordinated function with T cells against PD-L1-expressing tumor cells. In a study by Trefny et al., intratumoral NK cells from NSCLC patients expressed higher levels of PD-1 on their surface. These intratumoral NK cells were less functional than peripheral NK cells, correlating with their PD-1 expression. Treatment with PD-1 inhibitors reversed the PD-L1 mediated blockade of NK cell function, showing a good therapeutic function for NK cells (Trefny et al., 2020). As described above, no, or hardly any, decrease in peripheral NK cell percentage was observed in our study collective. Whether this is due to the immunotherapy or the reduced radiosensitivity of the NK cells, or both remains unclear. Interestingly, one study found a subset of peripheral NK cells expressing PD-1 in healthy persons (Pesce et al., 2017), whereas other studies only found upregulation in cancer patients. Until now, the mechanisms enabling PD-1 upregulation on NK cells in cancer patients remain undiscovered (Trefny et al., 2020). In the murine tumor model, it has been demonstrated that $PD\text{-}1^{+}$ NK cells are essential for realizing the complete therapeutic advantages of PD-1 checkpoint inhibitors (Hsu et al., 2018). Therefore, ICI research should not only center on T cells but also on NK cells. A comprehensive therapeutic approach is crucial for maximizing the benefits.

5.2. Neutrophil-Lymphocyte Ratio as a marker for SIR

To take an even broader look at the immune system of cancer patients, the neutrophil-lymphocyte ratio of bladder cancer patients was determined. The NLR can be calculated from the quotient of the absolute lymphocyte and neutrophil counts in the peripheral blood. Although neutrophils normally do not occur in the healthy bladder, they are present to an increased extent in the circulation of bladder cancer patients. Neutrophils can also be found in the tumor environment, where they have been shown to contribute to an immunosuppressive environment (Shaul et al., 2018). Neutrophils may be associated with tumor progression on different pathways. When direct irradiation has cytotoxic effects on cancer cells and also affects cells outside the irradiation field via the abscopal effect, the different effects of radiotherapy in vivo vs. in vitro can be attributed to the TME. An altered TME can modulate radiotherapy, leading to different prognoses depending on the therapy efficiency (Schernberg et al., 2017). Bladder cancer cells can secrete granulocyte-stimulating factor to trigger systemic inflammatory response (SIR), leading to increased myeloid cell recruitment and emigration of neutrophils from the bone marrow (Tachibana et al., 1995). This increased number of neutrophils leads to increased pro-angiogenic protease production, thus inducing proliferation, migration, and metastasis (Swierczak et al., 2015). Moreover, neutrophils can secrete more cytokines, such as IL-1β, TNF-α, and IL-12, to create chronic inflammation. Importantly, the additional release of arginase 1 inhibits the immune response of NK cells and T cells, creating an immune suppressive environment for the tumor to drive its progression (Hu et al., 2019; Uribe-Querol et al., 2015). Considering these correlations, it appears obvious that either an increased number of neutrophils or a decreased number of lymphocytes, which in turn is created by, among other things, an increased neutrophil count, generates an increased NLR that promotes tumor progression and decreased survival. In concordance with these findings, in a meta-analysis by Hu et al., with 11945 patients considered, an increased preoperative circulating NLR of bladder cancer patients who subsequently underwent radical cystectomy was associated with an unfavorable outcome (Hu et al., 2019). Another meta-analysis in 2016 concluded from 23 studies and 6240 patients that increased NLR correlated with poor overall and recurrence-free survival. NLR was then proposed as a potential biomarker to predict prognosis or response to therapy in bladder cancer patients (Marchioni et al., 2016). Furthermore, an association between

