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Hsp70 expression and the immune system as potential biomarkers for patients with brain tumours

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Outline

List of abbreviations

ATP	adenosine triphosphate
CAR	chimeric antigen receptor
CBTRUS	Central Brain Tumour Registry of the United States
CD	cluster of differentiation
CNS	central nervous system
CT	computer tomography
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FFPE	formaldehyde-fixed paraffin-embedded
Hsp70	heat shock protein 70
IDH	isocitrate dehydrogenase
IHC	immunohistochemistry
IL-2	interleukin-2
kDa	kilodalton
MGMT	O^6-methylalkylguanine-DNA-methyltransferase
MRI	magnet resonance imaging
NKG2D	natural-killer receptor group 2 member D
NOS	not otherwise specified
NSCLC	non-small cell lung carcinoma
OS	overall survival
PBS	phosphate-buffered saline
PFS	progression free survival

PD1	programmed cell death protein 1
RT	radiotherapy
WHO	world health organisation

Abstract

Heat shock protein 70 (Hsp70), a member of the 70-kDa heat shock protein family (HSP70) is ubiquitously expressed in different subcellular compartments and acts as a molecular chaperone. Apart from its physiological role, Hsp70 is known to be overexpressed in various types of tumours, where it can be found upregulated in the cytosol and on the plasma membrane. On the one hand, it effects proliferation, progression, and therapy resistance of cancer cells. On the other hand, Hsp70 and the 14-mer peptide TKD (TKDNNLLGRFELSG) in combination with low dose interleukin 2 can activate immune responses mediated by NK cells. In addition, Hsp70 can be used as a biomarker for the detection of different cancer types in early stages and monitoring of therapy response and relapse.

In this study, the potency of Hsp70 and the immune system as potential biomarkers for patients with different types of brain tumours was evaluated. Therefore, the cytosolic, membranous, and soluble expression of Hsp70 was measured in 116 patients with brain tumours using various techniques. By flow cytometry, the phenotype of lymphocyte subpopulations was measured in the peripheral blood. The tumours of the patients were graded according to the WHO classification of tumours of the central nervous system. In the acquired results, patients with grade IV tumours had significant higher concentrations of Hsp70 in the circulation than healthy donors. Especially those patients with large tumour volumes (>90cm^3) and large necrotic areas showed elevated Hsp70 serum levels. The immunohistochemical staining of grade IV tumours revealed a higher nuclear and cytosolic Hsp70 staining than that of lower grade gliomas. The absolute number of lymphocytes in the peripheral blood of patients with relapsing grade III brain tumours was significant lower compared to healthy donors. Furthermore, in grade IV glioblastoma patients the percentage of CD4 positive helper T cells was significantly lower than in healthy donors and that of regulatory T cells, although not significant, was higher than that of healthy donors. These data might provide a first hint of the immunosuppressive effect of high-grade gliomas on the peripheral blood lymphocytes. The higher percentages of NK cell subpopulations in the blood of patients with higher tumour grading, might be attributed to elevated concentrations of Hsp70 protein and inflammation markers in the circulation. The potential of Hsp70 to stimulate and upregulate activated NK cell subpopulations in an inflammatory microenvironment, could be confirmed by an incubation of peripheral blood lymphocytes with Hsp70 or an Hsp70 peptide (TKD) and IL-2, in vitro. In summary, the data acquired in this study

provide further information, how Hsp70 and the immune system could be used as potential biomarkers for patients with different types of brain tumours.

Zusammenfassung

Das stressinduzierbare Hitzeschockprotein 70 (Hsp70) ist ein Mitglied der HSP70-Familie mit einem Molekulargewicht von 72 kDa. Hsp70 ist evolutionär hochkonserviert und in unterschiedlichen subzellulären Kompartimenten von Pro- und Eukaryoten exprimiert. In seiner Funktion als molekulares Chaperon, gewährleistet es die Protein-Homöostase und sorgt für die korrekte Faltung neu synthetisierter Proteine. Darüber hinaus kann das eigentlich zytoplasmatisch exprimierte Hsp70 in Tumorzellen auf der Plasmamembran präsentiert und in die Zirkulation freigesetzt werden. Auf der einen Seite ist eine Überexpression von Hsp70 mit Therapieresistenz und Aggressivität von Tumoren assoziiert, andererseits kann Hsp70 in Verbindung mit niedrig dosiertem Interleukin-2 gegen den Tumor gerichtete Immunantworten auslösen. Bei einigen Tumorarten konnte Hsp70 als Biomarker etabliert werden, der Rückschlüsse über das Tumorvolumen, Progress und Therapieansprechen erlaubt. Ziel dieser Arbeit ist es, die Hsp70-Expression und das Immunsystem als potenzielle Biomarker für Patienten mit verschiedenen Hirntumoren zu bewerten. Dafür wurde die Menge an Hsp70 im Zytosol, auf der Membran von Tumorzellen und im Blut von 116 Patienten bestimmt, die gemäß der WHO-Klassifikation für Tumoren des zentralen Nervensystems klassifiziert wurden. Außerdem wurden die Subpopulationen der peripheren Blutlymphozyten der Patienten vergleichend analysiert. Im Vergleich zu Gesundspendern konnten bei Patienten mit Grad IV-Tumoren signifikant erhöhte Konzentrationen von Hsp70 im Serum gemessen werden. Dieser Befund zeigte sich vor allem bei Patienten mit großem Tumorvolumen (>90cm^3) und mit einem hohem Nekrose-Anteil im Tumor. Immunhistochemische (IHC) Färbungen der Tumore zeigten, dass Patienten mit Grad IV-Tumoren eine stärkere zytosolische und nukleäre Hsp70-Färbung aufwiesen als Patienten mit einem niedrigeren Tumorstadium. Außerdem zeigten Patienten mit Grad III-Rezidiven im Gegensatz zu Gesundspendern eine signifikant reduzierte absolute Anzahl an Lymphozyten im peripheren Blut. Auch die prozentual signifikant verminderten CD4-positiven T-Zellen bei Grad IV-Patienten und die erhöhten regulatorischen T-Zellen, tragen zum Bild eines veränderten und immunsuppressiven peripheren Immunsystem bei. Die erhöhten Prozentwerte unterschiedlicher NK-Zell-Subpopulationen mit höherem Tumorgrad könnten durch die

erhöhten Konzentrationen an zirkulierendem Hsp70 in Verbindung mit proinflammatorischen Zytokinen erklärt werden. In einem ex vivo Experiment konnte eine Aktivierung dieser NK-Zell-Populationen durch Hsp70, beziehungsweise TKD (Hsp70 Peptid), in Kombination mit niedrig dosiertem Interleukin-2, nachgestellt werden. Damit erlaubt diese Arbeit neue Rückschlüsse auf die Bewertung der Rolle von Hsp70 und dem Immunsystem als mögliche Biomarker für Patienten mit Hirntumoren in unterschiedlichen Stadien.

2. Introduction

2.1 Hsp70 (HSPA1A) a member of the stress protein family HSP70

Heat shock protein 70 (Hsp70), the major stress inducible member of the 70-kDa heat shock protein family (HSP70), is evolutionary highly conserved and ubiquitously expressed in different cellular compartments. (Radons, 2016) Hsp70 consists of an N-terminal ATPase domain with 45-kDa and a C-terminal substrate binding domain with 25-kDa, which binds hydrophobic peptides in an ATP-dependant manner. (Mayer & Bukau, 2005) In general, Heat shock proteins act as molecular chaperones, they prevent protein aggregation, support folding and unfolding of proteins under physiological conditions, and can be upregulated due to environmental stress, such as hyperthermia or ischemia. (Hartl, 1996) Apart from assisting protein homeostasis, Hsp70 interacts with signaling pathways, influencing apoptosis, proliferation and differentiation of cells. (Mayer & Bukau, 2005) As a result, high intracellular levels of Hsp70 can protect cells from apoptosis and lead to highly resistant tumour cell types. (Jaattela, Wissing, Kokholm, Kallunki, & Egeblad, 1998) In summary, Hsp70 not only supervises and assists protein folding, prevents protein aggregation, but also fulfils housekeeping functions by influencing protein homeostasis and apoptosis. (Beere & Green, 2001)

2.2 Role of Hsp70 in tumour cells

It is well known, that Hsp70 is upregulated and overexpressed in different types of tumours. (Calderwood, Khaleque, Sawyer, & Ciocca, 2006) For example in patients with breast, colon, liver and lung cancer, it has an impact on prognostic factors like tumour progression, proliferation and therapy resistance. (Ciocca et al., 1993) (Hwang et al., 2003) (Joo, Chi, & Lee, 2005) (Gunther et al., 2015)

Whereas in normal cells the expression of Hsp70 is restricted to the cytosol, in tumour cells Hsp70 accumulates in the cytosol, but also can be found on the plasma membrane. (Multhoff et al., 1997) (Jaattela, 1999) (Hantschel et al., 2000) By protecting tumour cells from apoptosis and by stabilization of the plasma membrane, high cytosolic and membranous Hsp70 levels can contribute to a more aggressive and therapy-resistant cancer type. (Nylandsted et al., 2004) (M. Shevtsov, Huile, &

Multhoff, 2018) It is very likely, that Hsp70 is not transported to the plasma membrane via the classical ER-Golgi transport system, but via a non-classical lipid vesicular pathway. Hsp70 has been found to interact with tumour-specific lipids which are not found in the plasma membrane of normal cells, such as globoyltriaosylceramide (Gb3). (Gehrmann et al., 2008) (M. Shevtsov et al., 2020)

Furthermore, membrane bound Hsp70 in the absence or presence of immunogenic peptides can enhance anti-tumour immune responses by activating and stimulating NK cells and cytotoxic T lymphocytes. (Multhoff et al., 1997) (Multhoff et al., 1999) (Schild, Arnold-Schild, Lammert, & Rammensee, 1999) Apart from Hsp70 protein, also a 14-mer peptide TKD (TKDNNLLGRFELSG) of the C-terminal substrate binding domain, in combination with low dose interleukin 2 (IL-2), can result in an upregulated cytotoxic activity of NK cells. (Multhoff et al., 2001) Activated NK cells recognize and kill membrane Hsp70 positive tumour cells by an uptake of the serine protease granzyme B via membranous Hsp70. (Gross, Koelch, DeMaio, Arispe, & Multhoff, 2003)

A treatment with NK cells, stimulated by TKD plus IL-2, in combination with PD1 inhibition, led to a significant reduction of the tumour volume and an improved overall survival in preclinical tumour mouse models. (M. Shevtsov et al., 2019) In a clinical phase II trial, Hsp70-peptide TKD plus IL-2 activated, autologous NK cells were adoptively transferred to enhance antitumoral immune responses in patients with Non-Small Cell Lung Cancer (NSCLC) after radio chemotherapy (RCTx). (Specht et al., 2015)

Beside its cytosolic or membranous localization, Hsp70 can also be found either free or lipid-bound in the extracellular milieu. It is assumed, that free Hsp70 origins from dying cells, and the lipid-bound, exosomal version of Hsp70, is actively secreted by viable cells. (Vega et al., 2008)

Compared to healthy individuals, patients with different diseases, such as infectious diseases and inflammatory processes also have elevated Hsp70 levels in the circulation. However, Hsp70 levels in tumour patients were found to be higher than in patients with infectious diseases. Therefore, Hsp70 in the serum can help to differentiate patients with different malignancies, for example patients with hepatocellular carcinoma and infectious liver diseases or multiple sclerosis. (Gehrmann et al., 2014) (Lechner, Buck, Sick, Hemmer, & Multhoff, 2018)

Depending on its subcellular or extracellular distribution, Hsp70 fulfils dual roles. On the one hand Hsp70 can lead to a more aggressive tumour type by protecting against apoptosis and enhancing therapy resistance, on the other hand it can activate immune responses against cancer cells and function as possible recognition site for immune cells. Depending on the intracellular, membranous or extracellular location, Hsp70 promotes different effects and functions. (M. Shevtsov et al., 2018)

2.3 Brain cancer and gliomas

In this project I received material of patients with different types of brain tumours, including diffuse astrocytoma and oligodendroglioma, anaplastic astrocytoma and oligodendroglioma, glioblastoma and giant cell glioblastoma. In general, gliomas are one of the most common tumours of the central nervous system (CNS), and have their origin in glial cells. (Ostrom et al., 2014) In total, there are four major types of glial cells in the central nervous system: astrocytes, oligodendrocytes, micro glia, and ependymal cells. A short overview of the function and characteristic of different glial cells can be seen in table 1. (Jakel & Dimou, 2017)

glial cell type	**characteristics**
Astrocytes	Maintain homeostasis (water and ion) Supply of neurons Part of the brain blood barrier
Oligodendrocytes	Coating of axons in the CNS Production of myelin
Micro glia	Phagocytic cells Immune response
Ependymal cells	Line spinal cord and ventricles

Table 1: Function and characteristics of different glial cells; (Jakel & Dimou, 2017)

2.3.1 Epidemiology

With approximately 80%, gliomas are the most frequent primary malignant brain tumours in adults. The incidence for gliomas in adults is about 5 cases per 100,000 persons. (Ostrom et al., 2014) Glioblastoma, the most common subtype among the glioma group in adults, has an incidence of approximately 3 per 100,000 persons and is diagnosed at older age with a median age of 64 years and a peak at 75-84 years. (Ostrom et al., 2014) (Tamimi & Juweid, 2017) According to the Central Brain Tumour Registry of the United States (CBTRUS), reporting Primary Brain and Central Nervous System Tumours, diagnosed in the United States between 2012-2016, ependymal tumours showed incidence of 0.43/ 100,000 persons, diffuse astrocytoma 0.46/ 100,000 persons, anaplastic astrocytoma 0.42/ 100000 persons, oligodendroglioma 0.23/ 100000 persons, anaplastic oligodendroglioma 0.11/ 100,000 persons and

glioblastoma 3.22/ 100,000 persons. For most glioma subtypes incidence is higher for males than females. (Ostrom et al., 2019)

2.3.2 Diagnosis

Since there are no biomarkers established for the detection of gliomas in early stages, magnet resonance imaging (MRI) has the highest relevance for diagnosing gliomas and determining response to treatment. (Ellingson et al., 2015) Positron emission tomography (PET), angiography, and computer tomography (CT) can support the planning of surgery and biopsy. Unspecific symptoms, occurring in patients with glioma, are focal neurological deficits, new onset of epilepsy and signs of increased cerebral pressure. (Weller et al., 2017)

2.3.3 WHO classification of tumours of the central nervous system

Regarding pathological diagnosis and molecular markers, gliomas are classified according to the WHO classification of Tumours of the Central Nervous System. The classification was updated in 2016 and focusses, apart from histological aspects, also on the expression of molecular markers, such as Isocitrate Dehydrogenase 1/ 2 (IDH 1/ 2), 1p/19q co-deletion status or 0^6-methylguanine-DNA-methyltransferase (MGMT). Addressing both, phenotypic and genotypic characteristics of tumours, the classification and integrated diagnosis reached a new level of objectivity and diagnostic reliability. (Louis et al., 2016) (Gupta & Dwivedi, 2017)

With higher grading, tumours show more malignant and aggressive characteristics and reproduce faster. (Louis et al., 2014) (Louis et al., 2016) (Weller et al., 2017) Table 2 presents an overview about the different tumour gradings in our collective of diffuse astrocytic and oligodendroglial and ependymal tumours, according to 2016 WHO classification.

grading				
group	**I**	**II**	**III**	**IV**
Diffuse astrocytic and oligodendroglial tumours		Diffuse astrocytoma (IDH mutant)	Anaplastic astrocytoma (IDH mutant)	Glioblastoma (IDH mutant/ IDH wildtype)
		Oligodendroglioma (IDH mutant, 1p19q co-deleted)	Anaplastic oligodendroglioma (IDH mutant, 1p19q co-deleted)	
Ependymal tumours	Subependymoma	Ependymoma	Anaplastic ependymoma	

Table 2: Tumour gradings of diffuse astrocytic, oligodendroglial and ependymal tumours; (Louis et al., 2016)

2.3.4 Therapy

The concept of treating gliomas is based on 3 major principles: surgery, radiotherapy, and drug therapy.

Using MRI navigation systems or neurochemical navigation with 5-aminolevulinic acid, a prodrug leading to an accumulation of fluorescent metabolites in gliomas, tumour identification is facilitated. This allows the resection of major parts of the tumour volume and prevents damage in the surrounding normal brain tissue. (Stummer et al., 2006) Especially during a surgical removal of tumours, located near brain areas, which are responsible for language and cognition, the use of mapping or evoked potentials can minimize neurological impairment. (Weller et al., 2017) However, due to the diffuse expansion and infiltration of glioma cells into healthy tissue, a complete resection of all glioma cells by surgery often is not possible. (Osswald et al., 2015) Therefore, regular magnet resonance imaging (MRI) of the brain is necessary for response assessment after surgery. (Vogelbaum et al., 2012)

In addition to surgery, radiotherapy (RT) is applied to increase local tumour control and survival. (Laperriere, Zuraw, Cairncross, & Cancer Care Ontario Practice Guidelines Initiative Neuro-Oncology Disease Site, 2002) Depending on the condition and prognosis, 50 – 60 Gy are applied in 1.8 – 2 Gy fractions. For older patients also lower dose of 15 x 2.67 Gy can be considered. Defined areas of gross tumour volume, clinical

target volume and final target volume is determined via magnet resonance imaging. (Niyazi et al., 2016) The use of stereotactic, intensity-modulated, or image-guided radiotherapy can reduce side effects in normal tissues. (Weller et al., 2017)

The application of chemotherapy complements the treatment of gliomas. Often Temozolomide, an alkylating agent with good penetration of the blood brain barrier, is applicated per os. (Wesolowski, Rajdev, & Mukherji, 2010) Apart from Temozolomide, Nitrosoureas and Vincristine are often used as chemotherapeutics to treat gliomas. (Lassman et al., 2011) (Brandes, Bartolotti, Tosoni, & Franceschi, 2016) (Weller et al., 2017)

2.3.5 Prognosis

Apart from tumour grading, age, status of performance, and extent of resection are important prognostic factors for gliomas. (Kita et al., 2009) (Weller et al., 2017)

Based on EUROCARE cancer registries and data sets from 2000 – 2002, survival of European patients with brain tumours could be estimated. (Sant et al., 2012) The 5-year relative survival of ependymoma and choroid plexus tumour was 83.5%, of astrocytoma NOS and other subtypes 38.5%, of anaplastic astrocytoma 15.8%, of oligodendroglioma 67.2%, of anaplastic oligodendroglioma 31.5%, and of glioblastoma 2.7%. In summary, patients with glioblastoma show an approximated overall survival (OV) of 15 months. (Hau, Stupp, & Hegi, 2007)

For evaluation of therapy responses and follow up, clinical neurological examination and magnet resonance imaging are used. (Weller et al., 2017)

2.4 Project design and aim of the dissertation

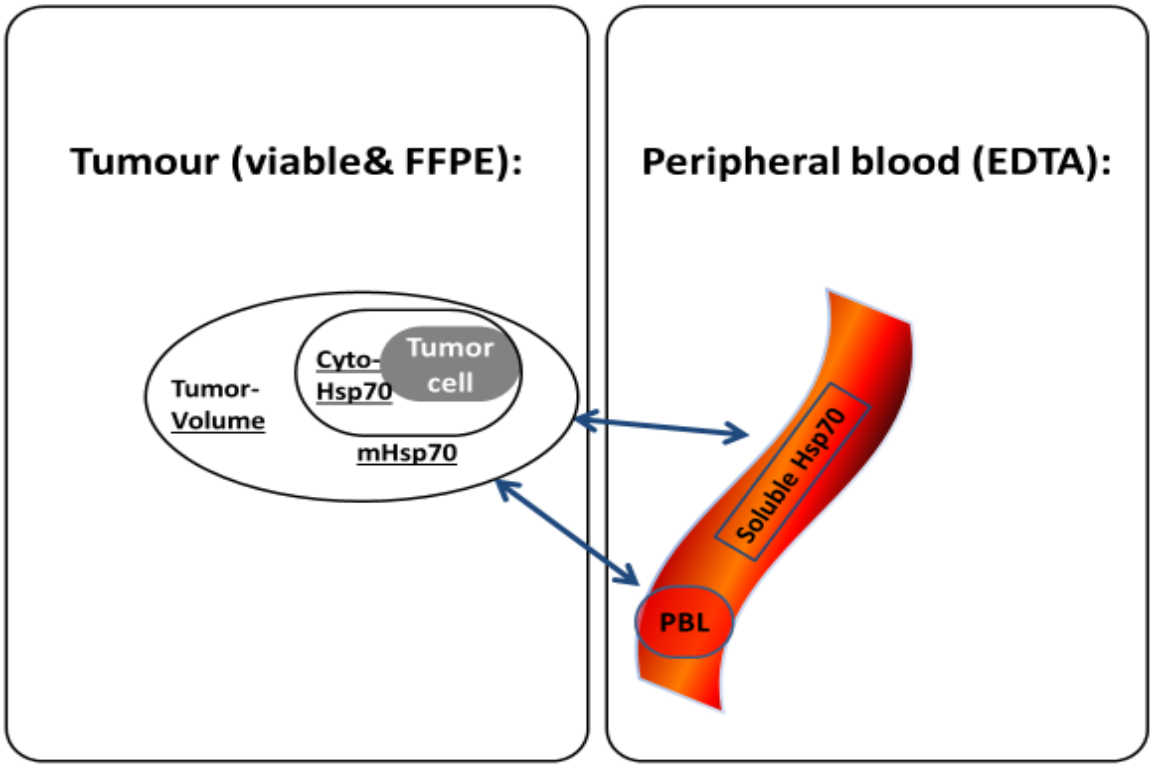

Figure 1: Summary of materials and samples, used for analysis

Figure 1 shows a summary about materials and samples, which were used in my project. In resected tumour material, I determined the Hsp70 expression [%] on the membrane of viable tumour cells by performing flow cytometry. Furthermore, I analysed the Hsp70 expression in nucleus and cytosol by staining 3 µm formalin-fixed paraffin-embedded slices of the tumours via immunohistochemistry. Finally, by magnet resonance imaging, the total tumour volume and the area of necrosis were determined. In the peripheral blood of patients, the concentration of soluble Hsp70 [ng/ml] was determined by ELISA, the composition of lymphocyte subpopulations [%] using flow cytometry. Furthermore, the number of absolute lymphocytes was determined. With this experimental design I tried to obtain further information about the potency of Hsp70, directly at tumour site and in the circulation, as a potential biomarker for patients with brain tumours. In addition, to develop a deeper understanding of the role of the immune system in brain tumour patients, the immunophenotype of patients with different tumour gradings was compared to that of healthy donors. Finally, I analysed clinical data like therapy response or overall survival of the patients.

