SERUM PROTEOMIC ANALYSES OF PATIENTS WITH BONE TUMOURS USING GEL-, LECTIN- AND MASS SPECTROMETRY-BASED STRATEGIES

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FACULTY OF MEDICINE
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ABSTRACT

Bone tumour refers to a neoplastic growth which originates from various types of skeletal tissues such as bone, surrounding soft tissues, muscles and ligaments. Bone tumours can be divided into two major groups, benign bone tumour and malignant bone tumour. Due to difficulty and invasive nature of the diagnosis, it is imperative to find new methods or technologies that could be used for early detection of these tumours in order to determine the best course of action for the patient. Currently, there are no known tumour marker (which has been approved by the U.S. Food and Drug Administration) for bone tumours, unlike breast cancer, ovarian cancer and prostate cancer. This study was directed to investigate if there were differences in proteome profiles of patients with various types of bone tumours compared to normal healthy individuals. It also aimed to identify proteins that were aberrantly expressed which may have potential to serve as biomarkers that could differentiate and detect various types of bone tumours. Analysis of silver stained 2DE protein expression profiles using image analysis software demonstrated different altered levels of several high abundance proteins in patients with osteosarcoma (OS), Ewing sarcoma (ES), chondrosarcoma (CS), pleomorphic sarcoma (PS) and giant cell tumour (GCT), relative to age-matched non-tumour healthy individuals. From the 2DE analysis, level serum amyloid A (SAA) demonstrated the highest altered abundance and was found to be significantly increased in patients with OS and PS. Apart from the silver stained 2DE analysis, pooled serum samples from patients with these tumours and healthy controls were also subjected to 2DE Western blotting analysis using enzyme-conjugated champedak galactose binding (CGB)- and champedak mannose binding (CMB)-lectins. Application of CGB- and CMB-lectins as probes enables the detection of O-glycosylated and N-glycosylated proteins, respectively. Three groups of common sarcoma, OS, CS and PS, were selected for further analysis to validate the increased level of SAA. Although the elevation of

SAA was not statistically significant in patients with CS, unlike OS and PS, it is a malignant tumour with a low metastatic rate and not very aggressive. A comparison can therefore be made between different types of sarcoma correlating to their aggressiveness. A similar trend of altered abundances was also observed when the levels of SAA in the subjects were determined using Western blot, enzyme-linked immunosorbent assay (ELISA) and SWATH™-MS (only in OS). Absolute quantification using multiple reaction monitoring (MRM) further demonstrated that the increased abundance of SAA in patients with OS, CS and PS was mainly attributed to isoform serum amyloid A1 (SAA1). In view of the different degrees of tumour malignancy in OS, CS and PS, this data suggests their apparent correlation with the levels of SAA in the patients and this offers potential to be used as biomarkers to aid early diagnosis of bone tumours, as well as in determining the risk of the malignant tumour.

ABSTRAK

Tumor tulang merujuk kepada pertumbuhan neoplastik yang berasal dari pelbagai jenis tisu seperti tulang, tisu lembut, otot dan ligamen. Tumor tulang boleh dibahagikan kepada dua kumpulan utama iaitu tumor tulang benain dan tumor tulang malignan. Oleh kerana cara-cara diagnosis yang invasif dan sukar, adalah amat penting untuk mencari kaedah baru atau teknologi yang boleh digunakan untuk pengesanan awal tumor tulang untuk menentukan tindakan yang terbaik untuk merawat pesakit. Pada masa ini, tidak seperti kanser payudara, ovari dan prostat, belum ada penanda tumor (yang telah diluluskan oleh U.S. Food and Drug Administration) untuk sebarang jenis tumor tulang. Dalam kajian ini, kami ingin menyiasat jika terdapat sebarang perbezaan dalam profil proteome pesakit dengan pelbagai jenis tumor tulang yang berbeza berbanding individu yang sihat. Kami berhasrat untuk mengenal pasti protein yang diekspres secara aberan yang mungkin mempunyai potensi sebagai penanda biologi yang boleh membezakan dan mengesan pelbagai jenis tumor tulang. Analisis ekspresi protein secara elektroforesis dua-dimensi (2DE) menggunakan teknik pewarnaan perak dan perisian analisis imej menunjukkan tahap yang berbeza-beza pada beberapa protein berkelimpahan tinggi dalam pesakit dengan osteosarkoma (OS), Ewing sarkoma (EW) , chondrosarkoma (CS), pleomorphic sarkoma (PS) dan tumor sel gergasi (GCT) , berbanding dengan subjek kawalan yang merupakan individu-individu bebas tumor dan sihat yang mepunyai umur yang berpadanan. Daripada analisis 2DE, aras serum amiloid A (SAA) menunjukkan peningkatan ketara yang signifikan dalam pesakit OS dan PS. Selain daripada analisis 2DE dengan teknik pewarnaan perak, sampel serum dikumpulkan daripada pesakit dengan pelbagai tumor tulang ini dan daripada subjek kawalan turut dianalisa menggunakan teknik pemedapan Western menggunakan lektin pengikat galaktosa cempedak (CGB) dan lektin pengikat manosa cempedak (CMB) yang dikonjugat bersama enzim. Penggunaan lektin CGB dan lektin CMB sebagai prob

membolehkan pengesanan protein berglikosilat-O dan protein berglikosilat-N. Tiga kumpulan sarkoma iaitu OS, CS dan OS, telah dipilih untuk analisis selanjutnya untuk mengesahkan peningkatan aras SAA. Walaupun peningkatan SAA tidak ketara secara statistik pada pesakit dengan CS, tidak seperti OS dan PS, ia adalah tumor malignan dengan kadar metastatik yang rendah dan tidak begitu agresif. Oleh itu perbandingan boleh dibuat di antara pelbagai jenis kanser tulang yang untuk menghubungkait aras SAA dengan tahap keagresifan mereka. Trend yang sama juga dapat diperhatikan apabila aras SAA didalam subjek diukur menggunakan kaedah pemedapan Western, asai imun terjerap enzime berangkai (ELISA) dan juga analisis SWATH™-MS (hanya dalam pesakit OS). Seterusnya, kuantifikasi mutlak menggunakan analisis pemantauan tindakbalas berganda (MRM) menunjukkan kenaikan aras isofom serum amiloid A1 (SAA1) adalah penyebab utama kenaikan SAA di dalam pesakit OS, CS dan PS. Memandangkan tumor OS, CS dan PS mempunyai darjah malignan yang berbeza-beza, data yang diperolehi menunjukan ianya mempunyai korelasi yang jelas dengan aras SAA di dalam pesakit dan ini menunjukkan potensi SAA untuk digunakan sebagai penanda biologi untuk membantu diagnosis awal tumor tulang, dan juga untuk menentukan risiko malignan tumor tersebut.

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Wan Izlina Wan Ibrahim
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LIST OF SYMBOLS AND ABBREVIATIONS

1DE	:	one-dimensional electrophoresis
2DE	:	two-dimensional electrophoresis
2D-DIGE	:	two-dimensional difference gel electrophoresis
AAT	:	alpha1-antitrypsin
ABG	:	alpha-1B-glycoprotein
ACN	:	acetonitrile
AHS	:	alpha-2-HS-glycoprotein
AIF1	:	allograft inflammatory factor 1
AP	:	alkaline phosphatase
APOA1	:	apolipoprotein A-I
APS	:	ammonium persulphate
ATP	:	adenosine triphosphate
B2G1	:	beta-2-glycoprotein 1
C3f	:	complement C3 (fragment)
CGB	:	champedak galactose binding
CMB	:	champedak mannose binding
CRYAB	:	crystallin α/β
CS	:	chondrosarcoma
CYC-1	:	cytochrome C1
DAB	:	3,3'-diaminobenzidine
DIA	:	data-independent acquisition
DNA	:	deoxyribonucleic acid
DTT	:	dithiothreitol
DVS	:	divinyl sulfone

EDTA-Na_2.2H_2O	:	ethylenediaminetetraacetic acid, disodium dihydrate
ELISA	:	enzyme-linked immunosorbent assay
ESI	:	electrospray ionization
EW	:	Ewing sarcoma
EZR	:	ezrin
FC	:	fold change
FDA	:	Food and Drug Administration
FDR	:	false discovery rate
g	:	gravity (relative centrifugal force)
GalNAc	:	N-acetyl-galactosamine
GCT	:	giant cell tumour
GPX1	:	glutathione peroxidase 1
H_2O	:	water
H_2O_2	:	hydrogen peroxide
HAP	:	haptoglobin
HCl	:	hydrocloric acid
HRP	:	horseradish peroxidase
HPX	:	hemopexin
HSP27	:	heat shock protein 27
IDA	:	information dependent acquisition
IEF	:	isoelectric focusing
IAA	:	iodoacetamide
Ig	:	immunoglobulin
IHC	:	immunhistochemistry
iTRAQ	:	Isobaric Tags for Relative and Absolute Quantitation
kDA	:	kilodalton

KH_2HPO_4	:	potassium dihydrogen phosphate
KNG	:	kininogen-1
LC	:	liquid chromatography
LRG	:	leucine-rich alpha-2-glycoprotein
mA	:	milliampere
MALDI-TOF	:	Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight
$MgCl_2$	:	magnesium chloride
M_r	:	theoretical mass
MRM	:	multiple reaction monitoring
mRNA	:	messenger RNA
MS	:	mass spectrometry
MS/MS	:	tandem mass spectrometry
m/z	:	mass to charge ratio
NaCl	:	sodium chloride
NaN_3	:	sodium azide
Na_2HPO_4	:	sodium hydrogen phosphate
OS	:	osteosarcoma
p	:	probability
pI	:	isoelectric focusing point
PRDX2	:	peroxiredoxin 2
PRX	:	thioredoxin peroxidase
PS	:	pleomorphic sarcoma
Q-TOF	:	quadrupole-time-of-flight
RNA	:	ribonucleic acid
RT-PCR	:	reverse transcription-polymerase chain reaction
SAA	:	serum amyloid A protein

SD	:	standard deviation
SDS	:	sodium dodecyl sulphate
SDS-PAGE	:	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SELDI-TOF	:	surface-enhanced laser desorption/ionisation time-of flight
SRM	:	single reaction monitoring
STR	:	serotransferrin
TEMED	:	N,N,N',N'-Tetramethyl-ethylenediamine
TTR	:	transthyretin
TUBA1C	:	tubulin-α1c
UBE2N	:	ubiquitin E2N
V	:	volt
VTDB	:	vitamin D binding protein
W	:	watt
WHO	:	World Health Organization
XIC	:	extracted ion chromatogram
ZAG	:	zinc-alpha-2-glycoprotein
$ZnCl_2$	:	zinc chloride
ZNF1133	:	zinc finger protein 133

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CHAPTER 1: INTRODUCTION

In general, there are increasing incidences of tumours, both benign and malignant cases, recorded each year. Late diagnosis, which is often the case, drastically reduces survival rate among patients. Early stages of cancer may not show any symptoms, and most of the times, patients only seek for treatment when the cancer has manifested the symptoms, which means it is already at an advanced stage and possibly spread to another organ. Therefore, timely and proper treatment cannot be delivered to the patients to escalate chances combat the disease.

Ideally, it is best if the tumour can be detected before the appearance of the symptoms, although in reality, this is almost impossible as patients will usually seek for advice and treatment when they are unwell. A screening process using a tumour marker, or biomarker, may assist in early detection of the tumour and therefore, it can improve the survival rate of the patient by timely intervention and deciding an accurate course of treatment for the patient. Biomarkers are substances released by tumour cells and sometimes, healthy cells, in response to the presence of benign as well as malignant tumours.

Bone tumour is one of the group of tumours that if treated early and correctly, can increase the survival probability. Important prognostic factors for bone tumour include the presence of metastasis, location of the tumour, size and also grade of the tumour. As there are various types of bone tumours, as well as soft tissue tumours, differentiating the type of tumour are also an important factor in managing the disease. Biomarkers that can detect and also differentiate various types of bone tumours are also useful not only for the screening process, but also as an indicator of response to therapy and medication.

Proteins, deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and metabolites are typical biomarkers that can be found in tissues and various bodily fluids such as blood, urine, saliva and cerebrospinal fluid. Proteomics are one of the commonly used methods in discovering protein biomarker candidates and they are generally suitable for these types of samples. Various proteomics platforms, including gel-based methods such as one- and two-dimensional electrophoresis (1DE and 2DE) and modified two-dimensional difference gel electrophoresis (2D-DIGE) as well as non-gel based approach such as liquid chromatography-mass spectrometry (LC-MS) have been used in biomarker research.

One of the major difficulties in studying the proteome of a biological sample is the complexity of the sample itself. Studying a specific fraction or a sub-proteome may facilitate the overall approach in discovering the biomarker for a particular disease. For example, the study of patient's serum glycoproteome fraction may enable a further understanding of the dynamic changes in the glycosylation profile in the patient when compared to a healthy control. Therefore in this study, champedak galactose binding (CGB) and champedak mannose binding (CMB) lectins were used to detect and analyse serum O-linked and N-linked glycoproteins profiles in the samples from patients with various types of bone tumours.

It is interesting to note that so far there is no specific tumour biomarker for bone tumour that has been approved for clinical use. The present study was performed to investigate if there are differences in the proteome profiles of patients with various types of bone tumours, *i.e.* osteosarcoma, (OS), Ewing sarcoma (ES), chondrosarcoma (CS), pleomorphic sarcoma (PS) and giant cell tumour (GCT), compared to normal healthy individuals. Although there are a number of publications mining the potential biomarkers for OS, EW and GCT (Wan-Ibrahim *et al.*, 2015), there has been no report

on any proteomic study on biomarkers for CS and PS. Identifying proteins that are aberrantly expressed may present their potential to serve as biomarkers that could differentiate and detect various types of bone tumours. It is hoped that this study can greatly facilitate in the discovery of candidate biomarkers for bone tumour diagnosis at an earlier stage.

Taking that into consideration, the specific objectives of the present study are:

1. to develop and analyse the high abundance silver stained and lectins stained 2DE profiles of patients with various types of bone tumours compared to normal healthy individuals within the comparable age range
2. to identify the aberrantly expressed proteins detected from silver stained 2DE experiment using mass spectrometry (MS) and further validate protein which may have potential as a biomarker using Western blotting and ELISA
3. to analyse protein profile of selected sample using non-gel based approach quantitative proteomics (SWATH™-MS)
4. to validate and do an absolute quantification of the aberrantly expressed protein discovered from 2DE silver stained analysis and SWATH™-MS using a mass spectrometry-based quantification approach (multiple reaction monitoring or MRM)

CHAPTER 2: LITERATURE REVIEW

2.1 Bone tumour

Bone tumour refers to a neoplastic growth which originates from various types of tissues such as bone, surrounding soft tissues, muscles and ligaments. It can be categorised as primary or secondary bone tumour, based on the origin of the tumour. Primary bone tumour starts from the bone itself while secondary bone tumour is caused by tumour that originates from other parts of the body such as lung, prostate or breast that has metastasised and spread to the bone (Giaccia & Erler, 2008). Bone tumours can be divided into two major groups, benign bone tumour and malignant bone tumour. Most of the time, benign bone tumours do not spread to the other tissues or organ, unlike the malignant bone tumour which is cancerous.

There are several different types of bone tumours and they are categorised based on the area of the bone, the affected surrounding tissues or the type of cells that form the tumour. Benign tumours, which are more common than malignant tumours, include bone tumours such as osteochondroma, enchondroma, osteoid osteoma, osteoblastoma, chondroblastoma and also chondromyxoid fibroma and GCT, as well as benign soft tissue tumours such as lipoblastoma and neurofibroma. Malignant bone tumours, on the other hand, or often termed "sarcomas", could be divided into two major groups depending on the origin of the tumour, *i.e.* bone sarcoma and soft tissue sarcoma. There are over 50 types of known sarcomas. Bone sarcomas include OS, EW, CS and fibrosarcoma (Robbins & Kumar, 1987). Some tumours may also develop from soft tissues such as fat, muscle, nerves or blood vessels. Rhabdomyosarcomas, neurofibrosarcoma and angiosarcomas are a few examples of soft tissue sarcomas. Most sarcomas have a predilection for a specific age group and they are quite rare, making up only 0.001% of all newly diagnosed cancers (Malawer *et al.*, 1998).

In this study, we focused on five types of tumours *i.e.* OS, EW, CS, PS and GCT.

2.1.1 Osteosarcoma (OS)

OS, also called osteogenic sarcoma, is the most common type of primary bone cancer (Gebhardt *et al.*, 2008). This tumour arises from mesenchymal cells and characterised by osteoblastic differentiation of the neoplastic cells. Majority of this neoplasm occurs in children and adult between the ages of 20 to 25 years. Only a small group is afflicted by the disease at the age of 50 years or more (Robbins & Kumar, 1987).

Possible causes or initiating factors of OS can be categorised into several groups: chemical agents, viruses, radiation and other miscellaneous reasons (Fuchs & Pritchard, 2002). The aetiology of OS is essentially unknown. Bassin and co-workers (2006) found that there was an association between fluoride exposure in drinking water during childhood and the incidence of OS. However, their finding is consistent only among the males. Several data which were published as early as 1972 had already suggested that viruses such as human OS virus (Reilly *et al.*, 1972) and Moloney murine sarcoma virus (Czitrom *et al.*, 1976; Olson & Capen, 1977) could induce OS.

2.1.2 Ewing sarcoma (ES)

ES, also known as primitive neuroectodermal tumour (PNET), is an extremely malignant tumour which arises within the marrow cavity of the bone. It consists of small round cell sarcomas showing a varying degree of neuroectodermal differentiation. Common locations for this tumour are the pelvis as well as the ribs. It usually arises in the diaphysis or metaphyseal-diaphyseal portion of long bones. This type of bone cancer usually affects the adolescent, from 10 to 15 years of age and almost 80% of the cases occur in patients younger than 20 years old (Bertoni *et al.*, 2002). With a slight predominance towards male, it can disseminate to other bones, soft tissues and also lung

(Bertoni *et al.*, 2002; Choi *et al.*, 2014). Worldwide, it is the second most common paediatric malignant bone tumour, after OS (Choi *et al.*, 2014; Kikuta *et al.*, 2009).

The aetiology of EW is still unknown but Cavazzana and co-workers (1987) suggested that there is a close relationship between EW and peripheral neural tumours. Other than genetic and environmental factors, the occurrence of EW was also hypothesised to be associated with cytomegalovirus, through *in utero* viral infection during the pregnancy (Cope, 2000). The current survival rate of ES is about 41% and the prognosis can be improved by monitoring the stage, anatomic location and the tumour size (Bertoni *et al.*, 2002).

2.1.3 Chondrosarcoma (CS)

CS is a malignant tumour with pure hyaline cartilage differentiation and the third most common primary malignancy of bone (Bertoni *et al.*, 2002). It has age-specific incidence rates, where it usually develops in adult up to the age of 75 with a slight male preponderance (Bertoni *et al.*, 2002). However, gender predilection does not exist for high grade CS (Gripp *et al.*, 1998). CS affects the long bone of the extremities, pelvis and ribs, manifested by local swelling and/or pain. However, the occurrence of this malignant tumour is very rare in the craniofacial bones and spine (Bertoni *et al.*, 2002).

The symptoms of this cancer are usually of long duration, which can take several months or years. The real causes of this tumour remain unknown but ionising radiation has been shown to induce CS in canine, following intraoperative radiotherapy (Barnes *et al.*, 1990). The prognosis is good for low grade volume of tumour and the five year survival rate for this tumour is 89% for patients with grade 1 tumour (Bertoni *et al.*, 2002). Local recurrences, as well as distant metastases, are seldom for low grade tumour. About 10% of the recurrence cases have an increase in the degree of malignancy (Bertoni *et al.*, 2002).

2.1.4 Pleomorphic sarcoma (PS)

PS, or used to be called malignant fibrous histiocytoma (MFH) is a malignant neoplasm of uncertain origin that arises both in soft tissue and bone (Chan *et al.*, 2008). PS is typically large deep-seated tumour which is progressive and has very rapid enlargement which can be very painful to the patient. Most cases occur in patients over 40 years old, and it is very rare to find this tumour in children and young adolescents. A slight male predominance exists in the patients (Bertoni *et al.*, 2002). Usual site occurrences of this tumour are the extremities, especially the lower limb and the trunk.

About 5% of the PS cases have metastases at presentation, usually to the lung. According to Chan *et al*. (2008), tumour size and location are important prognostic factors where late presentation with advanced disease was found to significantly affect the overall outcome. The general five year survival rate for this tumour is only 50 to 60% in patients with high grade PS, which is a really aggressive condition (Bertoni *et al.*, 2002).

2.1.5 Giant cell tumour (GCT)

GCT, also known as osteoclastoma, is quite uncommon and generally affects patients over the age of 20 (Zheng *et al.*, 1994). This benign tumour is rarely seen in patients below 10 years old. Unlike EW, CS and PS, GCT has a slight female predominance among the patients. The tumour lesion is characterised by having numerous multinucleated giant cells in the spindle cell stroma (Robbins & Kumar, 1987; Werner, 2006). It is composed of sheets of neoplastic ovoid mononuclear cells interspersed with uniformly distributed large, osteoclast-like giant cells. Patients are usually present with pain, swelling and limitation of joint movement.

Although GCT is a benign condition, it is a locally aggressive neoplasm, capable of distant metastases and can recur locally in up to 50% of the cases within two years

(Bertoni *et al.*, 2002; Hogendoorn *et al.*, 2010). Following treatment, local recurrence happens in approximately 25% of the patients. It is worth finding biomarkers for this disease as 5% of the patients with aggressive GCT (stage III) may face a possibility for the tumour to metastasise to the lung, and in 1-3% of the patients, spontaneous transformation to high grade malignancy can happen (Bertoni *et al.*, 1985; Hogendoorn *et al.*, 2010). Surgery (curettage) is the most common medical intervention if the tumour is not too aggressive (Campanacci *et al.*, 1987; Robbins & Kumar, 1987).

2.2 Bone tumour manifestation and diagnosis

The early stage of sarcoma usually manifest in a patient as a painless lump, but eventually, the patient will complain of pain in the affected area when it begins to spread to the nerves and muscles. Other early symptoms are local swelling, difficulty with normal movement, fever, weight loss, anaemia and sometimes spontaneous fracture. Soft tissue sarcomas are harder to detect as they can grow anywhere in the body. Due to the elasticity of the soft tissue, the tumour can also grow to a considerable size before the patient realises of its presence.

Plain radiographs can be used to detect a bone tumour in the initial diagnosis, which can suggest the aggressiveness of the tumour. Staging studies should be carried out using various methods, including bone scintigraphy, computed tomography scan, positron emission tomography scan or magnetic resonance imaging.

However, to confirm the diagnosis, a biopsy must be performed because it is usually the deciding factor in determining the decision about the surgery procedure for the bone tumour patients (Malawer *et al.*, 1998). It is also the only way to determine whether the tumour is cancerous and differentiate between a primary bone tumour and secondary bone tumour. Because it is usually painful, there are cases where patients have refused to undergo the biopsy procedure, which can cause them apprehension and anxiety. The

procedure must also be performed by a skilled surgeon and care must be taken to prevent the cancer from spreading to nearby tissues and organs.

If the tumour is cancerous, surgery is the best option to remove it, and the surgeon may suggest different types of surgery depending on the size of the tumour and location. They could be resection (removing part or all bone affected), curettage (scraping out the tumour from the bone without removing the bone itself, usually used for benign tumour) and limb salvage surgery (removing the cancer but still leaving some part of the limb for endoprosthesis). If limb salvage surgery is not possible, amputation may be needed which will definitely affect the normal function of the limb, and subsequently, the patient's quality of life. Therefore, early identification of the disease is important to reduce mortality risk and increase the limb salvage strategies (Jin *et al.*, 2007). Early detection of either recurrent or metastatic disease can also prompt initial decision and action to treat the tumour which may improve the prognosis (Fujiwara *et al.*, 2014).

2.3 Tumour marker and biomarker research

Due to the difficulty and invasive nature of the diagnosis, it is imperative to find new methods or technologies that could be used for early detection of bone tumour in order to determine the best course of action for the patient. Serum and plasma from blood and urine are the most frequently used samples in biomarker research because they are the easiest to obtain from the patients and the method to acquire them is normally considered as non-invasive, unlike tissue biopsy. These types of samples are well known to reflect various physiological and pathological states of the human body. There are also numerous biomarker studies that utilise tissue samples. However, they are not suitable for screening or early detection of the disease because the tissues typically come from patients that have already shown the symptoms of the tumour (Stephen *et al.*, 2013).

Currently, there is no known tumour marker, which has been approved by the United States Food and Drug Administration (USFDA), for bone sarcoma and soft tissue sarcoma, unlike breast cancer, ovarian cancer and prostate cancer. An elevated level of serum alkaline phosphatase released through osteoblastic activity in the bone tumour has been suggested as an indicator for the tumour presence (Robbins & Kumar, 1987). However, this enzyme was previously reported to be also elevated in patients with diseases of the liver, thus making it unsuitable for used as a bone tumour marker (Kaplan & Righetti, 1970; Moss, 1982). Alternatively, this enzyme may be more useful for monitoring the progression of the bone tumour, instead of its use as diagnostic marker.

A biological marker or biomarker is defined as a cellular indicator of the physiological state and also of a change during a disease process (Srinivas *et al.*, 2001). It is also a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Atkinson *et al.*, 2001). Biomarkers can either be proteins, DNA, RNA or even metabolites. In general, they can be classified into four main categories, depending on their intended applications. First is a diagnostic biomarker, which can be used as a tool in identifying diseases or abnormal conditions in patients. The second type is a prognostic biomarker, where it predicts tumour behaviour and therefore acts as an indicator of disease prognosis. The third type of biomarker is one that can be used in staging the disease, for example, in cancer. And finally, predictive biomarkers, which can be used to predict and monitor the clinical response following a treatment (Atkinson *et al.*, 2001).

Apart from the intended applications, biomarkers can also be classified according to their sources and functions, as explained by Baron (2012). The first is carcinogenesis

biomarkers, which are products of the neoplastic process directly produced by the tumour itself (e.g. mutated or hypermethylated DNA). The second group is response biomarkers, where the markers are generated when the body responds to the presence of cancer (e.g. antibodies, protein degradation products and acute phase reactants). The third is released biomarkers, which include physiological molecules that are released in abnormal amounts following anatomical or metabolic disruptions of carcinogenesis (e.g. blood in the stool or prostate specific antigen in serum). Lastly, risk biomarkers, which consist of molecular markers associated with, or supporting the carcinogenesis (e.g. increased hormone levels).

Ideally, biomarkers should possess certain characteristics to make them clinically valuable, such as easily measured, reliable, and able to be detected using a cost-effective assay without loss of neither analytical sensitivity nor specificity (Kulasingam & Diamandis, 2008). Up to 2013, Fuzery and co-workers listed 23 protein cancer biomarkers which had been approved by the USFDA, the majority of which are for breast cancer (Fuzery *et al.*, 2013). The other approved biomarkers are for testicular, pancreatic, ovarian, colorectal, thyroid, prostate and bladder cancers. Despite a lot of research has been carried out to identify biomarkers for bone tumours, none of the proposed candidates has so far been approved by USFDA for clinical settings.

2.4 Biomarker discovery strategies

Diagnostic, prognostic and predictive biomarkers are most sought after markers in bone tumour research, and this fact is also true for other cancer research in general. This is due to the urgent need in achieving a better clinical outcome for the patients (Ludwig & Weinstein, 2005). Presently, proteomic technologies are one of the most widely used approaches in biomarker discovery strategies.

The term ‘proteome’ was first coined by Marc Wilkins (Wasinger *et al.*, 1995), referring to the total protein complement of a genome. On the other hand, the term ‘proteomic’ refers to the analysis of the protein complement of the genome (Baak *et al.*, 2003). The general objective of proteomics study is to achieve a more global and integrated view of biology by studying all the proteins of a cell simultaneously rather than each one individually (Graves & Haystead, 2002). Compared to genomics approaches, proteomics offers wider avenues for research. This is due to the fact that the genome of an organism is almost at a constant, while proteome is always changing with time and from cell-to-cell, besides frequently subjected to posttranslational modifications.

There are three different approaches in proteomic studies: 1) protein expression proteomics, where it is a quantitative study of protein expression between samples that differ by some variable, 2) structural proteomics, which is to map the structure of protein complexes or the protein present in a specific cellular organelle, and 3) functional proteomics which is the study to characterise and to find information about the protein itself, such as protein signalling and protein-drug interaction (Graves & Haystead, 2002). In cancer research, proteomics has widely been applied in profiling the protein expression in the patients. Due to the versatility of the techniques in proteomics research, various types of samples including blood (serum and plasma), urine, cerebrospinal fluid, tear, saliva and tissues can be used in proteomics study.

A typical proteomics experiment usually involved the separation and isolation of protein from the sample, the acquisition of protein structure to characterise it, and finally, utilisation of annotated databases in order to identify the proteins. The most common and widely used techniques to separate and isolate the proteins are 1DE and 2DE. In 1DE, proteins are separated based on their molecular mass and this method can

be used to resolve proteins with molecular mass of 10 to 300 kDa. However, it has a limited resolving power, especially for more complex mixtures such as neat serum and crude cell lysate. To overcome this limitation, 2DE can be used instead as this method separates the proteins according to their net charge in the first dimension and to their molecular mass in the second. This added step provides a much better resolution for complex mixtures compared with 1DE. The separation according to these two properties also enables this method to resolve proteins that have undergone posttranslational modification (Jensen, 2004; Tomonaga *et al.*, 2004).

The identification of the protein spots resolved in the 2DE gel can be done using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) and database search. MALDI is a soft ionisation technique used in MS, to analyse biomolecules such as DNA, proteins, peptides as well as large organic molecules such as polymers and dendrimers. Samples for MALDI analysis must be mixed with a matrix that contains a chromophore, and applied to a metal plate. The mixture of sample-matrix will absorb the ultraviolet light radiated from a pulsed laser, which will trigger ablation and desorption of the sample and the matrix to form ions. The formed ions will be accelerated by a high voltage supply and allowed to drift down a flight tube, and each ion will be separated according to its time of ion flight, which is different according to their mass. At the end of the flight tube, the ions will be detected using a detector, and the data will be recorded as mass-to-charge (*m/z*) ratio value of the ion.

The 2DE has also been modified to generate its variant, 2D-DIGE. Using this, different protein samples can be resolved simultaneously in a single gel. Each protein sample is labelled with a different fluorescent dye having different excitation wavelength and the gel is scanned at the corresponding emission wavelength to generate

images of the individual samples. This method allows the introduction of labelled internal standard and therefore, minimises inter-gel variations.

Nonetheless, there are a few limitations to gel-based electrophoresis strategy for protein separation. Besides being time consuming and laborious, the 2DE experiment is usually unable to detect proteins of low abundance, especially if they have low molecular weights. High abundance proteins such as albumin and immunoglobulin frequently mask the presence of these proteins. The depletion of these high abundance proteins which masks the lower abundance proteins can facilitate in increasing the sensitivity of the detection. However, loss of other proteins may also occur during the process. Depletion of albumin, for example, may cause the loss of several proteins and cytokines due to their interaction with the albumin itself (Duan *et al.*, 2005; Granger *et al.*, 2005; Seibert *et al.*, 2005).

To overcome these limitations, there are other alternative platforms that can be used. A combination of liquid chromatography (LC) and mass spectrometry (MS), surface-enhanced laser desorption/ionisation time-of-flight MS (SELDI-TOF), SWATH™-MS, as well as single reaction monitoring (SRM) or multiple reaction monitoring (MRM) are among recent techniques developed in proteomics. These methods are able to analyse complex mixtures by way of a simpler workflow, enabling a greater number of samples to be analysed compared with the 2DE.

LC-MS involves physical separation and mass analysis techniques making it a very powerful analytical chemistry technique with high sensitivity and high specificity. The use of LC-MS allows the 'shot-gun proteomics' approach, a term used when an entire protein sample is subjected to proteolytic digestion yielding a highly complex mixture of peptides. The recovered peptides are subjected to LC system and in a typical reverse phase single dimensional LC, the separation is based on hydrophobicity. The peptides

pass through LC column, and the elution is later ionised before mass determination using MS. In the case of tandem MS (LC-MS/MS), the recovered peaks are further fragmented and analysed in the second MS. There are several advantages to shot-gun approach: 1) samples are reduced to peptides prior to separation and mass spectrometry analysis, providing no bias for molecular size of proteins that could be identified, 2) the technique provides a platform for high throughput analysis of protein/peptide samples, and 3) it can overcome the limitations of the 2DE-based approach and identify a different, and partially overlapping set of proteins in a proteome.

However, LC-MS on its own is incapable of determining the amount of each protein presents in the sample. An approach using Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) can be used to comparatively quantify the amount of these proteins. This chemical labelling method incorporates stable isotopes into an amine tagging reagent before the identification of the proteins using MS (Ross *et al.*, 2004). Currently, there are two sets of amine reactive isobaric tags available in the market, the 4-plex and 8-plex which allow the labelling of up to eight samples simultaneously. These tags are used to derivatise peptides at the N-terminus and the lysine side chains, and therefore labelling all the peptides in a digest mixture (Ross *et al.*, 2004). Upon fragmentation in MS/MS, these tags will give rise to unique reporter ions (*m/z*) that can be used to quantify the respective samples.

High-throughput relative protein quantification and identification can also be done using isotopic labelling of proteins or peptides and by means of label-free quantification of derived mass spectra. In label-free LC-MS method, the amount of peptides can be determined using the ion signal intensities in the sample, where the data are collected in full MS scan mode to reconstruct the elution profile of the ions, thus producing the extracted ion chromatogram (XIC) (Lange *et al.*, 2008; Wang *et al.*, 2003). The

abundance of the analyte can be determined relatively between two samples, which can be calculated based on the differences between the areas of the XICs of the two ions with the same mass (Parviainen *et al.*, 2013). The labelling of these ions with isotopes can provide a more accurate result, but the cost involved will be much higher than the label-free method, and complex sample processing for this method can lead to sample loss.

SELDI-TOF was first introduced in 1993 by Hutchens and Yip (Hutchens & Yip, 1993). This novel approach combines both retention and MS based on a relatively simple principle. A solid-phase chromatographic surface is used to capture and retain the proteins by adsorption, partition, electrostatic interaction or affinity chromatography (Issaq *et al.*, 2003). Proteins will be co-crystallized with the matrix on the target surface and later, a nitrogen laser can be used to subsequently ionise and their molecular masses are directly detected on the chip array by using TOF-MS (Issaq *et al.*, 2002). The SELDI's target surfaces are dissimilar to MALDI because the protein chip chromatographic surfaces in SELDI are designed specifically to retain proteins from complex mixtures according to their special properties such as hydrophobicity and charge.

Due to the unique surface chemistries of the chips, the system can be exploited to cater the used of different types of samples such as serum, blood, plasma, urine and cell lysates. SELDI-TOF is much more sensitive than the 2DE as it requires only a very small amount of sample per analysis. This system is also most valuable at profiling low molecular weight proteins (less than 20kDa) which cannot be delivered by 2DE (Issaq *et al.*, 2002). The versatility of ProteinChip Biology system allows it to be used for various applications. This platform has been successfully used to profile serum proteins as well as in the discovery of potential candidates of biomarker for lung cancer patients

(Yang *et al.*, 2005), renal cancer (Tolson *et al.*, 2004), endometrial cancer (Takano *et al.*, 2010), and gastric cancer (Qiu *et al.*, 2009). Petricoin and co-workers also suggested the serum proteomic pattern analysis using SELDI-TOF technology may be applied in medical screening in detecting all stages of ovarian cancer in high risk and general populations (Petricoin *et al.*, 2002).

SWATH™-MS analysis is a data-independent acquisition (DIA) mass spectrometric method that can generate a complete recording of the fragment ion spectra of all analytes in a biological sample in a single measurement. The samples are subjected to LC-MS/MS run for two major arms; the information dependent acquisition (IDA) analysis for discovery proteomics and SWATH™-MS analysis, for quantitative proteomics. SWATH™-MS data acquisition allows dynamic protein probing by re-mining the acquired data set without repeating the experiment. The data is acquired using a high resolution quadrupole-time-of-flight (Q-TOF) instrument by repeatedly cycling through a fixed precursor sequential isolation windows (swaths) which enable complete peptide fragment-ion coverage over the whole chromatographic elution range (Collins *et al.*, 2013; Gillet *et al.*, 2012; Liu *et al.*, 2013). The complex fragment ion maps obtained will be subjected to targeted data extraction strategy as described by Reiter *et al.* (2011).

A high-resolution scan of the data enabled us to do qualitative and quantitative analysis simultaneously, without the use of any tag, and therefore analyse different samples to establish a list of biomarkers in a very short time frame. Thus, SWATH™-MS allowed highly reproducible identification and quantification of a large number of analytes. It is a relatively new and emerging proteomic approach and had been used in quite a number of studies such as in glycoproteomics and cancer research due to its versatility and reliability (Liu *et al.*, 2014; Liu *et al.*, 2013; Zhang *et al.*, 2014).

If potential biomarkers have been discovered for targeting during the discovery phase, selective ion monitoring techniques can be applied for absolute quantitative measurement. Selection of the appropriate and unique parent/product ion pairs for the analyte of interest using a powerful triple quadrupole mass spectrometer is known as SRM or MRM, depending on the number of target ions screened (Kitteringham *et al.*, 2009; Lange *et al.*, 2008). This non-scanning technique, unlike in shot-gun approach, is highly selective and sensitive which allows the researcher to direct the instrument to specifically monitor and do absolute quantification of the target peptides or proteins of interest, even of low-abundance, in the complicated matrix (Anderson & Hunter, 2006; Lange *et al.*, 2008). MRM offers a rapid and specific quantification assay without the use of any antibodies, thus it has a huge potential to be used as a biomarker validation tool (Anderson & Hunter, 2006). MRM has been used to successfully quantify 45 proteins in human plasma, where 31 of them are putative biomarkers for cardiovascular disease (Kuzyk *et al.*, 2009). In a more recent study, Sung and co-workers developed a high-throughput MRM assay to quantify and differentiate different isoforms of serum amyloid A (SAA), a putative protein biomarker for patients with lung cancer (Sung *et al.*, 2012).

