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Expression Analysis of Heat Shock Proteins in Gastrointestinal Cancer

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ABSTRACT

Heat Shock Proteins (HSP) are highly conserved proteins that act as molecular chaperones, thereby controlling the proper folding of other proteins which contributes to maintaining cellular homeostasis and function. Deregulation of various HSPs has been shown to occur in various malignancies. In the present thesis the impact of the expression of the Heat Shock Proteins HSP27, HSP60, HSP70 and HSP90 in a large collective of primary resected gastrointestinal adenocarcinomas is investigated. The expression of the HSPs was determined by immunohistochemistry on a tissue microarray containing 108 esophageal adenocarcinomas, 348 gastric carcinomas and 355 colon carcinomas. The results of the immunohistochemical stainings were correlated with pathologic features (pT, pN category, distant metastases and grading) as well as patient´s survival. The observed expression patterns were variable ranging from negative to high. In esophageal adenocarcinomas, there was no association with HSP expression and clinicopathologic features. In gastric and colon carcinomas, the most notable findings were that high HSP90 expression was associated with a less aggressive tumor behavior. In contrast, high HSP27 and HSP70 expression were associated with worse clinical outcome in colon cancer, which was also independent from other pathologic factors in multivariate analysis. In conclusion, this thesis demonstrates that Heat Shock Proteins play a role in gastrointestinal adenocarcinomas, especially with regard to tumor biology which may also have impact on patients' outcome. Besides a potential usage as prognostic biomarkers, targeted therapies against specific Heat Shock Proteins or modulating the function of these molecular chaperones may be an interesting approach for alternative treatment of these tumors.

ZUSAMMENFASSUNG

Heat Shock Proteine (HSP) sind eine Familie hoch konservierter Proteine. Sie sind molekulare Chaperone, die die richtige Faltung anderer Proteine sicherstellen und dadurch Homöostase und Funktion der Zelle mit aufrechterhalten. Eine Deregulierung verschiedener HSPs wurde bereits für verschiedene Malignome nachgewiesen. In der vorliegenden Dissertationsschrift wurde der Einfluss der Expression von Heat Shock Protein HSP27, HSP60, HSP70 und HSP90 in einem großen Kollektiv primär resezierter, gastrointestinaler Adenokarzinome untersucht. Die Expression der HSPs wurde immunhistochemisch auf einem Tissue Micro Array aus 108 Ösophaguskarzinomen, 348 Magenkarzinomen und 355 Kolonkarzinomen bestimmt. Die Ergebnisse der immunhistochemischen Färbung wurden mit pathologischen Merkmalen (pT, pN Kategorie, Fernmetastasen und Grading) sowie dem Überleben der Patienten korreliert. Die beobachteten Expressionsmuster erstreckten sich von negativ bis hoch. Bei den Adenokarzinomen des Ösophagus bestand keine Assoziation zwischen HSP Expression und klinisch-pathologischen Merkmalen. Bei den Magen- und Kolonkarzinomen waren die bemerkenswertesten Ergebnisse, dass hohe HSP90 Expression mit weniger aggressivem Tumorwachstum verbunden war und im Gegensatz dazu hohe HSP27 und HSP70 Expression mit schlechterem klinischem Outcome bei Kolonkarzinomen assoziiert war. Dieses Ergebnis war in der multivariaten Analyse zusätzlich unabhängig von anderen pathologischen Merkmalen. Zusammenfassend konnte nachgewiesen werden, dass Heat Shock Proteine eine entscheidende Rolle in gastrointestinalen Karzinomen spielen, besonders in Hinblick auf die Tumorbiologie, was einen Einfluss auf das Outcome für die Patienten haben könnte. Neben einem möglichen Nutzen als prognostische Biomarker, könnten gezielte Therapien gegen spezifische Heat Shock Proteine oder die Modulation der Funktion dieser molekularen Chaperone interessante Ansätze für alternative Behandlungsverfahren bei diesen Tumoren darstellen.

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LIST OF ABBREVIATIONS

17-AAG	17-*N*-Allylamino-17-demethoxygeldanamycin
Apaf-1	Apoptosis protease-activating factor 1
ATP	Adenosine Triphosphate
CA	California
CI	Confidence Interval
DK	Denmark
DNA	Deoxyribonucleic acid
dest.	destilled
E-cadherin	epithelial cadherin
et al.	et alii
e.g.	exempli gratia; for example
FFPE	Formalin-fixed paraffin embedded (tissue)
G	Grading
gp96	glycoprotein 96
GRP	Glucose-Regulated Protein
HSF	Heat Shock Factor
HSP	Heat Shock Protein
IHC	Immunohistochemistry
IL	Illinois
Inc.	Incorporated
IRS	Immuno Reactivity Score
kDa	kiloDalton
M	Metastasis
MA	Massachusetts
MAGIC	Medical Research Council Adjuvant Gastric Infusional Chemotherapie
N	Node
p	p-value (p = probability)
PCR	Polymerase Chain Reaction
pH	Pondus Hydrogenii
pHSP	Phosphorylated Heat Shock Protein
pTNM	Pathological Tumor Node Metastasis (Classification for Tumor Staging)

R	Resection (Status)
RPPA	Reverse Phase Protein Array
SI	Staining Index
TMA	Tissue Micro Array
TNM	Tumor Node Metastasis
TRAIL	TNF related apoptosis inducing agent
UICC	Union Internationale Contre le Cancer
UK	United Kingdom
USA	United States of America
X^2	Chi-Square

1 INTRODUCTION

1.1 Heat Shock Proteins

Heat Shock Proteins (HSPs) are a class of highly conserved proteins that are expressed ubiquitarily in prokaryotic as well as in most compartments of eukaryotic cells. They are so called molecular chaperons. Hendrik et al. (1993: 354) describe a chaperon

> "*as a protein that binds to and stabilizes an otherwise unstable conformer of another protein and by controlled binding and release of the substrate protein, facilitates its correct fate in vivo: be it folding, oligomeric assembly, transport to a particular subcellular compartment, or controlled switching between active/inactive conformations.*"

In order to convert newly translated or denatured proteins to their active configuration form, HSPs are able to bind hydrophobic parts of the peptide sequence and release it in a controlled way, through an ATP-dependent process (Hartl 1996).

Heat Shock Proteins are separated into families following their approximate mass in kilo-Dalton (kDa). Major families are HSP100, HSP90, HSP70, HSP60, HSP40 and small Heat Shock Proteins. In each family there are constitutively expressed, as well as inducible forms (Jolly & Morimoto 2000).

Under stressful conditions, such as exposure to heat, Heat Shock Factors (HSFs) form trimers and bind to certain promoter regions of the DNA (Heat Shock Elements). This leads to rapidly increased transcription of HSP genes (Morimoto 1993).

In addition to the promotion of the reconversion of denatured proteins into their active form, HSP70 and HSP27 have antiapoptotic properties.

There are two different pathways for the induction of apoptosis. The extrinsic way is entered, when plasma membrane receptors of the TNF family are activated. This leads to direct activation of the caspases. Cytosolic stress signals lead to the intrinsic pathway of apoptosis, where the permeability of the outer mitochondrial membrane and the release of proapoptotic cytochrome c play a major role and lead through the formation of the apoptosome with apoptosis protease-activating factor-1 (Apaf-1) and procaspase 9 to controlled cell death. Different HSPs are able to manipulate the intrinsic pathway of apoptosis. For example, HSP 70 is able to inhibit the permeabilisation of the mitochondrial membrane and interfere with Apaf-1, while HSP27 impedes the liberation of cytochrome c (Garrido, et al. 2006, Garrido, et al. 2001).

In malignant cells, it is suggested that high expression levels of HSPs might lead to better resistance to physiologic stressors as well as chemotherapy and radiation. Indeed, overexpression of different families of Heat Shock Proteins have been shown for many different malignancies (Ciocca & Calderwood 2005).

1.2 Adenocarcinoma of the Esophagus

There are two main histological types of esophageal cancer: squamous-cell and adenocarcinoma. While incidence rates for squamous cell carcinoma are levelling or even decreasing in most parts of Europe in recent years, the incidence rate of esophageal adenocarcinoma is rising, and now outnumbers that of squamous-cell carcinoma in several countries (Bosetti, et al. 2008).

Reflux associated Barrett's metaplasia is considered as precursor of esophageal adenocarcinomas, but obesity and smoking seems to lead to a higher risk of tumor development, while a high intake of fruits and fibers as well as an infection with Helicobacter pylori appear to play a protective role (Pera, et al. 2005, Reid, et al. 2010).

Early-stage (T1a) adenocarcinoma has a very good prognosis and can be cured by endoscopic resection. However, most patients present with an already advanced disease have a far worse prognosis. Currently, locally advanced tumors are treated by a multimodal therapeutical concept including surgical resection and perioperative chemotherapy or radiochemotherapy. Main prognostic factors for those cases is the number of lymph nodes affected and the resection status (Behrens, et al. 2011).

1.3 Gastric cancer

Gastric cancer is particularly common in Eastern Asia. Although incidence rates are declining, it still accounts for 10% of overall cancer deaths (Jemal, et al. 2011).

Besides age and genetic factors, main risk factors for gastric cancer are Helicobacter pylori infection and dietary factors. High intake of dried, processed or salted meat and fish, pickled or smoked food as well as refined carbohydrates increases the risk of tumor development, while consumption of fresh fruits, vegetables and fibers leads to lower contraction rates (Catenacci, et al. 2011).

Prognosis of gastric cancer is mostly dependent on the operability of the tumor, represented by the Tumor Node Metastasis (TNM) classification of the Union Internationale Contre le Cancer (UICC). Another commonly used histologic classification system only used for gastric cancer is the Laurén classification, which classifies the tumor as intestinal, diffuse

or mixed type according to its growing habits. R0 surgery offers the best survival rates. Studies relating to the usefulness of neoadjuvant as well as adjuvant chemotherapy and radiation are currently executed. Although, recently concluded randomized trials, like the MAGIC (The Medical Research Council Adjuvant Gastric Infusional Chemotherapy) trial, show a survival benefit for perioperative chemotherapy in selected stages, overall survival is still poor due to the mostly advanced stages at time of diagnosis, as Blum et al. (2013) show in their review. They conclude that "*[s]tudies of molecular biology are highly encouraged to improve therapy and risk-stratification*" (Blum, et al. 2013: 269).

Similar to esophageal adenocarcinomas, alternative treatment strategies are highly demanded, since the percentage of patients who respond to conventional chemotherapy is rather low. The introduction of Her2-targeting treatment has been shown to prolong survival of patients with metastatic gastric cancer (Bang, et al. 2010). There is hope that development of further molecular based targeting and individualized treatment will lead to further improvement of prognosis of patients with this disease.

1.4 Colon cancer

Even though the death rate decreased in recent years because of screening programs and improvements in therapy, colorectal cancer is still one of the most common malignancies in western countries (Jemal, et al. 2011). Worldwide it accounted for 1.2 million cases and more than 600 000 deaths in 2008 (Ferlay, et al. 2010, Jemal, et al. 2011).

Modifiable risk factors for colorectal cancer include high intake of red meat, alcohol and smoking as well as low consumption levels of fresh fruit and vegetables, obesity and low physical activity (Chan & Giovannucci 2010).

The therapeutic concepts for colon and rectal cancer mainly base upon the disease stage ranging from surgery alone for early stage carcinomas to multimodal treatment in advanced stages (Catenacci, et al. 2011). The UICC staging (Sobin 2010) represents the most widely used categorization system, which bases on clinicopathological properties, such as tumor size, lymphatic node and organ metastases. However, there are considerable differences in a patients' outcome even within one single stage. Thus, for some cases conventional staging systems fail to predict accurately the prognosis of the patients (Mutch 2007).

In the last years, knowledge about the molecular genetics of colorectal cancer has increased significantly. Identification of molecules which may serve as prognostic and predictive biomarkers would greatly help to avoid toxic side effects and unnecessary costs caused by a better selection of patients for individualized therapeutic approaches in addition

or instead of the currently used stage based stratification process (Tejpar, et al. 2010). Furthermore, the design of specific drugs against those molecules may help to develop targeted therapeutic approaches.

1.5 Immunohistochemistry and Tissue Microarray

The expression profiles of the different HSPs in this thesis have been evaluated using immunohistochemical staining on microarrays of the different gastrointestinal carcinoma tissues. Immunohistochemistry (IHC) is a widely used technology to make the expression of certain proteins visible. Known problems are the semiquantitative nature of the technology and often observed inter observer variability. Nonetheless, IHC has found its way into clinical practice as well as research routine especially concerning cancer research.

To construct a tissue micro array (TMA) certain areas of interest (in this case cancer tissue in whole archival tissue sections) are first marked on HE slides of tissue sections, then core biopsies (in this study 0.6mm diameter) are retrieved of the donor tissues blocks and placed into a recipient wax block. In this way large TMAs containing samples of up to hundreds of different tissues can be manufactured, each containing not only the tissue sections in question but also negative and positive controls.

Obvious advantages of this technology are time and cost efficiency, as well as the possibility to use only small sections of the archival tissue, leaving the rest to possible future research. Additionally, many slides can be cut out of a single TMA-block, making further research, using the same tissue samples, simple and fast.

Further advantages of the TMA method in combination with IHC are, that all tissue specimen observed, as well as controls are on the same slide, making sure the antibody staining conditions are identical (Avninder, et al. 2008, Kallioniemi, et al. 2001).

