

# Quantitative Analysis of Liposomal Heat Shock Protein 70 (Hsp70) in the Blood of Tumor Patients Using a Novel LipHsp70 ELISA

# Stephanie Breuninger<sup>1</sup>, Janina Erl<sup>1</sup>, Clemens Knape<sup>1</sup>, Sophie Gunther<sup>1</sup>, Ivonne Regel<sup>2</sup>, Franz Rödel<sup>3</sup>, Udo S Gaipl<sup>4</sup>, Jun Thorsteinsdottir<sup>5</sup>, Lydia Giannitrapani<sup>6</sup>, Anne M Dickinson<sup>7</sup> and Gabriele Multhoff<sup>1,8\*</sup>

<sup>1</sup>Department of Radiation Oncology, Klinikum rechts der Isar, Technische Universität München, Ismaninger Str. 22, 81675 Munich, Germany

<sup>2</sup>Department of Surgery, Klinikum rechts der Isar, Technische Universität München, Ismaninger Str. 22, 81675 Munich, Germany

<sup>3</sup>Department of Radiation Therapy and Oncology, Universitätsklinikum Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany

<sup>4</sup>Department of Radiation Oncology, Universitätsklinikum Erlangen, Universitätsstr. 27, 91054 Erlangen, Germany

<sup>5</sup>Tumorbiological Laboratory, Department of Neurosurgery, Ludwig-Maximilians-University, Klinikum Grosshadern, Marchioninistr. 15, 81377 Munich, Germany <sup>6</sup>Biomedical Department of Internal Medicine & Specialities (DiBiMIS), University of Palermo, 90127 Palermo, Italy

<sup>7</sup>Cellular Therapies Facility Haematological Sciences (ICM), Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK

<sup>8</sup>Clinical Cooperation Group (CCG) "Innate Immunity in Tumor Biology", Helmholtz Zentrum München, German Research Center for Environmental Health Munich, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

### Abstract

Elevated levels of the stress-inducible heat shock protein 70 (Hsp70) in the peripheral circulation have been reported for many tumor entities. In line with these findings we have shown that Hsp70 membrane-positive tumor cells actively release Hsp70 in exosome-like lipid vesicles. Since most commercial Hsp70 Enzyme-Linked Immunosorbent Assays (ELISAs) are not validated for the detection of liposomal Hsp70 in serum, the lipHsp70 ELISA was established using the monoclonal antibody cmHsp70.1 as a detection reagent. This antibody has been reported to recognize free and membrane-bound Hsp70 on living tumor cells.

Validation of the ELISA showed a high assay precision and linearity in a concentration range of 0.36-17.4 ng/ml. A comparison of the recovery of spiked Hsp70 in buffer and serum samples revealed a significantly better recovery using the lipHsp70 ELISA compared to a commercial ELISA. With respect to lipid-associated Hsp70 a tenfold higher recovery was found with the lipHsp70 ELISA compared to the commercial ELISA. The analysis of blood samples of healthy human volunteers (n=114) revealed a mean serum Hsp70 concentration of 6.4 ± 2.7 and 2.8 ± 1.3 ng/ml, respectively, using the lipHsp70 and the control ELISA. No significant age-related differences in Hsp70 serum levels were detected. The lipHsp70 ELISA is equally suitable for serum and plasma and the measured Hsp70 concentrations were not impacted by food intake, repeated freezing and thawing of the sample or moderate hemolysis. A comparison of the Hsp70 levels in patients with head and neck, lung, colorectal, pancreatic cancer, glioblastoma or hematological malignancies and healthy human volunteers revealed significantly higher levels in tumor patients.

In summary, the lipHsp70 ELISA provides a highly sensitive and robust method for measuring liposomal and free Hsp70 in serum and plasma and thus could provide a useful tool for tumor detection and for monitoring the clinical outcome of patients.

**Keywords:** Hsp70; Lipid vesicles; Tumor exosomes; Tumor biomarker; ELISA

**Abbreviations:** ALL: Acute Lymphoid Leukemia; AML: Acute Myeloid Leukemia; AUC: Area Under the Curve; BAL: Biphenotypic Acute Leukemia; ca: Cancer; CI; Confidence Interval; CML: Chronic Myeloid Leukemia; ctrl: Control; CV: Co-efficient of Variation; ELISA: Enzyme-Linked Immunosorbent Assay; hematol mal: Hematological Malignancies; Hsc: Heat Shock Cognate protein; Hsp: Heat Shock Protein; LoB: Limit of Blank; LoD: Limit of Detection; mAb: monoclonal Antibody; MVB: Multivesicular Bodies; NHL: Non-Hodgkin Lymphoma; NSCLC: Non-Small Cell Lung Cancer; POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPS: 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine; ROC: Receiver Operating Characteristic; SCCHN: Squamous Cell Carcinoma of the Head and Neck; SCLC: Small Cell Lung Cancer; SD: Standard Deviation; SEM: Standard Error of the Mean

### Introduction

Heat Shock Proteins (Hsp) are molecular chaperones that play a key role in maintaining protein homeostasis and transport. Heat shock proteins with a molecular weight of approximately 70 kDa (Hsp70) are involved in assisting protein folding, preventing protein aggregation and transporting proteins across membranes [1,2]. The heat shock cognate

J Clin Cell Immunol ISSN: 2155-9899 JCCI, an open access journal protein 70 (Hsc70) and the major stress-inducible heat shock protein 70 (Hsc70), which are present in all nucleated eukaryotic cells, show a high sequence homology of 86% [3]. Following a variety of different stress stimuli, the synthesis of Hsc70 is moderately [4], while that of Hsp70 is highly upregulated in normal cells [1]. In contrast to normal cells, tumor cells frequently overexpress inducible Hsp70 already under physiological conditions [5] and present it on their plasma membrane [6]. Since Hsp70 on the membrane of tumor cells could not be removed with high salt, acid or basic washes [7,8], experimental evidence is

<sup>\*</sup>Corresponding author: Gabriele Multhoff, Department of Radiation Oncology, Klinikum rechts der Isar, Technische Universität München, Ismaninger Str. 22, 81675 Munich, Germany. Tel: 498941404514; Fax: 498941404299; E-mail: gabriele.multhoff@lrz.tum.de

Received September 10, 2014; Accepted October 09, 2014; Published October 16, 2014

**Citation:** Breuninger S, Erl J, Knape C, Gunther S, Regel I, et al. (2014) Quantitative Analysis of Liposomal Heat Shock Protein 70 (Hsp70) in the Blood of Tumor Patients Using a Novel LipHsp70 ELISA. J Clin Cell Immunol 5: 264. doi:10.4172/2155-9899.1000264

**Copyright:** © 2014 Breuninger S, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

given that Hsp70 is not merely associated with membrane proteins [9] but rather interacts with the lipid bilayer of the plasma membrane. Lipid profiling revealed that stress-inducible Hsp70 interacts with the tumor-specific lipid component globoyltriaosylceramide [7] under non-stressed conditions and with phosphatidylserine [8,10] following stress with high prevalence. We have generated a unique mouse monoclonal antibody (mAb) cmHsp70.1, which detects the lipid-associated membrane-bound form of Hsp70 on viable tumor cells. This antibody recognizes a non-conserved 8-mer epitope within the C-terminal oligomerization domain of the stress-inducible Hsp70, which is not recognized by other Hsp70 antibodies [11,12]. Regarding these findings, we assume that only a small part of the C-terminus, which is recognized by the cmHsp70.1 antibody, is accessible or exposed to the extracellular milieu of tumor cells.