2.5 Proteomics approaches in discovery of biomarkers

In cancer research, proteomics has widely been applied in profiling the protein expression in cancer patients using various types of samples including blood (serum and plasma), urine, cerebrospinal fluid, tear, saliva and tissues. The analysis of these profiles in normal versus cancer patients can provide useful insight to the researchers especially in order to find ways, for example, tumour biomarkers, to aid the diagnosis of cancer at a much earlier stage.

The majority of the proteomic biomarkers are of diagnostic value (Table 2.1). The most common proteomic platforms used in biomarker research for bone tumours were 2DE and 2D-DIGE. Recently, gel-free platforms such as SELDI-TOF and LC-MS have been very popular among proteomic researchers due to advantages offered where only a small amount of sample is required and the possibility of quantification. They also offer options for automations, and less laborious compared to 2DE.

2.5.1 Biomarkers for OS

As the most common type of bone tumour, the biomarkers for OS are the most extensively sought by researchers. Most of the reviewed studies concentrated in unearthing the diagnostic biomarkers for OS, followed by predictive biomarkers (Table 2.1). Majority of the studies used 2DE- and 2D-DIGE-based approaches and suggested various proteins as diagnostic, predictive and prognostic biomarkers (Folio *et al.*, 2009; Hua *et al.*, 2011; Jin *et al.*, 2007; Jin *et al.*, 2012; Kikuta *et al.*, 2010; Li *et al.*, 2010). Only two group of researchers utilised SELDI-TOF as the main technique (Li *et al.*, 2009; Li *et al.*, 2011; Li *et al.*, 2006).

Plasma level of serum amyloid A (SAA) was found to be significantly elevated in paediatric OS patients when compared to the benign bone tumour (Li *et al.*, 2006). SAA is secreted in response to inflammatory process and it is thought to be involved in tumourigenesis as positive acute phase reactant as SAA is normally present at low level in sera of healthy subjects (Malle *et al.*, 2009). In 2009, Folio *et al.* (2009) discovered that the amount of two proteins, crystallin α/β (CRYAB) and ezrin (EZR) were significantly higher in OS tissue when compared to normal bone tissue, particularly in

Table 2.1: Potential biomarkers for various bone tumours identified using proteomics

Type of bone tumour	Type of biomarker	Candidate biomarker(s)	Regulation	Sample	Technique(s)	Reference
OS	Diagnostic	SAA	↑	Plasma	-SELDI-TOF -Western blot	(Li *et al.*, 2006)
OS	Diagnostic	EZR CRYAB	↑	Tissue	-2D-DIGE -LC-ESI-MS/MS -RT-PCR for mRNA -Tissue microarray and immunohistochemistry (IHC)	(Folio *et al.*, 2009)
OS	Diagnostic	CYC-1	↑	Serum Cell lines	-SELDI-TOF -Gene microarray (cell lines)	(Li *et al.*, 2009)
OS	Diagnostic	ZNF133 TUBA1C	↑	Tissue	-2DE -MALDI-TOF MS -Western blot -IHC	(Li *et al.*, 2010)
OS	Diagnostic	Protein NDRG1	↑	Plasma membrane from cell line & tissue	-2DE -LC-ESI-MS/MS -IHC -Western blot	(Hua *et al.*, 2011)
OS	Diagnostic	Gelsolin	↓	Serum	-2D-DIGE -MALDI-TOF MS -Western blot -ELISA	(Jin *et al.*, 2012)

Table 2.1 continued

OS	Predictive Prognostic	SAA	↑	Serum	-2D-DIGE -MALDI-TOF MS -Western blot -ELISA	(Jin *et al.*, 2007)
OS	Predictive	PRDX2	↑ (poor prognosis)	Tissue	-2D-DIGE -LC-ESI-MS/MS -Western blot	(Kikuta *et al.*, 2010)
OS	Predictive	SAA TTR	↓ (poor prognosis)	Plasma	-SELDI-TOF - Western blot	(Li *et al.*, 2011)
EW	Prognostic	Nucleophosmin	↑ (poor prognosis)	Tissue	-2D-DIGE -LC-ESI-MS/MS -IHC	(Kikuta *et al.*, 2009)
GCT	Prognostic	GPX1 PRX AIF1 UBE2N HSP27	↑ (except for HSP27)	Tissue	-2DE -MALDI-TOF MS -Tissue microarray	(Conti *et al.*, 2011)

advanced stages of the disease. CRYAB protein has anti-apoptotic functions suggesting its involvement in the carcinogenesis of OS while EZR is a membrane-cytoskeleton linker protein that is involved in the growth and metastasis of various types of tumours (Folio *et al.*, 2009).

Other proteins found to be differently regulated in patients with OS and suggested as diagnostic biomarkers are cytochrome C1 (CYC-1), zinc finger protein 133 (ZNF133), tubulin-α1c (TUBA1C), protein NDRG1, and gelsolin (Hua *et al.*, 2011; Jin *et al.*, 2012; Li *et al.*, 2009; Li *et al.*, 2010). CYC-1 is a heme-containing subunit of the cytochrome b-c1 complex and mainly known as an important participant in mitochondrial ATP synthesis (Li *et al.*, 2009; Ow *et al.*, 2008). However, the relationship between this protein and tumourigenesis is relatively unknown. TUBA1C protein, a major cytoskeletal component, was suggested as a key protein involved in metastasis and progression of OS, and therefore might be valuable as prognostic indicator (Li *et al.*, 2010). Li *et al.* (2010) also demonstrated that ZNF133, a transcriptional repressor, was also upregulated in OS tissues, but the exact function and regulation of this protein is still unknown.

Another potential diagnostic biomarker for OS as discovered by Hua and co-workers (2011) is protein NDRG1, where it was found to be significantly upregulated. The specific biological function of NDRG1 is unknown, but there were studies that indicated this protein as a potential tumour suppressor protein (Angst *et al.*, 2006). In a separate study by Jin *et al*. (2012), the down-regulation of gelsolin was reported in OS, where they also suggested that this protein may act as a tumour suppressor. Gelsolin is involved in various biological functions such as apoptosis stimulator, cell motility, inflammation and wound healing (Jin *et al.*, 2012).

In studies carried out by three different research groups, SAA, peroxiredoxin 2 (PRDX2) and transthyretin (TTR) were suggested as predictive protein biomarkers in evaluating the efficacy of the treatment for patients with OS (Jin *et al.*, 2007; Kikuta *et al.*, 2010; Li *et al.*, 2011). Jin and co-workers (2007), in a study comparing the SAA expression in patients prior to treatment and post-chemotherapy, found that the level of this protein to be significantly lower after the treatment, as well post-surgery. Conversely, Li *et al*. (2011) reported that SAA protein was higher in good responders post-chemotherapy treatment. However, the former did not mention the response of the patients toward the chemotherapy (Jin *et al.*, 2007).

Aside from diagnostic and predictive biomarkers, SAA protein may also serve as prognostic biomarker, where the expression of SAA was significantly increased in relapsed patients with OS (Jin *et al.*, 2007). PDRX2 is an important protein in antioxidant defence mechanism and redox signalling process (Peskin *et al.*, 2007). The expression of PDRX2 is positively correlated with poor response to chemotherapy but the role of this protein in this reaction is still vague (Kikuta *et al.*, 2010). The expression of another protein, TTR was also significantly lower in good responder compared to poor responder to chemotherapy. TTR is involved in thyroxine and retinol-binding protein transportation and reacts to inflammation process. Yet, its down-regulation was also observed in ovarian cancer, suggesting the regulation of this protein is not merely because of inflammation, but is due to the presence of the tumour as well (Li *et al.*, 2011).

2.5.2 Biomarkers for other bone tumours - EW, GCT, CS and PS

The occurrence of EW is infrequent, and from the literature review that we had performed, we had only come across a single publication that reported proteomic biomarker for EW, where nucleophosmin was suggested as prognostic marker for this

disease (Kikuta *et al.*, 2009). Using 2D-DIGE as the main platform, the expression of nucleophosmin was found to be correlated with patients with poor prognosis. The functional role of nucleophosmin in the aetiology of EW is still unclear, but it is linked to a few proteins, including p53, and mutation to p53 protein is known in EW cases and it is associated with poor prognosis (Kikuta *et al.*, 2009; Nakatani *et al.*, 2012).

In 2011, using 2DE, Conti and co-workers (2011) suggested glutathione peroxidase 1 (GPX1), thioredoxin peroxidase (PRX), allograft inflammatory factor 1 (AIF1), ubiquitin E2N (UBE2N), and heat shock protein 27 (HSP27) as potential biomarkers in predicting the aggressive behaviour of GCT to local recurrences or to metastasis. These proteins are involved in various functions involving tumourigenesis such as oxidative stress, apoptosis, angiogenesis, cell proliferation and drug resistance (Conti *et al.*, 2011).

2.6 Lectins

Complexity of a sample presents a major challenge for comprehensive analysis of whole serum proteome through shot-gun analysis. To reduce the sample complexity, various techniques can be used to enrich a sub-proteome with specific properties or posttranslational modifications, such as organic fractionation, size exclusion, chemical extraction, affinity binding and immunochemical precipitation (Chen *et al.*, 2007).

Via affinity binding, lectins are suitable for enriching glycoconjugates fraction in the sample. They are structurally diverse carbohydrate-binding proteins of non-immune origin. Their affinities to carbohydrate ligands make these molecules as efficient tools to characterise and purify glycoconjugates (Abdul Rahman *et al.*, 2002; Mohamed *et al.*, 2008). Lectins can be found ubiquitously in animals, plants as well as microorganisms (Berg *et al.*, 2002). In animals, the main function of lectin is to assist cell to cell contact (Berg *et al.*, 2002). However, the exact function of lectins in plant remains uncertain,

but they are known to play a role in defense mechanisms of the plant against microorganisms and insects (Peumans & Vandamme, 1995; War *et al.*, 2012).

The seeds of *Artocarpus integer* fruit can produce two types of lectins, CGB lectin and CMB lectin, both discovered by Hashim and co-workers (Hashim *et al.*, 1991; Lim *et al.*, 1997; Lim *et al.*, 1998). Locally known as champedak, it is native to Southeast Asia and exudes distinct strong sweet smell when ripe. The brown seed is surrounded by an edible yellow flesh, with juicy and sweet, custard-like texture. CGB lectin has the ability to recognize specific glycosylation motifs [galactosyl (β1,3)N-acetyl-galactosamine (Galβ1,3GalNAc)] that are present in O-linked glycoproteins while CMB lectin has a high affinity for the core mannosyl residues of the N-glycans of glycoproteins. Previously, works by Hashim and co-workers had demonstrated that the CGB lectin binds to human IgA whilst CMB lectin was shown to have a selective strong interaction with human IgE and IgM, and a weak interaction with IgA2 (Hashim *et al.*, 1991; Lim *et al.*, 1997).

A number of glycosylation profiling and biomarker studies on various diseases, including cancers, have been carried out using CGB and CMB lectins over the years, as shown in Table 2.2. By using CGB lectin, Abdul-Rahman and co-workers (2007) had successfully demonstrated different altered expression O-glycoproteins compared to negative control women in sera of endometrial and cervical cancers. In 2008, Mohamed *et al*. used a combination of CGB lectin affinity chromatography and 2DE as well as 2DE-CGB lectin blot to profile O-glycoproteins in serum from patients with breast carcinoma, epithelial ovarian carcinoma, germ cell ovarian carcinoma, nasopharyngeal carcinoma and OS (Mohamed *et al.*, 2008).

CGB lectin was also used a probe to differentiate serum O-glycoprotein profiles between healthy non-pregnant women, women with normal pregnancy as well as

patients with molar pregnancy, also called as hydatidiform mole (Mohamed *et al.*, 2013). Hydatidiform mole is a condition occurs when an abnormal and benign mass of tissue from fertilized ovum develops inside the uterus, but will not develop into a fetus (Mohamed *et al.*, 2013). On the other hand, CMB lectins had been used to profile plasma and serum N-linked glycoproteins in patients with congenital hypothyroidism and nasopharyngeal carcinoma (Seriramalu *et al.*, 2010; Yong *et al.*, 2006).

Although serum and plasma are the typical samples used for lectin analysis, urine can also be used as demonstrated by Abdullah-Soheimi *et al*. (2010) and Mu *et al*. (2012) where urinary O-glycoproteins were profiled using CGB lectin via 2DE-lectin blot and CGB lectin affinity chromatography-2DE methods. Similar approaches were also applied by Jayapalan *et al*. to profile both serum and urine O-glycoproteins from patients from prostate cancer and benign prostatic hyperplasia (Jayapalan *et al.*, 2012; Jayapalan *et al.*, 2013).

Table 2.2: Applications of CGB and CMB lectins in proteomics profiling studies

Lectin	Disease/Condition	Type of sample	Method of detection	Reference
CGB	Endometrial adenocarcinoma Squamous cell cervical carcinoma Cervical adenocarcinoma	Serum	-2DE-Lectin blot	(Abdul-Rahman *et al.*, 2007)
	Breast carcinoma Epithelial ovarian carcinoma Germ cell ovarian carcinoma Nasopharyngeal carcinoma Osteosarcoma	Serum	-Lectin affinity column chromatography-2DE -2DE-Lectin blot	(Mohamed *et al.*, 2008)
	Normal pregnant women Hydatidiform mole	Serum	-2DE-Lectin blot	(Mohamed *et al.*, 2013)
	Ovarian carcinoma	Urine	-2DE-Lectin blot	(Abdullah-Soheimi *et al.*, 2010)
	Endometrial carcinoma	Urine	-2DE-Lectin blot -Lectin affinity column chromatography-LC-MS/S	(Mu *et al.*, 2012)
	Prostate cancer Benign prostatic hyperplasia	Serum & Urine	-Lectin affinity column chromatography-2DE -2DE-Lectin blot	(Jayapalan *et al.*, 2012; Jayapalan *et al.*, 2013)
CMB	Congenital hypothyroidism	Plasma	-2DE-Lectin blot	(Yong *et al.*, 2006)
	Nasopharyngeal carcinoma	Serum	-Lectin affinity column chromatography-2DE -2DE-Lectin blot	(Seriramalu *et al.*, 2010)

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Human serum samples

Serum samples were collected from newly diagnosed patients with primary bone tumours (prior initiation of therapy or surgery) at the Orthopaedic Clinic, University of Malaya Medical Centre, Kuala Lumpur. Ethical approval was obtained from the Medical Ethics Committee, University of Malaya Medical Centre (Appendix A). The nature of the study was described to the patients and consents were obtained before the collection of the samples (Appendix B). The diagnosis of all of the cases was histologically confirmed according to World Health Organization (WHO) histologic classification. Cases with metastasis presentation were excluded from this study. A total of 42 samples (age ranged from 6 to 78 years old) were collected for all five disease groups [OS (n=14), ES (n=5), CS (n=7), PS (n=8) and GCT (n=8)]. Non-tumour control samples (n=34) were collected from healthy individuals with their age ranging from 8 to 72 years old. As most bone tumours have a predilection for specific age group, therefore in this study, the non-tumour control samples were divided to form an age-matched control group for each disease group, as shown in Table 3.1. Throughout the study, patients with OS and ES were compared with Control 1 (C1), patients with CS and PS were compared with Control 2 (C2) and patients with GCT were compared with Control 3 (C3).

Six ml of venous whole blood was withdrawn from each individual and was allowed to clot by leaving it undisturbed at room temperature (22°C). The clot was separated from the serum within 3 h of blood collection by centrifugation at 1500 X g for 20 min. Following centrifugation, the serum was transferred and kept in aliquots of 100 µl (to avoid unnecessary freeze-thaw cycle) into clean polypropylene microcentrifuge tubes at -80°C.

Table 3.1: Sample size and age range of the non-tumour healthy controls and patients with bone tumours used in the study

Group	Age range (years)	Mean age in years (Mean ± SD)	Sex		Total number of samples
			Male	Female	
C1	8-29	20.18 ± 6.20	9	8	17
C2	30-72	47.68 ± 11.33	6	11	17
C3	20-72	38.43 ± 14.91	12	16	28
OS	8-22	15.36 ± 3.61	8	6	14
ES	6-23	18.00 ± 7.14	5	0	5
CS	37-73	51.29 ± 14.49	6	1	7
PS	41-78	59.50 ± 11.69	4	4	8
GCT	19-62	36.63 ± 14.96	4	4	8

3.1.2 General materials

3.1.2.1 Chemicals

i) *Fisher Scientific UK Limited, Leicestershire, UK*

Sodium dodecyl sulphate (SDS)

ii) *GE Healthcare Biosciences, Uppsala, Sweden*

Acrylamide

Amberlite® XAD4

Bromophenol blue

Coomassie Brilliant Blue R250

Dithiothreitol (DTT)

Glycerol 87%

Glycine

N,N'-methylenebisacrylamide

Urea

iii) *J. Kollin Chemicals, UK*

Ethanol

iv) *JT Baker®, Philadelphia, USA*

Acetic acid

v) *Merck KGaA, Darmstadt, Germany*

Acetonitrile (ACN)

Boric acid

Disodium phosphate (Na_2HPO_4)

Disodium tetraborate

Formic acid

Potassium dihydrogen phosphate (KH_2HPO_4)

Silver nitrate

Skimmed milk

Sodium acetate trihydrate

Sodium bicarbonate

Sodium carbonate

Sodium chloride

Sodium hydrogen carbonate

Tween® 20

vi) *Sigma Aldrich Company, St. Louis, USA*

Agarose

Ammonium bicarbonate

Ammonium persulphate (APS)

Ammonium sulphate

D-Galactose

Divinyl sulfone (DVS)

Ethanolamine

Formaldehyde

Gelatine

Glutaraldehyde

Hydrochloric acid 37%

Hydrogen peroxide (H_2O_2)

Iodoacetamide (IAA)

Mannose

Magnesium chloride ($MgCl_2$)

N,N,N',N'-Tetramethyl-ethylenediamine (TEMED)

Potassium chloride (KCl)

Potassium ferricyanide

Sodium acetate

Sodium azide (NaN_3)

Sodium borohydride

Sodium thiosulphate 5-hydrate

Sodium periodate

Triethanolamine

Triton™ X-100

Trizma® base

Zinc chloride ($ZnCl_2$)

α-cyano-4-hydroxycinnamic acid

β-mercaptoethanol

vii) *Systerm ChemAR, Selangor, Malaysia*

Ethylenediaminetetraacetic acid, disodium dihydrate (EDTA-$Na_2.2H_2O$)

Sodium hydroxide (NaOH)

3.1.2.2 Two-dimensional electrophoresis (2DE)

i) *GE Healthcare Biosciences, Uppsala, Sweden*

IPG Immobiline DryStrip (linear pH 4-7, 11 cm)

IPG buffer (pH 4-7)

DryStrip cover fluid

3.1.2.3 Enzymes and substrates

i) *BioRad Laboratories, USA*

3,3'-diaminobenzidine (DAB)

ii) *Promega, Madison, WI, USA*

Trypsin Gold (MS grade)

iii) *Roche Molecular Biochemicals, Mannheim, Germany*

Activated alkaline phosphatase (AP)

iv) *Sigma Aldrich Company, St. Louis, USA*

Activated horseradish peroxidase (HRP)

BCIP®/NBT tablets

3.1.2.4 Antibodies

i) *Abcam, Cambridge, UK*

Rabbit anti-human serum amyloid A (EPR4134)

Goat anti-rabbit IgG H&L (HRP) (ab97051)

3.1.2.5 Commercial kits

i) *Pierce, Rockford, Illinois, USA*

BCA Protein Assay Kit

ii) *Abcam, Cambridge, UK*

Serum amyloid A Human ELISA kit (ab100635)

3.1.2.6 Chromatographic media

i) *GE Healthcare Biosciences, Uppsala, Sweden*

Sepharose 4B

3.1.2.7 Membranes

i) *Pall, Ann Arbor, Michigan, USA*

Polyvinylidene difluoride (PVDF)

ii) *Advantec, Tokyo, Japan*

Nitrocellulose membrane

3.1.2.8 General buffers

i) *Phosphate-buffered saline (PBS) – 10X stock solution: 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2HPO_4, pH 7.2*

NaCl (80.0 g), KCl (2.0 g), Na_2HPO_4 (11.5g) and KH_2HPO_4 (2.0g) were dissolved in 800 ml distilled H_2O. The pH was adjusted to 7.2 and the solution was topped up with deionised distilled H_2O to 1 L.

ii) *PBS – 1X working solution, pH 7.2*

PBS stock solution (100 ml) was diluted with 900 ml of distilled H_2O and stored at room temperature.

3.1.2.9 General apparatus

Densitometer (Image Scanner III, GE Healthcare Biosciences, Uppsala, Sweden)

Multiphor Flatbed Electrophoresis System (GE Healthcare Biosciences, Uppsala, Sweden)

Gradient maker (SG30, Hoefer, USA)

Peristaltic pump (EYELA Micro Tube Pump MP-3, Tokyo Rikakikai, Tokyo, Japan)

Semidry transfer system - NovaBlot Kit of the Multiphor™ II Flatbed System (GE Healthcare Biosciences, Uppsala, Sweden)

Spectrophotometer - Hitachi U-2000 Spectrophotometer (Hitachi, Ibaraki, Japan)

Xevo™ Tandem Quadrupole Mass Spectrometer (Waters Corp, Manchester, UK)

2DE apparatus - SE 600 Ruby Electrophoresis System (GE Healthcare Biosciences, Uppsala, Sweden)

4800 Plus MALDI TOF/TOF™ analyser (AB SCIEX, Foster City, California)

Mini PROTEAN® Tetra Cell and PowerPac™ HC Power Supply (BioRad Laboratories, Hercules, USA)

3.2 Methods

3.2.1 Analysis of serum protein profiles using 2DE

Separation of proteins in neat serum samples was performed using 2DE. The general principles behind 2DE method involve a two-step procedure in order to separate a mixture of proteins. The first step is known as isoelectric focusing or IEF (first dimension), where it separates the proteins according to their isoelectric focusing points (pI). The second step is the standard sodium dodecyl sulphate polyacrylamide gel electrophoresis, or SDS-PAGE (second dimension), where the proteins can be separated according to their molecular weights. Multiphor™ II Flatbed System was used in the first dimension separation, where the IEF was performed on IPG Immobiline DryStrips (linear pH 4-7, 11 cm). The DryStrips were subjected to second dimension separation using Hoefer SE 600 Ruby electrophoresis system on gradient SDS gels (8-18%). Both systems used were from GE Healthcare Biosciences, Uppsala, Sweden.

3.2.1.1 First dimension electrophoresis

Standard solutions:

Sample buffer: 9 M urea, 60 mM dithiothreitol (DTT), 2% (v/v) IPG buffer, 0.5% (v/v) Triton™ X-100

The sample buffer was prepared by mixing 5.4 g urea, 0.092 g DTT, 0.2 ml IPG buffer (4-7), 0.05 ml Triton™ X-100 and topped up with deionised distilled H_2O to 10 ml. The solution was kept in aliquots of 200 µl and stored for up to 2 months at -20°C.

Rehydration solution: 8 M urea, 0.5% (v/v) IPG buffer, 0.5% (v/v) Triton™ X-100

The rehydration solution was prepared by mixing 4.809 g urea, 0.05 ml IPG buffer (4-7), 0.05 ml Triton™ X-100 and topped up with deionised distilled H_2O to 10 ml. A few grains of Orange G were added to the solution to aid visualization during rehydration

process. The solution was kept in aliquots of 1 ml at -20°C. Prior to use, 12 mM DTT was added and mixed to the rehydration solution.

Sample preparation and rehydration of Immobiline DryStrips

Sample preparation was carried out as described by Doustjalali *et al*. (2004). In this study, 7 µl of serum sample (~400 µg protein) was found to be sufficient to generate a good 2DE profile. Each serum sample was mixed in a ratio of 1:3 with the sample buffer and incubated at room temperature for 30 min. The mixture was next added with rehydration solution to 200 µl and left for another 30 min at room temperature. After the incubation, the sample mixture was distributed into a slot on a rehydration tray. Immobiline DryStrip (11 cm, pH 4-7) was carefully inserted into the slot on top of the mixture (the gel of the strip facing down). The inserted IPG DryStrips with the sample mixtures were covered with cover fluid to prevent evaporation and urea crystallization during rehydration. The tray was left at room temperature (22°C) for a minimum of 18 h to allow complete rehydration and uptake of the sample into the DryStrips.

First dimension run

The IEF instrument (Multiphor Flatbed Electrophoresis System, GE Healthcare Biosciences, Uppsala, Sweden) was set at 20°C using a thermostatic circulator. Cover fluid (5 ml) was pipetted onto the cooling ceramic plate. The DryStrip tray was placed on top of the ceramic plate and 10 ml of cover fluid was poured onto it. A plastic DryStrip aligner was used to position the rehydrated IPG DryStrips on the tray. Moistened paper wicks were placed across the cathode and anode ends of the aligned IPG DryStrips before applying the electrodes. The paper wicks at the electrodes were necessary to collect excessed proteins that were outside the pH range of interest and also to act as a desalting tool. The assembly was covered with cover fluid to ensure efficient

thermal contact and also to prevent drying out of the strip and crystallization of the urea during IEF. The first dimension of IEF for 11 cm IPG strip pH 4-7 was carried out following the manufacturer's recommendation (Table 3.2). Following the first dimension run, the focused strips were stored in screw-capped glass test tubes at -80°C.

Table 3.2: Electrophoresis phases for first dimension run of 2DE

Phase	V	mA	W	Duration (min)	V/h
1	300	2	5	30	-
2	3 500	2	5	Until completed	15 000

3.2.1.2 Second dimension electrophoresis

Standard solutions

Solution A: Monomer (30% acrylamide, 0.8% N,N'-methylenebisacrylamide)

Acrylamide (60 g) was mixed with 1.6 g N,N'-methylenebisacrylamide and made up to 200 ml with distilled H_2O. The solution was deionised using Amberlite, filtered and stored in an amber bottle at 4°C.

Solution B (4X resolving buffer): 1.5 M Tris-HCl, pH 8.8

Tris base (36.23 g) was dissolved in 150 ml distilled H_2O. The pH of the solution was adjusted to 8.8 using HCl and the solution was made up to 200 ml with distilled H_2O.

Solution C: 10% (w/v) SDS

SDS (10 g) was dissolved in 100 ml and the solution was stored at room temperature.

Solution D: 10% (w/v) ammonium persulphate (APS)

APS (2 g) was dissolved in 20 ml distilled H_2O. The solution was kept in aliquots of 200 µl and stored at -20°C. Each aliquot can be thawed once and the remainder must be discarded.

TEMED (N,N,N',N'-Tetramethyl-ethylenediamine)

SDS equilibration solution (SDS-EB)

Twenty ml of 4X resolving buffer, pH 8.8 was mixed with 144.14 g urea, 138 ml glycerol and 8 g SDS. The solution was made up to 400 ml with distilled H_2O.

Anode buffer: 0.37 M Tris-HCl, pH 8.8

Tris (136.2 g) was dissolved in 2 L distilled H_2O. The pH was adjusted to 8.8 with HCl.

Cathode buffer: 25 mM Tris, 198 mM glycine, 0.1% SDS

Tris (3.03 g) was mixed with 14.8 g glycine and 1 g SDS. Distilled H_2O (1 L) was added to the mixture. Buffer need to be freshly prepared prior to use.

Agarose sealing solution (0.5%)

Agarose (0.5 g) was dissolved in 100 ml cathode buffer. The mixture was heated in a microwave oven until the agarose was completely dissolved.

<u>Preparation of 8-18% gradient SDS-PAGE gel</u>

The gel caster was assembled according to the manufacturer's instruction (GE Healthcare Biosciences, Uppsala, Sweden). The gradient gel consists of two solutions: a) a light solution of 8% gel, b) a heavy solution of 18% gel (refer Table 3.3). Both

solutions were placed in separate chambers of a gradient maker (SG30, Hoefer, USA) and the valve was opened to mix both solutions in a tube connected to a peristaltic pump (EYELA Micro Tube Pump MP-3, Tokyo Rikakikai, Tokyo, Japan). The mixture was poured into the glass plate sandwich at a speed of 2.5 ml min^{-1}. The top of the gel was carefully laid with distilled H_2O to ensure an even surface formed during the gel polymerisation.

Table 3.3: Preparation of an 8-18% gradient gel for 2DE (for a single gel)

Solution	Gradient	
	Light solution (8%)	Heavy solution (18%)
Solution A (ml)	3.20	7.20
Solution B (ml)	3.00	3.00
Solution C (µl)	120	120
Sucrose (g)	-	1.80
Deionised distilled H_2O (ml)	5.64	Made up to 12.0
Solution D* (µl)	4	4
TEMED* (µl)	40	40
Total volume (ml)	12.00	12.00

* Added prior to use, directly into the gradient maker's chambers.

Equilibration of the focused IPG DryStrips

The focused IPG DryStrips must be equilibrated before being subjected to electrophoresis. The first equilibration solution was 1% (w/v) DTT in SDS equilibration buffer (SDS-EB). DTT was used to reduce the proteins in the sample. Five ml of this solution was pipetted into each tube containing the strip. The tubes were shaken gently on a rocking platform for 15 min. The first equilibration solution was discarded and replaced with 5 ml of the second SDS-EB containing 4.5% (w/v) iodoacetamide (IAA). The IAA acts as an agent to alkylate the proteins. The tubes were shaken gently again for 15 min, and the excess solution was discarded. The strips were quickly rinsed with

cathode buffer and inserted on top of the gradient gel. Melted agarose (0.5% agarose in cathode buffer mixed with a few grains of bromophenol blue) was used to seal the strips in place.

Second dimension run

The 2DE apparatus (SE 600 Ruby Electrophoresis System, GE Healthcare Biosciences, Uppsala, Sweden) was set at 16°C using a cooling circulator. The electrophoresis run was carried out in two phases, as shown below:

Table 3.4: Electrophoresis phases for second dimension run of 2DE

Phase	V	mA	W	Duration (min)
1	50	40/gel	25/gel	30
2	600	40/gel	25/gel	90-100

The electrophoresis run was stopped once the blue dye (from the agarose layer) reached approximately 1 cm from the bottom edge of the gel. The gels were carefully removed from the glass plates and placed in a container containing fixing solution (Section 3.2.1.3).

3.2.1.3 Silver staining of 2DE gels

The silver staining method of 2DE gels was carried out as described by (Heukeshoven & Dernick, 1988). All solutions were prepared fresh prior to staining process.

Standard solutions:

Fixing solution: 40% (v/v) ethanol, 10% acetic acid

Ethanol (400 ml) was mixed with 100 ml acetic acid and made up to 1 L with distilled H_2O.

Sensitizing solution: 30% (v/v) ethanol, 0.5 M sodium acetate, 8 mM sodium thiosulphate, 0.26% (v/v) glutaraldehyde

Sodium acetate trihydrate (68 g) and sodium thiosulphate 5-hydrate (2 g) were added to 300 ml of ethanol. The solution was made up to 1 L with distilled H_2O. Glutaraldehyde (2.6 ml) was added prior to use.

Silver solution: 5.9 mM silver nitrate, 0.02% (v/v) formaldehyde

Silver nitrate (1 g) was mixed with 1 L of distilled H_2O. Formaldehyde (200 µl) was added prior to use.

Developing solution: 0.24 M sodium carbonate, 0.2% (v/v) formaldehyde

Sodium carbonate (15 g) was mixed with 1 L of distilled H_2O. Formaldehyde (100 µl) was added prior to use.

Stopping solution: 40 mM EDTA-Na_2.$2H_2O$

EDTA-Na_2.$2H_2O$ (14.6 g) was mixed with 1 L of distilled H_2O.

Staining procedures

After the electrophoresis, the gels were immersed in fixing solution overnight on a platform shaker. The subsequent steps were also carried out on a platform shaker to ensure uniformity of the stain. Fixing solution was removed and the gels were placed in the sensitizing solution for 30 min. The sensitizing solution was discarded and the gels were washed thoroughly for three times with distilled H_2O (5 min each time). The silver solution was added to the gels and the reaction was carried out for 40 min. After the reaction, the gels were rinsed with developing solution to remove excess silver solution and fresh developing solution was added in the container. The development was stopped

(after about 10 min) by discarding the developing solution and adding the stopping solution. The developed gels were scanned using a densitometer (Image Scanner III, GE Healthcare Biosciences, Uppsala, Sweden).

MS compatible silver staining method

MS compatible silver staining method was performed as described by (Shevchenko *et al.,* 1996). The method is similar to silver staining method as described earlier, with the omission of glutaraldehyde in the sensitizing solution.

3.2.1.4 Image analysis

The analysis of the protein spots obtained from the 2DE gels was carried out using Image Master Platinum v7.0 software (GE Healthcare Biosciences, Uppsala, Sweden). The detection of the spots was done automatically while the spot editing to remove artefacts was done manually. All spots were analysed in terms of percentage volume contribution, where the volume percentage of a protein was taken against the total spot volumes of all proteins in each gel.

3.2.2 MS analysis

3.2.2.1 In-gel trypsin digestion

To identify the protein of interest obtained from the 2DE gels, the 2DE run of the serum sample was repeated and the resulted gels were stained with MS compatible silver staining method (See 3.2.1.3). The protein spots were carefully excised and the gel plugs were kept in ultrapure H_2O in clean polypropylene microcentrifuge tubes at -20°C until further use. Protein digestion was performed as described by Seriramalu *et al*. (2010).

Standard solutions

50 mM sodium thiosulphate solution

Sodium thiosulphate (0.079 g) was thoroughly dissolved in 10 ml of ultrapure H_2O.

100 mM ammonium bicarbonate solution

Ammonium bicarbonate (7.91 g) was dissolved in 1 L of ultrapure H_2O.

50 mM ammonium bicarbonate solution

Ten ml of 100 mM ammonium bicarbonate solution was mixed with 10 ml of ultrapure H_2O.

Destaining solution: 15 mM potassium ferricyanide in 50 mM sodium thiosulphate

Potassium ferricyanide (0.049 g) was added to 10 ml of 50 mM sodium thiosulphate solution.

Reducing solution: 10 mM DTT in 100 mM ammonium bicarbonate

DTT (0.015 g) was dissolved in 10 ml of 100 mM ammonium bicarbonate.

Alkylation solution: 55 mM IAA in 100 mM ammonium bicarbonate

IAA (0.102 g) was dissolved in 10 ml of 100 mM ammonium bicarbonate.

Washing solution: 50% acetonitrile (ACN) in 50 mM ammonium bicarbonate

Five ml of 100% ACN was mixed with 5 ml of 100 mM ammonium bicarbonate.

Dehydration solution: 100% ACN

Digestion solution: 10 ng/µl trypsin in 50 mM ammonium bicarbonate

Trypsin stock solution (20 µg/ml) was diluted to 10 ng/ µl with 50 mM ammonium bicarbonate.

Extraction solution 1: 50% ACN

Five ml of 100% ACN was mixed with 5 ml of ultrapure H_2O.

Extraction solution 2: 100% ACN

Note: Destaining, alkylation and reducing solutions were freshly prepared prior to use.

Digestion procedure

The gel plugs were destained at room temperature with 100 µl of destaining solution for 15 min or until they were transparent. The destaining solution was discarded and the proteins in the gel plugs were reduced with reducing solution (150 µl) for 30 min at 60°C. The gel plugs were cooled to room temperature before the alkylation process. Reducing solution was discarded and alkylation solution (150 µl) was added to the gel plugs prior to 20 min incubation at room temperature (in the dark). Alkylation solution was discarded and the gel plugs were washed thoroughly for three times with 500 µl washing solution. Dehydration solution (50 µl) was added to dehydrate the plugs before being subjected to vacuum centrifugation to remove as much ACN as possible. Protein in the dried gel plugs was digested with 25 µl digestion solution and incubated at 37°C for 18 h. Extraction of the peptides from the gel plugs was carried out twice by using 50% ACN (extraction solution 1) and 100% ACN (extraction solution 2). Fifty µl of extraction solution 1 was added to the tube containing the gel plugs and the tube was subjected to moderate shaking for 15 min. All liquid in the tube was transferred to a

fresh microcentrifuge tube and 50 µl of extraction solution 2 was added. The gel plugs were subjected to moderate shaking again for 15 min before transferring all the liquid into the aforementioned microcentrifuge tube. The solution in the tube was lyophilised in a vacuum centrifuge and kept at -20ºC until further use.

3.2.2.2 Sample preparation for MS

The lyophilised peptides were reconstituted in 0.1% formic acid and desalted using reversed phase ZipTip® pipette tips containing C18 resin (Millipore, MA, USA).

Standard solutions

Conditioning solution: 50% ACN in H_2O

Five ml of 100% ACN was mixed with 5 ml of ultrapure H_2O.

Sample preparation/Equilibration/Washing solutions: 0.1% formic acid in H_2O

One µl of formic acid was mixed with 1 ml of ultrapure H_2O.

Elution solution: 0.1% formic acid in 50% ACN

One µl of formic acid was mixed with 1 ml of 50% ACN.

