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Quantification and clinical relevance of soluble HLA class I molecules in the plasma of head neck cancer patients

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Ich widme diese Arbeit meiner Familie, ganz besonders meinen Eltern, meinen Freunden und Kollegen und allen Weggefährten, die diese Arbeit möglich gemacht haben und mich auf meinem Weg unterstützt haben. Ohne die meisten von euch wäre diese Arbeit nie möglich gewesen.

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Introduction

The immune system

The human immune system is responsible for the defense against external pathogens such as viruses or bacteria and is thus crucial for survival. Two parts of the immune system can be distinguished, the innate and the adaptive immune system.

The evolutionary older innate immune system provides a more general unspecific protection against potentially harmful substances and pathogens.[2] Skin and mucous membranes serve as physical barrier between potentially harmful exterior and the vulnerable interior of the organism. If this barrier is compromised a multitude of cells provide a second layer of defense. Phagocytes, such as macrophages, dendritic cells (DCs), and granulocytes are able to phagocyte, dismantle, and kill bacteria and fungi. Natural killer (NK) cells can identify and destroy potentially harmful body cells, and other mechanisms and further cells provide further direct and indirect defense mechanisms. This kind of defense depends mainly on the recognition of evolutionary consolidated molecules, such as distinctive components of bacterial or fungal cell membranes and cell walls, by a class of proteins named pattern-recognition receptors enabling a fast and effective way of recognition for potential dangers.[3] The detection of altered targets or harmful own body cells such as cancer cells by this system is limited, though.

The evolutionary newer adaptive immune system comprising B and T cells is better prepared for this task. It is on the one hand able to produce specific antibodies against extracellular targets and, on the other hand, specifically identify altered cells, for example virus-infected and malignant cells, and destroy them. This is enabled by stimulation and activation by the general immune reaction following the activation of the innate immune system and presentation with processed components of phagocytized structures from phagocytes.

T cells

Detection and eradication of harmful body cells, e.g. infected or cancer cells, is mediated by T cells.[4, 5] One way of detection of malignant cells by T cells is hereby mediated by the direct recognition of short tumor-associated peptides presented by human leucocyte

antigen (HLA) molecules on the surface of the malignant cells. Another, recently identified, mechanism of tumor killing is indirectly facilitated by the uptake of peptides from HLA-deficient tumor cells by antigen-presenting cells and HLA class II complex presentation to $CD4^+$ T cells, leading to stimulation and activation of the T cell and subsequently reprogramming of the tumor-associated myeloid cell network, enabling a remote mechanism of inflammatory tumor cell death.[6] T cells originate from hematopoietic stem cells in the bone marrow and undergo a process of maturation within the thymus, in which, by positive and negative selection, a unique T cell receptor (TCR) is created. Via their specific TCR, the T cells can bind to specific HLA-peptide complexes on the surface of all nucleated body cells. Those peptides are small fragments originating from proteins, which are digested into peptides by the proteasome, a protein complex responsible for degradation of proteins, and processed to be loaded upon the HLA, leading in the end to an HLA-peptide complex presented on the surface of the corresponding cell. Unaltered, endogenous peptides are recognized as such, and no immune response is induced. Non-endogenous or altered peptides, originating from virus-infected cells, but also from malignant cells, presented on HLA and bound by their corresponding TCR, can lead to stimulation and activation of T cells and, subsequently, induction of an immune response and eradication of the respective cell.

T cell receptor

T cells can be divided into two major subtypes, $CD4^+$ T cells and cytotoxic $CD8^+$ T cells.[7] Whereas, the role of $CD4^+$ T cells is essential in the activation, maturing, and shaping of an immune response, cytotoxic T cells are responsible for direct interaction with possible harmful body cells, such as virus-infected or malignant cells.[8]

The main protagonist of this interaction is the TCR, a heterodimeric receptor. The diversity of the TCR arises mainly from somatic genetic recombination of various DNA segments, called V(D)J recombination, in which three distinct gene segments (V, D and J) are nearly randomly rearranged, resulting hypothetically in up to 10^{15} different TCR clonotypes.[9, 10] During the maturation process of the TCR of each T cell in the thymus, a negative selection occurs, preventing the emergence of T cells bearing TCRs with a too high affinity to endogenous self-peptides or HLAs.[11] On the other hand, in a process of positive selection the binding of TCRs to HLA molecules, at least with a weak affinity,

lead to positive stimulation through the TCR, ensuring survival of these T cells and leading to apoptosis of T cells that are not able to bind HLAs.

After leaving the thymus, binding of T cells with their corresponding TCR to a fitting HLA-peptide complex lead to an initial signaling cascade together with the co-receptor CD3. Further costimulation through costimulatory proteins such as CD28 and different cytokines, such as Interleukin 1 (IL-1), leads finally to T cell activation, to induced apoptosis of the target cell and, further, an immune response.[12]

T cell exhaustion

In contrast to activation, there are also various ways that can lead to exhaustion of T cells. Inhibitory receptors, such as Programmed Cell Death 1 Protein (PD1), through activation via their corresponding ligand, can mediate direct apoptosis of antigen-specific T cells and, indirectly, inhibition of the same cells by reduced apoptosis of regulatory T cells.[13]

Additionally, soluble immunosuppressive mediators, for example cytokines, such as Interleukin 10 (IL-10) can directly promote T cell exhaustion. Other mediators, such as type I interferons, which are more commonly known for activating T cells, can also lead to immune suppression in chronic infection.[14, 15]

Furthermore, without corresponding costimulation T cells are unable to switch into an active state and, subsequently, in the target cell apoptosis will not be induced. Instead, repeated activation of the TCR, followed by missing co-activation of the T cell is suspected to lead to exhaustion of the T cell.[16, 17] The intensity as well as the duration of stimulation seems to play an important role and activation of sprout homologue 2 (SPRY2) and nuclear factor of activated T cells (NFAT), both TCR dependent pathways, are suspected to mediate the exhaustion.[18, 19]

Human leucocyte antigen

Genetically located on the short arm of chromosome 6, HLA molecules consist of 3 different classes, I, II, and III, with class I and II capable of binding and presenting antigens, while the class III gene locus encodes components of the innate immune system.[20] HLA class I, can be found on all nucleated cells and consisting of a heavy chain and the constant light chain ß2-microglobulin, can further be subdivided into HLA-A, -

B, and -C, all of which present a short sequence of 8-12 amino acids on the cell surface.[21, 22] Structurally, the heavy chain is composed of three α-subunits: α_1 and α_2, forming together the binding groove for peptides, and α_3, anchoring the complex to the cell membrane and interacting with the CD8 molecule on interacting T cells. Non-covalently bound together with the light chain β_2m and the presented peptide they form an HLA complex.[23]

In general, the presented peptides have their origin in a distinct antigen processing pathway derived by proteolysis of intracellular proteins.[24] Proteins within the cell are constantly ubiquitinated and thus marked for degradation (Figure 1A). After the degradation of the proteins by the proteasome, the resulting peptides are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) protein complex.[25] Under the influence of the immunostimulatory cytokines, such as Interferon gamma (IFN-γ), the proteasome is partly built with other subunits, making up the so called immunoproteasome.[26] This, in the end, leads, among other immunostimulatory effects, to an increased frequency of antigen presentation.[27] After loading of the peptides onto HLA class I within the ER, the complexes are transported via the Golgi apparatus, onto the cell membrane of every nucleated cell, thereby enabling presentation of the antigens to $CD8^+$ T cells.[21, 28]

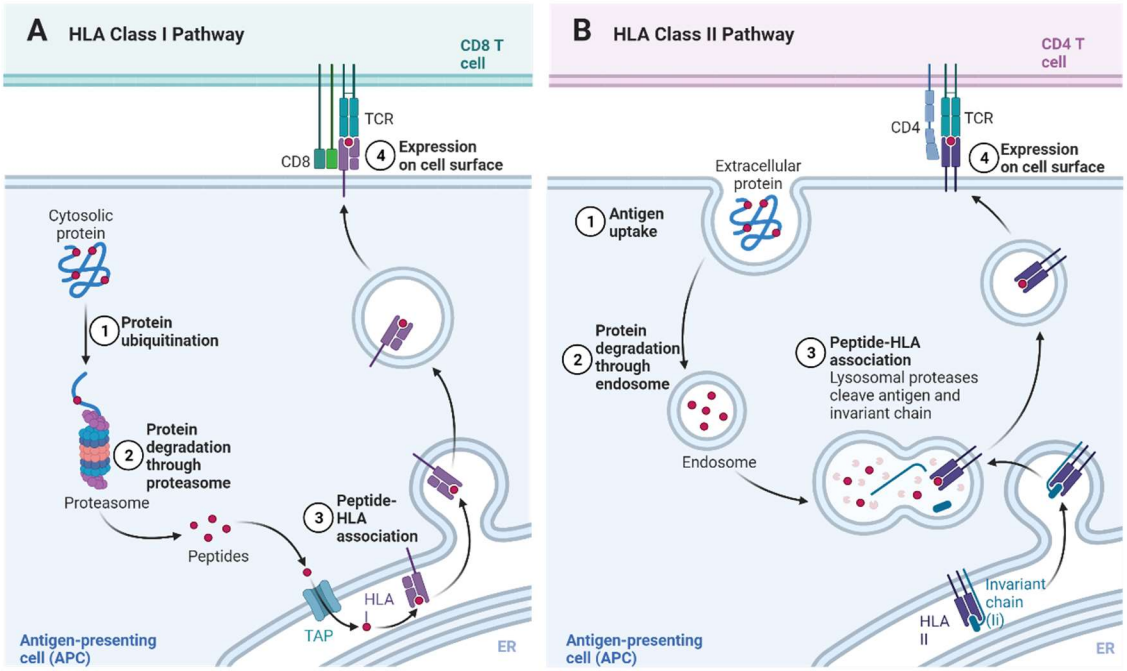

Figure 1: HLA Processing. (A) Antigen processing within the HLA class I pathway: Intracellular proteins are tagged for degradation (1) and degraded by the proteasome (2) into smaller fragments. After transport of these peptides into the ER via the transporter associated with antigen presentation (TAP), they are loaded onto HLA class I molecules within the ER (3). The HLA-peptide complexes are transported via the Golgi apparatus to the surface of the cells, where the vesicles merge with the plasma membrane and the HLA-peptide complex is subsequently able to be recognized by T cells via their TCR and CD8 molecule (4).
(B) Antigen processing within the HLA class II pathway: After endocytosis and degradation of extracellular proteins (1), late endosomes containing the degraded peptides (2), fuse with HLA class II loaded endosomes, originating from the ER, in which the binding site is blocked via an invariant chain (3). After fusion, the invariant chain is degraded, antigen is loaded on the binding site and the HLA-peptide complex is transported to the cell membrane, where the HLA class II complex is presented to $CD4^+$ T cells (4). Created with BioRender.com

Genetically, HLA molecules show a huge diversity especially in the binding grooves caused by polymorphisms. Each HLA allotype has its own binding groove with a unique binding motif. For HLA class I, the binding motif contains two anchor positions, that bind specific amino acids and thus fundamentally determine which peptides are presented on the HLA molecule.[29]

HLA class II molecules, similar to HLA class I, are genetically encoded by three polymorphic genes (HLA-DR, -DQ, and -DP), but provide different binding grooves for peptides, ranging in length from usually, but not limited to, 12 to 27 amino acids and less restriction to binding motifs leading to a more promiscuous peptide presentation.[30, 31] HLA class II molecules, unlike HLA class I molecules, are primarily expressed on antigen-presenting cells (APCs), comprising DCs, macrophages, and B cells, but are also expressed on malignant cells.[32, 33]