circulating neutrophils and the TME could be established because higher numbers of tumor-infiltrating neutrophils and NLR can both predict advanced pathological stage and poorer survival, so in this case, it is indeed possible to infer the TME from the processes in the peripheral blood (Joseph et al., 2019; K. Liu et al., 2018). Also, in NSCLC patients treated with PD-1/PD-L1 inhibitors, increased NLR was associated with shorter overall and progression-free survival, so the data are also promising in immunotherapy treatment. Furthermore, it was stated that NLR might be applicable as a biomarker to identify patients who could benefit more from anti-PD-1/PD-L1 treatment (Jiang et al., 2019). In our study collective, an NLR of 4.47 on average was determined before radioimmunotherapy, which increased significantly to 7.19 and remained consistently high after radioimmunotherapy. This increase in NLR may be explained by the previously described behavior of TME and neutrophils during radiotherapy. To compare and classify the measured NLRs prognostically, a cut-off range of 2.1 to 2.7 for a high NLR was defined based on the previously mentioned meta-analysis by Hu and colleagues (Hu et al., 2019). Interestingly, the mean value of the patients with the disease was already far above the cut-off for high NLR, but the standard deviation was almost as high as the actual value determined (4.47 ± 3.84). This is consistent with the idea that the NLR is a prognostic parameter and develops differently in different patients. Some limitations of the NLR determination should be noted. On the one hand, no standard cut-off for a high NLR could be found since a different value has been chosen in each study so far. Therefore, there are no consistent statements on prognosis estimates concerning high NLR values. Furthermore, due to logistical problems and the use of the method only in the further term of the project, the NLR could not be determined for all patients at all time points. Nonetheless, the increasing interest in therapy-determining biomarkers raises hope for further research and progress in this area. All meta-analyses presented found NLR to be a good predictive and prognostic ratio that can be easily determined. Its use with other biomarkers and adaptation to specific clinical situations results in another assessable factor on the way to optimizing therapy options and a consequently improved situation for cancer patients.

5.3. Baseline and dynamics of Hsp70 plasma levels and their importance for tumor detection and prognosis

Many different factors influence immunosurveillance. For example, individual immune cell subpopulations depend on cytokines and mediators secreted by other immune cells or cancer cells for their activation and proliferation. In addition, other signals from the immune system may also serve as triggers for increased or decreased immune responses. In this context, Hsp70 was further investigated in its role in the possible activation of the immune system for enhanced anti-tumor immunity. As mentioned above, cancer cells, unlike normal cells, can upregulate the expression of Hsp70 on their cell membrane and intracellular and thus can lead to higher levels of circulating Hsp70 in cancer patients. Like this, Hsp70 may influence prognosis and treatment response by modifying anti-tumor immunity (Multhoff et al., 2015; Radons, 2016; Radons et al., 2005). Therefore, in addition to analyzing the composition of different lymphocyte subpopulations and the SIR marker NLR, the concentration of Hsp70 was also determined in the plasma of patients with bladder cancer. In our collective, it was shown that patients with bladder cancer who had not yet received radioimmunotherapy had significantly higher Hsp70 plasma levels than healthy individuals. Patients who had already received radioimmunotherapy or radical cystectomy did not show significantly higher circulating Hsp70 than the healthy control group. Since Hsp70 is only increasingly expressed on the surface of tumor or stressed cells and tumor cells have specific lipid rafts to release Hsp70 from the cells, an association of elevated Hsp70 in the blood with an Hsp70 positive tumor or other stress response of the body can be associated. A study conducted by Yu et al. in 2013 demonstrated the expression of HSP70 in bladder tumors. Furthermore, a correlation of HSP70 with the behavior of bladder tumors was found, as immunohistochemical staining of bladder tumor tissue showed that a high expression of HSP70 is associated with a high recurrence rate in NMIBC (Yu et al., 2013).

Moreover, it was confirmed that blood Hsp70 levels correlate with intracellular Hsp70 levels and reflect Hsp70 expression on the membrane of tumor cells (Gehrmann et al., 2014; Gunther et al., 2015). Supporting our results, patients with squamous cell carcinoma of the lung and glioblastomas were also found to have elevated blood levels of Hsp70 at diagnosis compared with healthy individuals (Werner et al., 2021). At the time of diagnosis, Hsp70 can be considered to be released primarily from living tumor