3. Materials and Methods

3.1 Summary

In the following part “Materials and Methods”, a closer impression on the final patient collective and its characteristics is provided. Furthermore, the techniques and methods, I used to analyse different samples of patients with brain tumours, will be described.

To determine the percentages of the different lymphocyte subpopulations, Huyen Nguyen and I performed flow cytometry with peripheral blood of the patients on the day of their surgery or one day after, in collaboration with the Department of Neurosurgery. In total I performed flow cytometry with 102 blood samples. Taking only the patients at first diagnosis into focus, we analysed 78 patients by flow cytometry, 35 by Huyen Nguyen and 43 by me. Beside the composition of lymphocyte subpopulations and their percentages, I also counted the absolute number of lymphocytes after density gradient centrifugation by Ficoll at 39 patients at diagnosis and 17 with brain tumour recurrence. To study the strength and pattern of Hsp70 expression in the tumour cells, I stained 25 tumour slides for Hsp70, using immunohistochemistry. To evaluate the expression of Hsp70 on the surface of the tumour cells, flow cytometry was performed on 37 patients by different members of our group, including me. Analysis was done by Matthias Pilz and me. By ELISA, Sven Schmidt analysed the concentration of Hsp70 in the circulation of 56 patients. Finally, to determine the tumour volume and area of necrosis in cm^3, Isabelle Riederer, a radiologist of Klinikum rechts der Isar, TUM, analysed 51 magnet resonance images of patients with grade IV brain tumours. Clinical parameters like therapy concept, outcome and progress were collected by Melanie Barz, a neurosurgeon of Klinikum rechts der Isar, TUM.

The study was approved by the ethic committee of medical faculty of TUM with ethics vote number 2403/09.

In addition to the analysis of patient material, and to obtain a closer impression on the correlation of Hsp70 with the percentage of NK cells and their activation markers, I stimulated peripheral blood lymphocytes with TKD, a Hsp70 peptide (TKDNNLLGRFELSG), and Hsp70 ex vivo. (Gastpar et al., 2004)

The statistical analysis and evaluation of the data were done by me, using the programming language R, R studio version 3.5.2.

3.2 Patient collective

Between 2015 and 2019, in collaboration with the Department of Neurosurgery at Klinikum rechts der Isar, TUM, samples of patients with different types of brain tumours were collected. The patients were recruited to the study approved by the ethic committee of the medical faculty of TUM with ethics vote number 2403/09. Written consent was given by the patients with the formula, shown in appendix. In total 116 patients were included to the study, 89 at first diagnosis and 27 with relapse.

According to the WHO classification of Tumours of the Central Nervous System (Louis et al., 2016) and their pathological diagnosis, determined by the Department of Neuropathology at Klinikum rechts der Isar, TUM, the patients were scored into the different gradings I, II, III, IV.

One patient, 24 years, with ependymoma at diagnosis was scored as grade I. 14 patients with a median age of 37 years (age range: 23-56 years) were grade II, 4 with oligodendroglioma (2 first diagnosis and 2 relapse) and 10 with diffuse astrocytoma (6 at first diagnosis and 4 relapse). 26 patients with median age of 47 years (age range: 29-70 years) were grade III, 13 with anaplastic oligodendroglioma (8 at first diagnosis and 5 relapse) and 13 with anaplastic astrocytoma (6 at first diagnosis and 7 relapse). 75 patients with a median age of 62 years (age range: 21-89 years) were grade IV, 68 with glioblastoma (59 at first diagnosis and 9 relapse) and 7 with giant cell glioblastoma (all at first diagnosis). The median age and age range is summarized in table 3, WHO grading with pathological diagnosis in table 4.

WHO grade	median age [years]	age range [years]
I	24	/
II	37	23-56
III	47	29-70
IV	62	21-89

Table 3: Median age and age range of the patient collective

WHO grade	**n**	**first diagnosis**	***IDH-wildtype***	**relapse**	***IDH-wildtype***
I	**1**				
Subependymoma	1	1	*0*	0	*0*
II	**14**				
Oligodendroglioma	4	2	*0*	2	*0*
Diffuse astrocytoma	10	6	*0*	4	*1*
III	**26**				
Anaplastic oligodendroglioma	13	8	*0*	5	*0*
Anaplastic astrocytoma	13	6	*1*	7	*2*
IV	**75**				
Glioblastoma	68	59	*58*	9	*9*
Giant cell glioblastoma	7	7	*7*	/	*0*
∑	**116**	**89**		**27**	

Table 4: WHO grading and pathological diagnosis of the patient collective

The number of absolute lymphocytes was counted in 56 patients, 10 grade II (5 at first diagnosis and 5 with relapse), 14 grade III (6 at first diagnosis and 8 with relapse) and 32 grade IV (28 at first diagnosis and 4 with relapse), in giga per litre [G/l]. Certain lymphocyte subpopulations were determined in 75 patients at first diagnosis, 6 grade II, 13 grade III and 56 grade IV.

Hsp70 expression on the surface of tumour cells was analysed by flow cytometry in 37 patients, 3 grade II, 6 grade III and 28 grade IV at first diagnosis.

Immunohistochemistry staining for Hsp70 was performed in 25 slices, consisting of 1 grade I, 3 grade II, 4 grade III and 17 grade IV tumours at diagnosis.

The concentration of circulating Hsp70 [ng/ml] in the peripheral blood was measured in 68 patients at first diagnosis, 5 grade II, 12 grade III and 51 grade IV. Finally, tumour volume was defined in 44 patients with grade IV tumours at first diagnosis, area of necrosis at 27 grade IV tumours at first diagnosis.

The percentage of lymphocyte subpopulations of the patients' peripheral blood was compared to 16 healthy donors with a median age of 61 (age range: 21-85). The absolute lymphocyte counts were compared to 12 volunteers with a median age of 23 (age of range: 21-26). The concentration of Hsp70 in the circulation was measured in 150 healthy donors (median age of 40.5, age range: 20-74) by our group. The healthy donors were recruited to the study approved by the ethic committee of the medical faculty of TUM.

3.3 Immunohistochemistry staining for Hsp70 in brain tumours of different gradings

In collaboration with the Department of Neuropathology at Klinikum rechts der Isar, TUM, I received 3 µm formalin-fixed paraffin-embedded slices (n= 25) of brain tumours at diagnosis. The tumour gradings of our patient collective for the immunohistochemistry staining can be seen in table 5.

After heating the slices at 50-60°C for 30 minutes, they were dewaxed and hydrated in Xylol (Ref: 9713.3, ROTH) and descending concentrations of ethanol (ethanol absolute, Ref: 27694, Fischar; ethanol 96%, Ref: 27687, Fischar). The ethanol concentration of 70% and 50% were mixed using ethanol absolute and ddH_2O. To unmask the binding epitopes the samples were heated for 30 minutes in citrate buffer (Target Retrieval Solution, Ref: S1699, Dako; 1:10 dilution in ddH_2O). After being washed in ddH_2O for few minutes, H_2O_2 (3%) and sodium azide (0.1%) diluted in phosphate buffered saline (PBS, Ref: D8537-500ML, SIGMA Life Science), were added to reduce background staining and the activity of endogenous peroxidase. Between another two washing steps with ddH_2O and PBS, rabbit serum in a 5% dilution with antibody diluent (Ref: S2022, Dako) was used to block unspecific binding. In a next step, the samples were incubated with the primary antibody cmHsp70.1 (2,4 mg/ml, multimmune GmbH, Munich, Germany) at a 1:500 dilution with antibody diluent (Ref: S2022, Dako) over night at 4°C. At the next day, after washing with PBS, secondary HRP labelled polymer antibody (Envision + System HRP labelled polymer Anti-mouse, Ref: K4001, Dako) was added, followed by Diaminobenzidine Chromogen (DAB+ substrate buffer, Ref: K3468, Dako; DAB+ chromogen, Ref: K3468, Dako), restricted to 5 minutes. Finally, Haematoxylin and Eosin (Ref: T865.2, ROTH) were used for counterstaining and the slices were dehydrated with ascending concentrations of Ethanol and Xylol. The slices were embedded with a coverslip using Eukitt (Roti-Histokitt 100ml, Ref: 6638.1, ROTH). (Lobinger et al., 2021) Each immunohistochemistry staining procedure was accompanied by a negative control sample. The chemicals and antibodies used for the immunohistochemical staining can be seen in table 6.

WHO grading	**number of patients**	***IDH-wildtype***
I	1	*0*
II	3	*0*
III	4	*0*
IV	17	*17*

Table 5: WHO grading of the patient collective for immunohistochemistry

Name	**reference number**	**company**
Xylol	9713.3	ROTH
Ethanol absolute	27694	Fischar
EtOH 96%	27687	Fischar
Target Retrieval Solution 10X Concentrate	27660	Dako
Dako Pen	S2002	Dako
Wasserstoffperoxid 30%	S1699	Fluka
Natriumazid	S2002	ROTH
Antibody Diluent	95321	Dako
cmHsp70.1	04/09c; 2,4 mg/ml	multimmune GmbH
EnVision+ System- HRP Labelled Polymer	K4001	Dako
DAB+ SUBSTRATE BUFFER	K3468	Dako
DAB+ CHROMOGEN	K3468	Dako
Hämalaun	T865.2	ROTH
Roti-Histokitt 100ml	6638.1	ROTH
coverslip 21x21mm	10344081	Thermo Scientific
coverslip 24x60mm	15747592	Thermo Scientific
Menzel SUPERFROST PLUS	J1800AMNZ	Thermo Scientific

Table 6: Chemicals and antibodies used for immunohistochemistry

3.4 Using flow cytometry to analyse the expression of Hsp70 on the surface of brain tumour cells

To analyse the expression of Hsp70 on the surface of brain tumour cells of different gradings, I received tumour material of 37 patients in collaboration with the Department of Neurosurgery. The collective consisted out of 3 grade II, 6 grade III and 28 grade IV tumours at diagnosis. To keep the tumour cells viable, the whole procedure was done immediately and on ice. After aspiration of surplus material, the tumour sample was cut down with a scalpel in a petri dish. To obtain a single cell suspension, 8 minutes of incubation with trypsin was followed by filtration with a 70 µm strainer. Finally, after resuspension in phosphate buffered saline (PBS), the cells were aliquoted into 3 tubes and incubated with fluorescent-labelled antibodies for 30 minutes on ice. The combination of the used antibodies and further information like clone, reference number and company are shown in table 7 and 8. After washing with 10% FACS-Buffer (PBS witch 10% heat inactivated Foetal Calf Serum) and centrifugation at 500g (1500 rpm) the cell suspension was analysed by FACSCalibur flow cytometer (BD Biosciences). (Lobinger et al., 2021)

tube	antibody	ul
1	IgG1-FITC CD45-APC	5 1
2	Hsp70-FITC CD45-APC	2/20 1
3	MHC1-FITC CD45-APC	5 1

Table 7: Combination of antibodies for analysing the surface expression of Hsp70 on tumour cells

antibody	Clone	Reference number	company
IgG1-FITC	X40	345815	BD
CD45-APC	HI30	MHCD4505	Thermo Fisher
cmHsp70.1-FITC	Custom made		Multimmune
MHC1-FITC	W6/32	F5662	Sigma

Table 8: Clone, reference number and company of the antibodies

3.5 Using flow cytometry to analyse the composition of lymphocyte subpopulations

Using a FACSCalibur flow cytometer (BD Biosciences) the composition of lymphocyte subpopulations in the peripheral blood of the patients was analysed. From the Department of Neurosurgery, Klinikum rechts der Isar, TUM, I received EDTA blood at the day of the surgery of patients with different brain tumours at first diagnosis or with relapse. The flow cytometry was performed on the same day or within 24 h. Before adding 100 µl of EDTA blood, fluorescents-labelled antibodies were pipetted into 14 tubes. The combination, the antibodies were pipetted into the tubes can be seen in table 10. Further information about clone, reference number and company in table 11. Beside the isotype matched negative control antibodies like IgG1-FITC, IgG1-PE, IgG1-PerCP, IgG1-APC, antibodies for targeting T cells, such as CD3-PerCP, CD4-FITC, CD8-FITC, NK cells, such as CD56-FITC, CD94-FITC, NKG2D-PE, Nkp30-PE, Nkp46-PE, and B cells, such as CD19-PE were used. To obtain information about the percentage of regulatory T cells, FoxP3-PE, addressing the intranuclear transcription factor forkhead box P3, was pipetted into tube 13 and 14. (Khattri, Cox, Yasayko, & Ramsdell, 2003) After the blood was incubated for 15 minutes in the dark at room temperature, a washing step with 10% FACS-Buffer (PBS witch 10% heat inactivated Foetal Calf Serum) and centrifugation at 500 g (1500 rpm) followed. For elimination of red blood cells, a lysing buffer (1:9 dilution in $_{dd}H_2O$ of FACS Lysing Solution, Ref: 349202, BD) was added for 10 minutes, followed by a further washing procedure with 10% FACS-Buffer and centrifugation at 500g (1500 rpm). Tubes 1 to 11, ready to be analysed by flow cytometry, were measured within one hour. (Lobinger et al., 2021)

To analyse the percentage of regulatory T cells in the peripheral blood, the protocol continued with tube 12 to 14. Buffer A (1:10 dilution in $_{dd}H_2O$ of Component A, Ref: 51-9005451, BD) was added for fixation and Buffer C (1:50 dilution in Buffer A of Component B, Ref: 51-9005450, BD), following washing with 10% FACS-Buffer and centrifugation, for permeabilization. After 30 minutes in the dark at room temperature and further washing procedure, the cells were incubated with IgG1-PE and FoxP3-PE for 30 minutes. 50 000 cells in each tube were analysed and matched with a negative antibody control. The buffer and chemicals used for the analysis with flow cytometry can be seen in table 12.

The gating strategy can be seen in table 9. With respect to granularity, measured with side-scattered light, and size, measured with forward-scattered light, R1 defines lymphocytes, R2 monocytes and R3 granulocytes. The corresponding percentage of lymphocyte subpopulations are defined, as for specific markers positive fraction of the R1 lymphocyte gate. (Lobinger et al., 2021)

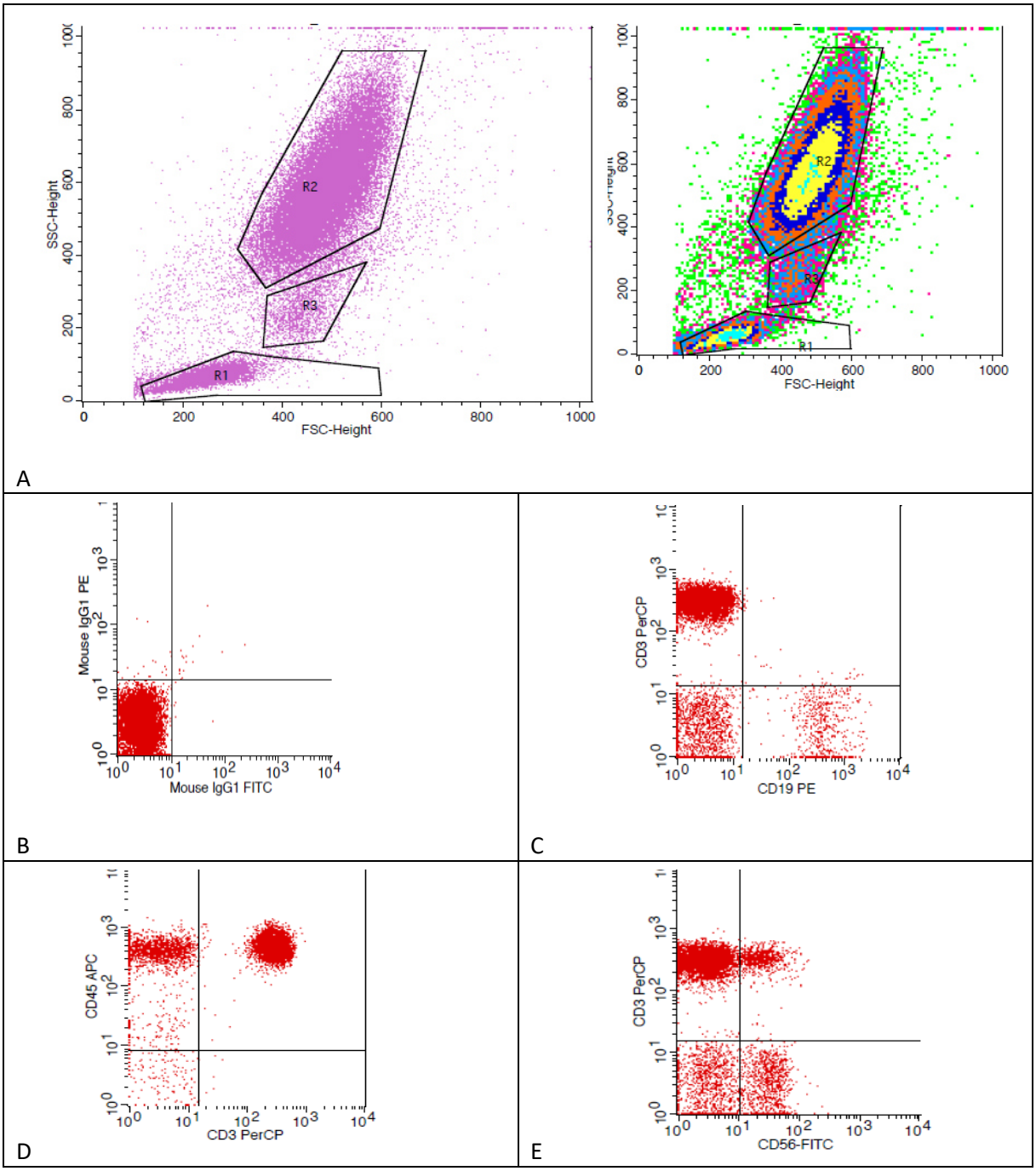

Table 9: A: R1 = Lymphocytes; B: Isotype; C: CD19+/CD3- B cells; D: CD3+/CD45+ T cells; E: CD56+/CD3- NK cells

tube	Antibody	µl
1	IgG1-FITC	5
	IgG1-PE	5
	IgG1-PerCP	5
	IgG1-APC	1
2	CD94-FITC	5
	CD56-PE	5
	CD3-PerCP	10
	CD45-APC	1
3	CD56-FITC	5
	CD19-PE	20
	CD3-PerCP	10
	CD45-APC	1
4	CD56-FITC	5
	CD16-PE	10
	CD3-PerCP	10
	CD45-APC	1
5	CD56-FITC	5
	NKG2D-PE	10
	CD3-PerCP	10
	CD69-APC	5
6	CD56-FITC	5
	Nkp30-PE	10
	CD3-PerCP	10
	CD69-APC	5
7	CD56-FITC	5
	NKP46-PE	10
	CD3-PerCP	10
	CD69-APC	5
8	CD94-FITC	5
	NKG2D-PE	10
	CD3-PerCP	10
	CD56-APC	10
9	CD94-FITC	5
	Nkp30-PE	10
	CD3-PerCP	10
	CD56-APC	10
10	CD94-FITC	5
	NKP46-PE	10
	CD3-PerCP	10
	CD56-APC	10
11	CD4-FITC	20
	CD8-PE	20
	CD3-PerCP	10
	CD45-APC	1
12	IgG1-FITC	5
	IgG1-PE (after fixation)	5
	IgG1-PerCP	5
	IgG1-APC	1
13	CD4-FITC	20
	CD3-PerCP	10
	CD25-APC	5
	FoxP3-PE (after fixation)	20
14	CD8-FITC	20
	CD3-PerCP	10
	CD25-APC	5
	FoxP3-PE (after fixation)	20

