Immunohistochemical Expression of Heat Shock Proteins in Fibroblastic/ Myofibroblastic Tumours of Extremities

THESIS SUBMITTED IN ACCORDANCE WITH THE REQUIREMENT OF THE UNIVERSITY OF LIVERPOOL FOR THE DEGREE OF MASTER IN PHILOSOPHY

By

Ayman Said Salim Al Amri

August 2017

Institute of Translational Medicine

Department of Molecular and Clinical Cancer Medicine

Abstract

BACKGROUND: Soft tissue sarcomas (STS) are rare heterogeneous human malignant neoplasms, accounting for 1% of all human malignancies, arising mostly from various mesenchymal tissue. Heat shock proteins (HSPs) are involved in cell proteostasis, and their expression by cancer cells allows the cells to survive, invade and undergo metastasis.

AIMS: The purpose of this thesis was to investigate the potential role of key HSPs (HSP27, HSP70 and HSP90) in fibroblastic/myofibroblastic tumours of the extremities.

METHODS: Expression of HSP27, HSP70 and HSP90 were analysed using immunohistochemistry on tissue sections from 35 cases of fibroblastic/myofibroblastic sarcomas and 8 cases of non-metastasising fibroblastic tumours. The expression of each protein was evaluated and its relationship with clinicopathological parameters examined.

RESULTS: HSP27 and HSP70 were expressed in all fibroblastic/myofibroblastic tumours, but only the expression of HSP70 was significantly higher in sarcomas compared to non-metastasising counterparts ($P < 0.05$). Also, there was a significant relationship between HSP70 expression and the grade of sarcomas ($P < 0.05$). However, no significant association found between HSP27 and HSP90 expression and clinicopathological features of sarcomas.

CONCLUSIONS: The findings of this study indicate that HSP70, and to a lesser extent HSP27, might have a role in sarcoma behaviour (as defined by the surrogates of tumour type and grade) and may provide prognostic information for clinicians in the future. Further studies are warranted to gain more insight into the role of these HSPs in the tumorigenesis of soft tissue sarcomas to determine whether they are possible markers of prognosis.

Table of Contents

List of Figures

List of Tables

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or another institute of learning.

Declaration of Originality

This thesis is a product of my own work in collaboration with the Musculoskeletal Science Research Group in the Department of Molecular and Clinical Cancer Medicine, produced during my time at the University of Liverpool. The thesis was written by me with guidance from my supervisors' Dr Margaret Roebuck, Dr Joseph Al Sousou and Professor Simon Frostick.

Acknowledgment

To start with, I have been extremely fortunate throughout my MPhil study to work with so many delightful people who have contributed positively to this study, and my sincere gratitude is due to them all for their help and support which have allowed the study to be conducted smoothly. I would like to express my deep appreciation and gratitude to my supervisors: Dr Margaret Roebuck, Dr Joseph Alsousou and Professor Simon Frostick. I thank them for their guidance, support, and encouragement. I have appreciated their patience, input, and positive criticism and comments throughout the development of this study. Also, I would also like to acknowledge all the staff of the Tissue Bio-Bank and Immunohistochemistry Lab for their warmth and help during my studies. My Special thanks is also dedicated to Dr Susha Varghese, pathologist, for her tremendous help in analysis and assessment of immunohistochemistry.

I would like to express my gratitude to my wonderful friends and colleagues: Haji Muhammad Khairul Azmi Bin Haji Abd Kadir and Mohammed Al Mutani for never giving up on me, thank you all from the bottom of my heart. Undoubtedly, the success of this study would not have been possible without my mother‟ prayers which have given me spiritual support. My special thanks also go to my sisters and brothers who believe in knowledge and consider the success of individuals a success for all.

Finally, special thanks and appreciation are due to my beloved wife Amal who has stood beside me, tolerated me and provided endless encouragement, support, and patience throughout my studies. My love and gratitude go to my children Osama and Idris who experienced with me the ups and downs during the duration of my studies.

Abbreviations

AB	Antibody
ABC	Avidin-Biotin Complex
AJCC	American Joint Committee on Cancer
ARM	Antigen Retrieval Method
ABD	ATPase-binding domain
BM	Basement Membrane
CA	Cancer Antigens
CAM	Cell – Cell Adhesion Molecule
CDK	Cyclin-dependent kinase
CNB	Core Needle Biopsy
CT	Computerised Tomography
DNA	Deoxyribonucleic Acid
EC	Endothelial cell
EMA	Epithelial Membrane Antigen
ECM	Extracellular Matrix
FFPET	Formalin-Fixed Paraffin-Embedded Tissue
FGF	Fibroblast Growth Factor
FISH	Fluorescence in Situ hybridization
FNCLCC	French Federation of Cancer Centres Sarcoma Group
FS	Fibromyxoid sarcoma
GRP78	glucose regulated protein-78
GSK	Glycogen synthase kinase
HIF	Hypoxia inducible factor
HOP	Heat organizing protein
HSF	Heat shock transcription factor protein

HSPs	Heat Shock Proteins
HSP90	Heat shock protein 90
HSP70	Heat shock protein 70
HSP27	Heat shock protein 27
IHC	Immunohistochemistry
IGF	Insulin-like growth factor
IL	Interleukin
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinases
MFS	Myxofibrosarcoma
MS	Myofibroblastic sarcoma
MIFS	Myxoinflammatory fibroblastic sarcoma
NF-κB	Nuclear factor κB
PBD	Peptide-binding domain
PDGF	Platelet-derived growth factor
PGF	Placental growth factor
PKB	Protein kinase B
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinases
STS	Soft tissue sarcoma
TAFs	Tumor-associated fibroblasts
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor

Chapter One

Introduction &

Literature review

1 Introduction and literature review

1.1 Soft tissue sarcoma (STS)

Soft-tissue sarcomas (STS) are a heterogeneous group of rare malignant tumours which arise from mesenchymal tissue in essentially every tissue of the body. They can differentiate along many tissue lineages, such as adipose, fibrous, cartilage, muscle or bone. Their natural behaviour is different from that of epithelial tumours (1). The biology of sarcomagenesis is not precisely understood, but models of stepwise progression to malignancy or in situ precursor lesions are not founded in the manner of many of the epithelial neoplasms. In opposition to epithelial tumours, sarcomas often do not arise from their corresponding mature adult tissue, and the progenitor cell remains unclear (2). Furthermore, soft tissue tumour behaviour is classified as benign, intermediate (locally aggressive), intermediate (rarely metastasizing), and malignant (3).

1.1.1 Incidence and Epidemiology

STSs account for approximately 1% of all adult malignancies, equating to approximately 1,500-2,000 cases a year in the UK (4). They can occur in any age group, however, the incidence of soft-tissue sarcomas rises significantly with age, with more than 65% of cases affecting patients aged 50 and above (5). A STS can occur in almost any site of the body, making it distinct amongst other human growths. The most common site of sarcoma is in the extremities (66%), with approximately two thirds of these occurring in the lower limbs (6). In 2010, there were 3298 newly diagnosed cases of soft tissue sarcoma in the UK, of which 51%

were in males and 49%were in females. Although the yearly incidence of soft tissue sarcoma is regularly higher in males than females, this difference is not statistically significant (4).

1.1.2 Aetiology and Risk factors

The aetiology of most soft tissue sarcoma remains unknown; however, it has been associated with genetic diseases, viral infections, and environmental factors (7). Furthermore, inherited diseases have been found to be related to sarcoma development. For example, Li-Fraumeni syndrome caused by germline mutations in the p53 tumour suppressor gene leads to sarcoma formation and neurofibromatosis Type 1 can also result in the development of peripheral nerve sheath tumours (8). Also, it is known that exposure to radiotherapy, as a treatment for other cancers, has an association with the development of STS. The most common sub-types of sarcoma in patients receiving radiotherapy are extra-skeletal osteogenic sarcoma (21%) and undifferentiated pleomorphic sarcoma (16%) (9).

1.1.3 WHO classification

As part of the International Classification of Diseases (ICD), the World Health Organisation (WHO) publishes classification systems for all types of cancer. The 'WHO Classification of Tumours of Soft Tissue and Bone', a sub classification of ICD covers sarcomas. This was revised in 2013 and published in a 4th Edition and provides a universal nomenclature which helps to ensure comparability of international clinical trials and translational research (10).

Although the WHO system depends on morphologic appearance to classify STS, the new system of classification includes much more detailed cytogenetic and molecular data in keeping with the exponentially increasing knowledge on the genetics of tumours. One important change is the removal of the term 'malignant fibrous histiocytoma' (MFH). This was previously one

of the most common diagnoses within soft-tissue sarcomas, but advances in molecular diagnosis have shown that these tumours should be classified with other more specific types of sarcoma as 'myxofibrosarcoma' (10). However, this leaves a small number of tumours that do not come under the diagnostic criteria of other specific types. Hence, the new WHO classification of soft tissue sarcomas has more than 50 histologic subtypes. A simplified classification of STSs by cell type differentiation is presented in Table 1.1.

Table 1.1 The WHO Classification of STSs.

WHO Classification of Soft Tissue Tumours	Most Common Sarcoma Subtype
Adipocytic tumours	Liposarcoma
Fibroblastic/myofibroblastic tumours	Myxofibrosarcoma, Low grade Fibromyxoid sarcoma, Myxoinflammatory fibroblastic sarcoma, Myofibroblastic sarcoma
Smooth-muscle tumours	Leiomyosarcoma
Skeletal-muscle tumours	Rhabdomyosarcoma
Vascular tumours	Angiosarcoma
Peripheral nerve tumours	Malignant Peripheral Nerve Sheath Tumour (MPNST)
Tumours of uncertain origin	Synovial sarcoma, Epithelioid sarcoma, Extra-skeletal Ewing sarcoma, Clear cell sarcoma

Adapted from WHO Classification of Tumours of Soft Tissue and Bone. 2013 (3)

The five most common STSs in adults are undifferentiated pleomorphic sarcoma (28%), liposarcoma (15%), leiomyosarcoma (12%), synovial sarcoma (10%), and malignant peripheral nerve sheath tumours (MPNST) (6%), whereas the residual 45 histological STS sub-types represent the remaining 29% (11).

1.1.4 Diagnosis of STS

Any patient with a suspected STS should have a triple assessment with clinical history, imaging and biopsy. In the UK, The National Institute for Health and Care Excellence (NICE) provides comprehensive guidelines for the management of soft tissue sarcoma. To achieve a successful diagnosis of STS, a collaboration between the specialist radiologists, surgeons, oncologists and pathologists is vital; prompt referral to specialist centres is designed to facilitate early and consistent diagnosis (12).

1.1.4.1 Clinical presentation and patient examination

Due to the various sites of origin of STS, it is difficult to clearly define the clinical features of the disease. Nonetheless, the most common presentation of a STS is as a painless mass increasing in size. The clinical history should inquire about a personal or family history of cancer or pre-malignant/inherited conditions such as neurofibromatosis, Li-Fraumeni Syndrome or Multiple Hereditary Exostoses, as well as other lesions. Clinical examination is the standard examination of any lump plus an assessment of the neurovascular status distally and palpation of both local and systemic lymph nodes. Approximately one third of sarcomas are superficial to the fascia, the remainder are deep. Usually, local symptoms develop late. A large tumour may cause compression of neural and vascular structures, resulting in radicular pain, venous stasis and lymphedema. In the advanced stage, the tumour may infiltrate the overlying skin, causing ulceration or necrosis (12, 13).

1.1.4.2 Imaging

Imaging investigations such as X-rays, ultrasound, CT scan, and magnetic resonance imaging (MRI) are also used to determine the anatomical location of the tumour, its size and infiltration into the surrounding tissues. A standard X-ray may be used to identify any connection with, or

erosion into, bone or calcification within the mass and ultrasound is often utilised in the primary care setting. NICE recommends urgent ultrasonography, within two weeks in adults and within two days for children and young people, for all unexplained lumps increasing in size (13). When the mass is smaller than 5 cm and superficial, ultrasound is appropriate, but when larger than 5 cm or deep, sarcoma centre referral and MRI scanning is mandatory. A MRI is preferred to CT for further information regarding size, location and extent of the tumour to help plan the biopsy and surgical intervention. For staging, a chest radiograph and CT thorax (plus or minus abdomen and pelvis) are required. An isotope bone scan has a limited role, whereas whole body MRI is an established technique in STS staging of myxoid liposarcoma because of their propensity for unusual metastases (14).

1.1.4.3 Biopsy

Despite advanced imaging techniques, there is only one reliable method of establishing whether a soft tissue tumour is benign or malignant, and that is by performing a biopsy on the tumour. Hence, a biopsy is the required initial procedure for all soft tissue lumps or masses that persist or increase in size. Numerous biopsy techniques are available, including: open incisional biopsy (where a portion of the tumour is removed), open excisional biopsy (where the entire tumour is removed), core needle biopsy (CNB), fine-needle aspiration (FNA), and core biopsy. Biopsy should be performed within the sarcoma diagnostic or treatment centre (12).

FNA is a convenient technique to perform, being a relatively short procedure that may be conducted under local anaesthetic in an outpatient clinic. However, it can be difficult to make an accurate primary diagnosis with FNA alone. It should be used in centres in which cytopathologists have experience in both the FNA technique and the interpretation of the results. The diagnostic accuracy of FNA, based on results of patients with primary tumours,

ranges from 60% to 96% (15). In other instances, FNA provides only a cytological sample with scant tissue, consequently, accurate typing and grading are not usually possible.

Core needle biopsy (CNB), usually under ultrasound guidance, is the method of choice, as it is a safe, accurate, and economical procedure for diagnosing STS. It provides an adequate sample for use in several diagnostic analyses, such as cytogenetic analysis and electron microscopy. Frequently, diagnosis is made on CNB material, and tumour subtype and grade can be determined in about 80% of core biopsies (16). Additionally, it is usually performed under CT or, less commonly, ultrasound guidance. CNB has been shown to be effective in the diagnosis, and sub-typing or grading of STS and, moreover, has lower morbidity. It also has a sensitivity of 82–100% and a specificity of 91–100% in STS, with complication rates of 0–1.1% (17).

This is a reliable diagnostic method for obtaining an adequate and representative sample of tissue for diagnosis. It is usually performed when a diagnosis has not been possible from fine-needle aspiration or core needle biopsy specimens. It is performed in a designated treatment centre, preferably by the same surgeon who will carry out the surgery, and hence, the procedure requires careful surgical planning. The direction of the incision should be orientated longitudinally along the extremity such that the resulting biopsy tract is easily excised at the time of the definitive tumour resection procedure (18).

Excision biopsy is sometimes straightforward and easily accessible at the outset, but this is usually reserved for tumours with certain features outside the sarcoma treatment centre, such as when the lump is superficial, well defined and < 3 cm in diameter (19). However, the benefits of excisional biopsy rarely exceed those of other biopsy techniques, and these procedures may also cause post-operative complications so ultimately, it would be best to perform a diagnostic biopsy first and delay the final surgical treatment (11).

1.1.5 Histology

Histological diagnosis of soft tissue tumours is known as being one of the most challenging areas in histopathology. For example, there is significant morphological overlap between soft tissue neoplasms and reactive lesions. Some aggressive sarcomas appear histologically bland while reactive conditions such as nodular fasciitis show potentially worrying characters including high cellularity and mitotic activity. Furthermore, A broad range of different soft tissue tumours have spindle cell morphology, and ancillary stains may be required to show specific differentiation features. Since identifying a histopathologic type of sarcoma is often difficult, some ancillary methods, such as immunohistochemistry and genetic analysis, are helpful in supporting the morphologic diagnosis.

1.1.5.1 Immunohistochemistry (IHC)

The diagnostic IHC technique was introduced in the early 1980s and it can localise molecules in tissues by observing histological staining due to the binding of specific antibodies (20). It is now a regular tool for evaluating the diagnosis and prognosis of tumours due to its relatively low cost, simplistic technique and the availability of a large number of increasingly sensitive and specific antibodies. In addition, IHC is important in the diagnosis of STSs because of their variety, with several lines of differentiation, and the common difficulty of diagnosis with numerous pseudosarcomatous benign lesions and non-mesenchymal malignant tumours (20).

The impact of IHC in pathology may be explained by three major advances, the availability of appropriate antibodies for routine formalin-fixed tissue, improvements in antigen retrieval methods (particularly heat-induced epitope retrieval which provides consistent and reliable results), and the availability of sensitive detection systems (21). However, IHC also has its limitations, since no IHC marker is wholly specific for any tumour and positive findings should

be taken in the context of the total antibody panel and the tumour morphology. For example, CD99, originally thought to be specific for Ewing sarcoma, is expressed to varying degrees in several tumours, including those in the Ewing sarcoma differential diagnosis, such as poorly differentiated synovial sarcoma (22). Also, CD117 (cKIT), positive in more than 95% of gastrointestinal stromal tumours (GISTs), is variably expressed in other tumours, such as angiosarcoma, Ewing sarcoma, synovial sarcoma, leiomyosarcoma, and MFH (23). In addition, sometimes the inappropriate antibody panels are selected because of incorrect light microscopic analysis, resulting in IHC results which are internally consistent, but completely inaccurate. Moreover, the continuing delineation of new entities and production of new antibodies will lead to the expansion of panels required for differential diagnosis, particularly when there are critical therapeutic options. The most important markers used in the differential diagnosis of STS and their diagnostic targets are listed in Table 1.2.

Table 1.2 Markers used in the differential diagnosis of STS.

IHC Markers	Diagnosis
Endothelial Markers	
CD31	Angiosarcoma, Kaposi sarcoma
CD34	Kaposi sarcoma, many vascular fibroblastic
Muscle Cell Markers	
Actin	Smooth and skeletal tumours, myofibroblastic
Desmin	Smooth and skeletal tumours
Calponin	Smooth muscle, myofibroblasts, myoepithelial, synovial sarcoma
H-caldesmon	Smooth muscle (Leiomyosarcoma)
Myoglobin	Rhabdomyosarcoma
Histiocytic Markers	
Lysozyme	Histiocytes, myelomonocytic cells
CD68	Histiocytes, macrophage, schwanoma
CD163	Histocytes

Other Markers	
S-100	Melanocytic, schwannian, chondroid
Keratin	Synovial and epithelioid sarcoma
Vimentin	Mesenchymal tumours, many poorly differentiated carcinoma
Osteocalcin	Osteosarcoma, osteoid material
CD99	Ewing sarcoma
Desmoplakin	Epithelial tumours in general, Ewing sarcoma

Adapted from Miettinen, 2003 (24)

Aggressive tumour phenotypes are characterised by vascular invasion, metastasis and local recurrence; however, this is more about research than current diagnostic work. For example, the factor VIII can be used to measure vessel density, Ki-67 is a marker of proliferation, and the p53, p27 and Bcl2 proteins are all related to the regulation of the cell cycle, hence, linked to apoptosis. Furthermore, the expression of CD44 and ezrin are associated with cell adhesion, and related to cell migration and metastasis (1, 24). No immunohistochemical marker is completely specific for any tumour and positive findings should be considered against the background of the total antibody panel as well as the tumour morphology (20).

1.1.5.2 Genetic Analysis

Occasionally, a diagnosis is not possible from morphologic features and IHC, therefore, the detection of sarcoma with specific genetic alterations, such as chromosomal translocation and complex karyotypes, represents an additional diagnostic and prognostics tool in STS (25). STS with recurrent chromosomal translocations can be classified into sub-types depending on the presence of fusion gene transcripts, for example, TLS-CHOP in myxoid or round cell liposarcoma, SYT-SSX (SYT-SSX1 or SYT-SSX2) in synovial sarcoma, EWS-ATF1 in clear cell sarcoma, and PAX-FKHR (PAX3-FKHR or PAX7-FKHR) in alveolar rhabdomyosarcoma. The fusion genes resulting from chromosomal translocations could be of clinical importance for diagnostic and prognostic information (1).

Kawai *et al.* suggested that the type of SYT-SSX fusion gene may be prognostic for survival in patients with synovial sarcoma. Another study found that 46% of synovial sarcomas have SYT-SSX1 and 36% have SYT-SSX2. The five-year survival for localised synovial sarcoma with the SYT-SSX2 was 48% as compared to 24% for patients with SYT-SSX1. Therefore, the type of SYT-SSX fusion gene appears to positively influence subsequent clinical behaviour in synovial sarcoma (26).

There are several techniques that can be used for cytogenetic analysis, but the main techniques are fluorescence *in–situ* hybridisation (FISH), reverse-transcriptase polymerase chain reaction (RT-PCR), and the more recent, gene expression profiling (1). It has been suggested that the molecular heterogeneity of the fusion transcript is useful in predicting the prognosis in particular sub-types of sarcoma. For example, fusion transcript type 1 EWS-FLI1 has been demonstrated to indicate better survival in patients with Ewing sarcoma than other fusion gene transcripts (27). Also, in alveolar rhabdomyosarcoma, the presence of PAX7-FOXO1A has been associated with a favourable prognosis, compared with the more common PAX3-FOXO1A (28). In patients with synovial sarcoma, a less clear impact has been noted among the fusion gene transcripts SYT-SSX1 and SYT-SSX2 (1).

In summary, genetic analysis appears promising, it involves highly complex techniques and has the potential to be useful for the differential diagnosis of sarcoma and the prediction of tumour behaviour. Additionally, the technical limitations associated with genetic analysis suggested that molecular evaluation be considered as an ancillary technique only. Genetic analysis findings should only be interpreted in the context of the morphologic features of a sarcoma (25, 29).

Genetic markers represent an increasingly important field in STS diagnosis and prognosis. In general, the oncogenes, which can induce malignant transformation and cell proliferation, have

been implicated in the development of STS. In the majority of cases, oncogene activation results from chromosomal rearrangements or gene amplifications. Changes in the microenvironment of the gene, for example, following epigenetic modifications, must also be considered. Example of oncogenes linked to STS are the C-KIT and PDGFRA mutations in GIST, and the fusion oncogene, such as SS18-SSX, in 90% of synovial sarcoma (27). In contrast, tumour suppressor genes (TSG) play a critical role in cell growth and direct the cell programme toward programmed cell death or apoptosis. Unlike oncogene activation, the loss or change in the TSG function commonly results from deletions or discrete mutations. Two major TSGs that are relevant to STS are the retinoblastoma gene (RBI) and the TP53 gene (tumour protein p53). Approximately 30–60% of STSs have been reported to harbour aberrations of the TP53 gene, including a sub-set of patients with germ-line mutations i.e. the Li-Fraumeni syndrome (30).

1.1.6 Prognostic factors

The overall STS prognosis in terms of survival is 50% for high-grade sarcoma and 90% for low-grade sarcoma (31). Five prognostic factors determine the outcome of STS, depending on advanced patient age, presence of metastases at presentation, tumour size, grade and depth. Of these, histological grade has been shown to be the most critical factor (32). These prognostic factors appear to have slightly different importance in different studies and in aspects of STS prognosis (local recurrence rate, distant metastasis, and tumour-related death). In most studies, poor prognostic factors for local recurrence were microscopically positive margins, tumour size and, in some studies, patient age over 50 years. Tumour size and grade are the major determinants of death from tumours (33, 34).

1.1.6.1 Grading of STS

Currently, the most important prognostic factor and the best indicator for accurate STS diagnosis is histological grade, and to help in this several grading systems have been developed. In the UK and Europe, the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) grading system is commonly adopted, which defines three grades (32). This system is based on three pathological parameters, degree of tumour differentiation compared with normal tissue, mitotic count, and amount of tumour necrosis, as shown in Table 1.4. In addition, the value of the malignancy grade as the prognostic variable for STS has been reported by many groups (35). These classical parameters have been proven to have value as prognostic markers for decades. As shown by Fletcher *et al.*, the mitotic index, the extent of tumour necrosis, and tumour size appear to be the most reliable predictors (8, 10).

Table 1.3 FNCLCC grading system: definition of parameters.

Tumour differentiation	
	Score 1: Sarcomas closely resembling normal adult mesenchymal tissue (e.g., low grade leiomyosarcoma) **Score 2: Sarcomas for which histological typing is certain (e.g., myxoid liposarcoma)** **Score 3: Embryonal and undifferentiated sarcomas, sarcomas of doubtful type (e.g., synovial sarcomas, PNET)**
Mitotic Count	
	Score 1: 0–9 mitoses per 10 HPF* Score 2: 10–19 mitoses per 10 HPF Score 3: ≥ 20 mitoses per 10 HPF
Tumour Necrosis	
	Score 0: no necrosis Score 1: <50% tumour necrosis Score 2: ≥50% tumour necrosis
Histological Grade	

	Grade 1: total score 2, 3 Grade 2: total score 4, 5 Grade 3: total score 6, 7, and 8

Adapted from Coindre *et al.* 2006 (36). PNET – primitive neuroectodermal tumour. * high power field (HPF) measuring 0.1734 mm.

To enhance the reproducibility and reliability of the system, the parameters are defined as exactly as possible to help to predict a chance of survival and distant metastatic rates of the patients with the tumours grade I–III. Nonetheless, many other grading systems are in use and are usually based on similar histological parameters, including the NCI (National Cancer Institute) three-grade system preferred in the USA. The Trojani/FNCLCC has confirmed slight superiority over NCI grading (37), and has been used to give the most complete survival data for STS to date. This is presented in Table 1.4.

Table 1.4 Survival rates in STS according to the Trojani/FNCLCC grading system.

Grade	5-year survival	10-year survival
I	90%	85%
II	70%	65%
III	45%	30%

Adapted from Kotilingam *et al.* 2006 (38).

1.1.6.2 Tumour size and site

The tumour size is of great importance in the prognosis. In this respect, STSs are divided into two groups according to whether they include T1 lesions, which are 5 cm or smaller, and T2 lesions, which are larger than 5 cm, since the 5-cm criterion has widespread acceptance as a poor prognostic variable. Prognosis is particularly poor for tumours larger than 10 cm at presentation due to a high rate of metastatic recurrence (39). In addition, some authors have

suggested that the further stratification of tumours larger than 5 cm would give more accurate prognostic information. A study of 316 patients who were divided on the basis of tumour size into four groups, demonstrated that the prognosis of each group differed substantially, with tumour sizes of less than 5 cm, 5 to less than 10 cm, 10 to 15 cm, and greater than 15 cm, the five-year survival rates were found to be 84%, 70%, 50%, and 33% (40). Additionally, a relationship was found between the location of the tumour and its size. Those STSs in the distal extremities are generally both superficial and small at the time of diagnosis and have a better prognosis than those found intra-abdominally, which often reach large proportions before they can be diagnosed (19).

1.1.6.3 Staging

The most commonly adopted staging system for soft-tissue sarcoma is provided by the American Joint Committee on Cancer (AJCC) (Table 1.5). This AJCC system uses the four criteria of tumour size, nodal status, grade, and metastasis (TNGM) to designate one of four disease stages (41).

Table 1.5 The American Joint Committee on Cancer Staging System, 7th edition.

Tumour Size (T)	
	T1 Primary tumour < 5cm (T1a – superficial; T1b – deep)
	T1 Primary tumour > 5cm (T2a – superficial; T2b – deep)
Lymph Nodes (N)	
	N0 No regional lymph nodes
	N1 Regional lymph nodes involved
Metastases (M)	
	M0 No metastases
	M1 Distant metastases
Histological Grade (G)	
	G1 Well differentiated

	G2 Moderately differentiated
	G3 Poorly differentiated
	G3 Poorly differentiated or undifferentiated
American Joint Committee on Cancer (AJCC) staging	
STAGE 1:	
	1A = low grade, small, superficial or deep (T1a-b, N0, M0, G1-2)
	1B = low grade, large, superficial (T2a-b, N0, M0, G1-2)
STAGE II:	
	IIA = low grade, large, deep (T2b, N0, M0, G1-2)
	IIB = high grade, small, superficial or deep (T1a-b, N0, M0, G3-4)
	IIC = high grade, large, superficial (T2a, N0, M0, G3-4)
STAGE III:	
	High grade, large, deep (T2B, N0, M0, G3-4)
STAGE IV:	
	Any metastasis (Any T, N1 or M1, Any (G)

Adapted from Edge *et al*., 2010 (41)

Overall, tumour grade and size are the most important determinants of prognosis, with approximately two-thirds of all sarcomas being high-grade. Another study claims that the prognostic risk for metastatic disease is mostly dependent on the histopathologic grade in the first two or three years, but after that, the size of the tumour becomes at least as significant as grade (42).

1.1.7 Treatment

In the UK, soft tissue sarcomas are managed in a specialised sarcoma centre by multidisciplinary teams, typically comprising sarcoma specialist surgeons, radiologists, medical and clinical oncologists, a specialist sarcoma histopathologist as well as specialist nurses and physiotherapists (12). Every patient with a soft-tissue sarcoma will require an individualised treatment plan and the management of newly-diagnosed sarcoma is not usually

straightforward, therefore, the therapeutic goals are improving survival, avoiding local recurrence, maximising function, and minimising morbidity. Treatment of STS is based on the size and grade, location of the tumour, and whether the tumour has metastasised. The options for treatment of STS are surgery, radiation therapy (local treatment), and chemotherapy (systemic treatment)(6).

1.1.7.1 Surgery

Surgical excision remains the cornerstone of therapy and is a dominant curative procedure with adjuvant (treatment given after the surgery to kill the remaining cancer cells) and neoadjuvant RT (treatment given before surgery) for STS in selected patients, except for those patients who are surgically inoperable or those who decline surgery if it entails unacceptable functional loss (43). The extent of surgery required, along with the optimum combination of radiotherapy, remains controversial, but the clinical and pathological factors of the specific tumour need to be considered by the surgeon to determine the treatment plan for individual patients. Therefore, increased understanding of sarcoma biology as the primary determinant of outcome has led to the decrease in radical amputations and the recognition of limb-sparing treatments, often using a multimodal team approach (12).

A recent study at the Memorial Sloan-Kettering Cancer Centre highlighted the need for collaborative, inventive approaches for sarcoma patients, as it appears to have reached that level with current therapy (44). In recent years, several more accurate descriptions of the surgical margin have been used and it is common practice to classify margins as positive or negative. A positive margin has been defined as one in which tumour cells are present at, or within, 1 mm of the resection margin, but which is otherwise negative. In some reports, margins have been documented as grossly positive, microscopically positive, close (5 mm or smaller) or negative (45); in others as clear (> 10 mm), close (1–9 mm) or positive (46).

Low grade tumours are usually treated by surgical excision with excellent functional prognosis, whereas surgery or radiation alone is usually inadequate for the management of high grade tumours more than 5 cm in size, locally recurrent, deep in investing fascia, having previous inadequate surgery or positive margins, and certain sub-types such as leiomyosarcoma or malignant peripheral nerve sheath tumour (12).

1.1.7.2 Radiotherapy

External beam RT is the most widely used modality, since its practice in now routine, and its application is technically less complex. In a randomised prospective study, it was reported that local control, conservative surgery with external beam RT is as effective as amputation in high-grade STS (47). RT is more commonly given post-operatively than pre-operatively. Post-operative RT has the following advantages: the operation can be performed without delay, the surgical specimen and margins can be accurately histologically analysed, and, in the event of wide margins, RT can be omitted. Nevertheless, pre-operative RT may be favoured in efforts to reduce the tumour size before surgery, with a view to minimising the resection area, reducing the seeding of viable tumour cells over the operative field and reducing the treatment volume.

However, a randomised trial comparing pre- and post-operative RT, with wound complications as an end point, found that pre-operative RT was associated with a greater risk of wound complications (48). Pre-operative RT and chemotherapy also cause tumour necrosis, thus confound histopathological typing and grading of the surgical specimen. Sequencing of RT with surgery has not been proved to provide a reliable benefit for outcome (49). Generally, pre-operative RT is recommended for large tumours in a difficult location to reduce their size and to make conservative surgery possible (50).

1.1.7.3 Chemotherapy

Chemotherapy has poorer results for sarcoma treatment, the effectiveness of neoadjuvant and adjuvant therapy for STS is still currently controversial, with little proof in the literature to support the use of either. However, it is considered for patients with high grade, large, and deep STS to improve patient survival based on the persisting poor survival outcome (50–60%, year survival) of high-risk STS patients, despite having good local disease control with surgery and radiotherapy (11).

According to a meta-analysis of 14 randomised trials comprising a total of 1,568 patients, doxorubicin-based adjuvant chemotherapy appeared to significantly increase the time to local or distant recurrence, improving the overall recurrence-free survival rate in adults with localised resectable STS (51). Significant absolute benefits were 6–10% at 10 years, and there was some evidence of a trend towards improved overall survival without a clear improvement in overall survival. In an Italian randomised co-operative trial, patients with high grade or recurrent sarcoma of extremities were randomised to undergo post-operative chemotherapy with epirubicin and ifosfamide or observation alone. After a median follow-up of 59 months, the treatment did significantly better in median overall survival (75 vs. 46 months) and the disease free period (48 vs. 16 months) (52).

In addition, there is further evidence of neoadjuvant chemotherapy benefitting survival in adult STS in the treatment of rhabdomyosarcoma because, unlike most sarcomas, great response rates are observed in this sub-type. The importance of neoadjuvant chemotherapy has been suggested, since it is sometimes possible to use the response of the sarcoma to such therapy as a guide for further treatment or for prognosis (53). A study conducted at the MD Anderson Cancer Center found that the sarcoma response to pre-operative chemotherapy gives reliable prognostic information, and can identify a sub-group of patients most likely to benefit from

conventional adjuvant chemotherapy or those who should be considered for second-line or experimental treatments (54).

Furthermore, chemosensitivity varies significantly between sub-types. Although most STSs are generally resistant to chemotherapy, smaller tumours have a higher growth fraction and are potentially more chemosensitive, whereas the larger the tumour becomes, the more likely chemoresistant clones will spontaneously arise (53).

1.1.8 Types of soft tissue tumours studied in this thesis

The focus of this study is on myxoid and myofibroblastic soft tissue tumours, which are well represented in our archival material of adult STS of extremities.

1.1.8.1 Myxoid soft tissue tumours

Myxoid soft-tissue tumours comprise a heterogeneous group of mesenchymal tumours characterised by the production of a copious extracellular myxoid matrix. Myxoid tissue is not a normal tissue in adults and is actively secreted by tumour cells in these lesions. It consists of a gelatinous mucopolysaccharide matrix of sulphated and non-sulphated glycosaminoglycans (55). Myxoid soft-tissue neoplasms encompass a heterogeneous group of benign, locally aggressive, and malignant lesions, commonly affecting the extremities. There is significant overlap in the clinical and pathologic characteristics of these tumours, making a precise diagnosis challenging. Current developments in understanding the genetic and molecular characteristics of these tumours have led to notable advances in their classification, diagnosis, and management. For instance, round cell liposarcomas have been reclassified as myxoid liposarcomas, and the myxoid variant of malignant fibrous histiocytoma has been reclassified as myxofibrosarcoma due to their similar cytogenetic profile (10, 56). The 4th edition of the WHO classification of tumours of soft tissue published in 2013, updating the former 2002 classification, proposed many changes in soft-tissue tumour classification and reported new genetic and molecular data for the tumours (3). A simplified classification of myxoid soft-tissue tumours based on the WHO classification is shown in Table 1-6.