cells and, therefore, may function as a biomarker for viable tumor mass (Gunther et al., 2015). During radiotherapy, Hsp70 is upregulated by irradiation, and, in addition, increased Hsp70 is released by apoptosis of irradiated tumor cells (Lindquist et al., 1988; Multhoff et al., 2015; Radons, 2016). After radiotherapy, a decrease of the Hsp70 concentration in the blood can be expected with the onset of tumor shrinkage. The individual development of kinetics during the treatment of bladder cancer patients depends on therapy response, the number of dying tumor cells, and radiation-induced inflammation. For example, in the study mentioned above of lung cancer patients, a significant drop in the circulating Hsp70 concentration was observed during and after radiotherapy. Nevertheless, there were individual outliers in this group in which the Hsp70 concentration increased (Werner et al., 2021). Similar observations were made in our study group after radioimmunotherapy, as there was a decreasing, but not significant, trend of Hsp70 level after radioimmunotherapy compared to the level before therapy. The decrease also meant no significant change could be detected compared to the healthy group. Twelve weeks after radical cystectomy, the mean value and standard deviation increased again, so no statistically significant statements could be made here. A limitation in presenting the mean value of circulating Hsp70 of the group suffering from bladder cancer is that some diseased patients had a low Hsp70 concentration. With a ROC curve, a 121 ng/ml cut-off was set to distinguish between high and low plasma Hsp70 levels of bladder cancer patients. Using this cut-off for Hsp70, an Hsp70 positive and an Hsp70 negative group could be formed, and the development of their Hsp70 profiles could be evaluated separately. Eight patients, representing 50% of the patients examined, could be classified as Hsp70+. Considering only patients with an initial Hsp70 value of 121 ng/ml or more before radioimmunotherapy, the highest mean value for Hsp70 and a more than 40-fold increase between Hsp70 positive and negative patients was observed at the first determined time point before radioimmunotherapy. The division into Hsp70 positive and negative groups and the large differences in the mean values of Hsp70 concentrations of both groups could be explained by the presence of Hsp70 positive and negative bladder tumors. It is important to note here that the value for the classification was determined based on the cut-off at the time of diagnosis when no therapy had yet been given. Even though all tumors were staged and graded before study inclusion and only similarly pathologically evaluated tumors were included, there appear to be differences in their expression of Hsp70. All eight Hsp70-positive patients

were examined in more detail concerning the individual development of their Hsp70 concentrations in peripheral blood to investigate this finding further. By excluding the Hsp70 negative patients, a clearer picture emerged, and it could be shown that, interestingly, the Hsp70 concentration of all Hsp70 positive patients decreased after radioimmunotherapy compared to the pre-radioimmunotherapy value. Unfortunately, the value after radioimmunotherapy could not be determined in one of eight patients, so only a collective of seven patients could be formed here. This drop in Hsp70 concentration after radioimmunotherapy indicates a consistent tumor response to the therapy and a reduced living tumor mass. The measurement at the time points after radioimmunotherapy took place on average approximately two to three weeks after completing the two months of therapy. This suggests that primary upregulated Hsp70 levels may recover from radiation stress within these 2 to 3 weeks and may reflect reduced tumor mass. In various studies of different patient groups with different cancers, this finding could be confirmed repeatedly. Thus, in squamous cell carcinomas of the head and neck region, a reduction of circulating Hsp70 concentration towards the healthy control collective could be observed after surgery and radiotherapy. Irradiated mice with tumors also showed a drop in Hsp70 after radiotherapy. In these patients and the previously mentioned patients with NSCLC, a correlation between tumor volume and circulating Hsp70 was additionally observed (Bayer et al., 2014; Gehrmann et al., 2014; Gunther et al., 2015). Overall, it can be said that by overcoming stress situations, patients with mHsp70 positive tumors have worse survival than patients with mHsp70 negative tumors (Pfister et al., 2007). The improved stress resistance seems to be a crucial prognostic factor. In line with evolutionary theory, Formenti and colleagues have described that tumor cells that have not been eliminated but are exposed to severe stress survive and, through genetic instability, generate a new, highly resistant generation of cells that carry a variety of protective and anti-apoptotic features (Formenti et al., 2013). Therefore, to assess the individual response to the removal of the tumor and the subsequent recovery process, the Hsp70 concentrations after radical cystectomy were examined. This time, blood sampling after radical cystectomy was always performed three months after surgery. In four patients, an increase in Hsp70 was observed after tumor removal, whereas a decrease was discovered in three patients. It is up for discussion whether chronic inflammation has developed in the patients with a rebounding Hsp70 concentration within the 12 weeks after radical cystectomy, tumor mass was not completely removed,