Table 10: Combination of antibodies

antibody	Clone	reference number	company
IgG1-FITC	X40	345815	BD
IgG1-PE	X40	345816	BD
IgG1-PerCP	X40	345817	BD
Mouse IgG1 APC Conjugate	Class I (ASR)	MG105	Thermo Fisher
FITC Mouse Anti-Human CD94	HP-3D9 (RUO)	555888	BD
CD56-FITC	NCAM16.2	345811	BD
FITC Mouse Anti-Human CD4	RPA-T4 (RUO)	555346	BD
FITC Mouse Anti-Human CD8	RPA-T8 (RUO)	555366	BD
CD56-PE	NCAM16.2	345812	BD
PE Mouse Anti-Human CD19	HIB19 (RUO)	555413	BD
PE Mouse Anti-Human CD16	3G8 (RUO)	555407	BD
anti-hNKG2D PE Conjugated Mouse	149810	FAB139P	R&D Systems
CD337(Nkp30)-PE	Z25	IM3709	Beckman Coulter
CD335(Nkp46)-PE	BAB281	IM3711	Beckman Coulter
PE Mouse Anti-Human CD8	RPA-T8 (RUO)	555367	BD
CD3-PerCP	SK7	345766	BD
Human CD45 APC Conjugate	HI30	MHCD4505	Thermo Fisher
CD69-APC	L78 (RUO)	340560	BD
APC Mouse Anti-Human CD56	B159 (RUO)	555518	BD
CD25-APC	2A3 (CE/IVD)	340907	BD
PE Mouse anti-Human FoxP3	259D/C7 (RUO)	349202	BD

Table 11: Clone, reference number and company of the antibodies used for flow cytometry

name	concentration	reference number	company
FACS™ Lysing Solution	10X	349202	BD
Dulbecco`s Phosphate Buffered Saline		D8537-500ML	SIGMA Life Science
Human FoxP3 Buffer A	10X	51-9005451	BD
Human FoxP3 Buffer B	50X	51-9005450	BD

Table 12: Buffer and chemicals used for analysis with flow cytometry

3.6 Ex vivo stimulation of peripheral blood lymphocytes with Hsp70 and TKD

Hsp70 protein and TKD, the 14-mer Hsp70 peptide (TKDNNLLGRFELSG), can enhance and upregulate the activity of NK cells. (Multhoff et al., 2001) (Gastpar et al., 2004) I illustrated this activation by stimulating peripheral blood lymphocytes of healthy human volunteer's ex vivo. Therefore, I measured the percentage of NK cells and their activation markers with flow cytometry, 5 days after the incubation with Hsp70 plus IL-2 and TKD plus IL-2.

The collective of probands consisted out of 4 healthy male volunteers with a median age of 23.5 and age range of 22-24 years. After collecting 8-13 ml of EDTA blood, the peripheral blood lymphocytes were separated using density gradient centrifugation with Ficoll. Figure 2 shows the mechanism of density gradient centrifugation and the experimental design. After centrifugation at 850 g (2000 rpm) for 20 minutes at room temperature with 10 ml Ficoll, the layer of lymphocytes could be pipetted off and resuspended in RPMI medium, followed by another centrifugation at 700 g (1800 rpm) for 15 minutes at room temperature. Finally, the cells were counted using trypan blue staining and resuspended in RPMI-1640 with L-glutamine, 10% Fetal Calf Serum, Penicillin G (100 IU/ml), and Streptomycin (100 µg/ml) at cell density of 5-8 x 10^6 PBL/ ml. The samples were either stimulated with 10 µg/ml Hsp70 plus 100 IU/ml IL-2 or 2 µg/ml TKD (TKDNNLLGRFELSG, Bachem AG, Bubendorf) plus 100 IU/ml IL-2. (Lobinger et al., 2021)

After 5 days of incubation with Hsp70 plus IL-2 or TKD plus IL-2, the cells were analysed using a FACSCalibur (BD Biosciences) flow cytometer. Characteristics of the used chemicals, like reference number and company can be seen in table 13. The percentage of lymphocyte subpopulations were measured as described earlier, using the antibody combination shown in table 14. 50 000 cells in each tube were analysed and matched with a negative antibody control.

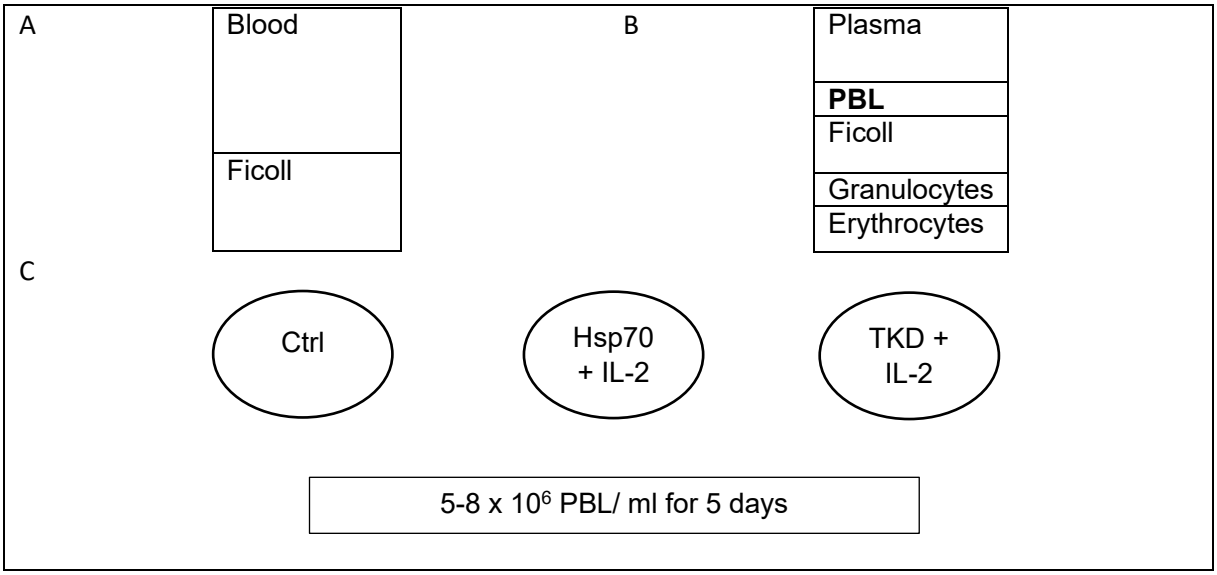

Figure 2: A: Layers before density gradient centrifugation; B: layers after density gradient centrifugation; C: Stimulation, Ctrl = Control

name	**clone**	**reference number**	**company**
IgG1-FITC	X40	345815	BD
IgG1-PE	X40	345816	BD
IgG1-PerCP	X40	345817	BD
Mouse IgG1 APC Conjugate	Class I (ASR)	MG105	Thermo Fisher
FITC Mouse Anti-Human CD94	HP-3D9 (RUO)	555888	BD
CD56-FITC	NCAM16.2	345811	BD
FITC Mouse Anti-Human CD4	RPA-T4 (RUO)	555346	BD
FITC Mouse Anti-Human CD8	RPA-T8 (RUO)	555366	BD
CD56-PE	NCAM16.2	345812	BD
PE Mouse Anti-Human CD19	HIB19 (RUO)	555413	BD
PE Mouse Anti-Human CD16	3G8 (RUO)	555407	BD
anti-hNKG2D PE Conjugated Mouse	149810	FAB139P	R&D Systems
CD337(Nkp30)-PE	Z25	IM3709	Beckman Coulter
CD335(Nkp46)-PE	BAB281	IM3711	Beckman Coulter
PE Mouse Anti-Human CD8	RPA-T8 (RUO)	555367	BD
CD3-PerCP	SK7	345766	BD
Human CD45 APC Conjugate	HI30	MHCD4505	Thermo Fisher
CD69-APC	L78 (RUO)	340560	BD
APC Mouse Anti-Human CD56	B159 (RUO)	555518	BD
CD25-APC	2A3 (CE/IVD)	340907	BD
Ficoll-Paque		17-5446-02	Cytiva
IL-2	100 IU/ml		Novartis
Hsp70	Custom made		Bachem AG
TKD	Custom made		Bachem AG

Table 13: Antibodies and chemicals used for the stimulation of PBL

tube	antibody	µl
1	IgG1-FITC	5
	IgG1-PE	5
	IgG1-PerCP	5
	IgG1-APC	1
2	CD94-FITC	5
	CD56-PE	5
	CD3-PerCP	10
	CD45-APC	1
3	CD56-FITC	5
	CD19-PE	20
	CD3-PerCP	10
	CD45-APC	1
4	CD56-FITC	5
	CD16-PE	10
	CD3-PerCP	10
	CD45-APC	1
5	CD56-FITC	5
	NKG2D-PE	10
	CD3-PerCP	10
	CD69-APC	5
6	CD56-FITC	5
	Nkp30-PE	10
	CD3-PerCP	10
	CD69-APC	5
7	CD56-FITC	5
	NKP46-PE	10
	CD3-PerCP	10
	CD69-APC	5
8	CD94-FITC	5
	NKG2D-PE	10
	CD3-PerCP	10
	CD56-APC	10
9	CD94-FITC	5
	Nkp30-PE	10
	CD3-PerCP	10
	CD56-APC	10
10	CD94-FITC	5
	NKP46-PE	10
	CD3-PerCP	10
	CD56-APC	10
11	CD4-FITC	20
	CD8-PE	20
	CD3-PerCP	10
	CD45-APC	1

Table 14: Combination of antibodies used for analysing the percentage of stimulated PBLs

3.7 Statistics

The statistical analysis was performed using the programming language R, R studio version 3.5.2. Normal distribution was tested with help of Shapiro-Wilk normality test with a value of $p < 0.05$. The significant decrease of CD4+ T cells [%] in grade IV patients compared to healthy donors, where the results followed normal distribution, was calculated using Anova and post-hoc Tukey test. Significant differences in the percentage of regulatory T cells and NK cells, the number of absolute lymphocytes and the expression of Hsp70 on the surface of tumour cells with different grading, not following normal distribution, were calculated using Kruskal Wallis test. With help of Anova and post-hoc Tukey test, the significant increase of Hsp70 concentration in the circulation of grade IV patients compared to healthy donors, again following normal distribution, was calculated. The significant higher concentration of Hsp70 in the circulation of grade IV patients with large tumours, was calculated using Kruskal Wallis test. Finally, the significant difference of Hsp70 levels in patients with high portion of necrosis in their tumours compared to healthy donors was analysed using Wilcoxon Rank test. The ex vivo stimulation with TKD and IL-2 resulted in a significant increase among CD69+ and CD94+ NK cells, as calculated using Tukey test. For all tests, a value of $p < 0.05$ was considered as statistically significant. The statistical data is summarized in table 15.

Statistically significant differences

[%] CD4+ T cells

> **Anova test**

	Df	Sum Sq	Mean Sq	F	**Pr(>F)**
Group	6	2138	356.4	2.24	**0.0444**

>**Tukey test**; multiple comparisons of means

	diff	lwr	upr	**p adj**
IV-H	-11.24	-22.02	-0.46	**0.0348825**

[%] regulatory T cells

>Kruskal Wallis test
Multiple comparison test after Kruskal-Wallis
p.value: 0.05

	obs.dif	critical.difference	
H-IIIrez	40.22	37.67	**TRUE**

[%] CD69+ NK cells

>Kruskal Wallis test
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons

	obs.dif	critical. difference	
H-IV	20.81	18.83	**TRUE**

[%] CD94+ NK cells

>Kruskal Wallis test
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons

	obs.dif	critical. difference	
H-IV	18.46	18.44	**TRUE**

[%] Hsp70 expression on cell surface

>**Kruskal Wallis test**
Multiple comparison test after Kruskal-Wallis
p.value: 0.05

	obs.	critical difference	
II-IV	12.74384	12.658	**TRUE**
III-IV	12.88669	12.658	**TRUE**

[ng/ml] Hsp70 concentration in circulation of patients with different gradings

>Anova test

	Df	Sum Sq	Mean Sq	F	**Pr(>F)**
Group	6	70.3	11.71	3.05	**0.000608**

>Tukey test; multiple comparisons of means

	diff	lwr	upr	**p adj**
IV-healthy	1.17	0.15	2.18	**0.0005289**

[ng/ml] Hsp70 concentration in circulation of patients with different tumour volume

>Kruskal Wallis test
Multiple comparison test after Kruskal-Wallis
p.value: 0.05

	obs.dif	critical difference	
healthy->90	35.22	34.71	**TRUE**

[ng/ml] Hsp70 concentration in circulation of patients with high necrosis

>Wilcoxon rank sum test (with continuity correction)
W = 646, **p-value = 0.04419**

[G/l] absolute lymphocyte counts	**>Kruskal Wallis test** Multiple comparison test after Kruskal-Wallis p.value: 0.05 obs.dif critical difference H-IIIrez 32.58 27.41 **TRUE**
[%] NK cells and progress	**>Kruskal Wallis test** Multiple comparison test after Kruskal-Wallis p.value: 0.01 obs.dif critical difference H-W 16.96 14.21 **TRUE**
Ex vivo stimulation CD69+ NK cells	**>Anova test** Df Sum Sq Mean Sq F **Pr(>F)** 2 260.0 130.01 9.261 **0.00654** **>Tukey test;** multiple comparisons of means. diff lwr upr **p adj** tkd-ctrl 9.21 1.81 16.60 **0.0172779** hsp70-ctrl 10.42 3.02 17.82 **0.0086595**
Ex vivo stimulation CD94+ NK cells	**>Anova test** Df Sum Sq Mean Sq F **Pr(>F)** 2 42.66 21.33 6.564 **0.0174** **>Tukey test;** multiple comparisons of means. diff lwr upr **p adj** tkd-ctrl 4.33 0.77 7.89 **0.0194216**

Table 15: Summary of statistical data

4. Results

In the following part, the results will be presented, which were acquired by analysing samples of patients with different types of brain tumours.

I investigated the function and role of Hsp70, well known to be overexpressed in various types of tumours, as a biomarker in brain tumour patients. Therefore, I analysed the expression of Hsp70 on the cell surface, evaluated the cytosolic Hsp70 expression by immunohistochemistry and measured its concentration in the peripheral blood. To obtain a better understanding of the effects of Hsp70 on immune surveillance, and how the Hsp70 expression can influence the composition of immune cells in brain tumour patients, I identified the absolute number of lymphocytes [G/l] and composition of single lymphocyte subpopulations [%] in the peripheral blood in our patients collective.

The number of patients, whose samples were used for the different experiments, and further characteristics, can be seen in table 16.

	grade I	**grade II**	**grade III**	**grade IV**	∑
Absolute lymphocyte counts		5/5	6/8	28/4	56
Composition of lymphocyte subpopulations		6/5	13/12	56/7	75
NK cells ~ progress				37	37
Hsp70 ~ surface		3	6	28	37
Hsp70 ~ IHC	1	3	4	17	25
Hsp70 ~ circulation		5	12	51	68
Hsp70 ~ tumour volume				44	44
Hsp70~ area of necrosis				27	27
Hsp70 ~ overall survival				34	34

Table 16: Number of patients [n] used for analysis; first diagnosis/ relapse

4.1 Hsp70 staining using immunohistochemistry

The Department of Neuropathology at rechts der Isar, TUM, provided tumour material of 25 brain tumours: 1 grade I, 3 grade II, 4 grade III and 17 grade IV tumours. The 3 µm formalin-fixed paraffin-embedded tumour sections were stained for Hsp70. After staining and incubation with the monoclonal cmHsp70.1 antibody, the evaluation was done using the software ImageScope, version 12.3.2.8013. Only tumour areas, not surrounding connective tissue was included in the evaluation. Negative control samples, being part of the staining process, were used as an internal reference. Analysis of the Hsp70 staining was done by Wu Zhiyuan and me in parallel.

Addressing the Hsp70 positive areas, two major distribution patterns could be distinguished. Some tumours were positive for Hsp70 only in the nucleus, whereas in others the nucleus and cytosol were positively stained for Hsp70. In figure 3 the different distribution patterns are shown. Lower grade tumours (grade I, II and III) only showed a nuclear staining pattern, whereas those of grade IV expressed Hsp70 in the nucleus and cytosol. (Lobinger et al., 2021)

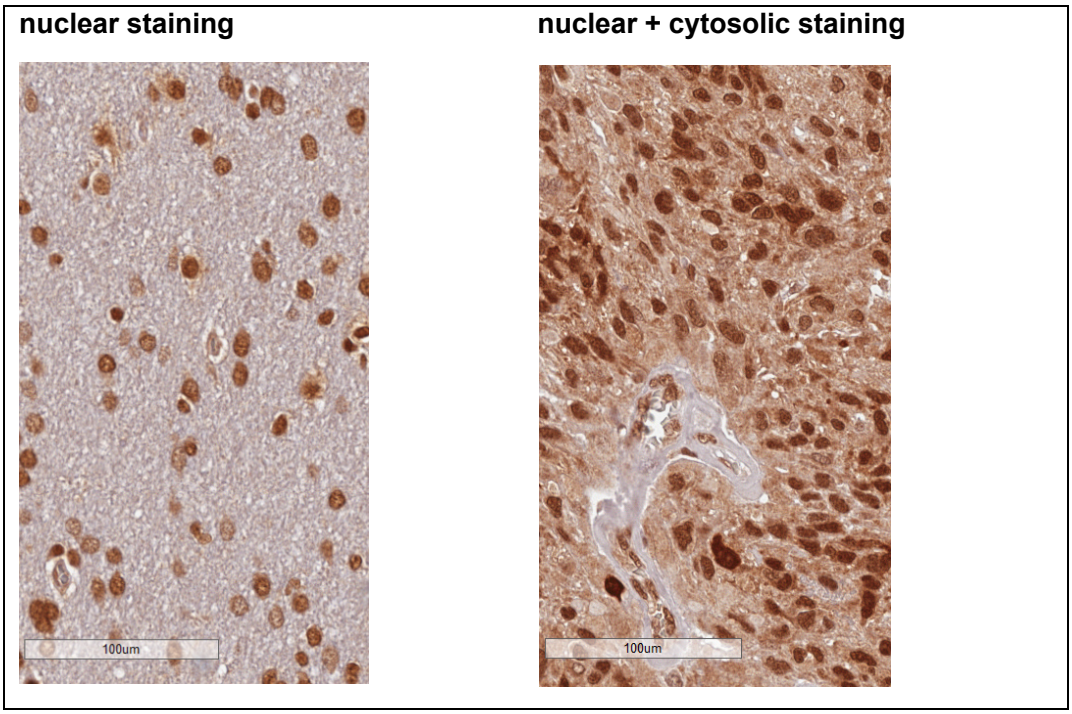

Figure 3: Distribution patterns of Hsp70 staining, nuclear (grade III tumour) and nuclear + cytosolic staining (grade IV tumour) (Lobinger et al., 2021)

The intensity of the Hsp70 staining was evaluated in the defined tumour area and classified as low (B) or as high intensity (C), blindly and independently by 2 researchers. Figure 4 shows representative pictures of tumour sections with a nuclear and figure 5 with a nuclear and cytosolic staining pattern.