Table 1.6 Classification of myxoid soft-tissue tumours (57)

Benign	Malignant
Myxoma	Myxofibrosarcoma
Myxolipoma	Low-grade fibromyxoid sarcoma
Angiomyxoma	Myxoinflammatory fibroblastic sarcoma
Acral fibromyxoma	Myxoid Liposarcoma
	Extraskeletal myxoid chondrosarcoma
	Ossifying fibromyxoid tumour

Adapted from Baheti et al, 2015 (57)

1.1.8.1.1 Myxofibrosarcoma (MFS)

Myxofibrosarcoma, previously known as the myxoid variant of malignant fibrous histiocytoma, is now considered as a distinct entity (58). Myxofibrosarcoma usually manifests in the sixth decade, with equal sex predilection and most commonly affects the extremities. It may be localised in the subcutaneous tissue, dermis, and intermuscular or intramuscular planes (59). Unlike other soft-tissue sarcomas, which are well-defined tumours, myxofibrosarcomas have an ill-defined infiltrative margin with an ability for centrifugal spread along fascial and vascular planes, which predisposes them to inadequate resections and high recurrence rates in 50–60% of cases. Local recurrence is associated with the resection margin and low-grade tumours can become high grade after recurrence, with a higher tendency for metastases (60, 61). MRI is the imaging modality of choice for myxofibrosarcomas. MFS appear heterogeneous on both T1- and T2-weighted images and lesions are T2 hyperintense with ill-defined infiltrative margins and inhomogeneous contrast enhancement (62). Metastases are more common in high-grade tumours, and lungs are most commonly affected. The differential diagnosis involves other myxoid neoplasms (e.g., myxoid liposarcoma and myxoinflammatory fibroblastic sarcoma), other soft-tissue tumours (e.g., undifferentiated pleomorphic sarcoma), and inflammatory conditions (e.g., nodular fasciitis) (63). The treatment of choice for

myxofibrosarcoma is wide surgical excision, with a 5-year survival rate in a recent study of 158 patients of 77% (60, 63).

1.1.8.1.2 Myxoinflammatory Fibroblastic Sarcoma (MIFS)

Myxoinflammatory fibroblastic sarcoma is a rare low-grade sarcoma first described in 1998 independently by three separate groups (64-66). It can affect both men and women equally, and all age groups. It characteristically involves the extremities and is often termed "acral myxoinflammatory fibroblastic sarcoma." It shows four components on pathologic analysis, proliferative fibroblasts (spindle cells), myxoid matrix, inflammatory components, and atypical giant cells. In the WHO classification, it is described as being characterised by the translocation t(1;10) (3, 10). Patients commonly present with a painless slowly growing mass, but some may present with pain or tenderness. On imaging, these tumours are predominantly subcutaneous, with well- or ill-defined and infiltrative lesions frequently located along the tendon sheath (67). Local recurrence is common, occurring in 28–51% of cases, while metastases are rare. A recent retrospective study of 104 cases described a single case of metastasis and 51% local recurrence in the 59 patients with follow-up (68). Due to the infrequent incidence of distant metastases, the WHO classification has introduced a new term for them, "atypical myxoinflammatory fibroblastic tumour," (10). Wide surgical excision of the tumour is the standard treatment, with a negative resection margin correlating with decreased recurrence (68).

1.1.8.1.3 Fibromyxoid sarcoma (FS)

Low-grade fibromyxoid sarcoma was first described by Evans in 1987 (also known as Evans tumour). It is a rare tumour that equally affects middle-aged men and women, commonly involving the extremities, trunk, and deep soft tissue (69, 70). Although FMS is characterised by relatively benign pathologic characteristics consisting of bland spindle cells with intermixed myxoid and fibrous stroma, it reveals aggressive behaviour with high rates of recurrence and

metastases (70). According to the recent WHO classification, MUC4 is a very sensitive and specific immunohistochemical marker for low-grade fibromyxoid sarcoma. Also, the presence of translocation t(7;16) and the formation of FUS/CREB3L2 fusion gene are also specific markers (3). Imaging reveals features consistent with the tumour's fibrous and myxoid content, with MRI showing the fibrous component to be T1 and T2 hypointense and the myxoid component to be T1 hypointense and T2 hyperintense (71). Wide surgical excision is the treatment of choice, local recurrence is common in the form of multiple lesions, and metastases usually affect the lungs (70).

1.1.8.1.4 Intramuscular Myxoma

Intramuscular myxoma is the most common benign myxoid tumour consisting of abundant myxoid stroma interspersed with benign spindle cells. Women are affected more commonly than men (mean patient age, 50–60 years). Although they are most frequently intramuscular (82%), they can also be intermuscular or subcutaneous. The lower limb is the most common site affected and patients typically present with a slowly growing painless mass. Association with fibrous dysplasia has been reported in Mazabraud syndrome, which is characterised by multiple myxomas with associated (usually polyostotic) fibrous dysplasia (72-74). On MRI, the lesions are well circumscribed with smooth or slightly lobulated margins, homogeneously hypointense in T1-weighted images, and extremely hyperintense in T2-weighted images, similar to water (75). The choice of treatment for intramuscular myxoma is wide surgical excision, with recurrence being rare (72).

1.1.8.2 Fibroblastic/Myofibroblastic soft tissue tumours

Tumours and tumour-like lesions of myofibroblasts may present a diagnostic challenge because of the uncertainties in identifying the myofibroblast, its attributes place it midway between a fibroblast and a smooth muscle cell, and since it appears capable of functional and phenotypic modulation (76). The main features of the myofibroblast include a spindled or stellate morphology; immunostaining for a-smooth muscle actin and the extra domain (76, 77).

However, since most diagnostic tumour pathology is based on phenotypic features of the excised lesion, it is an unavoidable fact that a wide range of soft tissue tumours show convincing myofibroblastic features. This type of lesion can be divided into four main groups: 1) the family of reactive fasciitis-like lesions, 2) a group of benign lesions (e.g., myofibrolastoma and dermatomyofibroma), 3) the locally aggressive fibromatoses (either superficial or deep fibromatosis) and, finally, 4) sarcomas showing myofibroblastic differentiation, the latter including myofibroblastic sarcoma (77, 78).

1.1.8.2.1 Myofibroblastic sarcoma (MS)

Myofibrosarcomas (myofibroblastic sarcomas) are rare neoplasms which are difficult to diagnose because myofibroblasts and fibroblasts appear morphologically similar in H&E sections, and reactive and neoplastic myofibroblasts, as well as some smooth muscle tumours, can be immunophenotypically indistinguishable. Myofibrosarcomas display a range of differentiation. Low- and intermediate-grade myofibrosarcomas are morphologically similar, and are distinct from pleomorphic myofibrosarcomas, which are high-grade neoplasms formerly classified as malignant fibrous histiocytomas. Other low-grade malignant tumours with myofibroblastic differentiation are inflammatory myofibroblastic tumours and infantile fibrosarcoma, which have specific genetic rearrangements and are not usually designated as myofibrosarcomas (76). The age range of patients with low-grade myofibrosarcoma (LGM) is

4–75 years, with a slight male predominance. The tumours range in size from 1.5 to 17 cm and most commonly occur in the head and neck region, proximal extremities and trunk, with occasional cases in the abdomen or pelvis (79). LGM usually arise in deep soft tissue, but some occur in the subcutis or submucosa. Although there can be partial circumscription, the growth pattern is predominantly infiltrative, in a fascicular, sheet-like, or occasionally storiform arrangement. Mitotic activity is variable but abnormal forms are usually absent (76). LGM are immunoreactive for actin (a-SMA and muscle-specific) in a multifocal pattern, fibronectin, calponin and less commonly (and more focally), desmin. Cytokeratin, epithelial membrane antigen (EMA), S100 protein and CD34 are almost always negative (80, 81). About a third of LGM recur locally, especially following incomplete excision. Metastasis, sometimes after a long period, has been reported in approximately 10% of published cases, usually to lung, but occasionally to other locations. These tumours are best managed by surgical excision, with the aim of achieving tumour-free resection margins (76, 77).

Pleomorphic high-grade myofibrosarcomas (PHGM) are pleomorphic sarcomas which display only fibroblastic and myofibroblastic differentiation. They are not identified in the WHO 2002 classification as a separate entity from pleomorphic malignant fibrous histiocytoma/undifferentiated pleomorphic sarcoma since they are morphologically indistinguishable, and immunohistochemistry or electron microscopy are required for their identification. There is, however, evidence that myofibroblastic differentiation in pleomorphic sarcomas is associated with more aggressive behaviour (82). Most PHGM arise in deep soft tissue in adults, predominantly in lower limb and trunk, with occasional cases in the head and neck (76).

Macroscopically, PHGM are large solid tumours with haemorrhage and necrosis. Histologically, they are composed of pleomorphic spindles and polygonal or large epithelioid

cells arranged in fascicular and storiform patterns, with frequently abnormal mitotic activity. PHGM are high-grade sarcomas with recurrence in a third of cases and metastasis in over 70%. These tumours, like pleomorphic sarcomas with myogenic differentiation, have a poorer outcome than undifferentiated sarcomas (77, 82).

1.1.8.2.2 Fibromatosis

The term fibromatosis includes a broad range of morphologically related lesions which are divided into superficial and deep subgroups. Superficial (fascial) fibromatoses include palmar fibromatosis (Dupuytren contracture), plantar fibromatosis (Ledderhose disease), penile fibromatosis (Peyronie disease) and knuckle pads (76). The lesions in this group are characterised by their small size, slow growth, origin from fascia/aponeurosis and less aggressive behaviour. Deep (musculoaponeurotic) fibromatoses include extra-abdominal fibromatosis (extra-abdominal desmoid), abdominal fibromatosis (abdominal desmoid) and intra-abdominal fibromatosis (intra-abdominal desmoid), which can be further subdivided into pelvic and mesenteric fibromatosis and Gardner syndrome. The deep fibromatoses arise from deep structures, attain large size and have relatively more rapid growth (83).

Histologically, fibromatoses are un-encapsulated spindle cell lesions with an infiltrative growth pattern. The cells are embedded in a collagenous stroma, arranged in long sweeping fascicles. The cells show no pleomorphism and mitotic figures are rare. The immunophenotype is positive for MSA and SMA and negative for desmin, h-caldesmon, and S-100. Seventy to 75% of cases show positivity for β-catenin (76, 84). The best method of treatment is excision by surgery. However, adjuvant therapy should be considered for cases with positive surgical margins, multiplicity of the tumour, inoperable tumours and severe adhesion (85).

1.1.9 The Microenvironment in Soft Tissue Sarcoma

The microenvironment of neoplasms is comprised of multiple stromal cell types and extracellular matrix proteins in addition to the cancer cells. Carcinomas are composed of two compartments, a stromal compartment of fibroblasts and myofibroblasts and an epithelial compartment of malignant epithelial cells. The stromal compartment is usually involved in functional and structural support of tumour growth (86). This desmoplastic benign stroma accounts for 30–90% of the specimens. Both compartments contribute to the tumour matrix regarding their respective gene products. In contrast, sarcomas mainly have only one compartment, the malignant mesenchymal cell that largely produces the sarcoma matrix and contributes essentially to its gene products. Although sarcomas also contain a benign stromal part, this component is often observed to be 1% of the specimens. Hence, the predominant compartment in sarcomas is comprised of the malignant mesenchymal cells (87).

These mesenchymal cells produce extracellular matrix (ECM) which is defined as a network of molecules, mostly proteins and carbohydrates which bind cells together. The main function of the ECM is to provide support and anchorage to cells. Furthermore, its components help in modulating other cell functions such as development, migration and proliferation (88). The ECM has various components including collagens, proteoglycans, hyaluronans, glycoproteins, elastin, fibronectins and laminins (89). Synthesis and degradation of the ECM is performed by a specialised function of mesenchymal cells, the matrix being the result of the balance between production and breakdown. However, biomechanical and biochemical signalling systems are integrated in this process. Biomechanical loading directs mesenchymal physiology, that is, differentiation, proliferation, cell shape and matrix constitution, whilst biochemical signalling coordinates the attachment and disconnection of adhesions according to other cellular activities

such as growth and proliferation. The cell matrix interacts via transmembrane receptors which are generically known as cell adhesion molecules (CAMs) such as integrin receptors (90).

In 1960, it was described how a primary tumour of sarcoma can evade the anticancer immune response by establishing an immune-privileged microenvironment (91). Then in the 1970s, Juda Folkman proposed that angiogenesis is essential for solid tumour growth (92). The significance of tumour cell intravasation into the vascular system has especially been indicated in sarcoma, where vascular invasion, as determined by the presence of tumour cells within any location having an endothelial lining, has been recognised as a prognostic factor for metastasis (93). Tomlinson *et al*. (1999) reported that the pattern of angiogenesis is different between sarcomas and carcinomas (87), confirming that the capillaries in carcinomas are clustered in bursts within the tumour stroma and that the microvessel density in these bursts can be used as a prognostic factor. In contrast, microvessel density in sarcomas was revealed to have a more homogeneous appearance. A more recent study reinforced this pattern of angiogenesis illustrating that angiogenesis was diffuse in high-grade STS and only present in 33% of the investigated specimens (94).

1.1.9.1 Hypoxia in STS

Hypoxia and oxidative damage is a common characteristic of many solid tumours owing to aberrant vascular function and rapid cell division. Tumour cells can survive under conditions of low oxygenation by initiating adaptive responses to match oxygen supply with metabolic, bioenergetic and redox demands (95). Clinical and experimental studies have long demonstrated that tumour hypoxia is associated with increased malignancy, poor prognosis, and resistance to radiation and chemotherapy (96). Patients with the most hypoxic soft tissue sarcomas have a poorer disease-specific and overall survival, with an increased likelihood of metastasis (97, 98).

Tumour cell adaptations to hypoxia largely depend on the transcription factor, hypoxia-inducible factor (HIF), which is activated in low oxygen conditions and is inactive when oxygen is sufficient (99). HIFs form heterodimeric complexes constituted of an oxygen-liable α subunit and a stable β subunit. Together these subunits bind hypoxia response elements (HREs) on several hundred genes that facilitate the adaptation to hypoxia (100) . Mammals have three isoforms of HIFα, of which HIF1α and HIF2α are the best characterised. HIF1α is ubiquitously expressed, whereas HIF2α and HIF3α are selectively expressed in specific tissue types including vascular endothelium and cells of myeloid lineage. HIF activity is controlled by the stabilization of HIF1α and HIF2α protein subunits, which increases as cells become more hypoxic (100). Like other solid tumours, as sarcomas relinquish their blood supply, hypoxia stabilises HIF1α and HIF2α, which bind to HIF, driving the transcription of important genes in many aspects of cancer biology including angiogenesis, epithelial-mesenchymal transition, invasion, metastasis, and resistance to radiation therapy and chemotherapy (101). Based on genome-wide chromatin immunoprecipitation studies, the estimated number of direct HIF target genes is greater than 800 (102).

Finally, there are many recent reports of a regulatory link between the cell oxygen sensors and heat shock pathways. The activation of HSPs is critical to adaptation to hypoxia and expression of inducible HSPs is regulated by heat shock factors (HSF), in particular HSF-1 (103). Baird *et al*. showed that HSF transcription is up-regulated during hypoxia due to direct binding by HIF-1 to HIF-1 response elements in a HSF intron. This rise in HSF transcripts is important for the full induction of HSPs during hypoxia and reoxygenation (104). HSPs give transient protection from stress, acting as chaperones by regulation of protein folding to ensure accurate conformation and translocation (105). This may explain the ability of malignant cells to sustain protein homoeostasis even in the harsh hypoxic microenvironment of the tumour.

Consequently, HSPs may account for the ability of tumour cells to tolerate genetic variations that would otherwise be lethal (103).

1.2 Heat Shock Proteins (HSPs)

1.2.1 Discovery of Heat Shock proteins (HSPs)

Among the many changes in cellular activity and physiology, the most remarkable event in stressed cells is the production of a highly conserved set of proteins, the heat shock or stress proteins (106). Intensive research into the structure and function of heat shock proteins (HSPs) has been ongoing for the past 30 years. They are of considerable interest since they have been shown to have a pivotal role in cell cycle progression and cell death (apoptosis), as well as being involved in many disease processes.

The term heat shock protein (HSP) is used to describe several families of proteins whose expression is induced by hyperthermia. The first description of this phenomenon arose from the observation that Drosophila larvae exhibited a novel puffing pattern on their giant salivary gland chromosomes when they were exposed to higher temperatures (107). Subsequently, Ritossa's early experiments demonstrated that these chromosome puffs were also induced in the midgut and hindgut of the larvae, indicating that the heat shock response was not tissue-specific. Furthermore, the same puffing pattern could be generated in isolated salivary glands by treatment with 2,4-dinitrophenol, sodium salicylate, sodium azide and release from anoxia. These early observations implied two important features of the heat shock response, firstly, the universality of the response in all cell types (not tissue-specific), and secondly, the variety of cellular stresses which will induce this pattern of specific gene expression (107).

For many years, all research on heat shock gene expression was performed solely using Drosophila larvae and it was not until the late 1970's that the ubiquity of the heat shock response was demonstrated. The same typical pattern of gene expression in response to heat shock was then shown in chicken embryo fibroblasts (108) and Escherichia Coli (109), and subsequently in yeast (110) and plants (111). The heat shock response has since been observed in all organisms examined, from archaebacteria to eubacteria, yeasts, plants, invertebrates and vertebrates, including humans.

Examination of the nucleic acid sequence of heat shock genes from distant species and protein analysis using monoclonal antibodies has demonstrated that the heat shock proteins are among the most abundant and highly conserved proteins in nature (112). Although the term ‘heat shock protein’ is still used widely, many environmental and pathophysiological stresses have been shown to induce the response, including heavy metals, ethanol, amino acid analogues, fever and viral or bacterial infection (113). For this reason, the heat shock proteins are frequently referred to as ‘stress proteins’. It should be noted that many of these inducers share a capacity to cause protein denaturation, a fact which hints at the major functional role of the heat shock proteins as molecular chaperones. This function is vital to the cell, both under normal circumstances and following cellular stress. Recently, the term ‘proteostasis’ was used to describe the function of chaperones controlling protein synthesis, folding, trafficking, aggregation, disaggregation, and degradation (114).

1.2.2 Nomenclature of HSPs

Some confusion still arises in the literature regarding the nomenclature used to describe heat shock proteins, molecular chaperones and a sub-group of molecular chaperones known as chaperonins. Heat shock proteins are also frequently referred to as ‘stress proteins’, a term

which reflects the fact that HSP expression is induced by a wide variety of cellular insults other than hyperthermia (113), and is therefore perhaps a more appropriate nomenclature.

Molecular chaperones are defined as a family of unrelated classes of protein which mediate the assembly of other cellular polypeptides, but are not components of the final functional polypeptide structure (115). As will be described, the ability of molecular chaperones to interact with other cellular proteins is vital to the cell, both during normal cell growth and when the cell is subjected to some form of environmental or pathophysiological insult. Many, but not all, molecular chaperones can be classified as heat shock proteins since their expression can be induced by hyperthermia and a variety of other cellular stresses. Similarly, most heat shock proteins are now known to perform a molecular chaperone function.

The term chaperonin is used to describe a specific sub-group of the molecular chaperones, comprising a class of sequence-related molecular chaperones found in all bacteria, mitochondria and plasmids examined so far (116). One of the remarkable features of the chaperonins is the very high degree of structural and functional homology between members of this group. Chaperonins are essential components of the cellular chaperone mechanism. Chaperonins have two-ring assemblies that comprise a central cavity to which unfolded polypeptides attach and where they reach the folded state. Hence, chaperonins able to prevent off-pathway reactions and help productive protein folding to the original state in ATP-dependent manner (117)

Finally, client proteins transiently non-covalently bind to HSP and this binding may be necessary for their function (118). To date, more than 200 HSP client proteins have been identified, involving almost all vital cellular activities and processes, including cell growth, proliferation and cell survival (119). For instance, HSP90 clients range from signalling protein

kinases through steroid hormone receptors and small G proteins to enzymes and components of the telomerase complex, and the list continues to grow (120).

1.2.3 Function of heat shock proteins as molecular chaperones

As stated above, HSPs are part of a group known as 'molecular chaperones', a nomenclature which clearly defines the function of these molecules which accompany unfolded proteins and polypeptides during their cellular transport in normal metabolism and help protect these proteins and polypeptides whenever they are subjected to stresses that may cause them to unfold (121).

A more detailed definition is that a 'molecular chaperone' is a protein that binds to and stabilises an otherwise unstable conformer of another protein, and by controlled binding and release of the substrate protein facilitates its correct fate *in vivo*, thus allowing folding, oligomeric assembly, transport to and between subcellular compartments, or controlled switching between active/inactive conformations (122). Molecular chaperones also bind to and prevent aggregation of denatured or partially folded proteins, assisting the correct folding of these proteins (123).

Under conditions of normal cellular growth, the many vital functions which are served by molecular chaperones derive from their ability to recognise and modulate the state of folding of other cellular proteins (112, 116). In the cell, nascent polypeptide chains emerge from ribosomes as unfolded linear chains and a comparable situation exists when polypeptide chains are transported across intracellular membranes in an extended state. In such an extended or partially folded state, hydrophobic surfaces become exposed, raising the possibility of aberrant inter- or intramolecular interactions and the formation of polypeptide aggregates. The major role of molecular chaperones appears to be the prevention of these incorrect intermolecular

associations between unfolded polypeptide chains, thereby preventing their aggregation and assisting in the maintenance of polypeptide chains in a translocation-competent state during transport across intracellular membranes (122).

During stress conditions, partially denatured misfolded proteins accumulate and their exposed hydrophobic regions lead them to aggregate. HSPs aid to prevent the change of the conformation of other proteins, protecting incorrectly folded proteins against aggregation. Upon recovery, they help the refolding of misfolded proteins, but they also can facilitate in the proteasomal degradation of peptides that cannot be refolded (124) (Fig. 1).

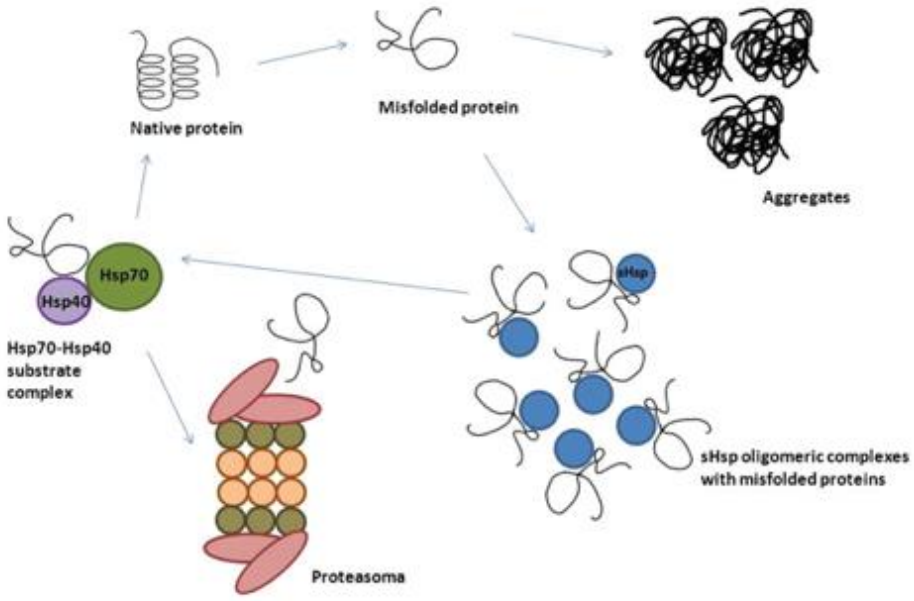

Figure 1.1 Chaperone functions of HSPs. Adapted from Toth et al., 2015 (125). During stress conditions, misfolded proteins accumulate and form large aggregates. However, some HSPs (like HSP70/HSP40 complex) can bind to misfolded proteins preventing their aggregation and facilitating their refolding. On the other hand, they also can assist in the proteosomal degradation of proteins that cannot be refolded.

1.2.4 Family of heat shock proteins

The expanding of members in the different HSP families and the inconsistencies in their nomenclature and classification have always led to confusion. Originally, mammalian HSPs were classified according to their molecular size (126). However, Kampinga *et al.* proposed new guidelines for the nomenclature and classification of human HSP families. Consequently, human HSPs are classified into the following groups: HSPB (small HSP), DNAJ (HSP40), HSPA(HSP70), HSPC (HSP90), HSPH (HSP110) and the chaperonin families HSPD/E (HSP60/HSP10). This nomenclature is based mainly on the more constant nomenclature assigned by the HUGO Gene Nomenclature Committee and adopted in the National Centre of Biotechnology Information Entrez Gene database for the heat shock gene (127). Table 1-7 presents a summary of these proteins, their cellular location and function.

High molecular weight HSPs are ATP-dependent chaperones, while small HSPs act in an ATP-independent fashion (128), with each HSP family member being expressed constitutively or regulated inductively and is targeted to different subcellular compartments (129). Constitutive activation of HSPs may occur in cells even in the absence of environmental stressors. For example, HSP90 can constitute up to 1% of total cellular protein in unstressed cells (130) indicating that these proteins have a role in maintaining protein conformation even under normal conditions.

Table 1.7 Summary of human HSPs according to their molecular weight, location and function.

HSP Family	Members	Molecular weight (KDa)/Sequence length (a.a.)	Intracellular Location
Small HSPs	HSP10	10/102	Mitochondria
	HSP27	22/205	Cytosol/Nucleus
	HSP40	38/340	Cytosol

HSP40/DNAJ family	DNAJA1	44/397	Nucleus/Cytosol
	DNAJA3	52/480	Cytosol
	CY40	40/370	Cytosol
HSP60	HSP60	61/573	Mitochondria
	CCT1	60/556	Cytosol Cytoskeleton
HSP70	HSP70	70/641	Cytosol
	HSP70-2	70/641	Cell surface
	HSP72	70/639	Cytosol
	HSP73(HSC70)	70/646	Cytosol
	Grp78	72/654	Endoplasmic reticulum
HSP90	HSP90	86/732	Cytosol
	HSP90B	84/724	Cytosol
	Grp97	92/803	Cytosol/Endoplasmic reticulum
	TRAP1	75/704	Mitochondria

(adapted from Khalil *et al*., 2011 (126))

1.2.4.1 HSPB (small heat shock protein) family

Small heat shock proteins (HSPB) are present in all type of cells and play vital roles in cell biology. They are fundamental components of the cellular protein quality control system, acting as the first line of defence against conditions that influence protein homeostasis and proteome stability (131). The small heat shock proteins have molecular weights in the range 16–40 kDa and are characterised by a conserved C-terminal domain of 100 amino acids, referred to as the alpha-crystallin domain (ACD) (132). From a structural point of view, HSPBs

can exist in the form of monomers and dimers, but can also assemble into large multimeric complexes that vary in size and contain up to 24–40 subunits. Association of monomers into large complexes, as well as dissociation of large oligomers into dimers and monomers, is modulated by sHSP post-translational modification, including phosphorylation, which, in turn, regulates sHSP functions (133). The mammalian HSPB family contains at least 10 members, the best-studied of which are HSPB1 (HSP27), HSPB4 (αA crystallin) and HSPB5 (α B-crystallin) (133, 134). Both HSPB1 and HSPB5 are constitutively expressed in a variety of tissues, however, their expression is up-regulated under stress conditions and in several diseases (134). Some members, like HSPB5, HSPB1, HSPB2 (MKBP), are highly expressed in cardiac and skeletal muscles (135).

Small HSPs functions are very diverse including chaperone-like activity and modulation of cytoskeleton stability. As a consequence of their role as chaperones towards diverse clients, which influences client fate (refolding or degradation) and due to their role as stabilizing agents of the cytoskeleton, sHSPs participate indirectly in the regulation of complex processes such as the response and adaptation to cell stress, thermotolerance, cell differentiation, cell movement, cell apoptosis, and development (136, 137). Thus, malfunction of HSPs can have adverse effects in many diseases, causing a wide range of pathologies including cardiomyopathy, myofibrillar myopathy, motor neuron diseases and cataracts (131). As the ATP level of the cells can reduce severely during stress conditions, these HSPBs act in an ATP independent manner. After stress recovery, when the ATP level has been returned, the sequestered damaged proteins can be transferred to ATP-dependent chaperone, like the HSPA/DNAJ (HSP70/HSP40) complex, which can facilitate the refolding or the degradation of these proteins (136, 138, 139).

1.2.4.2 DNAJ (HSP40) family

The DNAJ (HSP40) family is the largest human HSP family consisting of at least 50 members (127) characterised by a conserved, usually N-terminal J-domain, through which they bind to HSPA (HSP70) proteins. They are fundamental in protein folding, refolding and translocation as they are responsible for the stimulation of HSPA ATPase activity (140). DNAJ proteins can bind substrate peptides and transfer them to HSPA, while the J-domain facilitates ATP hydrolysis. Several members of DNAJ family also regulate the activity of other HSPs, like HSPC (HSP90) proteins. They can be found in various cell compartments, such as cytosol, nucleus, endoplasmic reticulum (ER), mitochondria, endosomes and ribosomes, with some of them showing tissue specific expression (140).

1.2.4.3 HSP60 (chaperonin) family

The chaperonin family of HSPs comprise two major subgroups, the GroEL (hsp60) family and members of the TCP-1 ring complex (TRiC) family, both of which have an essential function in promoting the ATP-dependent folding of proteins during normal growth and following cellular stress (127). Members of the GroEL family are found in mammalian mitochondria, where their major function is to promote the folding and assembly of imported proteins and the binding of heat-denatured mitochondrial proteins (112). The TRiC chaperonins are found in archaebacterial and the eukaryotic cytosol, and although fewer studies on the function of this subgroup have been reported, the available data on the structure of these proteins suggests that they are also important in providing a folding compartment for polypeptide assembly.

1.2.4.4 HSPA (HSP70) family

Members of this family are highly conserved ATPases, identified in both prokaryotes and most compartments of eukaryotic cells. The human HSP70 family consists of 13 members with similar structural and functional properties. HSPA1A (HSP70-1) and HSPA1B (HSP70-2) only vary in two amino acids, and probably because they are totally interchangeable proteins, are usually referred to as HSPA1 (HSP70) (127). In eukaryotes, there are four essential proteins, constitutively expressed HSC70 (HSP73 or HSPA8), mitochondrial mtHSP70, endoplasmic reticulum-localised GRP78/Bip and stress-inducible HSP70 (HSP72 or HSPA1) which is simply called HSP70 (141).

They have two main functional domains, an N-terminal ATPase-binding domain (ABD) responsible for substrate binding and refolding, and a C-terminal peptide-binding domain (PBD) to help the release of the client protein after ATP hydrolysis (142). HSP70 chaperone activity is regulated by different co-chaperones, e.g. Hip, CHIP or Bag-1. These co-chaperones bind to HSP70 and modulate its chaperone function by enhancing or reducing HSP70 affinity for substrates through the stabilisation of the ADP or ATP bound state of HSP70 (119).

1.2.4.5 HSPC (HSP90) family

HSPC (HSP90) family is the most well studied member of the heat shock protein families. Genetically, this family encodes five members (HSPC1-5) (127), which are the most abundant proteins in cells, producing 1–2% of the total cellular proteins. They can be found in different cell compartments such as cytosol, ER and the mitochondria (143). The most important members of the HSP90 family are inducible HSP90α (HSP90AA1) and constitutive HSP90β (HSP90AB1) isoforms (now also called HSPC1 and HSPC3, respectively), which are expressed by two distinct genes whose protein products are mainly cytoplasmic. The HSP90α was determined to be constitutively expressed at a low level, but strongly heat inducible. In

contrast, the HSP90β gene is expressed constitutively at a much higher level and is only weakly inducible following a heat shock (144). Members of the HSPC family are homodimers composed of three functional domains, an amino-terminal ATP binding domain, a charged middle linker domain, and a carboxyl-terminal dimerization domain. They are an ATP dependent molecular chaperon and there are over 200 HSP90 identified "client proteins" reported to interact with HSP90, compromising a variation of functional pathways for cell proliferation, growth and survival (144).

1.2.4.6 HSPH (HSP110) family

The human HSPH (HSP110) family contains three cytosolic and one ER specific members. They are highly homologous to HSPA proteins, but they have a longer linker region between the N-terminal ATPase domain and the C-terminal peptide-binding domain (127). A HSP110 family member has been described as a co-chaperone for HSP9. HSPH family members also cooperate with HSPA (HSP70) in protein folding, as they function as nucleotide exchange factors, removing ADP after ATP hydrolysis (145).

1.2.5 Transcriptional regulation of HSP activity

The induction of the expression of genes encoding HSPs in response to various stresses is regulated by a family of transcription heat shock factors (HSFs). Under stress conditions, these regulators activate the heat shock genes by binding to the heat shock elements found in the promoter region of the HSP genes. In humans, six types of HSFs have been identified namely HSF1, HSF2, HSF4, HSF5, HSFX and HSFY (146). HSF3 has been described for mice but not humans (147). The earliest-discovered and most widely studied HSF is HSF1, which plays a fundamental role in the heat shock response (148). HSF1 is expressed in almost all tissues and cell types, activated in response to different cellular stressors. while other members of the HSF

family are mainly involved in normal development, cell differentiation and life span regulation (149). HSFs are varied in size, but all of them contain an N-terminal DNA-binding domain, a hydrophobic oligomerization domain as well as a C-terminal transactivation domain (148).

The inactive, monomeric form of HSF is separated in the cytoplasm of unstressed cells by binding to various HSPs, such as HSPC (HSP90), HSPA (HSP70) or DNAJ (HSP40). During stress conditions, the amount of partially denatured proteins gradually increases, and these proteins upon binding to HSPs, release HSFs. The released HSFs undergo trimerisation, phosphorylation and translocate to the nucleus, where they bind to its target sites (heat-shock elements) in the regulatory region of heat shock induced genes and activate them. This activation is fast, the DNA-binding form of HSF can be identified within minutes following heat treatment (150). The activation of heat shock genes leads to increased expression of HSPs, which then associate with HSF. In this way, HSPs negatively regulate the expression of heat shock genes via an auto-regulatory loop. For example, the activity of HSF1 is down-regulated by the binding of HSP70 to its transactivation domain, resulting in the repression of heat shock gene transcription. Also, there is an interaction between HSP-binding factor 1 (HSPB1) with the active HSF1, thereby inhibiting the capacity of HSF-1 to bind DNA (148, 150).

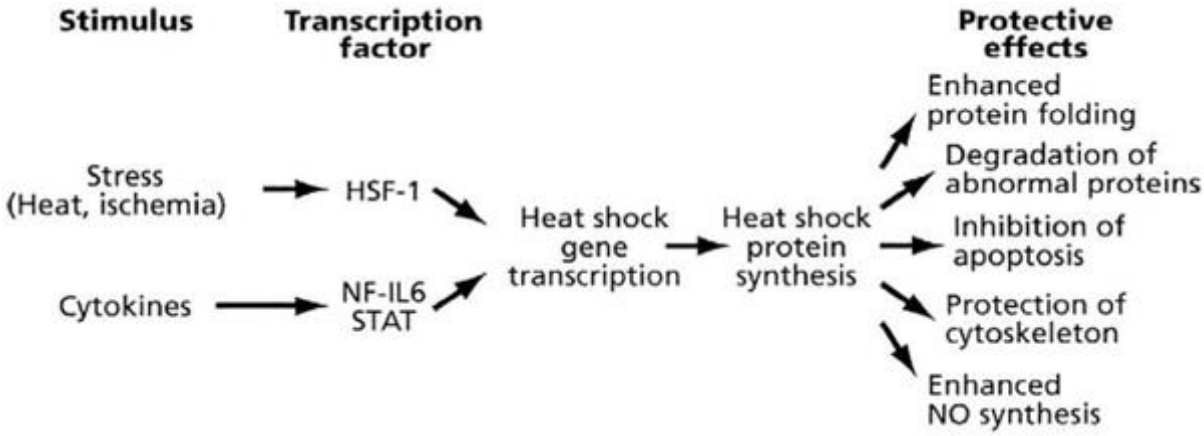

Figure 1.2 Activation of the heat shock protein (HSP) by specific stimuli and their protective effect. Adapted from Shi *et al*., 1998 (150).