and some tumor cells, were left in situ, or signs of recurrence were already evident. In a collective of 35 breast cancer patients, two patients with elevated Hsp70 serum levels for at least six weeks at the end of radiotherapy were found to have contralateral recurrence or distant metastases two years after radiotherapy. Since all patients were classified as tumor-free at the time of blood sampling, it can be assumed that the measured circulating Hsp70 originates from chronically inflamed normal tissue rather than from dying tumor cells (Ostheimer et al., 2017; Rothammer et al., 2019). In our collective, it can also be assumed that all patients were tumor-free at the time of blood sampling after surgery, but this cannot be proven due to the long-time interval between surgery and blood sampling. Assuming that tumor progression is driven by chronic inflammation (Jiang et al., 2014) and that this is represented by the presence of DAMPs such as Hsp70, an elevated Hsp70 can indicate chronic inflammation and an increased probability of progression. In a stressed, inflamed TME, these same DAMPs are released and can activate immune cells in the extracellular space and stimulate the repair of damaged tissue, thus supporting anti-tumor immunity (Hernandez et al., 2016; Pandolfi et al., 2016). However, a long-term increase in DAMPs is problematic because an immune tolerance can be generated, promoting tumor growth, metastasis, and recurrence (Klee et al., 2017). Thus, the measurement of the DAMP Hsp70 can be used to conclude these tumor properties precisely. The development of individual Hsp70 concentrations after tumor removal could provide conclusions about chronic inflammation, viable tumor mass, and the prognosis of individual patients. Several studies have already shown that Hsp70 can influence the activation of immune cells and, therefore, the composition of lymphocyte subpopulations. This has been observed, for example, in patients with NSCLC and glioblastoma (Gunther et al., 2015; Lobinger et al., 2021). Especially NK cells are interesting in this context since activated NK cells can detect membrane-bound Hsp70 and, in this way, also contribute to anti-tumor immunity via the Hsp70 pathway (Gross et al., 2003). Previously, low NK cell infiltration in squamous cell carcinoma of the head and neck and high Hsp70 expression was shown to predict poorer clinical outcomes (Stangl et al., 2018), while at the same time, in vitro, studies have shown that extracellular Hsp70 released by dying tumor cells promotes NK cell activation, proliferation, and even cytolytic activity when pro-inflammatory cytokines are present (Gastpar et al., 2005; Multhoff et al., 1996; Multhoff et al., 1999). Several studies recently found a correlation between NK cells and Hsp70 and an upregulation of different NK cell activation markers by Hsp70.

Moreover, the release of pro-inflammatory cytokines such as interleukin 2 induced by Hsp70 and other DAMPs and the corresponding interaction of NK cells with IL-2 and checkpoint inhibitors can further enhance the NK cell-mediated immune response. (Gross et al., 2003; Multhoff et al., 2001; Shevtsov et al., 2019). Further clinical studies have shown that the combined use of ex vivo Hsp70 and IL-2 stimulated autologous NK cells after radiochemotherapy in patients with colon and lung cancer restimulated the cytolytic activity of NK cells (Krause et al., 2004; Multhoff et al., 2020). This reactivation was impossible with IL-2 stimulation alone (Krause et al., 2004). As recent studies have shown, the density and expression of the activation markers CD94 and NKG2D were upregulated by Hsp70 stimulation (Gross et al., 2003). However, our study did not find a direct correlation between Hsp70 and NK cell kinetics. Comparison of the Hsp70+ and Hsp70- groups concerning their lymphocyte subpopulation composition revealed no relevant differences (data not shown). In conclusion, bladder cancer patients had significantly higher circulating Hsp70 than healthy patients at diagnosis, and 50% of all patients considered were defined as Hsp70+. All Hsp70+ patients showed a drop in their individual Hsp70 value after radioimmunotherapy and progressed in different directions three months after tumor removal. To what extent these different Hsp70 kinetics can predict prognosis remains to be seen until further data from the clinical development of the patients are available. Interestingly, in two Hsp70+ patients (pat. 9 and pat. 15, see Figure 25), a strong increase in Hsp70 could be measured at time point t2. In patient 9, a recurrence was detected at the time of the last Hsp70 measurement, when the value had risen again to a similar value as before the start of therapy. Seven months after the last Hsp70 measurement, patient 15 was found to have distant metastasis, with an Hsp70 value nearly three times higher than the value before therapy (t2 vs. t0). Unfortunately, no further measurement time points for Hsp70 are available due to the completed study protocol. Thus, the concrete relationship between recurrence and metastasis with increasing Hsp70 cannot be conclusively proven. Important for evaluating Hsp70 kinetics are the individually different baseline values since not the single value but the dynamics of the development of the Hsp70 concentration can provide information about the function of Hsp70 as a biomarker for therapy response and prognosis.