Nuclear staining:

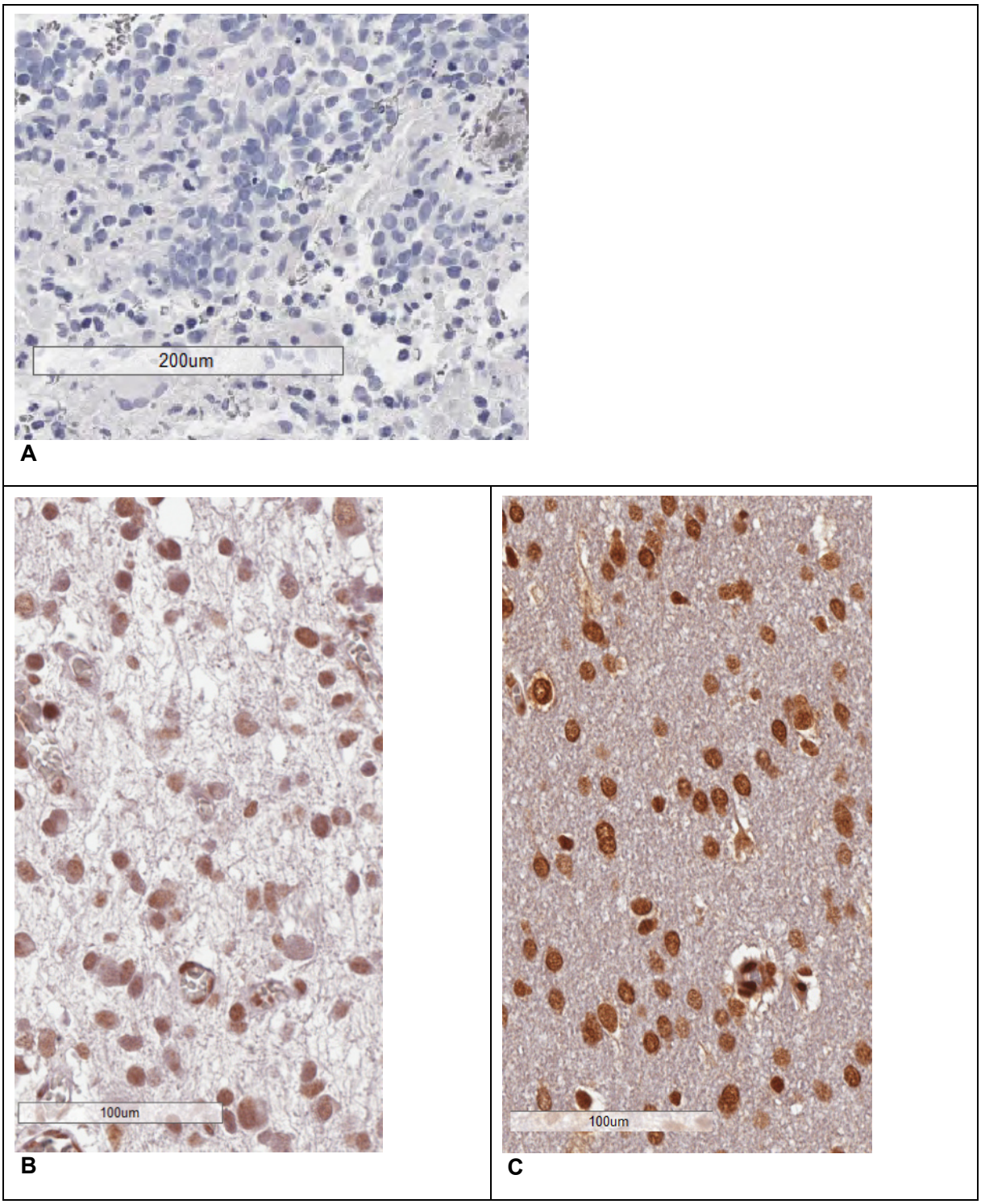

Figure 4: Nuclear staining, A: negative control, B: low intensity (grade II tumour), C: high intensity (grade II tumour) (Lobinger et al., 2021)

Nuclear plus cytosolic staining:

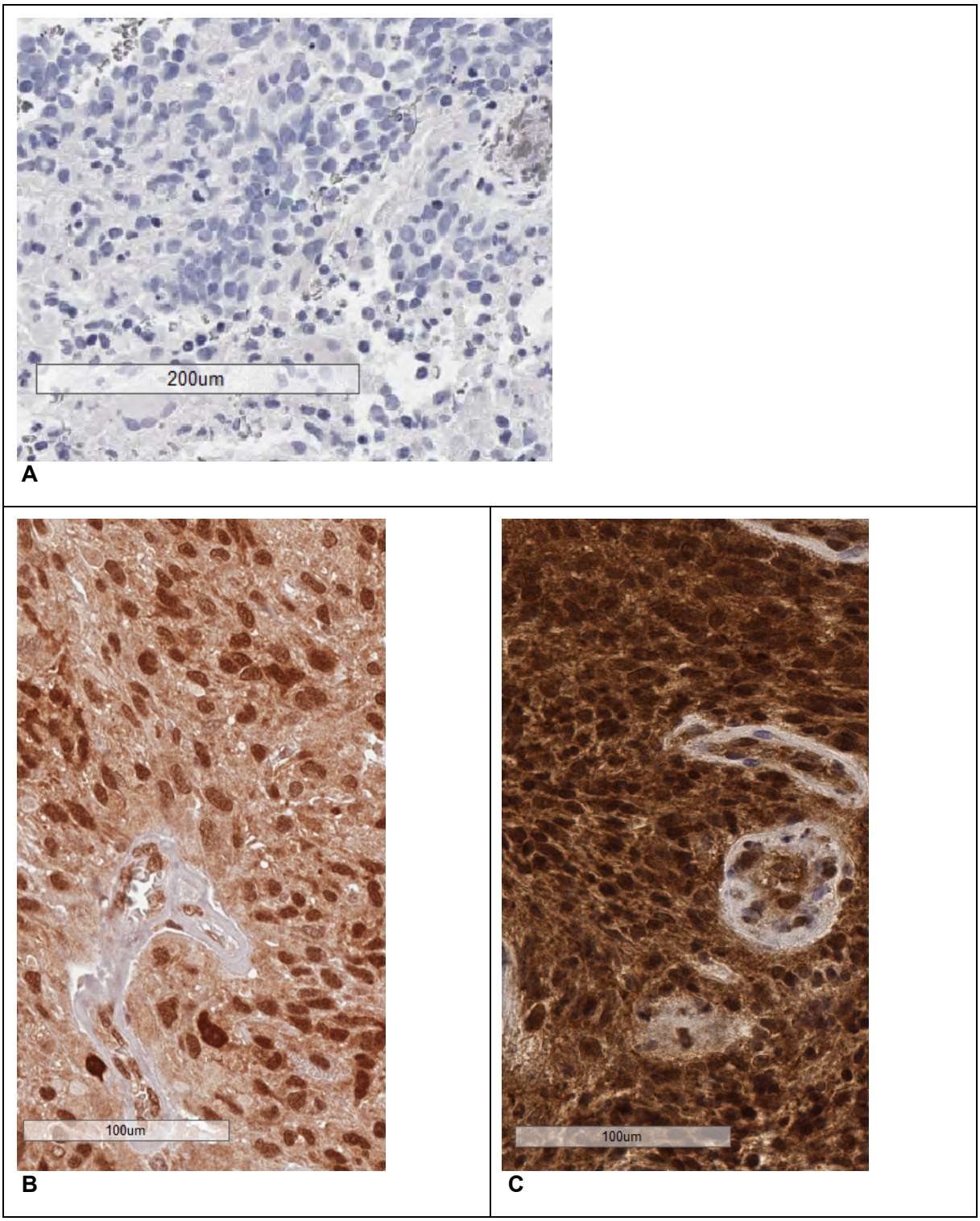

Figure 5: Nuclear plus cytosolic staining, A: negative control, B: low intensity (grade IV tumour), C: high intensity (grade IV tumour) (Lobinger et al., 2021)

Evaluating the sections based on staining intensity (low: < 50% positively stained cells in the tumour area, high: > 50% positively stained cells in the tumour area) and distribution pattern, I scored them into nuclear or nuclear plus cytosolic staining and low or high intensity.

In my findings, grade I and II tumours, including 2 sections with low and 2 with high intensity, and grade III tumours, including 1 section with low and 3 with high intensity, only showed nuclear staining. In contrast, in grade IV tumours, both distributions patterns could be found, 1 section with nuclear and 16 sections with nuclear and cytosolic Hsp70 expression. The grade IV tumour section with only nuclear expression showed a high intensity of the Hsp70 staining. Those with mixed expression pattern in nucleus and cytosol, include 5 sections with low and 11 sections with high intensity. (Lobinger et al., 2021)

grade IV glioma	**5 / 17**	**12 / 17**
grade III glioma	**1 / 4**	**3 / 4**
grade I+II glioma	**2 / 4**	**2 / 4**
	Hsp70 expression ***low (<50%)***	Hsp70 expression ***high (>50%)***

Table 17: Immunohistochemistry; Hsp70 expression of grade IV, grade III, grade II, and grade I (Lobinger et al., 2021)

Table 17 shows the analysis of the Hsp70 expression in tumour sections of lower grade glioma, including grade I, II and III, in contrast to sections of high-grade glioma, including grade IV. In total, 17 samples of grade IV gliomas were stained, 5 with low and 12 with high expression of Hsp70. Among grade III glioma, there were 3 sections with high and 1 with low Hsp70 expression. Grade I and II glioma showed equal amounts of sections with high and low Hsp70 staining. (Lobinger et al., 2021)

In table 18, the different distribution patterns of Hsp70 expression in high-grade and lower grade glioma can be seen. Whereas in high-grade glioma 16 out of 17 slides expressed Hsp70 in nucleus plus cytosol, in grade I, II and III glioma the nuclear expression is dominating. (Lobinger et al., 2021)

grade IV glioma	**1 / 17**	**16 / 17**
grade III glioma	**4 / 4**	**0 / 4**
grade I+II glioma	**4 / 4**	**0 / 4**
	Hsp70 expression ***nuclear***	Hsp70 expression ***nuclear + cytosolic***

Table 18: Immunohistochemistry; Hsp70 expression pattern of grade IV, grade III, grade II, and grade I (Lobinger et al., 2021)

The results of the immunohistochemistry staining intensity and distribution pattern are summarized in table 19.

Hsp70 expression	**nuclear**		**nuclear plus cytosolic**	
	low	*high*	*low*	*high*
grade I / II	2	2	/	/
grade III	1	3	/	/
grade IV	/	1	5	11

Table 19: Immunohistochemistry staining for Hsp70 (Lobinger et al., 2021)

4.2 Expression of Hsp70 on the surface of tumour cells

In collaboration with the Department of Neurosurgery, I received fresh tumour material of different types of brain tumours. After surgery, the samples were cooled down on ice and analysed within a few hours to keep as much tumour cells as possible viable. To determine the amount of Hsp70, expressed on the surface of tumour cells of grade II, grade III and grade IV patients, flow cytometry was performed by different members of our laboratory, including me.

Figure 6 shows the percentage of Hsp70 expression on the membrane of 3 grade II, 6 grade III and 28 grade IV tumours at first diagnosis. In my results the different gradings, without differing statistically significant from each other, did all show a positive percentage above 60% in average. (Lobinger et al., 2021)

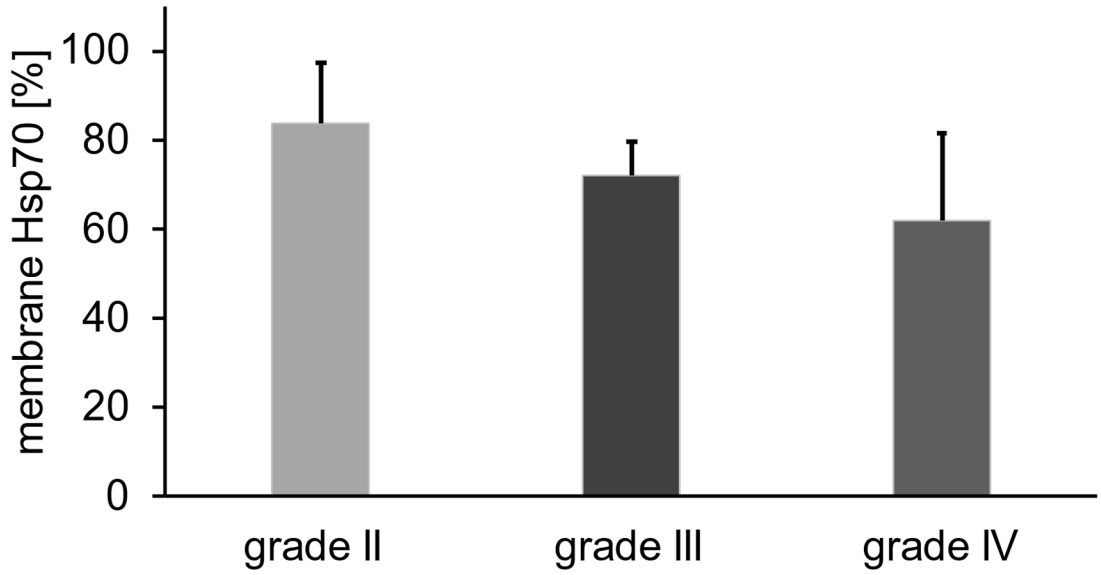

Figure 6: Percentage of Hsp70 expression on the membrane of tumour cells, patients at first diagnosis (Lobinger et al., 2021)

4.3 Hsp70 concentrations in the circulation of patients with brain tumours

4.3.1 Hsp70 serum levels in patients with different tumour grading

To investigate the role of circulating Hsp70 as a potential biomarker in patients with different types of brain tumours, the concentration of Hsp70 in the peripheral blood of 68 patients with different grading was analysed, using an Hsp70 ELISA technique.

Together with the data provided by Sven Schmidt, the patient collective included 5 grade II, 12 grade III and 51 grade IV patients at first diagnosis. The serum levels were compared to 150 healthy donors. The findings revealed that patients with grade IV brain tumours at diagnosis showed significantly higher Hsp70 mean serum levels (4.16 +/- 2.72 ng/ml, median of 3.48 ng/ml) in the circulation than a control collective with mean serum levels of 2.99 +/- 1.49 ng/ml and a median of 2.6 ng/ml, as calculated with Tukey Test ($p < 0.05$). Grade II and grade III patients with mean serum Hsp70 concentrations of 4.39 +/- 3.2 ng/ml (median of 3.14 ng/ml) and 3.75 +/- 1.85 ng/ml (median of 3.34 ng/ml), respectively, did not differ significantly from each other or from that of healthy control donors. (Lobinger et al., 2021) Figure 7 shows the serum levels of circulating Hsp70 in patients with brain tumours at first diagnosis with different gradings compared to healthy donors.

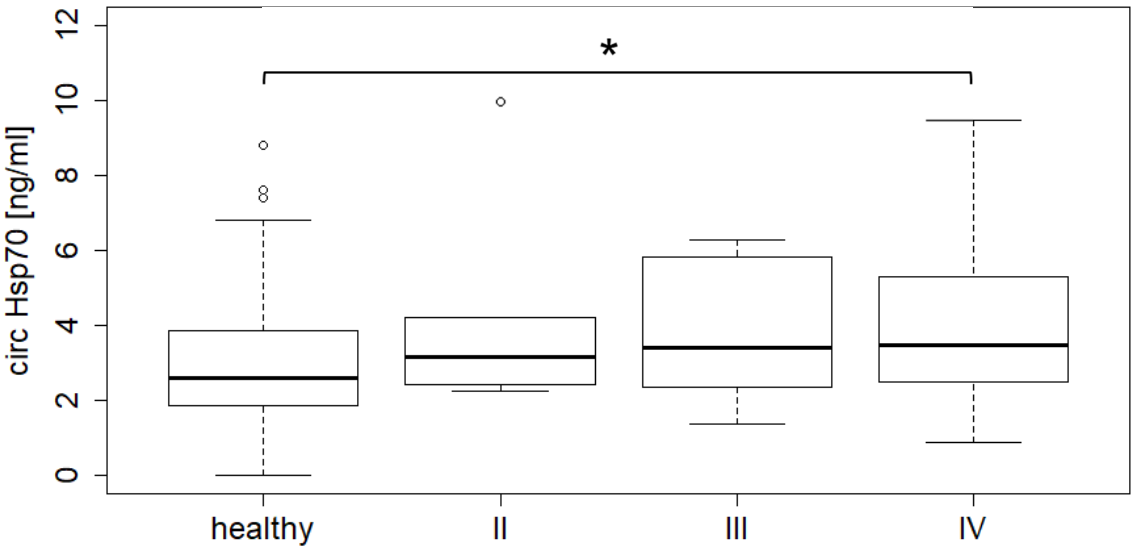

Figure 7: Hsp70 concentrations [ng/ml] in circulation of patients with different gradings at diagnosis (Lobinger et al., 2021)

4.3.2 Hsp70 serum levels in patients with different tumour volume

In mouse models increasing tumour volume resulted in higher concentrations of Hsp70 in circulation. (Bayer et al., 2014) Furthermore, also in patients with solid tumours like non-small-cell lung carcinoma (NSCLC), higher Hsp70 serum levels could be correlated with a higher tumour volume. (Gunther et al., 2015)

To investigate the effect of the tumour volume of brain tumours on the concentration of circulating Hsp70, Isabelle Riederer, a radiologist at Klinium rechts der Isar, determined the tumour volume of 44 grade IV patients at first diagnosis using magnet resonance imaging (MRI).

The patients were matched into groups with small (< 30 cm^3), medium (30 - 90 cm^3) and large (> 90 cm^3) tumour volume. The group with small volume consisted of 6 patients with a median tumour volume of 9.2 cm^3 and a range of 4.8 to 26 cm^3, those with a medium volume (16 patients) had a median of 71.7 cm^3 and ranged between 35.,3 – 81.8 cm^3. Finally, 22 patients with a median tumour volume of 140.9 cm^3 and a range of 99 – 222 cm^3 formed the group of patients with large tumour volumes. Details about tumour volume are summarized in table 20.

	small (<30 cm^3)	**medium (30-90 cm^3)**	**large (>90 cm^3)**
patients [n]	6	16	22
median [cm^3]	9.2	71.7	140.9
range [cm^3]	4.8 - 26	35.3 – 81.8	99 – 222

Table 20: Tumour volume of 43 patients with grade IV brain tumours (Lobinger et al., 2021)

Patients with large tumour volumes (mean serum levels of 4.34 +/- 3.18 ng/ml, median of 3.78 ng/ml) had significant higher concentrations of Hsp70 in the circulation than the 150 healthy donors (mean serum levels of 2.99 +/- 1.49 ng/ml, median of 2.6 ng/ml), calculated using Kruskal Wallis test with value of $p < 0.05$. Those with small (mean serum levels of 3.58 +/- 2.13 ng/ml, median of 2.89 ng/ml) and medium volume (mean serum levels of 3.66 +/- 1.92 ng/ml, median of 3.39 ng/ml) did not differ significantly. (Lobinger et al., 2021) The results are summarized in figure 8.

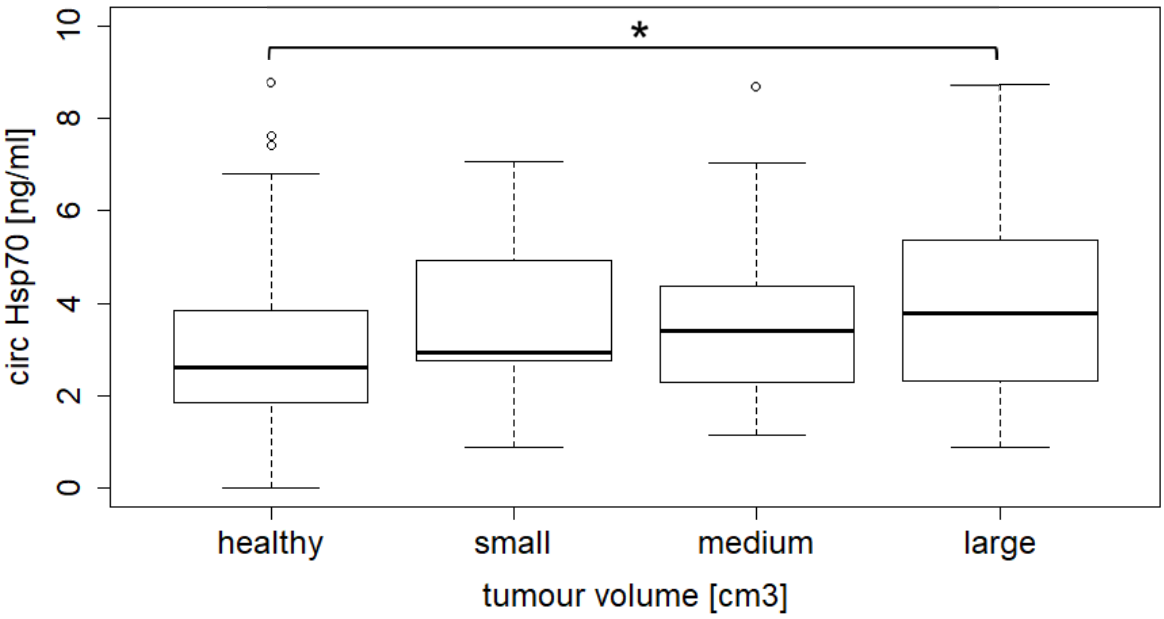

Figure 8: Tumour volume and Hsp70 concentration in circulation of grade IV patients at diagnosis (Lobinger et al., 2021)

4.3.3 The influence of necrosis in brain tumours on Hsp70 serum concentrations

The get a closer look how necrosis in brain tumours affects the concentration of Hsp70 in the circulation, Isabelle Riederer, radiologist at Klinikum rechts der Isar, TUM, defined the area of necrosis and tumour volume in 27 grade IV patients at first diagnosis, using magnet resonance imaging. I analysed the correlation of serum Hsp70, as measured by ELISA, and the proportion of necrosis in the tumours. Therefore, the patients were scored into 2 groups, one with high percentage (> 10%) and one with low percentage (< 10%) of necrosis in the tumour area. (Lobinger et al., 2021) The results are shown in figure 9.