An often voiced critic on TMA studies is whether the examination of small samples are exemplary for the rest of the tissue section, especially concerning intratumoral heterogeneity. But, as Kallioniemi, et al. (2001) state in their review: the aim in research is to gain relative, population-based frequencies of expression, not absolut information in order to form diagnosis and therapy strategy for a single case. Furthermore, Torhorst, et al. (2001: 2249) conclude, after they have evaluated four different TMAs and the whole tissue sections of 553 breast carcinomas immunohistochemically for expression of three different biomarkers, that *"contrary to expectations, tissue heterogeneity did not negatively influence the predictive power of the TMA results."*

1.6 Outline

Overall, this dissertation aims to evaluate the impact of the expression of the currently best characterized Heat Shock Proteins pHSP27, HSP27, HSP60, HSP70 and HSP90 on gastrointestinal malignancies.

The expression of the HSPs was evaluated by immunohistochemistry applied on tissue microarrays (TMAs) from samples of colon cancer, gastric cancer or adenocarcinoma of the esophagus assembled of 811 patients. The expression levels were correlated with clinicopathological parameters (UICC TNM classification and tumor grading) and patients' survival.

In short, this dissertation finds that in esophageal adenocarcinomas, there was no association with HSP expression and clinicopathologic features. In gastric and colon carcinomas, the most notable findings were that high HSP90 expression was associated with a less advanced tumor stage. In contrast, high HSP27 and HSP70 expression were associated with worse clinical outcome in colon cancer, which was also independent from other pathologic factors in multivariate analysis. This thesis therefore demonstrates that Heat Shock Proteins play a role in gastrointestinal adenocarcinomas, especially with regard to tumor biology which may also have impact on patients' outcome. Besides a potential usage as prognostic biomarkers, targeted therapies against specific Heat Shock Proteins or modulating the function of these molecular chaperones may be an interesting approach for alternative treatment of these tumors.

This thesis will proceed as follows. Chapter 2 presents the materials and methods used for this study. Chapter 3 will present results for the impact of the expression of pHSP27, HSP27, HSP60 and HSP70 on gastrointestinal malignancies. Chapter 4 discusses the findings while Chapter 5 concludes.

2 MATERIALS AND METHODS

2.1 Patients and Tissues

Formalin fixed, paraffin embedded archival cancer tissues from 355 patients with colon carcinoma, who underwent surgery between 1993 and 2005 at the Klinikum rechts der Isar of the Technische Universität München (Germany), from 348 patients with gastric carcinoma or carcinoma of the gastroesophageal junction, with primary resection between 1995 and 2005 as well at the Klinikum rechts der Isar of the Technische Universität München (Germany) and 108 patients with adenocarcinomas of the esophagus, operated between 1991 and 2006 at the same hospital, were investigated.

All patients had a primary resection without neoadjuvant radio- or chemotherapy and gave informed consent for the use of additional molecular analysis at the time of operation. The use of archival tissue for molecular analysis was approved by the Ethics Commission of the Faculty of Medicine of Technische Universität München (No. 2136/08 and 2056/08).

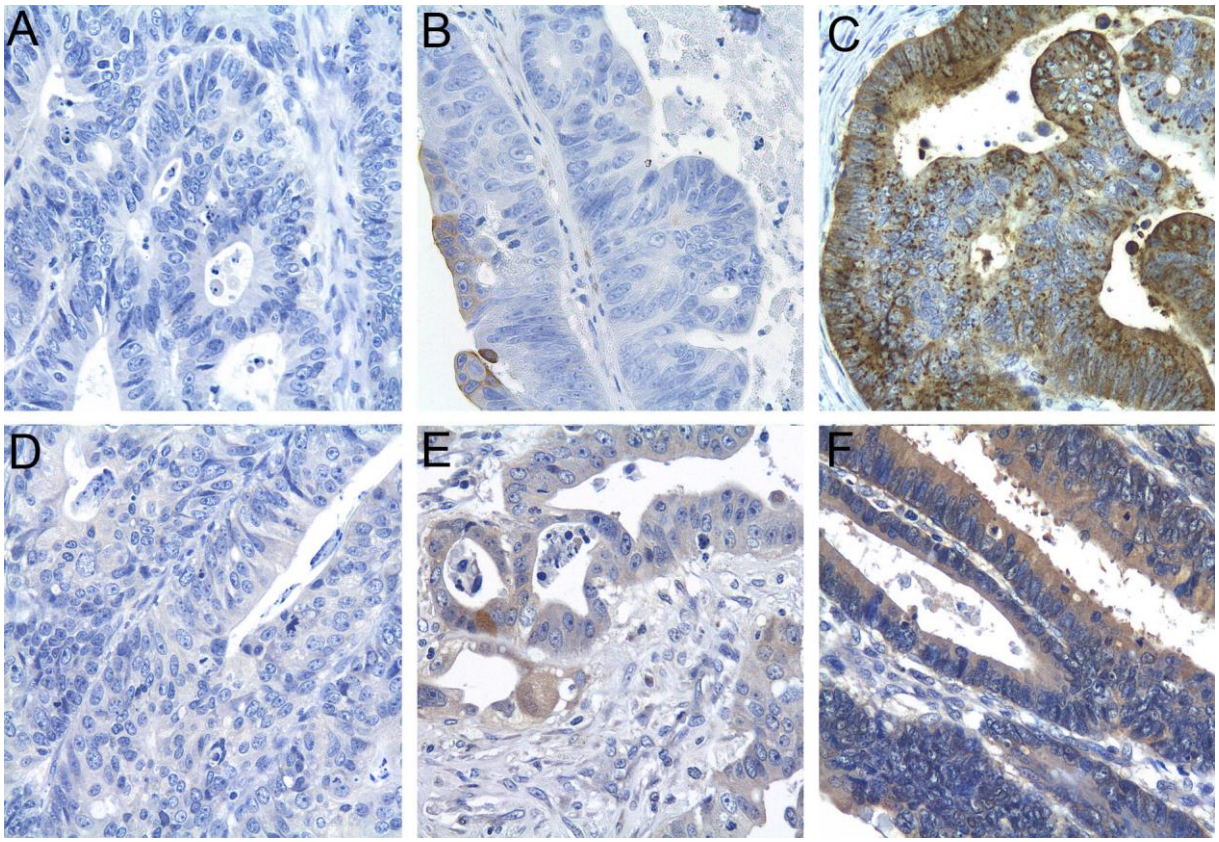
2.2 Immunohistochemistry

For the analysis of the expression of the HSPs, formalin fixed, paraffin embedded (FFPE) tissue was used. The resection specimens were opened immediately after surgery and fixed for at least 24 hours. Tissue microarrays were already available for esophageal adenocarcinomas and gastric carcinomas. For colon cancer, a tissue microarray was newly constructed for the purpose of this study: representative tumor sections were marked and a core needle biopsy specimen of 0.6 mm diameter was retrieved and placed in a recipient wax block by using a manual arrayer (Beecher Instruments; Sun Prairie, Wisconsin, USA).

For immunohistochemistry the paraffin blocks were freshly cut (3µm). After dewaxation and rehydration, heat-induced antigen retrieval using 10 mM citrate buffer, pH 6, was performed. Subsequent to H_2O_2 blocking using 3% H_2O_2 in aqua dest., as well as avidin biotin blocking (Avidin/Biotin Blocking Kit; Vector Laboratories, Inc., Burlingame, CA, USA), the sections were incubated with antibodies for HSP90 (mouse monoclonal; dilution 1:100; Abcam, Cambridge, UK), HSP70 (mouse monoclonal; prediluted; dilution 1:1; Abcam, Cambridge, UK), HSP60 (rabbit polyclonal; dilution 1:2000; Abcam, Cambridge, UK), HSP27 (mouse monoclonal; dilution 1:250; Cell Signaling Technology, Inc., Boston, MA, USA), pHSP 27^{Ser78} (rabbit polyclonal; dilution 1:500; Cell Signaling, Inc., Boston, MA, USA), pHSP 27^{Ser82} (rabbit polyclonal; dilution 1:100; Cell Signaling, Inc., Boston, MA, USA) or pHSP 27^{S15} (rabbit polyclonal; dilution 1:500; Abcam Cambridge, UK), followed by secondary biotinylated antibody. Immunodetection was performed with the Dako REAL™

Detection System Peroxidase/DAB+ kit (Dako, Glostrup, DK). Appropriate positive and negative controls were included in each reaction.

Figure 1 Examples of immunohistochemical stainings in colon carcinomas



Note: ***a*** *Low HSP27 expression (immunoreactivity score IRS=0);* ***b*** *Moderate HSP27 expression (IRS=2)* ***c*** *High HSP27 expression (IRS=9);* ***d*** *Low HSP70 expression (IRS=0)* ***e*** *Moderate HSP70 expression (IRS=3);* ***f*** *High HSP70 expression (IRS=8); Magnification x200. This figure was first published in Bauer, et al. (2012).*

As presented in Figure 1 above, cytoplasmic HSP expression was assessed based on the intensity of immunostaining and the percentage of stained tumor cells. The intensity was scored as 0 (no immunostaining), 1 (weak immunostaining), 2 (moderate immunostaining) or 3 (strong immunostaining). The percentage of stained tumor cells was scored as 0 (none), 1 (<10%), 2 (10-50%), 3 (51-80%) or 4 (>80%). Multiplication of the scores for intensity and percentage resulted in a staining index (SI) ranging from 0 to 12. Depending on the distribution of the staining index (terciles), the expression of HSPs was divided into low – moderate – high: For HSP27 staining index of 0 was defined as low, of 1-2 as moderate and 3-12 as high (Figure 1 A-C). Concerning phosphorylated HSP for $pHSP27^{S15}$ staining index of 0 was defined as low, 1-2 as moderate and 3-12 as high. For $pHSP27^{Ser78}$ and $pHSP^{27Ser82}$ only two groups were built: 0 as staining negative and 1 as staining positive. For HSP70, a

staining index of 0-1 was defined as low, a staining index of 2-3 moderate, of 4-12 as high (Figure 1 D-F). For HSP 60 expression staining index of 0-4 was defined as low, of 6 as moderate and of 8-12 as high. For HSP 90 expression a staining index of 0 was defined as low, of 1-3 as moderate and of 4-12 as high for colon carcinomas and adenocarcinomas of the esophagus, while for gastric carcinoma/carcinoma of the gastroesophageal junction only two groups were built: negative staining for less than 10% of stained cells and positive (Bauer, et al. 2012, Slotta-Huspenina, et al. 2012).

As noted in Bauer, et al. (2012: 199), to ensure the reliability of the staining results obtained from TMAs of HSP70, HSP60 and HSP27 immunohistochemical stainings of whole tumor sections with corresponding TMA cores were compared. For that purpose, a test-TMA was constructed from 28 gastrointestinal tumors (four esophageal squamous cell carcinomas, four esophageal adenocarcinomas, ten gastric carcinomas and ten colon carcinomas) which contained three cores of each tumor for comparison of the staining results of the three TMA cores with each other and with the corresponding whole tissue sections. From every specimen three tissue spots from different parts of the tumor were separately analysed. No differences at conventional levels of significance were revealed in the staining results between the three TMA cores of the test-TMA for all HSPs investigated (see Table 1). Given these homogenous staining patterns, it can be concluded for the present study to proceed with the chosen research design and to conduct as suggested the analysis of the large TMA of carcinoma samples for HSP27 and pHSP27 (pHSP27^{S15}, pHSP27^{Ser78}, pHSP27^{Ser82}), HSP60, HSP70 and HSP90.

Table 1 Test-TMA on intratumoral heterogeneity

HSP	Chi^2	Asymp. significance	N
HSP90	1.000	0.607	28
HSP70	0.877	0.645	27
HSP60	1.647	0.439	20
HSP27	3.304	0.192	26
pHSP27^{S15}	0.043	0.979	19
pHSP27^{Ser78}	2.000	0.368	16
pHSP27^{Ser82}	2.480	0.289	14

Note: Friedman test for differences between sets of three test-TMA cores.

2.3 Statistical Analysis

IBM SPSS Statistics 19.0 to 21.0 (SPSS Inc., Chicago, IL, USA) statistical software was used for statistical analysis. Associations between immunohistochemical expression patterns and pathological features were given in crosstabs and were evaluated with a χ^2-test. Correlations between staining results were determined by Spearman's correlation analysis. Differences between the test-tissue microarrays were analyzed using the Friedman test. Survival analysis was performed using Kaplan-Meier estimates, log rank tests and Cox's proportional hazards regression analysis (Held, et al. 2013, Weiß 2010). All tests were 2-sided, and the significance level was set at 5%.

3 RESULTS

In the following section, the impact of the expression of HSP90, HSP70, HSP27 and pHSP27 on gastrointestinal malignancies is reported, first for adenocarcinoma of the esophagus (Chapter 3.1), then for gastric cancer (Chapter 3.2) and for colon cancer (Chapter 3.3). The chapters each commence with a subchapter containing the description of patients and pathological findings, followed by a subchapter that presents HSP expression patterns and the correlation between expression patterns of different HSPs, a subchapter presenting the association between clinicopathological parameters and HSP expression levels following χ^2-tests and, finally, a subchapter presenting the association between patient survival and HSP expression following Kaplan-Meier estimates and multivariate Cox regression analysis.

3.1 Adenocarcinoma of the Esophagus

3.1.1 Patients and pathological findings

Table 2 Clinicopathological parameters in patients with adenocarcinoma of the esophagus

Sex	Male	%	Female	%		Total
	100	*92,6*	8	*7,4*		108
pT category	pT1	%	pT2	%	pT3/4	%	Total
	43	*39,8*	21	*19,4*	44	*40,7*	108
Lymph node metastases	Absent	%	Present	%			Total
	56	*51,9*	52	*48,1*			108
Distant metastases	Absent	%	Present	%			Total
	99	*91,7*	9	*8,3*			108
Grading	G1	%	G2	%	G3/4	%	Total
	5	*4,7*	45	*42,1*	57	*53,3*	107
Resection status	Complete	%	Incomplete	%			Total
	92	*85,2*	16	*14,8*			108

Note: Distribution of clinicopathological parameters in patients with adenocarcinoma of the esophagus and percentages of total cohort.

