IDENTIFICATION OF POTENTIAL SERUM AND URINE BIOMARKERS FOR PATIENTS WITH PROSTATE CANCER USING LECTIN-BASED PROTEOMIC ANALYSES

JAIME JACQUELINE JAYAPALAN

THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENT
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2015

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **Jaime Jacqueline Jayapalan** (I.C/Passport No:)

Registration/Matric No: **MHA100012**

Name of Degree: **Doctor of Philosophy**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

IDENTIFICATION OF POTENTIAL SERUM AND URINE BIOMARKERS FOR PATIENTS WITH PROSTATE CANCER USING LECTIN-BASED PROTEOMIC ANALYSES

Field of Study: **Cancer proteomic**

I do solemnly and sincerely declare that:

1) I am the sole author/writer of this Work;
2) This Work is original;
3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date

Subscribed and solemnly declared before,

Witness's Signature Date

Name:

Designation:

ABSTRACT

The lack of specificity of prostate specific antigen emphasizes the need for a highly specific, sensitive and reliable marker with effective diagnostic predictability in curbing prostate cancer (PCa) at an early stage. In the present study, candidate protein biomarkers were identified when serum and urine samples of patients with PCa, those with benign prostatic hyperplasia (BPH) and controls (for the latter) were profiled using gel- and lectin-based proteomics approaches.

In the first part of the study, significantly reduced levels of apolipoprotein AII and complement C3 beta chain fragment were demonstrated in the profiles of patients with PCa when their neat serum and those with BPH patients were separately subjected to two-dimensional electrophoresis (2-DE) and image analysis. Subsequently, when 2-DE resolved sera of subjects were electrotransferred onto NC membrane, probed with enzyme-conjugated champedak galactose binding (CGB) lectin and analysed by densitometry, only inter-alpha-trypsin inhibitor heavy chain 4 fragment (ITIH4f) was found to be significantly reduced in abundance in PCa patients. Alternatively, when pooled serum of patients were subjected to affinity isolation using immobilized CGB lectin and analysed by 2-DE and densitometry, enhanced abundance of high molecular weight kininogen, alpha-1-antitrypsin and transthyretin were detected in sera of PCa patients compared to those with BPH.

In the second part of the study, when acetone-precipitated urine of PCa and BPH patients as well as age-matched controls were separately subjected to 2-DE and image analysis, significantly lower abundance of urinary saposin B and two different fragments of ITI light chain (ITIL), namely, ITILf1 and ITILf2, were demonstrated in PCa patients compared to controls. Abundance of ITILf2 fragment was also found to be significantly decreased in patients with PCa compared to BPH patients. When image

analysis was similarly performed on 2-DE resolved urinary proteins that were electrotransferred on NC membranes and detected using CGB lectin, ITIH4f was significantly enhanced in urine of PCa patients compared to controls. This finding was in contrast to the data observed in sera of PCa subjects.

In the final part of the study, pooled serum and urine obtained from patients with BPH and PCa as well as controls were subjected either to lectin- or immuno-blotting techniques and densitometric analysis, as a means of validation. When probed with enzyme-conjugated CGB lectin or anti-bikunin + trypstatin antibody, levels of abundance of serum ITIH4f and urinary proteins including ITIH4f, ITILf1 and ITILf2 of subjects were generally comparable with the previously generated data via 2-DE, with little variation in levels of significance. However, when taken together as indices, densitometric ratio of (i) serum to urinary ITIH4f, (ii) urinary ITIH4f to ITILf1 and (iii) urinary ITIH4f to ITILf2 peptide fragments showed marked segregating differences in indices for different groups of subjects. Furthermore, individual mean ratio indices between all PCa and BPH patients or controls were found not to overlap thus, highlighting strong discriminatory powers of the indices in effectively differentiating PCa from non-cancer conditions. Nonetheless, potential use of these indices as diagnostic markers in early detection of PCa requires prior extensive validation on clinically representative populations.

ABSTRAK

Kekurangan kekhususan antigen spesifik prostat menekankan keperluan suatu penanda yang amat khusus, sensitif dan berguna berserta dengan kebolehramalan diagnostik yang berkesan untuk membendung kanser prostat (PCa) di peringkat awal. Dalam kajian ini, calon penanda protein telah dikenalpasti apabila sampel serum dan urin pesakit PCa, pengidap hiperplasia prostat benigna (BPH) dan subjek kawalan (bahagian kedua kajian) diprofil menggunakan pendekatan proteomik berasaskan gel dan lektin.

Pada bahagian pertama kajian, penurunan aras apolipoprotein AII dan pecahan rantai beta komplemen C3 yang signifikan telah ditunjukkan pada profil pesakit PCa apabila sampel serum mereka dan pesakit BPH dipisahkan melalui electroforesis dwi-dimensi (2-DE), diikuti dengan analisis imej. Seterusnya, apabila serum subjek yang telah dipisah melalui 2-DE dipindahkan secara elektro ke atas membran nitroselulosa (NC), diprob dengan lektin pengikat galaktosa cempedak (CGB) yang dikonjugat enzim dan dianalisa melalui densitometri, hanya serpihan rantai berat 4 inter-alfa-perencat tripsin (ITIH4f) didapati menurun jumlahnya dengan signifikan dalam pesakit PCa berbanding pesakit BPH. Secara alternatif, apabila sampel serum yang dikumpul daripada pesakit dipisahkan secara afiniti menggunakan lektin CGB yang diimobilisasikan dan dianalisa melalui 2-DE dan densitometri, peningkatan jumlah tiga kluster tompok protein, termasuk kininogen berberat molekul tinggi, alfa-1-antitripsin dan transtiretin dikesan dalam serum pesakit PCa berbanding pesakit BPH.

Pada bahagian kedua kajian, apabila urin pesakit PCa, pengidap BPH dan subjek kawalan yang berada dalam lingkungan usia yang sama yang dimendap aseton dipisahkan melalui 2-DE dan dianalisis, penurunan jumlah pengumpulan saposin B dan

dua pecahan ITI rantai ringan berbeza urin yang dinamai ITILf1 dan ITILf2 yang signifikan didedahkan dalam pesakit PCa berbanding kawalan. Selain itu, jumlah pecahan ITILf2 turut menurun dengan signifikan dalam pesakit PCa berbanding pengidap BPH. Apabila analisis imej dijalankan ke atas protein urin yang dipisah 2-DE yang telah dipindahkan ke atas membran NC dan dicam menggunakan lektin CGB, terdapat peningkatan ITIH4f yang signifikan dalam urin pesakit PCa berbanding kawalan. Penemuan ini adalah bertentangan dengan data diperoleh daripada analisis serum subjek PCa.

Pada bahagian akhir kajian, sampel serum dan urin terkumpul yang diperoleh daripada pesakit BPH dan pesakit PCa dan subjek kawalan telah dianalisis samaada menggunakan teknik pemblotan lektin atau imuno dan analisis densitometri sebagai kaedah pengesahan. Apabila diprob menggunakan lektin CGB terkonjugat enzim atau antibodi poliklonal anti-bikunin + tripstatin, paras jumlah ITIH4f serum dan protein urin termasuk ITILf1 dan ITILf2 pada kumpulan-kumpulan subjek didapati secara umumnya adalah sama dengan data yang dihasil sebelumnya melalui 2-DE, dengan sedikit perbezaaan pada paras signifikans. Walaubagaimanapun, apabila dilihat bersama sebagai indeks nisbah densitometri, pecahan peptida-peptida (i) ITIH4f serum kepada urin, (ii) ITIH4f urin kepada ITILf1 urin dan, (iii) ITIH4f urin kepada ITILf2 urin menunjukkan perbezaan pemisahan yang ketara bagi kumpulan subjek yang berbeza. Seterusnya, indeks purata nisbah individu di antara kesemua pesakit atau kawalan didapati tidak bertindih. Ini menonjolkan kuasa diskriminatori yang tinggi bagi indeks berkenaan untuk membezakan PCa daripada keadaan-keadaan bukan kanser yang lain secara berkesan. Walaubagaimanapun, potensi kegunaaan indeks ini bagi pengesanan PCa yang awal memerlukan pengesahan yang ekstensif ke atas jumlah subjek yang mewakili populasi klinikal.

ACKNOWLEDGEMENT

No man is an island. The significant outputs of this thesis are collective contributions of great many people. I am truly honoured to have met and worked alongside such personas.

Prof. Dr. Onn Haji Hashim, my supervisor... I have learned great many things under your supervision and as such, mere thanks can't possibly express the gratitude and respect I have for you. Nevertheless, thank you very much for spurring me in the right direction in becoming a better yet, conscientious scientist. Above all, thank you for giving me the opportunity and for trusting my abilities. For these alone, I am forever indebted to you.

Prof. Dr. Azad Hassan Abdul Razack, my co-supervisor… Thank you very much for your guidance and unwavering support that had made my journey of PhD, a tad easier. Your kind concerns and encouragements had always boosted me in the right direction and for that, *muchas gracias*.

Dr. Ng Keng Lim (Micheal)… Your immeasurable help and support throughout the course of project, particularly in acquiring the clinical samples is greatly appreciated. Indeed, a pleasure working with you.

Prof. Dr. M. Ravichandran and **Prof. Dr. Lalitha Pathabiraman**, my ex-supervisors… I simply could not miss mentioning you both at this defining moment of my life. I may appear far by distance and of course, my poor correspondences (sorry!), but you will always be held at the highest esteem in my thoughts. Thank you for nurturing in me the 'true-essence' of research and learning. You are both right, knowledge is indeed best, shared.

Mr. Mohanaraja, my best friend… Thank you for being around on both my best and worst days. 'Sanity' would have been a far-fetched idea without your encouraging words and kind gestures. Many thanks also, for keeping me going forward, always. One step at a time, indeed!

UM Research Grant… The financial support awarded by University of Malaya had indeed immeasurably propelled the progress of the project in quantum. Thank you!

Staff at the Department of Molecular Medicine, Medical Biotechnology Laboratory and TRUS-biopsy unit… Thank you for your constant support and help provided. A very special mention of **Ms. Jumriah Hiji** for her invaluable research assistance.

Colleagues and lab mates (Dr. Puteri Shafinaz, Thibashini, Wei Wei, Emida, Ramarao, Jesse, Izlina, Shahram and Francis)… I will always remember the easy camaraderie we have shared while working together in the lab. Thank you for making it memorable.

Last but not least, **my family**; mother - Annamarey, sisters - Rosemary & Magdelene, brother (-in law) - Ronie & Revindran and, nieces and nephew - Angie, Felicia and Marshal… From the bottom of my heart, I thank you all for the love, understanding and patience you have generously showered upon me. I am truly blessed.

"Success is not final, failure is not fatal: it is the courage to continue that counts."
Winston S. Churchill

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

1-DE	- one-dimensional electrophoresis
2-DE	- two-dimensional electrophoresis
AAG	- α1-acidic glycoprotein
AAT	- α_1-antitrypsin
ABG	- α_1-β glycoprotein
ACN	- acetonitrile
ACT	- α_1-antichymotrypsin
ACTH	- adrenocorticotropic hormone
AGP1	- α_1-acid glycoprotein 1
AHS	- α_2-HS glycoprotein
AJCC	- American Joint Committee on Cancer
Akt-1	- protein kinase B
ALB	- albumin
AMACR	- alpha-methylacyl-CoA racemase
AMBP	- protein AMBP
ANOVA	- analysis of variance
APOA1	- apolipoprotein A1
APOA2	- apolipoprotein A2
APOA4	- apolipoprotein A4
APOC3	- apolipoprotein C3
APOH	- apolipoprotein-H
APS	- ammonium persulfate
BCA	- bicinchoninic acid
BPH	- benign prostatic hyperplasia
BSA	- bovine serum albumin

C3	- complement C3
CD59	- CD59 glycoprotein
CDH	- cadherin-1
CGB	- champedak galactose binding
CHCA	- alpha-cyano-4-hydroxycinnamic acid
Da	- dalton
DAB	- 3,3'-diaminobenzidine
DNA	- deoxyribonucleic acid
DRE	- digital rectal examination
DTC	- disseminated tumour cell
DTT	- dithiothreitol
DVS	- divinyl sulfone
f	- fragment
Fuc	- fucose
G	- Gleason
Gal	- galactose
GalNAc	- N-acetylgalactosamine
Glc	- glucose
GlcNAc	- N-acetylglucosamine
GM2A	- ganglioside GM2 activator
GSTP1	- glutathione S-transferase pi 1
HAP	- haptoglobin
HCl	- hydrochloric acid
HDL-C	- high density lipoprotein-cholesterol
HPX	- hemopexin
HRP	- horseradish peroxidase

IAA	- iodoacetamide
ID	- identification
Ig	- immunoglobulin
IHC	- immunohistochemistry
ITI	- inter-α-trypsin inhibitor
ITIH	- inter-alpha-trypsin inhibitor heavy chain
ITIL	- inter-alpha-trypsin inhibitor light chain
kDa	- kilo dalton
KNG-1	- kininogen-1
LC	- liquid chromatography
LRG	- leucine-rich glycoprotein
M	- molar
m/z	- mass to charge ratio
Man	- mannose
miR	- microRNA
M_r	- relative molecular weight
MS	- mass spectrometry
MS/MS	- tandem mass spectrometry
MW	- molecular weight
NC	- nitrocelulose
NP	- normal phase
p	- probability
PBS	- phosphate-buffered saline
PCa	- prostate cancer
PCA3	- prostate cancer antigen 3
PGDS	- prostaglandin-H2 D-isomerase

pI	- isoelectric focusing point
PLC	- perlecan
PMF	- peptide mass fingerprint
ppm	- parts per million
PSA	- prostate specific antigen
PSAP	- proactivator polypeptide precursor
PSCA	- prostate stem cell antigen
PSP94	- prostate secretory protein
PTGS	- prostaglandin-endoperoxide synthase
PTM	- post-translational modification
QMSP	- quantitative methylation specific polymerase chain reaction
RBP	- retinol binding protein
RNA	- ribonucleic acid
RNS2	- non-secretory ribonuclease
rpm	- revolutions per minute
RT-PCR	- real time-polymerase chain reaction
S.E.M	- standard error of the mean
SD	- standard deviation
SDS-PAGE	- sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SELDI	- surface-enhanced laser desorption/ionization
SPA	- sinapinic acid
TBS	- tris-buffered saline
TFA	- trifluoroacetic acid
THP	- uromodulin
TNM	- tumour, lymph node, metastasis
ToF	- time of flight

TRF	- serotransferrin
TRUS	- transrectal ultrasonography
TTR	- transthyretin
UMMC	- University of Malaya Medical Centre
VDP	- vitamin-D binding protein
ZAG	- zinc-alpha glycoprotein

Abbreviations for standard units of measurement such as length, volume, mass, temperature and concentration were omitted from the list.

INTRODUCTION

Specific tumour markers for detecting prostate cancer is deemed necessary considering the lack of specificity of serum prostate specific antigen (PSA) as a biomarker (Barry, 2001; Giri *et al.*, 2007; Thompson *et al.*, 2004; Törnblom *et al.*, 1999) and inaccurate screening of the prostate of debilitating men via digital rectal examination (DRE) (Okotie *et al.*, 2007). Thus, relying on clinical findings of DRE and serum PSA in patients often gives rise to high rates of false-positive results in the diagnosis of PCa (Chou *et al.*, 2011; Okotie *et al.*, 2007). With this in mind, many attempts have been made at discovering novel biomarkers for both diagnosis (Benchikh *et al.*, 2010; Deras *et al.*, 2008; Jeronimo *et al.*, 2002; Salinas *et al.*, 2009; Vickers *et al.*, 2010) and prognosis (Ellinger *et al.*, 2008; Huizen *et al.*, 2005; Kollermann *et al.*, 2008; Michalaki *et al.*, 2004; Mitchell *et al.*, 2008; Sreekumar *et al.*, 2009; Whitesel *et al.*, 1984) of PCa. These efforts encompass all ranges of techniques (e.g., genetics, proteomic to bioinformatics) and biological materials (e.g., protein, DNA, RNA, microRNA, metabolite, disseminated tumour cell (DTC)) but none had thus far, provided solutions in addressing the current issue. Considering this, there is definitely an urgent need to develop an effective and less invasive method to identify highly sensitive tumour markers with potentials to detect the occurrence of PCa at an early stage as well as to discriminate benign prostatic hyperplasia (BPH), a benign condition of the prostate, and men with healthy prostate from PCa. This in turn, is believed to alleviate unnecessary distress and avoid unwarranted medical procedures of those with BPH and at the same time, catapulting successful clinical management and ensuring favourable outcomes among subjects with 'true positive' PCa.

Lectin is defined as carbohydrate-binding protein other than an enzyme or antibody (Barondes, 1988). Taking into consideration of its sugar binding capabilities,

lectins have been extensively utilised in the field of biomedical and biological researches (Peumans and van Damme, 1998). These includes ABO blood typing (Khan *et al.*, 2002), detection and classification of polyagglutination of erythrocytes (Horn, 1999), bacterial typing (Ascencio *et al.*, 1990; Cole *et al.*, 1984; Doyle *et al.*, 1984; Yakovleva *et al.*, 2011), karyotyping of chromosomes and evaluation of immunocompetence, bone marrow transplantation and enzyme replacement therapy (Sharon and Lis, 1993). Lectins are also used as neuron tracers (Gerfen and Sawchenko, 1985; Nakashima *et al.*, 2000) as well as for mediating cell separation intended for cell-based therapy (Dodla *et al.*, 2011; Wang *et al.*, 2011). As probes, lectins have been successfully utilised for identification and selection for mutants with altered cell surface glycoconjugate compositions (Stanley *et al.*, 1990) and *in situ* identification, localisation and characterisation of various *N*- and *O*-linked oligosaccharide moieties present in pathological tissues and cells (Allison, 1986; Kuhlmann *et al.*, 1983; Spicer and Schulte, 1992).

Glycosylation, a type of post-translational modification (PTM) that attaches saccharide moieties to proteins (or lipids), is the most common type of PTM in mammalian cells (Apweiler *et al.*, 1999; Wong, 2005). Glycosylated proteins in turn, modulates a great many number of biological processes such as host-pathogen interactions, inflammation and development, via changes in the structures, functions and interactions of proteins (Lisowska and Jaskiewicz, 2001). For example, the mucins, viscoelastic mucous gel layer containing large number of *O*-glycan chains lining the luminal surface of the gastrointestinal tract, serve as a protective barrier against the harsh luminal environment (Corfield *et al.*, 2000), whilst, glycophorin A that acts as decoy receptors, protects erythrocytes from infective agents like viruses and bacteria thus, eliciting host-protective interaction with pathogens (Baum *et al.*, 2002). On the other hand, alteration in structural characteristics of the glycan chains (Brockhausen *et*

al., 1998), and levels of glycosylated proteins have also been hugely correlated in manifestation and progression of various pathological conditions, including cancer (Dennis *et al.*, 1999; Nishimura *et al.*, 1995). Along this line, lectins had also served as a tool for detection of cancer-associated glycosylated proteins and/or specific glycoforms of the glycosylated-proteins, to facilitate discovery of cancer biomarkers for early diagnostic and prognostic prediction as well as novel therapeutic targets (Dennis *et al.*, 1999; Nishimura *et al.*, 1995) thus, maximising chances of survival of subjects with cancer with better quality of life.

The search for new tumour markers is highly accelerated through the use of proteomics (Verma and Srivastava, 2003; Wulfkuhle *et al.*, 2003) and glycoproteomics technologies (Kim and Misek, 2011). Instead of relying on single tumour markers, these approaches had unravelled series of newly identified biomarkers, which when analysed simultaneously may be able to discriminate different types of cancers, their stages or monitor the progress of cancer treatment towards patient's recuperation (Kim and Misek, 2011; Verma and Srivastava, 2003; Wulfkuhle *et al.*, 2003). Proteomic allows analysis of protein expression, differential protein modifications and aberration in localisation of protein (Srinivas *et al.*, 2001), directly at protein level, following transformation of healthy into neoplastic cells, during the development of cancer (Miles *et al.*, 2006).

Two-dimensional gel electrophoresis (2-DE) is the most commonly used gel-based proteomics technique for differential proteomic profiling and identification of candidate (glyco)proteins in biofluids that may be potentially used as cancer biomarkers (Chevalier, 2010). Advantages of 2-DE are numerous (Chevalier, 2010). 2-DE allows separation and fractionation of complex protein mixture (Klose, 1975; O'Farrell, 1975) extracted from tissues, cells, and other biological specimens in a single

gel with high resolution and efficiency. It also enables visualisation of hundreds of protein spots via staining (e.g., silver and fluorescent dyes such as SYPRO ruby and deep purple) (Chevallet *et al.*, 2008; Lopez *et al.*, 2000; Shevchenko *et al.*, 1996) and comparative analysis for protein of altered abundances between subject cohorts under study using appropriate software (e.g., Delta2D, Image Master, Melanie, PDQuest and Progenesis) (Marengo *et al.*, 2005; Millioni *et al.*, 2010; Rosengren *et al.*, 2003). Proteins resolved on 2-DE gels generally provide a spectrum of information of each protein such as molecular weight, pI, quantity and possible PTMs or processed forms of the same protein. Purification and identification of proteins of interests is also possible, if mass spectrometry (MS)-compatible stains are used (Lopez *et al.*, 2000; Shevchenko *et al.*, 1996). Last but not least, 2-DE also allows flexibility in workflow to such a great degree, that the resulting 2-DE gels serve as high-capacity 'storage system' for long-term archival of the resolved proteins (Beranova-Giorgianni, 2003).

Some of the biological fluids that have been mined for cancer biomarkers include serum or plasma, urine, cerebrospinal fluid, nipple aspirate fluid and tissue secretions. Among these, proteomic profiling of sera of subjects with cancer is the most popular choice of bodily fluids (Hanash *et al.*, 2008). Additionally, urine is also gaining popularity for use in proteomics workflows due to its non-invasive nature of sampling, convenience and cost-effectiveness (Hortin and Sviridov, 2007; Pisitkun *et al.*, 2006).

Alternatively, integration of lectin and lectin-based techniques to the commonly used conventional 2-DE approach serves as a beneficial tool for detection and evaluation of aberration of glycosylation. This is in part due to the lectins' distinct carbohydrates recognising and interacting capabilities (Peumans and van Damme, 1998). For example, lentil lectin interacts with fucosylated core region of bi- and tri-antennary complex type *N*-glycans (Sokolowski *et al.*, 1997) whilst, the lectin isolated

from elderberry binds specifically to the N-acetylglucosamine and sialic acid residues (Mach *et al.*, 1991). As such, lectins of plant origin particularly, had been widely used for investigation and identification of glycoproteins of altered structure (Brockhausen *et al.*, 1998; Sokolowski *et al.*, 1997) and abundance (Abdul-Rahman *et al.*, 2007; Abdullah-Soheimi *et al.*, 2010; Doustjalali *et al.*, 2006; Mohamed *et al.*, 2008; Mu *et al.*, 2012; Seriramalu *et al.*, 2010). Better yet, is the incorporation of lectins with various other techniques including affinity chromatography (Jung *et al.*, 2009; Seriramalu *et al.*, 2010), Western blot (Abdul-Rahman *et al.*, 2007; Rambaruth *et al.*, 2012), Bio-Plex assay (Li *et al.*, 2013a), protein array (Rosenfeld *et al.*, 2007), immunosorbent assays (Li *et al.*, 2013b) and surface enhanced laser desorption/ionisation-time of flight (SELDI-ToF)/MS (Zhang *et al.*, 2004) for comprehensive analysis of specific changes in glycosylation in biofluids, in the search for novel tumour glycoprotein markers (Abdul-Rahman *et al.*, 2007; Seriramalu *et al.*, 2010; Zhang *et al.*, 2004).

The lack of specificity of serum PSA and inaccurate DRE findings urgently calls for efficient and reliable biomarkers for diagnosis of PCa, which at the same time, discriminate PCa from other non-cancer conditions. With this in mind, the current study was undertaken to address setbacks of these currently used biomarkers via conventional 2-DE and lectin-based proteomic profiling of unfractionated serum and acetone-precipitated urine samples obtained from patients with PCa. Groups of men with BPH as well as age-matched subjects with neither history of prostatic complaints nor treatments were included in the study, to serve as controls for comparative analyses.

The present study was specifically performed:

1. to analyse differences in serum and urinary protein abundance in the 2-DE profile generated by silver analysis and lectin detection methods by image analysis using Image Master 2D Platinum Software between groups of subjects,

2. to identify the aberrantly expressed proteins and *O*-glycosylated proteins detected from the 2-DE experiments using MS,

3. to confirm the presence and identity of *O*-glycosylated urinary ITIH4 fragment of protein detected from the CGB-lectin probed 2-DE electrophoretic approach using CGB-lectin immobilised affinity column chromatography and 1D nano-LC coupled to MALDI ToF/ToF,

4. to validate the serum and urinary proteins and *O*-glycosylated protein of altered abundance detected from 2-DE and lectin detection methods using immuno- and lectin-blotting techniques.

LITERATURE REVIEW

2.1. PROSTATE

2.1.1. Cancer vs Benign

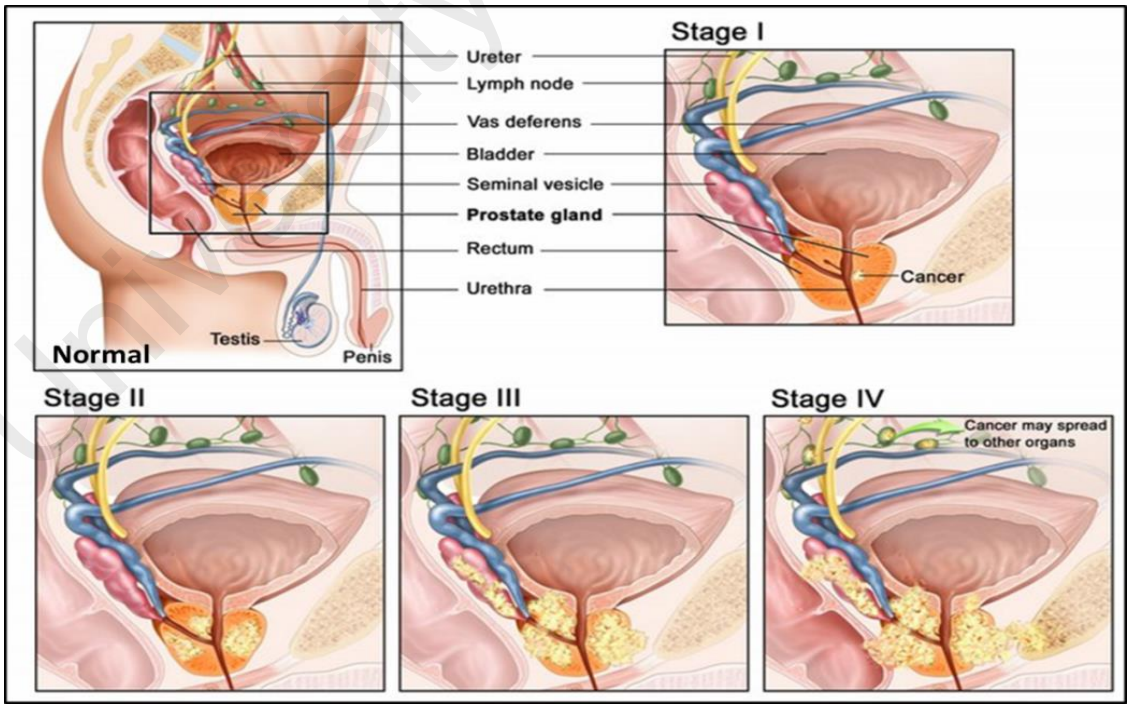
The prostate is an organ forming part of the male reproductive system. The gland resembles walnut in shape and size. It consists of both glandular (70%) and fibromuscular (30%) tissues, and located below and in front of the bladder and rectum, respectively. The thin, milky secretion produced by the prostate gives semen its characteristic colour and odour as well as play a role in the lubrication of the urethra and in protection, nourishment, and mobility of the sperm.

There are four main disorders of the prostate. These include prostatitis, prostatodynia, benign prostatic hyperplasia (BPH) and prostate cancer (PCa). Prostatitis is a condition presented by inflammation of the prostate and/or its surrounding area. Acute and chronic bacterial prostatic infections, urethral catheterisation, infection of the bladder, and abnormalities in the urinary tract may result in prostatitis.

Prostatodynia, on the other hand, is a chronic and painful prostatic condition but with no apparent evidences of infection caused by microorganisms, thus distinguishing itself from prostatitis. Although poorly understood, neurological disorder, viral and past bacterial infections, structural deformities of the urinary tract as well as prolonged physiological and psychological stress may predispose men, particularly above 50 years of age, to prostatodynia.

BPH, also known as benign prostatic hypertrophy, is an aging-associated condition (> than 50 years) and clinically presented by non-cancerous (benign) prostate gland enlargement due to excessive glandular and stroma proliferation of the prostate (Thorpe and Neal, 2003). This in effect, causes obstructive and irritable lower urinary tract symptoms of men with the benign disease.

PCa, a slow-growing tumour condition of the prostate, is also a disease that predominantly affects men aged more than 50 years. Like BPH, voiding dysfunction due to detrusor muscle underactivity and enlarged prostate gland are common clinical features of PCa. However unlike BPH, uncontrolled cellular growth and activities of prostate in subjects with PCa, often result in malignant prostatic glandular cells and stroma, instead (Guess, 2001). PCa are often asymptomatic until the disease progress to advanced stages of cancer. In some cases, subjects of this 'silent' cancer does demonstrates symptoms including complaints of pain in the lower pelvic area, back, hips and upper thighs, polyuria, hematuria, hematospermia, burning sensation during urination, urinary hesitancy, painful ejaculation and unexplained weight loss. Figure 2.1 shows the anatomical positioning of the prostate and progression of PCa featured by the size of the tumour and metastasis (e.g., bone and lymph node).



Adapted and modified from www.4urology.org

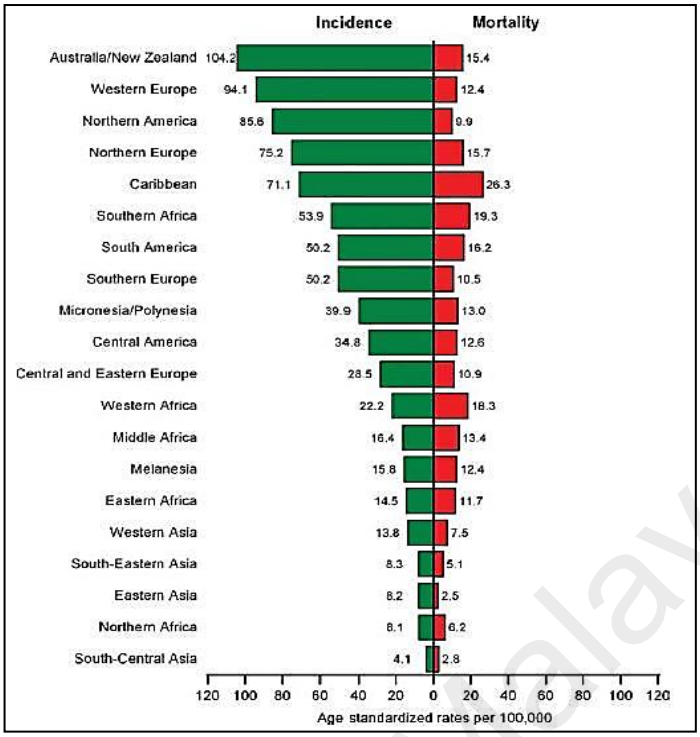
Figure 2.1. Normal versus cancer of the prostate.

2.1.2. Incidences of PCa

PCa is ranked as the sixth most frequent cancer and the second most commonly diagnosed cancers among men (Jemal *et al.*, 2011). Based on GLOBOCAN 2008 (Ferlay *et al.*, 2010), PCa incidence and mortality worldwide were estimated to approximately 899,000 and 258,000 cases, respectively (Figure 2.2) whilst, projected to increase in two-fold by 2030. Figure 2.2 exhibits age standardized prostate cancer incidence and mortality rates stratified according to world area.

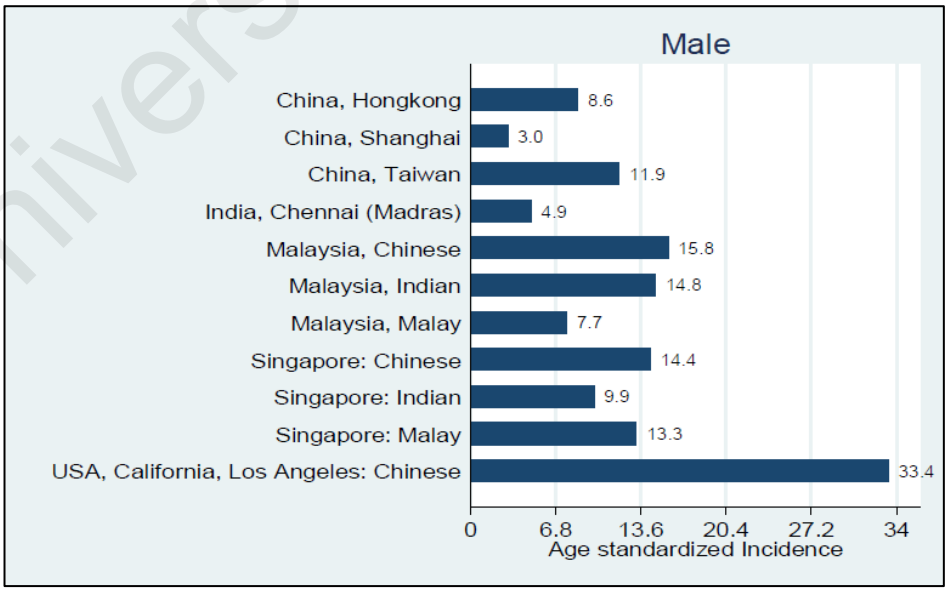
In Malaysia, PCa ranks fourth in the most frequent cancer in men after cancers of the bowel, lung and nasopharynx. The incidences account for 7.3% of the total reported cancer cases in male population. Men belonging to the Chinese ethnic group (15.8) had shown the highest age-adjusted incidences followed by the Malaysian Indians (14.8) and Malaysian Malays (7.7) per 100,000 population, in that order (Lim *et al.*, 2008). Figure 2.3 demonstrates international comparison of age-standardised incidences of PCa among men according to ethnic origins.

Of note, incidences of PCa were marked lower in Asian men compared to the western population as well as Asian men living in the North America and Europe (Metcalfe *et al.*, 2008; Miller *et al.*, 2008) with, however, higher proportion of advanced stage of PCa at the point of diagnosis. This had been postulated due to the sub-optimal diagnosis and management of subjects with PCa in these regions, in addition to genetic, lifestyle and environmental disparities (Ito, 2014), which will be further elaborated in Section 2.1.4.



Source: Globocan, 2008

Figure 2.2. Age-standardised PCa incidence and mortality rates worldwide.



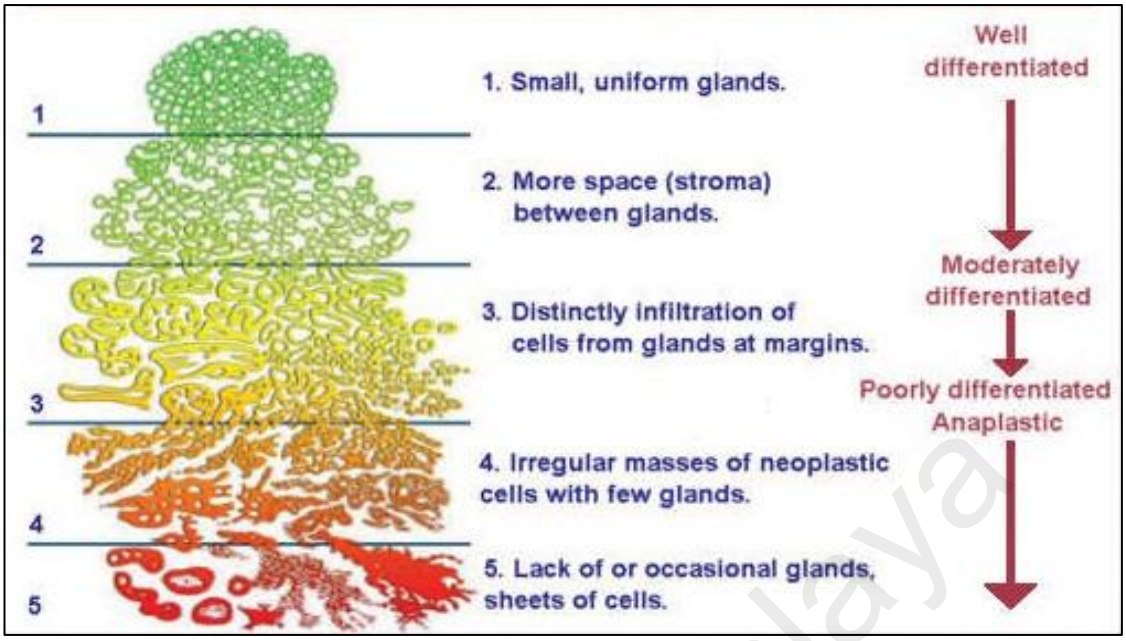
Source: Lim *et al*., 2008

Figure 2.3. Age-standardised PCa incidences by ethnic origin.

2.1.3. Stages of PCa

The designation of prostate cancer staging and grading are determined based on American Joint Committee Cancer (AJCC) Staging Manual (*AJCC cancer staging manual*, 2002) that take both the TNM scores and histopathological Gleason (G) into consideration. The TNM system is an expression of the anatomic extent of the primary tumour (T), absence or presence and extent of regional lymph node metastasis (N) and absence or presence of distant metastasis (M). The numerical subsets of the TNM components (T**0**, T**1**, T**2**… M**1**), on the other hand, indicates the progressive extent of the malignancy.

Gleason grading patterns (G1 to G5) is generally assigned based on the levels of glandular differentiation and pattern of tumour growth in the neoplastic prostatic stroma on a hematoxylin and eosin-stained tissue section as shown in Figure 2.4. The histologic score may range between 2 to 10, by summing the scores of two most common grade patterns of the tumour growth, thus, directly infers the number of histopathologic endpoints, including tumour size, margin status, and pathologic stage of PCa (Humphrey, 2004). G1 (scores - 2 to 3) and G2 (score - 3) demonstrates a well-differentiated tumour growth but with a less well-defined and circumscribed masses of stroma, in the latter. G3 shows moderately differentiated grade of tumour with ill-defined infiltrating edges, which are the most common pattern of growth of prostatic adenocarcinoma (scores - 5 to 7). A high-grade and poorly differentiated growth of carcinoma with raggedly infiltrative masses is indicated by G4 (score - 8). Last but not least, G5 (scores - 9 to 10) has the most pronounced pattern of poorly differentiated tumour growth that is highly associated with advanced stage of PCa and metastasis (Gleason, 1977). Table 2.1 shows the anatomic staging and prognostic grouping of subjects with PCa according to TNM, Gleason grading and levels of PSA.



Source: Adapted from Humprey, 2004.

Figure 2.4. Gleason's pattern scale.

2.1.4. Risk factors

PCa is an aging-associated disease of men, with a mean age of 67.7 at diagnosis (American Cancer Society, 2003). Nevertheless, the influence of PSA screening has been thought to contribute to the opposing trend of PCa incidences in men under age 50 years, to a certain extent (Li *et al.*, 2012). In terms of race, the Afro-Americans have been listed to show the highest rates of PCa worldwide (Jemal *et al.*, 2011), which may be in part due to their ancestry association to chromosome 8q24 (Bock *et al.*, 2009), followed by the whites, Hispanics and Asian or Pacific Islanders, in that order (American Cancer Society, 2003).

The risk for PCa was also found to be doubled by having a father or brother harbouring PCa and the magnitude of risk was further marked up to more than 10-fold when multiple first degree relatives are affected (Steinberg *et al.*, 1990). Aside from positive family history, genetic variation in the *RNASEL* gene at chromosome 1q24-25

Table 2.1. Anatomic and prognostic staging of PCa.

ANATOMIC STAGE/ PROGNOSTIC GROUP					
Group	**T**	**N**	**M**	**PSA (ng/ml)**	**Gleason**
I	T1a-c	N0	M0	< 10	≤ 6
	T2a	N0	M0	< 10	≤ 6
	T1-2a	N0	M0	X	X
IIA	T1a-c	N0	M0	< 20	7
	T1a-c	N0	M0	≥ 10 < 20	≤ 6
	T2a	N0	M0	≥ 10 < 20	≤ 6
	T2a	N0	M0	< 20	7
	T2b	N0	M0	< 20	≤ 7
	T2b	N0	M0	X	X
IIB	T2c	N0	M0	Any	Any
	T1-2	N0	M0	≥ 20	Any
	T1-2	N0	M0	Any	≤ 8
III	T3a-b	N0	M0	Any	Any
IV	T4	N0	M0	Any	Any
	Any	N1	M0	Any	Any
	Any	Any	M1	Any	Any

T, tumour; N, lymph node; M, metastasis; X, scores cannot be determined.

Source: AJCC Cancer Staging Manual, 2002.

(Meyer *et al.*, 2010), somatic and germline polymorphisms in the androgen receptor genes (e.g., CAG and GGN repeats (Stanford *et al.*, 1997) and Stu1 (Lu and Danielsen, 1996)), single nucleotide polymorphisms at Ala49Thr and Val89Leu of the *SRD5A2* gene (Izmirli *et al.*, 2011; Li *et al.*, 2003), germ line mutations in BRCA1 and BRCA2 (Liede *et al.*, 2004) and HPC1 (Ostrander and Stanford, 2000) have been invariably associated with increased risk of PCa.

Although the incidences of PCa are relatively lower among Asian men, interestingly, immigrants from the East Asian countries living in North America (Miller *et al.*, 2008) and Europe (Metcalfe *et al.*, 2008) had uncommonly shown higher incidences than that in their homeland. Such observation suggests influence of exogenous factors such as diet and lifestyle (Wolk, 2005) and at the same time, indicates improved healthcare and cancer registry systems in the Western world (Kimura, 2012).

In contrast to the beneficial effect of lycopene from tomatoes (Giovannucci *et al.*, 2002), dietary supplementation with vitamin E (Klein *et al.*, 2011) and high intake of animal fat diet (Huang *et al.*, 2012) and calcium (Chan *et al.*, 2001) may significantly increase the risk and catapult the progression of PCa, respectively. Low serum levels of hormone testosterone (Mearini *et al.*, 2013), high coital rates, particularly before 60 years of age (Dennis and Dawson, 2002), high baseline of body mass index (Calle *et al.*, 2003), smoking habit (Huncharek *et al.*, 2010), consistent and heavy consumption of alcohol (Fillmore *et al.*, 2009), occupational hazard to farming (Meyer *et al.*, 2007), polycyclic aromatic hydrocarbons (Rybicki *et al.*, 2006) and exposure to environmental agents like cadmium (Benbrahim-Tallaa *et al.*, 2007) and arsenic (Benbrahim-Tallaa and Waalkes, 2008) generally predisposes men to PCa.

2.1.5 Screening and diagnosis of PCa

Urinalysis, uroflowmetry, post-void residual urine, assessment of validated International Prostate Symptom Score questionnaire, DRE and quantitative measurement of serum PSA (Department of Health and Human Services, 1994) are some of the approaches employed in the investigation of lower urinary tract complaints of men. The combined use of DRE and levels of PSA however, have been implemented to serve as preliminary diagnosis of men with prostatic complaints (Howlader *et al.*, 2012; Omar and Tamin, 2011).

DRE is an internal examination of the lower rectum, where a healthcare professional uses a gloved and lubricated finger to palpate and examine for any abnormalities. Aside from disorders of prostate (e.g., BPH and PCa), DRE are also often performed for clinical presentation and symptoms which are indicative of gastrointestinal (e.g., faecal incontinence, rectal bleeding and tenesmus), neurological (e.g., spinal cord injuries) and female gynaecological disorders (e.g., malignancy of the reproductive organs, endometriosis and pelvic metastases and abscess) (Shirley and Brewster, 2011).

PSA, also known as human kallikrein 3, a serine protease belonging to the kallikrein family of proteases is produced by the prostatic epithelium at extremely high levels and secreted into the seminal fluid (approximately 0.5 - 2 g/L) (Wang *et al.*, 1981). A mature form of PSA glycoprotein consists of 237 amino acids and a carbohydrate chain thus, accounting for molecular weight of 28.4 kDa (Schaller *et al.*, 1987). In contrast to normal (prostate) condition, high amounts of PSA are released into the circulation from the diseased prostate thus, the apparent increase of serum PSA. Interestingly, PSA has also been previously reported to be expressed at low levels in extra-prostatic tissues such as urethral and anal glands, (Kamoshida and Tsutsumi,

1990) and breast (Stenman *et al.*, 1999) but, appeared not to contribute to its levels in the serum (Oesterling *et al.*, 1996). Gene expression and production of PSA in these tissues are most likely due to the regulation of steroid hormones including androgen, oestrogen, glucocorticoid and progestin (Diamandis and Yu, 1997).

Although widely used to provide provisional clinical opinion in monitoring as well as to detect asymptomatic men for PCa (Greene *et al.*, 2013; Schröder *et al.*, 2009; Wolf *et al.*, 2010), the rising controversies over the recommended use of PSA were thought to highlight more of potential risks of unnecessary pain and treatments (Chou *et al.*, 2011) as well as causing unwarranted medical complications such as impotence, urinary incontinence (Arkes and Gaissmaier, 2012), functional bowel disorders and infection than the "supposed benefits" of such exercise (Ablin, 2010; Basch *et al.*, 2012; Chustecka, 2010; Harris, 2011). Besides, there are also men with belligerent PCa featured biopsy results but had evidentially shown levels of PSA of less than 4 ng/ml (Giri *et al.*, 2007; Törnblom *et al.*, 1999). This is in part, due to lack of specificity of PSA, which was previously thought to be exclusive to PCa. Levels of PSA are seen to fluctuate in varying degrees at many instances. These include clinical conditions such as prostatitis, BPH and urinary tract infection, intake of certain drugs (e.g., finasteride and dutasteride), certain medical procedures (e.g., prostate biopsy and surgery), variations between inter-laboratories tests and not to mention PCa itself, where men who had the cancer had both demonstrated high (Barry, 2001) and lower levels of PSA (Thompson *et al.*, 2004; Törnblom *et al.*, 1999).

With this in mind, many improvements have been designed to further heighten the accuracy of PSA for the diagnosis of PCa. These include free versus total PSA (Ito *et al.*, 2003), PSA density of the transition zone (Aksoy *et al.*, 2003; Tang *et al.*, 2013), age-specific PSA reference ranges (Babaian *et al.*, 1992; Brawer *et al.*, 1992;

Collins *et al.*, 1993), PSA velocity (Vickers *et al.*, 2011), PSA doubling time (Sengupta *et al.*, 2005) and pro-PSA (Mikolajczyk *et al.*, 2000; Peyromaure *et al.*, 2005) yet, found inconclusive due to contradictory reports (Borer *et al.*, 1998; Giannarini *et al.*, 2008; Hoffman *et al.*, 2000; O'Brien *et al.*, 2009; Oesterling *et al.*, 1995; Partin *et al.*, 1996).

Therefore, considering the nature of PSA of being an organ-specific rather than a cancer-specific protein (Chou *et al.*, 2011), together with the relatively inaccurate DRE reports (Okotie *et al.*, 2007), diagnostic inferences and interpretations for PCa based on these findings are needed to be made with extreme caution. As such, in cases of suspicious DRE findings and raised levels of PSA, a transrectal ultrasonography (TRUS)-guided biopsy of the prostate gland is additionally performed, to rule out malignancy via histopathology examination as well as for grading and staging of PCa (Table 2.1), if found. In the event of a negative result, repeat biopsies are usually recommended (Djavan *et al.*, 2001; Durkan *et al.*, 2002) unless, the levels of serum PSA are found within the acceptable range (less than 4 ng/ml). The repeat biopsy is generally aimed at overcoming the low sensitivity and positive predictive value of the screening method thus, uncovering the 'missed' tumours (up to 20-30% of clinically significant cancer) harbouring at areas of which (e.g., anterior, midline-transitional zone or apex of the prostate gland), that are not routinely sampled by TRUS biopsy (Presti, 2009). Left undetected and/or untreated, invasion and metastasis of prostatic-cancer cells to distant parts of the body, particularly the bones and lymph nodes eventually occurs, with fatality.

Taking these into consideration and in view of the inefficient use of serum PSA and inaccurate DRE findings as diagnostic screening marker for PCa, which had markedly predispose men to the risk of overdiagnosis (Etzioni *et al.*, 2002; National Cancer Institute, 2007; Pashayan *et al.*, 2009), unnecessary post-biopsy complications

(Loeb *et al.*, 2011; Nam *et al.*, 2010; Wagenlehner *et al.*, 2013), psychological distress caused by negative biopsy results (Klotz, 1997; McNaughton-Collins *et al.*, 2004) and therapy (Stanford *et al.*, 2000; Trinh *et al.*, 2014; Wilt *et al.*, 2008), a superior detection method and/or biomarkers for PCa at an early onset is highly necessitated. This in turn, is believed to facilitate administration of timely and appropriate treatment strategies in curbing this life-threatening 'silent' disease.

2.2. LECTIN

2.2.1. Definition

Although the work on lectin, particularly the castor bean extracts and its heamagglutinating capabilities can be traced back to the 18th century (Stillmark, 1888), the term 'lectin' that originates from the Latin word *legere*, which means to choose or select, was first introduced by Boyd and Shapleigh in 1954. Lectin is defined as protein or glycoprotein substances, chiefly of, but not limited to plant origin, protein of non-immunoglobulin in nature, capable of specific recognition of and reversible binding to, carbohydrate (sugar) moieties of complex glycoconjugates without altering the covalent structure of any of the recognized glycosyl ligands and as such, causes agglutination and/or precipitation of cells (Kocourek and Harejsi, 1983). Or, in a simpler and concise definition as described by Barondes (1988), lectin is a carbohydrate-binding protein other than an enzyme or antibody.

