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Kinetics of Lymphocyte Subpopulations of HNSCC Patients before, after RCT and in Follow-up

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2 List of Abbreviations

ACT	Adoptive cell therapy
APC	Allophycocyanin
BME	Bitter melon extract
CMV	Cytomegalovirus
CT	Chemotherapy
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
Ctrl	Control(s)
CUP	Cancer of unknown primary tumor
DKTK	Deutsches Konsortium für Translationale Krebsforschung
e.g.	for example
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorter
Fas	First apoptosis signal receptor
FasL	Fas ligand
FCS	Fetal calf serum
FFPE	Formalin-fixed paraffin embedded
FITC	Fluorescein isothiocyanate
FoxP3	Transcription factor forkhead box P3
g	Gravity
GI	gastrointestinal
GM-CSF	Granulocyte macrophage colony-stimulating factor
Gy	Gray
HNSCC(s)	Head and neck squamous cell carcinoma(s)
HPV	Human papillomavirus
IMRT	Intensity-modulated radiotherapy
iNKT-cell(s)	Invariant NKT-cell(s)
m²/sqm	Square meter
MFI	Mean fluorescence intensity
mg	Milligram
MHC	Major histocompatibility complex
ml	Milliliter
MRI	Magnetic resonance imaging
MRT	Magnetic resonance tomography
µl	Microliter
n.d.	non-date
NCRs	Natural cytotoxicity receptors
NED	No evidence of disease
NK-cell(s)	Natural killer cell(s)
NKT-cell(s)	Natural Killer T-cell(s)
NPC(s)	Nasopharyngeal carcinoma(s)
OL	Oral leukoplakia
OSCC	Oral squamous cell carcinoma
Pat.	Patients
PBMC(s)	Peripheral blood mononuclear cell(s)
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll-protein
PET/CT	Positron emission tomography/ computer tomography
PTI	Panoramic tomographic image
RCT	Radiochemotherapy
Rec. Pat.	Recurrent patients
Reg. Mark.	Regulatory markers
rpm	Revolutions per minute
RT	Radiotherapy
t0	before RCT
t1	directly after RCT
t2	3 months after RCT
t3	6 months after RCT
T4	CD4+ T-cells
t5	at timepoint of relapse
T8	CD8+ T-cells
TAA(s)	Tumor-associated antigen(s)
TAM(s)	Tumor-associated macrophage(s)
TIL(s)	Tumor infiltrating lymphocyte(s)
Treg(s)	Regulatory T-cell(s)
vs.	versus
WHO	World health organization

3 Introduction

3.1 Head and Neck Squamous Cell Carcinoma (HNSCC)

Head and neck cancers consist of diverse types of malignant tumors. They can be categorized by the area of the head and neck in which they begin:

Carcinoma of the oral cavity

The oral cavity includes the lips, gingiva, buccal mucosa, hard and soft palate, retromolar trigone, front two-thirds of the tongue and the floor of the mouth beneath the tongue.

Pharyngeal carcinoma

The pharynx starts behind the nose and ends at the esophagus. It can be divided into three parts: nasopharynx/ epipharynx, oropharynx/ mesopharynx and laryngopharynx/ hypopharynx. (*Head and Neck Cancers*, n.d.) (Aumüller et al., 2017, p. 914)

Laryngeal carcinoma

The larynx is a short passageway below the pharynx. It contains the supraglottis, glottis with the vocal cords and subglottis. (Aumüller et al., 2017, p. 923)

Carcinoma of the paranasal sinuses and the nasal cavity

Paranasal sinuses, located near the nasal cavity, include the maxillary sinus, ethmoid sinus, frontal sinus as well as the sphenoid sinus. (Aumüller et al., 2017, pp. 1042-1043)

Salivary gland carcinoma

The three main pairs of salivary glands are the parotid gland, the submandibular gland and the sublingual gland. (Aumüller et al., 2017, p. 1018)

Most of the head and neck cancers are squamous cell carcinomas (HNSCCs). HNSCCs often arise in the epithelial lining of the oral cavity, oropharynx, larynx and hypopharynx. (Boyle, Levin, International Agency for Research on Cancer, & World Health Organization, 2008, p. 170) Other types of head and neck cancers, which are rarely seen, are e.g. adenocarcinomas, which originate from the salivary glands. (*Head and Neck Cancers*, n.d.)

Head and neck cancer is the sixth most common cancer worldwide with an estimated annual incidence rate of 563.826 (including 274.850 oral cavity carcinomas, 159.363 laryngeal carcinomas and 52.100 oropharyngeal carcinomas) and 301.408 deaths. (Jemal et al., 2011; Parkin et al., 2005)

In 2013, 13.000 men and 4.500 women are taken ill with head and neck cancers in Germany. The average age of disease onset is 66,2 years for German women and 63,8 years for German men. In 2013, the most common subtypes of head and neck cancers are carcinomas of the oral cavity, oropharyngeal carcinomas and laryngeal carcinomas. The relative 5-year-suvival rate in Germany is 61% for women and 51% for men. The incidence rate and the mortality rate of head and neck cancers in women after age standardization have increased in the long-term. In contrast, the rates of head and neck cancers in men remain steady at a high level or rather decreased in Germany. (‘Kapitel2_Epidemiologie.pdf’, n.d., pp. 57-59)

3.2 Risk Factors

For most head and neck cancers, alcohol and tobacco are well known risk factors. In regions with high consumption of alcohol and tobacco, the incidence rate is found to be higher. The combined intake of alcohol and tobacco has a greater effect on head and neck cancer risk than smoking or drinking alone, especially for oral and pharyngeal cancers. (Hashibe et al., 2009) Chronic tobacco or alcohol abuse shows an up to six times higher risk, a combination of both risk factors shows an up to 30 times higher risk to develop laryngeal cancers. (Altieri et al., 2002; Talamini et al., 2002) The contact between the cigarette and the lips, independent of the total consumption of tobacco, counts as a main risk of disease for lip cancer. (Perea-Milla López et al., 2003) Furthermore, chewing betel quid and tobacco also count as a substantial risk factor to develop oral cancer. (Critchley, 2003) Besides the consumption of tobacco and alcohol, an unbalanced diet with much meat, fat intake and fried food increases the risk for oral cavity carcinoma. (C Bosetti et al., 2002; Cristina Bosetti et al., 2003; Levi et al., 2000; Oreggia et al., 2001) Conversely, a well-balanced Mediterranean diet reduces the risk of disease. (C. Bosetti et al., 2003)

Several countries have witnessed a decline in head and neck cancers as a result of less consumption of tobacco in the past few decades. However, Canada, Denmark, the Netherlands, Norway, Sweden, the United States and the United Kingdom have had an increase in oropharyngeal and oral cavity cancers despite the declines in tobacco use since the 1980s. As a result of this matter, people come up to theories that human papillomavirus (HPV) infection might also affect the development of certain head and neck cancers. (Chaturvedi et al., 2013)

Human papillomavirus has been known to be an important cause of anogenital cancer. Only recently, HPV has been recognized as an etiologic cause of a subset of head and neck squamous cell carcinomas (HNSCCs). (D'Souza et al., 2007) Besides alcohol, tobacco and poor oral hygiene, the majority of HPV-associated HNSCCs are caused by HPV-16. (Dahlstrom et al., 2003; Herrero et al., 2003; Marur et al., 2010) But contrarily, HPV-16 is a prognostic marker for improved disease-free and overall survival and HPV-related oropharynx cancers tend to be more responsive to chemotherapy and radiation than HPV-negative oropharyngeal cancers. (Marur et al., 2010)

90% of the adult population worldwide is infected with a human herpesvirus called Epstein-Barr virus (EBV). After primary infection, EBV gains a life-long persistence in the host. EBV is associated with virtually all poorly or undifferentiated non-keratinizing nasopharyngeal carcinomas (NPCs) regardless of geographic origin and is present in all tumor cells. (Niedobitek, 2000)

3.3 Symptoms

As head and neck cancers are often painless at stage of early growth, patients show up with a few weeks' delay for medical clarification with a delay of treatment initiation. (Hollows et al., 2000; Pitiphat et al., 2002)

Head and neck tumors differ in their symptoms according to their location. (*Symptome bei Kopf-Hals-Tumoren*, n.d.)

Apart from the b-symptoms (fever, weight loss and night sweat), other general symptoms for all types of head and neck cancers include fatigue, loss of appetite, performance drop as well as swollen cervical lymph nodes. ('007-100OLl_S3_Mundhöhlenkarzinom_122012-122015-abgelaufen.pdf', n.d. pp. 19-20)

The alteration in color and consistence of the mucosa is a sign of a precancerous condition, such as leukoplakia or erythroplakia. Apart from that, oral cavity cancers can also occur as ulcers or cause swelling, pain, foetor ex ore, bleeding, tooth loosening, reduction of tongue mobility, numbness in tongue, lips and teeth, malocclusion, poor fit of dentures, restricted mouth-opening, difficulties in speaking and fractures of jawbone. (‘007-100OLl_S3_Mundhöhlenkarzinom_122012-122015-abgelaufen.pdf’, n.d. pp. 19-20)

Symptoms of pharyngeal and laryngeal carcinomas can be difficulty in swallowing and/or breathing, frequent headaches, tinnitus, chronical hoarseness, foreign body sensation as well as chronic cough.

Carcinomas of the paranasal sinuses and the nasal cavity often cause pain in upper teeth, epistaxis and chronic sinus infections which do not respond to antibiotics in many cases.

Patients, who suffer from salivary gland cancers, become symptomatic with swelling around the jawbone, in the submandibular or sublingual area, or numbness/paralysis of the mimic musculature. (*Head and Neck Cancers*, n.d.; *Symptome bei Kopf-Hals-Tumoren*, n.d.)

3.4 Diagnostics

The diagnostic process starts with anamnesis and clinical examination. With suspicion of head and neck cancer, the clinician would continue with diagnostic imaging such as endoscopy, laryngoscopy, nasoscopy, computer tomography and magnetic resonance tomography (MRT). (*Head and Neck Cancers*, n.d.)

For patients with advanced cancer, chest X-ray, or better computer tomography of the thorax and abdominal sonography should be considered. Regarding a potential treatment with radiotherapy, a panoramic tomographic image (PTI)/ orthopantomogram should be taken into account to examine the condition of patients’ teeth.

Positron emission tomography/ computer tomography (PET/CT) are basically not of use for primary diagnostic, but for the detection of “cancer of unknown primary tumor” (CUP), remote metastases and in case of suspected relapse.

As confirmation of tumor suspicion, a biopsy should be taken from the progression zone marginal of the tumor.

(‘007-100OLl_S3_Mundhöhlenkarzinom_122012-122015-abgelaufen.pdf’, n.d. pp. 20-28)

3.5 Staging

The staging depends on the UICC-TNM classification of the tumor (UICC: Union Internationale Contre le Cancer, TNM: Tumor-Node-Metastasis), which describes the anatomical expansion of the primary tumor, the presence of regional lymph node metastases and remote metastases with involvement of other organs.
('007-100OLl_S3_Mundhöhlenkarzinom_122012-122015-abgelaufen.pdf', n.d. p. 29)

Table 0: Tumor staging system according to UICC-TNM Classification
('007-100OLl_S3_Mundhöhlenkarzinom_122012-122015-abgelaufen.pdf', n.d. p. 75)

Stadium	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T1, T2 T3	N1 N0, N1	M0 M0
IVA	T1, T2, T3 T4a	N2 N0, N1, N2	M0 M0
IVB	T4b Every T	Every N N3	M0 M0
IVC	Every T	Every N	M1

T-Classification (Primary Tumor)
TX primary tumor cannot be assessed
T0 no evidence of primary tumor
Tis carcinoma in situ
T1 greatest tumor extension 2 cm
T2 tumor extension 2-4 cm
T3 greatest tumor extension > 4cm
T4 infiltration of adjacent anatomical structures

N-Classification (Regional Lymph Node Metastases)
NX regional lymph nodes cannot be assessed
N0 no evidence of regional lymph node metastases
N1 metastasis in solitary ipsilateral lymph node up to 3 cm
N2a metastasis in solitary ipsilateral lymph node, 3-6 cm
N2b metastases in multiple ipsilateral lymph nodes up to 6 cm
N2c metastases in bilateral or contralateral lymph nodes up to 6 cm
N3 metastasis/metastases over 6 cm

M-Classification
MX remote metastases cannot be assessed
M0 no evidence of remote metastases
M1 remote metastasis/metastases

R-Status
RX presence of residual tumor cannot be assessed
R0 no evidence of residual tumor
R1 microscopic residual tumor
R2 macroscopic residual tumor

Grading
GX degree of differentiation cannot be assessed
G1 well differentiated
G2 moderately differentiated
G3 poorly differentiated
G4 undifferentiated

L (Lymph Vessel Invasion)
L0 no evidence of lymph vessel invasion
L1 presence of lymph vessel invasion

V (Vein Invasion)
V0 no evidence of vein invasion
V1 microscopic vein invasion
V2 macroscopic vein invasion

Histological Tumor Type
Prototypic HNSCC
Basaloid HNSCC
Spindle cell HNSCC
Papillary HNSCC
Verrucous HNSCC
Lymphoepithelial HNSCC

Important parameters for staging and prognosis are tumor localization, macroscopic tumor size, histological tumor type according to WHO, histological tumor grade, depth of invasion, invasion of lymph vessels and blood vessels, perineural invasion, local penetrated structures and R-status. (Kruse Gujer, Jacobsen, & Grätz, 2013, p. 148; Pai & Westra, 2009) ('007-100OLl_S3_Mundhöhlenkarzinom_122012-122015-abgelaufen.pdf', n.d. pp. 28-30, 73-74)

At time of diagnosis, 2% of patients with squamous cell carcinomas of the head and neck already have remote metastases, which are often located in the lung and bones. (Kruse Gujer et al., 2013)

A higher grading of tumor brings along a worse prognosis with more distant metastases. (Fortin et al., 2001) High categories in the TNM-Classification as well as tumor thickness of more than 4mm correlate with a worse forecast. (Carinci et al., 1998; O'Brien et al., 2003)

Papillary and verrucous HNSCCs have a better prognosis, spindle cell and basaloid HNSCCs are associated with a worse prognosis. (Barnes et al., 2007)

Cervical lymph node metastases, especially in level IV and V, and extracapsular tumor growth are associated with a worse forecast. (Woolgar et al., 2003)

HPV positive patients, who suffer from oropharyngeal cancers, are associated with a younger age, lower risk factors (less consumption of tobacco and alcohol), characteristic basaloid morphology of the tumor, poor tumor grade, increased sensitivity toward radiotherapy and a favorable outcome. (Gillison et al., 2000; Lindel et al., 2001)

3.6 Therapy

Possibilities of curative therapy include:

- Surgery
- Radiotherapy
- Radiochemotherapy
- Combination of surgery, radio- and chemotherapy

Individual therapy for each patient depends on tumor type, location and size of the primary tumor, morbidity and general health of the patient.

Surgery

Surgery should be performed under the consideration of tumor-free resection margin (R0-resection) and postoperative life quality.

If there is a clinical suspicion of cervical lymph node metastases (cN+), a modified radical neck-dissection should be carried out. ('007-100OLl_S3_Mundhöhlenkarzinom_122012-122015-abgelaufen.pdf', n.d. pp. 33-40)

In 20-40% of patients with oral cavity cancer, occult metastases are detected in the cervical lymph nodes. Patients with clinically unobtrusive cervical lymph nodes (cN0), independent of T-classification, should undergo a selective neck-dissection. Without a prophylactical neck-dissection, the prognosis of patients with metastases, which occurred at a later timepoint, is restricted, even if a radical neck-dissection is conducted later on. (McGuirt et al., 1995)

Radiotherapy (RT)

There are several types of radiotherapy:

- Percutaneous radiotherapy (ionizing radiation coming from external)
- Afterloading/ brachytherapy (radiation source next to tumor, radiation goes directly into the tumor)
- Conventionally fractionated radiotherapy (total dose 70 Gy, 1,8 - 2,0 Gy daily, 5x per week)
- Accelerated radiotherapy (> 10 Gy per week)
- Hyperfractionated radiotherapy (1,1 – 1,2 Gy, 2x daily)
- IMRT (intensity-modulated radiotherapy)

('007-100OLl_S3_Mundhöhlenkarzinom_122012-122015-abgelaufen.pdf', n.d. pp. 43-44)

The primary percutaneous radiotherapy should be hyperfractionated or accelerated. In contrast to conventionally fractionated radiotherapy, these two other methods lead to a better locoregional tumor control, but temporarily higher radiation toxicity. (Hliniak et al., 2002; Stuschke & Thames, 1997)

IMRT (intensity-modulated radiotherapy) can reduce radiation toxicity, e.g. through dose reduction on the parotid gland to prevent xerostomia. (Chao et al., 2001)

Radiochemotherapy (RCT)

The combination of radio- and chemotherapy brings along survival benefit for patients in comparison to sole radiotherapy. Especially patients with advanced, inoperable, non-metastatic squamous cell carcinomas under the age of 70, would improve more from primary radiochemotherapy than from sole radiotherapy. (Pignon et al., 2007)

The chemotherapy in the primary radiochemotherapy should contain cisplatin. ('007-100OLl_S3_Mundhöhlenkarzinom_122012-122015-abgelaufen.pdf', n.d. p. 45)

Postoperative radio- or chemoradiotherapy should be carried out in HNSCC patients with advanced T-classification (T3/T4), close or positive margins, invasion of blood vessels, nerve invasion, lymph node involvement as well as extracapsular nodal disease. Patients with histopathological criteria of high risk (resection margin < 5mm and/or extracapsular nodal disease) would improve from postoperative radiochemotherapy with cisplatin.

Postoperative radiotherapy should be started as soon as possible and conventionally fractionated. Tumors with moderate risk should receive 54-60 Gy in 27-30 fractions over 5,5-6 weeks and tumors with high risk of relapse should receive 66 Gy in 33 fractions over 6,5 weeks. (Bernier et al., 2004; J. S. Cooper et al., 2004; Peters et al., 1993) ('007-100OLl_S3_Mundhöhlenkarzinom_122012-122015-abgelaufen.pdf', n.d. p.48)

Palliative therapy for patients with good general condition comprises chemotherapy with cisplatin and cetuximab, for those with reduced general condition, monotherapy is recommended. ('007-100OLl_S3_Mundhöhlenkarzinom_122012-122015-abgelaufen.pdf', n.d. p.56)

3.7 Scientific Issue and Objective

Patients with locally advanced HNSCCs have a 5 year survival rate of 40–60%. (Ferlay et al., 2015) These mortality rates are still not satisfying despite the progress in medicine over the past decades. In inoperable cases, primary RCT is another treatment option left for head and neck cancer patients. As every squamous cell carcinoma of the head and neck is different, it is difficult to predict how they respond to treatment. (Stangl et al., 2018) Therefore, reliable biomarkers, which are able to predict outcome of therapy at an early timepoint, are urgently needed to stratify patients with respect to their prognosis and to guide adaptations for the treatment.

For example, in the paper "Heat shock protein 70 and tumor-infiltrating NK-cells as prognostic indicators for patients with squamous cell carcinoma of the head and neck after radiochemotherapy: A multicenter retrospective study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG)" two pre-treatment measures were detected, which are associated with poor prognosis of HNSCC patients after surgery and RCT. High Hsp70 expression and low numbers of tumor-infiltrating NK-cells in formalin-fixed paraffin embedded (FFPE) tumor specimens of patients have the highest negative predictive value in overall survival (OS), local progression-free survival (LPFS) and distant metastases-free survival (DMFS). The poor prognostic value of high Hsp70 expression was also observed in patient cohorts with p16-, p53- and HPV16-DNA negative tumors. (Stangl et al., 2018)

Human papillomavirus (HPV)-positivity in patients with HNSCC has been associated with improved response and superior survival rates after radiotherapy and radiochemotherapy compared to HPV-negative patients. (Ang et al., 2010) The immune phenotype (CD8high/PD-L1high) is more common in HPV16-positive tumors and associated with favorable outcome. HPV16-positive tumors express more immune markers and in conjunction with CD8, PD-L1

constitutes an independent prognostic marker in patients with HNSCC after surgery and RCT. (Balermpas et al., 2017)

Consequently, PD-1/PD-L1 immune checkpoint inhibitors could be considered to be used in combination with RCT in HNSCC patients. (Balermpas et al., 2017) Indeed, PD-L1 antibodies such as MPDL3280A, MEDI4736 and MSB0010718C have also been shown to cause disease responses in early-phase clinical trials in a number of malignancies including tumor types such as bladder cancer, head and neck cancer and GI malignancies. (Freeman et al., 2011; Herbst et al., 2013; Powles et al., 2014; Segal et al., 2014) Interestingly, patients with tumors which express PD-L1 have higher response rates to PD-1/PD-L1 blockade than those which do not express PD-L1. (Grosso et al., 2013; Topalian et al., 2012; Weber et al., 2013)

High levels of CD8+ tumor infiltrating lymphocytes (TILs) also constitute an independent prognostic marker in HNSCC patients treated with adjuvant radiochemotherapy, which indicates the antitumor activity of CD8-positive TILs. (Balermpas et al., 2016)

All these potential prognostic markers in response to adjuvant radiochemotherapy mentioned above are collected from FFPE of patients who underwent surgery. Therefore, other prognostic biomarkers in response to primary radiochemotherapy, which can be detected in the peripheral blood of patients, are needed for inoperable patients.

It is known that RT and CT result in several changes in immune-related parameters such as composition, phenotype and function of immune cells. And immune cells can be regarded as reliable biomarkers because their abundance or activation status often forecasts how well patients respond to a particular treatment regimen. (Coffelt & de Visser, 2015) The effects of oncological therapies on lymphocyte subpopulations have been of great interest as therapy-induced changes in immune cell homeostasis might interfere with anti-tumor activity. (P. J. Schuler et al., 2013)

Thus, we investigated the composition of lymphocyte subgroups in the peripheral blood of HNSCC patients treated with primary RCT before and at different timepoints after RCT.

Last but not least, the detailed knowledge of immunity phenomena of radiotherapy and chemotherapy can also provide possible starting points for additional immunomodulatory therapies, which can be combined with RT and CT. The link between RCT and immunotherapy is of great importance as new synergetic treatment methods could be discovered to improve patients' clinical outcome and survival.

4 Material and Methods

4.1 Study Collective

Thirty-five patients, four out of these suffered from a relapse at a later timepoint, with histologically confirmed HNSCC of the oral cavity, oro- and hypopharynx, who received primary radiochemotherapy, were recruited into the HNprädBio study from DTKT (Deutsches Konsortium für Translationale Krebsforschung). This trial was approved by the ethical committees of all eight DKTK partner sites.

All patients have HNSCC at stadium III or IV without any remote metastases and general condition according to WHO 0-2 (WHO classification in appendix). They received primary radiochemotherapy, intensity modulated radiotherapy with a total radiation dose of 69-72 Gy on the boost volume, >49 Gy on the elective volume and chemotherapy with cisplatin, with a cumulative total dose at least 180mg/m² body surface. The boost volume includes the primary tumor and suspected lymph nodes in the diagnostic imaging. Tumor volume and lymphatic pathways on both sides rank among the elective volume. Thermoplastic masks were used for immobilization. Contrast-enhanced computer tomography-, MRI- or PET-CT-scans were used for treatment planning and for follow up.

Blood (20ml EDTA blood) was collected from patients at five different timepoints: before RCT (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after RCT and at time of suspicion of recurrence or metastases or rather after their histological examination (t5), with a median time of 9 months (range: 7-11 months) after t0.

Blood samples of twenty-two age-related healthy blood donors with a median age of 64 years (range: 27-80 years) were collected as a control collective.

4.2 Flow Cytometry/ FACS (Fluorescence Activated Cell Sorter)

Flow cytometry is a method of measuring multiple chemical and physical characteristics of particles by optical means. The samples are in suspensions of single cells or other particles in a suitable buffer, usually PBS/10% FCS (fetal calf serum), to ensure that each cell is measured independently.

Flow cytometer was first used in the 1950s, many innovations have been made and nowadays, the flow cytometer is able to detect up to 30 parameters, all measured on the same cell in a matter of seconds. A typical cytometer consists of fluidics system, optical system and electronics. The fluidics system transports the sample from the sample tube to the flow cell. Once the sample goes through the flow cell and passes the laser, the sample is either sorted (in case of FACS) or transferred to waste. The optical system includes excitation light sources, lenses and filters used to move and collect light around the instrument, and the detection system which generates the photocurrent. The electronics digitalize and process the photocurrent from the detector, which is saved for subsequent analysis.

Antibody stained cells are distributed to a single cell suspension and aspirated through a nozzle of the flow cytometer. After excitation with laser light, the light beam illustrates a single cell, which causes the light to scatter. The light scatter, depending where the light is collected with respect to its path, can be measured as forward angle scatter (FSC) and side angle scatter (SSC). At the same time, light from the laser will also excite fluorophores to produce a fluorescence emission. All these parameters can be detected by the electronics component of the flow cytometer and the no longer needed sample is carried by the fluidics system to the

waste container. In a cell sorter, the cells would be passed to a collection tube and used for further experiments.
These following parameters can be measured:

- Cell count
- Cell sorting (with FACS)
- Relative size (Forward Angle Scatter - FSC), measured along the axis of the incoming light
- Granularity or complexity (Side Angle Scatter - SSC), measured in 90° direction to the excitation light
- Fluorescence intensity (FL1, FL2, FL3, FL4) of the fluorochrome molecule (e.g. APC, PerCP, FITC or PE)

(*How a Flow Cytometer Works*, n.d.)

In my experiment, these following lymphocyte subpopulations were analyzed by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences):

- B-lymphocytes in %
- T-lymphocytes (T-helper-cells, Cytotoxic T-cells, Natural killer T-cells, Regulatory T-cells) in %
- Natural killer cells in %
- Subsets of Natural killer cells (Regulatory Markers) in % / MFI
- Subsets of Natural killer cells (CD56/CD16) in %

A representative instrument setting of the FACSCalibur for the analysis of lymphocyte subpopulations is summarized in Table 1.

4.3 Procedure

100µl of EDTA-whole blood was mixed with fluorescence-labeled, undiluted antibody combinations in Eppendorf tubes (Table 2: Antibody panel and antibody combinations). After that, the tubes were incubated in the dark at room temperature for 15 minutes. Then the mixtures in Eppendorf tubes were washed in flow cytometry buffer (PBS buffer with 10% heat-inactivated fetal calf serum, Sigma F7524) to avoid unspecific binding of antibodies and centrifugated at 1500rpm=500g at room temperature for 5 minutes. After that, supernatants were discarded and FACS lysing solution with 1+9 dilution with H2O was added into each Eppendorf tube. Then the tubes were vortexed shortly, incubated in the dark at room temperature for 10 minutes and centrifugated at 1500rpm=500g at room temperature for 5 minutes. Subsequently, supernatants were discarded and again, the tubes were washed with flow cytometry buffer, centrifuged at 1500rpm=500g at room temperature for 5 minutes. Supernatants were discarded afterwards and finally, the Eppendorf tubes were analyzed by FACS (fluorescence activated cell sorter).

Regulatory T-cells were analyzed after fixation with Human FoxP3 buffer A (BD Pharmingen, Category number 51-9005451), permeabilization with Human FoxP3 buffer A and B (BD Pharmingen, Human FoxP3 Buffer B, Category number 51-9005450), dye with fluorescence-labeled antibodies and washing procedure.

The measurements were analyzed by the software BD CellQuest Pro. The percentage of population positive for each marker was determined with the help of quadrant statistics. The population of interest (here lymphocytes) was gated and compensations were made to avoid signal overspill. Percentage of lymphocyte subpopulations is defined as the percentage of cells within the lymphocyte gate. MFI describes the mean fluorescence intensity on the surface of cells.

Table 1

Representative instrument settings of FACSCalibur instrument (BD) for measuring blood samples by flow cytometry

Cytometer Type	FACSCalibur			
Detectors/Amps				
	Detector	Voltage	AmpGain	Mode
P1	FSC	E00	1.60	Lin
P2	SSC	368	2.00	Lin
P3	FL1	585	1.00	Log
P4	FL2	607	1.00	Log
P5	FL3	628	1.00	Log
P6	FL1-A		1.00	Lin
P7	FL4	616		Log
Threshold				
Primary Parameter	FSC			
Value	100			
Secondary Parameter	None			
Compensation				
FL1	-	0.3 %	FL2	
FL2	-	50.3 %	FL1	
FL2	-	0.0 %	FL3	
FL3	-	8.6 %	FL2	
FL3	-	0.0 %	FL4	
FL4	-	5.6 %	FL3	

Table 2

Antibody panel and antibody combinations

Specificity	Antibody	Company	Cat. No.	Volume (µl)
Ctrl	IgG1-FITC	BD	345815	5
	IgG1-PE	BD	345816	5
	IgG1-PerCP	BD	345817	5
	IgG1-APC	Caltag/Invitrogen	MG 105	1
T/NK	CD94-FITC	BD	555888	5
	CD56-FITC	BD	345811	5
	CD4-FITC	BD	555346	20
	CD8-FITC	BD	555366	20
B/T/NK	CD56-PE	BD	345811	5
	CD19-PE	BD	555413	20
	CD16-PE	BD	555407	10
T/NK	NKG2D-PE	R&D	FAB139P	10
	NKp30-PE	BC	PN IM 3709	10
	NKp46-PE	BC	PN IM 3711	10
	CD8-PE	BD	555366	20
T	CD3-PerCP	BD	345766	10
T/NK	CD45-APC	Caltag/Invitrogen	MHCD 4505	1
	CD69-APC	BD	340560	5
	CD56-APC	BD	555518	10
	CD25-APC	BD	340907	5

Abbreviations: Ctrl, Controls; BD, Becton Dickinson Biosciences; BC, Beckmann Coulter;

4.4 Statistics

Statistical differences between sets of data were evaluated by IBM SPSS Statistics software (IBM GmbH, Ehningen, Germany). For data sets following a normal distribution paired/unpaired T-test was used. In not normally distributed data sets Wilcoxon rank sum test was used for dependent, Mann-Whitney-U test for independent samples. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples, values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples. Data sets were considered as statistically significantly different at $p \leq 0.05$.