Table 2 above presents clinicopathological parameters in patients with adenocarcinoma of the esophagus: 100 (92.6%) patients were male, only eight (7.4%) female. Mean age at the time of resection was 64±10 years, with a range from 38 to 83 years (median: 65 years). Tumor grading was available only in 107 of 108 cases. 5 (4.7%) tumors were graded as G1 (well differentiated), 45 (42.1%) as G2 (moderately differentiated) and 57 (53.3%) as G3-4 (poorly differentiated). All tumors were classified according to UICCs TNM-classification current at time of operation (Sobin & Compton 2010). 43 (39.8%) tumors were classified as pT1, 21 (19.4%) as pT2 and 44 (40.7%) as pT3. Lymph node metastases were present in 52 (48.1%) cases and distant metastases in 9 (8.3%) cases. Complete resection, was achieved in 92

(85.2%) cases. Mean overall survival time was 39.6 months (95%-KI: 26.2-52.9; range: 0 to 164 months).

3.1.2 HSP expression

For HSP90 106 of the tumor samples were evaluable: 26 (24.5%) showed low, 34 (32.1%) moderate and 46 (43.4%) high staining intensity (see as well Table 4). For HSP70 106 tumors could be evaluated: 63 (59.4%) were classified as low, 31 (29.2%) as moderate and 12 (11.3%) as showing high staining results (see Table 5). 104 tumors could be analyzed for HSP60: 72 (69.2%) showed low, 20 (19.2%) moderate and 12 (11.5%) high staining intensity (see Table 6). For HSP27, 107 could be analyzed: 34 (31.8%) showed low, 36 (33.6%) moderate and 37 (34.6%) high staining results (see Table 7). For pHSP27^{S15} 106 cases were evaluable: 71 (67.0%) showed low, 28 (26.4%) moderate and 7 (6.6%) high staining results. For pHSP27^{Ser78} and pHSP27^{Ser82}, 106 and respectively 105 of the tumors could be analyzed: only one (0.9%) was positive for pHSP27^{Ser78}, while 12 (11.4%) showed positive staining for pHSP27^{Ser82} (see Table 8).

Table 3 below presents the correlation between expression patterns of different HSPs in adenocarcinomas of the esophagus: Significant correlations between HSP expression levels were only present for HSP90 and HSP60 (p=0.042). Significant correlation between HSP expression and pHSP expression were seen for: pHSP27^{Ser82} with HSP90 (p=0.010) and HSP27 (p=0.001), as well as pHSP27^{S15} with HSP70 (p=0.004) and HSP27 (p=0.002). Significant intercorrelation between different pHSPs were seen for pHSP27^{S15} , pHSP27^{Ser82} (p<0.001), pHSP27^{Ser78} and pHSP27^{Ser82} (p=0.005), but for pHSP27^{S15} and pHSP27^{Ser78} the correlation did just not reach statistical significance.

Table 3 Correlation between expression patterns of different HSPs in adenocarcinomas of the esophagus

p-values	HSP90	HSP70	HSP60	HSP27	pHSP27^{S15}	pHSP27^{Ser78}	pHSP27^{Ser82}
HSP90		0.473	0.042*	0.167	0.052	0.294	0.010**
HSP70	0.473		0.600	0.674	0.004**	0.421	0.464
HSP60	0.042*	0.600		0.998	0.914	0.060	0.276
HSP27	0.167	0.674	0.998		0.002**	0.233	0.001**
pHSP27^{S15}	0.052	0.004**	0.914	0.002**		0.050	<0.001**
pHSP27^{Ser78}	0.294	0.421	0.060	0.233	0.050		0.005**
pHSP27^{Ser82}	0.010**	0.464	0.276	0.001**	<0.001**	0.005**	

*Note: P-values following Spearman's correlation analysis (2-sided), with * (**) indicating significance at 5% (1%) level.*

3.1.3 Association between clinicopathological parameters and HSP expression

HSP expression levels were correlated with the clinicopathological parameters pT category, lymph node metastases, distant metastases and grading as shown in Table 3 to 7. Table 4 presents HSP90 expression in adenocarcinomas of the esophagus and pathological parameters, Table 5 HSP70 expression, Table 6 HSP60 expression, Table 7 HSP27 expression and Table 8 pHSP27 expression.

The only significant association observed links high HSP60 expression and high pT category ($p=0.028$) (Table 6). While the other associations did not reach conventional levels of significance, the association between high HSP27 expression and high pT category came close ($p=0.052$).

Table 4 HSP90 expression in adenocarcinomas of the esophagus and pathological parameters

	HSP90				
	Low	*Moderate*	*High*	*Total*	*P-value (χ^2)*
pT Category					
pT1	9	12	21	42	0.091
pT2	4	4	13	21	
pT3-4	13	18	12	43	
Total	26	34	46	106	
Lymph node metastases					
Absent	14	13	27	54	0.184
Present	12	21	19	52	
Total	26	34	46	106	
Distant metastases					
Absent	25	30	42	97	0.551
Present	1	4	4	9	
Total	26	34	46	106	
Grading					
G1	3	1	1	5	0.094
G2	13	10	22	45	
G3-4	10	23	23	56	
Total	26	34	46	106	

Note: Crosstabs of clinicopathological parameters and HSP90 expression with p-values from a χ^2-test.

Table 5 HSP70 expression in adenocarcinomas of the esophagus and pathological parameters

	HSP70				
	Negative/low	*Moderate*	*High*	*Total*	*P-value (χ^2)*
pT Category					
pT1	27	9	6	42	0.683
pT2	12	7	2	21	
pT3-4	24	15	4	43	
Total	63	31	12	106	
Lymph node metastases					
Absent	32	17	5	54	0.740
Present	31	14	7	52	
Total	63	31	12	106	
Distant metastases					
Absent	58	29	10	97	0.542
Present	5	2	2	9	
Total	63	31	12	106	
Grading					
G1	2	3	0	5	0.565
G2	25	13	6	44	
G3-4	35	15	6	56	
Total	62	31	12	105	

Note: Crosstabs of clinicopathological parameters and HSP70 expression with p-values from a χ^2-test.

Table 6 HSP60 expression in adenocarcinomas of the esophagus and pathological parameters

	HSP60				
	Negative/low	*Moderate*	*High*	*Total*	*P-value (χ^2)*
pT Category					
pT1	34	3	3	40	0.028
pT2	11	8	2	21	
pT3-4	27	9	7	43	
Total	82	20	12	104	
Lymph node metastases					
Absent	41	7	5	53	0.175
Present	31	13	7	51	
Total	72	20	12	104	
Distant metastases					
Absent	68	16	11	95	0.127
Present	4	4	1	9	
Total	72	20	12	104	
Grading					
G1	4	0	0	4	0.752
G2	29	9	5	43	
G3-4	38	11	7	56	
Total	71	20	12	103	

Note:Crosstabs of clinicopathological parameters and HSP60 expression with p-values from a χ^2-test.

Table 7 HSP27 expression in adenocarcinomas of the esophagus and pathological parameters

	HSP27				
	Negative/low	*Moderate*	*High*	*Total*	*P-value (χ^2)*
pT Category					
pT1	9	12	21	42	0.052
pT2	10	8	3	21	
pT3-4	15	16	13	44	
Total	34	36	37	107	
Lymph node metastases					
Absent	14	19	22	55	0.299
Present	20	17	15	52	
Total	34	36	37	107	
Distant metastases					
Absent	31	33	34	98	0.994
Present	3	3	3	9	
Total	34	36	37	107	
Grading					
G1	1	2	2	5	0.924
G2	15	13	16	44	
G3-4	17	21	19	57	
Total	33	36	37	106	

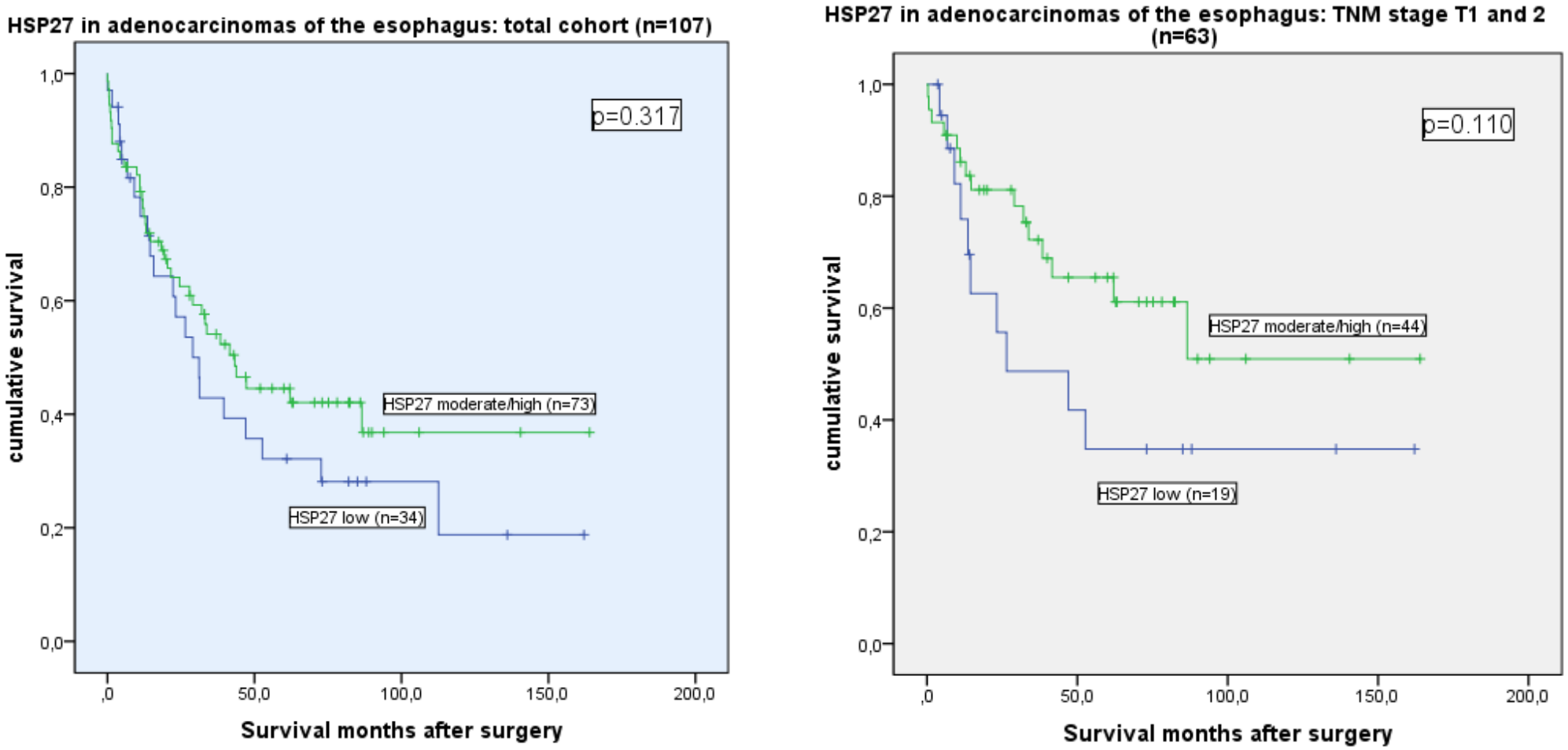
Note:Crosstabs of clinicopathological parameters and HSP27 expression with p-values from a χ^2-test.

Table 8 pHSP27 expression in adenocarcinomas of the esophagus and pathological parameters

	pHSP27^{Ser78}			**pHSP27^{Ser82}**			**pHSP27^{S15}**			
	Negative	*Positive*	*Total*	*Negative*	*Positive*	*Total*	*Negative/Low*	*Moderate*	*High*	*Total*
pT Category										
pT1	42	0	42	35	7	42	23	15	3	41
pT2	20	0	20	20	0	20	18	3	0	21
pT3-4	43	1	44	38	5	43	30	10	4	44
Total	105	1	106	93	12	105	71	28	7	106
Lymph node metastases										
Absent	54	0	54	46	7	53	36	14	4	54
Present	51	1	52	47	5	52	35	14	3	52
Total	105	1	106	93	12	105	71	28	7	106
Distant metastases										
Absent	96	1	97	84	12	96	66	24	7	97
Present	9	0	9	9	0	9	5	4	0	9
Total	105	1	106	93	12	105	71	28	7	106
Grading										
G1	5	0	5	5	0	5	3	1	0	4
G2	42	1	43	38	5	43	27	13	4	44
G3-4	57	0	57	49	7	56	40	14	3	57
Total	104	1	105	92	12	104	70	28	7	105

Note: Crosstabulation of clinicopathological parameters and pHSP27 expression levels. Stars indicate $p<0.05$ from a χ^2-test.

Figure 2 Survival of patients with adenocarcinoma of the esophagus and HSP27 expression levels



Note: Kaplan-Maier survival estimates of patients with adenocarcinoma of the esophagus (left panel: total cohort, n=107; right panel: TNM state T1 and 2, n=63). Cumulative survival of patients with moderate and high HSP27 expression (upper curve) and with low HSP27 expression (lower curve).

3.1.4 Association between patient survival and HSP expression

In a log rank test all clinicopathological parameters were statistically significantly associated with patient survival ($p<0.001$ for pT category, presence of lymph node metastases, and resection status, $p=0.030$ for presence of distant metastases and $p=0.081$ for grading).