2.2.2. Source of lectins

Lectins are ubiquitously present in nature. They had been sourced from invertebrate and vertebrate animals, microorganisms and plants as well. Table 2.2 exhibits a list of selected lectins from various sources.

Lectins of fauna origin are also known as endogenous lectins. The works of Charcot and Robin (1853), and Leyden (1872) dating back to the 18th century, which culminates in the identification of Charcot–Leyden crystals protein (now known as galectin-10) (Swaminathan *et al.*, 1999) in eosinophil-mediated pathological conditions such as allergic reactions and parasitic infections, is probably the first animal (mammalian) lectin discovered, though, not as a 'lectin'. Lectins from animals have been previously isolated from various sources including tissue, serum, venom, egg and other biofluids from invertebrates (e.g., perivitelline and heamolymph) (Cohen, 1984; Kilpatrick, 2002). There are eight well-established structurally distinct families of intra- and extra-cellular type of vertebrate lectins. The calnexin family, M-type, L-type and P-type are examples of intracellular lectins, whilst, C-type, R-type, siglecs and galectins, the extracellular lectins. The former is located in the luminal compartments of the secretory pathway. On the other hand, the latter exists either as membrane-bound or in soluble forms (e.g., biofluids). In terms of biological functions, the former is involved in the trafficking, sorting and targeting of maturing glycoproteins whilst the latter, mediates cell adhesion, cell signalling, glycoprotein clearance and pathogen recognition. Newer additions to these existing families of lectins are the F-box lectins, ficolins, chitinase-like lectins, F-type lectins and intelectins. Amongst these lectins, some were noted to offer complementary functional-potential to the well-established lectin families denoted above. (Drickamer and Taylor, 1998).

Table 2.2. List of lectins from various sources.

Source		Abbreviation	Specificity
Common name	**Taxonomic name**		
ANIMAL			
Atlantic cod	*Gadus morhua*	Galectin-1	β-galactoside linked moieties
Burgundy snail	*Helix promatia*	HPA	α-N-acetylgalactosamine
Horseshoe crab	*Limulus polyphemus*	LPA	N-acetyl neuraminic acid
Yellow slug	*Limax flavus*	LFA	N-acetyl neuraminic acid
MICROORGANISM			
-	*Burkholderia cenocepacia*	BC2L-C N-ter dom	Lewis Y
-	*Chromobacterium violaceum*	CV-IIL	Fucosylated blood group epitopes
-	*Pseudomonas aeruginosa*	PA-IL	αGal4Gal
-	*Pseudomonas aeruginosa*	PA-IIL	Lewis a
-	*Ralstonia solanacearum*	RS2L	Oligomannose
PLANT			
Elderberry	*Sambucus nigra*	SNA	Neu5Acα2-6Gal(NAc)-R
Gorse	*Ulex europaeus*	UEA I	Fucα1-2Gal-R
Hairy vetch	*Vicia villosa*	VVA	GalNAcα-Ser/Thr (Tn-Antigen)
Jackbean	*Canavalia ensiformis*	Con A	α-Man, α-Glc
Jackfruit	*Artocarpus integrifolia*	AIL	(Sia)Galβ1-3GalNAcα1-Ser/Thr (T-Antigen)
Lentil	*Lens culinaris*	LcH	Fucosylated core region of bi- and triantennary complex type N-glycans
Orange peel fungus	*Aleuria aurantia*	AAL	Fucα1-2Galβ1-4(Fucα1-3/4)Galβ1-4GlcNAc; R2-GlcNAcβ1-4(Fucα1-6)GlcNAc-R1
Wheat	*Triticum vulgaris*	WGA	GlcNAcβ1-4GlcNAcβ1-4GlcNAc, Neu5Ac

The very first microbial glycan-binding protein lectin identified was the hemagglutinin in influenza virus by Alfred Gottschalk (1957), based on its ability to aggregate erythrocytes. Since then, numerous other microbial lectins (e.g., virus, bacteria and protozoa) have been described. These include the adhesins or heamagglutinins-surface lectins, receptor or ligand lectins and toxins, which are the secreted form of lectins. Pioneering work on bacterial surface lectin had resulted in the denotation of its function in facilitating attachment of bacteria to host cells and colonisation (Sharon and Lis, 1972). With that, bacterial surface lectins are often termed as adhesins whilst, its interacting partners on the surface of the host cells, as receptors or ligands. Amongst many, the cholera toxin, Bt toxin and Shiga toxin produced by *Vibrio cholera, Bacillus thuringiensis and Shigella dysenteria* respectively, are some examples of bacterial toxins that interacts with glycan-binding subunit on the host-cell surfaces during infections.

Like animal lectins, discovery of plant lectin similarly dates back to the 18th century (Stillmark, 1888). Amongst the sources, lectins of plant origin are the largest and best-categorised group of lectins. To date, there are more than a thousand plant species including the mono- and dicotyledons, molds and lichens, have been reported to possess lectins. Most of the plants stockpiles lectins at the highest levels in their seeds (Lis and Sharon, 1986; Putzai *et al.*, 1983) whilst some, in tissues including leaves, roots, flower, sap, barks, rhizosomes, bulbs, tubers and stems (Rudiger, 1988). Of note, there are also plants that release lectin into its plant products such as the *Hevea brasiliensis* - rubber tree. It had been observed to release the lectin, hevein, in latex sap when a sliver of bark is removed (Giordani *et al.*, 1999). The distribution and localisation of seed lectins are most predominant in the parenchyma of the cotyledons (e.g., *Pisum sativum*) or endosperm (e.g., *Ricinus communis*). Nevertheless, there are

also those that were merely confined to the primary axis - plumule and radical without the cotyledon (e.g., *Euphorbia heterophylla*).

2.2.3. Classification of lectins

The first classification scheme designed particularly for plant lectins was based on the carbohydrate-binding specificities of the lectins. As such, plant lectins may be categorised either as galactose (Gal)/N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), mannose (Man) and/or glucose (Glc) or maltose, L-fucose (L-Fuc), sialic acid-specific or complex glycan-binding lectins (Lis and Sharon, 1986). This form of classification however, must be treated with caution due to its limitations. This is because plant lectins may display broad range of binding specificities, with most of them demonstrating higher affinity for oligosaccaharides than for simple sugars. Additionally, structurally different lectins may also recognise same sugars thus, the mixed binding specificities. Also of note, are the pronounced preferences of plant lectins for 'foreign'-interacting glycans. For example, all chitin-binding lectins recognise carbohydrates that are common in fungi and insects but not in plants. (Peumans and van Damme, 1998).

Lectins have also been characterised based on their distinct protein folding domains/structural similarities and evolutionary relatedness of proteins (Peumans and van Damme, 1998). Via this categorization, seven families of lectin were distinguished. These include legume monocot mannose binding lectins, chitin-binding proteins with hevein domain, type 2-ribosome inactivating proteins, β-prism lectins, the Cucurbitaceae phloem lectins and the Amaranthaceae lectins. The former four types of lectin families encompass the majority of all currently known plant lectins.

The legume lectins are the best known lectin family. Structurally, all legume lectins have canonical twelve-stranded beta-sandwich framework (e.g., *Canavalia ensiformis*, *Maackia amurensis*, *Ulex europaeus* and *Vicia villosa*). The monocot mannose binding lectin belonging to a single superfamily of evolutionary related proteins on the other hand, contains tertiary structure consisting of three sequential β-sheet sub-domains (I, II and III) related by a pseudo 3-fold axis of symmetry (e.g., amaryllis, garlic bulbs lectin and snowdrop lectin). Distinctly, the chitin binding proteins has conserved glycine and cysteines residues domain of 43 amino acids to serve distinct binding specificity for oligosaccharides of N-acetylglucosamine (e.g., pokeweed lectin, *Urtica dioica* and wheat germ) whilst, the type 2-ribosome inactivating proteins are chimerolectins consisting of polynucleotide:adenosine glycosidase domain that are arrayed in tandem with a carbohydrate-binding domain (e.g., plant families Euphorbiaceae, Fabaceae and Sambucaceae).

The β-prism plant lectin which is also known as Jacalin or Jacalin like proteins is a galactose-specific non-legume plant lectin. It consists of 3 - 4 stranded sheets, lined perpendicular to the 3-fold axis duplication (e.g., *Artocarpus integrifolia*, jacalin, *Maclura pomifera*) and exerts heterogeneity, in terms of carbohydrate-binding specificity. The Cucurbitaceae phloem lectins are protein dimers that are made up of two identical subunits of about 24 kDa (e.g., *Cucurbita argyrosperma*, winter squash, and cucumber), and has high specificity to oligomers of GlcNAc. Last but not least, the Amaranthaceae lectins which are relatively the smallest plant lectin family after Cucurbitaceae phloem lectins family, is characterized by the β-trefoil folds with internal 3-fold axis of symmetry structure. It has high specificity for *O*-linked glycans. Examples of β-trefoil lectin include amaranthin, castor bean ricin B, ebulin and mistletoe lectin.

Nevertheless, with the dawn of molecular cloning, a more consistent classification of lectins on the basis of structural and evolutionary relatedness, but with greater emphasis on the highly conserved amino acid sequence motifs in the 'carbohydrate-recognition domains' had emerged (Dodd and Drickamer, 2001). Based on this, lectins may be classified into two distinct categories; Category I define lectin families with structural and/or evolutionary sequence similarities whilst category II, houses lectin-like proteins without established evolutionary classification as shown in Table 2.3. Of note, some of the protein families does share structural or sequence similarities with animal lectins.

2.2.4. Functions of plant lectins

Despite its ubiquity, the biological roles of plant lectins remain speculative. Considering the plentiful harvest of lectins from storage tissues (e.g., seeds or vegetative storage tissues), it has been suggested that lectins may serve as (carbohydrate) storage proteins that contribute sources of nitrogen for the development of plants (Peumans and Van Damme, 1995), as well as to maintain seed dormancy. Lectins have also been unequivocally associated with cell organisation, embryo morphogenesis, growth and elongation of cell walls and transport of carbohydrates (Moreira *et al.*, 1991) as well as in pollen recognition (Knox *et al.*, 1976).

Amongst all supposition, the other most notable function of plant lectins is the defence against fungal, viral and bacterial pathogens as well as animal (insect) predators (Rudiger and Gabius, 2001) either via active or passive defence mechanism of actions (Peumans and Van Damme, 1995). Plant lectins execute active defence mechanism of actions via the recognition of pathogens through their carbohydrate-binding specificities and thus, sequentially triggering host-immune responses (e.g.,

Table 2.3. Classification of lectins and lectin-like proteins.

Category I
β-prism lectins - Jacalin-related lectins
C-type lectins (e.g., calcium-dependent lectins such as selectins and collectins)
Eel fucolectins
Ficolins - fibrinogen/collagen-domain-containing lectins
Garlic and snowdrop lectins and related proteins
Galectins (formerly known as S-type lectins)
Hyaluronan-binding proteins or hyaladherins
I-type lectins - Immunoglobulins superfamily members including the Siglec family
Amoeba lectins - Jacob and related chitin-binding proteins
L-type lectins - plant legume seed lectins, ERGIC-53 in ER-Golgi pathway, calnexin family
M-type lectins -α-mannosidase-related lectins (e.g., EDEM)
N-type lectin nucleotide phosphohydrolases with glycan-binding and apyrase domains
P-type (e.g., mannose-6-phosphate receptors)
R-type (e.g., ricin, other plant lectins and GalNAc-SO4 receptors)
Tachylectins from horseshoe crab *Tachypleus tridentatus*
Hevein-domain lectins (e.g., wheat germ agglutinin and hevein)
Xenopus egg lectins/eglectins
Category II
Some annexins
Pentraxins with pentavalent domain structure
Some laminin G domains, recognizing glycans on α-dystroglycan
CD11b/CD18 recognizes fungal glucans and exposed GlcNAc residues on glycoproteins
Complement factor H, recognizes cell-surface sialic acids as “self”
Tumour necrosis factor-α, binds to oligomannose N-glycans
Interleukins - IL-1α, IL-1β, IL-4, IL-6, and IL-7 bind various glycans
Amphoterin binds carboxylated N-glycans

Source: Data extracted and modified from Varki *et al*., 2009.

inhibition of bacterial, viral and fungal activities). The passive-type of defence mechanism includes physical barriers, morphological and biochemical adaptations. Accumulation of toxic low molecular weight compound in the whole plant or susceptible tissues is an example of biochemical adaptation (Peumans and Van Damme, 1995).

On another note, characteristic properties of lectin in adhesion and agglutination highly suggest its role in establishing symbiotic and pathogenic interaction between glycoconjugates of hosts, which may interfere with the normal functioning of the host. For example, recognition and binding of legume root lectin to *Rhizobium spp.* or *Bradyrhizobium spp.* institutes Rhizobium-legume symbiosis thus, resulting in the formation of nitrogen-fixing root nodules (Diaz *et al.*, 1989). On the same note, binding of tomato lectin to mucosal cells of pathogens causes host-resistances to acid and proteolysis (Kilpatrick *et al.*, 1984).

2.2.5. Application of lectins

Distinct carbohydrate moieties recognising and interacting capabilities of lectins have been greatly exploited in the field of biomedical and biological researches as well as in agricultural plant protection (Peumans and van Damme, 1998).

The inception of ABO blood typing using plant lectins was by Boyd in 1945, via his observation on blood-type A specific agglutinating capabilities of lima beans (*Phaseolus lunatus*). To date, there are over 100 blood-type specific lectins of various origins that have been reported to demonstrate specificities for blood types A, B, O, AB, M or N, and subtypes A1 or A2 (D'Adamo, 2014). Among these, the most common plant lectins used for ABO typing include *Vicia cracca, Dolichos biflouros, Falcata*

japonica, Griffonia simplicifolia and *Ulex europaeus,* that specifically agglutinate erythrocytes with A, A1, A2, B and H antigens, respectively (Khan *et al.*, 2002).

Besides this, lectins are also frequently used in the field of transfusion medicine for detection and classification of polyagglutination of erythrocytes (Horn, 1999) and for diagnosis and management of T antigen activation and related disorders in neonates and children (e.g., necrotising enterocolitis and atypical haemolytic uraemic syndrome). Other clinical uses of lectins are seen in karyotyping of chromosomes, evaluation of immunocompetence, bone marrow transplantation and enzyme replacement therapy (Sharon and Lis, 1993).

Another marked ability of lectins is the induction of mitogenesis of cells, particularly the T and B lymphocytes, macrophages and neutrophils (Goldstein *et al.*, 1980). With this in mind, a large number of research has been conducted to comprehend blastogenesis of lymphocytes as well as biochemical and physical alterations associated cellular mitogenesis (Lim *et al.*, 1998; Maciel *et al.*, 2004; Oda *et al.*, 1991; Powell and Leon, 1970). These cumulative findings in turn, had led to the utilisation of lectins for treatment of cancer via mitogenic lectin therapy (Chan *et al.*, 2012), as well as anti-tumour cytotoxic therapy (Valadez-Vega *et al.*, 2011) and targeted tumour therapy (Sancho *et al.*, 2008).

Use of specific lectins had also served in mediating cell separation for the purpose of basic research as well as in cell-based therapy applications. Initial such works was reported by Reisner *et al*., (1976), based on his successful attempts at separating B and T cells of mouse lymphocytes using *Glycine max* agglutinin. In the recent years, Wang *et al*., (2011) had used fucose-specific lectin, *Ulex europaeus,* to identify, sort, and separate human pluripotent cells from heterogeneous cell populations

whilst, Dodla *et al*., (2011) had used *Vicia villosa* agglutinin for the isolation of human neural progenitor cells.

It is also possible to recognise and differentiate strains of bacterial species using lectin (Etaler, 1978). Bacterial typing is made possible by lectin via the recognition of 'exclusive' carbohydrate-rich surface antigens or receptors on these organisms. In the past, lectins (e.g., *Arachis hypogaea*, *Bauhinia purpurea*, *Solanum tuberosum*, *Triticum vulgaris* and *Glycine max*) have been successfully used to type bacterial strains of *Helicobacter pylori* (Ascencio *et al.*, 1990), *Campylobacter jejuni* (Yakovleva *et al.*, 2011), *Bacillus spp.* (Cole *et al.*, 1984) and *Neisseriae spp.* (Doyle *et al.*, 1984).

Lectins are also used to identify and select mutants with altered cell surface glycoconjugate compositions. One of the earliest reports of such use was by Stanley (1981). Based on the characteristics and unique patterns of lectin-resistance properties exhibited by the glycosylation mutants in Chinese hamster ovary cells to the cytotoxicity of plant lectins (e.g., wheat germ agglutinin), he had concluded that lectin does offer solutions for independent selection protocols for each type of mutant. On another occasion, castor bean lectin (*Ricinus communis*) was used for identification and selection of seven new mutants ricin-resistant Chinese hamster ovary cells (Stanley *et al.*, 1990).

Advances in genetic engineering and in receptor biology had led to the emergence of selective tumour cell killing using lectin-toxic conjugates in cancer therapy with greater efficacy, compared to the conventional treatment modalities including surgery, chemo- and radiation therapy. Toxic conjugates are either constructed using antibodies and/or their fragments (e.g., immunotoxin) or other carrier proteins (e.g., epidermal growth factor, fibroblast growth factor and transferrin)

(Fracasso *et al.*, 2004). Regardless, more importantly, these toxic conjugates must be resistant to chemical modifications and conjugation, endogenous proteolytic activity as well as able to deliver toxic substance efficiently when the target is reached, to facilitate effective killing of the target cells (Dosio *et al.*, 2011). In addition to deglycosylated A chain of ricin-, truncated diphtheria toxin- and pseudomonas exotoxin A-containing immunotoxins, saporin-S6, a type 1-ribosome-inactivating protein isolated from the seeds of *Saponaria officinalis* had also been extensively used for conjugation in pre-clinical studies against hematological (e.g., myeloma, lymphomas and leukemias) and solid tumours (e.g., cancers of the cervix, bladder, pancreas and prostate) (Polito *et al.*, 2011).

The use of lectins as cyto- and histochemical characterisation tool has also been previously described (Allison, 1986; Kuhlmann *et al.*, 1983). Due to high carbohydrate binding specificity, lectins have been used as cyto- and histochemical probes, which allow *in situ* identification, localisation and characterisation of various *N*- and *O*-linked oligosaccharides present in pathological tissues and cells (Spicer and Schulte, 1992). Along that line, lectins have been successfully used in characterisation of glycosaccharides in lysosomal storage diseases, including GM1-gangliosidosis, Krabbe disease, Gaucher disease and fucosidosis (Alroy and Lyons, 2014; Alroy *et al.*, 1985), colon adenocarcinoma of the human rectum (Madrid *et al.*, 2000) and normal cells of human gall bladder (Madrid *et al.*, 1994).

Plant lectins have also been noted to have high affinity for membrane-associated glycoconjugates on neurons. As such, plant lectins such as wheat germ agglutinin (Nakashima *et al.*, 2000) and phytohemagglutinin (Gerfen and Sawchenko, 1985) which are considered as superior neuronal tracers, have been extensively exploited for tracing chemically specified circuits in the central nervous system thus, the

mapping of neuronal pathways for better understanding of brain functions. The precise anterograde and retrograde-directional transport of lectins are traced from the point of injection, via horseradish peroxidase- (HRP) or radioactive-labelled lectin detection methods (Sharon and Lis, 1993). For example, HRP-conjugated wheat germ agglutinin had served as a beneficial transneuronal marker for visual and olfactory pathways in monkey (Itaya and van Hoesen, 1982) and rat (Shipley, 1985), respectively. Other types of lectin used for neuroanatomical studies include soybean agglutinin, ricin and volkensin. However, the latter two types of lectin are rather known as 'suicide transport' tracer as it had been observed to cause apparent neuronal morphological aberration and apoptosis (Wiley *et al.*, 1982) and degeneration (Heath *et al.*, 1997) in rat, respectively. Later, the combination of conventional neuroanatomical tracing method with wheat germ agglutinin transgene technology had greatly enhanced the investigations of brain-neural networks (Yoshihara *et al.*, 1999).

The well-defined interaction between a lectin and its carbohydrate ligand further facilitates the structural definition of target glycoconjugates/glycans (e.g., glycoproteins, polysaccharides and glycolipids) (Brockhausen *et al.*, 1998; Peumans and van Damme, 1998). However, due to the extreme levels of microheterogeneity of carbohydrate moieties, its characterisation is often challenging. Nevertheless, by exploiting the narrow specificity of lectins, detection of subtly varied structures of carbohydrates is made possible. For example, Shibuya *et al.*, (1987) had successfully studied the microheterogeneity patterns in fetuin and α1-acidic glycoprotein (AAG) sialylation using *Sambucus nigra* lectin.

Isolation and characterisation of glycoconjugates via the use of lectin is particularly important considering the significant correlation of altered levels of glycoproteins in manifestation and progression of various diseases. Although

containing comparable levels of *N*-linked bi-antennary oligosaccharides with the controls, serum IgG of subjects with rheumatoid arthritis were found to demonstrate lower abundance of terminal galactose (Parekh *et al.*, 1988a), whilst in other studies, differential levels of galactosylation were considered indicators for diseases including juvenile rheumatoid arthritis (Parekh *et al.*, 1988b), tuberculosis (Rook *et al.*, 1991) and chronic inflammatory disease such as systemic lupus erythematosus and Crohn's disease (Tomana *et al.*, 1988).

Association between altered levels of glycoproteins have also been observed in studies investigating the onset, progression and metastasis of cancers (Dennis *et al.*, 1999; Nishimura *et al.*, 1995). In this case, lectins had served as a beneficial tool in detection of tumour-associated glycoproteins of altered abundances thus, facilitating potential diagnostic and prognostic predictions as well as therapeutic interventions for cancer. For this, lectins have been invariably incorporated with other techniques such as affinity chromatography, Western blotting, enzyme-linked immunosorbent assay (ELISA) and array-types to enhance detection of glycoproteins that are generally of lower in abundance. For example, concanavalin A - immobilised affinity chromatography employed prior to 2-DE, had resulted in successful profiling of serum *N*-glycosylated sub-proteome with improved resolution in subjects with colorectal carcinoma (Rodriguez-Pineiro *et al.*, 2004), respectively. A more advanced use of affinity chromatography technique is the multi-lectin affinity chromatography. Serial multi-lectin affinity chromatography has been used to demonstrate comprehensive detection and changes in abundances in the serum or plasma of subjects with breast cancer (Zeng *et al.*, 2011). Aside from gel-based solid support, magnetic beads are also used to facilitate enrichment and analysis of specific glycoproteome (Abdul Rahman *et al.*, 17 August 2006) that may be associated with diseases (Loo *et al.*, 2010).

Detection of glycoproteins on either electroblotted membranes or arrays on the other hand, are made possible via the use of lectin conjugates such as enzymes, biotin, fluorescent dyes, digoxigenin, colloidal gold and radioactive markers (Badia and Querol, 1988; Kijimoto-Ochiai *et al.*, 1985; Li *et al.*, 1993; Versura *et al.*, 1986; Yoshikawa *et al.*, 1987). On another note, soybean agglutinin lectin captured CA-S27 monoclonal antibody sandwich enzyme-linked immunosorbent assay (ELISA) has been used to detect serum CA-S27, a novel carbohydrate antigen in patients with cholangiocarcinoma (Silsirivanit *et al.*, 2013).

2.2.6. Lectin of interest: champedak galactose binding lectin

Artocarpus integer, locally known as champedak, has distinct sweet aroma and its inner core filled with brown seeds that are covered with yellow fleshy pulp. In 1991, Hashim *et al.*, had first harvested a lectin from the seeds of champedak. Due to its affinity for galactose residues of the *O*-linked oligosaccaharide moieties, inherently, the lectin was termed as, champedak galactose binding (CGB) lectin (Hashim *et al.*, 1991). Later, Lim *et al*., (1997) had successively isolated another lectin from the seeds of champedak which has high affinity for core mannosyl residues of the *N*-linked oligosaccaharide moieties and as such, designated as champedak mannose binding (CMB) lectin. Structurally, CGB lectin comprises two non-covalently linked protein subunits with M_r of 13 and 16 kDa whilst, the CMB lectin is composed of four subunits of polypeptides with M_r of 64 kDa. A recent follow-up X-ray crystallography analysis of structures and binding specificity of CGB and CMB lectin's had also corroborated the previous data reported for these lectins (Gabrielsen *et al.*, 2014).

Although CGB lectin is similar in many ways to jacalin, which is also an IgA1-reactive and D-galactose binding lectin but harvested from the seeds of jackfruit

(*Artocarpus integrifolia*) (Roque-Barreira and Campos-Neto, 1985), the lectin is more homogenous in both structures and functions. CGB lectin isolates from various clones exhibited consistency and uniformity in interaction with IgA1 but not with IgA2, IgD, IgG and IgM of human serum (Hashim *et al.*, 1993; Hashim *et al.*, 1992; Hashim *et al.*, 1991). However, jacalin isolated from seeds of different origins such as those from Brazil, Japan and Manila have been reported to display differences in structures and reactivity to subclasses of immunoglobulins (Aucouturier *et al.*, 1988; Kobayashi *et al.*, 1988). Hashim *et al*., (1994) had also demonstrated the effect of CGB lectin on the functional activity of guinea-pig complement. In this study, CGB lectin were found to consume complement thus, decreased the complement-triggered haemolysis of sensitised erythrocytes of sheep.

Besides this, the capability of CGB lectin to probe for disease-related *O*-glycosylated proteins using techniques including immunodiffusion, affinity chromatography and lectin-blotting were tested as well. For example, probing of 2-DE resolved nitrocellulose membrane (NC) blots of sera and urine from subjects with ovarian, endometrial, cervical and prostate cancers using CGB lectin had resulted in detection and identification of a number of serum and urinary *O*-glycosylated proteins with biomarker potentials (Abdul-Rahman *et al.*, 2007; Abdullah-Soheimi *et al.*, 2010; Jayapalan *et al.*, 2012; Jayapalan *et al.*, 2013; Mohamed *et al.*, 2008; Mu *et al.*, 2012). Interestingly in another study, differential abundances of galactose and sialic acid residues in the sera of subjects with IgA nephropathy detected using CGB lectin had contributed to the postulation of structural aberration of *O*-glycans of IgA1 that may attribute to the etiopathogenesis of IgA nephropathy (Mestecky *et al.*, 1993; Shuib *et al.*, 1998).

In terms of reactivity to human immunoglobulins, the CMB lectin on the other hand, had shown strong reactions to IgE and IgM but weak binding to IgA2 (Lim *et al.*, 1997). Aside from this, CMB lectin had also demonstrated profound mitogenic activity by stimulating cellular proliferation of murine T cells but not B lymphocytes (Lim *et al.*, 1998). When CMB lectin was similarly used as probes to detect *N*-glycosylated proteins on 2-DE resolved NC blots of sera or plasma of subjects with nasopharyngeal carcinoma (Seriramalu *et al.*, 2010) and congenital hypothyroidism (Yong *et al.*, 2006), a number of glycoproteins were found to be differentially altered in abundances. The former investigation had shown reduced abundances of serum α-2 macroglobulin and complement factor B whilst, in the latter study, significantly up- and down-regulated abundances of plasma fibrinogen and haptoglobin β-chains, was respectively observed in the patients' cohorts.

2.3. PROTEOMICS

2.3.1. Definition

'Proteome' connotes the whole complement of PROTEins encoded and produced by genOME of an organism or a cellular system. The levels of these translated proteins, which may be post-translationally modified and subjected to pathophysiological changes, vary according to the predisposed stimuli (Wasinger *et al.*, 1995). As such, 'proteomics' refers to large-scale comprehensive study of a specific proteome, in terms of identity, abundance, variation, modification, structure, function, and interacting and networking partners, in order to provide wholesome information of the proteins expressed in health and disease (Wilkins *et al.*, 1996).

2.3.2. Area of proteomics

Proteomics, a high-throughput branch of biochemistry, is deemed to contribute insightful and 'direct' description of cellular functions as well as dysfunctions of biological processes. As such, proteomics had indeed superseded the strength and contributions of genomic era via its capability in generating large-scale information on protein-protein interactions, organelle composition, protein activity patterns and differential protein profiles thus, reflecting the 'true' status of a cell (Petricoin *et al.*, 2004). Yet, proteomics often faces challenges due to the inherent attributes of proteomes, which has high level of complexity. Factors including genetic capability of one gene encoding for more than one protein, limited and variability of samples material, dynamic range of more than 10^6 -fold due to synthesis, degradation or protein-protein interaction (Corthals *et al.*, 2000), a plethora of pre-, co- and post-translational modifications (e.g., glycosylation, phosphorylation and acetylation) (Darie, 2013), vast types of tissues and variation in protein abundances due to developmental and/or temporal specificity and cellular distress, which may be caused by diseases and treatment regiments had profoundly contributed to the complexity of proteomes. However, combine use of multiple proteomics approaches were thought to provide valuable merits in overcoming these drawbacks (Tyers and Mann, 2003).

There are many sub-divisions of proteomics (Tyagi *et al.*, 2010). These include structural proteomics, expression proteomics, interaction or functional proteomics and proteome informatics. Structural proteomics integrates structural maps of proteins and protein domains in 3D conformation reflecting of protein interactions and functions garnered from individual protein to protein complexes and its associated microenvironment at cellular levels, into a common framework (Sali *et al.*, 2003). In-depth analysis of protein structures is made possible by the use of X-ray

crystallography, nuclear magnetic resonance spectroscopy, *in silico* screening (Kamionka *et al.*, 2002), electron microscopy and electron tomography. Collectively, these technologies had greatly contributed to studies involving drug discovery, prediction of proteins' 3D structure from genomic sequence and identification of structurally-related orthologs of proteins.

Expression proteomics provide quantitative maps of expression of proteins either from cells or tissues. In this field, differential expression of proteins are used as the basis for identification of disease markers, biological pathways of normal and disease states as well as to assist in the study of pharmacodynamics of drugs intended for drug targets. Of note, 2-DE and MS are the most commonly used techniques for the investigation of expression proteomics (James, 1997; Wilkins *et al.*, 1996). It may also be incorporated with techniques such as affinity chromatography and Western blotting for comprehensive analysis of protein aberration due to a disease state. ELISA, protein array and Bio-Plex assays on the other hand, are used to provide information on the changes of levels of proteins being assessed, quantitatively.

Interaction or functional proteomics, on the other hand, provide physical maps of cellular localisation as well as interactions between proteins for characterisation of protein networks and determination of their functions. The proteome-wide biochemical assays was pioneered by Field and Song (1989) using novel two-hybrid genetic system to detect protein-protein interaction, regardless of strength of the interaction or abundances of the proteins involved. On this basis, various array-based approaches have been later adopted, to investigate protein activities such as protein localisation in the cell through green fluorescent protein signals, protein interactions through fluorescence resonance energy transfer (Tavare *et al.*, 2001), protein modifications and enzymatic activities.

Proteome informatics branch of proteomics as a tool for molecular biology and biomedicine was first coined by Mathias Mann in 1996. This field offers statistically sound bioinformatics algorithms, software tools and strategies toward accurate and comprehensive analysis of proteomics data (Palagi *et al.*, 2006). Proteome informatics tools are made available for all ranges of proteomics techniques. These include tools for post-separation analyses (2-DE - Delta2D, ImageMaster2D Platinum, Progenesis; liquid chromatography (LC) /MS - Decyder MS, Mapquant, Msight), protein identification by peptide mass fingerprint (PMF) (e.g., MASCOT, Aldente, MS-Fit) and protein fragmentation fingerprint (e.g., Spectrum Mill, SEQUEST, X!Tandem), *de novo* sequencing (e.g., AUDENS, PEAKS, PepNovo), and quantitative analyses (e.g., DTA Select, MSQuant, EXPRESS). Proteome informatics tools are also available for post-processing of MS/MS data (e.g., Trans-Proteomic Pipeline, Scaffold).

2.3.3. Techniques of proteomics

Techniques employed for separation of proteins for proteomic analysis may be divided into gel-based and gel-free separations (Scherp *et al.*, 2011). The gel-based approaches include one-dimensional gel electrophoresis (1-DE), 2-DE, and 2D difference in-gel electrophoresis (DIGE) whilst, the gel-free methods includes SELDI-ToF/MS, nano LC-MS/MS, capillary electrophoresis, multidimensional protein identification technology (MudPIT) and protein microarray, to name a few. After considering the advantages and disadvantages of these approaches as outlined in Table 2.4, they may either be used independently or in combination for optimal separation and analysis of proteins and proteomes of interests.

The most commonly used integrated technology of separation and MS for proteomic analysis are 2-DE followed by identification of proteins using matrix-assisted laser desorption/ionisation - time of flight (MALDI-ToF/ToF). Ideal separation and analysis of proteins may also be achieved via affinity capture on ProteinChip array followed by SELDI-ToF/MS analysis or by a LC system-coupled to either an online or offline mass spectrometer. In the following section, only the techniques that were predominantly used in this study are elaborated at length.

1- and 2-DE

1-DE experiments generally offer information on the MW and purity of proteins and its subunits, if present, at a relatively faster rate due to the limited separation area. To a certain extent, the technique is also able to detect PTMs such as glycosylation and phosphorylation by subjecting the samples for prior chemical, enzymatic (Sriyam *et al.*, 2008) or radiolabelling (Larsen *et al.*, 2001) procedures or via immunodetection, following electrophoretic separation (Anderson and Peck, 2008).

2-DE technique, an extension of 1-DE on a larger gel format, was first introduced by Klose (1975) and O'Farell (1975) in an attempt to produce high resolution protein profile of various tissues harvested from mouse and *Escherichia coli*, respectively. The proteins are generally separated in 2 steps according to two independent physico-chemical properties; isoelectric point (pI) and molecular weight (MW) and later, stained using either silver, Coomasie or fluorescent dyes for visualisation of the electrophoresed proteins. With that, 2-DE provides resolution and visualisation of hundreds of proteins on a single gel, simultaneously, and at the same time, delivers a spectrum of pertinent information of each protein, such as its molecular weight, pI, quantity and possible PTMs. One other advantage of 2-DE is the ability to

separate the workflow to be conducted at different space and time whilst, the resulting 2-DE gels serve as high-capacity 'storage system' for long-term archival of proteins, for further purification and identification by MS (Beranova-Giorgianni, 2003). Taken together, gel-based proteomics serves a comprehensive platform for detection and identification of proteins in biological fluids that may be potentially used for discovery of cancer biomarkers (Chevalier, 2010).

SELDI-ToF/MS

ProteinChip array technology, SELDI-ToF/MS was developed as an alternative to 2-DE. It usually involves rapid analysis of small (intact) molecules ranging between 2 - 20 kDa with high-throughput. Owing to the integrated technology of separation and analysis, SELDI-ToF/MS is able to enrich proteins from crude samples at femtomole levels via affinity capture on the surface of the array that is modified either chemically or biologically and at the same time, analyse the expression profiles of the proteins by a linear ToF/MS (Hutchens and Yip, 1993). The chemical characteristics of the surface may bear hydrophobic, hydrophilic, anionic, cationic or metal affinity properties whilst, antibodies, lectins (Ueda *et al.*, 2009) and DNA may serve as biological surfaces. Analysis of the mass spectra profiles using variety of statistical and bioinformatics techniques recognises discriminating patterns of proteins between groups of sample that may be potentially used as markers for diagnosis (Petricoin *et al.*, 2002b) or prognosis (Yanagisawa *et al.*, 2003) of cancers and other diseases (Hong *et al.*, 2008; Tomosugi *et al.*, 2006) as well as for biochemical profiling of serum high-density lipoproteins variants (Dayal and Ertel, 2002).

Table 2.4. Advantages and disadvantages of proteomics technologies.

	Gel-based methods		Gel-free methods		
	2-DE	**2D-DIGE**	**SELDI, MALDI**	**LC-MS/MS**	**Microarray, Protein array**
Advantages	• Separation of large number of proteins	• Separation of large number of proteins • Requires small amount of proteins • Reproducible • Quantitative	• Automated • Requires small amount of proteins • Quantitative	• Automated • Requires small amount of proteins • Quantitative	• Automated • Requires small amount of proteins • High-throughput • Reproducible • Sensitive for detection of PTMs
Disadvantages	• Not-automated • Limitation for very large and small M_r of proteins • Requires large amount of proteins	• Not-automated • Limitation for very large and small M_r of proteins	• Lower detection of low abundance proteins	• Lower detection of low abundance proteins	• Limitation for total number of proteins analysed

Source: Data adapted and modified from Suehara *et al*., 2012.

Despite its strong potential in providing an alternative avenue for rapid and minimally invasive approach for screening, diagnosis and prognosis of diseases, particularly cancer (Adam *et al.*, 2002; Petricoin *et al.*, 2002b; Qu *et al.*, 2002), the suitability of SELDI-ToF/MS as profiling approach and its exceptional sensitivities and specificities had created controversies (Baggerly *et al.*, 2004; Diamandis, 2003; Diamandis, 2004; Sorace and Zhan, 2003) and cast a huge setback for method.

Nano-LC and tandem mass spectrometry (MS/MS)

Nanoscale LC coupled to MS/MS is an ideal setup for the analysis of enzymatically-digested purified or complex peptide mixture particularly, in sample-limited situations. The use of chromatographic columns with small (µm) internal diameter renders the heightened sensitivity of the technique (Gaspari and Cuda, 2011). In a nano LC system, peptides are forced by a mobile phase (e.g., water-acetonitrile) at high pressure through a column packed with a stationary phase to facilitate separation and thus, introduces the samples to MS (e.g., online - electrospray ionisation (ESI) or, offline - MALDI target spotter). The use of hydrophobic type of stationary phase with its corresponding polarity of the mobile phase is termed reverse-phase liquid chromatography whilst, stationary phase with hydrophilic surfaces are designated as normal phase liquid chromatography.

An improvisation to the pioneering method of LC is the incorporation of two- (Wolters *et al.*, 2001) or three-dimensional (Prins *et al.*, 2001) chromatographic separations to the trypsin digested protein samples prior to MS. This intends to extend the capability of the technique in detecting proteins of very low abundance (lower than ng/mL in serum). Additionally, metal-coded affinity tags (Bergmann *et al.*, 2012) and stable-isotope labelling of proteins either via metabolic (Conrads *et al.*, 2002),

enzymatic (Yao *et al.*, 2001) or chemical modifications facilitates both absolute and relative quantification of proteins of interest.

The ToF, quadrupole, ion trap and hybrids such as QTrap and QToF are the most commonly used types of mass analyser. In a typical ToF type of mass analyser, immobilised enzyme-digested protein samples in an energy absorbing matrix on a plate are first ionised, accelerated at high voltage through a flight tube at which the ions are separated, and detected according to their velocity and time they take to travel the length of tube, in terms of mass to charge ratio (m/z). Based on these data, a mass spectrum, which is an intensity vs m/z graph, is subsequently generated. Owing to the high specificity and sensitivity, MS-based proteomics provides excellent option for separation, identification as well as quantitation of small amounts of proteins in complex mixtures thus, overcomes drawbacks of 2-DE (Aebersold and Mann, 2003). The resulting MS and/or MSMS data of high mass accuracy also does facilitate the determination of molecular structures and formulae of small molecules. In this study, MALDI tandem-time-of-flight (MALDI-TOF/TOF) mass spectrometer was used to enable ultra-high throughput, high sensitivity, and high-energy collision-induced dissociation to garner peptide-sequence information of proteins that were significantly altered in abundances.

Western blotting

Rapid detection, characterisation and quantitation of proteins (e.g., ng levels) is made possible by the use of Western blotting technique, via antigen-antibody recognition and its non-covalent linkages (Gallagher and Chakavarti, 2008). Western blotting involves electrotransfer of proteins from SDS-PAGE gel onto a solid support such as NC, polyvinylidene fluoride or nylon membranes. Visualisation of the target proteins is achieved via the use of chromogenic (e.g., 3, 3'-diaminobenzidene, TMB,

BCIP-NBT) or chemiluminescent substrates (e.g., Pierce ECL), following immunoprobing of the membrane using specific and matching primary and labelled-secondary antibodies pair (e.g., polyclonal or monoclonal).

The membranes mentioned above differ in protein-binding ability as well as in the effects of duration of incubation and detergents or agents used on the resulting quantity of proteins bound (Euan and Brian, 1989) and therefore, needed to be carefully selected. For example, NC membrane has high affinity and retention abilities for proteins but not recyclable whilst, polyvinylidene fluoride membrane has lower protein binding properties but possessed other valued assets including resistance to heat, chemicals and corrosion thus, allowing re-probing of the membrane (Mahmood and Yang, 2012). Nylon membrane, on the other hand, is better suited for nucleic acid binding application.

Lectin (Eastern) blot

Proteins translated from mRNA undergo a series of covalent processing events known as PTM. PTM may either involve addition or removal of a certain functional groups, protein or lipids, alteration in the chemical nature of amino acids, or, by causing structural changes (Bradshaw and Stewart, 1994). Together, these results in marked changes in the properties of proteins and as such, involved in the regulation of cellular functions including localisation, turn-over and interaction with other molecules (e.g., DNA, co-factors and lipids). Additionally, differential expression of protein PTMs is deemed as a potential clinical indicator of a disease state (Mann and Jensen, 2003). Figure 2.5 demonstrates the most common types of PTMs.

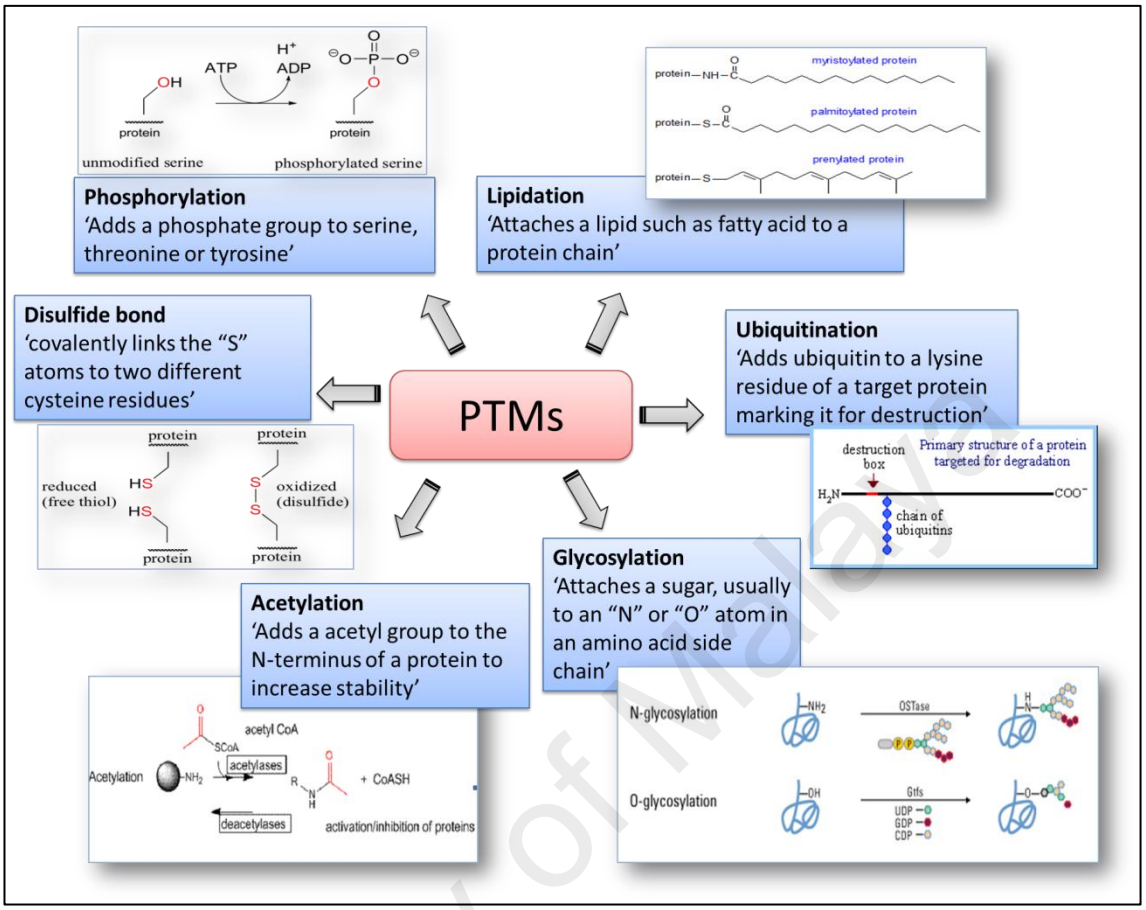


Figure 2.5. Common types of PTMs.

Eastern blot technique is an extension of Western blotting that is used to detect PTMs such as lipids, phosphate group (PO_4) and carbohydrate epitopes on proteins (Freeze, 1993; Shan *et al.*, 2001). As in Western blotting, samples are similarly separated in a 1- or 2-DE gel and transferred onto a membrane but detected using respective PTM-appropriate probes. For example, nitrophospho-molybdate methyl green (Thomas *et al.*, 2009) is used to detect phosphorylation whilst concanavalin A, for the detection of mannose-containing glycans (Seriramalu *et al.*, 2010). To that effect, specific detection of glycosylation type of PTM using lectin is referred as lectin or eastern blotting (Freeze, 2001) (see Section 3.13.1).

Lectin-immobilised affinity chromatography

Considering the complexity and vast dynamic range of proteins in biofluid samples like serum, pre-fractionation or separation of proteins in complex mixture (e.g., from cells, sub-cellular fractions, column fractions or immunoprecipitates) is often the key step in proteomics. Pre-fractionation may be achieved either by depletion of proteins of high abundance such as albumin (Lei *et al.*, 2008) and IgG (Pieper *et al.*, 2003) using immunoaffinity chromatography techniques (Anderson *et al.*, 2004) or, by enriching sub-proteome of interests, which is usually low in abundance (Florens *et al.*, 2008) using lectins (Abdul Rahman *et al.*, 2002).

Lectin-immobilised affinity chromatography allows separation and purification of glycoproteins based on specificity for sugar moieties (Varki *et al.*, 2001) (Table 2.1). The resulting glycoprotein-rich eluate is commonly used for structural characterisation of glycoprotein carbohydrate chains (Haselbeck *et al.*, 1990) or, identification of lectin-binding glycoproteins (Abdul-Rahman *et al.*, 2007). However, the technique does have some limitations. This includes interferences from non-specific interaction between the analytes and immobilised lectin as well as the availability of multiple-binding sites of 'a glycoprotein' that facilitates extreme stronghold interactions with the immobilised lectin thus, causing difficulty during elution.

There are many different types of commercially available and ready-to-use lectin immobilised columns for purification of glycoproteins. To name a few, concanavalin A has high binding specificity for glycoproteins with terminal mannose or glucose residues (αMan > αGlc > GlcNAc) whilst, wheat germ agglutinin exhibits strong interaction with chitobiose core of *N*-linked oligosaccharides [GlcNAc $(\beta 1,4GlcNAc)_{1\text{-}2}$ > βGlcNac] or sialic acid residues. Aside from this, there are those that are packed in laboratories. These include CGB-lectin immobilised onto Sepharose 4B (Abdul Rahman *et al.*, 2002) and immobilised CMB-lectin Sepharose 4B

(Seriramalu *et al.*, 2010) for isolation and purification of *O*- and *N*-glycosylated proteins, respectively.

2.3.4. Application of proteomics in clinical research

Clinical proteomics, a sub-discipline of proteomics is defined as systematic large-scale identification of disease-specific molecular biomarker patterns and therapeutic targets on clinical biofluid materials and its application in improving patient care, as well as public health through better assessment of disease susceptibility, prevention, diagnosis and prognosis, and selection of appropriate disease-countermanding therapeutic regiments (Granger *et al.*, 2004). In other words, applications of proteomics in clinical research play an important role in the identification of disease-specific, pharmacogenomics as well as therapeutic monitoring biomarkers. With that in mind, proteomics technologies have been readily exploited in various fields of researches of clinical medicine particularly, in search of disease-reflecting protein biomarkers. Proteomics have been applied in the fields of haematology (Cristea *et al.*, 2004), neurotrauma (Zhu *et al.*, 2013), renal diseases (Luczak *et al.*, 2011), neurology (De Masi *et al.*, 2013), foetal and maternal medicine (Cho and Diamandis, 2011), autoimmune disease (López-Pedrera *et al.*, 2009), cardiovascular disease (Majek *et al.*, 2011), diabetes (Sundsten and Ortsäter, 2009) and cancer (Gregorc *et al.*, 2014).

Biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention (Biomarkers Definition Working Group, 2001). Simple parameters from pulse, blood pressure to complex laboratory-based investigations using cells, tissues and biofluids are classified as biomarkers.

Identification of unique protein biomarkers for cancer along with those indicating efficacious therapeutic intervention, lies in the understanding and real-time assessment of the perturbed biological processes that orchestrates the onset and progression of the disease (Miles *et al.*, 2006). Neoplastic events occurring during cancer brings about tangible and detectable changes at protein levels, either by rendering aberration in protein expression, modification or localisation (Srinivas *et al.*, 2001). As such, application of proteomics technologies expands opportunities and possibilities for better understanding as well as wholesome-appraisal of these events. This, in turns aids in the discovery of biomarkers for risk assessment, diagnostics, prognostics and recurrences as well as therapeutic responses of cancer via the measurement and analysis of these altered levels of proteins.

Although lacking in specificity and sensitivity, proteins including PSA, alpha-fetoprotein and carcinoembryogenic antigen are still being used for diagnosis of cancers of the prostate, liver and gastrointestinal tract, respectively. Markers such as MTDH, SCC-A and HE4 have been previously identified for indicating progression, metastasis and poor prognosis of cancers including gall bladder adenocarcinoma (Liu and Yang, 2013), cervical carcinoma (Brioschi *et al.*, 1991) and ovarian cancer (Trudel *et al.*, 2012), respectively. On the other hand, monitoring of responses to therapy of patients with cancers of pancreas and ovary as well as melanoma has been previously suggested via the measurement of proteins CA19-9 (Ballehaninna and Chamberlain, 2012), CA125 (Pignata *et al.*, 2011) and S100B (Harpio and Einarsson, 2004), respectively.