An Hsp70 membrane-positivity has been found in a large variety of different tumor entities such as head and neck, lung, colorectal, pancreas, breast carcinomas and hematological malignancies, but not on the corresponding normal tissues [13,14]. In addition, Hsp70 membrane-positive tumor cells but not their Hsp70-negative counterparts release lipid vesicles that contain large amounts of Hsp70 in their lumen and carry Hsp70 on their lipid surface [15]. In line with these in vitro findings, serum Hsp70 levels were found to be elevated in cancer patients compared to healthy individuals [16]. However, depending on the Hsp70 ELISA test system that was used to determine Hsp70 in serum, the detected Hsp70 levels varied from pg/ml [17-19] to several  $\mu g/ml$  [20]. This finding might be due to the fact that most commercially available ELISA kits are optimized and validated for the detection of free Hsp70 in buffer but not in serum. Furthermore, if undiluted serum is used in the ELISA test [21], matrix effects need to be considered that might negatively influence the detection of Hsp70.

Presently, two major pathways are discussed as possible mechanisms for the secretion of Hsp70 by tumor cells. On the one hand, Hsp70 can be released by dying cells as a free protein, on the other hand, evidence is accumulating that a major proportion of extracellular Hsp70 is actively released by living tumor cells with an intact plasma membrane via vesicular export [15]. Protein profiling of tumor-derived lipid vesicles revealed the presence of cytosolic and endosomal proteins including Hsp70 and Rab-4 and the absence of Endoplasmic Reticulum (ER)-derived proteins. In accordance with these findings, ER/Golgi perturbing drugs like monensin and brefeldin A did not negatively impact the release of Hsp70-containing vesicles from tumor cells [22]. The floating properties on a sucrose gradient, the small size and the high acetylcholinesterase activity of these tumor-derived vesicles characterized them as exosomes [15]. The formation of exosomes involves Multivesicular Bodies (MVBs), which are formed by an inward budding of the endosomal membrane [23]. After fusion of the MVBs with the plasma membrane, exosomes with a size of 50-100 nm are secreted into the extracellular space [24]. Apart from tumor cells, a variety of cell types have been described to release exosomes, including different hematopoietic cells, intestinal epithelial cells, Schwann cells, neuronal cells, adipocytes and fibroblasts [25,26] and therefore, exosomes can be found in several different biological fluids like serum, plasma, urine, breast milk, ascites, synovial fluid and broncho-alveolar lavage fluid [25]. Since exosomes are produced by a double inversion of the plasma membrane, protein content and orientation of proteins in the exosomal membrane reflect that of the plasma cell membrane from which they are derived [27]. As a result, tumor cells that express Hsp70 on their plasma membrane secrete exosomes that also exhibit Hsp70 on their surface [15]. Similarly, the protein composition in the exosomal lumen reflects that of the cytosol of the respective cell. Page 2 of 10

Therefore, it is assumed that exosomes derived from normal cells carry low amounts of Hsp70, whereas exosomes from tumor cells with a high cytosolic Hsp70 content contain high amounts of Hsp70 in their lumen and present it on their lipid surface [23].

To quantify free as well as lipid-bound Hsp70 derived from exosomes in the serum of tumor patients, we developed the novel lipHsp70 sandwich ELISA. This ELISA specifically detects the inducible form of Hsp70 and does not cross-react with the highly homologous constitutive form Hsc70. The detection reagent in the lipHsp70 ELISA is the cmHsp70.1 monoclonal antibody, which is directed against an epitope that is exposed on the cell surface of tumor cells and tumorderived exosomes [28,29]. The buffer systems, detergents and antibodies used in the novel lipHsp70 ELISA were optimized and validated for the detection of both free and lipid-bound Hsp70 in serum and plasma of human donors and compared to a commercially available Hsp70 ELISA as an internal control.

# Material and Methods

# Collection of plasma and serum samples

Blood samples (9 ml) were taken from 114 healthy human volunteers and patients with head and neck cancer (n=23), lung cancer (n=22), colorectal cancer (n=44), pancreatic cancer (n=46), gliobastoma (n=30) or hematological malignancies (n=32), who provided informed, written consent. Patient characteristics are summarized in tables 2 and 3. Blood was collected in one EDTA KE tube and one serum separator tube (S-Monovette, Sarstedt, Nümbrecht, Germany) and mixed by gently inverting the tube. For plasma separation, EDTA blood was centrifuged at 1,500 g for 15 min. For serum collection, blood was allowed to clot for 15 min at room temperature and serum was separated by centrifugation at 750 g for 10 min. Serum from leukemia patients was allowed to clot for two to three hours and separated by centrifugation at 380 g for 5 min. Serum and plasma were stored in 150 µl aliquots at -80°C. Approval of the study was obtained by the Ethics Committees of the universities that are involved in the study. All procedures were in accordance with the Helsinki Declaration of 1975 as revised in 2008.

To validate the ELISA, the interference factors food intake of the blood donor, repeated freezing and thawing and hemolysis of the serum samples were tested. To test the influence of food intake, serum samples were collected from healthy human individuals before and 2 h after intake of a high-fat diet. Repeated freezing and thawing procedures of up to ten cycles were performed on the serum samples. In order to study the impact of hemolysis on the assay precision, erythrocytes were isolated from the blood of healthy donors by density gradient centrifugation using LSM1077 (PAA, Cölbe, Germany). Erythrocytes were lysed by applying shear stress and the corresponding serum samples were spiked with increasing amounts of the lysed erythrocytes. The hemoglobin content of the spiked serum samples was analyzed by measuring the absorbance of the samples at 562 nm, 578 nm and 598 nm. The hemoglobin concentration was calculated as described elsewhere [30].

Parameters	Performance
Linear range (ng/ml)	0.36-17.41
Intra-assay precision (%CV)	5.2-8.1
Inter-assay precision (%CV)	1.0-18.0
Recovery (%): Buffer/Serum	101 ± 3 / 78 ± 3
Limit of Detection (ng/ml)	0.31

Table 1: Assay performance characteristics of the lipHsp70 ELISA.

Page 3 of 10

		Healthy donors	Head & neck cancer	Lung cancer	Colorectal carcinoma	Pancreatic cancer	Glioblastoma	Hematological malignancies
Number (n)		114	23	22	44	46	30	32
Gender (M/F)		67/47	21/2	16/6	26/18	26/20	14/16	25/7
Age	Mean	42.9	62.5	66.1	64.2	69.8	56.2	41.7
	Range	20-74	36-83	48-88	29-81	44-90	25-77	19-64
	SD	14.6	12.2	10.3	13.0	40.6	14.1	12.2
	Median	41.5	61.0	66.5	67.5	73.0	59.0	42.0
lipHsp70 ELISA	Mean Hsp70 (ng/ml)	6.4	12.4	16.8	11.0	14.8	67.6	11.1
	SD	2.7	6.1	16.2	5.2	7.3	37.5	9.0
ROC	AUC (CI 95%)	-	0.80	0.78	0.81	0.86	0.92	0.62
	p-value	-	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.03
	Sensitivity (%)	-	78	73	70	85	90	41
	Specificity (%)	-	75	75	75	75	75	75

Abbreviations: AUC: Area Under the Curve; M:Male; F: Female; CI: Confidence Interval; ROC: Receiver Operating Characteristic; SD: Standard Deviation

 Table 2: Age, gender and Hsp70 levels of healthy donors and patients.