Structurally, HLA class II molecules consist of four extracellular domains (α1, α2, β1, and β2), with the peptide-binding grooves formed by the α1 and β1 domains.[34, 35] Unlike HLA class I, peptides on HLA class II are often of extracellular origin. Therefore, extracellular proteins are internalized for example by macrophages via phagocytosis followed by the degradation into peptides by proteases.[21, 28]

In order for the HLA class II complex to be loaded with the peptide, the not yet loaded complexes are capped as they pass through the ER by an invariant chain in the peptide-binding groove, which is degraded by the proteases only after fusion with the peptide-containing endosomes, allowing binding of the peptides (Figure 1B).[21]

In general, peptides of intracellular and extracellular origin are presented on HLA class I and HLA class II molecules, respectively. However, cross presentation, a process in which peptides of extracellular origin can be presented on HLA class I is found in DCs, macrophages and B cells, therefore enabling presentation of exogenous peptides in a self-manner to other immune cells. This contributes heavily to the anti-cancer capacities of the immune system.[36]

Soluble HLA

In addition to being expressed on the cell surface, HLA molecules together with their respective peptide can also be found in soluble form (sHLA) in the blood and other body fluids, such as sweat, saliva, tears and urine.[37, 38, 39]. Three distinct mechanisms have been identified that lead to the release of membrane-bound HLA molecules (mHLA) into their soluble form: alternative splicing, proteolytic processing and exosomal shedding (**Fehler! Verweisquelle konnte nicht gefunden werden.**).[40, 41, 42]

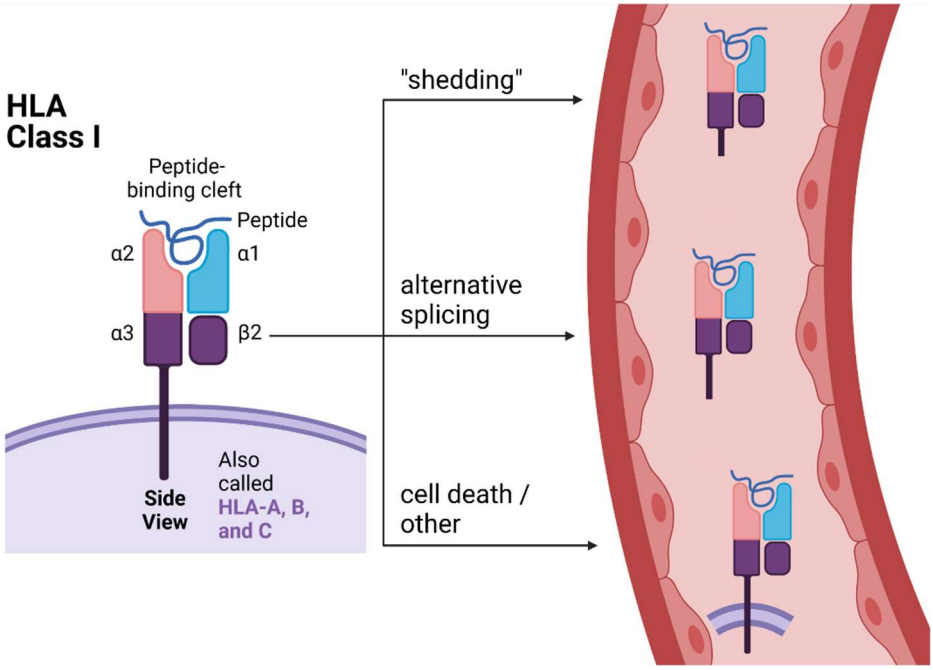

Figure 2: Origin of sHLA. Membrane-bound HLA molecules (mHLA), consisting of the heavy chain with the short cytoplasmic, the transmembrane and the α1, α2, and α3 domains as well as the light chain β2 microglobulin, are processed in three different ways into soluble HLA (sHLA): shedding, alternative splicing and cell death and other mechanisms that lead to parts of the membrane being shed into the blood stream. Shedding of mHLA by proteolytic cleavage leads to the smallest molecules (35-37 kDa), alternative splicing to a construct of 39-41 kDa size, and, lastly, cell death or membrane blebbing to a size of 44-46 kDa [1]. Created with BioRender.com

Proteolytic cleavage of mHLA leads to the smallest construct with a size of 35-37 kDa, leaving almost only the α1-3 domains together with the β2 microglobulin domain intact with a very short to no transmembrane region and no cytoplasmic domain left.[43] This process of cleavage is mediated by a Zn(2+)-dependent, membrane-bound metalloprotease.[42, 44]

Furthermore, alternative RNA splicing pathways can lead to molecules sized between 39-41 kDa, which lack the transmembrane domain and are secreted by the cells.[41]

Lastly, shedding from the cell membrane leads to the largest construct with a size of 44-46 kDa. In this case a fully intact HLA molecule, consisting also of cytoplasmic and transmembrane region, can be detected.[45] The process of release can be active and energy-dependent.[46] Within the blood this form is mainly found on exosomes. In fact, it has long been assumed that the largest proportion of sHLA in the blood is accounted for by this form, but it has been shown that only a small fraction actually originates from this form of shedded sHLA.[47]

Role of sHLA in the immune response

To date, the function of sHLA has not been conclusively elucidated. There are several theories in connection with malignant diseases: It was shown that sHLA is able to induce apoptosis in cytotoxic T cells and also NK cells.[48, 49]

Different studies suggest that this interaction is TCR-dependent, while in other sources the mechanism is described as CD8-mediated.[49, 50]

A possible and, therefore widely discussed mechanism of action to influence and suppress immune cells is an active secretion of sHLA by the tumor cells into the blood. The discussions focus mainly on the possibility of sHLA binding repeatedly TCRs on T cells without corresponding costimulation, leading to inactivity or even exhaustion of the T cells. In a further study it could be shown that sHLA is also able to cause a longer tolerance of the transplant in transplanted patients, presumably by inertia of the T cells.[39]

On the other hand, it was also shown that sHLA can activate T cells. This mechanism is based on an exchange of peptides from sHLA and mHLA on T cells, leading to self-activation of T cells, rendering it difficult to determine if the effects of sHLA on immune cells can be generally seen as stimulating or suppressive.[51]

What has been shown, however, is that in certain tumor types, the concentration of sHLA is associated with tumor burden and, in some cases, clinical prognosis.[52]

For example, in various studies of hematological diseases such as non-Hodgkin's lymphoma and multiple myeloma, it has been shown that the concentration of sHLA in

the blood of patients is elevated compared to healthy subjects and that the levels of concentration also correlates with clinical prognosis.[53, 54, 55]

Head and neck cancer

In 2020, head and neck cancer (HNC) was the seventh most common cancer type worldwide with an incidence of 660,000 and a mortality of 325,000 cases with a suspected increasing incidence.[56, 57] The group of head and neck tumors includes various subtypes of tumors that can be located in the oral cavity, nasal cavity with paranasal sinuses, pharynx, larynx, salivary glands and lymph nodes of the head and neck. While alcohol and tobacco consumption are the primary risk factors, recent discoveries detected a strong association of human papillomavirus and head and neck squamous cell carcinoma (HNSCC), the most common subtype of HNC.[58] Further risk factors, aside from general factors such as radiation exposure or age, are Epstein-Barr virus (EBV) for nasopharyngeal cancer and gastroesophageal and laryngopharyngeal reflux disease (GERD and LRPD) for the area of the upper airway and throat.[59, 60, 61]

Staging

Depending on the affected anatomical site, the tumors are classified into four different stages (I - IV) according to the American Joint Committee on Cancer staging manual and the Union for International Cancer Control as proposed by the World Health Organization (WHO).[62] For example in HNSCC, stage I contains tumors smaller than 2 cm with no infiltration of nearby structures, lymph nodes or metastases, while stage IV tumors are infiltrating nearby structures, affecting multiple lymph nodes or showing multiple metastases.[63] Especially distant metastases indicate a poor prognosis for the patient.

Treatment

For early stages of squamous cell carcinomas, the choice of treatment depends on location and anatomical accessibility of the tumor. Often, primary surgery followed by radiation therapy is recommend, yielding five-year overall survival rates of 70% to 90%.[64] In case of close (<2mm) or positive surgical margins, reresection is recommended.[65] The choice of treatment depends on the site of tumor and its requirements. Oral cavity tumors are, for example, optimally treated with surgery, while patients with many comorbidities

and/or surgically difficult treatable tumors profit more from a definitive radiotherapy approach.[66]

In cases of locoregionally advanced disease, due to complexity depending on involved sites, size, progression, patients' status and a multitude of further factors, the respective treatment should be discussed and decided in interdisciplinary tumor boards.[67] Often surgery is still a viable option if the tumor is resectable and organ-preservation can be achieved, while chemotherapy or radiochemotherapy become equivalent beneficial or even preferred options.[68, 69, 70]

The most common chemotherapies used are Cisplatin or Carboplatin, interfering with DNA replication, 5-Flurouracil, blocking the synthesis of the nucleotide pyrimidine thymidylate, and/or taxanes, disrupting microtubule function, all interfering with cell proliferation. [71, 72, 73, 74]

In case of locally recurrent disease, open surgical salvage in addition to surgical resection becomes the first-line treatment, whereby frequency and management of complications is increasing depending on the choice of initial treatments.[75] Radiation treatment is conducted postoperative, if not already administered initially or if reirradiation is indicated. After initial chemotherapeutic regiments and subsequent tumor recurrence, the benefit of further classical chemotherapeutic approaches is often limited.

Lastly, treatment of patients with systemic metastatic or recurrent head neck cancer is challenging and accompanied by a poor median survival of 6 to 15 months.[76]

Thus made clear, with advanced tumors stages and/or with disease recurrence, the benefit of surgical approaches decreases, while chemotherapeutic regiments increase in relevance. A few years ago, prognosis of patients with progressing diseases that were not surgically or radiochemotherapeutically treatable and were initially treated with classical chemotherapy or under treatment, was very limited.[77]

Nowadays, approaches involving the immune system, like Cetuximab, and monoclonal antibody targeting Epidermal Growth Factor Receptor (EGFR), showed significant improvements in overall survival and is therefore approved for treatment. [78]

Likewise, a more immunogenic approach is given in the utilization of Pembrolizumab and Nivolumab, both also monoclonal antibodies, this time targeting and inhibiting Programmed Cell Death Protein 1 (PD-1), a protein found highly expressed on the surface of many tumor cells. PD-1, expressed on T and B cell binds to its respective ligand, PD-L1, a protein highly expressed on the surface of many tumor cells, leading to inhibition of those cells. By binding of PD-1 via Pembrolizumab/Nivolumab, this mechanism of cancer immune evasion is blocked, leading to improved overall survival in patients with metastatic and recurrent head and neck cancer. [79]

Similar to squamous cell carcinoma of the head and neck, treatment of other tumor entities in this area is multimodal, depending on the tumor entity, differentiation, location and extent of the tumor and other patient and clinic associated factors.[77]

Aim of the thesis

The ability of the immune system to actively fight tumor cells has gained importance in research and the treatment of patients in recent years. More and more new approaches are aiming to activate the immune system specifically or non-specifically and on preventing and counteracting the immunosuppressive effect of tumors on the immune system. sHLA molecules are suspected to mediate immunosuppressive mechanisms by repetitive binding to TCRs. This study focuses on the establishment and refinement of a method for the measurement of sHLA concentrations in the plasma of tumor patients. Furthermore, the established method was applied for the analysis of sHLA plasma levels in HNC patients to gain insights into the clinical relevance of sHLA in HNC.