Figure 2 above presents Kaplan-Maier estimates for the survival of patients with adenocarcinoma of the esophagus and HSP27 expression levels: A low HSP27 expression level was associated with bad prognosis in Kaplan-Meier estimates, but this did not reach statistical significance ($p=0.317$) (see Figure 2 above, left panel). In subgroup analysis cases with pT category 1 and 2 this association became more pronounced, but still didn´t reach statistical significance ($p=0.110$) (see Figure 2 above, right panel). For other HSP expression levels no association to patient survival could be observed.

3.2 Gastric Cancer

3.2.1 Patients and pathological findings

As can be seen from Table 9 below, 222 (63.8%) of all 348 patients with gastric cancer were male, 126 (36.2%) female. Age at time of operation ranged from 29 to 100 years, with a mean age of 67 ± 12 years (median 69 years).

According to Lauréns classification 154 tumors were of intestinal type, 60 mixed and 111 of diffuse type. 23 tumors were unclassifiable. Only one tumor (0.3%) was graded as G1 (well differentiated), 54 (15.5%) as G2 (moderately differentiated) and 293 (84.2%) as G3-4 (poorly differentiated).

All tumors were reclassified to the current UICC TNM classification (2010) (Sobin & Compton 2010). 25 (7.2%) tumors were classified as T1, 31 (8.9%) as T2, 113 (32.5%) as T3 and 179 (51.4%) as T4. Lymph node metastases were present in 262 (75.3%) cases and distant metastases in 88 (25.3%) cases.

Complete, macroscopic and microscopic resection was achieved in 198 (56.9%) cases. Median overall survival was 19 months (95% confidence interval: 14-23 months; mean overall survival: 45 months, CI: 38-51 months).

Table 9 Clinicopathological parameters in patients with gastric carcinomas

Sex	Male	*%*	Female	*%*			Total
	222	*63.8*	126	*36.2*			348
pT category	pT1	*%*	pT2	*%*	pT3/4	*%*	Total
	25	*7.2*	31	*8.9*	292	*83.9*	348
Lymph node metastases	Absent	*%*	Present	*%*			Total
	79	*23.2*	262	*76.8*			341
Distant metastases	Absent	*%*	Present	*%*			Total
	260	*74.7*	88	*25.3*			348
Grading	G1	*%*	G2	*%*	G3/4	*%*	Total
	1	*0.3*	54	*15.5*	293	*84.2*	348
Resection status	Complete	*%*	Incomplete	*%*			Total
	198	*56.9*	150	*43.1*			348

Note: Distribution of clinicopathological parameters in patients with gastric carcinomas and percentages of total cohort.

3.2.2 HSP expression

For HSP90 it was possible to evaluate 323 cases: 198 (61.3%) showed a negative, 125 (38.7%) a positive staining result (see Table 11). All cases could be analyzed for HSP70 expression: 230 (66.1%) showed a low, 85 (24.4%) a moderate and 33 (9.5%) a high staining intensity (see Table 12). 301 cases were evaluated for HSP60: low staining could be observed in 203 (67.4%) cases, moderate staining in 24 (8.0%) and high staining results in 74 (24.6%) cases (see Table 13). For HSP27 316 cases could be analyzed: 182 (57.6%) with low, 52 (16.5%) with moderate and 82 (25.9%) with high staining intensity (see Table 14). It was possible to evaluate 334 cases for $pHSP27^{S15}$: low staining intensities were present in 149 (44.6%) cases, moderate in 98 (29.3%) and high staining rates in 87 (26.1%) cases. For $pHSP27^{Ser78}$ only 5 (1.5%) out of 333 cases showed positive staining, for $pHSP27^{Ser82}$ 33 (10.0%) out of 331 cases (see Table 15). Table 10 below presents the correlation between expression patterns of different HSPs in gastric carcinomas. The only significant correlations were: between HSP90 and HSP60 ($p<0.001$), between $pHSP27^{S15}$ and HSP60 ($p<0.001$) as well as HSP70 ($p<0.001$) and between $pHSP27^{Ser82}$ and HSP70 ($p=0.007$) as well as HSP27 ($p=0.001$).

Table 10 Correlation between expression patterns of different HSPs in gastric carcinomas

p-values	HSP90	HSP70	HSP60	HSP27	pHSP27^{S15}	pHSP27^{Ser78}	pHSP27^{Ser82}
HSP90		0.855	<0.001**	0.123	0.914	0.953	0.213
HSP70	0.855		0.053	0.083	<0.001**	0.692	0.007**
HSP60	<0.001**	0.053		0.346	<0.001**	0.613	0.402
HSP27	0.123	0.083	0.346		0.109	0.138	0.001**
pHSP27^{S15}	0.914	<0.001**	<0.001**	0.109		0.280	0.153
pHSP27^{Ser78}	0.953	0.692	0.613	0.138	0.280		0.450
pHSP27^{Ser82}	0.213	0.007**	0.402	0.001**	0.153	0.450	

*Note: P-values following Spearman's correlation analysis (2-sided), with * (**) indicating significance at 5% (1%) level.*

3.2.3 Association between clinicopathological parameters and HSP expression

HSP expression levels were correlated with the clinicopathological parameters pT category, lymph node metastases, distant metastases and grading as shown in Table 11-Table 15. Table 11 presents HSP90 expression in gastric carcinomas and pathological parameters, Table 12 HSP70 expression, Table 13 HSP60 expression, Table 14 HSP27 expression and Table 15 pHSP27 expression.

HSP90 expression was significantly associated with all clinicopathological parameters tested. High HSP90 expression was associated with low pT classification ($p<0.001$), absence of lymph node metastases ($p=0.023$), absence of distant metastases ($p=0.011$) and low grading ($p=0.025$). Those correlations taken together seem to suggest that high HSP90 expression levels occur especially in early stage gastric malignancies. High expression levels of HSP70 and HSP60 were both associated with low grading ($p=0.017$ for HSP70 and $p=0.004$ for HSP60). Additionally, high HSP60 expression was associated with absence of lymph node metastases ($p=0.032$). There were no significant associations of HSP27, pHSP27^{Ser78} or pHSP27^{Ser82} with any of the clinicopathological parameters investigated. In contrast pHSP27^{S15} was as well associated with low grading ($p=0.001$). Given that high expression of four different HSPs/pHSPs (HSP 90, HSP70, HSP60 and pHSP27 $^{\mathbf{S15}}$) were associated with highly differentiated gastric malignancies, this might indicate a systematic relationship.

Table 11 HSP90 expression in gastric carcinomas and pathological parameters

	HSP90			
	Negative	*Positive*	*Total*	*P-value (χ^2)*
pT Category				
pT1	4	19	23	0.000
pT2	16	14	30	
pT3-4	178	92	270	
Total	198	125	323	
Lymph node metastases				
Absent	41	40	81	0.023
Present	157	85	242	
Total	198	125	323	
Distant metastases				
Absent	138	103	241	0.011
Present	60	22	82	
Total	198	125	323	
Grading				
G1	1	0	1	0.025
G2	24	29	53	
G3-4	173	96	269	
Total	189	125	323	

Note:Crosstab of clinicopathological parameters and HSP90 expression with p-values from a χ^2-test.

Table 12 HSP70 expression in gastric carcinomas and pathological parameters

	HSP70				
	Negative/low	*Moderate*	*High*	*Total*	*P-value (χ^2)*
pT Category					
pT1	18	4	3	25	0.606
pT2	20	9	2	31	
pT3-4	192	72	28	292	
Total	230	85	33	348	
Lymph node metastases					
Absent	52	23	11	86	0.347
Present	178	62	22	262	
Total	230	85	33	348	
Distant metastases					
Absent	170	64	26	260	0.826
Present	60	21	7	88	
Total	230	85	33	348	
Grading					
G1	1	0	0	1	0.017
G2	25	22	7	54	
G3-4	204	63	26	293	
Total	169	85	33	348	

Note:Crosstab of clinicopathological parameters and HSP70 expression with p-values from a χ^2-test.

Table 13 HSP60 expression in gastric carcinomas and pathological parameters

	HSP60				
	Negative/low	*Moderate*	*High*	*Total*	*P-value (χ^2)*
pT Category					
pT1	13	0	10	23	0.085
pT2	20	2	6	28	
pT3-4	170	22	58	250	
Total	203	24	74	301	
Lymph node metastases					
Absent	48	4	28	80	0.032
Present	155	20	46	221	
Total	203	24	74	301	
Distant metastases					
Absent	155	16	58	229	0.499
Present	48	8	16	72	
Total	203	24	74	301	
Grading					
G1	1	0	0	1	0.004
G2	31	0	23	54	
G3-4	171	24	51	246	
Total	203	24	74	331	

Note:Crosstab of clinicopathological parameters and HSP60 expression with p-values from a χ^2-test.

Table 14 HSP27 expression in gastric carcinomas and pathological parameters

	HSP27				
	Negative/low	*Moderate*	*High*	*Total*	*P-value (χ^2)*
pT Category					
pT1	13	4	6	23	0.473
pT2	15	3	10	28	
pT3-4	154	45	66	265	
Total	182	52	82	316	
Lymph node metastases					
Absent	50	11	18	79	0.494
Present	132	41	64	237	
Total	182	52	82	316	
Distant metastases					
Absent	137	38	61	236	0.947
Present	45	14	21	80	
Total	182	52	82	316	
Grading					
G1	1	0	0	1	0.793
G2	33	9	11	53	
G3-4	148	43	71	262	
Total	182	52	82	316	

Note:Crosstab of clinicopathological parameters and HSP27 expression with p-values from a χ^2-test.

Table 15 pHSP27 expression in gastric carcinomas and pathological parameters

	pHSP27^{Ser78}			**pHSP27^{Ser82}**			**pHSP27^{S15}**			
	Negative	*Positive*	*Total*	*Negative*	*Positive*	*Total*	*Negative/Low*	*Moderate*	*High*	*Total*
pT Category										
pT1	24	0	24	22	2	24	9	10	6	25
pT2	31	0	31	25	5	30	17	5	8	30
pT3-4	273	5	278	251	26	277	123	83	73	279
Total	328	5	333	298	33	331	149	98	87	334
Lymph node metastases										
Absent	81	2	83	75	8	83	31	26	27	84
Present	247	3	250	223	25	248	118	72	60	250
Total	328	4	333	398	33	331	149	98	87	334
Distant metastases										
Absent	244	4	248	222	24	246	109	71	71	251
Present	84	1	85	76	9	85	40	27	16	83
Total	328	5	333	298	33	331	149	98	87	334
Grading										
G1	1	0	1	1	0	1	0*	0*	1*	1*
G2	52	1	53	47	7	54	12*	18*	24*	54*
G3-4	275	4	279	250	26	276	137*	80*	62*	279*
Total	328	5	333	298	33	331	149*	98*	87*	334*

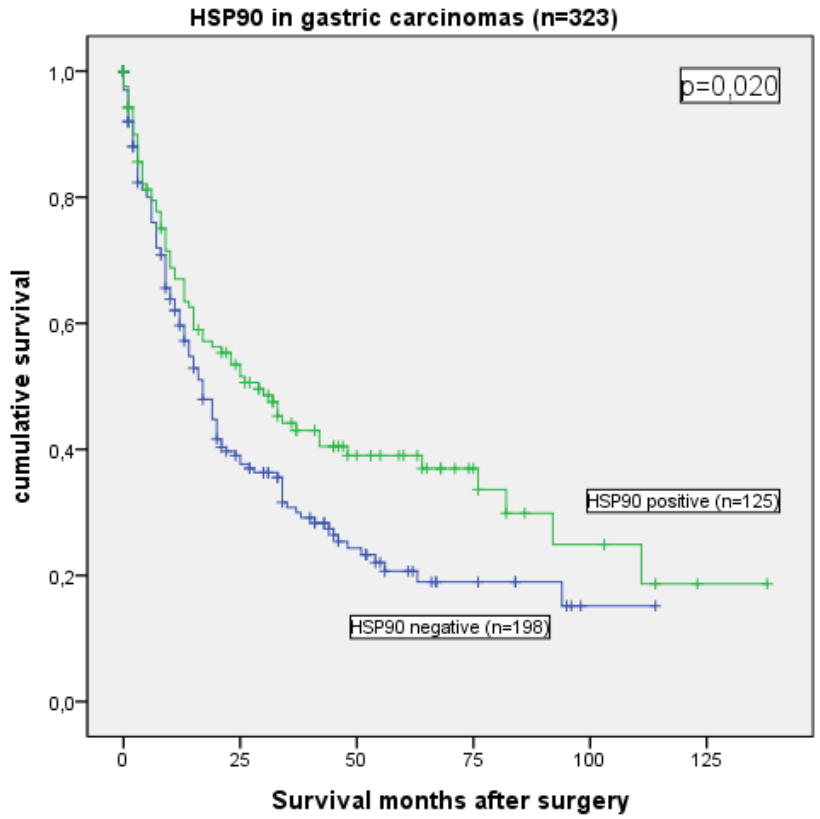
Note: Crosstabulation of clinicopathological parameters and pHSP27 expression levels. Stars indicate $p<0.05$ from a χ^2-test.

3.2.4 Association between patient survival and HSP expression

pT category (p<0.001), presence of lymph node and distant metastases (p<0.001 each), complete resection (p<0.001) and Laurens classification (p=0.001) were highly significantly associated with patient survival in log rank test. There was no significant association with grading. HSP90 expression was significantly associated with better survival rates (p=0.020) (see Figure 3) and moderate-high expression levels of HSP60 was as well associated with better prognosis, but this did not reach statistical significance (p= 0.064) (see Figure 4).