Patient #	Tumor location	Histology	Staging		Grading	
1	hypopharynx	SCCHN	T1	N0	M0	G2
2	hypopharynx	SCCHN	Т3	N2b	M0	G3
3	hypopharynx	SCCHN	T1	N0	M0	G3
4	larynx	SCCHN	Т3	N2	M0	G2
5	larynx	SCCHN	Т3	N0	M0	G3
6	larynx	SCCHN	T4	N1	M0	G3
7	larynx	SCCHN	Т3	N0	M0	G2
8	larynx	SCCHN	T4a	N0	M0	G3
9	naso/oro/hypo pharynx	SCCHN	T4c	N2c	MO	G2
10	naso/oro/hypo pharynx	SCCHN	T3-4a	N2c	M0	G3
11	nasopharynx	SCCHN	T2	N0	M0	G1
12	oral cavity	SCCHN	T1	N0	M0	G2
13	oro/hypopharynx	SCCHN	T2	N2b	M0	G3
14	oro/hypopharynx	SCCHN	T2	N2a	M0	G2
15	oropharynx	SCCHN	T4a	N2c	M0	G3
16	oropharynx	SCCHN	Т3	N0	M0	G3
17	oropharynx	SCCHN	T1	N2b	M0	G2
18	oropharynx	SCCHN	T1a	N0	M0	G2
19	oropharynx	SCCHN	T4	N0	M0	G2
20	oropharynx	SCCHN	T2	N2b	M0	G3
21	oropharynx	SCCHN	T4	N0	M0	G3
22	oropharynx	SCCHN	T2	N1	M0	G3
23	paranasal sinus	SCCHN	T2	N0	MO	G3

Abbreviations: SCCHN:Squamous Cell Carcinoma of the Head and Neck

Table 3a: Clinico-pathological characteristics of head and neck cancer patients.

#### **Recombinant Hsp70**

His-tagged Hsp70 protein was isolated from a Sf9 insect cell system (Orbigen, San Diego, CA, USA). Briefly, Sf9 cells were transfected with baculovirus containing cDNA coding for human Hsp70 protein with a His-tag on the N terminus (Orbigen, San Diego, CA, USA). Cell lysates were loaded on Ni-sepharose columns (GE Healthcare, Chalfont St. Giles, UK) in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4). His-tagged Hsp70 was eluted with increasing concentration of elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4). Fractions containing high amounts of Hsp70 were pooled and the buffer was exchanged using PD-10 desalting columns (GE Healthcare, Chalfont St. Giles, UK). Protein amount was determined using a BCA protein kit (Pierce, Thermo, Rockford, IL, USA) and aliquots were stored at -20 $^{\circ}$ C.

As a further control, recombinant Hsp70 without His-tag was purchased from Stressgen (ADI-NSP-555, Enzo Life Sciences, Farmingdale, NY, USA).

#### Antibody biotinylation

The monoclonal mouse antibody cmHsp70.1 (multimmune, Munich, Germany) was biotinylated using EZ-link sulfo NHS-LCbiotin (Thermo, Rockford, IL, USA). The antibody was incubated with a 40-fold molar excess of biotin for 1 h at room temperature. The remaining free biotin was removed using Zeba spin desalting columns (Thermo). Protein concentration was determined with a BCA protein kit (Pierce, Thermo) and aliquots were stored at 4°C.

# Preparation of lipid vesicles

Lipid vesicles were prepared as described previously [31]. Briefly, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, Avanti Polar Lipids, Alabaster, AL, USA) and 1-palmitoyl-2-oleoyl-snglycero-3-phospho-L-serine (POPS, Avanti Polar Lipids), dissolved in chloroform, were mixed in a molar ratio of 8:2 and dried under nitrogen gas. Lipids were rehydrated in 25 mM Tris/HCl pH 7.4, 250 mM NaCl at 10 mg lipid per 1 ml of buffer for 1.5 h at room temperature. The Mini Extruder (Avanti Polar Lipids) was assembled according to the manufacturer's instructions and the lipid suspension was pushed through a 100 nm polycarbonate membrane 13 times to achieve uniformly sized vesicles. Vesicle concentration was adjusted to 1 mg/ml with 1 mM Bis/Tris buffer pH 7.4 and 100 µl of the suspension were incubated with 1 µg of recombinant Hsp70 protein for 30 min at room temperature. After addition of an equal amount of ultrapure water the vesicle suspension was centrifuged at 200,000 g for 2 h at 4°C in an ultracentrifuge. The pellet was resuspended in PBS and stored at 4°C. Hsp70-containing lipid vesicles and empty control vesicles were used for further tests within 24 hours.

#### **SDS-PAGE and Western blot analysis**

Lysed Hsp70-containing vesicles and defined concentrations of recombinant Hsp70 were loaded onto a 10% acrylamide gel under reducing conditions and blotted onto nitrocellulose membranes. The protein was detected using the monoclonal antibody cmHsp70.1 (multimmune, Munich, Germany). Bound antibodies were visualized using a horseradish peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark) and a chemiluminescence developing kit (Pierce, Thermo, Rockford, IL, USA). The Hsp70 protein content of the vesicles was quantified by densitometry and compared to the signals generated by defined amounts of a recombinant Hsp70 protein that were run on the same gel.

# LipHsp70 ELISA

96-well MaxiSorp Nunc-Immuno plates (Thermo, Rochester, NY) were coated overnight with 2 µg/ml rabbit polyclonal antibody (Davids, Biotechnologie, Regensburg, Germany), directed against human recombinant Hsp70, in sodium carbonate buffer (0.1 M sodium carbonate, 0.1 M sodium hydrogen carbonate, pH 9.6). After washing three times with phosphate buffered saline (PBS, Life Technologies, Carlsbad, CA, USA) with 0.05% Tween-20 (Calbiochem, Merck, Darmstadt, Germany), the wells were blocked with 2% milk powder (Carl Roth, Karlsruhe, Germany) in PBS for 1.5 h at 27°C. Following another washing step, serum samples diluted 1:5 in CrossDown Buffer (Applichem, Chicago, IL, USA) were added to the wells for 2 h at 27°C. Then the wells were washed again and incubated with 4 µg/ml of the biotinylated mouse monoclonal antibody cmHsp70.1 (multimmune, Munich, Germany) in 2% milk powder in PBS for 2 h at 27°C. Finally, after another washing step, 0.2 µg/ml horseradish peroxidase-conjugated streptavidin (Pierce, Thermo, Rockford, IL, USA) in 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was added for 1 h at 27°C. Binding was quantified by adding substrate reagent (R&D Systems, Minneapolis, MN, USA) for 30 min at 27°C and absorbance was read at 450 nm, corrected by absorbance at 570 nm, in a Microplate Reader (BioTek, Winooski, VT, USA). An Hsp70 eight point standard was included into each ELISA test using 0-50 ng/ml recombinant Hsp70 diluted in CrossDown Buffer.

As a control, Hsp70 serum levels were also determined using the DuoSet<sup>®</sup> IC Human/Mouse/Rat Total Hsp70 ELISA (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol.

### **ELISA validation**

Linearity was evaluated according to the Clinical Laboratory Standards Institute (CLSI) guideline EP6-A. Briefly, six solutions of different Hsp70 concentrations were analyzed with the ELISA and their relative concentration was plotted against the system output (concentration according to ELISA measurement). First-, second- and third-order models were then fitted to the data and a *t*-test was applied to the non-linear coefficients of the second- and third-order models using SigmaPlot 12.5 software. If none of the non-linear coefficients were significant (p>0.05), the dataset was considered linear.

To determine intra-assay precision, control serum samples from two different donors were run in 20 replicates on a single plate. Interassay precision was assessed by running control serum samples in duplicate on three different days. The concentration was determined for each sample and the Co-efficients of Variation (CVs) were calculated.