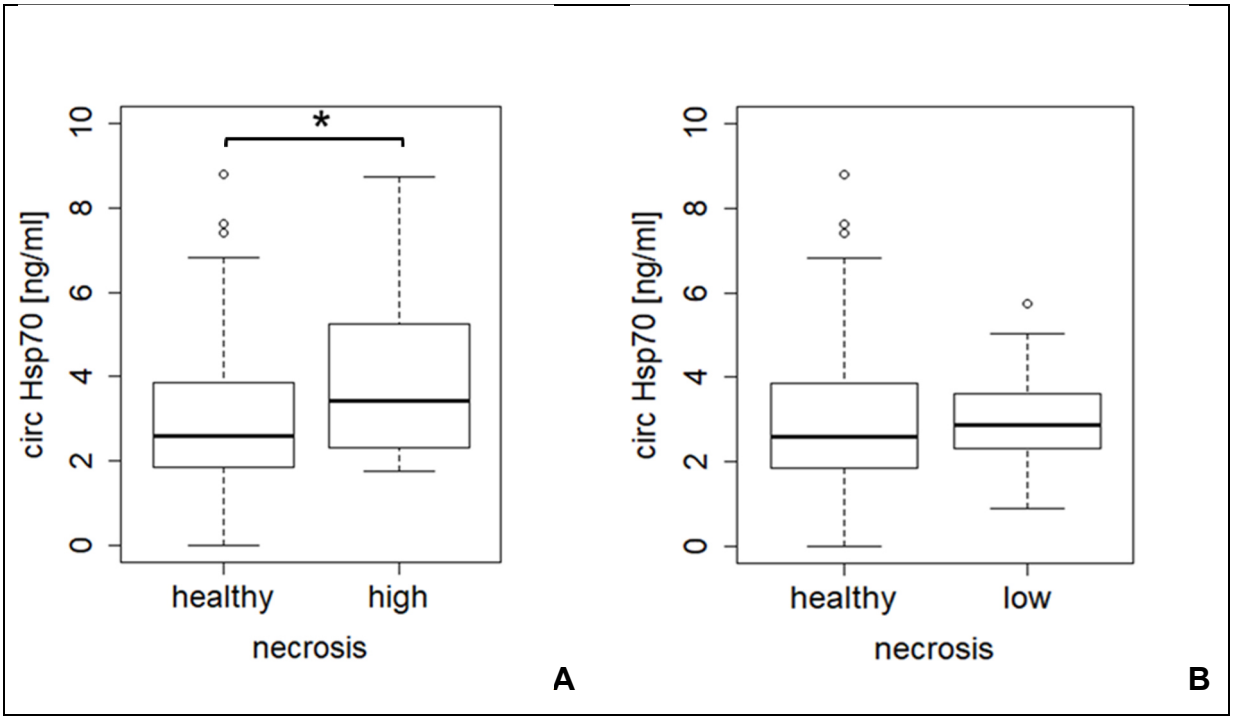

Figure 9 Correlation of necrosis in brain tumours and circulating Hsp70 concentrations in grade IV patients at diagnosis; A: > 10% necrosis; B: < 10% necrosis. (Lobinger et al., 2021)

According to my findings 13 patients with high percentage of necrosis (> 10%) showed significant higher concentrations of Hsp70 in the circulation (mean serum levels of 4.31 +/- 2.41 ng/ml, median of 3.42 ng/ml) than the control collective (mean serum levels of 2.99 +/- 1.49 ng/ml, median of 2.6 ng/ml), as calculated using Wilcoxon rank Test ($p < 0.05$). For the patient collective with low percentage of necrosis (< 10%), including 14 patients, no significant differences in the concentration of Hsp70 (mean serum levels of 3.02 +/- 1.43 ng/ml, median of 2.87 ng/ml) were observed compared to healthy donors. (Lobinger et al., 2021)

4.3.4 Kaplan-Meier analysis

Kaplan-Meier analysis of 34 patients with glioblastoma, treated with Radio chemotherapy after surgery according to Stupp-regimen, was done. (Stupp et al., 2005) Patients with a concentration of Hsp70 in the circulation at diagnosis above 3.5 ng/ml (n=16) tend to lower survival probability than those with concentrations below 3.5 ng/ml (n=18), although not statistically significant, as shown in figure 10. (Lobinger et al., 2021)

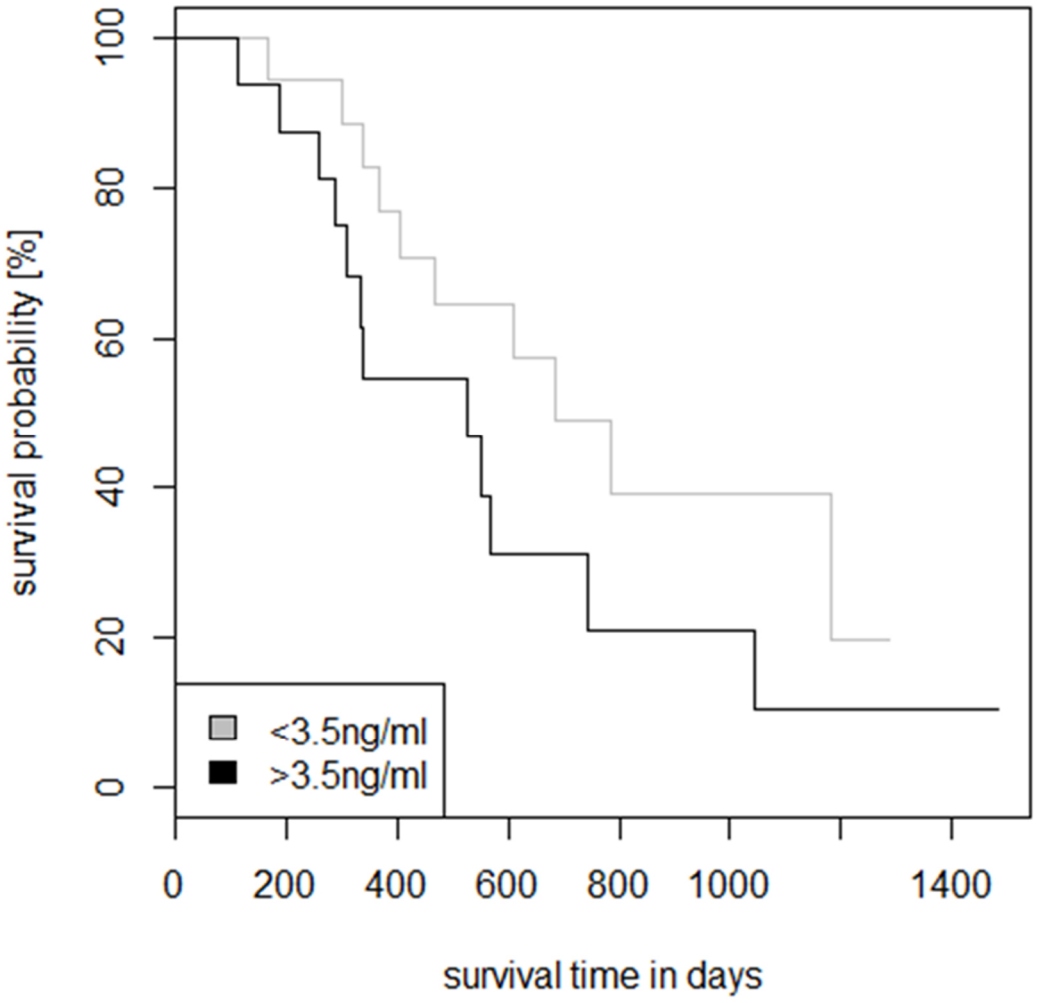

Figure 10: Kaplan-Meier analysis of circ. Hsp70 and survival probability (Lobinger et al., 2021)

4.4 Absolute number of lymphocytes

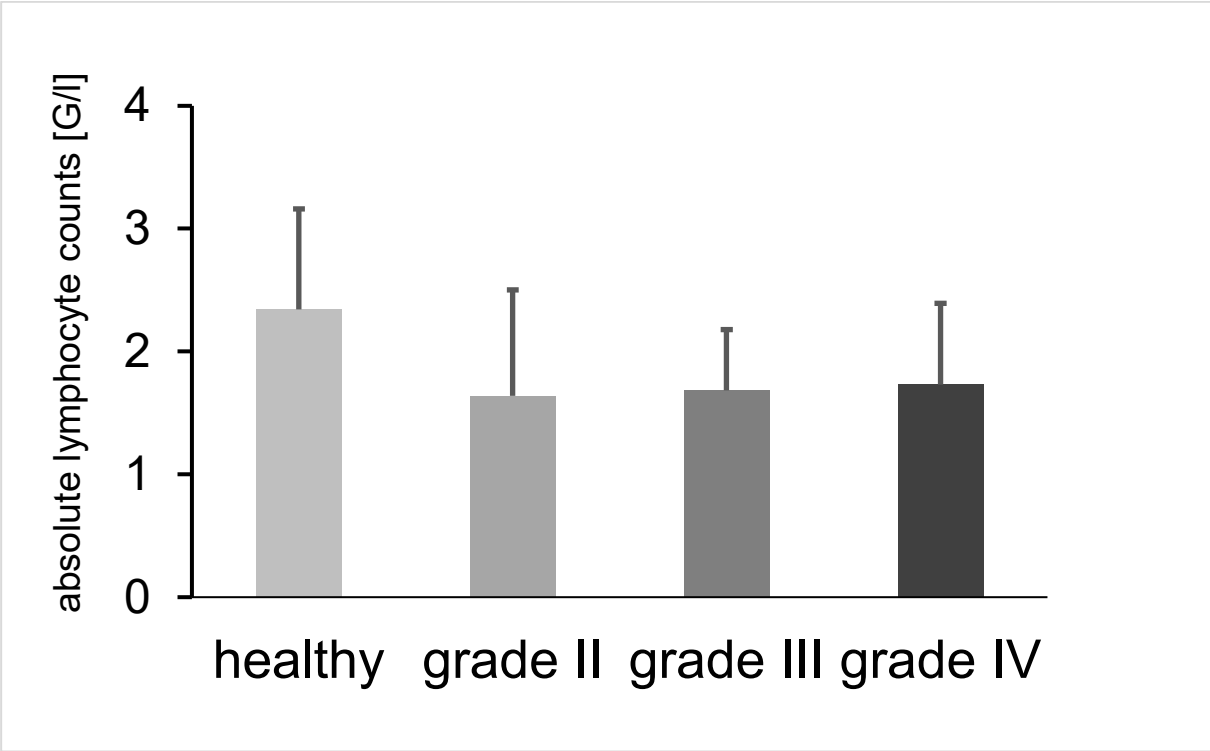

Figure 11: Absolute numbers of lymphocytes in G/l, patients at first diagnosis

Absolute number of lymphocytes, counted after density gradient centrifugation with Ficoll, was determined in 39 patients with different types of brain tumours at first diagnosis. The collective consisted out of 5 grade II, 6 grade III and 28 grade IV patients at diagnosis. Shown in figure 11, compared to healthy donors (2.34 +/- 0.82 G/l), grade II (1.64 +/-0.86 G/l), grade III (1.68 +/- 0.50 G/l) and grade IV patients (1.73 +/- 0.66 G/l) showed a smaller number of absolute lymphocytes, although not significant.

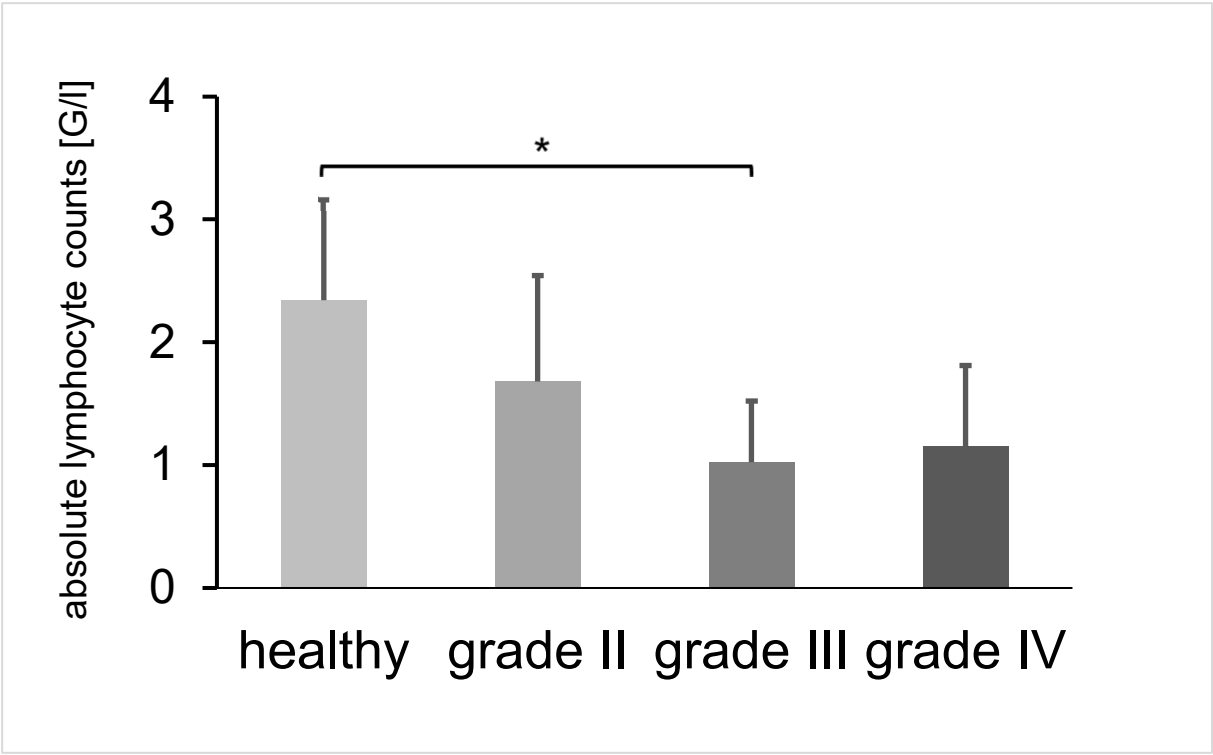

Figure 12: Absolute numbers of lymphocytes in G/l, patients with relapse

Apart from patients at first diagnosis, absolute lymphocytes were counted also in 17 patients with brain tumour recurrence. Presented in figure 12, 5 grade II (1.68 +/- 1.00 G/l), 8 grade III (1.03 +/- 0.61 G/l) and 4 grade IV patients (1.15 +/- 0.66 G/l) with relapse, showed smaller numbers of absolute lymphocytes than the control collective. Compared to healthy donors, grade III relapse patients had significant lower numbers of lymphocytes ($p < 0.05$), as calculated by the Kruskal Wallis test. The control collective consisted out of 12 healthy individuals.

4.5 Composition of lymphocyte subpopulations

With flow cytometry the percentage of key lymphocyte subpopulations was measured. Beside B cells, several T cell populations and NK cells plus NK cell activation markers were analysed and compared to a healthy control collective of 16 volunteers. (Lobinger et al., 2021)

4.5.1 B cells

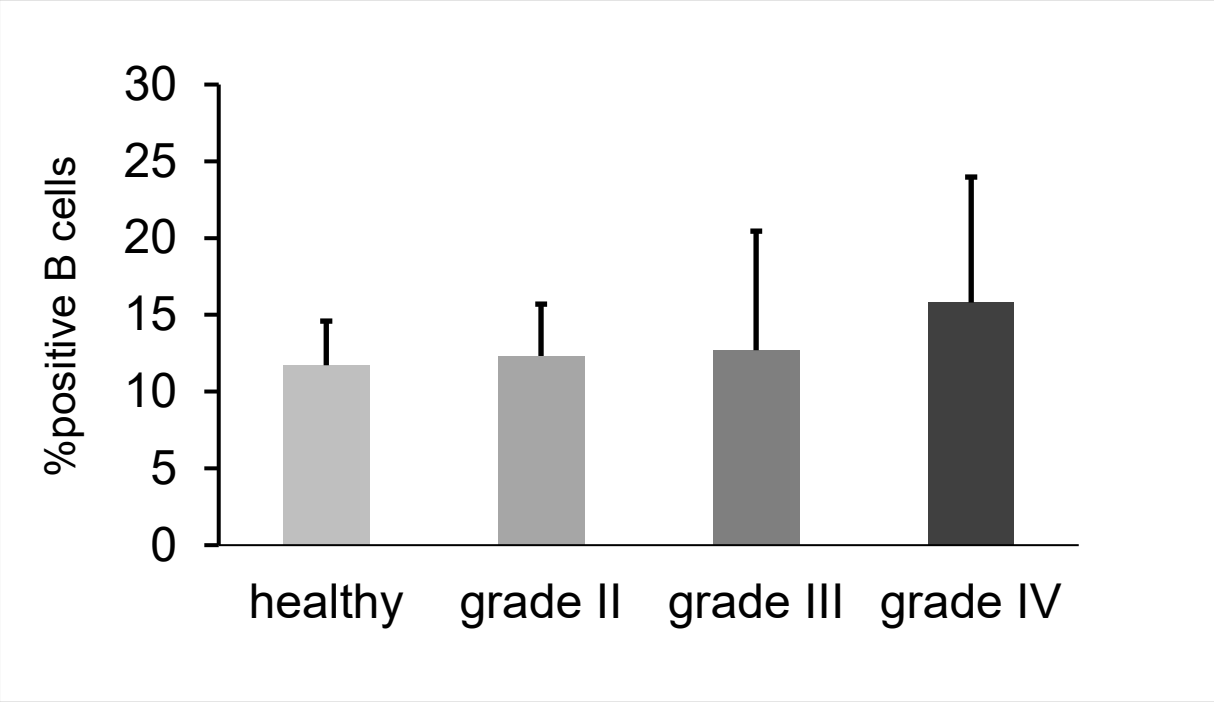

Figure 13: Percentage of CD19 positive B cells, patients at first diagnosis (Lobinger et al., 2021)

The percentage of CD19 positive B cells was measured by flow cytometry in EDTA blood of 74 patients at first diagnosis, including 5 grade II, 13 grade III and 56 grade IV patients. There was no major difference between tumour patients and control collective, apart from slightly higher percentages of B cells in grade IV patients, although not significant. The results are summarized in figure 13. For patients with brain tumour recurrence, not shown in the figure, there was no significant difference. (Lobinger et al., 2021)

4.5.2 T cells

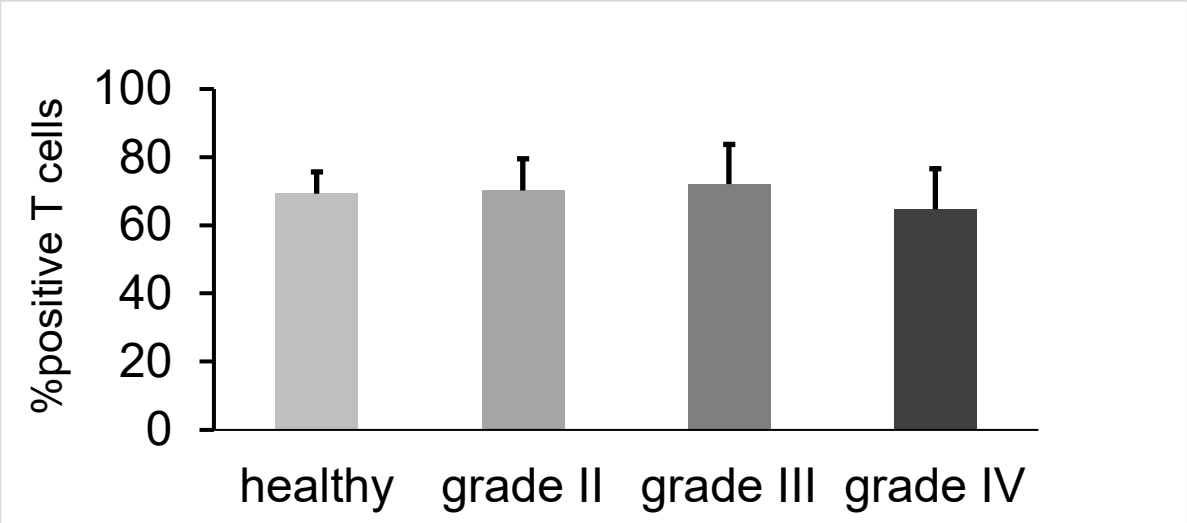

Figure 14: Percentage of CD3 positive T cells, patients at first diagnosis (Lobinger et al., 2021)