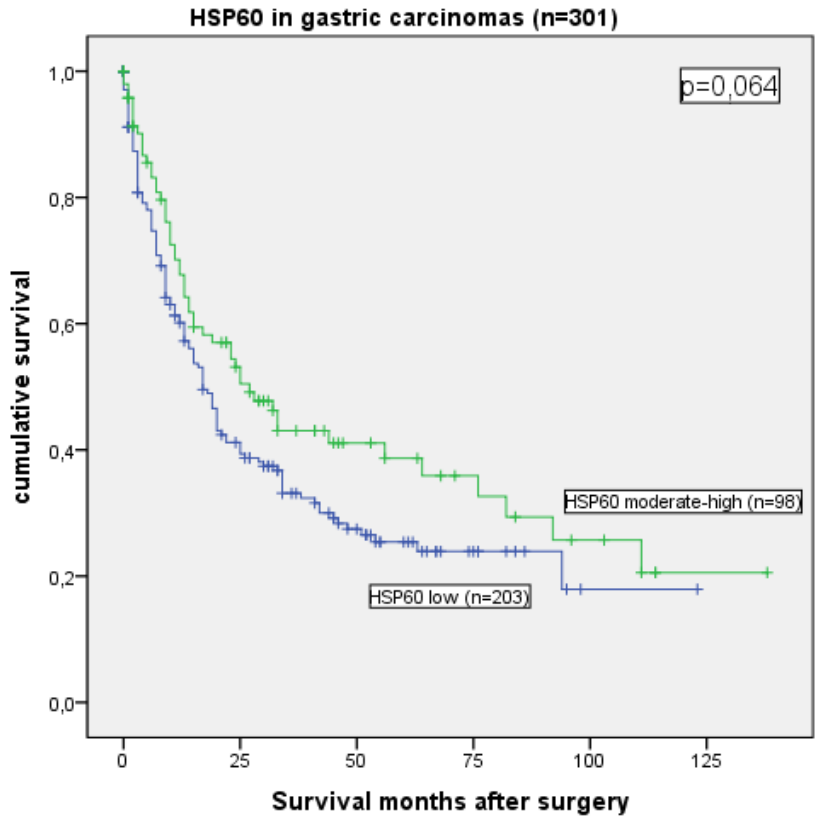
In multivariate cox regression analysis of all clinicopathological parameters and HSP expression levels, significantly associated with patient survival, only depth of tumor invasion (pT category) and presence of lymph node metastases were independent adverse prognostic factors (p<0.001 each) (see Table 16 below). The association of High HSP90 expression and better survival rates doesn't surprise, if the results shown in Chapter 3.2.3 are taken into account: They indicate a link between high HSP90 expression levels and early stage carcinomas. This fits into the picture of high HSP90 expression not being an independent prognostic factor.

Figure 3 Survival of patients with gastric cancer and HSP90 expression



Note: Kaplan-Maier survival estimates of patients with gastric cancer (total cohort, n=323); cumulative survival of patients with positive HSP90 expression (upper curve) and negative HSP90 expression (lower curve).

Figure 4 Survival of patients with gastric cancer and HSP60 expression



Note: Kaplan-Maier survival estimates of patients with gastric cancer (total cohort, n=301); cumulative survival of patients with moderate/high HSP60 expression (upper curve) and low HSP60 expression (lower curve).

Table 16 Multivariate analysis for gastric carcinomas

		95.0% CI for exp(B)		
Factor	Exp(B)	Min	Max	P-value
pT category	1.625	1.266	2.087	<0.001
pN category	1.533	1.282	1.832	<0.001
p/cM category	1.409	0.992	2.001	0.056
Resection status	1.206	0.933	1.560	0.153
Tumor grading	0.866	0.582	1.291	0.481
HSP90 expression	1.015	0.747	1.378	0.925

Note: Cox regression analysis of clinicopathological parameters and HSP90 expression level; 95% confidence intervals and p-values shown.

3.3 Colon Cancer[1]

3.3.1 Patients and pathological findings

Table 17 below presents clinicopathological parameters in patients with colon carcinomas: 200 of the patients were male (56%), 155 female (44%). The mean age was at 65 ± 12 years (median: 66, range: 25 to 91). Tumor staging was accomplished using the UICC TNM-classification current at the time of resection. 21 were adjudged as pT1 (5.9%), 62 as pT2 (17.5%), 193 as pT3 (54.4%) and 79 as pT4 (22.3%). Lymph node metastases were present in 140 (39.4%) and organ metastases in 56 patients (15.8%). Tumor grading was G1 (well differentiated) in 3 cases (0.8%), G2 (moderately differentiated) in 230 cases (64.8%) and G3-G4 (poorly differentiated) in 122 cases (34.3%). Information about adjuvant treatment was available for 337 patients. 121 patients (35.9%) received adjuvant treatment, among them 106 of 131 patients with lymph node or distant metastases at the time of surgery. Mean overall survival of all patients was 65 ± 37.7 months. Mean follow up time for surviving patients was 87 ± 27.6 months.

Table 17 Clinicopathological parameters in patients with colon carcinomas

Sex	Male	*%*	Female	*%*		Total
	200	*56.3*	155	*43.7*		355
pT category	pT1	*%*	pT2	*%*	pT3/4 *%*	Total
	21	*5.9*	62	*17.5*	272 *76.7*	355
Lymph node metastases	Absent	*%*	Present	*%*		Total
	214	*60.5*	140	*39.5*		354
Distant metastases	Absent	*%*	Present	*%*		Total
	299	*84.2*	56	*15.8*		355
Grading	G1	*%*	G2	*%*	G3/4 *%*	Total
	3	*0.8*	230	*64.8*	122 *34.3*	355
Resection status	Complete	*%*	Incomplete	*%*		Total
	306	*86.2*	49	*13.8*		355

Note: Distribution of clinicopathological parameters in patients with colon carcinomas and percentages of total cohort.

3.3.2 HSP expression

353 cases could be analysed for HSP90: 96 (27.2%) were classified as low, 152 (43.1%) as moderate and 105 (29.7%) as high (see Table 19). For HSP70 353 cases were evaluable: 96 (27.2%) showed low, 116 (32.9%), moderate and 141 (39.9%) high staining intensity (see Table 20). 351 cases were evaluable for HSP60: 108 (30.8%) with low, 24 (6.8%) with

[1] The results for colon cancer and HSP70, HSP 60 and HSP27, have previously been published in Bauer, et al. (2012).

moderate and 219 (62.4%) with high staining intensity (see Table 21). 350 cases were analysed for HSP27 expression: 169 (48.3%) showed low, 103 (29.4%) moderate and 78 (22.3%) high staining intensity (see Table 22). For pHSP27^{Ser15} 351 cases were evaluable, of which 135 (38.5%) showed low, 111 (31.6%) moderate and 105 (29.9%) high staining intensity. For pHSP27^{Ser78} and pHSP27^{Ser82} 350 cases and 342 cases, respectively could be analysed. Only 4 (1.1%) were staining positive for pHSP27^{Ser78} and 32 (9.4%) for pHSP27^{Ser82} (see Table 23).

As presented in Table 18, significant correlations could be observed between HSP27 and pHSP27^{S15} as well as pHSP27^{Ser82} ($p<0.001$ each) and HSP 90 was significantly correlated with HSP27 ($p=0.021$) respectively with pHSP^{27S15} ($p<0.001$). Additionally, HSP70 expression was correlated with pHSP^{27S15} expression ($p<0.001$). Highly significant intercorrelation between different pHSPs could be observed between pHSP27^{Ser78} and pHSP27^{Ser82} ($p=0.005$).

Table 18 Correlation between expression patterns of different HSPs in colon carcinomas

P-values	HSP90	HSP70	HSP60	HSP27	pHSP27^{S15}	pHSP27^{Ser78}	pHSP27^{Ser82}
HSP90		0.070	0.298	0.021*	<0.001**	0.911	0.384
HSP70	0.070		0.722	0.164	<0.001**	0.835	0.174
HSP60	0.298	0.722		0.837	0.292	0.783	0.812
HSP27	0.021*	0.164	0.837		<0.001**	0.084	<0.001**
pHSP27^{S15}	<0.001**	<0.001**	0.292	<0.001**		0.141	0.129
pHSP27^{Ser78}	0.911	0.835	0.783	0.084	0.141		0.005**
pHSP27^{Ser82}	0.384	0.174	0.812	<0.001**	0.129	0.005**	

*Note: P-values following Spearman's correlation analysis (2-sided), with * (**) indicating significance at 5% (1%) level.*

3.3.3 Association between clinicopathological parameters and HSP expression

HSP expression levels were correlated with the clinicopathological parameters pT category, lymph node metastases, distant metastases and grading as shown in Table 19 to Table 23. Table 19 presents HSP90 expression in colon carcinomas and pathological parameters, Table 20 HSP70 expression, Table 21 HSP60 expression, Table 22HSP27 expression and Table 23 pHSP27 expression.

HSP27, HSP60 and HSP70 expression was neither associated with tumor category (UICC pT category), nor presence of lymph node metastases or distant metastases (Table 20 to Table 22). In contrast, high HSP90 expression was associated with absence of distant ($p=0.001$) and lymph node metastases ($p=0.046$) (Table 19). This finding is in line with the

results in gastric carcinomas showing an association between high HSP90 expression and early stage carcinomas as well.

Additionally, low HSP27^{S15} expression was associated with absence of lymph node metastases (p= 0.048) and low HSP27^{Ser82} expression was associated with the absence of distant metastases (p= 0.005). Moreover, low HSP27^{Ser82} expression was associated with better tumor differentiation grade (p= 0.039) (Table 23).

Table 19 HSP90 expression in colon carcinomas and pathological parameters

	HSP90				
	Negative/low	*Moderate*	*High*	*Total*	*P-value (χ^2)*
pT Category					
pT1	4	5	12	21	0.082
pT2	15	28	19	62	
pT3-4	77	119	74	270	
Total	96	152	105	353	
Lymph node metastases					
Absent	54	84	75	213	0.046
Present	41	68	30	139	
Total	95	152	105	352	
Distant metastases					
Absent	71	128	98	297	0.001
Present	25	24	7	56	
Total	96	152	105	353	
Grading					
G1	2	0	1	3	0.456
G2	58	98	73	230	
G3-4	36	54	31	120	
Total	96	152	105	353	

Note:Crosstab of clinicopathological parameters and HSP90 expression with p-values from a χ^2-test.

Table 20 HSP70 expression in colon carcinomas and pathological parameters

	HSP70				
	Negative/low	*Moderate*	*High*	*Total*	*P-value (χ^2)*
pT Category					
pT1	4	5	11	20	0.162
pT2	19	26	17	62	
pT3-4	73	85	113	271	
Total	96	116	141	353	
Lymph node metastases					
Absent	59	77	77	213	0.175
Present	37	39	63	139	
Total	96	116	140	352	
Distant metastases					
Absent	81	103	114	298	0.217
Present	15	13	27	55	
Total	96	116	141	353	
Grading					
G1	57	82	94	233	0.218
G2	39	34	47	120	
G3-4	96	116	141	353	
Total	57	82	94	233	

Note:Crosstab of clinicopathological parameters and HSP70 expression with p-values from a χ^2-test.

Table 21 HSP60 expression in colon carcinomas and pathological parameters

	HSP60				
	Negative/low	*Moderate*	*High*	*Total*	*P-value (χ^2)*
pT Category					
pT1	9	3	8	20	0.234
pT2	20	3	39	62	
pT3-4	79	18	172	269	
Total	108	24	219	351	
Lymph node metastases					
Absent	63	13	136	212	0.686
Present	44	11	83	138	
Total	107	24	219	350	
Distant metastases					
Absent	86	23	187	296	0.111
Present	22	1	32	55	
Total	108	24	219	351	
Grading					
G1	3	0	0	3	0.033
G2	61	16	151	228	
G3-4	44	8	68	120	
Total	108	24	219	351	

Note:Crosstab of clinicopathological parameters and HSP60 expression with p-values from a χ^2-test.

Table 22 HSP27 expression in colon carcinomas and pathological parameters

	HSP27				
	Negative/low	*Moderate*	*High*	*Total*	*P-value (χ^2)*
pT Category					
pT1	10	4	6	20	0.157
pT2	38	13	11	62	
pT3-4	121	86	61	268	
Total	169	103	78	350	
Lymph node metastases					
Absent	103	63	47	213	0.987
Present	65	40	31	136	
Total	168	103	78	349	
Distant metastases					
Absent	141	89	66	296	0.805
Present	28	14	12	54	
Total	169	103	78	350	
Grading					
G1	1	0	2	3	0.237
G2	114	69	45	228	
G3-4	54	34	31	119	
Total	169	103	78	350	

Note:Crosstab of clinicopathological parameters and HSP27 expression with p-values from a χ^2-test.

Table 23 pHSP27 expression in colon carcinomas and pathological parameters

	pHSP27^{Ser78}			**pHSP27^{Ser82}**			**pHSP27^{S15}**			
	Negative	*Positive*	*Total*	*Negative*	*Positive*	*Total*	*Negative/Low*	*Moderate*	*High*	*Total*
pT Category										
pT1	20	0	0	19	1	20	6	6	8	20
pT2	60	2	2	58	2	60	27	20	15	62
pT3-4	266	2	2	233	29	262	102	85	82	269
Total	346	4	350	310	32	342	135	111	105	351
Lymph node metastases										
Absent	211	2	213	194	16	210	93*	68*	52*	213*
Present	134	2	136	115	16	131	42*	43*	52*	137*
Total	345	4	349	309	32	341	135*	111*	104*	350*
Distant metastases										
Absent	293	3	296	266	22	288	113	94	90	297
Present	53	1	54	44	10	54	22	17	15	54
Total	346	4	350	310	32	342	135	111	105	351
Grading										
G1	3	0	3	3	0	3	0	1	2	3
G2	227	2	229	208	16	224	87	76	66	229
G3-4	116	2	118	99	16	115	48	34	37	119
Total	346	4	350	310	31	342	135	111	105	351

Note: Crosstabulation of clinicopathological parameters and pHSP27 expression levels. Stars indicate $p<0.05$ from a χ^2-test.