Material and methods

Materials

Chemicals

Table 1: Chemicals

Description	Manufacturer
cOmplete Protease Inhibitor Cocktail	Roche (Basel, Switzerland)
Sodium bicarbonate	Merck (Darmstadt, Germany)
Sodium carbonate	Merck (Darmstadt, Germany)
Dulbecco's phosphate-buffered saline	Gibco - Thermo Fisher Scientific (Waltham, USA)
Bovine serum albumin (BSA)	Sigma-Aldrich (St. Louis, USA)
Phosphoric acid	Merck (Darmstadt, Germany)
TMB Substrate A	Biolegend (San Diego, USA)
TMB Substrate B	Biolegend (San Diego, USA)
Flex-T™ HLA Class I ELISA Control	Biolegend (San Diego, USA)
Tween 20	Carl Roth (Karlsruhe, Germany)
Ethanol (70% in ddH_2O)	SAV Liquid Production (Flintsbach a.Inn, Germany)

Antibodies

Table 2: Antibodies

Description	Target	Manufacturer
Mouse IgG2a, κ purified anti-human HLA-A,B,C Antibody clone W6/32	HLA class I histocompatibility antigen, A alpha chain	Biolegend (San Diego, USA)
Mouse IgG1, κ horse radish peroxidase anti-human β2-microglobulin Antibody clone 2M2	HLA class I histocompatibility antigen, β2-microglobulin	Biolegend (San Diego, USA)

Soluble HLA constructs

Table 3: Soluble HLA constructs

Description	Manufacturer
Flex-T™ HLA Class I ELISA Control	Biolegend (San Diego, USA)
HLA-A*24 RFPPTPPLF monomer	In-house

Devices

Table 4: Devices

Description	Manufacturer
Barnstead GenPure Pro	Thermo Fisher Scientific (Waltham, USA)
ELISA reader Spectramax 340	Molecular Devices (Sunnyvale, USA)
Freezer Skadi -80°C	Telstar (Woerden, Netherlands)
Freezer GlassLine -20°C	Liebherr (Bulle, Switzerland)
Heraeus Centrifuge Megafuge 1.0R	Thermo Fisher Scientific (Waltham, USA)
Multi-channel pipette 200 µl	Abimed (Langenfeld, Germany)
One-channel pipettes 2, 10, 200, 1000 µl	Abimed (Langenfeld, Germany)
Multi-Dispenser Pipette Repeater M4	Eppendorf (Hamburg, Germany)
Pipet aid Pipetboy acu 2	Integra Biosciences (Fernwald, Germany)
Refrigerator GlassLine 4°C	Liebherr (Bulle, Switzerland)
Mini Centrifuge GMC-060	LMS (Tokyo, Japan)
Sorvall RC-5C Plus Superspeed Centrifuge	Kendro Laboratory Products (Asheville, USA)
ZX3 Advance Vortex Mixer	VELP Scientifica Srl (Usmate, Italy)

Software

Table 5: Software

Description	Manufacturer
ELISA reader SoftMax Pro 2.2	Molecular Devices (Sunnyvale, USA)
GraphPad Prism 9	GraphPad Software (San Diego, USA)
RECIST 1.1	Mint Medical GmbH (Heidelberg, Germany)
Microsoft Office Professional Plus 2019	Microsoft Corporation (Redmond, USA)
Inkscape 1.1	Inkscape Project (New York, USA)

General material

Table 6: General material

Description	Manufacturer
Tubes 50 ml	Greiner Bio-One (Frickenhausen, Germany)
Glass bottles (250 ml, 500 ml, 2l)	Schott AG (Mainz, Germany)
Disposable pipettes (5, 10, 25 ml)	Becton Dickinson (Franklin Lakes, USA)
Safe-Lock reaction tubes (0.5, 1.5, 2 ml)	Eppendorf (Hamburg, Germany)
Microlon™ Polystyrene 96-well ELISA Microplates	Greiner Bio-One (Frickenhausen, Germany)
Sealing film ROTILABO	Roth (Karlsruhe, Germany)
Costar® 50 mL + 100 ml Reagent Reservoirs	Corning Incorporate (Corning, USA)

Methods

Plasma samples

Peripheral blood (EDTA tubes) samples from patients with advanced stage head neck cancer (HNC cohort 1) and patients with recently diagnosed and surgically treatable head neck cancer (HNC cohort 2) were collected at the Department of Internal Medicine, University Hospital Tübingen, Germany, and the ENT Clinic, University Hospital Tübingen, Germany, respectively. Peripheral blood (EDTA tubes) samples from healthy volunteers (HVs) were collected at the Department of Immunology, Tübingen, Germany and the University Hospital Tübingen, Germany. Donor characteristics of HVs were collected by questionnaire-based assessment. Informed consent was obtained in accordance with the Declaration of Helsinki protocol. The study was approved by and performed according to the guidelines of the local ethics committees (431/2012B02, 406/2019B02). Plasma was separated by centrifugation for 10 min at 1200 g without break at room temperature (RT). The supernatant was collected and transferred into a Superspeed Centrifuge tube. Protease Inhibitor Cocktail (1:25) was added, followed by centrifugation for 10 min at 12000 g at 4°C and the supernatant was stored at -80°C.

Refinement of sHLA quantification by ELISA

To quantify sHLA class I levels in plasma an already existing ELISA protocol was adopted and refined. For this, every step of the protocol was investigated and adapted if necessary. Changes were made to physical handling, washing procedures, temperature, and time of various incubation steps, blocking, dilution of samples, origin, and concentration of standard, plate manufacturer, substrate solution manufacturer, and concentration, washing buffer concentrations, and stopping solution concentrations.

Quantification of sHLA by enzyme-linked immunosorbent assay (ELISA)

The principle is shown in Figure 3**:** In a first step a solution with capturing antibodies is added to wells of a 96 well plate (1). The antibodies, after binding to the surface, a step labeled coating, are then able to bind their respective antigen (2), which is added in a second step. Subsequently, a second solution with a Horse-radish-peroxidase (HRP)-coupled antibody, specific for the antigen, is added (3). Binding site on the antigen of both antibodies is different, enabling simultaneously binding of both antibodies. To visualize binding to the antigen and to quantify amount of antigen, TMB solution is added

(4), leading to enzymatic reaction with the HRP, converting the colorless TMB substrate into colored product, subsequently followed by stopping the reaction with an acidic solution. The final product within the wells enables quantification of antigen within the respective well via an ELISA reader.

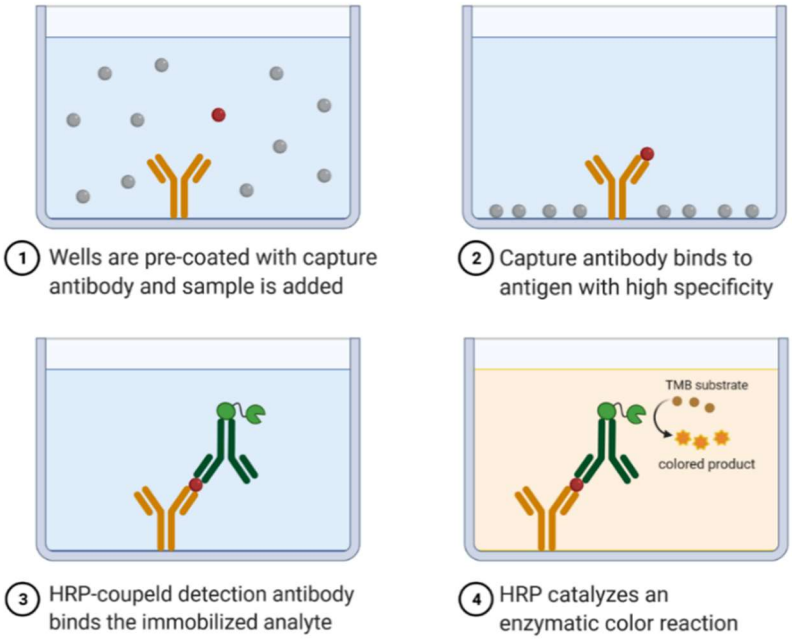

Figure 3: Principle of enzyme-linked immunosorbent assay (ELISA). Wells are pre-coated with antibodies (1), meant to specifically bind to target antigen. After sample is added and target antigen (2) is bound, a second, HRP-coupled detection antibody is added (3), which enables catalyzation of a reaction, processing TMB substrate into a colored product, leading to measurable and quantifiable signal (4). Created with BioRender.com

For quantification of sHLA in the plasma of HNC patients and HVs, flat-bottomed high binding 96-well microplates (Greiner, Microlon®, 655061) were used. After coating with 100 µl of anti-human HLA-A, -B, -C antibody (clone W6/32, Biolegend) diluted 1:100 in 100 mM carbonate bicarbonate coating buffer (Na_2CO_3, 3.53 mg/ml and $NaHCO_3$, 5.6 mg/ml) per well and incubation overnight at 4°C, the plates were washed four times with phosphate-buffered saline (PBS) supplemented with 0.5% Tween-20 and subsequently blocked with 200 µl 3% bovine serum albumin (BSA) PBS per well for 2 h at RT. If not stated otherwise all subsequent washing steps were conducted with the same washing solution. A standard HLA monomer (Biolegend, 280301) was used as a standard starting with a concentration of 100 ng/ml and subsequently diluted seven times in 1:2 dilution steps. Plasma samples were diluted 1:200 with 0.5% BSA-PBS. After four washing steps,

100 µl of each standard dilution, diluted samples, and blank sample (0.5% BSA-PBS) were loaded per well in triplicates and incubated for 2 h at RT. The plates were washed four times and 100 µl per well of horseradish peroxidase (HRP) anti-human ß2-microglobulin antibody (clone 2M2, Biolegend 280303) as detection antibody in a 1:800 dilution in 0.5% BSA-PBS was added. After incubation for 90 min at RT, the plates were washed six times and 100 µl TMB substrate (1:1 of TMB substrate A and B) was added per well. After incubation for 15 to 30 min at RT in the dark, the reaction was stopped with 100 µl 1m phosphoric acid (H_3PO_4) per well. The optical density (OD) was measured at 450 nm wavelength using a SpectraMax Plus 384 (Molecular Devices) ELISA reader. Analysis was done in triplicates. The standard curve was calculated by using the four-parameter algorithm to plot the mean absorbance of standards against the known concentration of standards on a logarithmic scale. Results were depicted as µg/ml.

Software and statistical analysis

The mass and location of the primary tumor, the number and size of metastases, the mass of the ten largest metastases, size dynamics of the primary tumor and metastases over time, and location of tumor relapse were determined using RECIST 1.1 for each patient.

For statistical analysis, GraphPad Prism 9 (GraphPad Software) was used. *P* values of < 0.05 were considered statistically significant.

Results

Refinement of ELISA for the quantification of sHLA plasma levels

The ELISA used for the quantification of sHLA plasma levels were previously established in the lab of Prof. Reinhild Klein (Department of Hematology, Oncology, Clinical Immunology and Rheumatology, University Hospital Tübingen), which had to be adapted and refined for the use in our lab. Therefore, every step of the protocol was investigated and adapted if necessary.