5 Results

The percentage of lymphocyte subpopulations is defined as the percentage of cells within the lymphocyte gate.

MFI describes the mean fluorescence intensity on the surface of cells.

In the comparisons between recurrent patients and controls, as well as recurrent patients and patients, we could not detect any significances in the lymphocyte subpopulations due to the small number of recurrent patients (n=4). In addition, there was only one value at timepoint t2. Thus, only tendencies are described in the group of recurrent patients.

Figures represent the composition of lymphocyte subpopulations of controls, patients and recurrent patients at different timepoints t0, t1, t2, t3 and t5 of RCT. The boxplots comprise median value as well as mean value (x = mean value). The mere significances, which are illustrated in the figures, are between t0 and other timepoints of RCT of patients as well as between controls and all timepoints of RCT of patients.

In NK-cell subgroups with MFI values, other significances, e.g. significances between different timepoints among each other, are also presented in the figures.

Abbreviations: pat, patients; rec, recurrent patients; ctrl, controls; vs., versus;

5.1 Subsets of Lymphocytes in %

Table 3A – Course of Lymphocyte Subsets of Pat. throughout RCT in %

Composition of lymphocyte subpopulations in % (mean value ± standard deviation) in head and neck cancer **patients without relapse n=31** before (t0), after RCT (t1), 3 months after (t2) and 6 months after (t3). Values at different timepoints during RCT of each patient were considered as dependent samples (Wilcoxon rank sum test). **Significantly different values (t0 vs. tx) are indicated in bold (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).**

Time	t0	t1	t2	t3
B-cells in % CD3-/CD19+	8.2 ± 4.29	**3.85 ± 3.25*** **	6.84 ± 4.68	9.8 ± 5.51
T-cells in % CD3+/CD56-	68.37 ± 14.34	66.1 ± 14.86	**57.9 ± 14.03** **	**55.17 ± 11.9*** **
T-helper-cells in % CD3+/CD4+	46.04 ± 12.38	41.6 ± 11.65	**25.9 ± 10.17*** **	**26.07 ± 9.84*** **
Cytotoxic T-cells in % CD3+/CD8+	16.78 ± 7.03	18.36 ± 7.39	**23.93 ± 10.76*** **	**20.47 ± 9.44***
Natural killer T-cells in % CD3+/CD56+	4.3 ± 3.18	**6.73 ± 4.45*** **	6.54 ± 4.32	**6.45 ± 4.77** **
Regulatory T-cells in % CD3+/CD4+ CD25+/FoxP3+	6.86 ± 3.47	6.02 ± 2.88	**10.36 ± 5.1** **	**10.14 ± 6.81** **
Natural killer cells in % CD3-/CD56+	10.93 ± 11.36	12.51 ± 11.8	**15.16 ± 11.09** **	**16.8 ± 10** **

Table 3B – Lymphocyte Subsets of Pat. vs. Ctrl in %

Composition of lymphocyte subpopulations in % (mean value ± standard deviation) in healthy **controls (Ctrl) n=22** and head and neck cancer **patients n=31** before (t0), after RCT (t1), 3 months after (t2) and 6 months after (t3). Values at different timepoints of patients in comparison with values of controls were considered as independent samples (Mann-Whitney-U test). **Significantly different values (Ctrl vs. tx) are indicated in bold (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).**

	Ctrl	t0	t1	t2	t3
B-cells in % CD3-/CD19+	10.83 ± 2.71	**8.2 ± 4.29***	**3.85 ± 3.25*****	**6.84 ± 4.68****	9.8 ± 5.51
T-cells in % CD3+/CD56-	67.29 ± 7.41	68.37 ± 14.34	66.1 ± 14.86	**57.9 ± 14.03****	**55.17 ± 11.9*****
T-helper-cells in % CD3+/CD4+	48.82 ± 9.24	46.04 ± 12.38	**41.6 ± 11.65***	**25.9 ± 10.17*****	**26.07 ± 9.84*****
Cytotoxic T-cells in % CD3+/CD8+	10.83 ± 3.81	**16.78 ± 7.03****	**18.36 ± 7.39*****	**23.93 ± 10.76*****	**20.47 ± 9.44*****
NKT-cells in % CD3+/CD56+	2.46 ± 1.95	**4.3 ± 3.18***	**6.73 ± 4.45*****	**6.54 ± 4.32*****	**6.45 ± 4.77*****
Regulatory-T-cells in % CD3+/CD4+ CD25+/FoxP3+	9.92 ± 4.19	**6.86 ± 3.47****	**6.02 ± 2.88*****	10.36 ± 5.1	10.14 ± 6.81
NK-cells in % CD3-/CD56+	11.7 ± 5.15	10.93 ± 11.36	12.51 ± 11.8	15.16 ± 11.09	**16.8 ± 10***

Table 4 – Lymphocyte Subsets of Rec. Pat. vs. Ctrl in %

Composition of lymphocyte subpopulations in % (mean value ± standard deviation) in healthy **controls (Ctrl) n=22** and head and neck cancer **patients with relapse n=4** before (t0), after RCT (t1), 3 months after (t2) and at time of relapse (t5). Values at different timepoints during RCT of each recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). **Significantly different values (t0 vs. tx); (Ctrl vs. tx) are indicated in bold (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).**

	Ctrl	t0	t1	t2	t5
B-cells in % CD3-/CD19+	10.83 ± 2.71	10.09 ± 12.55	5.47 ± 6.55	1.89 (single value)	6.63 ± 4.06
T-cells in % CD3+/CD56-	67.29 ± 7.41	68.03 ± 17.13	84.29 ± 13.73	69.74 (single value)	52.7 ± 6.84
T-helper-cells in % CD3+/CD4+	48.82 ± 9.24	25.89 ± 14.3	38.26 ± 8.78	31.32 (single value)	31.74 ± 8.12
Cytotoxic T-cells in % CD3+/CD8+	10.83 ± 3.81	23.81 ± 13.64	20.9 ± 19.83	35.1 (single value)	18.82 ± 9.35
NKT-cells in % CD3+/CD56+	2.46 ± 1.95	8.67 ± 10.93	9.93 ± 13.18	24.02 (single value)	9.31 ± 10.05
Regulatory-T-cells in % CD3+/CD4+ CD25+/FoxP3+	9.92 ± 4.19	7.45 ± 3.41	6.25 ± 5.34	0.39 (single value)	7.37 ± 2.66
NK-cells in % CD3-/CD56+	11.7 ± 5.15	3.81 ± 3.72	6.32 ± 3.96	2.56 (single value)	9.73 ± 5.5

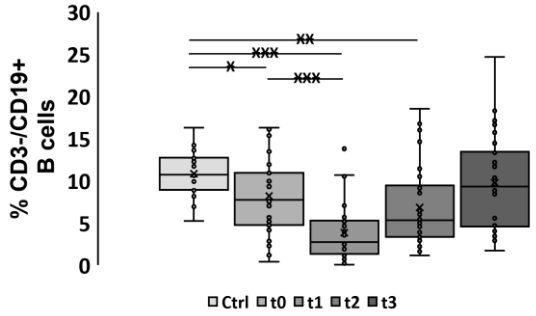
5.1.1 CD3-/CD19+ B-Cells in %

B-lymphocytes, as the crucial component of humoral immunity, function in immunoglobulin (Ig) production, antigen presentation and secretion of pro-inflammatory cytokines and human squamous cell carcinomas of the head and neck show hallmarks of B-cell infiltration. (Affara et al., 2014; Gunderson & Coussens, 2013) In our study, B-cells seem to be the most sensitive lymphocyte subpopulation to radiochemotherapy.

As shown in Table 3A and Figure 1, there was a striking drop in the percentage of CD3-/CD19+ B-cells of head and neck cancer patients (n=31) directly after RCT at t1 (t0 vs. t1: 8.2 ± 4.29% vs. 3.85 ± 3.25%, ***p ≤ 0.001), but B-cells recovered throughout t2 (t1 vs. t2: 3.85 ± 3.25% vs. 6.84 ± 4.68%, *p ≤ 0.05) and t3 (t2 vs. t3: 6.84 ± 4.68% vs. 9.8 ± 5.51%, **p ≤ 0.01).

Interestingly, patients (n=31) had significantly lower % of B-cells in respect of the total amount of lymphocytes at timepoints t0, t1 and t2 compared to healthy controls (ctrl vs. t0: 10.83 ± 2.71% vs. 8.2 ± 4.29%, *p ≤ 0.05; ctrl vs. t1: 10.83 ± 2.71% vs. 3.85 ± 3.25%, ***p ≤ 0.001; ctrl vs. t2: 10.83 ± 2.71% vs. 6.84 ± 4.68%, **p ≤ 0.01).
(Figure 1, Table 3A and 3B)

Like B-cells in patients, B-cells of recurrent patients are also sensitive to RCT. The data of recurrent patients (n=4) also showed a drop in % of B-cells at t1 and t2 (t0 vs. t1: 10.09 ± 12.55% vs. 5.47 ± 6.55%, t1 vs. t2: 5.47 ± 6.55% vs. 1.89%), but B-cells of recurrent patients tended to have a slower recovery until the relapse at t5 (6.63 ± 4.06%) in comparison with patients without recurrence (t2: 6.84 ± 4.68%, t3: 9.8 ± 5.51%). Furthermore, patients with relapse at t5 had less % of B-cells than controls (ctrl vs. t5: 10.83 ± 2.71% vs. 6.63 ± 4.06%).
(Figure 2, Table 4)



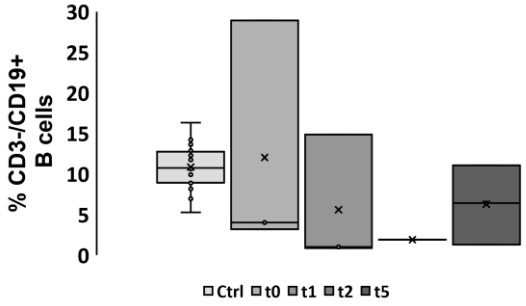


Figure 1: % B-cells, Ctrl vs. Patients **Figure 2: % B-cells, Ctrl vs. Recurrent Patients**

Percentages of CD3-/CD19+ B-cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

5.1.2 CD3+/CD56- T-Cells in %

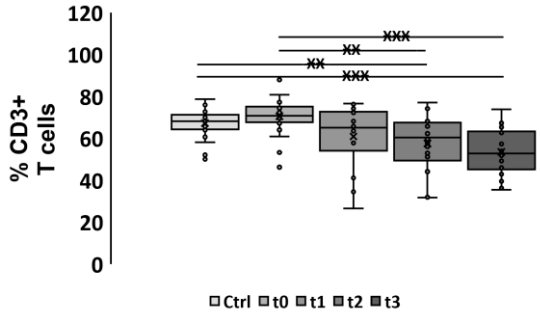
In our study, patients as well as recurrent patients received primary RCT with the chemotherapeutic agent cisplatin. It is known that cisplatin's systemic toxicity causes reduction of T-cell proliferation. (Sfikakis et al., 1996)

During the course of RCT, the percentage of CD3+/CD56- T-cells of patients dropped significantly (t0 vs. t2: 68.37 ± 14.34% vs. 57.9 ± 14.03%, **p ≤ 0.01; t0 vs. t3: 68.37 ± 14.34%

vs. 55.17 ± 11.9%, ***p ≤ 0.001; t1 vs. t2: 66.1 ± 14.86% vs. 57.9 ± 14.03%, *p ≤ 0.05). In the same way, data of head and neck cancer patients showed significantly lower % of T-cells than controls at timepoints of t2 and t3 (ctrl vs. t2: 67.29 ± 7.41% vs. 57.9 ± 14.03%, **p ≤ 0.01, ctrl vs. t3: 67.29 ± 7.41% vs. 55.17 ± 11.9%, ***p ≤ 0.001).
(Figure 3, Table 3A and 3B)

Likewise, the % of T-cells of recurrent patients also decreased, even under the % of T-cells of healthy controls and patients at all timepoints, until t5 (t0 vs. t5: 68.03 ± 17.13% vs. 52.7 ± 6.84%, ctrl vs. t5: 67.29 ± 7.41% vs. 52.7 ± 6.84%), but the course during RCT was different and there was a noticeable rise at t1. The % of T-cells of recurrent patients at t1 was even higher than those of controls and patients at all timepoints (rec t0 vs. rec t1: 68.03 ± 17.13% vs. 84.29 ± 13.73%, rec t1 vs. ctrl, pat t0, pat t1, pat t2, pat t3: 84.29 ± 13.73% vs. 67.29 ± 7.41%, 68.37 ± 14.34%, 66.1 ± 14.86%, 57.9 ± 14.03%, 55.17 ± 11.9%).
(Figure 3 and 4, Table 3B and 4)

Thus, suggesting that recurrent HNSCC patients are more likely to show abnormalities of T-cell subpopulations. (Johnson et al., 1984) Besides head and neck cancer patients, patients with myeloma, breast -, ovarian - as well as liver cancer have also been observed to have abnormalities in T-cell counts. (Kay et al., 2001; Melichar, Tousková, Solichová, Králicková, & Kopecký, 2001; Schröder, Vering, Stegmüller, & Strohmeier, 1997; Wolf et al., 1985)



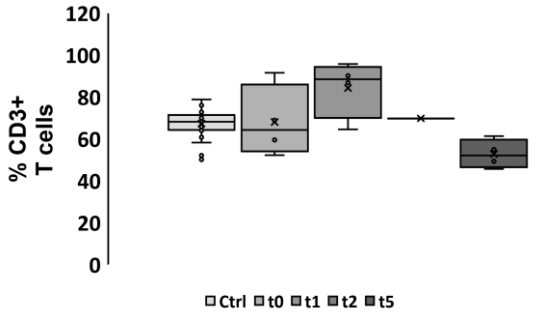


Figure 3: % T-cells, Ctrl vs. Patients **Figure 4: % T-cells, Ctrl vs. Recurrent Patients**

Percentages of CD3+/CD56- T-cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

5.1.3 CD3+/CD4+ T-Helper-Cells in %

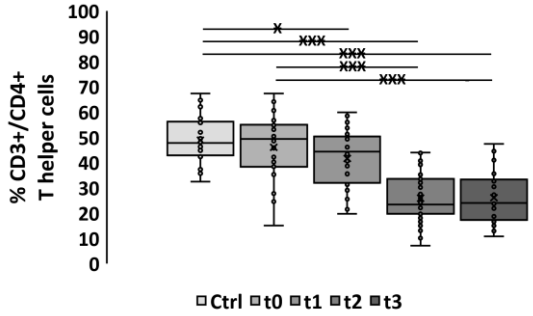
CD3+/CD4+ T-helper-cells made up to 2/3 of the total amount of T-cells and patients had a course of CD3+/CD4+ T-helper-cells similar to that of CD3+/CD56- T-cells during RCT and in the follow-up. The % of T-helper-cells dropped significantly at t2 and t3 (t0 vs. t2: 46.04 ± 12.38% vs. 25.9 ± 10.17%, ***p ≤ 0.001; t0 vs. t3: 46.04 ± 12.38% vs. 26.07 ± 9.84%, ***p ≤ 0.001; t1 vs. t2: 41.6 ± 11.65% vs. 25.9 ± 10.17%, ***p ≤ 0.001) and HNSCC patients had lower % of T-helper-cells at all timepoints than controls (ctrl vs. t0: 48.82 ± 9.24% vs. 46.04 ± 12.38 %, ctrl vs. t1: 48.82 ± 9.24% vs. 41.6 ± 11.65%, *p ≤ 0.05; ctrl vs. t2: 48.82 ± 9.24% vs. 25.9 ± 10.17%, ***p ≤ 0.001; ctrl vs. t3: 48.82 ± 9.24% vs. 26.07 ± 9.84%, ***p ≤ 0.001).

Remarkably, the % of T-helper-cells of patients at t2 and t3 were almost half of that of t0 and of controls (ctrl, t0 vs. t2, t3: 48.82 ± 9.24%, 46.04 ± 12.38 % vs. 25.9 ± 10.17%, 26.07 ± 9.84%).
(Figure 5, Table 3A and 3B)

Like T-cells of patients, T-helper-cells of HNSCC patients did not recover 6 months after RCT, which suggest that effects of the tumor or anti-tumor therapy on the homeostasis of lymphocytes can persist months after RCT.

Recurrent patients had a much lower % of T-helper-cells at t0 than patients at t0 and controls (ctrl vs. rec t0: 48.82 ± 9.24% vs. 25.89 ± 14.3%, pat t0 vs. rec t0: 46.04 ± 12.38% vs. 25.89 ± 14.3%) and they had lower % of T-helper-cells at all timepoints than controls (ctrl vs. t0, t1, t2, t5: 48.82 ± 9.24% vs. 25.89 ± 14.3%, 38.26 ± 8.78%, 31.32%, 31.74 ± 8.12%) although this lymphocyte subpopulation tended to grow at t1 (t0 vs. t1: 25.89 ± 14.3% vs. 38.26 ± 8.78%). (Figure 5 and 6, Table 3B and 4)

A low % of T-helper-cells before treatment at t0 might be a possible marker for HNSCC patients, suggesting the likelihood of these patients to suffer from recurrent disease at a later timepoint.



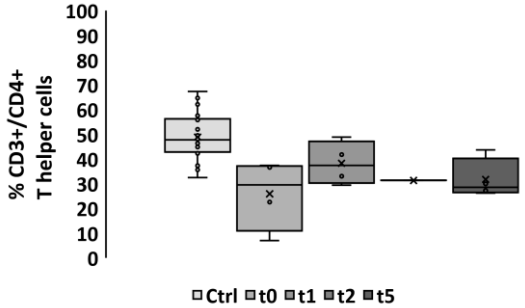


Figure 5: % T-helper-cells, Ctrl vs. Patients **Figure 6: % T-helper-cells, Ctrl vs. Recurrent Patients**

Percentages of CD3+/CD4+ T-helper-cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

5.1.4 CD3+/CD8+ Cytotoxic T-Cells in %

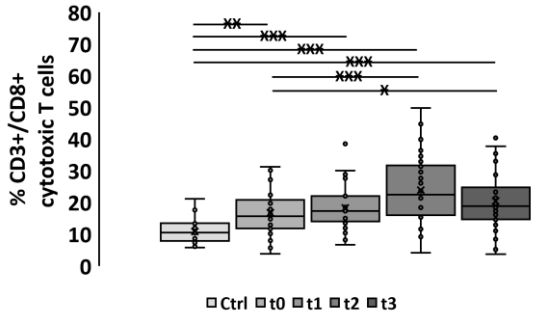
In contrast to T-helper-cell subpopulation, HNSCC patients had significantly higher % of CD3+/CD8+ Cytotoxic T-cells at all timepoints t0, t1, t2 and t3 than controls; and patients at t2 even reached values more than twice of those of controls (ctrl vs. t0: 10.83 ± 3.81% vs. 16.78 ± 7.03%, **p ≤ 0.01; ctrl vs. t1: 10.83 ± 3.81% vs. 18.36 ± 7.39%, ***p ≤ 0.001; ctrl vs. t2; 10.83 ± 3.81% vs. 23.93 ± 10.76%, ***p ≤ 0.001; ctrl vs. t3: 10.83 ± 3.81% vs. 20.47 ± 9.44%, ***p ≤ 0.001). Interestingly, the % of Cytotoxic T-cells of patients increased during and after RCT significantly with an exceptional drop at t3 (t0 vs. t2: 16.78 ± 7.03% vs. 23.93 ± 10.76%, ***p ≤ 0.001; t0 vs. t3: 16.78 ± 7.03% vs. 20.47 ± 9.44%, *p ≤ 0.05; t1 vs. t2: 18.36 ± 7.39% vs. 23.93 ± 10.76%, ***p ≤ 0.001; t2 vs. t3: 23.93 ± 10.76% vs. 20.47 ± 9.44%, *p ≤ 0.05). (Figure 7, Table 3A and 3B)

As CD8+ T-cells are potent mediators of antitumor immunity (Ho et al., 2002), a significant rise in the % of Cytotoxic T-cells of HNSCC patients throughout RCT, and also compared to controls, suggests the anti-tumor immune reaction along with higher % of CD8+ T-cells in HNSCC patients before, during and in the follow-up of RCT.

Recurrent patients also tended to have higher % of Cytotoxic T-cells than controls, values at t0 and t2 were more than twice higher than those of controls. (ctrl vs. t0: 10.83 ± 3.81% vs. 23.81 ± 13.64%, ctrl vs. t1: 10.83 ± 3.81% vs. 20.9 ± 19.83%, ctrl vs. t2: 10.83 ± 3.81% vs. 35.1%, ctrl vs. t5: 10.83 ± 3.81% vs. 18.82 ± 9.35%). Overall, recurrent patients at t0, t1 and

t2 had higher % of Cytotoxic T-cells than patients (rec t0, rec t1, rec t2 vs. pat t0, pat t1, pat t2, pat t3: 23.81 ± 13.64%, 20.9 ± 19.83%, 35.1% vs. 16.78 ± 7.03%, 18.36 ± 7.39%, 23.93 ± 10.76%, 20.47 ± 9.44%) and at t2, they had a greater amount of Cytotoxic T-cells in respect of the total quantity of lymphocytes than patients at all timepoints (rec t2 vs. pat t0, pat t1, pat t2, pat t3: 35.1% vs. 16.78 ± 7.03%, 18.36 ± 7.39%, 23.93 ± 10.76%, 20.47 ± 9.44%). Furthermore, there were a noticeable increase at t2 and a drop at t5 (rec t1 vs. rec t2: 20.9 ± 19.83% vs. 35.1%, t2 vs. rec t5: 35.1% vs. 18.82 ± 9.35%).
(Figure 7 and 8, Table 3B and 4)

Hence, a higher % of Cytotoxic T-cells before treatment at t0 might be a possible marker for HNSCC patients, suggesting the likelihood of these patients to suffer from recurrent disease at a later timepoint.



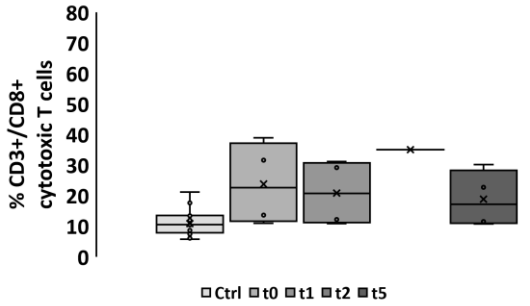


Figure 7: % Cytotoxic T-cells, Ctrl vs. Patients **Figure 8: % Cytotoxic T-cells, Ctrl vs. Recurrent Patients**
Percentages of CD3+/CD8+ Cytotoxic T-cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

5.1.5 CD3+/CD56+ Natural Killer T-Cells in %

As NKT-cells share some receptors and functions between NK-cells and T-lymphocytes, they make a functional link between innate and adaptive antitumor immunity. (Lanier, 1998; Yokoyama, Kim, & French, 2004) NKT-cells exhibit, additionally to NK-cell activities, characteristics of both Th1 and Th2 CD4+ T-lymphocytes. Therefore, they may have a dual function in immune regulation and immune surveillance against tumors. (Brutkiewicz & Sriram, 2002)

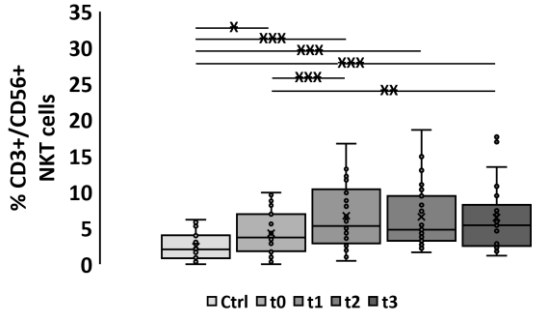
Like Cytotoxic T-cells, CD3+/CD56+ Natural killer T-cells of HNSCC patients grew during and after RCT with great significance (t0 vs. t1: 4.3 ± 3.18% vs. 6.73 ± 4.45%, ***p ≤ 0.001; t0 vs. t3: 4.3 ± 3.18% vs. 6.45 ± 4.77%, **p ≤ 0.01). Already at timepoint t0, there were almost twice as much of % NKT-cells in patients as in controls (ctrl vs. t0: 2.46 ± 1.95% vs. 4.3 ± 3.18%, *p ≤ 0.05) and at all timepoints from t0 to t3, the % of NKT-cells of patients were higher than that of controls (ctrl vs. t1: 2.46 ± 1.95% vs. 6.73 ± 4.45%, ***p ≤ 0.001; ctrl vs. t2: 2.46 ± 1.95% vs. 6.54 ± 4.32%, ***p ≤ 0.001; ctrl vs. t3: 2.46 ± 1.95% vs. 6.45 ± 4.77%, ***p ≤ 0.001).
(Figure 9, Table 3A and 3B)

In the same way, patients with relapse at t0 had approximately twice as much of NKT-cells in % as patients at t0 (rec t0 vs. pat t0: 8.67 ± 10.93% vs. 4.3 ± 3.18%) and an almost 4-times higher % of NKT-cells than controls (ctrl vs. rec t0: 2.46 ± 1.95% vs. 8.67 ± 10.93%). Altogether,

recurrent patients showed a higher % of NKT-cells than controls and patients at all timepoints (ctrl vs. rec t0, rec t1, rec t2, rec t5: 2.46 ± 1.95% vs. 8.67 ± 10.93%, 9.93 ± 13.18%, 24.02%, 9.31 ± 10.05%; rec t0, rec t1, rec t2, rec t5 vs. pat t0, pat t1, pat t2, pat t3: 8.67 ± 10.93%, 9.93 ± 13.18%, 24.02%, 9.31 ± 10.05% vs. 4.3 ± 3.18%, 6.73 ± 4.45%, 6.54 ± 4.32%, 6.45 ± 4.77%). The most striking rise was at t2, where the % of NKT-cells reached 24.02%, which was almost 12-times higher than the % of NKT-cells in healthy controls. Additionally, there was also a noticeable drop at t5 (t2 vs. t5: 24.02% vs. 9.31 ± 10.05%).
(Figure 9 and 10, Table 3B and 4)

Our patients as well as recurrent patients had, already before RCT, a more than twice or rather 4-times higher % of NKT-cells than controls and the % of NKT-cells of patients increased with great significance throughout RCT, which suggest the presence of altered immune balance before any treatment, probably favorable for the host, and the ability of primary RCT to boost HNSCC patients' immune system.

Moreover, as the value of recurrent patients at t0 was particularly high, it could be used as a possible marker to predict relapses of patients before initiation of any treatment, which might facilitate doctors to modify the treatment to achieve a better outcome at an early timepoint.



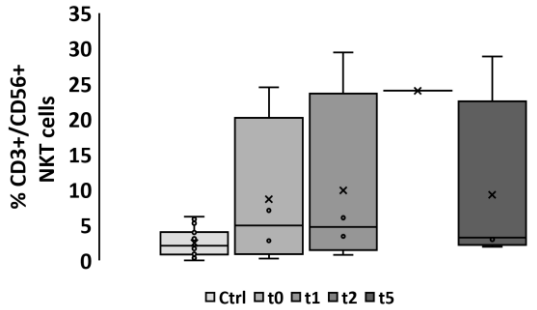


Figure 9: % NKT-cells, Ctrl vs. Patients **Figure 10: % NKT-cells, Ctrl vs. Recurrent Patients**
Percentages of CD3+/CD56+ Natural Killer T-cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

5.1.6 CD3+/CD4+/CD25+/FoxP3+ Regulatory T-Cells in %

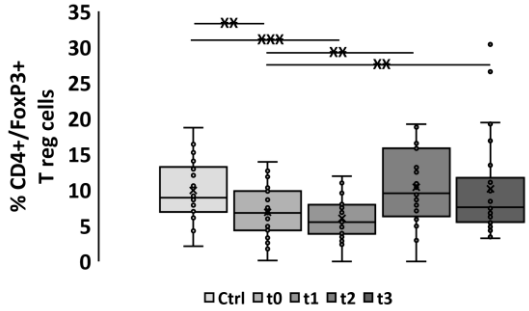
The transcription factor forkhead box P3 (FoxP3), which is considered to be the most specific marker for Regulatory T-cells so far, is a crucial intracellular molecule for the development and function of Tregs. Up to now, functions of Tregs are diverse and not completely understood.