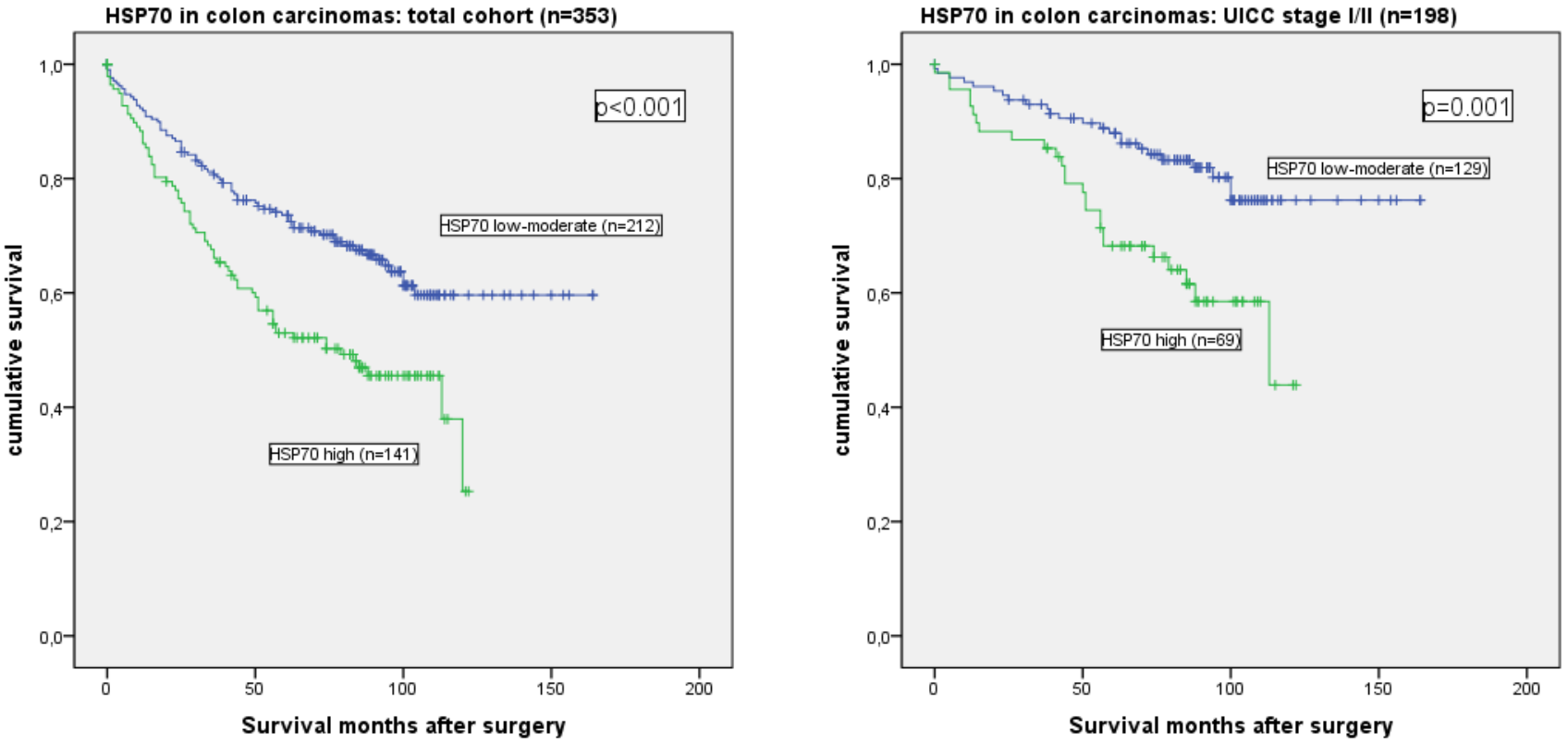
3.3.4 Association between patient survival and HSP expression

As previously partly published in Bauer, et al. (2012: 200), in univariate analysis, the factors UICC pT category, presence of lymph node or distant metastases at the time of surgery, resection status and tumor differentiation (grading) were associated with patients' overall survival ($p<0.001$ each). Remarkably, low HSP70 expression was as well highly significantly associated with better overall survival rates in Kaplan-Maier estimates ($p<0.001$, Figure 5). This was true for the whole cohort (Figure 5, left panel), but became even more pronounced when only the subgroup of UICC stage I/II were analysed (Figure 5, right panel). High HSP27 expression was also associated with bad prognosis. This did not reach statistical significance ($p=0.189$, Figure 6, left panel) for the whole cohort, but in the subgroup of patients with left sided carcinomas ($p=0.035$, Figure 6, right panel). On the contrary, high HSP90 expression was significantly associated with better survival in univariate analysis ($p=0.032$, Figure 7).

In multivariate analysis, the factors higher pT category ($p=0.020$), presence of lymph node metastases ($p<0.001$) or distant metastases ($p=0.009$), incomplete tumor resection ($p=0.020$) and remarkably high HSP70 expression ($p=0.006$) were independent adverse prognostic factors for overall survival. HSP90 expression was no independent prognostic factor (Table 24A), which does not surprise, given the associations observed in Chapter 3.3.3 indicating a link between early stage colon carcinomas and high HSP90 expression. Additionally, these findings mirror those found in gastric cancer (comp. Chapter 3.2.3). In left sided carcinomas (n=282) only, high HSP27 expression also was an adverse independent prognostic factor ($p=0.018$), in addition to high HSP70 expression ($p=0.008$), pT category ($p=0.019$), lymph node metastases ($p<0.001$) and distant metastases ($p=0.019$) (Table 24 B).

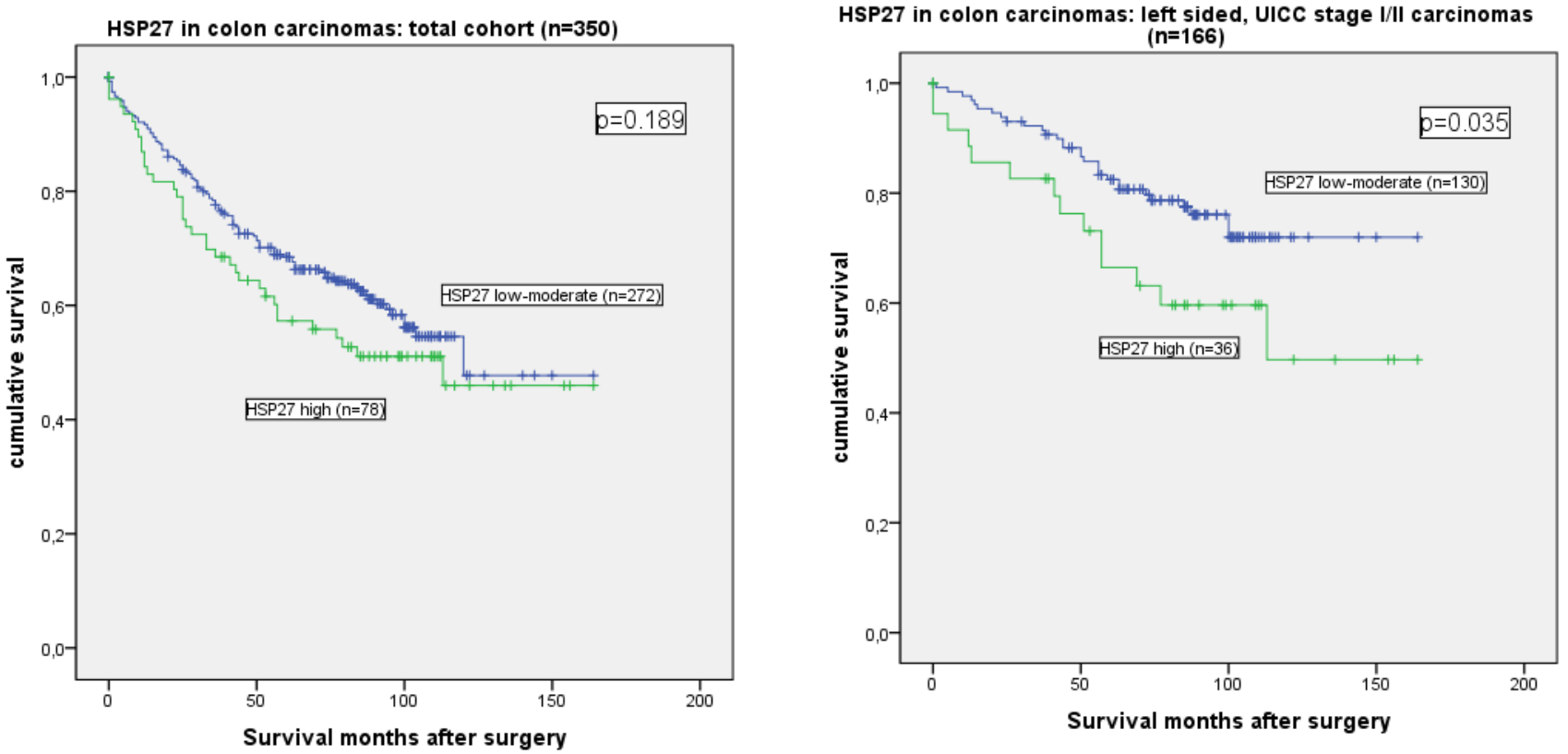
Subgroup analysis of UICC stage I/II patients (n=210) revealed HSP70 expression as the only independent prognostic factor ($p<0.001$, Table 24C) apart from resection status ($p=0.007$), which was superior to the depth of tumor invasion (UICC pT category; $p=0.351$). This was also the case with regard to left side carcinomas of UICC stage I/II only. In this particular group of patients (n=175), high HSP27 expression also had adverse prognostic impact (Figure 6, right panel) and was an independent prognostic factor ($p=0.013$) besides HSP70 ($p=0.002$) (Table 24D).

Figure 5 Survival of patients with colon cancer and HSP expression: HSP70 expression and survival in total cohort and in UICC stage I/II



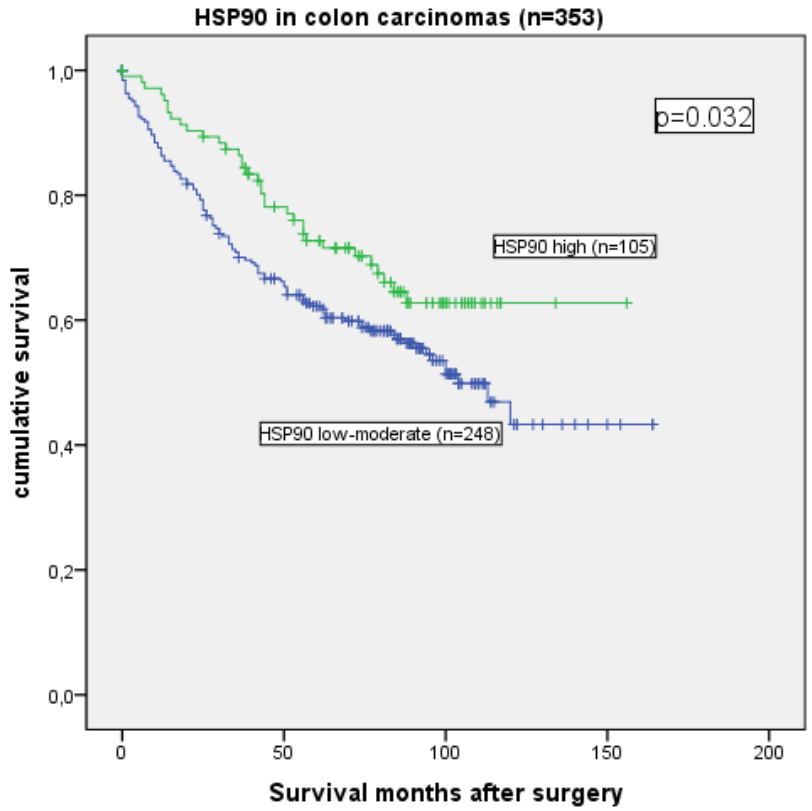
Note: Kaplan-Maier survival estimates of patients with colon cancer (left panel: total cohort, n=353; right panel: UICC stage I/II, n=198). Cumulative survival of patients with low-moderate HSP70 expression (upper curve) and with high HSP70 expression (lower curve).

Figure 6 Survival of patients with colon cancer and HSP expression: HSP27 expression and survival in total cohort and left sided UICC stage I/II carcinomas



Note: Kaplan-Maier survival estimates of patients with colon cancer (left panel: total cohort, n=350; right panel: left sided UICC stage I/II, n=166). Cumulative survival of patients with low and moderate HSP27 expression (upper curve) and with high HSP27 expression (lower curve).

Figure 7 Survival of patients with colon cancer and HSP90 expression



Note: Kaplan-Maier survival estimates of patients with colon cancer (total cohort, n=353). Cumulative survival of patients with high HSP90 expression (upper curve) and with low to moderate HSP90 expression (lower curve).