The Limit of Detection (LoD) was established according to the Clinical Laboratory Standards Institute (CLSI) guideline EP17-A as summarized by Armbruster and Pry [32]. Briefly, OD values of 36 blank samples and 36 samples with a low Hsp70 concentration (0.63 ng/ml) were converted to concentrations by back-calculating against the standard curve. The Limit of Blank (LoB) was calculated according to the following equation:  $LoB=\mu_B+1.645 \sigma_B$ , where  $\mu_B$  and  $\sigma_B$  are the mean and standard deviation of the blank measurements, respectively.

Finally, the Limit of Detection (LoD) was calculated according to the following equation: LoD=LoB+1.645  $\sigma_s$ , where  $\sigma_s$  is the standard deviation of the low sample measurements.

Recovery was assessed by spiking defined amounts of the respective standard Hsp70 into 1:5 diluted serum samples. The Hsp70 concentration of the serum alone was subtracted from the measured value and recovery was calculated as the ratio of observed concentration versus expected.

# Results

# Hsp70 ELISA validation: Calibration curve, intra- and interassay precision, limit of detection and recovery

Hsp70 is frequently overexpressed in tumor cells and can be actively released in lipid vesicles by viable tumor cells. Therefore, elevated Hsp70 serum levels have potential utility as biomarkers for the detection of viable tumor mass and to measure the response to therapeutic interventions. However, commercially available Hsp70 ELISAs are neither optimized for the measurement of serum Hsp70 nor of lipid-associated Hsp70. Therefore, for the novel lipHsp70 ELISA, blocking reagents, antibody combinations and serum diluent had to be optimized. A 1:5 dilution of the serum samples was found to be optimal to reduce unfavorable matrix effects, which are observed with undiluted serum. As an internal control, a commercial ELISA was used in parallel.

A representative calibration curve with eight measuring points and the fitting equation are shown in Figure 1A. A four-parameter fit model was applied to obtain the function describing a sigmoid curve. In 20 independent experiments, the highest concentration of the standard yielded a mean OD value of 2.82 and a standard deviation of 0.34. Linearity was assessed by comparing linear and nonlinear polynomial fitting of the relationship between true and observed concentrations of six Hsp70 samples. The lipHsp70 ELISA was linear in a concentration range from 0.36-17.41 ng/ml (Figure 1B). To determine assay precision, intra- and inter-assay runs were performed with control serum samples from two and five healthy donors, respectively, and the Co-efficients of Variation (CVs) were calculated. Intra-assay precision CVs ranged from 5.2% to 8.1%, and inter-assay precision CVs varied between 1.0% and 18.0% with a mean of 10.9%. The Limit of Detection (LoD) for the lipHsp70 ELISA was 0.3ng/ml.

Recovery was determined by spiking recombinant Hsp70 protein in buffer and serum samples using either the lipHsp70 or the commercially available ELISA as a control. When 2.5 ng/ml Hsp70 protein from Enzo Life Sciences were spiked into dilution buffer, the lipHsp70 ELISA recovered significantly higher amounts of the expected Hsp70 protein  $(2.53 \pm 0.09 \text{ ng/ml}, 101 \pm 3\% \text{ recovery})$  compared to the control ELISA  $(1.43 \pm 0.07 \text{ ng/ml}, 57 \pm 3\% \text{ recovery})$  (Figure 1C). To determine the recovery of Hsp70 in serum samples, Hsp70 (2.5 ng/ml and 5 ng/ml) of the respective standards was spiked into serum samples derived from four different healthy volunteers. With an average recovery of 78 ± 3%, the lipHsp70 ELISA showed a significantly higher recovery of the spiked Hsp70 compared to the control ELISA with 50 ± 3% (Figure 1D). The details of the assay performance are summarized in Table 1.

### Hsp70 serum levels in healthy human volunteers

Serum samples from 114 healthy human volunteers at different ages (age range 20-74, Table 2) were analyzed to determine the basal Hsp70 levels in blood. To minimize matrix effects, serum was diluted 1:5 in CrossDown Buffer prior to analysis. Significantly higher basal

Page 5 of 10



Figure 1: Validation of the lipHsp70 ELISA. (A) Representative eight point calibration curve obtained from the lipHsp70 ELISA using a four-parameter fit model (inset). The OD (mean corrected) is indicated on the Y-axis and the Hsp70 concentration (ng/ml) on the X-axis. (B) Linearity of the lipHsp70 ELISA was assessed by comparing first-, second- and third-order model fits of a relative concentration vs. system output plot. Linearity was shown within a concentration range of 0.36-17.4 ng/ml. (C) Recovery of free Hsp70 in buffer: 2.5 ng/ml Hsp70 was spiked into dilution buffer. The lipHsp70 ELISA recovered 2.53 ± 0.09ng/ ml (left panel) and thus revealed a significantly better recovery (101 ± 3%, right panel) compared to the control ELISA (1.43 ± 0.07 ng/ml, left panel; 57 ± 3%, right panel). The data show the mean of n=20 tests. (D) Recovery of free Hsp70 in serum: 2.5 ng/ml and 5 ng/ml Hsp70 were spiked intoserum (diluted 1:5). The lipHsp70 ELISA recovered 1.98  $\pm$  0.12 ng/ml and 3.82  $\pm$  0.12 ng/ml for 2.5 and 5 ng/ml (left panel) and thus revealed a significantly better recovery (mean 78  $\pm$  3%, right panel) compared to the control ELISA (1.41  $\pm$  0.07 ng/ml and 2.18  $\pm$ 0.09 ng/ml, left panel; mean 50 ± 3%, right panel). Black bars: lipHsp70 ELISA, white bars: control ELISA, dashed line: expected amount of Hsp70. p<0.05, \*\*p<0.001 (t-test).

levels ( $6.4 \pm 2.7 \text{ ng/ml}$ ) could be detected with the lipHsp70 ELISA compared to the control ELISA ( $2.8 \pm 1.3 \text{ ng/ml}$ ) (Figure 2A). The 25th and 75th percentiles were 4.5 ng/ml and 7.7 ng/ml for the lipHsp70 ELISA and 2.0 ng/ml and 3.1 ng/ml for the control ELISA, respectively. As shown in Figure 2B, no correlation was found between the basal Hsp70 serum levels and the age of the donors using both ELISAs.

### Comparison of the detection of Hsp70 in serum and plasma

To test whether the lipHsp70 ELISA is suitable for measuring Hsp70 levels in both serum and plasma, samples were taken in parallel from four healthy donors and measured using the lipHsp70 ELISA. For this experiment, donors with different basal levels of Hsp70 were chosen. For all four donors, the Hsp70 levels in plasma did not differ significantly from those in the corresponding serum samples. These data indicate that both serum and plasma can be used to measure

#### Influence of interference factors on the detection of Hsp70

Different factors in the donor's lifestyle or in the sample preparation could have an impact on the measurement of Hsp70 in serum [33]. In order to determine the robustness of the lipHsp70 ELISA, the influence of food intake of the donor, repeated freezing and thawing of the serum sample and hemolysis was tested.

Serum samples from seven healthy individuals were taken before and two hours after intake of a high-fat diet. In all donors, Hsp70 serum levels did not differ significantly before and after food intake (Figure 4A).