Desalting procedure

ZipTip® was set to 10 µl micropipette and the column was first conditioned with 10 µl conditioning solution. The conditioning of the column was repeated three times. The column was subsequently equilibrated with 10 µl equilibration solution, thrice. Binding of the peptide sample to the C18 resin was performed by repeatedly aspirating and dispensing 10 µl of the sample for 10 times. The column was washed thrice with 10 µl washing solution before elution of the sample. The peptides were

eluted from the column by pipetting the ZipTip® column into 1.5 µl elution solution repeatedly for 10 times without introducing air into the column. The eluted peptide was mixed with an equal amount of α-cyano-4-hydroxycinnamic acid matrix (5 mg/ml) and 0.7 µl of the mixture was immediately pipetted onto the Opti-TOF® 384-well MALDI plate insert (AB SCIEX, Foster City, California). The spots were allowed to dry completely before the plate was analysed using the 4800 Plus MALDI TOF/TOF™ analyser (AB SCIEX, Foster City, California).

3.2.2.3 Identification of proteins

The proteins were identified using MASCOT search engine (Matrix Science Ltd, London, UK). The data obtained was searched against *Homo sapien* entries in the Swiss-Prot database. The parameters were set as follows: enzyme – trypsin; missed cleavage – 1; variable modification – 2; (i) carbamidomethylation of cysteine, and (ii) oxidation of methionine; MS precursor ion mass tolerance – 100 ppm; MS/MS fragment ion mass tolerance – 0.2 Da, and inclusion of monoisotopic masses only.

3.2.3 Analysis of serum glycoproteome using lectins

3.2.3.1 Purification of CGB and CMB lectins

The crude lectin was isolated from the seeds of champedak (*Artocarpus integer*) fruits obtained from a local market using an in-house method developed in our laboratory as described by Abdul Rahman *et al*. (2002) and Hashim *et al*. (2001). The fresh champedak seeds were cleaned and dried at 37°C. The seeds were grounded to a fine powder before suspended in PBS pH 7.2 at 1:20 ratio and stirred for 24 h at 4°C. The mixture was centrifuged at 1500 X g (4°C) for 15 min and the supernatant was collected. The supernatant was subjected to ammonium sulphate precipitation (60%) while being stirred for 2 h at 4°C. The mixture was centrifuged again 1500 X g (4°C)

for 15 min and the pellet (crude extract) was collected and dissolved in minimal volume of cold 1X PBS pH 7.2. The crude extract was dialysed against 0.5X PBS pH 7.2 for 5 times (about 48 h) and was kept in aliquot of 1 ml at -20°C until use.

3.2.3.2 Preparation of galactose and mannose chromatography columns

Sugar affinity chromatography was used to purify the lectin from the crude extract of champedak seed. Galactose sugar coupled with the activated Sepharose 4B column was used to isolate the CGB lectin and mannose sugar coupled with the Sepharose 4B column was used to isolate the CMB lectin in the extract.

Activation of Sepharose 4B

Sepharose 4B (100 ml) was washed with 1 L distilled H_2O in a sintered glass funnel. The gel slurry was filtered under suction to a moist cake and transferred into a glass beaker. The gel was suspended in 100 ml 0.5 M sodium carbonate and stirred slowly in a fume hood at room temperature. Divinyl sulfone or DVS (10 ml) was gradually added over a period of 15 min to activate the gel. The gel suspension was stirred continuously for 1 h and washed extensively with distilled H_2O to remove the acid. The acidity of the filtrate was measured using a pH paper (due to corrosive nature of DVS) until it was no longer acidic.

Coupling of galactose and mannose to activated Sepharose 4B

The CGB lectin and CMB lectin were isolated from the crude extract of champedak seed using columns containing activated Sepharose 4B which were coupled with galactose sugar and mannose, respectively. The DVS activated Sepharose 4B (20 ml) was mixed with an equal volume of 20% w/v D-galactose or D-mannose in 0.5 M sodium carbonate. The mixture was left to stir continuously for 24 h at room

temperature. The gel was washed extensively with 2 L of distilled H_2O and later with 2 L of 0.5 M sodium bicarbonate. The washed gel was suspended in 0.5 M sodium bicarbonate that contained 5% β-mercaptoethanol (to block excess vinyl reactive groups) and was left to stir for 2 h in a fume hood at room temperature. The gel was washed again with 2 L of distilled H_2O followed with 2 L of PBS. The immobilised sugar-coupled Sepharose 4B gel was packed into polypropylene columns and stored at 4°C.

3.2.3.3 Isolation and purification of CGB and CMB lectins via column chromatography

The CGB and CMB lectins were isolated and purified from the crude lectin extract of champedak seed (see 3.2.3.1) using the galactose- and mannose-coupled Sepharose 4B columns (see 3.2.3.2). To isolate the CGB lectin, the galactose column was first pre-equilibrated with PBS buffer before applying it with 5 ml of crude lectin extract. PBS pH 7.2 was added to wash the unbound substances. The unbound fractions of 10 ml each was collected and the absorbance for each fraction was monitored at 280 nm with a spectrophotometer (Hitachi U-2000 Spectrophotometer, Hitachi, Ibaraki, Japan) with PBS served as the blank. Once the absorbance had fallen to the baseline (A280 < 0.005), the bound fractions (containing CGB lectin) were eluted with 0.8 M galactose in PBS pH 7.2. Bound fractions of 10 ml each was collected and the absorbance of each was monitored at 280 nm with 0.8 M galactose in PBS served as the blank. Fractions with high absorbance values were pooled and dialysed against six changes of PBS pH 7.2 at 4°C. The dialysed bound fractions were lyophilised to concentrate the CGB lectin and were stored at -20°C until use. To isolate the CMB lectin, the crude extract was applied to the mannose column and similar procedures were followed with the exception of the elution solution, where 0.8 M mannose in PBS pH 7.2 was used

instead, to elute out the CMB-bound fraction from the mannose column. The CMB-bound fractions were similarly dialysed, lyophilised and stored -20°C until further use.

3.2.3.4 Assessing the purity of CGB and CMB lectins using SDS-PAGE

The concentration of the lectins was determined using a commercial BCA protein assay kit (Pierce™ BCA Protein Assay Kit, Rockford, Illinois) according to manufacturer's instruction, before subjected to SDS-PAGE to determine the purity of the isolated lectins. SDS-PAGE was carried out using 18% separating tris-glycine gel.

Standard solutions

All solutions were stored at 4°C, unless stated otherwise.

Solution A: 30% acrylamide, 0.8% N,N'-methylenebisacrylamide

This solution was prepared as previously described in Section 3.2.1.2.

Solution B (4X resolving buffer): 1.5 M Tris-HCl, pH 8.8

This solution was prepared as previously described in Section 3.2.1.2.

Solution C: 10% (w/v) SDS

This solution was prepared as previously described in Section 3.2.1.2.

Solution D: 10% (w/v) APS

This solution was prepared as previously described in Section 3.2.1.2.

Solution E: TEMED

Solution F: 0.5 M Tris-HCl, pH 6.8

Tris (6.1 g) was dissolved in 50 ml distilled H_2O and the pH was adjusted to 6.8 using HCl. The solution was made up to 100 ml with distilled H_2O.

4X SDS-PAGE sample buffer: 62.5 mM Tris-HCl pH 6.8, 10% (w/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT and 0.01% (w/v) bromophenol blue

Solution F (2.5) ml was mixed with 2 ml glycerol, 400 mg SDS, 200 mg DTT and a few grains of bromophenol blue. The solution was made up to 20 ml with distilled H_2O.

SDS-PAGE electrophoresis buffer: 25 mM Tris, 198 mM glycine, 0.1% (w/v) SDS, pH 8.3

Tris (1.22 g) was mixed with 5.94 g glycine and 0.4 g SDS. The solution was made up to 1 L with distilled H_2O and pH was adjusted to 8.3.

Preparation of the separating and the stacking gels

The separating gel (18%) was prepared as shown in Table 3.5. The mixture was pipetted into the gel cassette, leaving 2 cm space from the top for the stacking gel. The top of the gel was carefully layered with distilled H_2O to get an even gel surface. The gel was left to completely polymerised and the H_2O overlay was removed. The stacking gel mixture was loaded on top of the separating gel, a comb was inserted and was allowed to polymerise.

Table 3.5: Preparation of separating gel (18%) and stacking gel (4%) for SDS-PAGE

Solution	Volume	
	Separating gel (18%)	Stacking gel (4%)
Solution A (ml)	12.00	0.65
Solution B (ml)	5.00	-
Solution C (µl)	200	50
Solution D* (µl)	100	25
Solution E* (µl)	6.6	5.0
Solution F (ml)	-	1.25
Distilled H_2O (ml)	2.69	3.05
Total volume (ml)	20.00	5.03

*Added prior to use.

SDS-PAGE run

The lectin (~60 µg each of CGB and CMB lectins) was mixed with sample buffer at a ratio of 3:1 in a microcentrifuge tube. The sample mixture was heated to 100°C for 10 min and left to cool at room temperature. The mixture was loaded into a well next to a broad range molecular weight marker. The electrophoresis run was carried out using Mini PROTEAN® Tetra Cell and PowerPac™ HC Power Supply (BioRad Laboratories, Hercules, USA) at a constant voltage of 60 V for the first 20 min and then the voltage was increased to 90 V until the blue dye front reached 1 cm from the bottom of the gel. The gel was removed from the cassette and placed in a container. Hot Coomassie Blue stain (10% (v/v) acetic acid, 0.1% (w/v) Coomassie Brilliant Blue R250) was poured and the container was left to shake on a rocking platform for 30 min. The staining solution was removed and the gel was rinsed with 20 ml destaining solution (10% (v/v) acetic acid solution). The destaining solution was added and the container was left to shake on the rocking platform overnight. The destained gel was scanned using a densitometer (Image Scanner III, GE Healthcare Biosciences, Uppsala, Sweden).

3.2.3.5 Preparation of enzyme conjugated CGB and CMB lectins

(a) ***CGB lectin conjugated with activated horseradish peroxidase (HRP) (CGB-HRP)***

The conjugation of CGB lectin to HRP was carried out as described by Hudson and Hay (1980).

Standard solutions

0.1 M sodium periodate

Sodium periodate solution was freshly prepared by dissolving 21.39 mg of sodium periodate in 1 ml of distilled H_2O.

1 mM sodium acetate buffer, pH 4.4

Sodium acetate trihydrate (13.608 g) was dissolved in 90 ml of distilled H_2O and subsequently, 0.437 ml of 1 M glacial acetic acid was added. The solution was brought up to 100 ml with distilled H_2O.

0.1 M sodium carbonate buffer, pH 9.5

Sodium carbonate (5.3 g) and sodium hydrogen carbonate (4.2 g) were dissolved in 300 ml of distilled H_2O. The pH was adjusted to 9.5 and the solution was brought up to 500 ml with distilled H_2O.

0.4% (w/v) sodium borohydride

Sodium borohydride (4 mg) was dissolved in 1 ml of cold distilled H_2O. The solution was kept cold until use.

0.1 M borate buffer, pH7.4

The borate solution was prepared by dissolving 9.54 g disodium tetraborate in 250 ml of distilled H_2O. Boric acid (24.73 g) was added to 4 L of distilled H_2O to make the boric acid solution. The pH of the borate acid was adjusted to 7.4 with the addition of approximately 115 ml of borate solution.

Conjugation of CGB lectin to HRP

HRP solution was prepared by dissolving 4 mg of HRP with 1 ml of distilled H_2O. Fresh 0.1 M sodium periodate solution (200 µl) was added to the HRP solution and the mixture was stirred for 20 min at room temperature and subsequently dialysed overnight against 0.1 mM sodium acetate buffer at 4°C. The pH of the dialysed mixture was adjusted to 9.5 by adding about 25 µl of 0.1 M sodium carbonate buffer. One ml of CGB-lectin (2 mg/ml) was added to the mixture and stirred for 2 h at 4°C before the addition of 100 µl fresh sodium borohydride solution to reduce the remaining free enzyme. The conjugated CGB-HRP was kept at 4°C or diluted with 60% glycerol in borate buffer at 1: 1 ratio for longer storage life.

(b) ***CMB lectin conjugated with alkaline phosphatase (AP) (CMB-AP)***

Standard solutions

0.1 M sodium carbonate/hydrogen carbonate buffer, pH 9.4

Sodium carbonate (5.3 g) and sodium hydrogen carbonate (4.2 g) were added to 200 ml of distilled H_2O and the pH of the solution was adjusted to 9.4. The buffer was made up to 500 ml using distilled H_2O.

0.2 M sodium borohydride

Sodium borohydride (8 mg) was dissolved in 1 ml of cold distilled H_2O and maintain at 4°C until use. This solution was freshly prepared prior to use.

2 M trithethanolamine solution, pH 8.0

Triethanolamine (2.66 ml) was added to 3 ml of distilled H_2O. The pH was adjusted to 8.0 with 25% HCl and made up to 10 ml with distilled H_2O.

1 M glycine solution, pH 7.0

Glycine (0.75 g) was dissolved in 6 ml of distilled H_2O and the pH was adjusted to 7.0 with 0.1 M NaOH. The solution was made up to 10 ml with distilled H_2O.

Triethanolamine buffer, pH 7.6 (50 mM triethanolamine solution pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 10 mM glycine, 0.1% w/v NaN_3)

Triethanolamine (1.6 µl), NaCl (21.92 g), $MgCl_2$ (0.51 g), $ZnCl_2$ (0.034 g), glycine (1.88 g), and NaN_3 (2.5 g) were dissolved in 1.5 L of distilled H_2O. The pH was adjusted to 7.6 and the buffer was made up to 2.5 L with distilled H_2O.

Conjugation of CMB lectin to AP

Fifty µl of CMB lectin (7 mg/ml in carbonate buffer) was added to 100 µl AP (6 times the concentration of CMB lectin). The mixture was incubated overnight at 4°C. The reaction was terminated with the addition of 20 µl of 2M triethanolamine (pH 8.0) and 40 µl of cold sodium borohydride. The solutions were mixed well and incubated at 4°C for 30 min. Five µl of 2M triethanolamine was added and the mixture was further incubated at 4°C for 2 h. To stabilize the conjugate, 10 µl of 1 M glycine (pH 7) was added to the mixture before subjected to extensive dialysis against five changes (500 ml

each) of triethanolamine buffer (pH 7.6) at 4°C. The conjugated CMB-AP was kept in aliquots of 15 µl in microcentrifuge tubes and stored at -80°C until use. For a longer storage life, BSA (10 mg/ml) and NaN_3 (1 mg/ml) can be added to the CMB-AP mixture.

3.2.3.6 Western blotting and detection of serum glycoproteins

Proteins from pooled serum samples from each control group (C1, C2, and C3) and bone tumours (OS, EW, CH, PL and GCT) were first resolved using 2DE (as described in Section 3.2.1 before being subjected to Western blot analysis using enzyme-conjugated CGB and CMB lectins. The 2DE gels were transferred electrophoretically onto nitrocellulose membranes and probed with CGB-HRP and CMB-AP to detect the serum O-glycosylated and N-glycosylated proteins, respectively.

Standard solutions

Transfer buffer (40 mM glycine, 0.1 M Tris, 0.038 (w/v) SDS, 20 % (v/v) methanol)

Glycine (2.93 g), Tris (5.81 g), SDS (0.375 g) and 200 ml methanol were first dissolved in 900 ml of distilled H_2O and then made up to 1 L with distilled H_2O.

Tris buffered saline 10X (TBS)

Tris base (24 g) and NaCl (88 g) were dissolved in 900 ml of distilled H_2O. The pH of the buffer was adjusted to 7.6 with HCl and distilled H_2O was added to a final volume of 1 L. For a 1X solution, 1 part of TBS 10X was mixed with 9 parts of distilled H_2O.

Tris buffered saline-Tween-20, TBS-T (TBS with 0.1% (v/v) Tween-20)

Tween-20 (1 ml) was added to 1 L of 1X TBS and the solution was mixed thoroughly.

Electrophoretic transfer

The electrophoretic transfer of the 2DE gels onto nitrocellulose membranes (0.45 µm) was carried out using a semidry transfer system (NovaBlot Kit of the Multiphor™ II Flatbed System, GE Healthcare Biosciences, Uppsala, Sweden). The graphite anode plate was saturated with transfer buffer before placing the transfer stack onto it. The transfer stack consists of three layers of filter paper, followed by the membrane, the gel and another three layers of filter paper before placing the graphite cathode plate on top of the stack, which was also saturated with transfer buffer. All the filter papers, gel and membrane were pre-soaked with transfer buffer. The transfer stack was flattened with a roller to remove all trapped air bubbles. The transfer was carried out for 2 h at a constant current of 0.8 mM/cm^2. After the transfer, the membrane was immersed in 3% gelatine in TBS-T for 2 h to prevent non-specific background binding. The membrane was washed extensively thrice (10 min each) with TBS-T.

For the detection of O-glycosylated proteins, the membrane was incubated with CGB-HRP in TBS-T (1 µg/ml) at 4°C overnight. The membrane was washed again thrice before the colour development. The membrane was developed with freshly prepared 3,3'-diaminobenzidine (DAB) substrate (BioRad Laboratories, USA) consisting 50 mg DAB and 100 µl of 3% hydrogen peroxide (H_2O_2) in 100 ml 1X TBS and the reaction was stopped by washing the membrane twice with distilled H_2O.

For the detection of N-glycosylated proteins, the membrane was incubated with CMB-AP (1:10000 dilution) in cold TBS-T at 4°C overnight. The membrane was washed again thrice before the colour development using BCIP®/NBT tablet. The reaction was stopped after sufficient colour development was achieved by washing the membrane twice with distilled H_2O. All membranes were air dried prior to

densitometric scanning. The image analysis of the protein spots obtained from the membranes was carried out as described in Section 3.2.1.4.

3.2.4 Validation for serum amyloid A (SAA) using Western blot and ELISA

SAA, a protein that exhibited the most significant change in abundance in the patients with OS and PS (as detected from silver 2DE analysis) was selected to be validated using Western blot and ELISA. Three group of sarcomas were selected in the validation study *i.e.* OS, CS and PS, as well as their respective control groups, C1 and C2.

3.2.4.1 Validation of SAA using SDS-PAGE and Western blot analysis

(a) ***Detection of SAA using SDS-PAGE***

Pooled serum samples from each control groups (C1 and C2) and patients' samples (OS, CS and PS) were analysed by Western blotting to confirm the expression of significantly altered proteins attained from the 2DE analysis. Total protein concentrations were determined for each sample to ensure equal amount of samples (60 µg) were loaded onto the gel. Protein concentrations were determined using a commercial protein assay kit (Pierce™ BCA Protein Assay Kit, Rockford, Illinois) according to manufacturer's instruction.

The SDS-PAGE was carried out as outlined Section 3.2.3.4. The percentage of the Tris-glycine gel used was 12.5% (Table 3.6). Samples (60 µg) were mixed with sample buffer at a ratio of 3:1 in a microcentrifuge tube. The sample mixture was heated to 100°C for 10 min and was left to cool at room temperature. The mixture was loaded into a well next to a broad range molecular weight marker. The electrophoresis run was carried out at a constant voltage of 60 V for the first 20 min and then the voltage was

increased to 90 V until the blue dye front reached 1 cm from the bottom of the gel. The gel was removed from the cassette and subjected to electrophoretic transfer.

Table 3.6: Preparation of separating gel (12.5%) and stacking gel (4%) for SDS-PAGE

Solution	Volume	
	Separating gel (12.5%)	Stacking gel (4%)
Solution A (ml)	8.33	0.65
Solution B (ml)	5.00	-
Solution C (µl)	200	50
Solution D* (µl)	100	25
Solution E* (µl)	6.6	5
Solution F (ml)	-	1.25
Distilled H_2O (ml)	6.36	3.05
Total volume (ml)	20.00	5.03

*Added prior to use

(b) ***Western blotting and detection of SAA using SAA antibody***

Standard solutions

Transfer buffer (40 mM glycine, 0.1 M Tris, 0.038 (w/v) SDS, 20 % (v/v) methanol)

Glycine (2.93 g), Tris (5.81 g), SDS (0.375 g) and 200 ml methanol were first dissolved in 900 ml of distilled H_2O and then made up to 1 L with distilled H_2O.

TBS (10X)

Tris base (24 g) and NaCl (88 g) were dissolved in 900 ml of distilled H_2O. The pH of the buffer was adjusted to 7.6 with HCl and distilled H_2O was added to a final volume of 1 L. For a 1X solution, 1 part of TBS 10X was mixed with 9 parts of distilled H_2O.

TBS-T (TBS with 0.1% (v/v) Tween-20)

Tween-20 (1 ml) was added to 1 L of 1X TBS and the solution was mixed thoroughly.

Electrophoretic transfer

Polyvinylidene difluoride (PVDF) membrane was used and the electrophoretic transfer of the SDS-PAGE gel onto membrane was carried out using a semidry transfer system (NovaBlot Kit of the Multiphor™ II Flatbed System, GE Healthcare Biosciences, Uppsala, Sweden). The graphite anode plate was saturated with transfer buffer before placing the transfer stack onto it. The transfer stack consists of three layers of filter paper, followed by the membrane, the gel and another three layers of filter paper before placing the graphite cathode plate on top of the stack, which was also saturated with transfer buffer. All the filter papers and gel were pre-soaked with transfer buffer while the PVDF membrane was pre-soaked in 100% methanol prior to the transfer. The transfer stack was flattened with a roller to remove all trapped air bubbles. The transfer was carried out for 1 h at a constant current of 0.8 mM/cm^2.

Following the transfer, the membrane was immersed in 5% w/v skimmed milk in TBS-T for 2 h to prevent non-specific background binding. The membrane was washed extensively thrice (10 min each) with TBS-T. To detect the SAA protein, the membrane was incubated overnight at 4°C in rabbit anti-human SAA antibody at a dilution of 1:2000. The membrane was washed extensively again with TBS-T and incubated with HRP-conjugated goat anti-rabbit IgG at 1:5000 dilution for 2 h at room temperature. The membrane was washed thrice and developed using DAB as described in Section 3.2.3.6. The membranes were air dried prior to densitometric scanning and analysed using ImageJ v1.46r software (http://imagej.nih.gov/ij).

3.2.4.2 Validation of SAA using ELISA

Sandwich ELISA was performed to measure the concentration of SAA in the serum samples. The assay was performed according to manufacturer's instruction (Serum amyloid A Human ELISA kit, ab100635, Abcam, Cambridge, UK).

3.2.5 Quantitative proteomics by SWATH™-MS

Quantitative SWATH™-MS analysis was carried out on two selected samples, control C1 and patients with OS. Pooled serum samples from each of these groups were first subjected to albumin and IgG depletions to remove the high abundance albumin and immunoglobulins and enrich the low abundance serum proteins.

3.2.5.1 Albumin and IgG depletion

ProteoExtract™ Albumin/IgG Removal kit from Calbiochem® was used according to the manufacturer's instruction. Protein concentrations of the depleted samples were determined using a commercial protein assay kit (Pierce™ BCA Protein Assay Kit, Rockford, Illinois) according to manufacturer's instruction. The depleted samples were then lyophilised prior to tryptic digestion.

3.2.5.2 In-solution tryptic digestion

Standard solutions

Digestion buffer: ~50 mM ammonium bicarbonate

Ten mg of ammonium bicarbonate was dissolved in 2.5 ml of ultrapure H_2O.

Reducing buffer: ~100 mM DTT

Eight mg of DTT was dissolved in 500 µl of ultrapure H_2O. This solution must be freshly prepared prior to use.

Alkylation buffer: ~100 mM IAA

Nine mg of IAA was dissolved in 500 µl of ultrapure H_2O. This solution must be freshly prepared and kept in dark prior to use.

Trypsin solution: 10 ng/µl trypsin in 50 mM ammonium bicarbonate

Trypsin stock solution (20 µg/ml) was diluted to 10 ng/ µl with 50 mM ammonium bicarbonate.

In-solution tryptic digestion

In-solution tryptic digestions of the depleted samples were carried out according to the optimized method established in our laboratory. The digestion buffer (300 µl of 50 mM ammonium bicarbonate) and reducing buffer (30 µl of 100 mM DTT) were added to a 1.5 ml microcentrifuge tube. The lyophilised proteins were reconstituted in 200 µl of ultrapure H_2O (to a final concentration of 2 mg/ml protein) and added into the tube. The final volume was adjusted to 540 µl with ultrapure H_2O. The mixture was incubated at 95°C for 5 min and was allowed to cool at room temperature. Alkylation buffer (60 µl of 100 mM IAA) was added and the tube was incubated in the dark at room temperature for 20 min. Trypsin (Promega, Madison, WI, USA) was added to the protein mixture with a ratio of enzyme to protein of 1:20 (w/w) and the digestion was carried out for 18 h at 37 °C. The digested proteins were kept at -20°C until further analysis.

3.2.5.3 LC-MS/MS run and SWATH™ acquisition

The LC-MS/MS run was carried out for both C1 and OS lyophilized samples. The samples were reconstituted in 20 µl of 50 mM ammonium bicarbonate and 1 µl of the protein solution was loaded onto a microfluidic trap column packed with ChromXP

C18-CL 3 µm particles (300 Å nominal pores size; equilibrated in 0.1 % formic acid/5 % ACN) at 5 µl/min using an Eksigent NanoLC Ultra® 2D cHiPLC system. An analytical (15 cm x 75 µm ChromXP C18-CL3) microfluidic column was then switched in line and peptides separated using linear gradient elution of 0-80 % ACN over 90 min (300 nl/min). Separated peptides were analysed using an AB SCIEX 5600 Triple TOF® mass spectrometer equipped with a Nanospray III ion source. The following ionization source parameters were used; the ion spray voltage was set at 2400 V, curtain gas at 22 L/min, ion source gas at 8 L/min and an interface heater temperature setting of 150 ºC. Data acquisition was subjected to two separate acquisition, i) Information Dependent Acquisition (IDA) for discovery proteomics and ii) SWATH™ for quantitative proteomics.

In IDA mode, data acquisition performed using an MS/MS switch criteria included ions of m/z >200 amu, the charge state of +2 to +5, intensity >40 cps and the top 30 ions meeting this criterion were selected for MS/MS per cycle. For quantitative proteomics, each sample was subjected to technical triplicate using SWATH™ acquisition on AB SCIEX Triple TOF® 5600 mass spectrometer. The instrument was regulated to allow for a quadrupole resolution of 25 Da/mass selection over a precursor mass range of 350 – 1250 Da.

The raw data generated was analysed with ProteinPilot™ v4.5 software against a Swiss-Prot *Homo sapien* database and peptide and protein identities determined subject to strict bioinformatics criteria that included the use of a decoy database to calculate the false discovery rate (FDR). An FDR cut-off of 1 % was applied and the filtered dataset was further analysed manually to exclude redundant peptides and known contaminants. The following ProteinPilot™ search parameters were used; IAA selected as cysteine alkylation, trypsin digestion, instrument specific settings for AB SCIEX Triple TOF®

5600 mass spectrometer (MS tolerance 0.05 Da, MS/MS tolerance 0.1 Da, charge state +2-5), biological modification probabilistic features on, thorough ID algorithm, and detected protein threshold of 0.05. The ProteinPilot™ data were used to create an ion library for subsequent SWATH™ data processing.

The SWATH™ acquisition data were imported into and processed using SWATH™ microapplications, PeakView™ with SWATH™ processing and MarkerView™. PeakView™ software enable the exploration and interpretation of mass spectral data with specific tools for processing accurate mass data, structural interpretation and batch analysis. MarkerView™ software reviewed and subjected the peak integration data to a statistical analysis to determine the dysregulation of the proteins in the samples.

3.2.6 Multiple reaction monitoring (MRM) analysis

Absolute quantification of the two isoforms of SAA present in the samples, serum amyloid A1 (SAA1) and serum amyloid A2 (SAA2) was carried out by MRM analysis. Pooled samples from selected group of controls (C1 and C2) and tumour patients (OS, CS and PS) were subjected to in-solution tryptic digestion.

3.2.6.1 In-solution tryptic digestion

In-solution tryptic digestion was carried out similarly as described in Section 3.2.5.2, but on a smaller scale. The digestion buffer (15 µl) and reducing buffer (1.5 µl) were added into a 500 µl microcentrifuge tube. Pooled neat serum sample from each group (containing 100 µg total protein) was added into the tube. The final volume was adjusted to 27 µl with ultrapure H_2O. The mixture was incubated at 95°C for 5 min and was allowed to cool at room temperature. Alkylation buffer (3 µl) was added and the tube was incubated in the dark at room temperature for 20 min. Trypsin solution was added to the protein mixture with a ratio of enzyme to protein of 1:20 (w/w) and the

digestion was carried out for 18 h at 37°C. The digested proteins were lyophilized and reconstituted in 20 µl 0.1% formic acid in H_2O (v/v).

3.2.6.2 MRM analysis

Four peptides were selected for this targeted MRM analysis, as proposed in the publication by Sung and co-workers (Sung *et al.*, 2012). Two unlabelled synthetic peptides were selected as external standards for the targeted proteins *i.e.* SAA1-1 and SAA1-2, which were found to be unique for SAA1 (P0DJI8) and the other two, SAA2-1 and SAA2-2, unique for SAA2 (P0DJI9). The peptides were synthesized commercially by Chiron Mimotopes (Clayton, Victoria, Australia) at more than 90% purity. The amino acid sequences of these peptides and their transitions for the MRM experiment are listed in Table 3.7. These peptides were dissolved in ultrapure H_2O to an initial concentration of 10 mg/ml. Each peptide was diluted to a series of different concentrations (0-20 µg/ml) to construct a standard curve.

The MRM analysis was carried out using Waters ACQUITY UPLC coupled with a Xevo™ Tandem Quadrupole Mass Spectrometer (Waters Corp, Manchester, UK). The injection volume for all samples and the synthetic peptides was 10 µL. Peptides were separated in the Waters ACQUITY UPLC BEH column (2.1 X 100 mm, 1.7 µm) using two mobile phases, *i.e.* mobile phase A, consisted of 0.1% formic acid in H_2O and mobile phase B, which was made of 0.1% formic acid in ACN. The peptide elution gradient was delivered at 0.2 ml/min with column temperature set at 40ºC, and executed as follows: 10% B at start, 30% B at 7 min, 90% B at 8 min, 10 % B at 10.1 min and

Table 3.7: Sequence for the synthetic pure peptides specific for protein SAA1 and SAA2 and their MRM transitions used for the experiment

Protein	Peptide	Peptide sequence	Molecular mass (Da)	Precursor ion *(m/z)*	Product ion *(m/z)*	Cone voltage (V)	Collision energy (V)
SAA1	SAA1-1	GPGGVWAAEAISDAR	1456.2	729.00	554.00	25	30
					561.00	25	30
					632.00	25	30
	SAA1-2	FFGHGAEDSLADQAANEWGR	2177.1	727.00	732.00	24	28
					803.00	24	28
					1046.00	24	30
SAA2	SAA2-1	GPGGAWAAEVISNAR	1454.8	728.40	859.00	25	30
					930.00	25	30
					1116.00	25	30
	SAA2-2	GAEDSLADQAANK	1288.6	645.00	646.00	25	4
					717.00	25	25
					830.00	25	25

65

stop time at 12 min. The electrospray ionization was carried out under positive mode and the cone and collision energy were adjusted to get the optimum ionization and fragmentation of the peptides. The capillary voltage was maintained at 1.8 kV and the source temperature was kept constant at 120ºC. The nebulizing gas used was nitrogen, with the desolvation gas flow being maintained at 800 L/hr and cone gas flow at 5 L/hr. Argon was used as the collision gas for the collision-induced dissociation. The determination of the transition for each peptide was based on the method developed by (Sung *et al.*, 2012). The first highest transition was used as the quantifier and the second and the third highest transitions were used as the qualifier. Quantification was done based on the peak area. The acquired data was analysed using Masslynx software version 4.1.

3.2.7 Statistical analysis

All values in this study are expressed as mean ± S.E.M (standard error mean). The Statistical Package for Social Sciences (SPSS) version 22.0 (IBM Corporation, New York, USA) was used to analyse the data. The independent-sample t-test was used to analyse the differences between the means. Levene's Test For Equality of Variances was used to assess the homogeneity of the variances. A *p*-value of less than 0.05 was considered as significant.

CHAPTER 4: RESULTS

4.1 Analysis of serum protein profiles using 2DE

The unfractionated serum from non-tumour control samples (C1, C2 and C3) and five diseased groups (OS, ES, CS, PS and GCT) were resolved using 2DE and the results show complex protein profiles containing hundreds of spots (Figure 4.1). The developed profiles demonstrated an analogous pattern to the standard Swiss-Prot ExPASy plasma protein reference map (Sanchez *et al.*, 1995).

Based on the visual comparison between the protein reference map and the 2DE gel, 15 proteins were selected for analysis due their presence at high abundance and were well resolved in the 2DE gels. The proteins were alpha 1-antitrypsin (AAT), alpha-1B-glycoprotein (ABG), alpha-2-HS-glycoprotein (AHS), apolipoprotein A-I (APOA1), beta-2-glycoprotein 1 (B2G1), fragment of complement C3 (C3f), haptoglobin (HAP), hemopexin (HPX), kininogen-1 (KNG), leucine-rich alpha-2-glycoprotein (LRG), SAA, serotransferrin (STR), transthyretin (TTR), vitamin D binding protein (VTDB) and zinc-alpha-2-glycoprotein (ZAG). C3f protein was identified as a protein fragment (at ~34 kDa) as indicated by its lowered position in the 2DE gels (Figure 4.1) compared to the parent protein, protein C3, which has a theoretical molecular weight (Mr) of 187.03 kDa. The identities of these 15 proteins were confirmed using mass spectrometry analysis as shown in Table 4.1, indicating the details of the identified proteins *i.e.* the protein name, their abbreviation, Swiss-Prot accession number, Mr, calculated pI, the number of peaks matched, the protein score (MASCOT score) and percentage coverage. Protein with MASCOT score greater than 65 was considered as confidently identified ($p<0.05$).

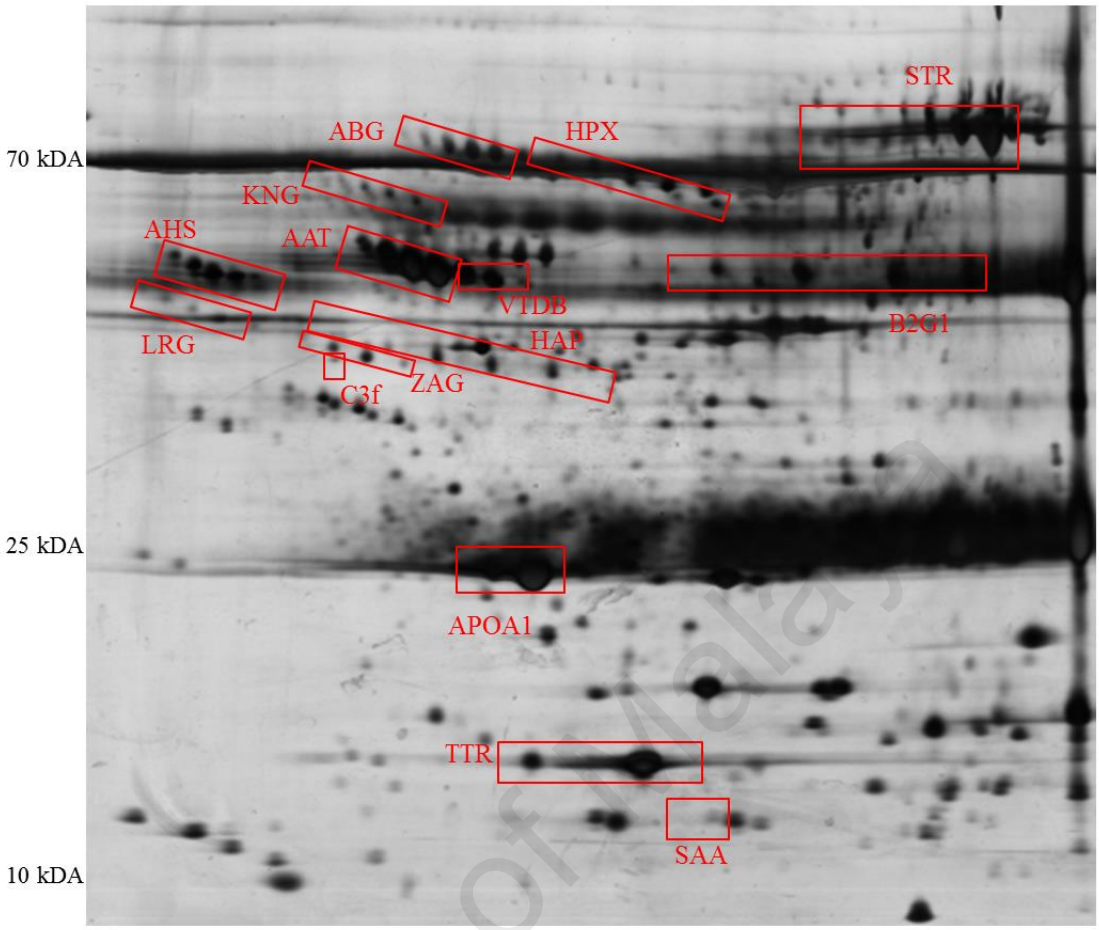


Figure 4.1: Typical representative of serum protein profile using silver stained 2DE

Serum sample was resolved using 2DE and the results show complex protein profiles containing hundreds of spots. The profiles acquired demonstrated a comparable pattern to the standard Swiss-Prot ExPASy plasma protein reference. The acidic side of the 2DE gel is to the left and relative molecular mass declines from the top. Visually, proteins with higher expression have higher intensity and vice versa.