In some of the solid tumors such as melanomas, cervical -, renal - and breast cancers, high FoxP3+ Regulatory T-cell infiltration of tumor sites correlates significantly with shorter overall survival, whereas FoxP3+ Tregs are associated with improved survival in colorectal, head and neck as well as esophageal cancers. (Shang, Liu, Jiang, & Liu, 2015)

Although patients at t0 and t1 had significantly lower % of CD3+/CD4+/CD25+/FoxP3+ Regulatory T-cells than controls (ctrl vs. t0: 9.92 ± 4.19% vs. 6.86 ± 3.47%, **p ≤ 0.01; ctrl vs. t1: 9.92 ± 4.19% vs. 6.02 ± 2.88%, ***p ≤ 0.001), the % of Regulatory T-cells rose significantly in the course of RCT, in particular at t2, with exceptional little drops at t1 and t3 (t0 vs. t2: 6.86 ± 3.47% vs. 10.36 ± 5.1%, **p ≤ 0.01; t0 vs. t3: 6.86 ± 3.47% vs. 10.14 ± 6.81%, **p ≤ 0.01; t1

vs. t2: 6.02 ± 2.88% vs. 10.36 ± 5.1%, ***p ≤ 0.001) (t0 vs. t1: 6.86 ± 3.47% vs. 6.02 ± 2.88%, t2 vs. t3: 10.36 ± 5.1% vs. 10.14 ± 6.81%).
(Figure 11, Table 3A and 3B)

Interestingly, recurrent patients had lower % of Regulatory T-cells than controls at all timepoints (ctrl vs. t0: 9.92 ± 4.19% vs. 7.45 ± 3.41%, ctrl vs. t1: 9.92 ± 4.19% vs. 6.25 ± 5.34%, ctrl vs. t2: 9.92 ± 4.19% vs. 0.39%, ctrl vs. t5: 9.92 ± 4.19% vs. 7.37 ± 2.66%), but recurrent patients at t0 had a higher % of Regulatory T-cells than patients at t0 (rec t0 vs. pat t0: 7.45 ± 3.41% vs. 6.86 ± 3.47%). In addition, there was a striking fall at t2, but this should be interpreted with caution as there was only one value available at t2 (t1 vs. t2: 6.25 ± 5.34% vs. 0.39%). Regulatory T-cells in patients with relapse recovered throughout RCT, but more slowly than Regulatory T-cells in patients; and recurrent patients at t5 had a little less % of Regulatory T-cells than at t0 (t0 vs. t5: 7.45 ± 3.41% vs. 7.37 ± 2.66%).
(Figure 12, Table 4)



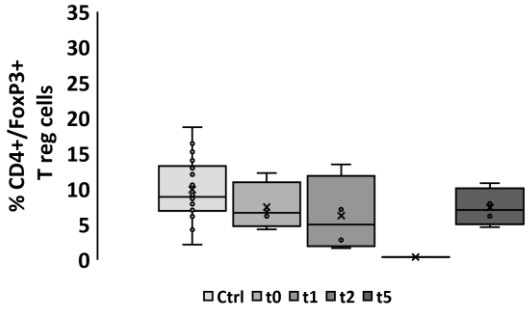


Figure 11: % Regulatory T-cells, Ctrl vs. Patients **Figure 12: % Regulatory T-cells, Ctrl vs. Recurrent Patients**
Percentages of CD3+/CD4+/CD25+/FoxP3+ Regulatory T-cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

5.1.7 CD3-/CD56+ Natural Killer Cells in %

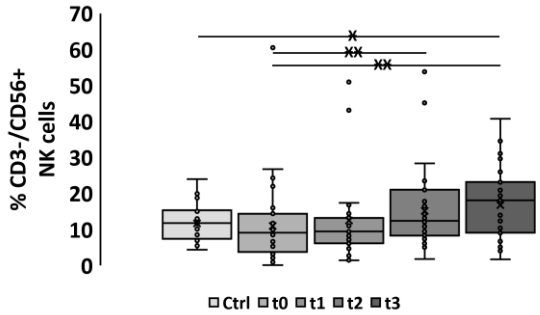
NK cells are important effectors of the innate immune system that are able to directly lyse transformed or virus-infected cells without prior sensitization or MHC class restriction. (Konjevic et al., 2012) They have the ability to attack tumor cells and have been long thought to play a crucial role in anti-tumor immunity. (S. Kim, Iizuka, Aguila, Weissman, & Yokoyama, 2000) Our findings described a significant increase in the % of NK-cells throughout RCT, which might reflect the effect of RCT on patients' immune competence to improve anti-tumor activity.

The % of CD3-/CD56+ Natural killer cells of patients increased from t0 to t3 significantly (t0 vs. t2: 10.93 ± 11.36% vs. 15.16 ± 11.09%, **p ≤ 0.01; t0 vs. t3: 10.93 ± 11.36% vs. 16.8 ± 10%, **p ≤ 0.01, t1 vs. t2: 12.51 ± 11.8% vs. 15.16 ± 11.09%, *p ≤ 0.05) and patients at t1, t2 and t3 had a greater % of NK-cells than controls (ctrl vs. t1: 11.7 ± 5.15% vs. 12.51 ± 11.8%, ctrl vs. t2: 11.7 ± 5.15% vs. 15.16 ± 11.09%, ctrl vs. t3: 11.7 ± 5.15% vs.16.8 ± 10%, *p ≤ 0.05).
(Figure 13, Table 3A and 3B)

Like NK-cells of patients, NK-cells of recurrent patients rose during RCT with an exceptional drop at t2 (t0 vs. t1: 3.81 ± 3.72% vs. 6.32 ± 3.96%, t0 vs. t5: 3.81 ± 3.72% vs. 9.73 ± 5.5%) (t1 vs. t2: 6.32 ± 3.96% vs. 2.56%), but recurrent patients had much lower % of NK-cells than controls and patients at all timepoints before, during and after RCT, in particular at t0 and t2

(rec t0, rec t2 vs. ctrl, pat t0, pat t1, pat t2, pat t3: 3.81 ± 3.72%, 2.56%, vs. 11.7 ± 5.15%, 10.93 ± 11.36%, 12.51 ± 11.8%, 15.16 ± 11.09%, 16.8 ± 10%).
(Figure 13 and 14, Table 3B and 4)

In our study, data of recurrent patients at t5, at time of relapse, showed a higher % of NK-cells than they had before the treatment at t0. The rise of NK-cells of recurrent patients directly after RCT might be caused by immune-enhancing effects of RCT. But overall, the % of NK-cells of patients with relapse were still lower than those of patients and controls, which might indicate the impairment of immune function in recurrent patients as they failed to fight against the tumor.



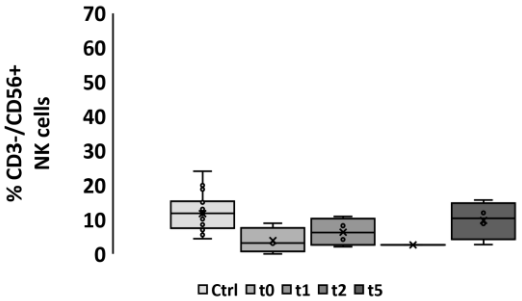


Figure 13: % NK-cells, Ctrl vs. Patients **Figure 14: % NK-cells, Ctrl vs. Recurrent Patients**

Percentages of CD3-/CD56+ Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

5.2 Subsets of Natural Killer Cells (Regulatory Markers) in %

Table 5A – Course of NK-Cell Subsets (Reg. Mark.) of Pat. throughout RCT in % / MFI

Composition of Natural killer cell subpopulations in % and MFI (mean value ± standard deviation) in head and neck cancer **patients without relapse n=31** before (t0), after RCT (t1), 3 months after (t2) and 6 months after (t3). Values at different timepoints during RCT of each patient were considered as dependent samples (Wilcoxon rank sum test). **Significantly different values (t0 vs. tx) are indicated in bold (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).**

Time	t0	t1	t2	t3
NK-cells in % CD3-/CD94+	7.95 ± 6.06	9.9 ± 9.58	**12.21 ± 8.98****	**13.19 ± 7.92*****
NK-cells in % CD3-/CD56+	10.93 ± 11.36	**12.51 ± 11.8***	**15.16 ± 11.09****	**16.8 ± 10****
NK-/T-cells in % CD56+/CD69+	2.28 ± 1.91	**3.68 ± 3.64****	3.5 ± 2.1	**4.84 ± 6.6****
NK-cells in % CD3-/NKG2D+	10.56 ± 7.52	13.65 ± 11.24	**16.5 ± 11.52****	**17.46 ± 10.36*****
NK-cells in % CD3-/NKp30+	8.08 ± 6.84	9.91 ± 10.63	**13.42 ± 9.88****	**15.35 ± 10.32*****
NK-cells in % CD3-/ NKp46+	9.95 ± 7.18	12.09 ± 11.61	**15.69 ± 11.57****	**16.92 ± 10.83*****
NK-cells (MFI) CD3-/CD94+	66.81 ± 22.32	63.73 ± 22.47	72.28 ± 23.64	76.29 ± 17.17
NK-cells (MFI) CD3-/CD56+	54.08 ± 17.09	54.3 ± 16.51	53.58 ± 15.55	50.7 ± 13.7
NK-cells (MFI) CD56+/CD69+	10.99 ± 19.74	15.41 ± 36.62	12.57 ± 24.8	9.34 ± 18.53
NK-cells (MFI) CD3-/NKG2D+	58.34 ± 22.6	53.72 ± 14.35	53.18 ± 10.89	52.26 ± 11.18
NK-cells (MFI) CD3-/NKp30+	75.34 ± 33.27	75.11 ± 29.85	80.06 ± 29.91	79.54 ± 30.67
NK-cells (MFI) CD3-/ NKp46+	159.93 ± 64.31	146.98 ± 63.55	**189.5 ± 71.82****	175.16 ± 65.8

Table 5B – NK-Cell Subsets (Reg. Mark.) of Pat. vs. Ctrl in % / MFI

Composition of Natural killer cell subpopulations in % and MFI (mean value ± standard deviation) in healthy **controls (Ctrl) n=22** and head and neck cancer **patients n=31** before (t0), after RCT (t1), 3 months after (t2) and 6 months after (t3). Values at different timepoints of patients in comparison with values of controls were considered as independent samples (Mann-Whitney-U test). **Significantly different values (Ctrl vs. tx) are indicated in bold (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).**

	Ctrl	t0	t1	t2	t3
NK-cells in % CD3-/CD94+	7.42 ± 3.71	7.95 ± 6.06	9.9 ± 9.58	**12.21 ± 8.98***	**13.19 ± 7.92****
NK-cells in % CD3-/CD56+	11.7 ± 5.15	11.92 ± 13.3	14.59± 16.17	16.74 ± 14.13	17.56 ± 11.48
NK-/T-cells in % CD56+/CD69+	1.95 ± 2.64	2.28 ± 1.91	**3.68 ± 3.64****	**3.5 ± 2.1***	**4.84 ± 6.6*****
NK-cells in % CD3-/NKG2D+	13.14 ± 5.05	10.56 ± 7.52	13.65 ± 11.24	16.5 ± 11.52	17.46 ± 10.36
NK-cells in % CD3-/NKp30+	8.86 ± 4.75	8.08 ± 6.84	9.91 ± 10.63	**13.42 ± 9.88***	**15.35 ± 10.32***
NK-cells in % CD3-/ NKp46+	11.97 ± 5.12	9.95 ± 7.18	12.09 ± 11.61	15.69 ± 11.57	**16.92 ± 10.83***
NK-cells (MFI) CD3-/CD94+	61.92 ± 17.71	66.81 ± 22.32	63.73 ± 22.47	72.28 ± 23.64	76.29 ± 17.17
NK-cells (MFI) CD3-/CD56+	51.56 ± 11.39	54.08 ± 17.09	54.3 ± 16.51	53.58 ± 15.55	50.7 ± 13.7
NK-cells (MFI) CD56+/CD69+	3.17 ± 0.97	**10.99 ± 19.74****	**15.41 ± 36.62****	**12.57 ± 24.8****	**9.34 ± 18.53***
NK-cells (MFI) CD3-/NKG2D+	78.87 ± 18.69	**58.34 ± 22.6****	**53.72 ± 14.35*****	**53.18 ± 10.89*****	**52.26 ± 11.18*****
NK-cells (MFI) CD3-/NKp30+	64.87 ± 19.44	75.34 ± 33.27	75.11 ± 29.85	**80.06 ± 29.91***	**79.54 ± 30.67***
NK-cells (MFI) CD3-/ NKp46+	154.24 ± 48.06	159.93 ± 64.31	146.98 ± 63.55	**189.5 ± 71.82***	175.16 ± 65.8

Table 6 – NK-Cell Subsets (Reg. Mark.) of Rec. Pat. vs. Ctrl in % / MFI

Composition of Natural killer cell subpopulations in % and MFI (mean value ± standard deviation) in healthy **controls (Ctrl) n=22** and head and neck cancer **patients with relapse n=4** before (t0), after RCT (t1), 3 months after (t2) and at time of relapse (t5). Values at different timepoints during RCT of each recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). **Significantly different values (t0 vs. tx); (Ctrl vs. tx) are indicated in bold (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).**

	Ctrl	t0	t1	t2	t5
NK-cells in % CD3-/CD94+	7.42 ± 3.71	4.46 ± 2.47	7.7 ± 4.33	2.18 (single value)	10.33 ± 6.63
NK-cells in % CD3-/CD56+	11.7 ± 5.15	3.81 ± 3.72	6.32 ± 3.96	2.56 (single value)	9.98 ± 5.87
NK-/T-cells in % CD56+/CD69+	1.95 ± 2.64	1.42 ± 0.57	4.29 ± 2.67	2.77 (single value)	3.99 ± 4.1
NK-cells in % CD3-/NKG2D+	13.14 ± 5.05	5.94 ± 1.85	7.79 ± 4.53	4.62 (single value)	13.6 ± 7.12
NK-cells in % CD3-/NKp30+	8.86 ± 4.75	2.81 ± 3.22	6.11 ± 6.09	1.55 (single value)	9.88 ± 7.54
NK-cells in % CD3-/ NKp46+	11.97 ± 5.12	4.45 ± 3.43	6.42 ± 4.83	2.25 (single value)	13.38 ± 8.74
NK-cells (MFI) CD3-/CD94+	61.92 ± 17.71	63.12 ± 27.76	62.35 ± 18.72	45.47 (single value)	65.16 ± 13.31
NK-cells (MFI) CD3-/CD56+	51.56 ± 11.39	39.32 ± 8.93	35.38 ± 5.87	37.21 (single value)	42.24 ± 14.76
NK-cells (MFI) CD56+/CD69+	3.17 ± 0.97	57.83 ± 41.95	50.11 ± 31.42	3.23 (single value)	56.12 ± 35.65
NK-cells (MFI) CD3-/NKG2D+	78.87 ± 18.69	82.39 ± 35.59	45.49 ± 12.66	52.48 (single value)	44.97 ± 1.98
NK-cells (MFI) CD3-/NKp30+	64.87 ± 19.44	94.59 ± 33.25	65.42 ± 12.38	74.04 (single value)	73.38 ± 25.34
NK-cells (MFI) CD3-/ NKp46+	154.24 ± 48.06	178.7 ± 32.95	126.45 ± 33	113.51 (single value)	156.24 ± 34.47

NK-cell function is associated with a vast network of inhibitory and activating signals. Under normal immune surveillance, NK-cells have inhibitory receptors that recognize MHC class I molecules as their cognate ligands; these receptors include killer Ig-like receptors (KIR)-L, LIR-1/ILT-2, LAIR-1 and the CD94/NKG2A heterodimer. Cytotoxicity occurs if stimulatory signals outweigh inhibitory signals by a critical threshold. Several of these activating receptors have been characterized, including NKG2C, NKG2D, and the natural cytotoxicity receptors NKp30, NKp44 and NKp46. (C. Bottino, Moretta, & Moretta, 2006; Hudspeth, Silva-Santos, & Mavilio, 2013; Sun & Lanier, 2011; Vivier, Tomasello, Baratin, Walzer, & Ugolini, 2008)

Studies have demonstrated that low NK-cell activity leads to high incidence of tumor occurrence and metastasis, and its degree correlates with invasiveness of malignancy. (Takeuchi et al., 2001) Contrarily, high NK-cell activity has been shown to correlate with lower incidence of tumors and NK-cell infiltration in certain tumors, e.g. in melanomas and HNSCCs, is associated with better oncological outcome. (Schleypen et al., 2006; Vivier, Ugolini, Blaise, Chabannon, & Brossay, 2012)

Overall, the % of all subtypes of Natural killer cells of patients increased with significance in the course of RCT (t0 vs. t3; all NK-cell subtypes). At t3, the % of all subgroups of NK-cells of patients were higher than that of controls (ctrl vs. t3; all NK-cell subtypes).
(Figure 15, 17, 19, 21, 23 and 25, Table 5A and 5B)

Similarly, the % of all subgroups of Natural killer cells in recurrent patients also tended to grow throughout RCT (t0 vs. t1, t0 vs. t5; all NK-cell subtypes). At t0, recurrent patients had lower % of NK-cells of all subtypes than controls (ctrl vs. t0; all NK-cell subtypes). At t5, patients with relapse had higher % of all subtypes of NK-cells, except of the CD3-/CD56+ NK-cell subpopulation, than controls (ctrl vs. t5; all NK-cell subtypes without CD3-/CD56+ NK-cells). Furthermore, recurrent patients had lower % of all subtypes of NK-cells at t0 and t5 than patients at t0 and t3 (rec t0 vs. pat t0, rec t5 vs. pat t3; all NK-cell subtypes). Interestingly, increases at t1 and t5, as well as drops at t2 were noticeable in all NK-cell subtypes of recurrent patients (t0 vs. t1, t1 vs. t2, t2 vs. t5; all NK-cell subtypes).
(Figure 15-26, Table 5B and 6)

Our findings suggested that RCT can increase anti-tumor function of NK-cells in HNSCC patients as well as in recurrent patients by increasing the % of expressions of activation marker such as CD69, NKG2D, NKp30 and NKp46 on NK-cells.

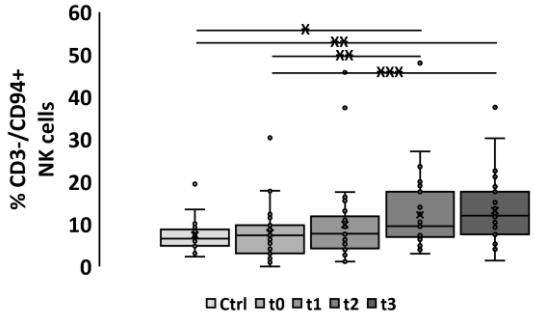
We also observed that the % of all NK-cell subsets of recurrent patients were lower than those % of all NK-cell subtypes of patients before RCT as well as at time of relapse, which might be a possible marker to help clinicians to foresee the relapse and to adjust the treatment at an early timepoint.

CD3-/CD94+ NK-cells in %

CD94 receptor can be subdivided into CD94/NKG2A and CD94/NKG2C receptors, which bind with human leukocyte antigen (HLA)-E-peptide complex. CD94/NKG2A receptor mediates inhibition and CD94/NKG2C receptor activation of the NK-cell-mediated cytotoxicity. (Braud et al., 1998; Lanier, Corliss, Wu, & Phillips, 1998)

The % of CD3-/CD94+ Natural killer cells of HNSCC patients rose throughout RCT (t0 vs. t2: 7.95 ± 6.06% vs. 12.21 ± 8.98%, $^{**}p \leq 0.01$; t0 vs. t3: 7.95 ± 6.06% vs.13.19 ± 7.92%, $^{***}p \leq 0.001$; t1 vs. t2: 9.9 ± 9.58% vs. 12.21 ± 8.98%, $^{*}p \leq 0.05$). At t2 and t3, patients had significantly more CD3-/CD94+ NK-cells in respect of the total amount of lymphocytes than controls (ctrl vs. t2: 7.42 ± 3.71% vs. 12.21 ± 8.98%, $^{*}p \leq 0.05$; ctrl vs. t3: 7.42 ± 3.71% vs. 13.19 ± 7.92%, $^{**}p \leq 0.01$).
(Figure 15, Table 5A and 5B)

In the same way, the % of CD3-/CD94+ Natural killer cells of recurrent patients tended to increase from t0 to t5 (t0 vs. t5: 4.46 ± 2.47% vs.10.33 ± 6.63%), but there was a drop at t2 (rec t1 vs. rec t2: 7.7 ± 4.33% vs. 2.18%) and recurrent patients had lower % of CD3-/CD94+ Natural killer cells at t0 and t2 than controls and patients at all timepoints (rec t0, rec t2 vs. ctrl, pat t0, pat t1, pat t2, pat t3: 4.46 ± 2.47% , 2.18% vs. 7.42 ± 3.71% , 7.95 ± 6.06% , 9.9 ± 9.58% , 12.21 ± 8.98% , 13.19 ± 7.92%). Furthermore, recurrent patients also had lower % of CD3-/CD94+ NK-cells at t0 and t5 than patients at t0 and t3 (rec t0 vs. pat t0: 4.46 ± 2.47% vs. 7.95 ± 6.06%, rec t5 vs. pat t3: 10.33 ± 6.63% vs. 13.19 ± 7.92%).
(Figure 15 and 16, Table 5B and 6)



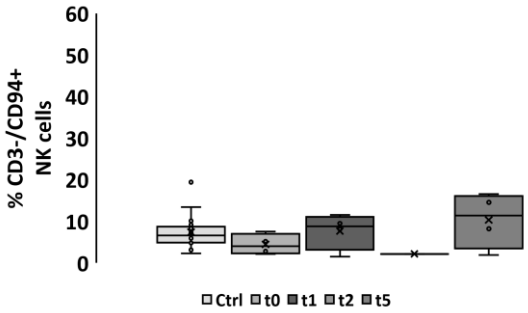


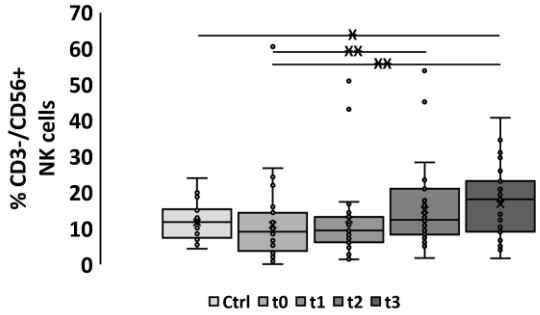
Figure 15: % CD3-/CD94+ NK-cells, Ctrl vs. Patients **Figure 16: % CD3-/CD94+ NK-cells, Ctrl vs. Recurrent Patients**
Percentages of CD3-/CD94+ Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

CD3-/CD56+ NK-cells in %

NK cells are important effectors of the innate immune system that are able to directly lyse transformed or virus-infected cells without prior sensitization or MHC class restriction. (Konjevic et al., 2012) They have the ability to attack tumor cells and have been long thought to play a crucial role in anti-tumor immunity. (S. Kim, Iizuka, Aguila, Weissman, & Yokoyama, 2000)

Like other NK-cell subgroups, CD3-/CD56+ Natural killer cells of patients grew significantly throughout RCT (t0 vs. t1: 11.92 ± 13.3% vs. 14.59 ± 16.17%, *p ≤ 0.05; t0 vs. t2: 11.92 ± 13.3% vs. 16.74 ± 14.13%, **p ≤ 0.01; t0 vs. t3: 11.92 ± 13.3% vs. 17.56 ± 11.48%, **p ≤ 0.01).
(Figure 17, Table 5A)

And recurrent patients showed the same course as patients (t0 vs. t1, t5: 3.81 ± 3.72% vs. 6.32 ± 3.96%, 9.98 ± 5.87%), apart from the drop at t2 (t1 vs. t2: 6.32 ± 3.96% vs. 2.56 %).
(Figure 18, Table 6)



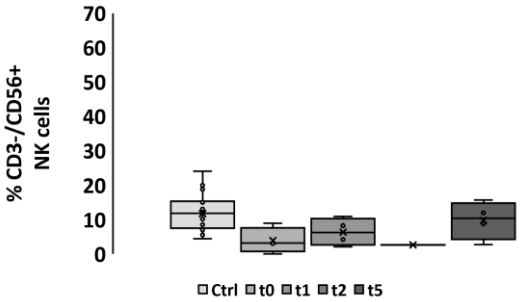


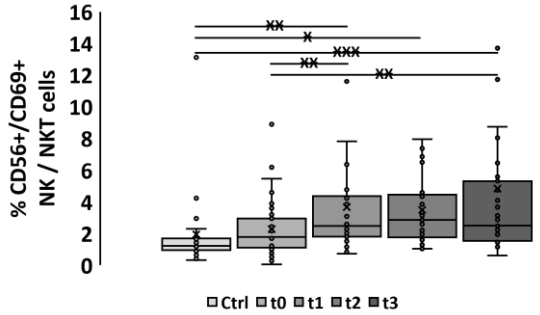
Figure 17: % CD3-/CD56+ NK-cells, Ctrl vs. Patients **Figure 18: % CD3-/CD56+ NK-cells, Ctrl vs. Recurrent Patients**
Percentages of CD3-/CD56+ Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

CD56+/CD69+ NK-/T-cells in %

CD56+ is an important effector marker on NK- and T-lymphocytes and CD69+ an early activation marker on NK-lymphocytes. (Cohavy & Targan, 2007; Millrud et al., 2012)

Like other NK-cell subgroups, the % of CD56+/CD69+ Natural killer cells/T-cells of patients increased significantly from t0 to t3 (t0 vs. t1: 2.28 ± 1.91% vs. 3.68 ± 3.64%, **p ≤ 0.01; t0 vs. t3: 2.28 ± 1.91% vs. 4.84 ± 6.6%, **p ≤ 0.01). At t1, t2 and t3, patients had significantly more CD56+/CD69+ NK-/T-cells in respect of the total amount of lymphocytes than controls (ctrl vs. t1: 1.95 ± 2.64% vs. 3.68 ± 3.64%, **p ≤ 0.01; ctrl vs. t2: 1.95 ± 2.64% vs. 3.5 ± 2.1%, *p ≤ 0.05; ctrl vs. t3: 1.95 ± 2.64% vs. 4.84 ± 6.6%, ***p ≤ 0.001).
(Figure 19, Table 5A and 5B)

In total, the % of CD56+/CD69+ NK-/T-cells in recurrent patients also grew throughout RCT (t0 vs. t5: 1.42 ± 0.57% vs. 3.99 ± 4.1%). Interestingly, there was a noticeable rise from t0 to t1 (t0 vs. t1: 1.42 ± 0.57% vs. 4.29 ± 2.67%) and a visible drop from t1 to t2 (t1 vs. t2: 4.29 ± 2.67% vs. 2.77%).
(Figure 20, Table 6)



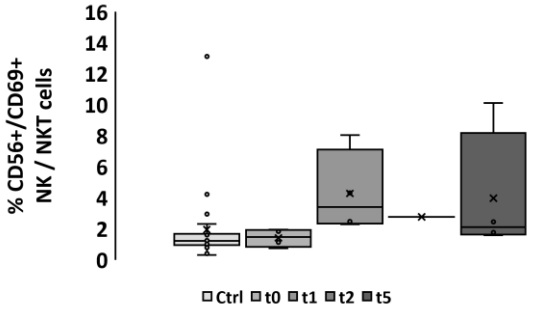


Figure 19: % CD56+/CD69+ NK-/T-cells, Ctrl vs. Patients **Figure 20: % CD56+/CD69+ NK-/T-cells, Ctrl vs. Recurrent Patients**
Percentages of CD56+/CD69+ Natural killer-/T-cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U

test). Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

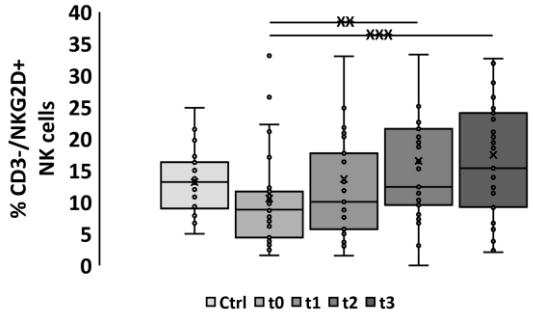
CD3-/NKG2D+ NK-cells in %

Natural cytotoxicity receptors (NCRs) belong to major lysis receptors in the NK-cell. NCRs include three immunoglobulin-like proteins: NKp46 (NCR1; CD335), NKp44 (NCR2; CD336) and NKp30 (NCR3; CD337). (Enk & Mandelboim, 2014; Noronha, Harman, Wagner, & Antczak, 2012) And NK-cell-activating receptors, such as NKG2D, NKp30, NKp44 and NKp46, have been shown to be particularly important for NK-cell recognition and lysis of target, e.g. for lysis of tumor cells. (Carbone et al., 2005; D. Pende et al., 2001; Daniela Pende et al., 2005; Vivier et al., 2011)

NKG2D receptor is not only expressed by NK-cells, but also by activated Cytotoxic T-cells and activated macrophages. Depending on the level of NKG2D ligands expressed on tumor cells, anti-tumor response can be induced by potent priming of Cytotoxic T-cells and sensitization of NK-cells. (Diefenbach, Jensen, Jamieson, & Raulet, 2001) For example, NKG2D receptor recognizes two different families of ligands: MHC class I chain-related molecules (MICA and MICB) and UL-16-binding proteins (ULBP). (Cristina Bottino, Castriconi, Moretta, & Moretta, 2005) NK-cells can recognize these NKG2D ligands on malignant tumor cells and eradicate them. (González, Groh, & Spies, 2006)

The % of CD3-/NKG2D+ Natural killer cells of HNSCC patients also rose significantly throughout RCT (t0 vs. t2: 10.56 ± 7.52% vs. 16.5 ± 11.52%, **p ≤ 0.01; t0 vs. t3: 10.56 ± 7.52% vs. 17.46 ± 10.36%, ***p ≤ 0.001; t1 vs. t2: 13.65 ± 11.24% vs. 16.5 ± 11.52%, *p ≤ 0.05). (Figure 21, Table 5A)