5.4. Conclusion and Outlook

Our findings highlight the significance and promise of adopting a comprehensive perspective of the immune system for comprehending cancer, its evolution, and advancement, as well as for identifying improved methods for diagnosis and treatment. Despite considerable advancements in the field of immuno-oncology, numerous aspects of the interaction between pro and anti-tumor effects remain to be thoroughly comprehended. Currently, research is devoted to the relatively new field of immunotherapy to modify and improve the body's anti-tumor immunity. Although immunotherapies, such as ICIs, have improved the outcome of patients with cancer (Pardoll, 2012), there are still problems with the universal use of immunomodulatory drugs. As PD-1/PD-L1 ICIs can reinstate the immune response against malignant cells and clinical use is growing fast, there are still challenges that need to be overcome to establish the new hope of cancer treatment further. The main problem is autoimmunity and the associated adverse events (Teixidor et al., 2019). Some treatment trials using Nivolumab have resulted in severe adverse events, including patient death (Zhao et al., 2018). Since not all patients can benefit optimally from immunotherapy but other forms of therapy, such as radiotherapy, we must improve our understanding of the individual patient's immune system to make more precise statements about the response to treatment and the corresponding prognosis. Joseph and colleagues suggested one way to better personalize immunotherapy to specific patient groups in 2016. They showed that irradiation before immunotherapy is associated with a higher absolute neutrophil count and lower absolute lymphocyte and thus influences the response to immunotherapy. As in our study, combined use of both therapies should be used cautiously. Patients should be evaluated if they show a promising outcome, e.g., by their absolute lymphocyte counts (Joseph et al., 2016).

As previously discussed, the composition of lymphocyte subpopulations, the NLR, and the protein Hsp70 offer further opportunities to improve clinical decision-making regarding treatment and follow-up trials. The emergence of lymphocyte subpopulations, each serving distinct or even conflicting functions in pro- or anti-cancer immunity, their interconnected dynamics, and their concurrent impact and reliance on the kinetics of NLR and Hsp70 are just scratching the surface of how the immune system self-regulates. This interaction presents fresh opportunities for cancer research. In this study, the immunophenotype of 16 bladder cancer patients was

determined, and the effect of cancer on the immune system was investigated in the whole study population and individually. A reduction in the percentage of B cells was found compared to healthy patients, which was associated with the hypothesis of increased B cell migration into the tumor milieu, which in turn influenced the increased recruitment of T cells into the TME by increased antigen presentation. However, in the $CD45^+$ T cell population, a percentage increase was found in diseased compared to healthy patients, mainly reflected in the subpopulation of $CD8^+$ cytotoxic T cells, whereas $CD4^+$ T cells showed a decrease throughout therapy. This increase of $CD45^+$ and $CD8^+$ T cells could depend on using the PD-1 checkpoint inhibitor Nivolumab and the decrease of $CD4^+$ on the solid increase of immunosuppressive regulatory T cells but cannot be proven. This shift in cell balance and the impact on anti-tumor immunity can be transferred to NK cells, as Nivolumab also targets them. While the percentage of $NKG2D^+$ NK cells decreased compared to healthy NK cells, the percentages of $CD56^+$, $NKp30^+$, $NKp46^+$, and $CD94^+$ NK cells remained the same. The fact that NK cells behave differently depending on the tumor entity makes interpretation difficult in this small study population. However, both immunotherapy and the reduced sensitivity of NK cells to irradiation may impact their development.

Subsequently, the NLR was considered a marker for systemic inflammation, which provides information about immunostimulatory lymphocytes and immunosuppressive neutrophils. In our patients, significantly higher NLR compared to healthy persons can be reflected in an increase in neutrophils and a decrease in lymphocytes and thus shifts the immune balance into the negative. This effect influences the TME and, therefore, prognosis and outcome. In addition, the biomarker circulating Hsp70 was determined, which was increased by a multiple in patients with the disease. Nevertheless, an Hsp70 positive and negative group could be formed, and a uniform decrease in Hsp70 after radioimmunotherapy was measurable for all the patients of the Hsp70 positive group. Twelve weeks after tumor removal, the Hsp70 concentrations developed individually in different directions, so it remains to be shown whether this results in a prognostic relevance over a more extended period. There are indications of a correlation, as metastasis or a recurrent tumor were detected in two out of eight patients with an elevated level of Hsp70 following tumor removal. In the sense of translational research, all these findings improve the knowledge about the early detection of cancer, its prognosis estimation, and predictions about the response to specific proposed treatments. Overall, a solid immunophenotype profile of the

considered bladder cancer patients could be established, which, combined with the ongoing data collection on progression-free survival and individual occurrence of recurrences, will allow even more insights into potential biomarkers such as absolute lymphocyte counts, the composition of lymphocyte subpopulations, NLR and Hsp70 in the future. Further work in the field of immuno-oncology will pave the way for a holistic understanding of cancer, creating new opportunities for early tumor detection and a new spectrum for curative therapeutic approaches.

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