Several problems had to be overcome to make the assay reliable and sensitive. Like in most assays, it proved beneficial to prepare all necessary solutions freshly in advance. Effects of outliers were reduced by usage of triplicates. Incubation temperature was set to RT (ideally 19-21°C, except for capture antibody incubation step at 4°C) to reduce the number of outliers and overall stability. Rough physical handling of the plate in any step had to be avoided, otherwise leading to overall reduced values OD at 450 nm (OD_{450}) and more outliers. Preventing evaporation during incubation further improved quality by

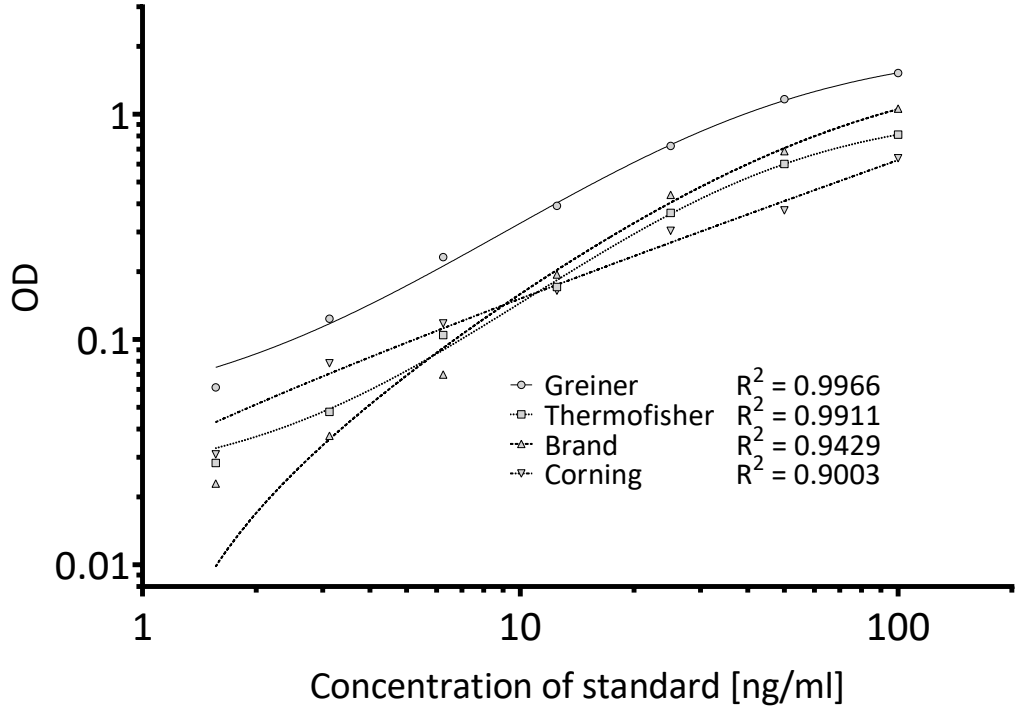

Figure 4: Comparison of standard curves for sHLA quantification in plasma samples using different 96 well plates from different manufacturers. Absorbance (optical density (OD) at 450 nm) was plotted as a function of the concentration of the HLA standard monomer. Dots present mean values of triplicates. Blank mean values were subtracted from all measured absorbances. The resulting standard curve was created by 4-PL nonlinear regression analysis.

reducing the number of outliers in the outer wells of the plate. Blocking of unspecific binding sites by the usage of 3% BSA-PBS and increased numbers of washing steps before substrate addition improved the sensitivity by lowering background signal. Stopping the reaction with phosphoric acid (H_3PO_4) proved to be superior to other solutions in terms of OD yield. Furthermore, high-binding plates (Greiner 655061 (Microlon high bind)) were superior to other commercially available plates in terms of OD yield and coefficient of determination (R^2) (Figure 4). For further experiments the Microlon high bind plates from Greiner were used. With these refinements, we were able to establish an ELISA with blank OD_{450} values between 0.05 and 0.07 while reaching maximal values of 2.1 OD_{450} for the highest concentration of standard.

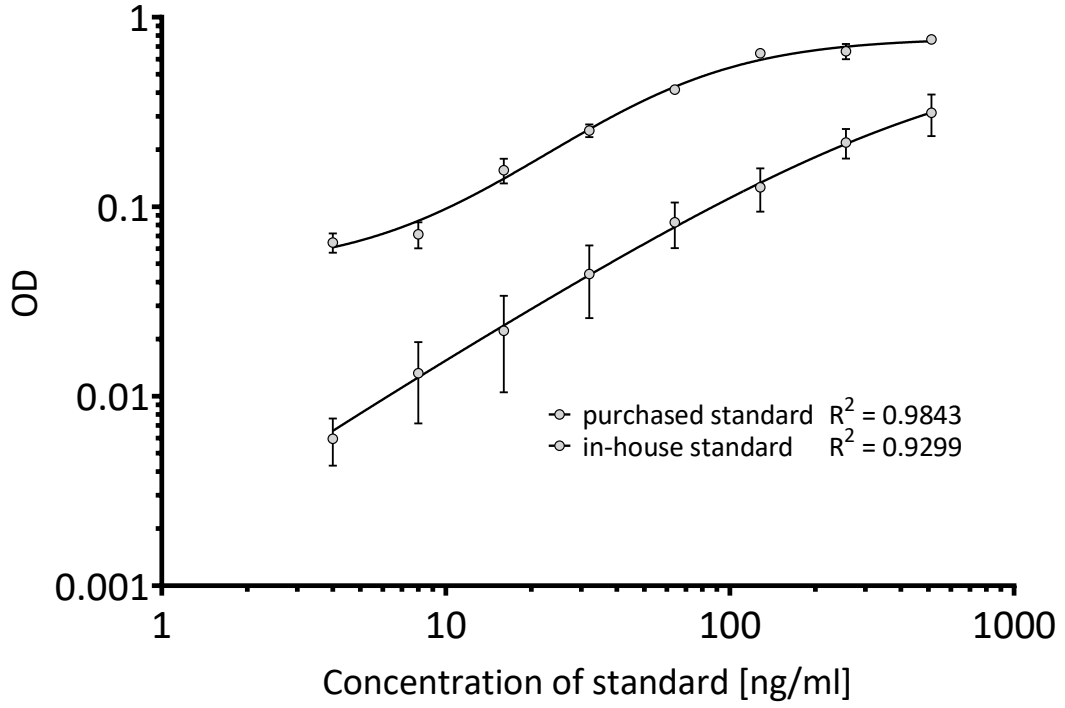

Figure 5: Comparison of standard curves for sHLA quantification of commercially available HLA class I standard and in-house manufactured standard (HLA-A*24 RFPPTPPLF monomer). Absorbance (optical density (OD) at 450 nm) was plotted as a function of the concentration of the HLA standard monomer. Dots present mean values of triplicates with whiskers showing standard derivation (SD). Blank mean values were subtracted from all measured absorbances. The resulting standard curve was created by 4-PL nonlinear regression analysis.

Compared to an in-house generated HLA-A*24 complex, a commercially available HLA class I standard showed higher ODs, higher R^2, and less variation in SDs (Figure 5). For further experiments only the commercially available HLA class I standard was used.

For the quantification of sHLA levels, the use of an appropriate and reproducible standard curve is necessary. Therefore, different HLA standard monomers with different dilutions were analyzed (Figure 6). A starting concentration of 100 ng/ml standard with seven 1:2 dilution steps of the purchased standard yielded the best results in our analysis. With this standard curve, the detectable range of our optimized ELISA covers a range of 1.5 ng/ml to 100 ng/ml (Figure 6). Almost every plasma sample diluted 1:200 was within the linear range of this standard curve. The inter- and intra-assay coefficient of variability (CV) of the refined ELISA assay is 4.087 (n = 3) and 3.117 (n = 32), respectively.

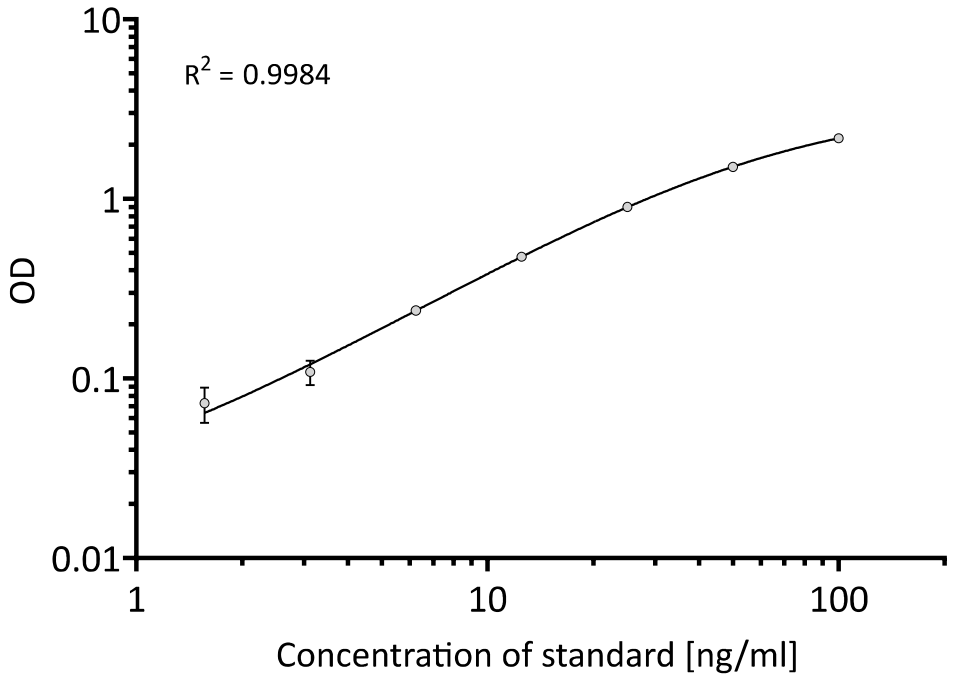

Figure 6: Representative standard curve for sHLA quantification in plasma samples using the refined ELISA. Absorbance (optical density (OD) at 450 nm) was plotted as a function of the concentration of the HLA standard monomer. Dots present mean values of triplicates with whiskers showing standard derivation (SD). Blank mean values were subtracted from all measured absorbances. The resulting standard curve was created by 4-PL nonlinear regression analysis.

Characteristics of HNC patient and control cohort

For this study, we analyzed sHLA plasma levels in two different cohorts of HNC patients (HNC cohort 1, n = 28; HNC cohort 2, n = 22) and in a control cohort of HVs (n = 61, Table 6). HNC cohort 1 comprised patients with metastatic disease under different treatment regiments who had been diagnosed in median 2.6 years ago (range 4 months - 18 years). Patients in HNC cohort 2 were recently diagnosed with HNSCC tumors and blood samples were drawn prior to therapy. Median age of HNC patients was 64 (range 53 - 83 years) and 62 years (range 42 - 85 years) for cohort 1 and 2, respectively, whereas the median age of the control cohort was 35 years (range 22 - 68 years). There are considerably more men in the HNC cohorts (79% cohort 1, 86% cohort 2), whereas they are underrepresented in the control group (28%). The most common primary tumor localization was throat (68% cohort 1, 82% cohort 2).

Table 7: Donor characteristics of HNC cohorts and HVs.

Characteristics	**HNC cohort 1**	**HNC cohort 2**	**HV**
Participants - n	28	22	61
Age - years			
Median	64	62	35
Range	53 - 83	42 - 85	22 - 68
Sex - n (%)			
Female	6 (21)	3 (14)	44 (72)
Male	22 (79)	19 (86)	17 (28)
Body mass index			
Median	n.a.	n.a.	23.1
Range	n.a.	n.a.	16.9 - 39.1
Primary tumor localization - n (%)			
Oral cavity/nose	8 (31)	4 (18)	-
Throat	19 (68)	18 (82)	-
n.a.	1	0	-
Metastases - n (%)			
Yes	22 (79)	9 (41)	-
No	6 (21)	12 (55)	-
n.a.	0	1	-
Initial therapy - n (%)			
Surgery	18 (61)	17 (82)	-
Laser surgery	2 (7)	2 (9)	-
Radiochemotherapy	4 (14)	3 (14)	-
Immunotherapy	2 (7)	0 (0)	-
Radiatio	3 (11)	0 (0)	-
Histology of tumor - n (%)			
HNSCC	22 (79)	22 (100)	-
Other	4 (14)	0 (0)	-
n.a.	2 (7)	0 (0)	-

n, number; HNC, head and neck cancer; n.a., not available; HNSCC, head and neck squamous cell carcinoma.