Table 4.1: MS identification of serum proteins that were analysed in the study using MALDI TOF/TOF

Protein ID	Matched protein identity	Accession Number (Swiss-Prot)	Theoretical Mass - M_r (Da)	Theoretical pI	No of peaks matched	MASCOT score	Sequence Coverage (%)
AAT	Alpha1-antitrypsin	P01009	44223	5.37	23	504	51
ABG	Alpha-1B-glycoprotein	P04217	54220	5.56	16	198	27
AHS	Alpha-2-HS-glycoprotein	P02765	39300	5.43	15	444	35
APOA1	Apolipoprotein A-I	P02647	28061	5.27	28	716	56
B2G1	Beta-2-glycoprotein 1	P02749	38273	8.34	16	213	43
C3f[a)]	Complement C3	P01024	187030	6.02	22	150	8
HAP	Haptoglobin	P00738	45177	6.13	15	202	25
HPX	Hemopexin	P02790	51512	6.57	19	370	31
KNG	Kininogen-1	P01042	71912	6.34	24	370	25
LRG	Leucine-rich alpha-2-glycoprotein	P02750	38154	6.45	9	167	24
SAA	Serum amyloid A protein	P0DJI8	13532	6.28	11	217	63
STR	Serotransferrin	P02787	77000	6.81	38	716	38
TTR	Transthyretin	P02766	15877	5.52	9	425	63
VTDB	Vitamin D binding protein	P02774	51183	5.17	17	308	42
ZAG	Zinc-alpha-2-glycoprotein	P25311	34237	5.71	24	353	44

a) Identified as a protein fragment as indicated by its lowered position in the 2DE gels compared to the parent protein

Table 4.2 shows the protein occurrence of 15 proteins in all three control groups and disease groups. AAT, ABG, AHS, APOA1, B2G1, HAP, HPX, STR, TTR, VTDB and ZAG proteins were constantly present in all samples (100%). Three proteins, C3f, KNG and LRG, were present in at least 50% of all cases. However, the rate of presence of SAA protein in the control groups, C1, C2 and C3, was considerably lower (less than 60%) compared to its presence in the disease groups. Figure 4.2, 4.3 and 4.4 show representative profiles of serum proteins from patients with OS, ES, CS, PS, and GCT, with their respective control groups C1, C2 and C3.

The digitised images of silver stained 2DE gels were analysed using Image Master Platinum v7.0 software. Spot volume analysis was carried out on the 15 selected proteins (AAT, ABG, AHS, APOA1, B2G1, C3f, HAP, HPX, KNG, LRG, SAA, STR, TTR, VTDB and ZAG) as shown in Figure 4.1. The homogeneity of the data was assured using Levene's test and the level of significance were determined using independent-samples t-test. In this study, all spots were analysed in terms of percentage volume contribution, where the volume percentage of a protein was taken against the total spot volumes of all proteins in each gel. Figure 4.5 demonstrates the mean percentage volume contribution of all of the analysed protein (mean ± SEM for biological replicates) in each disease group compared to their respective age-matched control group. In patients with OS, seven proteins demonstrated significantly altered abundance compared to the control group C1 (Figure 4.5a). They were AAT, APOA1, HPX, SAA, STR, TTR and VTDB, in which AAT, SAA and VTDB were found to be significantly up-regulated whilst APOA1, HPX, STR and TTR were found to be significantly down-regulated in the patients.

Table 4.2: Protein occurrence in control groups and bone tumour groups from silver 2DE analysis

Protein	C1		C2		C3		OS		EW		CS		PS		GCT	
	(n=17)	%	(n=17)	%	(n=28)	%	(n=14)	%	(n=5)	%	(n=7)	%	(n=8)	%	(n=8)	%
AAT	17	100.0	17	100.0	28	100.0	14	100.0	5	100.0	7	100.0	8	100.0	8	100.0
ABG	17	100.0	17	100.0	28	100.0	14	100.0	5	100.0	7	100.0	8	100.0	8	100.0
AHS	17	100.0	17	100.0	28	100.0	14	100.0	5	100.0	7	100.0	8	100.0	8	100.0
APOA1	17	100.0	17	100.0	28	100.0	14	100.0	5	100.0	7	100.0	8	100.0	8	100.0
B2G1	17	100.0	17	100.0	28	100.0	14	100.0	5	100.0	7	100.0	8	100.0	8	100.0
C3f[a)]	15	88.2	13	76.5	22	78.6	12	85.7	4	80.0	7	100.0	8	100.0	8	100.0
HAP	17	100.0	17	100.0	28	100.0	14	100.0	5	100.0	7	100.0	8	100.0	8	100.0
HPX	17	100.0	17	100.0	28	100.0	14	100.0	5	100.0	7	100.0	8	100.0	8	100.0
KNG	17	100.0	17	100.0	28	100.0	14	100.0	5	100.0	5	71.4	5	62.5	8	100.0
LRG	17	100.0	17	100.0	28	100.0	14	100.0	4	80.0	6	85.7	5	62.5	8	100.0
SAA	3	17.6	10	58.8	12	42.9	12	85.7	5	100.0	5	71.4	5	62.5	6	75.0
STR	17	100.0	17	100.0	28	100.0	14	100.0	5	100.0	7	100.0	8	100.0	8	100.0
TTR	17	100.0	17	100.0	28	100.0	14	100.0	5	100.0	7	100.0	8	100.0	8	100.0
VTDB	17	100.0	17	100.0	28	100.0	14	100.0	5	100.0	7	100.0	8	100.0	8	100.0
ZAG	17	100.0	17	100.0	28	100.0	14	100.0	5	100.0	7	100.0	8	100.0	8	100.0

[a)] Identified as a protein fragment as indicated by its lowered position in the 2DE gels compared to the parent protein

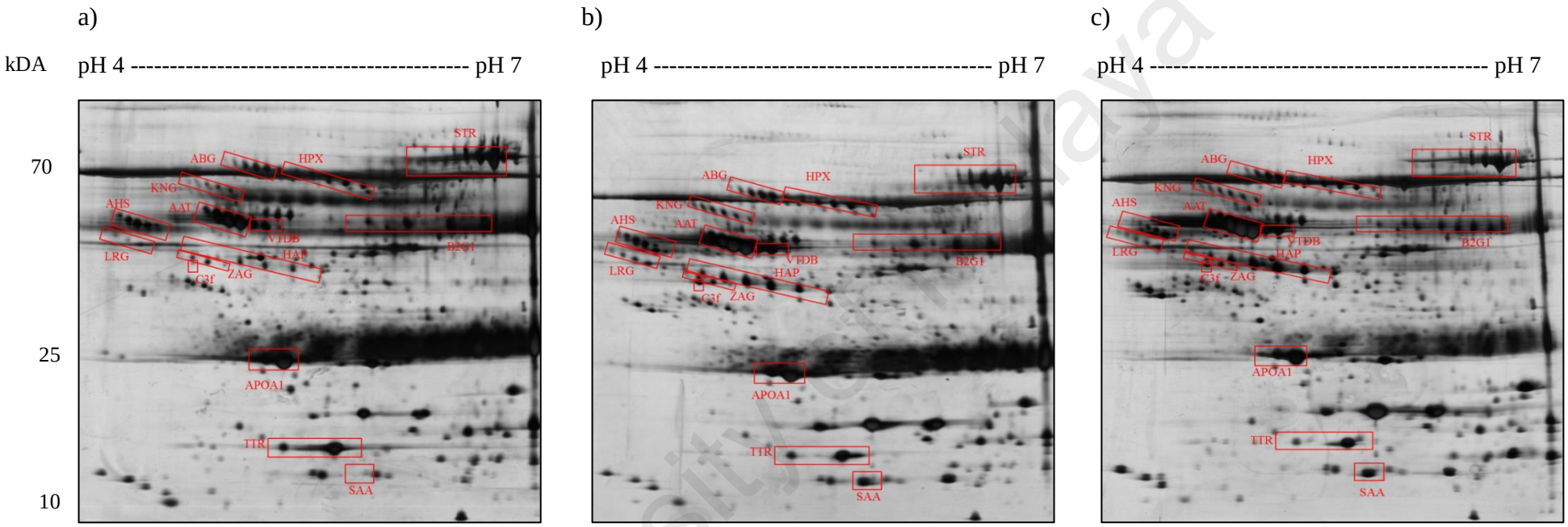


Figure 4.2: Typical serum protein profiles of healthy control C1 and patients with OS and ES using silver stained 2DE

Whole serum samples healthy subjects and patients with OS and ES were subjected to 2DE and silver staining. Panels demonstrate typical serum protein profiles of (a) a healthy individual from control group C1, (b) patient with OS and (c) patient with ES. For all panels, the acidic side of the 2DE gel is to the left and relative molecular mass declines from the top.

72

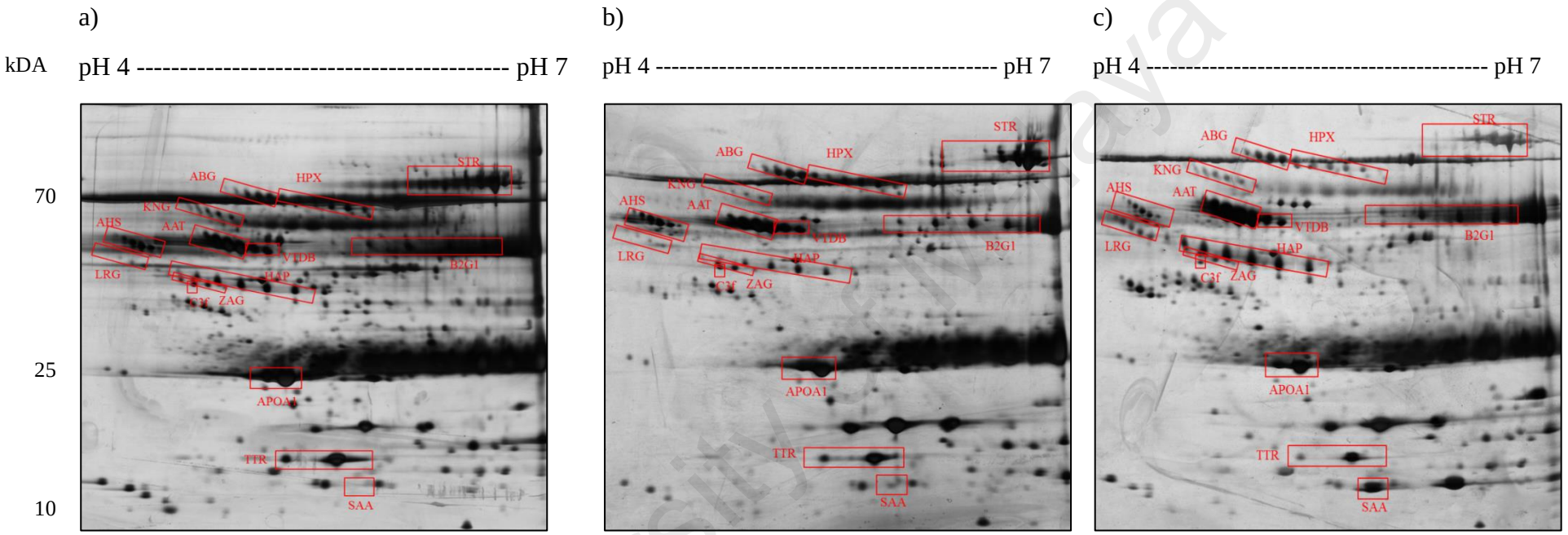


Figure 4.3: Typical serum protein profiles of healthy control C2 and patients with CS and PS using silver stained 2DE

Whole serum samples healthy subjects and patients with CS and PS were subjected to 2DE and silver staining. Panels demonstrate typical serum protein profiles of (a) a healthy individual from control group C2, (b) patient with CS and (c) patient with PS. For all panels, the acidic side of the 2DE gel is to the left and relative molecular mass declines from the top.

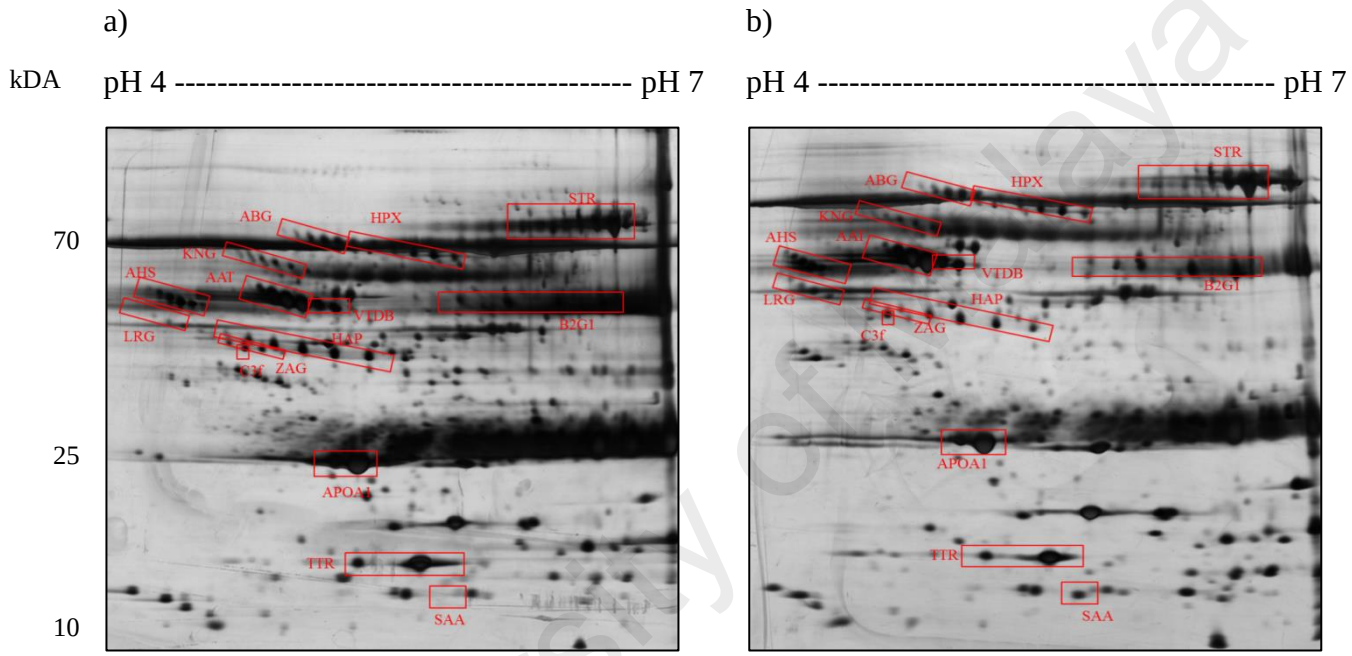
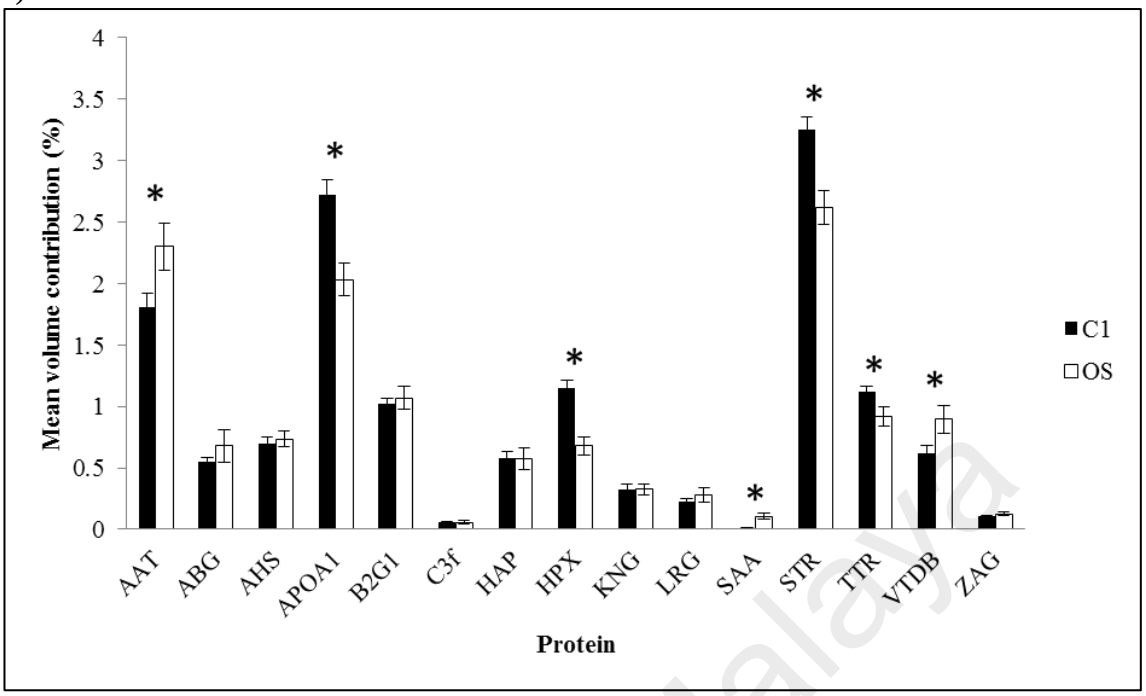


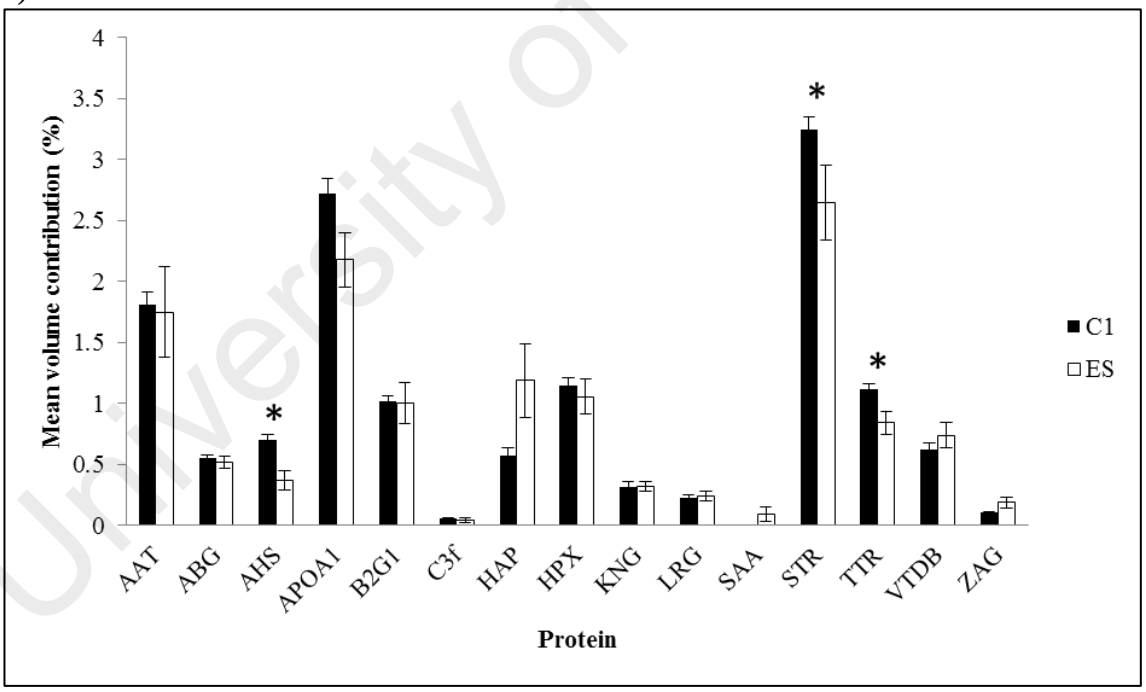
Figure 4.4: Typical serum protein profiles of healthy control C3 and patients with GCT using silver stained 2DE

Whole serum samples healthy subjects and patients with GCT were subjected to 2DE and silver staining. Panels demonstrate typical serum protein profiles of (a) a healthy individual from control group C3 and (b) patient with GCT. For all panels, the acidic side of the 2DE gel is to the left and relative molecular mass declines from the top.

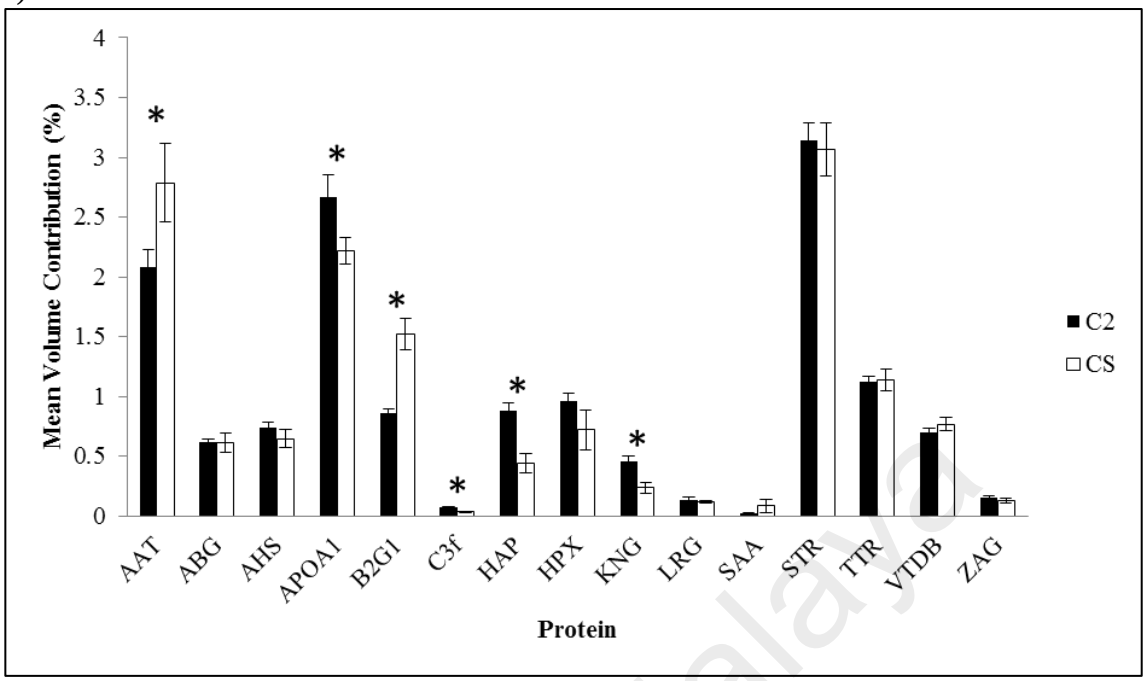
a)



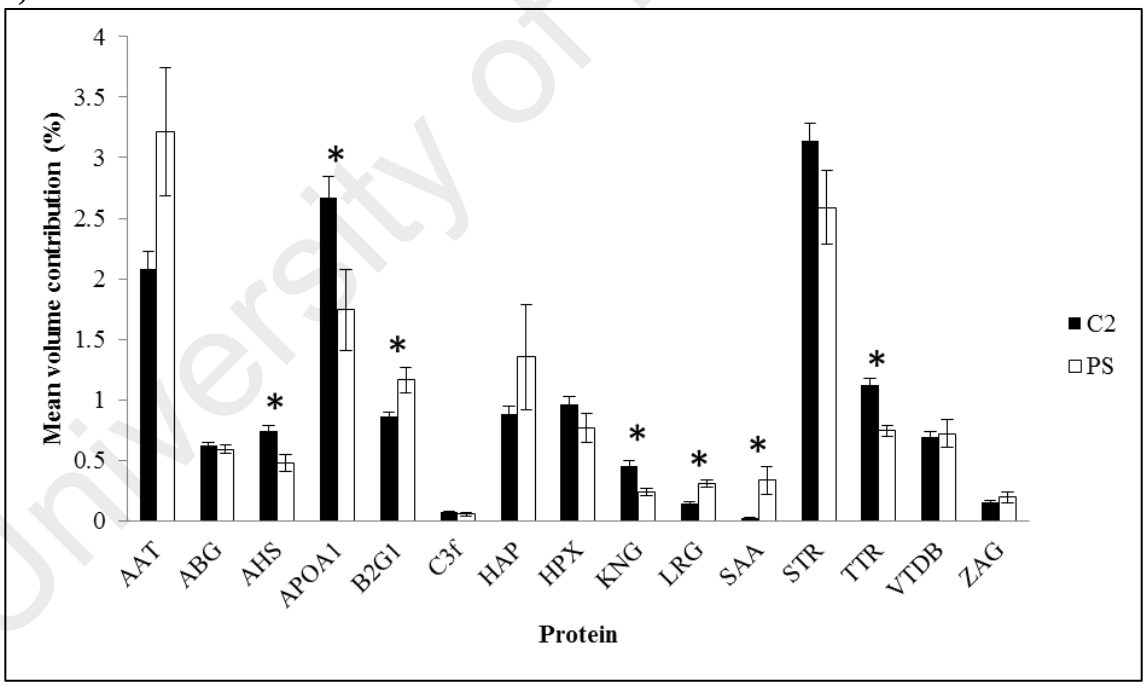
b)



c)



d)



e)

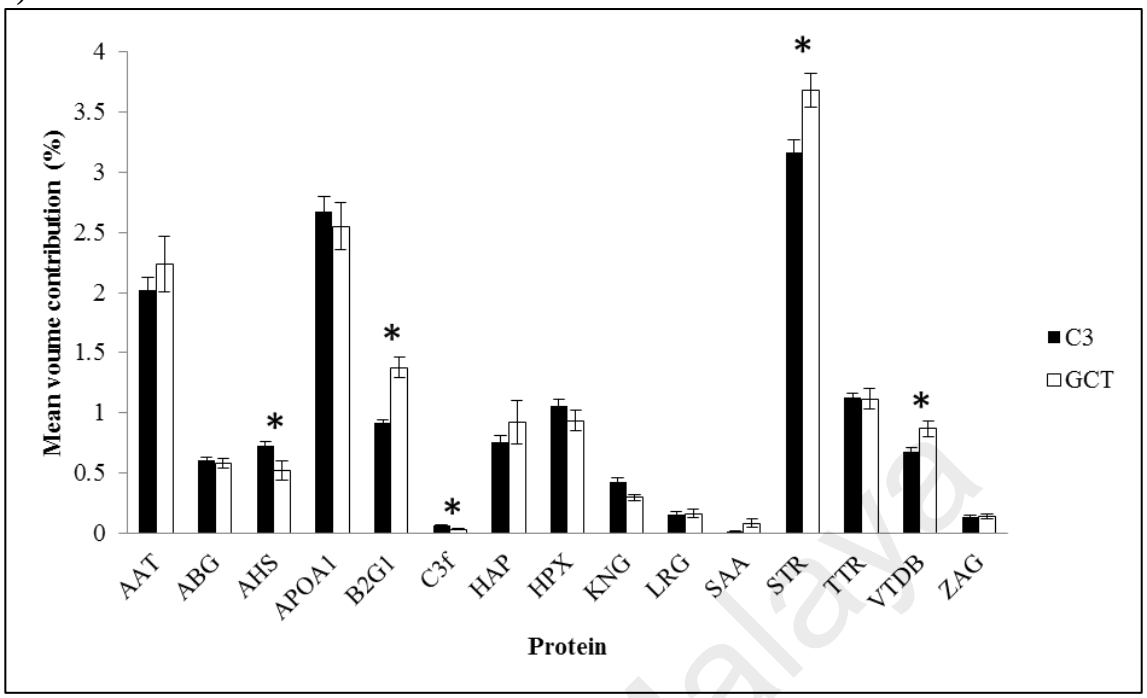


Figure 4.5: Mean percentage of volume contribution of 2DE resolved serum proteins

The mean percentage of volume contribution of 15 protein clusters were analysed using Image Master Platinum v7.0 software from the silver stained 2DE gels (mean ± S.E.M. of biological replicates). Densitometry analysis was performed on AAT, ABG, AHS, APOA1, B2G1, C3f, HAP, HPX, KNG, LRG, SAA, STR, TTR, VTDB and ZAG. Panels demonstrate mean percentage of volume contribution of (a) healthy individuals from control group C1 and patients with OS, (b) healthy individuals from control group C1 and patients with ES, (c) healthy individuals from control group C2 and patients with CS, d) healthy individuals from control group C2 and patients with PS, and e) healthy individuals from control group C3 and patients with GCT. Asterisk (*) denotes protein with significant alteration in abundance compared to the control group (p<0.05).

In patients with ES, only three proteins were found to exhibit significantly altered abundance compared to the control group, C1 (Figure 4.5b). These three proteins, AHS, STR and TRR were found to be significantly down-regulated in the patients. None of the analysed proteins demonstrated significant up-regulation compared to the control.

In patients with CS, six proteins *i.e.* AAT, APOA1, B2G1, C3f, HAP and KNG demonstrated significantly altered expression compared to the control group, C2 (Figure 4.5c). Among these, AAT and B2G1 were found to be significantly up-regulated and the rest were significantly down-regulated in the patients.

Seven proteins demonstrated significantly altered abundance in patients with PS, compared to the age-matched control C2 (Figure 4.5d). B2G1, LRG and SAA were found to be significantly up-regulated in the patients while AHS, APOA1, KNG and TTR were found to be down-regulated.

In the final group of bone tumour assessed, GCT, five proteins showed significantly altered abundance compared with the age-matched control group C3 (Figure 4.5e). The percentage mean volume contribution of three proteins, B2G1, STR and VTDB were found to be significantly higher in the patients' serum, while two proteins, AHS and C3f were found to be significantly lower compared to the control.

To summarise the differences in the abundance of the proteins in the diseased groups compared to the control groups, fold change (FC) difference for each protein was calculated. FC is a value describing the ratio of the measured value for an experimental sample to the value of the control sample. If this number was less than one the (negative) reciprocal is listed. The relative abundance of the analysed serum proteins in each disease group compared with their respective controls is demonstrated in Table 4.3.

Table 4.3: Relative abundances of serum proteins from silver 2DE analysis in patients with bone tumours compared to their respective controls

Protein	C1 vs OS		C1 vs ES		C2 vs CS		C2 vs PS		C3 vs GCT	
	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value
AAT	1.27	0.037	-1.03	0.834	1.34	0.031	1.54	0.073	1.11	0.350
ABG	1.23	0.312	-1.07	0.572	-1.01	0.950	-1.05	0.522	-1.05	0.593
AHS	1.05	0.678	**-1.90**	**0.003**	-1.15	0.272	**-1.55**	**0.003**	-1.40	0.019
APOA1	-1.34	0.001	-1.25	0.055	-1.21	0.047	**-1.53**	**0.015**	-1.05	0.652
B2G1	1.04	0.660	-1.02	0.881	**1.76**	**0.000**	1.35	0.003	**1.51**	**0.000**
C3f	1.03	0.924	-1.14	0.708	**-2.05**	**0.026**	-1.29	0.325	**-1.88**	**0.031**
HAP	-1.00	0.982	2.07	0.109	**-2.00**	**0.001**	1.54	0.311	1.23	0.389
HPX	**-1.69**	**0.000**	-1.09	0.533	-1.33	0.126	-1.25	0.153	-1.13	0.304
KNG	1.01	0.959	1.02	0.954	**-1.93**	**0.023**	**-1.92**	**0.005**	-1.44	0.070
LRG	1.25	0.392	1.10	0.728	-1.20	0.466	**2.23**	**0.000**	1.01	0.961
SAA	**14.29**	**0.002**	13.24	0.203	3.96	0.299	**15.86**	**0.031**	5.26	0.115
STR	-1.24	0.001	-1.23	0.027	-1.02	0.798	-1.21	0.082	1.16	0.021
TTR	-1.22	0.031	-1.32	0.018	1.02	0.867	**-1.50**	**0.000**	-1.01	0.878
VTDB	1.44	0.031	1.19	0.328	1.10	0.335	1.03	0.843	1.29	0.02
ZAG	1.14	0.359	1.75	0.133	-1.20	0.453	1.27	0.331	1.00	0.991

Relative abundance of serum protein was calculated from mean percentage volume contribution obtained from the silver 2DE analysis. Proteins that met both criteria as potential biomarkers (FC $\geq$ 1.5 and $p < 0.05$) are indicated in bold. Negative (-) value indicates down-regulation.

In patients with OS, out of the 15 proteins analysed, only two proteins, HPX and SAA, met these criteria. The expression of HPX was decreased by 1.69-fold (p<0.001). On the other hand, the expression of SAA was up-regulated by almost 15.0-fold (p=0.002) compared to its age-matched control C1. In patients with ES, only a single protein, AHS, met these criteria. AHS was down-regulated by 1.90-fold (p=0.003) in the patients. Four proteins, B2G1, C3f, HAP and KNG, were found to be significantly altered (FC>1.5) in patients with CS. B2G1 was increased by 1.76-fold while the expression of C3f, HAP and KNG were decreased by about 2-fold compared to the control group, C2. In patients with PS, a total of six proteins showed significant alterations with FC>1.5. LRG and SAA were found to be increased by 2.23-fold and 15.86-fold respectively. KNG was found to be decreased by 1.92-fold and the other three proteins, AHS, APOA1 and TTR were decreased by about 1.5-fold. In a benign bone tumour, GCT, only two proteins met these criteria. They were B2G1, which was increased by 1.51-fold and C3f, which was decreased by 1.88-fold in the patients, compared to the control C3.

4.2 Analysis of serum glycoproteome using lectins

Apart from silver stained 2DE analysis, pooled serum samples from bone tumour patients and healthy control groups were also subjected to Western blotting analysis using enzyme-conjugated CGB and CMB lectins. The purified CGB and CMB lectins were used to detect human serum O-glycosylated and N-glycosylated glycoproteins using a combination of 2DE and immunoblotting techniques as described in Section 3.2.3. The application of lectins as probes enables the detection of glycosylated polypeptides in the resolved 2DE profiles. CGB lectin binds to the O-glycosylated proteins while CMB lectin binds to the N-glycosylated proteins.

4.2.1 Isolation and purification of champedak seed lectin (CGB and CMB lectins)

The crude extract of champedak seed was obtained as described in Section 3.2.3.1 and was subjected to galactose-Sepharose 4B affinity column chromatography to isolate the CGB and CMB lectin (as described in Section 3.2.3.3). Absorbance of the unbound fractions (flow through fractions) and bound fractions of CGB and CMB lectins, which were eluted with 0.8 M galactose and 0.8 M mannose in PBS pH 7.2 respectively, was monitored at 280 nm.

Figure 4.6 shows the typical elution profile of the CGB lectin using the galactose-Sepharose 4B affinity column. Fraction 1 to 17 contained the unbound fractions. The column was washed extensively with PBS to wash all the unbound substances and the absorbance of each fraction was monitored until it reached the baseline (fraction 18 to 33). Elution was started (indicated by the arrow) and the absorbance was monitored until it reached the baseline again (starting from fraction 34 to 55). These fractions containing the CGB lectin were pooled and dialysed to remove the sugar before being subjected to lyophilisation to concentrate the CGB lectin.

Similar to the typical elution profile of CGB lectin, Figure 4.7 shows the typical elution profile of the CMB lectin using the mannose-Sepharose 4B affinity column. Fraction 1 to 17 contained the unbound fractions. The column was washed extensively with PBS to wash all the unbound substances and the absorbance of each fraction was monitored until it reached the baseline (fraction 18 to 20). Elution was started at fraction 21 (as indicated by the arrow) and the absorbance was monitored until it reached the baseline again (fraction 40). Fractions containing the isolated CMB lectin were pooled and dialysed to remove the sugar before being subjected to lyophilisation.

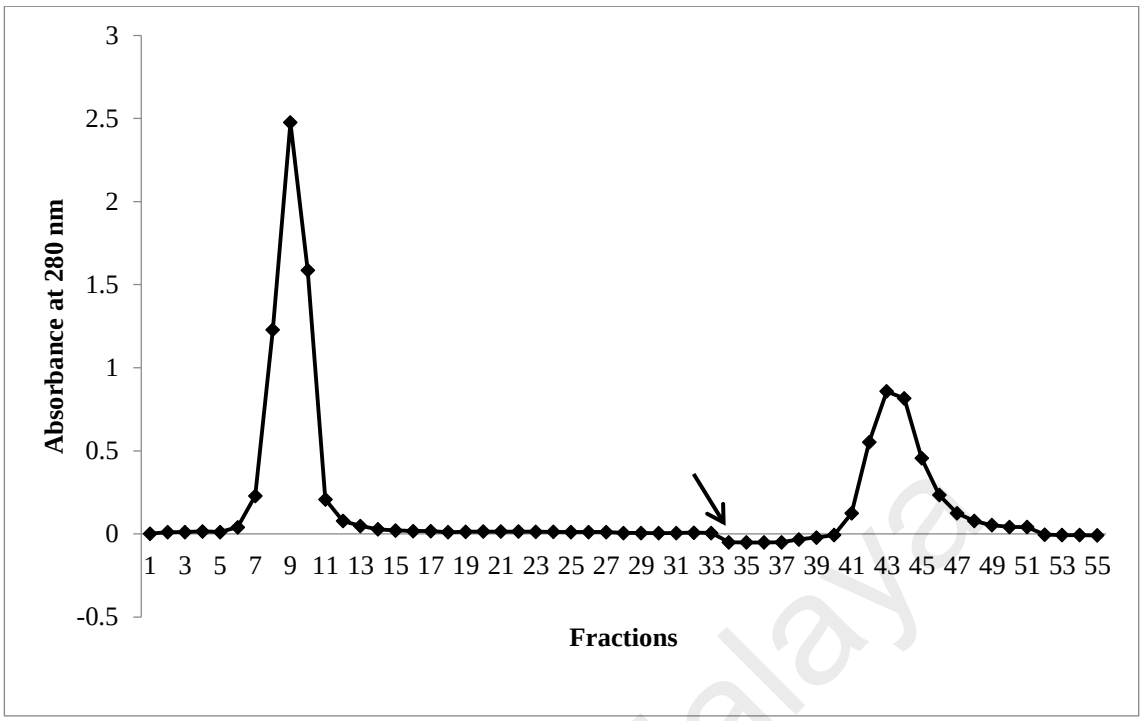


Figure 4.6: Elution profile of CGB lectin from galactose-Sepharose 4B affinity column

This graph demonstrates the elution profile of CGB lectin when crude extract of champedak seeds was applied into galactose-Sepharose 4B affinity column. The unbound fractions of 10 ml each were collected and the absorbance for each fraction was monitored at 280. Once the absorbance had fallen to the baseline ($A_{280} < 0.005$), the bound fractions (containing CGB lectin) were eluted with 0.8 M galactose in PBS pH 7.2 (indicated with an arrow). Bound fractions of 10 ml each were collected and the absorbance for monitored at 280 nm with 0.8 M galactose in PBS served as the blank. Fractions with high absorbance values were pooled and dialysed against PBS 7.2 with 6 changes at 4°C. The dialysed bound fractions were lyophilised to concentrate the CGB lectin.