Table 24 Multivariate analysis of colon carcinomas

A Total patient cohort (n=353)

		95.0% CI for exp(B)		
Factor	Exp(B)	Min	Max	P-value
pT category	1.352	1.049	1.742	0.020
pN category	1.773	1.412	2.225	<0.001
p/cM category	2.287	1.232	4.246	0.009
Resection status	1.429	1.059	1.930	0.020
Tumor grading	1.312	0.942	1.826	0.108
HSP70 expression	1.603	1.142	2.251	0.006
HSP27 expression	1.373	0.937	2.012	0.104
HSP90 expression	0.973	0.647	1.464	0.896

B Left sided carcinomas (n=282)

		95.0% CI for exp(B)		
Factor	Exp(B)	Min	Max	P-value
pT category	1.396	1.057	1.843	0.019
pN category	1.77	1.391	2.253	<0.001
p/cM category	2.282	1.146	4.544	0.019
Resection status	1.381	0.982	1.942	0.064
Tumor grading	1.27	0.887	1.819	0.191
HSP70 expression	1.632	1.134	2.349	0.008
HSP27 expression	1.624	1.085	2.43	0.018

C UICC stage I/II carcinomas (n=210)

		95.0% CI for exp(B)		
Factor	Exp(B)	Min	Max	P-value
pT category	1.084	0.78	1.505	0.632
Resection status	1.653	1.15	2.375	0.007
Tumor grading	1.053	0.605	1.833	0.855
HSP70 expression	2.503	1.487	4.213	0.001
HSP27 expression	1.765	0.998	3.121	0.051

D UICC stage I/II left sided carcinomas (n=175)

		95.0% CI for exp(B)		
Factor	Exp(B)	Min	Max	P-value
pT category	1.193	0.837	1.702	0.329
Resection status	1.487	0.984	2.246	0.06
Tumor grading	0.986	0.539	1.803	0.964
HSP70 expression	2.475	1.411	4.341	0.002
HSP27 expression	2.165	1.18	3.971	0.013

Note: Panels A-D show results from cox regression analysis of clinicopathological parameters and HSP90/70/27 expression levels (as indicated) for the total cohort (Panel A) and the subsamples indicated in the headline for panels B-D; 95% confidence intervals and p-values shown.

4 DISCUSSION

4.1 Methods

Immunohistochemistry is commonly used as a way to detect the expression of certain proteins. It has found its way in daily clinical routine, as a supplementary diagnostic tool, especially in cancer diagnosis and subtype analysis and for targeted therapy approaches. This concerns many malignancies including lymphomas, breast and gynecologic cancers, soft tissue tumors, gastrointestinal malignancies, melanoma and lung malignancies (Boyd, et al. 2013, de Deus Moura, et al. 2013, Ferringer 2012, Fisher 2011, Lee 2013, Szutowicz & Dziadziuszko 2010, Taliano, et al. 2013).

It is also widely used in research. This section will discuss the advantages of immunohistochemistry in combination with tissue microarrays, as used in this study.

To create a tissue microarray it is necessary to mark the section of the tissue, which is mostly an archival formalin-fixed paraffin-embedded tissue, that contains the relevant part of the tissue (normal, tumor or specific areas within tumors) and afterwards to transfer it into a paraffin recipient block, using a tissue arrayer. Possible limitations of this technique are the loss or shifting of individual tissue cores and possible miss of heterogeneity of the tumoral protein expression on the tissue core (Avninder, et al. 2008). But Sauter shows in his review the feasibility of the approach:

> "*[N]umerous studies comparing the results of TMA studies with the findings from conventional large sections have shown that all well-established associations between molecular markers and tumor phenotype or patient prognosis can be reproduced with TMAs even if only one single 0.6 mm tissue spot is analyzed*"(Sauter 2010: 27).

In order to analyze the suitability of the TMA technique, especially with regard to intratumoral heterogeneity of staining patterns, a test TMA was assembled including 28 samples of colon, gastric and esophagus carcinomas, that were included in the study. From every specimen three tissue spots from different parts of the tumor were separately analysed for statistical difference using the Friedman test. This did not show any significant differences across the TMA cores for the investigated markers (p-values ranging from 0.192 to 0.979, see Table 1 in Chapter 2.2). Demonstrating homogenous staining patterns, the large TMA of carcinoma samples could be analysed for HSP27 and pHSP27 (pHSP27^{S15}, pHSP27^{Ser78}, pHSP27^{Ser82}), HSP60, HSP70 and HSP90.

These findings support the suitability of the method for the analyses of the present work. The additional practical advantages of the method, its cost efficiency and time saving nature (Avninder, et al. 2008, Moch, et al. 2001), made it possible to analyse large numbers for this thesis: Altogether, it was possible to include 811 tumor samples through this technique.

As an additional benefit, it was possible to use the tissue microarray data and the collected survival data for future studies (comp. Bauer, et al. 2012, Berezowska, et al. 2013, Drecoll, et al. 2014, Slotta-Huspenina, et al. 2012).

4.2 Adenocarcinoma of the Esophagus

This study is about the expression of the heat shock proteins HSP27, HSP60, HSP70 and HSP90 in a large collection of primary resected adenocarcinomas of the esophagus, gastric carcinomas and colon carcinomas and the correlation of the expression patterns with pathologic features and patient survival. Increased expression of heat shock proteins have been reported to occur in many human malignancies and show association with biological tumor behavior (Calderwood, et al. 2006, Ciocca & Calderwood 2005, Khalil, et al. 2011).

However, in this study it was not possible to provide evidence for any associations of HSP expression and patient survival time for adenocarcinomas of the esophagus based on immunohistochemistry. The only statistically significant association between HSP expression and clinicopathological parameters in those cases was found between elevated levels of HSP60 and bigger tumor size ($p=0.028$).

Other studies examining the influence of HSP expression on patient survival in esophageal carcinoma are scarce for adenocarcinoma, for squamous cell carcinoma data exists to a slightly bigger extent: For squamous cell carcinoma Kawanishi, et al. (1999) reported an association of low expression levels of HSP70 as well as HSP27 and poor postoperative outcome. Faried, et al. (2004) reported a positive outcome for patients with high HSP60 expression, but could not detect an association between HSP90 expression and survival time, again examining squamous cell carcinoma. Farkas, et al. (2011) examined different tumor-associated proteins in relation to response rates to neoadjuvant radiochemotherapy. Among others HSP90 expression was decreased in the responding squamous cell esophageal carcinoma tumors. Wu, et al. (2009) as well could not show an association between HSP90 expression in esophageal carcinomas (squamous cell and adenocarcinomas) and survival time. However, they could detect that the inhibition of HSP90 in two different cancer cell lines resulted in the inhibition of cell proliferation. Additionally, they describe an augmented

response to γ-photon radiation and reported that of the 25 adenocarcinomas they analyzed, 56% showed at least low staining intensities and 4% moderate ones, 40% showed only faint staining for HSP90, while squamous cell carcinoma showed a more pronounced difference in HSP90 expression between normal epithelium and carcinoma cells.

In this study much higher staining results were observed: HSP90 showed the highest expression with 75.5% moderate-high staining intensities, HSP27 showed 68.2% of moderately-highly stained tumors, HSP70 40.5% and HSP60 30.7%.

This overexpression of HSP, especially of HSP90, could make it a possible target for individualized therapeutic approaches. Barrott and Haystead (2013) describe HSP90 as a counterintuitive target in cancer therapy, being an ubiquitary expressed protein that constitutes up to 1-3% of total cell proteins and has no known alterations in malignant cells. They conclude that this is the reason HSP90 is not mentioned in two recent high profile publications on new approaches in molecular cancer therapy (Hanahan & Weinberg 2011, Koboldt D 2012). Still HSP90 inhibitors turn up in different clinical studies showing promising results: Barrott and Haystead (2013) identify three factors that may contribute to HSP90 being nevertheless an auspicious target:

First, many studies document an overexpression in malignant tissues, were it is induced in order to support coping mechanisms in the hypoxic and nutrition deprived microenvironment.

Second, although HSP90 interaction with co-chaperones are extremely complex and manifold and therefor far from understood, the fact that HSP90 inhibitors seem to target preferentially tumor HSP90, leads Barrott and Haystead (2013) to the conclusion of different activation levels and interactions in normal and malignant cells. They suggest a possible interaction of HSP90 with mutant oncoproteins such as BCR-ABL and human epidermal growth factor receptor 2 (Her2) (Barrott & Haystead 2013). This assumption is in line with the finding of Berezowska, et al. (2013), who could show a highly significant correlation between HSP90 and Her 2 expression in our collective of gastric carcinomas.

Third, Barrott and Haystead (2013) mention the ectopic occurrence either on the cell surface or secreted into the extracellular compartment. This opens several pathways supporting cell migration and invasion, including the activation of plasminogen (Barrott & Haystead 2013) and the down-regulation of E-cadherin, which has been shown to lead to enhanced migration and invasion as shown for colon carcinoma cell lines (Chen, et al. 2013). This ectopic expression of HSP90 could be a likely target for HSP90-inhibitors.

The finding in this thesis of higher HSP90 expression levels than reported before might support the role as a possible target in esophageal adenocarcinoma as well.

Similarly, Multhoff (2007) confirmed the occurrence of plasma membrane-bound proteins for HSP70 in a variety of cancer cells but not in their corresponding normal tissue. This extracellular HSP70 can trigger anti-tumor immune response (Guzhova, et al. 2013, Multhoff 2007, Multhoff, et al. 2012). These findings lead to the conclusion that further studies should focus not only on total expression levels, but as well on the location of HSP expression. Intracellular HSP may well, through its chaperoning function and anti-apoptotic property, play a reinforcing role on tumor cell survival and thereby be a negative prognostic factor, while extracellular HSP may lead to enhanced anti-tumor immune response and thereby be a protective factor. These differences in the role HSPs play due to their location may explain some of the discrepancies in the outcomes of studies analyzing the impact of HSP expression on patient overall survival time.

Given the strong and not jet fully understood interferences between function and expression of different HSP-classes, another notable approach at identifying patients, who might profit from a targeted therapy using HSP- (and GRP-)inhibitors was made by Slotta-Huspenina, et al. (2012), using the immunohistochemistry data generated for this dissertation in addition to newly generated data of reverse phase protein arrays and real time quantitative PCR on the same tissue micro array. They observed an influence of different expression patterns of many cellular chaperones, not only the up/down regulation of a single HSP-class. Examining 92 cases out of the collective of adenocarcinomas of the esophagus used in this thesis, they could show two different profiles of HSP and Glucose-Regulated Protein (GRP) expression with prognostic relevance in reverse phase protein arrays (RPPA), but could not recognize them in immunohistochemistry or real-time quantitative PCR, which matches the results of Chapter 3.1.4. A first patient group showing high expression levels of pHSPs ($pHSP27^{S15}$, $pHSP27^{Ser78}$ and $pHSP27^{Ser82}$) and low expression levels of GRP78, GRP94 and HSP60 had significantly better overall survival times than a second group showing reversed expression patterns (Slotta-Huspenina, et al. 2012). In a following work, Slotta-Huspenina, et al. (2013) could establish a tie between HSP/GRP expression levels and the response to neoadjuvant chemotherapy. A group (similar of that with better prognosis in the first paper) with low expression of HSP90, HSP27 and pHSP27 and high expression of GRP78, GRP94, HSP60 and HSP70 showed significantly better response to the applied chemotherapy than a group showing inverse expression patterns. Similar to the first study, the RPPA results could not be

seen by other methods such as immunohistochemistry, suggesting that subtle differences of quantitative protein expression may not reliably be detectable by immunohistochemistry (Slotta-Huspenina, et al. 2013).

4.3 Gastric Cancer

In the present study a significant association between higher expression levels of HSP70 and HSP60 and tumor-grading could be detected ($p=0.017$ and $p=0.004$ respectively). HSP60 expression was additionally associated with the absence of lymph node metastases ($p=0.032$).

Even more notably, an association between HSP90 expression, small tumor category ($p<0.001$), absence of metastases ($p=0.023$ for lymph node metastases and $p=0.011$ for distant metastases) as well as well differentiated tumors in grading ($p=0.025$) could be established. All those parameters point at the possibility, that HSP90 may be especially overexpressed in early tumor stages or biologically less aggressive tumors, as stated before. The finding, that HSP90 expression was associated with better prognosis, but is no independent prognostic factor, is in this respect not surprising. The results for HSP90 expression in colon carcinoma support these results. Another study could show an association between HSP90α expression and lymph node metastases (Zuo, et al. 2003).

Giaginis, et al. (2009), examining 66 patients with gastric carcinoma, too, found in their study an association between HSP90 expression and tumor size ($p=0.020$) and furthermore with longer overall survival. They even identified HSP90 expression as a possible positive independent prognostic factor. They argue, that

> *"(t)he present data that HSP-90 expression is associated with improved survival deserves special attention, as it could be implied that loss of chaperoning, such as HSP-90, may lead to a more aggressive phenotype, thus leading to poor prognosis or resistance to therapy."(Giaginis, et al. 2009: 8)*

But even if it should prove true in further studies that HSP90 overexpression is a positive prognostic factor, an overexpression of HSP90 could still be targeted for therapeutical approaches as argued previously. Notably, two studies could show an inhibition of tumor growth by HSP90 inhibition in gastric cancer xenocrafts (Lang, et al. 2007, Lu, et al. 2013).