Interestingly, increases at t1 and t5 as well as a drop at t2 were noticeable in recurrent patients (t0 vs. t1: 5.94 ± 1.85% vs. 7.79 ± 4.53%, t0 vs. t5: 5.94 ± 1.85% vs. 13.6 ± 7.12%, t1 vs. t2: 7.79 ± 4.53% vs. 4.62%), whereby the % of CD3-/NKG2D+ NK-cells at t0, t1 and t2 were much lower than those of patients at all timepoints throughout RCT and of controls (rec t0, rec t1, rec t2 vs. ctrl, pat t0, pat t1, pat t2, pat t3: 5.94 ± 1.85%, 7.79 ± 4.53%, 4.62% vs. 13.14 ± 5.05%, 10.56 ± 7.52%, 13.65 ± 11.24%, 16.5 ± 11.52%, 17.46 ± 10.36%).
(Figure 21 and 22, Table 5B and 6)



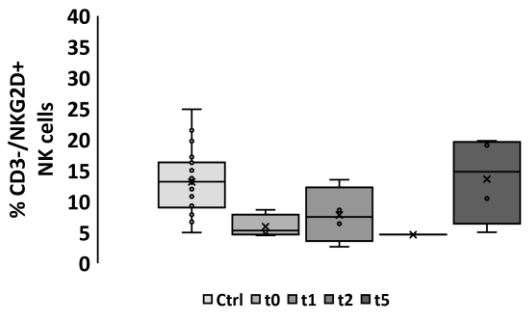


Figure 21: % CD3-/NKG2D+ NK-cells, Ctrl vs. Patients **Figure 22: % CD3-/NKG2D+ NK-cells, Ctrl vs. Recurrent Patients**
Percentages of CD3-/NKG2D+ Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

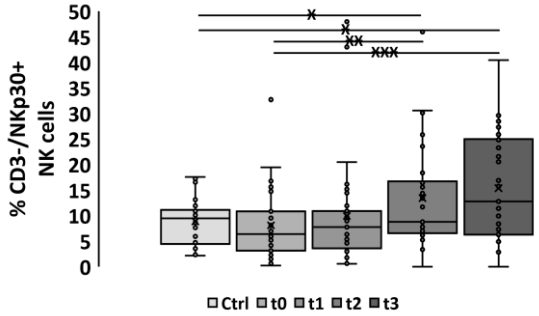
CD3-/NKp30+ NK-cells in %

Natural cytotoxicity receptors (NCRs) belong to major lysis receptors in the NK-cell. NCRs include three immunoglobulin-like proteins: NKp46 (NCR1; CD335), NKp44 (NCR2; CD336) and NKp30 (NCR3; CD337). (Enk & Mandelboim, 2014; Noronha et al., 2012) And NK-cell-activating receptors, such as NKG2D, NKp30, NKp44, and NKp46, have been shown to be particularly important for NK-cell recognition and lysis of the target. (Carbone et al., 2005; D. Pende et al., 2001; Daniela Pende et al., 2005)

NKp30 is important for mediating tumor immunosurveillance in several clinical settings. One of its ligands, B7H6, is expressed on many types of tumor cells, but absent on normal tissues. Interaction of NKp30 with B7H6 has been shown to improve degranulation of NK-cells. (H. Wang, Zheng, Wei, Tian, & Sun, 2012)

A significant increase in the % of CD3-/NKp30+ Natural killer cell subpopulation of patients could be observed (t0 vs. t2: 8.08 ± 6.84% vs. 13.42 ± 9.88%, **p ≤ 0.01; t0 vs. t3: 8.08 ± 6.84% vs. 15.35 ± 10.32%, ***p ≤ 0.001; t1 vs. t2: 9.91 ± 10.63% vs. 13.42 ± 9.88%, **p ≤ 0.01) and the % of CD3-/NKp30+ NK-cells of patients at t2 and t3 were significantly higher than that of controls (ctrl vs. t2: 8.86 ± 4.75% vs. 13.42 ± 9.88%, *p ≤ 0.05; ctrl vs. t3: 8.86 ± 4.75% vs. 15.35 ± 10.32%, *p ≤ 0.05).
(Figure 23, Table 5A and 5B)

In recurrent patients, the % of CD3-/NKp30+ NK-cells at t0, t1 and t2 were much lower than those of controls and patients at all timepoints (rec t0, rec t1, rec t2 vs. ctrl, pat t0, pat t1, pat t2, pat t3: 2.81 ± 3.22%, 6.11 ± 6.09%, 1.55% vs. 8.86 ± 4.75%, 8.08 ± 6.84%, 9.91 ± 10.63%, 13.42 ± 9.88%, 15.35 ± 10.32%). In addition, the rise at t1 and t5 as well as the drop at t2 were noticeable (t0 vs. t1: 2.81 ± 3.22% vs. 6.11 ± 6.09%, t0 vs. t5: 2.81 ± 3.22% vs. 9.88 ± 7.54%, t1 vs. t2: 6.11 ± 6.09% vs. 1.55%).
(Figure 23 and 24, Table 5B and 6)



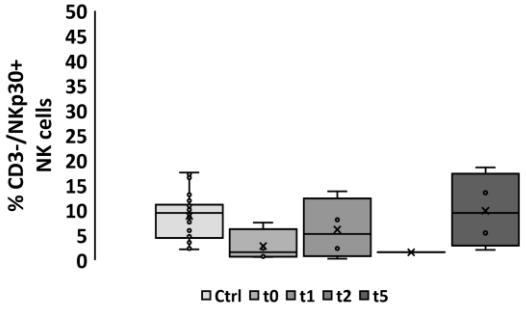


Figure 23: % CD3-/NKp30+ NK-cells, Ctrl vs. Patients **Figure 24: % CD3-/NKp30+ NK-cells, Ctrl vs. Recurrent Patients**
Percentages of CD3-/NKp30+ Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

CD3-/NKp46+ NK-cells in %

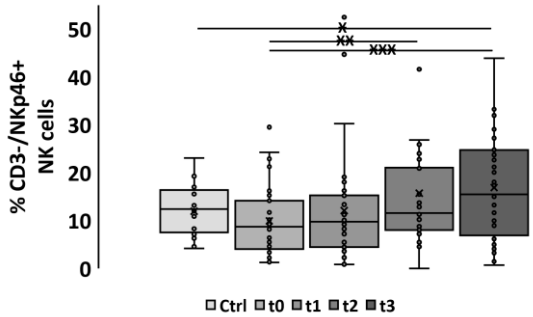
Natural cytotoxicity receptors (NCRs) belong to major lysis receptors in the NK-cell. NCRs include three immunoglobulin-like proteins: NKp46 (NCR1; CD335), NKp44 (NCR2; CD336) and NKp30 (NCR3; CD337). (Enk & Mandelboim, 2014; Noronha et al., 2012) NK-cell-

activating receptors, such as NKG2D, NKp30, NKp44, and NKp46, have been shown to be particularly important for NK-cell recognition and lysis of the target. (Carbone et al., 2005; D. Pende et al., 2001; Daniela Pende et al., 2005) In contrast to NKp30 and NKp44, which have similar cellular functions, NKp46 is structurally distinct from the other two molecules and is located in a different region of the genome. It is stably expressed on both resting and activated NK-cell, and is considered as an unique NK-cell marker. (Enk & Mandelboim, 2014; Noronha et al., 2012)

It was demonstrated that NKp46 is critically involved in influenza, tumorigenesis and diabetes. (Gazit et al., 2006; Gur et al., 2010; Halfteck et al., 2009) Moreover, NKp46 is described to be the key receptor in controlling the spread of various primary tumors in melanoma, lymphoma and other carcinomas. (Glasner et al., 2012)

Like other NK-cell subgroups, the % of CD3-/NKp46+ Natural killer cells of HNSCC patients rose significantly (t0 vs. t2: 9.95 ± 7.18% vs. 15.69 ± 11.57%, **p ≤ 0.01; t0 vs. t3: 9.95 ± 7.18% vs. 16.92 ± 10.83%, ***p ≤ 0.001, t1 vs. t2: 12.09 ± 11.61% vs. 15.69 ± 11.57%, *p ≤ 0.05). At t3, patients had significantly more CD3-/NKp46+ NK-cells in respect of the total amount of lymphocytes than controls (ctrl vs. t3: 11.97 ± 5.12% vs. 16.92 ± 10.83%, *p ≤ 0.05).
(Figure 25, Table 5A and 5B)

Apart from an exceptional drop at t2 (t1 vs. t2: 6.42 ± 4.83% vs. 2.25%), patients with relapse also had increased CD3-/NKp46+ NK-cells throughout RCT (t0 vs. t1: 4.45 ± 3.43% vs. 6.42 ± 4.83%, t0 vs. t5: 4.45 ± 3.43% vs. 13.38 ± 8.74%). Additionally, they had lower % of CD3-/NKp46+ NK-cells at t0, t1 and t2 than controls and patients at all timepoints (rec t0, rec t1, rec t2 vs. ctrl, pat t0, pat t1, pat t2, pat t3: 4.45 ± 3.43%, 6.42 ± 4.83%, 2.25% vs. 11.97 ± 5.12%, 9.95 ± 7.18%, 12.09 ± 11.61%, 15.69 ± 11.57%, 16.92 ± 10.83%).
(Figure 25 and 26, Table 5B and 6)



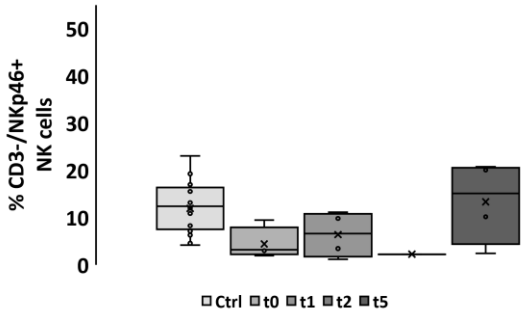


Figure 25: % CD3-/NKp46+ NK-cells, Ctrl vs. Patients **Figure 26: % CD3-/NKp46+ NK-cells, Ctrl vs. Recurrent Patients**
Percentages of CD3-/NKp46+ Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

5.3 Subsets of Natural Killer Cells (Regulatory Markers) in MFI

Table 5A – Course of NK-Cell Subsets (Reg. Mark.) of Pat. throughout RCT in % / MFI

Composition of Natural killer cell subpopulations in % and MFI (mean value ± standard deviation) in head and neck cancer **patients without relapse n=31** before (t0), after RCT (t1), 3 months after (t2) and 6 months after (t3). Values at different timepoints during RCT of each patient were considered as dependent samples (Wilcoxon rank sum test). **Significantly different values (t0 vs. tx) are indicated in bold (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).**

Time	t0	t1	t2	t3
NK-cells in % CD3-/CD94+	7.95 ± 6.06	9.9 ± 9.58	**12.21 ± 8.98****	**13.19 ± 7.92*****
NK-cells in % CD3-/CD56+	10.93 ± 11.36	**12.51 ± 11.8***	**15.16 ± 11.09****	**16.8 ± 10****
NK-/T-cells in % CD56+/CD69+	2.28 ± 1.91	**3.68 ± 3.64****	3.5 ± 2.1	**4.84 ± 6.6****
NK-cells in % CD3-/NKG2D+	10.56 ± 7.52	13.65 ± 11.24	**16.5 ± 11.52****	**17.46 ± 10.36*****
NK-cells in % CD3-/NKp30+	8.08 ± 6.84	9.91 ± 10.63	**13.42 ± 9.88****	**15.35 ± 10.32*****
NK-cells in % CD3-/ NKp46+	9.95 ± 7.18	12.09 ± 11.61	**15.69 ± 11.57****	**16.92 ± 10.83*****
NK-cells (MFI) CD3-/CD94+	66.81 ± 22.32	63.73 ± 22.47	72.28 ± 23.64	76.29 ± 17.17
NK-cells (MFI) CD3-/CD56+	54.08 ± 17.09	54.3 ± 16.51	53.58 ± 15.55	50.7 ± 13.7
NK-cells (MFI) CD56+/CD69+	10.99 ± 19.74	15.41 ± 36.62	12.57 ± 24.8	9.34 ± 18.53
NK-cells (MFI) CD3-/NKG2D+	58.34 ± 22.6	53.72 ± 14.35	53.18 ± 10.89	52.26 ± 11.18
NK-cells (MFI) CD3-/NKp30+	75.34 ± 33.27	75.11 ± 29.85	80.06 ± 29.91	79.54 ± 30.67
NK-cells (MFI) CD3-/ NKp46+	159.93 ± 64.31	146.98 ± 63.55	**189.5 ± 71.82****	175.16 ± 65.8

Table 5B – NK-Cell Subsets (Reg. Mark.) of Pat. vs. Ctrl in % / MFI

Composition of Natural killer cell subpopulations in % and MFI (mean value ± standard deviation) in healthy **controls (Ctrl) n=22** and head and neck cancer **patients n=31** before (t0), after RCT (t1), 3 months after (t2) and 6 months after (t3). Values at different timepoints of patients in comparison with values of controls were considered as independent samples (Mann-Whitney-U test). **Significantly different values (Ctrl vs. tx) are indicated in bold (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).**

	Ctrl	t0	t1	t2	t3
NK-cells in % CD3-/CD94+	7.42 ± 3.71	7.95 ± 6.06	9.9 ± 9.58	**12.21 ± 8.98***	**13.19 ± 7.92****
NK-cells in % CD3-/CD56+	11.7 ± 5.15	11.92 ± 13.3	14.59± 16.17	16.74 ± 14.13	17.56 ± 11.48
NK-/T-cells in % CD56+/CD69+	1.95 ± 2.64	2.28 ± 1.91	**3.68 ± 3.64****	**3.5 ± 2.1***	**4.84 ± 6.6*****
NK-cells in % CD3-/NKG2D+	13.14 ± 5.05	10.56 ± 7.52	13.65 ± 11.24	16.5 ± 11.52	17.46 ± 10.36
NK-cells in % CD3-/NKp30+	8.86 ± 4.75	8.08 ± 6.84	9.91 ± 10.63	**13.42 ± 9.88***	**15.35 ± 10.32***
NK-cells in % CD3-/ NKp46+	11.97 ± 5.12	9.95 ± 7.18	12.09 ± 11.61	15.69 ± 11.57	**16.92 ± 10.83***
NK-cells (MFI) CD3-/CD94+	61.92 ± 17.71	66.81 ± 22.32	63.73 ± 22.47	72.28 ± 23.64	76.29 ± 17.17
NK-cells (MFI) CD3-/CD56+	51.56 ± 11.39	54.08 ± 17.09	54.3 ± 16.51	53.58 ± 15.55	50.7 ± 13.7
NK-cells (MFI) CD56+/CD69+	3.17 ± 0.97	**10.99 ± 19.74****	**15.41 ± 36.62****	**12.57 ± 24.8****	**9.34 ± 18.53***
NK-cells (MFI) CD3-/NKG2D+	78.87 ± 18.69	**58.34 ± 22.6****	**53.72 ± 14.35*****	**53.18 ± 10.89*****	**52.26 ± 11.18*****
NK-cells (MFI) CD3-/NKp30+	64.87 ± 19.44	75.34 ± 33.27	75.11 ± 29.85	**80.06 ± 29.91***	**79.54 ± 30.67***
NK-cells (MFI) CD3-/ NKp46+	154.24 ± 48.06	159.93 ± 64.31	146.98 ± 63.55	**189.5 ± 71.82***	175.16 ± 65.8

Table 6 – NK-Cell Subsets (Reg. Mark.) of Rec. Pat. vs. Ctrl in % / MFI

Composition of Natural killer cell subpopulations in % and MFI (mean value ± standard deviation) in healthy **controls (Ctrl) n=22** and head and neck cancer **patients with relapse n=4** before (t0), after RCT (t1), 3 months after (t2) and at time of relapse (t5). Values at different timepoints during RCT of each recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). **Significantly different values (t0 vs. tx); (Ctrl vs. tx) are indicated in bold (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).**

	Ctrl	t0	t1	t2	t5
NK-cells in % CD3-/CD94+	7.42 ± 3.71	4.46 ± 2.47	7.7 ± 4.33	2.18 (single value)	10.33 ± 6.63
NK-cells in % CD3-/CD56+	11.7 ± 5.15	3.81 ± 3.72	6.32 ± 3.96	2.56 (single value)	9.98 ± 5.87
NK-/T-cells in % CD56+/CD69+	1.95 ± 2.64	1.42 ± 0.57	4.29 ± 2.67	2.77 (single value)	3.99 ± 4.1
NK-cells in % CD3-/NKG2D+	13.14 ± 5.05	5.94 ± 1.85	7.79 ± 4.53	4.62 (single value)	13.6 ± 7.12
NK-cells in % CD3-/NKp30+	8.86 ± 4.75	2.81 ± 3.22	6.11 ± 6.09	1.55 (single value)	9.88 ± 7.54
NK-cells in % CD3-/ NKp46+	11.97 ± 5.12	4.45 ± 3.43	6.42 ± 4.83	2.25 (single value)	13.38 ± 8.74
NK-cells (MFI) CD3-/CD94+	61.92 ± 17.71	63.12 ± 27.76	62.35 ± 18.72	45.47 (single value)	65.16 ± 13.31
NK-cells (MFI) CD3-/CD56+	51.56 ± 11.39	39.32 ± 8.93	35.38 ± 5.87	37.21 (single value)	42.24 ± 14.76
NK-cells (MFI) CD56+/CD69+	3.17 ± 0.97	57.83 ± 41.95	50.11 ± 31.42	3.23 (single value)	56.12 ± 35.65
NK-cells (MFI) CD3-/NKG2D+	78.87 ± 18.69	82.39 ± 35.59	45.49 ± 12.66	52.48 (single value)	44.97 ± 1.98
NK-cells (MFI) CD3-/NKp30+	64.87 ± 19.44	94.59 ± 33.25	65.42 ± 12.38	74.04 (single value)	73.38 ± 25.34
NK-cells (MFI) CD3-/ NKp46+	154.24 ± 48.06	178.7 ± 32.95	126.45 ± 33	113.51 (single value)	156.24 ± 34.47

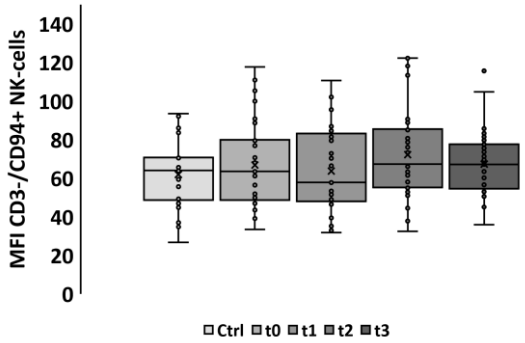
Relative comparisons can be made with MFI, but one might be careful that other factors are not responsible for the differences in MFI. Generally, the data of MFI in subgroups of NK-cells of patients remained almost at a steady level and showed less statistical significance throughout RCT.
(Figure 27-38, Table 5A, 5B and 6)

CD3-/CD94+ NK-cells in MFI

CD94 receptor can be subdivided into CD94/NKG2A and CD94/NKG2C receptors, which bind with human leukocyte antigen (HLA)-E-peptide complex. CD94/NKG2A receptor mediates inhibition and CD94/NKG2C receptor activation of the NK-cell-mediated cytotoxicity. (Braud et al., 1998; Lanier, Corliss, Wu, & Phillips, 1998)

Patients had higher MFI values of CD3-/CD94+ NK-cells at all timepoints than controls (ctrl vs. t0, t1, t2, t3: 61.92 ± 17.71 vs. 66.81 ± 22.32, 63.73 ± 22.47, 72.28 ± 23.64, 76.29 ± 17.17). Furthermore, the only significance in the subgroup of CD3-/CD94+ Natural killer cells of patients was the rise at t2 (t1 vs. t2: 63.73 ± 22.47 vs. 72.28 ± 23.64, *p ≤ 0.05).
(Figure 27, Table 5A and 5B)

In recurrent patients, a sharp drop at t2 could be observed (t1 vs. t2: 62.35 ± 18.72 vs. 45.47).
(Figure 28, Table 6)



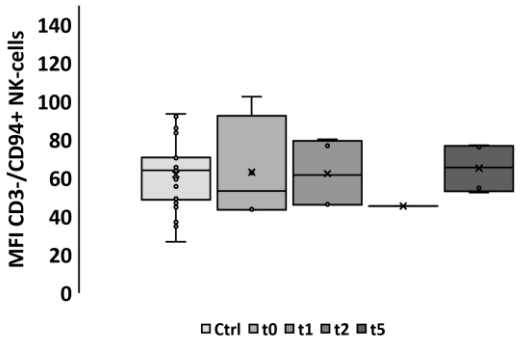


Figure 27: MFI CD3-/CD94+ NK-cells, Ctrl vs. Patients **Figure 28: MFI CD3-/CD94+ NK-cells, Ctrl vs. Recurrent Patients**
MFI (Mean Fluorescence Intensity) of CD3-/CD94+ Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of MFI of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test).

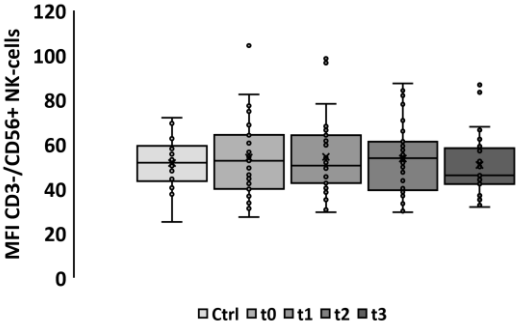
CD3-/CD56+ NK-cells in MFI

NK cells are important effectors of the innate immune system that are able to directly lyse transformed or virus-infected cells without prior sensitization or MHC class restriction. (Konjevic et al., 2012) They have the ability to attack tumor cells and have been long thought to play a crucial role in anti-tumor immunity. (S. Kim, Iizuka, Aguila, Weissman, & Yokoyama, 2000)

In HNSCC patients, a drop was visible in the subgroup of CD3-/CD56+ NK-cells from t0 to t3 (t0 vs. t3: 54.08 ± 17.09 vs. 50.7 ± 13.7).
(Figure 29, Table 5A)

Interestingly, patients with relapse had lower MFI values than controls and patients at all timepoints (rec t0, rec t1, rec t2, rec t5 vs. ctrl, pat t0, pat t1, pat t2, pat t3: 39.32 ± 8.93, 35.38 ± 5.87, 37.21, 42.24 ± 14.76 vs. 51.56 ± 11.39, 54.08 ± 17.09, 54.3 ± 16.51, 53.58 ± 15.55, 50.7 ± 13.7) and the drop at t1 as well as increases at t2 and t5 were noticeable (t0 vs. t1:

39.32 ± 8.93 vs. 35.38 ± 5.87, t1 vs. t2: 35.38 ± 5.87 vs. 37.21 t2 vs. t5: 37.21 vs. 42.24 ± 14.76).
(Figure 29 and 30, Table 5B and 6)



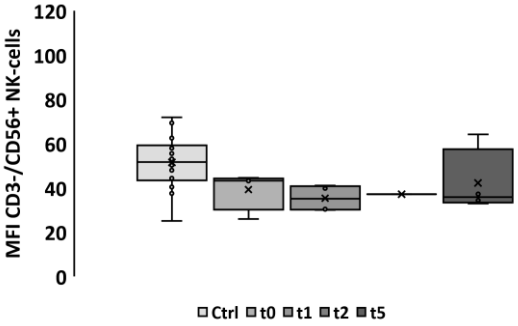


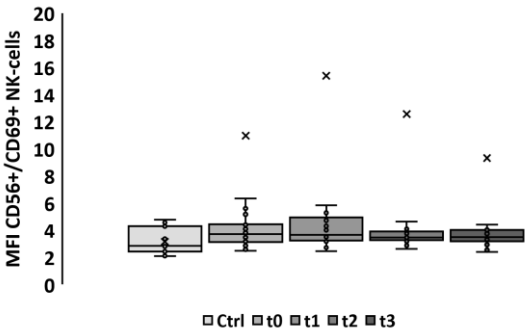
Figure 29: MFI CD3-/CD56+ NK-cells, Ctrl vs. Patients **Figure 30: MFI CD3-/CD56+ NK-cells, Ctrl vs. Recurrent Patients**
MFI (Mean Fluorescence Intensity) of CD3-/CD56+ Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of MFI of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test).

CD56+/CD69+ NK-/T-cells in MFI

CD56+ is an important effector marker on NK- and T-lymphocytes and CD69+ an early activation marker on NK-lymphocytes. (Cohavy & Targan, 2007; Millrud et al., 2012)

At all timepoints, patients had significantly higher MFI values of CD56+/CD69+ NK-/T-cells than controls (ctrl vs. t0: 3.17 ± 0.97 vs. 10.99 ± 19.74, **p ≤ 0.01; ctrl vs. t1: 3.17 ± 0.97 vs. 15.41 ± 36.62, **p ≤ 0.01; ctrl vs. t2: 3.17 ± 0.97 vs. 12.57 ± 24.8, **p ≤ 0.01; ctrl vs. t3: 3.17 ± 0.97 vs. 9.34 ± 18.53, *p ≤ 0.05). Furthermore, an increase at t1 (t0 vs. t1: 10.99 ± 19.74 vs. 15.41 ± 36.62) as well as drops at t2 and t3 could be observed (t1 vs. t2: 15.41 ± 36.62 vs. 12.57 ± 24.8, t2 vs. t3: 12.57 ± 24.8 vs. 9.34 ± 18.53).
(Figure 31, Table 5A and 5B)

Remarkably, recurrent patients had much higher MFI at t0, t1 and t5 than controls and patients at all timepoints (rec t0, rec t1, rec t5 vs. ctrl, pat t0, pat t1, pat t2, pat t3: 57.83 ± 41.95, 50.11 ± 31.42, 56.12 ± 35.65 vs. 3.17 ± 0.97, 10.99 ± 19.74, 15.41 ± 36.62, 12.57 ± 24.8, 9.34 ± 18.53) and a sharp drop at t2 was observable (t1 vs. t2: 50.11 ± 31.42 vs. 3.23).
(Figure 31 and 32, Table 5B and 6)



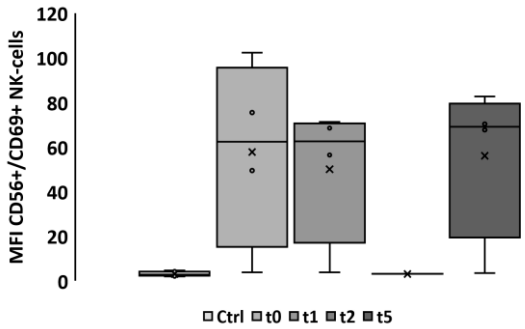


Figure 31: MFI CD56+/CD69+ NK-/T-cells, Ctrl vs. Patients **Figure 32: MFI CD56+/CD69+ NK-/T-cells, Ctrl vs. Recurrent Patients**
MFI (Mean Fluorescence Intensity) of CD56+/CD69+ Natural killer-/T-cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of MFI of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at

different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test).

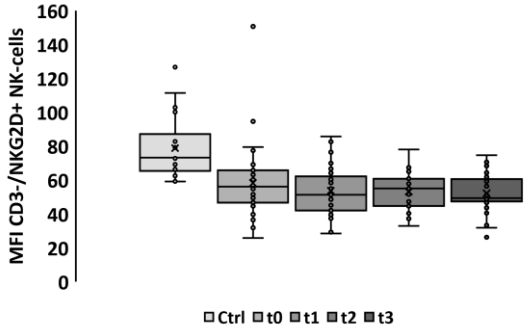
CD3-/NKG2D+ NK-cells in MFI

Natural cytotoxicity receptors (NCRs) belong to major lysis receptors in the NK-cell. NCRs include three immunoglobulin-like proteins: NKp46 (NCR1; CD335), NKp44 (NCR2; CD336) and NKp30 (NCR3; CD337). (Enk & Mandelboim, 2014; Noronha, Harman, Wagner, & Antczak, 2012) And NK-cell-activating receptors, such as NKG2D, NKp30, NKp44 and NKp46, have been shown to be particularly important for NK-cell recognition and lysis of target, e.g. for lysis of tumor cells. (Carbone et al., 2005; D. Pende et al., 2001; Daniela Pende et al., 2005; Vivier et al., 2011)

NKG2D receptor is not only expressed by NK-cells, but also by activated Cytotoxic T-cells and activated macrophages. Depending on the level of NKG2D ligands expressed on tumor cells, anti-tumor response can be induced by potent priming of Cytotoxic T-cells and sensitization of NK-cells. (Diefenbach, Jensen, Jamieson, & Raulet, 2001) For example, NKG2D receptor recognizes two different families of ligands: MHC class I chain-related molecules (MICA and MICB) and UL-16-binding proteins (ULBP). (Cristina Bottino, Castriconi, Moretta, & Moretta, 2005) NK-cells can recognize these NKG2D ligands on malignant tumor cells and eradicate them. (González, Groh, & Spies, 2006)

In contrast to most of the NK-cell subgroups, MFI of CD3-/NKG2D+ NK-cells of patients dropped throughout RCT (t0 vs. t3: 58.34 ± 22.6 vs. 52.26 ± 11.18) and MFI of patients at all timepoints were significantly lower than that of controls (ctrl vs. t0: 78.87 ± 18.69 vs. 58.34 ± 22.6, **p ≤ 0.01; ctr. vs. t1: 78.87 ± 18.69 vs. 53.72 ± 14.35, ***p ≤ 0.001; ctrl vs. t2: 78.87 ± 18.69 vs. 53.72 ± 14.35, ***p ≤ 0.001; ctrl vs. t3: 78.87 ± 18.69 vs. 52.26 ± 11.18, ***p ≤ 0.001). (Figure 33, Table 5A and 5B)

At t0, recurrent patients had higher values of MFI than controls and patients at all timepoints (rec pat t0 vs. ctrl, pat t0, pat t1, pat t2, pat t3: 82.39 ± 35.59 vs. 78.87 ± 18.69, 58.34 ± 22.6, 53.72 ± 14.35, 53.18 ± 10.89, 52.26 ± 11.18). Moreover, a sharp drop at t1 could be observed in recurrent patients (t0 vs. t1: 82.39 ± 35.59 vs. 45.49 ± 12.66).
(Figure 33 and 34, Table 5B and 6)



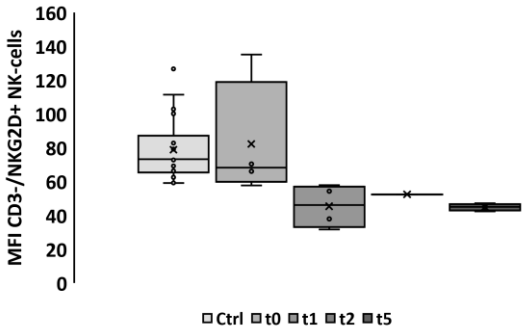


Figure 33: MFI CD3-/NKG2D+ NK-cells, Ctrl vs. Patients **Figure 34: MFI CD3-/NKG2D+ NK-cells, Ctrl vs. Recurrent Patients**
MFI (Mean Fluorescence Intensity) of CD3-/NKG2D+ Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of MFI of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test).