The percentage of CD3 positive T cells was determined in 75 patients at first diagnosis, including 6 grade II,13 grade III and 56 grade IV patients. Shown in figure 14 there was no significant difference compared to each other and the healthy individuals, apart from slightly lower percentage in grade IV patients. (Lobinger et al., 2021)

CD8 positive T cells (figure 15) were measured in 6 grade II, 13 grade III and 54 grade IV patients at first diagnosis, but showed no major difference compared to the control collective. (Lobinger et al., 2021)

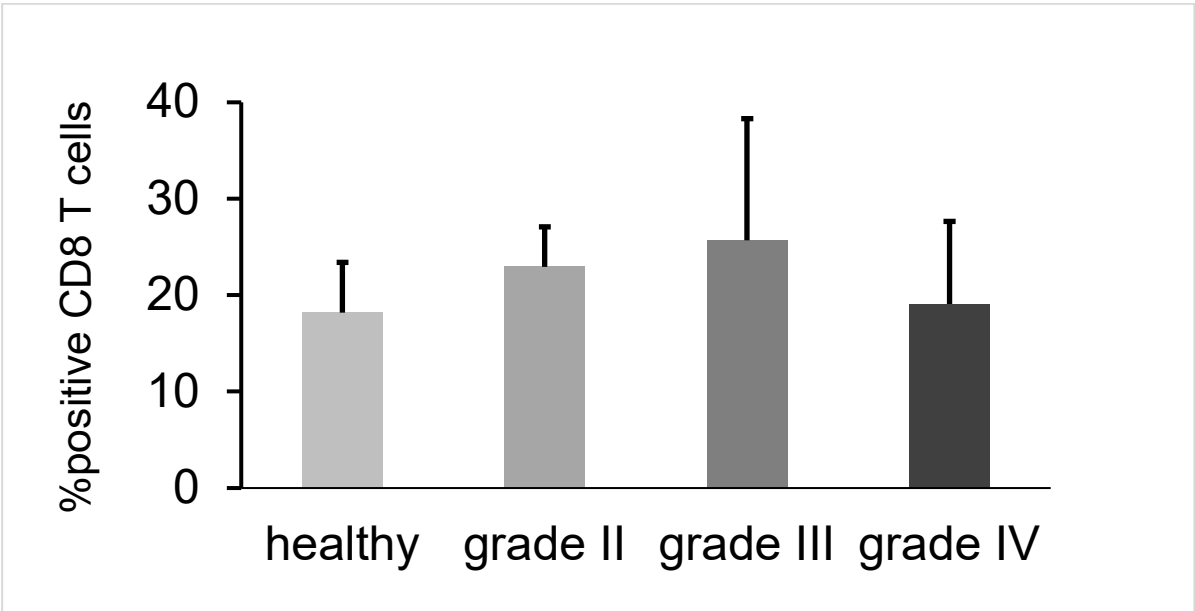

Figure 15: Percentage of CD8 positive T cells, patients at first diagnosis (Lobinger et al., 2021)

CD4 positive T cells in grade IV patients at first diagnosis, as well as those with relapse, had significant lower percentages than healthy donors, as calculated with Anova and post-hoc Tukey test ($p < 0.05$). (Lobinger et al., 2021) As shown in figure 16, CD4 positive T cells, were analysed in 6 grade II, 13 grade III and 54 grade IV patients at first diagnosis.

T cell subpopulations determined in patients with brain tumour recurrence did not differ significantly from those at first diagnosis.

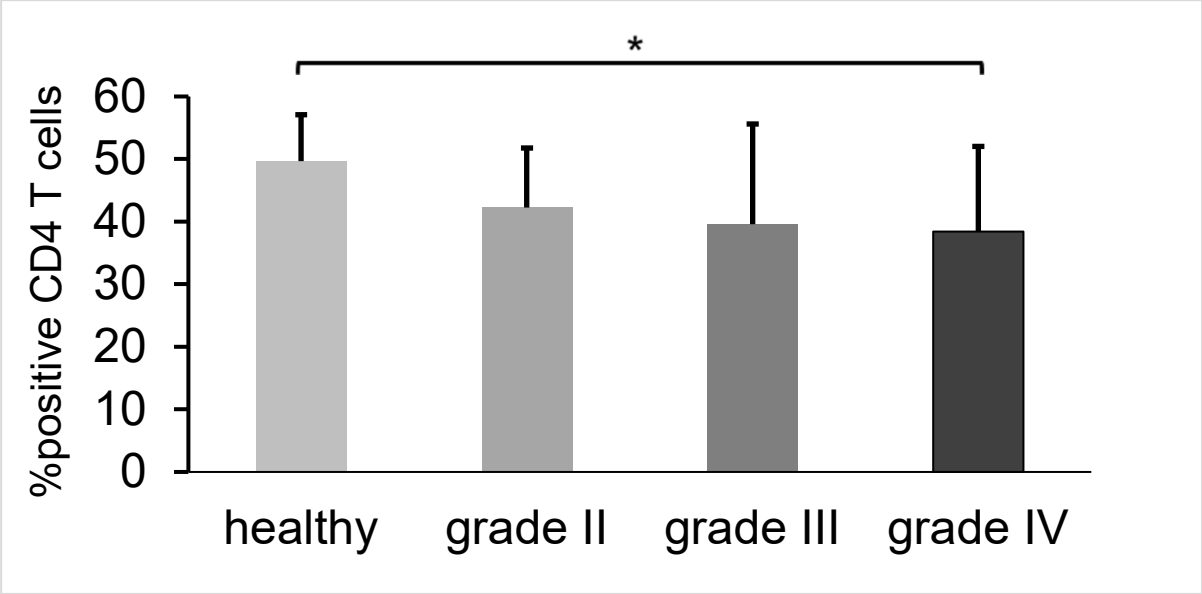

Figure 16: Percentage of CD4 positive T cells, patients at first diagnosis (Lobinger et al., 2021)

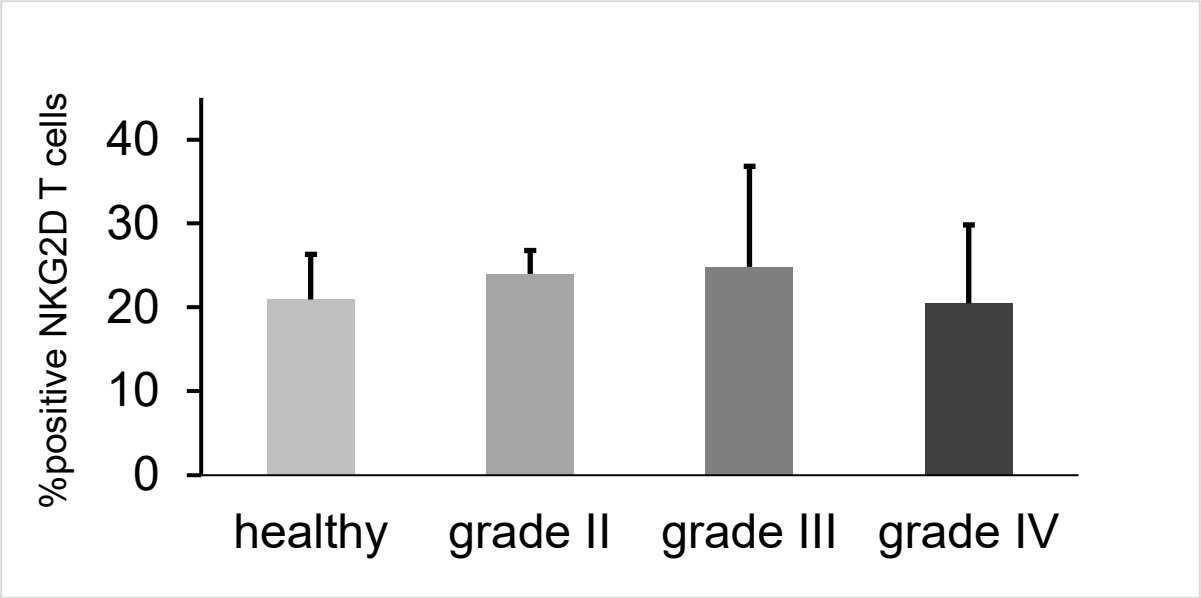

Figure 17: Percentage of NKG2D/CD3 positive T cells, patients at first diagnosis

NKG2D positive T cells were identified in 5 grade II, 13 grade III and 55 grade IV patients at diagnosis. There were no significant differences between different tumour gradings and the healthy control collective, as shown in figure 17. Also, for patients with brain tumour recurrence, there were no significant differences. (Lobinger et al., 2021)

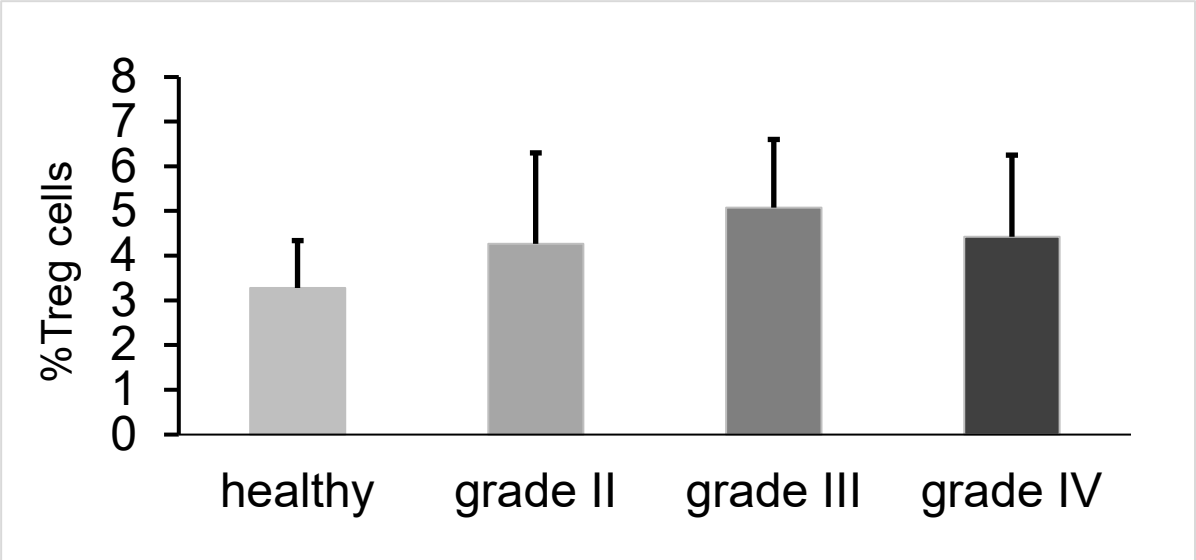

Figure 18: Percentage of CD4/CD25 positive regulatory T cells, patients at first diagnosis

Regulatory T cells, positive for CD4/CD25/FoxP3, were measured in 72 patients with first diagnosis, including 6 grade II, 13 grade III and 53 grade IV patients. Compared to the healthy control collective, the patients of all different gradings showed higher percentage of regulatory T cells in their peripheral blood. The data are shown in figure 18. The percentage of regulatory T cells in patients with brain tumour recurrence showed higher values than those at first diagnosis. Grade III patients with a brain tumour recurrence had significant higher percentage compared to the healthy control collective, as calculated with Kruskal Wallis ($p < 0.05$). The percentage of 5 grade II, 12 grade III and 7 grade IV patients with relapse are shown in figure 19. (Lobinger et al., 2021)

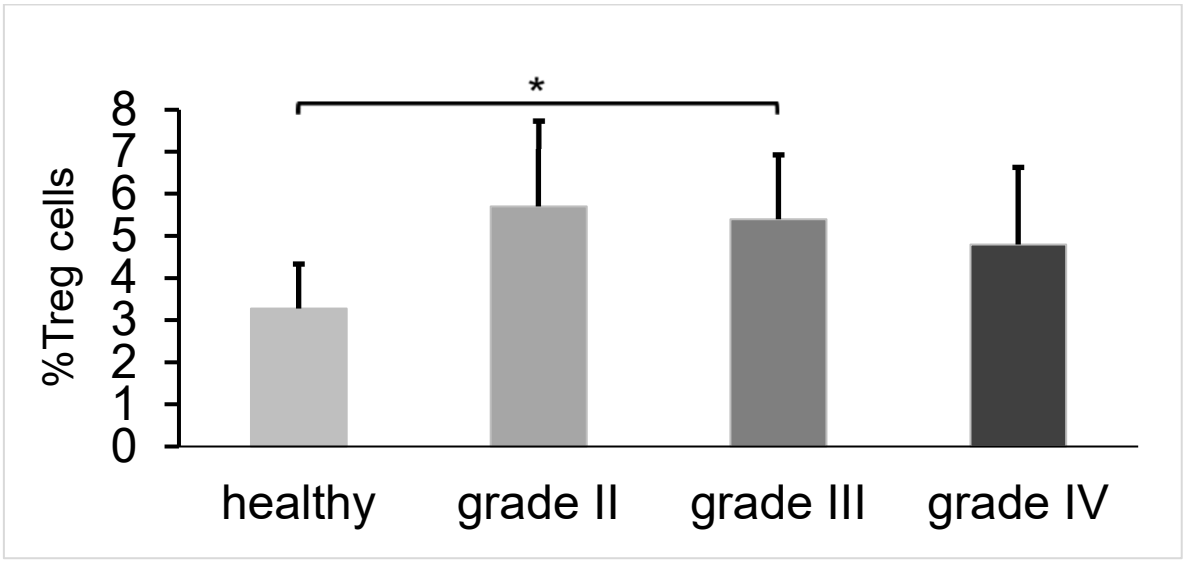

Figure 19: Percentage of CD4/CD25 positive regulatory T cells, patients with relapse

4.5.3 NK cells

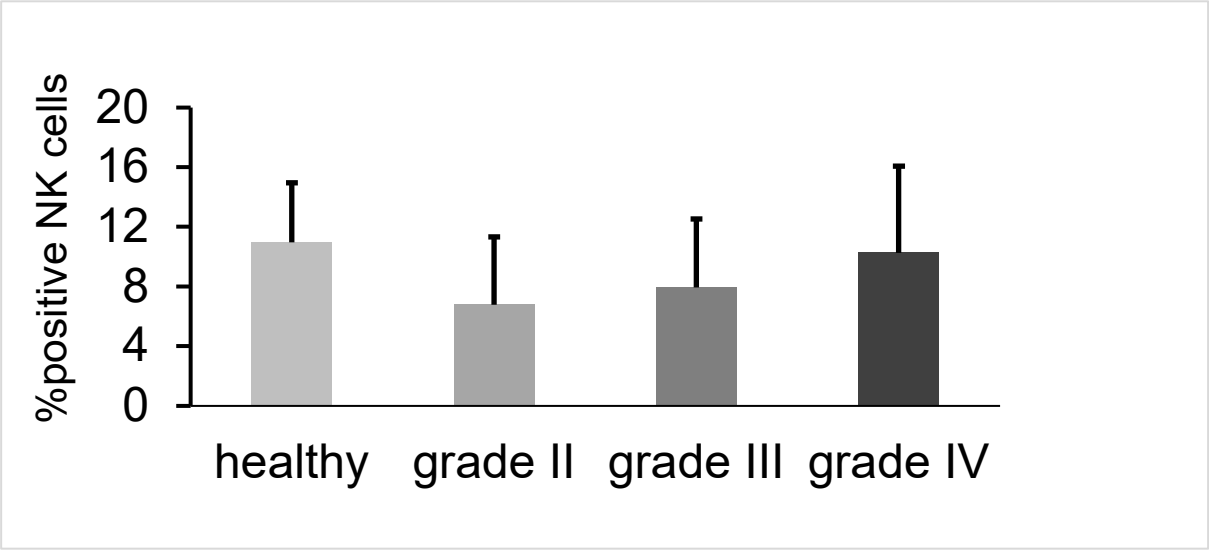

Figure 20: Percentage of CD56 positive/ CD3 negative NK cells, patients at first diagnosis

CD56 positive NK cells were measured in 5 grade II, 13 grade III and 56 grade IV patients. Compared to the healthy control collective, there is no significant difference in the percentage of CD56 positive NK cells. However, as shown in figure 20, a trend is visible: with higher tumour grading the percentage of NK cells appear to increase. A similar trend can also be seen for other NK cell activation markers, like CD94, Nkp30, Nkp46, NKG2D and CD69. (Lobinger et al., 2021)

CD94 positive NK cells, as shown in figure 21, in grade IV patients significantly higher than in healthy donors, calculated with Kruskal Wallis test ($p < 0.05$), were analysed at 5 grade II, 13 grade III, 55 grade IV patients. (Lobinger et al., 2021)

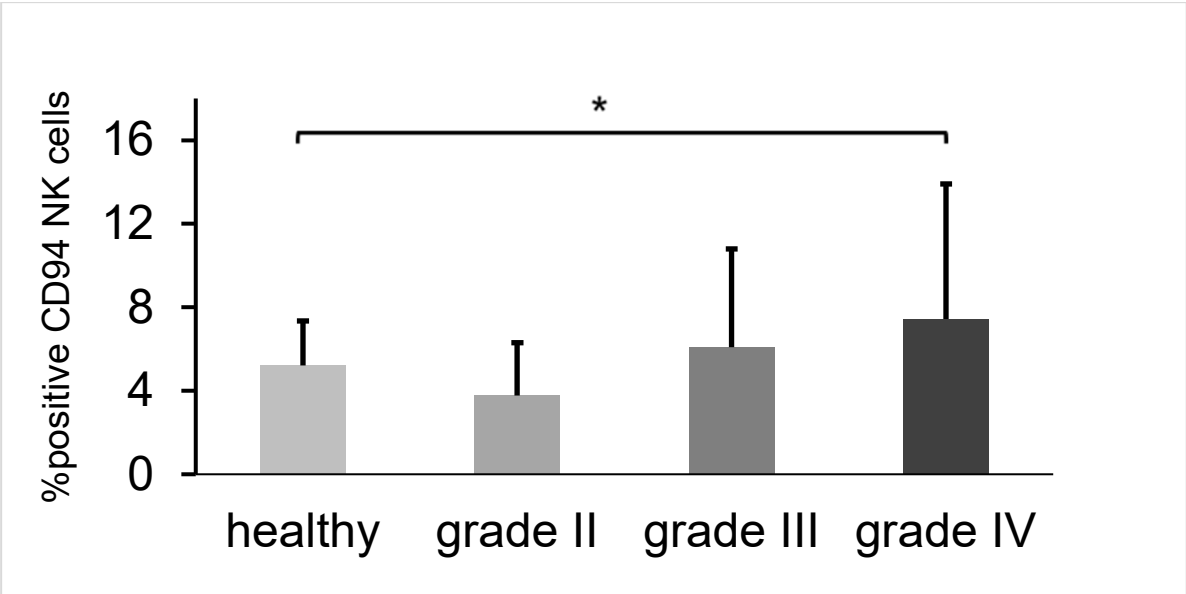

Figure 21: Percentage of CD94 positive NK cells, patients at first diagnosis (Lobinger et al., 2021)