Bodily fluids that have been mined for cancer biomarkers thus far, include serum or plasma, urine and other tissue-specific bio-fluids such as exfoliated skin cells, saliva, oral rinses, sputum, bronchoalveolar lavage, exhaled breath, nipple aspirate, ductal lavage, fine needle aspirates, gastric fluid, bile, pancreatic juice, stool, urine,

expressed prostatic secretions, seminal fluid, ovarian epithelial brushes/washes, menstrual flow, secretions or swaps of anatomic sites, circulating leukocytes, bone marrow aspirates and cerebrospinal fluid. Among these, the dynamic nature of the circulatory system and its constituents that may be altered during pathogenesis of cancer and as such, reflected in the circulation, makes serum or plasma the ideal choice of biofluid (Hanash *et al.*, 2008) for proteomics-based cancer biomarker studies. Aside from this, urine, containing ultrafiltrate of plasma as well as proteins and peptide components that are shed from the urinary tract (Hortin and Sviridov, 2007) is also fast gaining popularity as biofluid for proteomic workflows. This is most likely due to the attractive non-invasive and cost-effective attributes of urine sample collection (Pisitkun *et al.*, 2006). Table 2.5 demonstrates some of the investigations conducted using various types of bio-fluids on a variety of proteomic platform for identification of potential biomarkers for various types of cancer.

Table 2.5. Candidate biomarkers identified from bio-fluids in various cancer using proteomics technologies.

Bio-fluid	Biomarker	Type of cancer	Method	Reference
Plasma	Increased level of HAPβ, SAP, AAT, ACT, AAG, RBP and TTR.	Breast cancer	2-DE, Edman sequencing	Chahed *et al*., 2004
Plasma	Increased level of serum amyloid protein A.	Osteosarcoma	SELDI-ToF/MS, WB	Li *et al*., 2006
Albumin/IgG depleted serum	Increased level of TTR.	Pancreatic ductal adenocarcinoma	2D-DIGE, MALDI-ToF–MS, WB, ELISA, IHC	Chen *et al*., 2013
AffiGel-blue depleted serum	Increased level of four isoforms of HAP and two isoforms of AAT.	Infiltrating ductal breast carcinomas	2-DE, MALDI-ToF–MS, IHC	Hamrita *et al*., 2009
Serum	Increased level of pigment epithelium-derived factor.	Prostate cancer	2D-DIGE and LC-MS/MS	Byrne *et al*., 2009
Serum	Increased level of ceruloplasmin.	Nasopharyngeal carcinoma	2-DE, MALDI-ToF–MS, IHC, ELISA	Doustjalali *et al*., 2006
Serum	Increased level of ABG, ATR and ZAG. Decreased level of AAT and KNG.	Cervical cancer	2-DE, MALDI-ToF–MS, Lectin affinity chromatography, ELISA	Abdul-Rahman *et al*., 2007
Urine	Increased level of CD90.	Prostate cancer	ICAT-MS & LC-SRM-MS	True *et al*., 2010
Urine	Increased level of fragments of ALB. Decreased level of CD59, KNG-1 and ITIH4f.	Ovarian carcinoma	2-DE, MALDI-ToF–MS, WB	Abdullah-Soheimi *et al*., 2010
Urine	Increased level of ZAG, AAG and CD59. Decreased level of fragment of nebulin.	Endometrial cancer	2-DE, MALDI-ToF–MS, WB, Lectin affinity chromatography, LC-MS/MS	Mu *et al*., 2012

Table 2.5 continued…

Bio-fluid	Biomarker	Type of cancer	Method	Reference
Expressed prostatic secretion-urine	Increased level of PARK7 and 14-3-3σ. Decreased level of TGM4, LTF, ANPEP, MME and TIMP1.	Prostate cancer	MudPIT, LTQ Orbitrap XL-FTMS, 1-DE, WB	Principe *et al*., 2012
Saliva	Increased level of AAT, HAP, C3, HPX and TTR.	Oral squamous cell carcinoma	2-DE, MALDI-ToF/MS, IHC, ELISA	Jessie *et al*., 2013
Nipple aspirate fluid	Increased level of lipophilin B, β-globin, HPX and VDB. Decreased level of AHS.	Breast cancer	ICAT-MS, WB	Pawlik *et al*., 2006
CSF	Decreased level of gelsolin.	Astrocytoma	2-DE, MALDI-ToF/MS, IHC	Ohnishi *et al*., 2009
Gastric fluid	Increased level of alpha-defensin. Decreased level of pepsinogen C and pepsin A activation peptide.	Gastric cancer	SELDI-ToF/MS	Kon *et al*., 2008
Sputum	Increased level of enolase.	Lung cancer	1-DE, LC-MS/MS, WB, ELISA	Yu *et al*., 2014
Bile	Increased level of SSP411.	Cholangiocarcinoma	2D LC-MS/MS, WB, IHC	Shen *et al*., 2012
Ascites fluid	Identification of 52 candidate biomarkers (e.g., CD 59, CLU, PSAP and LRG).	Ovarian cancer	2D LC-LTQ/MS	Kuk *et al*., 2009

Biomarkers - AAT, α1-antitrypsin; ACT, α1-antichymotrypsin; AHS, α2-HS-glycoprotein; ALB, albumin; C3, complement C3; CD59, CD59 glycoprotein; CLU, clusterin; HAP β, haptoglobin β-chain; HPX, hemopexin; ITIH4f, fragment of inter-alpha-trypsin inhibitor heavy chain H4; LRG, leucine- rich α2-glycoprotein; PSAP, proactivator polypeptide precursor; RBP, retinol binding protein; SAP, serum amyloid P; TTR, transthyretin; VDB, vitamin D-binding protein; ZAG, zinc-alpha glycoprotein; Proteomics technologies - FTMS, fourier transform mass spectrometer; ICAT, isotope-coded affinity tag; IHC, immunohistochemistry; LTQ, linear ion trap; MudPIT, multidimensional protein identification technology; SRM, single reaction monitoring.

MATERIALS & METHODS

3.1. ARTOCARPUS FRUITS

The fruits of champedak used to obtain the lectins were bought from a local market as the structure and glycoprotein binding specificity of lectins isolated from seeds of several different cultivars of champedak had been previously demonstrated to be similar (Hashim *et al.*, 1993). The CGB lectin was extracted from the champedak seeds as described in Section 3.6.1.

3.2. HUMAN SERUM AND URINE SAMPLES

Collection of serum and urine samples was done at the Prostate-TRUS Biopsy clinic, University of Malaya Medical Center (UMMC), Kuala Lumpur. Samples were collected from newly diagnosed patients with stage I or stage II PCa, patients with BPH and age-matched control subjects. The designation of stages of PCa of the patients were based on AJCC staging manual (2002) stage groupings (Table 2.3) . Subjects with BPH represent those with inflammation and without carcinoma whilst, age-matched subjects with neither history of prostatic complaints nor treatments, served as controls in the study.

Approval from Medical Ethics Committee of UMMC, which operates according to the Declaration of Helsinki (Millum *et al.*, 2013) was obtained prior to conducting the study (Appendix II). All subjects were made to understand the purpose and risks of the study prior to obtaining their informed consent. Relevant clinical and demographic information were recorded.

Approximately 3 ml of blood was collected into plain BD vacutainers (Becton, Dickinson & Co, New Jersey, USA). The specimens were left to stand at room

temperature for 30 minutes prior to centrifugation (Centromix II – BL, Indiana, USA) at 3500 rpm for 20 minutes (Henry, 1979). Serum samples were then carefully separated and kept at -80°C for subsequent experiments.

Urine samples were collected in sterile plastic containers (Ningbo Sofine Electric Co. Ltd., Zhejiang, China) and immediately added with sodium azide to make a final concentration of 20 mM, as a means of preservation. The samples were centrifuged (Eppendorf, Hamburg, Germany) at 10,000 rpm for 15 minutes at 4°C. The resulting supernatant was dialysed against distilled water for 6 changes, at 4°C within 3 days using snakeskin pleated dialysis tubing (Thermo Fisher Scientific, Rockford, USA) for removal of salt and impurities (Oh *et al.*, 2004). Urinary proteins were finally aliquoted, freeze-dried (Labconco, Missouri, USA) and stored at –80°C until further use.

To ensure complete removal of undesirable substances as well as to enable concentration and purification of the urinary proteins, acetone precipitation was performed as previously described (Botelho *et al.*, 2010). Lyophilized urine was re-dissolved in 100 µl of 1× PBS, pH 7.2. Four times of the sample volume of cold (–20°C) acetone was added, vortexed and incubated overnight at –20°C. This was followed by centrifugation for 15 minutes at 13,000 × *g* at 4°C. Supernatant containing the interfering substances was carefully removed. Pellet was washed briefly with 100 µl of cold 90% acetone and centrifuged. The resulting precipitated urinary protein pellet was finally air-dried and re-dissolved in sample rehydration buffer (described in Section 3.10.1) that is compatible with downstream application.

3.3. GENERAL MATERIALS

All materials used in this study are listed together in accordance to their respective manufacturers.

3.3.1. Chemicals

i. *GE Healthcare Biosciences, Uppsala, Sweden*

Acrylamide (MW: 71.08 g/mol)

Amberlite® XAD4

Bromophenol blue (MW: 669.99 g/mol)

Coomassie Brilliant Blue (R250)

Dithiothreitol (DTT)

Glycerol 87% (MW: 92.09 g/mol)

Glycine (MW: 75.07 g/mol)

N,N'-methylene-bis-acrylamide (MW: 154.17 g/mol)

Urea (MW: 60.06 g/mol)

ii. *Sigma Aldrich Company, St. Louis, USA*

Acetone (MW: 58.08 g/mol)

Agarose

Ammonium persulfate (APS) (MW: 228.20 g/mol)

Ammonium sulphate (MW: 132.14 g/mol)

D-Galactose (MW: 180.16 g/mol)

Divinyl sulfone (DVS) (MW: 118.15 g/mol)

EDTA- disodium salt dihydrate (MW: 372.24)

Ethanolamine (16.9 M) (MW: 61.08 g/mol)

Formaldehyde 37%

Hydrochloric acid 37%

Iodoacetamide (IAA) (MW: 185.00 g/mol)

N,N,N',N'-Tetramethyl-ethylenediamine (TEMED) (MW: 116.21 g/mol)

Sodium acetate (MW: 82.0343 g/mol)

Sodium azide (MW: 65.01 g/mol)

Sodium hydroxide (MW: 40.00 g/mol)

Sodium thiosulphate (MW: 158.11 g/mol)

Sodium periodate (FW: 213.90 g/mol)

Trizma®Base (MW: 121.14 g/mol)

iii. *JT Baker®, Philadelphia, USA*

Acetic acid (MW: 60.05 g/mol)

iv. *Merck, Darmstadt, Germany*

Acetonitrile (ACN) (MW: 41.05 g/mol)

Boric acid (MW: 61.83 g/mol)

di-Sodium tetraborate (MW: 201.22 g/mol)

Formic acid 98-100%

Silver nitrate (MW: 169.88 g/mol)

Sodium acetate trihydrate (MW: 36.08 g/mol)

Sodium bicarbonate (MW: 84.01 g/mol)

Sodium carbonate (MW: 105.99 g/mol)

Sodium chloride (MW: 58.44 g/mol)

Trifluoroacetic acid (MW: 114.02 g/mol)

Tween® 20 (MW: 1228.00 g/mol)

v. *J. Kollin Chemicals, UK*

Ethanol (MW: 46.07 g/mol)

vi. *Fisher Scientific UK Limited, Leicestershire, UK*

di-Sodium hydrogen orthophosphate (MW: 177.99 g/mol)

Potassium chloride (MW: 74.54 g/mol)

Potassium dihydrogen phosphate (MW: 136.09 g/mol)

Sodium dodecyl sulphate (SDS) (MW: 288.38 g/mol)

3.3.2. Commercial kits and assays

i. *Siemens Medical Solutions Diagnostics, Tarrytown, USA*

Creatinine_2 (CREA_2) assay

Prostate specific antigen (PSA) assay

ii. *Thermo Fisher Scientific, Rockford, USA*

Pierce BCA protein assay kit

iii. *Nacalai Tesque Inc., Kyoto, Japan*

Peroxidase stain DAB kit

3.3.3. Materials for 2-DE

i. *GE Healthcare Biosciences, Uppsala, Sweden*

Drystrip cover fluid

IPG buffer pH 4-7

IPG Immobiline dry strips 11 cm (pH 4-7 and 3-10)

Pharmalyte™ 3-10 for IEF

3.3.4. Materials for SELDI-ToF/MS

i. *BioRad Laboratories Inc., Hercules, USA*

Energy absorbing matrix molecules - Sinapinic acid (SPA)

NP20 ProteinChip array

Standard peptide array

3.3.5. Materials for MS

i. *AB Sciex, Framingham, USA*

Mass Standards Kit for Calibration

ii. *Sigma Aldrich Company, St. Louis, USA*

Energy absorbing matrix molecules - α-cyano-4-hydroxycinnamic acid

3.3.6. Chromatographic media

i. *GE Healthcare Biosciences, Uppsala, Sweden*

Cyanogen bromide-activated Sepharose™ 4B

Sepharose 4B

3.3.7. Membrane

i. *Schleicher and Schuell, Dassel Germany*

Nitrocellulose membrane

3.3.8. Enzyme and substrates

i. *Sigma Aldrich Company, St. Louis, USA*

3,3'- Diaminobenzidine (DAB)

Peroxidase from horseradish (HRP)

ii. *Promega, Madison, USA*

Trypsin Gold, MS grade

3.3.9. Protein marker and ladder

i. *Thermo Fisher Scientific, Rockford, USA*

Spectra multicolour low range protein ladder

Spectra multicolour broad range protein ladder

3.3.10. Serological reagents

i. *Biorbyt Ltd. Cambridge, UK*

Goat anti-human ITIH4

ii. *Abcam, Cambridge, UK*

Rabbit anti-human bikunin

iii. *Meridian Life Science Inc., Santa Cruz, USA*

HRP-conjugated donkey anti-goat IgG

HRP-conjugated goat anti-rabbit IgG

3.4. STANDARD SOLUTIONS

10× Phosphate-buffered saline (PBS): 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2HPO_4, pH 7.2

NaCl	80.00 g
KCl	2.00 g
Na_2HPO_4	11.50 g
KH_2HPO_4	2.00 g

The pH solution was adjusted to 7.2, made up to 1 L with distilled water and stored at room temperature.

1× PBS, pH 7.2

PBS (100 ml of 10×), pH 7.2 was diluted with 900 ml of distilled water and stored at room temperature.

PBS-Tween 20 (PBS-T)

One ml of Tween 20 was diluted with 999 ml of 1× PBS and stored at room temperature.

1× Tris Buffered Saline (TBS): 100 mM Tris-HCl, 0.9% HCl

Trizma®Base	12.11 g
NaCl	9.0 g

Trizma®Base was dissolved in 900 ml distilled water and the pH of the solution was adjusted to 7.5 by the addition of concentrated hydrochloric acid (HCl). NaCl was then added to the solution, made up to 1 L with distilled water and stored at room temperature.

TBS-Tween 20 (TBS-T): 100 mM Tris-HCl, 0.9% HCl, 0.1% Tween 20

One ml of Tween 20 was diluted with 999 ml of 1× TBS and stored at room temperature.

3.5. ASSAYS

3.5.1. Biochemical assays

Quantitative measurements of serum creatinine and PSA in the subjects' serum samples were performed according to the manufacturer's instructions using ADVIA 2400 Chemistry and ADVIA Centaur Systems (Siemens Medical Solutions Diagnostics, Tarrytown, USA), respectively. Table 3.1 demonstrates the demographic and clinical characteristics of subjects involved in the study.

3.5.2. Bicinchoninic acid (BCA) protein assay

The BCA protein assay reduces Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Smith *et al.*, 1985). The purple-coloured reaction product of this assay was formed by chelation of two molecules of BCA with one cuprous ion and thus, exhibiting a strong absorbance at 570 nm.

The protein content in the samples was determined using Pierce BCA protein assay kit according to manufacturer's instructions. The absorbance readings of bovine serum albumin (BSA) standard, serum and lyophilised urine samples, CGB lectin and affinity chromatography enriched eluates were measured at 570 nm using Microplate Reader Model 680 (BioRad Laboratories, Hercules, USA). Dilution scheme as shown in Table 3.2 was adapted for construction of BSA standard calibration curve.

Table 3.1. Demographic and clinical characteristics of subjects.

Subgroups	Serum (First part of the study)		Urine (Second part of the study)		
	BPH	PCa	Control	BPH	PCa
N	8	8	15	20	13
Ethnicity (M:C:I)[a]	1:5:2	2:5:1	2:10:3	6:10:4	1:9:3
Age (years)[b]	65.3 ± 5.4	69.0 ± 3.6	64.8 ± 7.2	66.2 ± 5.6	69.6 ± 5.6
PSA (ng/mL)[b]	6.7 ± 1.7	29.6 ± 48.5	1.9 ± 1.0	6.7 ± 1.8	11.2 ± 7.7
Serum creatinine (µmol/L)[b,c]	NA	NA	91.9 ± 10.4	93.7 ±19.3	89.8 ± 20.6

a) M:C:I refers to Malay: Chinese: Indian.

b) Values expressed in mean ± SD.

c) Serum creatinine values of 60-110 µmol/L is considered normal in men.

NA, not applicable.

Table 3.2. Dilution scheme for standard microplate BCA assay.

Volume of diluents (µl)	Volume of BSA stock (µl)	Final BSA concentration (µg/ml)
100	0	Blank
97.5	2.5	50
95.0	5.0	100
92.5	7.5	150
90.0	10.0	200
87.5	12.5	250
85.0	15.0	300
82.5	17.5	350
80.0	20.0	400
77.5	22.5	450

3.6. PURIFICATION OF CGB LECTIN

3.6.1. Extraction of crude lectin from seeds of champedak

The extraction of crude lectin from champedak seeds was performed as previously described (Abdul Rahman *et al.*, 2002). Seeds of *Artocarpus integer* were washed and dried for 24 hours at 37°C. They were then ground to powder using a blender and suspended in of 10% (w/v) of the seeds with 1× PBS, pH 7.2. The suspension was left stirring (Global Lab, Seoul, Korea) overnight at 4°C and centrifuged (Thermo Scientific, Rockford, USA) at 8000 × *g* for 15 minutes at 4°C. The resulting supernatant was then isolated and precipitated with ammonium sulphate (Sigma Aldrich Company, St. Louis, USA) at 60% saturation (w/v) for 30 minutes at room temperature. The mixture was again centrifuged at 8000 × *g* for 15 minutes at 4°C. The resulting pellet containing crude extract of champedak lectin was re-dissolved in ice-cold 1× PBS, pH 7.2 prior to washing (dialysis) against 4 L of 1× PBS, pH 7.2 for 48 hours. The crude lectin extract was stored at –20°C.

3.6.2. Preparation of galactose Sepharose 4B column

Purification of lectins from crude extract was performed by utilising galactose affinity chromatography. Preparation of galactose Sepharose 4B column involved activation of Sepharose 4B with divinyl sulfone (DVS) (Jaramillo *et al.*, 2012) and coupling of D-galactose to the activated-Sepharose 4B gel matrix (Pohleven *et al.*, 2012).

Solutions and buffers

0.5 M Sodium carbonate

Sodium carbonate	10.6 g
Double distilled water	200 ml

The solution was stored at room temperature.

0.5 M sodium bicarbonate

Sodium bicarbonate	84.0 g
Double distilled water	2 L

The solution was stored at room temperature.

20% (w/v) of D-galactose in 0.5 M sodium carbonate

D-galactose	4.0 g

The above was dissolved in 20 ml of 0.5 M sodium carbonate.

3.6.2.1. Activation of Sepharose 4B with DVS

Activation of Sepharose 4B with DVS was performed as previously described (Hermanson *et al.*, 1992). DVS renders the chromatographic media, Sepharose 4B, active via its reactive vinyl groups and thus, enables the immobilisation of (galactose) sugar through hydroxyl linkages.

A hundred ml of Sepharose 4B was washed with distilled water in a sintered glass funnel, suction dried to a wet cake and transferred into a 500 ml beaker. The moist gel was re-suspended in 100 ml of 0.5 M sodium carbonate and stirred slowly. Activation of the gel was performed by slowly adding 10 ml of DVS to the suspension over a period of 15 minutes in fumehood, with constant stirring. The mix was continuously stirred for another hour. This is followed by extensive washing of the gel with distilled water until the filtrate was no longer acidic which was subsequently used for coupling with galactose.

3.6.2.2. Coupling of D-galactose to DVS-activated Sepharose 4B

Twenty ml of DVS-activated Sepharose 4B gel was suspended in an equal volume of 20% (w/v) of D-galactose in 0.5 M sodium carbonate. The mixture was stirred at room temperature for 24 hours before the gel was washed successively with 2 L of distilled water and 0.5 M sodium bicarbonate. The gel was later re-suspended in 0.5 M sodium bicarbonate containing 2 ml of β-mercaptoethanol by stirring for 2 hours in a fumehood to block excess vinyl reactive groups. The gel was then washed with 2 L of water followed by same volume of 1× PBS, pH 7.2. The resulting galactose-coupled Sepharose 4B gels were finally packed into polypropylene columns (2.8 cm in diameter) to a bed volume of 16 cm in height.

3.6.3. Purification of CGB lectin

Solution

0.8 M D-galactose

D-galactose	28.8 g

The above was dissolved in 200 ml of 1× PBS, pH 7.2.

Methodology

Purification of CGB lectin was performed as illustrated in Figure 3.1. Fifty ml of the crude lectin extracts was applied into a pre-equilibrated D-galactose-coupled DVS-activated Sepharose 4B immobilised affinity column. The initial flow-through was applied again into the column in order to maximise the retention of CGB lectin. The column was washed extensively with 1× PBS, pH 7.2. Unbound fractions of 50 ml were collected and the absorbance was constantly monitored at 280 nm using U-2000 Spectrophotometer (Hitachi, Tokyo, Japan) until a baseline reading was reached (Abs 280 nm < 0.005). This was followed by elution of bound CGB lectins using 0.8 M D-galactose in 1× PBS, pH 7.2. The absorbance monitoring was continued for the bound fractions as well. The bound fractions with readings of high absorbance were pooled, dialysed against 1× PBS, pH 7.2 and lyophilised for subsequent applications.

3.7. ASSESSMENT OF PURIFIED CGB LECTIN

3.7.1. Determination of concentration of CGB lectin

The concentration of the lyophilized CGB lectin was estimated using BCA protein assay as described in Section 3.5.2. Serially diluted stock concentration of BSA at 2 mg/ml served as standard for the BCA assay.

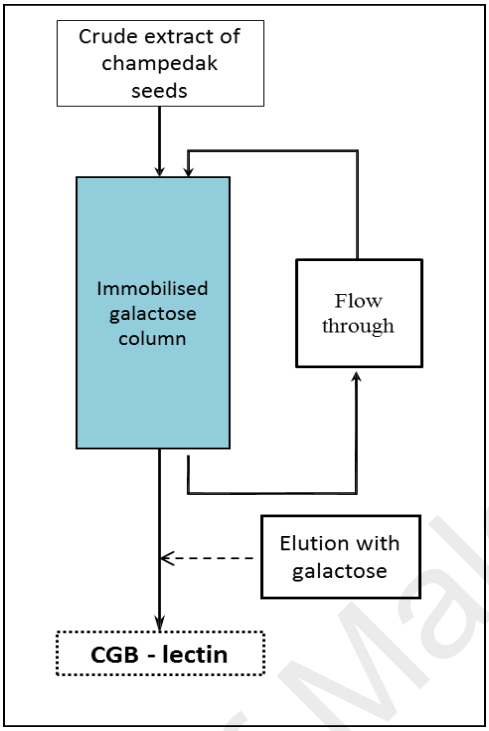


Figure 3.1. Purification workflow for CGB lectin.

3.7.2. Determination of purity of CGB lectin

The purity of the isolated CGB lectin was assessed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as well as SELDI-ToF/MS. The latter was used for profiling of CGB lectin with better accuracy.

3.7.2.1. SDS-PAGE

The isolated CGB lectin was electrophoresed on a 18% SDS-PAGE gel and stained using hot Coomassie Blue stain. The molecular weight of the electrophoresed CGB lectin was estimated by comparing to the spectra multicolour low range protein ladder.

Solutions and buffers

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

Acrylamide	60 g
N, N'-methylenebisacrylamide	1.8 g
Double distilled water	topped up till 200 ml

The solution was deionised using Amberlite® XAD4, filtered and stored in dark bottle at 4°C.

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

Trizma® Base	36.23 g
Double distilled water	150 ml

The pH of the solution was adjusted to 8.8 using hydrochloric acid (HCl), topped up till 200 ml and stored at 4°C.

Solution C: 10% (w/v) SDS

SDS	10 g
Double distilled water	100 ml

The solution was stored at room temperature.

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

APS	2 g
Double distilled water	20 ml

APS was kept in aliquots of 0.2 ml at –20°C.

Solution E: N, N, N', N'-tetrametylethylenediamine (TEMED)

Solution F: 0.5 M Tris-HCl, pH 6.8

Trizma® Base	6.1 g
Double distilled water	50 ml

The solution was adjusted to pH 6.8 using HCl, topped up till 100 ml and stored at 4°C.

4× 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

0.5 M Tris-HCl, pH 6.8	2.5 ml
Glycerol	2.0 ml
SDS	400 mg
DTT	200 mg
Bromophenol blue	few grains

All the above was mixed well and made up to 20 ml with double distilled water. Subsequently, 1 ml aliquots were prepared and stored at –20°C.

SDS Electrophoresis Buffer: 25mM Tris, 192mM glycine, 0.1% (w/v) SDS, pH 8.3

Trizma Base	3.03 g
Glycine	14.4 g
SDS	1.0 g

The solution was adjusted to pH 8.3 using HCl, topped up to 1 L and stored at 4°C.

Hot Coomassie Blue stain: 10% (v/v) acetic acid, 0.1% (w/v) Coomassie Brilliant Blue

The stain solution was kept at room temperature and heated up to 90°C for 5 minutes, prior to use. The stain solution was recycled for up to 3 times use.

Destaining solution: 10% (v/v) acetic acid solution

Methodology

i) Preparation of stacking and resolving gels

The 18% resolving gel mixture was prepared according to Table 3.3. The resolving gel mixture was carefully pipetted into the glass plate sandwich and subsequently, overlaid with distilled water to achieve an even gel surface. Once the gel was polymerized, the water overlay was removed and replaced with stacking gel mixture. A gel comb was inserted into the stacking gel layer and was left to polymerise.

Table 3.3. Resolving and stacking gels preparation for SDS-PAGE.

Solutions^	Resolving gel		Stacking gel
	10%	18%	
Solution A (ml)	6.66	12	0.65
Solution B (ml)	5	5	-
Solution C (µl)	200	200	50
Solution D* (µl)	100	100	25
Solution E* (µl)	6.6	6.6	5
Solution F (ml)	-	-	1.25
Mili Q water (ml)	8.03	2.69	3.05
Total volume (ml)	20	20	5.03

^ The volumes of solutions are sufficient for the preparation of 2 gels.

* Added prior to use only.

ii) SDS-PAGE

Samples were mixed at a ratio of 1:1 with 4× SDS-PAGE sample buffer and boiled at 80°C for 5 minutes. Approximately 7 µl of sample was loaded into each well alongside a protein ladder marker. The electrophoresis was performed using Mini-PROTEAN® Tetra Cell and PowerPac™ HC Power Supply (BioRad Laboratories, Hercules, USA). The run condition was initially set at 50 Volt (V) for 30 minutes and

later, switched to 90 V till the blue dye front was about 1 cm from the bottom of the gel. The gel was finally stained using Hot Coomassie Brilliant Blue stain.

iii) Hot Coomassie staining

Coomassie dye allows detection of more than 100 ng of proteins (Weiss *et al.*, 2009b). In acidic buffer conditions, Coomassie dye binds to basic and hydrophobic residues of proteins and thus, changing from dull reddish-brown to intense blue colour.

Following electrophoresis, the gels were rinsed with distilled water to remove residual SDS that may interfere with dye-binding. The Coomassie stain solution was heated up to 90°C and poured into a container containing the electrophoresed gel. The container was left to shake on a rocking platform for 30 minutes. The stain solution was removed from the gel and rinsed with 20 ml of destaining solution to remove excess unbound Coomassie dye from the gel matrix. Fresh destain solution was added to the container and a piece of KimWipe (Kimberley-Clark Professional, Roswell, USA) was placed on the top of the solution to accelerate the destaining procedure. The gel was left to shake on the rocking platform overnight before being rinsed in distilled water and scanned using Image Scanner III (GE Healthcare, Uppsala, Sweden).

3.7.2.2. SELDI-ToF/MS analysis

As illustrated in Figure 3.2, the SELDI-ToF/MS technique involves a biochemical properties-dependent, non-covalent binding of molecules of interest in complex biological samples to the surface of a ProteinChip array (Hutchens and Yip, 1993). The bound molecules on the array will then be analysed using the ProteinChip SELDI reader (PSC 4000) (Bio-Rad Laboratories, Hercules, USA).

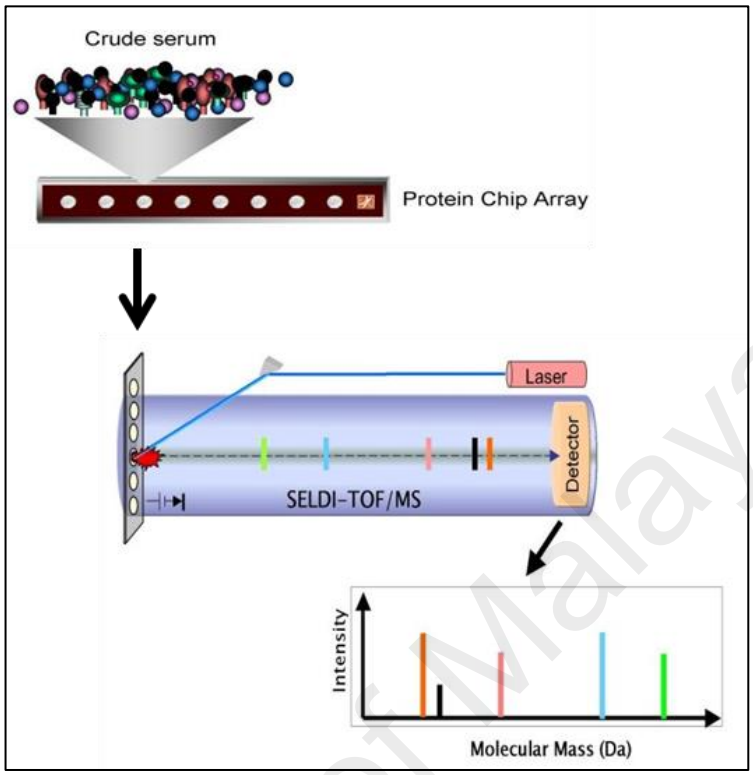


Figure 3.2. SELDI-ToF/MS analysis.

Solution for matrix

Energy absorbing matrix molecules – Sinapinic acid (SPA)

SPA in 50% acetonitrile (ACN) (v/v) 0.5% formic acid (FA).

Methodology

NP (normal phase) 20 ProteinChip array was used to analyse the purity of the eluted CGB lectin. The NP20 ProteinChip array has a general protein binding surface that allows proteins to bind through hydrophilic and charged residues, including serine, threonine and lysine, and as such, renders it suitable for purity assessment for purified CGB lectin.

One µl of the saturated energy absorbing SPA matrix solution was first added to a NP20 ProteinChip array spot and left to air-dry for 5 minutes at room temperature. Subsequently, 1 µl of 1 mg/ml CGB lectin at a dilution of 1:50 was added to the spot and similarly air-dried. This was followed by addition of 1 µl matrix onto the same arrays' spot and thus forming a sandwich preparation of matrix and CGB lectin. The spotted array was left to dry completely for 20 minutes at room temperature and subsequently analysed using ProteinChip SELDI reader (PSC 4000). Data collection was carried out in positive mode. The resulting spectrum was calibrated using standard peptides array consisting of arginine 8-vasopressin (1,084.25 Da), somatostatin (1,637.90 Da), dynorphin A (2,147.50 Da), adrenocorticotropic hormone (ACTH) (2,933.50 Da), beta-endorphin (3,465.00 Da) and arginine-insulin (5,960.80 Da).

3.8. HRP-CONJUGATED CGB LECTIN

The conjugation of HRP to CGB lectin was performed according to the previously established protocol (Hudson and Hay, 1980).

Standard buffers and solutions

0.1 M Sodium periodate

Sodium periodate	21.39 mg

Sodium periodate was dissolved in 1 ml of double distilled water. The solution was prepared fresh prior to use.

1 mM Sodium acetate buffer, pH 4.4

Sodium acetate	8.20 g/ L
Acetic acid	6.0 g/L

Sodium acetate solution was mixed with acetic acid solution at a ratio of 1:2. The solution was diluted with double distilled water to a final concentration of 1 mM.

0.1 M Sodium carbonate buffer, pH 9.5

Sodium carbonate	10.6 g/L
Sodium hydrogen carbonate	8.4 g/L

Sodium carbonate solution was added to the sodium hydrogen carbonate solution until pH 9.5 was obtained.

0.4% (w/v) Sodium borohydride

Sodium borohydride	4.0 mg

Sodium borohydride was dissolved in 1 ml of double distilled water.

0.1 M borate buffer, pH 7.4

di-Sodium tetraborate	9.54 g/ 250 ml
Boric acid	24.73 g/ 4 L

Approximately 115 ml of borate solution was added to 4 L of boric acid to solution until pH reaches 7.4.

Methodology

Four mg of HRP was dissolved in 1 ml of double distilled water. Next, 200 µl of 0.1 M sodium periodate solution was added to the HRP solution resulting in a green coloured solution. The solution was then stirred for 20 minutes at room temperature and dialyzed against 0.1 mM sodium acetate buffer, pH 4.4 at 4°C, overnight. The pH of the HRP dialysate was then raised to 9.5 by the addition of approximately 25 µl of 0.1 M sodium carbonate buffer, pH 9.5. One ml of 2 mg/ml CGB lectin was immediately added to the mix and left to stir for 2 hours at 4°C. Free enzyme of HRP, if present, was reduced by addition of 100 µl of 0.4% (w/v) sodium borohydride and left to stand at 4°C for another 2 hours. Finally, the HRP-conjugated CGB lectin was dialysed overnight against 0.1 M sodium borate buffer, pH 7.4 at 4°C. The conjugated products were diluted with equal volume of 60% glycerol in 0.1 M borate buffer, pH 7.4 and stored in aliquots at 4°C.

3.9. CGB LECTIN AFFINITY COLUMN CHROMATOGRAPHY

3.9.1. Preparation of CGB lectin affinity column

Solutions

0.1 M sodium bicarbonate, pH 8.5

Sodium bicarbonate	8.4 g

The solution was adjusted to pH 8.5 using HCl and topped up till 1 L with distilled water.

1.0 M sodium chloride

Sodium chloride	70.13 g

Sodium chloride was dissolved and topped up till 1.2 L with distilled water.

1.0 M ethanolamine, pH 9.0

Ethanolamine	11.8 ml

Ethanolamine was slowly mixed with distilled water to 200 ml and pH adjusted to 9.0 using HCl via the use of pH indicator strip (Sigma Aldrich Company, St. Louis, USA).

Methodology

Four g of cyanogen bromide-activated Sepharose 4B gel was rehydrated overnight in ice-cold double distilled water at 4°C by gentle stirring. About 12 ml of the rehydrated gel (settled volume) was washed with 2 L of ice-cold distilled water and 1 L of ice-cold 0.1 M sodium bicarbonate, pH 8.5, successively. The gel was suction dried into a moist cake using a sintered glass funnel and Buchner suction. This was followed by gentle stirring of the gel cake with 12 ml of CGB lectin at 4 mg/ml (with 4 mg/ml protein concentration) at 4°C for 20 hours. Following incubation, the mixture was promptly filtered. The initial filtrate was collected to assess the column preparation efficiency whilst, the (CGB lectin-coupled gel matrix) retentate was subsequently washed with 200 ml of 1.0 M sodium chloride and 200 ml of distilled water. The gel was suction-dried and left stirring in 100 ml of 1.0 M ethanolamine, pH 9.0 for an hour at room temperature in a fumehood. This was followed by an extensive washing with 1 L of 1.0 M sodium chloride and distilled water, respectively. Finally, 3 ml of gel bed was packed into disposable columns (Thermo Scientific, Rockford, USA) for subsequent isolation of *O*-glycosylated proteins from respective serum and urine samples.

To assess the non-specific interaction between biological samples and chromatographic matrix, a similar affinity column was prepared and affinity chromatography was subsequently performed using uncoupled activated and inactivated Sepharose 4B gel matrix.

3.9.2. Efficiency of CGB lectin immobilisation

The efficiency of CGB lectin affinity column that was prepared was determined based on the successful immobilisation of CGB lectin to CNBr-activated Sepharose 4B gel matrix. This was estimated using the following formulae.

$$\text{Percentage of bound lectin} = \frac{(\text{Concentration of lectin})_i - (\text{Concentration of lectin filtrate})_f}{(\text{Concentration of lectin})_i} \times 100\%$$

where, i = initial conditions and f = final conditions.

3.9.3. Isolation of *O*-glycosylated protein using CGB lectin affinity column chromatography

The immobile phase of the column was made-up of CNBr-activated Sepharose 4B gel. It was covalently linked to the CGB lectin thus, forming an intricate matrix that has high affinity towards *O*-glycosylated proteins. Samples (mobile phase) containing the target molecule were bound to the CGB lectin sites whilst, other molecules that do not possess the property or recognition for adherence were washed away. Finally, the entrapped *O*-glycosylated proteins was displaced and eluted out using sugar at high concentration. Figure 3.3 demonstrates the workflow involved in isolation of *O*-glycosylated protein using CGB lectin affinity column chromatography.

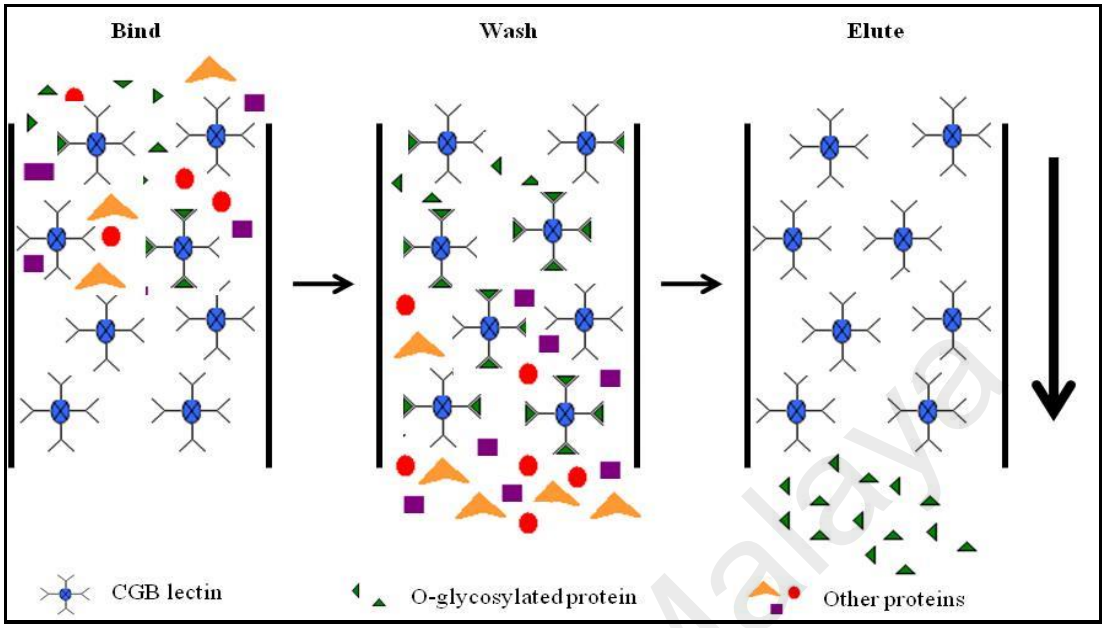


Figure 3.3. Affinity column chromatography.

Solution and buffer

0.1 M melibiose in 1× PBS, pH 7.2

Melibiose	3.42 g

Melibiose was dissolved in 100 mL of 1× PBS, pH 7.2 and stored at 4°C.

4 L of 0.5× PBS, pH 7.2

200 ml of 10× PBS, pH 7.2 was diluted to 4 L using distilled water.

Methodology

The CGB lectin columns were pre-equilibrated with 1× PBS, pH 7.2 with five times bed volume (~15 ml). Then, 200 µl of pooled neat serum and/or approximately 500 µg of dialysed pooled urine samples[1] (as described in Section 3.2) were applied separately into columns. The flow through was allowed to pass through the column

[1] This was intended for nano LC-MALDI ToF/ToF experiment.

twice to ensure maximal binding of *O*-glycosylated proteins to immobilised CGB lectin. The column was washed with 1× PBS, pH 7.2 and fractions of 5 ml were collected. The fractions were monitored for their absorbance at 280 nm using a quartz spectrophotometer cell (BioRad Laboratories, California, USA) and U-2000 Spectrophotometer (Hitachi, Tokyo, Japan). Elution of (bound) *O*-glycosylated protein enriched fractions was performed using 0.1 M melibiose in 1× PBS, pH 7.2. The fractions were similarly assessed spectrophotometrically for traces of bound proteins.

Bound protein fractions with high absorbance reading were pooled and dialyzed against 4 L of 0.5× PBS for 72 hours using dialysis tubing cellulose membrane (Sigma Aldrich Company, St. Louis, USA). The dialysate was finally desalted and concentrated using Vivaspin column concentrator (Sartorius Stedim Biotech, Göttingen, Germany) and kept at –20°C for subsequent experiments. Chromatographic separation profiles of each type and group of samples were constructed by plotting absorbance at 280 nm versus number of fractions (Figures 4.9 and 4.20).

3.10. 2-DE

2-DE involves isoelectric focusing (IEF) of protein complex mass in their native state according to pI and later, by second-dimensional electrophoresis (SDS-PAGE) which separates the proteins according to their molecular weight. In this study, 2-DE was performed according to the optimised condition as demonstrated in Table 3.4 for proteomic profiling of neat serum and acetone precipitated urine as well as enriched *O*-glycosylated proteins isolated using CGB lectin affinity column chromatography (see Section 3.9.3).

Table 3.4. Optimised conditions for 2-DE.

Sample	Optimised concentration (µg)	2DE					
		1st Dimension			2nd Dimension		
		Strip	Buffers	Run condition	SDS-PAGE gel	Buffers	Run condition
Neat serum	800	IPG Immobiline Drystrip pH 4-7, 11 cm	Sample buffer: 30 min Rehydration solution with IPG buffer pH 4-7: 30 min	**Phase I:** 300 V, 2 mA, 5 W, 30 min **Phase II:** 3500 V, 2 mA, 5 W, 15 kV/h	8–18% gradient	**Anode chamber:** Anode buffer **Cathode chamber:** SDS electrophoresis buffer*	**Phase I:** 50 V, 40 mA, 15 W/gel, 30 min **Phase II:** 600 V, 40 mA, 25 W/gel, 1-2 hour(s)
Serum *O*-glycosylated protein	60						
Acetone precipitated urine	100	IPG Immobiline Drystrip pH 3-10, 11 cm	Rehydration solution with Pharmalyte pH 3-10 for IEF: 2 hours	**Phase I:** 300 V, 2 mA, 5 W, 30 min **Phase II:** 3500 V, 2 mA, 5 W, 12 kV/h	12.5% homogenous	**Anode chamber:** SDS electrophoresis buffer **Cathode chamber:** SDS electrophoresis buffer*	**Phase I:** 50 V, 17 mA, 15 W/gel, 30 min **Phase II:** 600 V, 25 mA, 15 W/gel, 2-3 hours

* Prepared fresh prior to use.

3.10.1. First dimensional electrophoresis – IEF

Buffers

Sample buffer: 9 M urea, 60 mM DTT, 2% (v/v) IPG buffer pH 4-7, 0.5% (v/v) Triton X-100

Urea	13.5 g
DTT	250.0 mg
IPG buffer pH 4-7	0.5 ml
Triton X-100	0.13 ml

All the above was mixed and made up to 25 ml with double distilled water and kept in aliquots of 200 ml at –20°C.

Rehydration solution with IPG buffer pH 4-7: 8 M urea, 0.5% (v/v) IPG buffer pH 4-7, 0.5% (v/v) Triton X-100

Urea	12.0 g
IPG Buffer pH 4-7	0.13 ml
Triton X-100	0.13 ml
A few grains of Orange G.	

The solution was made up to 25 ml by addition of double distilled water and kept in one ml aliquots at –20°C. Prior to use, 12 mM DTT was added to the solution.

Rehydration solution with Pharmalyte pH 3-10: 8 M urea, 0.5% v/v Pharmalyte pH 3–10, 0.5% v/v NP-40

Urea	12.0 g
Pharmalyte pH 3-10	0.13 ml
NP-40	0.13 ml
Few grains of Orange G.	

The solution was made up to 25 ml by addition of double distilled water and kept in one ml aliquots at –20°C. Prior to use, 12 mM DTT was added to the solution.

3.10.1.1. Sample preparation and rehydration

Serum and urine samples were prepared according to Doustjalali *et al*., (2004) and Abdullah-Soheimi *et al*., (2010) with little modification for the first dimensional separation. The concentration of neat serum, acetone precipitated urine and *O*-glycosylated serum proteins were optimised to 800 µg, 100 µg and 60 µg, respectively to generate high resolution separation and reproducible protein profiles.

Serum proteins were mixed with appropriate volumes of sample buffer (three times volume of samples) and incubated at room temperature for 30 minutes. Subsequently, rehydration solution with IPG buffer pH 4-7 was added to the mixture to a final volume of 200 µl and again incubated at room temperature for 30 minutes. On the other hand, 200 µl of rehydration solution with Pharmalyte pH 3-10 was directly added to the air-dried acetone precipitated urine samples prior to incubation at room temperature for 2 hours. The sample mixtures were then loaded into the immobiline drystrip reswelling tray (GE Healthcare, Uppsala, Sweden) and appropriate drystrips (Table 3.4) was carefully lowered with the gel side down onto the sample mixture. Three ml of drystrip cover fluid was pipetted into sample slot to minimize evaporation of sample mixture and urea crystallization. The immobiline drystrips was left to stand for 18 hours at room temperature to ensure complete rehydration and uptake of sample prior subjecting it to IEF using Multiphor™ II Flatbed electrophoresis system and Electrophoresis Power Supply EPS-3501 XL (GE Healthcare, Uppsala, Sweden).

3.10.1.2. First dimensional run

The temperature of IEF apparatus was conditioned at 18°C using a cooling circulator (Grant Instrument Ltd., Cambridge, UK). Five ml of drystrip cover fluid was pipetted onto the cooling plate and the immobiline drystrip tray was positioned on the cooling plate (without bubbles) with the respective anode (red) and cathode (black) electrode leads on the tray connected to the Multiphor unit. About 10 ml of drystrip cover fluid was poured into the immobiline drystrip tray. An immobiline drystrip aligner with 12-groove-side-up was placed into the tray on top of the cover fluid. The rehydrated IPG strips were then transferred to the grooves of the immobiline drystrip aligner. Distilled water moistened electrode strips were placed across the cathodic and anodic ends of the aligned IPG immobiline strips whilst, the electrodes were aligned over the electrode strips. Finally, the IPG immobiline strips were covered with cover fluid to ensure good thermal contact. All apparatus used were from GE Healthcare Biosciences (Uppsala, Sweden).

IEF was performed according to the pH and length of IPG strips used, as recommended by the manufacturer (Table 3.4). Once completed, the focused strips were individually stored at –80°C in screw-cap tubes.

3.10.2. Second dimensional electrophoresis – SDS-PAGE

Solution and buffers

Solutions for SDS-PAGE such as solution A (monomer), solution B (4× resolving buffer), solution C (10% SDS) and solution D (10% APS) were prepared as described in Section 3.7.2.1. Additionally, the following was prepared:

SDS equilibration buffer: 50 mM Tris-HCl pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS

Tris-HCl, pH 8.8	25 ml
Urea	180.2 g
Glycerol	172.5 ml
SDS	10.0 g

The solution was made up to 500 ml using double distilled water and stored at room temperature. Prior to use, 1% (w/v) DTT and 4.5% (w/v) IAA are added consecutively to the buffer solution for the intended two-step equilibration.

Anode buffer: 0.37 M Tris-HCl, pH 8.8

Tris-HCl	136.2 g

The solution was made up to 3 L with distilled water, following pH adjustment of the buffer to 8.8 using HCl.

SDS electrophoresis buffer: 25 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS

Tris-HCl	3.03 g
Glycine	14.4 g
SDS	1.0 g

The solution was made up to 1 L with distilled water. Buffer was prepared fresh prior to use.

0.5% Agarose sealing solution

Agarose	0.25 g
SDS electrophoresis buffer	50 ml
Few grains of bromophenol blue.	

The mixture was swirled to disperse the agarose and heated in a microwave oven until the agarose was completely dissolved.