Recently, a study disclosing contradictory data on the association between HSP90 expression and patient survival has been published: Wang, et al. (2013) analyzed a large cohort of 322 patients with advanced gastric cancer and the HSP90 expression immunohistochemically. In their case, 69.6% of their tumor sample showed an overexpression of HSP90, in stark contrast to the findings in this thesis. HSP90 expression

levels were significantly higher in patients with increased tumor size (>5cm; $p<0.001$), pT-category ($p<0.001$), presence of lymph node metastases ($p<0.001$) and higher clinical staging ($p<0.001$). They included 157 patients in survival analysis. In Kaplan-Meier survival analysis they found that HSP90 overexpression was associated with bad prognosis, concerning both overall and recurrence free survival ($p<0.001$ each). With multivariate Cox proportional hazard models they could even show HSP90 as an independent prognostic factor for their cohort ($p=0.015$ for recurrence free survival, $p=0.042$ for overall survival) (Wang, et al. 2013). More studies concerning HSP90 expression in gastric cancer could help to clarify those contradictory results. But still, those results of Wang, et al. (2013) point even stronger at HSP90 as a possible target in anti-cancer therapy for patients with gastric cancer and all show an overexpression of HSP90 in gastric tumor tissue.

For HSP27 and gastric cancer only sparse data exists: in contrary to the finding of no significant association of HSP27 expression and eight clinicopathologic parameters of patient survival in this study, a study including 86 gastric carcinoma patients detected a correlation of HSP27 expression and presence of more than six metastatic lymph nodes, as well as an association with shorter overall survival in univariate analysis, although this did not reach statistical significance in multivariate analysis (Kapranos, et al. 2002).

For HSP70 expression in gastric cancer two studies, including 164 respectively 81 patients, exist, showing no notable correlations between HSP70 expression and clinicopathological parameters (Isomoto, et al. 2003, Maehara, et al. 2000), except an association between HSP70 expression and better differentiated carcinomas ($p<0.001$) (Isomoto, et al. 2003). Both studies detected no influence of HSP70 expression levels on patient prognosis (Isomoto, et al. 2003, Maehara, et al. 2000). All those findings are in line with the results presented in this thesis.

Another study including 60 patients examined only expression levels of HSP72 in gastric mucosal and cancer tissue, but did not perform a survival analysis of the patients. HSP72 expressed in 90% of the cancer tissues. In cancer tissue taken from lymph node metastases ($n=45$; $p<0.05$) and distant metastases ($n= 34$; $p<0.05$) HSP72 expression was present in 100%. They interpret this finding as an indication, that

> *"there is a close correlation between the overexpression of HSP72 (and gp96) and progression of gastric carcinomas. The high level expression of HSP72 (and gp96) may be useful as diagnostic and prognostic markers for gastric carcinoma.*" (Wang, et al. 2007: 40)

A recent large study including 458 patients detected a correlation between HSP70 expression and a large variety of clinicopathological parameters including again better differentiation of tumors ($p=0.007$), intestinal type carcinoma in Lauren classification ($p<0.001$), low pT category ($p<0.001$), lower pN category ($p<0.001$) and earlier pTNM stage in general ($p<0.001$). In survival analysis they could show a worse prognosis for patients, whose tumors expressed HSP70 in a subset of patients with early gastric cancers ($p=0.009$). This did not reach statistical significance for the whole cohort. But for early gastric cancers it was even an independent prognostic factor ($p=0.024$) (Kang, et al. 2013).

This is in line with the recent findings of Lee, et al. (2013), who could show a correlation between high HSP70 expression and poor prognosis in a subset of patients with intestinal type carcinomas, though this, again, did not reach statistical significance for the total cohort of the gastric carcinomas they analyzed.

Those results might point at the possibility that HSP70 might have a prognostic impact for some patients with gastric cancer, but not for all. More studies helping to identify those patients, who could profit from the identification of HSP70 expression levels, would be helpful.

4.4 Colon Cancer

For colon and colorectal carcinomas and HSP60 expression, there are only very scarce data. One small study exists, showing an association between HSP60 expression and the presence of lymph node metastases (Cappello, et al. 2005). As discussed in Bauer, et al. (2012: 202f.), data about HSP70 expression in human cancer tissue exist to a slightly higher extent: HSP70 expression has been reported to be elevated in colon cancer in several, albeit small studies, indicating an association of high HSP70 expression with aggressive tumor behavior (Hwang, et al. 2003, Kanazawa, et al. 2003, Wang, et al. 2005).

Interestingly, externalization of HSP70 onto the cell membrane has been proven to be tumor specific in colon cancer cells and appears to correlate with patient prognosis (Pfister, et al. 2007). The comparison between normal and neoplastic tissue was not the primary aim of this study. However, some TMA cores contained normal colonic tissue. In these samples, the HSP70 expression in the non-neoplastic tissue seemed generally to be extremely weak and a shift of the localization of HSP70 expression in any tumor cells was not conspicuous (neither to the nucleus nor the plasmamembrane), but it might be possible, that immunohistochemistry is no adept method to depict membranous staining. This approach of Pfister et al. (2007) might be promising to follow up on using other study designs.

High serum HSP70 levels have been shown to be associated with poor clinical outcomes of colon cancer patients (Kocsis, et al. 2011). However, the neoplastic origin of soluble HSP70 has not yet been proven and systemic mechanisms like the immunoresponse of the host towards cancer cells may cause increased HSP70 levels in non-neoplastic cells as well.

Concerning HSP27 expression in colorectal cancers a very interesting and large study has recently been published by Tweedle, et al. (2010), which is discussed later in this section.

For HSP90, an enhancement of apoptosis in cancer cell lines as well as in colorectal cancer xenocrafts co-treated with TRAIL (TNF related apoptosis inducing agent) and the HSP90 inhibitor 17-AAG has been described (Saturno, et al. 2013) as well as significantly reduced tumor invasiveness in vitro and reduced tumor growth in vivo through HSP90 inhibition (Moser, et al. 2007). In relation to HSP90 expression and patient survival, Chen, et al. (2011) could show a significant association between high HSP90α expression and the presence of metastases as well as poor prognosis.

In the present study, high expression of HSP27 was observed in 22% of the cases, high expression of HSP60 was observed in 62% of the cases, high tumoral HSP70 could be detected in 40% and HSP90 expression was present in 39%. The study also investigated phosphorylation of HSP27 which could be observed for $pHSP27^{Ser15}$ in over 60% of the cases with 30% showing high staining intensity. For $pHSP27^{Ser78}$ and $pHSP27^{Ser82}$ only small subsets of cases showed immunoreactivity. This thesis could not demonstrate significant associations between clinicopathologic features like UICC pT or pN category and HSP27, HSP 60 or HSP70 expression, only an association between the presence of lymph node and distant metastases and the phosphorylation of HSP27, indicating an influence of aggressive tumor behaviour on phosphorylation of stress proteins. Additionally, an association between high HSP90 expression and absence of metastatic processes could be detected, indicating either earlier tumor stages or less aggressive tumoral behaviour, as shown for gastric cancer as well. But it was not possible to reproduce some findings of others in our large cohort of colon cancer patients.

However, and most importantly, this study was able to demonstrate the prognostic value of high HSP70 expression, which was associated with worse clinical outcomes for the patients. HSP70 expression was shown to be an independent prognostic factor in the whole patient cohort and especially in the group of UICC stage I/II patients. As discussed in Bauer, et al. (2012: 203f.), according to current therapeutic concepts this group of patients is usually

treated by surgery alone, while in stage III tumors surgical resection followed by adjuvant chemotherapy is the treatment of choice. In stage II disease, however, the role of adjuvant CTX remains controversial due to its minimal efficacy. However, it is recognized that up to 30% of stage II patients will experience recurrence after resection. Staging alone seems to have limitations as the only determinant of treatment strategies (Andre & de Gramont 2004, Benson, et al. 2004, Marshall, et al. 2007).

Unfortunately, at present no morphologic or molecular markers have been identified which can provide relevant prognostic information and which therefore can be used for risk stratification (Mutch 2007, Tejpar, et al. 2010). In this context the association of high tumoral HSP70 expression with bad clinical outcome as presented in this thesis can be considered a promising observation in order to identify prognostic biomarkers for colon cancer. In particular, as these findings were apparent also in the subgroup analysis of patients without lymph node or distant metastases (i.e. UICC stages I/II), where HSP70 expression was an independent prognostic factor for survival and even superior to UICC pT category. Of note, the expression pattern of HSPs showed a cytoplasmic staining for all HSPs. A specific membranous staining which could be indicative for a externalisation of HSP70 like it has been described by Pfister, et al. (2007) was not observable. However, these findings support that cytoplasmic HSP levels may be of impact for patients prognosis and tumor cell specific HSP expression can be visualised by simple immunostaining in a reproducible manner.

Interestingly, a significant association between high HSP27 expression and bad prognosis could be demonstrated as well. However, this phenomenon could only be found in the cohort of patients with left sided carcinomas. Tweedle at al. report that overexpression of HSP27 was associated with poor survival in rectal cancer (Tweedle, et al. 2010), which is in line with these findings. This interesting finding may be explained by the different genetic molecular background of right sided vs. left sided colon cancer with microsatellite instability and CpG island methylator phenotypes on the one hand and chromosomal instability on the other hand (Iacopetta 2002, Marx, et al. 2008), and the close genetic similarity of left sided colon cancer to rectal cancer. In contrast to HSP70 the biological effects of HSP27 may therefore differ depending from the interaction with the genes involved in carcinogenesis. Nevertheless, these findings could also confirm the impact of HSP27 on tumor behavior in colorectal cancer.

Moreover, an association between overexpression of HSP90 and good prognosis could be determined (p=0,032), though this finding was not independent in multivariate analysis (p=0.896). This is in contrast to the findings of an association between high HSP90 mRNA

expression and poor prognosis in 56 colorectal carcinomas by Chen, et al. (2011), mentioned earlier in this section.

High expression of HSPs in tumors may also influence their responsiveness towards targeted anti-HSP therapies or conventional antitumoral therapies like chemotherapy and radiation (Khalil, et al. 2011). Langer, et al. (2008) have demonstrated that high HSP27 expression was associated with better tumor response to neoadjuvant chemotherapy in esophageal adenocarcinomas. In cell line experiments, upregulation of HSP27 and downregulation of HSP60 and 70 was observed in colon cancer cell lines after 5FU treatment (Wong, et al. 2008), and high pretreatment HSP27 expression was associated with 5FU resistance (Tsuruta, et al. 2008). Choi, et al. (2007a) report a correlation of high HSP27 expression and decreased sensitivity to irinotecan of colon cancer cells, which was also confirmed by tissue based analysis of cancer specimens of a small patient cohort (Choi, et al. 2007b). Notwithstanding, there is still a lack of a comprehensive investigation about the role of HSPs concerning response to conventional adjuvant or neoadjuvant treatment in colon and rectal cancer, respectively.

As already outlined in Bauer, et al. (2012: 204), in the last years, novel therapeutic agents which lead to the inhibition of heat shock proteins have been developed and have already emerged to be powerful anti-tumoral agents in preclinical settings (Dudeja, et al. 2009, Jego, et al. 2013). The most investigated drugs are Geldanamycin (17-AAG) and radiciol which act as an inhibitor of HSP90. These substances have been demonstrated to have antitumoral effects alone, and dependent from or interacting with other molecules like HER2 and EGFR (Banerji 2009, Fukuyo, et al. 2010, Jego, et al. 2013). Clinical studies of HSP90 inhibitors alone and in combination with other chemotherapeutic drugs are underway. For HSP70 it would be desirable to overcome the unfavorable prognostic impact of elevated HSP tumoral levels which are associated with an antiapoptotic effect by inhibition of the molecule. HSP70 antagonists like quercetin and triplotide have been developed and caused inhibition of tumor growth in pancreatic and colon cancer cells in vitro (Aghdassi, et al. 2007, Phillips, et al. 2007, Yuan, et al. 2006). Similar experiments have been conducted concerning HSP27 inhibition (Dudeja, et al. 2009). However, this approach has emerged to be challenging due to the structural complexity of this molecule (Jego, et al. 2013) including the frequently observed phosphorylation state which also could be observed in our study. Therefore, although representing a promising approach the application of HSP27 inhibitors, as well as of HSP70 inhibitors – alone or as combination with other anticancer drugs – has not reached preclinical levels yet.

In summary, this thesis detected the expression of HSP27, HSP60, HSP70 and HSP90 in a significant subset of colon carcinomas. High HSP90 expression was associated with absence of lymph node and distant metastases and better prognosis. High HSP27 and HSP70 expression could be demonstrated to be associated with worse clinical outcome, which was particularly the case when regarding left sided, non-metastasized carcinomas only. Therefore, determination of tumoral HSP27 and HSP70 may be used as additional biomarker for risk stratification in colon cancer patients especially in UICC stage I/II patients. These findings warrant validation of the impact of HSP27 and HSP70 expression in an independent collective of colon carcinomas. Moreover, a potential role of HSP27 or HSP70 expression concerning response to conventional chemotherapy and/or HSP targeted therapies may also be the target of future investigations.

5 CONCLUSION

Taken together, in this thesis the expression of the Heat Shock Proteins HSP27, HSP60, HSP70 and HSP90 in a large collection of gastrointestinal adenocarcinomas, comprising esophageal, gastric and colon carcinomas, is investigated. The expression of the HSPs was analyzed by immunohistochemistry on a tissue microarray. The expression patterns were variable ranging from negative to high. In esophageal adenocarcinomas, there was no association with HSP expression and clinicopathologic features. In gastric and colon carcinomas, high HSP90 expression was associated with a less aggressive tumor behavior. In contrast, HSP70 expression was associated with worse clinical outcome in colon cancer, high HSP27 expression with worse clinical outcome in left sided colon cancer – both these findings were also independent from other pathologic factors in multivariate analysis. In conclusion, it could be demonstrated in this thesis that Heat Shock Proteins play a role in gastrointestinal adenocarcinomas, especially with regard to tumor biology which may also have impact on patients' outcome. Besides a potential usage as prognostic biomarkers, targeted therapies against specific Heat Shock Proteins or modulating the function of these molecular chaperones may be an interesting approach for alternative treatment of these tumors.

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