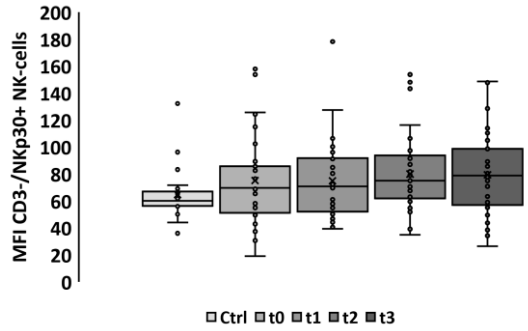
CD3-/NKp30+ NK-cells in MFI

Natural cytotoxicity receptors (NCRs) belong to major lysis receptors in the NK-cell. NCRs include three immunoglobulin-like proteins: NKp46 (NCR1; CD335), NKp44 (NCR2; CD336) and NKp30 (NCR3; CD337). (Enk & Mandelboim, 2014; Noronha et al., 2012) And NK-cell-activating receptors, such as NKG2D, NKp30, NKp44, and NKp46, have been shown to be particularly important for NK-cell recognition and lysis of the target. (Carbone et al., 2005; D. Pende et al., 2001; Daniela Pende et al., 2005)

NKp30 is important for mediating tumor immunosurveillance in several clinical settings. One of its ligands, B7H6, is expressed on many types of tumor cells, but absent on normal tissues. Interaction of NKp30 with B7H6 has been shown to improve degranulation of NK-cells. (H. Wang, Zheng, Wei, Tian, & Sun, 2012)

At all timepoints, patients had much higher MFI values of CD3-/NKp30+ NK-cells than controls (ctrl vs. t0, t1: 64.87 ± 19.44 vs. 75.34 ± 33.27, 75.11 ± 29.85, ctrl vs. t2: 64.87 ± 19.44 vs. 80.06 ± 29.91, *p ≤ 0.05; ctrl vs. t3: 64.87 ± 19.44 vs. 79.54 ± 30.67, *p ≤ 0.05).
(Figure 35, Table 5B)

Interestingly, recurrent patients had higher MFI values at t0 than controls and patients at all timepoints (rec pat t0 vs. ctrl, pat t0, pat t1, pat t2, pat t3: 94.59 ± 33.25 vs. 64.87 ± 19.44, 75.34 ± 33.27, 75.11 ± 29.85, 80.06 ± 29.91, 79.54 ± 30.67) and a sharp drop at t1 was noticeable (t0 vs. t1: 94.59 ± 33.25 vs. 65.42 ± 12.38).
(Figure 35 and 36, Table 5B and 6)



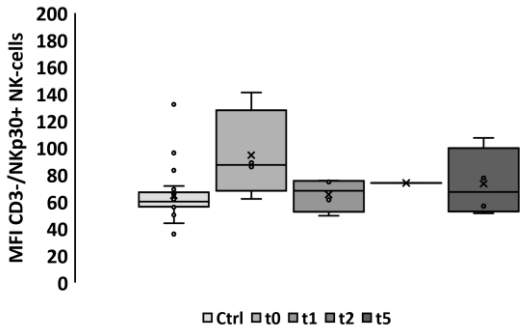


Figure 35: MFI CD3-/NKp30+ NK-cells, Ctrl vs. Patients **Figure 36: MFI CD3-/NKp30+ NK-cells, Ctrl vs. Recurrent Patients**
MFI (Mean Fluorescence Intensity) of CD3-/NKp30+ Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of MFI of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test).

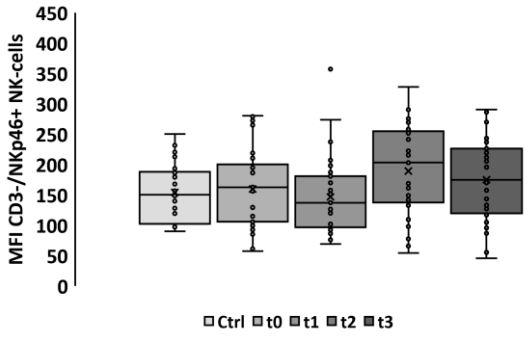
CD3-/NKp46+ NK-cells in MFI

Natural cytotoxicity receptors (NCRs) belong to major lysis receptors in the NK-cell. NCRs include three immunoglobulin-like proteins: NKp46 (NCR1; CD335), NKp44 (NCR2; CD336) and NKp30 (NCR3; CD337). (Enk & Mandelboim, 2014; Noronha et al., 2012) NK-cell-activating receptors, such as NKG2D, NKp30, NKp44, and NKp46, have been shown to be particularly important for NK-cell recognition and lysis of the target. (Carbone et al., 2005; D. Pende et al., 2001; Daniela Pende et al., 2005) In contrast to NKp30 and NKp44, which have similar cellular functions, NKp46 is structurally distinct from the other two molecules and is located in a different region of the genome. It is stably expressed on both resting and activated NK-cell, and is considered as an unique NK-cell marker. (Enk & Mandelboim, 2014; Noronha et al., 2012)

It was demonstrated that NKp46 is critically involved in influenza, tumorigenesis and diabetes. (Gazit et al., 2006; Gur et al., 2010; Halfteck et al., 2009) Moreover, NKp46 is described to be the key receptor in controlling the spread of various primary tumors in melanoma, lymphoma and other carcinomas. (Glasner et al., 2012)

MFI of CD3-/NKp46+ NK-cells of patients rose significantly at t2 (t0 vs. t2: 159.93 ± 64.31 vs. 189.5 ± 71.82, **p ≤ 0.01; t1 vs. t2: 146.98 ± 63.55 vs. 189.5 ± 71.82, ***p ≤ 0.001) and patients at t2 had significantly higher values of MFI of CD3-/NKp46+ NK-cells than controls (ctrl vs. t2: 154.24 ± 48.06 vs. 189.5 ± 71.82, *p ≤ 0.05).
(Figure 37, Table 5A and 5B)

Interestingly, patients with relapse at t0 had higher values of MFI than controls and patients at t0 (rec t0 vs. ctrl, pat t0: 178.7 ± 32.95 vs. 154.24 ± 48.06, 159.93 ± 64.31). Moreover, drops at t1 and t2 as well as an increase at t5 were visible (t0 vs. t1: 178.7 ± 32.95 vs. 126.45 ± 33, t1 vs. t2: 126.45 ± 33 vs. 113.51, t2 vs. t5: 113.51 vs. 156.24 ± 34.47).
(Figure 37 and 38, Table 5B and 6)



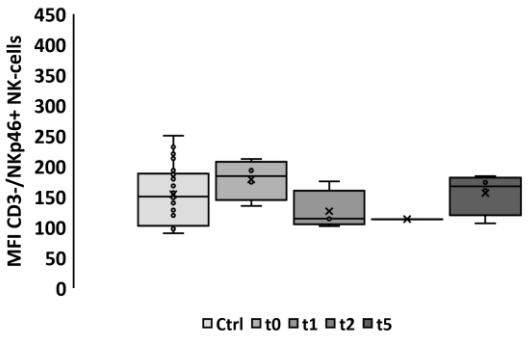


Figure 37: MFI CD3-/NKp46+ NK-cells, Ctrl vs. Patients **Figure 38: MFI CD3-/NKp46+ NK-cells, Ctrl vs. Recurrent Patients**
MFI (Mean Fluorescence Intensity) of CD3-/NKp46+ Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of MFI of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test).

5.4 Subsets of Natural Killer Cells (CD56/CD16) in %

Table 7A – Course of NK-Cell Subsets (CD56/CD16) of Pat. throughout RCT in %

Composition of Natural killer cell subpopulations in % (mean value ± standard deviation) in head and neck cancer **patients without relapse n=31** before (t0), after RCT (t1), 3 months after (t2) and 6 months after (t3). Values at different timepoints during RCT of each patient were considered as dependent samples (Wilcoxon rank sum test). **Significantly different values (t0 vs. tx) are indicated in bold (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).**

Time	t0	t1	t2	t3
NK-cells in % CD56bright/CD16-	2.1 ± 2.32	1.48 ± 2.08	2.19 ± 1.97	1.94 ± 2.18
NK-cells in % CD56bright/CD16+	4.73 ± 5.57	**3.2 ± 3.63***	3.87 ± 4.35	3.8 ± 3.32
NK-cells in % CD56dim/CD16-	5.43 ± 10.01	4.51 ± 6.08	2.8 ± 3.3	6.5 ± 14.41
NK-cells in % CD56dim/CD16+	82.63 ± 15.8	86.08 ± 12.92	88.13 ± 9.97	85.01 ± 16.11
NK-cells in % CD56-/CD16+	0.01 ± 0.15	0.11 ± 0.62	0.04 ± 0.21	0.08 ± 0.28

Table 7B – NK-Cell Subsets (CD56/CD16) of Pat. vs. Ctrl in %

Composition of Natural killer cell subpopulations in % (mean value ± standard deviation) in healthy **controls (Ctrl) n=22** and head and neck cancer **patients n=31** before (t0), after RCT (t1), 3 months after (t2) and 6 months after (t3). Values at different timepoints of patients in comparison with values of controls were considered as independent samples (Mann-Whitney-U test). **Significantly different values (Ctrl vs. tx) are indicated in bold (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).**

	Ctrl	t0	t1	t2	t3
NK-cells in % CD56bright/CD16-	1.83 ± 1.14	2.1 ± 2.32	1.48 ± 2.08	2.19 ± 1.97	1.94 ± 2.18
NK-cells in % CD56bright/CD16+	2.52 ± 1.34	**4.73 ± 5.57***	3.2 ± 3.63	3.87 ± 4.35	3.8 ± 3.32
NK-cells in % CD56dim/CD16-	2.8 ± 1.8	5.43 ± 10.01	4.51 ± 6.08	2.8 ± 3.3	6.5 ± 14.41
NK-cells in % CD56dim/CD16+	87.27 ± 5.97	82.63 ± 15.8	86.08 ± 12.92	88.13 ± 9.97	85.01 ± 16.11
NK-cells in % CD56-/CD16+	0 ± 0	0.01 ± 0.15	0.11 ± 0.62	0.04 ± 0.21	0.08 ± 0.28

Table 8 – NK-Cell Subsets (CD56/CD16) of Rec. Pat. vs. Ctrl in %

Composition of Natural killer cell subpopulations in % (mean value ± standard deviation) in healthy **controls (Ctrl) n=22** and head and neck cancer **patients with relapse n=4** before (t0), after RCT (t1), 3 months after (t2) and at time of relapse (t5). Values at different timepoints during RCT of each recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test). **Significantly different values (t0 vs. tx); (Ctrl vs. tx) are indicated in bold (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).**

	Ctrl	t0	t1	t2	t5
NK-cells in % CD56bright/CD16-	1.83 ± 1.14	0.92 ± 0.61	0.72 ± 0.42	0 (single value)	1.75 ± 2.47
NK-cells in % CD56bright/CD16+	2.52 ± 1.34	1.95 ± 2.19	1.04 ± 0.52	3.31 (single value)	1.13 ± 0.82
NK-cells in % CD56dim/CD16-	2.8 ± 1.8	4.45 ± 5.08	7.22 ± 4.69	4.13 (single value)	2.26 ± 1.72
NK-cells in % CD56dim/CD16+	87.27 ± 5.97	89.2 ± 9.06	87.44 ± 7.13	90.08 (single value)	90.97 ± 7.52
NK-cells in % CD56-/CD16+	0 ± 0	0 ± 0	0 ± 0	0 (single value)	0.8 ± 1.61

CD56bright (≈ 10%) and CD56dim (≈ 90%) NK-cells are reported to have diverse functional characteristics including cytolytic activity, response to activating agents, cytokine profile and trafficking to secondary lymphoid organs. (M. A. Cooper, Fehniger, & Caligiuri, 2001) Briefly, CD56bright NK-cells mediate low or no cytotoxicity, proliferate in response to picomolar levels of IL-2 and produce high levels of inflammatory cytokines. Conversely, CD56dim/CD16bright NK-cells show potent cytotoxicity functions, have a distinct cytokine and chemokine profile and express abundant killer inhibitory receptors (KIR). (Colonna et al., 1997; M. A. Cooper, Fehniger, & Caligiuri, 2001)

In our study, the largest proportion of subgroups of CD56/CD16 Natural killer cells in controls, patients and recurrent patients was formed by CD56dim/CD16+ NK-cells. These made up around 90% of NK-cells.

CD56bright/CD16- NK-cells in %

In the NK-cell subset, bright CD56 staining (CD56bright) defines elevated potential for cytokine production, while weaker fluorescence intensity (CD56dim) defines enhanced cytotoxicity together with a mature differentiation state. (M. A. Cooper, Fehniger, Turner, et al., 2001; Parrish-Novak et al., 2000) CD56bright/CD16- NK-cells are characterized as highly proliferative, immature regulatory cells, which can interact with neighboring immunocompetent cells in lymphoid tissues as they preferentially migrate to secondary lymphoid organs. (Chan et al., 2007; Juelke et al., 2010)

Although CD56bright NK-cells are described to secrete pro-inflammatory cytokines rather than mediating cytotoxicity, these NK-cells are able to efficiently eliminate Hsp70 membrane-positive tumor cells. (Stangl et al., 2018)

The % of CD56bright/CD16- NK-cells of controls was similar to those of patients at all timepoints, but recurrent patients had lower % of CD56bright/CD16- NK-cells at all timepoints than controls and patients at t0, t2 and t3 (rec t0, rec t1, rec t2, rec t5 vs. ctrl, pat t0, pat t2, pat t3: 0.92 ± 0.61%, 0.72 ± 0.42%, 0%, 1.75 ± 2.47% vs. 1.83 ± 1.14%, 2.1 ± 2.32%, 2.19 ± 1.97%, 1.94 ± 2.18%). After initial drops at t1 and t2, CD56bright/CD16- NK-cells in recurrent patients increased until t5 (t0 vs. t1: 0.92 ± 0.61% vs. 0.72 ± 0.42%, t1 vs. t2: 0.72 ± 0.42% vs. 0%, t2 vs. t5: 0% vs. 1.75 ± 2.47%).
(Figure 39 and 40, Table 7B and 8)

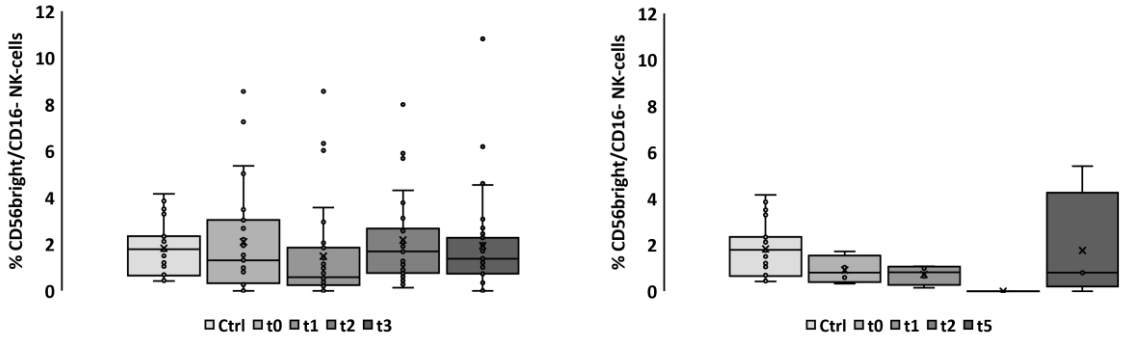


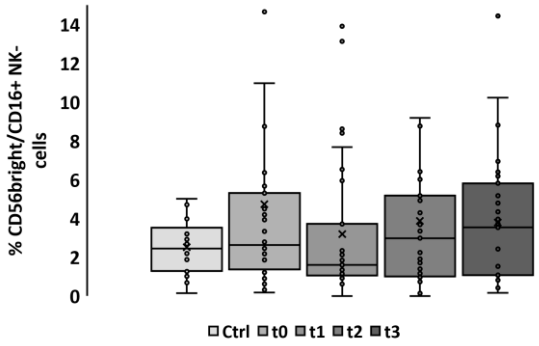
Figure 39: % CD56bright/CD16- NK-cells, Ctrl vs. Patients **Figure 40: % CD56bright/CD16- NK-cells, Ctrl vs. Recurrent Patients**
Percentages of CD56bright/CD16- Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test).

CD56bright/CD16+ NK-cells in %

CD16+/CD56bright NK-cells play a role in tumor immunity and bacterial infections. They show strong cytotoxicity against not only MHC class I negative, but also MHC class I positive tumors. (Takahashi et al., 2007)

HNSCC patients at t0 had almost twice as much of % CD56bright/CD16+ NK-cells as controls (ctrl vs. t0: 2.52 ± 1.34% vs. 4.73 ± 5.57%, *p ≤ 0.05), but CD56bright/CD16+ NK-cells in patients dropped significantly at t1 (t0 vs. t1: 4.73 ± 5.57% vs. 3.2 ± 3.63%, *p ≤ 0.05).

Interestingly, a rise at t2 as well as a drop at t5 in CD56bright/CD16+ NK-cells were visible in recurrent patients (t1 vs. t2: 1.04 ± 0.52% vs. 3.31%, t2 vs. t5: 3.31% vs. 1.13 ± 0.82%). (Figure 41 and 42, Table 7A, 7B and 8)



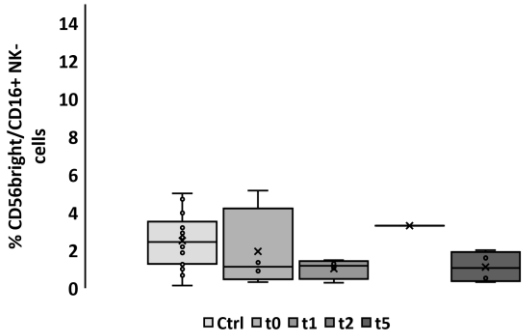


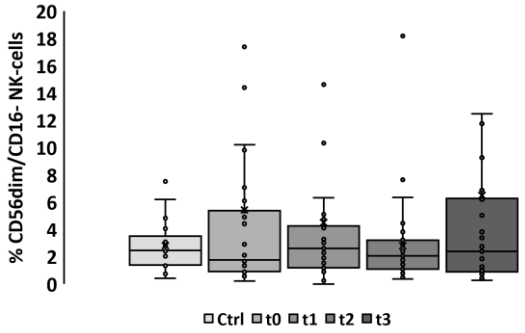
Figure 41: % CD56bright/CD16+ NK-cells, Ctrl vs. Patients **Figure 42: % CD56bright/CD16+ NK-cells, Ctrl vs. Recurrent Patients**
Percentages of CD56bright/CD16+ Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test).

CD56dim/CD16- NK-cells in %

CD56low/CD16low Natural killer cells are multifunctional cells. (Penack et al., 2005) And CD56low NK-cells in lymph nodes and tonsils are functionally and phenotypically different from circulating CD56low NK-cells. (Björkström et al., 2010; Ferlazzo et al., 2004) Takahashi et al described that CD56dim/CD16- cells are unconventional NK-cells that vigorously proliferate in response to cytokines involved in NK cell maturation, e.g. IL-2, IL-15, or IL-21. Similar to CD56bright/CD16- NK-cells, this NK-cell subset is able to produce large amounts of IFN-g and FAS ligand when stimulated. (Takahashi et al., 2007)

The % of CD56dim/CD16- NK-cells of patients at t0 was almost as twice as much of that of controls (ctrl vs. t0: 2.8 ± 1.8% vs. 5.43 ± 10.01%) and increased throughout RCT with exception of drops at t1 and t2 (t0 vs. t3: 5.43 ± 10.01% vs. 6.5 ± 14.41%, t0 vs. t1: 5.43 ± 10.01% vs. 4.51 ± 6.08%, t1 vs. t2: 4.51 ± 6.08% vs. 2.8 ± 3.3%).
(Figure 43, Table 7A and 7B)

In the same way, patients with relapse had a greater % of CD56dim/CD16- NK-cells at t0 than controls (ctrl vs. t0: 2.8 ± 1.8% vs. 4.45 ± 5.08%). In the course of RCT, a rise at t1 as well as drops at t2 and t5 were noticeable (t0 vs. t1: 4.45 ± 5.08% vs. 7.22 ± 4.69%, t1 vs. t2: 7.22 ± 4.69% vs. 4.13%, t2 vs. t5: 4.13% vs. 2.26 ± 1.72%). Thus, recurrent patients had less % of CD56dim/CD16- NK-cells at t5 than at t0 or controls (t5 vs. ctrl, t0: 2.26 ± 1.72% vs. 2.8 ± 1.8%, 4.45 ± 5.08%). (Figure 44, Table 8)



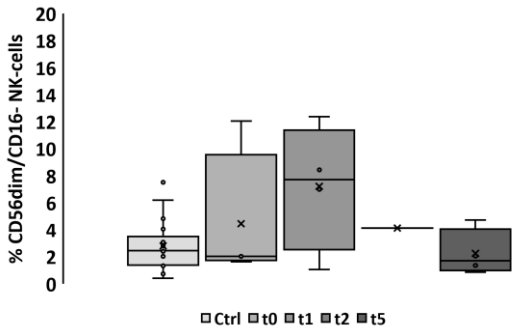


Figure 43: % CD56dim/CD16- NK-cells, Ctrl vs. Patients **Figure 44: % CD56dim/CD16- NK-cells, Ctrl vs. Recurrent Patients**
Percentages of CD56dim/CD16- Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test).

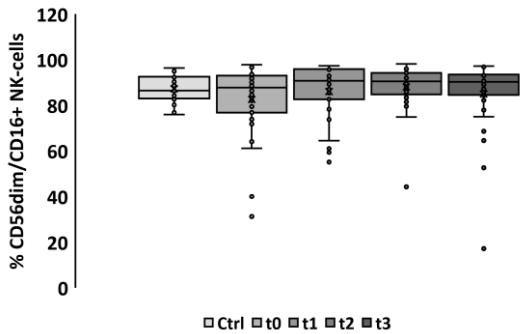
CD56dim/CD16+ NK-cells in %

According to receptor repertoire and expression levels, phenotypically distinct NK-cell subpopulations have been identified in different tissues, they likely represent specialized NK-cell subgroups capable of mediating different functions and are endowed with distinct migratory properties. (M. A. Cooper, Fehniger, & Caligiuri, 2001; Nagler, Lanier, Cwirla, & Phillips, 1989)

CD56dim/CD16bright NK-cells, which produce low levels of cytokines, predominantly mediate natural cytotoxicity, while the CD56bright/CD16dim subset plays a role in immune regulation through a high cytokine secretion potential. (Konjevic et al., 2012) Furthermore, CD56dim/CD16+ NK-cells are able to exert cytotoxic functions and promote immune responses as they home toward inflammatory sites. (Hanna et al., 2004)

The % of CD56dim/CD16+ NK-cells of patients at t0 was less than that of controls (ctrl vs. t0: 87.27 ± 5.97% vs. 82.63 ± 15.8%), but CD56dim/CD16+ NK-cells of patients increased at t1 and t2 (t0 vs. t1: 82.63 ± 15.8% vs. 86.08 ± 12.92%, t1 vs. t2: 86.08 ± 12.92% vs. 88.13 ± 9.97%) and dropped at t3 (t2 vs. t3: 88.13 ± 9.97% vs. 85.01 ± 16.11%).

Interestingly, CD56dim/CD16+ NK-cells of recurrent patients stayed almost at the same level. (Figure 45 and 46, Table 7A, 7B and 8)



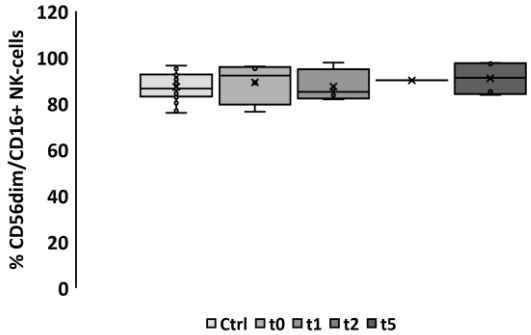


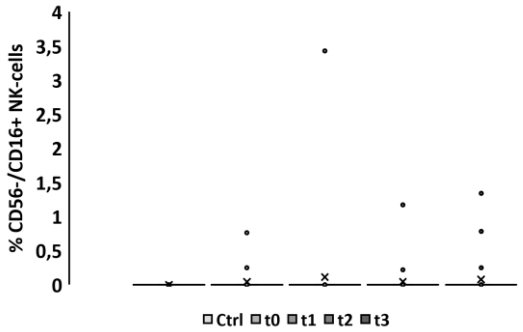
Figure 45: % CD56dim/CD16+ NK-cells, Ctrl vs. Patients **Figure 46: % CD56dim/CD16+ NK-cells, Ctrl vs. Recurrent Patients**
Percentages of CD56dim/CD16+ Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test).

CD56-/CD16+ NK-cells in %

The presence of CD56-/CD16+ NK-cells was observed in virus-infected patients and in healthy cord blood. (Gaddy & Broxmeyer, 1997; X. Lu et al., 2008) Furthermore, studies reported that CD56-/CD16+ NK-cells were not only expanded in hepatitis C virus-infected patients, but also in HIV-positive patients. (Alter et al., 2005; Mavilio et al., 2005)

Remarkably, HNSCC patients had a higher % of CD56-/CD16+ NK-cells than controls at all timepoints (ctrl vs. t0, t1, t2, t3: 0 ± 0% vs. 0.01% ± 0.15%, 0.11 ± 0.62%, 0.04 ± 0.21%, 0.08 ± 0.28%).

Interestingly, the % of CD56-/CD16+ NK-cells of recurrent patients kept a steady level at zero until the increase at t5 (t2 vs. t5: 0% vs. 0.8 ± 1.61%).
(Figure 47 and 48, Table 7B and 8)



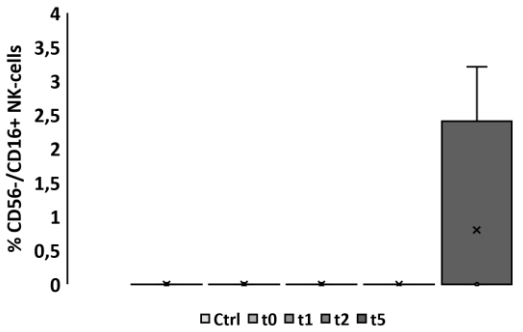


Figure 47: % CD56-/CD16+ NK-cells, Ctrl vs. Patients **Figure 48: % CD56-/CD16+ NK-cells, Ctrl vs. Recurrent Patients**
Percentages of CD56-/CD16+ Natural killer cells in healthy controls, patients and recurrent patients with HNSCC before (t0), directly after RCT (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5). The data show mean values ± standard deviation of the percentage of positively stained cells. Values at different timepoints during RCT (t0, t1, t2, t3, t5) of each patient and recurrent patient were considered as dependent samples (Wilcoxon rank sum test), values at different timepoints of patients and recurrent patients in comparison with values of controls as independent samples (Mann-Whitney-U test).

6 Discussion

Immune cells can infiltrate a developing tumor mass and either promote, hold it in a state of growth dormancy or inhibit tumorigenesis. In this way, tumors are imprinted by the immunologic environment in which they form. This process is also called “cancer immunoediting”. (Shankaran et al., 2001) The extent of host immune-deficiency directly correlates with the level of cancer immunoediting. (O’Sullivan et al., 2012) Some studies indicate that patients with HNSCCs have dysfunctional immune cells. (Wanebo et al., 1993) We have shown that HNSCC patients, treated with primary RCT, have altered lymphocyte homeostasis, which already exists before the treatment with primary RCT and persists for months after RCT.

The alteration of human lymphocyte phenotypes in the peripheral blood can be used for evaluation of systemic immune status of patients. It provides the opportunity for serial tracing of immunological changes before, during and after treatment. Furthermore, age, gender, nutritional status, autoimmune diseases, drug abuse as well as variations in geographic area may also affect lymphocyte subtypes in the peripheral blood of patients. (Lee et al., 2010)

Before discussing the changes of each lymphocyte subset we have discovered throughout the primary RCT, we first want to talk about how RT/CT works and mention some of their interactions with the immune system described in the literature.