3.10.2.1. Preparation of SDS PAGE gel

In this study, 8-18% gradient gel was used for 2-DE proteomic profiling of serum proteins whilst, 12.5% homogenous gels for urinary proteins. The use of gradient gels renders wider overall separation and larger linear separation range, in addition to offering enhanced resolution due to the spot sharpening effect (minimised diffusion) as proteins migrate into pores of decreasing size (Molloy and McDowell, 2005). On the other hand, a homogenous gel offers better resolution for a particular M_r range (e.g., 12.5% acrylamide concentration for M_r of 14-100 kDa range of protein separation) (Heumann, 2003).

a) 8-18% gradient polyacrylamide gel

Glass plates (16 × 18 cm) were assembled as sandwich in the gel caster according to the manufacturer's instruction. A 1 mm thick gradient gel was prepared according to the solution composition (Table 3.5) consisting of a low (8%) and high (18%) percentages of gel solutions using a gradient makers (Model SG 30, Hoefer, USA). Gradient maker consists of 2 chambers joined by a narrow connector at their bases with an outlet at the base of the second chamber. Solutions were placed in the designated chambers of the gradient maker and constantly stirred before the valve was opened to allow mixing of both solutions. Draining from the outlet chamber (high percentage of gel solution) was progressively diluted with low percentage of gel solution from inlet chamber until the gradient marker emptied the outflowing material.

The gradient maker was connected to a peristaltic pump - EYELA (Tokyo Rikakikai Co., Tokyo, Japan) by tubing with a canula at one end to regulate a constant flow of 2.5 ml per minute of the mixed solution into the glass plate sandwich. Finally, the gel was immediately overlaid with distilled water to create a flat gel surface as well as to minimise exposure to oxygen, and allowed to polymerise overnight.

b) 12.5% homogenous polyacrylamide gel

Glass plates (16 × 18 cm) were similarly assembled as described above. A 1 mm thick homogenous gel consisting of uniform polyacrylamide concentration of 12.5% was prepared according to the solution composition as shown in Table 3.5. The solution mixture was filled into the glass plate sandwich to 3 to 10 mm below the top of the glass plates, overlaid with distilled water and allowed to polymerise overnight.

3.10.2.2. Equilibration of IPG immobiline drystrips

Equilibration of IPG immobiline drystrips was performed in two steps using SDS equilibration buffer (EB), prior to second-dimensional run. The first step involved equilibration of strips in EB with 1% (w/v) DTT. DTT preserves the fully reduced state of denatured, un-alkylated proteins in the sample. Fifty mg of DTT was dissolved in 5 ml of SDS-EB for each strip. Once dissolved, the solution was pipetted into a tube containing the IPG immobiline drystrip and shaken gently on a rocking platform for 15 minutes. The DTT containing EB was then discarded and replaced with second change of EB consisting of 4.5% (w/v) IAA instead, in 5 ml of EB (for each strip) with a few grains of bromophenol blue (tracker dye). IAA alkylates thiol group on proteins in the samples and thus, preventing its re-oxidation during electrophoresis. The second step of

equilibration with IAA was similarly carried out for 15 minutes, after which the solution was discarded. The IPG immobiline drystrip was immediately rinsed with freshly prepared SDS electrophoresis buffer, laid on the surface of appropriate gel (Table 3.5) and sealed with 0.5% agarose sealing solution.

Table 3.5. Gradient and homogenous gels preparation.

Solutions^	Gradient (8-18%)		Homogenous (12.5%)
	Light	Heavy	
Solution A	6.4 ml	14.4 ml	20.85 ml
Solution B	6 ml	6 ml	12.5 ml
Solution C	240 µl	240 µl	500 µl
Solution D*	40 µl	40 µl	15.9 µl
Solution E*	4 µl	4 µl	16.5 µl
Sucrose	-	3.6 g	250 µl
Mili Q water	11.36 ml	Topped up to 24 ml	~ 50 ml

^ The volume of solutions is sufficient for preparation of two (14 × 14 cm) gels.

* Added directly into the chambers containing high and low percentages of solutions.

3.10.2.3. Second dimensional run

The 2-D molecular weight-based electrophoresis was carried out using SE 600 Ruby Electrophoresis System (GE Healthcare, Uppsala, Sweden) linked to a cooling circulator (Grant Instrument Ltd., Cambridge, UK) and Power Supply-EPS601 (GE Healthcare, Uppsala, Sweden) at 18°C. The electrophoresis was performed in two phases using appropriate run condition and buffers as shown in Table 3.4. The electrophoresis was terminated once the tracker dye reaches the bottom of the gel. The

gels were carefully removed and subjected to either silver staining procedure or, electrotransferred onto a NC membrane. The upper corner nearest to the pointed end of the IPG immobiline strip was marked at each gel, to identify the acidic end of proteins.

3.11. SILVER STAINING OF 2-DE GELS

Silver staining is the most sensitive colorimetric method, allowing detection of protein at the range of ng (Weiss *et al.*, 2009b). The technique involves deposition of metallic silver onto the surface of a gel at the location of protein spots. Silver ions interact and bind with specific protein functional groups such as the carboxylic acid groups (Asp and Glu), imidazole (His), sulfhydryls (Cys), and amines (Lys). The silver ions are then reduced to metallic silver, resulting in brown-black colour following addition of developing solution.

In this study, silver staining of the 2-DE gels was performed as previously described (Yan *et al.*, 2000) with minor modification. The method was based on the method developed by Heukeshoven and Dernick (1988) but omitted the use of glutaraldehyde in the sensitisation step and formaldehyde in the silver impregnation step. This ensured compatibility for the ionization method for the subsequent MS. All solutions were prepared fresh prior to staining with particular care to avoid contamination by keratin and other extraneous proteins.

Solutions (The following solutions are sufficient for staining of 4 gels).

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

Ethanol	200 ml
Acetic acid	50 ml

The solution was made up to 500 ml with double distilled water.

Sensitising solution: 30% (v/v) ethanol, 0.5 M sodium acetate trihydrate, 12.7 mM sodium thiosulphate

Ethanol	150 ml
Sodium acetate trihydrate	34.0 g
Sodium thiosulphate	1.0 g

Solution was made up to 500 ml of double distilled water.

Silver solution: 14.7 mM silver nitrate

Silver nitrate	1.25 g

Solution was made up to 500 ml with double distilled water.

Developing solution: 0.24 M sodium carbonate, 0.04% (v/v) formaldehyde

Sodium carbonate	12.5 g

Solution was made up to 500 ml with double distilled water. Prior to use, 200 µl of formaldehyde was added.

Stopping solution: 40 mM EDTA-sodium dihydrate

EDTA-sodium dihydrate	14.6 g

Solution was made up to 500 ml with double distilled water.

Preserving solution: 10% (v/v) glycerol

Methodology

Following 2-DE, gel slabs were fixed in fixing solution for 30 minutes to remove interfering compounds such as SDS and also, to allow protein precipitation. The gels were then immersed in sensitising solution for 30 minutes and washed with double distilled water for 5 minutes. The wash step was repeated thrice. The sensitisation and rinse step increases sensitivity and contrast of the staining method. The silver impregnation step was carried out next by immersing the gel in silver solution for 20 minutes. The gels were then rinsed twice with double distilled water for 1 minute to remove excess silver solution and immersed in freshly prepared developing solution. The rinses and development served to build the silver metal image. Once the protein spots were developed, the gels were soaked in stopping solution for 30 minutes and rinsed twice with double distilled water. This terminated the development from excessive background formation and also, removes excess silver ion and other chemicals from the gel surface. Developed gels were stored in preserving solution at 4°C for MS analysis. The entire silver staining method was carried out on an orbital shaker (BioLab, UbiTechpark, Singapore) which was set at a constant speed of 50 rpm. Resulting silver-stained images of gels were promptly scanned and subsequently analysed using Image Master 2D-Platinum software (GE Healthcare, Uppsala, Sweden).

3.12. WESTERN BLOTTING

The Western blotting technique involves electrotransfer of proteins from the electrophoresed 2-DE gels or one-dimensional SDS PAGE gels onto a NC membrane (Kurien and Scofield, 2009) using a semidry transfer system, Novablot Kit for the Multiphor™ II Flatbed Electrophoresis System.

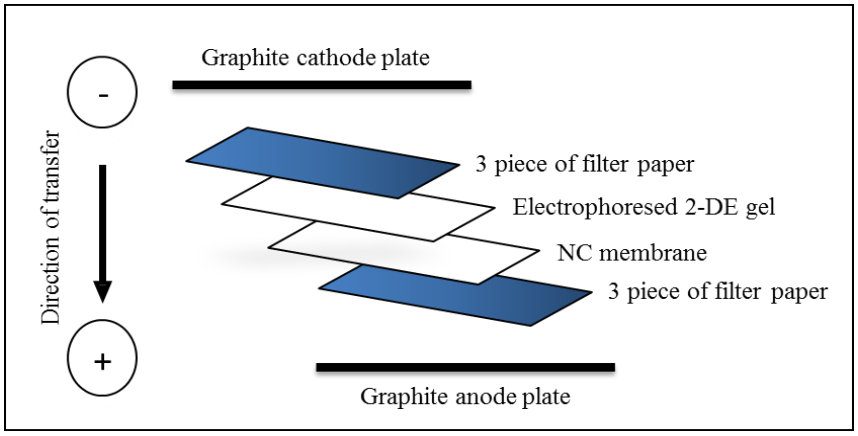


Figure 3.4. Western blotting sandwich-assembly.

Buffer

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	0.38 g
Methanol	200 ml

The above was dissolved, made up to 1 L with distilled water and stored at room temperature.

Methodology

Six pieces of filter paper (Schleicher and Schuell, Dassel, Germany) and a piece of NC membrane (0.45 mM; ADVANTEC, Toyo Roshi Kaisha, Tokyo, Japan) were carefully cut according to the size of the gel of interest. Transfer buffer pre-soaked filter papers, NC membrane and gel of interest were sandwich-arranged onto a transfer buffer-saturated graphite anode plate as illustrated in Figure 3.4. A roller was

used to carefully roll on the sandwich ensemble to remove trapped air bubbles. The transfer buffer-saturated graphite cathode plate was then placed on top of the sandwich. The electrotransfer was finally performed for 2 hours at a constant current 0.8 mA/cm^2.

3.13. DETECTION OF PROTEIN SPOTS ON NC BLOT

3.13.1. Detection of *O*-glycosylated proteins using HRP-conjugated CGB lectin

Buffer and solution

Blocking buffer: 3% (w/v) gelatine in TBS-T

Gelatine	3.0 g
TBS-T (see Section 3.4)	100 ml

The mixture was swirled to disperse gelatine and heated in a microwave oven until completely dissolved.

HRP colour development substrate solution

3,3'-diaminobenzidine (DAB)	75.0 mg
3% hydrogen peroxide	150 µl
1× TBS (see Section 3.4)	150 ml

The solution was made fresh prior to use.

Methodology

Detection of electrotransferred *O*-glycosylated serum and urinary proteins on NC membrane was performed using HRP-conjugated CGB lectin (see Section 3.8) as previously described (Abdul-Rahman *et al.*, 2007). Following Western blotting, NC membrane was blocked with TBS-T containing 3% gelatine for an hour on a shaker at

room temperature. The membrane was then washed thrice with TBS-T for 10 minutes each time. This was followed by an overnight incubation of the membrane with 1 μg/ml HRP-conjugated CGB lectin in TBS-T at 4°C. The washing step was again performed, 3 times for 10 minutes each, to remove unbound/excess conjugates, if present. The membranes were finally developed colorimetrically using freshly prepared DAB-substrate solution. The reaction was stopped by washing the membrane twice with distilled water. The membrane was briefly air dried before being scanned and subsequently analysed using Image Master 2D Platinum software.

3.13.2. Detection of proteins using HRP-conjugated secondary antibodies

Methodology

Immunodetection of targeted serum and urinary proteins on electrotransferred one-dimensional SDS-PAGE gels (Section 3.7.2.1) was performed as previously described by Jungblut *et al*., (1999) with slight modification. Following Western blotting, the membrane was blocked with 5% skim milk in 1× PBS-T for 1 hour at room temperature and later, incubated overnight with primary antibodies in 1× PBS-T containing 1% skim milk at 4°C. The membrane was then washed thrice with 1× PBS-T for 5 minutes and subjected to incubation with HRP-conjugated secondary antibodies in 1× PBS-T for 1 hour at room temperature. The membrane was again washed thrice with PBS-T for 5 minutes. The membranes were finally developed using Peroxidase stain DAB kit and the images captured for subsequent analysis using Image Master 2D Platinum software. Optimised concentrations of antibodies used for immunodetection are demonstrated in Table 3.6.

Table 3.6. Optimised concentrations of antibodies.

Sample	Primary antibodies	Dilution	Secondary antibodies	Dilution
Serum	Goat anti-human ITIH4	1:10,000	HRP-conjugated donkey anti-goat IgG	1:20,000
Urine	Goat anti-human ITIH4	1:10,000	HRP-conjugated donkey anti-goat IgG	1:10,000
	Rabbit anti-human bikunin	1:10,000	HRP-conjugated goat anti-rabbit IgG	1:10,000

3.14. IMAGE ANALYSIS

Levels of abundance of serum and urinary proteins and CGB lectin affinity chromatography enriched O-glycosylated proteins on silver stained 2-DE gels as well as CGB lectin-probed NC blots were analysed in terms of percentage of volume contribution using Image Master 2D Platinum software. The percentage volume contribution refers to volume percentage of a protein taken against the total spot volume of all proteins including the un-resolved peptides in each gels and as such, normalised the protein spots.

The detection of spots was performed automatically using default parameters including, smooth – 2, saliency – 1 and min area – 5; whilst, spot editing and removal of artefacts were performed manually. Analysis was restricted to distinctly resolved proteins spots only. 'Common housekeeping' proteins and/or fragments such as albumin (ALB) and immunoglobulins that constitute approximately 60 - 97% of the

total serum protein (Ahmed *et al.*, 2003; Chen *et al.*, 2005) were excluded from analysis.

3.15. MASS SPECTROMETRY

3.15.1. In-gel trypsin digestion

Solutions (All solutions were prepared fresh prior to digestion with particular care to avoid contamination by keratin and other extraneous protein).

50 mM Sodium thiosulphate

Sodium thiosulphate	0.158 g

The above was dissolved in 20 ml of double distilled water.

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

Potassium ferricyanide	0.049 g

The above was dissolved in 10 ml of 50 mM sodium thiosulphate.

100 mM Ammonium bicarbonate

Ammonium bicarbonate	0.396 g

The above was dissolved in 50 ml of double distilled water.

Reducing solution: 10 mM DTT in 100mM ammonium bicarbonate

DTT	0.0154 g

The above was dissolved in 10 ml of 100 mM ammonium bicarbonate.

Alkylating solution: 55 mM IAA in 100mM ammonium bicarbonate

IAA	0.1018 g

The above was dissolved in 10 ml of 100 mM ammonium bicarbonate.

Washing solution: 50% ACN in 100 mM ammonium bicarbonate

Equal volume of 100% ACN was mixed with 100 mM ammonium bicarbonate.

50 mM ammonium bicarbonate

Equal volume of 100 mM ammonium bicarbonate was mixed with double distilled water.

Enzyme: 6 ng/µl trypsin in 50 mM ammonium bicarbonate

Working solution, 6 ng/µl trypsin was prepared using 50 mM ammonium bicarbonate.

Sample reconstituting buffer - 0.1% formic acid (FA)

One µl of FA was dissolved in 1 ml of double distilled water.

Energy absorbing matrix molecules: α-cyano-4-hydroxycinnamic acid (CHCA)

CHCA	6 mg

CHCA was dissolved in 70% ACN (v/v) containing 0.1% FA.

Methodology

Protein spots of interest were carefully excised from 2-DE gels and subjected to in-gel trypsin digestion and MS/MS using 4800 Plus MALDI ToF/ToF analyser (Applied Biosystems, Foster City, USA). Optimisation for mass assignment, calibration, resolution and sensitivity of the analyser was performed using 4700 Proteomic Analyzer Mass Standard Kit according to the manufacturer's instructions.

The in-gel trypsin digestion was performed as previously described (Seriramalu *et al.*, 2010). The excised gel plugs were destained using 15 mM potassium ferricyanide in 50 mM sodium thiosulphate for 15 minutes at room temperature. Following this, the gel plugs were first reduced using 10 mM DTT in 100 mM ammonium bicarbonate for 30 minutes at 60°C and later, alkylated using 55 mM IAA in 100 mM ammonium bicarbonate for 20 minutes in the dark. The gel plugs was subsequently washed three times with 50% ACN in 100 mM ammonium bicarbonate and dehydrated using 100% ACN. The gel plugs were then digested using 6 ng/µl trypsin gold MS grade in 50 mM ammonium bicarbonate at 37°C, overnight. The extraction of proteins was performed using 50% ACN and 100% ACN subsequently, yielding pooled trypsin-digested peptides in ACN. Finally, the peptides were lyophilized and reconstituted with 0.1% formic acid for subsequent desalting procedure.

The desalting procedure was carried out using C_{18} reversed phase media ZipTip column (Millipore, Billerica, USA) according to manufacturer's instructions. The desalted peptides and CHCA matrix solution (6 mg/ml) were mixed at 1:1 ratio prior to spotting (0.7 µl) onto an Opti-Tof 384 well insert (Applied Biosystems, Foster City, USA).

3.15.2. Identification of proteins via database search

Identification of proteins was performed using MASCOT search engine (Perkins *et al.*, 1999). The MS and MS/MS spectra obtained was searched against *Homo sapiens* entries in Swiss-Prot database (Last update: January 31, 2013, containing 539,616 sequences) according to the following selection parameters: enzyme - trypsin, missed cleavage - 1, variable modification - 2; carbamidomethylation of cysteine and 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. A Mascot protein score greater than 42 indicates identity and extensive homology ($p < 0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

3.16. NANO LC-MS/MS

The identity of CGB lectin probed *O*-glycosylated urinary proteins on NC membranes was initially determined by visual comparison to the previously published work of Soheimi *et al*., (2010) and Mu *et al*., (2012). Later, this was validated by subjecting CGB lectin affinity chromatography enriched *O*-glycosylated urinary proteins (see Section 3.9.3) to in-solution trypsin digestion prior to reverse phase (C_{18}) chromatographic separation using an offline Tempo™ LC MALDI (Applied Biosystems, Foster City, USA) and profiled using 4800 Plus MALDI ToF/ToF analyser.

Solutions and buffers (All solutions were prepared fresh prior to digestion with particular care to avoid contamination by keratin and other extraneous protein).

50 mM Ammonium bicarbonate

Ammonium bicarbonate	10 mg

The above was dissolved in 2.5 ml of double distilled water.

Reducing solution: 100 mM DTT in 50mM ammonium bicarbonate

DTT	8.0 mg

The above was dissolved in 500 µl of 100 mM ammonium bicarbonate.

Alkylating solution: 100 mM IAA in 50mM ammonium bicarbonate

IAA	9.0 mg

The above was dissolved in 500 µl of 100 mM ammonium bicarbonate.

Enzyme: 0.1 µg/µl of trypsin in 50 mM ammonium bicarbonate

Trypsin at 0.1 µg/µl trypsin was prepared using 50 mM ammonium bicarbonate.

Energy absorbing matrix molecules: α-cyano-4-hydroxycinnamic acid (CHCA)

CHCA	6.0 mg

CHCA was dissolved in 70% ACN (v/v) in 0.1% FA.

Methodology

The concentrated *O*-glycosylated urinary proteins were subjected to in-solution trypsin digestion. The concentrate, at 100 µg (approximately 10 µl), was first reduced in 1.5 µl of 100 mM DTT in 50 mM ammonium bicarbonate for 5 minutes at 95°C. The mixture was alkylated next, using 3 µl of 100 mM IAA in 50 mM

ammonium bicarbonate for 20 minutes in the dark. Trypsin digestion was carried out using 2 µl of 0.1 µg/µl of trypsin in 50 mM ammonium bicarbonate overnight at 37°C. The resulting trypsin digested peptide mixture (10 µl) was then loaded into a Chromolith CapRod reverse phase - 18 monolithic capillary, 150 µm × 50 mm (Merck, Darmstadt, Germany) for separation using linear gradient of two mobile phases for a total run time of 57 minutes (Table 3.7) on an offline Tempo™ LC MALDI. The peptide eluates were automatically pre-mixed with equal volumes of 6 mg/ml CHCA in 70% ACN in 0.1% TFA and directly deposited onto an Opti-ToF LC-MALDI insert (Applied Biosystems, Foster City, USA). The droplets were then air-dried and analysed in reflector detector mode on a 4800 Plus MALDI ToF/ToF analyser. The resulting mass spectra were processed and interpreted using Protein Pilot™ Software 2.0.1 (Applied Biosystems, Foster City, USA) with selection parameters as previously denoted (see Section 3.15.2).

Neural network prediction of mucin type GalNAc *O*-glycosylation sites in the CGB lectin affinity chromatography enriched urinary proteins were determined using the NetOGlyc 4.0 server (Steentoft *et al.*, 2013).

Table 3.7. Linear gradient flow applied for LC.

Time (min)	% Channel A	% Channel B
0	98	2
2	98	2
42	65	35
45	50	50
46	30	70
51	30	70
53	98	2
57	98	2

3.17. STATISTICAL ANALYSES

All values are expressed in mean ± SEM (standard error of the mean), unless otherwise stated. The Statistical Package for Social Sciences (SPSS) version 21.0 (IBM Corporation, New York, USA) was used to analyse the data. The Student's t-test and ANOVA were used (where applicable) to compare means of percentage of volume contribution of the protein spots between groups of subjects for data sets that had shown a normal distribution. Test of homogeneity was used to assess the sample distribution. A *p* value of less than 0.05 was considered significant, unless otherwise stated.

RESULTS

4.1. ISOLATION, PURIFICATION AND ASSESSMENT OF CGB LECTIN FROM CHAMPEDAK SEEDS

4.1.1. Isolation and purification of CGB lectin

Extraction of crude lectins from champedak seeds was performed as described in Section 3.6.1. The crude lectin extract was applied into a galactose-Sepharose 4B affinity column for the isolation and purification of CGB lectin as described in Section 3.6.3. The column was thoroughly washed with PBS, pH 7.2 to remove unbound substances. Absorbance of the flow-through fractions were spectrophotometrically monitored at 280 nm until a baseline reading was obtained. The bound CGB lectin was eluted using 0.8 M galactose in PBS, pH 7.2. Figure 4.1 illustrates an elution profile of the CGB lectin using galactose-Sepharose 4B affinity column.

4.1.2. Assessment of purified CGB lectin

Elute fractions of CGB lectin with high absorbance reading collected from the initial experiment (Figure 4.1) were pooled and freeze-dried prior to biochemical assessment which was carried out to determine the quality of the purified CGB lectin as described in Sections 3.7.1 and 3.7.2. The assessment includes determination of concentration and purity of the CGB lectin prepared.

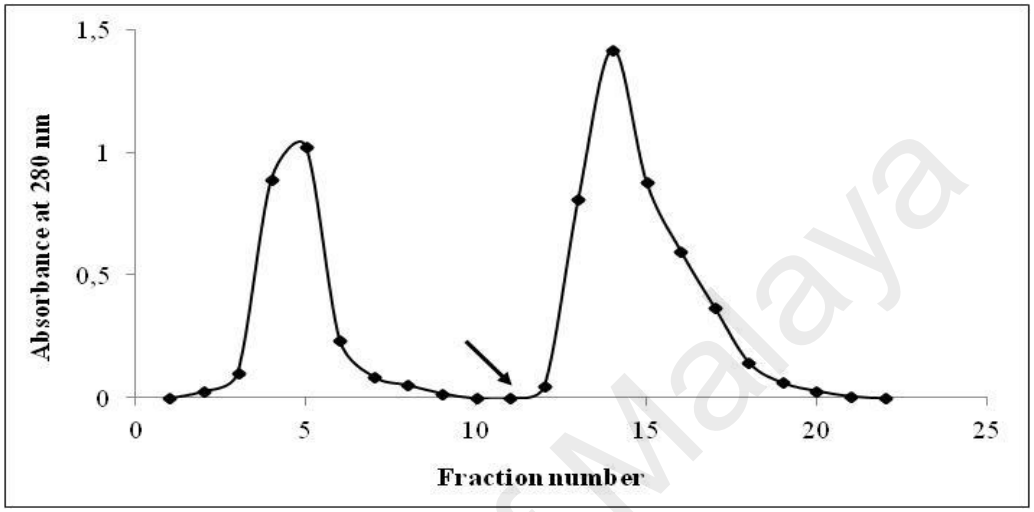


Figure 4.1. Purification of CGB lectin from crude extracts of champedak seeds.

Illustrated is a purification profile of CGB lectin obtained when crude extracts of champedak seeds were subjected to an immobilised galactose-Sepharose 4B affinity column chromatography. The fractions were collected in 5 ml fractions and were monitored spectrophotometrically at absorbance 280 nm. The unbound fractions were washed thoroughly using PBS, pH 7.2 until the baseline value was reached. Elution of the CGB lectin was performed to completion using 0.8 M galactose in PBS, pH 7.2. Arrow indicates the start of galactose elution.

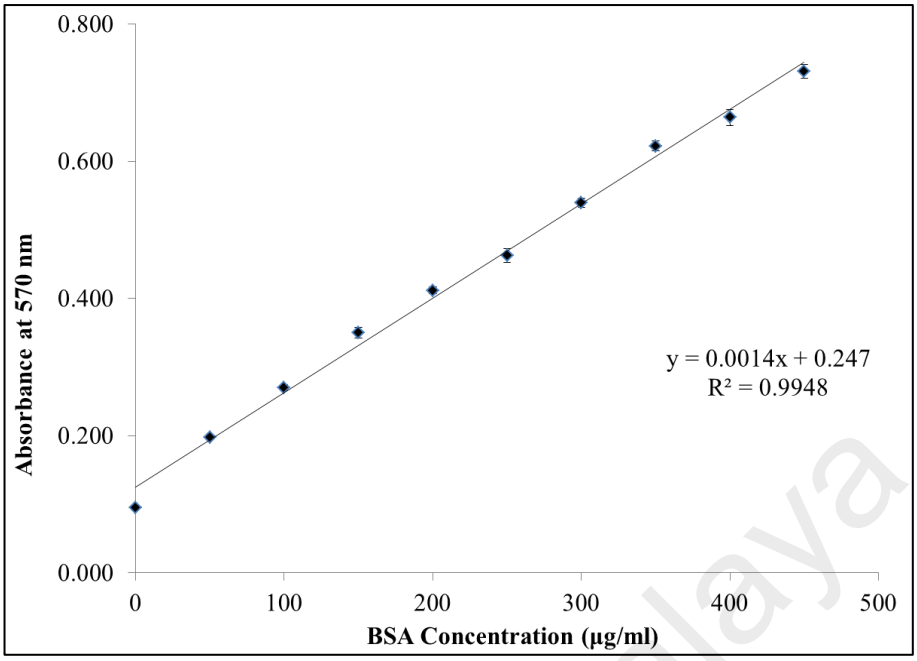
4.1.2.1. Concentration of CGB lectin

The concentration of the lyophilized CGB lectin was estimated using bicinchoninic protein assay (Thermoscientific, Rockford, USA). A standard calibration curve of absorbance values over ten concentrations (ranging from 0 – 450 µg/ml) of bovine serum albumin (BSA) was constructed. Figure 4.2 illustrates a standard calibration curve constructed following methodology as described in Section 3.5.2. Both standard solutions and purified CGB lectin were assayed in triplicates. According to the equation derived from the constructed BSA calibration curve, the lyophilized and purified CGB lectin measured 3.5 mg/ml in concentration. The concentrations of sera and urine samples were similarly estimated for 2-DE and affinity column chromatography applications.

4.1.2.2. Purity of CGB lectin

The purity of the isolated CGB lectin was assessed using SELDI-ToF/MS as described in Section 3.7.2.2. The spectrum for CGB lectin was generated following a mass calibration using the standard peptide array (BioRad, Hercules, USA). Figure 4.3 illustrates the mass calibration spectra of standard proteins and its calibration equation. Peptides of known mass including arginine 8-vasopressin, somatostatin, dynorphin A, ACTH, beta-endorphin and arginine-insulin (M_r ranging between 1.084 to 5,960 Da) were used for calibration. The calibration equation was generated by assigning these known masses to the corresponding peaks observed in the spectrum. As desired, in the present study, a calibration equation with residual errors of less than 300 parts per million (ppm) was observed. This indicates a better fit of the equation to the calibration and samples' data.

A)



B)

BSA Standard	Absorbance			
(ug/ml)	Reading 1	Reading 2	Reading 3	Mean ± S.E.M
0 (Blank)	0.095	0.094	0.097	0.095 ± 0.001
50	0.199	0.199	0.196	0.198 ± 0.001
100	0.273	0.276	0.262	0.270 ± 0.004
150	0.364	0.351	0.336	0.350 ± 0.008
200	0.411	0.422	0.403	0.412 ± 0.006
250	0.470	0.476	0.443	0.463 ± 0.010
300	.533	0.533	0.553	0.540 ± 0.007
350	0.611	0.620	0.636	0.622 ± 0.007
400	0.666	0.683	0.643	0.664 ± 0.012
450	0.745	0.745	0.712	0.731 ± 0.010

Figure 4.2. Typical BSA colour response standard curve.

Panel A illustrates a standard curve for BSA, constructed according to the manufacturer's instruction. A dilution scheme, ranging from 50 to 450 µg/ml was opted for dilution of BSA stock concentration (2 mg/ml) with PBS, pH 7.2. The absorbance of the complex formed was read at 570 nm and reported in mean ± S.E.M. The CGB lectin concentration was determined based on the equation generated for the colour response standard curve.

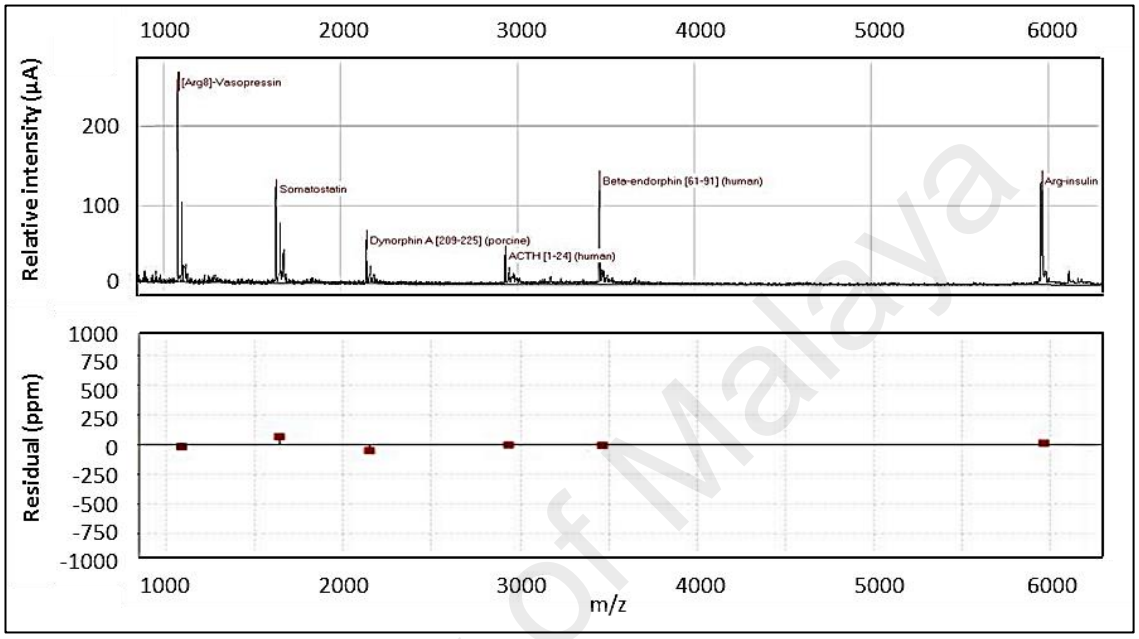


Figure 4.3. Mass calibration spectra derived from SELDI-ToF/MS analysis.

Mass calibration spectrum which was generated using ProteinChip peptide mass calibration kit (BioRad, Hercules, USA) is as illustrated above. Calibration was performed using standard peptides of known masses including arginine 8-vasopressin (1,084.25 Da), somatostatin (1,637.90 Da), dynorphin A (2,147.50 Da), ACTH (2,933.50 Da), beta-endorphin (3,465.00 Da) and arginine-insulin (5,960.80 Da). The calibration equation was generated by assigning these known masses to their corresponding peaks in the spectrum.

Figure 4.4 (panel A) demonstrates SELDI-ToF/MS derived spectral profile of CGB lectin. Two peaks at approximately 14.6 kDa and 16 kDa highly corresponds to the previously published reports using SDS-PAGE technique (Abdul Rahman *et al.*, 2002; Hashim *et al.*, 1993; Hashim *et al.*, 1991). Also observed, are doubly charged ions of the CGB lectin at an approximate mass of 7.3 kDa and 8 kDa, respectively. Aside from this, no other peak was observed within the spectra and thus, indicating a pure isolate of CGB lectin. The gel view of SDS-PAGE resolved CGB lectin isolates similarly showed a profile consisting of two bands with similar relative molecular weights (Figure 4.4 - panel B). The lower M_r bands (< 10 kDa) that correspond to the peaks of doubly charged ions of CGB lectin were not visible due to technical limitations.

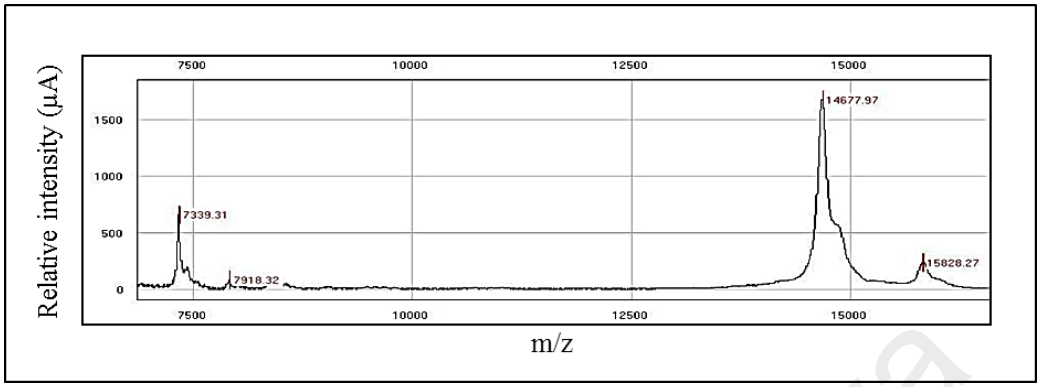
The purified CGB lectin was subsequently used as probes for detection of human serum and urinary *O*-glycosylated proteins using techniques including affinity column chromatography and Western blotting.

4.2. COMPREHENSIVE PROTEOMIC PROFILING OF HUMAN SERUM

All subjects recruited for the study were selected based on the pre-requisite biochemical and clinical characteristics including levels of PSA and serum creatinine, aside from being age-matched groups of subjects. The demographic and clinical characteristics of the subjects from whom serum and urine samples were obtained for the study are summarized in Table 3.1.

Subjects involved comprised Malaysians of Malay, Chinese and Indian ethnicity. Recruitment of subjects belonging to the Chinese ethnicity was apparently most predominant in all groups, particularly the PCa sub-group. Interestingly, this observation is in coherence with incidences of PCa in Malaysia marking Chinese at

A)



B)

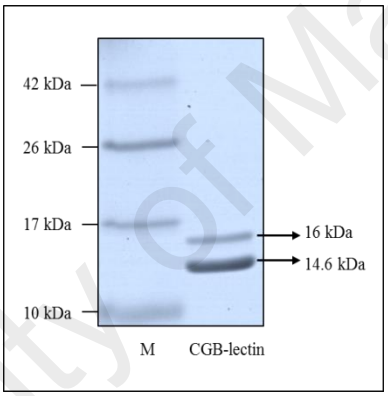


Figure 4.4. Affinity purified CGB lectin.

Panel A illustrates a spectrum of purified CGB lectin when subjected to SELDI-ToF/MS analysis. The NP20 array was spotted with 1 µl of CGB lectin at an optimised concentration of 1 mg/ml. Peaks at approximately 14.6 kDa and 16 kDa denote the singly charged ions of the purified CGB lectin, whilst peaks at approximately 7.3 kDa and 8 kDa denote the doubly charged ions corresponding to its singly charged CGB lectin peaks. Panel B illustrates the corresponding SDS-PAGE analysis of the affinity purified CGB lectin that resolved into two bands at approximately 14.6 kDa and 16 kDa, when subjected to the 18% SDS-PAGE under reducing condition and stained with Coomasie Blue. The SDS-PAGE gel was calibrated using the spectra multicolor low range protein ladder (M) (Thermo Scientific, Rockford, USA) consisting of 42 kDa, 26 kDa, 17 kDa and 10 kDa recombinant proteins.

13.6, followed by Indian at 7.8 and last but not least, the Malays at 4.0, per 100,000 population (Benbrahim-Tallaa and Waalkes, 2008).

Quantitative measurement of PSA performed in serum of subjects with PCa and those with BPH demonstrated an overlapping distribution, further in agreement with the lack of sensitivity of PSA. On the other hand, control subjects were carefully selected to meet the stated guidelines for levels of serum PSA which is less or equals to 4 ng/ml (Thompson *et al.*, 2004). Those subjects demonstrating levels of serum PSA more than the above-mentioned ranges were excluded from the study.

Serum creatinine, a diagnostic marker reflective of renal function was also taken into consideration while recruiting subjects and sampling of urine. Under normal circumstances, kidney freely filters creatinine, a waste by-product of muscle metabolism at a constant rate together with fluids, salt and other waste or toxin (Kaplan and Pesce, 2009). However in cases of kidney-related disorders, the creatinine-filtering ability of the kidney would cease and thus, results in increased levels of creatinine in the sera. Having this in mind, the levels of serum creatinine for all subjects recruited in the latter part of the study were duly measured and only those with levels of serum creatinine within the specified reference range of 60 - 110 µmol/L (Howard, 1989)**,** which is indicative of a normally-functioning kidney, were selected for the study.

4.2.1. Typical 2-DE serum protein profiles of patients with BPH and those with PCa generated by 2-DE

The 2-DE separation enables the serum proteins to be resolved according to their isoelectric focusing points (pI) and molecular masses (M_r) for their maximal appraisal in both presence and distribution in the gel profiles. Profiling of human neat serum samples by 2-DE optimised at a concentration of 800 µg followed by silver

staining of the gels had resulted in the separation of more than hundreds of protein spots. Although a comparable profile to the standard serum proteome map (Sanchez *et al.*, 1995) was generated, the identities of clusters of protein spots, particularly those that were altered in abundance, were further confirmed by MS and database search (Table 4.1).

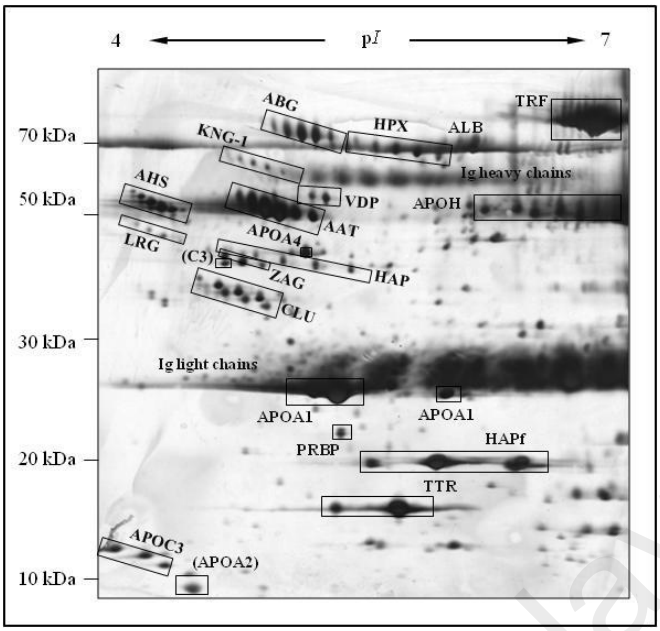
Figure 4.5 demonstrates representative 2-DE profiles generated from neat sera of patients with BPH (panel A) and PCa (panel B), respectively. Proteins that were well resolved in the gels include ALB, the heavy and light chains of immunoglobulins such as IgA, IgG and IgM, AHS, α_1-β glycoprotein (ABG), AAT, HAP, kininogen (KNG-1) serotransferrin (TRF), HPX, vitamin-D binding protein (VDB), apolipoprotein-H (APOH), apolipoprotein A4 (APOA4), LRG, ZAG, RBP 4, apolipoprotein A1 (APOA1), TTR, apolipoprotein C3 (APOC3), complement C3 (C3) and apolipoprotein A2 (APOA2).

4.2.2. Image analyses of 2-DE generated serum protein profiles of patients with BPH and those with PCa

Variability in the fractionation of light and heavy chains of immunoglobulins renders inconsistency to the patterns of 2-DE gel profiles (Doustjalali *et al.*, 2004). Therefore, in this study, these proteins were discounted from comparative image analyses. Similarly albumin, a protein of high abundance in the serum, was also excluded from the analyses.

The silver stained 2-DE profiles of serum samples of patients with BPH (n = 8) as well as those with PCa (n = 8) were scanned using a densitometer. The images were analysed using Image Master 2D Platinum software (Version 7.0) and subjected to statistical analyses. The percentage of volume contribution analysis was performed on a

A)



B)

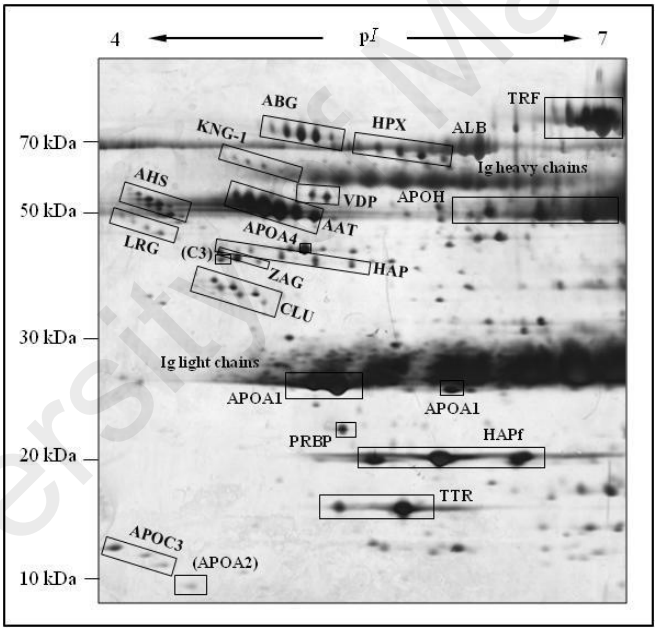


Figure 4.5. Typical silver stained 2-DE protein profiles of sera from patients with BPH and PCa.

Neat serum samples obtained from patients with BPH and those with PCa were subjected to 2-DE and silver staining. Panel A demonstrates a silver stained 2-DE protein profile of serum from a patient with BPH whilst panel B, a patient with PCa. Clusters of protein spots that were clearly resolved were as labelled. The proteins of altered abundance are marked in brackets. For all panels, the acidic sides of the 2-DE gels are to the left and relative M_r declines from the top. 'f' denotes protein fragments as indicated by its position in the 2-DE gels compared to the M_r of the parent protein.

total of 20 well-resolved clusters of protein spots. These include AAT, ABG, AHS, APOA1, APOA2, APOA4, APOC3, APOH, C3, CLU, HAP, HAPf, HPX, KNG-1, LRG, RBP, TRF, TTR, VDB and ZAG. Student t-test was used to analyse the significance of differences of protein expression between patients with BPH and those with PCa. A *p* value of less than 0.05 was considered significant.

Volume analysis of the 20 clusters of protein spots in the 2-DE profiles of patients with BPH and those with PCa demonstrated altered abundance of two serum proteins namely, APOA2 and C3 (Figure 4.6). Significantly decreased level of protein APOA2 (–0.4 fold, $p = 0.009$) and C3 (–0.35 fold, $p = 0.002$) was detected in the profiles of patients with PCa.

4.2.3. Typical HRP-conjugated CGB lectin generated 2-DE serum *O*-glycosylated protein profiles of patients with BPH and those with PCa

HRP-conjugated CGB lectin that was prepared as described in Section 3.8 was utilised to detect serum glycoproteins that were similarly resolved by 2-DE. 2-DE-resolved serum proteins from patients with BPH and those with PCa were electrophoretically transferred onto a NC membrane by Western blotting (see Section 3.12) and incubated with HRP-conjugated CGB lectin solution. The use of CGB lectin specifically facilitates detection of *O*-linked oligosaccharides of glycoproteins, which are linked through hydroxyl linkages to their serine or threonine residues. This therefore, excludes detection of non- and other than *O*-glycosylated proteins such as albumin, ACT (Seriramalu *et al.*, 2010) and light chains of immunoglobulin that inadvertently masked the detection of serum proteins with relatively lower abundance.

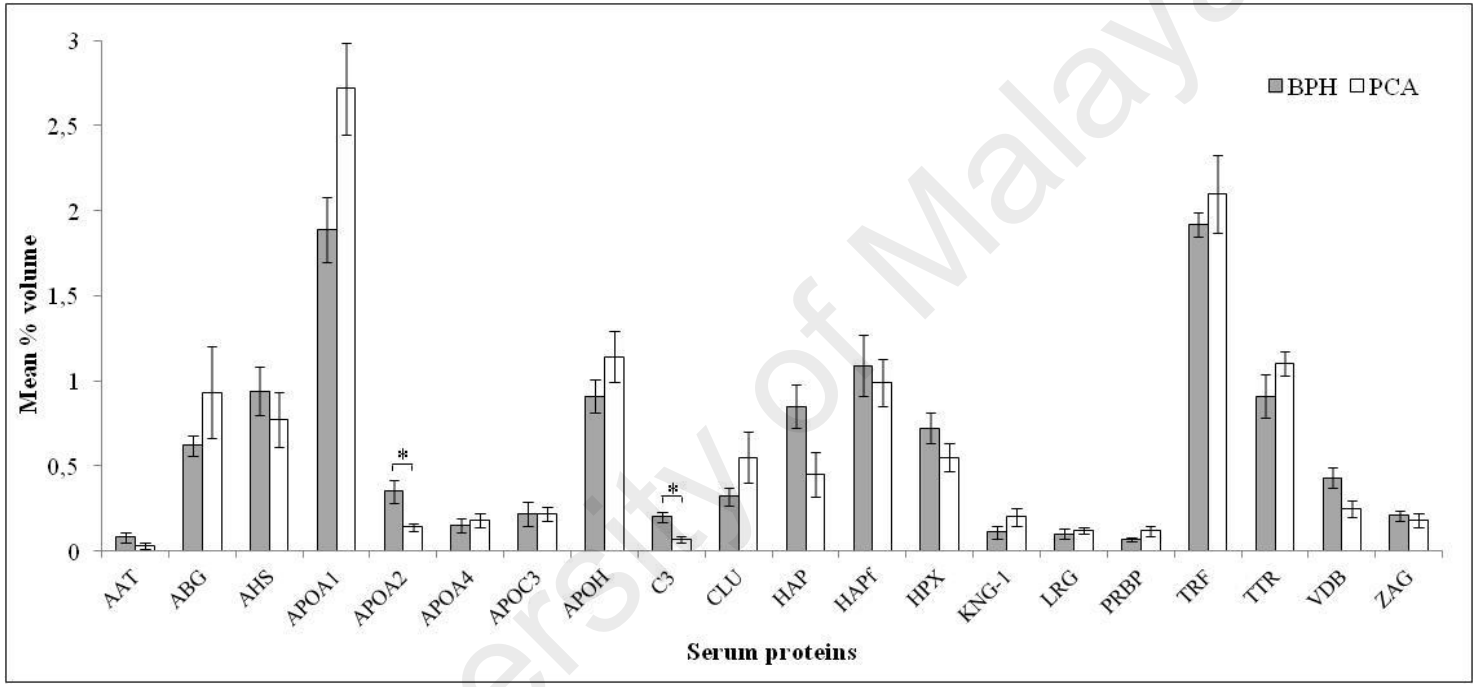


Figure 4.6. Mean percentage of volume contribution of 2-DE resolved serum proteins.

Percentage of volume contribution of protein spots were analysed using ImageMaster™ 2D Platinum software, version 7.0. Densitometry analysis was performed on a total of 20 well-resolved clusters of protein spots including AAT, ABG, AHS, APOA1, APOA2, APOA4, APOC3, APOH, C3, CLU, HAP, HAPf, HPX, KNG-1, LRG, RBP, TRF, TTR, VDB and ZAG. Asterisk denotes proteins with significant alteration in abundance in terms of the percentage of volume contribution.

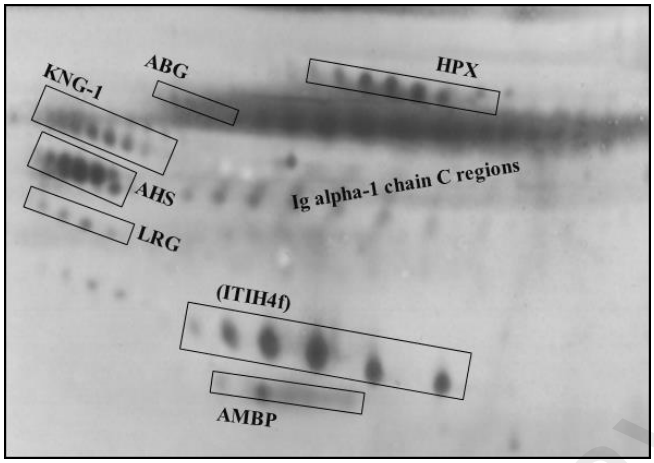
Figure 4.7 demonstrates the 2-DE profiles of serum *O*-glycosylated proteins of patients with BPH (panel A) and those with PCa (panel B) that were generated using HRP-conjugated CGB lectin as probe. The resolved profiles of glycoprotein spots were initially identified through visual comparison with the standard SWISS ExPASy plasma protein map (Sanchez *et al.*, 1995), the silver stained 2-DE profiles that were developed earlier in this study and other previous publications (Abdul-Rahman *et al.*, 2007; Mohamed *et al.*, 2008). Spots that were visually identified include heavy chains of Ig, ABG, AHS, HPX, KNG-1, LRG, alpha-1 microglobulin/bikunin precursor (AMBP), 35 kDa fragment of ITIH4 (ITIH4f).