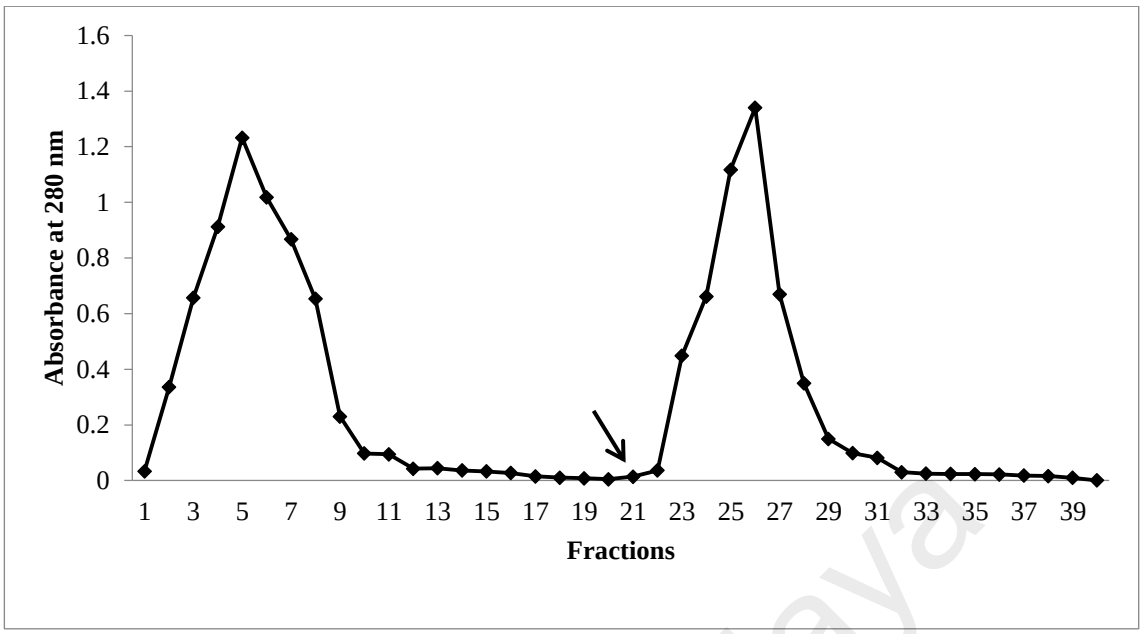


Figure 4.7: Elution profile of CMB lectin from mannose-Sepharose 4B affinity column

This graph demonstrates the elution profile of CMB lectin when crude extract of champedak seeds was applied into mannose-Sepharose 4B affinity column. The unbound fractions of 10 ml each were collected and the absorbance for each fraction was monitored at 280. Once the absorbance had fallen to the baseline ($A_{280} < 0.005$), the bound fractions (containing CMB lectin) were eluted with 0.8 M mannose in PBS pH 7.2 (indicated with an arrow). Bound fractions of 10 ml each were collected and the absorbance for monitored at 280 nm with 0.8 M mannose in PBS served as the blank. Fractions with high absorbance values were pooled and dialysed against PBS 7.2 with 6 changes at 4°C. The dialysed bound fractions were lyophilised to concentrate the CMB lectin.

The lyophilised CGB and CMB lectins were subjected to SDS-PAGE (as described in section 3.2.3.4) to assess the purity and quality of the isolated lectins. Using 18% homogenous separating gel, the electrophoresis was carried out and the resolved gel was stained with Coomassie Blue. Figure 4.8 shows the protein profile of the lectins under reduced conditions. The CGB lectin was resolved into two bands (Lane 3) where each band had a relative M_r of 14.5 kDa and 16.0 kDa respectively. The protein profile of the CGB lectin on SDS-PAGE obtained here was in agreement as observed in previous studies (Hashim *et al.*, 1993; Hashim *et al.*, 1991). Lane 2 shows a single band with a M_r of 15.0 kDa which is the characteristic of CMB lectin (Lim *et al.*, 1997; Lim *et al.*, 1998). The absence of other bands on both lanes indicates that the isolated lectins were pure.

4.2.2 Detection of serum glycosylated proteins from the CGB- and CMB-generated blots

Similar to the 2DE gels, the digitised images of CGB- and CMB-generated blots were analysed using Image Master Platinum v7.0 software. The homogeneity of the data was assured using Levene's test and the level of significance were determined using independent-sample t-test. In this study, all spots were analysed in terms of percentage volume contribution, where the volume percentage of a protein was taken against the total spot volumes of all proteins in each gel.

4.2.2.1 Detection of serum O-glycosylated proteins from CGB-generated blots

Spot volume analysis was carried out on seven well resolved protein clusters as shown in Figure 4.9. These proteins were AHS, alpha-1-microglobulin precursor (AMBP), HPX, inter-alpha-trypsin inhibitor heavy chain 4 fragment (ITIH4f), KNG, LRG and VTDB. The numbers of well-resolved protein clusters were considerably

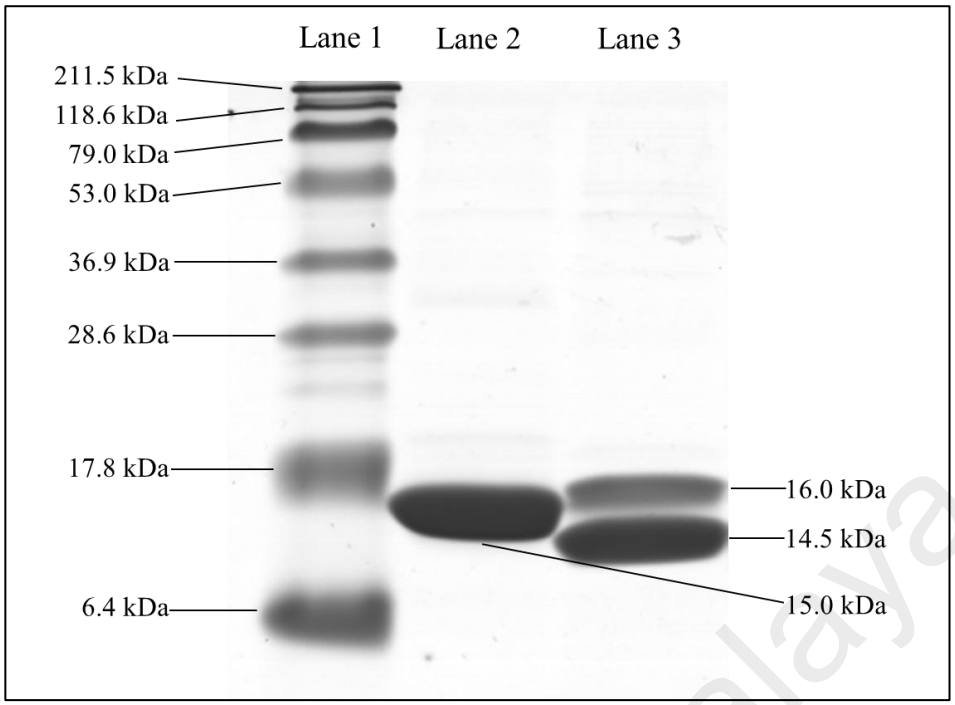


Figure 4.8: Determination of CGB and CMB lectins purity using SDS-PAGE

This figure shows the protein profile of CGB and CMB lectin when subjected to an 18% SDS-PAGE run under denaturing and reducing conditions. The CMB lectin was resolved into a single band (Lane 2) showing a relative M_r of 15 kDa. The CGB lectin was resolved into two bands (Lane 3) where each band had a M_r of 14.5 kDa and 16 kDa respectively. No other visible band was present in these lanes indicating the isolated lectins were pure. Lane 1 depicted molecular weight markers consisting of myosin (211.5 kDa), β-galactosidase (118.6 kDa), bovine serum albumin (79.0 kDa), ovalbumin (53 kDa), carbonic anhydrase (36.9 kDa), soybean trypsin inhibitor (28.6 kDa), lysozyme (17.8 kDa) and aprotinin (6.4 kDa).

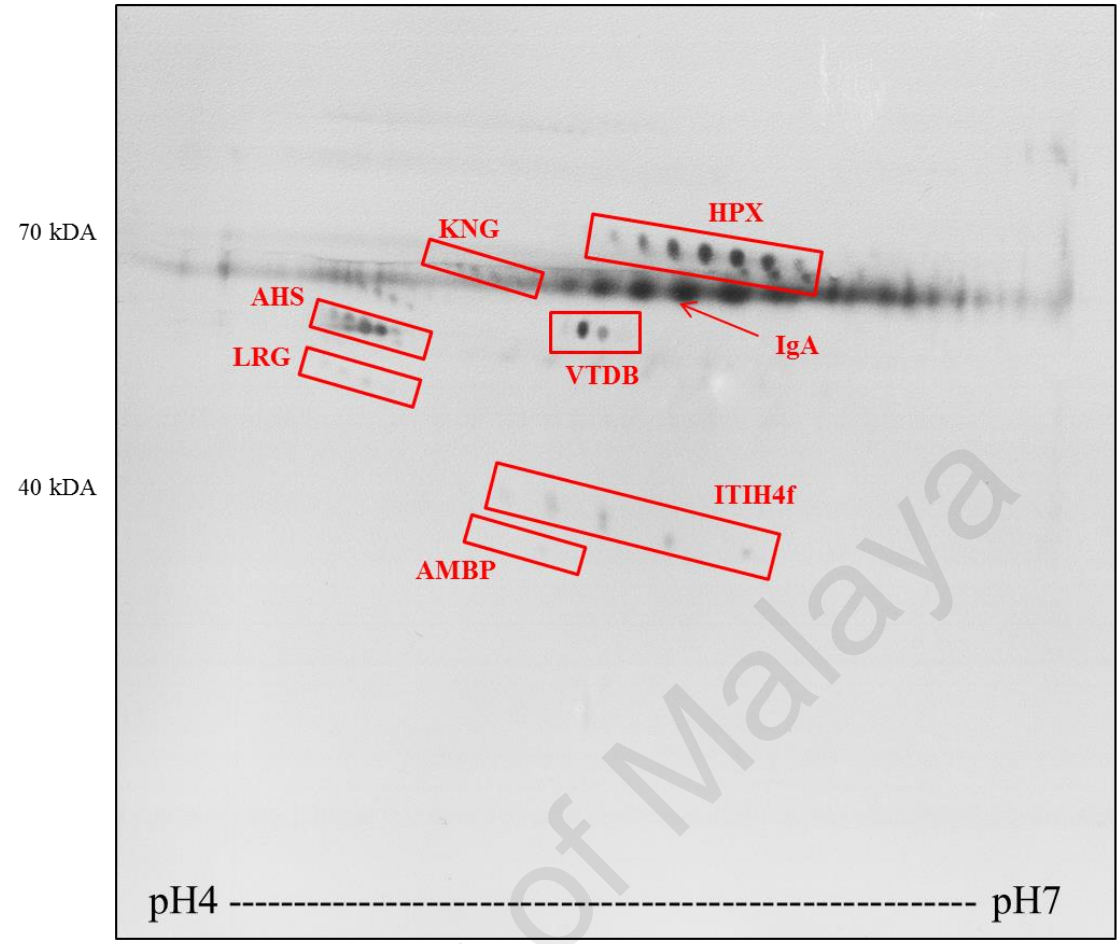


Figure 4.9: Typical representative of serum proteins using enzyme-conjugated CGB lectin on 2DE blot

CGB lectin conjugated to HRP was used as a probe to detect O-glycosylated proteins from pooled serum samples that were subjected to 2DE and Western blotting onto nitrocellulose membrane. The acidic side of the blot is to the left and relative molecular mass declines from the top.

lower than those obtained from silver 2DE gels. The identities of these proteins were confirmed based on visual comparison with the earlier studied silver 2DE profile, with the exception of AMBP and ITIH4f. The identity of AMBP was confirmed by MS analysis, using a gel plug which was excised from its corresponding spot on silver stained 2DE gel (Table 4.4) and the ITIH4f protein cluster was confirmed of its identity based on a previous finding by our laboratory (Mohamed *et al.*, 2008).

Figure 4.10, 4.11 and 4.12 show profiles of serum O-glycosylated proteins from pooled serum samples of patients with OS, ES, CS, PS, and GCT, with their respective control groups C1, C2 and C3. Similar to 2DE profile analysis, FC difference for each protein was calculated and the relative abundance of the analysed serum proteins in each disease group compared with their respective controls is demonstrated in Table 4.5.

Figure 4.13 demonstrates the analysed proteins in terms of percentage mean volume contribution (mean ± SEM for three technical replicates) for each disease group compared to their respective age-matched control group. In patients with OS, only a single protein, AHS, demonstrated significantly altered abundance compared to the control C1 (Figure 4.13a). The mean percentage contribution of AHS observed in the control samples was 4.27% while in the patients was 2.72%. The abundance of AHS was significantly down-regulated in the patient by about 1.5-fold (Table 4.5). AMBP, ITIH4f and LRG were not detected in the control, but the absence was not statistically significant. Meanwhile, none of the analysed proteins was found to be significantly altered in patients with ES, when compared to their control group (Figure 4.13b). AMBP and ITIH4f were both found to be absent in the patients.

Table 4.4: Identification of AMBP using MALDI TOF/TOF analysis

Protein ID	Matched protein identity	Accession Number (Swiss-Prot)	Theoretical Mass - M_r (Da)	Theoretical pI	No of peaks matched	MASCOT score	Sequence Coverage (%)
AMBP	Protein AMBP	P02760	38974	5.95	11	221	29

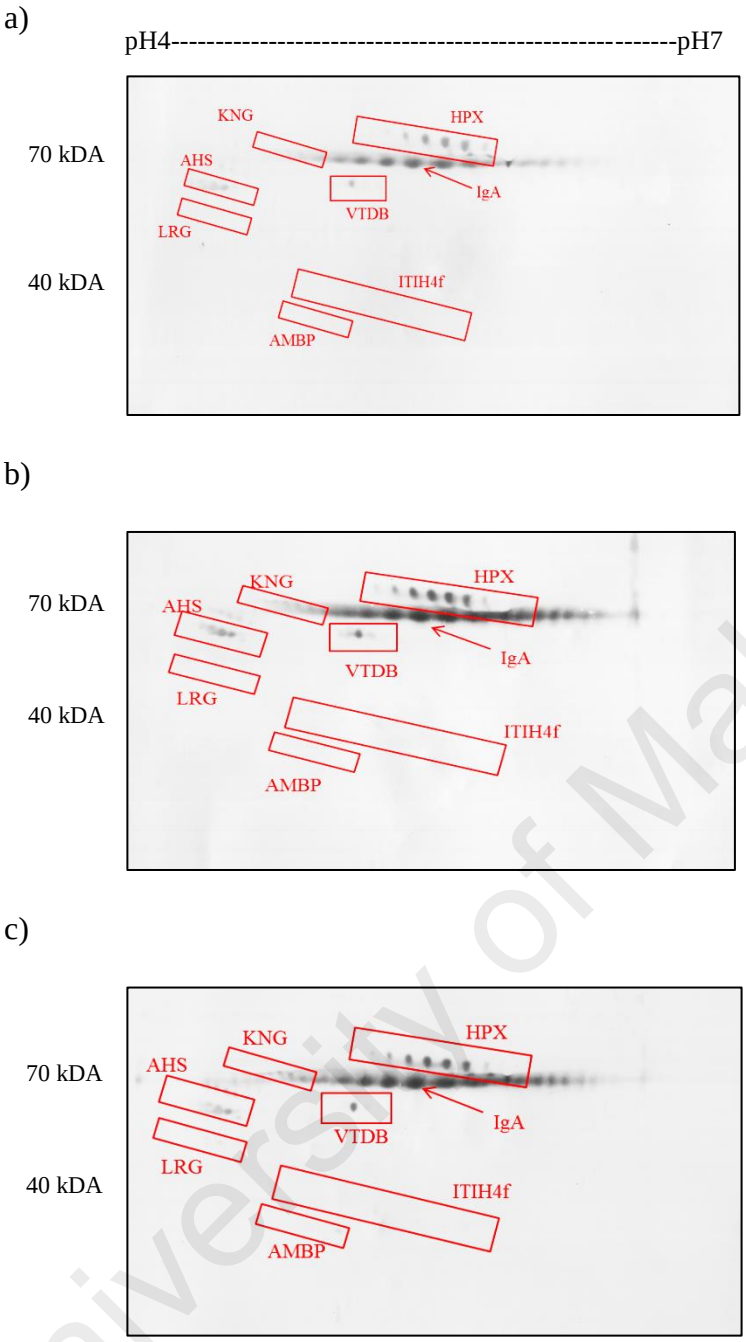
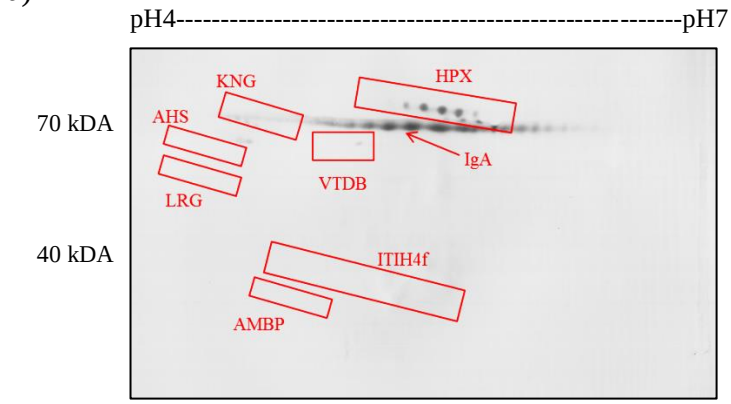


Figure 4.10: Profiles of serum O-glycosylated serum proteins of pooled healthy control C1, patients with OS and patients with ES

Unfractionated pooled serum from each group of samples was subjected to 2DE, Western blotting and detection with HRP-conjugated CGB lectin. Panels demonstrate typical serum protein profiles of (a) control group C1, (b) patients with OS and (c) patients with ES. For all panels, the acidic side of the 2DE gel is to the left and relative molecular mass declines from the top.

a)



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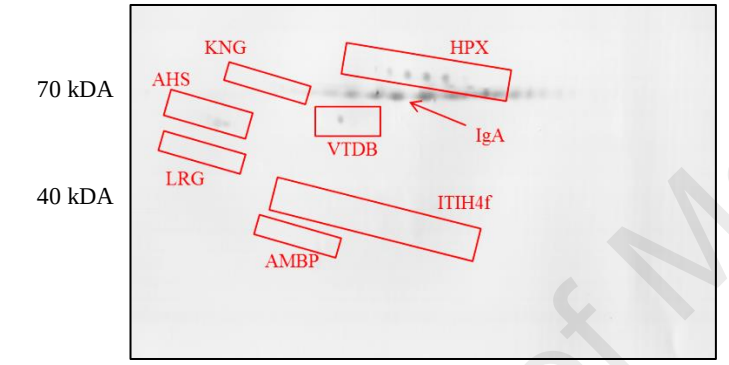

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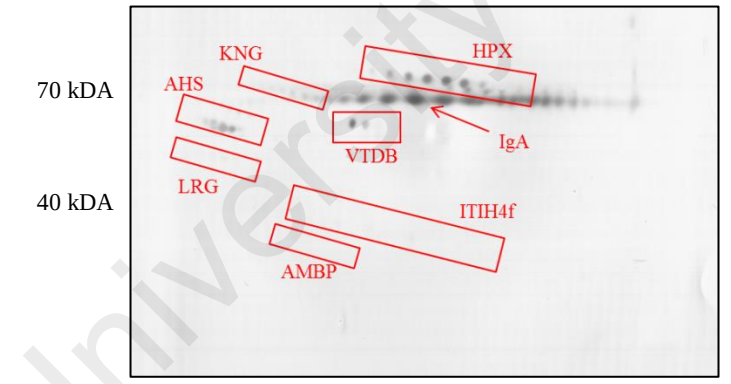
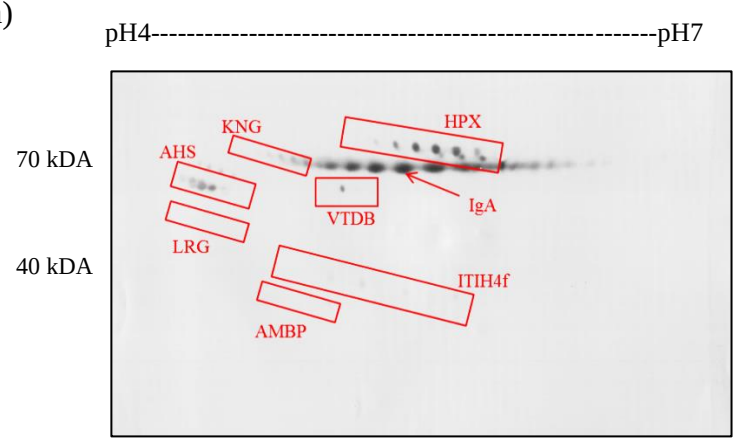


Figure 4.11: Profiles of serum O-glycosylated serum proteins of pooled healthy control C2, patients with CS and patients with PS

Unfractionated pooled serum from each group of samples was subjected to 2DE, Western blotting and detection with HRP-conjugated CGB lectin. Panels demonstrate typical serum protein profiles of (a) control group C2, (b) patients with CS and (c) patients with PS. For all panels, the acidic side of the 2DE gel is to the left and relative molecular mass declines from the top.

a)



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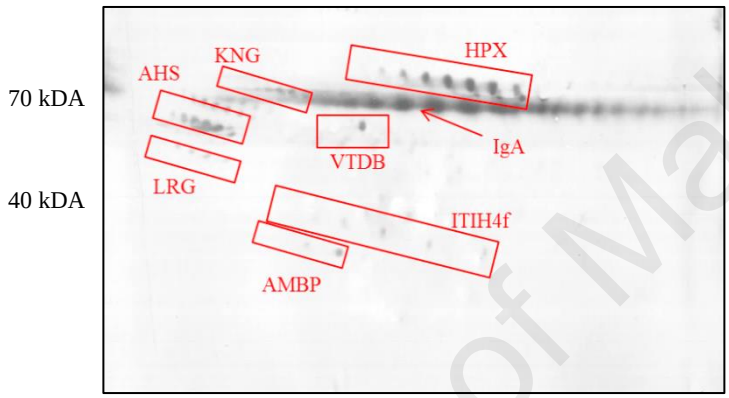
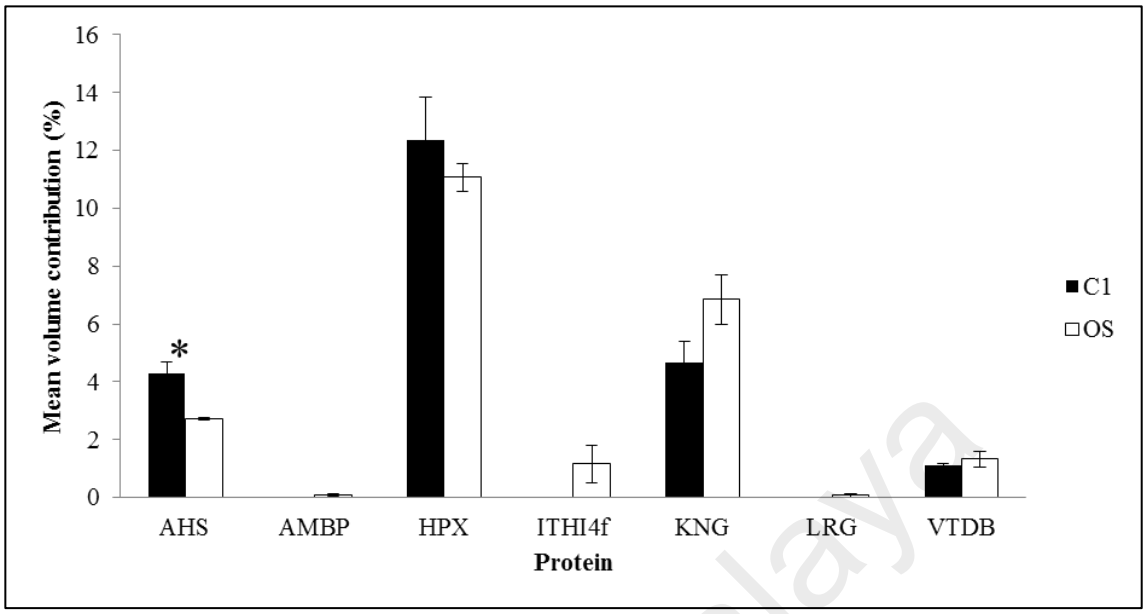


Figure 4.12: Profiles of serum O-glycosylated serum proteins of pooled healthy control C3 and patients with GCT

Unfractionated pooled serum from each group of samples was subjected to 2DE, Western blotting and detection with HRP-conjugated CGB lectin. Panels demonstrate typical serum protein profiles of (a) control group C3 and (b) patients with GCT. For all panels, the acidic side of the 2DE gel is to the left and relative molecular mass declines from the top.

a)



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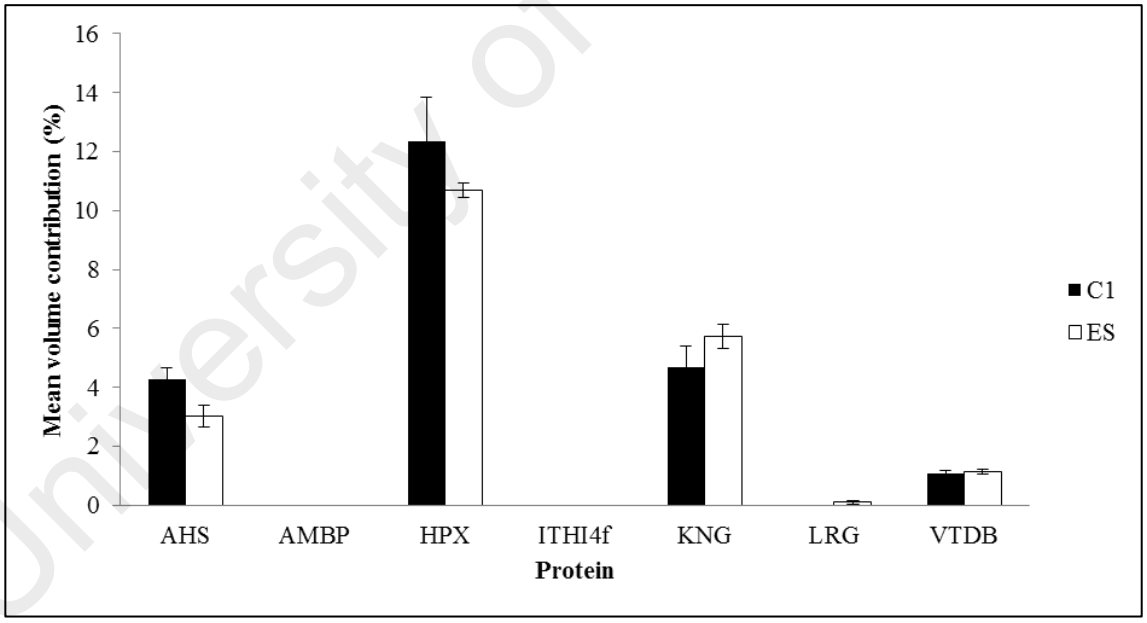

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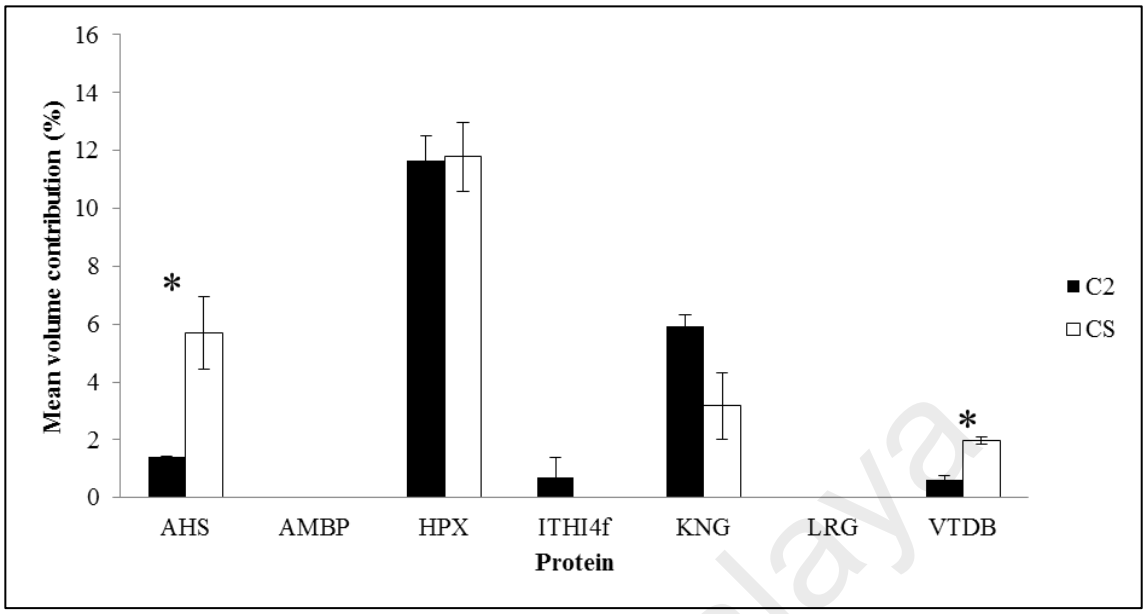
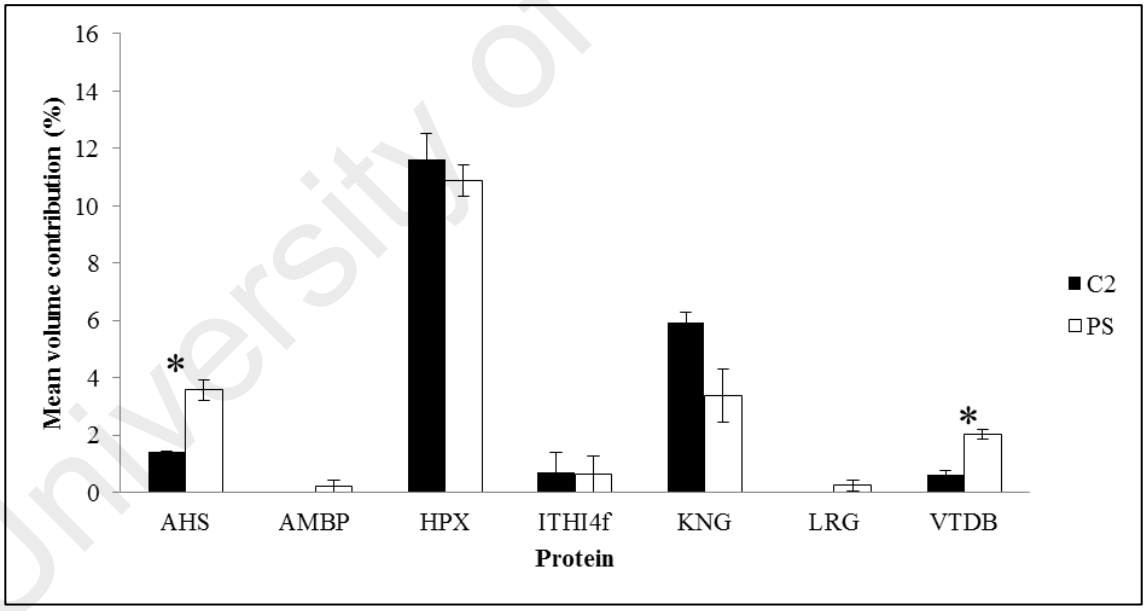


d)



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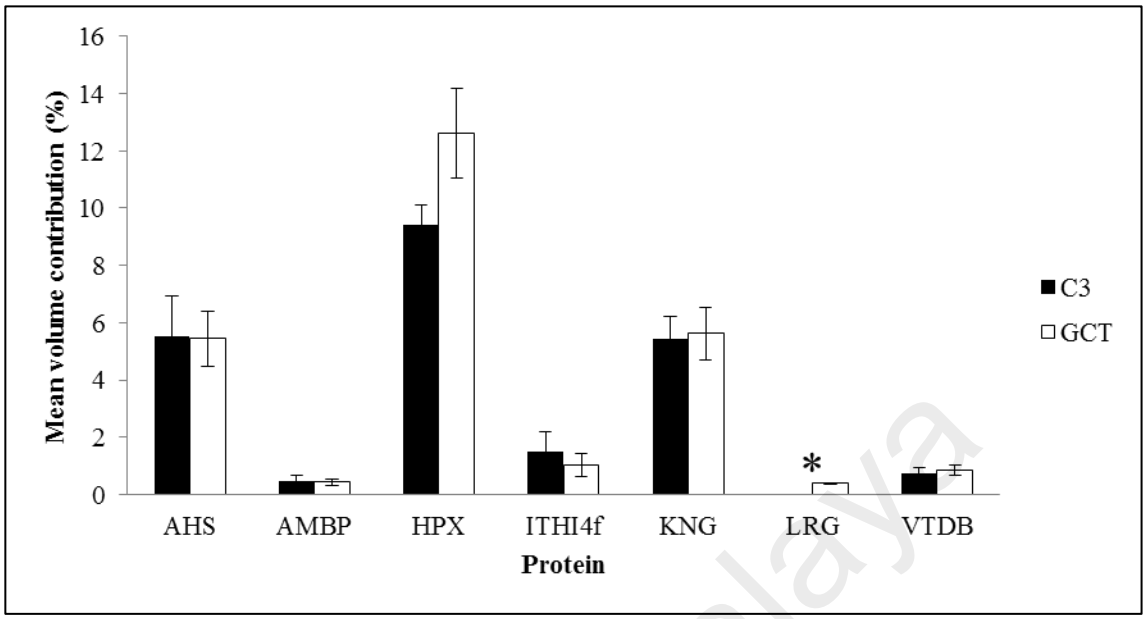


Figure 4.13: Mean percentage of volume contribution of serum O-glycosylated proteins from CGB-generated blots

The mean percentage of volume contribution of seven serum O-glycosylated proteins were analysed using Image Master Platinum v7.0 software from CGB-generated blots (mean ± S.E.M. of quadruplicate analysis). Densitometry analysis was performed on AHS, AMBP, HPX, ITIH4f, KNG, LRG and VTDB. Panels demonstrate mean percentage of volume contribution of pooled samples from (a) control group C1 and patients with OS, (b) control group C1 and patients with ES, (c) control group C2 and patients with CS, d) control group C2 and patients with PS, and e) control group C3 and patients with GCT. Asterisk (*) denotes protein with significant alteration in abundance compared to the control group (p<0.05).

Table 4.5: Relative abundances of serum proteins from CGB-generated blots in patients with bone tumours compared to their respective controls

Protein	C1 vs OS		C1 vs ES		C2 vs CS		C2 vs PS		C3 vs GCT	
	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value
AHS	**-1.57**	**0.029**	-1.41	0.065	**4.02**	**0.035**	**2.53**	**0.004**	-1.01	0.966
AMBP	+ (n.a)	0.185	n.d	n.a	n.d	n.a	+ (n.a)	0.437	-1.12	0.805
HPX	-1.12	0.435	-1.16	0.305	1.01	0.926	-1.07	0.487	1.34	0.110
ITIH4f	+ (n.a)	0.179	n.d	n.a	- (n.a)	0.423	-1.08	0.958	-1.45	0.570
KNG	1.47	0.104	1.23	0.258	-1.87	0.107	-1.74	0.079	1.03	0.886
LRG	+ (n.a)	0.391	+ (n.a)	0.201	n.d	n.a	+ (n.a)	0.335	+ (n.a)	0.001
VTDB	1.22	0.416	1.04	0.741	**3.15**	**0.001**	**3.21**	**0.002**	1.15	0.688

Relative abundance of serum protein was calculated from mean percentage volume contribution obtained from the analysis of the CGB-generated blots. Proteins that met both criteria as potential biomarkers (FC $\geq$ 1.5 and $p < 0.05$) are indicated in bold. Positive (+) value indicates up-regulation. Negative (-) value indicates down-regulation. n.a = not available, n.d = not detected.

In patients with CS, two proteins, AHS and VTDB were significantly altered compared to its age-matched control group, C2 (Figure 4.13c). These two proteins were significantly up-regulated in the patients, by 4.02-fold (p=0.035) and 3.15-fold (p=0.001), respectively. Similar to control C1, AMBP and LRG were not present in the control C2. These proteins were also not detected in the patients.

The same proteins, AHS and VTDB, also showed significantly enhanced expression in patients with PS, compared to the control (Figure 4.13d). The percentages mean volume contribution of AHS in the control group and disease group were 1.41% and 3.57%, respectively, an increase of 2.53-fold (p=0.004) compared to the control group (Table 4.5). The abundance of VTDB was also significantly increased by 3.21-fold (p=0.002) in the disease group. AMBP and LRG proteins were only present in the patients, not in the control group, a similar pattern observed in patients with OS.

In the final group of bone tumour assessed, GCT, only a single protein, LRG, showed a significantly altered abundance in patients' sample compared to the age-matched control C3. LRG was not detected in the control and the mean volume contribution was 0.39% in the disease group (Figure 4.13e). Due to the absence of LRG in the control, the FC could not be determined in this case (Table 4.5). Similar to the 2DE profile, the percentage mean volume contribution of this protein was found to be significantly higher in the patients' serum when compared to the control.