Free-radical producing is utilized in radiation therapeutic approaches and effects of ionizing radiation produce DNA damage and cause consequent cell death. (DeVita et al., 2015) RT can kill cancer cells not only by a direct cytotoxic effect, but also by modifying tumor microenvironment and generating inflammation through direct and indirect effects on immunologic processes. (Formenti & Demaria, 2013; Reynders & De Ruysscher, 2015)

On the one hand, radiotherapy is perceived as a generally immunosuppressive modality in the oncology community because of the well-known application of whole-body radiation to ablate patients’ immune system in preparation for allogeneic transplant. But on the other hand, effects of local radiotherapy on tumors are rapidly emerging as opportunities to remodel and enhance immunity against cancer. (Formenti & Demaria, 2013; Gough & Crittenden, 2009)

Effects of ionizing radiation on T-cells in tumor response were first suggested in 1979 in experiments demonstrating reduced therapeutic efficacy in irradiated mice that lacked a normal T-cell repertoire. (Slone et al., 1979)

However, the goal of local radiotherapy is to cause tumor cell destruction to limit the progress of tumor, while minimizing the damage of normal tissues. The destruction of tumor cells is associated with a great amount of tumor antigens in form of necrotic and apoptotic tumor cells as well as cellular debris, which have the potential to stimulate immune response. Local tumor irradiation enhances antigen presentation within tumor-draining lymph nodes and increases the numbers of T-cells within tumor-draining lymph nodes that secrete IFN-γ upon tumor peptide stimulation. Radiation further elevates the numbers of immune cells trafficking towards and infiltrating the tumors, including tumor-infiltrating CD8+ lymphocytes (TILs) which secrete IFN-γ upon tumor peptide stimulation and lyse tumor cell targets. (Chakraborty et al., 2003; Foss, 2002; Garnett et al., 2004; Kotera et al., 2001; Lugade et al., 2005, 2008; Matsumura & Demaria, 2010; Reits et al., 2006)

Through radiotherapy, structural changed proteins, known as tumor associated antigens (TAAs), can be overexpressed and death receptors, such as Fas/CD95, MHC I and co-stimulatory molecules on certain tumor cells can also be upregulated. As a result, the immune system is able to recognize these tumor cells and eliminate them. (Friedman, 2002; Reynders & De Ruysscher, 2015; Santin et al., 1996; Sheard et al., 1997) Another impact of RT, mediated by the immune system, is a phenomenon called “abscopal effect”, which describes

that local irradiation of a single tumor site leads to reduction of non-irradiated distant metastases. (Ma et al., 2010)

On the contrary, radiation can also be immunosuppressive as it can activate latent transforming growth factor β. (Barcellos-Hoff et al., 1994; Jobling et al., 2006) Some papers also describe that radiation enhances the immunosuppressive and protumorigenic phenotype of macrophages. (Chiang et al., 2012; Tsai et al., 2007)

However, Apetoh et al suggested that irradiation and chemical drugs may complement each other in fulfilling the requirements for molecular signals of immunogenic cell death. (Apetoh et al., 2007)

One of the most promising single chemotherapeutic agent used against HNSCC has been cisplatin. It leads to DNA strand breakage during replication via intra- and interstrand cross-links in DNA via covalent bonds with the platinum molecule. (D. Li et al., 2002) In addition to the direct effects of chemotherapeutic agents on cancer cells, their efficacy is influenced by the interplay between tumor cells and immune components as well. (Coffelt & de Visser, 2015) Chemotherapy can either destroy cancer cells by causing them to elicit an immune response and enhance their susceptibility to immune attack, or stimulate anticancer immune effectors in a direct manner or by subverting immunosuppressive mechanisms. (Zitvogel et al., 2011)

But it is not easy to understand this interplay, e.g. on the one hand tumor-associated macrophages (TAMs) interfere with or augment the therapeutic activity of several types of chemotherapy, but on the other hand some papers report that increased TAM+ abundance and low CD8+ T-cell abundance in human breast tumors are associated with poor response to neoadjuvant chemotherapy and interestingly, splenic macrophages have also been implicated in conferring systemic resistance to cisplatin in subcutaneous cell line models via secretion of lysophospholipids which alter the DNA damage response. (DeNardo et al., 2011; De Palma & Lewis, 2013; Houthuijzen et al., 2014) Thus, several studies suggest that the synergism between TAM inhibition and chemotherapy may be beneficial for several types of cancer. (Coffelt & de Visser, 2015)

Moreover, tumor cells divide asynchronously and haphazardly, which means that only a small proportion of cancer cells can be influenced by cytotoxic drugs, leaving a large proportion unable to be affected by the agents. (Coventry & Ashdown, 2012) However, cytotoxic agents do not only affect cancer cells, but also kill synchronously dividing normal cells, especially rapidly dividing normal cell populations, such as cells in the bone marrow and cause myelosuppression. As cells of the innate immune system are particularly sensitive to cytotoxic agents, CT can lead to decreased peripheral neutrophil and monocyte counts in the circulation and in tissues. Furthermore, both CD4+ as well as CD8+ T-cells are also highly sensitive to the effects of cytotoxic agents. Indeed, not only CT can cause immunosuppression, but also RT can result in the death of immunologic cells, e.g. intravascular cells that circulate through the blood vessels in and around the cancer mass. (Coventry & Ashdown, 2012)

In conclusion, not only RT and CT, but also surgery cause cell death and cell injury which are associated with “danger signals” issued to the immune system, both innate and adaptive, and result in immune reactivity. (Coventry & Ashdown, 2012) But the answer to whether the immune response following those therapies mentioned above is activated or suppressed, is unclear. The answer to this question may lie in reports of the “bimodality” of action of numerous and various cytokines and chemokines of the human immune system and in the “time-dependent” dynamic nature of the immune response. (Coventry & Ashdown, 2012; Hatzivassiliou et al., 2010; Knoechel et al., 2005; Ribas & Flaherty, 2011; Sosman et al., 2012)

After a little introduction on various effects of RT and CT on the immune system, which are not yet completely understood, we present and discuss our findings, outlined in **bold,** in the following:

6.1 Subsets of Lymphocytes in %

6.1.1 CD3-/CD19+ B-Cells in %

B-lymphocytes, as the crucial component of humoral immunity, function in immunoglobulin (Ig) production, antigen presentation and secretion of pro-inflammatory cytokines. Studies have revealed that increased systemic humoral immune responses, including increased production of Ig can lead to chronic inflammation associated with increased cancer risk. Cancer patients often develop antibodies to TAAs; however, production of these does not necessarily mean protection, but instead anti-tumor antibodies can enhance tumor growth by promoting protumor immune responses and protecting malignant cells from Cytotoxic T-cell-mediated killing, which often correlates with poor prognosis and decreased survival for several human cancer types. (Gunderson & Coussens, 2013; Tan & Coussens, 2007)

Human squamous cell carcinomas of the vulva and head and neck show hallmarks of B-cell infiltration. Interestingly, B-cell-deficient mice have reduced ability to support tumor growth. B-cells facilitate squamous cell carcinoma progression in K14-HPV16 mice through antibody-mediated activation of Fc receptors on TAMs and mast cells, stimulating their proangiogenic abilities. Indeed, the combination of platinum-based chemotherapy or paclitaxel together with anti-CD20 antibodies in orthotopic squamous cell carcinoma elicits stasis of established tumors, while chemotherapy or B-cell depletion as single regimens are completely ineffective. (Affara et al., 2014; Andreu et al., 2010; de Visser et al., 2005)

Conversely, other studies suggest that a great number of peritumoral B-cells in lymph node metastases of patients with oro- and hypopharyngeal carcinoma is associated with favorable outcome indicating that relevant interaction between tumors and humoral immune system occurs in lymph nodes rather than at primary tumor site and a low % of circulating CD19+ B-cells in patients with nasopharyngeal carcinoma is associated with poorer 5-year progression-free survival and 5-year overall survival than patients with a high % of CD19+ B-cells. (Pretscher et al., 2009; T. Xu et al., 2014) Interestingly, patients with multiple myeloma with higher baseline levels of blood CD19+ B-cells have a longer survival and higher CD19+ blood levels are positively associated with patients' survival before CT, two years after CT and at relapse. (Kay et al., 2001)

In our study, we observed a striking drop in the percentage of CD3-/CD19+ B-cells of head and neck cancer patients (n=31) directly after RCT at t1 (t0 vs. t1: 8.2 ± 4.29% vs. 3.85 ± 3.25%, ***p ≤ 0.001), but B-cells recovered 6 months after RCT. (Figure 1, Table 3A)

These results correlate with observations from other studies, where B-cells seemed to be the most radiosensitive lymphocyte subpopulation, but there was no decline in immunoglobulin levels. (Belka et al., 1999) In breast cancer patients, the % of B-cells dropped during RT, but recovered within 6 months after RT, possibly by B-cell precursors which originated from the non-irradiated bone marrow. (Sage et al., 2016) The percentage of B-cells registered in the peripheral blood of patients with mammary carcinoma prior to treatment was also significantly higher than that of patients at the end of 3 weeks after chemotherapy treatment with cyclophosphamide. (Smit et al., 1979) RCT reduces the % of circulating B-cells in HNSCC patients, but whether B-cell functions are impaired or whether they migrate from the circulation into tumor site is unknown and should be further studied. No detectable decline in immunoglobulin levels after RT might be a hint that B-cell functions might not be impaired after RT.

In our study, patients (n=31) had significantly lower % of B-cells in respect of the total amount of lymphocytes at timepoints t0, t1 and t2 (before, directly after and 3 months

after RCT) in comparison to healthy controls (n=22) (ctrl vs. t0: 10.83 ± 2.71% vs. 8.2 ± 4.29%, *p ≤ 0.05; ctrl vs. t1: 10.83 ± 2.71% vs.3.85 ± 3.25%, ***p ≤ 0.001; ctrl vs. t2: 10.83 ± 2.71% vs. 6.84 ± 4.68%, **p ≤ 0.01). (Figure 1, Table 3A and 3B)

Other studies also demonstrated that HNSCC patients had a lower % of B-lymphocytes than healthy controls in the peripheral blood. (Andrade et al., 2013; Boucek et al., 2010; Kaffenberger et al., 1995) But a higher percentage of CD19+-B-cells, that expressed the activation marker HLA-DR, was detected in HNSCC patients compared to controls. Tumor production of growth factors and B-cell chemoatractives might be eliciting lymphocyte migration into tumor site and metastasis and could explain the reduction of B-cells in the peripheral blood during HNSCC. (Andrade et al., 2013) And a higher % of HLA-DR+ B-cells might be able to enhance antitumor activity.

Our data of recurrent patients (n=4) also showed a drop in % of B-cells at t1 and t2 and B-cells of recurrent patients tended to have a slower recovery until the relapse at t5 in comparison with patients. Furthermore, patients with relapse at t5 had less % of B-cells than controls. (Figure 2, Table 4)

In another study, an elevation in the % of CD19+ B-cells was observed in patients with oral leukoplakia (OL) relative to healthy people. (Lee et al., 2010) In contrast to patients with OL, a decline in CD19+ B-cells was noted in patients with recurrent and advanced OSCC compared to healthy controls. Possible explanation could be the early induction of B-cell immune response to tumor antigens, followed by accumulation of genotoxic events to B-cells and bone marrow due to cytotoxic therapies at a late stage, which leads to impairment of B-cell proliferation and function. A decline in CD19+ B-cells might be a contributing factor for malignant transformation of OL to OSCC. (Lee et al., 2010) Furthermore, a lower level of B-cells (CD3+/CD19+) in the group of patients with locoregional metastases could be identified compared with patients at N0 stage. (Boucek et al., 2010) This, together with the slow recovery of recurrent patients after RCT in our study, indicate an impairment of the immune system of recurrent patients, which differs from patients without relapse and healthy controls.

6.1.2 CD3+/CD56- T-Cells in %

During the course of RCT, the percentage of CD3+/CD56- T-cells dropped significantly (t0 vs. t2: 68.37 ± 14.34% vs. 57.9 ± 14.03%, **p ≤ 0.01; t0 vs. t3: 68.37 ± 14.34% vs. 55.17 ± 11.9%, ***p ≤ 0.001). In the same way, the data of head and neck cancer patients showed significantly lower % of T-cells than controls at timepoints of t2 and t3 (ctrl vs. t2: 67.29 ± 7.41% vs. 57.9 ± 14.03%, **p ≤ 0.01, ctrl vs. t3: 67.29 ± 7.41% vs. 55.17 ± 11.9%, ***p ≤ 0.001). (Figure 3, Table 3A and 3B)

Nollert et al also demonstrated a significant drop of total CD3+ T-cell counts of stage IV HNSCC patients during RCT with carboplatin (70 mg/sqm) and a total radiation dose of 66 Gy. But conversely to our findings, these cells recovered 7 weeks after RCT and patients in our study did not recover even 6 months after RCT. (Nollert et al., 1999) A cumulative total dose of at least 180mg/m² body surface of cisplatin in our study might offer an explanation for the steady decrease of CD3+ T-cells during and after RCT, as it is known that cisplatin's systemic toxicity causes reduction of T-cell proliferation. (Garzetti et al., 1994; Sfikakis et al., 1996) Interestingly, an increase in the average percentage of T-cells in HNSCC patients could be observed after RT as well as surgery. (Olkowski & Wilkins, 1975) And patients previously treated with surgery and adjuvant RT had lower CD3+ T-cell counts compared to patients, who only had surgery. (B. Hathaway et al., 2005a)

On the one hand, one study reported about no significant change of % CD3+ T-cells observed in breast cancer patients with RCT, who received chemotherapeutic agents including 5-

fluorouracil, epirubicin and cyclophosphamide. (Sage et al., 2016) But on the other hand, some studies observed diminished absolute CD3+ T-cell counts or % of CD3+ T-cells in breast cancer and other cancer patients, who received RT, CT or RCT, after treatment or in comparison with healthy controls. (Belka et al., 1999; Mozaffari et al., 2009; Smit et al., 1979)

We could not detect any significant differences in the % of CD3+ T-cells between HNSCC patients and controls. In the same way, some studies described a significant decrease in the absolute counts of CD3+ T-cells of HNSCC patients compared with controls, but not percentages. (B. Hathaway et al., 2005a; Boucek et al., 2010) In contrast, other studies reported that HNSCC patients prior to treatment had a lower % of T-cells than controls. (Kaffenberger et al., 1995; Olkowski & Wilkins, 1975)

Possible explanation for this might be that the % of lymphocyte subsets is dependent on the total count of lymphocytes, which might be altered in HNSCC patients and this transition is particularly after anticancer therapies visible.

In comparison to patients, the % of T-cells of recurrent patients also decreased, even under the % of T-cells of healthy controls and patients at all timepoints, until t5 (t0 vs. t5: 68.03 ± 17.13% vs. 52.7 ± 6.84%, ctrl vs. t5: 67.29 ± 7.41% vs. 52.7 ± 6.84%), but the course during RCT was different and there was a noticeable rise at t1. The % of T-cells of recurrent patients at t1 was even higher than that of controls and patients at all timepoints. (Figure 3 and 4, Table 3B and 4)

Others also demonstrated that recurrent HNSCC patients tended to have a lower % of circulating T-cells than patients, who have remained free of disease, and healthy controls. (Johnson et al., 1984) It could be observed that patients with active disease had significantly lower CD3+ T-cell counts than those with non-evident disease. (B. Hathaway et al., 2005a) Thus, suggesting that recurrent HNSCC patients are more likely to show abnormalities of T-cell subpopulations. (Johnson et al., 1984) The paradoxical rise directly after RCT at t1 might be a possible immune response of recurrent patients to RCT.

Patients with HNSCC, some with active disease and some treated with surgery, surgery with adjuvant RT or surgery with adjuvant CT at least 12 months previously, had a higher % of circulating Fas+ CD3+ T-cells compared to normal controls and these T-cells were more apoptotic. Fas/FasL interactions in HNSCC patients might lead to an increased turnover rate of lymphocytes, which might be responsible for functional lymphocyte imbalance and reduced immune competence. (Hoffmann et al., 2002)

Besides head and neck cancer patients, patients with myeloma, breast -, ovarian - as well as liver cancer have also been observed to have abnormalities in T-cell counts. (Kay et al., 2001; Melichar et al., 2001; Schröder et al., 1997; Wolf et al., 1985)

Decreased T-cell counts could reflect effects of the disease process on long-lasting T-cell imbalance, in addition, it could also be treatment-mediated. Such persistent changes in homeostasis of T-cell subpopulations might adversely affect antitumor responses and favor recurrence. As a result, therapies designed to increase lymphocyte counts in HNSCC patients should be considered. (Iris Kuss et al., 2004)

6.1.3 CD3+/CD4+ T-Helper-Cells in %

CD3+/CD4+ T-helper-cells made up to 2/3 of the total amount of T-cells and patients had a course of CD3+/CD4+ T-helper-cells similar to that of CD3+/CD56- T-cells during RCT and in the follow-up. The % of T-helper-cells dropped significantly at t2 and t3 (t0 vs. t2: 46.04 ± 12.38% vs. 25.9 ± 10.17%, ***p ≤ 0.001; t0 vs. t3: 46.04 ± 12.38% vs. 26.07 ± 9.84%,

*****p ≤ 0.001; t1 vs. t2: 41.6 ± 11.65% vs. 25.9 ± 10.17%, ***p ≤ 0.001) and at all timepoints, HNSCC patients had lower % T-helper-cells than controls (ctrl vs. t0: 48.82 ± 9.24% vs. 46.04 ± 12.38 %, ctrl vs. t1: 48.82 ± 9.24% vs. 41.6 ± 11.65%, *p ≤ 0.05; ctrl vs. t2: 48.82 ± 9.24% vs. 25.9 ± 10.17%, ***p ≤ 0.001; ctrl vs. t3: 48.82 ± 9.24% vs. 26.07 ± 9.84%, ***p ≤ 0.001). Remarkably, the % of T-helper-cells of patients at t2 and t3 were almost half of that of t0 and of controls (ctrl, t0 vs. t2, t3: 48.82 ± 9.24%, 46.04 ± 12.38 % vs. 25.9 ± 10.17%, 26.07 ± 9.84%). (Figure 5, Table 3A and 3B)**

In the same way, other studies have reported that patients with HNSCC had significantly less % of CD4+ T-cells than controls. (Chikamatsu et al., 2007) Hathaway et al. described a significant decrease in the absolute counts of CD4+ T-cells, but not percentages compared to healthy controls. (B. Hathaway et al., 2005a) In particular, the percentage of CD4+ T-cells of stage III and IV HNSCC patients before initiation of any treatment was much lower than in healthy subjects. (Bose et al., 2008) Melioli et al might give reasons for the findings above with their observations of severe proliferative defect of the CD4+ subpopulation. (Melioli et al., 2003)

On the contrary, the percentage of CD4+ circulating T-cells in HNSCC patients at time of diagnosis was significantly increased compared to healthy controls in Boucek et al. (Boucek et al., 2010)

One study also reported that in comparison with controls, HNSCC patients showed significant increases in the activation status of CD4+ T-cells as well as higher migration potentials of lymphocytes, but there were no significant differences in the frequency of CD4+ T-cells in HNSCC patients and negative controls. (Lee et al., 2010)

For the course of CD4+ T-helper-cells throughout RCT, we provide the same explanation as used in CD3+ T-cells as CD4+ T-helper-cells made up to 2/3 of the total amount of T-cells. In Nollert et al, a significant drop of total CD4+ T-cell counts of stage IV HNSCC patients during RCT with carboplatin (70 mg/sqm) and a total radiation dose of 66 Gy was also demonstrated. But conversely to our findings, these cells recovered 7 weeks after RCT and patients in our study did not recover even 6 months after RCT. (Nollert et al., 1999) A cumulative total dose of at least 180mg/m² body surface of cisplatin in our study might offer an explanation for the steady decrease of CD4+ T-cells during and after RCT, as it is known that cisplatin's systemic toxicity causes reduction of T-cell proliferation. (Garzetti et al., 1994; Sfikakis et al., 1996)

Similarly, HNSCC patients previously treated with surgery and adjuvant RT had lower CD4+ T-cell counts compared to patients, who only had surgery, which then again had lower CD4+ T-cell counts than healthy controls. (B. Hathaway et al., 2005b; Iris Kuss et al., 2004) In another study, the percentage of CD4+ T-helper-cells decreased in head and neck patients during and after definite and adjuvant RT. (Wolf et al., 1985)

For patients with other cancer types, the level of total CD4+ T-cell number of cancer patients also declined during RT, but recovered more slowly than CD8+ T-cells. (Belka et al., 1999) Our data also show that T-helper-cells did not recover 6 months after RCT, which suggest that effects of the tumor or anti-tumor therapies on the homeostasis of lymphocytes can persist months after tumor removal and/or any other lymphoablative therapy.

Interestingly, the percentage and absolute counts of CD3+/CD4+ helper T-cells rose in breast cancer patients during RT and RCT. (Mozaffari et al., 2009; Sage et al., 2016) The absolute number of T-helper-cells of breast cancer patients, who received RCT, was significantly decreased compared to patients, who only received RT, and healthy controls. (Mozaffari et al., 2009) But expressions of intracellular cytokines (IFN-gamma, IL-2, IL-4) in CD4+ and CD8+ T-cells were significantly higher in RCT patients compared to patients, who only received RT. (Mozaffari et al., 2009)

Some reports even show associations of T-cell subset abnormalities with patients' clinical outcomes. In advanced breast cancer patients, the subgroup with an increased CD4/CD8 ratio had a better chance of responding to neoadjuvant chemotherapy. Patients with multiple myeloma with higher baseline levels of blood CD4+ T-cells had a longer survival. (Kay et al., 2001; Murta et al., 2000)

Recurrent patients had a much lower % of T-helper-cells at t0 than patients at t0 and controls (ctrl vs. rec t0: 48.82 ± 9.24% vs. 25.89 ± 14.3%, pat t0 vs. rec t0: 46.04 ± 12.38% vs. 25.89 ± 14.3%) and they had lower % of T-helper-cells at all timepoints than controls although this lymphocyte subpopulation tended to grow at t1. (Figure 5 and 6, Table 3B and 4)

Recurrent HNSCC patients are more likely to show abnormalities of T-cell subpopulations. (Johnson et al., 1984) Two studies confirmed that CD4 lymphopenia was associated with poor survival and prognosis in metastatic cancer. (Péron et al., 2013, p. 4; Trédan et al., 2013) Not surprisingly, recurrent HNSCC patients tended to have a lower % as well as the lowest absolute counts of circulating T-helper-cells compared to patients, who have remained free of disease. (B. Hathaway et al., 2005a; Johnson et al., 1984; Lee et al., 2010) Likewise, it could be observed that patients with active disease had significantly lower CD4+ T-cell counts than those with non-evident disease. (B. Hathaway et al., 2005a) And an obvious decrease in CD4+ T-cells was found in advanced OSCC patients compared to healthy controls. (Lee et al., 2010)

Thus, a low % of T-helper-cells before treatment at t0 could be a marker for HNSCC patients, suggesting the likelihood of these patients to suffer from recurrent disease at a later timepoint.

The paradoxical rise directly after RCT at t1 might be a possible immune response of recurrent patients to RCT.

6.1.4 CD3+/CD8+ Cytotoxic T-Cells in %

At all timepoints t0, t1, t2 and t3, HNSCC patients had significantly higher % of CD3+/CD8+ Cytotoxic T-cells than controls, and patients at t2 even reached values more than twice of those of controls (ctrl vs. t0: 10.83 ± 3.81% vs. 16.78 ± 7.03%, **p ≤ 0.01; ctrl vs. t1: 10.83 ± 3.81% vs. 18.36 ± 7.39%, ***p ≤ 0.001; ctrl vs. t2; 10.83 ± 3.81% vs. 23.93 ± 10.76%, ***p ≤ 0.001; ctrl vs. t3: 10.83 ± 3.81% vs. 20.47 ± 9.44%, ***p ≤ 0.001). The % of Cytotoxic T-cells of patients increased during and after RCT significantly with an exceptional drop at t3 (t0 vs. t2: 16.78 ± 7.03% vs. 23.93 ± 10.76%, ***p ≤ 0.001; t0 vs. t3: 16.78 ± 7.03% vs. 20.47 ± 9.44%, *p ≤ 0.05; t1 vs. t2: 18.36 ± 7.39% vs. 23.93 ± 10.76%, ***p ≤ 0.001; t2 vs. t3: 23.93 ± 10.76% vs. 20.47 ± 9.44%, *p ≤ 0.05). (Figure 7, Table 3A and 3B)

In contrast to our data, other studies observed no significant difference in % of CD8+ T-cells of head and neck cancer patients at time of diagnosis in comparison with controls. (Boucek et al., 2010; Lee et al., 2010) But HNSCC patients showed significant increases in the activation status of CD4+ and CD8+ T-cells as well as higher migration potentials of lymphocytes. (Lee et al., 2010)

Furthermore, Hathaway et al. described a significant decrease in the absolute counts of CD8+ T-cells of HNSCC patients, but not percentages in comparison with healthy individuals. (B. Hathaway et al., 2005b) It has been reported that the percentage of lymphocyte subsets is predictive of survival in head and neck cancer as elevated T4/T8 ratios and low percent T8 cell levels are significantly associated with decreased disease-free survival. (Wolf et al., 1987)

Our data exhibit a significant rise in the % of Cytotoxic T-cells of HNSCC patients throughout RCT and also compared to controls, which possibly suggests the anti-tumor immune reaction along with higher % of CD8+ T-cells in HNSCC patients before, during and in the follow-up of RCT as well as the successful immune boost through RCT at t1.

In contrast to our data, a significant drop of total CD8+ T-cell counts of stage IV HNSCC patients during RCT with carboplatin (70 mg/sqm) and a total radiation dose of 66 Gy was demonstrated in Nollert et al and these cells recovered 7 weeks after RCT. (Nollert et al., 1999) Carboplatin as a chemotherapeutic agent might have other effects on patients' immune cells than cisplatin. Furthermore, the accelerated radiochemotherapy with carboplatin induced lymphopenia and functional impairment of cellular immunity, followed by compensatory cytokine reaction. (Nollert et al., 1999)

Similarly, the percentage and the absolute number of CD3+/CD8+ Effector T-cells decreased in breast cancer patients during RT and RCT with chemotherapeutic agents including 5-fluorouracil, epirubicin and cyclophosphamide. (Mozaffari et al., 2009; Sage et al., 2016) And these patients had smaller quantity of CD8+ T-cells than healthy controls. (Mozaffari et al., 2009) At the same time, expressions of intracellular cytokines (IFN-gamma, IL-2, IL-4) in CD4+ and CD8+ T-cells were significantly higher in RCT patients compared to breast cancer patients, who only received RT, and healthy controls. (Mozaffari et al., 2009)

In Kuss et al, the absolute count of CD8+T-cells recovered after treatment (surgery, surgery with adjuvant RT, surgery with adjuvant RCT) in most patients with no evidence of disease (NED), although CD8+ T-cell count was significantly decreased in patients versus controls, especially in patients with active disease. (Iris Kuss et al., 2004)

Likewise, the absolute counts of Cytotoxic T-cells decreased in cancer patients, but these cells recovered faster than CD4+ T-cells and reached normal levels immediately after the end of RT. (Belka et al., 1999) This indicates that CD8+ T-cells are less radiosensitive than CD4+ T-cells. (B. Hathaway et al., 2005a) In our study, the rise in the % of CD8+ T-cells of patients as well as of recurrent patients 3 months after RCT at t2 might indicate the fast recovery of Cytotoxic T-cells after RCT.

Hoffmann et al might offer an explanation for the observations above as they described that patients with HNSCC, some with active disease and some treated with surgery, surgery with adjuvant RT or surgery with adjuvant CT at least 12 months previously, had a higher % of circulating Fas+ CD3+ T-cells compared to normal controls and that significantly greater proportions of $CD8^+$ T-cells than $CD4^+$ T-cells were apoptotic. Fas/FasL interactions in HNSCC patients might lead to an increased turnover rate of lymphocytes, which might be responsible for functional lymphocyte imbalance and reduced immune competence. (Hoffmann et al., 2002)

In comparison with healthy controls, patients with HNSCC showed expansion of CD8+ effector subsets, specifically CD8+CD45RO−CD27− or CD8+CD28− subpopulation, which was accompanied by the rapid demise of these cells by apoptosis. (I Kuss et al., 2003; Tsukishiro et al., 2003) Logically, if the number of lymphocytes is low, proliferation will increase and more cells are generated and if the number of lymphocytes is high, excess lymphocytes die.(Surh & Sprent, 2002) All this possibly explains the decrease of CD8+ T-cells in cancer patients as well as their fast recovery after treatment.