When the DAB-developed proteins on blots were visually compared, all showed comparable intensities, with the exception of ITIH4f. The abundance of ITIH4f in the blot profiles of patients with PCa was markedly mild in intensity compared to those with BPH.

4.2.4. Image analyses of HRP-conjugated CGB lectin generated serum *O*-glycosylated protein profiles of patients with BPH and those with PCa

A total of seven consistently well-resolved clusters of protein spots were selected for image analyses. These include, ABG, AHS, HPX, KNG-1, LRG, AMBP and ITIH4f. Densitometry analysis of CGB lectin detected serum protein profiles confirmed the significantly decreased level of ITIH4f (–0.29 fold, $p = 0.006$) in patients with PCa ($n = 8$) compared to patients with BPH ($n = 8$) (Figure 4.8). Aside from ITIH4f, no other *O*-glycopeptides showed significant alteration in abundance between the groups of subjects.

A)



B)

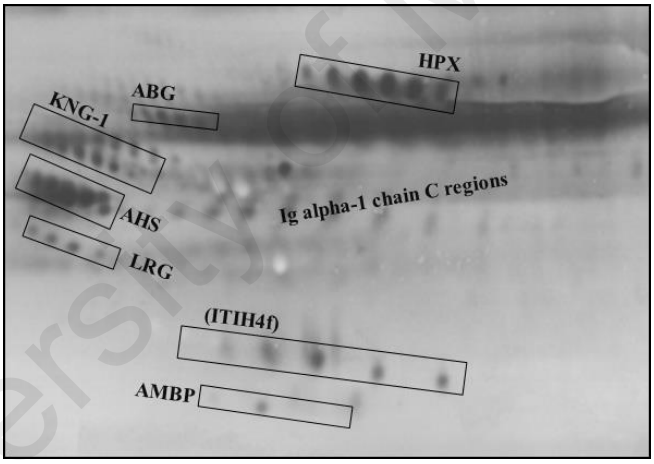


Figure 4.7. Detection of *O*-glycosylated serum proteins using enzyme-conjugated CGB lectin.

Enzyme-conjugated CGB lectin was used to probe for the *O*-glycosylated proteins from sera that were subjected to 2-DE and transferred onto NC membranes. Panel A demonstrates a DAB-developed *O*-glycosylated serum proteins profile from patients with BPH whilst panel B, patients with PCa. The protein spot of altered abundance is marked in bracket.

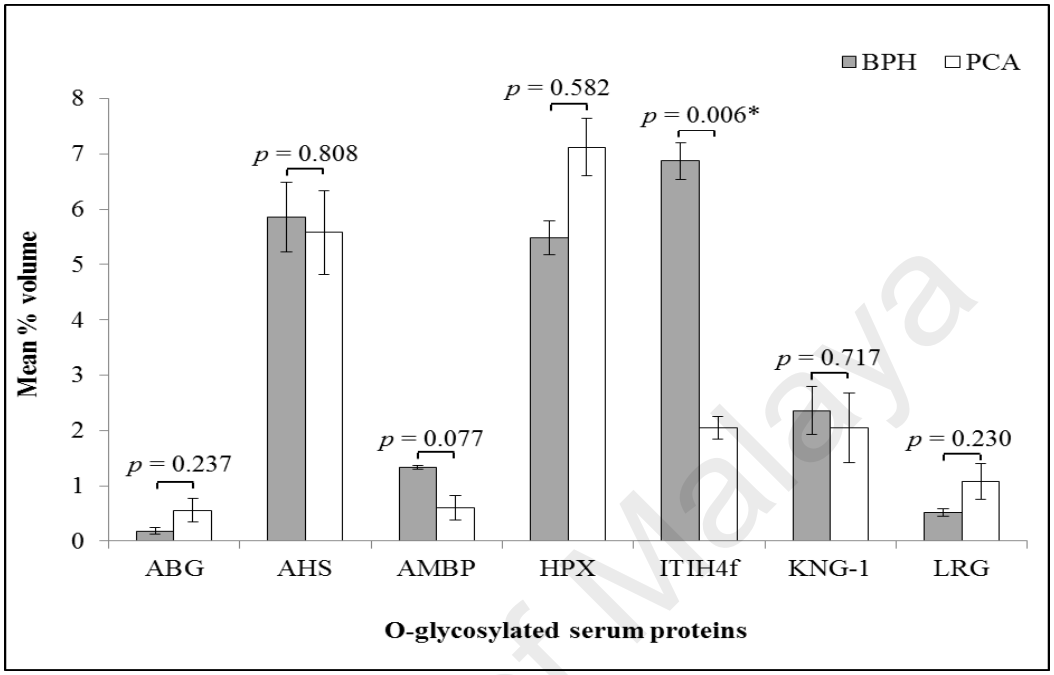


Figure 4.8. Mean percentage of volume contribution of HRP-conjugated CGB lectin detected serum *O*-glycosylated proteins.

Panel demonstrates analysis of *O*-glycosylated proteins detected by enzyme-conjugated CGB lectin. Percentage of volume contribution of protein spots was analysed using ImageMaster™ 2D Platinum software, version 7.0. Densitometry analysis was performed on 7 clusters of protein spots including ABG, AHS, AMBP, HPX, ITIH4f, KNG-1 and LRG. Asterisk denotes *O*-glycosylated proteins with significant alteration in abundance in terms of the percentage of volume contribution.

4.2.5. 2-DE serum *O*-glycosylated protein profiling using CGB lectin affinity column chromatography

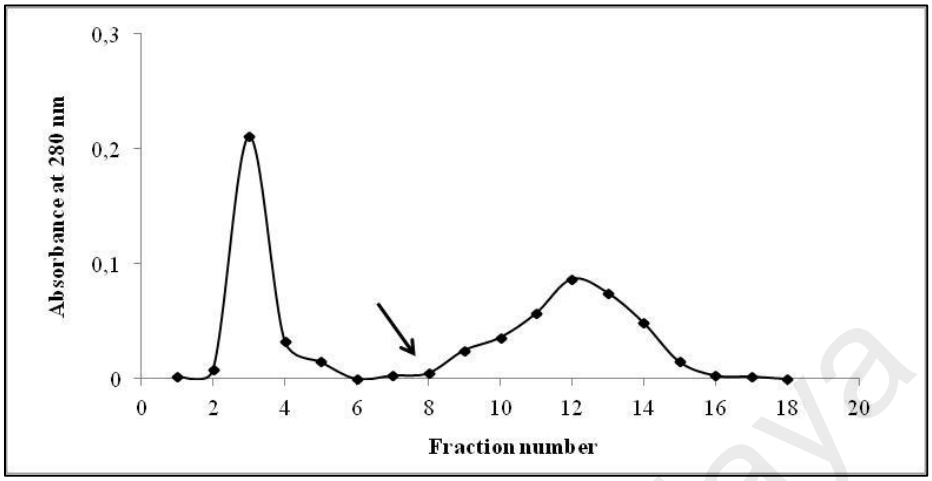
4.2.5.1. Efficiency of CGB lectin immobilisation

Purified CGB lectin was coupled to CNBr-activated Sepharose 4B matrix as described in Section 3.9.1. Immobilisation was performed with 12 ml CGB lectin at a concentration of 4 mg/ml. The CGB lectin binding efficiency was subsequently assessed according to Section 3.9.2. Based on the formulae, approximately 93.6% of the activated matrix support in the 3 ml column was successfully coupled with purified CGB lectin.

4.2.5.2. Isolation of serum *O*-glycosylated protein by CGB lectin affinity column chromatography

Pooled serum samples was passed through the CGB lectin conjugated Sepharose 4B columns and the fractions containing the *O*-glycosylated protein were eluted using 0.1 M melibiose in PBS, pH 7.2 (see Section 3.9.3). This method was performed to reduce the complexity of serum sample as well as to enrich the serum *O*-glycosylated proteins subset that may have been masked in the initial experiment. Figure 4.9 demonstrates elution profiles of pooled serum samples obtained from patients with BPH (panel A) and those with PCa (panel B). The ratio of bound: total protein fractions were similar for all pooled samples, which were approximately 0.05 and 0.06, respectively, indicating similar recovery of proteins for both pooled serum samples. Non-specific interaction between pooled serum proteins from patients with BPH and those with PCa (n = 16) and chromatography media was tested using un-coupled activated and inactivated chromatographic matrix, and none was detected (Figure 4.10: panel A & B, respectively).

A)



B)

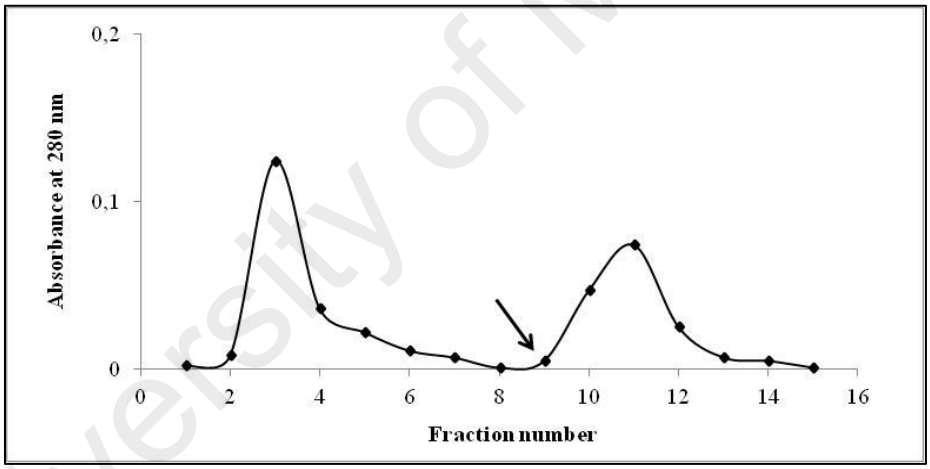
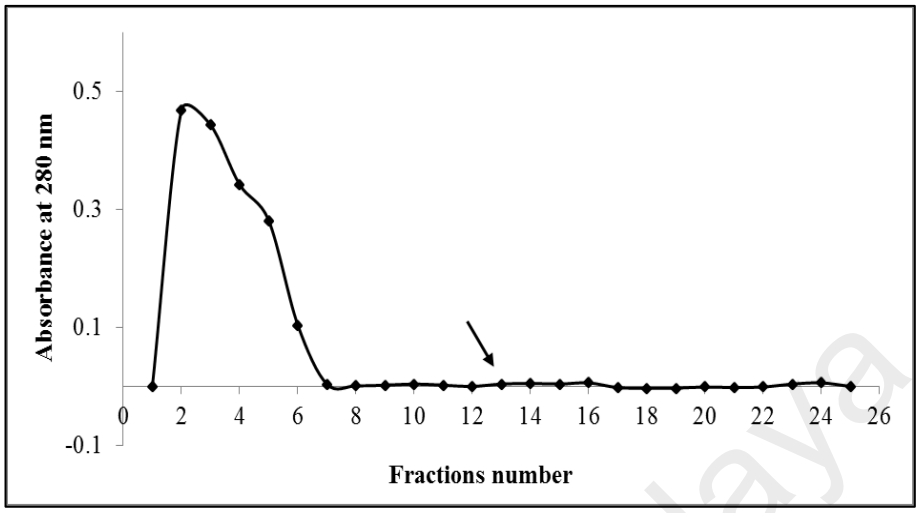


Figure 4.9. Typical affinity separation profiles of pooled sera obtained from patients with BPH as well as from those with PCa.

Pooled serum samples from patients with BPH (panel A) and those with PCa (panel B) were separately subjected to CGB lectin affinity column chromatography. Two hundred µl of pooled serum samples was loaded into the 3 ml column and the *O*-glycosylated proteins were eluted with 0.1 M melibiose in PBS, pH 7.2, as described in Section 3.9.3. Collected fractions were monitored by measurement of absorbance at 280 nm. The arrows indicate start of elution with 0.1 M melibiose in PBS, pH 7.2.

A)



B)

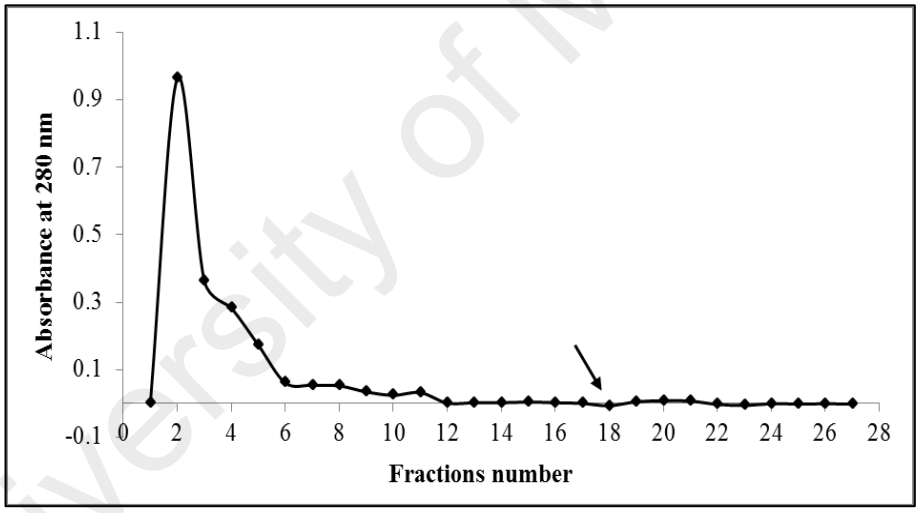


Figure 4.10. Affinity separation profiles of possible interactions between serum proteins and chromatographic matrix.

Pooled serum samples from patients with BPH and PCa were subjected to affinity chromatography using un-coupled activated (panel A) and inactivated (panel B) Sepharose 4B matrix. Two hundred µl of pooled serum samples was loaded into the 3 ml gel bed and the collected fractions were monitored by measurement of absorbance at 280 nm. The arrow indicates start of elution with 0.1 M melibiose in PBS, pH 7.2.

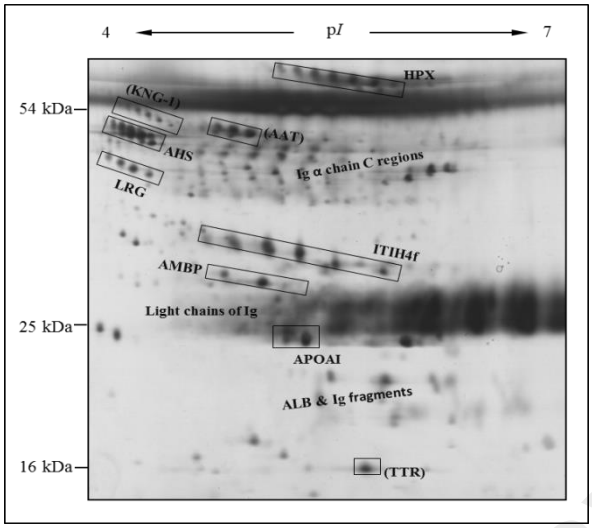
4.2.5.3. Typical CGB lectin affinity column chromatography generated serum *O*-glycosylated protein profiles of pooled serum of patients with BPH and those with PCa

The 2-DE profiles of enriched melibiose-eluted fractions from pooled serum samples from patients with BPH as well as from those with PCa were similarly generated by subjecting the fractions to 2-DE and silver staining as described in Section Sections 3.10 and 3.11, respectively. Figure 4.11 demonstrates the 2-DE profiles of pooled serum *O*-glycosylated protein of patients with BPH (panel A) and those with PCa (panel B) generated using CGB lectin affinity chromatography. The resolved profiles of *O*-glycosylated protein spots were initially identified through visual comparison with the silver-stained 2-DE profiles of the previously published report (Doustjalali *et al.*, 2004).

4.2.5.4. Image analyses of serum *O*-glycosylated protein profiles of pooled serum of patients with BPH and those with PCa

Nine clearly resolved clusters of *O*-glycosylated peptides were selected for spot volume analysis (Figure 4.12). When the 2-DE gels were scanned and analysed by densitometry, three clusters of protein spots including the AAT (+2.5 fold, $p = 0.007$), KNG-1 (+3.1 fold, $p = 0.006$) and TTR (+2.2 fold, $p = 0.001$) were found to be significantly enhanced in the sera of patients with PCa compared to those with BPH. The mass spectrometric data confirming the identities of proteins of altered abundance are displayed in Table 4.1.

A)



B)

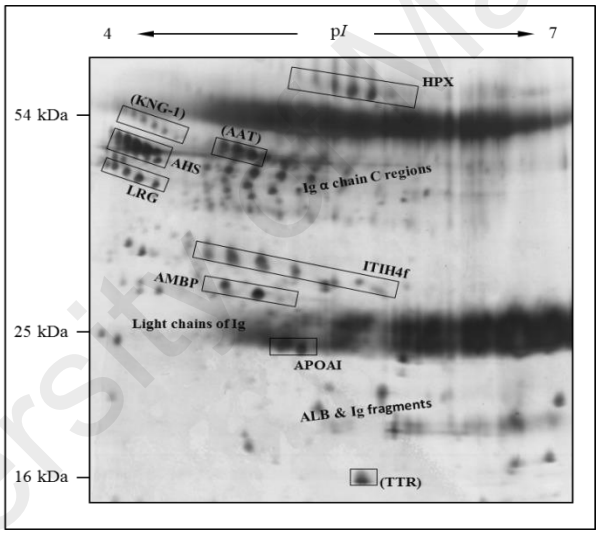


Figure 4.11. Detection of *O*-glycosylated serum proteins using CGB lectin affinity chromatography.

Pooled neat serum samples were subjected to CGB lectin affinity chromatography. Analysis of the CGB lectin bound fractions was performed by 2-DE and silver staining. Panel A demonstrates CGB lectin affinity chromatography isolated *O*-glycosylated serum proteins profile from patients with BPH whilst panel B, patients with PCa. Identities of proteins that were altered in abundance were confirmed by MS and database search (Table 4.1). Acidic sides of the 2-DE gels are to the left and M_r declines from the top. The clusters of protein spots of altered abundance are in brackets.

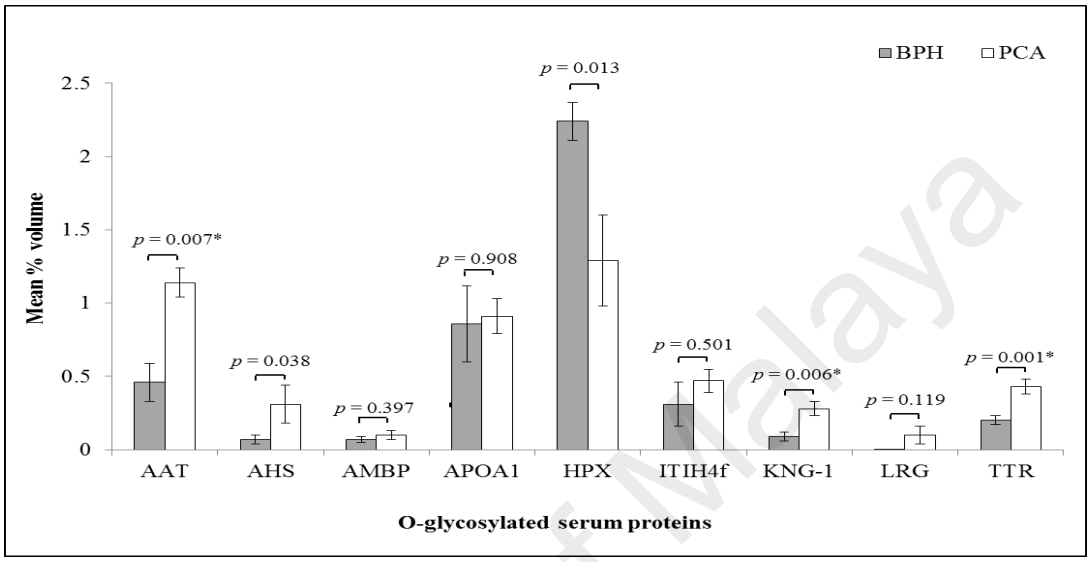


Figure 4.12. Mean percentage of volume contribution of serum *O*-glycosylated proteins.

Panel demonstrates analysis of *O*-glycosylated proteins detected by CGB lectin affinity chromatography. Percentage of volume contribution of protein spots was analysed using ImageMaster™ 2D Platinum software, version 7.0. Densitometry analysis was performed on nine clusters of protein spots including AHS, AAT, AMBP, APOA1, HPX, ITIH4f, KNG-1, LRG and TTR. Asterisk denotes *O*-glycosylated proteins with significant alteration in abundance in terms of the percentage of volume contribution.

4.2.6. Identification of serum proteins obtained from MS-compatible silver stained 2-DE gels

MS analysis was performed on all serum proteins with altered abundance as well as *O*-glycosylated proteins ($p < 0.05$) detected using CGB lectin affinity chromatography resolved by 2-DE. The methodology opted for trypsin digestion is described in Section 3.15.1. To enable and maximize confident MS results; the concentration of the serum applied for 2-DE workflow was doubled, the spots of interests was pooled from all similar gel and a modified MS-compatible silver staining was performed as previously described (Yan *et al.*, 2000).

The serum proteins identified were APOA2, C3, ITIH4f, AAT, KNG-1 and TTR. A Mascot protein score greater than 29 indicates identity and extensive homology ($p < 0.05$). Mass spectrometric details on the identified proteins in terms of accession number as listed in the Swiss-Prot database, theoretical mass, calculated pI, Mascot score, number of matched peptides and percentage of sequence coverage are enumerated in Table 4.1. The peptide mass fingerprint (PMF) of the identified protein spots are shown in Figure 4.13. Its respective MS/MS derived peptide sequences of the aberrantly expressed proteins are illustrated in Table 4.2.

Table 4.1. MS identification of serum (*O*-glycosylated) protein spots of significant altered abundance detected in 2-DE profiles.

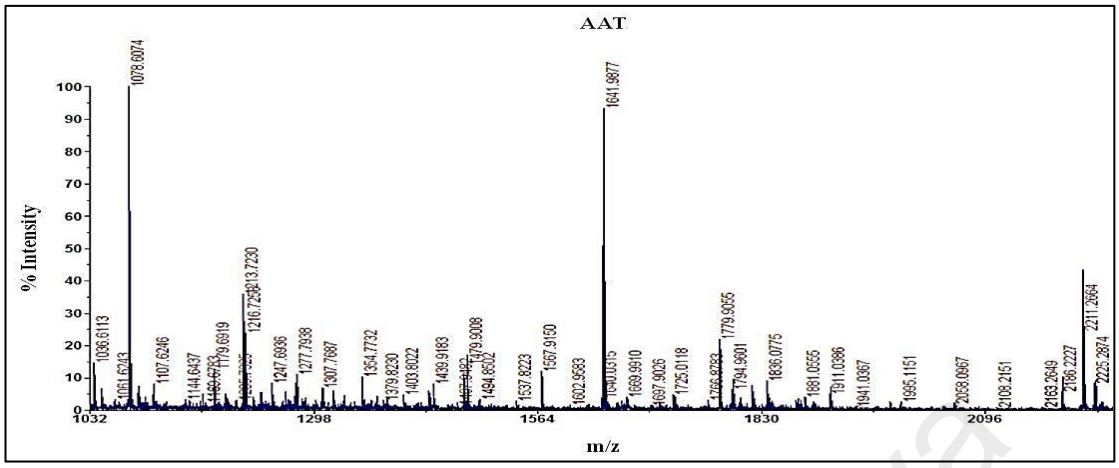
Spot ID[(a)]	Protein ID	Accession number[(b)]	M_r (Da)	pI	Mascot Score	Matched peptides	Sequence Coverage (%)
AAT	Alpha-1 antitrypsin	P01009	46,707	5.37	239	4	13
APOA2	Apo-lipoprotein A-II	P02652	11,168	6.26	156	3	41
C3[(c)]	Complement C3	P01024	187,030	6.02	460	7	5
ITIH4[(c)]	Inter-alpha-trypsin inhibitor heavy chain H4	Q14624	103,261	6.51	566	5	10
KNG-1	High–molecular weight kininogen	P01042	71,912	6.34	307	7	13
TTR	Transthyretin	P02766	15,877	5.52	336	4	34

a) Spot ID are as in Figure 4.5.

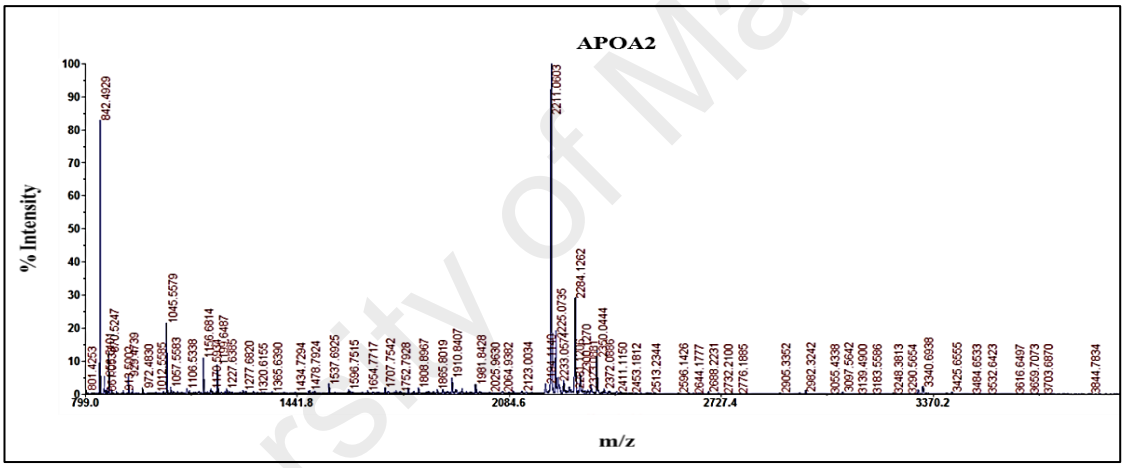
b) Accession numbers are from the Swiss-Prot database (http://www.uniprot.org)

c) Identified as protein fragment as indicated by its position in the 2-DE gels compared to the parent protein.

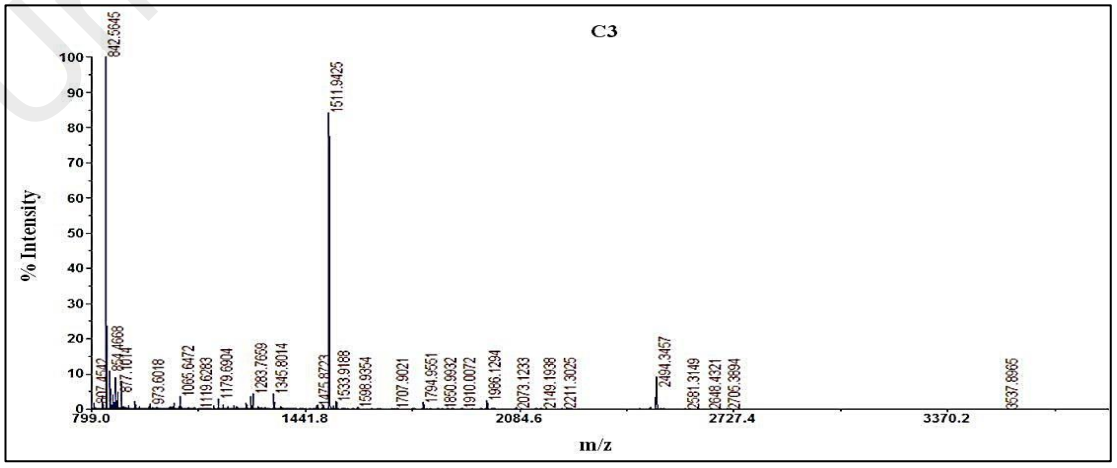
A)



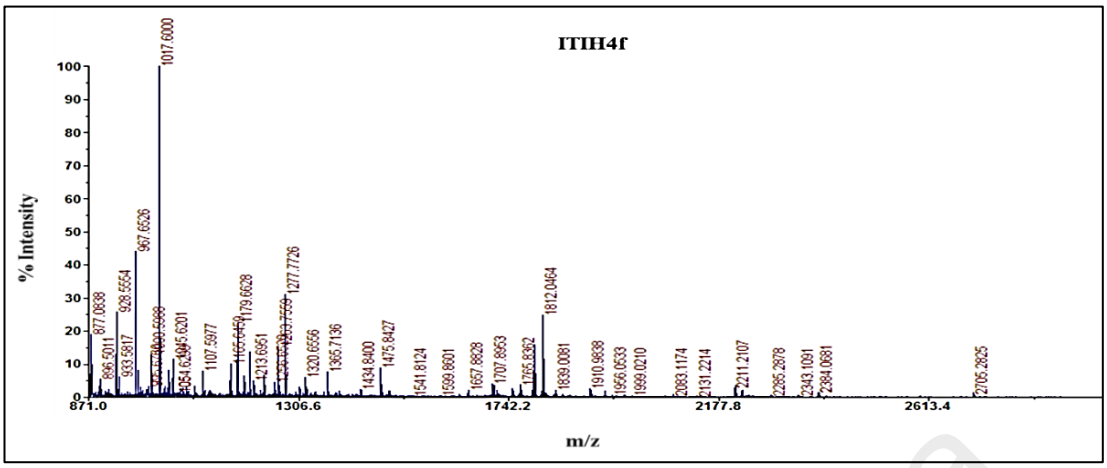
B)



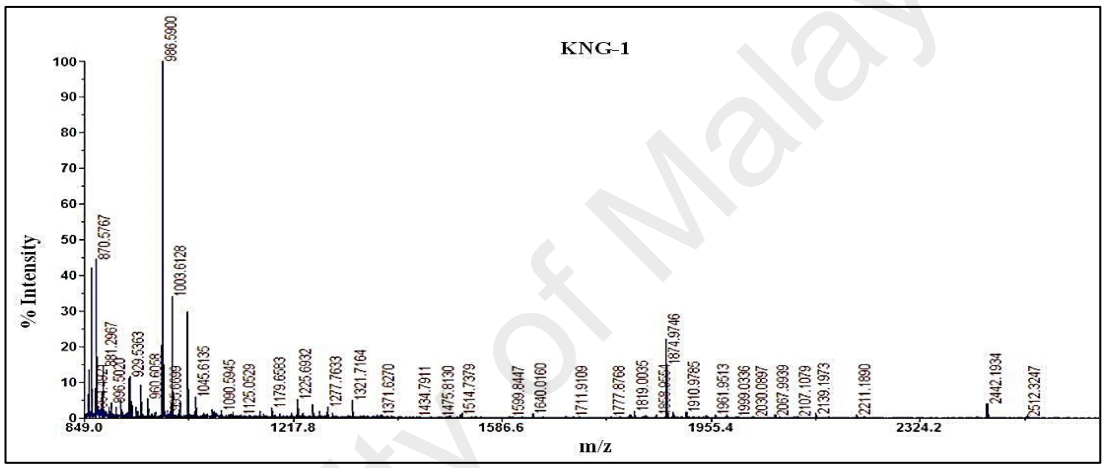
C)



D)



E)



F)

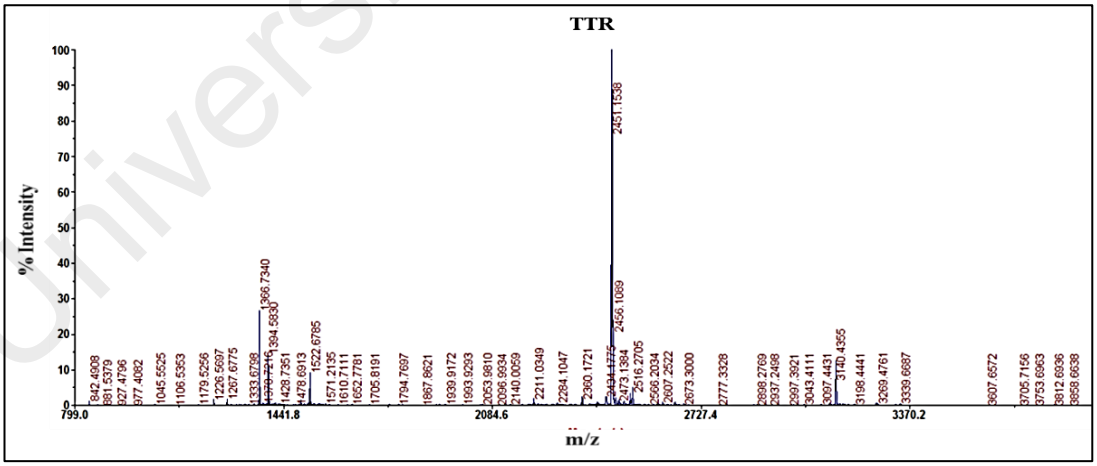


Figure 4.13. PMF of trypsin digested serum peptides.

Gel plugs excised from the protein resolved 2-DE gels were trypsin digested and mass spectrometrically analysed. Identities of the proteins of altered abundance including (panel A) AAT, (panel B) APOA2, (panel C) C3, (panel D) ITIH4f, (panel E) KNG-1 and (panel F) TTR, were confirmed using 4800 Plus MALDI ToF/ToF analyser.

Table 4.2. MS/MS derived peptide sequences of significantly altered serum proteins.

Spot/ Cluster ID	Peptide sequence	Amino acid	Ion score
AAT	K.TDTSHHDQDHPTFNK.I	35 - 49	65
	K.ITPNLAEFAFSLYR.Q	50 - 63	77
	K.LYHSEAFTVNFGDTEEAKK.Q	161 - 179	57
	K.FLENEDRR.S	299 - 306	41
APOA2	K.EPCVESLVSQYFQTVTDYGK.D	27 - 46	101
	K.VKSPELQAEAK.S	52 - 62	35
	K.SKEQLTPLIK.K	68 - 77	19
C3	R.LVAYYTLIGASGQR.E	531 - 544	58
	R.EVVADSVWVDVK.D	545 - 556	92
	R.QPVPGQQMTLK.I	574 - 584	7
	K.IEGDHGAR.V	585 - 592	27
	R.VVLVAVDK.G	593 - 600	15
	K.IWDVVEK.A	616 - 622	37
	K.DYAGVFSDAGLTFTSSSGQQTAQR.A	634 - 657	182
ITIH4f	R.QGPVNLLSDPEQGVEVTGQYER.E	754 - 775	130
	K.ETLFSVMPGLK.M	778 - 788	60
	K.NPLVWVHASPEHVVVTR.N	789 - 805	103
	R.FSSHVGGTLGQFYQEVLWGSPAASDDGR.R	867 - 894	207
	R.RLDYQEGPPGVEISCWSVEL.-	911 - 930	67
KNG-1	K.YNSQNQSNNQFVLYR.I	44 - 58	93
	K.TVGSDTFYSFK.Y	65 - 75	51
	K.AATGECTATVGKR.S	102 - 114	28
	R.QVVAGLNFR.I	188 - 196	28
	K.SLWNGDTGECTDNAYIDIQLR.I	220 - 240	66
	K.ICVGCPR.D	263 - 269	12
	K.YFIDFVAR.E	317 - 324	31
TTR	R.GSPAINVAVHVFR.K	42 - 54	69
	R.KAADDTWEPFASGK.T	55 - 68	104
	K.AADDTWEPFASGK.T	56 - 68	77
	K.ALGISPFHEHAEVVFTANDSGPR.R	101 - 123	86

4.3. COMPREHENSIVE PROTEOMIC PROFILING OF HUMAN ACETONE PRECIPITATED URINARY PROTEINS

4.3.1. Typical 2-DE generated acetone precipitated urinary protein profiles of control male subjects

2-DE of the human acetone precipitated urine samples optimised at a protein amount of 100 µg demonstrated resolution of more than 50 clusters of protein spots. A comparable profile to the urinary proteome map with those previously published (Thongboonkerd *et al.*, 2002) was generated. Nevertheless, the identities of all consistently resolved clusters of protein spots were first confirmed by MS and database search, for convenience of subsequent densitometry analysis. Table 4.3 shows the list of identified urinary proteins that was consistently detected in 2-DE profiles.

Figure 4.14 illustrates a representative profile of 2-DE resolved and silver stained acetone precipitated urinary proteins obtained from a control subject following methods as described in Section 3.10 and 3.11, respectively. Loss of proteins such as KNG-1 and immunoglobulins was evident when current 2-DE urinary proteome profiles were compared to other previous publications from our laboratory (Abdullah-Soheimi *et al.*, 2010; Mu *et al.*, 2012). This may have been contributed by the inclusion of acetone precipitation method in this study. On the other hand, urinary protein precipitation with acetone had both improved the reproducibility and resolution of the 2-DE gels. Proteins that were consistently resolved in the gels include ALB, heavy and light chains of immunoglobulins, uromodulin (THP), cadherin-1 (CDH), AHS, alpha-1 acid glycoprotein 1 (AGP1), prostaglandin-H2 D-isomerase (PGDS), ZAG, TRF, ITIH4f, AMBP, perlecan (PLC), ganglioside GM2 activator (GM2A), CD59, non-secretory ribonuclease (RNS2), PSAP, KNG-1 and LRG. Proteins such as AMBP, CD59, ITIH4 and PSAP were found in fragments of their native form based on the marked differences in M_r and pI of the spots observed, which may be in part of the actions of proteases.

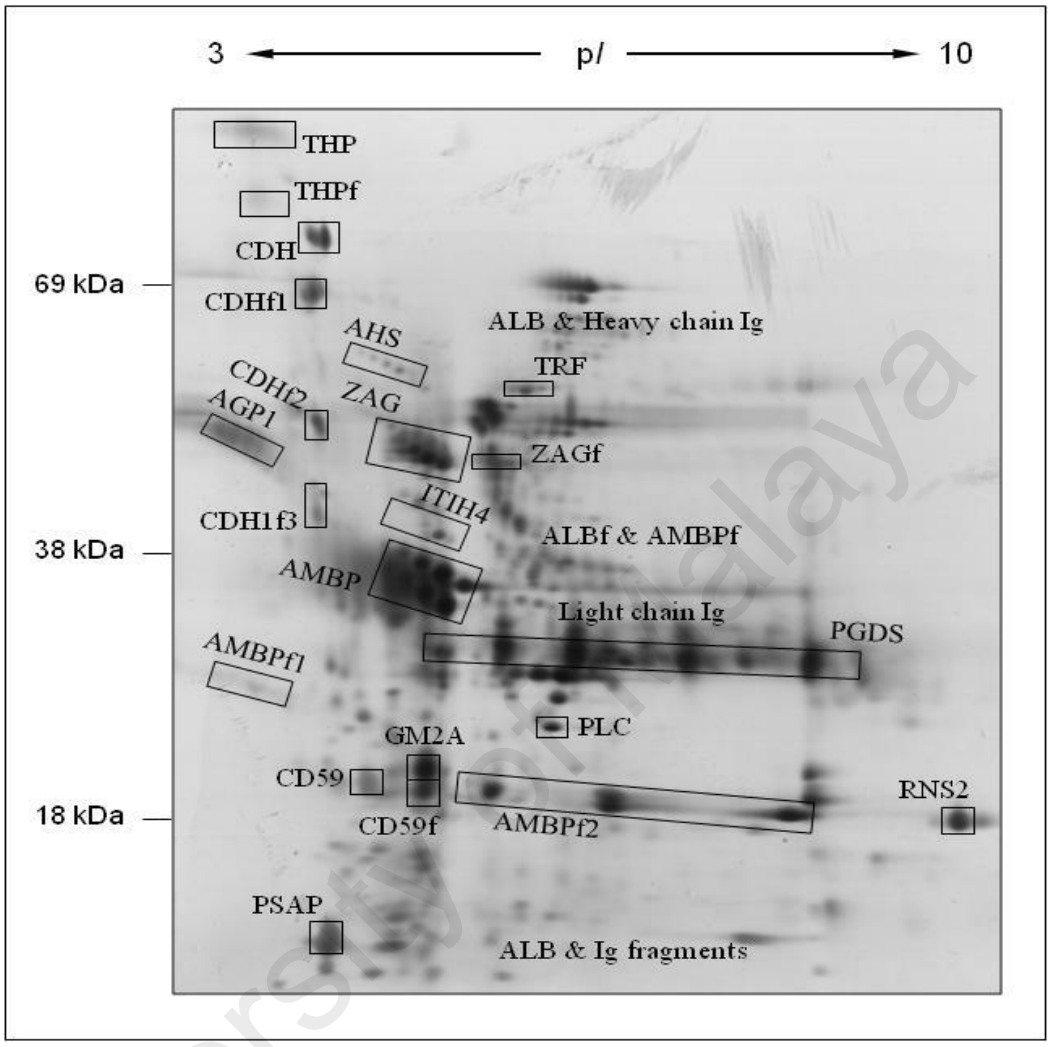


Figure 4.14. Typical silver stained 2-DE protein profiles of acetone precipitated urine proteins.

Acetone precipitated urine samples obtained from control subjects were subjected to 2-DE and silver staining. The identities of well-resolved clusters of protein spots that were determined by MS and database search are as labelled. Details of the identified proteins are listed in Table 4.3 (page 131). The acidic sides of the 2-DE gels are to the left and relative M_r declines from the top. Proteins denoted as 'f' are protein fragments as indicated by its position in the 2-DE gels compared to the parent protein.

4.3.2. Image analyses of 2-DE generated acetone precipitated urine protein profiles of patients with BPH and those with PCa as well as controls

The silver stained 2-DE profiles of acetone precipitated urine samples of patients with BPH (n = 20) and those with PCa (n = 13) as well as control subjects (n = 15) were scanned using a densitometer and analysed by Image Master 2D Platinum software, version 7.0. Statistical analysis was performed to analyse the significance distribution of percentage of volume contribution of a total of 21 well-resolved urinary clusters of protein spots between the 2-DE profiles of patients with BPH and those with PCa and control subjects ($p < 0.05$). The urinary proteins assessed include AGP1, AHS, AMBP, AMBPf1, AMBPf2, CD59, CD59f, CDH, CDHf1, CDHf2, CDHf3, GM2A, PGDS, PLC, PSAP, RNS2, THP, THPf, TRF, ZAG and ZAGf.

Normal urine also contains small amounts of heavy and light chains of immunoglobulins (Vaughan *et al.,* 1967) and modified albumin (Wiggins *et al.,* 1985) or fragments of albumin (Lafitte *et al.,* 2002) but are of unknown biological significance particularly, the latter (Eppel *et al.,* 2000). Therefore, these clusters of protein spots were excluded from the densitometry analysis. Volume analysis of the 21 clusters of urinary protein spots in the 2-DE profiles of patients with BPH, those with PCa and control subjects are shown in Figure 4.15. Significantly increased level of proteins AMBPf1 (+2.3 fold, p = 0.005), AMBPf2 (+2.2 fold, p = 0.004) and PSAP (+2.2 fold, p = 0.002) were observed among the controls when compared to patients with PCa. Apart from this, only AMBPf2 was seen to be of lower abundance among subjects with PCa compared to BPH patients (+2.2 fold, p = 0.004). Figure 4.16 illustrates the corresponding cropped images showing the urinary proteins of altered abundance from 2-DE gels.

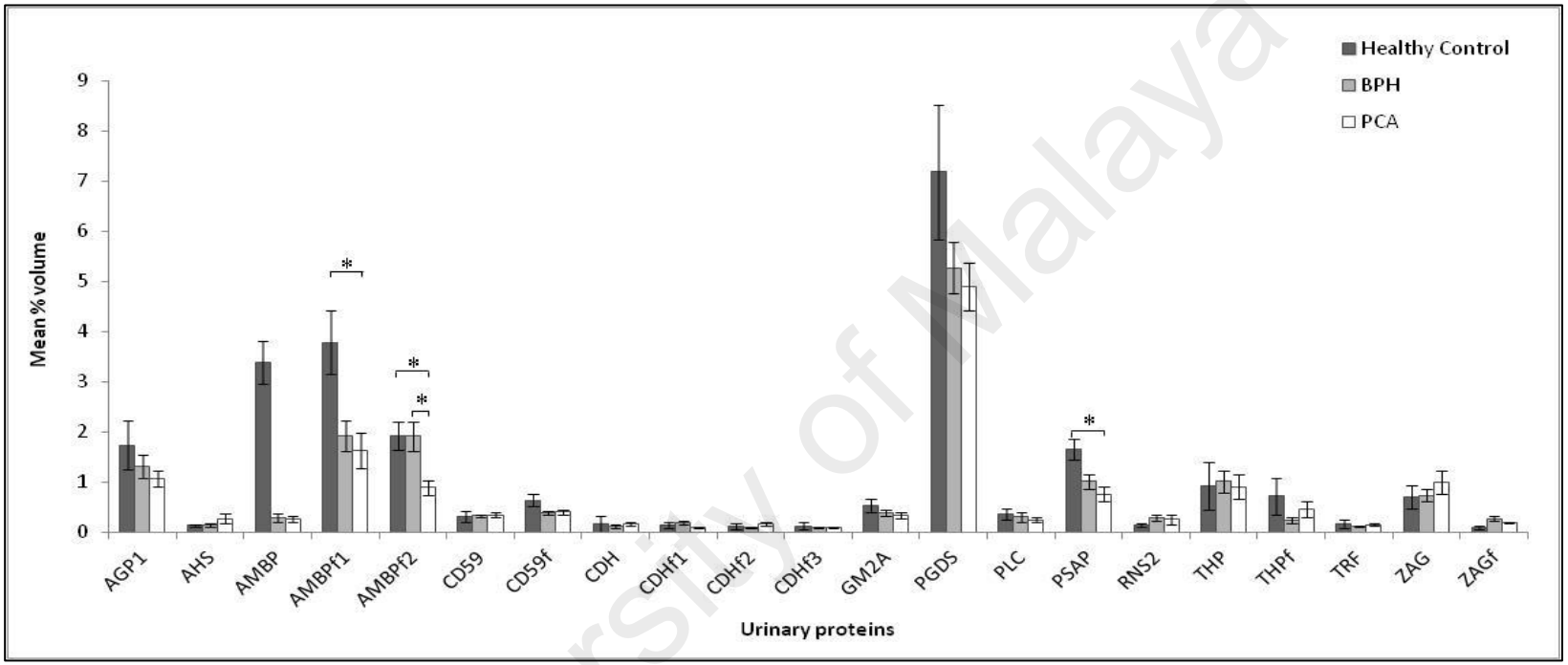


Figure 4.15. Mean percentage of volume contribution of 2-DE resolved acetone precipitated urine proteins.

Percentage of volume contribution of urinary protein spots detected in the 2-DE gels was analysed using ImageMaster™ 2D Platinum software, version 7.0. Densitometry analysis was performed on a total of 21 well-resolved clusters of protein spots including AGP1, AHS, AMBP, AMBPf1, AMBPf2, CD59, CD59f, CDH, CDHf1, CDHf2, CDHf3, GM2A, PGDS, PLC, PSAP, RNS2, THP, THPf, TRF, ZAG and ZAGf. Asterisk denotes urinary proteins of significant alteration in abundance in terms of the percentage of volume contribution.

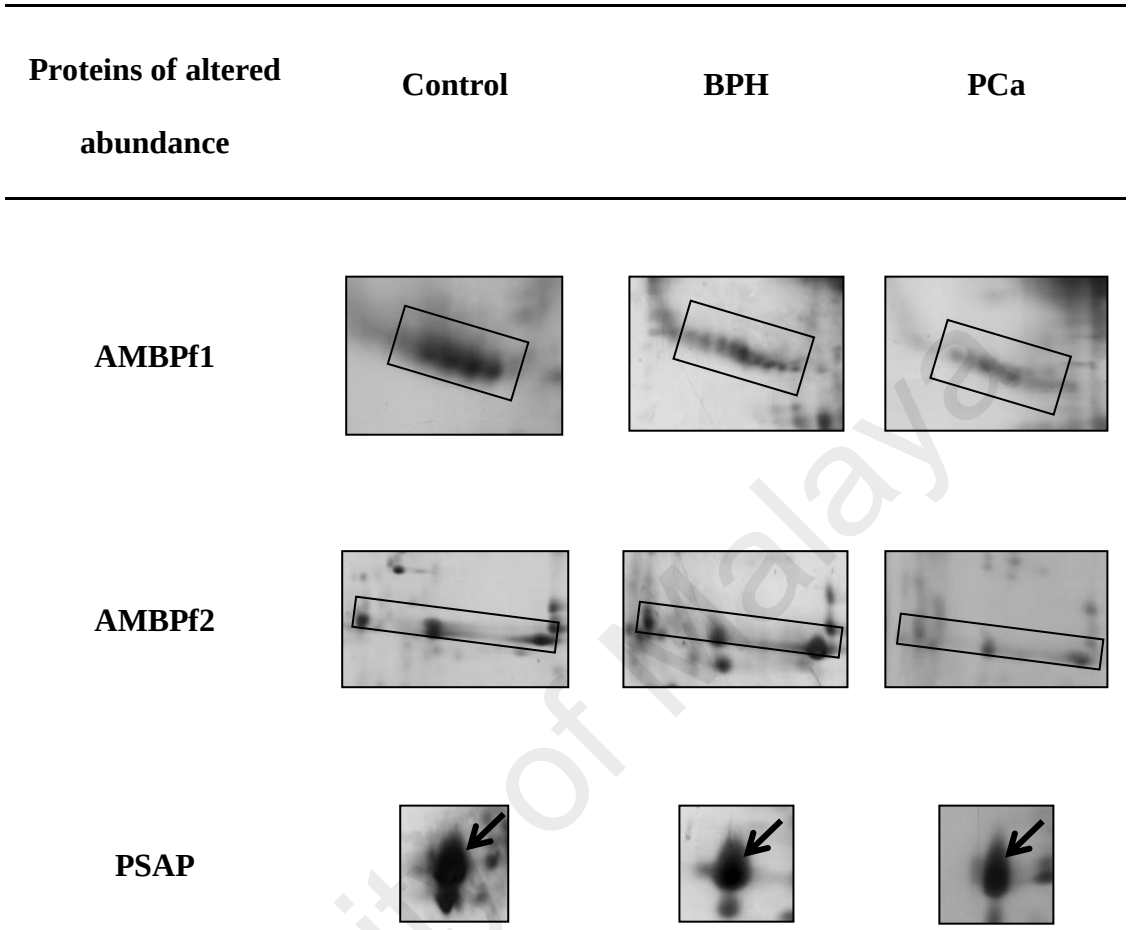


Figure 4.16. Cropped silver stained 2-DE images of urinary proteins of altered abundance from control subjects and patients with BPH and those with PCa.