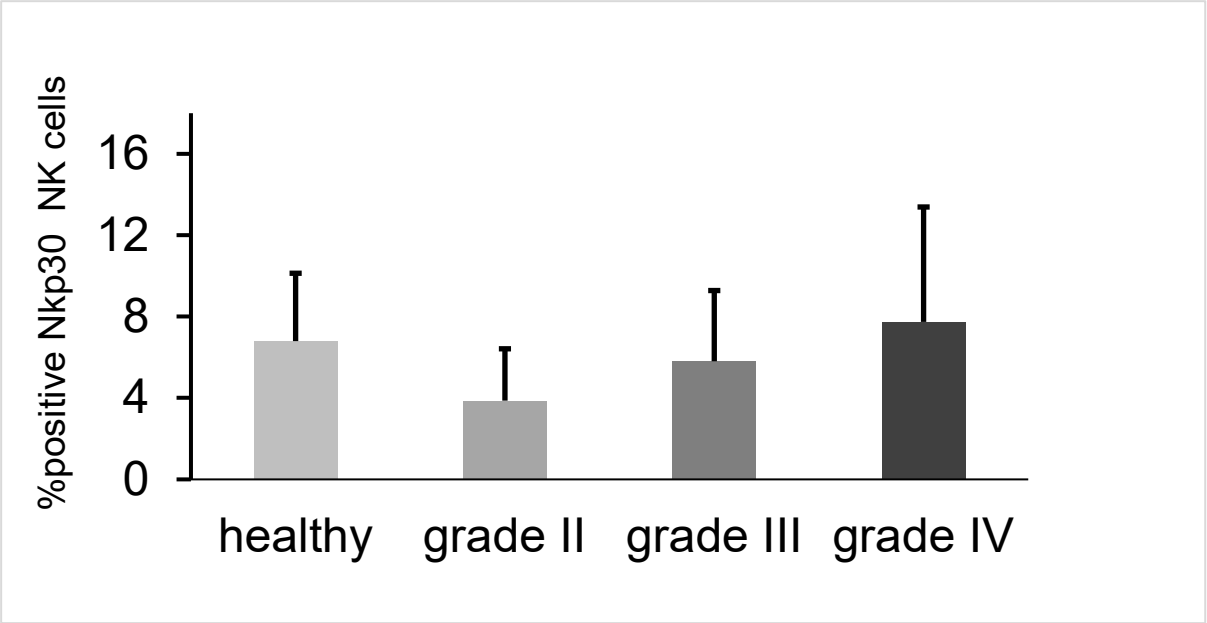

Figure 22: Percentage of Nkp46 positive NK cells, patients at first diagnosis

Nkp30 positive and Nkp46 positive NK cells were measured at 6 grade II, 13 grade III and 55 grade IV patients at diagnosis. For both markers, rising percentage of activated NK cells with higher tumour grading can be seen, shown in figure 22 and 23. (Lobinger et al., 2021)

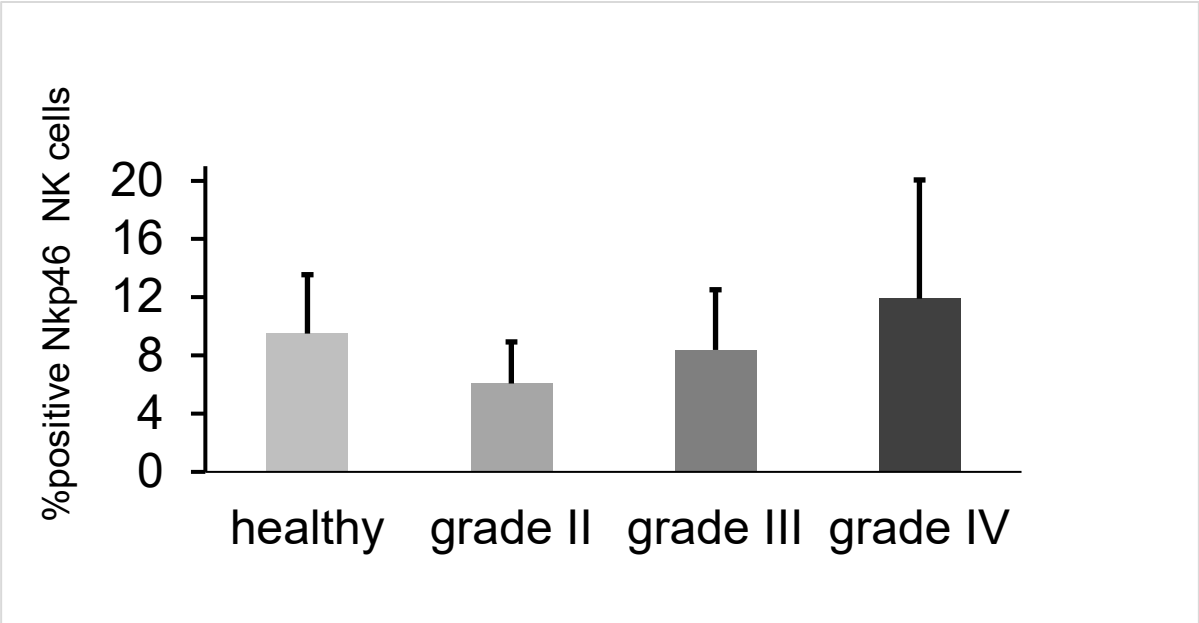

Figure 23: Percentage of Nkp46 positive NK cells, patients at first diagnosis

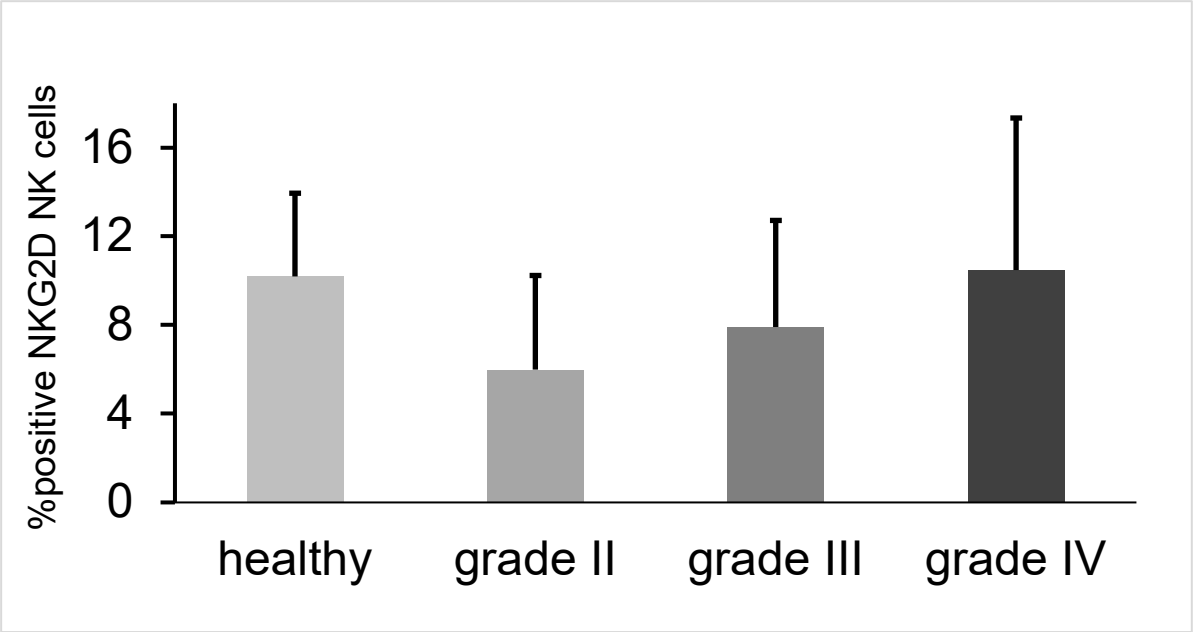

Figure 24: Percentage of NKG2D positive NK cells, patients at first diagnosis

NK cells, positive for NKG2D, were measured in 5 grade II, 13 grade III and 55 grade IV patients at diagnosis, as shown in figure 24. Although not significant, patients with higher tumour grading, showed elevated percentages of NKG2D positive NK cells. CD69 positive NK cells, analysed in 5 grade II, 13 grade III and 53 grade IV patients at diagnosis, are represented in figure 25. The percentage of CD69 NK cells in grade IV patients is significantly higher than in healthy donors, calculated with Kruskal Wallis test ($p < 0.05$). (Lobinger et al., 2021)

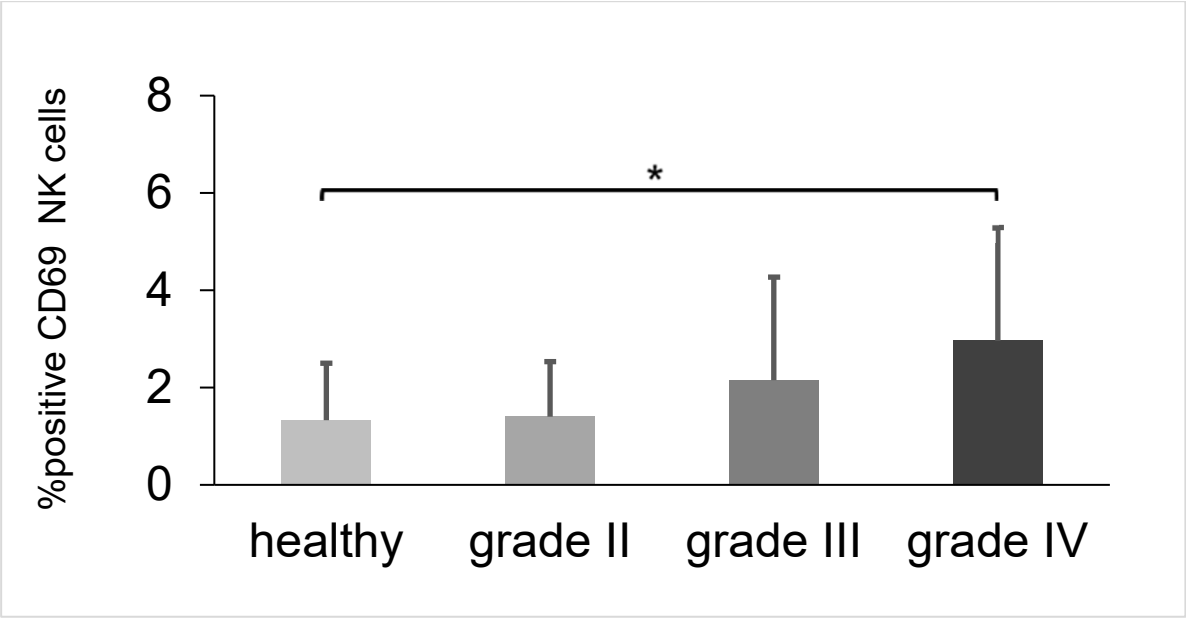

Figure 25: Percentage of CD69 positive NK cells, patients at first diagnosis (Lobinger et al., 2021)

4.5.4 NK cells and progress

The percentage of NK cell subpopulations were determined in glioblastoma patients with (n=10) and without (n=27) progress in the first 6 months after diagnosis, treated with surgery and Radio chemotherapy according to Stupp-regimen and compared to healthy donors (n=15). (Stupp et al., 2005) Shown in figure 26, CD56+ and CD94+ NK cells did not differ significantly from each other and healthy donors. Although, CD56+ and CD94+ NK cells were slightly higher in patients without progress (11.90+/-8.86 % and 7.09+/-5.62 %) compared to healthy donors (10.97+/-4-00 % and 5.22+/-2.13 %) and to patients with progress (9.67+/-5.03 % and 6.89+/-5.21 %). CD69+ NK cells were significantly elevated in patients without progress compared to healthy donors (3.82+/-3.34% vs. 1.33+/-1.17%), calculated with Kruskal Wallis test ($p < 0.01$), and slightly higher than in patients with progress (2.47+/-1.41 %). (Lobinger et al., 2021)

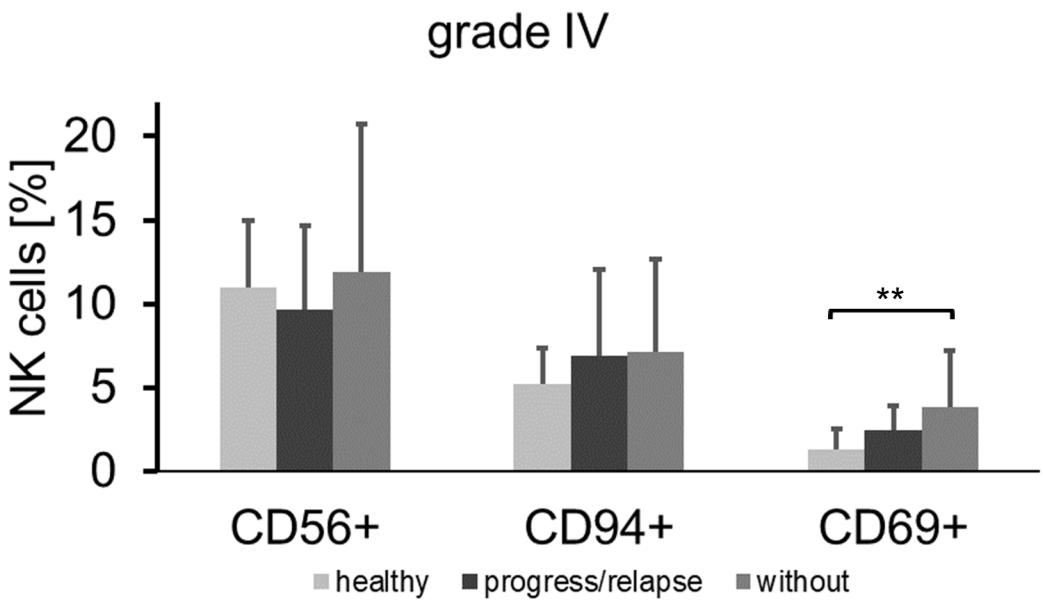

Figure 26: Percentage of NK cells in grade IV patients with and without progress 6 months after diagnosis (Lobinger et al., 2021)

4.6 Ex vivo Stimulation with Hsp70 and TKD

Hsp70 and the 14-mer peptide TKD in the presence of IL-2 can enhance the cytolytic activity and function of NK cells against Hsp70 positive tumours. (Multhoff et al., 1997) (Gastpar et al., 2004) Therefore, to demonstrate the capability of Hsp70 and TKD to activate NK cells, I stimulated peripheral blood lymphocytes ex vivo and measured the percentage of certain NK cells populations subsequently.

After isolating peripheral blood lymphocytes using Ficoll density gradient centrifugation, 5 - 8 x 10^6 cells were either incubated with Hsp70 plus IL-2 or TKD plus IL-2 (100 IU IL-2/ml). On day 5, flow cytometry was performed to analyse the percentage of different lymphocyte subpopulations. In the composition of B cells and T cells, such as CD4 positive, CD8 positive or NKG2D positive, there was no major difference between stimulated lymphocytes and unstimulated cells. (Lobinger et al., 2021)

With respect to NK cells, the percentage of CD56 positive cells stimulated with Hsp70 plus IL-2 (5.31 +/- 3.96 %, median of 6.98 %) and TKD plus IL-2 (5.47 +/- 3.72 %, median of 5.25 %), as shown in figure 27, increased compared to the control value (2.28 +/- 1.93 %, median of 1.62 %), although not significant. (Lobinger et al., 2021)

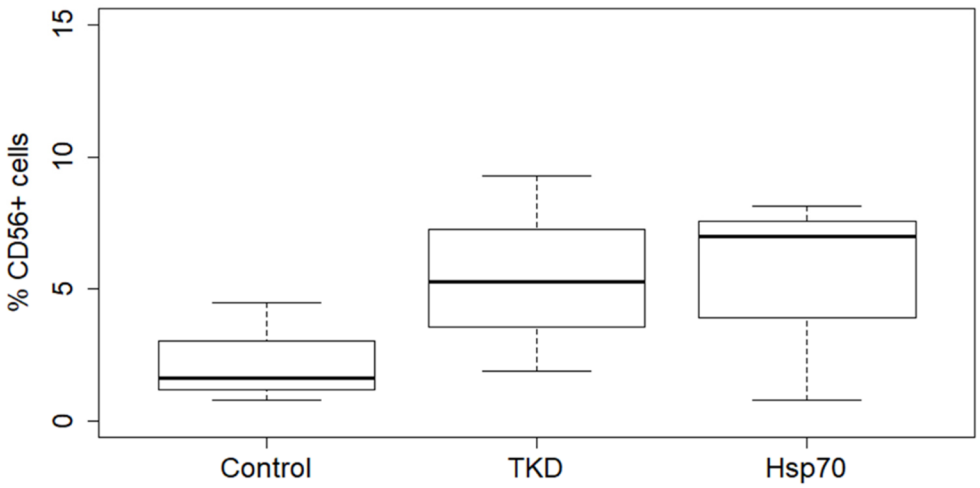

Figure 27: Stimulation with TKD/IL-2 and Hsp70/IL-2, CD56 positive NK cells (Lobinger et al., 2021)

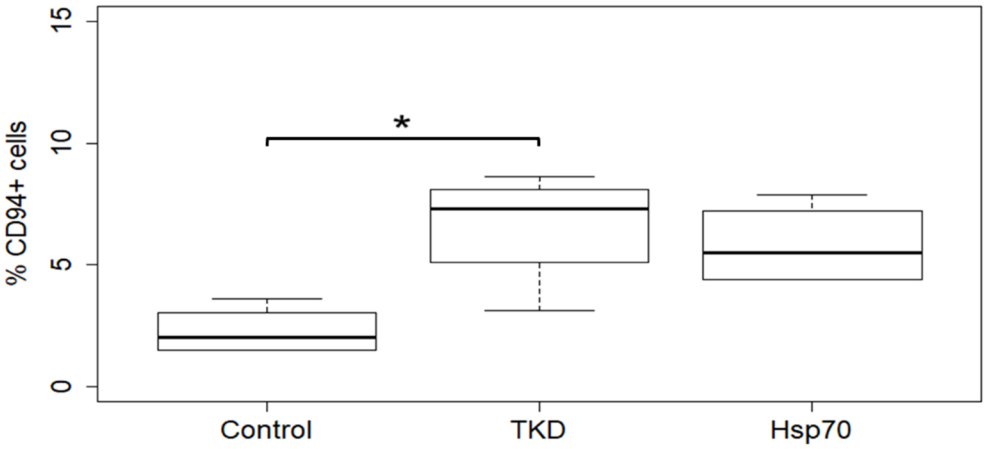

Figure 28: Stimulation with TKD/IL-2 and Hsp70/IL-2, CD94 positive NK cells (Lobinger et al., 2021)

CD94 positive NK cells, as represented in figure 28, showed also higher percentages compared to the control (2.27 +/- 0.86%, median of 2 %), after being stimulated with Hsp70 plus IL-2 (5.81 +/- 1.49%, median of 5.49 %) or TKD plus IL-2 (6.6 +/- 2.08%, median of 7.32 %). (Lobinger et al., 2021) The stimulation with TKD and IL-2 resulted in a significant increase as calculated using Tukey test ($p < 0.05$). Figure 29 shows the effect on CD69 positive NK cells after stimulation with TKD plus IL-2 (9.48 +/- 3.85%, median of 10.32 %) and Hsp70 plus IL-2 (10.69 +/- 4.09 %, median of 11.22 %), compared to unstimulated cells (0.27 +/- 0.24%, median of 0.18 %). TKD and Hsp70 stimulated lymphocytes had significantly higher percentages of CD69 positive NK cells than the control, as calculated using Tukey test ($p < 0.05$ and $p < 0.01$, respectively). (Lobinger et al., 2021)

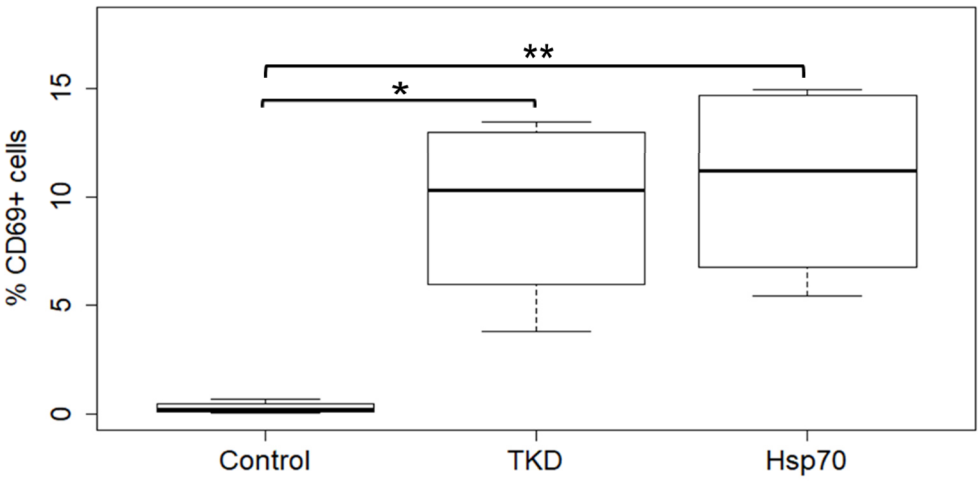

Figure 29: Stimulation with TKD/IL-2 and Hsp70/IL-2, CD69 positive NK cells (Lobinger et al., 2021)

5. Discussion

Lymphocytes as active players of the immune system, maintain an important role in developing, growing and progression of cancer. (Gonzalez, Hagerling, & Werb, 2018) Not just since the noble price for medicine and physiology which was awarded in 2018 to James P. Allison and Tasuku Honjo by their discovery of important immune regulatory mechanisms, a variety of different approaches of immunotherapy, for example checkpoint inhibitors or T cells with chimeric antigen receptors (CAR), got access to cancer treatment and were tested and established in clinical trials. (Guo, 2018) (Kruger et al., 2019) This shows the importance and significance of understanding the role of the immune system in different tumour entities.