What should be mentioned is that T-cell-mediated anti-tumor reactivity during active HNSCC might be highly or differently expressed at inflammatory sites of the tumor which might not be detectable in the peripheral blood. (Andrade et al., 2013)

Although most of the data we have found disagree with our findings, one study demonstrated that the percentage of CD8+ Cytotoxic T-cells tended to increase in head and neck patients during and after definite and adjuvant RT. (Wolf et al., 1985)

Recurrent patients also tended to have a higher % of Cytotoxic T-cells than controls, the values at t0 and t2 were more than twice higher than those of controls. (ctrl vs. t0: 10.83 ± 3.81% vs. 23.81 ± 13.64%, ctrl vs. t1: 10.83 ± 3.81% vs. 20.9 ± 19.83%, ctrl vs. t2: 10.83 ± 3.81% vs. 35.1%, ctrl vs. t5: 10.83 ± 3.81% vs. 18.82 ± 9.35%). Overall, recurrent patients at t0, t1 and t2 had higher % of Cytotoxic T-cells than patients (rec t0, rec t1, rec t2 vs. pat t0, pat t1, pat t2, pat t3: 23.81 ± 13.64%, 20.9 ± 19.83%, 35.1% vs. 16.78 ± 7.03%, 18.36 ± 7.39%, 23.93 ± 10.76%, 20.47 ± 9.44%) and at t2, they had a higher percentage of Cytotoxic T-cells than patients at all timepoints (rec t2 vs. pat t0, pat t1, pat t2, pat t3: 35.1 vs. 16.78 ± 7.03%, 18.36 ± 7.39%, 23.93 ± 10.76%, 20.47 ± 9.44%). Furthermore, there were a noticeable increase at t2 and a drop at t5 (rec t1 vs. rec t2: 20.9 ± 19.83% vs. 35.1%, t2 vs. rec t5: 35.1% vs. 18.82 ± 9.35%). (Figure 7 and 8, Table 3B and 4)

Recurrent HNSCC patients are more likely to show abnormalities of T-cell subpopulations. (Johnson et al., 1984) On the one hand, similar to our findings, it was reported that recurrent HNSCC patients tended to have a higher % of circulating Cytotoxic T-cells than patients who had remained free of disease. (Johnson et al., 1984) And a higher proportion of CD8+/CD54+ T-cells (CD54, also known as intercellular adhesion molecule 1, ICAM-1, is an adhesion molecule on T-cells) could represent a relevant biomarker associated with metastasis in HNSCC patients. (Andrade et al., 2013) In the same way, it was observed that the increase in CD8+ T-cell subsets in patients at time of diagnosis directly correlated with the level of tumor cell differentiation/ histological grading. (Boucek et al., 2010)

But on the other hand, patients with recurrent disease had the lowest absolute counts of CD8+ T-cells compared to other HNSCC patients in Hathaway et al. (B. Hathaway et al., 2005a) And this might be the result of T-cell apoptosis caused by tumor cells of patients with head and neck squamous cell carcinoma during progression. (Saito et al., 1999) Pretscher et al observed a reduced number of peritumoral CD8+ T-cells in metastatic lymph nodes as compared to uninvolved regional nodes of oro- and hypopharyngeal cancer patients suggesting a local down-modulation of cellular immunity. (Pretscher et al., 2009)

Furthermore, Lee et al described that the % of CD8+ T-cells dropped in recurrent cancer but increased in advanced OSCC patients compared to healthy individuals. (Lee et al., 2010)

On the contrary, the percentage of CD8+ T-cells of stage III and IV HNSCC patients before initiation of any treatment was much lower than that in healthy subjects in Bose et al. (Bose et al., 2008) And after surgery, HNSCC patients with stage IV disease showed a profound depression of CD8+ T-cell count without recovery, which might suggest that patients might fail to effectively compensate for the loss of CD8+ T-cells in advanced disease. (Iris Kuss et al., 2004)

It is hypothesized that once cancer induces death or functional abnormalities in Cytotoxic T-cells and alters their homeostasis, restoration of immune balance becomes very difficult. Additionally, persistent apoptosis and altered lymphocyte homeostasis might explain the inability of these “cured” patients to establish effective antitumor responses and might be the reason for frequent recurrences of cancer after therapy. (J.-W. Kim et al., 2004)

6.1.5 CD3+/CD56+ Natural Killer T-Cells in %

CD3+/CD56+ Natural killer T-cells of HNSCC patients also grew throughout RCT with great significance (t0 vs. t1: 4.3 ± 3.18% vs. 6.73 ± 4.45%, ***p ≤ 0.001; t0 vs. t3: 4.3 ± 3.18% vs. 6.45 ± 4.77%, **p ≤ 0.01). Already at timepoint t0, there were almost twice as much of % NKT-cells in patients as in controls (ctrl vs. t0: 2.46 ± 1.95% vs. 4.3 ± 3.18%, *p ≤ 0.05). (Figure 9, Table 3A and 3B)

In the same way, patients with relapse at t0 had twice as much of % NKT-cells as patients at t0 and an almost 4-times higher % of NKT-cells than controls. Altogether, recurrent patients showed a higher % of NKT-cells than controls and patients at all timepoints. The most striking rise was at t2, where the % of NKT-cells reached 24.02%, which was almost 12-times higher than the % of NKT-cells in healthy controls. (Figure 9 and 10, Table 3B and 4)

As NKT-cells share some receptors and functions between NK-cells and T-lymphocytes, they make a functional link between innate and adaptive antitumor immunity. (Lanier, 1998; Yokoyama et al., 2004) NKT-cells exhibit, additionally to NK-cell activities, characteristics of both Th1 and Th2 CD4+ T-lymphocytes. Therefore, they may have a dual function in immune regulation and immune surveillance against tumors. (Brutkiewicz & Sriram, 2002) Hence, it can be interpreted that they have a crucial role in tumor defense and their usage in ACT can improve patients' outcome.

Our patients as well as recurrent patients had, already before RCT, a more than twice or rather 4-times higher % of NKT-cells than controls and the % of NKT-cells of patients increased with great significance throughout RCT, which suggested the presence of altered immune balance before any treatment, probably favorable for the host, and the ability of primary RCT to boost the immune system of HNSCC patients.

As the value of recurrent patients at t0 was particularly high, it could be used as a possible marker to predict relapses of patients before initiation of any treatment, which might facilitate doctors to modify the treatment to achieve a better outcome at an early timepoint.

Furthermore, Kobayashi et al observed that in patients with stage IV HNSCC, the number of circulating NKT-cells did not decrease in comparison with age- and gender-matched healthy controls and that the population of NKT-cells remained stable after radiation therapy. IFN-gamma production from NKT-cells directly after RT was impaired after stimulation with exogenous ligand, but their proliferative responses as well as anti-tumor activity was improved in comparison with NKT-cells collected before radiation therapy. As a result, NKT-cells seem to be radiation resistant and might be suitable for adjuvant immunotherapy combined with RT to eliminate remnant cancer cells in patients with HNSCC. (Kobayashi et al., 2010)

But conversely to our findings, others described that the percentage of CD3+/CD56+ NKT-cells of stage III and IV HNSCC patients within the lymphocyte gated population prior to treatment was significantly lower than that seen for healthy individuals. (Bose et al., 2008) In Molling et al, an age-dependent decrease in circulating Va24+Vb11+ NKT-cell numbers in both healthy controls and cancer patients was demonstrated and females had higher NKT-cell levels compared to males. (Molling et al., 2005) And cancer patients had fewer circulating Va24+Vb11+ NKT-cell numbers than in age- and gender-matched healthy controls. This decrease was independent of tumor type or tumor load and was not able to be restored after tumor removal by means of surgery or radiotherapy. (Molling et al., 2005)

Invariant NKT-cells (iNKT) are a subgroup of NKT-cells, that express a highly restricted T-cell receptor and respond to CD1d-restricted lipid ligands.(Juno et al., 2012) It was published that invariant Natural killer T-cell (iNKT-cell) numbers were diminished in the peripheral blood of cancer patients compared with controls and that a severe deficiency in circulating iNKT-cells was related to poor clinical outcome in response to RT in HNSCC patients. (Molling et al., 2007) Likewise, patients with tumor-infiltrating iNKT-cells had a better prognosis than patients whose tumors did not have iNKT-cell infiltration. (Metelitsa et al., 2004)

Therefore, as NKT-cells can be activated via antigenic recognition of the glycolipid alpha-galactosylceramide (alphaGalCer) (Godfrey et al., 2000), adoptive transfer of NKT-cells from transgenic mice or of alphaGalCer-loaded dentritic cells can lead to inhibition of tumor development in various models, depending on activation of the innate and/or the adaptive arm

of the immune system. (Akutsu et al., 2002; Shin et al., 2001; Smyth et al., 2005; Stewart et al., 2003)

6.1.6 CD3+/CD4+/CD25+/FoxP3+ Regulatory T-Cells in %

Patients at t0 and t1 had significantly lower % of CD3+/CD4+/CD25+/FoxP3+ Regulatory T-cells than controls (ctrl vs. t0: 9.92 ± 4.19% vs. 6.86 ± 3.47%, **p ≤ 0.01; ctrl vs. t1: 9.92 ± 4.19% vs. 6.02 ± 2.88%, ***p ≤ 0.001). In the course of RCT, the % of Regulatory T-cells rose, in particular at t2, significantly. (t0 vs. t2: 6.86 ± 3.47% vs. 10.36 ± 5.1%, **p ≤ 0.01; t0 vs. t3: 6.86 ± 3.47% vs. 10.14 ± 6.81%, **p ≤ 0.01; t1 vs. t2: 6.02 ± 2.88% vs. 10.36 ± 5.1%, ***p ≤ 0.001). (Figure 11, Table 3A and 3B)

The transcription factor forkhead box P3 (FoxP3), which is considered to be the most specific marker for Regulatory T-cells so far, is a crucial intracellular molecule for the development and function of Tregs. Up to now, functions of Tregs are diverse and not completely understood.

In some of the solid tumors such as melanomas, cervical -, renal - and breast cancers, high FoxP3+ Regulatory T-cell infiltration of tumor sites correlates significantly with shorter overall survival, whereas FoxP3+ Tregs are associated with improved survival in colorectal, head and neck as well as esophageal cancers. (Shang et al., 2015)

Solid tumors (head and neck cancer, colon cancer) as well as hematologic malignancies, in which the presence of tumor-infiltrating FoxP3+ Tregs is associated with favorable clinical outcome, are tumors heavily infiltrated by inflammatory immune cells, such as neutrophils and macrophages, which facilitate tumor progression by producing growth factors or inflammatory cytokines. Therefore, Tregs might be able to inhibit tumor-promoting inflammation. (Badoual et al., 2006; Carreras et al., 2006; H-B Jie et al., 2013; Schottelius & Dinter, 2006)

Contrarily, increased tumor-infiltrating and peripheral Tregs were associated with a negative prognosis of head and neck cancer patients in other studies. (Hyun-Bae Jie et al., 2015; Weed et al., 2015) The ability of tumor-infiltrating Tregs to kill effector NK- and CD8+ T-cells in a granzyme B- and perforin-dependent manner had been demonstrated. (Cao et al., 2007) In addition, tumor-infiltrating immunosuppressive Tregs in HNSCC patients expressed CTLA4 on their cell surface. (H-B Jie et al., 2013) Furthermore, intratumoral Tregs exhibited more potently immunosuppressive activity than circulating Tregs suggesting that tumor-infiltrating Tregs were more immunosuppressive than peripheral Tregs. (H-B Jie et al., 2013) However, expressions of antigens on Tregs might be different in circulating and tumor-infiltrating Tregs and might not be necessarily detectable in the peripheral blood.

In Boucek et al, HNSCC patients with all stages (T1 – T4) and different histological gradings were individually compared at time of diagnosis and no significant differences in Tregs could be detected. (Boucek et al., 2010)

An elevation in Regulatory T-cells in the peripheral circulation and at tumor site was reported in patients with HNSCC compared with healthy people and these results seem to be in agreement with the majority of published data for other human cancers. (Albers et al., 2005; Boucek et al., 2010; Chikamatsu et al., 2007; Curiel et al., 2004; Liyanage et al., 2002; Schaefer et al., 2005) Moreover, the % of CD4+ CD25+ Foxp3+ Regulatory T-cells was significantly higher among HNSCC PBMCs than in PBMCs from healthy people. (Bose et al., 2008) And not only an increase in frequency, but also in suppression of Treg function at primary tumor location as well as in the peripheral blood of head and neck cancer patients could be observed. (P. J. Schuler et al., 2013; Strauss et al., 2007)

One study had similar findings as we do. They observed a slight decline in CD4+CD25+ T-cells of OSCC patients relative to healthy controls. (Lee et al., 2010) However, fewer Tregs in the blood circulation of HNSCC patients might suggest the possibility of Tregs migrating to tumor site.

The effects of cancer therapies on Regulatory T-cells are not well understood. Like in our study, the rise in frequency of Tregs could also be found in HNSCC patients treated with adjuvant RCT in Schuler et al and Strauss et al. (P. J. Schuler et al., 2013; Strauss et al., 2007) Moreover, RCT decreased the frequency of peripheral CD4+ T-cells, but increased that of CD4+CD39+ Tregs, which remained elevated for more than three years. (P. J. Schuler et al., 2013) Kachikwu et al observed that CD3+/CD4+/CD25+/Foxp3+ Regulatory T-cells increased after radiation as they were more radioresistant than other lymphocyte subsets. (Kachikwu et al., 2011) This resistance might be caused by the existence of protective mechanisms, e.g. multi-drug transporter pumps and/ or enhanced capability for DNA-repair. (Borst et al., 2008; Kuo et al., 2007; P. Schuler et al., 2010) On the contrary, Schaefer et al suggested that Tregs in HNSCC patients and healthy individuals were more sensitive to apoptosis than other T-cell subtypes. (Schaefer et al., 2005)

Interestingly, the percentage of FoxP3+ Regulatory T-cells increased in patients with mammary carcinoma during RT and RCT. (Sage et al., 2016) But the absolute number of Regulatory T-cells of breast cancer patients, who received RCT, was significantly decreased compared to patients, who only received RT, and healthy controls. (Mozaffari et al., 2009)

It was also reported that CT could reduce the number of circulating Tregs in end-stage cancer patients and additionally enhance T- and NK-cell functions. (Ghiringhelli et al., 2007)

Recurrent patients had lower % of Regulatory T-cells than controls at all timepoints, but a higher % of Regulatory T-cells than patients at t0. There was a striking drop at t2, but this should be interpreted with caution as there was only one value available at t2. (Figure 12, Table 4)

In our study, recurrent patients had slightly more Tregs before RCT at t0 than patients at t0, which was not significant as there were only 4 patients with relapse. Similarly, in Boucek et al, the group of patients with relapse at a later timepoint was compared with the group without evidence of the disease at an interval of 1 year after the end of the therapy and a significant increase in levels of Tregs was observed in recurrent patients at time of diagnosis. (Boucek et al., 2010)

Moreover, our recurrent HNSCC patients had lower % of Tregs than controls at all timepoints, which might indicate the possibility of Tregs migrating to tumor site.

Diverse functions of Regulatory T-cells are not fully understood, but some reports about the success of combination of conventional therapies (surgery, RT, CT) with therapy forms inhibiting Tregs seem to be promising. For example, depletion of Tregs using anti-CD25 antibodies synergizes with chemotherapy, including platinum-containing agents and etoposide, to reduce tumor growth in subcutaneous, transplantable models. (Sahu et al., 2014; Wu et al., 2011) Additionally, Strauss et al suggested that the rise in Tregs could be regarded as a problem, as there is the possibility that these cells interfere with antitumor immune reactions and inhibit responses to immunotherapies. (Strauss et al., 2007)

6.1.7 CD3-/CD56+ Natural Killer Cells in %

The % of CD3-/CD56+ Natural killer cells of patients increased significantly throughout RCT (t0 vs. t2: 10.93 ± 11.36% vs. 15.16 ± 11.09%, **p ≤ 0.01; t0 vs. t3: 10.93 ± 11.36% vs.

16.8 ± 10%, **p ≤ 0.01) and patients at t3 had a significantly greater % of NK-cells than controls (ctrl vs. t3: 11.7 ± 5.15% vs.16.8 ± 10%, *p ≤ 0.05). (Figure 13, Table 3A and 3B)

NK-cells have the ability to attack tumor cells and have been long thought to play a crucial role in anti-tumor immunity. (S. Kim et al., 2000) Our findings described a significant increase in the % of NK-cells throughout RCT, which might reflect the effect of RCT on patients' immune competence to improve anti-tumor activity. In Lee et al, an elevation in CD56+ NK-cells could be detected in patients with oral leukoplakia relative to healthy controls. (Lee et al., 2010) Moreover, the reduction of T-cell compartment might account for the large percentage of NK-cells, because the percentage of lymphocyte subpopulations is defined as the percentage of cells within the lymphocyte gate.

Boucek et al reported that the % of NK-cells was decreased in HNSCC patients at time of diagnosis compared to controls. (Boucek et al., 2010) In opposition to our study, a significant drop of total NK-cell counts of stage IV HNSCC patients during RCT with carboplatin (70 mg/sqm) and a total radiation dose of 66 Gy was demonstrated in Nollert et al and these cells recovered 7 weeks after RCT. (Nollert et al., 1999) The accelerated radiochemotherapy with carboplatin was described to induce lymphopenia and functional impairment of cellular immunity, followed by compensatory cytokine reaction. (Nollert et al., 1999) Furthermore, carboplatin as a chemotherapeutic agent might have other effects on patients' immune cells than cisplatin, which was used in our study. Contribution to the efficacy of particular chemotherapeutics had also been reported for cytotoxic lymphocytes, including CD8+ T-cells and NK-cells. (Coffelt & de Visser, 2015) And several commonly used chemotherapeutic drugs such as paclitaxel, cisplatin and doxorubicin were able to upregulate mannose-6-phosphate receptors on tumor cells of mouse and human origin and promote NK- and CD8+ T-cell killing as they increased permeability to granzyme B of tumor cells.(Ramakrishnan et al., 2010)

Interestingly, the percentage of NK-cells dropped during RT and RCT in breast cancer patients, but recovered to initial levels 6 months after treatment. (Sage et al., 2016) NK-cell cytotoxicity was significantly higher in breast cancer patients with RCT compared to patients, who only received RT, and healthy controls. (Mozaffari et al., 2009) In cancer patients, the absolute counts of Natural killer cells decreased and reached normal levels immediately after the end of RT. (Belka et al., 1999)

NK-cells of patients with relapse also rose during RCT with an exceptional drop at t2, but they had a much lower % of NK-cells than controls and patients at all timepoints, in particular at t0 and t2. (Figure 13 and 14, Table 3B and 4)

In our study, data of recurrent patients at t5, at time of relapse, showed a higher % of NK-cells than they had before the treatment at t0. The rise of NK-cells of recurrent patients directly after RCT might be caused by immune-enhancing effects of RCT. But overall, the % of NK-cells of patients with relapse were still lower than those of patients and controls, which might indicate the impairment of immune function in recurrent patients as they failed to fight against the tumor.

6.2 Subsets of Natural Killer Cells (Regulatory Markers) in % and MFI

CD3-/CD94+; CD56+/CD69+; CD3-/NKG2D+; CD3-/NKp30+; CD3-/NKp46+

Relative comparisons can be made with MFI, but one might be careful that other factors are not responsible for the differences in MFI. Generally, the data of MFI in subgroups of NK-cells of patients remained almost at a steady level and showed less statistical significance throughout RCT.

Overall, the % of all subtypes of Natural killer cells of patients increased significantly in the course of RCT (t0 vs. t3; all NK-cell subtypes). At t3, the % of all subgroups of NK-cells of patients were higher than that of controls (ctrl vs. t3; all NK-cell subtypes). (Figure 15, 17, 19, 21, 23 and 25, Table 5A and 5B)

All subgroups of Natural killer cells in recurrent patients also tended to grow throughout RCT (t0 vs. t1, t0 vs. t5; all NK-cell subtypes). At t0, recurrent patients had lower % of NK-cells of all subtypes than controls (ctrl vs. t0; all NK-cell subtypes). At t5, patients with relapse had higher % of all subtypes of NK-cells than controls (ctrl vs. t5; all NK-cell subtypes). Recurrent patients had lower % of all subtypes of NK-cells at t0 and t5 than patients at t0 and t3 (rec t0 vs. pat t0, rec t5 vs. pat t3; all NK-cell subtypes). Interestingly, drops at t2 were noticeable in all NK-cell subtypes in recurrent patients (t0 vs. t1, t1 vs. t2, t2 vs. t5; all NK-cell subtypes). (Figure 15-26, Table 5B and 6)

NK-cell function is associated with a vast network of inhibitory and activating signals. Under normal immune surveillance, NK-cells have inhibitory receptors that recognize MHC class I molecules as their cognate ligands; these receptors include killer Ig-like receptors (KIR)-L, LIR-1/ILT-2, LAIR-1 and the CD94/NKG2A heterodimer. Cytotoxicity occurs if stimulatory signals outweigh inhibitory signals by a critical threshold. Several of these activating receptors have been characterized, including NKG2C, NKG2D, and the natural cytotoxicity receptors NKp30, NKp44 and NKp46. (C. Bottino et al., 2006; Hudspeth et al., 2013; Sun & Lanier, 2011; Vivier et al., 2008)

Studies have demonstrated that low NK-cell activity leads to high incidence of tumor occurrence and metastasis, and its degree correlates with invasiveness of malignancy. (Takeuchi et al., 2001) Contrarily, high NK-cell activity has been shown to correlate with lower incidence of tumors and NK-cell infiltration in certain tumors, e.g. in melanomas and HNSCCs, is associated with better oncological outcome. (Schleypen et al., 2006; Vivier et al., 2012)

At t0, the % of all NK-cell subsets of patients in our study were not significantly distinguishable from the % of all NK-cell subtypes of controls. Interestingly, some reported that NK-cell activity of patients with primary squamous cell carcinoma was lower than that of normal controls and patients with benign tumors. In contrast to our results, NK-cell activity of patients with recurrent squamous cell carcinoma was lower than that of normal controls. Furthermore, it was reported that primary squamous cell carcinoma patients as well as non-recurrent patients with a stage of T3 or T4 showed lower NK-cell activity than patients with stages T1-T2. We also observed that the % of all NK-cell subsets of recurrent patients were lower than those % of all NK-cell subtypes of patients before RCT as well as at time of relapse, which might be a possible marker to help clinicians to foresee the relapse and to adjust the treatment at an early timepoint. All these results indicate that patients with oral maxillofacial squamous cell carcinoma have impairment of NK-cell function and this impairment is even greater in patients with advanced tumors. (Vinzenz & Micksche, 1986; J. M. Wang, 1989)

In patients with NPC, decreased expression levels of the NKG2D ligand UBLP4 were associated with significantly poorer overall survival, progression-free survival and distant metastasis-free survival than those with preserved levels of ULBP4. (Y. Xu et al., 2017)

In HNSCC patients, impaired NK-cell cytotoxicity was related to lower expression of NKG2D, NKp30 and NKp46 receptors and not to a drop in frequency of NK-cells. IRX-2, a primary biologic containing multiple cytokines, which had been used for the therapy of head and neck squamous cell carcinoma (HNSCC) with promising clinical results, was effective in enhancing NK-cell cytotoxicity and protecting NK-cell function in HNSCC patients. (Freeman et al., 2011; Schilling et al., 2012; *Science of IRX-2—IRX Therapeutics*, n.d.)

As significant anti-tumor effects require a high dose of cisplatin, which can result in a systemic toxicity and immunosuppression, such as decreased NK-cell activity and T-cell proliferation,

combination with other forms of therapy is needed. (Garzetti et al., 1994; Sfikakis et al., 1996) For example, a synergy of combined cisplatin and IL-2 gene transfer can achieve release of tumor antigen caused by cisplatin-induced cell death coupled with IL-2 activation of NK-cells and cytotoxic lymphocytes. (D. Li et al., 2002)

Unlike our findings, it was observed that surgery as well as CT tended to inhibit NK-cell activity. (J. M. Wang, 1989) Interestingly, the percentages of all NK-cells as well as NK-cell subpopulations carrying regulatory receptors CD56+, CD94+, NKG2D+, NKp30+, NKp46+ dropped during RT and RCT in breast cancer patients, but recovered to initial levels 6 months after treatment. (Sage et al., 2016)

Our findings suggested that RCT can increase anti-tumor function of NK-cells in HNSCC patients as well as in recurrent patients by increasing the % of expressions of activation marker such as CD69, NKG2D, NKp30 and NKp46 on NK-cells. Hence, combination of RCT with immunotherapy might be advantageous to increase anti-tumor toxicity of NK-cells in HNSCC patients.

CD3-/CD94+ NK-cells in %

CD94 receptor can be subdivided into CD94/NKG2A and CD94/NKG2C receptors, which bind with human leukocyte antigen (HLA)-E-peptide complex. CD94/NKG2A receptor mediates inhibition and CD94/NKG2C receptor activation of the NK-cell-mediated cytotoxicity. (Braud et al., 1998; Lanier et al., 1998)

The % of CD94+ NK-cells in patients as well as in recurrent patients rose throughout RCT.

Interestingly, it was reported that CD94 transcripts implied a better prognosis in nasal-type extranodal NK/T-cell lymphoma. (Lin et al., 2003) But studies observed that expansion of CMV-mediated NKG2C+ NK-cells in liver-transplanted patients was associated with the development of de novo head and neck or colorectal tumors. (Achour et al., 2014) And there was a trend of reduced risk of NPC in individuals who were homozygous for both HLA-E/01:03 and NKG2C deletion. (L. Li et al., 2015)

CD56+/CD69+ NK-/T-cells in %

Several reports suggest that CD56 is an important effector marker of NK- and T- lymphocytes. (Cohavy & Targan, 2007)

In our study, HNSCC patients at t0 had lower % of early activation marker CD69+ expression on NK-/T-cells than controls, but the % increased after RCT resulting in a higher % of CD69+ NK-/T-cells in HNSCC patients at t3 than controls. This suggests that NK-/T-cell activity of HNSCC patients can be influenced by RCT.

In Millrud et al, patients with HNSCC had a higher percentage of T-cell subsets and NK-cells expressing the early activation marker CD69+ compared to healthy controls, which could be correlated to both tumor burden and spread to lymph nodes. (Millrud et al., 2012)

In patients with recurrent and advanced oral squamous cell carcinoma, CD56+/CD69+ NK-/T-cells were elevated. (Lee et al., 2010) In the same way, recurrent patients with oral squamous cell carcinoma had an increase in CD56+/CD69+ NK-/T-cells relative to untreated patients, indicating that the immune system might respond with sensitivity to exogenous or endogenous stress/danger signal to stimulate NK-cell activity against tumor growth. (Lee et al., 2010) In

agreement, increased levels of CD69+ NK-/T-cells had been shown in patients with advanced HNSCC and resulted in poor prognosis as well as low long-term survival. (Aarstad et al., 2006, 2015)

In contrast to the findings above, our recurrent patients had before RCT and at time of relapse a lower % of CD69+ NK-/T-cells than HNSCC patients, but an increase in the % of this subgroup throughout RCT was visible suggesting the immunomodulatory effect of RCT.

CD3-/NKG2D+ NK-cells in %

Natural cytotoxicity receptors (NCRs) belong to major lysis receptors in the NK-cell. NCRs include three immunoglobulin-like proteins: NKp46 (NCR1; CD335), NKp44 (NCR2; CD336) and NKp30 (NCR3; CD337). (Enk & Mandelboim, 2014; Noronha et al., 2012) And NK-cell-activating receptors, such as NKG2D, NKp30, NKp44 and NKp46, have been shown to be particularly important for NK-cell recognition and lysis of target, e.g. for lysis of tumor cells. (Carbone et al., 2005; D. Pende et al., 2001; Daniela Pende et al., 2005; Vivier et al., 2011)

NKG2D receptor is not only expressed by NK-cells, but also by activated Cytotoxic T-cells and activated macrophages. Depending on the level of NKG2D ligands expressed on tumor cells, anti-tumor response can be induced by potent priming of Cytotoxic T-cells and sensitization of NK-cells. (Diefenbach, Jensen, Jamieson, & Raulet, 2001) For example, NKG2D receptor recognizes two different families of ligands: MHC class I chain-related molecules (MICA and MICB) and UL-16-binding proteins (ULBP). (Cristina Bottino et al., 2005) NK-cells can recognize these NKG2D ligands on malignant tumor cells and eradicate them. (González et al., 2006)

In patients with laryngeal squamous carcinoma, NKG2D ligands overexpression and their interaction with NKG2D on NK-cells mediated anti-laryngeal cancer immune response. (X. M. Chen et al., 2008) On the one hand, expression of NKG2D ligands on multidrug-resistant nasopharyngeal carcinoma cell line was correlated with NK-cell-mediated lysis, but on the other hand persistent exposure to tumor cells expressing NKG2D ligands could lead to downregulation of NKG2D receptor expression and reduction of NK-mediated cytolysis, which suggested that enhancing the expression of NKG2D might promote NK-cell-mediated cytolysis. (Guo et al., 2007; Mei et al., 2007)

Moreover, typical levels of NKG2D ligands naturally found on most tumors were suboptimal in inducing anti-tumor immunity, which raised the possibility of immunotherapy by engineering cells with higher levels of ligands to boost anti-tumor reaction. (Diefenbach et al., 2001) In addition, IL-17A was able to improve the cytotoxic functions of NK-cells against tumor cells by augmenting the expression of cytotoxic molecules, for example, tumor necrosis factor alpha, interferon-gamma, perforin and granzyme B as well as activation receptors such as NKp46, NKp44 and NKG2D on NK-cells. These findings might also be able to support the development of novel cancer immunotherapy strategies. (L. Lu et al., 2013)

Interestingly, immunonutrition enriched with arginine, omega-3 fatty acids and RNA significantly enhanced antibody-dependent cell-mediated cytotoxic activity as a NK-cell function, upregulated the expression of NKG2D and increased the frequency of CD56dim NK-cells in patients with gastric and esophageal cancer. (Maruyama et al., 2011)

In our study, RCT resulted in a rise in the % of NKG2D+ NK-cells in HNSCC patients as well as in recurrent patients indicating that RCT could be combined with other types of therapy, e.g. immunotherapy to enhance NK-cell cytotoxicity against tumor cells.