Cropped images demonstrate acetone precipitated 2-DE resolved and silver stained urinary proteins of altered abundance from control subjects, and patients with BPH and those with PCa. The identity of clusters of protein spots were confirmed by MS and database search (Table 4.3).

4.3.3. MS identification of 2-DE resolved acetone precipitated urinary proteins

MS analysis was performed on all consistently 2-DE resolved clusters of protein spots as described in Section 3.15. A Mascot protein score greater than 27 indicates identity and extensive homology ($p < 0.05$) of the protein identified. Mass spectrometric data of the identified proteins in terms of accession number as listed in the Swiss-Prot database, theoretical mass, calculated pI, Mascot score, number of matched peptides and percentage of sequence coverage are shown in Table 4.3. The PMF and MS/MS derived peptide sequences of aberrantly expressed proteins are illustrated in Figure 4.17 and Table 4.4, respectively.

Proteins AMBP and PSAP that was detected were most likely truncated fragments of their native forms (AMBP - 38,974 Da; PSAP - 58,074 Da) based on their locations in 2-DE gels (Figure 4.14). Further scrutiny into their MS/MS derived peptide sequences showed that the identified fragments indeed matched those of saposin B, which is derived from PSAP, and those of inter-alpha-trypsin inhibitor light chains (ITIL) or bikunin regions, which is derived from its parent protein, AMBP (Table 4.4).

Table 4.3. MS identification of urinary proteins detected in 2-DE profiles.

Spot ID[(a)]	Protein ID	Accession number[(b)]	M_r (Da)	Calc. pI	Mascot Score	Matched peptides	Sequence Coverage (%)
AGP1	Alpha-1 acid glycoprotein 1	P02763	23,497	4.93	236	4	16
AHS	Alpha-2-HS glycoprotein	P02765	39,300	5.43	117	2	7
AMBP	Protein AMBP	P02760	38,974	5.95	144	3	10
AMBPf1*	**Protein AMBP**	**P02760**	**38,974**	**5.95**	**342**	**5**	**14**
AMBPf2*	**Protein AMBP**	**P02760**	**38,974**	**5.95**	**278**	**6**	**14**
CD59	CD59 glycoprotein	P13987	14,168	6.02	126	2	18
CD59f*	CD59 glycoprotein	P13987	14,168	6.02	80	2	18
CDH1*	Cadherin-1	P12830	97,396	4.58	99	3	6
CDH1f1*	Cadherin-1	P12830	97,396	4.58	140	3	5
CDH1f2*	Cadherin-1	P12830	97,396	4.58	147	2	4
CDH1f3*	Cadherin-1	P12830	97,396	4.58	99	3	6
GM2A	Ganglioside GM2 activator	P17900	20,809	5.17	100	1	10
ITIH4*	Inter-alpha-trypsin inhibitor heavy chain 4	Q14624	103,293	6.51	38	2	4
LRG	Leucine-rich alpha-2-glycoprotein	P02750	38,154	6.45	70	2	8
PGDS	Prostaglandin-H2 D-isomerase	P41222	21,015	7.66	306	4	32
PLC*	Perlecan	P98160	468,528	6.06	369	7	1
RNS2	Non-secretory ribonuclease	P10153	18,342	9.1	215	5	21

Table 4.3., continued…

Spot ID[(a)]	Protein ID	Accession number[(b)]	M_r (Da)	Calc. pI	Mascot Score	Matched peptides	Sequence Coverage (%)
PSAP*	**Proactivator polypeptide**	**P07602**	**58,074**	**5.06**	**78**	**3**	**5**
THP	Uromodulin	P07911	69,714	5.05	608	14	22
TRF*	Serotransferrin	P02787	77,000	6.81	496	14	19
ZAG	Zinc-alpha-2-glycoprotein	P25311	33,851	5.57	663	9	29

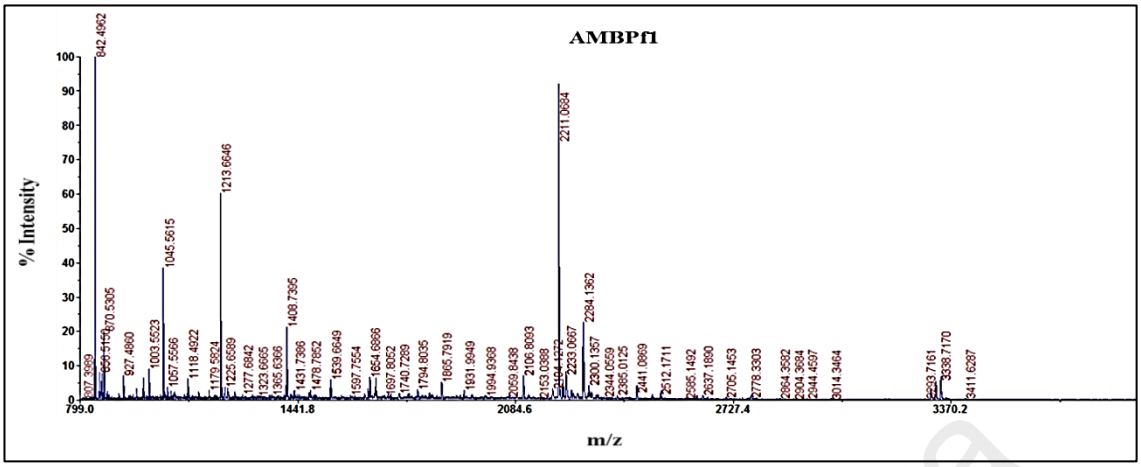
* Identified as protein fragment as indicated by its position in the 2-DE gels compared to the parent protein.

a) Spot ID are as in Figure 4.14.

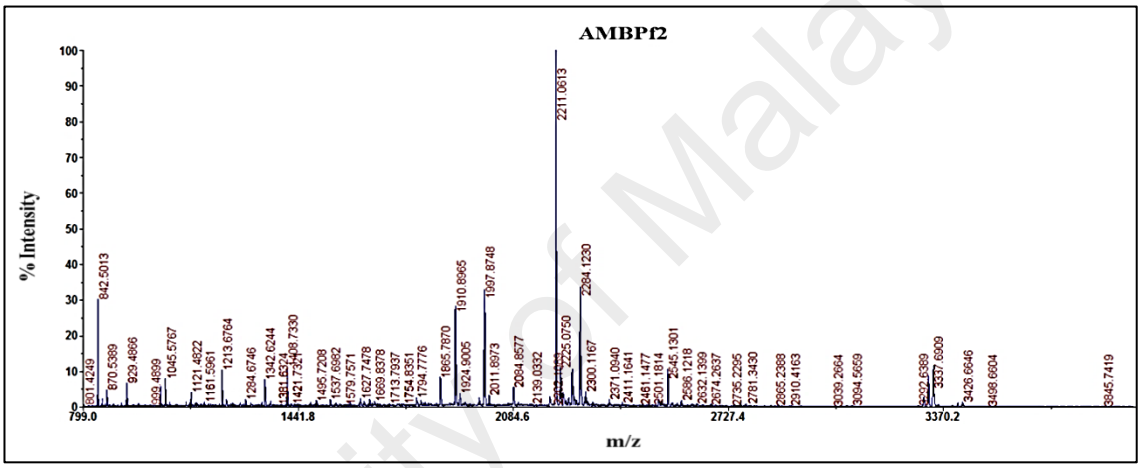
b) Accession numbers are from the Swiss-Prot database (http://www.uniprot.org).

c) Significantly altered urinary proteins are in bold.

A)



B)



C)

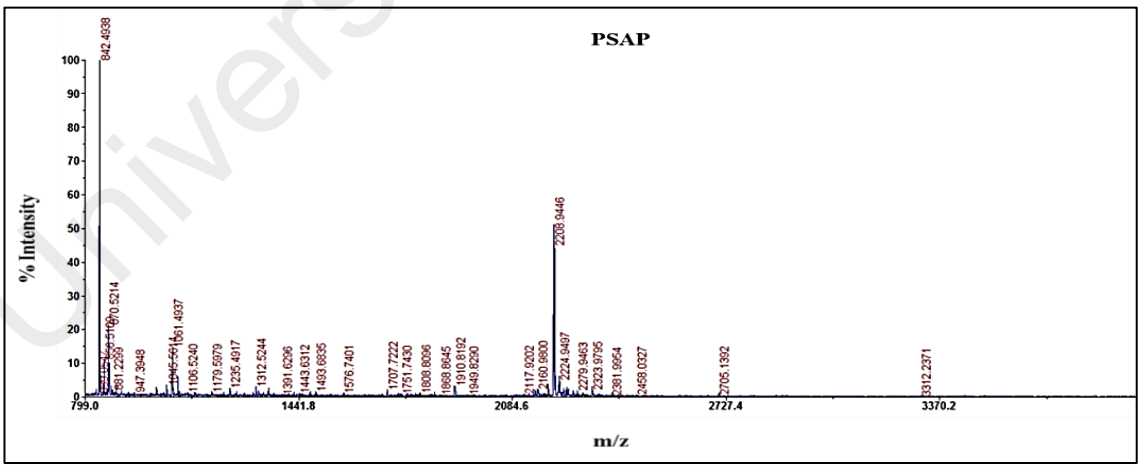


Figure 4.17. PMF of trypsin digested urinary peptides.

Clusters of protein of spots of interest excised from resolved 2-DE gels were tryptic digested and analysed by a mass spectrometer. Identities of proteins of altered abundance including (panel A) AMBPf1, (panel B) AMBPf2 and (panel C) PSAP were confirmed using 4800 Plus MALDI ToF/ToF analyser.

Table 4.4. MS/MS derived peptide sequences of urinary proteins of altered abundance.

Spot/Cluster ID	Peptide sequence	Amino acid	Ion score
AMBPf1	K.KEDSCQLGYSAGPCMGMTSR.Y	227 - 246	75
	K.EDSCQLGYSAGPCMGMTSR.Y	228 - 246	113
	R.TVAACNLPIVR.G	283 - 293	57
	R.AFIQLWAFDAVK.G	298 - 309	84
	K.FYSEKECR.E	327 - 334	12
AMBPf2	K.KEDSCQLGYSAGPCMGMTSR.Y	227 - 246	14
	K.KEDSCQLGYSAGPCMGMTSR.Y	227 - 246	42
	K.EDSCQLGYSAGPCMGMTSR.Y	228 - 246	80
	K.ECLQTCR.T	276 - 282	29
	R.TVAACNLPIVR.G	283 - 293	57
	R.AFIQLWAFDAVK.G	298 - 309	71
PSAP	K.DNGDVCQDCIQMVTDIQTAVR.T	193 - 213	17
	R.LGPGMADICK.N	233 - 242	61
	R.LGPGMADICK.N	233 - 242	33

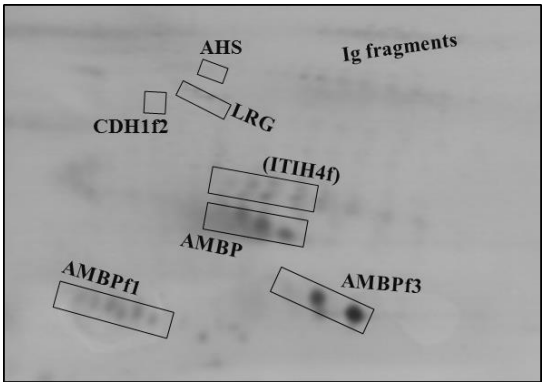
4.3.4. Typical HRP-conjugated CGB lectin generated 2-DE urinary *O*-glycosylated protein profiles of control subjects and patients with BPH and those with PCa

The 2-DE resolved urinary proteins of patients with BPH, those with PCa and controls subjects were similarly subjected to electroblotting and HRP-conjugated CGB lectin detection as previously described (Abdul Rahman *et al.*, 2002). Figure 4.18 demonstrates the 2-DE profiles of urinary *O*-glycosylated proteins of patients' with PCa and those with BPH as well as control subjects that were generated using HRP-conjugated CGB lectin as probe. A total of seven clusters of *O*-glycosylated urinary protein spots were consistently detected in all lectin-developed profiles. The profile closely resembled the CGB lectin blot urinary protein profiles of Soheimi *et al*., (2010) and Mu *et al*., (2012). Proteins detected include the heavy chains of IgA, AHS, AMBP, AMBPf1, AMBPf3, CDH1f2, LRG and ITIH4f. The use of HRP-conjugated CGB lectin had facilitated the unmasking of *O*-glycosylated proteins that was not significantly detected in the previous experiments.

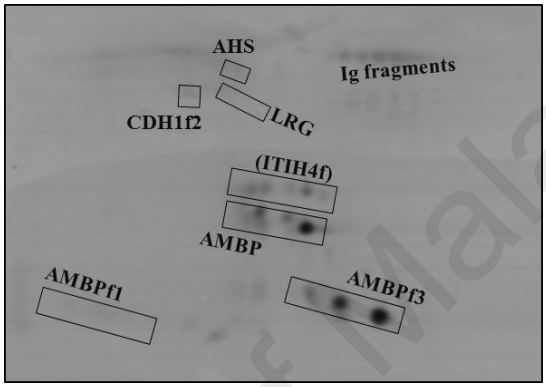
4.3.5. Image analyses of HRP-conjugated CGB lectin generated 2-DE urinary *O*-glycosylated protein profiles of patients with BPH and those with PCa as well as control subjects

A total of 7 clusters of urinary *O*-glycosylated protein spots were assessed for aberrance in abundance. The densitometry analysis of the HRP-conjugated CGB lectin detected urinary protein profiles of patients with BPH (n = 20) and those with PCa (n = 13) as well as control subjects (n = 15) demonstrated enhanced level of ITIH4f in the patients with PCa compared to control subjects (+4.5 fold, p = 0.006) (Figure 4.19).

A)



B)



C)

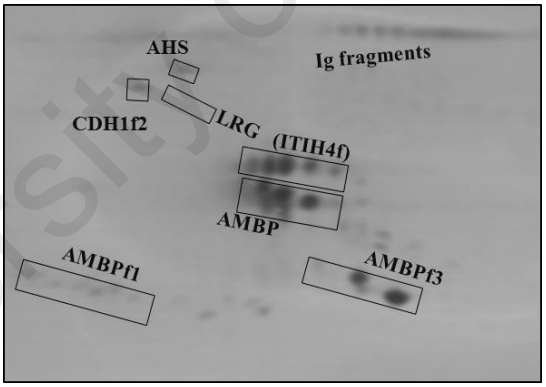


Figure 4.18. Detection of *O*-glycosylated urinary proteins using HRP-conjugated CGB lectin.

HRP-conjugated CGB lectin was used to probe *O*-glycosylated proteins from urine of subjects that were subjected to 2-DE and later, electroblotted onto NC membranes. Identities of the proteins detected were made based on visual comparison of the blot profiles with those reported by Soheimi *et al*., (2010) and Mu *et al*., (2012). Panels A, B and C demonstrate HRP-conjugated CGB lectin detected *O*-glycosylated urinary protein profiles from control subjects, patients with BPH and those with PCa, respectively. The proteins spots of altered abundance are marked in bracket.

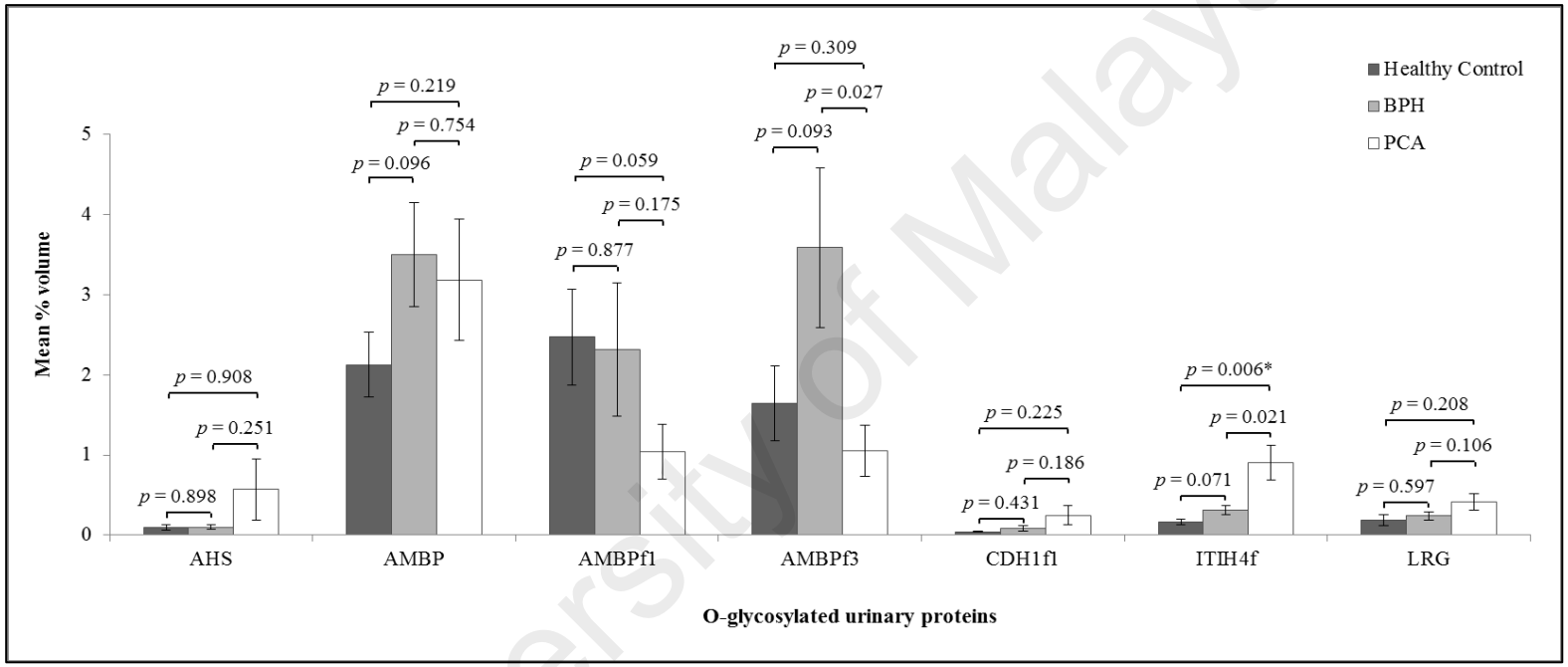


Figure 4.19. Mean percentage of volume contribution of CGB lectin detected urinary *O*-glycosylated protein.

Panel demonstrates analysis of *O*-glycosylated proteins detected by HRP-conjugated CGB lectin in urine of subjects. Percentage of volume contribution of protein spots was analysed using ImageMaster™ 2D Platinum software, version 7.0. Densitometry analysis was performed on 7 clusters of protein spots including AHS, AMBP, AMBPf1, AMBPf3, CDH1f2, LRG and ITIH4f. Asterisk denotes *O*-glycosylated proteins with significant alteration in abundance in terms of the percentage of volume contribution.

4.3.6. Confirmation of presence of ITIH4f in urine using CGB lectin affinity column chromatography and nano-LC MS/MS

4.3.6.1. Isolation of urinary *O*-glycosylated proteins

To validate the presence of ITIH4f in urine samples that was observed in the HRP-conjugated CGB lectin generated 2-DE urinary *O*-glycosylated protein blot profiles (Figure 4.18), pooled concentrated urinary protein from controls (n = 15) were subjected to CGB lectin affinity chromatography as described in Section 3.9.3. Figure 4.20 demonstrates elution profiles of affinity separation of the pooled urine samples. The ratio of bound to total protein fractions was 0.15. Pooled urine was used in these experiments due to the limited availability of samples. The eluted bound fractions containing *O*-glycosylated urinary protein were subsequently subjected to in-solution trypsin digestion and profiling by nano LC MS/MS as described in Section 3.16.

To investigate whether the urinary proteins had non-specifically and/or directly interacted with the chromatographic matrix, a similar experiment was performed but using uncoupled activated and inactivated Sepharose 4B. Figures 4.21 demonstrate no non-specific interaction between pooled urinary proteins with both the uncoupled activated (panel A) and inactivated (panel B) chromatographic matrices, respectively.

4.3.6.2. Profiling and identification of isolated urinary *O*-glycosylated proteins by nano-LC MS/MS

Based on 99.9% of detected protein threshold confidence and competitor error margin at 2.00, a total of 21 *O*-glycosylated proteins were collectively profiled and identified from pooled urinary samples of control subjects (Table 4.5). These include ITIH4f (bold) and thus, in agreement with the initial visual comparison to the previously

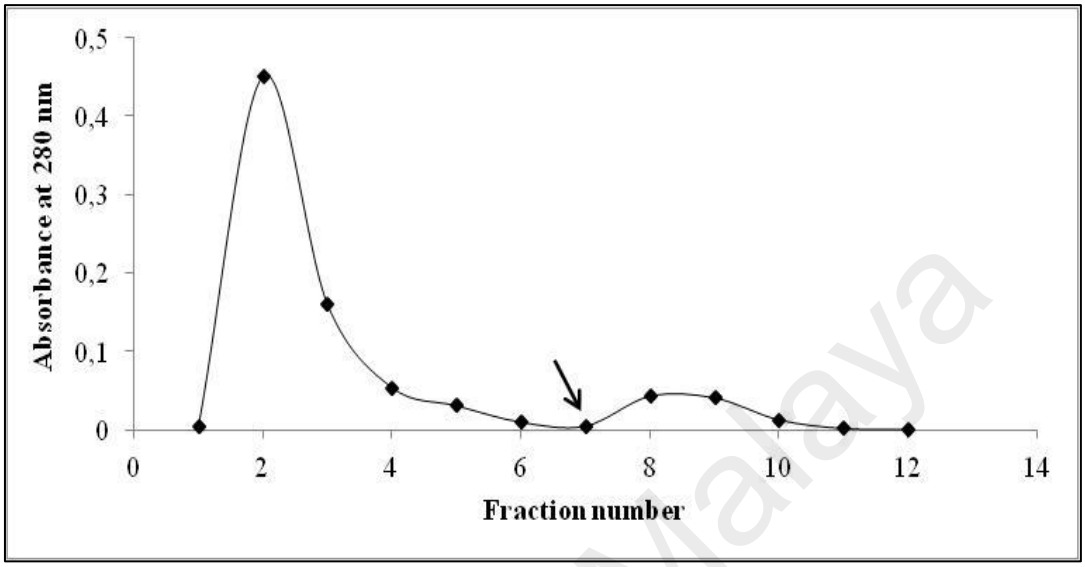
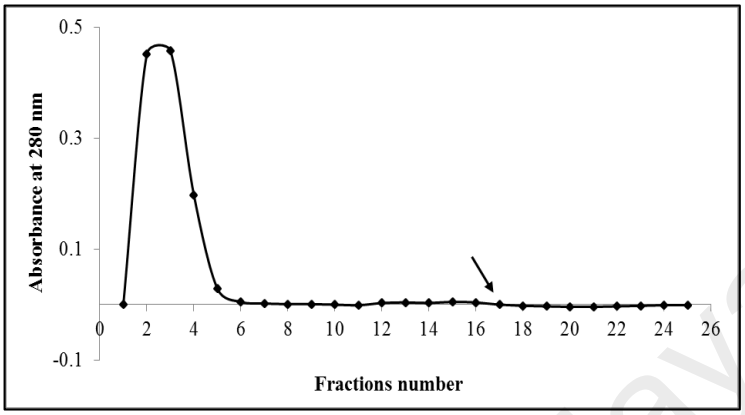


Figure 4.20. Typical affinity separation profile of pooled urine samples obtained from control subjects.

Pooled urine samples from control subjects (n = 15) were subjected to CGB lectin affinity chromatography. The fractions containing *O*-glycosylated urinary proteins were eluted with 0.1 M melibiose in PBS, pH 7.2 as described in Section 3.9.3. Collected fractions were monitored spectrophotometrically by measurement of absorbance at 280 nm. Arrow indicates start of elution with 0.1 M melibiose in PBS, pH 7.2.

A)



B)

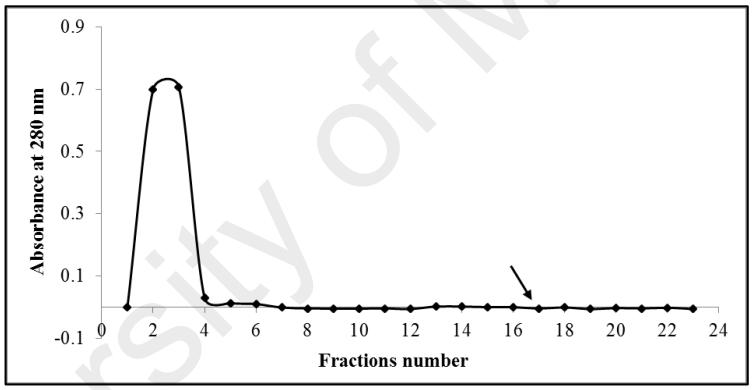


Figure 4.21. Affinity profiles of pooled urinary proteins and uncoupled activated and inactivated chromatographic matrices.

Pooled urine samples from control subjects (*n* = 15) were subjected to affinity chromatography using uncoupled activated (panel A) and inactivated (panel B) Sepharose 4B matrices. Pooled concentrated urinary samples were loaded separately into a 3 ml gel beds of uncoupled activated and inactivated Sepharose 4B matrices. Collected fractions were monitored by measurement of absorbance at 280 nm. Arrow indicates start of elution with 0.1 M melibiose in PBS, pH 7.2.

published work of Soheimi *et al*., (2010) and Mu *et al*., (2012). Table 4.5 demonstrates the list of O-glycosylated proteins isolated, separated and identified using CGB lectin affinity chromatography and nano LC MS/MS. The predictions of mucin type GalNAc O-glycosylation sites in the isolated urinary proteins were determined using NetOGlyc 4.0 server. Table 4.6 on the other hand, shows the respective MS/MS derived peptide sequences of ITIH4f.

4.4. VALIDATION OF PROTEIN ABUNDANCE IN SERUM AND URINE SAMPLES USING WESTERN BLOTTING - LECTIN AND/OR ANTIBODY DETECTION METHODS

In view of the interest and potential applications of serum ITIH4f and urinary proteins including ITIH4f, AMBPf1 (also known as ITILf1) and AMBPf2 (also known as ITILf2) as biomarkers, a validation of their altered abundance in the serum and urine samples were performed via lectin- and/or immuno-blotting techniques as described in Sections 3.13.1 and 3.13.2, respectively. Detection using HRP-conjugated CGB lectin was opted for validation of serum and urinary ITIH4f. On the other hand, immunodetection was performed using commercially available polyclonal antibodies against urinary ITIL proteins.

Table 4.5. List of urinary *O*-glycosylated proteins.

Protein Name	Accession No[a]	Subcellular Location	Glycan[b]
Polymeric Ig receptor	P01833	Membrane	Potential *O*-linked
Vasorin	Q6EMK4	Membrane	Potential *O*-linked
CD55	P08174	Membrane	Potential *O*-linked
Protein AMBP	P02760	Secreted	*O*-linked
LDL receptor	P01130	Membrane	Potential *O*-linked
IGL@ protein	Q6GMX4	Unknown	Potential *O*-linked
CD44 antigen	P16070	Membrane	*O*-linked
Hyaluronic acid receptor 1	Q9Y5Y7	Membrane	*O*-linked
Peptidase inhibitor 16	Q6UXB8	Membrane	*O*-linked
Ig J chain	P01591	Secreted	Potential *O*-linked
Tenascin-X	P22105	Secreted	Potential *O*-linked
Alpha-1B glycoprotein	P04217	Secreted	Potential *O*-linked
Apolipoprotein D	P05090	Secreted	Potential *O*-linked
Amyloid beta A4 protein	P05067	Membrane	*O*-linked
Kininogen-1	P01042	Secreted	*O*-linked
Phosphoinositide-3-kinase interacting protein 1	Q96FE7	Membrane	*O*-linked
Inter-alpha-trypsin inhibitor heavy chain 4	**Q14624**	**Secreted**	***O*-linked**
Ig kappa chain C region	P01834	Unknown	Potential *O*-linked
Ig alpha-1 chain C region	P01876	Unknown	*O*-linked
Ig lambda-1 chain C regions	P0CG04	Unknown	*O*-linked
Ig lambda-2 chain C regions	P0CG05	Unknown	Potential *O*-linked

a) Accession numbers are from the Swiss-Prot database (http://www.uniprot.org).

b) The types of glycan are as annotated in Uniprot database (http://www.uniprot.org) and the predictions of mucin type GalNAc *O*-glycosylation sites in the isolated urinary proteins were determined using NetOGlyc 4.0 server.

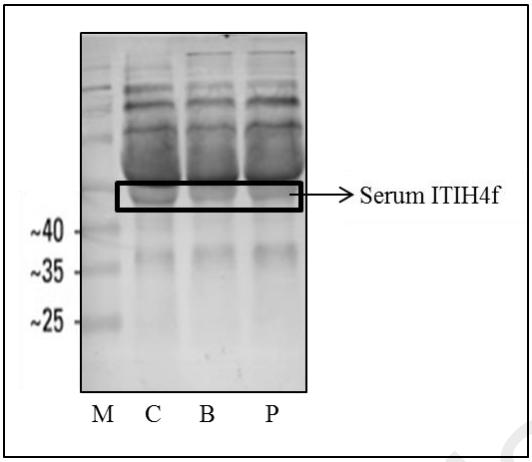
Table 4.6. MS/MS derived peptide sequences of ITIH4f.

Cluster ID	Peptide Sequence	Amino acid	Ion score
ITIH4f	R.QGPVNLLSDPEQGVEVTGQYER.E	754 - 775	114
	K.AGFSWIEVTFK.N	778 - 788	59
	K.NPLVWVHASPEHVVVTR.N	789 - 805	81
	K.WKETLFSVMPGLK.M	814 - 826	11
	K.ETLFSVMPGLK.M	816 - 826	39
	K.ETLFSVMPGLK.M	816 - 826	3
	K.TGLLLLSDPDK.V	832 - 842	15

4.4.1. Detection of serum and urinary ITIH4f protein

Representative blot images of pooled serum and urine samples obtained from patients with BPH and those with PCa as well as control subjects resulted from Western blotting with subsequent CGB lectin detection is demonstrated in Figure 4.22. Panel A demonstrates a representative electrotransferred NC membrane consisting of serum proteins that were probed with HRP-conjugated CGB lectin. Following HRP-DAB substrate reaction, an irreversibly stained dark brown band with a M_r of approximately 50 kDa indicative of ITIH4f was detected on each lane, representing each group of subjects. When the detected bands were subjected to densitometric analysis, the levels of ITIH4f appeared lowest in the pooled sera of subjects with PCa (Table 4.7). Panel B, on the other hand, exhibits a representative blot image of CGB lectin detected urinary proteins on electrotransferred NC membrane. Similarly, dark brown ~50 kDa bands, indicative of ITIH4f, were subjected to densitometric analysis. The abundance of urinary ITIH4f protein was significantly highest in the pooled urine samples of subjects with PCa ($p < 0.05$) (Table 4.7).

A)



B)

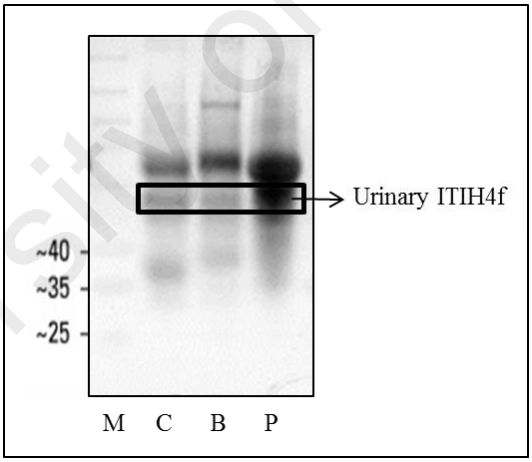


Figure 4.22. Representative blot images of serum and urinary ITIH4f detected using CGB lectin.

Pooled serum and urine samples from control subjects (C), patients with BPH (B) and those with PCa (P) were separated on 10% SDS-PAGE gels. Detection of the glycosylated proteins electro-adsorbed onto NC membrane was performed using HRP-conjugated CGB lectin. Panels A and B represent lectin blots for serum and urinary proteins, respectively. The SDS-PAGE gel was calibrated using broad range protein ladder (M) (Thermo Scientific, Rockford, USA).

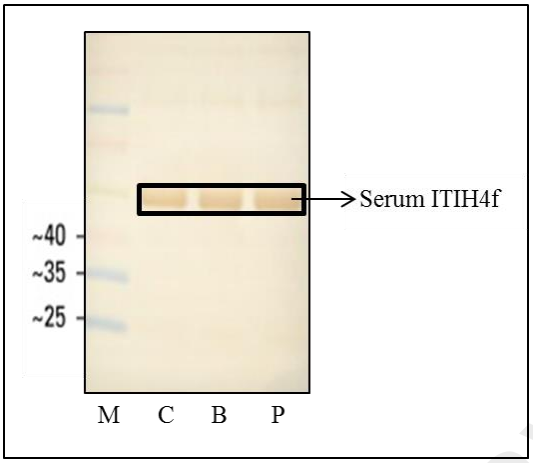
4.4.2. Confirmation of ITIH4f protein bands

In order to confirm the location or M_r of ITIH4f on the NC membranes, similar Western blotting experiments were repeated but with subsequent probing of NC membranes using commercially available polyclonal antibodies against serum and urinary ITIH4f proteins. Representative blot images resulted from the Western blotting with subsequent antibody detection of the pooled serum and urine samples obtained from patients with BPH and those with PCa as well as control subjects are demonstrated in Figure 4.23. Panels A and B demonstrate representative electrotransferred NC membranes consisting of serum and urinary proteins that were probed with anti-ITIH4, respectively. Following HRP- DAB substrate reaction, protein bands of approximately 50 kDa corresponding to the blot images resulted from probing with CGB lectin (Figure 4.22) were detected on each lane representing each group of subjects.

4.4.3. Detection of urinary ITILf1 and ITILf2 proteins

To validate the different altered abundance of ITILf1 and ITILf2 in urine of controls and patients with BPH and PCa, a Western blotting analysis was performed using anti-bikunin + trypstatin. Representative blot image resulted from the experiment are shown in Figure 4.24. Following HRP-DAB substrate reaction, bands with M_r of approximately 25 kDa and 18 kDa, which are indicative of ITILf1 and ITILf2 proteins respectively, were detected on each lane representing each group of subjects. When the detected bands were subjected to densitometric analysis, the levels of ITILf1 was significantly least most intense among subjects with PCa compared to control subjects, whilst that of ITILf2, was significantly lowest in subjects with PCa compared to those with BPH ($p < 0.05$). Table 4.7 demonstrated summarised results of validation performed for selected serum and urinary proteins of altered abundance.

A)



B)

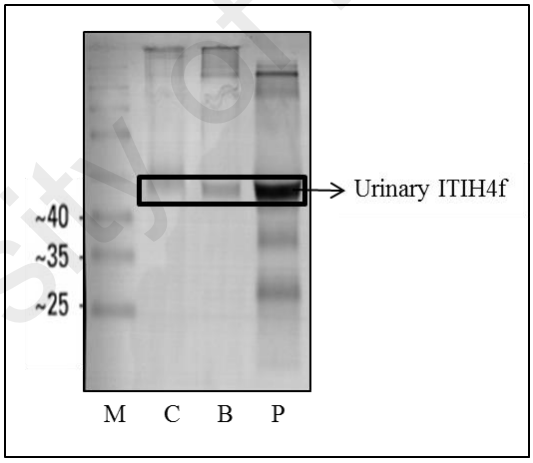


Figure 4.23. Representative blot images for serum and urinary ITIH4f detected using anti-ITIH4.

Pooled serum and urine samples from control subjects (C), patients with BPH (B) and PCa (P) were separated on 10% SDS-PAGE gels. Immunodetection of the proteins electro-adsorbed onto NC membrane was performed using anti-ITIH4. Panels A and B represent immunoblots for serum and urinary proteins, respectively. The SDS-PAGE gel was calibrated using broad range protein ladder (M) (Thermo Scientific, Rockford, USA).

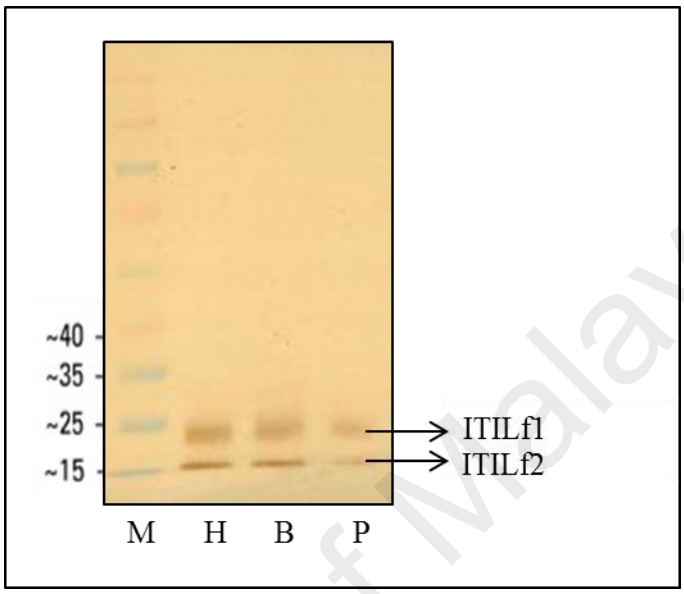


Figure 4.24. Representative blot image for urinary ITIL fragments detected using immunodetection method.

Pooled urine samples from control subjects (H), patients with BPH (B) and those with PCa (P) were separated on 10% SDS-PAGE gel. Immunodetection of ITIL protein fragments electro-adsorbed onto NC membrane was performed using anti-bikunin + trypstatin. The SDS-PAGE gel was calibrated using broad range protein ladder (M) (Thermo Scientific, Rockford, USA).

Table 4.7. Indices relating to the levels of serum and urinary fragment proteins of ITIH4 and ITIL.

Parameter	Mean % volume*			*p* value
	Control	BPH	PCa	
Serum ITIH4f¥	0.49 ± 0.07	0.29 ± 0.03	0.22 ± 0.07	0.097
Urine ITIH4f¥	0.60 ± 0.05	0.65 ± 0.01	3.66 ± 0.24[a]	< 0.001
Mean ratio[1]	0.82	0.45	0.06	
Range of ratio	0.69 – 0.94	0.41 – 0.50	0.04 – 0.08	
Urine ITIH4f¥	0.60 ± 0.05	0.65 ± 0.01	3.66 ± 0.24[a]	< 0.001
Urine ITILf1∞	0.74 ± 0.07	0.52 ± 0.07	0.39 ± 0.01[b]	0.014
Mean ratio[2]	0.81	1.25	9.31	
Range of ratio	0.74 – 0.94	1.10 – 1.46	8.71 – 9.50	
Urine ITIH4f¥	0.60 ± 0.05	0.65 ± 0.01	3.66 ± 0.24[a]	< 0.001
Urine ITILf2∞	0.38 ± 0.09	0.40 ± 0.04	0.12 ± 0.04[c]	0.040
Mean ratio[3]	1.57	1.63	31.12	
Range of ratio	1.28 – 2.02	1.45 – 2.01	23.14 – 47.45	

*Data presented in mean ± S.E.M of the mean percentage volume of protein.

¥CGB lectin detection; ∞Immunodetection.

Indices : [1]Ratio of mean % volume of serum ITIH4f to urine ITIH4f;
[2]Ratio of mean % volume of urine ITIH4f to urine ITILf1;
[3]Ratio of mean % volume of urine ITIH4f to urine ITILf2.

Significance ($p < 0.05$) : [a]PCa compared to BPH & controls;
[b]PCa compared to controls;
[c]PCa compared to BPH.

DISCUSSION

5.1. CGB LECTIN

Isolation of CGB lectin of good quality, in terms of concentration and purity is an important factor for all subsequent downstream applications. These include the preparation for affinity column chromatography and also, for the use of CGB lectin as conjugated probes for the detection of *O*-glycosylated proteins in human serum and urine. In the past, the capability of CGB lectin in uniquely recognizing and binding to the *O*-linked Galβ1-3GalNAc structure of IgA1 and C1 inhibitor molecules (Hashim *et al.*, 1994; Hashim *et al.*, 1993; Hashim *et al.*, 1991) have been exploited to study the altered abundance of serum and urinary proteins obtained from groups of patient with various types of cancers (Abdul-Rahman *et al.*, 2007; Abdullah-Soheimi *et al.*, 2010; Mohamed *et al.*, 2008; Mu *et al.*, 2012) and other benign conditions (Mohamed *et al.*, 2013) in our laboratory.

Considering these, the concentration and purity of the isolated CGB lectin was first determined in this study. Using commercially available BCA protein assay kit and subsequent construction of standard curve for BSA, the concentration of CGB lectin was estimated to 3.5 mg/ml. On the other hand, the profiling of CGB lectin showed two distinct peaks and/or bands at approximately 14.6 kDa and 16 kDa when subjected to SELDI-ToF/MS and one-dimensional (18%) SDS-PAGE under reducing condition and thus, confirming the effectiveness of the purification method used. The resolution of CGB lectin into two peaks and/or bands was in agreement with previous reports from our laboratory (Abdul Rahman *et al.*, 2002; Hashim *et al.*, 1991), if not for the incongruence in the M_r. This minor slight in M_r of the peaks and/or bands could probably be due to the differences in the choice of percentage of resolving gels used for the assessment. Previously, Hashim *et al.*, (1991) had meticulously characterised the

structural composition of CGB lectin to dissociate into identical primary structure of two types of non-covalently linked subunits, α and β^1, and due absence of disulphide bonds between them. Characteristic dissimilarity of the subunits was however noted, in terms of the resulting differential patterns of glycosylation (Aucouturier *et al.*, 1989; Kabir *et al.*, 1993; Ruffet *et al.*, 1992).

Additionally, the spectrum resulted from SELDI-ToF/MS analysis had also demonstrated peaks of doubly charged molecular ions of CGB lectin at approximately half the initial m/z values; M_r of 7.3 kDa and 8 kDa, respectively. This is because, proteins with M_r greater than 1200 Da and/or in their native state (or containing significant amount of protein folding) that has many sites for protonation are known to produce multiply charged molecular-related ions in positive ionisation MS mode (Ashcroft, 1997). On the other hand, these doubly charged peaks representing bands were not observed in the resolved SDS-PAGE gel. This is due to the use of SDS and a thiol reducing agent (DTT) in the sample preparation. SDS forms complexes with proteins whilst, the reducing agent disrupts disulfide bonds between and within proteins, allowing complete denaturation and dissociation. Additionally, heat treatment in the presence of SDS and reducing agent completely eliminates the effects of native charge and protein structure and as such, unlike in native PAGE separations, electrophoretic mobility of the CGB lectin depends primarily on its molecular weight and not mass-to-charge ratio. Secondly, the optimum separation range for 18% SDS-PAGE gels used for profiling of CGB lectin is between 10 - 50 kDa, therefore, the lower molecular weight protein of doubly charged CGB lectin at approximately 7.3 and 8.0 kDa was poorly resolved. Nevertheless, owing to the high sensitivity and feasibility of use of SELDI-ToF/MS (Lau *et al.*, 2011), the technique was favoured over SDS-PAGE for the determination of relatively low M_r protein of CGB lectin.

5.2. PROFILING OF SERUM PROTEINS OF PATIENTS WITH PCa AND THOSE WITH BPH

PSA is still being used to provide provisional clinical opinion in monitoring as well as to detect asymptomatic men with PCa (Greene *et al.*, 2013; Schröder *et al.*, 2009; Wolf *et al.*, 2010) despite rising controversies and evidences on its lack of specificity (Chou *et al.*, 2011). Considering the downsides of levels of serum PSA as biomarker for PCa, myriad studies have attempted to identify markers to distinguish men with aggressive PCa from indolent ones, in serum, urine and tissue-based media using various genomic and proteomics tools as well as novel bioinformatics approaches for both diagnostic and prognostic predictability of PCa with accuracy.

For example, circulating serum proteins including acid phosphatase and interleukin-6, and tissue proteins including protein kinase B (Akt-1) (Ayala *et al.*, 2004) and Ki-67 antigen (Pollack *et al.*, 2004) have been previously shown to serve as predictive prognostic markers whilst, serum kallikreins and PSA as diagnostic markers. There were also serum and tissue protein markers reported to indicate progression of PCa. These include prostate secretory protein (PSP94) (Huizen *et al.*, 2005) and prostate stem cell antigen (PSCA) (Reiter *et al.*, 1998), respectively. On the other hand, DNA-based type of biomarker for diagnosis of PCa were also numerous. To name a few, these include single nucleotide polymorphisms (SNPs) at various loci (e.g., rs1447295, rs1859962, rs1800629, rs2348763) (Nam *et al.*, 2009), glutathione S-transferase pi 1 (GSTP1) (Jeronimo *et al.*, 2002) and prostaglandin-endoperoxide synthase (PTGS) (Ellinger *et al.*, 2008). It is indeed noteworthy, as the latter is also known for demonstrating dual role as both diagnostic and prognostic marker for PCa. In addition, the expression and significance of TMPRSS2/ERG fusion (Wang *et al.*, 2006), golgi membrane protein 1 (Varambally *et al.*, 2008), serine peptidase inhibitor

Kazal type 1 (Tomlins *et al.*, 2008), telomerase reverse transcriptase (Crocitto *et al.*, 2004), trefoil factor 3 (Garraway *et al.*, 2004), α-methylacyl coenzyme A racemase (AMACR) (Zehentner *et al.*, 2006) and prostate cancer antigen 3 (PCA3) (Deras *et al.*, 2008) messenger RNAs also does add new perspective to detection as well as to foretell the course of PCa. Interestingly, differentially expressed metabolites including 8-hydroxydeoxyguanosine (Chiou *et al.*, 2003) and sarcosine (Sreekumar *et al.*, 2009) were also reported as emerging biomarkers to assist in identification of aggressive tumours for radical treatments. Last but not least, microRNA (Mitchell *et al.*, 2008) as well as bone DTC (Kollermann *et al.*, 2008) had also demonstrated potential prognostic predictability for PCa. Table 5.1 enumerates a list of selected biomarkers for distinguishing PCa from BPH.

Utilisation of proteomic approaches had greatly expanded the opportunity for identification of new potential biomarkers for PCa. In line with this, various sample types including serum, urine, prostatic fluid, cell lysates and nuclear matrices have been previously investigated using conventional 2-DE methodology. A protein D expressed in the prostatic fluid was found exclusively in patients with PCa (Grover and Resnick, 1995) whilst, several urinary (Grover and Resnick, 1997) and nuclear matrix proteins (Partin *et al.*, 1993) were altered in abundance in patients with PCa compared to control subjects. In another study, Alaiya *et al.*, (Alaiya *et al.*, 2001), had shown differential expression of an array of proteins (e.g., proliferating cell nuclear antigen, calreticulin, elongation factor 2, oncoprotein 18 (V), tropomyosin-1 and cytokeratin 18) between subjects with PCa and those with BPH in cell lysates obtained from post-surgical prostatic tissues. With the advent of gel-free proteomics approaches including ProteinChip immunoassay (Xiao *et al.*, 2001), SELDI-ToF/MS (Banez *et al.*, 2003; Cazares *et al.*, 2002; Petricoin *et al.*, 2002a) and LC-MS/MS based methods such as ICAT (Griffin *et al.*, 2003), SILAC (Everley *et al.*, 2004) and iTRAQ

Table 5.1. Biomarkers for PCa.

Markers	Sample / Approach	Type of biomarker	Application	References
Acid phosphatase	Serum	Protein	Prognostic	Whitesel *et al*., 1984
Kallikrein markers	Serum, Predictive model	Protein	Diagnostic	Benchikh *et al*., 2010; Vickers *et al*., 2010
Interleukin-6	Serum	Protein	Prognostic	Michalaki *et al*., 2004
PSCA	Tissue / RT-PCR & In situ hybridisation	Protein	Progression	Reiter *et al*., 1998
PSP94	Serum / Mouse model	Protein	Progression	Huizen *et al*., 2005
Akt-1	Tissue / Microarray & IHC	Protein	Prognostic	Ayala *et al*., 2004
Ki-67 Antigen	Tissue / IHC	Protein	Prognostic	Pollack *et al*., 2004
PSA	Serum	Protein	Diagnostic	Howlader *et al*., 2012; Omar *et al*., 2011
SNPs at loci	Blood	DNA	Diagnostic	Salinas *et al*., 2009; Zheng *et al*., 2008
GSTP1	Plasma & urine / QMSP	DNA	Diagnostic	Jeronimo *et al*., 2002
PTGS	Tissue / PCR	DNA	Diagnostic & prognostic	Ellinger *et al*., 2008
AMACR	Blood and urine / RT-PCR	RNA	Diagnostic & prognostic	Zehentner *et al*., 2006
PCA3	Urine	RNA	Diagnostic	Bussemakers *et al*., 1999; Deras *et al*., 2008
miR-141	Serum	MicroRNA	Prognostic	Mitchell *et al*., 2008
Sarcosine	Urine	Metabolite	Progression	Sreekumar *et al*., 2009
Bone - DTC	Bone marrow aspirate / IHC	DTC	Prognostic	Kollermann *et al*., 2008

Key: RT-PCR, real time-polymerase chain reaction; IHC, immunohistochemistry; QMSP, quantitative methylation specific PCR; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

(Glen *et al.*, 2008; Rehman *et al.*, 2012), many other novel potential markers for differentiating PCa from non-malignant conditions were identified as well.