**Figure 2:** Hsp70 serum levels in healthy human volunteers determined with the lipHsp70 and control Hsp70 ELISA. (A) Serum samples were taken from 114 healthy human volunteers and Hsp70 levels were determined comparatively with the lipHsp70 and the control Hsp70 ELISA (ctrl ELISA). The lipHsp70 ELISA detected significantly higher Hsp70 concentrations (6.4 ± 2.7 ng/ml) in the serum than the ctrl ELISA(2.8 ± 1.3 ng/ml). Lines inside the box plots show the median value, upper and lower boundaries indicate the 25<sup>th</sup> and the 75<sup>th</sup> percentile, whiskers indicate the 10th and the 90th percentile, respectively. "'p<0.001 (Mann-Whitney Rank Sum Test).(B) The Hsp70 serum levels determined with the lipHsp70 (upper panel) and the control ELISA (lower panel) showed no correlation with the age of the donors.

Page 6 of 10







**Figure 4:** Influence of interference factors on the detection of Hsp70 in serum using the lipHsp70 ELISA. (A) Serum samples of seven healthy individuals were taken before (dark grey bar) and two hours after intake of a high-fat diet (light grey bar). Hsp70 serum levels were determined using the lipHsp70 ELISA. No significant differences in the Hsp70 serum values were detected before and after food intake. (B) Serum samples were subjected to three repeated cycles of freezing and thawing and Hsp70 levels were determined after each cycle. No significant differences in the Hsp70 values were detected after repeated freezing and thawing using the lipHsp70 ELISA. (C) Serum samples were spiked with increasing amounts of lysed, autologous erythrocytes and Hsp70 levels were determined using the lipHsp70 ELISA (n=3). Up to a hemoglobin concentration of 9.6 mg/dl in the serum the Hsp70 values remained unaffected; higher serum hemoglobin concentrations resulted in a significant increase in the Hsp70 values. "p<0.01, ""p<0.001 (*t*-test).

Serum samples from four healthy individuals with different basal levels of Hsp70 (2.3-9.1 ng/ml with the lipHsp70 ELISA and 1.4-2.1 ng/ml with the control ELISA) were subjected to three cycles of freezing and thawing and Hsp70 levels were determined after each cycle (Figure 4B). Even after ten cycles, the measured Hsp70 values did not change significantly (data not shown).

To test the influence of free hemoglobin in serum on the ELISA measurements, serum derived from three healthy individuals was spiked with increasing amounts of lysed erythrocytes. The hemoglobin

content of the samples was analyzed and correlated with the measured Hsp70 levels. Free hemoglobin at a concentration of up to 9.6 mg/dl did not significantly change the measured Hsp70 values. In contrast, hemoglobin concentrations above 9.6 mg/dl resulted in a non-specific increase of the Hsp70 values (Figure 4C).

# Detection of lipid-bound Hsp70

To test the ability of both ELISA tests to measure lipid-bound Hsp70, artificial POPS/POPC lipid vesicles were produced and loaded with recombinant Hsp70. The amount of lipid-associated Hsp70 that was determined with the lipHsp70 ELISA showed an excellent correlation with the Hsp70 concentration that was determined by Western blotting (data not shown). However, a comparison of the levels of liposomal Hsp70 using the lipHsp70 ELISA and the control ELISA revealed large differences. The recovery of liposomal Hsp70 using the lipHsp70 ELISA was 76  $\pm$  5%, whereas that of the control ELISA was only 7  $\pm$  1%. These data indicate that the detection of lipid-associated Hsp70 was more than 10-fold better with the lipHsp70 ELISA than with the control ELISA (Figure 5).

#### Hsp70 serum levels in patients with different tumor entities

Hsp70 levels were measured in the serum of patients with head and neck cancer (n=23), lung cancer (n=22), colorectal cancer (n=44), pancreatic cancer (n=46), glioblastoma (n=30) or hematological malignancies (n=32) (Table 3A-F) and compared to the Hsp70 levels in healthy donors (n=114). The mean Hsp70 serum levels in patients of all tumor entities were significantly higher than those of the healthy donors (Figure 6A). Receiver Operating Characteristic (ROC) curve analysis was performed by comparing serum Hsp70 levels of healthy donors with those of the different patient cohorts (Figure 6B). The Area Under the Curve (AUC; CI 95%) and sensitivity for a cut-off value of 7.7 ng/ml (derived from the 75<sup>th</sup> percentile of the healthy donors) is summarized in Table 2. The specificity was 75% for all patient groups.

# Discussion

Blood-borne biomarkers have potential utility for the detection of tumors, monitoring tumor growth and assessing the outcome of antitumor therapies [34-36], but many have been criticized for their lack of specificity and selectivity [37-39]. Although heat shock (stress) proteins are commonly considered as being intracellular molecules, elevated levels of Hsp70 have been detected in the supernatants of cultured



**Figure 5:** Recovery of lipid-bound Hsp70 using the lipHsp70 ELISA and the control Hsp70 ELISA. Artificial POPS/POPC lipid vesicles were produced and loaded with recombinant Hsp70. Recovery of lipid-bound Hsp70: according to quantitative Western blot analysis (data not shown) the lipid vesicles were loaded with 2,100 ng/ml Hsp70. The lipHsp70 ELISA recovered 1,610 ± 292 ng/ml (left panel) and thus revealed a significantly better recovery (76 ± 5%, right panel) compared to the control ELISA (155 ± 36 ng/ml, left panel; 7 ± 1%, right panel). The data show the mean of n=3 tests. Black bars: lipHsp70 ELISA, white bars: control ELISA." p<0.001 (*t*-test).

Patient #	Histology	Staging			Grading
1	NSCLC adeno	T2	N2	M1	G3
2	NSCLC adeno	T4	N2	M0	G2
3	NSCLC adeno	T2	N2	M0	G3
4	NSCLC adeno	Т3	N3	M1	G3
5	NSCLC adeno	T4	N2	M1	G3
6	NSCLC adeno	T2	N0	M0	G2
7	NSCLC adeno	T1	N3	M0	G3
8	NSCLC adeno	T2	N2	M1	G2
9	NSCLC squamous	Т3	N3	M1	G3
10	NSCLC squamous	Т3	N3	M1	G3
11	NSCLC squamous	T4	N0	M0	G2
12	NSCLC squamous	T1	N0	M0	G3
13	NSCLC squamous	T4	N2	M0	G3
14	NSCLC squamous	T4	N3	M1	G3
15	NSCLC squamous	Т3	N3	M0	G3
16	NSCLC squamous	Т3	N2	M1	G3
17	NSCLC squamous	T4	N2	M1	G3
18	NSCLC squamous	Т3	N2	M1	G2
19	SCLC	limited			-
20	SCLC	extensive			-
21	SCLC	extensive -			-
22	SCLC	extensive -			-

Abbreviations: NSCLC:Non-Small Cell Lung Cancer; SCLC:Small Cell Lung Cancer

Table 3b: Clinicopathological characteristics of lung cancer patients.

tumor cells [16] and also in the peripheral circulation of patients with cancer and other diseases [40]. Levels of circulating heat shock proteins, including Hsp70 might therefore serve as useful biomarkers for disease in a number of clinical settings.

Extracellular Hsp70 exists either as a free protein, as a protein in association with lipid vesicles such as exosomes [15] and lysosomal endosomes [41] or in the context of cholesterol-rich microdomains [42]. The minor part of extracellular Hsp70 is free Hsp70, which is mostly derived from dying cells. Only combined treatment modalities such as radiation plus hyperthermia have been shown to increase the release of free Hsp70 by dying cells [43]. The major proportion of extracellular Hsp70, which is derived from living, metabolically active tumor cells, is bound to small lipid vesicles such as exosomes, which are actively released by a large variety of human tumor cell types [8,15]. Lipid-bound, exosomal Hsp70 could therefore be an interesting novel biomarker, which might better reflect the presence and size of viable tumor masses in patients and their response to treatment. However, most commercially available Hsp70 ELISA systems are optimized and validated for the analysis of free Hsp70 protein in buffer systems, and it is therefore not too surprising that different "in-house" and commercially available Hsp70 assays have reported different Hsp70 levels [17-21]. A prerequisite for measuring the absolute levels of Hsp70 in patient blood is therefore an assay that reliably detects both free and liposomal Hsp70.