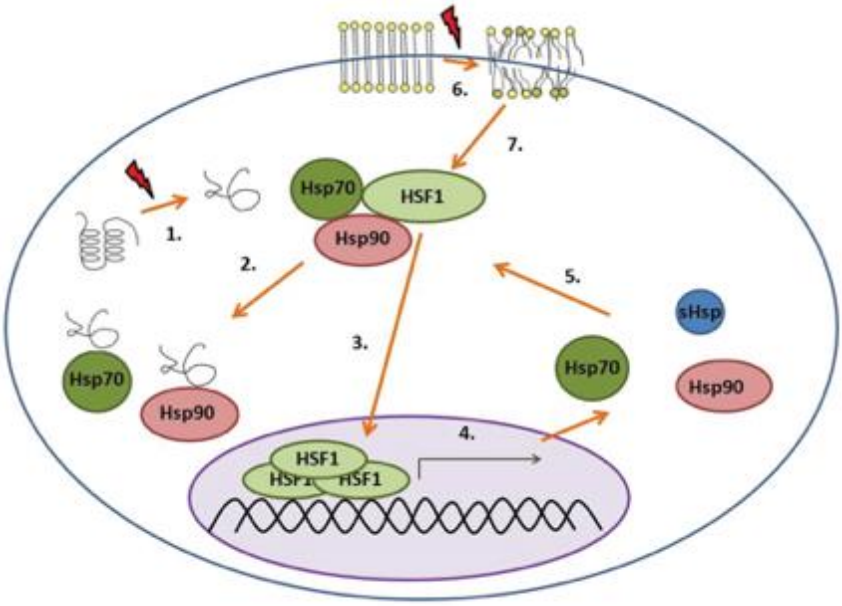

Figure 1.3 Transcriptional regulation of heat shock proteins. Adapted from Toth et al., 2015 (125). The inactive form of HSF is segregated in the cytoplasm of unstressed cells by binding to different HSPs, such as HSPC (HSP90) or HSPA (HSP70). During stress conditions, the amount of denatured proteins increases (1), which can bind to HSPs, thus releasing the HSFs (2). The released HSFs undergo trimerization, phosphorylation and translocate to the nucleus (3), where they bind to the HSE of the promoters of heat shock induced genes and activate them (4). The newly synthesized HSPs than associate with HSF, thereby negatively regulate their own expression via an autoregulatory loop (5). On the other hand there is an alternative, membrane-associated "thermosensor" that can initiate heat shock gene activation. During heat stress, the membrane fluidity rapidly increases (6), which can activate stress sensing and signalling pathways leading to the elevated expression of HSPs, and this transcription activation is also mediated by HSF1 (7).

1.2.6 The role of heat shock proteins in cancer

There is overwhelming evidence that heat shock proteins are expressed at elevated levels in a range of cancers and form an encouraging environment that is essential for tumour development (151, 152). HSP expression appears essential in many of the distinctive traits of malignant cells as described by Douglas Hanahan and Robert Weinberg in their article in 2000, "The hallmarks of cancer" , including uncontrolled proliferation, insensitivity to anti-growth signals, avoidance of cell senescence, evading apoptosis, as well as the acquisition of powerful capacities for angiogenesis and metastasis (153, 154).

1.2.6.1 Tumour proliferation and avoidance of senescence

Cell proliferation in adult tissues is not a default state and most cells have long ceased growing at this stage. In cancer, growth control is deregulated and proliferation resumes (155). Most of the receptors and enzymes that constitute the cascade are oncogenic when expressed at elevated levels or activated through mutation. Many of these proteins are clients of HSP90, therefore, amplification of HSP90 is permissive for unrestrained proliferation (156). Thus, HSP90 chaperone complexes maintain the signalling circuitry that underlies the capacity of many cancers for independent growth (157). There is some suggestion that HSP70 is also required in a similar way, because inactivation of this chaperone led to inhibition of proliferation in murine mammary tumour cells (158).

HSPs contribute to limitless proliferation by avoidance of senescence. Normal cells resist transformation by having a limited number of permitted divisions (159). This system is based on the lack of replication of chromosome ends at each cell division; the capping structures at the chromosome ends become progressively shortened, leading to arrest of further division and

cell senescence (160, 161). Cancer cells evade the senescence program by deploying the enzyme telomerase, which replaces the shortening ends of telomeres (161). HSP90 binds to telomerase and is required for its efficient function (162), thus, HSP90 might deter senescence by chaperoning telomerase and overcoming the erosion of telomeres over time when expressed to high levels, as in cancer.

In addition, one of the principal factors that controls the development of cancer is p53, a protein with a role in mediating growth arrest and apoptosis in response to DNA damage. Expression of HSP70 and HSP90 increases to high levels in tumours with mutated p53 and both chaperones may have roles in stabilizing the altered conformation of the mutant p53 (163, 164). Also, HSP27 and HSP70 inhibit the effector arm of the senescence pathway by reducing the effectiveness of p53 in promoting cell senescence (165, 166). P53 transcriptionally upregulates cell cycle protein p21, which directly arrests proliferation and this process is inhibited by high levels of HSP70 (167).

1.2.6.2 Angiogenesis

Under hypoxic conditions, tumour cells are able to deploy HIFs, proteins that can sense the low oxygen environment and mediate the expression of growth factors, such as vascular endothelial growth factor (VEGF), thereby increases the growth of the tumour capillary network and angiogenesis (168).

Also, several critical mediators in this angiogenic signalling pathway, including HIF, VEGF-receptor and Nuclear factor-κB (NF-κB) are dependent upon HSPs for their function (169). Indeed, HIF-1 is stabilised by HSP90 in hypoxic conditions (170). HSP27 becomes proangiogenic when released from tumour cells and can bind to receptors, stimulating VEGF transcription through an alternative pathway involving the factor NF-κB (171).

1.2.6.3 Invasion and metastasis

Tumour metastasis is a complex process in which cancer cells acquire specific capabilities (154) and elevated expression of each of the HSPs strongly promotes metastasis. An increase in HSP90 is associated with metastasis, largely due its capacity to chaperone focal adhesion kinase, integrin linked kinase and the receptor tyrosine kinases, ErbB2 and MET (172). HSP90 was found on the cell surface and in conditioned medium of tumour cells, where it acted as a molecular chaperone to help in the activation of matrix metalloproteinase-2 (MMP-2) leading to elevated tumour invasiveness. Activated MMP-2 protease digests many of the essential ECM components surrounding tumour tissue, thereby facilitating tumour invasion (172, 173).

In addition, HSP27 expression also favours metastasis though its effects on a process known as the epithelial-mesenchymal transition (EMT), in which cells switch from a compact shape to a spindle shape and gain enhanced cell motility (174).

1.2.6.4 Anti-apoptosis

Two main pathways for apoptosis induction have been recognised, intrinsic and extrinsic. The hallmarks of the intrinsic pathway are mitochondrial involvement and the production of the 'apoptosome'. In the intrinsic pathway, cell death signals cause the release of the cytochrome complex (Cyt c) from the mitochondria, which then attaches to the apoptosis protease activating factor-1 (Apaf-1), inducing oligomerization and ultimate recruitment of procaspase-9. Apoptosome formation results in the activation of caspase-9, which triggers the caspase pathway by activating the downstream caspase-3 (175). Cancer cells have been shown to deploy a range of mechanisms to evade apoptosis (176). Remarkably, HSP27, HSP70 and HSP90 have been shown to interact directly with protein intermediates in the apoptosis pathways and are potent inhibitors of apoptosis (176, 177). HSP27 inhibits apoptosis through its capacity to block multiple steps in these death pathways, including inhibiting cytochrome c

and SMAC Diablo release from mitochondria, as well as antagonizing caspases 3 and 9 (178, 179). HSP70 is likewise a versatile inhibitor, blocking the c-jun kinase pathway of programmed cell death and interrupting cytochrome c release from mitochondria (177, 180). Also, HSP70 functions at the post-mitochondrial level by interacting with the apoptosis-inducing factor (AIF) and Apaf-1 (181). HSP90 and its co-chaperones also modulate and mediate tumour cell apoptosis via interaction with Akt kinase, tumour necrosis factor (TNF) receptor, HER2/ErbB2 receptor, and NF-κB (176, 182).

The way that various HSPs behave in the cell death machinery is still controversial and the detailed signalling pathways that lead to apoptosis are far from being understood. However, HSPs 27, 70 and 90 are now established anti-apoptotic proteins (176, 177).

1.2.6.5 Resistance to chemotherapy

Since the cytoprotective effect of HSPs is essential for cancer cell survival, it is not surprising that HSPs targeted therapy is considered as a potential pharmacological intervention strategy for cancer treatment. To that end, there are currently thirteen HSP90 inhibitors being clinical evaluated in cancer patients (183). Most investigations to identify HSP90 inhibitors have concentrated on ligands binding to the N-terminal ATP-binding site, which interrupts HSP90's ATPase activity and the ongoing ATP-dependent folding cycle. As this cyclic event requires multiple co-chaperone proteins, inhibition results in the destabilization, ubiquitination, and ultimately, proteasomal degradation of the client proteins simultaneously causing the disruption of multiple oncogenic signalling pathways.

However, one of the crucial problems with HSP90 inhibition is the increase of HSP70 after treatment, which can reduce cell death induced by HSP90 inhibitors and therefore buffer their anti-tumour efficacy in the clinic. These disappointing results may be related to inherent HSP90 inhibitors toxicity and induction of HSP70 by calcium mobilization and activation of the TGF-

β signalling pathway. Thus, HSP70 inhibition combined with anti-HSP90 compounds may eliminate the toxic side-effects of HSP90 inhibitors, enhancing the synergistic efficacy for the treatment of cancer.

1.2.7 Expression of studied HSPs (HSP27, HSP70 & HSP90) in various tumour types

In the literature, there is overwhelming evidence that heat shock proteins are expressed at elevated levels in a range of cancers (126, 152). This thesis focuses on HSP27, HSP70, and HSP90, the most widely reported chaperones in the literature.

1.2.7.1 HSP27

HSP27 (HSPB1) is a member of the small HSP family and acts as an ATP-independent chaperone. The primary structure of HSP27 is highly homologous to other members of the HSP family, containing the conserved α-crystalline domain and differing in the C- and N-terminal regions. HSP27 is expressed in all human tissues (184), mainly residing in the cytosol (185). In the field of oncology, HSP27 has been found intracellularly and the extracellularly, with increased levels detected in various types of cancer such as ovarian, prostate, brain and breast cancers (126, 152, 186).

1.2.7.2 HSP70

The HSP70 superfamily consists of at least 13 members. There are four major proteins, constitutively expressed HSC70 (HSP73 or HSPA8), mitochondrial mtHSP70, endoplasmic reticulum-localised GRP78/Bip and stress-inducible HSP70 (HSP72 or HSPA1), simply referred to as HSP70 (127). The extensively studied inducible HSP70 (HSPA1A or HSP72) has ATP-dependent chaperone properties (187) and is over-expressed in various tumours such

as gastric adenocarcinomas (188), hepatocarcinoma (189) and oesophageal cancer (190). Myung *et al*. studied HSP70 expression in human tumour cell lines and showed that HeLa cells, osteosarcoma, A-673 (rhabdomyosarcoma), CaOv-3 (ovarian adenocarcinoma), as well as malignant melanoma expressed HSP70 (191).

1.2.7.3 HSP90

HSP90 belongs to another important class of the HSPC family and is a highly abundant ATP-dependent chaperone protein expressed by all eukaryotic cells (127, 192). It accounts for 1–2% of total cellular proteins, increasing upon induction from baseline levels to 4–6%. It has two main isoforms, HSP90α (inducible form) and HSP90β (constitutive form) (144). Like other HSPs, HSP90 over-expression has been related to the prognosis and evolution of neoplasia. Moreover, this family of HSPs is considered the classic chaperone family in cancer (126, 152, 193).

1.3 Thesis Hypothesis

1.3.1 The Rationale of the current study

Soft tissue sarcomas are a rare and a heterogeneous group of malignancies originating from mesenchymal tissue. The rarity of soft tissue sarcomas, coupled with their diversity, is challenging for researchers to understand their natural history, prognosis and management. Hence, further research is essential to understand the cellular and molecular variability of soft tissue sarcomas, which may provide insights into the clinical heterogeneity of the disease and its pathology. Among the many changes in cellular activity and physiology, the most remarkable event in stressed cells is the production of a highly-conserved set of proteins called heat shock proteins (HSPs). Intensive research into the structure and function of HSPs has been ongoing for the past 20 years. They are of considerable interest since they have been shown to have a pivotal role in cell cycle progression and cell death (apoptosis), and to be involved in many disease processes (152). In addition, they found to be increased in many solid tumours and haematological malignancies (126).

Among the heat shock proteins, HSP27, HSP70 and HSP90 are the most commonly studied. They are potent anti-apoptotic proteins, associating with essential apoptotic factors, thereby blocking this cell death process at different levels (176). There are several reports about the prognostic role of anti-apoptotic HSP27, HSP70 and HSP90 in various tumour types. Furthermore, it has been reported that over-expression of HSPs is associated with poor prognosis, such as HSP27 in breast malignancy (193), HSP70 in pancreatic adenocarcinoma and HSP90 in gastrointestinal stromal tumours (194). According to Uozaki *et al*., the over-expression of HSP27 has the strongest negative prognostic value for conventional osteosarcoma (195). Despite the importance of HSP activity in various tumours, little is known

about the role of HSPs in soft tissue sarcomas. In a proteomic study, Suehara *et al*. demonstrated that the expression levels of HSP27 were associated with histological grading and patient survival in leiomyosarcoma (196). Recently, Bekki *et al*. reported that HSP90 is highly expressed in undifferentiated pleomorphic sarcoma (UPS) using immunohistochemistry, concluding that it is a poor prognostic factor (197).

1.3.2 Hypothesis

Based on the literature review, given the importance of HSPs (notably HSP27, HSP70 and HSP90) in malignancies and their participation in different hallmarks of cancer, it is hypothesised that HSPs (particularly HSP27, HSP70 and HSP90) are highly expressed in myofibroblastic/fibroblastic sarcoma, and there is a relationship between overexpression of these HSPs and the prognostic clinical pathological features of soft tissue sarcomas.

1.3.3 Null Hypothesis

The hypotheses are individually or collectively not true.

1.3.4 Aim & objective

1.3.4.1 Primary aim

The primary aim of the present study is to evaluate the expression of selected HSPs (HSP27, HSP70, HSP90) in adult myofibroblastic/fibroblastic sarcomas of the extremities by immunohistochemistry (IHC).

1.3.4.2 Secondary aims

1. To study the association between the expression of HSPs and the subtype of myofibroblastic/fibroblastic sarcoma.
2. To compare the expression of HSPs between sarcoma and non-metastasising soft tissue tumours in general.
3. To assess the relationship between the expression of these HSPs and the grade of sarcomas (high grade vs low grade).
4. To investigate the relationship between the expression of HSPs with other clinicopathological parameters (age, gender, tumour site, tumour size and metastasis).

Chapter Two

Materials & Methods

2 Materials & Methods

2.1 Patients and tissue samples

The study was conducted under the Liverpool Musculoskeletal Biobank ethical approval (NRES North West Liverpool Central Reference 15/NW/0661) and sponsored by the University of Liverpool (reference UoL001223). Informed patient consent for using the samples for research purposes was obtained from all patients prior to surgery.

2.1.1 Patient samples

Soft tissue samples were obtained from adult soft tissue neoplasms of the extremity resected from patients who attended the Northwest Orthopaedic Oncology service in Liverpool from 2006 to 2016 and who had no history of preoperative radiotherapy or chemotherapy.

The inclusion criteria for patients were:

- Adult with soft tissue sarcoma of the extremities
- Consented
- No prior treatment with radiotherapy or chemotherapy
- Detailed clinical information available.

Detailed clinical pathological parameters (age and sex, tumour site, size, stage, grade and distant metastasis) for most of the cases were taken directly from the surgical pathology report and patients' files. The histological diagnosis of soft tissue tumours was made by a specialist sarcoma pathologist at Royal Liverpool University Hospital based on World Health Organization (WHO) criteria and widely accepted morphological and immunohistochemically characteristics. For histological grading, the French Fédération Nationale des Centres de Lutte

Contre le Cancer (FNCLCC) system was used. Tumours were classified as upper extremity if they were located at, or distal to, the clavicle, lower extremity if they were at, or distal to, the pelvic brim. The maximum macroscopic size of the tumour was obtained from the pathology report.

2.1.2 Tissue samples

All tissue samples were archival paraffin-embedded, formalin-fixed tissue blocks from adult soft tissue neoplasms of the extremity resected from patients who attended the Northwest Orthopaedic Oncology service in Liverpool from 2006 to 2016, obtained from the Liverpool Tissue bank.

The criteria for tissue selection were:

1. Availability of informed consent.
2. Blocks of tissue from the most appropriate region of the tumour by a senior sarcoma pathologist consultant:
    - representing tumour cells
    - representing the highest grade in the sample
    - away from necrotic tissue
    - avoid of tumour capsule or reactive tissue
3. Sufficient tumour tissue present.
4. Availability of relevant clinical information.

Once the blocks had been selected, sequential 4 μm sections of paraffin block were cut using a rotary microtome (Microm, Oxford, UK) from the corresponding formalin-fixed, paraffin-embedded (FFPE) tissue blocks.

2.2 Haematoxylin and eosin (H&E) staining procedure

One section from each FFPE tissue block was stained with haematoxylin and eosin by the Liverpool Tissue Biobank used for histological assessment.

2.3 Immunohistochemistry (IHC)

2.3.1 Basics of immunohistochemistry

Immunohistochemistry (IHC) is a powerful method for localizing specific antigens in formalin-fixed, paraffin-embedded (FFPE) tissues based on antigen-antibody interaction (198). It is now identified as an essential element and an important tool, both in diagnostic and research-orientated cellular pathology. The technique involves the detection of specific or very selective cellular epitopes with an antibody and appropriate labelling system. Immunohistochemistry can be performed on cytological compounds, frozen sections and paraffin-embedded histological sections (199, 200). The basic steps of an immunohistochemistry protocol are detailed in appendix B.

2.3.1.1 Fixation and section cutting

The process of fixation is essential for tissue and antigen preservation as it stabilises the proteins. The general principle is that the fixatives form cross-links between proteins, thereby stabilising the cytoskeletal structure. Formaldehyde (formalin) is the fixative of choice for routine histology, therefore, any retrospective studies using patient samples will involve the use of immunohistochemistry on formalin-fixed, paraffin-embedded blocks. The aldehydes form cross-links between protein molecules, the reaction being with the basic amino acid lysine (200, 201). All tissues used in this study are formalin-fixed, paraffin-embedded blocks.

Generally, unless otherwise specified, sections for IHC were cut at 3 μm, 4 μm or 5 μm. Thicker sections may cause difficulty during staining, creating problems in interpretation due to the multi-layering of cells. In this study, sequential 4 μm sections of the paraffin block were cut using a rotary microtome from the corresponding formalin-fixed, paraffin-embedded (FFPE) tissue blocks. This was performed by an experienced technician from the tissue bank at the University of Liverpool using a microtome (Microm). The tissues were then placed on labelled superior Adhesive Slides (Apex, Leica, UK) and dried overnight in a 37°C oven.

2.3.1.2 Antigen retrieval (AR)

Antigen retrieval is the method by which antigens that have been masked through fixation and processing, are unmasked prior to immunostaining. There are two main methods for antigen retrieval, proteolytic and heat-mediated. Of these two, heat mediated antigen retrieval (HMAR) is the most effective on most antigens (201). For this study, HMAR was performed using the Dako PT-Link with a high pH. The protocol for PT-Link antigen retrieval is in appendix C.

The main advantage of HMAR over proteolytic digestion is that heating times to retrieve antigens tend to be uniform, regardless of the amount of time spent in fixative. This is in contrast to the variability in digestion times required when using enzymes. The main pitfall with HMAR is that extreme care must be taken not to allow the sections to dry, as this destroys antigenicity (200).

2.3.1.3 Blocking of endogenous peroxidase

Peroxidase results in the decomposition of hydrogen peroxide and is a common property of all haemoproteins, myoglobin, cytochrome, and catalases. Using the immunohistochemical method with one of the reagents labelled with peroxidase requires blocking of the endogenous peroxidase present in tissues, which would give a false positive reaction with the applied

chromogen (201). In this study, this process was performed by pre-treating sections with Dako Envision-Flex block (Dako, UK Ltd.) which exhausts the endogenous enzyme.

2.3.1.4 Candidate proteins (primary antibodies)

An antigen can be defined as a molecule (protein, carbohydrate, or lipid) that binds with an antibody, and is usually composed of a number of epitopes or antigenic determinant groups. An epitope consists of a small amino acid sequence which binds to the variable region of the antibody. Due to differences in their manufacture, a monoclonal antibody will only recognise one epitope on an antigen, whereas a polyclonal antibody will recognise many epitopes on an antigen. The vast majority of primary antibodies available for use on human tissue are made in either rabbits or mice. Generally, monoclonal antibodies are preferable to polyclonal antibodies, as they tend to be more specific (200).

In this study, expression of selected heat shock proteins was examined in soft tissue tumours using immunohistochemistry. The expression of HSP27, HSP70 & HSP90 was investigated to examine their potential role in soft tissue sarcoma and to gain an insight into the pathobiology of connective tissue tumours.

The first antibody is anti-HSP27 primary antibody from Abcam Ltd (UK) is a mouse monoclonal (G3.1) to heat shock 27 kDa protein, **HSPB1**. It reacts with mouse, rat and human antigens. Mouse monoclonal IgG1 is suitable for use as an isotype control with this antibody. HSPB1 is localised mainly in cytoplasm and detected in many tissues, including gallbladder, smooth and skeletal muscles. This antibody was used in immunohistochemistry on human tissue in previous research (202, 203).

The second antibody is anti-HSP70 primary antibody from Abcam, a mouse monoclonal (5A5) to heat shock 70 kDa protein 1B, **HSPA1B**. This antibody recognises human and mouse

antigens. Epitope mapping with a panel of HSP70 deletion mutants suggests that the epitope recognised is located between amino acids 122–264 of human HSP 70, a region that has been shown to be involved in ATP binding. Mouse monoclonal IgG1, is suitable for use as an isotype control with this antibody. HSPA1B is testis-specific and localised in the cytoplasm. This antibody was used in immunohistochemistry on human tissue in previous research (204, 205).

The third antibody in this study is anti-HSP90 antibody from Abcam, a mouse monoclonal (AC88) to heat shock 84.7 kDa protein, **HSP90 alpha** and 83.2 kDa protein, **HSP90 beta**. It recognises both HSP alpha and beta in human. The epitope of this antibody has been mapped to amino acid residues 604–697 of the human HSP90 sequence. It is localised in the cytoplasm and nucleus. This antibody was used in immunohistochemistry on human tissue in previous research (206).

Antibodies for use in immunohistochemistry were optimised by trial runs covering a range of concentrations and dilutions (1:100, 1:250 & 1:500) using a PT-link antigen retrieval (AR) method. Table 2-1 summarises the primary antibodies of the HSPs used in this study.

Table 2.1 List of HSP primary antibodies used in the study

Primary Antibody	Source	Isotype	Clonality	Clone number	Dilution used
Anti-HSP27 Antibody	Abcam (ab2790)	IgG1	Mouse Monoclonal	G3.1	1:500
Anti-HSP70 Antibody	Abcam (ab2787)	IgG1	Mouse Monoclonal	5A5	1:100
Anti-HSP90 Antibody	Abcam (ab13492)	IgG1	Mouse Monoclonal	AC88	1:500 (10 µg/ml)

2.3.1.5 Tissue controls used in the study

Staining control cases were included across experiments to check for the consistency and reliability of the immunostaining technique. According to the literature, Human Protein Atlas and manufacturer's recommendations, the positive controls were gallbladder tissue for HSP70, skin for HSP27 and testis for HSP90. For negative controls, all staining steps were applied to the same positive control tissue except that the mouse non-immune IgG (Dako) was substituted in place of the primary mouse antibody at the same dilution as the respective primary antibody. Figure 2.1 shows positive and negative tissue controls for various HSPs.

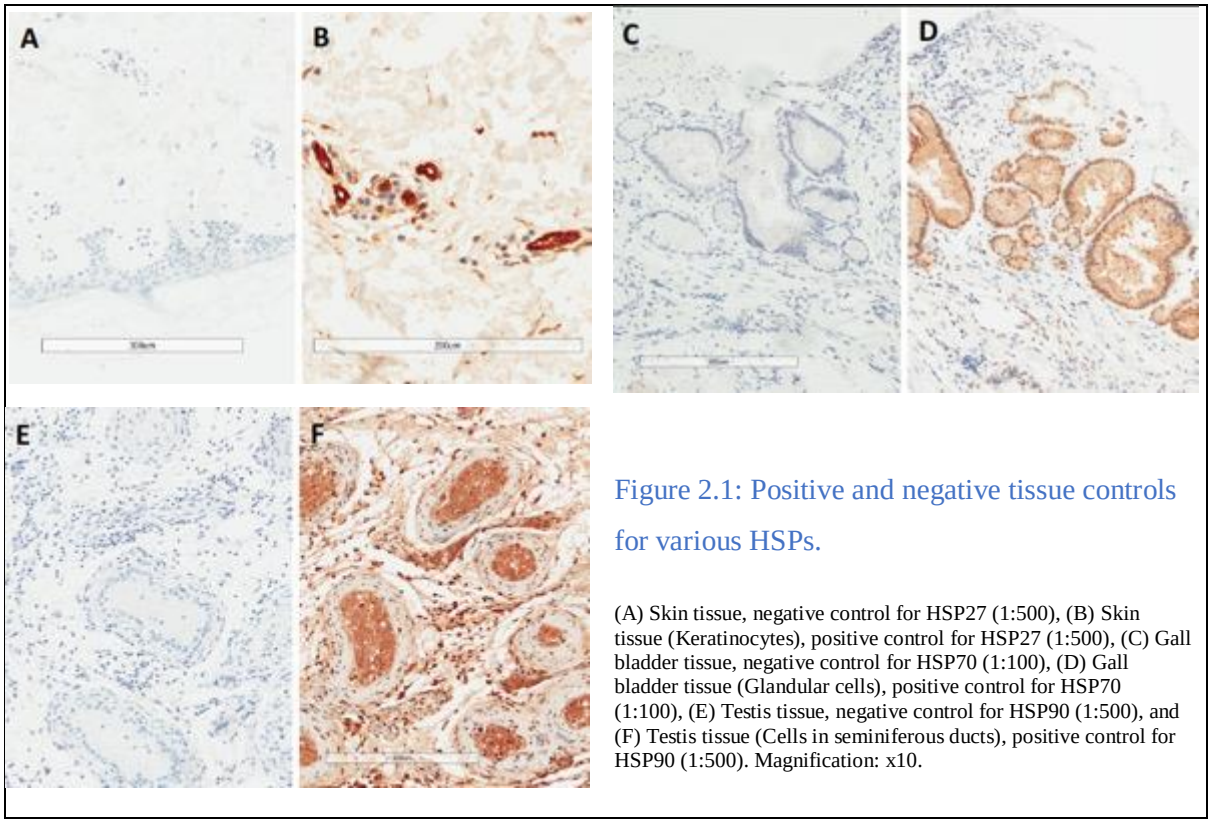

Figure 2.1: Positive and negative tissue controls for various HSPs.

(A) Skin tissue, negative control for HSP27 (1:500), (B) Skin tissue (Keratinocytes), positive control for HSP27 (1:500), (C) Gall bladder tissue, negative control for HSP70 (1:100), (D) Gall bladder tissue (Glandular cells), positive control for HSP70 (1:100), (E) Testis tissue, negative control for HSP90 (1:500), and (F) Testis tissue (Cells in seminiferous ducts), positive control for HSP90 (1:500). Magnification: x10.

2.3.1.6 Staining methods

There is a wide variety of immunohistochemical staining methods available today, but the best and most reliable of these are the avidin-biotin and polymer-based systems (200). In this study, a polymer-based immunohistochemical method was chosen for the best possible staining.

Polymer-based methods can be either a two- or three-layer system. In the two-layer system, the secondary antibody is part of the polymer molecule and the secondary antibody is conjugated to the polymer as a large number of enzyme molecules. The EnVision kit, available from Dako, is an example of a two-layer polymer system used in this study (Figure 2.2). In the three-layer system, the secondary antibody is applied unconjugated, then the tertiary antibody which is conjugated to the polymer along with enzyme molecules. The main advantage of polymer-based systems is that they can be used on tissues containing a lot of endogenous biotin without producing background staining (200).

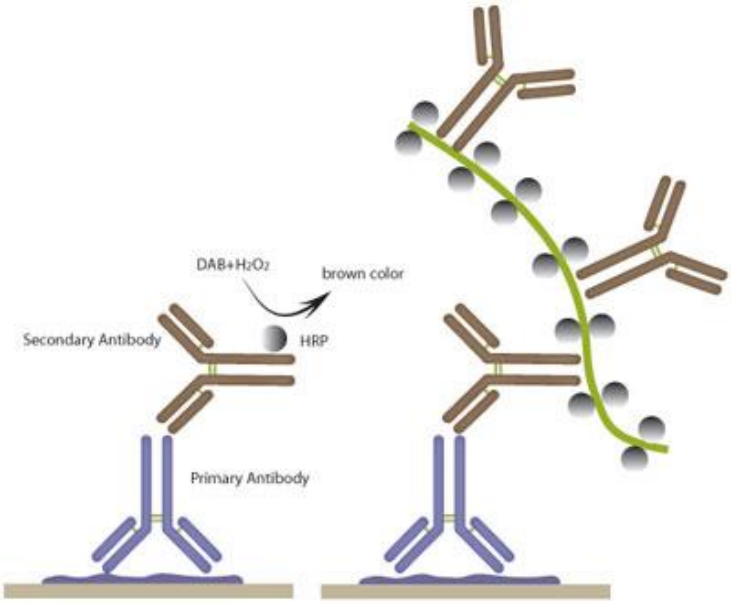

Figure 2.2 The basic immunohistochemistry sequence - a polymer-based method.

2.3.1.7 Enzyme labels and chromogens

Enzymes are the most widely used labels in immunohistochemistry, and incubation with a chromogen using a standard histochemical method produces a stable, coloured reaction end-product suitable for the light microscope. In addition, the variety of enzymes and chromogens available allow the user a choice of colour for the reaction end-product. Horseradish peroxidase labelling + DAB (3,3′diaminobenzidine tetrahydrochloride) were used in this study and remain the most commonly used combination of enzyme and chromogen in immunocytochemistry. DAB precipitates to a brown reaction end-product when in the presence of peroxidase and hydrogen peroxide (hydrogen peroxide is in solution with the DAB). A by-product of the reaction of hydrogen peroxide (the substrate) with peroxidase (the enzyme) is an oxygen radical, which acts on DAB and precipitates it at the antigenic site.

2.3.1.8 Immunostaining procedure using a Dako autostainer

Immunohistochemistry was performed on 4 µm sections cut from the corresponding formalin-fixed, paraffin-embedded (FFPE) tissue blocks and the slides were stained on a Dako autostainer, an automated slide processing system designed to automate manual staining methods routinely used in immunocytochemistry. Briefly, study tissue sections with control tissues were run in each assay performed. Slides were labelled with antibody, dilution and date using a pencil. Then, slides were placed in the PT-Link (Dako), heated to 96°C, held for 20 minutes, and returned to 65°C (approx. 1 hour 10 minutes). After that, slides were washed with Flex-Wash buffer for 5–15 min and then placed on staining tray.

Following antigen retrieval, the slides were stained on an autostainer (Dako UK Ltd, Ely, Cambridgeshire, UK). Endogenous peroxidases were blocked with a proprietary peroxidase

blocking solution (Dako) applied to each section for 10 min. Sections were then washed in Flex wash buffer, prior to incubation with the monoclonal primary antibodies for HSP27, HSP70 and HSP90 (Abcam, UK Ltd) at the appropriate dilution (Table 2.4) for 30 min at room temperature. The dilution of the primary antibodies was chosen by testing a range of dilutions on positive and negative control sections. Negative control sections were incubated with mouse IgG1(Dako, UK Ltd) at the same concentration as the primary antibody. Bound antibody was detected using the Dako mouse linker and incubated for 20 min, then, DAB (Dako flex system) was added to each slide and incubated at room temperature for 20 min. Finally, the sections were counterstained with haematoxylin, dehydrated through the ethanol series and cleared in xylene in the fume hood, and mounted with DPX resinous mountant (Merck Chemicals Ltd, Nottingham, UK). A trained person supervised the staining procedure to ensure all the steps are performed correctly. The slides were examined for the quality of staining by the supervisor. The staining was satisfactory and there was no need to repeat any marker.

2.4 Immunohistochemistry (IHC) analysis and assessment

2.4.1 Automated quantitative analysis

Following IHC staining, all slides were scanned digitally using an Aperio Image Scanner. Multiple snapshots (digital TIFF images) of the chosen area were captured at x10 magnification. The number of images varied from 5–10, depending on the size of the selected area. Areas of necrotic tumour or those with excess tissue oedema or haemorrhage were avoided. In order to eliminate assessment bias, all snapshot images were blinded to the investigator.

Image analysis was performed using Image J software (version Fiji-wind64). The markers were quantified as percentage of area of section stained according to the technique of Hartig *et al.* using image J software (207). This technique relies on choosing the colour deconvolution that

eliminates background noise. For colour detection, the colour selection is used to select and reserve the positive colour pixels while the background colour pixels are eliminated. After that, H DAB vector used to isolate the DAB stain. Staining can be assessed by setting "threshold" using the thresholding tool in ImageJ software. Then, the software measures the total DAB percentage (positive colour pixels) of section stained area, which is related to the specific tissue, based on the antibody used.With my supervisor (Dr Joseph Alsousou), we created a macro code (see appendix D) that will automatically perform the colour deconvolution and select the brown DAB stain, set the threshold and measure the area fraction and DAB intensity. The average of DAB intensity percentage of stained area for each case was taken When run on many slide samples, the results matched the expected manual calculation and visual inspection. However, our automated analytic method (ImageJ) did not consider cellularity of tumours which might lead to some calculation bias.

2.4.2 Semi-quantitative scoring

For the purpose of this study, IHC slides were also analysed semi-quantitatively by two observers; an inexperienced investigator (the author) and a pathologist (Dr Susha Varghese). They were viewed blind to the clinical and pathological diagnosis. High inter-observer agreement was confirmed using Cohen's Kappa coefficient ($k = 0.91$, $P < 0.001$). In case of inter-observer differences, consensus was achieved through simultaneous reassessment of the respective specimen by both investigators.

At least five fields were used to evaluate expression of HSPs for each case under a standard light microscope (x400) or digital image(40x). Then, a semi-quantitative estimate was made using a immunoreactive score (IRS) as described by Remmele *et al*.,1987 (208). Expression of HSPs was assessed by determination of IHC staining based on the intensity of

immunostaining and the percentage of stained tumour cells. Immunostaining was scored as 0 (no immunostaining), 1 (weak immunostaining), 2 (moderate immunostaining), or 3 (strong immunostaining). The percentage of positive tumour 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 the immunoreactive score (IRS) ranging from 0 to 12 (Table 2-2). For the statistical analysis, expression of HSPs was dichotomised into either low expression (score 0–3), or high expression (score 4–12). Similar method of analysis for IHC staining of HSPs was used in many previous cancer studies including breast and colon cancers (209-211).