4.2.2.2 Detection of serum N-glycosylated proteins from CMB-generated blots

CMB-generated blots showed considerably higher number of protein spots compared to CGB-generated blots (Figure 4.14). The acquired profile was quite similar to that of silver 2DE, with the notable absence of low molecular weight proteins.

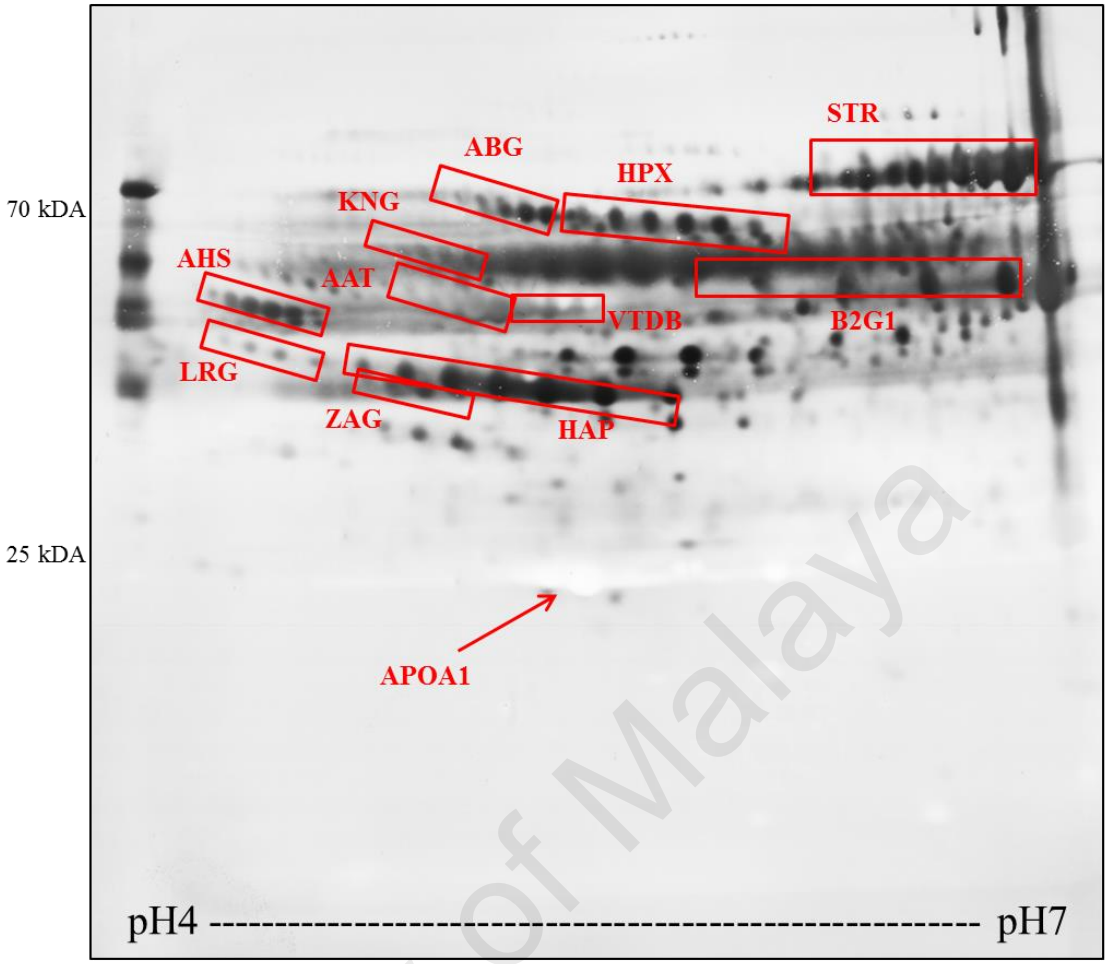


Figure 4.14: Typical representative of serum proteins using enzyme-conjugated CMB lectin on 2DE blot

CMB lectin conjugated to AP was used as a probe to detect N-glycosylated proteins from pooled serum samples that were subjected to 2DE and Western blotting onto nitrocellulose membrane. The acidic side of the blot is to the left and relative molecular mass declines from the top.

Spot volume analysis was carried out on 11 well resolved protein clusters and the identities of these proteins were confirmed visually based on the silver 2DE profile. These proteins were AAT, ABG, AHS, B2G1, HAP, HPX, KNG, LRG, STR, VTDB and ZAG. Figure 4.15, 4.16 and 4.17 show profiles of serum N-glycosylated proteins from pooled serum samples from patients with OS, ES, CS, PS, and GCT, with their respective control groups C1, C2 and C3. FC difference for each protein was calculated and the relative expression of the analysed serum proteins in each disease group compared with their respective controls is demonstrated in Table 4.6.

In patients with OS, the expression of all of the proteins analysed showed statistically significant differences compared to the control group C1, with the exception of HPX (Figure 4.18a). Eight proteins, AAT, ABG, AHS, B2G1, LRG, STR, VTDB and ZAG were significantly up-regulated in the patients, while two proteins, HAP and KNG were down-regulated. Out of these 10 proteins, six proteins demonstrated fold changes of more than 1.5 with *p*-value of lower than 0.05. They were AAT, ABG, B2G1, HAP, LRG and VTDB (Table 4.6). The highest fold change was exhibited by AAT, where this protein was up-regulated by 4.60-fold (p=0.005) in patients with OS.

Six proteins were found to be significantly up-regulated in patients with ES. They were AAT, ABG, AHS, B2G1, LRG and VTDB (Figure 4.18b). From the FC analysis, all of these proteins were significantly increased by more than 1.5-fold in the patients, compared to the control group C2 (Table 4.6). Similar to OS, the highest fold change was exhibited by AAT (FC=2.77, p=0.008), where the percentages mean volume contribution of this protein in the control and the disease sample were 1.08% and 3.00%, respectively.

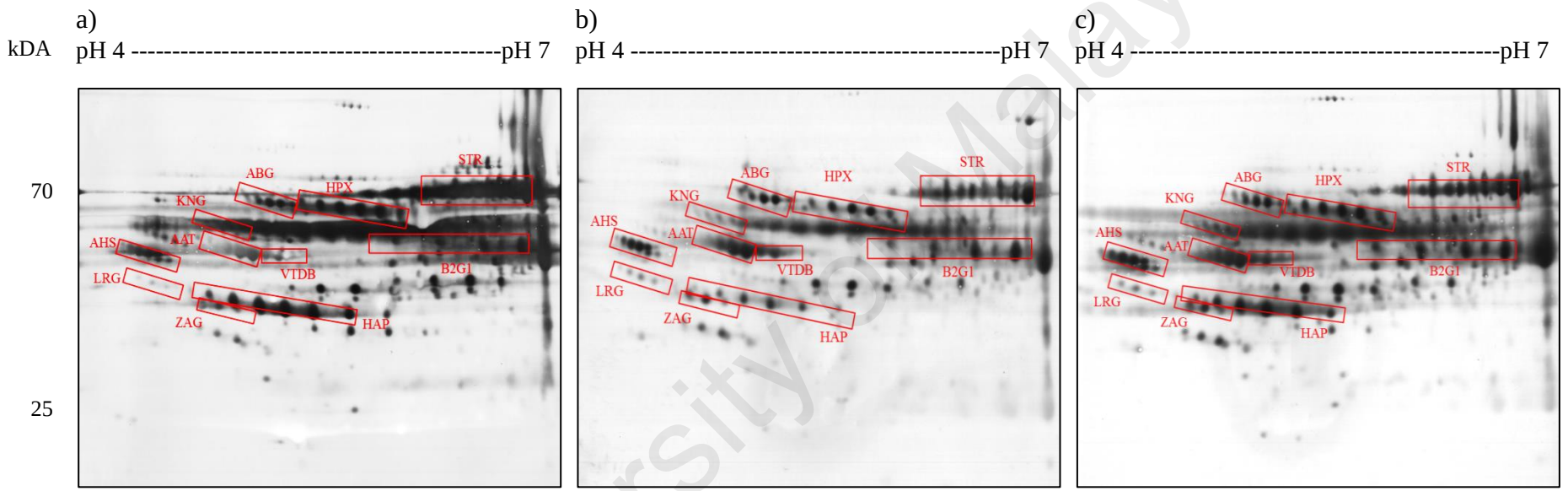


Figure 4.15: Profiles of serum N-glycosylated serum proteins of pooled healthy control C1, patients with OS and patients with ES

Unfractionated pooled serum from each group of samples was subjected to 2DE, Western blotting and detection with AP-conjugated CMB lectin. Panels demonstrate typical serum protein profiles of (a) control group C1, (b) patient with OS and (c) patient with ES. For all panels, the acidic side of the 2DE gel is to the left and relative molecular mass declines from the top.

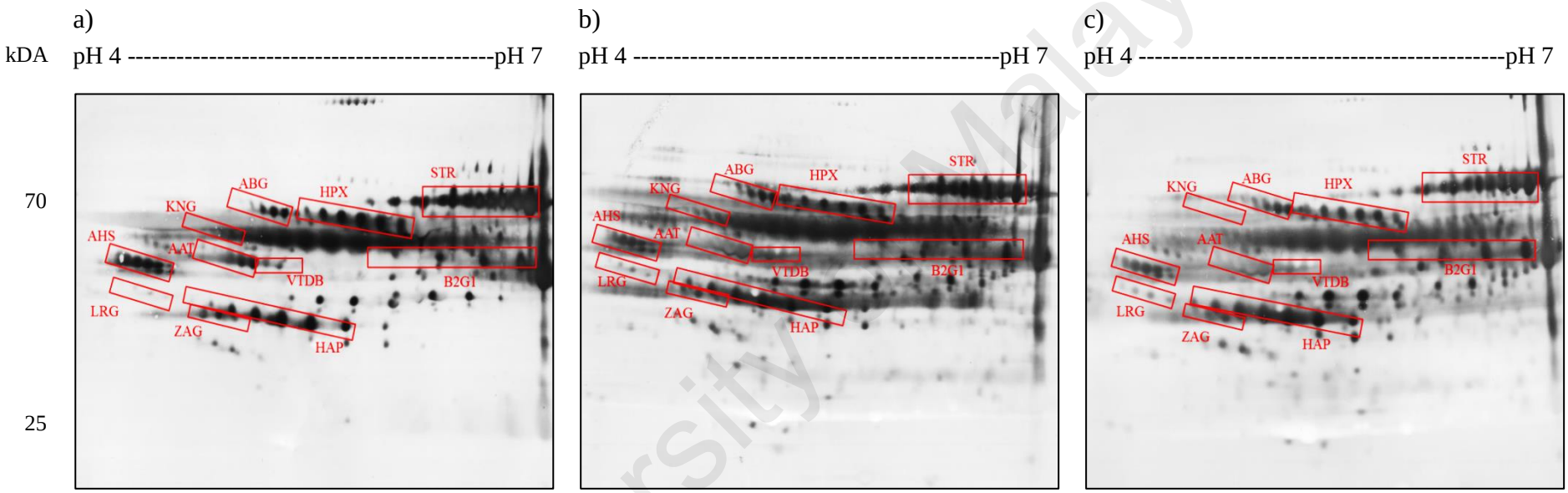


Figure 4.16: Profiles of serum N-glycosylated serum proteins of pooled healthy control C2, patients with CS and patients with PS

Unfractionated pooled serum from each group of samples was subjected to 2DE, Western blotting and detection with AP-conjugated CMB lectin. Panels demonstrate typical serum protein profiles of (a) control group C2, (b) patient with CS and (c) patient with PS. For all panels, the acidic side of the 2DE gel is to the left and relative molecular mass declines from the top.

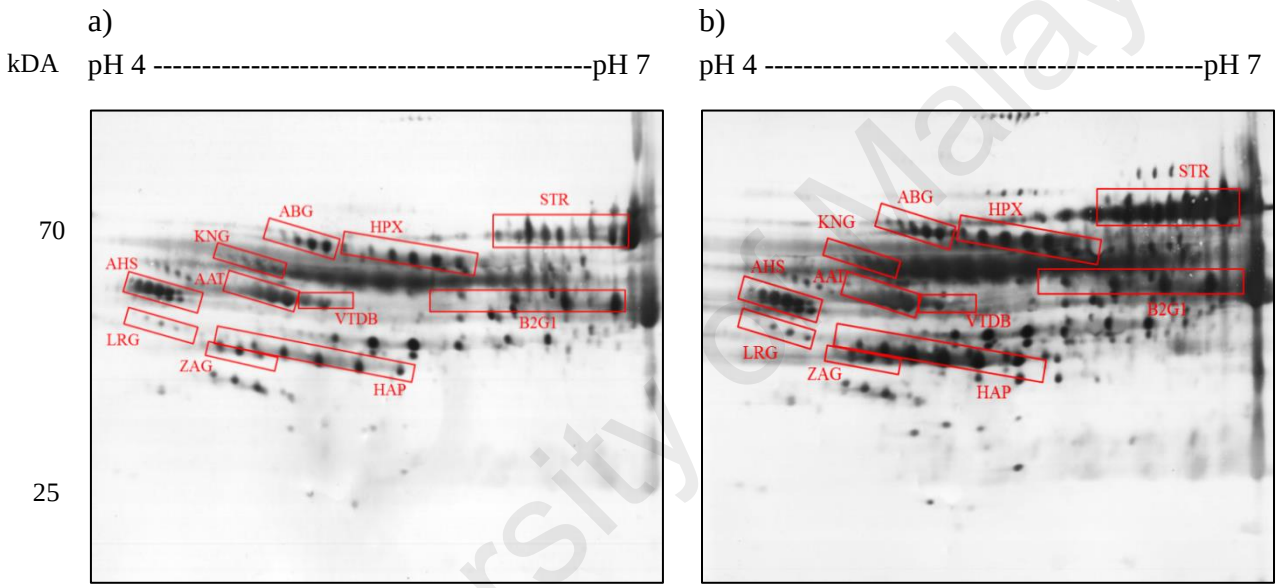


Figure 4.17: Profiles of serum N-glycosylated serum proteins of pooled healthy control C3 and patients with GCT

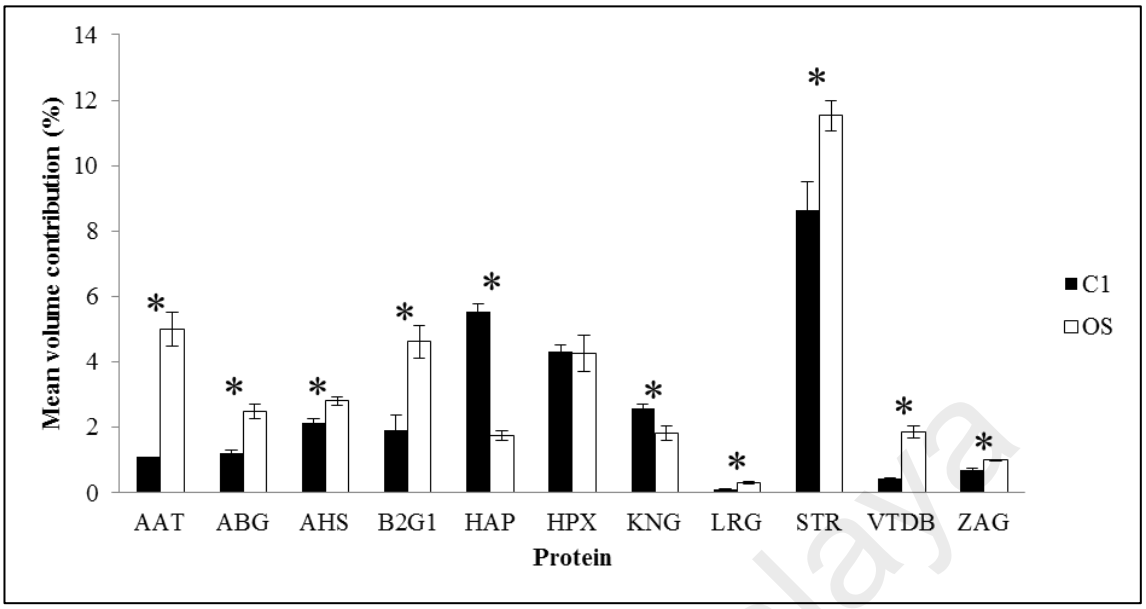
Unfractionated pooled serum from each group of samples was subjected to 2DE, Western blotting and detection with AP-conjugated CMB lectin. Panels demonstrate typical serum protein profiles of (a) control group C3 and (b) patient with GCT. For all panels, the acidic side of the 2DE gel is to the left and relative molecular mass declines from the top.

Table 4.6: Relative abundances of serum proteins from CMB-generated blots in patients with bone tumours compared to their respective controls

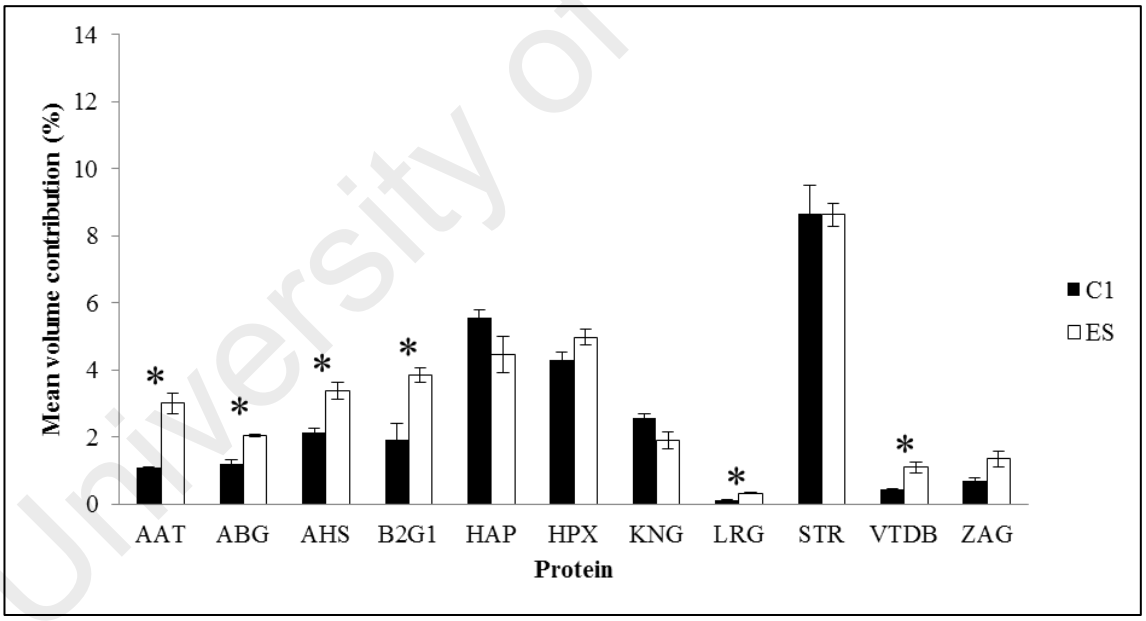
Protein	C1 vs OS		C1 vs ES		C2 vs CS		C2 vs PS		C3 vs GCT	
	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value
AAT	**4.60**	**0.005**	**2.77**	**0.008**	-1.70	0.083	-1.67	0.142	-1.34	0.166
ABG	**2.07**	**0.005**	**1.70**	**0.000**	-1.08	0.644	-1.09	0.610	-1.36	0.105
AHS	1.32	0.016	**1.58**	**0.010**	**-1.93**	**0.001**	**-2.18**	**0.001**	-1.10	0.370
B2G1	**2.41**	**0.012**	**2.01**	**0.011**	-1.07	0.700	1.23	0.148	1.18	0.292
HAP	**-3.17**	**0.000**	-1.24	0.165	-1.10	0.532	1.02	0.874	**1.93**	**0.011**
HPX	-1.01	0.968	1.16	0.106	**-1.79**	**0.039**	**-1.75**	**0.041**	-1.02	0.737
KNG	-1.41	0.049	-1.36	0.066	**-1.62**	**0.017**	**-2.42**	**0.002**	-1.46	0.161
LRG	**2.47**	**0.004**	**2.64**	**0.001**	**2.50**	**0.001**	**2.24**	**0.046**	**1.56**	**0.015**
STR	1.33	0.025	-1.00	0.993	-1.19	0.115	-1.24	0.110	-1.05	0.660
VTDB	**4.42**	**0.001**	**2.59**	**0.023**	1.84	0.073	**1.55**	**0.024**	-1.38	0.068
ZAG	1.42	0.008	1.93	0.083	1.02	0.941	-1.06	0.787	1.32	0.010

Relative abundance of serum protein was calculated from mean percentage volume contribution obtained from the analysis of the CMB-generated blots. Proteins that met both criteria as potential biomarkers (FC $\geq$ 1.5 and $p < 0.05$) are indicated in bold. Positive (+) value indicates up-regulation. Negative (-) value indicates down-regulation. n.a = not available, n.d = not detected.

a)



b)



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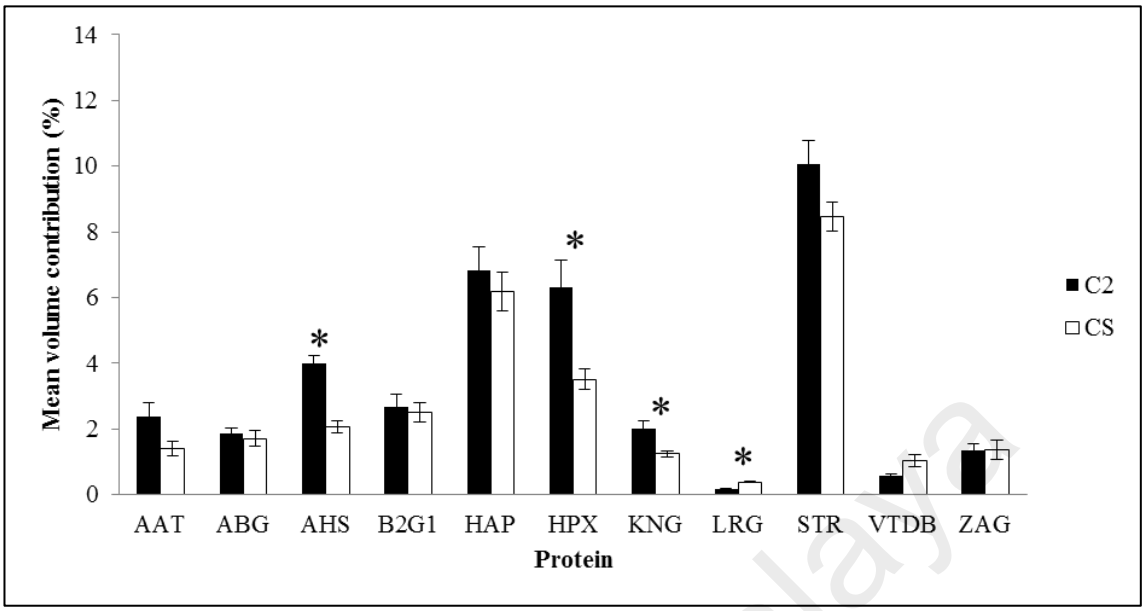

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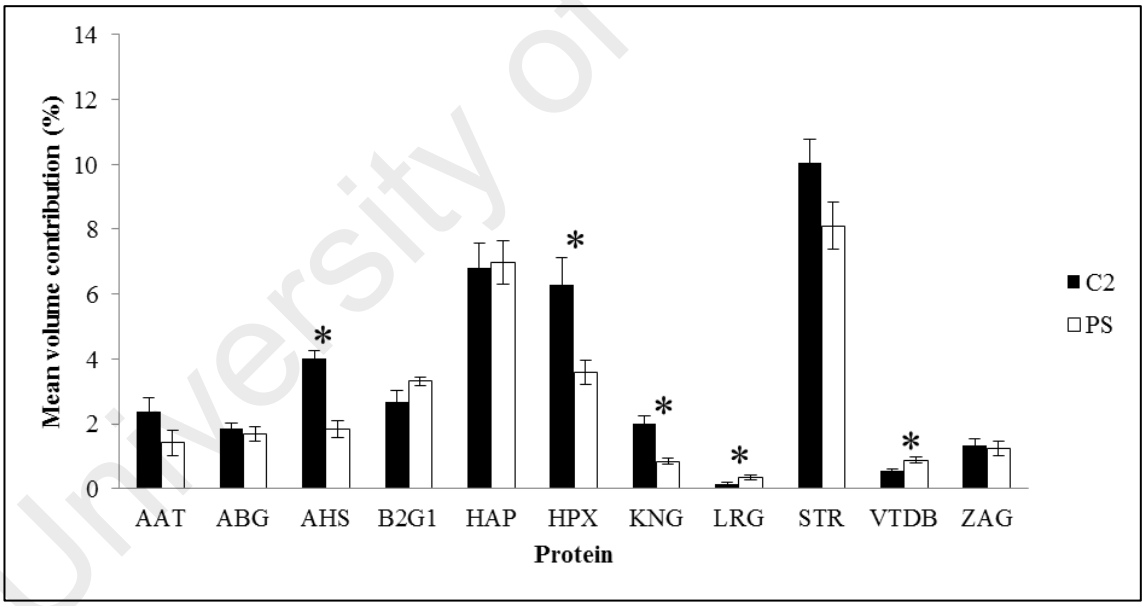

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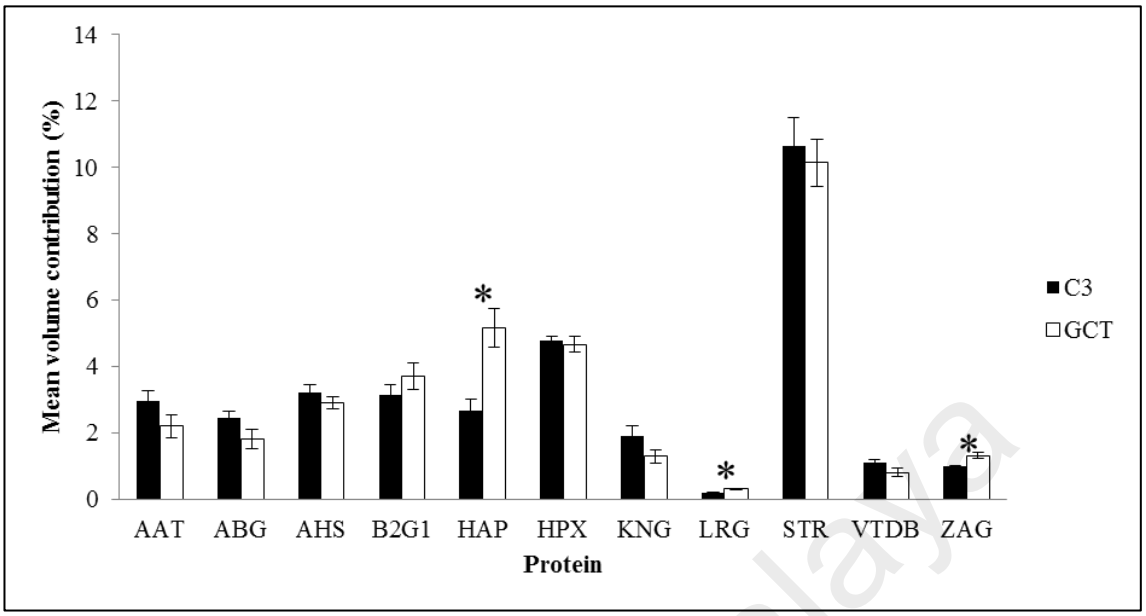


Figure 4.18: Mean percentage of volume contribution of serum N-glycosylated proteins from CMB-generated blots

The mean percentage of volume contribution of 11 serum N-glycosylated proteins were analysed using Image Master Platinum v7.0 software from CMB-generated blots (mean ± S.E.M. of quadruplicate analysis). Densitometry analysis was performed on AAT, ABG, AHS, B2G1, HAP, HPX, KNG, LRG, STR, VTDB and ZAG. Panels demonstrate mean percentage of volume contribution of pooled samples from (a) control group C1 and patients with OS, (b) control group C1 and patients with ES, (c) control group C2 and patients with CS, d) control group C2 and patients with PS, and e) control group C3 and patients with GCT. Asterisk (*) denotes protein with significant alteration in abundance compared to the control group (p<0.05).

CMB-generated blots developed from patients with CS, four proteins were found to be significantly altered with a FC of more than 1.5 compared to their age-matched control C2. AHS, HPX and KNG were down-regulated, whilst LRG was up-regulated in the patients (Figure 4.18c, Table 4.6). An increase of 2.50-fold (p=0.001), which was the highest observed in this group was exhibited by LRG, where the mean percentages volume contribution of this protein in the control C2 and the patients with CS were 0.15% and 0.38%, respectively.

A similar pattern was observed in patients with PS. AHS, HPX and KNG were significantly down-regulated and LRG was up-regulated, along with another protein, VTDB (Figure 4.18d). All of these proteins exhibited fold changes of more than 1.5 with a significant *p*-value of less than 0.05. The highest FC was exhibited by KNG (Table 4.6), where this protein was significantly down-regulated in the patients (FC=-2.42, *p*=0.002).

In patients with GCT, only three proteins, HAP, LRG and ZAG, were shown to be significantly altered in abundance (Figure 4.18e). These proteins were found to be significantly up-regulated in the patients. However, only HAP and LRG were found to be significantly altered with more than 1.5-fold difference in patients with GCT (Table 4.6).

4.3 Validation for SAA using Western blot and ELISA

The increased levels of SAA in patients with three types of malignant bone tumours *i.e.* OS, CS and PS were validated using Western blot and ELISA. OS and PS groups were selected because, from the silver 2DE analysis, the level of SAA was found to be significantly higher in the patients with these cancers. While the increase of SAA from the silver 2DE analysis was not statistically significant in patients with CS, this malignant sarcoma is not very aggressive and has a low metastatic rate.

For easier evaluation, Figure 4.19 demonstrates cropped images of SAA spots in six representatives silver 2DE gels from the earlier analysis of each group of samples, *i.e.* healthy adolescents (C1) and patients with OS, healthy adults (C2), CS patients and PS patients. The SAA spots in 2DE gels of patients with PS appeared darkest and most intense among the disease groups, followed by OS and CS. However, they were almost absent in the 2DE gels of both control groups. When the analysis was performed using Image Master Platinum v7.0 software (Figure 4.20), in the case of patients with OS, a difference of almost 15-fold was detected when their mean percentage of volume contribution (0.103 ± 0.025) was compared to their age-matched controls (0.007 ± 0.002). Meanwhile, the mean percentages of volume contribution for SAA spots in C2, patients with CS and patients with PS were 0.021 ± 0.005, 0.083 ± 0.055 and 0.334 ± 0.117, respectively (Figure 4.20). It appeared that the levels of SAA in PS patients were almost 16-fold higher than their age-matched controls but only 4-fold difference was detected in patients with CS. In addition, there was also a 3-fold difference in the levels of SAA between the two control groups, with a higher mean percentage of volume contribution in the adult subjects (C2), which was statistically significant ($p = 0.014$).

4.3.1 SAA expression analysed by Western blot

To validate the altered abundance of SAA in patients with OS, CS and PS, Western blot analysis was performed. Figure 4.21a shows the expression of SAA in pooled samples on the PVDF membranes. The 14 kDA SAA band were clearly overexpressed in patients with OS and PS compared to their respective age-matched controls. This is generally comparable with the earlier results obtained from the 2DE analysis.

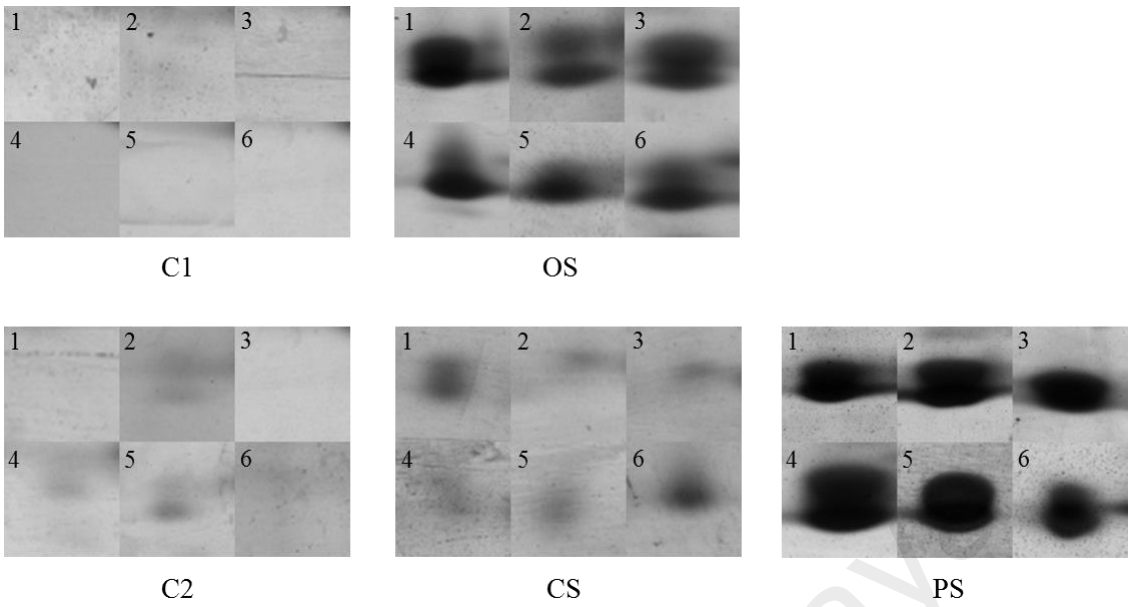


Figure 4.19: Cropped images of SAA spots and densitometry analysis of 2DE gels

Six representative biological replicates of SAA spots from different 2DE gels for healthy adolescents (C1), patients with OS, healthy adults (C2), CS patients and PS patients.

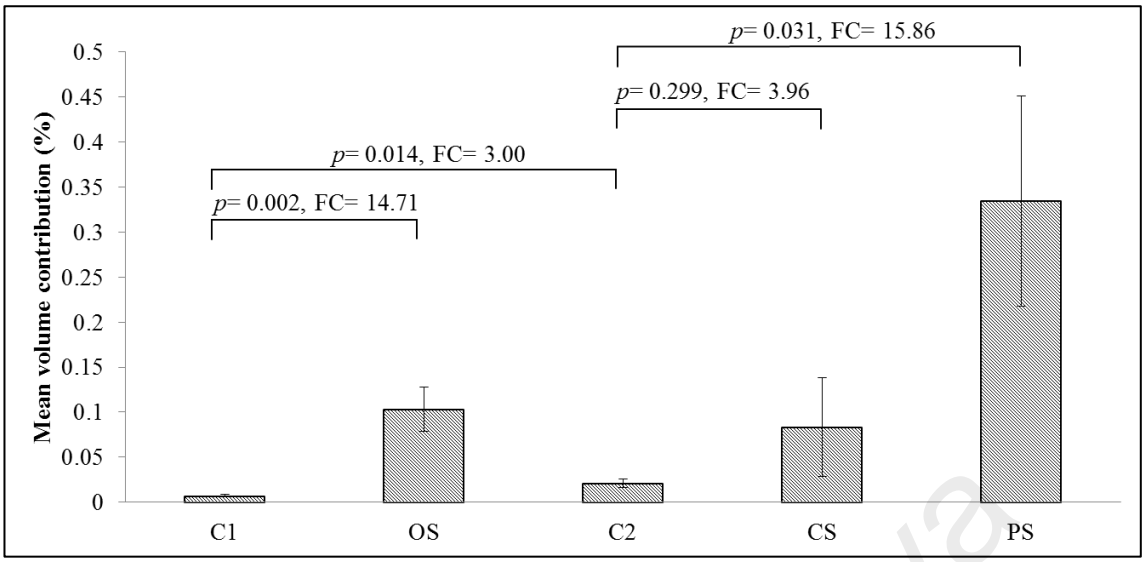
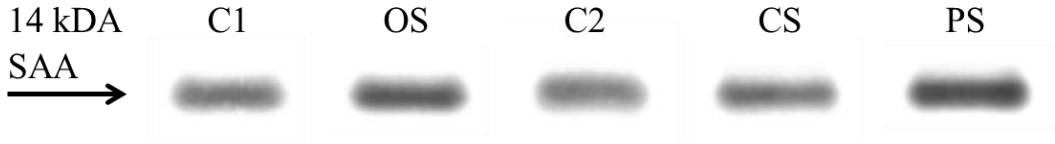


Figure 4.20: Densitometry analysis of SAA spot on 2DE gels

Mean percentage of volume contribution of SAA as analysed by densitometry from 2DE gels (mean ± S.E.M.).

a)



b)

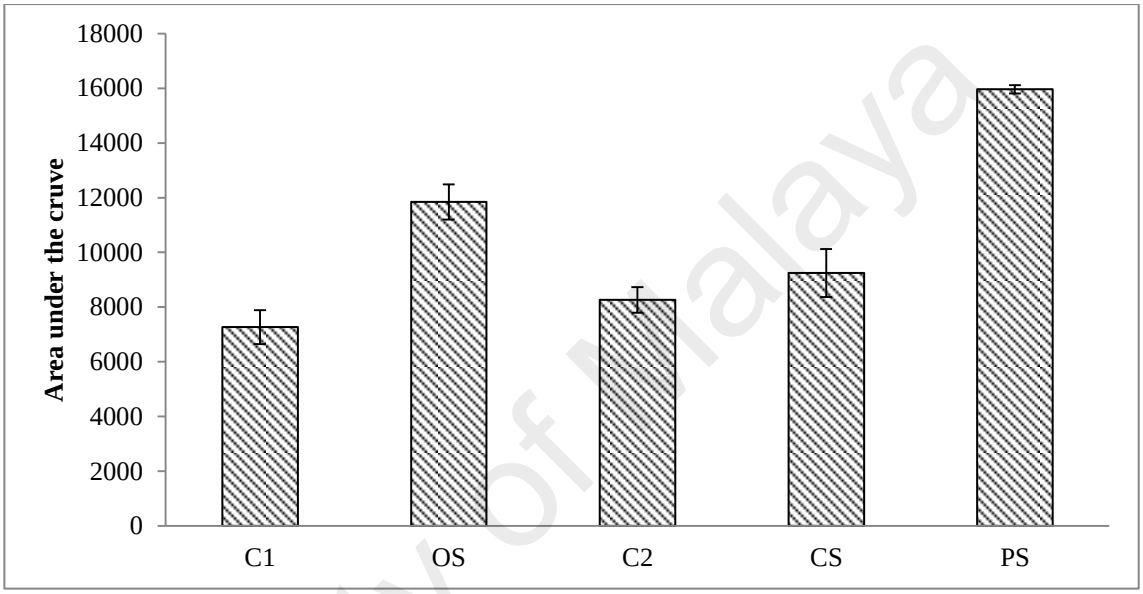


Figure 4.21: Western blot analysis of SAA

Panels demonstrate a) SAA band (14 kDa) detected by antisera against SAA protein and b) densitometry analysis of Western blot images using ImageJ software (mean ± SD of duplicate analysis).