We have previously reported on the development and validation of a mouse monoclonal antibody, which is able to bind to a form of Hsp70 that is selectively located in the plasma membrane of viable tumor cells (cmHsp70.1) [29], but also detects free Hsp70 in Western blots. This antibody was validated as a detection reagent for liposomal and free Hsp70 in the lipHsp70 ELISA, which is described herein. The lipHsp70 ELISA allows the quantification of Hsp70 in serum and plasma, and is less susceptible to matrix effects that are often caused by serum components. The validation experiments (summarized in Table 1) indicate high assay precision and linearity in the relevant concentration range. The recovery of spiked Hsp70 in buffer and serum samples was significantly higher with the lipHsp70 ELISA compared to that of a commercial ELISA. The most prominent differences in the recovery of Hsp70 were detected with respect to liposomal Hsp70, in that the lipHsp70 ELISA recovered tenfold more of the liposomal Hsp70 than the commercial ELISA. An explanation for this observation is a differential capacity of the monoclonal antibodies to recognize the lipid-associated form of Hsp70 as outlined schematically in Figure 7.

Membrane-bound Hsp70 is often located in detergent-resistant microdomains or lipid rafts [8,44]. As most commercially available

Patient #	Staging			Grading
1	uT3	uN+	MX	G2
2	uT3	uN0	cM0	G2
3	cT3	cN+	cM0	G2
4	cT3	cN1	cM0	G2
5	cT3	cN1	cM1	G2
6	cT3	cN0	cM0	G2
7	cT3	cN0	cM0	G2
8	uT3	uN0	cM0	G2
9	cT3	cN2	cM0	G2
10	cT3	uN0/cN+	MO	
11	cT3	cN+	cM0	G2
12	uT3	cNX	cM0	G2
13	cT3	cN1	cM0	G2
14	uT3	uN1	cM0	G2
15	uT3	cN0	cM0	G2
16	cT2	cN1	cM0	G2
17	cT3	cN2	MX	G2
18	cT3	cN2	cM0	G2
19	cT3a	cN2	cM0	
20	cT3-4	cN+	cM0	G2
21	cT3	cN0	cM0	G2
22	cT3	cN1	cM0	G3
23	uT3c	N0	cM0	G2
24	uT3	uN0	cM0	G2
25	cT4	cN1	cM0	G2
26	uT3	uN2	cM0	G2
27	uT3	uN1	cM0	G2
28	uT3	uN1	cM0	G2
29	uT3	N+	cM0	G2
30	cT3	cN0	cM0	
31	cT3	cN1	cM0	G2
32	cT3	cN2	cM1	G2
33	Т3	N2	MO	G2
34	cT3	cN0	MO	G2
35	uT3	uN0	cM0	
36	cT3	cN+	cM0	
37	cT3	cN0	cM0	
38	cT2	uN0	MO	G2
39	cT3	cN+	cM0	G2
40	uT3	uN1	cM0	G2
41	uT3b-4	cN+	cM0	
42	uT3	uN1	cM0	G2
43	cT3	cN+	cM0	G2
44	cT3	uN1	cM0	G2

Abbreviations: C:Staging by Clinical Examination; u:Staging by Ultrasonography Table 3c: Clinico-pathological characteristics of colorectal carcinoma patients.

#### Page 8 of 10

Patient #	Tumor Location	Staging		Grading	
1	body	Т3	N0	M0	G2
2	body/tail	T2	N1	M1	-
3	body/tail	T4	N2	M1	-
4	body/tail	T4	N2	M1	-
5	body/tail	T1	N1	M1	-
6	body/tail	T4	N2	M1	-
7	body/tail	Т3	N1	M0	-
8	body/tail	T4	N2	M1	-
9	body/tail	T4	N3	M1	-
10	head	Т3	N1	M0	G2
11	head	T4	N1	M0	G2
12	head	Т3	N0	M0	G2
13	head	Т3	N1	M0	G1
14	head	Т3	N1	M0	G2
15	head	Т3	N0	M0	G1
16	head	Т3	N1	M0	G2
17	head	T1	N0	M0	G3
18	head	Т3	N1	M0	G3
19	head	Т3	N1	M0	G3
20	head	Т3	N1	M0	G3
21	head	T4	N2	M1	-
22	head	Т3	N1	M0	-
23	head	Т3	N2	M1	-
24	head	Т3	N2	M1	-
25	head	T2	N1	M0	-
26	head	Т3	N2	M1	-
27	head	T2	N1	M1	-
28	head	T2	N0	M0	-
29	head	Т3	N1	M0	-
30	head	T2	N0	M0	-
31	head	T2	N1	M0	-
32	head	T4	N2	M1	-
33	head	T2	N1	M1	-
34	head	Т3	N1	M1	-
35	head/body/tail	T4	N2	M1	-
36	head/tail	T1	N0	M0	G1
37	head/uncinate process	T2	N0	M0	-
38	head/uncinate process	Т3	N2	M1	-
39	head/uncinate process	T2	N0	M0	-
40	head/uncinate process	T4	N2	M1	-
41	tail	T4	N1	M0	G3
42	tail	Т3	N1	M0	G2
43	tail	Т3	N0	M0	G2
44	tail	T4	N3	M1	-
45	uncinate process	Т3	N1	M0	-
46	uncinate process	T2	N0	M0	-

Patient #	Tumor Origin	Grading
1	primary	G4
2	primary	G4
3	primary	G4
4	primary	G4
5	primary	G4
6	primary	G4
7	primary	G4
8	primary	G4
9	primary	G4
10	primary	G4
11	primary	G4
12	primary	G4
13	primary	G4
14	primary	G4
15	primary	G4
16	primary	G4
17	primary	G4
18	primary	G4
19	primary	G4
20	primary	
21	secondary	G4
22	secondary	G4
23	secondary	G4
24	secondary	G4
25	secondary	G4
26	secondary	G4
27	secondary	G4
28	secondary	G4
29	secondary	G4
30	secondary	

Patient #	Hematological disease		
1	ALL		
2	AML		
3	AML		
4	AML		
5	AML		
6	AML		
7	AML		
8	AML		
9	AML		
10	AML		
11	AML		
12	BAL		
13	CML		
14	Hodgkin lymphoma		
15	Hodgkin lymphoma		
16	Hodgkin lymphoma		
17	MDS & MPS		
18	Multiple myeloma		
19	Multiple myeloma		
20	Multiple myeloma		
21	MDS		
22	MDS		
23	MDS		
24	MDS		
25	MDS		
26	MDS		
27	MDS		
28	NHL		
29	NHL		
30	NHL		
31	NHL		
32	Secondary acute leukemia		

Abbreviations: ALL:Acute Lymphoid Leukemia; AML:Acute Myeloid Leukemia; BAL:Biphenotypic Acute Leukemia; CML:Chronic Myeloid Leukemia; MDS: Myelodysplastic Syndrome; MPS:Myeloproliferative Syndrome; NHL: Non-Hodgkin Lymphoma

Table 3f: Clinico-pathological characteristics of patients with hematological malignancies.