Table 2.2 Immunoreactive score (IRS) according to Remmele and Stegner, 1987 (208)

A (Percentage of positive cells)	**B (Intensity of staining)**	**IRS score (multiplication of A and B)**
0 = no positive cells	0 = no colour	0-1 = negative
1 = < 10% of positive cells	1 = mild reaction	2-3 = weak
2 = 10-50% positive cells	2 = moderate reaction	4-8 = moderate
3 = 50-80% positive cells	3 = intense reaction	9-12 = strong
4 = > 80% positive cells		

2.4.3 Relationship between automated quantitative analysis and semi-quantitative scoring

The relationship between automated quantitative analysis (% of staining area) and semi-quantitative scoring (IRS scoring) was investigated using Spearman's rank order correlation. There was a positive correlation between the two variables ($p < 0.05$), with high levels of automated % staining area associated with higher IRS.

2.5 Clinical follow-up

Patients had follow-up appointments every 3 months for the first 3 years after surgery, every 6 months for the next 2 years, and yearly thereafter. The presence or absence of recurrence was confirmed by computed tomography (CT) or magnetic resonance imaging (MRI) every 12 months after surgery. Follow-up was calculated from the day of primary surgery and overall survival (OS) was calculated from the date of surgery to the date of death.

2.6 Statistical analysis

Statistical analyses were performed using the software package SPSS, version 22.0 (SPSS, Chicago, IL), at the University of Liverpool with the advice and assistance of my supervisors. In all statistical analyses, the initial step was to assess whether the data were normally distributed. This is performed automatically by SPSS, which superimposes a normal curve on the data histogram and using Shapiro Wilk's test. Wherever there was uncertainty or two data sets were to be compared in which there were both normally and non-normally distributed data, non-parametric statistics were used. This means that the analyses might be less sensitive than had it been possible to apply parametric statistics, but this was considered an advantage as it decreased the likelihood of obtaining false positive relationships between biomarker expression and biological/clinical significance. All data were recorded onto datasheets in Microsoft Office Excel Software and then transferred to the SPSS (Statistical Package for Social Sciences) version 22. Different tests were used for different aspects of the analysis as these analyses were better applied to each data set/ combination of data sets. Thus:

- Parametric data were described as means +/- standard deviation, whilst non-parametric data were reported as median with ranges. With regards to categorical data, they were presented in number & percentages.
- Categorical data were compared by chi-square or Fisher's exact tests.

- The Mann-Whitney U test was used to test for differences between two independent groups on a continuous measure.
- The Kruskal Wallis test was used to test for differences between more than two independent groups on continuous variables.
- The Spearman's rank order test was used to test correlation between two non-parametric continuous variables.
- OS was calculated using the Kaplan-Meier method. Death from disease was taken as the endpoint for disease-specific survival, patients dying from other causes were censored at the date of death.

Results were deemed statistically significant at a P value of less than 0.05 (2-tailed).

Chapter Three

Results

3 Results

3.1 Normality test for continuous variables

This study had categorical, ordinal and continuous variables, requiring different statistical analysis for the different type of variables. For continuous variables, it is important to assess if data is normally distributed (parametric) or not (non-parametric), because different statistical tests are used for parametric and non-parametric data. The Shapiro-Wilk statistical test was used to assess the normality of the distribution of the continuous data. In this study, a P value less than 0.05 was considered significant, which suggests violation of the assumption of normality (Table 3-1). Hence, non-parametric tests were used for continuous variables.

Table 3.1 Normality tests for continuous variables

	Shapiro-Wilk		
	Statistic	df	Sig.
Age (year)	0.922	43	0.006
Size of tumour (mm)	0.916	43	0.004
HSP27 staining	0.912	43	0.003
HSP70 staining	0.933	43	0.015
HSP90 staining	0.620	43	0.000

3.2 Clinical and pathological characteristics of patients and tumour types

This study included 43 patients with myofibroblastic/fibroblastic tumours of the extremities, 35 patients (81.4%) had sarcoma tumours, and only eight patients (18.4%) had non-metastasising tumours. The subtypes of tumours were myxofibrosarcomas (n = 26), myofibroblastic sarcomas (n = 3), fibromyxoid sarcomas (n = 3), myxoinflammatory fibroblastic sarcomas (n = 3), myxomas (n = 3) and fibromatosis (n = 3) as shown in Figure 3.1.

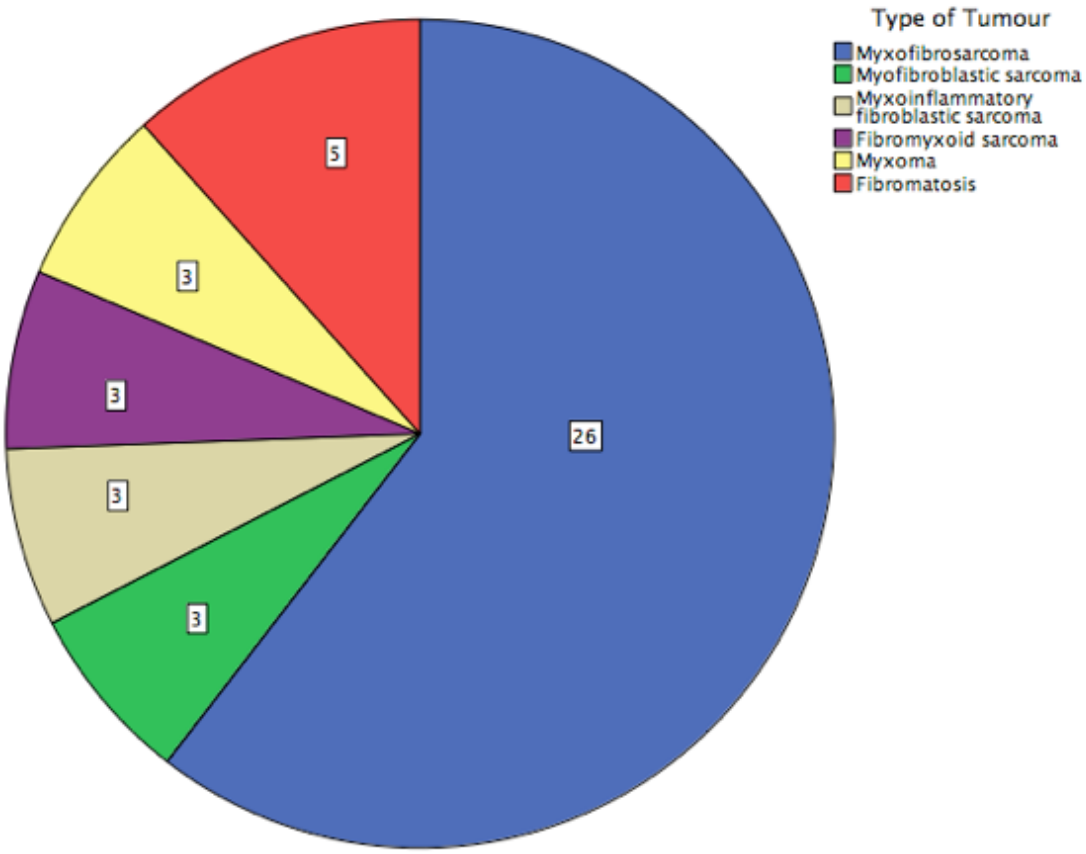

Figure 3.1 Number of cases for each type of tumour

3.2.1 Clinicopathological parameters of soft tissue sarcomas

There were 35 myofibroblastic/fibroblastic sarcomas included in this study, 17 (48.6%) were men, and 18 (51.4%) were women. The median age of patients with sarcomas was 70.0 years at diagnosis (range 27–94 years). The median maximum diameter of a tumour was 81.1 mm (range 35–150 mm). Tumours were histologically FNCLCC grade I in 7 (20%), grade II in 5 (14.3%) and grade III in 24 (68.7%). The tumour was located in the upper limb in 11 patients (31.4%) and the lower limb in 24 (68.5%) patients. A total of 8 patients (22.8%) developed metastatic disease, of which, 7 had FNCLCC grade III at presentation and one patient had a grade II tumour. Three patients had metastasis at the time of diagnosis. Metastases were in the lung in seven patients and groin lymph node in one. Table 3.2 summaries clinicopathological features for patients with STSs.

Table 3.2 Clinical and pathological characteristics for patients with sarcomas.

		MFS (n=26)	**MS (n=3)**	**MIFS (n=3)**	**FS (n=3)**	**Total (n=35)**
Gender	Male	11	1	3	2	17 (48.6)
	Female	15	2	0	1	18 (51.4)
Age (years)	Median	69.0	81.0	75.0	59.0	70.0
	Range	27-94	80-83	68-82	54-68	27–94
Site	Upper Limb	6	1	2	2	11 (31.4)
	Lower Limb	20	2	1	1	24 (68.6)
Size (mm)	Median	84.0	72.0	80.0	61.0	81.0
	Range	35-150	58-100	50-100	55-65	35–150
Grade	Low Grade	3	1	0	2	7 (20)
	Intermediate Grade	3	0	1	1	5 (14.3)
	High Grade	20	2	2	0	24 (68.7)
Metastasis	No Metastasis	19	3	2	3	24 (88.6)
	Metastasis	7	0	1	0	8 (11.4)

Number (%), MFS (myxofibrosarcoma), MS (myofibroblastic sarcoma), MIFS (myxoinflammatory fibroblastic sarcoma), FS (fibromyxoid sarcoma).

3.2.2 Clinicopathological parameters of Non-metastasising Tumors

There were eight non-metastasising myofibroblastic/fibroblastic tumours included in this study, four (50%) males and four (50%) females. The median age of patients was 41.8 years at diagnosis (range 24–65 years). These tumours had a median size of 50.6 mm (range 40-58mm), six tumours (75%) were in the lower limb and two (25%) were in the upper limb extremity. Table 3.3 summaries clinicopathological features for patients with non-metastasising tumours.

Table 3.3 Clinical and pathological characteristics for patients with non-metastasising tumours

		Myxoma (n=3)	**Fibromatosis (n=5)**	**Total (n=8)**
Gender	Male	3	1	4 (50)
	Female	0	4	4 (50)
Age (years)	Median	60.6	54.33	41.8
	Range	58-65	50-58	24-65
Site (mm)	Upper Limb	0	2	2 (25)
	Lower Limb	3	3	6 (75)
Size	Median	30.6	3	50.6
	Range	24-38	40-56	40-58

Number (%).

3.2.3 Comparison between myofibroblastic/fibroblastic sarcomas and their non-metastasising counterparts

Comparison of clinicopathological features between myofibroblastic/fibroblastic sarcomas and their non-metastasising counterparts are summarised in table 3-4.

Table 3.4 Clinicopathological comparison between myofibroblastic/fibroblastic sarcomas and their non-metastasising counterparts

		Total	Myofibroblastic/ Fibroblastic Sarcomas	Non-metastasising tumours	P-value
			(n=35)	(n=8)	
Gender					0.94
	Male	21	17	4	
	Female	22	18	4	
Age (years)					**0.001***
	Median		74.0	36.8	
	Range		27-94	24-65	
Site					0.72
	Upper Limb	13	11	2	
	Lower Limb	30	24	6	
Size (mm)					**0.002***
	Median		80.0	50.0	
	Range		25-150	40-58	

Number (%). Statistical analysis based on Fisher's exact test for categorical variables, Mann-Whitney test for continuous variables, $p < 0.05$ indicates a significant association.

Fisher's exact test revealed that there was no statistical significance difference ($p = 0.94$) in gender between myofibroblastic /fibroblastic sarcomas (male = 17, female = 18) and non-metastasising tumours (male = 4, female = 4). Furthermore, there was also no statistically

significant (p = 0.72) association between the tumour site and type of tumour soft tissue sarcoma tumours (upper limb =11, lower limb = 24) and non-metastasising tumours (upper limb = 2, lower limb = 6).

However, a Mann-Whitney U test revealed a significant difference (U = 31.000, Z = -3.403, p = 0.001) in the age of sarcoma patients (median = 74, n = 35) and patients with non-metastasising tumours (median = 36, n = 8). The effect size (r value) is 0.5, and this would be considered a strong effect size according to Cohen's criteria (212). Myofibroblastic/fibroblastic sarcoma had a higher median age of patients (median = 74) than non-metastasising tumours (median = 36). Similarly, a Mann-Whitney U test revealed a statistical significant difference (U = 41.000, Z = -3.098, p = 0.001) in the size of sarcoma patients (median = 80, n = 35) and patients with non-metastasising tumours (median = 50, n=8). The r value is 0.4 and considered a medium effect size. Soft tissue sarcoma tumours had a higher median size (median = 80) than non-metastasising tumours (median = 50). Figure 3.2 illustrates comparison of patient age and tumour size between myofibroblastic/fibroblastic sarcomas and their non-metastasising counterparts.

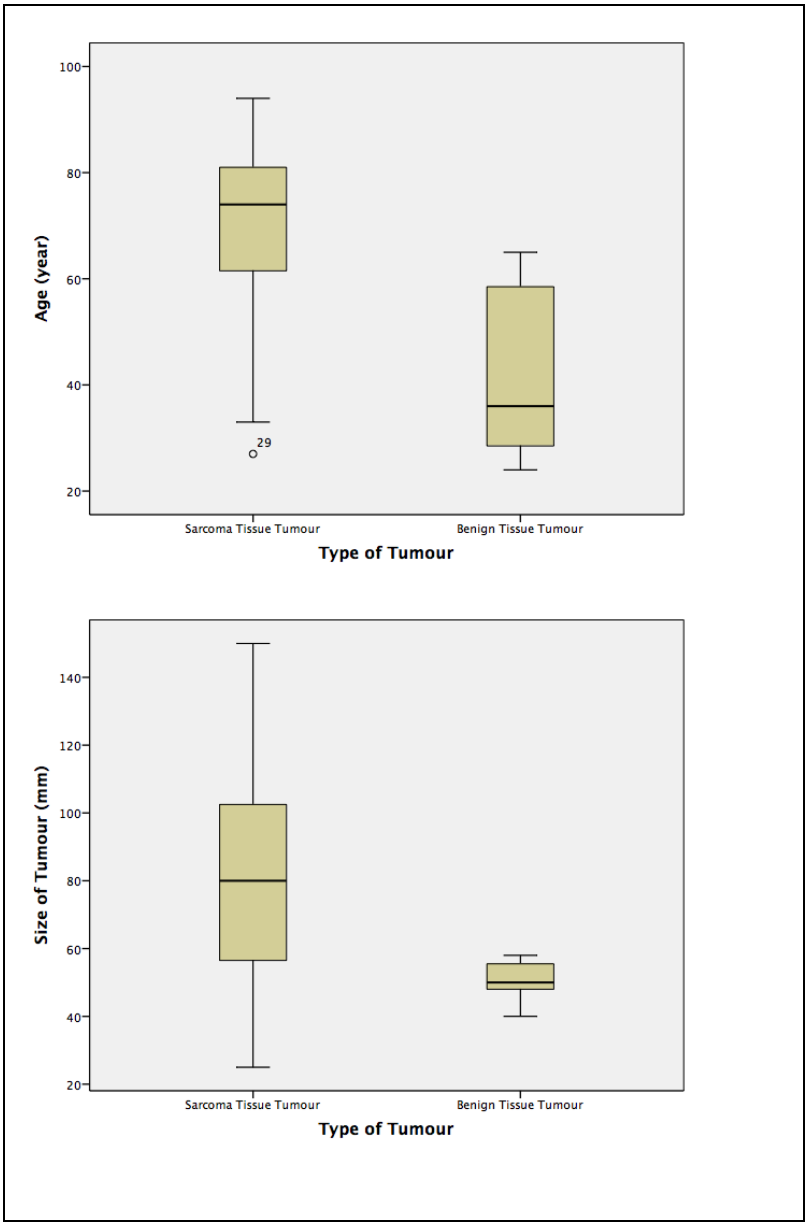

Figure 3.2 Comparison of patient age and tumour size between myofibroblastic/fibroblastic sarcoma and their non-metastasising counterparts' tumours. Sarcoma patients had a higher median age and a higher median tumour size than non-metastasising tumours ($p<0.05$).

3.3 Overview of IHC staining of HSPs in neoplastic and non-neoplastic cells

IHC staining of HSPs were demonstrated in non-neoplastic and neoplastic cells. The distribution and amount of stain in non-neoplastic cells are detailed in Table 3-5. The staining patterns of malignant cells are the subject of this section of the thesis. In terms of analysis, the vast majority of cells present in tumour tissue were neoplastic cells, other cells present in low numbers were macrophages and endothelial cells.

Immunoreactive staining of anti-HSP27 was detected mainly in the cytoplasm of tumour cells. However, IHC staining for anti-HSP27 was positive in non-neoplastic cells such as endothelial cells, inflammatory cells and fibroblasts. Furthermore, it was found that HSP27 was expressed in the extracellular matrix. Figure 3.3 shows an example of HSP27 staining in myxofibrosarcoma.

IHC staining of anti-HSP70 was found in both the nucleus and cytoplasm of tumour cells. Although HSP70 staining is negative in most non-neoplastic cells, such as endothelial cells and inflammatory cells, it does show some trace staining in the extracellular matrix. Figure 3.4 shows an example of IHC staining of anti-HSP70 in myxofibrosarcoma and demonstrates the pattern of staining in neoplastic and non-neoplastic cells.

Similarly, IHC staining of HSP90 was detected in both nucleus and cytoplasm of tumour cells. However, endothelial and inflammatory cells were also positive for HSP90 staining. Nevertheless, it was almost negative in the extracellular matrix. Figure 3.5 shows an example of IHC staining of HSP90 in myxofibrosarcoma and demonstrates the pattern of staining in neoplastic and non-neoplastic cells.

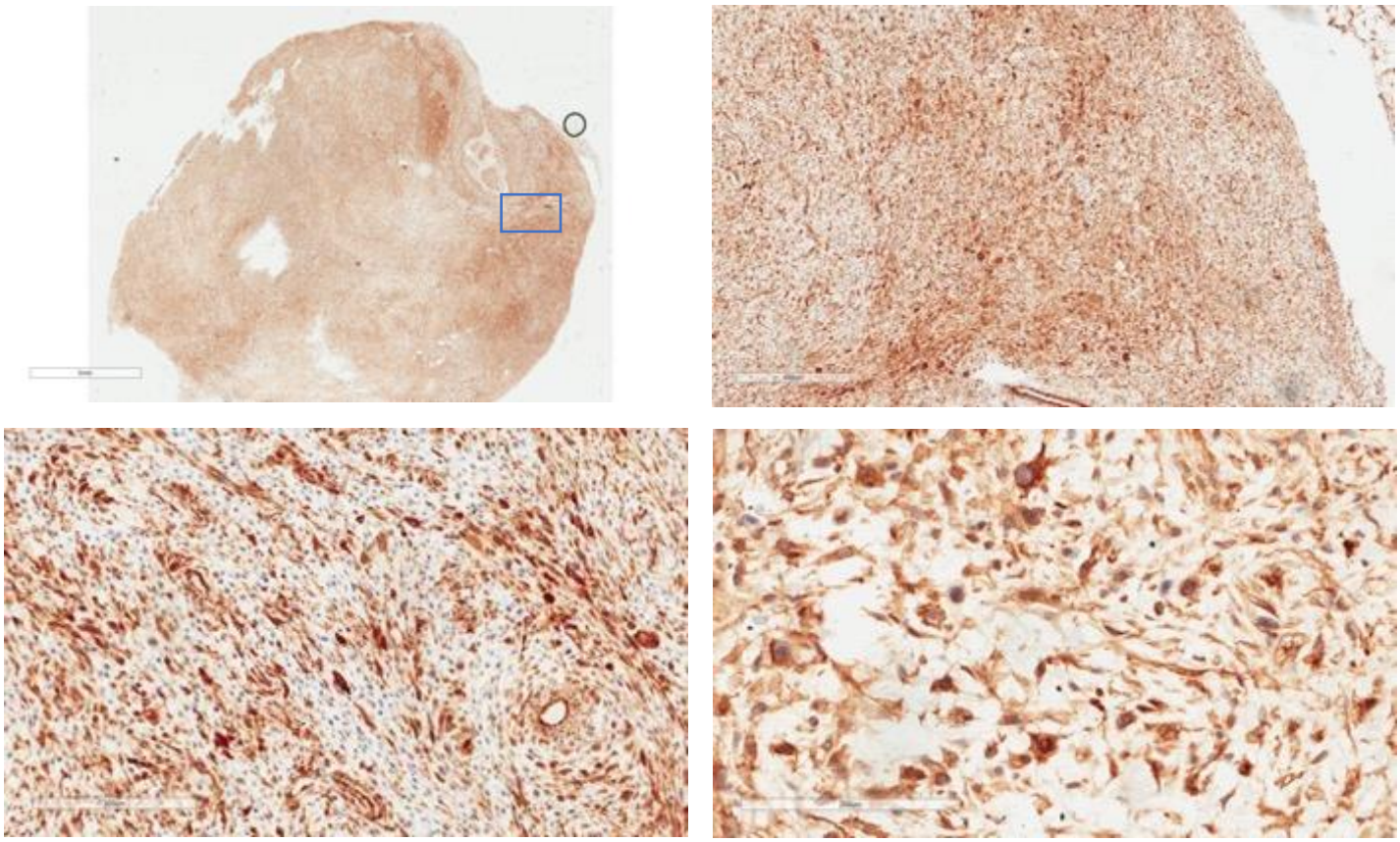

Figure 3.3 Example of IHC staining of HSP27 in myxofibrosarcoma showing the pattern of staining in neoplastic and non-neoplastic cells. Different magnifications, x2, x4, x10 and x40. IHC staining of anti-HSP27 was detected mainly in the cytoplasm of tumour cells. However, IHC staining for anti-HSP27 was also positive in non-neoplastic cells such as endothelial cells, inflammatory cells and fibroblasts.

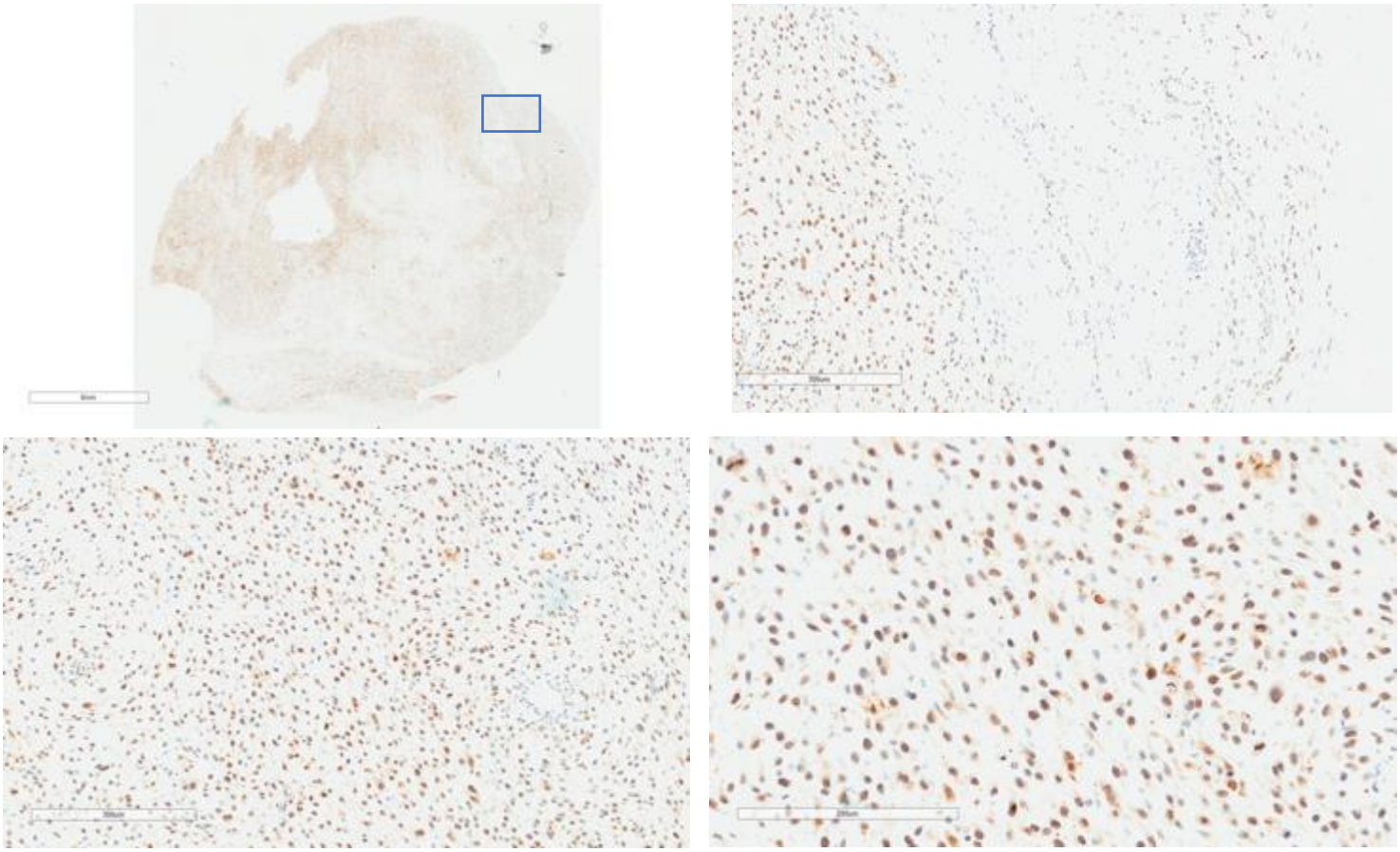

Figure 3.4 Example of IHC staining of HSP70 in myxofibrosarcoma showing the pattern of staining in neoplastic and non-neoplastic cells. Different magnifications, x2, x4, x10 and x40. IHC staining of anti-HSP70 was found in both the nucleus and cytoplasm of tumour cells. However, HSP70 staining is negative in most non-neoplastic cells, such as endothelial cells and inflammatory cells.

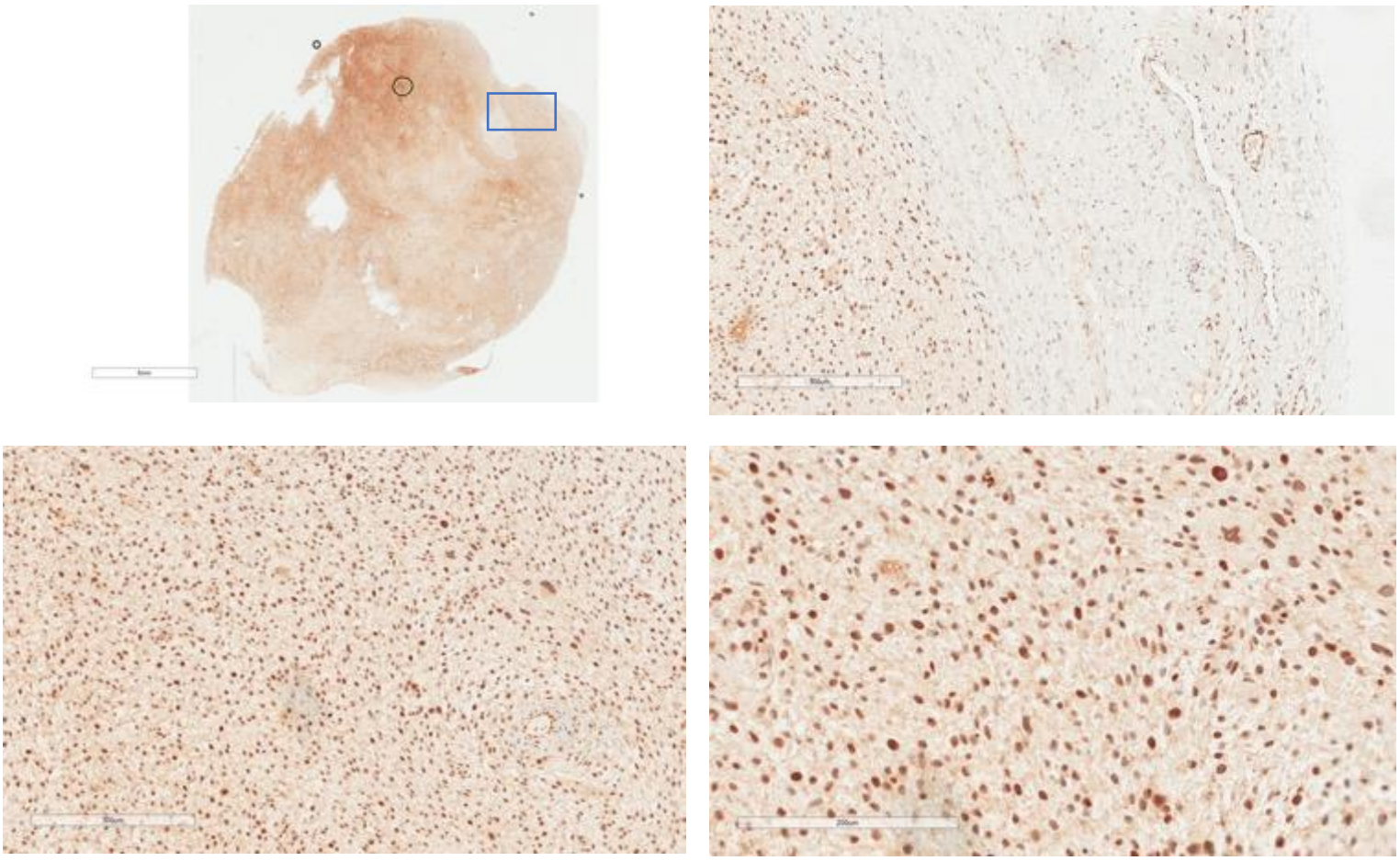

Figure 3.5 Example of IHC staining of HSP90 in myxofibrosarcoma showing the pattern of staining in neoplastic and non-neoplastic cells. Different magnifications, x2, x4, x10 and x40. IHC staining of HSP90 was detected in both nucleus and cytoplasm of tumour cells. However, endothelial and inflammatory cells were also positive for HSP90 staining

3.4 HSP27 expression

3.4.1 Expression of HSP27 in different tumour types

IHC staining of anti-HSP27 was positive in all tumour types included in this study. However, a Kruskal-Wallis Test revealed that there is no significant statistical difference in HSP27 expression across the tumour subtypes (Chi-Square = 6.05, df = 5, P = 0.2). Table 3-6 summarises staining of HSP27 in various type of tumours and figure 3.7 shows an example of the HSP27 staining in various type of the tumours.

Table 3.5 Summary of HSP27 staining in different type of tumours

	HSP27 Staining (%)*			
	Median	Range	Mean	SD
Myxofibrosarcoma (n=26)	28.09	1.09-93.42	29.76	20.96
Myofibroblastic sarcoma (n=3)	78.34	31.61-85.31	65.09	29.20
Myxoinflammatory fibroblastic sarcoma (n=3)	47.38	1.89-94.30	47.86	46.21
Fibromyxoid sarcoma (n=3)	39.47	12.13-51.27	34.29	20.08
Myxoma (n=3)	14.91	12.18-26.54	17.88	7.63
Fibromatosis (n=5)	21.09	0.67-42.81	19.83	18.52

*% of area of section stained using automated analysis

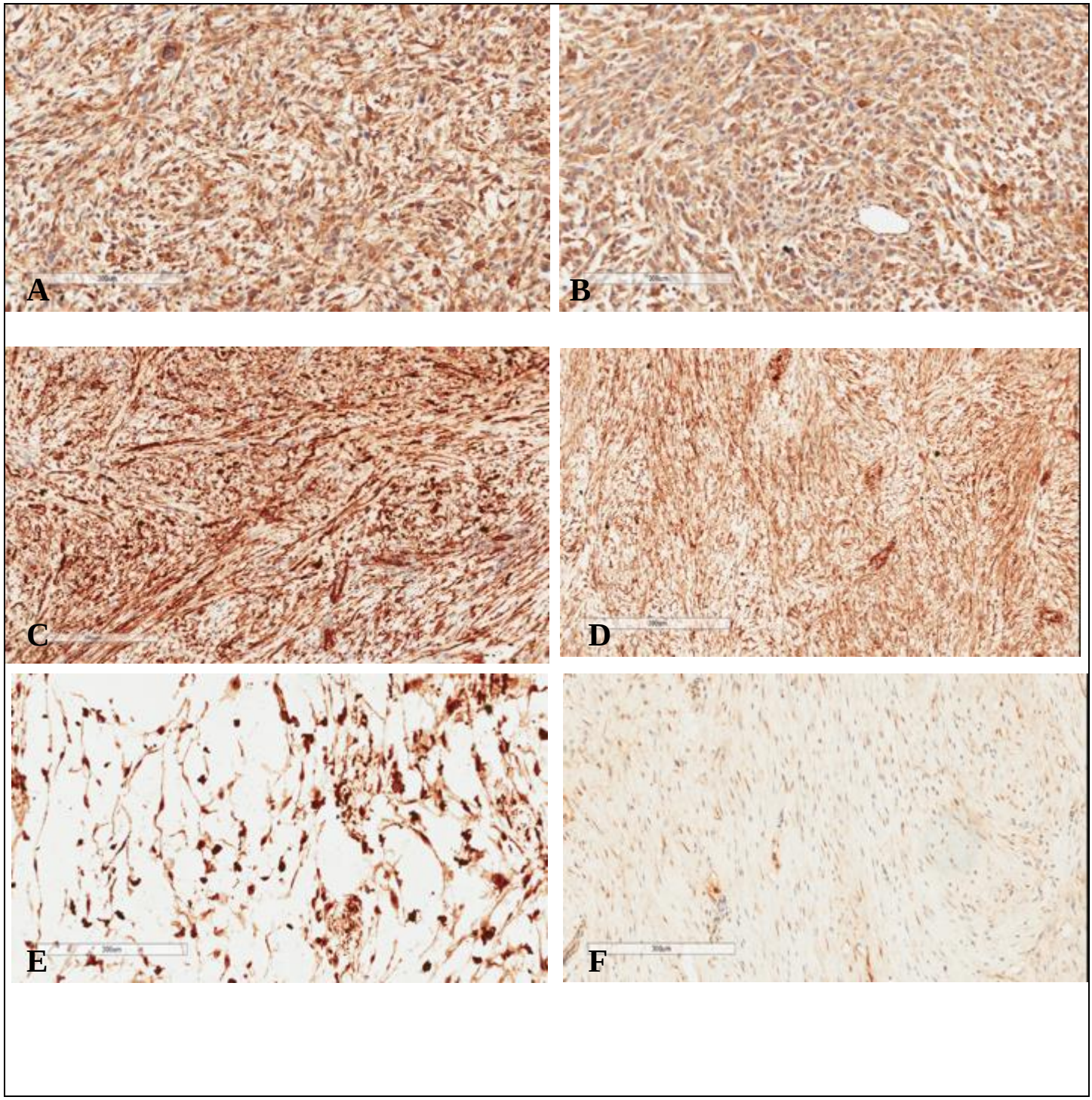

Figure 3.3 Examples of expression of HSP27 in different tumour types. Magnification scale: 300 µm.

A) HSP27 expression in Myxofibrosarcoma. B) HSP27 expression in Myofibroblastic sarcoma. C) HSP27 expression in MIFS. D) HSP27 expression in Fibromyxoid sarcoma. E) HSP27 expression in Myxoma. F) HSP27 expression in Fibromatosis.

3.4.2 Comparison of HSP27 expression between STS tumours and non-metastasising tumours

Overall expression of HSP27 in soft tissue sarcoma (median = 31.61, n = 35) was higher than in non-metastasising tumours (median = 18.00, n = 8). However, there is no statistically significantly difference in HSP27 expression between two groups using a Mann-Whitney U Test (Z = - 1.67, p = 0.9).