The intensity of each band from the blot was determined using ImageJ software. Areas under the intensity curve were plotted in a histogram for all control groups and patients groups (Figure 4.21b). The intensities of serum SAA band in patients with OS and PS were markedly increased compared to their respective controls by 1.6-fold and 1.9-fold, respectively. In patients with CS, the intensity was similar to that observed in the control (1.1-fold).

4.3.2 Determination of SAA expression level by ELISA

The level of serum SAA from the healthy control groups and patients with OS, CS and PS was also determined using sandwich ELISA. Generally, the results show a comparable pattern with 2DE and Western blot analysis (Figure 4.22). The mean concentrations of serum SAA in C1 subjects and paediatric patients with OS were 17.88 ± 1.60 ng/mL and 19.58 ± 3.38 ng/mL, respectively. Meanwhile, the mean concentrations of serum SAA for C2 subjects, patients with CS and patients with PS were 16.18 ± 1.22 ng/mL, 23.11 ± 4.82 ng/mL and 27.03 ± 5.11 ng/mL, respectively. From the ELISA test, only the increase exhibited in patients with PS was found to be statistically significant compared to its age-matched control C2 (p=0.01).

4.4 Quantitative proteomics by SWATH™-MS

The SWATH™-MS analysis was performed at AB Sciex Singapore courtesy of Analisa Resources (M) Sdn Bhd. At that stage of the study, only sufficient numbers of samples from patients with OS and control C1 managed to be collected and therefore, these groups were selected SWATH™-MS analysis. Pooled serum samples from each of these groups were subjected to albumin and IgG depletions to enrich the low abundance serum proteins. The depleted samples were lyophilised prior to tryptic digestion and LC-MS/MS run and SWATH™-MS acquisition.

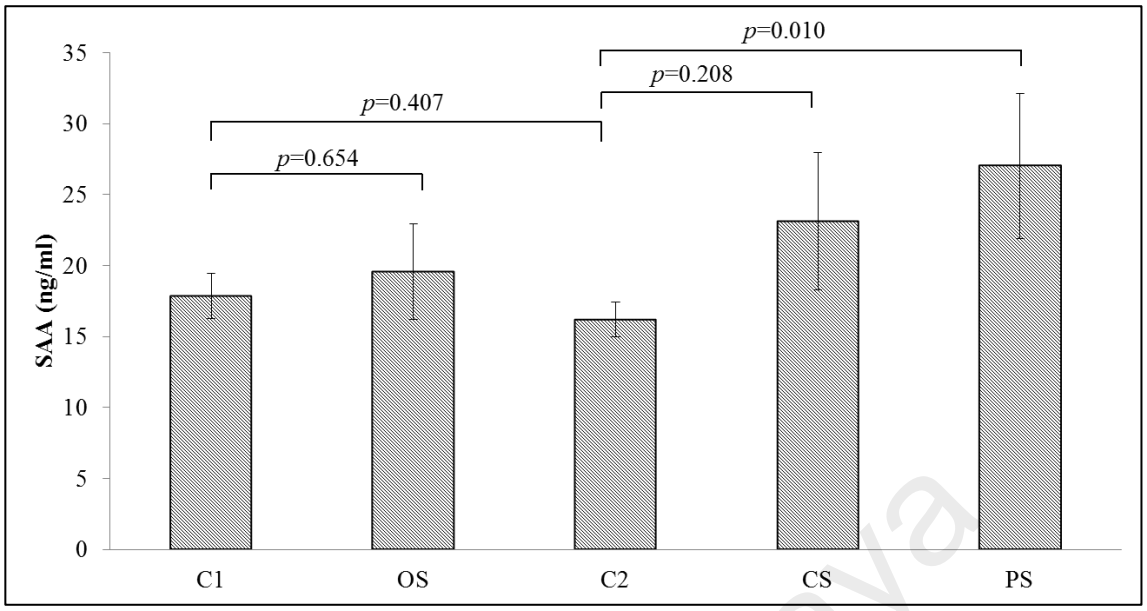


Figure 4.22: ELISA of SAA

Sandwich ELISA was performed to estimate the concentration of SAA in sera of control groups as well as patients with OS, CS and PS (mean ± S.E.M. of biological replicates).

4.4.1 Discovery proteomics using information dependent acquisition (IDA)

Using the ProteinPilot™ v4.5 software, at 1% FDR cut-off, we had successfully identified and quantified 205 unique proteins from the combination of both control C1 and OS samples, of which 7129 distinct peptides and 90561 unique spectral were detected.

4.4.2 Quantitative proteomics using SWATH™-MS acquisition

MS peak extraction was carried out using the PeakView™ software. For each selected peptides, MS/MS spectrum was generated. An example of a SWATH™ MS/MS spectrum is depicted in Figure 4.23 which shows the spectrum generated for peptide FFGHGAEDSLADQAANEWGR from SAA protein (IPI00552578.2). The ion library MS/MS spectrum (pink) from IDA was aligned with SWATH™ acquisition peak apex (blue) to get the peak area in order to generate quantitative values. Figure 4.24a and 4.24b show examples of extracted ion chromatogram (XIC) from both C1 and OS samples. The collection of full scan high-resolution MS/MS enables selection of the best fragments or transitions for further quantitative analysis.

The data was subsequently exported into MarkerView™ software for statistical analysis. Figure 4.25 shows the volcano plot generated from the software which depicted the *p*-values vs log fold change for each 205 proteins (identified as the proteins' IPI) detected. The significant level was set at $p<0.05$ which equals to >log 0.3 and <log -0.3 on the x-axis of the volcano plot. Figure 4.26a shows an example of protein regulation profile plot generated from the software for SAA, which was found to be significantly up-regulated ($p<0.05$) in OS sample as compared to the control C1 sample. Figure 4.26b shows an example of protein regulation profile for isoform 1 of complement factor H (IPI00029739.5), which was significantly down-regulated in patients with OS ($p<0.05$).

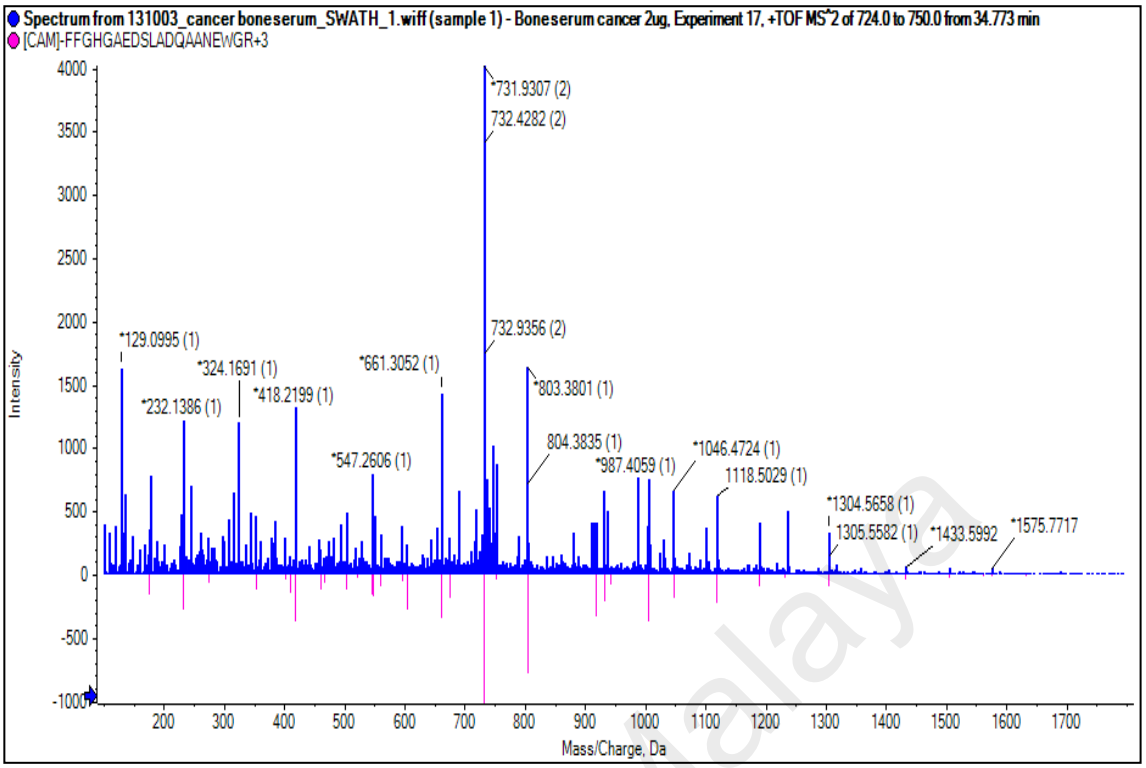
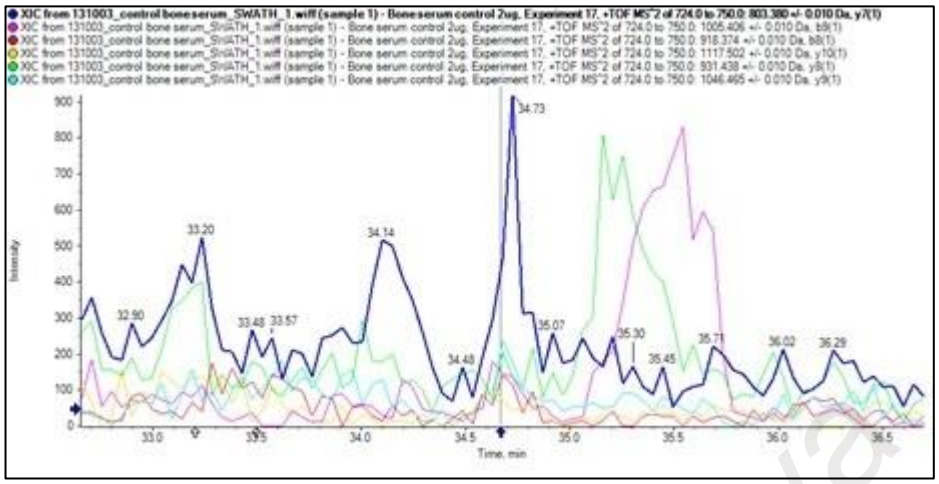


Figure 4.23: MS/MS spectrum of SAA protein

An example of MS/MS spectrum of SAA protein (peptide sequence: FFGHGAEDSLADQAANEWGR) is shown here. The ion library MS/MS spectrum (pink) from IDA was aligned with SWATH™ acquisition peak apex (blue) to get the peak area in order to generate quantitative values.

a)



b)

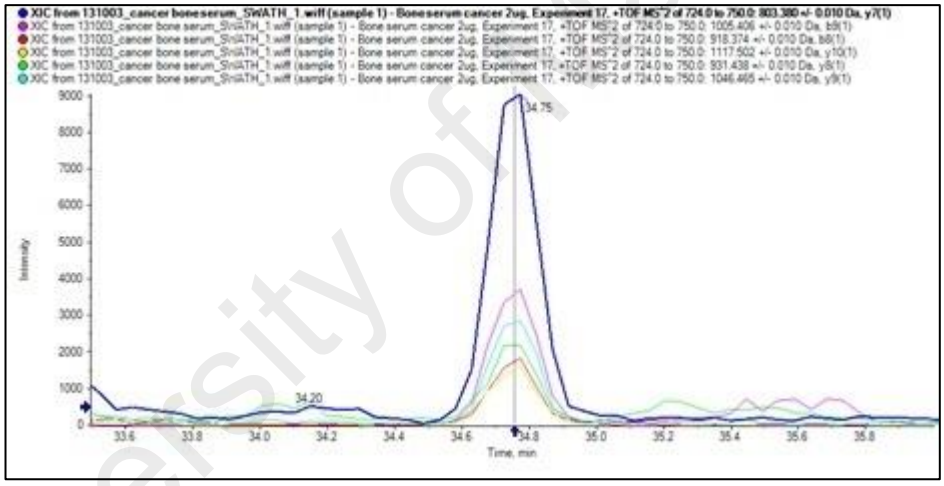


Figure 4.24: Extracted ion chromatogram (XIC) from SWATH-MS acquisition

Panels demonstrate examples of XICs from both (a) C1 and (b) OS samples. The best fragments or transitions from the collection of full scan high resolution MS/MS were selected for the quantitative analysis.

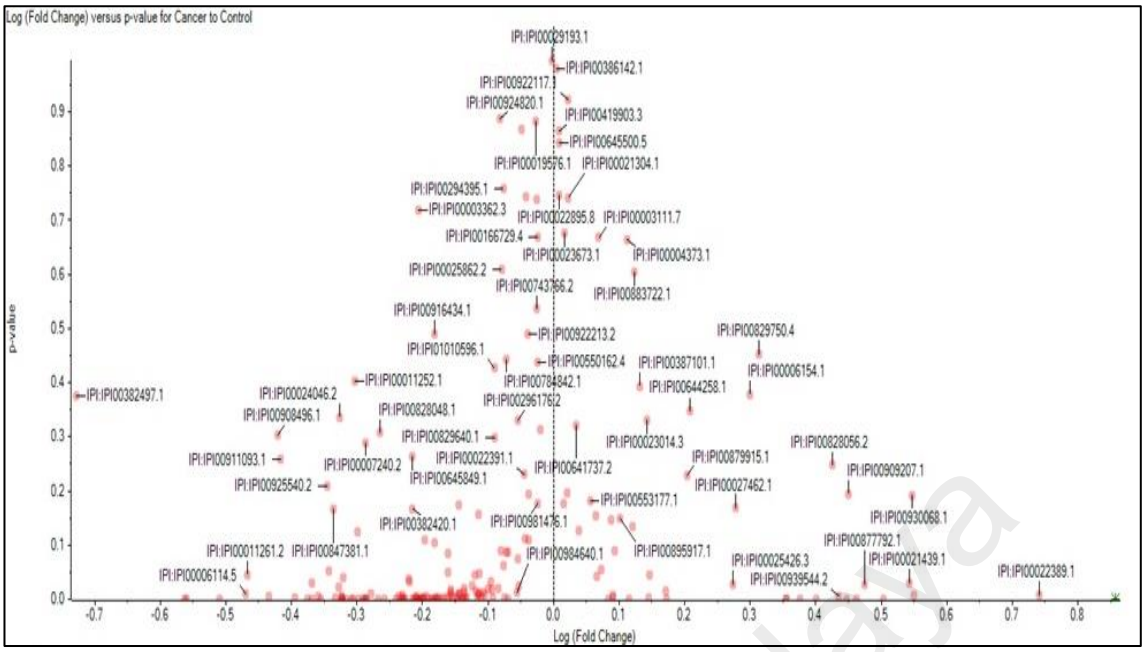
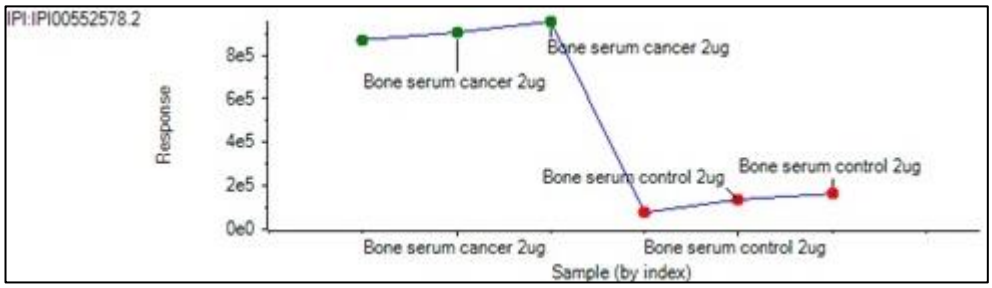


Figure 4.25: Volcano plot of the identified and quantified proteins from SWATH™ analysis

Volcano plot depicted the *p*-values vs log fold change for each 205 proteins detected. The positive side of the graph shows all the up-regulated proteins in the OS sample when compared with control group C1 and the negative side shows the down-regulated proteins.

a)



b)

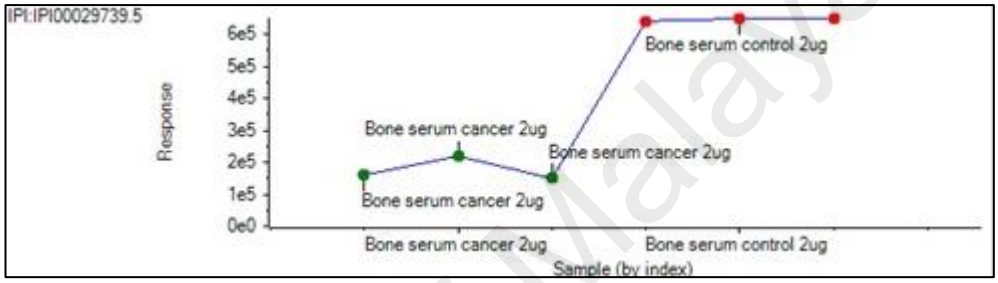


Figure 4.26: Protein regulation profile from the SWATH™ analysis

Panels demonstrate protein regulation profiles of significantly altered proteins (a) SAA (IPI00552578.2) and (b) isoform of complement factor H (IPI00029739.5). Red dots indicate control C1 sample and green dots indicate OS sample (triplicate analysis).

To summarise the differences in the expression of the proteins in the OS sample when compared to the control sample C1, FC for each protein was calculated using MarkerView™ software. In this method, a FC threshold of 2.0 or greater and a *p*-value of 0.05 or less were established as cut-off criteria for potential biomarker candidates. The relative expression of the analysed serum proteins in OS group compared with the control C1 is shown in Table 4.7. Among the 205 proteins identified, 8 proteins were found to be significantly up-regulated in OS sample (Table 4.7a), where the highest FC was demonstrated by SAA (FC=7.20), followed by serum amyloid A2 isoform a, haemoglobin subunit alpha HBA, haemoglobin subunit delta, haemoglobin subunit beta, uncharacterized protein CA1, LRG and alpha-1-acid glycoprotein 1. Fifteen proteins were observed to be significantly down-regulated in OS sample (Table 4.7b), when compared to the control. Isoform 1 of complement factor H was the most down-regulated protein (FC=3.70), followed by isoform 1 of gelsolin, insulin-like growth factor-binding protein complex acid labile subunit isoform 1 precursor, complement C5, B2G1, butyrylcholinesterase isoform CRA_b, apolipoprotein M, kallistatin, plasminogen, alpha-2-antiplasmin, apolipoprotein A-IV, isoform 1 of lipoprotein L1, cDNA FLJ77744, heparin cofactor 2 and isoform 1 of attractin.

Table 4.7: Significantly altered expression of serum proteins in patients with OS compared to control C1

a) Up-regulated proteins

Protein	Gene symbol	International Protein Index (IPI)	Fold change (FC)	*p*-value
Serum amyloid A protein	SAA1, SAA2	IPI00552578.2	7.20	2.42×10^{-5}
Serum amyloid A2 isoform a	SAA1, SAA2	IPI00006146.4	3.19	7.16×10^{-5}
Hemoglobin subunit alpha	HBA1, HBA2	IPI00410714.5	2.90	1.39×10^{-5}
Hemoglobin subunit delta	HBD	IPI00473011.3	2.80	7.13×10^{-6}
Hemoglobin subunit beta	HBB	IPI00654755.3	2.52	7.13×10^{-6}
Uncharacterized protein	CA1	IPI00977761.1	2.38	4.00×10^{-4}
Leucine-rich alpha-2-glycoprotein	LRG1	IPI00022417.4	2.27	3.70×10^{-4}
Alpha-1-acid glycoprotein 1	ORM1	IPI00022429.3	2.26	2.20×10^{-4}

b) Down-regulated proteins

Protein	**Gene symbol**	**International Protein Index (IPI)**	**Fold change (FC)**	***p*-value**
Isoform 1 of Complement factor H	CFH	IPI00029739.5	-3.70	2.74×10^{-5}
Isoform 1 of Gelsolin	GSN	IPI00026314.1	-3.57	7.91×10^{-5}
Insulin-like growth factor-binding protein complex acid labile subunit isoform 1 precursor	IGFALS	IPI00925635.1	-3.23	7.05×10^{-7}
Complement C5	C5	IPI00032291.2	-2.50	1.33×10^{-3}
Beta-2-glycoprotein 1	APOH	IPI00298828.3	-2.38	4.30×10^{-4}
Butyrylcholinesterase, isoform CRA_b	BCHE	IPI00025864.5	-2.33	4.30×10^{-4}
Apolipoprotein M	APOM	IPI00030739.1	-2.27	6.80×10^{-4}
Kallistatin	SERPINA4	IPI00328609.3	-2.17	1.77×10^{-3}
Plasminogen	PLG	IPI00019580.1	-2.13	2.38×10^{-3}
Alpha-2-antiplasmin	SERPINF2	IPI00879231.1	-2.08	5.44×10^{-5}
Apolipoprotein A-IV	APOA4	IPI00304273.2	-2.08	1.60×10^{-4}
Isoform 1 of Apolipoprotein L1	APOL1	IPI00514475.5	-2.08	4.70×10^{-4}
cDNA FLJ77744, highly similar to Homo sapiens kallikrein B, plasma (Fletcher factor) 1 (KLKB1), mRNA	KLKB1	IPI00966520.1	-2.08	4.08×10^{-3}
Heparin cofactor 2	SERPIND1	PI00879573.1	-2.04	2.14×10^{-5}
Isoform 1 of Attractin	ATRN	IPI00027235.1	-2.04	8.94×10^{-5}

4.5 MRM analysis of SAA1 and SAA2 isoforms in the neat serum

Two isoforms of SAA, SAA1 and SAA2 were subjected to MRM analysis to determine the absolute quantity of each isoform. For each of the isoforms, two unlabelled synthetic peptides were selected as external standards for the targeted proteins, *i.e.*, peptides SAA1-1 and SAA1-2 were used to detect isoform SAA1 and peptides SAA2-1, and SAA2-2 were used to detect isoform SAA2 (Table 3.7 in Materials and Methods). The concentrations of SAA1-1, SAA1-2, SAA2-1, and SAA2-2 peptides in pooled serum samples of the three groups of patients with different types of bone cancers (OS, CS and PS) and the two different groups of control subjects (C1 and C2) were determined based on standard curves plotted for each synthetic peptide (Figure 4.27).

Based on the standard curves plotted (Figure 4.27), the concentration of SAA1-1, SAA1-2, SAA2-1, and SAA2-2 peptides in pooled serum from control groups as well as from patients, were determined (Tables 4.8a-b, Figure 4.28). The concentrations of SAA1-1 peptide were apparently quite similar in all groups of subjects analysed. This peptide was found to be marginally increased when their levels in patients with OS, CS and PS were compared to their respective age-matched control groups. However, the concentrations of SAA1-2 peptide appeared higher in all three groups of bone cancer patients when compared to their respective controls. The highest fold change of the SAA1-2 peptide was observed in patients with PS, which was elevated by 2.8-fold, while patients with OS and those with CS demonstrated similar fold changes. On the other hand, comparison was not possibly made for the SAA2 isoform as both the SAA2-1 and SAA2-2 peptides were not detected in C2 and patients with OS.

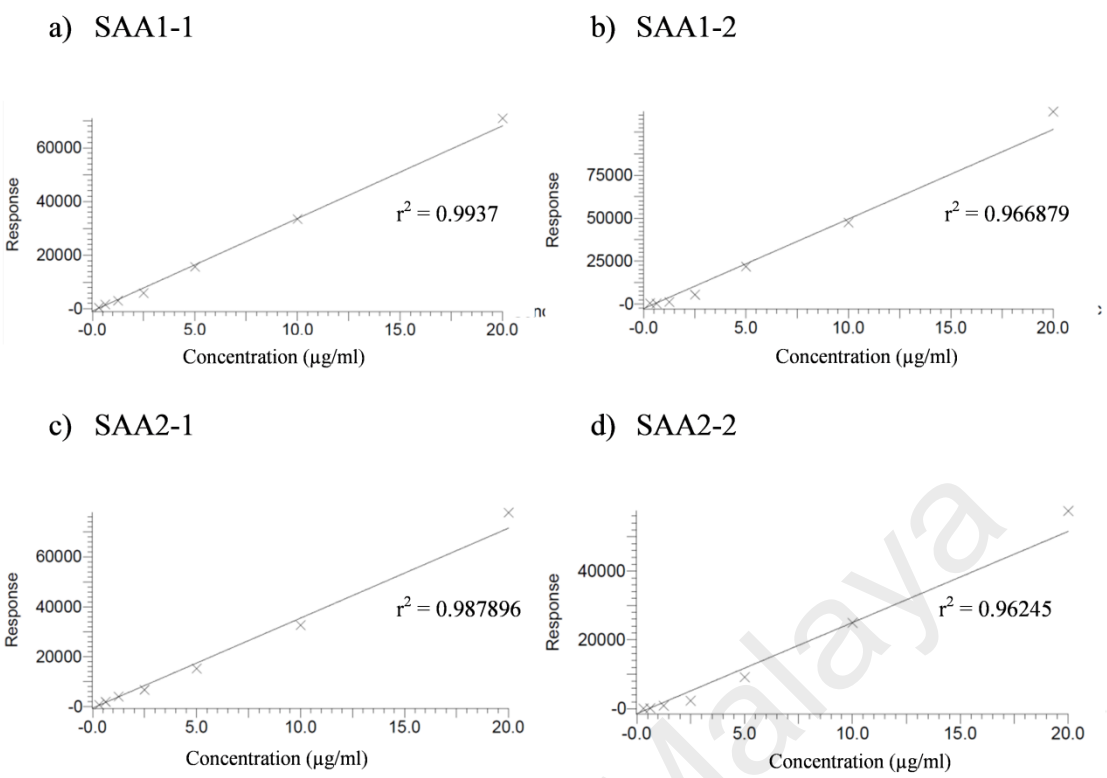


Figure 4.27: Standard curves of the pure peptides using MRM analysis

Standard curves were constructed for each peptide that is specific for SAA1 (peptide SAA1-1 and SAA1-2) and SAA2 (peptide SAA2-1 and SAA2-2). The x-axis represents the concentration of the pure peptides in µg/ml.

Table 4.8: The concentration of target peptides for SAA1 and SAA2 proteins in serum

a)

Protein	Target peptide	C1	OS	
		Concentration (µg/ml)	Concentration (µg/ml)	FC
SAA1	SAA1-1	0.2321	0.2341	1.0086
	SAA1-2	0.5125	0.7882	1.5380
SAA2	SAA2-1	0.1456	n.d	-
	SAA2-2	0.5455	n.d	-

b)

Protein	Target peptide	C2	CS		PS	
		Concentration (µg/ml)	Concentration (µg/ml)	FC	Concentration (µg/ml)	FC
SAA1	SAA1-1	0.2311	0.2308	1.0013	0.2310	1.0004
	SAA1-2	0.5113	0.7768	1.5192	1.4199	2.7770
SAA2	SAA2-1	n.d	0.1500	-	0.1489	-
	SAA2-2	n.d	n.d	-	n.d	-

The concentration of target peptides for SAA1 and SAA2 proteins in serum from all the controls and patients with PS, CS and OS were determined using MRM analysis. FC was calculated compared to their respective controls. (n.d = not detected).

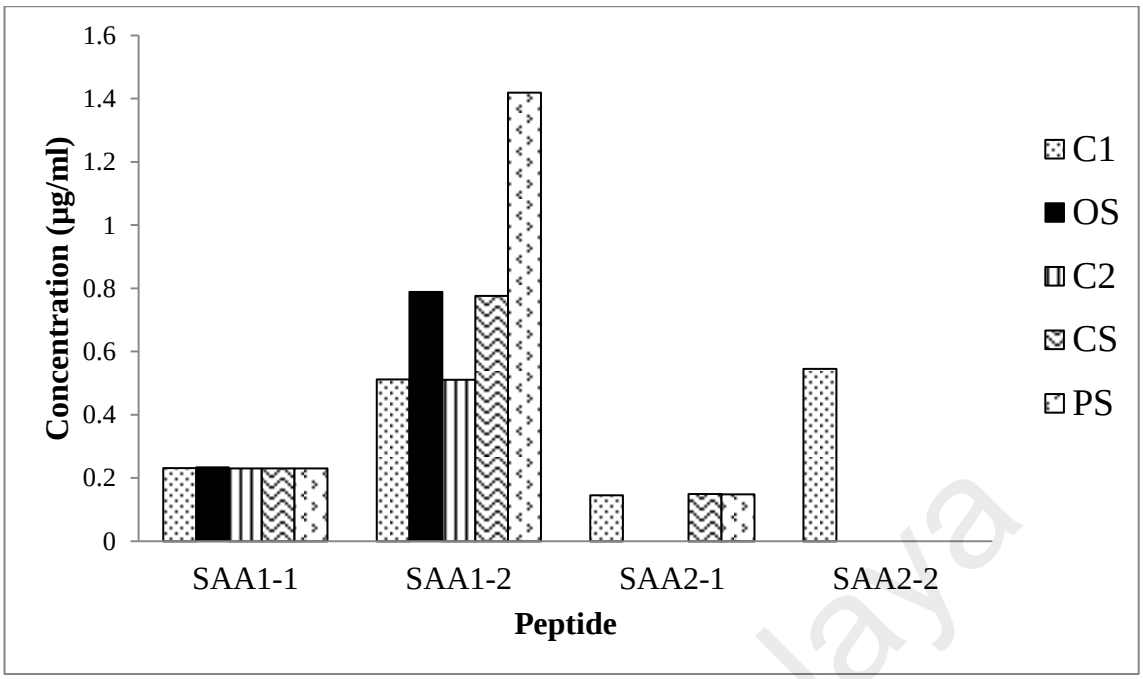


Figure 4.28: Concentrations of SAA1-1, SAA1-2, SAA2-1 and SAA2-2 peptides determined by the MRM experiment

The concentrations of SAA peptides in pooled samples from healthy control groups (C1 and C2) and patients with OS, CS and PS were determined using MRM analysis. (Only a single experiment was carried out for each sample and therefore, standard deviation could not be calculated.)

CHAPTER 5: DISCUSSION

5.1 Analysis of serum protein profiles using silver 2DE and lectin approaches

Analysis of the 2DE protein expression profiles using the image analysis software demonstrated different altered levels of several high abundance proteins in patients with OS, ES, CS, PS and GCT, relative to age-matched non-tumour healthy individuals, C1, C2 and C3. In the silver stained gels, 15 well resolved proteins were selected for analysis because of their high abundance presence. These proteins were AAT, ABG, AHS, APOA1, B2G1, C3f, HAP, HPX, KNG, LRG, SAA, STR, TTR, VTDB and ZAG. The initial identities of these proteins were obtained from comparing the acquired profile to the standard SWISS ExPASy plasma protein reference (Sanchez *et al.*, 1995). Mass spectrometry analysis was later performed to confirm their identities.

Apart from silver stained 2DE analysis, pooled serum samples from the patients and healthy controls were also subjected to Western blotting analysis using enzyme-conjugated CGB and CMB lectins. Glycosylation is a common form of posttranslational modification that occurs on proteins. O-glycosylation and N-glycosylation are the main forms of protein glycosylation. In O-glycosylation, the addition of sugar, or glycan, occur at a serine (Ser) and threonine (Thr) residues while in N-linked glycosylation, the glycan is attached to an asparagine (Asn) residue in tripeptide consensus sequon Asn-X-Ser/Thr where X can be of any amino acid, except for proline. The application of CGB and CMB lectins as probes enables the detection of O-glycosylated and N-glycosylated proteins, respectively. Blots developed with HRP-conjugated CGB lectin showed different profiles compared to 2DE blots developed with AP-conjugated CMB lectin, because CGB lectin binds to the Galβ1,3GalNAc motifs that are present in O-glycosylated peptides while CMB lectin binds to the core mannosyl residues of the N-glycosylated peptides.

The use of lectins also enables the reduction of sample complexity in order to determine the expression of proteins which could not be detected or well resolved in silver 2DE gel. In the CGB-generated blots from the pooled samples for the respective control and disease groups, a total of seven protein clusters were detected and subjected to spot volume analysis. The protein clusters were AHS, AMBP, HPX, ITIH4, KNG, LRG and VTDB. All of these proteins, except for LRG, have been reported in previous publications to contain O-linked glycosylation sites on their peptides (Table 5.1). However, by using NetOGlyc 4.0 server, a software that can predict potential O-glycosylation site for a protein, there are five potential sites for O-linked glycosylation on the amino acid sequence for LRG (Table 5.1). AMBP and ITIH4f proteins were not able to be clearly seen in the silver 2DE gels. However, on CGB-generated blots, ABMP and ITIH4f protein clusters were prominent and easily identified compared to their expression in silver 2DE gels. Both of these proteins contain quite a number of potential O-glycosylation sites as predicted by the software.

There were higher numbers of protein spots detected on CMB-generated blots compared to the CGB-generated blots, and the protein profiles of CMB-generated blots are almost similar to the silver 2DE profiles. This result shows that N-linked glycosylation is a more common type of protein glycosylation in human serum, compared to O-linked glycosylation. Almost 90% of serum glycoproteins contain the N-glycosylation sites (Apweiler *et al.*, 1999). Most of the proteins present in the silver 2DE gel were also shown to be well-resolved on the CMB blot. They were AAT, ABG, AHS, B2G1, HAP, HPX, KNG, LRG, STR, VTDB and ZAG. All of these proteins have also been reported in several publications to contain experimentally annotated N-linked glycosylation sites in their structure (Table 5.1). NetNGlyc Server 1.0 was used to predict N-glycosylation sites for these proteins and they contain at least one potential

Table 5.1: Previously reported and predicted glycosylation sites in the analysed proteins

Accession Number (Swiss Prot)	Protein ID	Type	Publication(s)	Prediction (amino acid position)*
P01009	AAT	O-linked	-	35,37,38,46,337
		N-linked	(Kolarich *et al.*, 2006; Kreunin *et al.*, 2007; Nilsson *et al.*, 2009)	70,107,271 ,414
P04217	ABG	O-linked	-	351
		N-linked	(Bunkenborg *et al.*, 2004; Kreunin *et al.*, 2007; Nilsson *et al.*, 2009)	44,179,363,371
P02765	AHS	O-linked	(Halim *et al.*, 2012)	252,256,257,270,280,293,297,319, 325,328,330,334,339,341,346
		N-linked	(Bunkenborg *et al.*, 2004; Halim *et al.*, 2012; Nilsson *et al.*, 2009)	156,176
P02760	AMBP	O-linked	(Kreunin *et al.*, 2007)	18,24,145,215,222,225,230
		N-linked	(Halim *et al.*, 2012; Kreunin *et al.*, 2007)	36,115,250
P02647	APOA1	O-linked	-	14185,221,228
		N-linked	(Bunkenborg *et al.*, 2004)	No sites predicted
P02749	B2G1	O-linked	-	32,33,149,152,189,259,263,272, 330,331
		N-linked	(Bunkenborg *et al.*, 2004; Nilsson *et al.*, 2009)	162,183,193,253
P01024	C3f	O-linked	-	648,649,650,654,672,742,749,885, 887,1212,1366
		N-linked	(Bunkenborg *et al.*, 2004)	85,939
P00738	HAP/ HAPf	O-linked	-	67,126,316,317,323
		N-linked	(Bunkenborg *et al.*, 2004; Kreunin *et al.*, 2007; Nilsson *et al.*, 2009)	184,207,211,241
P02790	HPX	O-linked	(Halim *et al.*, 2013; Nilsson *et al.*, 2009; Takahashi *et al.*, 1984)	24,29,30,40,181,242,247,248,339
		N-linked	(Bunkenborg *et al.*, 2004; Halim *et al.*, 2013; Nilsson *et al.*, 2009; Takahashi *et al.*, 1984)	64,187,240,246,453

Table 5.1 continued

H7C0L5	ITIH4f	O-linked	(Halim *et al.*, 2012)	418,420,460,471,478,484,494,495, 524,529,530,537,547,548,550,551, 553,675,679,685,694
		N-linked	(Bunkenborg *et al.*, 2004; Liu *et al.*, 2005)	77,375,435#
P01042	KNG		(Nilsson *et al.*, 2009)	137,386,390,400,401,403,407,408, 419,426,521,526,527,528,531,532, 533,535,538,542,546,550,557,559, 571,577,583,604,609,610,611,621, 623
		N-linked	(Bunkenborg *et al.*, 2004; Kellermann *et al.*, 1986; Nilsson *et al.*, 2009)	48,205,294
P02750	LRG	O-linked	-	2,3,5,11,129
		N-linked	(Bunkenborg *et al.*, 2004; Nilsson *et al.*, 2009)	79,186
P0DJI8	SAA	O-linked	-	106
		N-linked	-	No sites predicted in this sequence
P02787	STR	O-linked	-	306,561
		N-linked	(Bunkenborg *et al.*, 2004; Nilsson *et al.*, 2009)	432,630
P02766	TTR	O-linked	-	18
		N-linked	(Bunkenborg *et al.*, 2004)	No sites predicted in this sequence.
P02774	VTDB	O-linked	(Christiansen *et al.*, 2007; Rehder *et al.*, 2009; Yamamoto *et al.*, 1996)	92,95,98,100,113,135,192,214,330
		N-linked	-	288
P25311	ZAG	O-linked	-	197
		N-linked	(Bunkenborg *et al.*, 2004)	112,128,259

* The prediction of O-glycosylation site was determined using NetOGlyc 4.0 server at http://www.cbs.dtu.dk/services/NetOGlyc/ .
The prediction of N-glycosylation site was determined using NetNGlyc 1.0 server at http://www.cbs.dtu.dk/services/NetNGlyc/ .
For any protein that has more than one isoform, the amino acid sequence of the first isoform listed in UniProt website was submitted for predicted for the glycosylation site.