Soluble HLA plasma levels do not associate with demographics and vary over time

For the characterization of sHLA plasma levels in relation to different demographics including age, body mass index (BMI), and gender we first analyzed the plasma of 61 HVs (Table). Plasma levels of sHLA did not correlate with age (Figure 7A) or BMI (Figure 7B) and did not differ according to gender (Figure 7C). Longitudinal assessment of sHLA plasma levels were performed for three HVs at four different time points within a range of 20 weeks revealing relatively stable sHLA levels over time (Figure 7D).

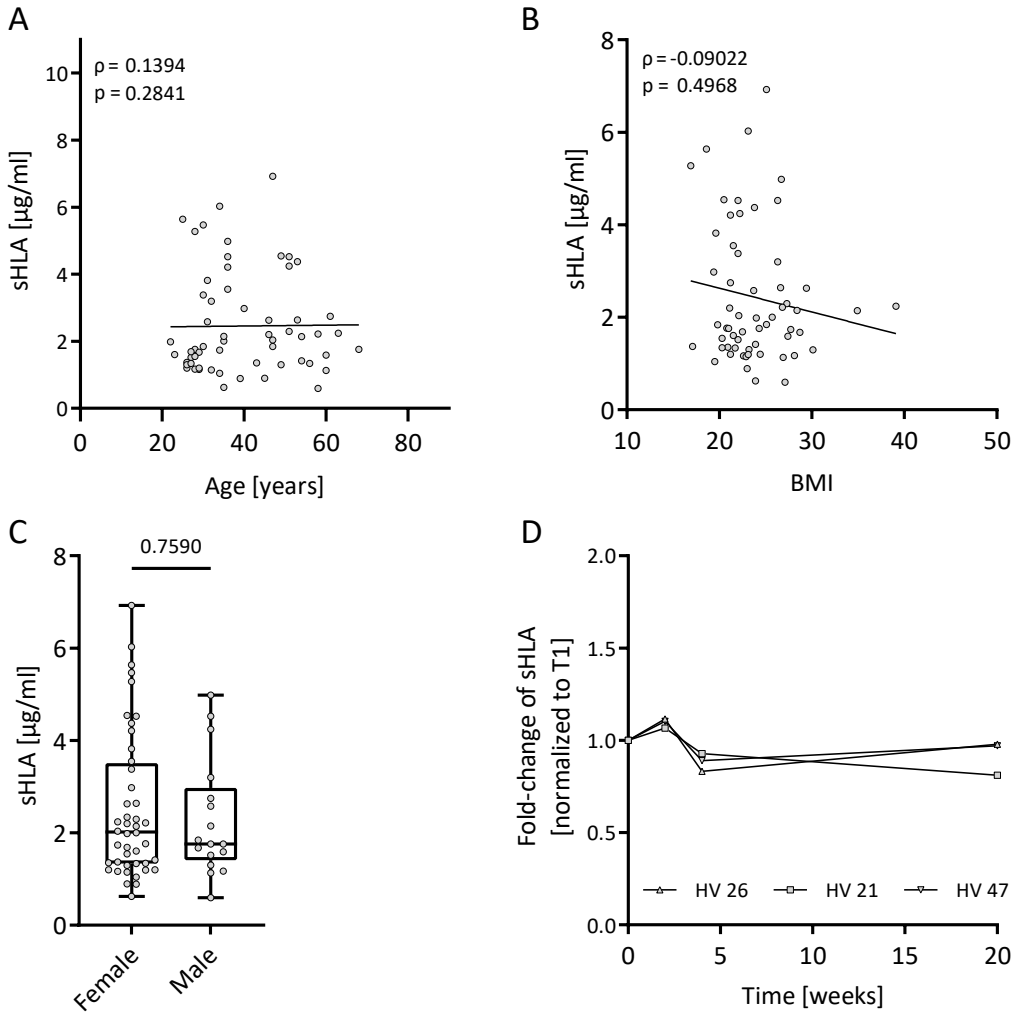

Figure 7: Characterization of sHLA plasma levels in HVs. (A, B) Correlation analysis of sHLA plasma levels to (A) age and (B) body mass index (BMI) of HVs (n = 61, linear regression, Spearman's rho (ρ) and p-value). (C) Comparison of sHLA levels between male (n = 17) and female (n = 44) HVs. Boxes represent median, 25th and 75th percentiles, whiskers are minimum and maximum, two-sided Mann-Whitney *U* test. (D) Longitudinal assessment of sHLA plasma levels in three HVs at different time points depicted as fold-change normalized to the first sample collection (T1). Each dot represents the sHLA plasma level of one donor quantified by enzyme-linked immunosorbent assay (ELISA). Lines connect measurement on different time points of the same donor.

The independence of sHLA plasma levels from age and gender observed in the cohort of HVs could also be validated in the HNC cohort 1 (Figure 8A, B).

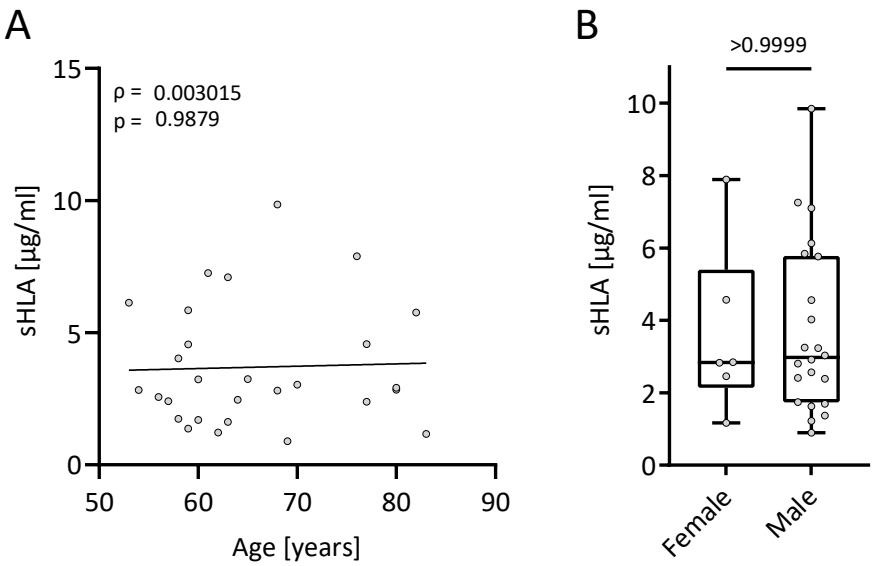

Figure 8: Plasma levels of sHLA in HNC patients according to demographics. (A) Correlation analysis of sHLA plasma levels to age of HNC patients (HNC cohort 1, n = 28, linear regression, Spearman's rho (ρ) and p-value). (B) Soluble HLA level comparison between male (n = 22) and female (n = 6) HNC patients. Boxes represent median, 25th and 75th percentiles, whiskers are minimum and maximum, two-sided Mann-Whitney *U* test. Each dot represents the sHLA plasma level of one donor quantified by enzyme-linked immunosorbent assay (ELISA).

Soluble HLA levels are increased in HNC patients but independent of tumor characteristics or therapy

Quantification of sHLA plasma levels of HNC cohort 1 (n = 28) were compared to the control cohort (HV, n = 61, **Fehler! Verweisquelle konnte nicht gefunden werden.**). The sHLA plasma levels were significantly increased in HNC cohort 1 (median 2.00 µg/ml, range 0.89 - 9.85 µg/ml, p = 0.0063) compared to HVs (median 2.88 µg/ml, range 0.60 - 6.92 µg/ml). We further compared sHLA plasma levels in HNC cohort 1 according to different tumor characteristics at the time of diagnosis such as primary tumor size, lymph node infestation and metastasis staging. The cohort was subdivided in four different groups according to the primary tumor size: up to 2 cm (n = 4, median 3.64 µg/ml), between 2 and 4 cm (n = 6, median 2.65 µg/ml), more than 4 cm (n = 7, median 2.81 µg/ml), or initial advanced local staging (n = 7, median 5.76 µg/ml). No significant differences in sHLA plasma levels were observed according to initial tumor staging (Figure 10A). Levels of sHLA in plasma of patients with (n = 14, median 3.79 µg/ml) or without (n = 10, median 4.11 µg/ml) lymph node involvement (Figure 10B)

and with (n = 7, median 1.63 µg/ml) or without (n = 18, median 3.08 µg/ml) distant metastases (Figure 10C) at the time of diagnosis did not differ according to initial lymph node involvement or metastases status.

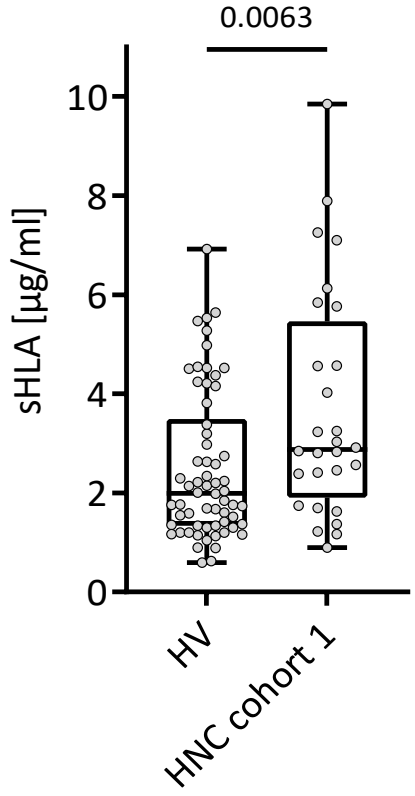

Figure 9: Comparison of sHLA plasma levels in HVs and HNC cohort 1. Comparison of sHLA levels between HVs (n = 62) and HNC cohort 1 (n = 28). Boxes represent median, 25th and 75th percentiles, whiskers are minimum and maximum, two-sided Mann-Whitney *U* test. Each dot represents the sHLA plasma level of one donor measured by enzyme-linked immunosorbent assay (ELISA).

Levels of sHLA in HNC cohort 1 did not differ between patients undergoing first line treatment chemotherapy (n = 4, median 3.47 µg/ml), checkpoint inhibitor therapy (n = 14, median 3.24 µg/ml), a combination of chemotherapy and EGFR antibodies (n = 8, median 2.69 µg/ml), or sole radiotherapy (n = 1, median 2.38 µg/ml).