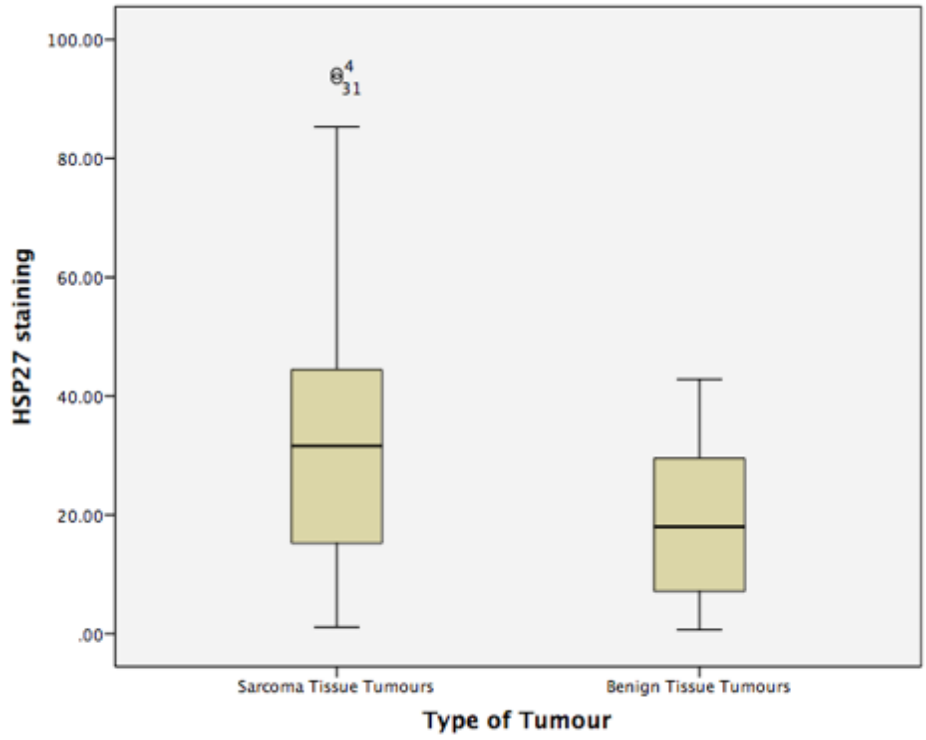

Figure 3.4 Comparison of HSP27 staining between STS tumours and non-metastasising tumours. There was no statistically significantly difference between two groups

3.4.3 Relationship between HSP27 expression and the grade of STSs

The relationship between HSP27 expression and grade of STSs was investigated using Spearman's rank order correlation. Although, high levels of HSP27 were associated with a higher grade of soft tissue sarcoma, this was not statistically significant (rs = 0.29, p = 0.08). Figure 3.10 demonstrates an example of HSP27 expression in different grade of STSs.

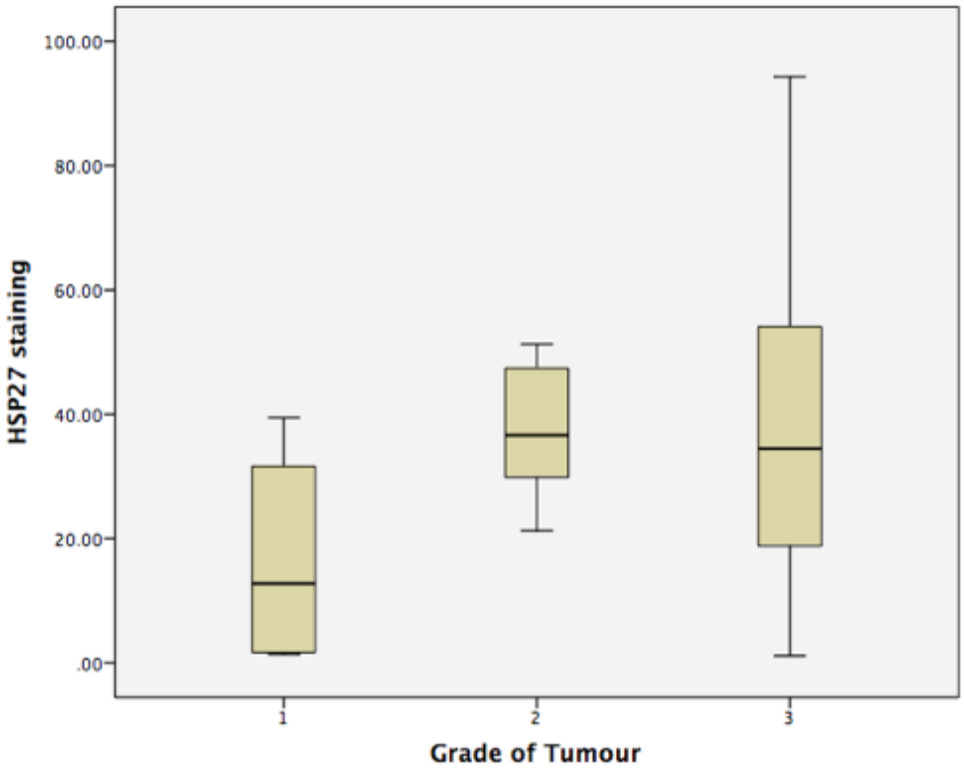

Figure 3.5 Relationship between HSP27 staining and the grade of STS. There was no statistically significant.

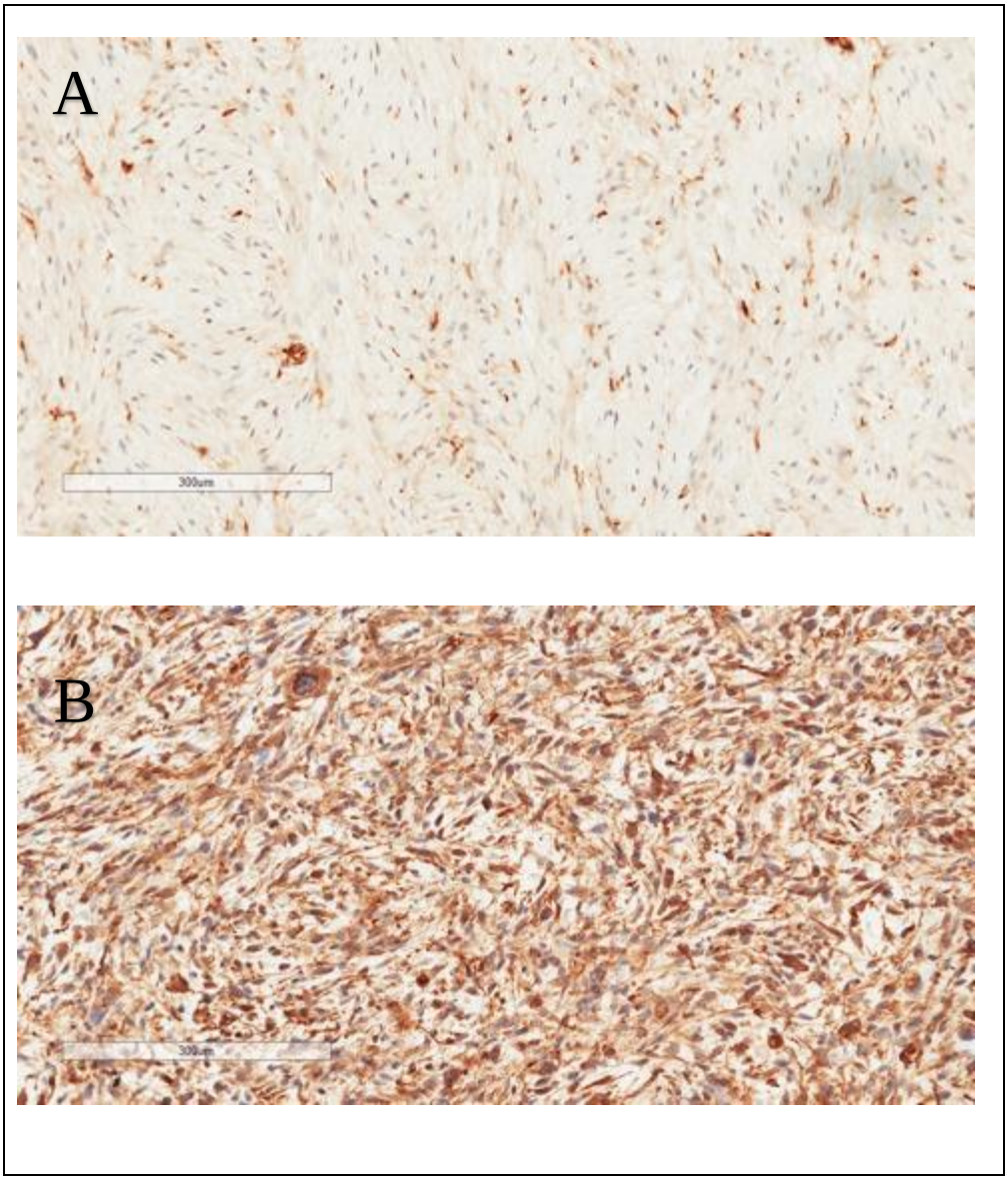

Figure 3.6 HSP27 expression in different grade of Myxofibrosarcoma.

A) Low expression of HSP27 in low grade myxofibrosarcoma. B) Strong expression of HSP27 in high grade of myxofibrosarcoma.

3.4.4 Relationship between HSP27 expression and clinicopathological features

Table 3-7 illustrates the relationship between the percentage of HSP27 expression and clinical features of soft tissue tumours included in this study.

Table 3.6 Relationship between HSP27 expression and clinicopathological features of tumours

	HSP27 staining (%)				
	Median	Minimum	Maximum	Mean	Standard Deviation
Male (n=21)	31.61	1.09	94.30	30.41	21.25
Female (n=22)	24.79	.67	93.42	33.16	27.31
Upper Limb (n=13)	39.47	.67	93.42	35.86	25.61
Lower Limb (n=30)	26.56	1.09	94.30	30.07	23.93
No Metastasis (n=4)	29.09	.67	93.42	30.06	22.58
Metastasis (n=39)	44.82	11.78	94.30	48.93	36.98

*% of area of section stained using automated analysis

The relationship between HSP27 expression and the age of patients was investigated using Spearman's rank order correlation. There was no statistically significant correlation between the two parameters (r_s = 0.17, p = 0.2). Also, Spearman's test showed that there was no statistically significant correlation between HSP27 expression and the size of a tumour (r_s = -0.029, p = 0.8).

A Mann-Whitney U Test revealed no significant difference (z = -.17, p = 0.8) in the HSP27 expression of males (median = 31.61, n = 21) and females (median = 24.79, n = 22). Also, a Mann-Whitney U Test revealed no significant difference (z = -.95, p = 0.3) in the HSP27 expression between upper limb tumours (median = 39.47, n = 13) and lower limb tumours (median = 26.56, n = 30).

Although median percentage of HSP27 expression metastatic group was higher than the non-metastatic group, a Mann-Whitney U Test revealed that there was no significant difference (z

= -.96, p = 0.4) in the HSP27 expression between non-metastatic patients (median = 29.09, n = 31) and metastatic patients (median = 44.82, n = 4).

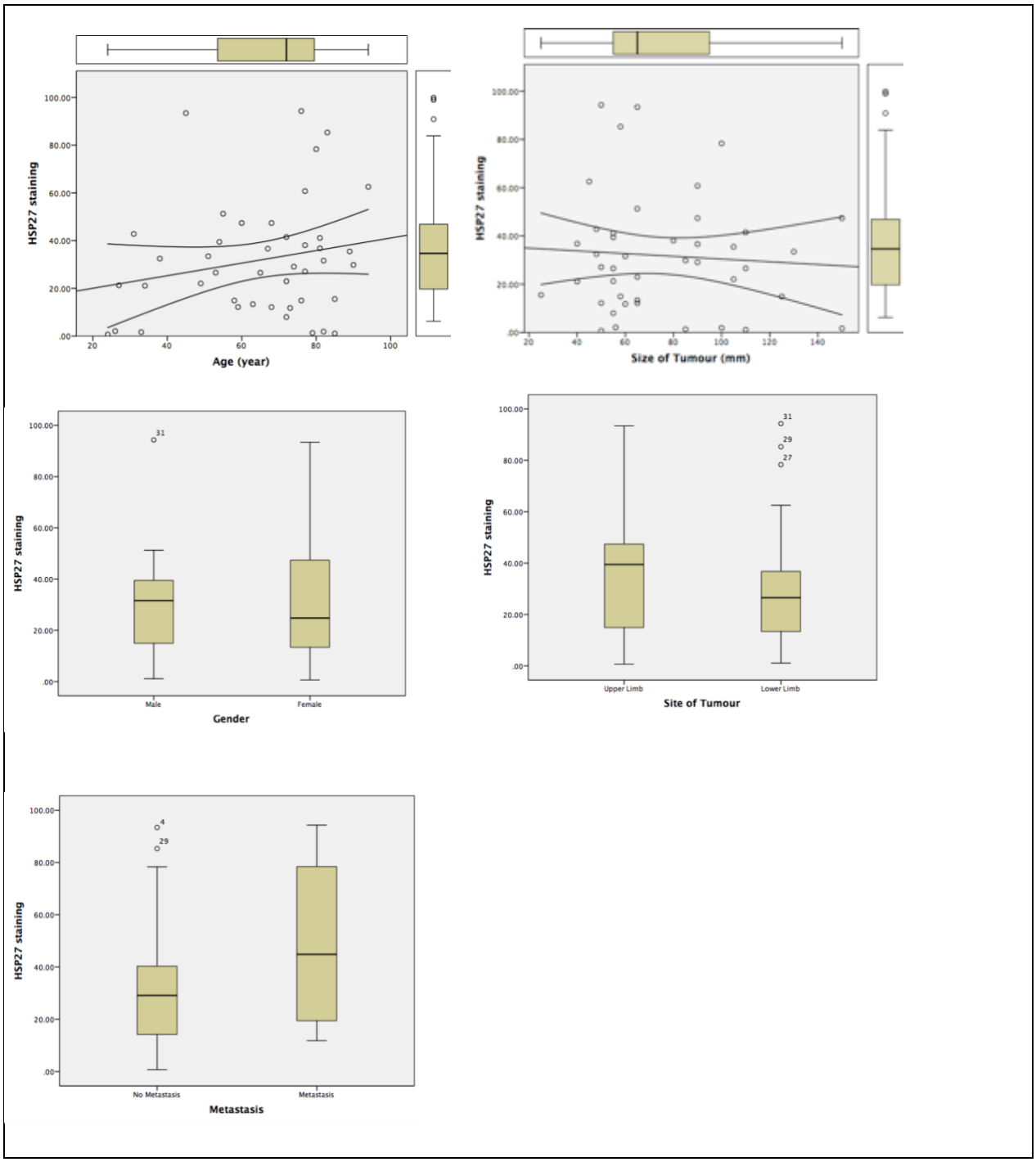

Figure 3.7 Relationship between HSP27 expression (% of staining area) and clinical features (patient age, size of tumour, gender, tumour site and metastasis). Not significant statistically.

3.5 HSP70 expression

3.5.1 Expression of HSP70 in different tumour types

Table 3-8 summarises staining of HSP70 in various type of tumours. A Kruskal-Wallis Test revealed that there was a significant statistical difference in HSP70 expression across six tumour subtypes included in this study (Chi-Square = 11.4, df = 5, p = 0.044). Myxofibrosarcoma tumours had a higher median HSP70 expression (median = 19.22) than the other groups. Pairwise comparisons were executed using Dunn's procedure with a Bonferroni correction for multiple comparisons. Values were derived from mean ranks unless specified otherwise and adjusted p-values were reviewed. The post hoc analysis revealed a statistically significant difference (p= 0.006) between the myxofibrosarcoma group (median = 19.22) and myxoma group (median = 3.86). However, no significant difference was found between other types of tumours.

Table 3.7 HSP70 expression in different tumour types

	HSP70 staining (%)				
	Median	Minimum	Maximum	Mean	SD
Myxofibrosarcoma (n=26)	19.22	.22	54.26	20.86	12.36
Myofibroblastic sarcoma (n=3)	11.90	10.26	21.56	14.57	6.11
Myxoinflammatory fibroblastic sarcoma (n=3)	12.30	11.57	32.42	18.76	11.83
Fibromyxoid sarcoma (n=3)	5.66	5.21	19.77	10.21	8.28
Myxoma (n=3)	3.86	2.46	5.38	3.90	1.46
Fibromatosis (n=5)	6.46	2.09	24.46	10.05	8.63

*% of area of section stained using automated analysis

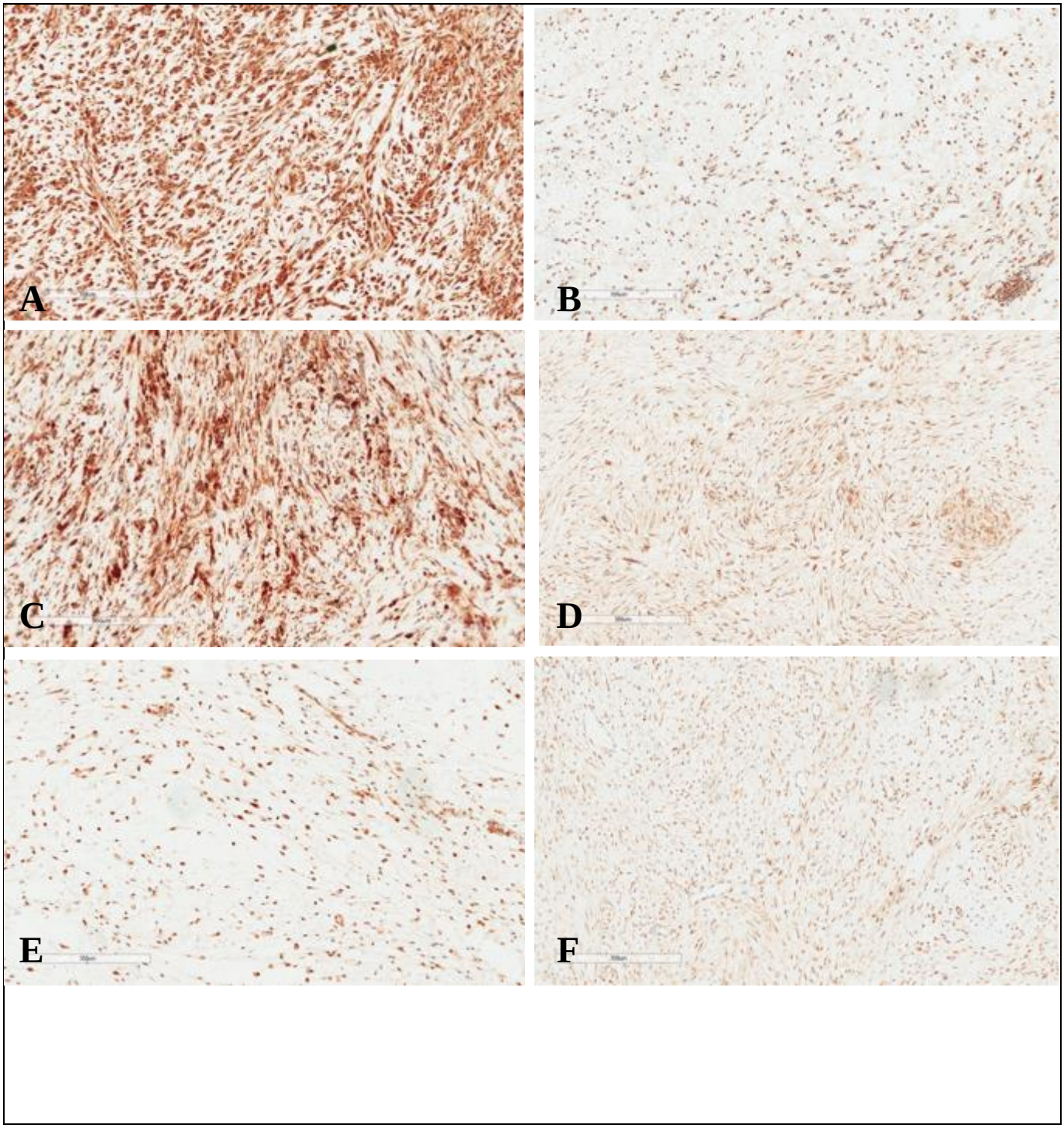

Figure 3.8 Examples of IHC staining of HSP70 in different type of tumours.

A) HSP70 expression in myxofibrosarcoma, B) HSP70 expression in myofibroblastic sarcoma, C) HSP70 expression in myxoinflammatory fibroblastic sarcoma, D) HSP70 expression in fibromyxoid sarcoma, E) HSP70 expression in myxoma, and F) HSP70 expression in fibromatosis. Magnification scale: 300 µm.

3.5.2 Relationship between HSP70 expression and grade of STSs

The relationship between HSP70 expression and grade of STSs was investigated using Spearman's rank order correlation. There was a medium, positive correlation between the two variables ($r_s = 0.33$, $p = 0.02$), with high levels of HSP70 associated with a higher grade of soft tissue sarcoma (Figure 3.10). An example of HSP70 expression in a different grade is demonstrated in figure 3-11.

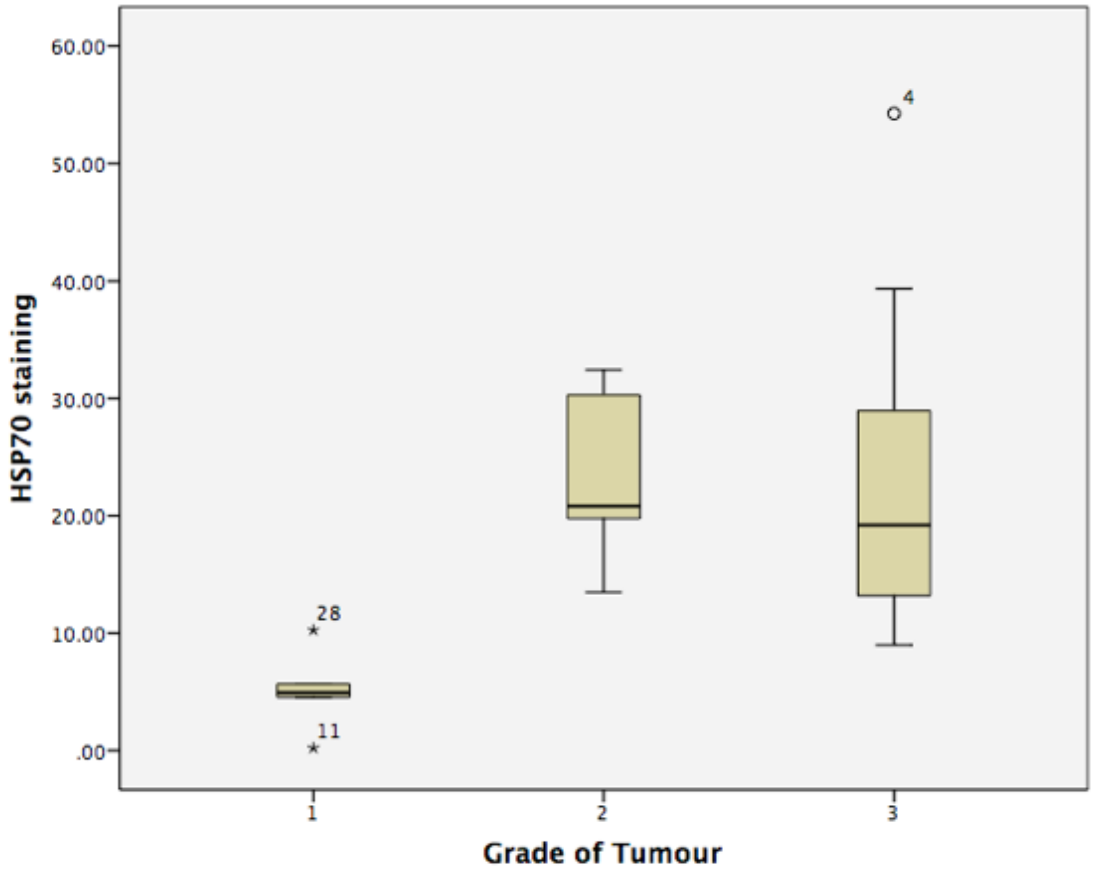

Figure 3.9 Relationship between HSP70 staining and the grade of STS. There was a positive correlation between the two variables.

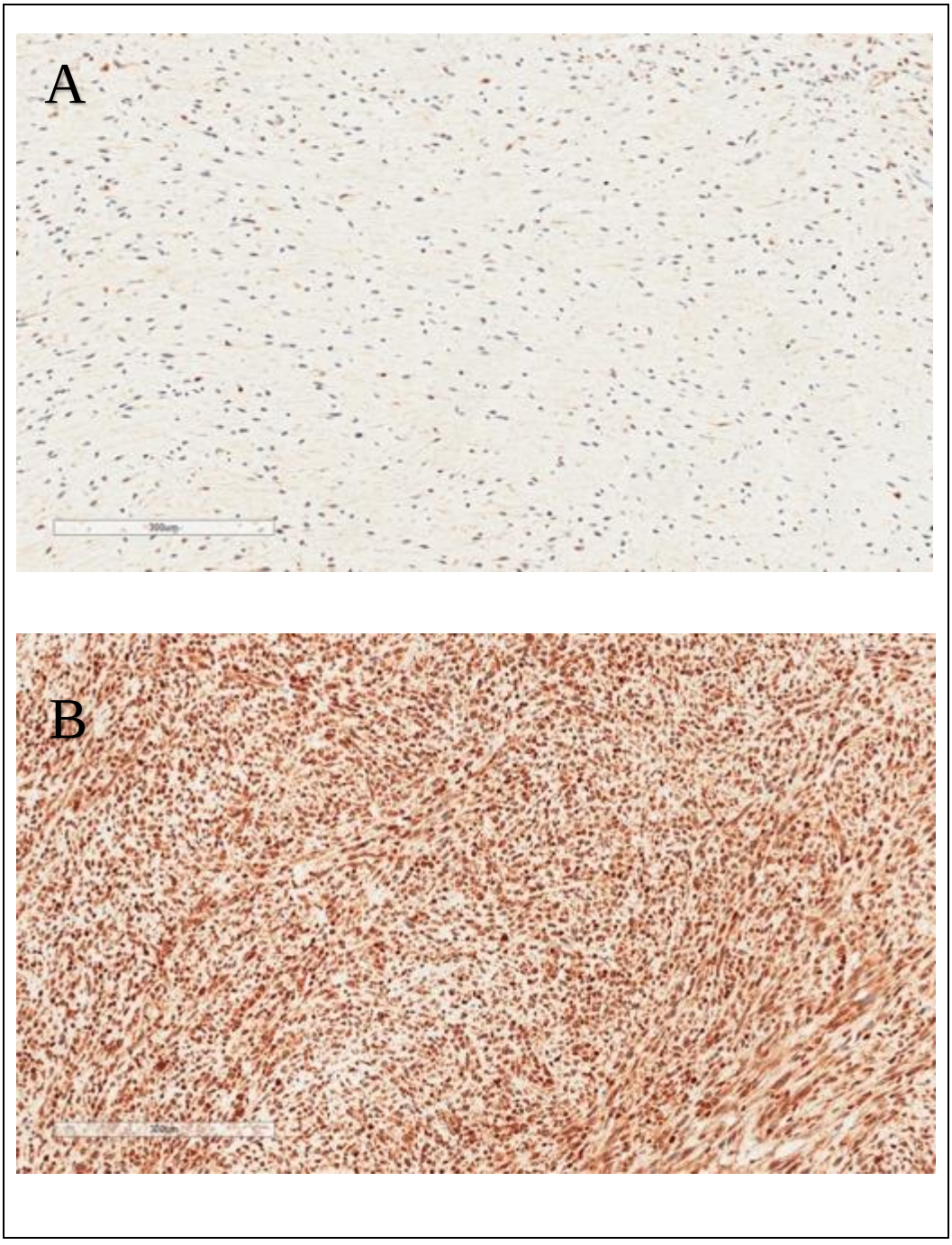

Figure 3.10 HSP70 expression in different grade of Myxofibrosarcoma.

A) Low expression of HSP70 in low grade myxofibrosarcoma and B) strong expression of HSP70 in high grade of myxofibrosarcoma.

3.5.3 Comparison of HSP70 expression between STSs and non-metastasising tumours

Soft tissue sarcoma tumours have a higher median expression of HSP70 (median = 16.50) than non-metastasising soft tissue tumours (median = 5.90). A Mann-Whitney U Test revealed that there was a statistical significant difference (U = 52.00, z = -2.746, p = 0.005) in the expression of HSP70 between STS tumours (median = 16.50, n = 35) and non-metastasising soft tissue tumours (median = 5.90, n = 8). The r value was 0.4, a medium effect size using Cohen's criteria. Figure 3.12 illustrates the comparison of HSP70 expression between STSs and non-metastasising tumours.

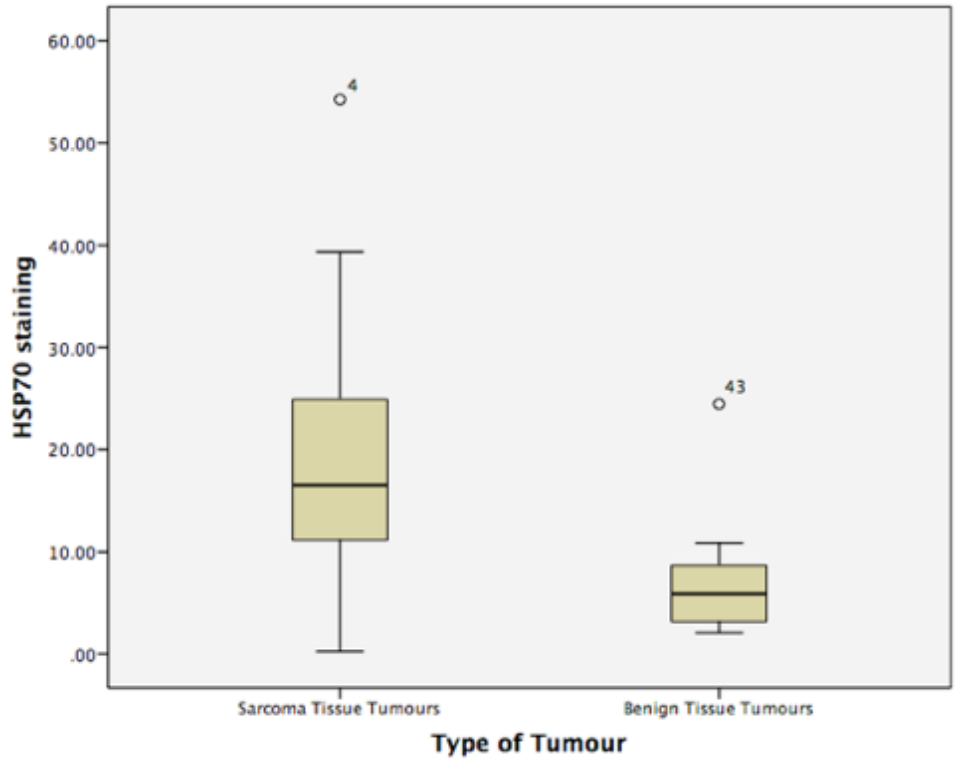

Figure 3.11 Comparison of HSP70 staining between STS tumours and non-metastasising tumours. There was a statistically significant difference in HSP70 expression between two groups.

3.5.4 Relationship between HSP70 expression and clinicopathological features

Table 3-9 illustrates the relationship between the percentage of HSP70 staining and clinicopathological features of soft tissue tumours included in this study.

Table 3.8 Relationship between HSP70 expression and clinicopathological features of tumours

	HSP70 staining (%)				
	Median	Minimum	Maximum	Mean	Standard Deviation
Male (n=21)	13.49	2.46	34.69	15.69	10.05
Female (n=22)	16.69	.22	54.26	18.43	13.49
Upper Limb (n=13)	14.08	5.21	54.26	18.68	13.20
Lower Limb (n=30)	14.22	.22	39.34	16.40	11.42
No Metastasis (n=39)	14.31	.22	54.26	17.46	12.37
Metastasis (n=4)	13.22	8.99	18.41	13.46	3.93

*% of area of section stained using automated analysis

The relationship between HSP70 expression and the age of patients was investigated using Spearman's rank order correlation. There was no statistically significant correlation between the two parameters ($r_s = 0.13$, $p = 0.39$). Furthermore, a Mann-Whitney U Test revealed no statistical significant difference ($z = -.65$, $p = 0.51$) in the HSP70 expression of males (median = 13.49, n = 21) and females (median = 16.69, n = 22). In addition, there was no statistical significant difference ($z = -0.39$, $p = 0.7$) in the HSP70 expression between upper limb tumours (median = 14.08, n = 13) and lower limb tumours (median = 14.22, n = 30).

The relationship between HSP70 expression and the size of tumours was investigated using Spearman's rank order correlation. There was no statistically significant correlation between the two variables ($r_s = 0.218$, $p = 0.16$).

A Mann-Whitney U Test revealed no significant difference (z = -.37, p = 0.73, r =0.05) in the HSP70 expression between non-metastatic patients (median = 14.31, n = 39) and metastatic patients (median = 13.22, n = 4).

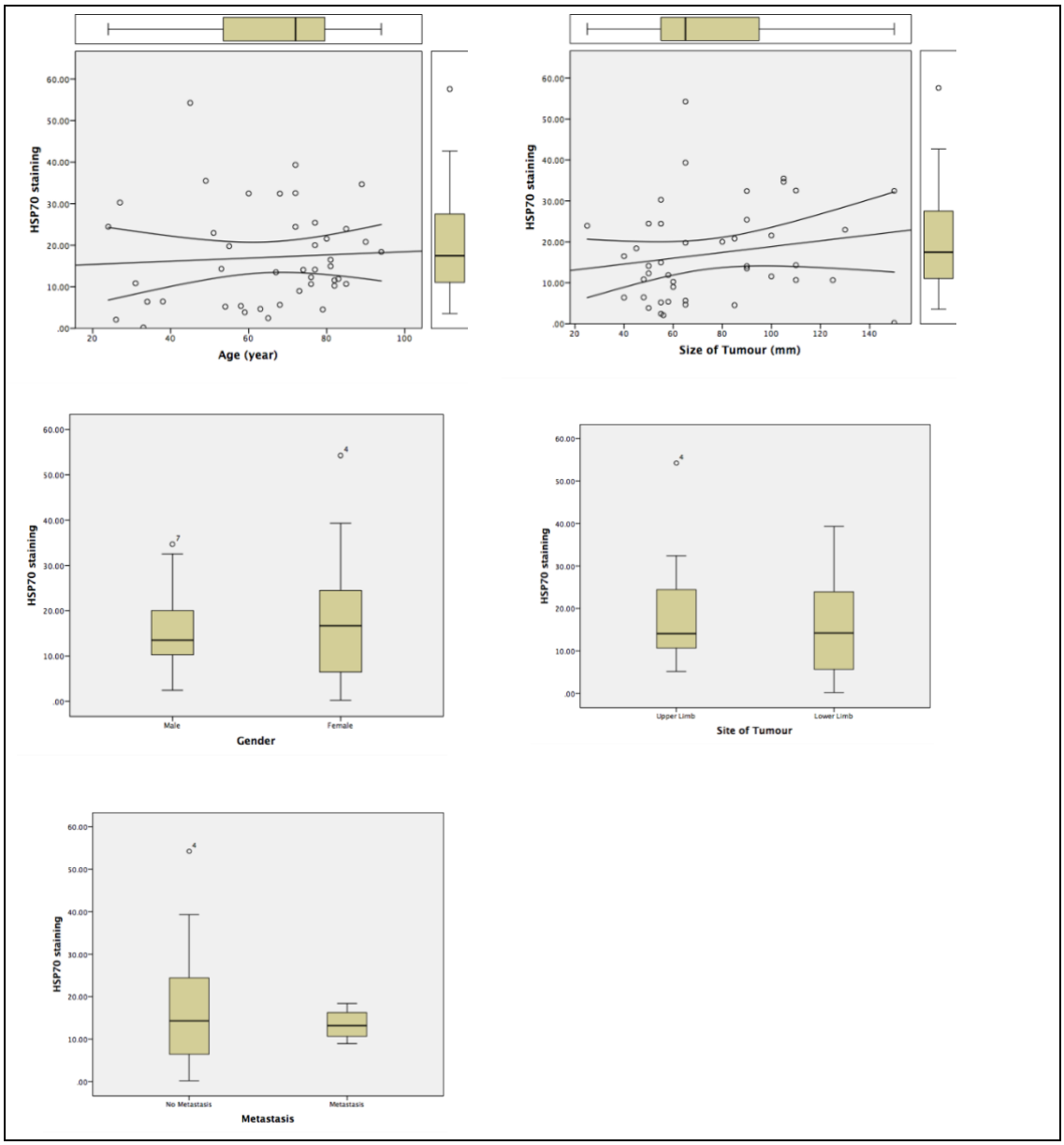

Figure 3.12 Relationship between HSP70 expression (% of staining area) and clinical features (patient age, size of tumour, gender, tumour site and metastasis). Not significant statistically.