Unfortunately, none thus far, had poised to be a definitive stand-alone predictive marker of PCa. Having explored and understand the limitations of these studies, in this study, protein and glycosylated protein profiles of serum and urine samples of subjects with PCa hand in hand, via gel- and lectin-based proteomic platforms were investigated. This is intended to provide a better opportunity for an early non-invasive detection which would in turn, lead to a better prognosis and wider therapeutic options for subjects with PCa.

Human plasma or serum proteome houses the entire subset of human proteins including the secreted tissue proteins and immunoglobulin sequences with an extraordinary dynamic range of more than 10 orders of magnitude as well as those synthesised within the plasma or serum (Anderson and Anderson, 2002; Thadikkaran *et al.*, 2005). Changes in structures and/or abundance of plasma or serum proteins had been extensively used as means of diagnosis and prognosis of diseases as well as therapeutic monitoring. With the pressing need to discriminate PCa from BPH due to similarities in clinical presentations of these conditions, the first part of the present study was initiated by subjecting an optimised concentration of the whole sera (800 µg) from subjects with BPH and those with PCa to 2-DE, with the intention to identify proteins of altered abundance, if present. The 2-DE resolved gels were either silver stained or transferred to a NC membrane for the detection of *O*-glycosylated serum proteins for the differential analysis of protein spots or clusters based on their percentage of volume contribution.

An array of well-resolved high abundance serum proteins were consistently observed in the silver-stained 2-DE separated unfractionated serum samples obtained

from patients with PCa and those with BPH. These serum proteins include AAT, ABG, AHS, CLU, HAP, KNG-1, LRG, CFH, VDP, TRF, APOA2, C3 and ZAG. However, with exceptions of two serum proteins, namely, APOA2 and complement C3, none of the other high abundance serum proteins were found to be differentially expressed when 2-DE protein profiles generated from the sera of patients with BPH were compared with those generated from PCa patients' sera. Mass spectrometry analysis had confirmed the identities of proteins of altered abundance - APOA2 and C3 beta chain fragment, respectively.

APOA2, the second most common protein in high density lipoprotein (HDL) is involved in the metabolism and reverse transport of cholesterol (HDL-C) to the liver. Its deficiency has been previously reported to result in conditions including hypercholesterolaemia (Kalopissis *et al.*, 2003; Maiga *et al.*, 2014) and neurodegenerative diseases such as Alzheimer's (Kawano *et al.*, 1995) and senile dementia (Kuriyama *et al.*, 1994). Genetic polymorphism of *APOA2* gene, has also been invariably linked to plasma HDL-C levels and/or composition (Brousseau *et al.*, 2002; Welch *et al.*, 2004), which in turns confers to incidences of insulin resistance (Zaki *et al.*, 2014) and metabolic disorders (Zaki *et al.*, 2013). APOA2 is also known to inhibit HDL remodelling by cholesteryl ester transfer protein and thus, leading to an increase in size and composition of HDL (Rye *et al.*, 2003; Zhong *et al.*, 1994) and augment monocytes-host responses to LPS during sepsis (Thompson *et al.*, 2008).

The association between the status of lipid metabolising proteins including various subsets of apolipoproteins and predisposition to cancer had long been corroborated. Along that line, proteins of lipid metabolic pathways including APOA2 (Jayapalan *et al.*, 2012; this thesis) , APOD (Aspinall *et al.*, 1995), APOE (Lehrer, 1998), APOJ (Trougakos and Gonos, 2002), fatty acid synthase (Myers *et al.*, 2001) and

circulating cholesterol (Freeman and Solomon, 2004) have been extensively implicated in PCa. Interestingly, as seen in this study, cancer-associated decline of levels of APOA2 has been previously reported in subjects with pancreatic cancer (Ehmann *et al.*, 2007). Conversely, in another study, overexpression of an isoform of serum APOA2 at approximately 8.9 kDa was also reported to pose as a discriminating factor for subjects with PCa from those without, using SELDI-ToF/MS. Although the mechanism of involvement of APOA2 in the pathogenesis of PCa is poorly understood, some data had paradoxically suggested the role play of proteins of lipid metabolic pathway via cellular proliferation or apoptosis (Trougakos and Gonos, 2002; Vogel *et al.*, 1994). As such, lower abundance of APOA2 in patients with PCa observed in the present study bears consistencies with this hypothesised role, possibly in suppression of PCa at an early stage. Secondly, the lower levels of APOA2 observed in this study may also be due to disturbed activity or balance of proteases including metalloproteinases, cathepsins, trypsin and protease inhibitors in the tumour microenvironment (Ehmann *et al.*, 2007).

C3, the second serum protein of altered abundance that was detected in the present study, is a complement protein where both the classical and alternative pathways converge. Besides playing a central role in the activation of both the classical and alternative pathways of complement, it also sub-serves a number of critical functions including solubilisation of immune complexes, enhancing bacterial killing either through membrane attack-complex formation or opsonophagocytosis, and potentiation of humoral immune response. Therefore, subjects with low levels of C3 are generally susceptible to severe infections caused by *Streptococcus pneumoniae*, *Hemophilus influenzae*, and *Neisseria meningitidis* (Reis *et al.*, 2006; Walport, 2003) as well as to recurrence of infection (Bonnin *et al.*, 1993). On another note, C3 was postulated to offer beneficial neuroprotective role via clearance of amyloid beta plaque deposition

and modulation of microglia or macrophage phenotypes in amyloid precursor protein transgenic mice (Maier *et al.*, 2008).

Nonetheless, it is also interesting to note that complement cascade C3, C3a, C3a-des arginine (the anaphylatoxin C3a that lacks the C-terminal arginine) and C3d have been suggested to facilitate major features of carcinogenesis including angiogenesis, invasion and metastasis and sustained cellular proliferation via growth factors or production of cytokines (Rutkowski *et al.*, 2010). Having said that, C3a fragment at approximately 9 kDa, has been previously reported to be elevated in patients with hepatocellular carcinoma (Kanmura *et al.*, 2010), breast cancer (Fan *et al.*, 2010) and lymphoid malignancies (Miguet *et al.*, 2006) whilst C3a-des arginine fragment, in colorectal cancer (Fentz *et al.*, 2007; Ward *et al.*, 2006) using SELDI-ToF/MS. This is certainly compatible with the diminished amounts of C3 seen in the sera of PCa patients in this study. On another note, observation of decreasing levels of C3 fragment in men with advancing stage of squamous cell carcinoma of penis highly indicates the prognostic capability of C3 fragments (Ornellas *et al.*, 2012).

Aberration in abundances of glycoproteins in cancer (Dennis *et al.*, 1999; Nishimura *et al.*, 1995) has been thought to serve a valuable avenue for identification of potential biomarkers for its diagnosis, prognosis and therapeutic monitoring. In essence of this, precise detection of aberration in glycoprotein abundance is of paramount importance. Considering the threat posed by the broad dynamic range of more than 10 orders of magnitude of serum or plasma, in the past, enhanced detection and resolution of lower abundance proteins (Anderson and Anderson, 2002) were successfully achieved either by immunosubtraction of high abundance, interfering or contaminating proteins (Jenkins *et al.*, 2008), incorporation of other chromatographic methodologies such as size exclusion, ion exchange, affinity binding and hydrophobic interaction prior

to 2-DE, or via utilising lectins as probes for detection of post-translationally-modified subset of proteins, depending on the carbohydrate binding specificity of the lectin used. With this in mind, works in our laboratory had previously demonstrated significant *N*- and *O*-glycosylated proteins of altered abundance with cancer biomarker potentials via the use of HRP-conjugated lectins serving as probes, alongside 2-DE workflow. The types of cancer that had been correlated with aberration in the abundance of *O*-glycosylated proteins, thus far, include those of the endometrium (Mu *et al.*, 2012), ovary (Abdullah-Soheimi *et al.*, 2010), cervix (Abdul-Rahman *et al.*, 2007) and bone (Mohamed *et al.*, 2008).

When enzyme-conjugated CGB lectin was used to detect 2-DE separated *O*-glycosylated proteins on NC membrane, ITIH4f at approximately 50 kDa, was the sole cluster of protein found to be significantly altered in abundance. Unlike APOA2 and C3, the identity of ITIH4f was initially determined by visual comparison to the previously published works (Abdul-Rahman *et al.*, 2007; Mohamed *et al.*, 2008; Mohamed *et al.*, 2013). Later, it was isolated by immobilized CGB lectin affinity chromatography, subjected to 2-DE, trypsin digestion and finally, identified using MS/MS. The marked differences in the experimental M_r of ITIH4f (~ 50 kDa) than the previously reported 35 kDa *O*-glycopeptide fragment (Saguchi *et al.*, 1995) could be due to the addition of carbohydrates in the form of serine or threonine-linked oligosaccharides to the fragment protein (a glycosylation type of PTM) and as such, contribute to the increase in the M_r of the protein fragment. The abundance of ITIH4f was found to be significantly lower in patients with PCa compared to those with BPH. This finding appears to be in contrast with the initial expectation on the basis of the previous reports of our laboratory, as PCa is also now known to be associated with increased levels of serum oestradiol, the predominant form of oestrogen (Carruba, 2007).

Data from our laboratory have previously shown significant enhanced levels of ITIH4f selectively in patients with cancers (Abdul-Rahman *et al.*, 2007; Mohamed *et al.*, 2008) and benign conditions (Mohamed *et al.*, 2013) that are associated with increased levels of oestrogens. In these cancers, oestrogen which is also a regulator involved in kallikrein gene expression (Myers and Clements, 2001; Yousef and Diamandis, 2001) was thought to induce overexpression of circulating serum kallikrein (Diamandis *et al.*, 2000; Myers and Clements, 2001) that will subsequently lead to increased cleavage of native kallikrein-sensitive 120 kDa ITIH4 (Mohamed *et al.*, 2008) into its 85 and 35 kDa fragments (Saguchi *et al.*, 1995). The latter fragment of which, is *O*-glycosylated and thus, detected by the CGB lectin.

Therefore, until the recent association linking levels of oestrogen to PCa (Ho *et al.*, 2006; Ho *et al.*, 2011), increased levels of oestrogen has always been a suspected carcinogen confined to cancers of women such as breast (Russo and Russo, 2006) and endometrial (Amant *et al.*, 2005; Carlson *et al.*, 2012) cancer. Based on *in vivo* and *in vitro* studies, oestrogen was thought to contribute to prostate tumourigenesis (Nelles *et al.*, 2011) either via estrogenic imprinting (Huang *et al.*, 2004b; Prins *et al.*, 2001) and epigenetic modifications (Feinberg *et al.*, 2006), direct genotoxicity (Ho and Roy, 1994), hyperprolactinaemia (Fernandez *et al.*, 2010), inflammation (Balkwill and Mantovani, 2001) and immunologic changes (Inoue *et al.*, 2000), and oestrogen receptor-mediated actions (Bonkhoff and Berges, 2009). As such, the data of the present study that had demonstrated inverse correlation of levels of serum ITIH4f observed in patients with PCa and the postulated contributory effects of oestrogen in tumourigenesis of the prostate is rather puzzling.

However, on another note, the different altered levels of ITIH4f in PCa and BPH patients further suggests the contributing effects of androgen since the

hormone is also up-regulated in patients with PCa and that both hormones oestrogen and androgen are believed to be the causing factors for prostate malignancy (Risbridger *et al.*, 2003). Secondly, the accelerated excretion of ITIH4f triggered by a mechanism, which is not clearly understood at this time (Jayapalan *et al*., 2013; this thesis), may have resulted in lower levels of serum ITIH4f in patients with PCa as well. Since ITIH4f has also been detected in the urine of patients with PCa in the subsequent study of this thesis, further discussion on this peptide is elaborated in Section 5.3.

In the present study, the use of immobilized CGB lectin affinity column chromatography was opted as an enrichment technique (Mechref *et al.*, 2008) for the isolation of *O*-glycosylated serum proteins that may have been masked by other high abundance proteins or, were not well-resolved using former techniques. Besides, it also facilitates confirmation of the identity of *O*-glycosylated proteins detected on CGB lectin-blot. Binding efficiency of the purified CGB lectin as ligand onto cyanogen bromide-activated Sepharose 4B gel matrix via covalent immobilisation was estimated at 93.6%, on average, and considered sufficient for the interaction between the ligand and *O*-glycosylated serum proteins during affinity chromatography. This is because, whilst higher lectin load enhances the capacity of affinity media, it may also cause significant non-specific binding through hydrophobic and/or ionic protein-protein interaction (Hermanson *et al.*, 1992; Mechref *et al.*, 2007). Additionally, parallel experiments using controlled column containing un-coupled activated and inactivated Sepharose 4B gel matrix, similar to that was reported by Roop-ngam *et al*., (2012) were performed to detect and subtract backgrounds of non-specific bindings. The experiments demonstrated no interaction between serum (and urinary proteins) to the chromatographic matrix and as such, non-specific adsorption of these proteins had very little or no contribution to the resulting protein eluates.

By subjecting the 2-DE profiles of the *O*-glycosylated proteins isolated using CGB lectin affinity column chromatography of patients with PCa and those with BPH to densitometric analysis, significantly enhanced abundance of three different glycosylated proteins, namely, KNG-1, AAT and TTR were demonstrated in the serum of patients with PCa compared to those with BPH. However unlike KNG-1, a known *O*-glycosylated protein, clusters of protein spots of AAT and TTR were not detected in the earlier Western blotting experiments that had used HRP-conjugated CGB lectin as probe. These proteins are generally not known to be *O*-glycosylated (Liu *et al.*, 2005). Nevertheless, it was indeed interesting to find several predicted *O*-glycosylated sites within the protein sequences of AAT and TTR using the NetOGlyc 4.0 software (Steentoft *et al.*, 2013).

KNG-1 is an inhibitor of thiol proteases and generally plays a role blood coagulation, hemostasis and being an acute-phase reactant protein, in inflammatory responses. Significant reduction in abundance of serum KNG-1 in patients with breast cancer (Doustjalali *et al.*, 2004), endometrial cancer, squamous cell cervical carcinoma, cervical adenocarcinoma (Abdul-Rahman *et al.*, 2007) and urine of subjects with ovarian cancer (Abdullah-Soheimi *et al.*, 2010) via 2-DE approach have been previously reported. Similar reduced levels of KNG-1 but by using immunochemical techniques have also been detected in the plasma of patients with gastrointestinal cancer as well (Roeise *et al.*, 1990). Paradoxically, the present data demonstrating elevated levels of serum KNG-1 of those patients succumbed to PCa appeared to be in contradiction to these findings. The contrasting abundance in the levels of KNG-1 is most likely attributed to the differences in the ratio of the sex steroid hormones of men and women cancer patients. Secondly, KNG-1 has also been invariably associated with endothelial cell migration and proliferation as well as angiogenesis (Colman *et al.*, 2000; Hayashi *et al.*, 2002; Zhang *et al.*, 2000). Endogenous kinin generated from low molecular weight

kininogen via the action of tissue kallikrein-kinin system was reported to enhance angiogenesis in chronic and proliferative granuloma and stromal cells around the tumour in a rat sponge model (Hayashi *et al.*, 2002). Since angiogenesis-mediating mechanism is generally activated at the early stage of tumour growth, increased amounts of KNG-1 seen in the sera of patients with early stage of PCa in this study may have been similarly physiologically elevated to enhance the tumour-associated angiogenesis. Additionally, KNG-1 had been implicated to exhibit anti-bacterial activities (Nordahl *et al.*, 2005) as well as anti-thrombotic role via inhibition of plasminogen activator inhibitor-1 function (Chavakis *et al.*, 2002).

The second protein of altered abundance that was isolated from CGB lectin affinity chromatography, AAT, is a serine protease inhibitor and an acute phase reactant protein that is responsible in emitting negative feedback during inflammation and/or infection responses (Chan *et al.*, 2007). It also functions as a physiologic inhibitor of blood coagulation (Kase and Pospisil, 1983) as well as in hemostasis (Emmerich, 2009). Deficiency in AAT is indicative of disorders of the lung (DeMeo and Silverman, 2004; Yang *et al.*, 2005) and liver (Kaplan and Cosentino, 2010) whilst, increase in AAT have been suggested to pose as potential markers for detection of cancer. Over the years, significant correlation of elevated levels of serum or plasma AAT had been made between various cancer types including those of the lung (Ferrigno *et al.*, 1995; Topic *et al.*, 2011), prostate (El-Akawi *et al.*, 2008), cervix (Kloth *et al.*, 2008), ovary (Warwas *et al.*, 1986), breast (Hamrita *et al.*, 2009), pancreas (Tountas *et al.*, 1985), liver (Dabrowska *et al.*, 1997), larynx (Krecicki and Leluk, 1992), colorectum (Perez-Holanda *et al.*, 2014) as well as the head and neck tumour (Brkic *et al.*, 2011) and Hodgkin's lymphoma (Dabrowska *et al.*, 1992) either for its diagnostic, prognostic or therapeutic outcome potentials. Since AAT is also known to inhibit angiogenesis and tumour growth (Huang *et al.*, 2004a), its increased level in the sera of subjects with PCa

observed in this study, may be in effect, the body's defence mechanism against the development of prostate tumour at the early stage of cancer.

TTR is another protein of altered abundance that was isolated from the CGB lectin affinity chromatography. Although not generally known to be *O*-glycosylated, the retention of TTR by the CGB lectin affinity column is likely related to indirect interactions with other *O*-glycosylated serum proteins. TTR is rather known to bind indiscriminately to many aromatic compounds (Bekki *et al.*, 2009; Weiss *et al.*, 2009a). In this study particularly, it could have been retained due to the interaction with the retinol-retinol binding protein 4 complexes or the sex hormone binding globulin, known to possess the *O*-linked oligosaccharides (Raineri *et al.*, 2002).

Generally, TTR offers a specific measure for an early detection of malnutrition (Shenkin, 2006) in patients with immunodeficiency, diabetes, renal disease and pneumonia. However in acute-phase responses following infection, inflammation or malignancy, the levels of TTR has been observed to be down-regulated, possibly due to pathophysiological or metabolic changes. Along that line, TTR has been earlier identified as a potential biomarker for ovarian (Gericke *et al.*, 2005; Zhang *et al.*, 2004), colorectal (Fentz *et al.*, 2007) and lung cancers (Ding *et al.*, 2014; Liu *et al.*, 2009).

In contrast, higher levels of TTR was also seen in sera of patients with lung cancer (Maciel *et al.*, 2005; Tran *et al.*, 2008; Wang *et al.*, 2012) and aqueous humor of patients with primary open-angle glaucoma (Grus *et al.*, 2008) thus, showing consistency to the data of the current study, in terms of levels of altered abundance of TTR in patients with PCa. Although the mechanism is not clearly understood, increased level of TTR observed in the sera of patients with PCa may have been contributed by the abnormally proliferating cancer cell of the prostate to supplement the deteriorating levels of TTR during development of PCa (Liu *et al.*, 2007).

5.3. PROFILING OF URINARY PROTEINS OF PATIENTS WITH PCa AND THOSE WITH BPH AS WELL AS CONTROLS

The second part of the current study involves experimental-designs mirroring the initial proteomic profiling of serum samples but on optimised concentration of acetone precipitated urinary proteins from healthy subjects, patients with BPH and those with PCa. Urine, albeit containing 1000-fold lower in protein concentration compared to plasma or serum, represents most of the plasma proteins. It also has increased proportions of low molecular weight protein and peptide components particularly those of the urinary tract (Hortin and Sviridov, 2007). In addition, the urinary proteome is also known to alter as results of pathophysiological conditions, and as such, provides an ideal alternative to plasma or serum for analysis (Veenstra *et al.*, 2005). In addition to this, urine sample was also selected for its attractive features of non-invasiveness, ease of attainability and cost-effective sampling method (Pisitkun *et al.*, 2006). The adaptation of acetone precipitation method on the other hand, had resulted in concentrated and purified urinary protein contents (Lovrien and Matulis, 1997) which was intended for the 2-DE proteomic profiling. The inclusion of age-matched healthy subjects as 'non- cancer' controls and a slight increase in the sample size in this second part of the study had favourably enhance the specificity as well as sensitivity of the potentially identified biomarkers.

This part of the study was initiated by subjecting an optimised concentration of acetone precipitated urinary proteins (100 µg) from patients with BPH and those with PCa as well as control subjects to 2-DE and silver staining, to investigate the differential levels of altered abundance of urinary proteins. The 2-DE resolved silver-stained urinary protein profiles of patients with BPH and those with PCa and control subjects were generally comparable with those detected in urine of patients with endometrial

cancer (Mu *et al.*, 2012) but with little loss in proteins such as immunoglobulin gamma chains and KNG-1. The adaptation of acetone precipitation to the pre-processing of the urine samples had very likely contributed to the loss due to less than complete recovery of proteins (Puchades *et al.*, 1999; Srivastava and Srivastava, 1998; Thongboonkerd *et al.*, 2002). Although, the levels of acetone (four parts of cold acetone) employed in this study were generally acceptable (Botelho *et al.*, 2010; Davidsson *et al.*, 1999), but may not have been optimal for some proteins (Srivastava and Srivastava, 1998; Thongboonkerd *et al.*, 2002). Nevertheless, incorporation of this method had certainly improved resolution and consistency of the 2-DE gels (Jayapalan *et al.*, 2013; this thesis).

When comparative densitometric analysis was performed on the 2-DE resolved urinary protein profiles of patients and control subjects, different altered levels of abundance of three proteins were observed. Significantly lower abundance of PSAPf, AMBPf1 and AMBPf2 was demonstrated in the urine of PCa patients compared to control subjects. Abundance of AMBPf2, on the other hand, was also significantly different when comparison was made between 2-DE profiles generated from urine of patients with PCa and those with BPH.

The identities of proteins of altered abundance, including PSAPf, AMBPf1 and AMBPf2 were confirmed by MS. A detailed study of MS/MS-derived amino acid sequences of both urinary AMBPf1 and AMBPf2 that were detected in the present study indicates extensive sequence homology to inter-alpha-trypsin inhibitor light chain (ITIL) region of AMBP (amino acids 206 - 352). Four of the peptide hit sequences appeared in both AMBPf1 and AMBPf2 cluster of spots that were analysed. This, together with their differences of molecular weights and pIs, suggests that urinary AMBPf1 and AMBPf2 were the ITIL products of the different proteolytic processing of

AMBP. As such, urinary AMBPf1 and AMBPf2 were henceforth, referred as ITILf1 and ITILf2, respectively for convenience of ensuing discussion.

PSAP is a 68 - 73 kDa lysosomal compartmental precursor glycoprotein (Kishimoto *et al.*, 1992), ubiquitously present either as secretory or integral membrane proteins in secretory fluids (e.g.: cerebrospinal fluid, seminal plasma, human milk, pancreatic juice, blood plasma and bile) (Hineno *et al.*, 1991) and tissues (e.g., brain, testes, kidney, spleen and liver) (O'Brien *et al.*, 1988). Initially expressed as a single precursor PSAP, all four saposins A, B, C and D are by-products of proteolytic processing of PSAP within the lysosomes with diverse biological involvement. The urinary PSAPf spot detected in the present study was most likely the saposin B peptides cleaved from the prosaposin precursor (Kishimoto *et al.*, 1992). This was deduced based on the observed experimental mass and MS/MS-derived amino acid sequences of the protein (Table 4.4).

Aside from exerting essential neurotrophic and myelinotrophic activities (Misasi *et al.*, 2009), PSAP is also a well-known pleiotropic growth factor (Wu *et al.*, 2012). Overexpression of PSAP in breast cancer cell lines was thought to promote tumour growth in breast cancer by stimulating the oestrogen receptor alpha-mediated signalling axis (Wu *et al.*, 2012). Similarly, amplified levels of PSAP were also demonstrated to promote carcinogenesis and progression of the prostate but through the activation of multiple signal transduction pathways and anti-apoptotic effect in metastatic androgen-independent prostate cancer cell lines (Koochekpour *et al.*, 2005). In another study, influence of up-regulation of androgen receptors, PSA expression and cellular activity in androgen-sensitive human prostate adenocarcinoma cells were also thought to contribute to PCa (Koochekpour *et al.*, 2007). Conversely, down-regulation of PSAP was shown to significantly decrease adhesion, migration and invasion of

metastatic prostate cancer cells via lysosomal proteolysis-dependent pathway by RNA interference (Hu *et al.*, 2010). Although the mechanism is poorly understood, it was believed that the decreased level of urinary saposin B of PCa patients observed in this study may be related to the reduced levels of proteolytic processing of PSAP in the patients compared to control subjects.

ITIL, also known as bikunin, a split-product of proteolytically cleaved AMBP precursor containing two domains of Kunitz-type proteinase inhibitor with a theoretical M_r of 25 - 26 kDa, is released in free-form into the circulation (Fries and Blom, 2000; Gebhard *et al.*, 1990). Generally, the free-form bikunin is filtered in the glomeruli of the kidney and reabsorbed in the tubuli. However, due to physiological or pathological duress, the levels of reabsorption were thought to be compromised hence, the apparent high levels of bikunin in the urine (Blom *et al.*, 1997). Additionally, these high levels were also hypothesised to have been contributed by the secretion of free-form bikunin from the tumour cells itself, in cancer (Hochstrasser *et al.*, 1989).

Nevertheless, this is not the first time the long-term association of bikunin as being the major component of cancer-associated proteinuria (Chawla *et al.*, 1992; Chawla *et al.*, 1984) has been thwarted. To the best of knowledge, the present study was the first to report on the lower levels of ITIL peptides in the urine of patients with PCa compared to controls, particularly in terms of its diagnostic potential (Jayapalan *et al*., 2013; this thesis). Aside from this, the reduced abundance of AMBPf2 peptide observed in urine of patients with PCa compared to those with BPH, does offer a new dimension to the specificity of the identified potential marker.

As observed in this study, down-regulation of bikunin in urine of patients with bladder cancer (Tsui *et al.*, 2010) has been previously reported as well. The mechanism of action of bikunin in cancer prevention lies in the suppression of various

signalling cascades (Kobayashi *et al.*, 2001; Matsuzaki *et al.*, 2004). As such, increased level of bikunin has been previously shown to prevent progression of ovarian cancer (Suzuki et al., 2003; Tanaka et al., 2003) by inhibiting expression of tumour cell-associated plasmin activity and urolinase-plasminogen activator expression at the levels of gene and protein (Kobayashi et al., 2000; Kobayashi et al., 2001) in ovarian carcinoma cell line. Therefore, the lower levels of bikunin detected in urine of patients with PCa in the present study possibly reflects localised cancer within the prostate gland thus, in line with the stages of cancer of the recruited PCa patients (stage I and II).

In the second approach, the 2-DE electrophoresed gels were electrotransferred onto a NC membrane for the detection *O*-glycosylated urinary proteins using HRP-conjugated CGB lectin. By subjecting the urinary *O*-glycosylated protein resolved on the lectin-detected NC membranes to densitometry analysis, significantly enhanced level of ITIH4f (at approximately 50 kDa) was noted in the urine of PCa patients compared to control subjects. No tangible differences in levels of other *O*-glycosylated urinary proteins were detected between patients with BPH and healthy controls. Despite many attempts, the identity of urinary ITIH4f on the NC membranes was unable to be confirmed using on-membrane trypsin digestion technique (Luque-Garcia and Neubert, 2009; Mu *et al.*, 2012). Because of that, ITIH4f was initially determined by visual comparison to that of the optimised acetone precipitated urinary protein profiles. Later, *O*-glycosylated urinary proteins was first isolated using CGB lectin affinity chromatography, trypsin digested (in-solution) and finally, the presence of ITIH4f in urine was confirmed by nano LC-MS/MS.

Contrary to the ITIL peptides, the lectin-detected ITIH4f appeared to be overexpressed in the urine of patients with PCa as opposed to the controls and patients with BPH. ITIH4, a single chain acute-phase glycoprotein with an apparent M_r of 120-

kDa belongs to the inter-α-trypsin inhibitor (ITI) family of serine protease inhibitors (Fujita *et al.*, 2004). Unlike its other ITIH family members, ITIH4 does not interact with ITIL due to the lack of a C-terminus region peptide sequence that is required for ITIL binding (Nishimura *et al.*, 1995; Salier *et al.*, 1996). Interestingly, ITIH, which is typically linked to ITIL via estrified chondroitin 4-sulfate, has been shown to be down-regulated in multiple solid tumours (Hamm *et al.*, 2008), and this is also in agreement with the lower level of urinary ITIL that was detected in patients with PCa in the present study. Another unique property of ITIH4 is the kallikrein-released bradykinin-like domain perched on its C-terminal end (Nishimura *et al.*, 1995), which in turns, allows fragmentation of the 120 kDa ITIH4 native protein by plasma kallikrein (Saguchi *et al.*, 1995). The resulting fragmented patterns of 85 kDa and 35 kDa ITIH4fs have been invariably associated with various diseased conditions including cancer (Song *et al.*, 2006), inflammation (Pineiro *et al.*, 2004), normal and hydatiform molar pregnancy (Mohamed *et al.*, 2013), amyotrophic lateral sclerosis (Tanaka *et al.*, 2013), acute ischaemic stroke (Kashyap *et al.*, 2009) and many others. When referred to the earlier findings of the present study, data on elevated levels of ITIH4f in the urine of patients with PCa neatly corroborates to the low levels of the serum ITIH4f in PCa patients as substantial amount of the peptide may have been excreted in the urine in order to clear the excess breakdown product of the ITIH4, which is the 35 kDa ITIH4f in the sera. Interestingly, the data of the present study also appear to be in direct contrast to what was previously reported for ovarian carcinoma, in which the ITIH4f was significantly enhanced in the patients' sera (Mohamed *et al.*, 2008) but lowered in abundance in the their urine (Abdullah-Soheimi *et al.*, 2010).

5.4. POTENTIAL USE OF INDICES RELATING TO LEVELS OF SERUM AND URINARY FRAGMENT PROTEINS OF ITIH4F AND ITILS

When taken together, the data of the present study strongly highlights the potential use of the serum and urinary peptides in the diagnosis of PCa. The significant altered abundance of peptides in patients with PCa compared to those with BPH and controls reflects their specific use to discriminate PCa from non-cancer conditions. This is particularly so, when the abundance of serum to urinary ITIH4f as well as those of urinary ITIH4f to urinary ITIL peptides were taken into consideration, as the parameters involved had demonstrated a reciprocal trend. To validate these data, pooled serum and urine samples from patients with PCa, those with BPH and controls were separately subjected to 1-DE and Western blotting in the third part of the study. Proteins in the respective groups of subjects were detected using CGB lectin and/or antibodies against ITIH4 and bikunin + trypstatin before being analysed by densitometry. Differences in protein abundance were expressed in mean percentage of volume contribution of the resulting bands.

Data generated from the validation study generally substantiated the levels of abundance of serum and urinary ITIH4f as well as those of urinary ITIL peptides (Table 4.7), with little variation in the levels of significance. Although not significant, lowest levels of serum ITIH4f was observed in pooled serum of patients with PCa compared to those with BPH and controls. Similar were the case for the apparently lower levels of urinary ITIH4f peptide in pooled urine of patients with BPH, and higher levels of urinary ITILf2 peptide in pooled urine of controls, which were insignificant when compared to pooled urine of patients with PCa (Table 4.7). These discrepancies are most likely due to the effect of technical interferences. It had been widely documented that statistically ‘small’ yet, significant differences in expression are lost during pooling

of samples, and as such, minimises the effects of biological variation (Zhang and Gant, 2005).

Nevertheless, when the data was collectively used as parameters for calculation of indices relating to levels of serum and urinary fragment proteins in terms of, (i) abundance ratio of serum to urinary ITIH4f peptides, (ii) urinary ITIH4f to urinary ITILf1 peptides and (iii) urinary ITIH4f to urinary ITILf1 peptides, marked segregating differences in indices for the different groups of subjects were obtained. Individual mean ratio indices between all patients with PCa and those with BPH or controls were found not to overlap thus, showing strong discriminatory powers between PCa patients from those with BPH or control subjects. This is also compatible with the study by Yurkovetsky *et al*., (2006) that had shown synergistic effects of multiple markers in dramatically improving the sensitivity of cancer detection.

Therefore, in principle, the data of this study highlights the strong potentials of these peptide marker indices for discriminating patients with PCa at an early stage from those with non-cancer conditions of the prostate. At the same time, these markers could also pave way for inception of novel therapeutic interventions in clinical management of patient, in addition to avoiding unwarranted medical procedures and alleviating needless anguish of those with BPH. However, in spite of the compelling evidences that had been demonstrated in the present study, the technical and methodological limitations of the present study has to be acknowledged and need to be carefully addressed in a separate study involving large clinically representative populations, using feasible and time-effective techniques such as the Bio-Plex and protein array methodologies.

CONCLUSION

Using 2-DE gel-based approach together with silver staining detection method, an attempt to establish 2-DE profiles of high abundance serum and acetone-precipitated urinary proteins of patients with PCa, those with BPH and control subjects (for the latter) were methodically performed. Comparative image master analyses between the silver-stained serum 2-DE profiles of these subjects had resulted in marked reduction in protein abundance of APOA2 and C3 beta chain fragment in serum of patients with PCa compared to those with BPH. On the other hand, image analysis of the silver-stained urinary protein 2-DE profiles of these subjects showed significantly lower abundance of urinary saposin B, ITILf1 and ITILf2 peptide fragments in patients with PCa compared to the controls. In addition, the levels of ITILf2 peptide fragment was also significantly lower in patients with PCa compared to those with BPH.

Profiles of 2-DE serum and urinary *O*-glycosylated proteins of patients with PCa, those with BPH and control subjects (for the latter) were successfully generated using 2-DE in combination with HRP-conjugated CGB lectin. Via image analysis, marked aberration in the abundance of *O*-glycosylated proteins was only observed for ITIH4f peptide in both serum and urine 2-DE-lectin blot profiles of patients with PCa compared to those of non-cancer conditions. Contrastingly, significantly lower levels of serum ITIH4f and enhanced levels of ITIH4f peptide were observed in the urine of patients with PCa compared to those harbouring non-cancer conditions. This reciprocal trend of altered abundance of peptide fragments may be readily exploited for efficient diagnostic predictability of PCa.

Additionally, profiles of 2-DE serum *O*-glycosylated proteins of patients with PCa and those with BPH were also successfully generated by incorporating CGB lectin affinity chromatography prior to 2-DE separation. Using this method, several additional

clusters of *O*-glycosylated protein spots that were not previously detected, were found resolved in 2-DE profiles of the subjects. Comparative image analysis of silver-stained 2-DE profiles of serum *O*-glycosylated proteins between patients with PCa and those of benign condition, demonstrated altered levels of three different proteins. These include elevated levels of KNG, AAT, and TTR in sera of patients with PCa. Identities and/or presence of proteins of altered abundance were confirmed by MS analysis.

As a means of validation, different levels of serum ITIH4f and abundance of urinary ITIH4f, ITILf1 and ITILf2 in groups of subjects were further confirmed using either lectin- and immuno-blotting techniques. The levels of abundances of these proteins in groups of subjects were generally comparable to the initial data obtained via 2-DE approach, with little variation in the levels of significance. However, when considered as indices relating to the levels of serum and urinary fragment proteins of ITIH4f and ITILs, in terms of, (i) abundance ratio of serum to urinary ITIH4f, (ii) urinary ITIH4f to ITILf1 and (iii) urinary ITIH4f to ITILf2, marked segregating differences in indices for each group of subjects were obtained. In essence, these peptide marker indices that had highlighted its strong discriminatory power between PCa patients from those with BPH or controls may be potentially used for diagnosis of early stage PCa as well as pave a way for inception of novel therapeutic interventions in clinical management of patient with PCa. At the same time, this could also play a fundamental role in avoiding unwarranted medical procedures. Nevertheless, further validation of these promising markers on clinically representative populations, using feasible and time-effective techniques is paramount.

REFERENCES

Abdul-Rahman, P. S., Lim, B. K. and Hashim, O. H. (2007). Expression of high-abundance proteins in sera of patients with endometrial and cervical cancers: Analysis using 2-DE with silver staining and lectin detection methods. *Electrophoresis, 28*(12), 1989-1996.

Abdul Rahman, M., Karsani, S. M., Othman, I., Abdul Rahman, P. S. and Hashim, O. H. (2002). Galactose-binding lectin from the seeds of champedak (*Artocarpus integer*): Sequences of its subunits and interactions with human serum *O*-glycosylated glycoproteins. *Biochem Biophys Res Commun, 295*(4), 1007-1013.

Abdul Rahman, P. S., Lee, E. S., Hashim, O. H. and Shuib, A. S. (17 August 2006). *Application of lectin coated magnetic beads in the proteomic profiling of human serum glycoproteins.* Paper presented at the 31st Annual Conference of The Malaysian Society of Biochemistry and Molecular Biology, Bangi, Malaysia.

Abdullah-Soheimi, S., Lim, B. K., Hashim, O. H. and Shuib, A. (2010). Patients with ovarian carcinoma excrete different altered levels of urine CD59, kininogen-1 and fragments of inter-alpha-trypsin inhibitor heavy chain 4 and albumin. *Proteome Sci, 8*(1), 58.

Ablin, R. J. (2010). The great prostate mistake. *The New York Times*.

Adam, B. L., Qu, Y., Davis, J. W., Ward, M. D., Clements, M. A., Cazares, L. H., Semmes, O. J., Schellhammer, P. F., Yasui, Y., Feng, Z. and Wright, G. L., Jr. (2002). Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. *Cancer Res, 62*(13), 3609-3614.

Aebersold, R. and Mann, M. (2003). Mass spectrometry-based proteomics. *Nature, 422*(6928), 198-207.

Ahmed, N., Barker, G., Oliva, K., Garfin, D., Talmadge, K., Georgiou, H., Quinn, M. and Rice, G. (2003). An approach to remove albumin for the proteomic analysis of low abundance biomarkers in human serum. *Proteomics, 3*(10), 1980-1987.

AJCC cancer staging manual. (2002). (6th ed.). New York: Springer.

Aksoy, Y., Oral, A., Aksoy, H., Demirel, A. and Akcay, F. (2003). PSA density and PSA transition zone density in the diagnosis of prostate cancer in PSA gray zone cases. *Ann Clin Lab Sci, 33*(3), 320-323.

Alaiya, A. A., Oppermann, M., Langridge, J., Roblick, U., Egevad, L., Brindstedt, S., Hellstrom, M., Linder, S., Bergman, T., Jornvall, H. and Auer, G. (2001). Identification of proteins in human prostate tumor material by two-dimensional gel electrophoresis and mass spectrometry. *Cell Mol Life Sci, 58*(2), 307-311.

Allison, R. T. (1986). Lectins in diagnostic histopathology: A review. *Med Lab Sci, 43*(4), 369-376.

Alroy, J. and Lyons, J. A. (2014). Lysosomal storage diseases. *JIEMS, 2*.

Alroy, J., Ucci, A. A. and Warren, C. D. (1985). Human and canine fucosidosis: A comparative lectin histochemistry study. *Acta Neuropathol, 67*(3-4), 265-271.

Amant, F., Moerman, P., Neven, P., Timmerman, D., Van Limbergen, E. and Vergote, I. (2005). Endometrial cancer. *Lancet, 366*(9484), 491-505.

American Cancer Society. (2003). Cancer facts & figures. Atlanta, Georgia: American Cancer Society.

Anderson, J. C. and Peck, S. C. (2008). A simple and rapid technique for detecting protein phosphorylation using one-dimensional isoelectric focusing gels and immunoblot analysis. *Plant J, 55*(5), 881-885.

Anderson, N. L. and Anderson, N. G. (2002). The human plasma proteome: History, character, and diagnostic prospects. *Mol Cell Proteomics, 1*(11), 845-867.

Anderson, N. L., Anderson, N. G., Haines, L. R., Hardie, D. B., Olafson, R. W. and Pearson, T. W. (2004). Mass spectrometric quantitation of peptides and proteins using stable isotope standards and capture by anti-peptide antibodies (SISCAPA). *J Proteome Res, 3*(2), 235-244.

Apweiler, R., Hermjakob, H. and Sharon, N. (1999). On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim Biophys Acta, 1473*(1), 4-8.

Arkes, H. R. and Gaissmaier, W. (2012). Psychological research and the prostate-cancer screening controversy. *Psychologic Sci,* 10.1177/0956797612437428.

Ascencio, F., Guruge, J. L., Ljungh, Å., Mégraud, F., Wei, S. and Wadström, T. (1990). Lectin typing of *Helicobacter pylori*. In P. Malfertheiner and H. Ditschuneit (Eds.), *Helicobacter pylori, Gastritis and Peptic Ulcer* (pp. 104-109). Berlin Heidelberg: Springer

Ashcroft, A. E. (1997). Ionization methods in organic mass spectrometry. In N. W. Barnett (Ed.), *Analytical Spectroscopy Monographs* (pp. 176). London: RSC Publishing.

Aspinall, J. O., Bentel, J. M., Horsfall, D. J., Haagensen, D. E., Marshall, V. R. and Tilley, W. D. (1995). Differential expression of apolipoprotein-D and prostate specific antigen in benign and malignant prostate tissues. *J Urol, 154*(2 Pt 1), 622-628.

Aucouturier, P., Pineau, N., Brugier, J. C., Mihaesco, E., Duarte, F., Skvaril, F. and Preud'homme, J. L. (1989). Jacalin: A new laboratory tool in immunochemistry and cellular immunology. *J Clin Lab Anal, 3*(4), 244-251.

Aucouturier, P., Pineau, N. and Preud'Homme, J. L. (1988). A simple procedure for the isolation of human secretory IgA of IgA1 and IgA2 subclass by a jackfruit lectin, jacalin, affinity chromatography. *Mol Immunol, 25*(3), 321-322.

Ayala, G., Thompson, T., Yang, G., Frolov, A., Li, R., Scardino, P., Ohori, M., Wheeler, T. and Harper, W. (2004). High levels of phosphorylated form of Akt-1 in prostate cancer and non-neoplastic prostate tissues are strong predictors of biochemical recurrence. *Clin Cancer Res, 10*(19), 6572-6578.

Babaian, R. J., Miyashita, H., Evans, R. B. and Ramirez, E. I. (1992). The distribution of prostate specific antigen in men without clinical or pathological evidence of prostate cancer: Relationship to gland volume and age. *J Urol, 147*(3 Pt 2), 837-840.

Badia, M. and Querol, E. (1988). Detection and purification of bovine herpes virus 1 glycoproteins by lectin affinity. *J Virol Methods, 22*(1), 23-29.

Baggerly, K. A., Morris, J. S. and Coombes, K. R. (2004). Reproducibility of SELDI-ToF protein patterns in serum: Comparing datasets from different experiments. *Bioinformatics, 20*(5), 777-785.

Balkwill, F. and Mantovani, A. (2001). Inflammation and cancer: Back to Virchow? *Lancet, 357*(9255), 539-545.

Ballehaninna, U. K. and Chamberlain, R. S. (2012). The clinical utility of serum CA 19-9 in the diagnosis, prognosis and management of pancreatic adenocarcinoma: An evidence based appraisal. *J Gastrointest Oncol, 3*(2), 105-119.

Banez, L. L., Prasanna, P., Sun, L., Ali, A., Zou, Z., Adam, B. L., McLeod, D. G., Moul, J. W. and Srivastava, S. (2003). Diagnostic potential of serum proteomic patterns in prostate cancer. *J Urol, 170*(2 Pt 1), 442-446.

Barondes, S. H. (1988). Bifunctional properties of lectins: Lectins redefined. *Trends Biochem Sci, 13*(12), 480-482.

Barry, M. J. (2001). Prostate specific antigen testing for early diagnosis of prostate cancer. *N Engl J Med, 344*(18), 1373-1377.

Basch, E., Oliver, T. K., Vickers, A., Thompson, I., Kantoff, P., Parnes, H., Loblaw, D. A., Roth, B., Williams, J. and Nam, R. K. (2012). Screening for prostate cancer with prostate-specific antigen testing: American Society of Clinical Oncology Provisional Clinical Opinion. *J Clin Oncol, 30*(24), 3020-3025.

Baum, J., Ward, R. H. and Conway, D. J. (2002). Natural selection on the erythrocyte surface. *Mol Biol Evol, 19*(3), 223-229.

Bekki, K., Takigami, H., Suzuki, G., Tang, N. and Hayakawa, K. (2009). Evaluation of toxic activities of polycyclic aromatic hydrocarbon derivatives using *in vitro* bioassays. *J Healt Sci, 55*(6), 601-610.

Benbrahim-Tallaa, L., Liu, J., Webber, M. M. and Waalkes, M. P. (2007). Estrogen signaling and disruption of androgen metabolism in acquired androgen-independence during cadmium carcinogenesis in human prostate epithelial cells. *Prostate, 67*(2), 135-145.

Benbrahim-Tallaa, L. and Waalkes, M. P. (2008). Inorganic arsenic and human prostate cancer. *Environ Health Perspect, 116*(2), 158-164.

Benchikh, A., Savage, C., Cronin, A., Salama, G., Villers, A., Lilja, H. and Vickers, A. (2010). A panel of kallikrein markers can predict outcome of prostate biopsy following clinical work-up: An independent validation study from the European Randomized Study of Prostate Cancer Screening, France. *BMC Cancer, 10*, 635.

Beranova-Giorgianni, S. (2003). Proteome analysis by two-dimensional gel electrophoresis and mass spectrometry: Strengths and limitations. *Trends Analyt Chem, 22*(5), 273-281.

Bergmann, U., Ahrends, R., Neumann, B., Scheler, C. and Linscheid, M. W. (2012). Application of metal-coded affinity tags (MeCAT): Absolute protein quantification with top-down and bottom-up workflows by metal-coded tagging. *Anal Chem, 84*(12), 5268-5275.

Biomarkers Definition Working Group. (2001). Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. *Clin Pharmacol Ther, 69*(3), 89-95.

Blom, A. M., Falkenberg, C., Sjöquist, M., Eriksson, U. J., Åkerström, B. and Fries, E. (1997). Increase of bikunin and α1-microglobulin concentrations in urine of rats during pregnancy is due to decreased tubular reabsorption. *Biochimica Biophysica Acta, 1361*(2), 198-202.

Bock, C. H., Schwartz, A. G., Ruterbusch, J. J., Levin, A. M., Neslund-Dudas, C., Land, S. J., Wenzlaff, A. S., Reich, D., McKeigue, P., Chen, W., Heath, E. I., Powell, I. J., Kittles, R. A. and Rybicki, B. A. (2009). Results from a prostate cancer admixture mapping study in African-American men. *Hum Genet, 126*(5), 637-642.

Bonkhoff, H. and Berges, R. (2009). The evolving role of oestrogens and their receptors in the development and progression of prostate cancer. *Eur Urol, 55*(3), 533-542.

Bonnin, A. J., Zeitz, H. J. and Gewurz, A. (1993). Complement factor I deficiency with recurrent aseptic meningitis. *Arch Intern Med, 153*(11), 1380-1383.

Borer, J. G., Sherman, J., Solomon, M. C., Plawker, M. W. and Macchia, R. J. (1998). Age specific prostate specific antigen reference ranges: Population specific. *J Urol, 159*(2), 444-448.

Botelho, D., Wall, M. J., Vieira, D. B., Fitzsimmons, S., Liu, F. and Doucette, A. (2010). Top-down and bottom-up proteomics of SDS-containing solutions following mass-based separation. *J Proteome Res, 9*(6), 2863-2870.

Boyd, W. C. and Shapleigh, E. (1954). Antigenic relations of blood group antigens as suggested by tests with lectins. *J Immunol, 73*(4), 226-231.

Bradshaw, R. A. and Stewart, A. E. (1994). Analysis of protein modifications: Recent advances in detection, characterization and mapping. *Curr Opin Biotechnol, 5*(1), 85-93.

Brawer, M. K., Chetner, M. P., Beatie, J., Buchner, D. M., Vessella, R. L. and Lange, P. H. (1992). Screening for prostatic carcinoma with prostate specific antigen. *J Urol, 147*(3 Pt 2), 841-845.

Brioschi, P. A., Bischof, P., Delafosse, C. and Krauer, F. (1991). Squamous-cell carcinoma antigen (SCC-A) values related to clinical outcome of pre-invasive and invasive cervical carcinoma. *Int J Cancer, 47*(3), 376-379.

Brkic, S., Salkic, A., Nuhbegovic, S., Brkic, F. and Umihanic, S. (2011). Influence of surgical therapy on serum values of alpha 1-antitrypsin in patients with head and neck tumors. *Med Arh, 65*(2), 99-101.

Brockhausen, I., Schutzbach, J. and Kuhns, W. (1998). Glycoproteins and their relationship to human disease. *Acta Anat (Basel), 161*(1-4), 36-78.

Brousseau, T., Dupuy-Gorce, A. M., Evans, A., Arveiler, D., Ruidavets, J. B., Haas, B., Cambou, J. P., Luc, G., Ducimetiere, P., Amouyel, P. and Helbecque, N. (2002). Significant impact of the highly informative (CA)n repeat polymorphism of the APOA-II gene on the plasma APOA-II concentrations and HDL subfractions: The ECTIM study. *Am J Med Genet, 110*(1), 19-24.

Byrne, J. C., Downes, M. R., O'Donoghue, N., O'Keane, C., O'Neill, A., Fan, Y., Fitzpatrick, J. M., Dunn, M. and Watson, R. W. (2009). 2D-DIGE as a strategy to identify serum markers for the progression of prostate cancer. *J Proteome Res, 8*(2), 942-957.

Calle, E. E., Rodriguez, C., Walker-Thurmond, K. and Thun, M. J. (2003). Overweight, obesity and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med, 348*(17), 1625-1638.

Carlson, M. J., Thiel, K. W., Yang, S. and Leslie, K. K. (2012). Catch it before it kills: Progesterone, obesity, and the prevention of endometrial cancer. *Discov Med, 14*(76), 215-222.

Carruba, G. (2007). Estrogen and prostate cancer: An eclipsed truth in an androgen-dominated scenario. *J Cell Biochem, 102*(4), 899-911.