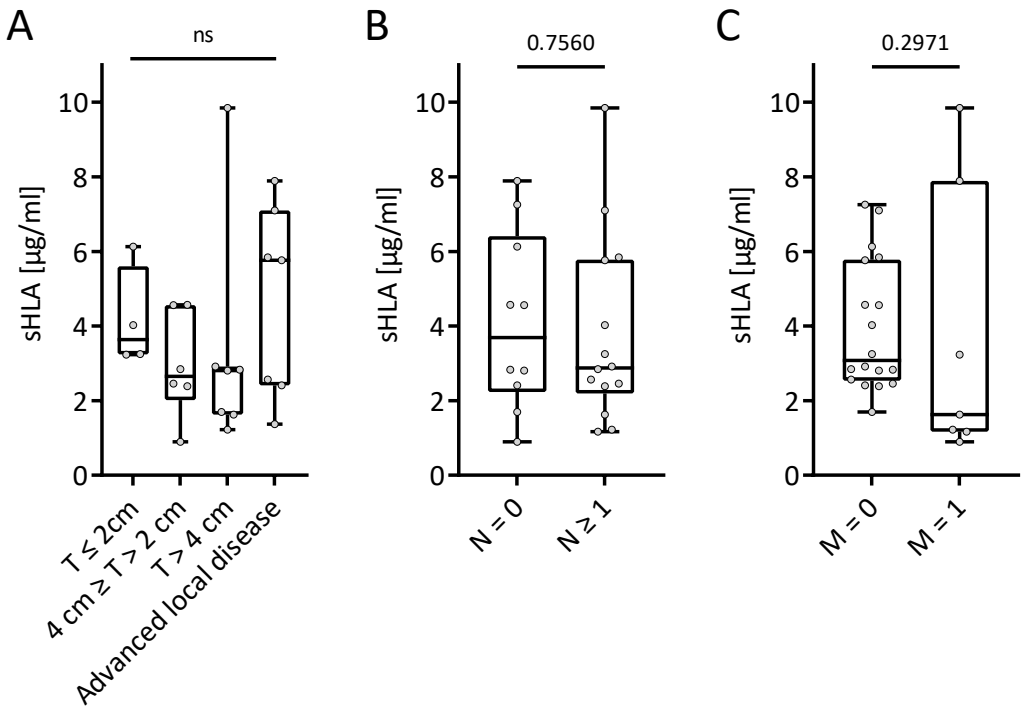

Figure 10: Comparison of sHLA levels in HNC cohort 1 regarding the initial tumor staging at the time of diagnosis. (A) Comparison analysis of sHLA plasma levels between groups of patients with different initial tumor size staging (HNC cohort 1, Boxes represent median, 25th and 75th percentiles, whiskers are minimum and maximum, Kruskal-Wallis test with Dunn's multiple comparison test). (B, C) Plasma level of sHLA comparison between patients with initially (B) no (n = 10) or at least one lymph node infestation (n = 14) and (C) no (n = 18) or at least one metastasis (n = 7). Boxes represent median, 25th and 75th percentiles, whiskers are minimum and maximum, two-sided Mann-Whitney *U* test. Each dot represents the sHLA plasma level of one donor quantified by enzyme-linked immunosorbent assay (ELISA).

Time-dependent variability of sHLA plasma levels is increased in HNC patients

Next, we investigated the influence of time since diagnosis on sHLA plasma levels in HNC cohort 1. No correlation of sHLA levels with time since diagnosis was observed (Figure 12A). Longitudinal quantification of sHLA plasma levels from up to 14 different time points of 19 patients from HNC cohort 1 and four HVs revealed higher intraindividual sHLA plasma level changes over time in HNC patients compared to HVs (highest fold-change of 2.01 in HNC cohort 1 compared to 1.19 in HV, Figure 12B).

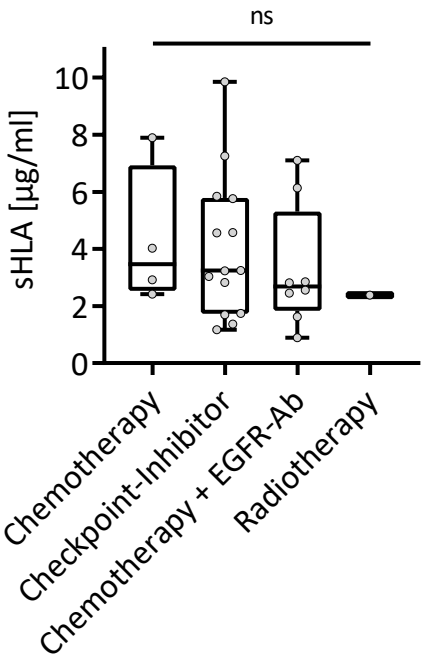

Figure 11: Comparison of sHLA levels in HNC cohort 1 regarding their first line treatment. Comparison analysis of sHLA plasma levels between groups of patients with different initial treatment (HNC cohort 1, Boxes represent median, 25th and 75th percentiles, whiskers are minimum and maximum, Kruskal-Wallis test with Dunn's multiple comparison test)**.** Each dot represents the sHLA plasma level of one donor quantified by enzyme-linked immunosorbent assay (ELISA).

Soluble HLA levels correlate with tumor mass and metastases

In a next step, we investigated the role of the current state of disease in HNC cohort 1 on the levels of sHLA in plasma. Patients suffering from distant metastatic relapse (n = 17, median 3.03 µg/ml) had significantly (p = 0.0008) increased levels of sHLA compared to patients with locoregional relapse (n = 11, median 2.56 µg/ml, Figure 13A). However, for patients with distant metastatic progression, the specific organ site of the relapse has no influence on sHLA levels (Figure 13B). Furthermore, patients presented with a high number of metastases (≥ 10 metastases, n = 11, median 4.29 µg/ml) showed significantly higher levels (p = 0.037) of sHLA than those with fewer metastases (n = 17, median 2.4 µg/ml, Figure 13C). In line, sHLA levels correlate significantly but weakly with the tumor mass at the time of sample collection (p = 0.0321, Figure 13D).

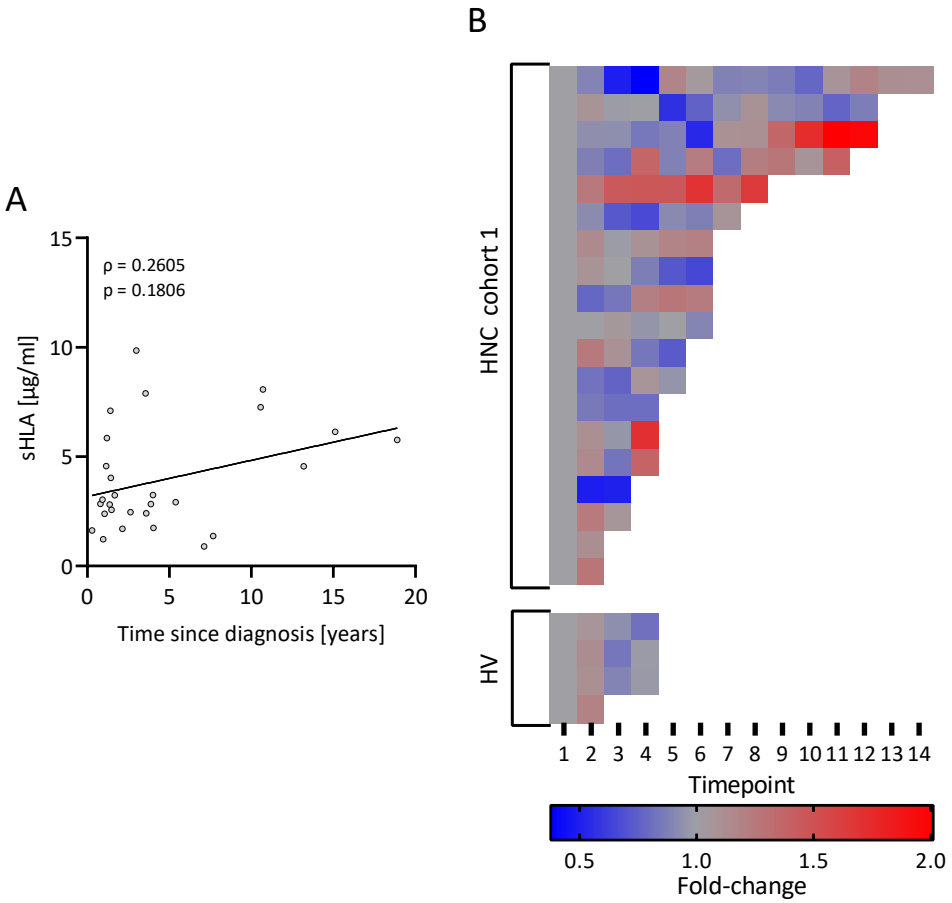

Figure 12: Plasma sHLA levels in HNC cohort 1 over time. (A) Correlation analysis of sHLA plasma levels since initial diagnosis in HNC cohort 1 (n = 28, linear regression, Spearman's rho (ρ) and p-value). (B) Heatmap of increasing (red) or decreasing (blue) sHLA plasma levels in follow-up samples of HNC cohort 1 and HVs quantified by enzyme-linked immunosorbent assay (ELISA) and displayed as fold-change to time point 1 of each respective individual.

To investigate the role of current disease progression, sHLA levels of patients with a progression of tumor mass at the time of sample collection (n = 5, median 4.65 µg/ml) were compared to patients in remission or with stable disease (n = 11, median 2.83 µg/ml, Soluble HLA plasma levels are not increased at early stages of disease in HNC patients

To investigate the role of HNC in early stages of disease, sHLA levels in plasma of patients with recently diagnosed HNC, prior to treatment, (HNC cohort 2, n = 21) were analyzed. In line with the results in HNC cohort 1 and HVs, sHLA levels did not correlate with age (Figure 15A) and did not differ according to gender (male, n = 18, median 2.22 µg/ml; female, n = 3, median 2.31 µg/ml, Figure 15B). Furthermore, no differences in sHLA plasma levels were observed between patients with grade I and II (n = 6, median 1.99 µg/ml) or grade III and IV (n = 8, median 2.31 µg/ml) tumors (Figure 15C).

Figure 14A) showing no difference in sHLA regarding disease progression. This was further investigated by comparing sHLA levels to the fold-change of tumor burden in up to 3 follow-up samples of nine different patients of HNC cohort 1 (Figure 14B).

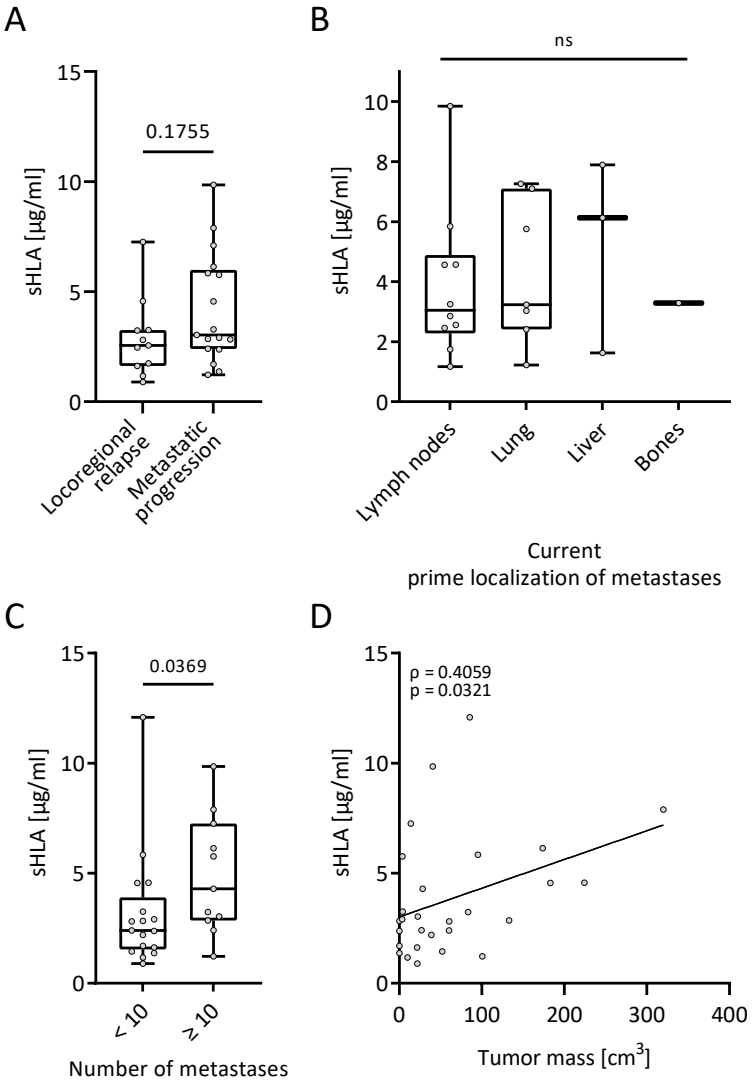

Figure 13: Influence of current tumor characteristics on sHLA plasma levels in HNC cohort 1. (A, B) Plasma sHLA level comparison between patients with (A) locoregional relapse (n =11) or metastatic progression (n = 17) and (C) less than (n = 17) or at least 10 (n = 11) metastases. Boxes represent median, 25th and 75th percentiles, whiskers are minimum and maximum, two-sided Mann-Whitney *U* test. (B) Comparison analysis of sHLA plasma levels between groups of patients with different current primary burden of tumor (Boxes represent median, 25th and 75th percentiles, whiskers are minimum and maximum, Kruskal-Wallis test with Dunn's multiple comparison test)**.** Current prime localization of metastases contains primary location (n = 2), lymph nodes (n = 10), lung (n = 7), live (n = 3) r, and bones (n = 1). (D) Correlation analysis of sHLA plasma levels with current tumor mass in HNC cohort 1 (n = 28, linear regression, Spearman's rho (ρ) and p-valu*e*). Each dot represents the sHLA plasma level of one donor measured by enzyme-linked immunosorbent assay (ELISA).