3.6 HSP90 Expression

3.6.1 Expression of HSP90 in different tumour subtypes

Table 3.10 summarises expression of HSP90 in various type of tumours. A Kruskal-Wallis Test revealed that there is no significant statistical difference in HSP90 expression across six tumour subtypes included in this study (Chi-Square = 2.85, df = 5, p = 0.72). An example of IHC staining of HSP90 in different type of tumours is demonstrated in figure 3.14.

Table 3.9 Expression of HSP90 in different tumour subtypes

	HSP90 staining (%)				
	Median	Minimum	Maximum	Mean	Standard Deviation
Myxofibrosarcoma (n=26)	1.59	.12	51.57	6.33	11.37
Myofibroblastic sarcoma (n=3)	10.19	1.61	14.89	8.90	6.73
Myxoinflammatory fibroblastic sarcoma (n=3)	1.12	.58	10.96	4.22	5.84
Fibromyxoid sarcoma (n=3)	.18	.17	2.95	1.10	1.60
Myxoma (n=3)	2.48	.11	9.24	3.94	4.74
Fibromatosis (n=5)	.38	.13	25.31	5.42	11.12

*% of area of section stained using automated analysis

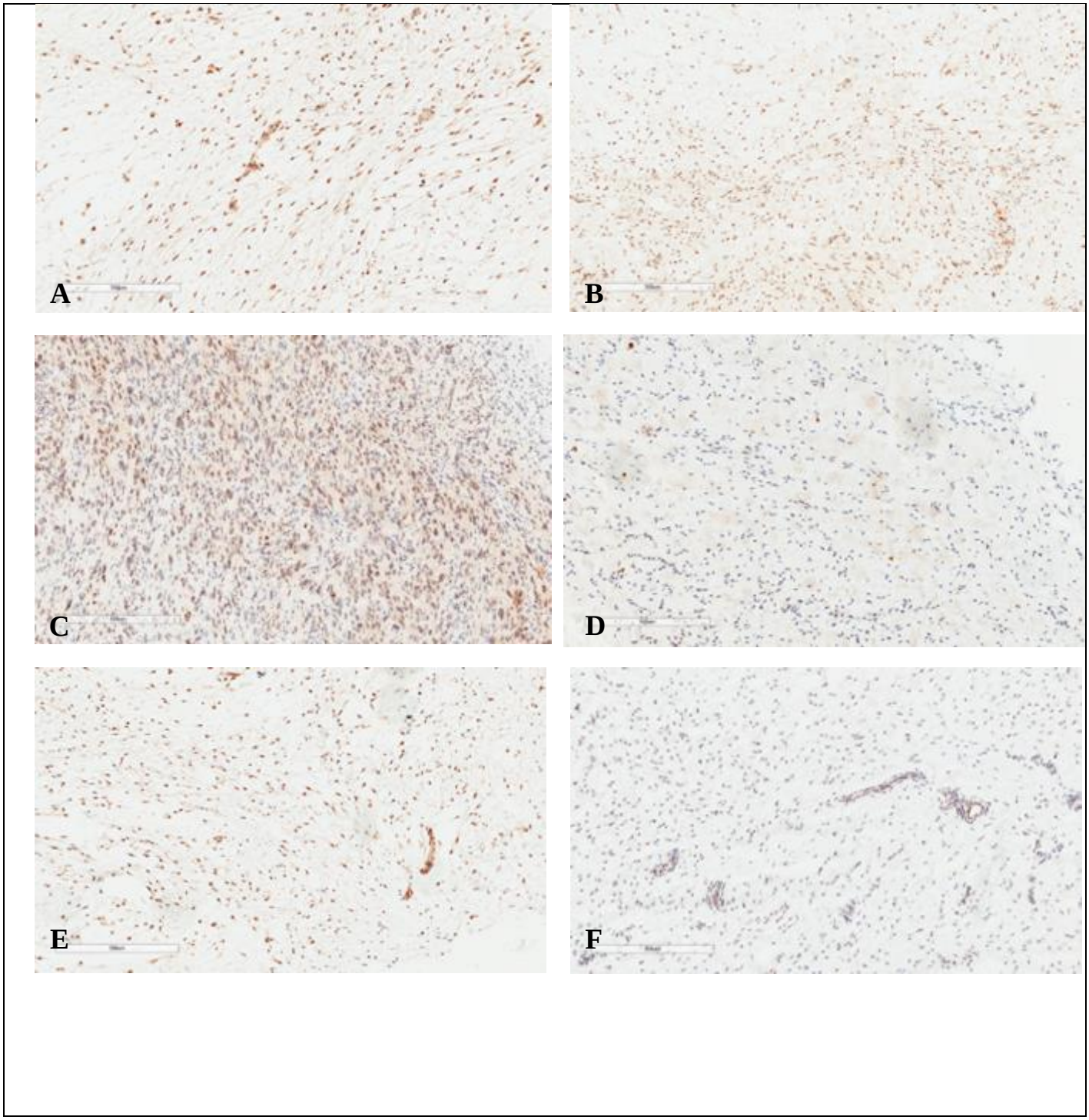

Figure 3.13 Examples of IHC staining of HSP90 in different type of tumours.

A) HSP70 expression in myxofibrosarcoma, B) HSP90 expression in myofibroblastic sarcoma, C) HSP90 expression in myxoinflmmatory fibroblastic sarcoma, D) HSP90 expression in fibromyxoid sarcoma, E) HSP90 expression in myxoma, and F) HSP90 expression in fibromatosis. Magnification scale: 300 µm.

3.6.2 Relationship between HSP90 expression and grade of STSs

The relationship between HSP90 expression and grade of STSs was investigated using Spearman's rank order correlation, with no significant correlation between the two variables ($r_s = 0.14$, $p = 0.34$). Figure 3.15 illustrates the relationship between HSP90 expression and the grade of STSs.

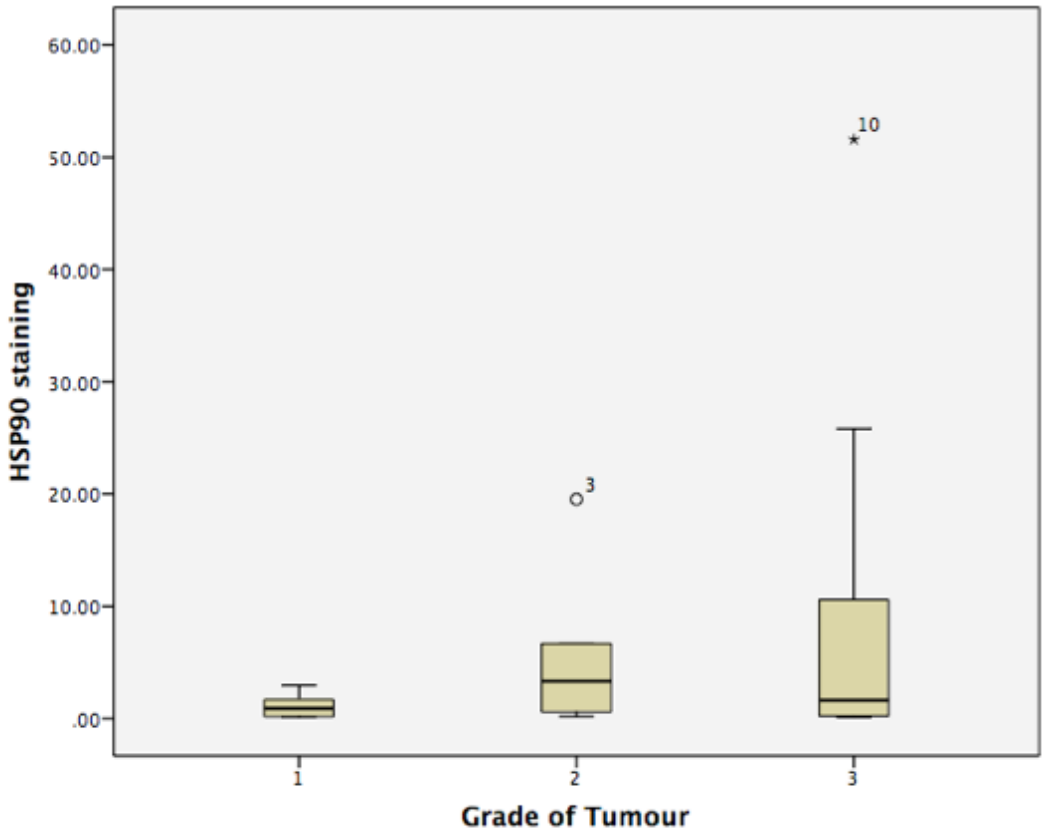

Figure 3.14 Relationship between HSP90 staining and the grade of STS. There was no significant relationship.

3.6.3 Comparison of HSP90 expression between STSs and non-metastasising tumours

A Mann-Whitney U Test revealed that there is no statistical significant difference (U = 118.00, z = -0.687, p = 0.51, r = 0.1) in the expression of HSP70 between STS tumours (median = 1.61, n = 35) and non-metastasising soft tissue tumours (median = 0.75, n = 8). Figure 3.16 illustrates the comparison of HSP90 expression between STSs and non-metastasising tumours.

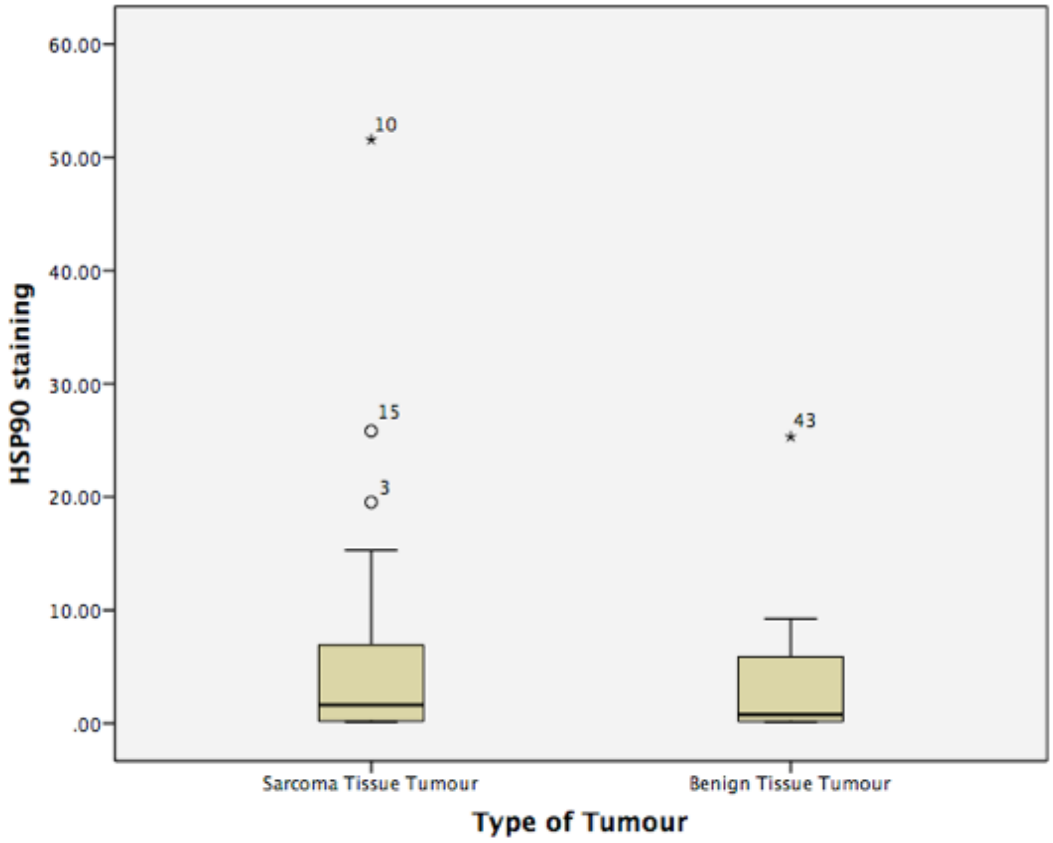

Figure 3.15 Comparison of HSP90 staining between STS tumours and non-metastasising tumours. There was no significant difference between two groups.

3.6.4 Relationship between HSP90 expression and clinicopathological features

Table 3.11 illustrates the relationship between the percentage of HSP70 staining and clinicopathological features of soft tissue tumours included in this study. A Mann-Whitney U Test detected no significant difference (U = 179.00, z = -1.26, p = 0.2) in the HSP90 expression of males (median = 0.58, n = 21) compared to females (median = 2.36, n = 22).

Table 3.10 Relationship between HSP90 expression and clinicopathological features

	HSP90 staining (%)				
	Median	Minimum	Maximum	Mean	Standard Deviation
Male (n=21)	.58	.11	51.57	5.40	11.69
Female (n=22)	2.36	.12	25.82	6.04	7.93
Upper Limb (n=13)	1.12	.13	51.57	8.76	15.02
Lower Limb (n=30)	1.59	.11	25.82	4.41	6.38
No Metastasis (n=39)	1.12	.11	51.57	5.66	10.20
Metastasis (n=4)	6.23	1.24	11.74	6.36	5.77

*% of area of section stained using automated analysis

Also, the relationship between HSP90 expression and the age of patients was investigated using Spearman's rank order correlation, with no statistically significant correlation between the two parameters (r_s = 0.06, p = 0.69). Similarly, there was no statistically significant correlation (r_s = -0.18, p = 0.22) between HSP90 expression and the size of tumours.

A Mann-Whitney U Test revealed no statistical significant difference (z = -0.71, p = 0.48) in the HSP90 expression between upper limb tumours (median = 1.12, n = 13) and lower limb tumours (median = 1.59, n=30). Likewise, there was no significant difference (U = 50.00, z =

-1.17, p = 0.26) in the HSP90 expression between the non-metastatic patient group (median = 1.12, n = 39) and metastatic patient group (median = 6.23, n=4).

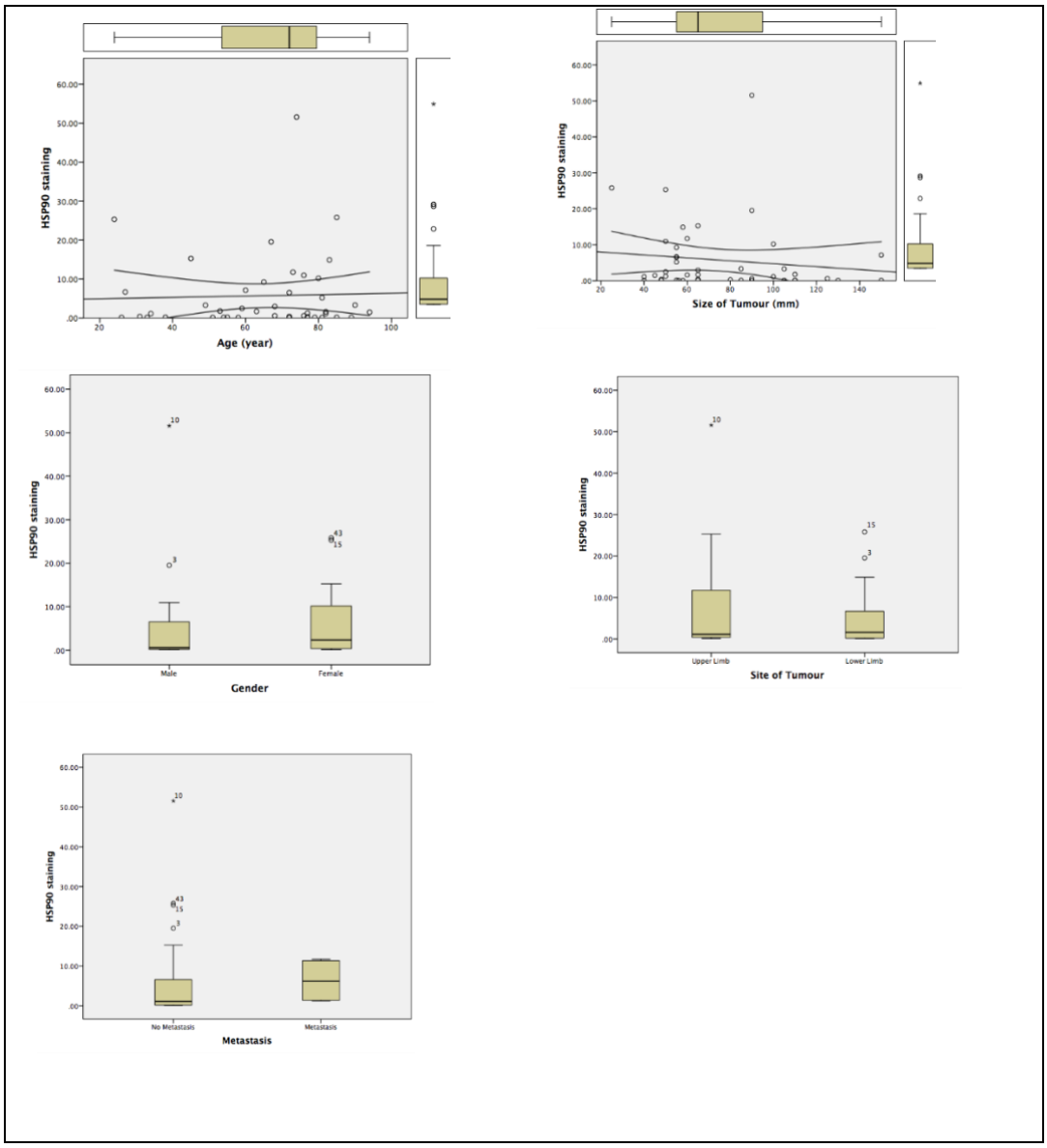

Figure 3.16 Relationship between HSP90 expression (% of staining area) and clinicopathological features (patient age, size of tumour, gender, tumour site and metastasis). Not significant statistically.

3.7 Correlations between the expression of heat shock proteins

The relationship between HSP27 expression and HSP70 expression was investigated using Spearman's rank order correlation. There was a medium, positive correlation between the two variables (r_s = 0.43, p = 0.004), with high levels of HSP27 associated with higher levels of HSP70. On the other hand, there was no significant correlation between HSP27 expression and HSP90 expression (r_s = 0.69, p = 0.65), or between HSP70 expression and HSP90 expression (r_s = 0.16, p = 0.3). Figure 3.18 shows the correlations between the expression of the heat shock proteins.

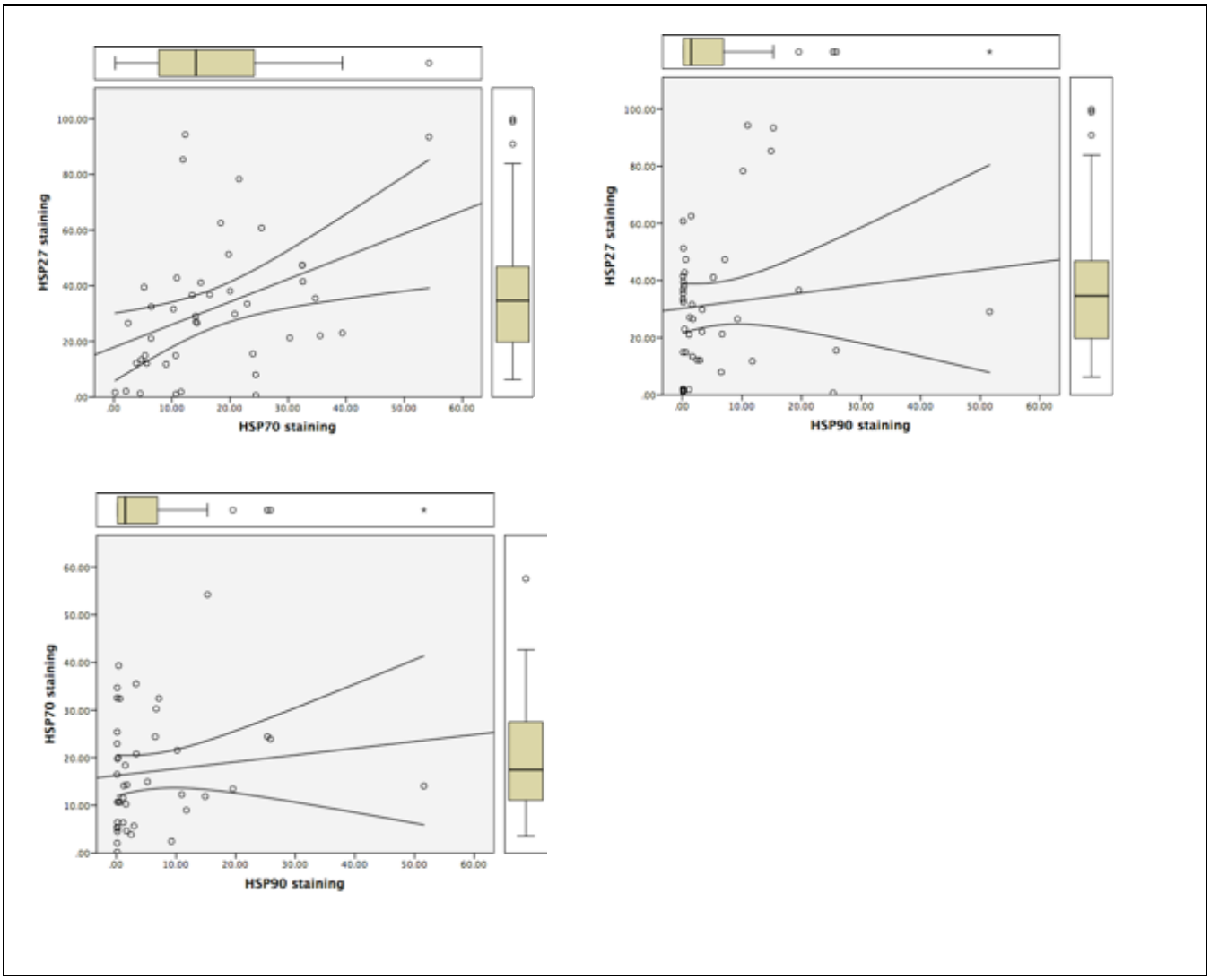

Figure 3.17 Correlations between the expression of the heat shock proteins. There was only a positive correlation between HSP27 expression and HSP70 expression.

3.8 Survival analysis

Overall survival in the 35 cases of soft tissue sarcomas ranged from 2 to 120 months. Patients were classified as alive (n = 24), dead (n = 8), or lost to follow-up (n = 3). The Kaplan-Meier method (the log-rank test) was used for analysis of survival, revealing no significant association between survival and HSP27, HSP70, or HSP90 expressions. However, there was only a significant relationship between metastasis and survival (p = 0.001). Analysis of all clinicopathological variables for overall survival in STS patients is illustrated in table 3.12.

Table 3.11 Analysis of all clinicopathological variables for overall survival in STS patients

Variable	P value
Gender	0.42
Age	0.76
Site	0.66
Size	0.07
Grade	0.19
Metastasis	**0.001***
HSP27	0.13
HSP70	0.48
HSP90	0.58

Kaplan-Meier method using log-rank test for statistical significance; $p < 0.05$.

Chapter Four

Discussion

4 Discussion

Soft tissue sarcomas are a rare and a heterogeneous group of malignancies that originate from mesenchymal tissue. Their rarity, coupled with their variety, is challenging for investigators to understand their natural history, prognosis and management. Soft tissue sarcoma tumours are well known for their aggressiveness and resistance to anticancer therapy.

The heat shock proteins (HSP) constitute a superfamily of chaperone proteins present in all cells and in all cell compartments, operating in a complex interplay with a synergistic/overlapping multiplicity of functions, even though the common effect is cell protection and cell survival. Several reasons explain the need for investigating HSPs in STSs: (1) these molecules function as chaperones of tumorigenesis, (2) they are associated with disease aggressiveness and with resistance to anticancer therapies including radiotherapy and chemotherapy, and (3) they can be used as targets for therapies.

However, only limited work has been conducted on the role of HSPs in connective tissue tumours, so much of the current knowledge about HSPs relates to carcinomas rather than sarcomas. The work presented in this thesis is based on the hypothesis that similar mechanisms of HSPs may be involved in tumorigenesis and aggressiveness in sarcomas as in carcinomas. Hence, the present study was performed to clarify the expression of those HSPs in soft tissue sarcomas. This work focused on the expression of HSP27, HSP70 and HSP90 in myofibroblastic/fibroblastic sarcoma (STS) of extremities, with a final goal to provide further insight on the biological behaviours of STS by focusing on the correlations between the expression of each protein and clinicopathological parameters. To the best of our knowledge, this is the first study to investigate the three selected HSPs (HSP27, HSP70 and HSP90) in adult myofibroblastic/fibroblastic sarcomas of extremities.

Due to the rarity of sarcomas, the present study focused on archived tissue to obtain sufficient material for the study. The material was formalin-fixed and paraffin embedded, and there was limited clinical and pathological data available. These constraints limited the techniques that could be employed to examine the expression of the chosen biomarkers, to immunohistochemistry (IHC) and *in situ* hybridization. Immunohistochemistry was chosen because by studying the expression of protein, one of the unknowns in investigating HSPs, translation of mRNA into protein, was eliminated.

Although the majority of results were not clear cut, a number of significant findings did emerge from the present study. In view of the fact that there is a lack of similar data on STS, it was considered appropriate for a discussion of the study findings and comparison of the results to other types of tumours in the literature.

4.1 Discussion of the study findings and those described in the literature

Surrogates were used to assess the importance of HSP expression and regulation to tumour behaviour in the absence of any specific markers. Of particular interest, was the relationship of expression of HSPs to histological grading, which is the best indicator of the aggressiveness of those tumours to date. In addition, tumours of different levels of malignancy (from benign to highly aggressive) were compared to identify patterns of biomarker expression that most clearly related to tumorigenesis. A total of 43 tumours were available for this study as archival formalin-fixed, paraffin-embedded tissue blocks.

4.1.1 Clinicopathological Parameters

With regard to clinicopathological features of sarcoma tumours and non-metastasising tumours, this study showed that patient age and size of tumour were statistically significantly

different between these two groups (p = 0001), as malignant tumours occurred in older patients and had a larger size than non-metastasising tumours. Overall, the incidence of soft-tissue sarcomas increases significantly with age, with more than 65% of cases occurring in patients aged 50 and over (5). Furthermore, there was a significant correlation between the size of tumour and grade of soft tissue sarcoma; a large tumour size associated with a higher grade of soft tissue sarcoma (p = 0.04). Similarly, a study by Stojadinovic *et al*. showed that a large tumour size was consistently associated with a higher grade and a poor prognosis of STS (39).

4.1.2 Expression of HSP27

In this study, the staining of HSP27 was positive in all types of soft tissue tumours. Although HSP27 was more highly expressed in sarcoma tumours, particularly myofibroblastic sarcoma and MIFS compared to non-metastasising tumours, there was no significant statistical difference in expression between the non-metastasising and sarcoma tumours. Similarly, there was a trend for high HSP27 expression in high-grade STSs and metastatic group; however, this was not statistically significant. Furthermore, there were no statistically significant differences in relation to age, gender, tumour size, tumour site and survival outcome.

In the oncology field, many studies have found that HSP27 was overexpressed in a wide range of malignant cells and tissue compared to normal tissue. For example, Wataba *et al*. in an IHC study found that HSP27 was overexpressed in hyperplastic endometrium and carcinoma compared to normal endometrium tissue (213). Also, in a study of biopsy samples from prostate cancer patients, expression of intracellular HSP27 expression in prostate cancer tissue was up-regulated compared to the controls (214). Rui *et al*. also demonstrated that HSP27 levels in serum were significantly higher in breast cancer patients compared to healthy controls (215). Similarly, Grzegrzolka *et al*. found that HSP27 expression was significantly higher in 101

patients with invasive ductal breast carcinoma compared to 20 patients with benign fibrocystic lesions (209).

Many studies have investigated the correlation of HSP27 expression with grade and poor survival in many cancer tissues. For instance, King *et al*. examined expression of HSP27 in 58 patients with hepatocellular cancer and adjacent non-cancerous liver tissue by IHC, finding a significantly higher distribution of HSP-27 expression in HCC tissues compared with adjacent non-cancerous liver tissues and that HSP-27 expression was positively correlated with a histologic tumour grade and poor survival rate (216). HSP27 expression was also strongly correlated with poor survival in patients with rectal tumours (217). Assimakopoulou *et al.* reported that HSP27 expression was correlated with histological grades of astrocytoma (218). Similarly, Moon *et al.* found that significantly high levels of HSP27 expression in conventional osteosarcoma compared to low-grade osteosarcoma and it was significantly related to distant metastasis with a tendency toward poor survival (219). Recently, Shuangjiang *et al.* found in their meta-analysis that high HSP27 expression can be an effective biomarker for predicting poor clinicopathological parameters of non-small cell lung cancer, including the degree of differentiation, lymphatic metastasis, TNM stage, histological subtypes and tumour size (220).

In contrast, HSP27 expression has been associated with good prognosis in endometrial adenocarcinomas, oesophageal cancer, and in malignant fibrous histiocytomas. Muzio *et al.* analysed 79 cases of oral squamous cell carcinoma and 10 cases of normal mucosa for HSP27 expression by IHC. They found that patients with reduced HSP27 expression had more aggressive (higher grade and poorly differentiated) carcinoma, with a poor survival rate (221). In malignant fibrous histiocytoma, Tetu *et al*. (1992) evaluated by IHC the prognostic influence of the expression of HSP27 in 43 malignant fibrous histiocytomas, finding that HSP-27

expression was associated with a more favourable prognosis, and a significant correlation was observed with overall survival and metastasis-free survival (222).

Taken together, these results suggest that HSP27 plays a role in tumorigenesis and is a marker of aggressiveness of STSs, but not useful in diagnostic immunopathology. However, further experimentation is needed to investigate the biological functions of HSP27 proteins, that is, potential pleiotropic function in soft tissue sarcoma.

4.1.3 Expression of HSP70

In broad terms, this study showed that HSP70 expression was higher in myofibroblastic/fibroblastic sarcomas than the non-metastasising counterpart. Unlike HSP27, there was a significant statistical difference in the expression of HSP70 between sarcomas and non-metastasising tumours ($p < 0.05$). Similar to HSP27, there was a positive correlation between expression of HSP70 and the grade of STSs, with high levels of HSP70 associated with a higher grade of STSs. However, there was no significant relationship between HSP70 expression and patient age, gender, tumour location, tumour size, metastasis and survival.

There is extensive evidence in the literature that HSP70 is overexpressed in many cancers, and that high expression of this chaperone correlates with increased tumour grade and poor prognosis. A comparative pilot study of protein profiles of human hepatocellular carcinoma and non-tumour human liver samples showed that HSP70 is over expressed (2 fold) in the tumour cells compared to the non-tumor tissue (223). Isomoto *et al*. demonstrated that there is a significant correlation between HSP70 and HSP40 expression and histopathological typing of gastric cancer and concluded that these HSPs are less frequently expressed in undifferentiated carcinomas than in differentiated types (194). In colon cancer, Bauer *et al*. studied 355 primary resected colon carcinoma by IHC and found that high HSP70 expression

is associated with tumour grade, distant metastasis and poor clinical outcome in colon cancer (210). In bladder cancer, Syrigos *et al.* found that HSP70 overexpression correlated positively with the grade and stage of bladder carcinoma. Also, HSP-70 expression correlated closely with a poor outcome of 58 patients with bladder carcinoma. Furthermore, HSP70 overexpression correlated with increased cell proliferation rate and malignancy (224). Alexious *et al*. reported a significant positive correlation between proliferation rate index and aggressive subtype of medulloblastomas (225).

In contrast, there are some tumour types where a lower expression of HSP70 is observed compared with adjusted normal tissue and there is no correlation between HSP70 levels and survival. For example, in patients with renal cancer, Santarosa *et al*. (1997) reported that HSP70-1 upregulation was associated with a better prognosis, irrespective of stage and histological grade (226). Similarly, Ramp *et al*. (2007) showed that HSP70 expression was decreased from well (G1) to poorly differentiated renal cell carcinoma (227). In oesophageal squamous cell carcinoma, Nakajima *et al*. found that HSP70 expression is frequently reduced in 102 patients with oesophageal squamous cell carcinoma and reduction of HSP 70 expression was significantly correlated with poor prognosis (228). In chondrosarcoma, Trieb *et al*. investigated IHC expression of HSP72 in 37 chondrosarcoma sections and 10 chondroma sections, finding a decreased HSP72 expression in chondrosarcoma correlating with low differentiation (229).

4.1.4 Expression of HSP90

HSP90 expression was rather different to that of HSP27 and HSP70; there is less expression of HSP90 across all type of tumours. Furthermore, there was no relationship between HSP90 expression and clinicopathological parameters and survival.

Our results are in agreement with previous studies suggesting a decreased expression of HSP90 in some types of tumours. Wang *et al.* (2014) investigated HSP90 expression in 32 cases of myxofibrosarcoma and 29 myxoid liposarcomas (MLS) by IHC. Only 4 cases of myxofibrosarcoma showed scattered HSP90 positivity, while all cases of MLS showed positive staining for HSP90. No correlation was found between expression of HSP90 and tumour size or grade (230). Nabu *et al.* (1998) reported that high expression of HSP90 was significantly correlated with lower tumour grade and a favourable prognosis in 44 endometrial carcinoma patients (231). Zagouri *et al*. (2010) reported that there was a statistically significant decrease in HSP90 expression in infiltrative lobular carcinomas of the breast of 32 patients compared to the adjacent normal breast ducts and lobules tissue (232).

On the other hand, there is a large body of evidence supporting that HSP 90 is over-expressed in many cancers and is associated with high grade and poor survival. HSP90 has been found elevated in medulloblastoma, and there was a significant positive correlation between the expression of HSP90 and poor survival (225). In breast cancer, significant increased expression of HSP90 has been found in ductal carcinomas and associated with the higher malignant phenotype and poor survival (232, 233). Furthermore, Lim *et al*. reported that HSP90 expression showed a strong association with poor prognostic factors of hepatocellular carcinoma, vascular invasion and metastasis (234). Similar findings were observed in bladder cancer, where the HSP90 level was correlated with a high-grade tumour (235).

In this study, HSP90 was expressed in very low levels across all types of soft tissue tumours and there was no association between its expression, prognosis and other clinical variables, which may indicate that HSP90 may only play a minor role in soft tissue tumours.

4.2 Data relating to sarcoma tumour behaviour

In this study, attempts have been made to correlate the expression of different biomarkers with clinical outcome in soft tissue sarcomas. To date, most of the prognostic aspects of the behaviour of a particular patient's tumour are related back to the likelihood data derived from pooling information from groups of tumours, and there is nothing that can be used to predict the behaviour of a patient-specific tumour. However, it is the biological behaviour of neoplastic cells within tumours that are responsible for the resultant clinical outcomes.