Table 3d: Clinico-pathological characteristics of pancreatic cancer patients.

sample dilution buffers for ELISAs contain non-ionic detergents to dissolve lipid vesicles, it is likely that a proportion of serum Hsp70 remains associated with lipids after treatment (Figure 7A). The continued association of Hsp70 with lipid components could inhibit the binding of Hsp70-specific antibodies that are used in commercial kits or influence the conformation of Hsp70 such that binding of the antibody does not occur. In contrast, the documented ability of the cmHsp70.1 antibody to detect the membrane-bound conformation of Hsp70 on viable tumor cells (Figure 7B) suggests that it can also recognize lipid-associated Hsp70. Since a major proportion of serumderived Hsp70 is bound to lipid vesicles, the lipHsp70 ELISA is more



Figure 6: Hsp70 serum levels in patients with different tumor entities compared to healthy controls. (A) Serum samples were taken from healthy human volunteers (n=114) and patients withs quamous carcinomas of the head and neck (n=23), lung cancer (n=22), colorectal cancer (n=44), pancreatic cancer (n=46), glioblastoma (n=30) or haematological malignancies (n=32). Patient characteristics are summarized in Table 3. Significantly higher Hsp70 levels were found in all tumour patient cohorts compared to the healthy controls. Lines inside the box plots show the median value, upper and lower boundaries indicate the 25<sup>th</sup> and the 75<sup>th</sup> percentile, whiskers indicate the 10<sup>th</sup> and the 90<sup>th</sup> percentile, respectively. \*p<0.05, \*\*\*p<0.001 (Mann-Whitney Rank Sum Test). (B) ROC curve analysis was performed on the data shown in (A). AUC, sensitivity and specificity data are summarized in Table 2.



Figure 7: Lipid-association of Hsp70 in exosomes and in the plasma membrane of living tumor cells. (A) An incomplete solubilisation of lipid vesicles such as exosomes with detergents used in ELISA tests can lead to Hsp70-lipid complexes. Lipid-bound Hsp70 that exerts a different conformation can only be detected by cmHsp70.1 mAb but not by other Hsp70-specific antibodies. (B) The cmHsp70.1 mAb detects Hsp70 on the plasma membrane of living tumor cells. This lipid-bound Hsp70 in exosomes. Therefore, we speculate that the recovery of lipid-bound Hsp70 with the lipHsp70 ELISA is better than with the control ELISA.

Page 9 of 10

appropriate for the measurement of circulating Hsp70 that is derived from viable tumor cells.

The basal levels of Hsp70 in the serum of 114 healthy human donors were found to be significantly higher with the lipHsp70 ELISA compared to the commercial ELISA. The results obtained with the lipHsp70 ELISA remained unaffected by food intake of the blood donor and repeated freezing and thawing of the serum samples, thereby facilitating the use of this assay in the clinical setting. The lipHsp70 ELISA also tolerated moderate hemolysis up to a hemoglobin concentration of 9.6 mg/dl, with concentrations above this level resulting in a non-specific increase of the measured Hsp70 values. The higher Hsp70 levels in healthy donors that were measured with the lipHsp70 ELISA are most likely due to the fact that hematopoietic cells such as B cells, T cells, dendritic cells, mast cells and platelets, as well as intestinal epithelial cells, Schwann cells, neuronal cells, adipocytes and fibroblasts have all been reported to release exosomes that contain low amounts of Hsp70 in their lumen [25]. Furthermore, significantly higher Hsp70 levels were detected in the serum of patients with various different tumor entities compared to healthy controls, which is not too surprising given that tumor cells exhibit higher cytosolic Hsp70 levels and actively release high amounts of Hsp70 in lipid vesicles. Notably, we observed distinct differences with elevated concentrations of circulating Hsp70 in patients with different tumor entities. Although these data require future analysis and validation with extended cohorts of patients, these relationships may provide evidence on different expression patterns not only between individual patients but also between different tumor entities.

# Conclusion

In summary, the reliability and robustness of the lipHsp70 ELISA together with its ability to detect higher levels of Hsp70 in the circulation of patients with cancer makes this method a promising tool for monitoring the presence and size of viable tumor mass, as well as therapeutic outcomes.

#### Acknowledgement

The authors would like to thank Dr. Christine Bayer and Rihab Gam for their valuable help during data acquisition and in providing patient data.

The work of Gabriele Multhoff was supported in part by grants of the Deutsche Forschungsgemeinschaft (SFB824/2 B4; DFG INST95/980-1FUGG, INST411/37-1FUGG), the DFG Cluster of Excellence: Munich-Centre for Advanced Photonics (MAP), the Bundesministerium für Forschung und Technologie (BMBF Innovative Therapies, 01GU0823; BMBF m<sup>4</sup> Spitzencluster, 16EX1021C; BMBF Kompetenzverbund Strahlenforschung 02NUK038A), European Union (EU-CELLEUROPE 315963) and the Wilhelm-Sander Stiftung (2012.078.1). Udo Gaipi's work was funded by the German Research Foundation (DFG-Graduiertenkolleg 1660: Key signals of the adaptive immune response and GA 1507/1-1), the German Federal Ministry of Education and Research (BMBF; m4 Cluster, 16EX1021R and GREWIS, 02NUK017G), and the European Commissions (DoReMi, European Atomic Energy Community's Seventh Framework Programme (FP7/2007-2011) under grant agreement n° 249689). The work of Franz Rödel was funded by the German Research Foundation (DFG-Graduiertenkolleg 1657: Molecular and cellular responses to ionizing radiation), the German Federal Ministry of Education and Research (BMBF; m4 Cluster, 16EX1021J and GREWIS, 02NUK017F), and the European Commissions (DoReMi, grant agreement 249689).

#### Authors' Contribution

The authors Stephanie Breuninger and Janina Erl contributed equally to this work.

#### References

- 1. Lindquist S, Craig EA (1988) The heat-shock proteins. Annu Rev Genet 22: 631-677.
- Hartl FU (1996) Molecular chaperones in cellular protein folding. Nature 381: 571-579.