Soluble HLA plasma levels are not increased at early stages of disease in HNC patients

To investigate the role of HNC in early stages of disease, sHLA levels in plasma of patients with recently diagnosed HNC, prior to treatment, (HNC cohort 2, n = 21) were analyzed. In line with the results in HNC cohort 1 and HVs, sHLA levels did not correlate with age (Figure 15A) and did not differ according to gender (male, n = 18, median 2.22

µg/ml; female, n = 3, median 2.31 µg/ml, Figure 15B). Furthermore, no differences in sHLA plasma levels were observed between patients with grade I and II (n = 6, median 1.99 µg/ml) or grade III and IV (n = 8, median 2.31 µg/ml) tumors (Figure 15C).

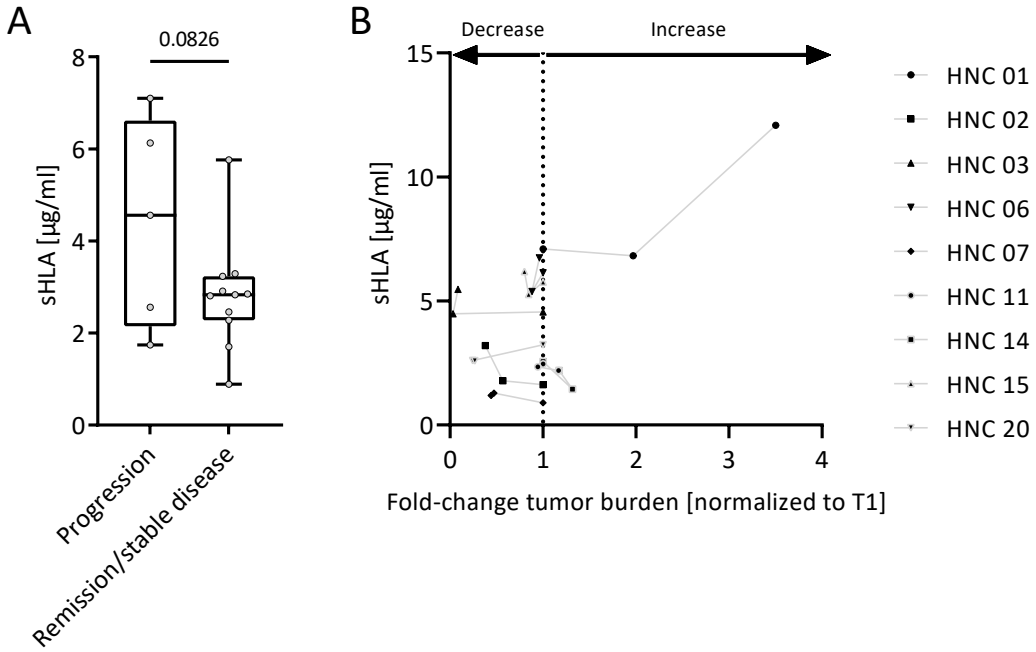

Figure 14: Influence of stage of disease on sHLA plasma levels in HNC cohort 1. (A) Levels of sHLA comparison between HNC cohort 1 with disease in progression (n = 5) or in remission/stable disease (n = 11). Boxes represent median, 25th and 75th percentiles, whiskers are minimum and maximum, two-sided Mann-Whitney *U* test. Each dot represents the sHLA plasma level of one donor measured by enzyme-linked immunosorbent assay (ELISA). (B) Longitudinal assessment of sHLA plasma levels in relation to the fold-change development of tumor burden. Measurements of tumor burden in 9 patients were set in relation to first assessed tumor burden of respective patients and compared to corresponding sHLA plasma levels. Dots represent the sHLA plasma level of donors measured by enzyme-linked immunosorbent assay (ELISA). Values below 1 on x-axis indicate a decrease in tumor burden. Lines connect measurements of individual patients.

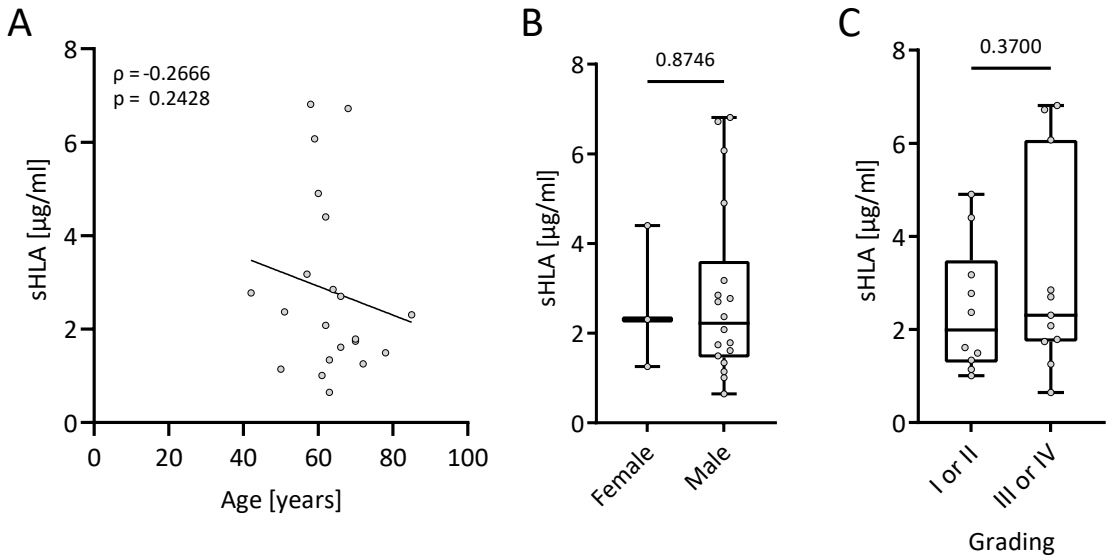

Figure 15: Plasma sHLA levels in patients with recently diagnosed and surgically treatable head neck cancer. (A) Correlation analysis of sHLA plasma levels with age of patients (n = 13, linear regression, Spearman's rho (ρ) and p-value). (B, C) Plasma sHLA level comparison between (A) male (n = 10) and female (n = 3) patients and (B) patients with I and II (n = 6) or III and IV (n = 8) tumor grading. Boxes represent median, 25th and 75th percentiles, whiskers are minimum and maximum, two-sided Mann-Whitney *U* test. Each dot represents the sHLA plasma level of one donor measured by enzyme-linked immunosorbent assay (ELISA).

Comparison of sHLA plasma levels of HVs (n = 62, median 2.00 µg/ml), HNC cohort 1 (n = 28, median 2.88 µg/ml) and HNC cohort 2 (n = 21, median 2.31 µg/ml) revealed a significant increase of sHLA levels in HNC cohort 1, but not in HNC cohort 2 compared to HVs (p = 0.0188,

Figure **16**).

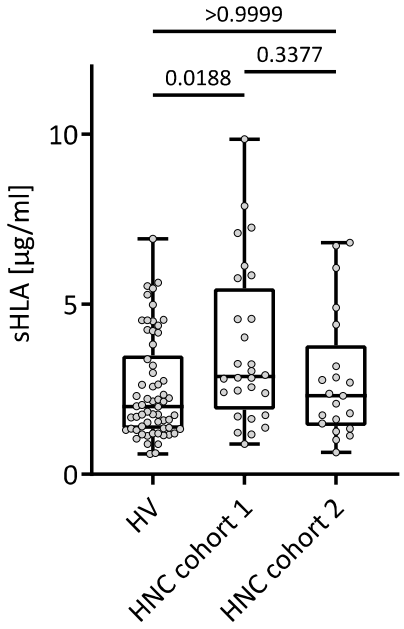

Figure 16: Comparison of sHLA levels of HVs, HNC cohort 1 and HNC cohort 2. Comparison analysis of sHLA plasma levels between groups of HVs (n = 62), HNC cohort 1 (n = 28) and HNC cohort 2 (n = 21). Boxes represent median, 25th and 75th percentiles, whiskers are minimum and maximum, Kruskal-Wallis test with Dunn's multiple comparison test**.** Each dot represents the sHLA plasma level of one donor measured by enzyme-linked immunosorbent assay (ELISA).

Discussion

Several studies have reported elevated plasma levels of sHLA in different cancer entities, as well as in various infectious and autoimmune diseases. [53, 54, 55, 80, 81, 82, 83, 84, 85, 86, 87, 88] Whereas, all those studies showed that elevated levels are associated with disease severity and outcome, the immunological role of sHLA in general and in particular in head neck cancer remains unclear.

To investigate the role of sHLA in HNC, we first established an ELISA assay for the quantification of sHLA plasma levels. The need for an in-house refinement and reestablishment of the ELISA for the quantification of sHLA plasma levels was given by the fact that the available protocol lacked consistency and sensitivity and resulted in higher levels of sHLA in HVs compared to literature.[52]

Before refinement, measurements would yield levels in the range of mg/ml, while several studies reported levels in the range of µg/ml down or even ng/ml. [54, 55, 81, 83, 84, 85, 86, 89] Commercially available kits offer even lower detection ranges in this regard, but were yielding unsatisfactory results in our attempts, therefore giving us an emerging need for refinement of our initial ELISA assay.[90]

After achieving satisfactory results in regression analyses with R^2 values > 0.995 and sufficient linearity, we validated the reliability of our assay. With both, inter-assay and intra-assay CV reaching values below 15% and 10%, respectively, we showed our assay to be reliable, consistent, and useful for future approaches regarding this topic.

In this study, we quantified total sHLA plasma levels in HNC patients with inoperable HNC under different therapies and HNC patients at primary diagnosis as well as in a cohort of healthy control donors. We could show elevated sHLA levels in advanced stage HNC patients compared to the control cohort.

In line with previous reports on sHLA serum levels in healthy individuals, demographic factors such as gender and age had no influence on sHLA plasma levels, which we were also able to show in our cancer cohorts.[91] However, we demonstrated that sHLA levels were increased in the plasma of patients with advanced HNC disease compared to HVs. But interestingly, plasma sHLA levels were not increased in recently diagnosed HNC

patients. To understand these differences between the two HNC cohorts, we looked deeper into the clinical data and searched for connections between initial staging at the point of diagnosis and their initial therapy. For both, initial staging as well as initial therapy, no differences in sHLA plasma levels were observed in HNC cohort 1, which is reasonable, due to the fact, that most of the patients were diagnosed several years ago, levels of sHLA are dynamic as already shown in healthy individuals, therefore implying that sHLA levels correlate with current state of disease, rather than giving conclusions about past stages of disease or their respective initial treatment. Conclusively, we thus investigated the role of the current tumor characteristics and metastases on sHLA plasma levels.