STS are a rare heterogeneous group of uncommon malignancies of mesenchymal origin, and new improved prognostic biomarkers are required to identify patients at high-risk for metastasis. Several known prognostic factors influence survival and outcome in sarcomas, such as location, age of the patient, surgical margins, tumour size, tumour subtypes, tumour grade genetic factors, unplanned excision and metastasis. However, the relevant prognostic value of each of these factors continues to be highly controversial (236).

The most important property influencing the clinical outcome of malignant tumours is their ability to invade and metastasise. Metastasis represents a significant clinical adverse predictor, since curative treatment is not usually achievable as conventional chemotherapy is limited in its effectiveness, with around 30% of all patients showing no improvement in their survival rates following treatment (237). Significantly, some tumours with the same lineage, histological diagnosis and grade do respond to chemotherapy, whilst other, apparently identical, tumours do not. Why this should be is unknown, but clearly demonstrates that the underlying susceptibility has therapeutic and prognostic implications.

Tumour grade is the most predictive time-honoured factor of distant metastatic disease and most likely to influence a decision to initiate treatment with adjuvant therapy. Hence, the

grading process is important in its ability to indicate clinical behaviour and the type of therapeutic intervention (2). Histological grading of STS consists of a low, intermediate and high grade. The higher the grade, the stronger the correlation with the occurrence of metastases and shorter patient survival. An earlier study found that the metastatic potential of low-grade sarcomas is 5–10%, of intermediate-grade 25–30%, and of high-grade is approximately 50–60% (32). Nonetheless, it is the impact of histological type which represents the principal confounding factor in grading, such that for some sarcomas, grade is less important than subtype in defining a behaviour such as synovial sarcoma (15). For others, the usual grading criteria are not applicable, for example, clear cell sarcoma tumour, which shows aggressive behaviour no matter what its histological appearance (2). The influence of tumour subtype has been considered an important factor that confounds grading systems in sarcomas. However, this may be related to other prognostic factors such as grade, in that some tumours such as synovial sarcoma are always high grade and others like well differentiated liposarcomas are invariably low grade (15).

Another prognostic factor that has been found to influence survival and metastasis in STS is age. In this respect, two studies have reported a better outcome for patients younger than 40 years compared to those older than 40 years at diagnosis (238, 239). Furthermore, tumour size is related to metastasis and survival in sarcomas; the smaller the tumour, the more likely it is to result in a universally good outcome. Geer *et al.* (1992) (240) provided support for the theory that in tumours of the extremities, small tumour size (< 5cm) carries a universally good prognosis, irrespective of grade, depth or location, and have found that surgical excision alone is usually sufficient to treat these tumours (240). Conversely, another study by Stojadinovic *et al.* (2002) shows that a large tumour size (> 10cm) has been consistently associated with a poor prognosis due to a high rate of metastatic recurrence (39).

It is widely held that by understanding the biology of cancer cells, it might be possible to individualise therapy and to produce much clearer and biologically more pertinent markers of tumour behaviour and potential response to specific treatments (154). In the current study, attempts have been initiated to identify such criteria in this poorly understood group of tumours by investigating potential interesting molecular biomarkers that have shown to have a pivotal role in tumorigenesis and aggressiveness in many types of cancer cell.

As in all early studies of cancer biology and its relationship to cell and tumour behaviour, little is known about the role of HSPs in soft tissue sarcomas. In a proteomic study, Suehara *et al.* demonstrated that the expression of HSP27 was associated with histological grading and patient survival in Leiomyosarcoma (196). Furthermore, Bekki *et al.* published recently that HSP90 is highly expressed in undifferentiated pleomorphic sarcoma (UPS) and is considered as a poor prognostic factor (197). Data of relevance to understanding how biomarker expression should be analysed, that is, the need to take into account expression of what at first might appear to be key biomarkers in benign tumours, have also been identified.

In this study, HSP expression was not significantly correlated with survival or metastatic disease, which might be due to the size of our sample or the follow-up interval. However, our data showed that high expression of HSP was associated with a clinical indicator of poor prognosis, the largest tumour diameter and the highest grade. This is the most significant clinical prognostic indicator for high risk of metastasis in STS (2). The biomarkers also showed differences in expression across the range of tumours. In general terms, some biomarker expression varied across the benign/malignant divide and others varied with the grade. In this discussion, variation across the benign/malignant divide is seen as evidence of an association with “malignant behaviour” (i.e. in some way the expression of the gene relates to the cellular changes associated with the development of a malignant phenotype). The expression of some

biomarkers not only varied across the benign/malignant divide, but also with the grade of the tumour. As increasing grade is, in general terms, associated with a poor outlook, these biomarkers are described as being associated with aggressiveness as well as the development of a malignant phenotype.

In these analyses, the distinctions made must be seen within the context of tumour group size and data spread, and as such, cannot be taken as definitive but rather, in a study such as this, one of the first of its type in the field, as providing insight into the behaviour of sarcoma cells and whole tumours. The precise significance of these data is difficult to assess. Creating ratios of two sets of numbers generated as these have been, is open to criticism but they nevertheless, do stress potential and actual differences between different tumour types.

4.3 Discussion on variation in expression of HSPs in tumours

The reason for the discrepancy in the expression of different HSPs in various tumours in the literature is not very clear. The differences might be explained by heterogeneity of the tumour, differences in the stage of the disease, treatment modalities, immunohistochemical methods, case selection, and possible differences in embedding and processing techniques. However, the heterogeneity of expression of HSPs raises the question of what factor(s) might influence the differential expression of HSPs in different type and grade of the tumour. Indeed, could the higher expression of HSPs be related to tumour necrosis, proliferation or the presence of a specific type of molecule. In particular, it raises the question “if we could establish the signalling mechanism for HSPs production, could this be a therapeutic target”.

HSP genes are activated at the transcriptional level by HSF1 and the capability of cells to react to stress by rising their HSP levels depends on the action of binding to the promoter regions of all HSP genes to activate their immediate and massive transcription (148, 186, 241). However,

the pathways of induction of HSPs in cancer are still under intense investigation, and no clear consensus has yet emerged. Such mechanisms may include transcription and translation of HSPs due to coupling of HSF1 expression to malignant cell signal transduction.

Furthermore, research on gene expression in neoplasm has noted that the coupling between transcription and translation is often interrupted. A study of HSP gene expression in prostate carcinoma showed that HSP mRNA expression and protein expression altered considerably and that the levels of some HSPs were increased, while HSP mRNA levels were not notably changed (242). However, a popular rationale for HSP increases in cancer is the "addiction to chaperones" hypothesis. According to this hypothesis, HSP increases are fed in cancer through the proliferation of mutated proteins due to the "mutator phenotype" correlated with cancer, to increases in total levels of mRNA translation that accompany transformation and raised protein expression due to the polyploidy of many cancerous cells (243, 244). Therefore, addiction is caused by the demand for HSPs to chaperone the raised protein load that accompanies transformation and the instability of many mutant proteins. As elevated protein expression and gene mutation are thought to be key drivers of tumour progression, an increased supply of molecular chaperones is needed to sustain the addiction. This is an attractive hypothesis as it links the findings in oncology with what is speculated to be the driving reason for HSP induction during stress. Though this mechanism is difficult to test, there is significant indirect evidence to suggest its merit, coming from studies of HSP inhibitors in cancer and there is considerable evidence gained by examining inhibitors of HSP90 *in vitro* studies. For example, animal experiments and clinical trials have suggested that inhibiting HSP90 using small molecule inhibitors causes the depletion of a broad spectrum of oncogenes (presumably due to unfolding and proteolysis) and inhibition of tumour growth(183).

Finally, confronted with such complex systems of production, activation and regulation, the prospect of unravelling the involvement of HSPs in sarcoma behaviour and the outcome is daunting. A criticism of this study might be that it is not all inclusive, but faced with this level of complexity and a general paucity of previous literature about HSP expression in sarcomas, the approach adopted here has been to extrapolate from what is known, working within the limits of the available material.

4.4 Limitations of this study

This is a study based on the use of immunohistochemical identification of biomarker expression in archived formalin-fixed, wax-embedded tissue, resulting in several limitations. First, the sample size was small due to the difficulty in finding more samples to assess more types of sarcoma and the rarity of tumours arising from soft tissues. Furthermore, this study relied on only one block from each tumour, which was taken for research only. Therefore, the concern was always related to the constraints of using archived tissue biopsy. In addition, the current study was limited by the relatively small sample size of some subgroups, which may not have been sufficient to overcome the influences of the histological heterogeneity, thus limiting the conclusive power of the study.

Due to the rarity of sarcomas, the present study focused on archived tissue to obtain sufficient material for the study. This material was formalin-fixed and paraffin embedded, with limited clinical and pathological data available. These constraints limited the techniques that could be employed to examine the expression of the chosen biomarkers to IHC and *in situ* hybridisation. IHC was chosen because by studying the expression of protein, one of the unknowns in investigating HSPs, translation of mRNA into protein, was eliminated.

Another concern is the limitations of IHC. IHC is a well-established, broadly accepted technique in both clinical and experimental medical research. However, IHC also has many limitations because it is a multi-step procedure and each step is vital. According to current IHC concepts, the challenges can be divided into three main variables, pre-analytical, analytical and post-analytical variables. Pre-analytical variables of IHC include any steps in obtaining the tissue sample, tissue processing, tissue fixation, and elements of tissue handling. Analytical variables include slide thickness, selection of antibody clones and their titration, determining the detection systems and antigen retrieval procedure. Finally, post-analytical variables of IHC include interpretation and reporting of the results. Despite all current recommendations, post-analytical variables are the most frivolous part of many studies using IHC. Hence, the most important step of IHC is the final scoring system. Although there is no gold standard for HSP expression scoring system, both automated analytic systems and semi-quantitative scoring systems were used in this study to reduce the subjectivity of values scores and increase repeatability of scoring systems. However, our automated analytic method (ImageJ) did not consider cellularity of tumours which might lead to some calculation bias. Moreover, it is expensive and time-consuming, and for these reasons, it was not possible to test for more HSPs and, therefore, conduct more comprehensive research into HSPs in STS tumours.

Another limitation is relating key molecules to one another. In the context of this study, HSPs are complexly activated and inhibited, so translating from IHC-expression into enzyme activity is impossible. However, there are real constraints on examining the activity of proteins in a very small area of tissue and IHC is the best way to address research questions that might then lead to the design of different and more appropriate experiments. The constraints of examining for HSP activity are related to production (whether all the proteins present in the cytoplasm are excreted), activation (some HSPs use other proteins, including HSPs to activate themselves)

and inhibition, hence, experiments were conducted to compare expression of the various molecules.

A final limitation of this study is inadequate access to patient data and absence of comprehensive follow-up information. Without such data, survival analyses are not possible. In cancer studies with an extended follow-up, there are data on early and late recurrences as well as metastases. This study has not "closed the loop" in respect of the issue of assessing whether HSP expression might assist in patient management prospectively. As such, it would be of great importance to conduct follow-up studies to determine whether, for instance, levels of HSP expression can help to assign patients to different treatment strategies or to identify prognostic factors that may lead to the identification of novel targets for therapy.

The limitations addressed above bring into focus the urgent need to improve the existing understanding of active biological processes and underlying mechanisms of disease progression in sarcoma. Possible ways forward are discussed under the further work in section 5.2.

Chapter Five

Conclusions & Future Work

5 Conclusion & Future Work

5.1 General Conclusions

Soft tissue sarcomas have generally been studied less extensively than carcinomas, so the advances in biological understanding that have led to improved diagnosis and treatment of carcinomas have yet to be translated into soft tissue sarcoma. Although rare, they represent an important group of cancers, of which the biology is relatively unknown and therefore, they require further investigation. However, because of their rarity, obtaining these tumours to study is difficult and, hence, any work that can be done on archived tissue could be very important.

This thesis examined the expression of HSP27, HSP70 and HSP90 in adult myofibroblastic/fibroblastic tumours of extremities using immunohistochemistry. The study showed differences in expression related to markers of behaviour and aggressiveness in these tumours that might form the basis of more focused investigations in the future.

In conclusion, there was a statistically significant difference in HSP70 expression between myofibroblastic/fibroblastic sarcoma and non-metastasising tumours; HSP70 expression was higher in sarcoma than non-metastasising counterparts. Also, HSP70 was positively correlated with the grade of STSs, with elevated levels of HSP70 associated with a higher grade of STSs. Although HSP27 expression was higher in malignant than benign tumours, there was no statistically significant. Also, there was no significant positive correlation between HSP27 expression and clinicopathological features of STSs. On the other hand, HSP90 expression is relatively low in all myofibroblastic/fibroblastic tumours compared to other studied HSPs. There was no statistically significant difference in HSP90 expression between sarcoma and non-metastasising tumours. Also, there was no correlation between HSP90 expression and

clinicopathological features of STSs. Finally, there was no significant relationship found between staining of any of the studied biomarkers and the age and sex of the patients.

The findings of this study indicate that HSP70 and, to a lesser extent, HSP27 might have a role in sarcoma behaviour (as defined by the surrogates of tumour type and grade) and may provide prognostic information for clinicians in the future. Further studies evaluating HSPs in sarcomas, within the newly described model, may provide a new avenue of treatment for sarcoma patients.

5.2 Future Work

HSPs are well-established as having a complex and key role in tumour growth, invasion, metastasis, and tumour progression. The findings presented in this thesis have raised several questions in areas that would provide interesting topics for future investigations. The key purpose of further study should explain the invasion and haematogenous spread of sarcomas in order to understand why some patients survive and others die. The results of this study have raised many issues that could guide further work. Some issues reflect the limitations of the study and the material available. The main suggestions for future work consequent upon these are:

- More sarcomas and their benign counterparts need to be tested. This study has shown that not all benign tumours express biomarkers in the same way and some behave in unexpected ways that affect how expression in malignant tumours should be interpreted.
- Even though IHC is one of the best methods for examining protein expression in the core tissue of sarcomas, other technologies may need to be applied to the problem of mechanisms of invasion. The key to this would be taking the findings from this *in vivo* study and testing them in cell-based *in vitro* systems.
- More comparisons will need to be made between gene expression and outcome (e.g. not just survival but perhaps also a response to drugs etc.).

- The key to overcoming some of these problems is the development of a dedicated soft tissue tumour bank, probably at the national level, of fresh and processed tissue and other samples (e.g. blood) from patients with these rare tumours to allow studies of sufficient size to be undertaken with statistical validity.

Some areas of further study directly relate to the findings, some require expansion, some further investigation and others putting into the context of an increasing knowledge base, the most urgent being functional studies to establish the role of HSPs in the STS. The key message of this thesis, as derived from the data presented, is that the HSPs are expressed in human STS of extremities in both benign and malignant tumours, but their function, particularly the roles of HSPs, is not completely understood. Therefore, functional studies are required to establish the role of the HSPs in the STS. This raises many questions such as, does blocking the expression of HSPs increase or decrease the activation of some HSPs and other enzymes, including HSPs to activate themselves? or does increasing the expression of HSPs increase or decrease tumour progression? Moreover, further investigation into the regulation of HSPs in human soft tissue sarcoma of extremities is required; there is much more to learn about how HSPs are regulated in STS. The effects of HSP expression in human STS and the role of HSF as inducer facilitators of HSPs activity have not been examined in detail, therefore, investigating the effect of different HSPs and HSF expression in human STS of extremities will further the understanding of how the HSPs and HSFs are regulated in STS.

These questions could be answered by investigations using more advanced methodologies than those possible in this study, such as the development of "knock-down" models of tumour cells for individual HSPs in a sequence-specific manner and thus, enable the function of each HSP to be investigated. Finally, the process of carcinogenesis involves a complex array of genetic and epigenetic alterations, which contribute to cancer pathogenesis (245), eventually resulting in a unique malignant tissue. This may alter the HSP context and behaviour dramatically,

therefore, it is important to determine whether changes in the expression of the HSPs at genomic or proteomic levels is of importance to cancer prevention, diagnosis, prognosis and treatment.

Appendices

Appendix A: LBIH Biobank Patient Consent Form

LBIH Biobank Consent Form

Collection and Storage of Samples for Research

I have read and understood the LBIH Biobank Patient Information Sheet (Version _____) on the above research project and have been given a copy to keep. I have had the opportunity to ask questions about the project and understand the benefits and risks of donating. ☐

I agree to give samples of tissue, blood, or any other appropriate biological sample from: ☐

- □ my current procedure or event
- □ any past procedure or event
- □ all future procedures or events

I agree that the LBIH Biobank will become custodian of this tissue, blood, and/or biological sample for use in regulated research projects. ☐

I agree that tissue bank staff can collect and store information on an ongoing basis for updating the tissue bank database from my health records for research that uses my samples and that this information may be viewed by regulatory authorities. I understand that some information may be held at different sources such as other NHS Trusts and Disease Registries. I understand that information about me will be treated confidentially and stored securely. ☐

I understand that any samples or information given to research groups will be anonymised and my identity will be protected. ☐

I agree that it may be appropriate for genetic assessment of the samples to be carried out to determine whether genetic makeup has any influence on my condition. ☐

I understand that some of these projects may be carried out by commercial organisations. I agree to samples being sent to research groups based in the UK, within the European Union (EU), and outside of the EU. ☐

I understand that I will not financially benefit if research using my samples leads to new treatments or medical tests. ☐

I agree to the use of the samples in research involving non-human models. ☐

I understand how the samples will be collected, that giving a sample is voluntary, and that I am free to with draw my approval for use of the samples at any time without giving reason and without my care or legal rights being affected. ☐

Version 1 LBIH Biobank Consent Form 7/1/2015
White copy to LBIH, Pink copy in patient's health record, Blue copy to patient
LBIH Biobank, 3rd Floor The Apex Building, University of Liverpool, L7 8TX

Appendix B: Basic steps of an immunohistochemistry protocol

fixation/processing/embedding

↓

section cutting/microtomy

↓

dewaxing sections and taking to water

↓

antigen retrieval

↓

peroxidase block

↓

primary antibody

↓

secondary antibody

↓

tertiary layer

↓

chromogen

↓

counterstain

↓

dehydrate, clear and mount sections

Appendix C: Immunohistochemistry Protocol - Using PT-Linker & DAKO Autostainer

This protocol is adapted from Liverpool Ocular Oncology Research Group (LOORG)

1. Turn on PT-Link and press "Run" to begin heating to 65°C (approx 20 minutes).
2. Label slides with antibody, dilution and date, using a pencil.
3. When 65°C is reached, place slides in staining rack. The PT Link will heat to 96°C, hold for 20 minutes, and return to 65°C (approx 1 hour 10 minutes)
4. Wash with Envision Flex-Wash buffer and place on DAKO.
5. Add 100µl Envision-Flex block and incubate at room temperature (RT) for **5 minutes**.
6. Bring reagents required for staining run to room temperature and then programmed all required agents.
7. Then, load the reagent vials according to the Reagent Layout Map, ensuring that enough reagent is in the vials to complete the programmed run.
8. Dilute primary antibody to appropriate concentration using antibody diluent (Envision-Flex). Add 100µl primary antibody, cover and incubate at room temperature for **30 minutes**.
9. Add 100µl of appropriate linker (i.e. mouse for mouse primary antibody) and incubate at room temperature for **15 minutes**.
10. Add 100µl of HRP and incubate at room temperature for **20 minutes**.
11. Make up DAB (make fresh each time, using empty autostainer vial), 1 drop of DAB per 1ml of DAB substrate buffer. Add 100µl DAB solution to each slide then incubate at room temperature for **20 minutes**. Place any waste DAB in the hazardous waste container attached to the autostainer then rinse tube well with a small volume of Milton.
12. Dip in distilled water.
13. Transfer slides to horse-shaped rack. **30 seconds - 1 minute** in haematoxylin. This time will vary depending on your haematoxylin batch!
14. Place rack into a bath of running tap water until the water runs clear.
15. Dip in and immediately remove rack from the acid alcohol.
16. Place rack back into the bath of running tap water until the slides are no longer pink.
17. Dip in Scott's tap water/ammonia water for **30 seconds**.
18. Place rack back into the bath of running tap water for **1 minute**.
19. Dehydrate through the ethanol series in the fume hood. This includes 5x IMS (Industrial Methylated Spirits) and 2x xylene. Agitate for **10 seconds** at each stage, vigorously.
20. Add one drop of mountant (Aquatex™) to a coverslip and apply to slide removing all air bubbles.

Appendix D: Macro Code for Image J analysis

```
//Image selection code
title = getTitle(); // get the title of the current image
imageOfInterest = title+"-(Colour_2)";  // the deconvolved imageName model
//Colour[1]:
  Red1=0.6500286;
  Green1=0.704;031
  Blue1=0.2860126;
//Colour[2]:
  Red2=0.26814753;
  Green2=0.57031375;
  Blue2=0.77642715;
//Colour[3]:
  Red3=0.7110272;
  Green3=0.42318153;
  Blue3=0.5615672;
run("Colour Deconvolution", "vectors=[User values] show [r1]=Red1 [g1]=Green1 [b1]=Blue1
[r2]=Red2 [g2]=Green2 [b2]=Blue2 [r3]=Red3 [g3]=Green3 [b3]=Blue3");
selectImage(imageOfInterest);
// select the imageOf Interest
// change brown image to b/w
run("8-bit");
run("Invert");
run("Subtract...", "value=50");
setAutoThreshold("Default dark");
run("Threshold...");
setOption("BlackBackground", false);
setThreshold(80,196);
run("Measure");
```

Each heat Shock protein staining had different set Threshold.

References

1. Jain S, Xu R, Prieto VG, Lee P. Molecular classification of soft tissue sarcomas and its clinical applications. International Journal of Clinical and Experimental Pathology. 2010;3(4):416-28.

2. Thway K. Pathology of soft tissue sarcomas. Clinical Oncology. 2009;21(9):695-705.

3. Fletcher CDM, Bridge JA, Hogendoorn P, Mertens F. WHO Classification of Tumours of Soft Tissue and Bone. Fourth Edition: International Agency for Research on Cancer; 2013.

4. Matthew F, Nicola D, Jackie C, Gill L, Rob G. Bone and soft tissue sarcomas UK incidence and survival: 1996 to 2010. National Cancer Intelligence Network 2013.

5. Dennis N, Francis M, Lawrence G. Soft tissue sarcoma incidence and survival: tumours diagnosed in England between 1985 and 2009. National Cancer Intelligence Network. 2012.

6. Goldberg BR. Soft tissue sarcoma: An overview. Orthopaedic Nursing. 2007;26(1):4-11.

7. Oniscu A, Salter D. Pathology of soft tissue tumours. Surgery (Oxford). 2016;34(9):436-9.

8. Fletcher C. Soft tissue tumours: Epidemiology, clinical features, histopathological typing and grading. Pathology and Genetics of Tumours of Soft Tissue and Bone. 2002.

9. Brady MS, Gaynor JJ, Brennan MF. Radiation-associated sarcoma of bone and soft tissue. Archives of Surgery. 1992;127(12):1379-85.

10. Fletcher CD. The evolving classification of soft tissue tumours–an update based on the new 2013 WHO classification. Histopathology. 2014;64(1):2-11.

11. Cormier JN, Pollock RE. Soft tissue sarcomas. CA: A Cancer Journal for Clinicians. 2004;54(2):94-109.

12. Dangoor A, Seddon B, Gerrand C, Grimer R, Whelan J, Judson I. UK guidelines for the management of soft tissue sarcomas. Clinical Sarcoma Research. 2016;6(1):20.

13. Cancer NCCf. Suspected cancer: recognition and referral. NICE Guideline. 2015.

14. Grimer R, Briggs T. Earlier diagnosis of bone and soft-tissue tumours. The Bone & Joint Journal. 2010;92(11):1489-92.

15. Somerhausen NdSA, Fletcher C. Soft-tissue sarcomas: an update. European Journal of Surgical Oncology (EJSO). 1999;25(2):215-20.

16. Hoeber I, Spillane AJ, Fisher C, Thomas JM. Accuracy of biopsy techniques for limb and limb girdle soft tissue tumors. Annals of Surgical Oncology. 2001;8(1):80-7.

17. Welker JA, Henshaw RM, Jelinek J, Shmookler BM, Malawer MM. The percutaneous needle biopsy is safe and recommended in the diagnosis of musculoskeletal masses. Cancer. 2000;89(12):2677-86.

18. Mankin HJ, Hornicek FJ. Diagnosis, classification, and management of soft tissue sarcomas. Cancer Control. 2005;12(1):5-21.

19. Grimer RJ. Size matters for sarcomas! The Annals of The Royal College of Surgeons of England. 2006;88(6):519-24.

20. Coindre J. Immunohistochemistry in the diagnosis of soft tissue tumours. Histopathology. 2003;43(1):1-16.

21. Chan J, editor Advances in immunohistochemistry: impact on surgical pathology practice. Seminars in Diagnostic Pathology; 2000.

22. Fisher C. Synovial sarcoma. Annals of Diagnostic Pathology. 1998;2(6):401-21.

23. Miettinen M, Lasota J. KIT (CD117): a review on expression in normal and neoplastic tissues, and mutations and their clinicopathologic correlation. Applied Immunohistochemistry & Molecular Morphology. 2005;13(3):205-20.

24. Miettinen MM. Diagnostic soft tissue pathology: Churchill Livingstone; 2003.

25. Antonescu C. The role of genetic testing in soft tissue sarcoma. Histopathology. 2006;48(1):13-21.

26. Kawai A, Woodruff J, Healey JH, Brennan MF, Antonescu CR, Ladanyi M. SYT–SSX gene fusion as a determinant of morphology and prognosis in synovial sarcoma. New England Journal of Medicine. 1998;338(3):153-60.

27. Tschoep K, Kohlmann A, Schlemmer M, Haferlach T, Issels R-D. Gene expression profiling in sarcomas. Critical Reviews in Oncology/Hematology. 2007;63(2):111-24.

28. Sorensen PH, Lynch JC, Qualman SJ, Tirabosco R, Lim JF, Maurer HM, et al. PAX3-FKHR and PAX7-FKHR gene fusions are prognostic indicators in alveolar rhabdomyosarcoma: a report from the children's oncology group. Journal of Clinical Oncology. 2002;20(11):2672-9.

29. Pfeifer J, Hill D, O'sullivan M, Dehner L. Diagnostic gold standard for soft tissue tumours: morphology or molecular genetics? Histopathology. 2000;37(6):485-500.

30. Hieken TJ, Gupta TD. Mutant p53 expression: a marker of diminished survival in well-differentiated soft tissue sarcoma. Clinical Cancer Research. 1996;2(8):1391-5.

31. Tukiainen E, Böhling T, Huuhtanen R. Soft tissue sarcoma of the trunk and extremities. Scandinavian Journal of Surgery. 2003;92(4):257-63.

32. Trojani M, Contesso G, Coindre J, Rouesse J, Bui N, De Mascarel A, et al. Soft-tissue sarcomas of adults; study of pathological prognostic variables and definition of a histopathological grading system. International Journal of Cancer. 1984;33(1):37-42.

33. Le Q-TX, Fu KK, Kroll S, Fitts L, Massullo V, Ferrell L, et al. Prognostic factors in adult soft-tissue sarcomas of the head and neck. International Journal of Radiation Oncology. 1997;37(5):975-84.

34. Pisters P, Leung D, Woodruff J, Shi W, Brennan MF. Analysis of prognostic factors in 1,041 patients with localized soft tissue sarcomas of the extremities. Journal of Clinical Oncology. 1996;14(5):1679-89.

35. Guillou L, Benhattar J, Bonichon F, Gallagher G, Terrier P, Stauffer E, et al. Histologic grade, but not SYT-SSX fusion type, is an important prognostic factor in patients with synovial sarcoma: a multicenter, retrospective analysis. Journal of Clinical Oncology. 2004;22(20):4040-50.

36. Coindre J-M. Grading of soft tissue sarcomas: review and update. Archives of Pathology & Laboratory Medicine. 2006;130(10):1448-53.

37. Hogendoorn PC, Collin F, Daugaard S, Dei Tos AP, Fisher C, Schneider U, et al. Changing concepts in the pathological basis of soft tissue and bone sarcoma treatment. European Journal of Cancer. 2004;40(11):1644-54.

38. Kotilingam D, Lev DC, Lazar AJ, Pollock RE. Staging soft tissue sarcoma: evolution and change. CA: A Cancer Journal for Clinicians. 2006;56(5):282-91.

39. Stojadinovic A, Leung DH, Allen P, Lewis JJ, Jaques DP, Brennan MF. Primary adult soft tissue sarcoma: time-dependent influence of prognostic variables. Journal of Clinical Oncology. 2002;20(21):4344-52.

40. Ramanathan RC, A'Hern R, Fisher C, Thomas JM. Modified staging system for extremity soft tissue sarcomas. Annals of Surgical Oncology. 1999;6(1):57-69.

41. Edge S. Byrd DR, editor; Compton CC, et al., editors. AJCC Cancer Staging Manual. 7th. New York, NY: Springer; 2010.

42. Fong Y, Coit DG, Woodruff JM, Brennan MF. Lymph node metastasis from soft tissue sarcoma in adults. Analysis of data from a prospective database of 1772 sarcoma patients. Annals of Surgery. 1993;217(1):72.

43. Chao AH, Mayerson JL, Chandawarkar R, Scharschmidt TJ. Surgical management of soft tissue sarcomas: Extremity sarcomas. Journal of Surgical Oncology. 2015;111(5):540-5.

44. Weitz Jr, Antonescu CR, Brennan MF. Localized extremity soft tissue sarcoma: improved knowledge with unchanged survival over time. Journal of Clinical Oncology. 2003;21(14):2719-25.

45. Herbert SH, Corn BW, Solin LJ, Lanciano RM, Schultz DJ, McKenna WG, et al. Limb-preserving treatment for soft tissue sarcomas of the extremities. The significance of surgical margins. Cancer. 1993;72(4):1230-8.

46. Mc Kee MD, Liu DF, Brooks JJ, Gibbs JF, Driscoll DL, Kraybill WG. The prognostic significance of margin width for extremity and trunk sarcoma. Journal of Surgical Oncology. 2004;85(2):68-76.

47. Yang JC, Chang AE, Baker AR, Sindelar WF, Danforth DN, Topalian SL, et al. Randomized prospective study of the benefit of adjuvant radiation therapy in the treatment of soft tissue sarcomas of the extremity. Journal of Clinical Oncology. 1998;16(1):197-203.

48. O'Sullivan B, Davis AM, Turcotte R, Bell R, Catton C, Chabot P, et al. Preoperative versus postoperative radiotherapy in soft-tissue sarcoma of the limbs: a randomised trial. The Lancet. 2002;359(9325):2235-41.

49. Davis A, O'sullivan B, Bell R, Turcotte R, Catton C, Wunder J, et al. Function and health status outcomes in a randomized trial comparing preoperative and postoperative radiotherapy in extremity soft tissue sarcoma. Journal of Clinical Oncology. 2002;20(22):4472-7.

50. Strander H, Turesson I, Cavallin-Ståhl E. A systematic overview of radiation therapy effects in soft tissue sarcomas. Acta Oncologica. 2003;42(5-6):516-31.

51. Collaboration SM-a. Adjuvant chemotherapy for localised resectable soft tissue sarcoma in adults. The Cochrane Database of Systematic Reviews. 2000(4):CD001419.

52. Frustaci S, Gherlinzoni F, De Paoli A, Bonetti M, Azzarelli A, Comandone A, et al. Adjuvant chemotherapy for adult soft tissue sarcomas of the extremities and girdles: results of the Italian randomized cooperative trial. Journal of Clinical Oncology. 2001;19(5):1238-47.

53. Scurr M, Judson I. Neoadjuvant and adjuvant therapy for extremity soft tissue sarcomas. Hematology/Oncology Clinics of North America. 2005;19(3):489-500.

54. Pezzi CM, Pollock RE, Evans HL, Lorigan JG, Pezzi TA, Benjamin RS, et al. Preoperative chemotherapy for soft-tissue sarcomas of the extremities. Annals of Surgery. 1990;211(4):476.

55. van Roggen G, Fletcher M. Myxoid tumours of soft tissue. Histopathology. 1999;35(4):291-312.

56. Nishio J, Iwasaki H, Nabeshima K, Naito M. Cytogenetics and molecular genetics of myxoid soft-tissue sarcomas. Genetics Research International. 2011;2011.

57. Baheti AD, Tirumani SH, Rosenthal MH, Howard SA, Shinagare AB, Ramaiya NH, et al. Myxoid soft-tissue neoplasms: comprehensive update of the taxonomy and MRI features. American Journal of Roentgenology. 2015;204(2):374-85.

58. Kaya M, Wada T, Nagoya S, Sasaki M, Matsumura T, Yamaguchi T, et al. MRI and histological evaluation of the infiltrative growth pattern of myxofibrosarcoma. Skeletal Radiology. 2008;37(12):1085-90.

59. Mentzel T, Calonje E, Wadden C, Camplejohn RS, Beham A, Smith MA, et al. Myxofibrosarcoma: clinicopathologic analysis of 75 cases with emphasis on the low-grade variant. The American Journal of Surgical Pathology. 1996;20(4):391-405.

60. Sanfilippo R, Miceli R, Grosso F, Fiore M, Puma E, Pennacchioli E, et al. Myxofibrosarcoma: prognostic factors and survival in a series of patients treated at a single institution. Annals of Surgical Oncology. 2011;18(3):720-5.

61. Willems SM, Debiec-Rychter M, Szuhai K, Hogendoorn PC, Sciot R. Local recurrence of myxofibrosarcoma is associated with increase in tumour grade and cytogenetic aberrations, suggesting a multistep tumour progression model. Modern Pathology. 2006;19(3):407-16.

62. Lefkowitz RA, Landa J, Hwang S, Zabor EC, Moskowitz CS, Agaram NP, et al. Myxofibrosarcoma: prevalence and diagnostic value of the "tail sign" on magnetic resonance imaging. Skeletal Radiology. 2013;42(6):809-18.

63. Ghazala C, Agni N, Ragbir M, Dildey P, Lee D, Rankin K, et al. Myxofibrosarcoma of the extremity and trunk. The Bone & Joint Journal. 2016;98(12):1682-8.

64. Meis-Kindblom JM, Kindblom L-G. Acral myxoinflammatory fibroblastic sarcoma: a low-grade tumor of the hands and feet. The American Journal of Surgical Pathology. 1998;22(8):911-24.

65. Michal M. Inflammatory myxoid tumor of the soft parts with bizarre giant cells. Pathology-Research and Practice. 1998;194(8):529-33.

66. Montgomery EA, Devaney KO, Giordano TJ, Weiss SW. Inflammatory myxohyaline tumor of distal extremities with virocyte or

Reed-Sternberg-like cells: a distinctive lesion with features simulating inflammatory conditions, Hodgkin's disease, and various sarcomas. Modern Pathology. 1998;11(4):384-91.

67. Narváez JA, Martinez S, Dodd LG, Brigman BE. Acral myxoinflammatory fibroblastic sarcomas: MRI findings in four cases. American Journal of Roentgenology. 2007;188(5):1302-5.

68. Laskin WB, Fetsch JF, Miettinen M. Myxoinflammatory fibroblastic sarcoma: a clinicopathologic analysis of 104 cases, with emphasis on predictors of outcome. The American Journal of Surgical Pathology. 2014;38(1):1-12.

69. Evans HL. Low-grade fibromyxoid sarcoma: a report of two metastasizing neoplasms having a deceptively benign appearance. American Journal of Clinical Pathology. 1987;88(5):615-9.