To obtain a better understanding of the immune system and its composition in brain cancer patients, I counted their absolute number of lymphocytes and analysed certain subpopulation in comparison to healthy individuals in a similar age group. Furthermore, the link between the expression of Hsp70 in different cell compartments and a possible activation of the immune system, especially of certain NK cell markers, was part of this study. (Lobinger et al., 2021)

The activation and proliferation of immune cells, especially NK cells, relates to the expression of Hsp70 and its location intracellular, on the plasma membrane and in the extracellular space. Therefore, beside analysing the composition of certain lymphocyte subpopulations and the absolute number of lymphocytes in patients with gliomas, I analysed the level and concentration of Hsp70 in these different cellular compartments. As mentioned above and in contrast to normal cells, cancer cells can show an upregulated expression of cytosolic and membranous bound Hsp70 and can lead to higher levels of circulating Hsp70 in cancer patients, influencing prognosis, therapy resistance and anti-apoptotic activity. (Jaattela et al., 1998) (Ciocca & Calderwood, 2005) (N. Murakami et al., 2015)

Recent studies, which investigated the role of Hsp70 in patients with glioma, indicate that overexpression of Hsp70 in different cellular compartments is potentially positive correlated with tumour grade, and could serve a potential prognostic biomarker. (Beaman, Dennison, Chatfield, & Phoenix, 2014) (Ceccarelli et al., 2016) Furthermore, there is evidence, that glioblastoma cells express Hsp70 on their plasma membrane. (Thorsteinsdottir et al., 2017)

One part of this study was the evaluation of the cytosolic Hsp70 expression by immunohistochemical staining. It is known, that especially primary glioblastoma cells, in contrast to surrounding tissue, low grade glioma and secondary glioblastoma cells, can show a strong positive staining for Hsp70. (Thorsteinsdottir et al., 2017) Whereas one study, including primary and secondary glioblastoma patients, did not find any association between Hsp70 expression in immunohistochemistry and progression free survival or overall survival, another study shows significant better progression free survival and overall survival in patients with high expression of cytosolic Hsp70 levels by immunohistochemistry. (Thorsteinsdottir et al., 2017) (Lammer et al., 2019) In my findings, only grade IV tumours showed a strong nuclear and cytosolic staining for Hsp70, in contrast to grade I, II and III tumours, which only showed a nuclear staining. The staining in general, according to literature, seemed to be stronger in high-grade tumours than in lower grade glioma, where some of the positive stained cells could be derived from reactive astrocytes. (Kawamoto et al., 2007) Further stratification of our collective in patients with primary and secondary glioblastoma tumours could complement the findings. (Lobinger et al., 2021)

Beside the analysis of Hsp70 via immunohistochemistry, the membranous expression can be determined via flow cytometry on viable cells with intact cell membrane. (M. Shevtsov et al., 2018) In my results the membranous Hsp70 expression of different gradings all show a positive percentage above 60% in average. The lower expression of Hsp70 on the plasma membrane of high-grade brain tumours could contribute to a reduced immunogenicity of this tumour type. Immunohistochemical staining of high-grade glioma tumours revealed a higher Hsp70 expression in the cytosol than lower grade glioma and a higher release of Hsp70 into the extracellular milieu. These findings might indicate that extracellular Hsp70 in the blood might be derived from the plasma membrane. However, the evaluation of membrane Hsp70 expression by flow cytometry, is limited and biased by the selection of representative tumour parts by the surgeon. (Lobinger et al., 2021)

Beside the membranous and cytosolic localisation, free Hsp70 levels in the circulation can also be used to distinguish tumour patients from those with infectious diseases and from healthy individuals. Thereby extracellular Hsp70 might serve as a potential biomarker. (Yeh et al., 2009) (Gehrmann et al., 2014)

In our collective, patients with grade IV gliomas had significant higher concentrations of Hsp70 in their serum than healthy individuals. By classifying those patients with grade IV tumours into groups with different tumour volume, there is evidence for an association of the tumour volume and the concentration of Hsp70 in the circulation. Especially patients with tumours larger than 90 cm^3 showed significantly higher Hsp70 concentrations in serum than healthy donors. Furthermore, particularly grade IV patients, whose tumours had a high fraction of necrosis, larger than 10% of total tumour mass, had significantly higher concentrations of Hsp70 in the circulation. Therefore, I assume that circulating Hsp70 to some extent, might be released by dying cells via necrosis. The necrotic material including other DAMPS could lead to the upregulation of inflammation markers, such as Interleukin 2. (Lobinger et al., 2021)

Hsp70 in the circulation, either lipid-bound or free, has the potential to stimulate and influence immune response and NK cell activity. (Asea et al., 2000) (Delneste et al., 2002) Especially the effect of Hsp70 on NK cells and the stimulation of different NK cell activation marker has been demonstrated recently. (Multhoff et al., 2001) (Elsner et al., 2007) (Specht et al., 2015) (M. Shevtsov et al., 2019)

Our ex vivo experiments mimic the elevated Hsp70 levels in combination with inflammation markers, such as IL-2, in patients with high-grade tumours, by incubating peripheral blood lymphocytes of healthy individuals with Hsp70 or TKD plus IL-2. The increased percentage of different peripheral NK cell populations with higher tumour grading in our patient collective, could be explained by the higher amount of circulating Hsp70 plus inflammation markers like IL-2, and as a result, intensified stimulation, and activation. (Lobinger et al., 2021)

The immunogenicity of tumours is heterogeneous and depends on the tumour subtype and the patient's constitution. (Gomez & Kruse, 2006) Furthermore, it is known that certain types of cancer can supress the antitumour immune responses, for example by the secretion of certain mediators, expression of immune checkpoint inhibitors or upregulation of regulatory T cells and myeloid derived suppressive cells (MDSCs). (Zitvogel, Tesniere, & Kroemer, 2006) (Blankenstein, Coulie, Gilboa, & Jaffee, 2012) The absolute lymphocyte counts can show an association with outcome and prognosis of patients with different tumour types, like breast cancer, lung cancer or ovarian cancer. (Milne et al., 2012) (Tredan et al., 2013) (Cho, Oh, Chun, Noh, & Lee, 2016)

In our findings the tumour patients showed lower absolute number of lymphocytes (G/l) in circulation than the control collective, especially those patients who relapsed had significantly lower lymphocyte counts than the healthy donors. This could be a further hint on the important role of the immune system in controlling tumour development, process, and growth. (Lobinger et al., 2021)

However, the validity of the number and composition of immune cells in the peripheral blood of patients with brain tumours is limited, due to the special central nervous system microenvironment and blood brain barrier, controlling and restricting access and exit of lymphocytes and mediators to the tumour site. (Brown, Carter, Ottaviani, & Mulholland, 2018) Evaluating the condition and integrity of the blood brain barrier, for example by imaging, could provide more information about the connection of peripheral immune phenotype and composition of the immune system in microenvironment directly at the tumour field. Moreover, determining the composition of immune cells directly at the tumour site and in its environment, for example by immunohistochemistry staining of tumour slides for certain immune cell markers or analysing tumour infiltrating lymphocytes by flow cytometry, could contribute to a better understanding of the immune phenotype in brain cancer patients. This aspect is part of ongoing studies in our laboratory.

Taking previous studies into consideration, the genetical constitution of brain tumours, for example IDH status, can affect the composition of tumour infiltrating lymphocytes and the immunogenicity, as well. (Berghoff et al., 2017) Therefore, a further stratification of tumours into different genetical subtypes could improve the significance of our data.

Addressing certain lymphocyte subpopulations, patients with gliomas can show decreased percentage of T cells in circulation and different further abnormalities in their immune phenotype, like a lower fraction of CD4 positive T cells. (McVicar, Davis, & Merchant, 1992) (Fecci et al., 2007) Furthermore, as mentioned above, patients with malignancies can show higher fraction of regulatory T cells, contributing to an immunosuppressive phenotype in peripheral blood, but especially as tumour infiltrating lymphocytes in tumour microenvironment. (Beyer & Schultze, 2006) (Nishikawa & Sakaguchi, 2010) (Liyanage et al., 2002) (Ichihara et al., 2003) For patients with gliomas, the impact and significance of regulatory T cells is topic of ongoing discussions. Whereas some studies assume, that presence of regulatory T cells at the

tumour microenvironment is not correlated with altered survival and prognosis, other argue, that increased fraction of regulatory T cells among tumour infiltrating lymphocytes, may affect the appearance of recurrence, outcome and is correlated with higher tumour grading. (Heimberger et al., 2008) (Jacobs et al., 2010) (Humphries, Wei, Sampson, & Heimberger, 2010) (Han et al., 2014) (Sayour et al., 2015) (Mu et al., 2017) The level of regulatory T cells in peripheral blood of glioma patients can show reduced absolute number, but higher fraction in the remaining CD4 positive cell pool compared to healthy donors. (Fecci et al., 2006) (Sonabend, Rolle, & Lesniak, 2008) (Wiencke et al., 2012) (Yue et al., 2014)

In our patient collective, in which patients were compared to healthy individuals showed significant lower percentage of CD4 positive T cells, as well as a higher portion of regulatory T cells, significant only for those with relapse, gives a further hint on the immunosuppressive and abnormal immune phenotype of patients with brain tumours. Analysis of the fraction of regulatory T cells among the tumour infiltrating lymphocytes could complement the findings. (Lobinger et al., 2021)

The role of B cells in patients with glioma is also still subject of various discussion. In the tumour microenvironment of glioblastoma patients, they may have an impact on antitumor immunity, the activation of T cells and prognosis. (Candolfi et al., 2011) (Martinez-Lage et al., 2019) (Klopfenstein, Truntzer, Vincent, & Ghiringhelli, 2019) In our collective, the fraction of B cells in the peripheral blood of patients with brain tumours did not differ from that of healthy individuals, apart from slightly higher percentage in grade IV patients, although not significant.

The next lymphocyte subpopulation, which I analysed in this study, are NK cells. Whereas one study denies an association of NK cells in the composition of tumour infiltrating lymphocytes with tumour differentiation and biology, another shows that the presence of NK cells, infiltrating the tumour microenvironment, is more common in high grade glioblastoma than in low grade glioma. (Stevens, Kloter, & Roggendorf, 1988) (Yang, Han, Sughrue, Tihan, & Parsa, 2011) Because of their ability to kill glioblastoma cells and their effect on progression and survival, especially in the presence of a certain NK cell receptor allele, NK cell-based immunotherapy got into focus beside to established treatment procedures like surgery, chemotherapy and radiotherapy. (Castriconi et al., 2009) (Dominguez-Valentin et al., 2016) Different approaches of NK cell-based immunotherapy are already part of clinical or preclinical studies for the

treatment of glioblastoma patients. (Zhang et al., 2016) (T. Murakami et al., 2018) (Burger et al., 2019)

In our collective, the percentage of NK cells in the peripheral immune phenotype of patients with glioma showed no significant differences to healthy donors, apart from significant higher percentage of CD69 and CD94 positive NK cells in grade IV patients. However, as mentioned above, with higher tumour grading, the percentage of NK cells and their activation markers in the peripheral blood raised. This could be explained by higher concentrations of Hsp70 and inflammation markers in circulation. (Lobinger et al., 2021)

The potential of Hsp70 to modulate immune response, is object of research for the treatment of different tumour entities, for example in patients with Non-Small Cell Lung Cancer. (Specht et al., 2015) Furthermore, after successful treatment of glioblastoma in animal experiments, the intratumoral application of recombinant Hsp70 after surgery is already part of clinical trials. (M. A. Shevtsov, Pozdnyakov, et al., 2014) (M. A. Shevtsov, Kim, et al., 2014)

Taking first clinical parameters into analysis, glioblastoma patients with higher serum Hsp70 levels (>3.5 ng/ml) seem to have lower survival probability, although not statistically significant. This could be a first hint that more aggressive glioblastoma forms relate to higher circulating Hsp70 levels. Furthermore, glioblastoma patients of our collective without progress in the first 6 months after diagnosis, tend towards a higher percentage of NK cells than those with relapse or progress, although not statistically significant. Especially CD69 positive NK cells in patients without progress were significantly higher than in healthy donors. These findings provide a hint that elevated percentage of different NK cell populations at diagnosis can contribute to improved progression free survival and show once again the dualistic role of Hsp70. (Lobinger et al., 2021)

Taken together, the study provides a unique overview of the expression of Hsp70 in patients with glioma, analysed in different cellular compartments by various techniques, in correlation and connection with the immunophenotype in their peripheral blood and first clinical data. Information about clinical parameters like outcome and progression free survival of a larger number of patients and the analysis of tumour infiltrating lymphocytes are part of ongoing work in our team and can complement the findings and the significance of the role of Hsp70 as a potential biomarker.

6. List of references

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6.2 List of figures

6.3 List of tables

7. Appendix

7.1 Letter of acceptance

Klinik und Poliklinik für RadioOnkologie und Strahlentherapie

der Technischen Universität München

Klinik und Poliklinik für RadioOnkologie und Strahlentherapie

Klinikum rechts der Isar, Ismaninger Straße 22, 81675 München

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– Patienteninformation –

Gewinnung von Tumormaterial und Blut für die Erforschung der HSP70-Expression als Tumormarker

Sehr geehrte Patientin, sehr geehrter Patient,

bitte lesen Sie diese Information über die geplanten Untersuchungen im Rahmen des oben genannten Vorhabens sorgfältig durch. Für weitere Fragen stehen Ihnen die Prüfärzte gerne zur Verfügung.

Wir bitten Sie, an diesem Vorhaben teilzunehmen. Sie selbst haben davon keinen unmittelbaren Nutzen, helfen aber möglicherweise mit, ein Verfahren zu entwickeln, auf dessen Grundlage kranken Patienten eine innovative Therapieoption angeboten werden könnte.

Ziel der Studie

Ziel dieser Studie ist es, frisches Tumormaterial von Patienten auf das Vorhandensein von Hitze-Schock-Protein 70 (HSP70) zu untersuchen. Das Blut des Tumorpatienten wird funktionell und phänotypisch charakterisiert.

Einleitung

Es hat sich gezeigt, dass Tumorzelllinien häufig das HSP70 auf ihrer Zellmembran präsentieren. In der vorliegenden Studie soll überprüft werden, ob HSP70 auch auf soliden Tumoren von Patienten präsentiert wird.

Natürliche Killerzellen stellen eine Untergruppe der weißen Blutkörperchen dar und leisten einen wichtigen Beitrag in der körpereigenen Immunabwehr gegen Tumorerkrankungen. Die natürliche Funktion dieser Zellen ist leider nicht immer ausreichend, um die Ausbreitung einer Tumorerkrankung zu verhindern. Deshalb soll die Funktionalität Ihrer Immunabwehrzellen in einem etablierten Laborverfahren untersucht werden.

Ablauf der Studie

1. Tumoruntersuchung: Bei dem klinisch notwendigen operativen Eingriff (definitive Tumorresektion, ohne oder mit vorangegangener Therapie) wird eine Tumorprobe von maximal 0,5 cm^3 (etwa erbsengroß) entnommen. Dies beeinträchtigt in keinem Fall die weitere Diagnostik oder Therapie. Außerdem wird auch eine kleine Menge Referenzgewebe (Bindegewebe von maximal 0,5 cm^3) entnommen. Das erbsengroße Tumormaterial und Referenzgewebe wird in unserem Labor (Klinik und Poliklinik für Strahlentherapie und Radiologische Onkologie) auf das Vorhandensein von HSP70 untersucht. Völlig unabhängig davon wird das übrige entnommene Tumormaterial durch einen Pathologen (Institut für Allgemeine Pathologie und Pathologie Anatomie am Klinikum rechts der Isar der TU München) im Rahmen der Routinediagnostik feingeweblich untersucht.

2. Blutuntersuchungen: Wir benötigen von Ihnen 3 x 15 ml Ihres Blutes (entspricht der Menge von jeweils 3 Esslöffeln). Die Blutentnahme wird im Rahmen der allgemein notwendigen Therapievorbereitung und im Verlauf der Therapie erfolgen. Allgemein kann es bei der Blutentnahme (z.B. Armvenenblut) zu Schmerzen an der Einstichstelle, Entzündungen und Nachblutungen kommen. Manche Patienten können mit Übelkeit oder Schwindel auf eine Blutentnahme reagieren.

Abschließende Hinweise:

Um an dieser Studie teilzunehmen, ist Ihre Zustimmung notwendig. Stimmen Sie jedoch einer Teilnahme an dieser Studie nicht zu, wird Ihnen daraus in keiner Weise ein Nachteil entstehen. Auch zu einem späteren Zeitpunkt können Sie die Teilnahme jederzeit beenden.

Weitere ergänzend besprochene Punkte:

_____

_____

_____

_____

_____
_____
_____

Vertraulichkeit der Daten und Einblicknahme in die Krankenakte

Im Rahmen dieser Studie werden personenbezogene Daten erhoben, gespeichert und ausgewertet. Die Verwendung der Daten erfolgt nach gesetzlichen Bestimmungen. Nur wenn Sie die beiliegende Datenschutzerklärung unterschreiben, können sie an der Studie teilnehmen.

Weitere ergänzend besprochene Punkte:

_____
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– Datenschutzerklärung und Patienteneinwilligungserklärung –

Gewinnnung von Tumormaterial und Blut für die Erforschung der HSP70-Expression als Tumormarker

Hiermit erkläre ich,_____

dass ich durch Frau/Herrn _____

über das Ziel, das Wesen, die Bedeutung und die Tragweite des oben erwähnten Studienvorhabens aufgeklärt wurde.

Mein Arzt hat mich über die mit der Teilnahme an der Studie verbundenen Risiken informiert.

Informationen und Einwilligungserklärung zum Datenschutz:

Mir ist bekannt, dass bei dieser Studie personenbezogene Daten, insbesondere medizinische Befunde, über mich erhoben, gespeichert und ausgewertet werden sollen. Die Verwendung der Angaben über meine Gesundheit erfolgt nach gesetzlichen Bestimmungen und setzt vor der Teilnahme an der Studie folgende freiwillig abgegebene Einwilligungserklärung voraus.

1. Ich erkläre mich damit einverstanden, dass im Rahmen dieser Studie erhobene Daten, insbesondere Angaben über meine Gesundheit, in Papierform und auf elektronischen Datenträgern aufgezeichnet werden und namentlich (nichtanonymisiert) unabhängig von der Studie zurück an den betreuenden Arzt gehen.
   Soweit erforderlich, dürfen die erhobenen Daten anonymisiert (ohne Namensnennung) weitergegeben werden.

2. Ich bin darüber aufgeklärt worden, dass ich jederzeit die Teilnahme an der Studie beenden kann. Im Fall eines solchen Widerrufs meiner Einwilligung, an der Studie teilzunehmen, erkläre ich mich damit einverstanden, dass die bis zu diesem Zeitpunkt gespeicherten Daten ohne Namensnennung weiterhin verwendet werden dürfen.

3. Ich erkläre mich damit einverstanden, dass meine Daten nach Beendigung oder Abbruch der Studie mindestens zehn Jahre aufbewahrt werden. Danach werden meine personenbezogenen Daten gelöscht, soweit nicht gesetzliche oder satzungsmäßige Aufbewahrungsfristen entgegenstehen.

Ich bin darüber aufgeklärt worden, dass ich jederzeit meine Einwilligung zur Studienteilnahme ohne Angabe von Gründen widerrufen kann, ohne dass mir daraus Nachteile erwachsen.

Die oben genannten Punkte habe ich verstanden, alle von mir gestellten Fragen wurden mir beantwortet. Ich hatte Gelegenheit und ausreichend Zeit, mich für die Teilnahme an der Studie zu entscheiden. Dabei wurde ich nicht von meinem behandelnden Arzt oder einem anderen Klinikangehörigen beeinflusst.

Ich bin bereit, an der oben dargestellten HSP70-Screening-Studie teilzunehmen und willige in die Verarbeitung meiner personenbezogenen Daten unter Wahrung des Datenschutzes ein.

Eine Kopie der Patienteninformation und Einwilligungserklärung wurde mir ausgehändigt.

_____

Name der Patientin / des Patienten in Blockschrift ; Geburtsdatum

_____

Unterschrift der Patientin/des Patienten

_____

Unterschrift des Arztes

7.2 Acknowledgment

Firstly, I want to thank Prof. Dr. Gabriele Multhoff for the opportunity to develop this thesis and for the for great and everlasting support.

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Thanks to my partner, family and friends for emotional and financial support.

Finally, I want to thank all patients, although bearing a hard diagnosis, for participating at this study.

7.3 Curriculum Vitae

7.4 Publication:

“Potential Role of Hsp70 and Activated NK Cells for Prediction of Prognosis in Glioblastoma Patients”

Front. Mol. Biosci., 17 May 2021

Sec. Cellular Biochemistry