This sequence may not contain a signal peptide. Proteins without signal peptides are unlikely to be exposed to the N-glycosylation machinery and thus may not be glycosylated (in vivo) even though they contain potential motifs.

site of glycosylation in their amino acid sequence. This software works by distinguishing the glycosylated sequons from non-glycosylated sequons in the sequence.

Interestingly, the presence of white spots on the blots could be observed where the APOA1 protein supposed to be located (Figure 4.14). This protein is known to contain N-linked glycosylation sites (Bunkenborg *et al.*, 2004), although prediction on the amino acid sequence revealed no potential site for N-linked glycosylation to occur (Table 5.1). In the silver 2DE gels, APOA1 was displayed as very intense dark spots (Figure 4.1). An excess of protein or antibody on the blot is known to cause white band (negative band) in Western blot analysis, and this could be the case with APOA1 (Mahmood & Yang, 2012).

In this study, a few proteins exhibited dissimilar or contrasting regulation pattern between silver 2DE profiles and CGB- or CMB-generated blot profiles. For example, in patients with OS, HPX showed a significant down-regulation in silver 2DE gels but insignificant down-regulation in both CGB- and CMB-generated blots. The silver ions in the silver stain bind to the whole protein structure, whereas each lectin has the ability to only recognize specific glycosylation motif. Therefore, the expression of proteins in CGB- or CMB-generated blots cannot be used to directly correlate with the silver stained 2DE results. HPX had been reported to possess both O-linked (Halim *et al.*, 2013; Nilsson *et al.*, 2009; Takahashi *et al.*, 1984) as well as N-linked glycosylation sites (Bunkenborg *et al.*, 2004; Halim *et al.*, 2013; Nilsson *et al.*, 2009; Takahashi *et al.*, 1984). Therefore, the expression of the O-linked glycosylated sites represents only part of the whole protein structure, likewise with the N-linked glycosylated sites exhibited in CMB-generated blots. Due to the above reason, only proteins with significant dysregulation and met the biomarker criteria set in this study (FC>1.5 and $p<0.05$) observed from the silver 2DE analysis will be discussed here.

AHS, also known as fetuin-A, is a liver secretory glycoprotein, synthesized by hepatocytes. In this study, the level of AHS was found to be significantly down-regulated with FC>1.5 in patients with ES (p<0.01, -1.902-fold) and PS (p<0.01, -1.548-fold). AHS was shown to have opsonic properties by van Oss *et al.* (1974). Lebreton *et al.* (1979) showed evidence that human AHS is a negative acute phase reactant where the biological level decreases in diseases accompanied by an inflammatory process. This finding was supported by the fact that the concentration of AHS in serum and urine of various renal diseases was found to be significantly reduced when compared to the control samples (Kishore *et al.*, 1983). Schäfer *et al.* (2003) had identified serum AHS as an important inhibitor of ectopic calcification (inappropriate biomineralization occurring in soft tissues) in AHS-deficient mice. Plasma concentration of AHS was also found to be lower in patients with Paget's disease of bone, a chronic disorder that can cause deformity which involves abnormal bone destruction and regrowth (Ashton & Smith, 1980).

APOA1 is the major structural protein of high density lipoprotein (HDL). It is involved in lipid metabolism, mediating cellular cholesterol efflux and also acts as lecithin-cholesterol acyltransferase (LCAT) activator (Frank & Marcel, 2000). In this study, serum APOA1 was demonstrated to be significantly down-regulated in patients with OS (p<0.01), CS (p<0.05) and PS (p<0.05). However, the down-regulation of APOA1 of more than 1.5-fold was observed only in patients with PS (-1.53-fold). The down-regulation of APOA1 in serum of patients with insulin resistance/type-2 diabetes (Zhang *et al.*, 2004a) and also in patients with various tumours *i.e* ovarian, uterus and breast (Goufman *et al.*, 2006; Kozak *et al.*, 2005; Zhang *et al.*, 2004b) suggesting this protein as potential biomarkers. In ovarian cancer patients, APOA1 is thought to be a response towards the presence of the tumour (Zhang *et al.*, 2004b).

B2G1 is an apolipoprotein, formerly termed as apolipoprotein H, generally involved in lipid transport, the absorption and metabolism through the lymphatic and circulatory systems. This protein was found to be significantly up-regulated by more than 1.5-fold in patients with CS and GCT, both are bone tumour with lower degree of malignancy. However, from the SWATH™-MS analysis (which will be discussed in the next section), significant down-regulation of B2G1 was observed in the patients with malignant OS, similar to a report by Lokamani *et al*. (2014) in cervical squamous cell carcinoma. B2G1 is involved in the coagulation cascade and the decreased level was probably due to a high degree of coagulation process in cancer patients with higher degree of malignancy (Lokamani *et al.*, 2014; Murray, 1991; Sampson & Kakkar, 2002).

C3 is part of a group of plasma proteins that plays a major role in the activation of the complement system and contributes to the body's defence mechanism. It is primarily synthesized in the hepatocytes (Alper *et al.*, 1969). Antibodies or cellular surfaces activate the complement system and initiate an interaction cascade to produce membrane attack complex to cause direct cytolysis (de Bruijn & Fey, 1985). C3 involves in both the classical and alternative pathways of the activation. This study demonstrated that C3 was found to be significantly down-regulated in patients with CS ($p<0.05$, -2.053-fold) and GCT ($p<0.05$, -1.884-fold). Similarly, the expression of a carboxy terminal of C3 was also demonstrated to be lower in patients with hepatocellular carcinoma as compared to the control group (Steel *et al.*, 2003). The C3 expression was found to be up-regulated as well in the sera of patients with Alzheimer's disease and insulin resistance/type-2 diabetes (Zhang *et al.*, 2004a). Using SELDI-TOF MS and 2DE approaches, Lee *et al*. (2006) found that complement C3a (a product from proteolysis of C3) was elevated in serum of patients with chronic hepatitis C and

hepatitis C virus-related hepatocellular carcinoma. C3a was also seen to have an elevated level in colorectal cancer patients' serum as reported by Ward *et al*. (2006).

HAP is an acute phase reactant synthesized mainly in the hepatocytes (Theilgaard-Monch *et al.*, 2006). Initially, HAP is synthesized as a single polypeptide chain this tetrameric form is cleaved into two short α-chains and two long β-chains, linked via disulphide bonds, by proteolytic activity of complement C1r-like protein in the endoplasmic reticulum (Wicher & Fries, 2004). The plasma concentration of intact HAP can increases by several-fold following an infection or injury. One of the main functions of HAP is to be involved in the removal of haemoglobin from plasma where it complexed with the haemoglobin for the rapid clearance by tissue macrophages (Polticelli *et al.*, 2008). de Kleijn *et al*. (2002) had proven that HAP is expressed in the arteries and involved in arterial restructuring where it facilitates cell migration. Extensive bone resorption, a process involving the breakdown of bone by osteoclasts, occurs in CS (Jelthi *et al.*, 1987) and also GCT (Cowan & Singh, 2013). In GCT, the insufficient new bone formation may result from the presence of a high number of giant cells, which are bone-resorbing, osteoclast-like cells, and therefore promoting a higher rate of the bone resorption. HAP was found to stimulate bone resorption process via humoral mechanism (Lerner, 1994; Lerner & Frohlander, 1992).

HAP is also one of the most extensively studied acute phase proteins and numerous studies had suggested its potential as biomarkers. In this study, only serum of patients with CS was found to exhibit significant down-regulation of serum HAP ($p<0.01$, -2.001-fold). Similarly, HAP was found to be down-regulated in serum of patients with gastric cancer (Liu *et al.*, 2007). In contrast, it was demonstrated that serum HAP expression was found to be fourfold higher in patients with ovarian cancer as compared with normal controls (Ahmed *et al.*, 2004; Ahmed *et al.*, 2005). Significant elevation its

α-subunit also was discovered in the sera from ovarian cancer patients (Ye *et al.*, 2003). It was suggested that this protein may interfere with the immune system because it has similar structural and functional homology with human 7S immunoglobulins (Black & Dixon, 1968; Ye *et al.*, 2003). In contrast, Huang *et al*. (2006) reported a decreased expression of this protein's subunit in the serum of breast cancer patients. It was hypothesised that the increased of haemoglobin in the patients led to the decrease, where HAP acts as a haemoglobin scavenger (Huang *et al.*, 2006).

HPX is a serum acute phase reactant that possesses the highest binding affinity to heme among any known protein (Tolosano & Altruda, 2002). This protein is primarily expressed in the liver. It functions to bind heme and transports it to the liver for breakdown and iron recovery (Tolosano & Altruda, 2002). HPX can also be found in human saliva (Ramachandran *et al.*, 2006) and Kristiansen *et al*. (2004) had reported the presence of HPX in the bile fluid obtained from a patient with cholangiocarcinoma (malignancies of the biliary duct system). In this study, HPX was found to significantly down-regulated in the sera of OS patients as compared to the control (p<0.01, -1.7-fold). Similar finding was observed where HPX was found to be down-regulated in sera of pancreatic cancer patients (Yu *et al.*, 2005).

KNG or sometimes called as high molecular weight kininogen, is produced primarily by the liver. This protein is involved in blood coagulation system and fibrinolysis by inhibiting the thrombin- and plasmin-induced aggregation of thrombocytes in human (Pang *et al.*, 2010). In the present study, the expression of serum KNG was significantly decreased in patients with CS and PS by around 2-fold in both silver 2DE analysis and as well as in the CMB-generated blots. KNG is known as a negative acute phase reactant protein and similarly, its plasma level was found to be decreased in patients with advanced gastrointestinal cancer (Roeise *et al.*, 1990). It was also found to be

significantly reduced in other types of diseases such as breast cancer, endometrial cancer and cervical cancer (Abdul-Rahman *et al.*, 2007; Doustjalali *et al.*, 2004).

LRG is one of the trace proteins in human plasma. The function of LRG is unknown although a few reports had suggested LRG to play a role in cell adhesion (Kobe & Kajava, 2001; Takahashi *et al.*, 1985) and able to bind to cytochrome c (Cummings *et al.*, 2006). In this study, LRG was found to be significantly up-regulated in patients with PS by more than two-fold ($p<0.01$, +2.2-fold). Similarly, study of plasma proteomics of pancreatic cancer and lung cancer patients using multi-dimensional liquid chromatography and 2D-DIGE had also shown significant up-regulation of LRG (Kakisaka *et al.*, 2007; Okano *et al.*, 2006). Using ELISA, Andersen *et al.* (2010) had demonstrated that the level of serum LRG was significantly elevated in ovarian cancer patients compared to healthy women and women with benign gynaecological disease. The level of this protein was also found to be significantly increased in patients with endometrial adenocarcinoma, the most common type of endometrial cancer in women (Abdul-Rahman *et al.*, 2007).

In this study, the changes exhibited by SAA were the highest among the 15 analysed proteins. In general, from the 2DE analysis, the other proteins were found to be up-regulated or down-regulated by about 2-fold (Table 4.3), but on the other hand, SAA was found to be significantly elevated by about 14-fold and 16-fold in patients with OS and PS, respectively. For that reason, SAA was selected for further validation analyses (using Western blot, ELISA and MRM) and will be discussed in the next section.

TTR is a protein that possesses high affinity binding with thyroxine and tri-iodothyronine and it assists the plasma transport of retinol via its interaction with retinol binding protein (Zhang *et al.*, 2004b). It is synthesized in the hepatocytes (Blaner *et al.*, 1991) but Soprano *et al.* (1985) suggested that TTR may be synthesized in several

extrahepatic organs such as heart, skeletal muscle, stomach and spleen. In this study, it was found that the expression of TTR was significantly down-regulated in patients with OS ($p<0.05$), ES ($p<0.05$) and PS ($p<0.01$). Down-regulation of TTR of more than 1.5-fold was only observed in patients with PS (-1.502-fold). Using 2D-DIGE, Jin *et al*. (2007) reported a comparable finding, where the detected TTR in the serum of patients with OS was significantly down-regulated. Similarly, a proteomic study on sera of patients with lung cancer and benign lung diseases reported a down-regulation of TTR when compared with healthy controls (Liu *et al.*, 2007). Kozak *et al*. (2005) and Zhang *et al*. (2004b) both reported a down-regulation in the expression of serum TTR in ovarian cancer suggesting it can be used to facilitate the detection of this disease at an early stage and this finding was supported by an independent blinded study by Moore *et al*. (2006). The level of TTR in women with malignant ovarian tumour was compared with women with benign ovarian tumour and female controls with abdominal hernias, and the results show that TTR may improve the specificity of the diagnosis (Moore *et al.*, 2006).

In terms of glycosylation profiling using lectins, AMBP and ITIHf proteins were two proteins that were found to be well-resolved in the CGB-generated blots. AMBP was present in patient with OS, PS and GCT as well as in control C3. This protein was found to be increased in patients with OS and PS, but was decreased in patients with GCT. However, these differences were not significant when compared to the controls. This protein, exclusively expressed in the liver, functions as plasma serine proteinase inhibitor, endothelial cell growth, intracellular calcium level regulator and also as an inhibitor of kidney stone formation (Fries & Blom, 2000; Tyagi *et al.*, 2002). The gene coded for AMBP belongs to the family of human inter-alpha-inhibitor genes and the down-regulation of this gene was observed in kidney cancer (Hamm *et al.*, 2008).

ITIH4f was present in control C2, C3 as well as in patients with OS, CS, PS and GCT. With the exception of OS, the expression of this protein was decreased in patients with CS, PS and GCT, though insignificant compared to their age-matched control groups. Previous works in our laboratory had earlier reported about the dysregulation of serum ITIH4f in a few types of cancers (Jayapalan *et al.*, 2012; Mohamed *et al.*, 2008). These two studies used an immobilized CGB lectin affinity column, an alternative method to Western blotting, developed to resolve these glycosylated proteins on 2DE gels. Cyanogen bromide-activated Sepharose® 4B column incorporated with CGB-lectin was used to enrich the O-glycosylated proteins in order to profile any differences in the serum glycoproteomes between the patients and the controls (Jayapalan *et al.*, 2012; Mohamed *et al.*, 2008). In the previous study by Mohamed *et al*. (2008), ITIH4f was found to be elevated the patients with OS, but the increased level were found to be insignificant. However, significant enhanced expression of serum ITIH4f was observed in breast carcinoma, epithelial ovarian carcinoma, germ cell ovarian carcinoma (Mohamed *et al.*, 2008). The increased levels of this protein were deduced to be correlated with dysregulation of the hormonal activity *i.e*. increased levels of estrogens in the patients (Mohamed *et al.*, 2008). Conversely, a decreased expression of this protein in patients with prostate carcinoma was also reported (Jayapalan *et al.*, 2012). These contradicting results are most probably due the differences in the ratio of the sex steroid hormones between men and women cancer patients (Jayapalan *et al.*, 2012).

5.2 SAA as potential biomarker for sarcomas

From the silver 2DE analysis, serum SAA was found to be highly elevated in patients with OS and PS and the increased was statistically significant compared to their age-matched control groups. The protein was also increased in abundance (about 13-fold) in patients with ES, another type of a very aggressive paediatric bone sarcoma. However, the difference was found to be statistically insignificant. Small sample size (n=5) and

large variation of the SAA levels between the subjects could be the reason behind this observation.

Unlike the silver 2DE gels, SAA spot could not be detected in the CGB- and CMB-generated blots. Prediction of O-linked glycosylation motif on SAA by NetOGlyc 4.0 server revealed only a single potential glycosylated site which occurs at amino acid 106 (Ser) (Table 5.1). However, this predicted site may contain other moieties than the Galβ1,3GalNAc, which is specific for CGB lectin. Thus, this may be the reason as to why SAA could not be detected in the CGB-generated blot. On the other hand, NetNGlyc 1.0 server was used to analyse and predict potential N-glycosylation site in the amino acid sequence of the protein. From the analysis, it was discovered that SAA does not have any potential N-glycosylation site in its sequence (Table 5.1).

The altered abundance of serum SAA was also validated and confirmed using two other methods, *i.e*. Western blot and ELISA. Three groups of malignant bone sarcomas, OS, CS and PS, were selected for these analyses. The increased of SAA in patients with OS has been described before (Jin *et al.*, 2007; Li *et al.*, 2006). Therefore, the inclusion of this group in the current study is important as a confirmation and also as verification to the previous finding. In addition, the inclusion of CS group in the validation study allowed a comparison which could be made between different types of sarcomas and correlation to their aggressiveness. Although the increase of SAA was not statistically significant in patients with CS, unlike OS and PS, it is a type of malignant tumour but with a low metastatic rate and not very aggressive. From these tests, the SAA levels were demonstrated to be markedly high in patients with PS, and followed by patients with OS and patients with CS.

Jin and co-workers (Jin *et al.*, 2007) found the expression of SAA as the most highly up-regulated protein in patients with OS when compared with healthy controls by means

of 2D-DIGE. Li *et al*. (2006) suggested SAA as a plasma proteomic signature to differentiate between the malignant OS and benign osteochondroma. Plasma level of SAA was also found to be clearly elevated in paediatric OS patients when compared to the benign bone tumour (Li *et al.*, 2006). While the altered regulation of SAA in these tumours had been described by other studies, it is believed this was the first study that showed a significant increase of SAA in patients with PS compared to their healthy controls by means of proteomics. In other cancers, the SAA level was also found to be elevated in patients with nasopharyngeal carcinoma, uterine cervical carcinoma, ovarian carcinoma, non-small cell lung carcinoma, lung carcinoma, gastric carcinoma, prostate carcinoma and nephroblastoma (Bergamini *et al.*, 2014; Chan *et al.*, 2007; Cho *et al.*, 2004; Howard *et al.*, 2003; Menschikowski *et al.*, 2013; Moshkovskii *et al.*, 2005; Pan *et al.*, 2008; Ren *et al.*, 2014; Sung *et al.*, 2011; Wang *et al.*, 2012).

SAA is a major acute phase reaction protein and synthesized primarily in the liver (Uhlar & Whitehead, 1999). It is secreted in response to inflammatory process and normally present at low level in sera of healthy subjects (Biran *et al.*, 1986; Malle *et al.*, 2009). The human SAA is a polymorphic protein, exists in various isoforms encoded at four loci by different genes, *i.e*. SAA1, SAA2, SAA3 and SAA4 (Malle *et al.*, 2009; Uhlar & Whitehead, 1999). Human acute phase SAA1 and SAA2 proteins are encoded by the SAA1 and SAA2 genes, which are about 95% identical in their gene organization (Malle *et al.*, 2009; Uhlar & Whitehead, 1999). These two acute phase proteins have 90% homology in their amino acid sequences (Kluve-Beckerman *et al.*, 1988). There are 10 amino acids that are different between both isoforms (Figure 5.1). In humans, SAA3 is a pseudogene but in rodents it has been shown to be transcribed to SAA3 (Larson *et al.*, 2003). SAA4 encodes for a constitutive SAA protein that is expressed at a constant level in humans (Hagihara *et al.*, 2004; Ren *et al.*, 2014).

a) SAA1 (P0DJI8)

10	20	30	40	50
MKLLTGLVFC	SLVLGVSSRS	FFSFLGEAFD	GARDMWRAYS	DMREANYIGS
60	70	80	90	100
DKYFHARGNY	DAAKRGPGGV	WAAEAISDAR	ENIQRFFGHG	AEDSLADQAA
110	120			
NEWGRSGKDP	NHFRPAGLPE	KY		

b) SAA2 (P0DJI9)

10	20	30	40	50
MKLLTGLVFC	SLVLSVSSRS	FFSFLGEAFD	GARDMWRAYS	DMREANYIGS
60	70	80	90	100
DKYFHARGNY	DAAKRGPGGA	WAAEVISNAR	ENIQRLTGRG	AEDSLADQAA
110	120			
NKWGRSGRDP	NHFRPAGLPE	KY		

Figure 5.1 Amino acid sequences of acute phase SAA1 and SAA2

Both SAA1 and SAA2 consist of 122 amino acids, with the first 18 are signal peptides. Amino acids that are different between both isoforms are underlined.

There are many reports that indicated SAA is regulated by proinflammatory cytokines such as IL-1, IL-6 and TNFα, where they synergistically increase the level of SAA in inflammatory response (Castell *et al.*, 1988; Hagihara *et al.*, 2004; Hansen *et al.*, 2015; Raynes *et al.*, 1991). Lin and co-workers (2013) reported that the expression of IL-6 was significantly higher in OS tissues compared to normal bone and strongly correlated with tumour stage. The serum level of IL-1Ra, IL-6, IL-8 and TNFα were also found to be increased in patients with OS suggesting the role of proinflammatory cytokines in the development of OS (Xiao *et al.*, 2014). OS cells produce various growth factors such as transforming growth factor (TGF), insulin-like growth factor (IGF) and connective tissue growth factor (CTGF). The disruption in the regulation of these growth factors is known to expedite the cell proliferation (Broadhead *et al.*, 2011). Osteoclasts are the main bone-resorbing cells in normal as well as pathologic states (Roodman, 2001). IL-6, which may acts in concert with IL-1α, was found to induce bone resorption by stimulating the osteoclast formation (Ishimi *et al.*, 1990). Increased osteoclastic bone resorption in turn released the growth factors that can enhance tumour growth (Roodman, 2001). Osteoblast cells produce IL-6, in response to local bone-resorbing agents and this induces encourage bone resorption both alone and in combination with other bone-resorbing agents (Ishimi *et al.*, 1990).

The data of the present study appears to also support the association of SAA with tumour metastasis, as PS and OS, which were shown to associate with markedly high levels of SAA in the patients' sera, are known to be highly aggressive malignant neoplasms and often metastasise to other organs and become the cause of death. On the other hand, the increased amounts of SAA in patients with CS, which is a less aggressive sarcoma, were marginal compared to those detected in patients with PS and OS. Unlike PS and OS, CS is known to be a type of bone sarcoma that has a lower metastatic rate (Bertoni *et al.*, 2002; Hogendoorn *et al.*, 2010).

The high level of SAA has been suggested to increase the chance for a tumour to progress and metastasise (Urieli-Shoval *et al.*, 2000). In patients with OS, the metastasis of this tumour depends on the interaction between the bone matrix, OS cells, osteoblasts and osteoclasts (Broadhead *et al.*, 2011). The interaction between IL-6 and IL-6 receptor has also been shown to increase the expression of intercellular adhesion molecule-1 through integrin-linked kinase/Akt/c-Jun/AP-1-dependent pathways and this in turn induced the migration of human OS cells (Lin *et al.*, 2013). SAA also has been found to stimulate the production of matrix-metalloproteinase-9 by macrophages in lung cancer, which will assist the cancer cells to metastasise (Sung *et al.*, 2011). Although not much is known about the association of SAA with PS, it is possible that the mechanism of the metastasis formation is similar to these cancers.

In the 2DE analysis, the identification of the SAA spot in the gel by mass spectrometry analysis using MALDI-TOF/TOF was reported as SAA protein precursor with Swiss-Prot accession number of P0DJI8, having a theoretical mass of 13532 Da and theoretical pI of 6.28 (Table 4.1). These theoretical mass and pI matched the location of the spot observed in the 2DE gels (Figure 4.1). From the Western blot analysis, the SAA band detected was closer to the 15 kDa protein ladder mark, and it is therefore labelled and estimated as 14 kDa (Figure 4.21).

Without the signal peptide, the weight of a mature SAA protein is 11702 Da (from amino acid residues 19 to 122) (Howard *et al.*, 2003). However, SAA4 is known to be N-glycosylated and the glycosylation pattern was discovered to be influenced by polymorphism of the protein (Yamada *et al.*, 2014). The changes in the protein structures and posttranslational modifications to the SAA as well as incomplete unfolding may influence electrophoretic mobility during the 2DE and SDS-PAGE run.

Thus, it is possible for the protein to be resolved at a higher mass, particularly in the Western blot experiment, compared to the calculated weight of the mature SAA protein.

The theoretical pI of SAA1 and SAA2 are 5.89 and 9.10, respectively. However, in this study, the focus on pH 4-7 was chosen because earlier works by our laboratory had demonstrated that most of the serum proteins were best resolved in that particular range of pH. Consequently, it is possible that SAA2 was not able to be detected at all in the 2DE gel. Nevertheless, the experimental pI of most proteins resolved by 2DE usually and may differ from their theoretical values mainly because of protein modifications such as truncations, deletions and posttranslational modifications such as phosphorylation as well as glycosylation (particularly sialylation). Due to phosphorylations, a protein with a higher predicted pI (above 7.0) may experience a larger pI shift experimentally (by several units) compared to a protein with a pI of about 5.0 (Zhu *et al.*, 2005). Thus, it is likely that the shift exhibited by the SAA2 in the 2DE experiment was caused by phosphorylation, a common modification that occurs on this protein (Nel *et al.*, 1988).

Using 2DE and Western blot, it was not possible, however, to differentiate whether the SAA spot, or band, in the case of Western blot analysis, consists of either SAA1, SAA2 or possibly SAA4 or maybe a combination of two or more SAA protein isoforms, as they have similar theoretical molecular weight. In addition, the antisera used in Western blotting and ELISA experiments were also known not to be able to differentiate recognition of SAA1 and SAA2. The study of both SAA1 and SAA2, which are the acute phase SAA, could provide further understanding about the involvement of these proteins in inflammation and pathology of cancer. Most studies associating SAA with cancers did not make a distinction between these two acute phase isoforms (Chan *et al.*, 2007; Cho *et al.*, 2004; Howard *et al.*, 2003; Jin *et al.*, 2007; Li *et*

al., 2006; Menschikowski *et al.*, 2013; Pan *et al.*, 2008; Ren *et al.*, 2014; Sung *et al.*, 2011). However, due to unavailability of specific antibody that can distinguish these isoforms, other mass spectrometry methods such as SWATH™-MS and MRM can be used to overcome this disadvantage.

Among the various methods and techniques employed in the different approaches in proteomics studies, SWATH™-MS acquisition has been gaining traction as a powerful tool to quantitatively characterise multiple complex protein samples in a fast and efficient manner. SWATH™-MS data acquisition allows dynamic protein probing by re-mining the acquired data set at any time without repeating the experiment. Thus, SWATH™-MS acquisition enables highly reproducible identification and relative quantification of a large number of protein and peptides in the complex sample with good coverage of the proteome.

Out of 205 proteins identified in the analysis, 23 proteins were significantly dysregulated, in which eight were up-regulated in the patients with OS. From the database used, SAA (IPI00552578.2) demonstrated the highest altered level, which was found to be 7.2 times higher in the OS sample compared to the control sample C1. Another isoform of SAA, serum amyloid A2 isoform a (IPI00006146.4) was up-regulated by 3.19-fold in the patients. The protein accession numbers obtained from the SWATH™-MS analysis were given as International Protein Index (IPI), and upon further confirmation, SAA with IPI00552578.2 was found to have the same amino acid sequence as SAA1 (accession number P0DJI8) in the Swiss-Prot database. Serum amyloid A2 isoform a on the other hand, has the same amino acid sequence as SAA2 (accession number P0DJ19). This shows that SWATH™-MS analysis is able to detect and differentiate two isoforms of acute phase SAA present in the samples. However, as the differences were calculated by comparing the intensity between the patients' sample

and the control, this method only offers relative quantification, not an absolute quantification.

Apart from SWATH™-MS, MRM analysis was also performed to further explore the different isoforms of SAA that were elevated in all the three sarcomas (OS, CS and PS) that were analysed. Unlike SWATH™-MS, MRM is a non-scanning technique is a rapid, highly selective and sensitive method by directing the instrument to specifically monitor and do absolute quantification of target peptides or proteins of interest without the use of any antibodies, which is another critical advantage of this method. A peptide, or peptides, unique to the targeted protein must first be determined and selected in order to target the protein of interest. A triple quadrupole mass spectrometer was used to select and measure the appropriate and unique transitions of the targeted peptide in the sample. The intensity of each transition can be translated into concentration by comparing the value in the sample to a standard curve generated from various concentrations of a pure peptide.

Consistent with earlier data generated using 2DE, Western blot, ELISA, as well as SWATH™-MS, the MRM analysis also demonstrated the highest increase of SAA in patients with PS, followed by OS and CS. In all of these cases, the SAA increase was most likely attributed to the SAA1 isoform. Although MRM has better sensitivity and specificity compared to 2DE analysis, the fold changes for SAA observed in all three sarcomas from the MRM analysis were much lower compared to those exhibited by 2DE and SWATH™-MS. Most isoforms of human SAA possess similar experimental isoelectric points and molecular weights, and hence, they are probably represented as single spots in 2DE gels (Strachan *et al.*, 1989). The cumulative expression of all isoforms may contribute to the higher fold changes observed in the 2DE analysis, whereas in MRM analysis, each of the two specific isoforms, *i.e*. SAA1 and SAA2, was

independently targeted. Although SWATH™-MS could also detect both isoforms, the result from the analysis was constructed by matching the discovered peptides sequence coverage in the samples to the database, while in the MRM analysis, specific unique peptides for each isoform in the samples were targeted and quantified. Immunodepletion or sample enrichment, which was applied to the samples in the SWATH™-MS analysis, can facilitate in increasing the sensitivity of MRM, but this step is often not suitable for high-throughput clinical screening (Sung *et al.*, 2012).

In the MRM analysis, between the SAA1-1 and SAA1-2 peptides that were targeted to quantify the amounts of SAA1 in the samples, the concentrations of SAA1-2 were found to be varied and higher in all the sarcoma samples compared to their respective age-matched controls. In contrast, the concentrations of SAA1-1 peptide were observed to be similar in all groups of patients as well as the controls. The highest increment of SAA1-2 peptide was observed in patients with PS, by almost 2.8-fold compared to its control. Sung and co-workers suggested that the difference in the concentrations of both SAA1-1 and SAA1-2 peptides was related to the complexity of the serum samples, digestion efficiency of trypsin due to variation in the peptides sequences and possible posttranslational modifications, or different recovery rates from the desalting steps (Sung *et al.*, 2012). Similar to these results, the MRM data of Sung *et al*. (2012) also showed a stronger correlation between SAA1-2 peptide with the results of their ELISA validation test, and therefore this peptide was suggested to be a better target for MRM analysis for SAA.

In the case of the SAA2 isoforms, while the SAA2-2 peptide was solely detected in control C1 sample, trace amounts of SAA2-1 peptide were detected in patients with CS, PS and C1 subjects but not in the healthy adult controls (C2) and patients with OS. The absence of the SAA2-2 peptide in most of the samples analysed was probably due to the

difficulty of SAA2 protein to be digested into SAA2-2 peptide using trypsin (Sung *et al.*, 2012).

The higher amounts of serum SAA1 compared to SAA2 that was generally shown in this study are in agreement with the report of Kluve-Beckerman and co-workers (Kluve-Beckerman *et al.*, 1988). A similar finding was also observed in patients with lung cancer, whose concentrations of serum SAA1 were found to be higher than that of SAA2, but it was suggested that these two isoforms are actually co-regulated (Sung *et al.*, 2012). In a breast cancer animal model, SAA1 and SAA3 have been shown to be transcriptional targets of S100A4, a member of the calcium binding protein family which is also a metastasis binding protein (Grigorian *et al.*, 2008; Hansen *et al.*, 2015). These two proteins have been found to activate the matrix metalloproteinases and cytokines in an inflammatory reaction to prepare a microenvironment that is suitable for metastasis formation in the targeted organ (Hansen *et al.*, 2015). Hence, this suggests that the SAA1 proteins act as effectors for the metastasis-promoting functions of the S100A4 protein, linking the inflammation process with tumour progression (Hansen *et al.*, 2015), which may also be the case for OS, CS and PS, as reflected by the data from this study.

CHAPTER 6: CONCLUSION

From the silver 2DE comparative analysis of 15 high abundance serum proteins (AAT, ABG, AHS, APOA1, B2G1, C3f, HAP, HPX, KNG, LRG, SAA, STR, TTR, VTDB and ZAG), the difference in abundance of these proteins were demonstrated in five different types of bone tumours *i.e.* OS, ES, CS, PS and GCT, compared to their age-matched control groups, C1, C2 and C3. From the analysis, SAA protein was significantly altered in abundance among patients with highly malignant OS and PS. The magnitudes of fold differences of the up-regulated SAA in these two groups of patients were the highest among all the analysed proteins.

The high abundance O-linked and N-linked serum glycoproteome 2DE profiles of patients with OS, ES, CS, PS and GCT, as well as their age-matched control groups, were also successfully developed using lectin approaches, where enzyme-conjugated CGB-lectin specifically binds to the O-linked glycoproteins and enzyme-conjugated CMB-lectin binds to the N-linked glycoproteins. In the CGB-generated blots, a total of seven high abundance O-linked glycoproteins were analysed, five of which were also present in the silver stained 2DE gels *i.e.* AHS, HPX, KNG, LRG and VTDB, as well as two additional proteins, AMBP and ITIH4, that could not be clearly detected in the previously analysed silver stained 2DE gels. AHS and VDTB proteins exhibited significant altered abundances when comparison was made between patients with OS, CS and PS and their respective control groups.

Profiles of high abundance N-linked glycoproteins obtained from CMB-generated blots showed a higher number of well resolved proteins, and they were almost similar to that of observed in silver stained 2DE gels, with the absence of lower molecular weight proteins. Eleven proteins *i.e.* AAT, ABG, AHS, B2G1, HAP, HPX, KNG, LRG, STR, VTDB and ZAG were analysed, all of which were also present in the silver stained 2DE

gels. Although the expression pattern of these proteins was generally similar to the analysed silver stained 2DE gels, a few proteins were found to exhibit contrasting regulation pattern between silver 2DE profiles and CGB- or CMB-generated blot profiles.

From the silver stained 2DE profiles, SAA protein was found to be significantly altered in abundance in patients with OS, CS and PS, with the highest fold change was observed in patients with PS. Therefore, ELISA test and Western blot analysis specific for SAA protein were conducted to validate the increase of this protein in the samples. Higher amounts of SAA in patients with PS, OS and CS compared to their respective age-matched healthy non-tumour control subjects were apparent from these tests.

It is known that in human, acute SAA protein is present in two isoforms, SAA1 and SAA2. Initial investigation on pooled serum sample from patients with OS and its respective control C1 using SWATH™-MS analysis revealed that relative concentration of SAA1 was significantly increased in the patients' sample with the highest fold change exhibited, compared to the other detected proteins. Subsequent MRM analysis to target and absolutely quantify the specific peptides unique for each SAA1 and SAA2 isoforms in the samples from patients with OS, CS and PS further confirmed that the increased SAA, particularly isoform SAA1, was more apparent in patients with OS and PS, as opposed to those with CS. OS and PS are highly malignant tumours, and considerably more aggressive compared to CS. Therefore, the collected data is suggestive that the contrasting increased levels of SAA in patients with OS, CS and PS may be related to the different degrees of tumour malignancy.

Although there are already a number of publications suggesting potential diagnostic, prognostic and predictive protein biomarkers for patients with OS, ES and GCT, to the best of our knowledge, this is the first proteomics study in trying to find biomarkers for

patients with CS and PS. It is also the first study in simultaneously developing and comparing both O-linked and N-linked serum glycoproteome profiles of patients with these types of sarcomas to non-tumour healthy controls using lectin approaches. The study of the glycoproteome fractions enabled a further understanding of the dynamic changes in the glycosylation pattern of patients compared to normal individuals. Once validated, this array of proteins may offer tremendous potential to serve as protein fingerprint in distinguishing tumour types, facilitate the diagnosis and monitoring the disease. This work also represents the initial step in identifying the potential biomarkers that can contribute towards the early diagnosis of these tumours. Early detection combined with current treatment regimen can provide excellent prognosis in patients with cancerous tumours without detectable metastases.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications:

1. Wan-Ibrahim, W.I, Singh, V.A., Hashim, O.H. and Abdul-Rahman, P.S. (2015) Biomarkers for Bone Tumours: Discovery from Genomics and Proteomics Studies and Their Challenges. *Mol Med*. 21:861-872.
2. Wan-Ibrahim, W.I, Ashrafzadeh, A., Singh, V.A., Hashim, O.H. and Abdul-Rahman, P.S. (2016) Contrasting Increased Levels of Serum Amyloid A in Patients with Three Different Bone Sarcomas: An Indicator of Tumour Malignancy? *Electrophoresis.* 37:2328-2337.

Presentations:

1. Wan-Ibrahim W.I., Singh V.A., Karsani S., Hashim O.H. and Abdul-Rahman P.S. Glycoproteomic Profiling and Identification of Serum Protein Markers for Osteosarcoma: Champedak Lectin and LC-MS/MS Approach. HUPO 9th Annual World Congress, 19-23 September 2010, Sydney, Australia.
2. Wan-Ibrahim W.I., Singh V.A., Hashim O.H. and Abdul-Rahman P.S. Analysis of sera of patients with osteosarcoma using 2-dimensional electrophoresis-based proteomics. 22nd IUBMB & 37th FEBS Congress, 2-9 September 2012, Sevilla, Spain.