CD3-/NKp30+ NK-cells in %

Natural cytotoxicity receptors (NCRs) belong to major lysis receptors in the NK-cell. NCRs include three immunoglobulin-like proteins: NKp46 (NCR1; CD335), NKp44 (NCR2; CD336) and NKp30 (NCR3; CD337). (Enk & Mandelboim, 2014; Noronha et al., 2012) And NK-cell-activating receptors, such as NKG2D, NKp30, NKp44, and NKp46, have been shown to be particularly important for NK-cell recognition and lysis of the target. (Carbone et al., 2005; D. Pende et al., 2001; Daniela Pende et al., 2005)

NKp30 is important for mediating tumor immunosurveillance in several clinical settings. One of its ligands, B7H6, is expressed on many types of tumor cells, but absent on normal tissues. Interaction of NKp30 with B7H6 has been shown to improve degranulation of NK-cells. (H. Wang et al., 2012)

In our study, RCT resulted in a rise in the % of NKp30+ NK-cells in HNSCC patients as well as in recurrent patients indicating that RCT could be combined with other types of therapy, e.g. immunotherapy to enhance NK-cell cytotoxicity against tumor cells.

Interestingly enough, treatment of human NK-cell line (NK3.3) with bitter melon extract (BME) enhanced its ability to kill HNSCC cells as BME increased granzyme B accumulation and cell surface expression of NKp30 in BME treated NK3.3 cells, implicating an immunomodulatory role of BME. (Bhattacharya et al., 2017)

CD3-/NKp46+ NK-cells in %

Natural cytotoxicity receptors (NCRs) belong to major lysis receptors in the NK-cell. NCRs include three immunoglobulin-like proteins: NKp46 (NCR1; CD335), NKp44 (NCR2; CD336) and NKp30 (NCR3; CD337). (Enk & Mandelboim, 2014; Noronha et al., 2012) NK-cell-activating receptors, such as NKG2D, NKp30, NKp44, and NKp46, have been shown to be particularly important for NK-cell recognition and lysis of the target. (Carbone et al., 2005; D. Pende et al., 2001; Daniela Pende et al., 2005) In contrast to NKp30 and NKp44, which have similar cellular functions, NKp46 is structurally distinct from the other two molecules and is located in a different region of the genome. It is stably expressed on both resting and activated NK-cell, and is considered as an unique NK-cell marker. (Enk & Mandelboim, 2014; Noronha et al., 2012)

It was demonstrated that NKp46 was critically involved in influenza, tumorigenesis and diabetes. (Gazit et al., 2006; Gur et al., 2010; Halfteck et al., 2009) NKp46 was described to be the key receptor in controlling the spread of various primary tumors in melanoma, lymphoma and other carcinomas. (Glasner et al., 2012) Experiments in a NKp46-knockout mouse model raised the possibility that NKp46 receptor was crucial for controlling both cancer metastasis and influenza infection. (Gazit et al., 2006; Glasner et al., 2012)

Downregulated transcript expression of NKp46 gene as well as decreased expression of NK activating receptor NKp46 in both peripheral blood and tumor tissue in OSCC patients compared to controls indicated reduced NK-cell count and/or lower NK-cell activation. Furthermore, NKp46 expression was significantly lower in tumor tissue than in the peripheral blood in OSCC patients. (Dutta et al., 2015) These findings indicated that antigen expression on NK-cells in the peripheral blood might differ from antigen expression on NK-cells at tumor sites in quality and quantity, which might be caused by different tumor microenvironment.

In our study, RCT resulted in a rise in % of NKp46+ NK-cells in HNSCC patients as well as in recurrent patients indicating that RCT can improve NK-cell cytotoxicity. But at the same time, a higher expression of certain NK-cell activation markers in the circulation did not

necessarily mean an enhanced NK-cell activation at tumor site. Thus, it might be wise to combine RCT with other types of therapy, e.g. ACT, as transferred cells could be directly injected into tumor sites to enhance NK-cell cytotoxicity against tumor cells.

Furthermore, Lu et al reported that IL-17A was able to improve cytotoxic functions of NK-cells against tumor cells by augmenting the expression of cytotoxic molecules, for example, tumor necrosis factor alpha, interferon-gamma, perforin and granzyme B as well as activation receptors such as NKp46, NKp44 and NKG2D on NK-cells. These findings might also be able to support the development of novel cancer immunotherapy strategies. (L. Lu et al., 2013)

6.3 Subsets of Natural Killer Cells (CD56/CD16) in %

CD56bright/CD16-; CD56bright/CD16+; CD56dim/CD16-; CD56dim/CD16+; CD56-/CD16+

In our study, the largest proportion of subgroups of CD56/CD16 Natural killer cells in controls, patients and recurrent patients was formed by CD56dim/CD16+ NK-cells. These made up around 90% of NK-cells.

CD56bright (≈ 10%) and CD56dim (≈ 90%) NK-cells are reported to have diverse functional characteristics including cytolytic activity, response to activating agents, cytokine profile and trafficking to secondary lymphoid organs. (M. A. Cooper, Fehniger, & Caligiuri, 2001) Briefly, CD56bright NK-cells mediate low or no cytotoxicity, proliferate in response to picomolar levels of IL-2 and produce high levels of inflammatory cytokines. Conversely, CD56dim/CD16bright NK-cells show potent cytotoxicity functions, have a distinct cytokine and chemokine profile and express abundant killer inhibitory receptors (KIR). (Colonna et al., 1997; M. A. Cooper, Fehniger, & Caligiuri, 2001)

CD56bright/CD16- NK-cells in %

In the NK-cell subset, bright CD56 staining (CD56bright) defines elevated potential for cytokine production, while weaker fluorescence intensity (CD56dim) defines enhanced cytotoxicity together with a mature differentiation state. (M. A. Cooper, Fehniger, Turner, et al., 2001; Parrish-Novak et al., 2000) CD56bright/CD16- NK-cells are characterized as highly proliferative, immature regulatory cells, which can interact with neighboring immunocompetent cells in lymphoid tissues as they preferentially migrate to secondary lymphoid organs. (Chan et al., 2007; Juelke et al., 2010)

Although CD56bright NK-cells are described to secrete pro-inflammatory cytokines rather than mediating cytotoxicity, these NK-cells are able to efficiently eliminate Hsp70 membrane-positive tumor cells. (Stangl et al., 2018)

On the one hand, the percentage of CD56+ NK-cells (CD3-) of stage III and IV HNSCC patients within the lymphocyte gated population prior to treatment was significantly lower than that seen for healthy individuals. (Bose et al., 2008) But on the other hand, significantly down-regulated CD16 and up-regulated CD56 expressions on NK-cells were observed in patients with esophageal squamous cell carcinoma compared to healthy controls, which resulted in NK-cell dysfunction, and these expression levels were significantly restored to the levels of healthy controls after curative resections. (Watanabe et al., 2010)

In our study, the % of CD56bright/CD16- NK-cells of controls was similar to those of patients at all timepoints, but recurrent patients had lower % of CD56bright/CD16- NK-cells at all timepoints than controls and patients at t0, t2 and t3. After initial drops at t1

and t2, CD56bright/CD16- NK-cells of recurrent patients increased until t5. (Figure 39 and 40, Table 7B and 8)

A reduction of CD56bright/CD16- NK-cells in prostate cancer patients according to cancer stage progression was described in Koo et al. (Koo et al., 2013) Contrarily, Gogali et al described that CD3-/CD16-/CD56bright NK-cell infiltration was negatively associated with tumor stage in patients with papillary thyroid cancer. (Gogali et al., 2013) In Mamessier et al, an increased proportion of CD56bright/CD16- circulating NK-cells was observed in advanced breast cancer patients as well as patients with metastases and this subgroup was usually poorly represented in the peripheral blood of healthy donors. (Mamessier et al., 2013) Likewise, our recurrent patients also had an increase in the % of CD56bright/CD16- NK-cells at t5, at time of relapse.

Possible explanation for this might be that CD56dim NK-cells, which have a high turnover, have to be replaced by their precursor CD56bright NK-cells, resulting in the release of high numbers of these cells from the bone marrow and/or the lymph node. (Poli et al., 2009)

CD56bright/CD16+ NK-cells in %

CD16+/CD56bright NK-cells play a role in tumor immunity and bacterial infections. They exhibit potent antitumor immunity upon cytokine stimulation and show strong cytotoxicity against not only MHC class I negative, but also MHC class I positive tumors. (Takahashi et al., 2007; Wagner et al., 2017)

In our study, patients at t0 had almost twice as much of % CD56bright/CD16+ NK-cells as controls (ctrl vs. t0: 2.52 ± 1.34% vs. 4.73 ± 5.57%, *p ≤ 0.05), but CD56bright/CD16+ NK-cells of patients dropped significantly at t1 (t0 vs. t1: 4.73 ± 5.57% vs. 3.2 ± 3.63%, *p ≤ 0.05). (Figure 41 and 42, Table 7A, 7B and 8)

Likewise, in Mamessier et al, an increased proportion of CD56bright/CD16+ circulating NK-cells was observed in advanced breast cancer patients as well as in patients with metastases. Moreover, CD56bright/CD16+ NK-cells were also one of the main subsets found at tumor site although this subgroup was usually poorly represented in the peripheral blood of healthy donors. (Mamessier et al., 2013)

Similarly to our findings, one study observed that the postoperative proportion of CD16+/CD56+ circulating NK-cells in oral cancer patients decreased more significantly compared to values before operation. (Ye et al., 2004)

Our findings indicate a significantly altered hemostasis of NK-cells of patients before RCT compared with controls, which might be able to serve as a marker for detection of clinically unobtrusive HNSCC patients. Furthermore, RCT as well as surgery might have an influence on NK-cell hemostasis in HNSCC patients.

Conversely, other studies had different findings. In Grimm et al, there were significantly decreased levels of CD3−/CD16+/CD56+ NK-cells in OSCC patients before surgery compared with controls, but the total number of NK-cells significantly increased after surgery compared with healthy controls. (Grimm et al., 2016)

Moreover, no significant difference was observed in the % of CD16+/CD56+ NK-cells between oral cancer patients and controls in Manchanda et al. (Manchanda et al., 2006) In Yu et al, results showed that there was no significant difference in CD3–/CD16+/CD56+ expressions of NK-cells in OSCC patients, who received different treatments including CT and surgery,

suggesting no significant influence of different treatments on NK-cells of OSCC patients. (Yu et al., 2015)

CD56dim/CD16- NK-cells in %

CD56low/CD16low Natural killer cells are multifunctional cells. (Penack et al., 2005) And CD56low NK-cells in lymph nodes and tonsils are functionally and phenotypically different from circulating CD56low NK-cells. (Björkström et al., 2010; Ferlazzo et al., 2004) Takahashi et al described that CD56dim/CD16- cells were unconventional NK-cells that vigorously proliferate in response to cytokines involved in NK cell maturation, e.g. IL-2, IL-15, or IL-21. Similar to CD56bright/CD16- NK-cells, this NK-cell subset was able to produce large amounts of IFN-g and FAS ligand when stimulated. (Takahashi et al., 2007)

On the one hand, it was observed that CD56dim/CD16- NK-cells were prominent at tumor site of breast cancer patients, expressing low levels of activating receptors and low amounts of cytolytic molecules, indicating the inability to exert cytotoxic activity. (Mamessier et al., 2013) Possible explanation might be the exposition of NK-cells to soluble factors produced by the tumor, which elicited the decrease of cytokine production, activating and cytotoxic molecules, probably through local metalloproteases and/or other inhibitors secreted by tumor cells. (Mamessier et al., 2011)

But on the other hand, the presence of hematologic malignancies affected the frequency and functional ability of CD56low/CD16low Natural killer cells both at tumor site and in the peripheral circulation. (Stabile et al., 2015) However, evidence indicated that circulating CD56low/CD16- NK-cells were responsible for mediating natural cytotoxicity against human leukemia and lymphoma cells. (Penack et al., 2005)

The % of CD56dim/CD16- NK-cells of our patients at t0 was almost as twice as much of that of controls (ctrl vs. t0: 2.8 ± 1.8% vs. 5.43 ± 10.01%) and increased throughout RCT with exceptional drops at t1 and t2 (t0 vs. t3: 5.43 ± 10.01% vs. 6.5 ± 14.41%, t0 vs. t1: 5.43 ± 10.01% vs. 4.51 ± 6.08%, t1 vs. t2: 4.51 ± 6.08% vs. 2.8 ± 3.3%). (Figure 43, Table 7A and 7B)

Similarly, patients with relapse had a greater % of CD56dim/CD16- NK-cells at t0 than controls, but recurrent patients had less % of CD56dim/CD16- NK-cells at t5 than recurrent patients at t0 or controls. (Figure 44, Table 8)

As our HNSCC patients as well as recurrent patients had a higher % of CD56dim/CD16- NK-cells at t0 than controls, their immune system might be upregulated as a result of tumor presence. However, the drop in the % of CD56dim/CD16- NK-cells in recurrent patients at t5, at time of relapse, might suggest the reduction of immune competence against the tumor.

Likewise, in Mamessier et al, an increased proportion of CD56dim/CD16- circulating NK-cells was observed in advanced breast cancer patients as well as in patients with metastases; and CD56dim/CD16- NK-cells were also one of the main subsets found at tumor site although this subpopulation was usually poorly represented in the peripheral blood of healthy donors. (Mamessier et al., 2013)

CD56dim/CD16+ NK-cells in %

According to receptor repertoire and expression levels, phenotypically distinct NK-cell subpopulations have been identified in different tissues, they likely represent specialized NK-

cell subgroups capable of mediating different functions and are endowed with distinct migratory properties. (M. A. Cooper, Fehniger, & Caligiuri, 2001; Nagler et al., 1989)

CD56dim/CD16bright NK-cells, which produce low levels of cytokines, predominantly mediate natural cytotoxicity, while the CD56bright/CD16dim subset plays a role in immune regulation through a high cytokine secretion potential. (Konjevic et al., 2012) Furthermore, CD56dim/CD16+ NK-cells are able to exert cytotoxic functions and promote immune responses as they home toward inflammatory sites. (Hanna et al., 2004)

Interestingly, the % of CD56dim/CD16+ NK-cells of our patients at t0 was lower than that of controls (ctrl vs. t0: 87.27 ± 5.97% vs. 82.63 ± 15.8%). In the course of RCT, CD56dim/CD16+ NK-cells of patients increased at t1 and t2 (t0 vs. t1: 82.63 ± 15.8% vs. 86.08 ± 12.92%, t1 vs. t2: 86.08 ± 12.92% vs. 88.13 ± 9.97%) and dropped at t3 (t2 vs. t3: 88.13 ± 9.97% vs. 85.01 ± 16.11%).

Likewise**,** Mamessier et al described that the predominant subset in healthy controls, CD56dim/CD16+, was decreased in the circulation and at tumor site of advanced breast cancer patients and patients with metastases. (Mamessier et al., 2013) In Bose et at, the % of CD56 dim population was significantly decreased in stage III and IV HNSCC patients in comparison to healthy individuals. (Bose et al., 2008) And preferential apoptosis of NK-cells expressing low/dim levels of CD56 was observed in cancer patients, leading to low NK-cell activity, whereas only a small subset of CD56bright NK-cells underwent apoptosis. (Bauernhofer et al., 2003)

Gogali et al reported that CD3-/CD16+/CD56dim NK-cell tissue infiltration positively correlated with advanced stages of papillary thyroid cancer. (Gogali et al., 2013) **Similarly, our recurrent patients had a higher % of CD56dim/CD16+ NK-cells at t5 than before RCT, but with no striking significance.**

Interestingly, it was reported that immunonutrition enriched with arginine, omega-3 fatty acids and RNA significantly enhanced antibody-dependent cell-mediated cytotoxic activity as an NK-cell function, paralleling the upregulated expression of NKG2D and increased frequency of CD56dim NK-cells in patients with gastric and esophageal cancer. (Maruyama et al., 2011)

CD56-/CD16+ NK-cells in %

The presence of CD56-/CD16+ NK-cells was observed in virus-infected patients and in healthy cord blood. (Gaddy & Broxmeyer, 1997; X. Lu et al., 2008) Studies reported that CD56-/CD16+ NK-cells were not only expanded in hepatitis C virus-infected patients, but also in HIV-positive patients. (Alter et al., 2005; Mavilio et al., 2005)

In the same way, our HNSCC patients also had higher % of CD56-/CD16+ NK-cells at all timepoints than controls (ctrl vs. t0, t1, t2, t3: 0 ± 0% vs. 0.01 ± 0.15%, 0.11 ± 0.62%, 0.04 ± 0.21%, 0.08 ± 0.28%). (Figure 45 and 47, Table 7A and 7B)

In recurrent patients, the % of CD56-/CD16+ NK-cells increased at t5, at time of relapse. Similarly, Synderman et al reported that in HNSCC patients with cervical metastases, lymph node lymphocytes contained an increased proportion of CD16+ NK-cells. (Snyderman et al., 1991) Expansion of the population of CD3−/CD16+ Natural killer cells with heavily reduced “nonspecific” cytolytic capacities in the peripheral blood of HNSCC patients was observed in Melioli et at, suggesting that HNSCC was responsible for the expansion of large numbers of functionally impaired NK-cells resulting in a bad prognosis for these patients. (Melioli et al., 2003)

Conversely, it was also described that the percentage of CD16+ NK-cells (CD3-) of stage III and IV HNSCC patients within the lymphocyte gated population prior to treatment was significantly lower than that seen for healthy individuals. (Bose et al., 2008) And significantly down-regulated CD16 and up-regulated CD56 expressions on NK-cells were observed in patients with esophageal squamous cell carcinoma compared to healthy controls, resulting in NK-cell dysfunction and these expression levels were significantly restored to the levels of healthy controls after curative resections. (Watanabe et al., 2010)

6.4 Future Directions

Our study indicates the importance of monitoring cellular and humoral immunocompetence of HNSCC patients throughout the treatment and on a follow-up basis as it may offer possible early evaluation of the results of treatment, point out patients' outcome or even be helpful in selecting patients for combined immunotherapy. (Olkowski & Wilkins, 1975)

Furthermore, head and neck cancers tend to be more immunosuppressive than other cancer types. (Bose et al., 2008) Many studies have reported that the immune competence of patients suffering from advanced head and neck squamous cell carcinoma is frequently suppressed. (Bose et al., 2006; Z. Chen et al., 1999)

The levels of secreted Th1 cytokines IL-12, IFN-γ and TNF-α are significantly lower in HNSCC patients in stage III and IV prior to treatment in comparison with controls and levels of Th2 cytokines IL-4 and IL-10 are higher in stage III and IV HNSCC patients prior to treatment than in controls, which reflect the opposite of healthy controls. (Bose et al., 2008) However, dysregulation of T-cell subsets observed in various diseases, including malignancies, might be responsible for eliciting disease progression. (Chikamatsu et al., 2007) Indeed, evidence has accumulated suggesting that a shift from type 1 toward type 2 cytokine profile is associated with disease progression in HNSCC. (Agarwal et al., 2003; Lathers et al., 2003; Sparano et al., 2004)

In the same way, dysregulation in immune cells and cytokine secretion was reflected in the suppressed cytotoxic function of PBMCs of HNSCC patients with stage III and IV before any treatment, while they were tested on different cancer cell lines. (Bose et al., 2008) This might be due to diminished expression of cytotoxic molecules (perforin, granzymeB and FasL) on CD8+ T-cells and CD56+ NK-cells of patients compared with healthy individuals. (Bose et al., 2008)

In order to obtain an optimal clinical outcome, immunomodulation of HNSCC patients might be useful, thus, understanding the immune status of HNSCC patients supports finding the proper immunomodulator. (Bose et al., 2008)

However, some researches have already published some observations, but in our study, we report about the detailed immune status of HNSCC patients receiving primary RCT including lymphocyte subpopulations such as B-cells, T-cells, T-helper-cells, Cytotoxic T-cells, NKT-cells, Regulatory T-cells, NK-cells as well as subgroups with different regulatory marker expressions and CD56/CD16 (+/-/dim/bright) NK-cells.

Up to now, a great deal of studies has already demonstrated the possibilities of combination of conventional therapies such as surgery, RT/CT or RCT with relatively new therapy types, e.g. immunotherapy.

In adoptive cell therapy (ACT), autologous tumor infiltrating lymphocytes with anti-tumor activity are expanded in vivo and reinfused into patients with cancer, combined with high-dose IL-2 therapy. ACT appears to be an effective cancer immunotherapy, especially in patients

with metastatic melanoma. Prior to ACT, patients receive non-myeloablative chemotherapy as lymphodepletion regimen in order to improve clonal repopulation of anti-tumor T-cells and to eliminate Regulatory T-cells as well as endogenous lymphocytes, which compete with transferred cells for homeostatic cytokines (IL7, IL15).

In addition, CD4+ T-cells influence effector function and memory of CD8+ T-cells. Adoptive cell transfer of CD4+ T-cells together with tumor-reactive CD8+ T-cells into CD4+ T-cell-deficient patients with melanoma cause regression of tumor. As CD4+ T-helper-cells are able to produce IL12, they are important for the function and maintenance of CD8+ T-cell numbers. At the same time, this effect is diminished at presence of Regulatory T-cells, indicating that depletion of Regulatory T-cells improves tumor protection. (Antony et al., 2005; Dudley et al., 2005; Klebanoff et al., 2004)

Moreover, combination of RT/CT or RCT with ACT has proved to be advantageous for cancer patients. Chemo- as well as radiotherapy have been shown to increase recruitment of adoptively transferred T-cells to tumor sites and enhance antitumor responses. (Gupta et al., 2012; Ramakrishnan et al., 2010) Indeed, metastatic melanoma patients, who received ACT with non-myeloablative chemotherapy accompanied by 2 Gy or 12 Gy total body irradiation, had a higher response rate than those without radiotherapy, especially in case of 12 Gy total body irradiation. (Dudley et al., 2008) Vaccination with irradiated tumor cell lines, which were engineered to secrete GM-CSF by retroviral-mediated gene transfer, was able to mobilize dendritic cells, NKT-cells, B-cells, CD4+ and CD8+ T-cells in metastatic cancer patients as these were able to augment the antigen uptake and presentation of dying tumor cells in dendritic cells. (Hodi & Dranoff, 2006; Soiffer et al., 2003)

Other studies also show that with genetic modification of lymphocytes using retroviruses, which encode T-cell receptors, normal lymphocytes can be converted into tumor-reactive lymphocytes, causing tumor regression in patients with metastatic melanoma. These lymphocytes can mediate durable regression in big tumors as they express the transgene for a prolonged time. Furthermore, this method can also be used in patients for whom TILs are not available and genetically engineered cells, which are able to recognize TAAs expressed on various common cancers, can be created for the treatment of common epithelial cancers. (Morgan et al., 2006)

One study depicted that ACT could also be used in nasopharyngeal carcinoma (NPC). As EBV is associated with virtually all poorly or undifferentiated non-keratinizing nasopharyngeal carcinoma regardless of geographic origin and is present in all tumor cells, EBV specific Cytotoxic T-cells can be generated from patients with NPC irrespective of prior radio- or chemotherapy. And subsequently, some of the patients of this study showed remission. (Niedobitek, 2000; Straathof et al., 2005)

Human papillomavirus (HPV)-positivity in patients with HNSCC has been associated with improved response and superior survival rates after radiotherapy and radiochemotherapy compared to HPV-negative patients. (Ang et al., 2010) The immune phenotype (CD8high/PD-L1high) is more common in HPV16-positive tumors and is associated with favorable outcome. HPV16-positive tumors express more immune markers and in conjunction with CD8, PD-L1 constitutes an independent prognostic marker in patients with HNSCC after surgery and RCT. Consequently, PD-1/PD-L1 immune checkpoint inhibitors could be considered to be used in combination with RCT in HNSCC patients. (Balermpas et al., 2017)

In a variety of malignancies, immunologic checkpoint blockade with antibodies that target Cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and the programmed cell death protein 1 pathway (PD-1/PD-L1) has demonstrated promise. CTLA-4 is upregulated on the plasma membrane after T-cell activation in order to downregulate T-cell function. (Michael A. Postow et al., 2015) PD-1 is a negative regulator of T-cell activity when it is bound to one of its two ligands PD-L1 and PD-L2. (Ishida et al., 1992; Michael A. Postow et al., 2015)

CTLA-4 blockade caused by ipilimumab has resulted in an increase in the absolute lymphocyte count as well as in activation of peripheral T-cells which have been associated with overall survival in patients with melanoma. (Michael Andrew Postow et al., 2013) Anti-CTLA-4 antibodies combined with RT, however, caused complete regression of the majority of irradiated tumors and an abscopal effect in mice receiving a hypofractionated regimen, which indicate that dose of irradiation is important for synergistic effects of RT and immune checkpoint blockade therapy. (Dewan et al., 2009)

PD-L1 antibodies such as MPDL3280A, MEDI4736 and MSB0010718C have also been shown to cause disease responses in early-phase clinical trials in a number of malignancies including tumor types such as bladder cancer, head and neck cancer and GI malignancies. (Freeman et al., 2011; Herbst et al., 2013; Powles et al., 2014; Segal et al., 2014) Patients with tumors which express PD-L1 have higher response rates to PD-1/PD-L1 blockade than patients, who do not express PD-L1. (Grosso et al., 2013; Topalian et al., 2012; Weber et al., 2013)

CTLA-4 combinations with PD-1 blockade seem to be promising as a phase I study of ipilimumab and nivolumab in patients with melanoma resulted in impressive overall survival compared with historical data. (Callahan et al., 2017) Although no completed clinical trial exists for the use of CTLA-4 - combined with PD-1 blockade in head and neck cancer, preclinical data suggest that HNSCC patients would benefit from the combination as well. (Swanson & Sinha, 2015)

Finally, it is important to mention that in our study, the percentage of lymphocyte subpopulations is defined as the percentage of cells within the lymphocyte gate.

For example, a lower % of a certain lymphocyte subgroup does not necessarily mean lower absolute counts of this subset as the percentages of lymphocyte subsets are dependent on the total count of lymphocytes, which might be altered in HNSCC patients, particularly after anticancer therapies. Furthermore, the reduction of one cell subset compartment might account for the increase in the % of other cell subtypes.

Additionally, we would like to emphasize that due to low number of recurrent patients (n=4) (in particular at t2 only one value was available) we could not detect any significances. Hence, only tendencies are described in the group of recurrent patients.

7 Summary

Introduction: Head and neck cancer is the seventh most common cancer worldwide. In inoperable cases, primary radiochemotherapy is another treatment option left for head and neck squamous cell carcinoma (HNSCC) patients. Changes induced by RCT in immune cell homeostasis are of great interest as they might interfere with anti-tumor activity. Herein, we compared the composition of major lymphocyte subsets in the peripheral blood of controls (n=22), and of non-relapse (n=31) and relapse (n=4) HNSCC patients before and after RCT.

Methods: EDTA blood of non-recurrent HNSCC patients was collected before RCT (t0), directly after RCT (t1) and in the follow-up period 3 (t2) and 6 months (t3) after RCT. In recurrent patients, blood samples were taken at t0, t1, t2 and at the time of recurrence (t5). The blood of healthy human volunteers served as a control. The composition of major lymphocyte subpopulations was phenotyped by multiparameter flow cytometry.

Results: Non-relapse HNSCC patients had significantly lower proportions of CD19+ B-cells compared to healthy individuals already before initiation of any therapy (t0). B-cell counts dropped further until 3 months after RCT (t2) but reached initial levels 6 months after RCT (t3). In non-recurrent patients, the proportion of CD3+ T and CD3+/CD4+ T-helper-cells continuously decreased between t0 and t3, while that of CD8+ cytotoxic T-cells and CD3+/CD56+ NK-like T-cells (NKT) gradually increased in the same period of time. The percentage of CD4+/FoxP3+ regulatory T-cells (Tregs) dropped directly after RCT, but increased above initial levels in the follow-up period 3 (t2) and 6 (t3) months after RCT. At t0, non-recurrent patients had almost twice as much of CD56bright/CD16+ NK-cells as controls, which dropped significantly at t1. Relapse patients presented similar trends with respect to the percentages of B, T-cells and Tregs between t0 and t5, however, due to the small number of patients the data did not reach statistical significance. Compared to non-recurrent patients, CD4+ T-cells of recurrent patents appear to remain stably low, particularly at t0. The percentages of CD8+ cytotoxic T-cells und CD3+/CD56+ NKT-cells in relapse patients were higher than those in non-relapse patients, but they remained nearly unaltered over the course of RCT. While all NK cell subsets (CD3-/CD56+, CD56+/CD69+, CD3-/CD94+, CD3-/NKG2D+, CD3-/NKp30+, CD3-/NKp46+) increased continuously up to 6 months after RCT (t0-t3) in non-relapse patients, they remained stably low up to 3 months (t2) after RCT in relapse patients.

Conclusion: Non-relapse and relapse HNSCC patients, treated with primary RCT, have altered lymphocyte homeostasis, which already exists before start of therapy and persists for months in the follow-up period. Thus, monitoring the kinetics of lymphocyte subsets during RCT might provide a clue for the development of an early relapse, offer early evaluation of treatment outcome or might even help in the selection of patients that might profit from an additional immunotherapy.

8 Appendix

General condition according to WHO

There are five stages:

Stage 0 – patients are fully active and able to work and lead a normal live

Stage 1 – patients are limited to work; light labor is possible

Stage 2 – patients are still self-sufficient but incapacitated for work; they are bedridden and less than 50% of daily time is used as resting time

Stage 3 – self-sufficiency is strongly restricted; patients are dependent on nursing care and more than 50% of daily time is used as resting time

Stage 4 – patients are constantly bedridden and in need of care

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10 List of Figures

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