70. Evans HL. Low-grade fibromyxoid sarcoma: a clinicopathologic study of 33 cases with long-term follow-up. The American Journal of Surgical Pathology. 2011;35(10):1450-62.

71. Hwang S, Kelliher E, Hameed M. Imaging features of low-grade fibromyxoid sarcoma (Evans tumor). Skeletal Radiology. 2012;41(10):1263-72.

72. Charron P, Smith J. Intramuscular myxomas: a clinicopathologic study with emphasis on surgical management. The American Surgeon. 2004;70(12):1073-7.

73. Hashimoto H, Tsuneyoshi M, Daimaru Y, Enjoji M, Shinohara N. Intramuscular myxoma: a clinicopathologic, immunohistochemical, and electron microscopic study. Cancer. 1986;58(3):740-7.

74. Munksgaard PS, Salkus G, Iyer VV, Fisker RV. Mazabraud's syndrome: case report and literature review. Acta Radiologica Short Reports. 2013;2(4):2047981613492532.

75. Bancroft LW, Kransdorf MJ, Menke DM, O'Connor MI, Foster WC. Intramuscular myxoma: characteristic MR imaging features. American Journal of Roentgenology. 2002;178(5):1255-9.

76. Eyden B, Banerjee SS, Shenjere P, Fisher C. The myofibroblast and its tumours. Journal of Clinical Pathology. 2009;62(3):236-49.

77. Fisher C. Myofibroblastic malignancies. Advances in Anatomic Pathology. 2004;11(4):190-201.

78. Fletcher CD. Myofibroblastic tumours: an update. Verhandlungen der Deutschen Gesellschaft fur Pathologie. 1998;82:75-82.

79. Agaimy A, Wünsch PH, Schroeder J, Gaumann A, Dietmaier W, Hartmann A, et al. Low-grade abdominopelvic sarcoma with myofibroblastic features (low-grade myofibroblastic sarcoma): clinicopathological, immunohistochemical, molecular genetic and ultrastructural study of two cases with literature review. Journal of Clinical Pathology. 2008;61(3):301-6.

80. Montgomery E, Goldblum JR, Fisher C. Myofibrosarcoma: a clinicopathologic study. The American Journal of Surgical Pathology. 2001;25(2):219-28.

81. Cai Z-G, Pan C-C, Yu D-H, Feng Z-Z, Ma L, Zhao Y, et al. Myofibroblastic sarcomas: a clinicopathologic analysis of 15 cases and review of literature. International Journal of Clinical and Experimental Pathology. 2016;9(2):1568-77.

82. Montgomery E, Fisher C. Myofibroblastic differentiation in malignant fibrous histiocytoma (pleomorphic myofibrosarcoma): a clinicopathological study. Histopathology. 2001;38(6):499-509.

83. Escobar C, Munker R, Thomas J, Li B, Burton G. Update on desmoid tumors. Annals of Oncology. 2012;23(3):562-9.

84. Kotiligam D, Lazar A, Pollock RE, Lev D. Desmoid tumor: a disease opportune for molecular insights. Histology and Histopathology. 2008;23(1):117-26.

85. El-Haddad M, El-Sebaie M, Ahmad R, Khalil E, Shahin M, Pant R, et al. Treatment of aggressive fibromatosis: the experience of a single institution. Clinical Oncology. 2009;21(10):775-80.

86. Ehnman M, Larsson O. Microenvironmental targets in sarcoma. Frontiers in Oncology. 2015;5.

87. Tomlinson J, Barsky SH, Nelson S, Singer S, Pezeshki B, Lee MC, et al. Different patterns of angiogenesis in sarcomas and carcinomas. Clinical Cancer Research. 1999;5(11):3516-22.

88. Vaday GG, Lider O. Extracellular matrix moieties, cytokines, and enzymes: dynamic effects on immune cell behavior and inflammation. Journal of Leukocyte Biology. 2000;67(2):149-59.

89. Aumailley M, Gayraud B. Structure and biological activity of the extracellular matrix. Journal of Molecular Medicine. 1998;76(3):253-65.

90. Lukashev ME, Werb Z. ECM signalling: orchestrating cell behaviour and misbehaviour. Trends in Cell Biology. 1998;8(11):437-41.

91. Joyce JA, Fearon DT. T cell exclusion, immune privilege, and the tumor microenvironment. Science. 2015;348(6230):74-80.

92. Folkman J. Tumor angiogenesis: therapeutic implications. New England Journal of Medicine. 1971;285(21):1182-6.

93. Engellau J, Bendahl P-O, Persson A, Domanski HA, Åkerman M, Gustafson P, et al. Improved prognostication in soft tissue sarcoma: independent information from vascular invasion, necrosis, growth pattern, and immunostaining using whole-tumor sections and tissue microarrays. Human Pathology. 2005;36(9):994-1002.

94. West C, Brown N, Mangham D, Grimer R, Reed M. Microvessel density does not predict outcome in high grade soft tissue sarcoma. European Journal of Surgical Oncology. 2005;31(10):1198-205.

95. Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. Molecular Cell. 2010;40(2):294-309.

96. Bertout JA, Patel SA, Simon MC. The impact of O2 availability on human cancer. Nature Reviews Cancer. 2008;8(12):967-75.

97. Nordsmark M, Alsner J, Keller J, Nielsen OS, Jensen O, Horsman M, et al. Hypoxia in human soft tissue sarcomas: adverse impact on survival and no association with p53 mutations. British Journal of Cancer. 2001;84(8):1070.

98. Brizel DM, Scully SP, Harrelson JM, Layfield LJ, Bean JM, Prosnitz LR, et al. Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. Cancer Research. 1996;56(5):941-3.

99. Kaelin WG, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Molecular Cell. 2008;30(4):393-402.

100. Keith B, Johnson RS, Simon MC. HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression. Nature Reviews Cancer. 2012;12(1):9-22.

101. Semenza GL. Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. Trends in Pharmacological Sciences. 2012;33(4):207-14.

102. Heikkilä M, Pasanen A, Kivirikko KI, Myllyharju J. Roles of the human hypoxia-inducible factor (HIF)-3α variants in the hypoxia response. Cellular and Molecular Life Sciences. 2011;68(23):3885-901.

103. Ciocca DR, Arrigo AP, Calderwood SK. Heat shock proteins and heat shock factor 1 in carcinogenesis and tumor development: an update. Archives of Toxicology. 2013;87(1):19-48.

104. Baird NA, Turnbull DW, Johnson EA. Induction of the heat shock pathway during hypoxia requires regulation of heat shock factor by

hypoxia-inducible factor-1. Journal of Biological Chemistry. 2006;281(50):38675-81.

105. Young JC, Agashe VR, Siegers K, Hartl FU. Pathways of chaperone-mediated protein folding in the cytosol. Nature Reviews Molecular Cell Biology. 2004;5(10):781-91.

106. Schlesinger MJ, Ashburner M, Tissières A. Heat shock, from bacteria to man: Cold Spring Harbor Laboratory; 1982.

107. Ritossa F. A new puffing pattern induced by temperature shock and DNP in Drosophila. Cellular and Molecular Life Sciences. 1962;18(12):571-3.

108. Kelley PM, Schlesinger MJ. The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. Cell. 1978;15(4):1277-86.

109. Lemaux PG, Herendeen SL, Bloch PL, Neidhardt FC. Transient rates of synthesis of individual polypeptides in E. coli following temperature shifts. Cell. 1978;13(3):427-34.

110. McAlister L, Finkelstein DB. Heat shock proteins and thermal resistance in yeast. Biochemical and Biophysical Research Communications. 1980;93(3):819-24.

111. Barnett T, Altschuler M, McDaniel CN, Mascarenhas JP. Heat shock induced proteins in plant cells. Genesis. 1979;1(4):331-40.

112. Hartl FU. Molecular chaperones in cellular protein folding. Nature. 1996;381(6583):571.

113. Lindquist S, Craig E. The heat-shock proteins. Annual Review of Genetics. 1988;22(1):631-77.

114. Powers ET, Morimoto RI, Dillin A, Kelly JW, Balch WE. Biological and chemical approaches to diseases of proteostasis deficiency. Annual Review of Biochemistry. 2009;78:959-91.

115. Ellis RJ, Hemmingsen SM. Molecular chaperones: proteins essential for the biogenesis of some macromolecular structures. Trends in Biochemical Sciences. 1989;14(8):339-42.

116. Ellis RJ, Van der Vies SM. Molecular chaperones. Annual Review of Biochemistry. 1991;60(1):321-47.

117. Spiess C, Meyer AS, Reissmann S, Frydman J. Mechanism of the eukaryotic chaperonin: protein folding in the chamber of secrets. Trends in Cell Biology. 2004;14(11):598-604.

118. Barginear M, Van Poznak C, Rosen N, Modi S, Hudis C, Budman D. The heat shock protein 90 chaperone complex: an evolving therapeutic target. Current Cancer Drug Targets. 2008;8(6):522-35.

119. Jego G, Hazoumé A, Seigneuric R, Garrido C. Targeting heat shock proteins in cancer. Cancer Letters. 2013;332(2):275-85.

120. Citri A, Harari D, Shochat G, Ramakrishnan P, Gan J, Eisenstein M, et al. Hsp90 recognizes a common surface on client kinases. Journal of Biological Chemistry. 2006.

121. Kopeček P, Altmannová K, Weigl E. Stress proteins: nomenclature, division and functions. Biomedical Papers. 2001;145(2):39-47.

122. Hendrick JP, Hartl F-U. Molecular chaperone functions of heat-shock proteins. Annual Review of Biochemistry. 1993;62(1):349-84.

123. Fu X, Liu C, Liu Y, Feng X, Gu L, Chen X, et al. Small heat shock protein Hsp16. 3 modulates its chaperone activity by adjusting the rate of oligomeric dissociation. Biochemical and Biophysical Research Communications. 2003;310(2):412-20.

124. Becker J, Craig EA. Heat-shock proteins as molecular chaperones. European Journal of Biochemistry. 1994;219(1-2):11-23.

125. Tóth ME, Gombos I, Sántha M. Heat shock proteins and their role in human diseases. Acta Biologica Szegediensis. 2015;59(1):121-41.

126. Khalil AA, Kabapy NF, Deraz SF, Smith C. Heat shock proteins in oncology: diagnostic biomarkers or therapeutic targets? Biochimica et Biophysica Acta (BBA)-Reviews on Cancer. 2011;1816(2):89-104.

127. Kampinga HH, Hageman J, Vos MJ, Kubota H, Tanguay RM, Bruford EA, et al. Guidelines for the nomenclature of the human heat shock proteins. Cell Stress and Chaperones. 2009;14(1):105-11.

128. Didelot C, Lanneau D, Brunet M, Joly A-L, Thonel AD, Chiosis G, et al. Anti-cancer therapeutic approaches based on intracellular and extracellular heat shock proteins. Current Medicinal Chemistry. 2007;14(27):2839-47.

129. Schmitt E, Gehrmann M, Brunet M, Multhoff G, Garrido C. Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. Journal of Leukocyte Biology. 2007;81(1):15-27.

130. Kuhn DJ, Zeger EL, Orlowski RZ. Proteasome inhibitors and modulators of heat shock protein function. Update on Cancer Therapeutics. 2006;1(2):91-116.

131. Carra S, Alberti S, Arrigo PA, Benesch JL, Benjamin IJ, Boelens W, et al. The growing world of small heat shock proteins: from structure to functions. Cell Stress and Chaperones. 2017:1-11.

132. de Jong WW, Caspers G-J, Leunissen JA. Genealogy of the α-crystallin—small heat-shock protein superfamily. International Journal of Biological Macromolecules. 1998;22(3):151-62.

133. Peter E, Candido M. The small heat shock proteins of the nematode Caenorhabditis elegans: structure, regulation and biology. Small Stress Proteins: Springer; 2002. p. 61-78.

134. Kim KK, Kim R, Kim S-H. Crystal structure of a small heat-shock protein. Nature. 1998;394(6693):595-9.

135. Golenhofen N, Der Perng M, Quinlan RA, Drenckhahn D. Comparison of the small heat shock proteins αB-crystallin, MKBP,

HSP25, HSP20, and cvHSP in heart and skeletal muscle. Histochemistry and Cell Biology. 2000;122(5):415-25.

136. Sun Y, MacRae TH. The small heat shock proteins and their role in human disease. The FEBS Journal. 2005;272(11):2613-27.

137. Nakamoto H, Vigh L. The small heat shock proteins and their clients. Cellular and Molecular Life Sciences. 2007;64(3):294-306.

138. Garrido C, Schmitt E, Candé C, Vahsen N, Parcellier A, Kroemer G. HSP27 and HSP70: potentially oncogenic apoptosis inhibitors. Cell Cycle. 2003;2(6):578-83.

139. Kappé G, Franck E, Verschuure P, Boelens WC, Leunissen JA, de Jong WW. The human genome encodes 10 α-crystallin–related small heat shock proteins: HspB1–10. Cell Stress & Chaperones. 2003;8(1):53-61.

140. Qiu X-B, Shao Y-M, Miao S, Wang L. The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. Cellular and Molecular Life Sciences. 2006;63(22):2560-70.

141. Jäättelä M. Heat shock proteins as cellular lifeguards. Annals of Medicine. 1999;31(4):261-71.

142. Goloudina AR, Demidov ON, Garrido C. Inhibition of HSP70: a challenging anti-cancer strategy. Cancer Letters. 2012;325(2):117-24.

143. Csermely P, Schnaider T, So C, Prohászka Z, Nardai G. The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacology and Therapeutics. 1998;79(2):129-68.

144. Subbarao Sreedhar A, Kalmár É, Csermely P, Shen Y-F. Hsp90 isoforms: functions, expression and clinical importance. FEBS Letters. 2004;562(1-3):11-5.

145. Dragovic Z, Broadley SA, Shomura Y, Bracher A, Hartl FU. Molecular chaperones of the Hsp110 family act as nucleotide exchange factors of Hsp70s. The EMBO Journal. 2006;25(11):2519-28.

146. Xu Y-M, Huang D-Y, Chiu J-F, Lau AT. Post-translational modification of human heat shock factors and their functions: a recent update by proteomic approach. Journal of Proteome Research. 2012;11(5):2625-34.

147. Fujimoto M, Hayashida N, Katoh T, Oshima K, Shinkawa T, Prakasam R, et al. A novel mouse HSF3 has the potential to activate nonclassical heat-shock genes during heat shock. Molecular Biology of the Cell. 2010;21(1):106-16.

148. Morimoto RI. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes and Development. 1998;12(24):3788-96.

149. Björk JK, Sistonen L. Regulation of the members of the mammalian heat shock factor family. The FEBS Journal. 2010;277(20):4126-39.

150. Shi Y, Mosser DD, Morimoto RI. Molecularchaperones as HSF1-specific transcriptional repressors. Genes and Development. 1998;12(5):654-66.

151. Ciocca DR, Calderwood SK. Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. Cell Stress & Chaperones. 2005;10(2):86-103.

152. Lianos GD, Alexiou GA, Mangano A, Mangano A, Rausei S, Boni L, et al. The role of heat shock proteins in cancer. Cancer Letters. 2015;360(2):114-8.

153. The Hallmarks of Cancer. 2000.

154. Calderwood SK, Gong J. Heat shock proteins promote cancer: it's a protection racket. Trends in Biochemical Sciences. 2016;41(4):311-23.

155. Momeny M, Saunus JM, Marturana F, Reed AEM, Black D, Sala G, et al. Heregulin-HER3-HER2 signaling promotes matrix metalloproteinase-dependent blood-brain-barrier transendothelial migration of human breast cancer cell lines. Oncotarget. 2015;6(6):3932.

156. Kamal A, Thao L, Sensintaffar J, Zhang L, Boehm MF, Fritz LC, et al. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature. 2003;425(6956):407-10.

157. Agorreta J, Hu J, Liu D, Delia D, Turley H, Ferguson DJ, et al. TRAP1 regulates proliferation, mitochondrial function, and has prognostic significance in NSCLC. Molecular Cancer Research. 2014;12(5):660-9.

158. Gong J, Weng D, Eguchi T, Murshid A, Sherman M, Song B, et al. Targeting the hsp70 gene delays mammary tumor initiation and inhibits tumor cell metastasis. Oncogene. 2015;34(43):5460-71.

159. Campisi J. Aging, cellular senescence, and cancer. Annual Review of Physiology. 2013;75:685-705.

160. Hao X-D, Yang Y, Song X, Zhao X-K, Wang L-D, He J-D, et al. Correlation of telomere length shortening with TP53 somatic mutations, polymorphisms and allelic loss in breast tumors and esophageal cancer. Oncology Reports. 2013;29(1):226-36.

161. Jaskelioff M, Song W, Xia J, Liu C, Kramer J, Koido S, et al. Telomerase deficiency and telomere dysfunction inhibit mammary tumors induced by polyomavirus middle T oncogene. Oncogene. 2009;28(48):4225-36.

162. Toogun OA, DeZwaan DC, Freeman BC. The hsp90 molecular chaperone modulates multiple telomerase activities. Molecular and Cellular Biology. 2008;28(1):457-67.

163. Pinhasi-Kimhi O, Michalovitz D, Ben-Zeev A, Oren M. Specific interaction between the p53 cellular tumour antigen and major heat shock proteins. Nature. 1986;320(6058):182-5.

164. Wiech M, Olszewski MB, Tracz-Gaszewska Z, Wawrzynow B, Zylicz M, Zylicz A. Molecular mechanism of mutant p53 stabilization: the role of HSP70 and MDM2. PLoS One. 2012;7(12):e51426.

165. O'Callaghan-Sunol C, Gabai VL, Sherman MY. Hsp27 modulates p53 signaling and suppresses cellular senescence. Cancer Research. 2007;67(24):11779-88.

166. Gabai VL, Yaglom JA, Waldman T, Sherman MY. Heat shock protein Hsp72 controls oncogene-induced senescence pathways in cancer cells. Molecular and Cellular Biology. 2009;29(2):559-69.

167. Yaglom JA, Gabai VL, Sherman MY. High levels of heat shock protein Hsp72 in cancer cells suppress default senescence pathways. Cancer Research. 2007;67(5):2373-81.

168. Joseph JV, Conroy S, Pavlov K, Sontakke P, Tomar T, Eggens-Meijer E, et al. Hypoxia enhances migration and invasion in glioblastoma by promoting a mesenchymal shift mediated by the HIF1α–ZEB1 axis. Cancer Letters. 2015;359(1):107-16.

169. Bohonowych J, Gopal U, Isaacs J. Hsp90 as a gatekeeper of tumor angiogenesis: clinical promise and potential pitfalls. Journal of Oncology. 2010;2010.

170. Isaacs JS, Xu W, Neckers L. Heat shock protein 90 as a molecular target for cancer therapeutics. Cancer Cell. 2003;3(3):213-7.

171. Thuringer D, Jego G, Wettstein G, Terrier O, Cronier L, Yousfi N, et al. Extracellular HSP27 mediates angiogenesis through Toll-like receptor 3. The FASEB Journal. 2013;27(10):4169-83.

172. Tsutsumi S, Beebe K, Neckers L. Impact of heat-shock protein 90 on cancer metastasis. Future Oncology. 2009;5(5):679-88.

173. Miyajima N, Tsutsumi S, Sourbier C, Beebe K, Mollapour M, Rivas C, et al. The HSP90 inhibitor ganetespib synergizes with the MET kinase inhibitor crizotinib in both crizotinib-sensitive and-resistant MET-driven tumor models. Cancer Research. 2013;73(23):7022-33.

174. Pavan S, Musiani D, Torchiaro E, Migliardi G, Gai M, Cunto F, et al. HSP27 is required for invasion and metastasis triggered by hepatocyte growth factor. International Journal of Cancer. 2014;134(6):1289-99.

175. Takayama S, Reed JC, Homma S. Heat-shock proteins as regulators of apoptosis. Oncogene. 2003;22(56):9041.

176. Wang X, Chen M, Zhou J, Zhang X. HSP27, 70 and 90, anti-apoptotic proteins, in clinical cancer therapy (Review). International Journal of Oncology. 2014;45(1):18-30.

177. Lanneau D, de Thonel A, Maurel S, Didelot C, Garrido C. Apoptosis versus cell differentiation: role of heat shock proteins HSP90, HSP70 and HSP27. Prion. 2007;1(1):53-60.

178. Chauhan D, Li G, Hideshima T, Podar K, Mitsiades C, Mitsiades N, et al. Hsp27 inhibits release of mitochondrial protein Smac in multiple myeloma cells and confers dexamethasone resistance. Blood. 2003;102(9):3379-86.

179. Paul C, Simon S, Gibert B, Virot S, Manero F, Arrigo A-P. Dynamic processes that reflect anti-apoptotic strategies set up by HspB1 (Hsp27). Experimental Cell Research. 2010;316(9):1535-52.

180. Beere HM, Wolf BB, Cain K, Mosser DD, Mahboubi A, Kuwana T, et al. Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nature Cell Bioiology. 2000;2(8):469-75.

181. Garrido C, Brunet M, Didelot C, Zermati Y, Schmitt E, Kroemer G. Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. Cell Cycle. 2006;5(22):2592-601.

182. Zhang R, Luo D, Miao R, Bai L, Ge Q, Sessa WC, et al. Hsp90–Akt phosphorylates ASK1 and inhibits ASK1-mediated apoptosis. Oncogene. 2005;24(24):3954-63.

183. Trepel J, Mollapour M, Giaccone G, Neckers L. Targeting the dynamic HSP90 complex in cancer. Nature Reviews Cancer. 2010;10(8):537.

184. Sugiyama Y, Suzuki A, Kishikawa M, Akutsu R, Hirose T, Waye MM, et al. Muscle develops a specific form of small heat shock protein complex composed of MKBP/HSPB2 and HSPB3 during myogenic differentiation. Journal of Biological Chemistry. 2000;275(2):1095-104.

185. Simioni MB, De Thonel A, Hammann A, Joly A, Bossis G, Fourmaux E, et al. Heat shock protein 27 is involved in SUMO-2/3 modification of heat shock factor 1 and thereby modulates the transcription factor activity. Oncogene. 2009;28(37):3332-44.

186. Calderwood SK. Heat shock proteins in breast cancer progression–A suitable case for treatment? International Journal of Hyperthermia. 2010;26(7):681-5.

187. Seigneuric R, Mjahed H, Gobbo J, Joly A-L, Berthenet K, Shirley S, et al. Heat shock proteins as danger signals for cancer detection. Frontiers in Oncology. 2011;1:37.

188. Yoshihara T, Kadota Y, Yoshimura Y, Tatano Y, Takeuchi N, Okitsu H, et al. Proteomic alteration in gastic adenocarcinomas from Japanese patients. Molecular Cancer. 2006;5(1):75.

189. Lee I-N, Chen C-H, Sheu J-C, Lee H-S, Huang G-T, Yu C-Y, et al. Identification of human hepatocellular carcinoma-related biomarkers by two-dimensional difference gel electrophoresis and mass spectrometry. Journal of Proteome Research. 2005;4(6):2062-9.

190. Jazii FR, Najafi Z, Malekzadeh R, Conrads TP, Ziaee AA, Abnet C, et al. Identification of squamous cell carcinoma associated proteins by

proteomics and loss of beta tropomyosin expression in esophageal cancer. World Journal of Gastroenterology. 2006;12(44):7104.

191. Myung J-K, Afjehi-Sadat L, Felizardo-Cabatic M, Slavc I, Lubec G. Expressional patterns of chaperones in ten human tumor cell lines. Proteome Science. 2004;2(1):8.

192. Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. Nature Reviews Cancer. 2005;5(10):761-72.

193. Calderwood SK, Khaleque MA, Sawyer DB, Ciocca DR. Heat shock proteins in cancer: chaperones of tumorigenesis. Trends in Biochemical Sciences. 2006;31(3):164-72.

194. Isomoto H, Oka M, Yano Y, Kanazawa Y, Soda H, Terada R, et al. Expression of heat shock protein (Hsp) 70 and Hsp 40 in gastric cancer. Cancer Letters. 2003;198(2):219-28.

195. Uozaki H, Ishida T, Kakiuchi C, Horiuchi H, Gotoh T, Iijima T, et al. Expression of heat shock proteins in osteosarcoma and its relationship to prognosis. Pathology-Research and Practice. 2000;196(10):665-73.

196. Suehara Y, Kondo T, Fujii K, Hasegawa T, Kawai A, Seki K, et al. Proteomic signatures corresponding to histological classification and grading of soft-tissue sarcomas. Proteomics. 2006;6(15):4402-9.

197. Bekki H, Kohashi K, Maekawa A, Yamada Y, Yamamoto H, Harimaya K, et al. Elevated expression of HSP90 and the antitumor effect of an HSP90 inhibitor via inactivation of the Akt/mTOR pathway in undifferentiated pleomorphic sarcoma. BMC Cancer. 2015;15(1):804.

198. Taylor C, Burns J. The demonstration of plasma cells and other immunoglobulin-containing cells in formalin-fixed, paraffin-embedded tissues using peroxidase-labelled antibody. Journal of Clinical Pathology. 1974;27(1):14-20.

199. Schacht V, Kern JS. Basics of immunohistochemistry. The Journal of Investigative Dermatology. 2015;135(3):e30.

200. Patel HR, Arya M, Shergill I. Basic science techniques in clinical practice: Springer Science & Business Media; 2008.

201. Buchwalow IB, Böcker W. Immunohistochemistry: basics and methods: Springer Science & Business Media; 2010.

202. Wang A, Liu X, Sheng S, Ye H, Peng T, Shi F, et al. Dysregulation of heat shock protein 27 expression in oral tongue squamous cell carcinoma. BMC Cancer. 2009;9:167.

203. Ye H, Wang A, Lee BS, Yu T, Sheng S, Peng T, et al. Proteomic based identification of manganese superoxide dismutase 2 (SOD2) as a metastasis marker for oral squamous cell carcinoma. Cancer Genomics Proteomics. 2008;5(2):85-94.

204. Haines JD, Vidaurre OG, Zhang F, Riffo-Campos AL, Castillo J, Casanova B, et al. Multiple sclerosis patient-derived CSF induces transcriptional changes in proliferating oligodendrocyte progenitors. Multiple Sclerosis Journal. 2015;21(13):1655-69.

205. Khan KN, Kitajima M, Hiraki K, Fujishita A, Nakashima M, Masuzaki H. Involvement of hepatocyte growth factor-induced epithelial-mesenchymal transition in human adenomyosis. Biology of Reproduction. 2015;92(2):35.

206. Cheng W, Ainiwaer A, Xiao L, Cao Q, Wu G, Yang Y, et al. Role of the novel HSP90 inhibitor AUY922 in hepatocellular carcinoma: Potential for therapy. Molecular Medicine Reports. 2015;12(2):2451-6.

207. Hartig SM. Basic image analysis and manipulation in ImageJ. Current Protocols in Molecular Biology. 2013:14.5. 1-.5. 2.

208. Remmele W, Stegner H. A proposal for the standardization of the immunoreactive score (Irs) for the immunohistochemical demonstration of estrogen-receptors (Er-Ica) in breast-cancer. Pathologe. 1987;8(3):138-40.

209. Grzegrzółka J, Kurnol K, Piotrów P, Puła B, Kobierzycki C, Piotrowska A, et al. Hsp-27 expression in invasive ductal breast carcinoma. Folia Histochemica et Cytobiologica. 2012;50(4):527-33.

210. Bauer K, Nitsche U, Slotta-Huspenina J, Drecoll E, von Weyhern CH, Rosenberg R, et al. High HSP27 and HSP70 expression levels are independent adverse prognostic factors in primary resected colon cancer. Cellular Oncology. 2012;35(3):197-205.

211. Drecoll E, Nitsche U, Bauer K, Berezowska S, Slotta-Huspenina J, Rosenberg R, et al. Expression analysis of heat shock protein 90 (HSP90) and Her2 in colon carcinoma. International Journal of Colorectal Disease. 2014;29(6):663-71.

212. Cohen J. Statistical power analysis for the behavioral sciences . Hilsdale. NJ: Lawrence Earlbaum Associates. 1988;2.

213. Wataba K, Saito T, Fukunaka K, Ashihara K, Nishimura M, Kudo R. Over-expression of heat shock proteins in carcinogenic endometrium. International Journal of Cancer. 2001;91(4):448-56.

214. Miyake H, Muramaki M, Kurahashi T, Takenaka A, Fujisawa M, editors. Expression of potential molecular markers in prostate cancer: correlation with clinicopathological outcomes in patients undergoing radical prostatectomy. Urologic Oncology: Seminars and Original Investigations; 2010: Elsevier.

215. Rui Z, Jian-Guo J, Yuan-Peng T, Hai P, Bing-Gen R. Use of serological proteomic methods to find biomarkers associated with breast cancer. Proteomics. 2003;3(4):433-9.

216. King KL, Li AFY, Chau GY, Chi CW, Wu CW, Huang CL, et al. Prognostic significance of heat shock protein-27 expression in hepatocellular carcinoma and its relation to histologic grading and survival. Cancer. 2000;88(11):2464-70.

217. Tweedle EM, Khattak I, Ang CW, Nedjadi T, Jenkins R, Park BK, et al. Low molecular weight heat shock protein HSP27 is a prognostic

indicator in rectal cancer but not colon cancer. Gut. 2010;59(11):1501-10.

218. Assimakopoulou M, Varakis J. AP-1 and heat shock protein 27 expression in human astrocytomas. Journal of Cancer Research and Clinical Oncology. 2001;127(12):727-32.

219. Moon A, Bacchini P, Bertoni F, Olvi LG, Santini-Arawo E, Kim YW, et al. Expression of heat shock proteins in osteosarcomas. Pathology. 2010;42(5):421-5.

220. Li S, Zhang W, Fan J, Lai Y, Che G. Clinicopathological and prognostic significance of heat shock protein 27 (HSP27) expression in non-small cell lung cancer: a systematic review and meta-analysis. SpringerPlus. 2016;5(1):1165.

221. Muzio LL, Leonardi R, Mariggio M, Mignogna M, Rubini C, Vinella A, et al. HSP 27 as possible prognostic factor in patients with oral squamous cell carcinoma. Histology and Histopathology. 2004;19(1):119-28.

222. Têtu B, Lacasse B, Bouchard H-L, Lagacé R, Huot J, Landry J. Prognostic influence of HSP-27 expression in malignant fibrous histiocytoma: a clinicopathological and immunohistochemical study. Cancer Research. 1992;52(8):2325-8.

223. Matos JM, Witzmann FA, Cummings OW, Schmidt CM. A pilot study of proteomic profiles of human hepatocellular carcinoma in the United States. Journal of Surgical Research. 2009;155(2):237-43.

224. Syrigos KN, Harrington KJ, Karayiannakis AJ, Sekara E, Chatziyianni E, Syrigou EI, et al. Clinical significance of heat shock protein-70 expression in bladder cancer. Urology. 2003;61(3):677-80.

225. Alexiou GA, Vartholomatos G, Stefanaki K, Patereli A, Dova L, Karamoutsios A, et al. Expression of heat shock proteins in medulloblastoma: Laboratory investigation. Journal of Neurosurgery: Pediatrics. 2013;12(5):452-7.

226. Santarosa M, Favaro D, Quaia M, Galligioni E. Expression of heat shock protein 72 in renal cell carcinoma: possible role and prognostic implications in cancer patients. European Journal of Cancer. 1997;33(6):873-7.

227. Ramp U, Mahotka C, Heikaus S, Shibata T, Grimm M, Willers R, et al. Expression of heat shock protein 70 in renal cell carcinoma and its relation to tumor progression and prognosis. Histology and Histopathology. 2007;22(10-12):1099-108.

228. Nakajima M, Kuwano H, Miyazaki T, Masuda N, Kato H. Significant correlation between expression of heat shock proteins 27, 70 and lymphocyte infiltration in esophageal squamous cell carcinoma. Cancer Letters. 2002;178(1):99-106.

229. Trieb K, Kohlbeck R, Lang S, Klinger H, Blahovec H, Kotz R. Heat shock protein 72 expression in chondrosarcoma correlates with differentiation. Journal of Cancer Research and Clinical Oncology. 2000;126(11):667-70.

230. Wang T, Goodman MA, McGough RL, Weiss KR, Rao UN. Immunohistochemical analysis of expressions of RB1, CDK4, HSP90, cPLA2G4A, and CHMP2B is helpful in distinction between myxofibrosarcoma and myxoid liposarcoma. International Journal of Surgical Pathology. 2014;22(7):589-99.

231. Nanbu K, Konishi I, Komatsu T, Mandai M, Yamamoto S, Kuroda H, et al. Expression of heat shock proteins HSP70 and HSP90 in endometrial carcinomas: correlation with clinicopathology, sex steroid receptor status, and p53 protein expression. Cancer. 1996;77(2):330-8.

232. Zagouri F, Sergentanis T, Nonni A, Papadimitriou C, Pazaiti A, Michalopoulos NV, et al. Decreased Hsp90 expression in infiltrative lobular carcinoma: an immunohistochemical study. BMC cancer. 2010;10(1):409.

233. Pick E, Kluger Y, Giltnane JM, Moeder C, Camp RL, Rimm DL, et al. High HSP90 expression is associated with decreased survival in breast cancer. Cancer Research. 2007;67(7):2932-7.

234. Lim SO, Park SG, Yoo J-H, Park YM, Kim H-J, Jang K-T, et al. Expression of heat shock proteins (HSP27, HSP60, HSP70, HSP90, GRP78, GRP94) in hepatitis B virus-related hepatocellular carcinomas and dysplastic nodules. World Journal of Gastroenterology. 2005;11(14):2072-9.

235. Lebret T, Watson RWG, Molinié V, O'Neill A, Gabriel C, Fitzpatrick JM, et al. Heat shock proteins HSP27, HSP60, HSP70, and HSP90. Cancer. 2003;98(5):970-7.

236. Khatri VP, Goodnight JE. Extremity soft tissue sarcoma: controversial management issues. Surgical Oncology. 2005;14(1):1-9.

237. Van Glabbeke M, Van Oosterom A, Oosterhuis J, Mouridsen H, Crowther D, Somers R, et al. Prognostic factors for the outcome of chemotherapy in advanced soft tissue sarcoma: an analysis of 2,185 patients treated with anthracycline-containing first-line regimens—a European Organization for Research and Treatment of Cancer Soft Tissue and Bone Sarcoma Group Study. Journal of Clinical Oncology. 1999;17(1):150-.

238. Van Geel AN, Pastorino U, Jauch KW, Judson IR, Van Coevorden F, Buesa JM, et al. Surgical treatment of lung metastases: The European Organization for Research and Treatment of Cancer-Soft Tissue and Bone Sarcoma Group study of 255 patients. Cancer. 1996;77(4):675-82.

239. Robinson M, Sheppard M, Moskovic E, Fisher C. Lung metastasectomy in patients with soft tissue sarcoma. The British Journal of Radiology. 1994;67(794):129-35.

240. Geer RJ, Woodruff J, Casper ES, Brennan MF. Management of small soft-tissue sarcoma of the extremity in adults. Archives of Surgery. 1992;127(11):1285-9.

241. Wu C. Heat shock transcription factors: structure and regulation. Annual Review of Cell and Developmental Biology. 1995;11(1):441-69.

242. Tang D, Khaleque MA, Jones EL, Theriault JR, Li C, Hung Wong W, et al. Expression of heat shock proteins and heat shock protein messenger ribonucleic acid in human prostate carcinoma in vitro and in tumors in vivo. Cell Stress & Chaperones. 2005;10(1):46-58.

243. Fishel R, Lescoe MK, Rao M, Copeland NG, Jenkins NA, Garber J, et al. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. Cell. 1993;75(5):1027-38.

244. Dai C, Whitesell L, Rogers AB, Lindquist S. Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. Cell. 2007;130(6):1005-18.

245. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.