Even though the prime localization of tumor mass and local or distant relapses of disease did not influence levels of sHLA, the number of metastases was positively associated with it. Furthermore, considering that sHLA levels in HNC cohort 1 also correlated with the total measured tumor mass, it is arguable, that the total mass of the tumor, above a certain threshold, directly correlates with the levels of sHLA in plasma, likewise to the tumor marker Prostate-specific Antigen (PSA) that correlates also with tumor burden in patients with prostate cancer and is used as a marker für disease progression.[92] This is further in line with previous studies showing similar observations in multiple myeloma, in which levels of sHLA in plasma of patients correlates with staging of disease, which indirectly correlates with tumor mass.[55, 93] Interestingly this observation cannot be made for every tumor entity. Levels of sHLA, opposite to our results, inversely correlate with staging of disease in gastric cancer. This continuously prevents a clear conclusion about the association of sHLA and tumor mass in general.[89]

What we could show, was a trend towards increased levels when tumors are in progression rather than being stable or in regression. This effect of the tumor mass on sHLA plasma levels was confirmed by the low sHLA plasma levels within the HNC cohort 2, where the patients harbor only very small, resectable tumors. Concluding, this means, that while visible in patients with advanced disease and high tumor mass, sHLA levels do not seem to have a diagnostic benefit for patients with low overall tumor mass, therefore rendering the use of sHLA as marker for early detection of disease rather ineffective. On the other hand, sHLA might be used as a quantitative marker to survey

the increase or decrease of overall tumor mass in patients with advanced disease and rather moderate to high overall tumor mass, therefore giving rise to the possibility of a technically and economically advantageous method compared to established follow-up staging methods.

However, this approach must be critically evaluated, since we could also show, that levels of sHLA in plasma of tumor patients and healthy volunteers vary over time. sHLA levels were shown to also vary and be increased in patients with infectious disease, such as Covid-19.[91] In our study the fluctuations are more pronounced in the plasma of tumor patients compared to healthy volunteers, further complicating its use, therefore reducing the chance to use it in further approach like other tumor markers, such as PSA in prostate cancer: If the levels of PSA in serum are low and tend to be in the reference range, this marker can also be evaluated by its dynamics, for example, a higher than average increase, which can often be associated with a relapse of the tumor.[94] Due to the dynamic of sHLA in serum over time, a similar use seems not suitable and making it clear that the potential use of sHLA levels as a quantitative marker requires further research.

Possibly explanations for the more pronounced fluctuation of sHLA levels in plasma of tumor patients may be found in other reasons for increasing levels of sHLA: Inflammation, autoimmunological processes, and infection. Studies have shown that acute rejection of transplants, various infectious diseases or chronical inflammatory diseases can also lead to increase in sHLA levels in the blood. [1, 87, 95, 96, 97, 98] This suggests that besides the tumor mass, general inflammatory processes in the body of the patient, caused by infections or other chronical inflammation diseases but also by treatment such as chemotherapeutics or immunotherapeutics, may lead to increased and/or fluctuating sHLA levels, thus providing promising approaches for future studies.[98]

A limitation of this study is the focus on HLA class I. Further studies are needed to also investigate the role of sHLA class II molecules and their role in HNC. Especially comparison of levels and of sHLA class I and HLA class II would be interesting. Furthermore, the exact mechanism leading to the elevated levels in HNC patients with a larger tumor mass are still not answered. Possible explanations might be a passive release by cell death or an active secretion leading to immune suppression. The variable levels of sHLA over time within individual patients and the trend to increased sHLA levels during

tumor progression might be a hint for an active release of sHLA, when plasma sHLA levels are coupled to a certain degree to the activity of tumor cells and not their mass alone. If cell death would be leading to higher levels of sHLA we would expect overall sHLA levels to be equal or even higher in patients with tumor regression caused by cell death while the opposite seems to be true, and sHLA levels might correlate with mass and cellular metabolism of tumor cells, which is higher in patients with progression than patients with stable disease or in regression.

An interesting next step might be the mass spectrometry-based identification of peptides still bound on the sHLA molecules, the so-called soluble immunopeptidome, to go deeper into the role of sHLA in HNC.[99] Hereby, it would be interesting to compare the soluble immunopeptidome of HNC patients with the peptides bound on mHLA derived from tumor tissue. Identifying tumor-associated antigens and testing their ability to induce a T-cell dependent immune response would make them prime candidate for further immunotherapeutic approaches, e.g., in development of peptide vaccine approaches.[99] Furthermore, if tumor-associated antigens are also detectable on sHLA molecules, plasma or serum samples might be a viable choice as a liquid biopsy as shown by Bassani-Sternberg in 2010 indicating [100]. Firstly, because it could reduce the stress caused on patients and enabling almost limitless repeatable liquid biopsies, while, secondly, differences between the tumor-derived and the plasma-derived immunopeptidome might further indicate, that there is an active role of sHLA, likely in inhibiting the immune response.[101]Additionally, mapping the immunopeptidome in patients with HNC could reveal correlations between presented antigens, as well as their numbers, and clinical outcome, as was shown for other tumor entities. [102]

Furthermore, it would be interesting to investigate the role of exosomes. Since exosomes also contain sHLA on their surface and are might also be detected by our ELISA assay, it would be interesting to see if the ratios between exosomes and other forms of sHLA differ between patients and healthy cohorts. Exosomes do play an interesting role in the tumor immunosurveillance. It has been shown that tumor cells use exosomes to suppress the immune system.[103] At the same time, the role of sHLA on the surface of exosomes is controversial, as it has also been shown that the concentration of HLA on exosomes, especially in the case of HLA class II, is rather low.[104] Whether this is the same in the

case of head and neck tumors and what is presented on the HLA of the exosomes remains yet to be investigated.

Together, this study establishes a method for quantifying sHLA class I in plasma. With these methods we show that levels of sHLA are significantly higher in plasma from patients with head and neck tumors than in plasma from healthy individuals and that levels of sHLA correlate with overall tumor mass.

Summary

HLA peptides, presented on the cell surface, have also been found in small levels in soluble forms in body fluids comprising plasma. Soluble HLA levels have been shown to be increased in many cancer entities. However, the role of soluble HLA for cancer immune surveillance has not been conclusively resolved, which is of tremendous relevance with regard to upcoming peptide-based immunotherapy approaches for cancer treatment. However, inconsistent results across studies based on small and heterogenic cohorts as well as unreliable assay systems hamper the interpretation and evaluation of sHLA in cancer.

Thus, the first aim of this thesis was to, refining a method of quantifying of soluble HLA class I in plasma. In a next step we used this assay system to get first insight into the role of soluble HLA in head and neck cancer, an entity with little to no information on soluble HLA to date.

To gain insight into the role of soluble HLA, a reliable, sensitive and stable method of quantification was needed. An initially available ELISA protocol yielded values that were too high and too unstable, necessitating establishment and refinement of the procedure. After refinement we could present a protocol yielding reliable, stable levels with high sensitivity similar to those reported in the literature.

Using this assay, we quantified HLA class I levels in the plasma of patients with advanced stages of disease (cohort 1), who have been under treatment for a longer period of time, as well as patients with newly diagnosed tumors (cohort 2), who have not yet been treated. In comparison with a cohort of healthy volunteers, we could show an increased concentration of sHLA in the plasma of cohort 1, whereas no increased concentration was observed in cohort 2. In more detailed analysis, we observed that the amount of sHLA in cohort 1 correlates significantly with the tumor mass and the number of metastases in the individual patients as was already reported for other tumor entities.

In summary, we here described a reliable method for determining the concentration of sHLA in plasma samples and could show that the concentration of sHLA in the plasma of patients with head and neck tumors was elevated compared to healthy volunteers and correlates with the number of metastases and tumor mass.

Zusammenfassung

HLA-Moleküle, mit ihrem jeweilig präsentierten Antigen, normalerweise verankert auf der Zellmembran, können auch in geringen Konzentrationen in löslicher Form im Plasma nachgewiesen werden und scheinen bei unterschiedlichen Tumorerkrankungen erhöht zu sein. Die Rolle von löslichem HLA bei der Interaktion zwischen Immunsystem und Tumorzellen ist jedoch noch unklar. Im Hinblick auf immuntherapeutische Ansätze für die Krebsbehandlung vor allem im Bereich der T-Zell-basierten Immuntherapien ist dies jedoch von großer Relevanz. Unzuverlässige Messmethoden und uneindeutige Ergebnisse aus verschiedenen Studien, die auf kleinen und heterogenen Kohorten basieren, erschwerten bisher die Interpretation und Bewertung von löslichem HLA bei Tumorpatienten.

Daher war das erste Ziel dieser Arbeit die Etablierung und Weiterentwicklung einer Methode zur Quantifizierung von löslichen HLA Klasse I Molekülen im Plasma. Anschließend wurde unter Anwendung dieser Methode ein erster Einblick in die Rolle von löslichem HLA bei Patienten mit Kopf-Hals-Tumoren gegeben, einer Krebsart, über die bisher wenig Forschungsarbeiten über lösliches HLA gemacht wurden.

Um einen Einblick in die Rolle des löslichen HLAs zu erhalten, wurde eine zuverlässige, empfindliche und stabile Methode der Quantifizierung benötigt. Eine ursprünglich verfügbare ELISA-Methode lieferte zu hohe und zu instabile Werte, so dass die Methode neu etabliert und verfeinert werden musste. Die hier etablierte Methode liefert nun zuverlässige, stabile Werte mit einer hohen Empfindlichkeit, die den in der Literatur angegebenen Werten entsprechen.

Mit dieser Methode wurden die HLA-Klasse-I-Spiegel im Plasma von Patienten in fortgeschrittenen Krankheitsstadien (Kohorte 1), die bereits seit längerer Zeit behandelt wurden, sowie von unbehandelten Patienten mit neu diagnostizierten Tumoren (Kohorte 2), quantifiziert. Im Vergleich zu einer Kohorte von gesunden Probanden war eine erhöhte Konzentration von löslichem HLA im Plasma von Kohorte 1 nachweisbar, während in Kohorte 2 keine erhöhte Konzentration beobachtet werden konnte. In einer detaillierteren Analyse beobachteten wir, dass die Menge an löslichem HLA in Kohorte

1 signifikant mit der Tumormasse und der Anzahl der Metastasen bei den einzelnen Patienten korreliert, wie dies bereits für andere Tumorentitäten beschrieben worden ist.

Zusammenfassend präsentieren wir hier eine zuverlässige Methode zur Bestimmung der sHLA-Konzentration in Plasmaproben und konnten zeigen, dass die sHLA-Konzentration im Plasma von Patienten mit Kopf-Hals-Tumoren im Vergleich zu Plasma von gesunden Probanden erhöht ist und mit der Anzahl der Metastasen und der Tumormasse korreliert.

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Erklärung zum Eigenanteil der Dissertationsschrift

Die Arbeit wurde in der Abteilung für Peptid-basierte Immuntherapie unter Betreuung von Frau Prof. Juliane Walz durchgeführt.

Die Konzeption der Studie erfolgte in Zusammenarbeit mit Frau Dr. Annika Nelde und Frau Prof. Juliane Walz.

Sämtliche Versuche wurden von mir eigenständig durchgeführt.

Ich versichere, das Manuskript selbständig nach Anleitung durch Frau Dr. Annika Nelde verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Tübingen, den

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