Page 10 of 10

- Daugaard M, Rohde M, Jäättelä M (2007) The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. FEBS Lett 581: 3702-3710.
- 4. De Maio A (1999) Heat shock proteins: facts, thoughts, and dreams. Shock 11: 1-12.
- Jäättelä M (1999) Escaping cell death: survival proteins in cancer. Exp Cell Res 248: 30-43.
- Multhoff G, Botzler C, Wiesnet M, Müller E, Meier T, et al. (1995) A stressinducible 72-kDa heat-shock protein (HSP72) is expressed on the surface of human tumor cells, but not on normal cells. Int J Cancer 61: 272-279.
- Gehrmann M, Liebisch G, Schmitz G, Anderson R, Steinem C, et al. (2008) Tumor-specific Hsp70 plasma membrane localization is enabled by the glycosphingolipid Gb3. PLoS One 3: e1925.
- Vega VL, Rodríguez-Silva M, Frey T, Gehrmann M, Diaz JC, et al. (2008) Hsp70 translocates into the plasma membrane after stress and is released into the extracellular environment in a membrane-associated form that activates macrophages. J Immunol 180: 4299-4307.
- Gehrmann M, Marienhagen J, Eichholtz-Wirth H, Fritz E, Ellwart J, et al. (2005) Dual function of membrane-bound heat shock protein 70 (Hsp70), Bag-4, and Hsp40: protection against radiation-induced effects and target structure for natural killer cells. Cell Death Differ 12: 38-51.
- Arispe N, Doh M, Simakova O, Kurganov B, De Maio A (2004) Hsc70 and Hsp70 interact with phosphatidylserine on the surface of PC12 cells resulting in a decrease of viability. FASEB J 18: 1636-1645.
- Botzler C, Li G, Issels RD, Multhoff G (1998) Definition of extracellular localized epitopes of Hsp70 involved in an NK immune response. Cell Stress Chaperones 3: 6-11.
- Multhoff G, Hightower LE (2011) Distinguishing integral and receptor-bound heat shock protein 70 (Hsp70) on the cell surface by Hsp70-specific antibodies. Cell Stress Chaperones 16: 251-255.
- Hantschel M, Pfister K, Jordan A, Scholz R, Andreesen R, et al. (2000) Hsp70 plasma membrane expression on primary tumor biopsy material and bone marrow of leukemic patients. Cell Stress Chaperones 5: 438-442.
- Pfister K, Radons J, Busch R, Tidball JG, Pfeifer M, et al. (2007) Patient survival by Hsp70 membrane phenotype: association with different routes of metastasis. Cancer 110: 926-935.
- Gastpar R, Gehrmann M, Bausero MA, Asea A, Gross C, et al. (2005) Heat shock protein 70 surface-positive tumor exosomes stimulate migratory and cytolytic activity of natural killer cells. Cancer Res 65: 5238-5247.
- 16. De Maio A (2011) Extracellular heat shock proteins, cellular export vesicles, and the Stress Observation System: a form of communication during injury, infection, and cell damage. It is never known how far a controversial finding will go! Dedicated to FerruccioRitossa. Cell Stress Chaperones 16: 235-249.
- Lichtenauer M, Zimmermann M, Nickl S, Lauten A, Goebel B, et al. (2014) Transient hypoxia leads to increased serum levels of heat shock protein-27, -70 and caspase-cleaved cytokeratin 18. Clin Lab 60: 323-328.
- Lebherz-Eichinger D, Ankersmit HJ, Hacker S, Hetz H, Kimberger O, et al. (2012) HSP27 and HSP70 serum and urine levels in patients suffering from chronic kidney disease. Clin Chim Acta 413: 282-286.
- Fredly H, Reikvam H, Gjertsen BT, Bruserud O (2012) Disease-stabilizing treatment with all-trans retinoic acid and valproic acid in acute myeloid leukemia: serum hsp70 and hsp90 levels and serum cytokine profiles are determined by the disease, patient age, and anti-leukemic treatment. Am J Hematol 87: 368-376.
- Pockley AG, Shepherd J, Corton JM (1998) Detection of heat shock protein 70 (Hsp70) and anti-Hsp70 antibodies in the serum of normal individuals. Immunol Invest 27: 367-377.
- Njemini R, Demanet C, Mets T (2005) Comparison of two ELISAs for the determination of Hsp70 in serum. J Immunol Methods 306: 176-182.
- Bausero MA, Gastpar R, Multhoff G, Asea A (2005) Alternative mechanism by which IFN-gamma enhances tumor recognition: active release of heat shock protein 72. J Immunol 175: 2900-2912.
- 23. Mathivanan S, Ji H, Simpson RJ (2010) Exosomes: extracellular organelles important in intercellular communication. J Proteomics 73: 1907-1920.

- Théry C, Ostrowski M, Segura E (2009) Membrane vesicles as conveyors of immune responses. Nat Rev Immunol 9: 581-593.
- Record M, Subra C, Silvente-Poirot S, Poirot M (2011) Exosomes as intercellular signalosomes and pharmacological effectors. Biochem Pharmacol 81: 1171-1182.
- Anand PK (2010) Exosomal membrane molecules are potent immune response modulators. CommunIntegr Biol 3: 405-408.
- 27. Kharaziha P, Ceder S, Li Q, Panaretakis T (2012) Tumor cell-derived exosomes: a message in a bottle. Biochim Biophys Acta 1826: 103-111.
- Botzler C, Schmidt J, Luz A, Jennen L, Issels R, et al. (1998) Differential Hsp70 plasma-membrane expression on primary human tumors and metastases in mice with severe combined immunodeficiency. Int J Cancer 77: 942-948.
- Stangl S, Gehrmann M, Riegger J, Kuhs K, Riederer I, et al. (2011) Targeting membrane heat-shock protein 70 (Hsp70) on tumors by cmHsp70.1 antibody. Proc Natl Acad Sci USA 108: 733-738.
- Kahn SE, Watkins BF, Bermes EW Jr (1981) An evaluation of a spectrophotometric scanning technique for measurement of plasma hemoglobin. Ann Clin Lab Sci 11: 126-131.
- 31. Schilling D, Gehrmann M, Steinem C, De Maio A, Pockley AG, et al. (2009) Binding of heat shock protein 70 to extracellular phosphatidylserine promotes killing of normoxic and hypoxic tumor cells. FASEB J 23: 2467-2477.
- Armbruster DA, Pry T (2008) Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev 29 Suppl 1: S49-52.
- 33. Wenk RE (1998) Mechanism of interference by hemolysis in immunoassays and requirements for sample quality. Clin Chem 44: 2554.
- Madu CO, Lu Y (2010) Novel diagnostic biomarkers for prostate cancer. J Cancer 1: 150-177.
- 35. HiralesCasillas CE, Flores Fernández JM, Padilla Camberos E, Herrera López EJ, Leal Pacheco G, et al. (2014) Current status of circulating protein biomarkers to aid the early detection of lung cancer. Future Oncol 10: 1501-1513.
- Alvarez-Chaver P, Otero-Estévez O, Páez de la Cadena M, Rodríguez-Berrocal FJ, Martínez-Zorzano VS (2014) Proteomics for discovery of candidate colorectal cancer biomarkers. World J Gastroenterol 20: 3804-3824.
- 37. Loeb S (2014) Guideline of guidelines: prostate cancer screening. BJU Int 114: 323-325.
- Grunnet M, Sorensen JB (2012) Carcinoembryonic antigen (CEA) as tumor marker in lung cancer. Lung Cancer 76: 138-143.
- Hayes JH, Barry MJ (2014) Screening for prostate cancer with the prostatespecific antigen test: a review of current evidence. JAMA 311: 1143-1149.
- 40. Pockley AG, Henderson B, and Multhoff G (in press) Extracellular cell stress proteins as biomarkers of human disease. Biochem Soc Trans
- Mambula SS, Calderwood SK (2006) Heat shock protein 70 is secreted from tumor cells by a nonclassical pathway involving lysosomal endosomes. J Immunol 177: 7849-7857.
- 42. Broquet AH, Thomas G, Masliah J, Trugnan G, Bachelet M (2003) Expression of the molecular chaperone Hsp70 in detergent-resistant microdomains correlates with its membrane delivery and release. J Biol Chem 278: 21601-21606.
- 43. Schildkopf P, Frey B, Ott OJ, Rubner Y, Multhoff G, et al. (2011) Radiation combined with hyperthermia induces HSP70-dependent maturation of dendritic cells and release of pro-inflammatory cytokines by dendritic cells and macrophages. Radiother Oncol 101: 109-115.
- 44. Brown DA, London E (2000) Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J Biol Chem 275: 17221-17224.

**Citation:** Breuninger S, Erl J, Knape C, Gunther S, Regel I, et al. (2014) Quantitative Analysis of Liposomal Heat Shock Protein 70 (Hsp70) in the Blood of Tumor Patients Using a Novel LipHsp70 ELISA. J Clin Cell Immunol 5: 264. doi:10.4172/2155-9899.1000264