Cazares, L. H., Adam, B. L., Ward, M. D., Nasim, S., Schellhammer, P. F., Semmes, O. J. and Wright, G. L., Jr. (2002). Normal, benign, preneoplastic, and malignant prostate cells have distinct protein expression profiles resolved by surface enhanced laser desorption/ionization mass spectrometry. *Clin Cancer Res, 8*(8), 2541-2552.

Chahed, K., Hamrita, B., Mejdoub, H., Remadi, S., Chaïeb, A. and Chouchane, L. (2004). Two dimensional gel electrophoresis analyses of human plasma proteins. Association of retinol binding protein and transthyretin expression with breast cancer. *Gene Ther Mol Biol, 8*, 539-546.

Chan, E. D., Kaminska, A. M., Gill, W., Chmura, K., Feldman, N. E., Bai, X., Floyd, C. M., Fulton, K. E., Huitt, G. A., Strand, M. J., Iseman, M. D. and Shapiro, L. (2007). Alpha-1-antitrypsin (AAT) anomalies are associated with lung disease due to rapidly growing mycobacteria and AAT inhibits *Mycobacterium abscessus* infection of macrophages. *Scand J Infect Dis, 39*(8), 690-696.

Chan, J. M., Stampfer, M. J., Ma, J., Gann, P. H., Gaziano, J. M. and Giovannucci, E. L. (2001). Dairy products, calcium, and prostate cancer risk in the Physicians' Health Study. *Am J Clin Nutr, 74*(4), 549-554.

Chan, Y. S., Wong, J. H., Fang, E. F., Pan, W. and Ng, T. B. (2012). Isolation of a glucosamine binding leguminous lectin with mitogenic activity towards splenocytes and anti-proliferative activity towards tumor cells. *PLoS ONE, 7*(6), e38961.

Charcot, J. M. and Robin, C. (1853). Observation de leocythemie. *C R Mem Soc Biol, 5*, 44-50.

Chavakis, T., Pixley, R. A., Isordia-Salas, I., Colman, R. W. and Preissner, K. T. (2002). A novel antithrombotic role for high molecular weight kininogen as inhibitor of plasminogen activator inhibitor-1 function. *J Biol Chem, 277*(36), 32677-32682.

Chawla, R. K., Lawson, D. H., Ahmad, M. and Travis, J. (1992). Cancer-related urinary proteinase inhibitor, EDC1: A new method for its isolation and evidence for multiple forms. *J Cell Biochem, 50*(3), 227-236.

Chawla, R. K., Miller, F. W., Lawson, D. H. and Nixon, D. W. (1984). Urinary cancer-related protein EDC1 and serum inter-alpha trypsin inhibitor in breast cancer. *Tumour Biol, 5*(6), 351-363.

Chen, J., Chen, L.-J., Xia, Y.-L., Zhou, H.-C., Yang, R.-B., Wu, W., Lu, Y., Hu, L.-W. and Zhao, Y. (2013). Identification and verification of transthyretin as a potential biomarker for pancreatic ductal adenocarcinoma. *J Cancer Res Clin Oncol, 139*(7), 1117-1127.

Chen, Y. Y., Lin, S. Y., Yeh, Y. Y., Hsiao, H. H., Wu, C. Y., Chen, S. T. and Wang, A. H. (2005). A modified protein precipitation procedure for efficient removal of albumin from serum. *Electrophoresis, 26*(11), 2117-2127.

Chevalier, F. (2010). Highlights on the capacities of "gel-based" proteomics. *Proteome Sci, 8*(1), 23.

Chevallet, M., Luche, S., Diemer, H., Strub, J. M., Van Dorsselaer, A. and Rabilloud, T. (2008). Sweet silver: a formaldehyde-free silver staining using aldoses as developing agents, with enhanced compatibility with mass spectrometry. *Proteomics, 8*(23-24), 4853-4861.

Chiou, C. C., Chang, P. Y., Chan, E. C., Wu, T. L., Tsao, K. C. and Wu, J. T. (2003). Urinary 8-hydroxydeoxyguanosine and its analogs as DNA marker of oxidative stress: development of an ELISA and measurement in both bladder and prostate cancers. *Clin Chim Acta, 334*(1-2), 87-94.

Cho, C. K. and Diamandis, E. P. (2011). Application of proteomics to prenatal screening and diagnosis for aneuploidies. *Clin Chem Lab Med, 49*(1), 33-41.

Chou, R., Croswell, J. M., Dana, T., Bougatsos, C., Blazina, I., Fu, R., Gleitsmann, K., Koenig, H. C., Lam, C., Maltz, A., Rugge, J. B. and Lin, K. (2011). Screening for prostate cancer: A review of the evidence for the U.S. Preventive Services Task Force. *Ann Intern Med, 155*(11), 762-771.

Chustecka, Z. (2010). PSA discoverer says PSA screening is "Public Health Disaster". *Medscape Medical News*.

Cohen, E. (1984). Progress in clinical and biological research. In E. Cohen (Ed.), *Recognition Proteins, Receptor and Probes: Invertebrates.* (Vol. 157, pp. 207). New York: Alan, R. Liss.

Cole, H. B., Ezzell, J. W., Jr., Keller, K. F. and Doyle, R. J. (1984). Differentiation of *Bacillus anthracis* and other *Bacillus species* by lectins. *J Clin Microbiol, 19*(1), 48-53.

Collins, G. N., Lee, R. J., McKelvie, G. B., Rogers, A. C. and Hehir, M. (1993). Relationship between prostate specific antigen, prostate volume and age in the benign prostate. *Br J Urol, 71*(4), 445-450.

Colman, R. W., Jameson, B. A., Lin, Y., Johnson, D. and Mousa, S. A. (2000). Domain 5 of high molecular weight kininogen (kininostatin) down-regulates endothelial cell proliferation and migration and inhibits angiogenesis. *Blood, 95*(2), 543-550.

Conrads, T. P., Issaq, H. J. and Veenstra, T. D. (2002). New tools for quantitative phosphoproteome analysis. *Biochem Biophys Res Commun, 290*(3), 885-890.

Corfield, A. P., Myerscough, N., Longman, R., Sylvester, P., Arul, S. and Pignatelli, M. (2000). Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease. *Gut, 47*(4), 589-594.

Corthals, G. L., Wasinger, V. C., Hochstrasser, D. F. and Sanchez, J. C. (2000). The dynamic range of protein expression: A challenge for proteomic research. *Electrophoresis, 21*(6), 1104-1115.

Cristea, I. M., Gaskell, S. J. and Whetton, A. D. (2004). *Proteomics techniques and their application to hematology* (Vol. 103).

Crocitto, L. E., Korns, D., Kretzner, L., Shevchuk, T., Blair, S. L., Wilson, T. G., Ramin, S. A., Kawachi, M. H. and Smith, S. S. (2004). Prostate cancer molecular markers GSTP1 and hTERT in expressed prostatic secretions as predictors of biopsy results. *Urology, 64*(4), 821-825.

D'Adamo, P. (2014). Lecster Lectin Database. http://www.dadamo.com/

Dabrowska, M., Kemona, H., Prokopowicz, J. and Kiluk, S. (1992). Serum concentration of alpha-2-macroglobulin, alpha-1-antitrypsin and alpha-1-antichymotrypsin [correction of alpha-2-antichymotrypsin] in patients with Hodgkin's disease. *Mater Med Pol, 24*(1), 28-30.

Dabrowska, M., Mantur, M., Panasiuk, A. and Prokopowicz, J. (1997). Does the concentration of alpha 1-proteinase inhibitor reflect the transformation of liver cirrhosis to liver carcinoma? *Neoplasma, 44*(5), 305-307.

Darie, C. C. (2013). Post-translational modification (PTM) proteomics: Challenges and perspectives. *Mod Chem appl, 1*, e114.

Davidsson, P., Westman, A., Puchades, M., Nilsson, C. L. and Blennow, K. (1999). Characterization of proteins from human cerebrospinal fluid by a combination of preparative two-dimensional liquid-phase electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal Chem, 71*(3), 642-647.

Dayal, B. and Ertel, N. H. (2002). ProteinChip technology: A new and facile method for the identification and measurement of high-density lipoproteins apoA-I and apoA-II and their glycosylated products in patients with diabetes and cardiovascular disease. *J Proteome Res, 1*(4), 375-380.

De Masi, R., Pasca, S., Scarpello, R., Idolo, A. and De Donno, A. (2013). The clinical potential of blood-proteomics in multiple sclerosis. *BMC Neurol, 13*(1), 45.

DeMeo, D. L. and Silverman, E. K. (2004). Alpha1-antitrypsin deficiency. 2: Genetic aspects of alpha(1)-antitrypsin deficiency: phenotypes and genetic modifiers of emphysema risk. *Thorax, 59*(3), 259-264.

Dennis, J. W., Granovsky, M. and Warren, C. E. (1999). Glycoprotein glycosylation and cancer progression. *Biochim Biophys Acta, 1473*(1), 21-34.

Dennis, L. K. and Dawson, D. V. (2002). Meta-analysis of measures of sexual activity and prostate cancer. *Epidemiology, 13*(1), 72-79.

Department of Health and Human Services, U. S. (1994). Food and Drug Administration (FDA) approves test for prostate cancer Silver Spring, Madison: Department of Health and Human Services, United States.

Deras, I. L., Aubin, S. M., Blase, A., Day, J. R., Koo, S., Partin, A. W., Ellis, W. J., Marks, L. S., Fradet, Y., Rittenhouse, H. and Groskopf, J. (2008). PCA3: A molecular urine assay for predicting prostate biopsy outcome. *J Urol, 179*(4), 1587-1592.

Diamandis, E. P. (2003). Proteomic patterns in biological fluids: Do they represent the future of cancer diagnostics? *Clin Chem, 49*(8), 1272-1275.

Diamandis, E. P. (2004). Mass spectrometry as a diagnostic and a cancer biomarker discovery tool: Opportunities and potential limitations. *Mol Cell Proteomics, 3*(4), 367-378.

Diamandis, E. P., Yousef, G. M., Soosaipillai, A. R. and Bunting, P. (2000). Human kallikrein 6 (zyme/protease M/neurosin): A new serum biomarker of ovarian carcinoma. *Clin Biochem, 33*(7), 579-583.

Diamandis, E. P. and Yu, H. (1997). Non-prostatic sources of prostate-specific antigen. *Urol Clin North Am, 24*(2), 275-282.

Diaz, C. L., Melchers, L. S., Hooykaas, P. J. J., Lugtenberg, B. J. J. and Kijne, J. W. (1989). Root lectin as a determinant of host-plant specificity in the Rhizobium-legume symbiosis. *Nature, 338*(6216), 579-581.

Ding, H., Liu, J., Xue, R., Zhao, P., Qin, Y., Zheng, F. and Sun, X. (2014). Transthyretin as a potential biomarker for the differential diagnosis between lung cancer and lung infection. *Biomed Rep, 2*(5), 765-769.

Djavan, B., Ravery, V., Zlotta, A., Dobronski, P., Dobrovits, M., Fakhari, M., Seitz, C., Susani, M., Borkowski, A., Boccon-Gibod, L., Schulman, C. C. and Marberger, M. (2001). Prospective evaluation of prostate cancer detected on biopsies 1, 2, 3 and 4: When should we stop? *J Urol, 166*(5), 1679-1683.

Dodd, R. B. and Drickamer, K. (2001). Lectin-like proteins in model organisms: Implications for evolution of carbohydrate-binding activity. *Glycobiology, 11*(5), 71r-79r.

Dodla, M. C., Young, A., Venable, A., Hasneen, K., Rao, R. R., Machacek, D. W. and Stice, S. L. (2011). Differing lectin binding profiles among human embryonic stem cells and derivatives aid in the isolation of neural progenitor cells. *PLoS ONE, 6*(8), e23266.

Dosio, F., Brusa, P. and Cattel, L. (2011). Immunotoxins and anticancer drug conjugate assemblies: The role of the linkage between components. *Toxins, 3*(7), 848-883.

Doustjalali, S. R., Yusof, R., Govindasamy, G. K., Bustam, A. Z., Pillay, B. and Hashim, O. H. (2006). Patients with nasopharyngeal carcinoma demonstrate enhanced serum and tissue ceruloplasmin expression. *J Med Invest, 53*(1-2), 20-28.

Doustjalali, S. R., Yusof, R., Yip, C. H., Looi, L. M., Pillay, B. and Hashim, O. H. (2004). Aberrant expression of acute-phase reactant proteins in sera and breast lesions of patients with malignant and benign breast tumors. *Electrophoresis, 25*(14), 2392-2401.

Doyle, R. J., Nedjat-Haiem, F., Keller, K. F. and Frasch, C. E. (1984). Diagnostic value of interactions between members of the family Neisseriaceae and lectins. *J Clin Microbiol, 19*(3), 383-387.

Drickamer, K. and Taylor, M. E. (1998). Evolving views of protein glycosylation. *Trends Biochem Sci, 23*(9), 321-324.

Durkan, G. C., Sheikh, N., Johnson, P., Hildreth, A. J. and Greene, D. R. (2002). Improving prostate cancer detection with an extended-core transrectal ultrasonography-guided prostate biopsy protocol. *BJU Int, 89*(1), 33-39.

Ehmann, M., Felix, K., Hartmann, D., Schnolzer, M., Nees, M., Vorderwulbecke, S., Bogumil, R., Buchler, M. W. and Friess, H. (2007). Identification of potential markers for the detection of pancreatic cancer through comparative serum protein expression profiling. *Pancreas, 34*(2), 205-214.

El-Akawi, Z. J., Al-Hindawi, F. K. and Bashir, N. A. (2008). Alpha-1 antitrypsin (alpha1-AT) plasma levels in lung, prostate and breast cancer patients. *Neuro Endocrinol Lett, 29*(4), 482-484.

Ellinger, J., Bastian, P. J., Jurgan, T., Biermann, K., Kahl, P., Heukamp, L. C., Wernert, N., Muller, S. C. and von Ruecker, A. (2008). CpG island hypermethylation at multiple gene sites in diagnosis and prognosis of prostate cancer. *Urology, 71*(1), 161-167.

Emmerich, J. (2009). Alpha1-antitrypsin and the maintenance of hemostatic balance. *Haematologica, 94*(6), 762-763.

Eppel, G. A., Nagy, S., Jenkins, M. A., Tudball, R. N., Daskalakis, M., Balazs, N. D. and Comper, W. D. (2000). Variability of standard clinical protein assays in the analysis of a model urine solution of fragmented albumin. *Clin Biochem, 33*(6), 487-494.

Etaler, M. E. (1978). Distribution and function of plant lectins. . In I. E. Liener, N. Sharon and I. J. Goldstein (Eds.), *The Lectins, Properties, Ffunctions and Applications in Biology and Medicine* (pp. 371-435). Orlando: Academic Press.

Etzioni, R., Penson, D. F., Legler, J. M., di Tommaso, D., Boer, R., Gann, P. H. and Feuer, E. J. (2002). Overdiagnosis due to prostate-specific antigen screening: Lessons from U.S. prostate cancer incidence trends. *J Natl Cancer Inst, 94*(13), 981-990.

Euan, R. T. and Brian, A. B. (1989). Protein binding to nitrocellulose, nylon and PVDF membranes in immunoassays and electroblotting. *J Biochem Biophys Methods, 19*(2–3), 169-183.

Everley, P. A., Krijgsveld, J., Zetter, B. R. and Gygi, S. P. (2004). Quantitative Cancer Proteomics: Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) as a Tool for Prostate Cancer Research. *Mol Cell Proteomics, 3*(7), 729-735.

Fan, Y., Wang, J., Yang, Y., Liu, Q., Fan, Y., Yu, J., Zheng, S., Li, M. and Wang, J. (2010). Detection and identification of potential biomarkers of breast cancer. *J Cancer Res Clin Oncol, 136*(8), 1243-1254.

Feinberg, A. P., Ohlsson, R. and Henikoff, S. (2006). The epigenetic progenitor origin of human cancer. *Nat Rev Genet, 7*(1), 21-33.

Fentz, A. K., Sporl, M., Spangenberg, J., List, H. J., Zornig, C., Dorner, A., Layer, P., Juhl, H. and David, K. A. (2007). Detection of colorectal adenoma and cancer based on transthyretin and C3a-desArg serum levels. *Proteomics Clin Appl, 1*(6), 536-544.

Ferlay, J., Shin, H.-R., Bray, F., Forman, D., Mathers, C. and Parkin, D. M. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer, 127*(12), 2893-2917.

Fernandez, I., Touraine, P. and Goffin, V. (2010). Prolactin and human tumourogenesis. *J Neuroendocrinol, 22*(7), 771-777.

Ferrigno, D., Buccheri, G. and Camilla, T. (1995). Prognosis and lung-cancer - The contribution of plasma-proteins. *Oncol Rep, 2*(4), 637-641.

Fields, S. and Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature, 340*(6230), 245-246.

Fillmore, K. M., Chikritzhs, T., Stockwell, T., Bostrom, A. and Pascal, R. (2009). Alcohol use and prostate cancer: A meta-analysis. *Mol Nutr Food Res, 53*(2), 240-255.

Florens, L., Korfali, N. and Schirmer, E. C. (2008). Subcellular fractionation and proteomics of nuclear envelopes. *Methods Mol Biol, 432*, 117-137.

Fracasso, G., Bellisola, G., Castelletti, D., Tridente, G. and Colombatti, M. (2004). Immunotoxins and other conjugates: Preparation and general characteristics. *Mini Rev Med Chem, 4*(5), 545-562.

Freeman, M. R. and Solomon, K. R. (2004). Cholesterol and prostate cancer. *J Cell Biochem, 91*(1), 54-69.

Freeze, H. H. (1993). *Preparation and analysis of glycoconjugates.* New Jersey: John Wiley & Sons, Inc.

Freeze, H. H. (2001). *Lectin analysis of proteins blotted onto filters.* New Jersey: John Wiley & Sons, Inc.

Fries, E. and Blom, A. M. (2000). Bikunin - not just a plasma proteinase inhibitor. *Int J Biochem Cell Biol, 32*(2), 125-137.

Fujita, Y., Ezura, Y., Emi, M., Sato, K., Takada, D., Iino, Y., Katayama, Y., Takahashi, K., Kamimura, K., Bujo, H. and Saito, Y. (2004). Hypercholesterolemia associated with splice-junction variation of inter-a-trypsin inhibitor heavy chain 4 (ITIH4) gene. *J Hum Genet, 49*(1), 24-28.

Gabrielsen, M., Abdul-Rahman, P. S., Othman, S., Hashim, O. H. and Cogdell, R. J. (2014). Structures and binding specificity of galactose- and mannose-binding lectins from champedak: Differences from jackfruit lectins. *Acta Crystallogr F Struct Biol Commun, 70*(6), 709-716.

Gallagher, S. and Chakavarti, D. (2008). Immunoblot analysis. *J Vis Exp*, 10.3791/759(16).

Garraway, I. P., Seligson, D., Said, J., Horvath, S. and Reiter, R. E. (2004). Trefoil factor 3 is overexpressed in human prostate cancer. *Prostate, 61*(3), 209-214.

Gaspari, M. and Cuda, G. (2011). Nano LC-MS/MS: A robust setup for proteomic analysis. *Methods Mol Biol, 790*, 115-126.

Gebhard, W., Hochstrasser, K., Fritz, H., Enghild, J. J., Pizzo, S. V. and Salvesen, G. (1990). Structure of inter-alpha-inhibitor (inter-alpha-trypsin inhibitor) and pre-alpha-inhibitor: Current state and proposition of a new terminology. *Biol Chem Hoppe Seyler, 371*(Suppl), 13-22.

Gerfen, C. R. and Sawchenko, P. E. (1985). A method for anterograde axonal tracing of chemically specified circuits in the central nervous system: Combined *Phaseolus vulgaris*-leucoagglutinin (PHA-L) tract tracing and immunohistochemistry. *Brain Res, 343*(1), 144-150.

Gericke, B., Raila, J., Sehouli, J., Haebel, S., Konsgen, D., Mustea, A. and Schweigert, F. (2005). Microheterogeneity of transthyretin in serum and ascitic fluid of ovarian cancer patients. *BMC Cancer, 5*(1), 133.

Giannarini, G., Scott, C. A., Moro, U., Pertoldi, B., Beltrami, C. A. and Selli, C. (2008). Are PSA density and PSA density of the transition zone more accurate than PSA in predicting the pathological stage of clinically localized prostate cancer? *Urol Oncol, 26*(4), 353-360.

Giordani, R., Gachon, C., Buc, J., Regli, P. and Jacob, J. L. (1999). Antifungal action of *Hevea brasiliensis* latex. Its effect in combination with fluconazole on *Candida albicans* growth. *Mycoses, 42*(7-8), 465-474.

Giovannucci, E., Rimm, E. B., Liu, Y., Stampfer, M. J. and Willett, W. C. (2002). A prospective study of tomato products, lycopene and prostate cancer risk. *J Natl Cancer Inst, 94*(5), 391-398.

Giri, V. N., Beebe-Dimmer, J., Buyyounouski, M., Konski, A., Feigenberg, S. J., Uzzo, R. G., Hanks, G., Godwin, A. K., Chen, D. Y., Gordon, R., Cescon, T., Raysor, S. and Watkins-Bruner, D. (2007). Prostate cancer risk assessment program: A 10-year update of cancer detection. *J Urol, 178*(5), 1920-1924; discussion 1924.

Gleason, D. F. (1977). The Veterans Administration Cooperative Urological Research Group. Histologic grading and clinical staging of prostatic caricnoma. . In M. Tannenbaum (Ed.), *Urologic Pathology: The Prostate* (pp. 171–197). Philadelphia: Lea & Febiger.

Glen, A., Gan, C. S., Hamdy, F. C., Eaton, C. L., Cross, S. S., Catto, J. W. F., Wright, P. C. and Rehman, I. (2008). iTRAQ-Facilitated Proteomic Analysis of Human Prostate Cancer Cells Identifies Proteins Associated with Progression. *J Proteome Res, 7*(3), 897-907.

Goldstein, I. J., Hughes, R. C., Monsigny, M., Osawa, T. and Sharon, N. (1980). What should be called a lectin? *Nature, 285*(5760), 66-66.

Gottschalk, A. (1957). Neuraminidase: The specific enzyme of influenza virus and *Vibrio cholerae*. *Biochim Biophys Acta, 23*(3), 645-646.

Granger, C. B., Van Eyk, J. E., Mockrin, S. C., Anderson, N. L. and Members, o. b. o. t. W. G. (2004). National Heart, Lung and Blood Institute Clinical Proteomics Working Group Report. *Circulation, 109*(14), 1697-1703.

Greene, K. L., Albertsen, P. C., Babaian, R. J., Carter, H. B., Gann, P. H., Han, M., Kuban, D. A., Sartor, A. O., Stanford, J. L., Zietman, A. and Carroll, P. (2013). Prostate specific antigen best practice statement: 2009 update. *J Urol, 189*(Suppl 1), S2-S11.

Gregorc, V., Novello, S., Lazzari, C., Barni, S., Aieta, M., Mencoboni, M., Grossi, F., Pas, T. D., de Marinis, F., Bearz, A., Floriani, I., Torri, V., Bulotta, A., Cattaneo, A., Grigorieva, J., Tsypin, M., Roder, J., Doglioni, C., Levra, M. G., Petrelli, F., Foti, S., Viganò, M., Bachi, A. and Roder, H. (2014). Predictive value of a proteomic signature in patients with non-small-cell lung cancer treated with second-line erlotinib or chemotherapy (PROSE): A biomarker-stratified, randomised phase 3 trial. *Lancet Oncol, 15*(7), 713-721.

Griffin, T. J., Lock, C. M., Li, X.-j., Patel, A., Chervetsova, I., Lee, H., Wright, M. E., Ranish, J. A., Chen, S. S. and Aebersold, R. (2003). Abundance ratio-dependent proteomic analysis by mass spectrometry. *Anal Chem, 75*(4), 867-874.

Grover, P. K. and Resnick, M. I. (1995). Analysis of prostatic fluid: Evidence for the presence of a prospective marker for prostatic cancer. *Prostate, 26*(1), 12-18.

Grover, P. K. and Resnick, M. I. (1997). High resolution two-dimensional electrophoretic analysis of urinary proteins of patients with prostatic cancer. *Electrophoresis, 18*(5), 814-818.

Grus, F. H., Joachim, S. C., Sandmann, S., Thiel, U., Bruns, K., Lackner, K. J. and Pfeiffer, N. (2008). Transthyretin and complex protein pattern in aqueous humor of patients with primary open-angle glaucoma. *Mol Vis, 14*, 1437-1445.

Guess, H. A. (2001). Benign prostatic hyperplasia and prostate cancer. *Epidemiol Rev, 23*(1), 152-158.

Hamm, A., Veeck, J., Bektas, N., Wild, P. J., Hartmann, A., Heindrichs, U., Kristiansen, G., Werbowetski-Ogilvie, T., Del Maestro, R., Knuechel, R. and Dahl, E. (2008). Frequent expression loss of Inter-alpha-trypsin inhibitor heavy chain (ITIH) genes in multiple human solid tumors: A systematic expression analysis. *BMC Cancer, 8*, 25.

Hamrita, B., Chahed, K., Trimeche, M., Guillier, C. L., Hammann, P., Chaieb, A., Korbi, S. and Chouchane, L. (2009). Proteomics-based identification of alpha1-antitrypsin and haptoglobin precursors as novel serum markers in infiltrating ductal breast carcinomas. *Clin Chim Acta, 404*(2), 111-118.

Hanash, S. M., Pitteri, S. J. and Faca, V. M. (2008). Mining the plasma proteome for cancer biomarkers. *Nature, 452*(7187), 571-579.

Harpio, R. and Einarsson, R. (2004). S100 proteins as cancer biomarkers with focus on S100B in malignant melanoma. *Clin Biochem, 37*(7), 512-518.

Harris, G. (2011). U.S. panel says no to prostate screening for healthy men. *The New York Times*.

Haselbeck, A., Schickaneder, E., von der Eltz, H. and Hosel, W. (1990). Structural characterization of glycoprotein carbohydrate chains by using digoxigenin-labeled lectins on blots. *Anal Biochem, 191*(1), 25-30.

Hashim, O. H., Gendeh, G. S., Cheong, C. N. and Jaafar, M. I. (1994). Effect of *Artocarpus integer* lectin on functional activity of guinea-pig complement. *Immunol Invest, 23*(2), 153-160.

Hashim, O. H., Gendeh, G. S. and Jaafar, M. I. (1993). Comparative analyses of IgA1 binding lectins from seeds of six distinct clones of *Artocarpus integer*. *Biochem Mol Biol Int, 29*(1), 69-76.

Hashim, O. H., Kobayashi, K. and Taniguchi, N. (1992). Interaction of Artocarpus lectins with human IgA does not involve asparagine-linked oligosaccharide of the immunoglobulin. *Biochem Int, 27*(3), 423-429.

Hashim, O. H., Ng, C. L., Gendeh, S. and Nik Jaafar, M. I. (1991). IgA binding lectins isolated from distinct *Artocarpus species* demonstrate differential specificity. *Mol Immunol, 28*(4-5), 393-398.

Hayashi, I., Amano, H., Yoshida, S., Kamata, K., Kamata, M., Inukai, M., Fujita, T., Kumagai, Y., Furudate, S. and Majima, M. (2002). Suppressed angiogenesis in kininogen-deficiencies. *Lab Invest, 82*(7), 871-880.

Heath, P. R., Chessell, I. P., Sanders, M. W., Francis, P. T., Bowen, D. M. and Pearson, R. C. (1997). Neuronal degeneration by suicide transport following injection of volkensin into rat cerebral cortex. *Exp Neurol, 147*(1), 192-203.

Henry, J. B. (1979). *Clinical diagnosis and management by laboratory methods* (Vol. 1). Philadelphia: W.B Saunders Company.

Hermanson, G. T., Mallia, A. K. and Smith, P. K. (1992). *Immobilized affinity ligand techniques*. New York: Academic Press.

Heukeshoven, J. and Dernick, R. (1988). Improved silver staining procedure for fast staining in PhastSystem Development Unit. I. Staining of sodium dodecyl sulfate gels. *Electrophoresis, 9*(1), 28-32.

Heumann, K. G. (2003). *Handbook of elemental speciation: Techniques and methodology*. West Sussex: John Wiley & Sons Ltd.

Hineno, T., Sano, A., Kondoh, K., Ueno, S., Kakimoto, Y. and Yoshida, K. (1991). Secretion of sphingolipid hydrolase activator precursor, prosaposin. *Biochem Biophys Res Commun, 176*(2), 668-674.

Ho, S.-M., Leung, Y.-K. and Chung, I. (2006). Estrogens and antiestrogens as etiological factors and therapeutics for prostate cancer. *Ann N Y Acad Sci, 1089*(1), 177-193.

Ho, S. M., Lee, M. T., Lam, H. M. and Leung, Y. K. (2011). Estrogens and prostate cancer: Etiology, mediators, prevention, and management. *Endocrinol metab Clin North Am, 40*(3), 591-614, ix.

Ho, S. M. and Roy, D. (1994). Sex hormone-induced nuclear DNA damage and lipid peroxidation in the dorsolateral prostates of Noble rats. *Cancer Lett, 84*(2), 155-162.

Hochstrasser, K., Reisinger, P. W. M., Albrecht, G. J. and Wustrow, T. P. U. (1989). Proteinase inhibitors as tumor associated growth factors. *Laryngo Rhino Otol, 68*(01), 51-56.

Hoffman, R. M., Clanon, D. L., Littenberg, B., Frank, J. J. and Peirce, J. C. (2000). Using the free-to-total prostate-specific antigen ratio to detect prostate cancer in men with nonspecific elevations of prostate-specific antigen levels. *J Gen Intern Med, 15*(10), 739-748.

Hong, M., Zhang, X., Hu, Y., Wang, H., He, W., Mei, H., Yu, J., Guo, T. and Song, S. (2008). The potential biomarkers for thromboembolism detected by SELDI-ToF-MS. *Thromb Res, 123*(3), 556-564.

Horn, K. D. (1999). The classification, recognition and significance of polyagglutination in transfusion medicine. *Blood Rev, 13*(1), 36-44.

Hortin, G. L. and Sviridov, D. (2007). Diagnostic potential for urinary proteomics. *Pharmacogenomics, 8*(3), 237-255.

Howard, T. E. (1989). *Clinical chemistry*. New York: John Wiley and Sons.

Howlader, N., Noone, A. M., Krapcho, M., Neyman, N., Aminou, R., Altekruse, S. F., Kosary, C. L., Ruhl, J., Tatalovich, Z., Cho, H., Mariotto, A., Eisner, M. P., Lewis, D. R., Chen, H. S., Feuer, E. J. and Cronin, K. A. (2012). SEER Cancer Statistics Review, 1975-2009 (Vintage 2009 Populations). Bethesda: National Cancer Institute, US.

Hu, S., Delorme, N., Liu, Z., Liu, T., Velasco-Gonzalez, C., Garai, J., Pullikuth, A. and Koochekpour, S. (2010). Prosaposin down-modulation decreases metastatic prostate cancer cell adhesion, migration, and invasion. *Mol Cancer, 9*, 30.

Huang, H., Campbell, S. C., Nelius, T., Bedford, D. F., Veliceasa, D., Bouck, N. P. and Volpert, O. V. (2004a). Alpha1-antitrypsin inhibits angiogenesis and tumor growth. *Int J Cancer, 112*(6), 1042-1048.

Huang, L., Pu, Y., Alam, S., Birch, L. and Prins, G. S. (2004b). Estrogenic regulation of signaling pathways and homeobox genes during rat prostate development. *J Androl, 25*(3), 330-337.

Huang, M., Narita, S., Numakura, K., Tsuruta, H., Saito, M., Inoue, T., Horikawa, Y., Tsuchiya, N. and Habuchi, T. (2012). A high-fat diet enhances proliferation of prostate cancer cells and activates MCP-1/CCR2 signaling. *Prostate, 72*(16), 1779-1788.

Hudson, L. and Hay, F. C. (1980). *Practical immunology* (2 ed.). London: Blackwell Scientific Publication.

Huizen, I. V., Wu, G., Moussa, M., Chin, J. L., Fenster, A., Lacefield, J. C., Sakai, H., Greenberg, N. M. and Xuan, J. W. (2005). Establishment of a serum tumor marker for preclinical trials of mouse prostate cancer models. *Clin Cancer Res, 11*(21), 7911-7919.

Huncharek, M., Haddock, K. S., Reid, R. and Kupelnick, B. (2010). Smoking as a risk factor for prostate cancer: A meta-analysis of 24 prospective cohort studies. *Am J Public Health, 100*(4), 693-701.

Hutchens, T. W. and Yip, T.-T. (1993). New desorption strategies for the mass spectrometric analysis of macromolecules. *Rapid Commun Mass Spectrom, 7*(7), 576-580.

Inoue, K., Slaton, J. W., Eve, B. Y., Kim, S. J., Perrotte, P., Balbay, M. D., Yano, S., Bar-Eli, M., Radinsky, R., Pettaway, C. A. and Dinney, C. P. (2000). Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. *Clin Cancer Res, 6*(5), 2104-2119.

Itaya, S. K. and van Hoesen, G. W. (1982). WGA-HRP as a transneuronal marker in the visual pathways of monkey and rat. *Brain Res, 236*(1), 199-204.

Ito, K. (2014). Prostate cancer in Asian men. *Nat Rev Urol, 11*(4), 197-212.

Ito, K., Yamamoto, T., Ohi, M., Kurokawa, K., Suzuki, K. and Yamanaka, H. (2003). Free/total PSA ratio is a powerful predictor of future prostate cancer morbidity in men with initial PSA levels of 4.1 to 10.0 ng/mL. *Urology, 61*(4), 760-764.

Izmirli, M., Arikan, B., Bayazit, Y. and Alptekin, D. (2011). Polymorphisms of HPC2/ELAC2 and SRD5A2 (5alpha-Reductase Type II) genes in prostate cancer. *Balkan J Med Genet, 14*(1), 31-36.

James, P. (1997). Protein identification in the post-genome era: The rapid rise of proteomics. *Q Rev Biophys, 30*(4), 279-331.

Jaramillo, F. J. L., Mateo, F. H. and Gonzalez, F. S. (2012). *Vinyl sulfone: A multi-purpose function in proteomics*: InTech.

Jayapalan, J. J., Ng, K. L., Razack, A. H. A. and Hashim, O. H. (2012). Identification of potential complementary serum biomarkers to differentiate prostate cancer from benign prostatic hyperplasia using gel- and lectin-based proteomics analyses. *Electrophoresis, 33*(12), 1855-1862.

Jayapalan, J. J., Ng, K. L., Shuib, A. S., Razack, A. H. and Hashim, O. H. (2013). Urine of patients with early prostate cancer contains lower levels of light chain fragments of inter-alpha-trypsin inhibitor and saposin B but increased expression of an inter-alpha-trypsin inhibitor heavy chain 4 fragment. *Electrophoresis, 34*(11), 1663-1669.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E. and Forman, D. (2011). Global cancer statistics. *CA Cancer J Clin, 61*(2), 69-90.

Jenkins, R. E., Kitteringham, N. R., Greenough, C. and Park, B. K. (2008). Albumin and immunoglobulin depletion of human plasma. *Methods Mol Biol, 425*, 15-25.

Jeronimo, C., Usadel, H., Henrique, R., Silva, C., Oliveira, J., Lopes, C. and Sidransky, D. (2002). Quantitative GSTP1 hypermethylation in bodily fluids of patients with prostate cancer. *Urology, 60*(6), 1131-1135.

Jessie, K., Jayapalan, J. J., Ong, K. C., Abdul Rahim, Z. H., Zain, R. M., Wong, K. T. and Hashim, O. H. (2013). Aberrant proteins in the saliva of patients with oral squamous cell carcinoma. *Electrophoresis, 34*(17), 2495-2502.

Jung, K., Cho, W. and Regnier, F. E. (2009). Glycoproteomics of plasma based on narrow selectivity lectin affinity chromatography. *J Proteome Res, 8*(2), 643-650.

Jungblut, P. R., Grabher, G. and Stoffler, G. (1999). Comprehensive detection of immunorelevant Borrelia garinii antigens by two-dimensional electrophoresis. *Electrophoresis, 20*(18), 3611-3622.

Kabir, S., Aebersold, R. and Daar, A. S. (1993). Identification of a novel 4 kDa immunoglobulin-A-binding peptide obtained by the limited proteolysis of jacalin. *Biochim Biophys Acta, 1161*(2-3), 194-200.

Kalopissis, A. D., Pastier, D. and Chambaz, J. (2003). Apolipoprotein A-II: Beyond genetic associations with lipid disorders and insulin resistance. *Curr Opin Lipidol, 14*(2), 165-172.

Kamionka, M., Rehm, T., Beisel, H. G., Lang, K., Engh, R. A. and Holak, T. A. (2002). In silico and NMR identification of inhibitors of the IGF-I and IGF-binding protein-5 interaction. *J Med Chem, 45*(26), 5655-5660.

Kamoshida, S. and Tsutsumi, Y. (1990). Extraprostatic localization of prostatic acid phosphatase and prostate-specific antigen: Distribution in cloacogenic glandular epithelium and sex-dependent expression in human anal gland. *Hum Pathol, 21*(11), 1108-1111.

Kanmura, S., Uto, H., Sato, Y., Kumagai, K., Sasaki, F., Moriuchi, A., Oketani, M., Ido, A., Nagata, K., Hayashi, K., Stuver, S. O. and Tsubouchi, H. (2010). The complement component C3a fragment is a potential biomarker for hepatitis C virus-related hepatocellular carcinoma. *J Gastroenterol, 45*(4), 459-467.

Kaplan, A. and Cosentino, L. (2010). Alpha1-antitrypsin deficiency: Forgotten etiology. *Can Fam Physician, 56*(1), 19-24.

Kaplan, L. A. and Pesce, A. J. (2009). *Clinical chemistry* (5 ed.). St. Louis: Mosby.

Kase, F. and Pospisil, J. (1983). Physiologic inhibitors of blood coagulation. 2. Alpha 2-macroglobulin, alpha-antitrypsin and the C1 inhibitor of complement. *Folia Haematol Int Mag Klin Morphol Blutforsch, 110*(5), 634-650.

Kashyap, R. S., Nayak, A. R., Deshpande, P. S., Kabra, D., Purohit, H. J., Taori, G. M. and Daginawala, H. F. (2009). Inter-alpha-trypsin inhibitor heavy chain 4 is a novel marker of acute ischemic stroke. *Clin Chim Acta, 402*(1-2), 160-163.

Kawano, M., Kawakami, M., Otsuka, M., Yashima, H., Yaginuma, T. and Ueki, A. (1995). Marked decrease of plasma apolipoprotein AI and AII in Japanese patients with late-onset non-familial Alzheimer's disease. *Clin Chim Acta, 239*(2), 209-211.

Khan, F., Khan, R. H., Sherwani, A., Mohmood, S. and Azfer, M. A. (2002). Lectins as markers for blood grouping. *Med Sci Monit, 8*(12), Ra293-300.

Kijimoto-Ochiai, S., Katagiri, Y. U. and Ochiai, H. (1985). Analysis of N-linked oligosaccharide chains of glycoproteins on nitrocellulose sheets using lectin-peroxidase reagents. *Anal Biochem, 147*(1), 222-229.

Kilpatrick, D. C. (2002). Animal lectins: A historical introduction and overview. *Biochimica Biophysica Acta, 1572*(2–3), 187-197.

Kilpatrick, D. C., Graham, C., Urbaniak, S. J., Jeffree, C. E. and Allen, K. (1984). A comparison of tomato (*Lycopersicon esculentum*) lectin with its deglycosylated derivative. *Biochem J, 220*(3), 843-847.

Kim, E. H. and Misek, D. E. (2011). Glycoproteomics-based identification of cancer biomarkers. *Int J Proteomics, 2011*, 10.

Kimura, T. (2012). East meets West: Ethnic differences in prostate cancer epidemiology between East Asians and Caucasians. *Chin J Cancer, 31*(9), 421-429.

Kishimoto, Y., Hiraiwa, M. and O'Brien, J. S. (1992). Saposins: structure, function, distribution and molecular genetics. *J Lipid Res, 33*(9), 1255-1267.

Klein, E. A., Thompson, I. M., Tangen, C. M. and et al. (2011). Vitamin E and the risk of prostate cancer: The selenium and vitamin E cancer prevention trial (SELECT). *JAMA, 306*(14), 1549-1556.

Klose, J. (1975). Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. *Humangenetik, 26*(3), 231-243.

Kloth, J. N., Gorter, A., Fleuren, G. J., Oosting, J., Uljee, S., ter Haar, N., Dreef, E. J., Kenter, G. G. and Jordanova, E. S. (2008). Elevated expression of SerpinA1 and SerpinA3 in HLA-positive cervical carcinoma. *J Pathol, 215*(3), 222-230.

Klotz, L. H. (1997). PSAdynia and other PSA-related syndromes: A new epidemic - a case history and taxonomy. *Urology, 50*(6), 831-832.

Knox, R. B., Clarke, A., Harrison, S., Smith, P. and Marchalonis, J. J. (1976). Cell recognition in plants: Determinants of the stigma surface and their pollen interactions. *Proc Natl Acad Sci U S A, 73*(8), 2788-2792.

Kobayashi, H., Suzuki, M., Tanaka, Y., Hirashima, Y. and Terao, T. (2001). Suppression of urokinase expression and invasiveness by urinary trypsin inhibitor is mediated through inhibition of protein kinase C- and MEK/ERK/c-Jun-dependent signaling pathways. *J Biol Chem, 276*(3), 2015-2022.

Kobayashi, K., Kondoh, H., Hagiwara, K. and Vaerman, J. P. (1988). Jacalin: Chaos in its immunoglobulin-binding specificity. *Mol Immunol, 25*(10), 1037-1038.

Kocourek, J. and Harejsi, V. (1983). A note in the recent discussion on definition of the term "lectin". In T. C. Bog-Hansen and G. A. Spengler (Eds.), *Lectin: Biology, Biochemistry, Clinical Biochemistry*. Berlin: de Gruyter.

Kollermann, J., Weikert, S., Schostak, M., Kempkensteffen, C., Kleinschmidt, K., Rau, T. and Pantel, K. (2008). Prognostic significance of disseminated tumor cells in the bone marrow of prostate cancer patients treated with neoadjuvant hormone treatment. *J Clin Oncol, 26*(30), 4928-4933.

Kon, O., Yip, T.-T., Ho, M., Chan, W., Wong, W., Tan, S., Ng, W., Kam, S., Eng, A., Ho, P., Viner, R., Ong, H. and Kumarasinghe, M. P. (2008). The distinctive gastric fluid proteome in gastric cancer reveals a multi-biomarker diagnostic profile. *BMC Medical Genomics, 1*(1), 54.

Koochekpour, S., Lee, T. J., Wang, R., Culig, Z., Delorme, N., Caffey, S., Marrero, L. and Aguirre, J. (2007). Prosaposin upregulates AR and PSA expression and activity in prostate cancer cells (LNCaP). *Prostate, 67*(2), 178-189.

Koochekpour, S., Zhuang, Y.-J., Beroukhim, R., Hsieh, C.-L., Hofer, M. D., Zhau, H. E., Hiraiwa, M., Pattan, D. Y., Ware, J. L., Luftig, R. B., Sandhoff, K., Sawyers, C. L., Pienta, K. J., Rubin, M. A., Vessella, R. L., Sellers, W. R. and Sartor, O. (2005). Amplification and overexpression of prosaposin in prostate cancer. *Genes, Chromosom Cancer, 44*(4), 351-364.

Krecicki, T. and Leluk, M. (1992). Acute phase reactant proteins - An aid to monitoring surgical treatment of laryngeal carcinoma. *J Laryngol Otol, 106*(7), 613-615.

Kuhlmann, W. D., Peschke, P. and Wurster, K. (1983). Lectin - Peroxidase conjugates in histopathology of gastrointestinal mucosa. *Virchows Arch A Pathol Anat Histopathol, 398*(3), 319-328.

Kuk, C., Kulasingam, V., Gunawardana, C. G., Smith, C. R., Batruch, I. and Diamandis, E. P. (2009). Mining the ovarian cancer ascites proteome for potential ovarian cancer biomarkers. *Mol Cell Proteomics, 8*(4), 661-669.

Kurien, B. T. and Scofield, R. H. (2009). A brief review of other notable protein detection methods on blots. *Methods Mol Biol, 536*, 557-571.

Kuriyama, M., Takahashi, K., Yamano, T., Hokezu, Y., Togo, S., Osame, M. and Igakura, T. (1994). Low levels of serum apolipoprotein A-I and A-II in senile dementia. *Jpn J Psychiatry Neurol, 48*(3), 589-593.

Lafitte, D., Dussol, B., Andersen, S., Vazi, A., Dupuy, P., Jensen, O. N., Berland, Y. and Verdier, J. M. (2002). Optimized preparation of urine samples for two-dimensional electrophoresis and initial application to patient samples. *Clin Biochem, 35*(8), 581-589.

Larsen, M. R., Sorensen, G. L., Fey, S. J., Larsen, P. M. and Roepstorff, P. (2001). Phospho-proteomics: Evaluation of the use of enzymatic de-phosphorylation and differential mass spectrometric peptide mass mapping for site specific phosphorylation assignment in proteins separated by gel electrophoresis. *Proteomics, 1*(2), 223-238.

Lau, B. F., Aminudin, N. and Abdullah, N. (2011). Comparative SELDI-ToF/MS profiling of low-molecular-mass proteins from *Lignosus rhinocerus* (Cooke) Ryvarden grown under stirred and static conditions of liquid fermentation. *J Microbiol Methods, 87*(1), 56-63.

Lehrer, S. (1998). Possible relationship of the apolipoprotein E (ApoE) epsilon4 allele to prostate cancer. *Br J Cancer, 78*(10), 1398.

Lei, T., He, Q. Y., Wang, Y. L., Si, L. S. and Chiu, J. F. (2008). Heparin chromatography to deplete high-abundance proteins for serum proteomics. *Clin Chim Acta, 388*(1-2), 173-178.

Leyden, E. (1872). Zur Kenntniss des Bronchial-Asthma. *Archiv für pathologische Anatomie und Physiologie und für klinische Medicin, 54*(4), 324-344.

Li, D., Chiu, H., Chen, J., Zhang, H. and Chan, D. W. (2013a). Integrated analyses of proteins and their glycans in a magnetic bead-based multiplex assay format. *Clin Chem, 59*(1), 315-324.

Li, D., Chiu, H., Zhang, H. and Chan, D. (2013b). Analysis of serum protein glycosylation by a differential lectin immunosorbant assay (dLISA). *Clin Proteomics, 10*(1), 12.

Li, J., Djenaba, J. A., Soman, A., Rim, S. H. and Master, V. A. (2012). Recent trends in prostate cancer incidence by age, cancer stage and race, the United States, 2001-2007. *Prostate Cancer, 2012*, 8.

Li, W. P., Zuber, C. and Roth, J. (1993). Use of *Phaseolus vulgaris* leukoagglutinating lectin in histochemical and blotting techniques: a comparison of digoxigenin- and biotin-labelled lectins. *Histochemistry, 100*(5), 347-356.

Li, Y., Dang, T. A., Shen, J., Perlaky, L., Hicks, J., Murray, J., Meyer, W., Chintagumpala, M., Lau, C. C. and Man, T. K. (2006). Identification of a plasma proteomic signature to distinguish pediatric osteosarcoma from benign osteochondroma. *Proteomics, 6*(11), 3426-3435.

Li, Z., Habuchi, T., Mitsumori, K., Kamoto, T., Kinoshitu, H., Segawa, T., Ogawa, O. and Kato, T. (2003). Association of V89L SRD5A2 polymorphism with prostate cancer development in a Japanese population. *J Urol, 169*(6), 2378-2381.

Liede, A., Karlan, B. Y. and Narod, S. A. (2004). Cancer risks for male carriers of germline mutations in BRCA1 or BRCA2: A review of the literature. *J Clin Oncol, 22*(4), 735-742.

Lim, G. C. C., Rampal, S. and Yahaya, H. (2008). Cancer incidences in Peninsular Malaysia 2003-2005 (pp. 143-145). Kuala Lumpur: National Cancer Registry.

Lim, S. B., Chua, C. T. and Hashim, O. H. (1997). Isolation of a mannose-binding and IgE- and IgM-reactive lectin from the seeds of *Artocarpus integer*. *J Immunol Methods, 209*(2), 177-186.

Lim, S. B., Kanthimathi, M. S. and Hashim, O. H. (1998). Effect of the mannose-binding *Artocarpus integer* lectin on the cellular proliferation of murine lymphocytes. *Immunol Invest, 27*(6), 395-404.

Lis, H. and Sharon, N. (1986). Lectins as molecules and as tools. *Annu Rev Biochem, 55*, 35-67.

Lisowska, E. and Jaskiewicz, E. (2001). *Protein glycosylation, an overview.* (10.1002/9780470015902.a0006211.pub3). Chichester: John Wiley & Sons Ltd.

Liu, D. C. and Yang, Z. L. (2013). MTDH and EphA7 are markers for metastasis and poor prognosis of gallbladder adenocarcinoma. *Diagn Cytopathol, 41*(3), 199-205.

Liu, L., Liu, J., Dai, S., Wang, X., Wu, S., Wang, J., Huang, L., Xiao, X. and He, D. (2007). Reduced transthyretin expression in sera of lung cancer. *Cancer Sci, 98*(10), 1617-1624.

Liu, L., Sun, S., Liu, J., Wu, S., Dai, S., Wang, X., Huang, L., Xiao, X. and He, D. (2009). A new serum biomarker for lung cancer - transthyretin. *Zhongguo Fei Ai Za Zhi, 12*(4), 300-305.

Liu, T., Qian, W. J., Gritsenko, M. A., Camp, D. G., 2nd, Monroe, M. E., Moore, R. J. and Smith, R. D. (2005). Human plasma N-glycoproteome analysis by immunoaffinity subtraction, hydrazide chemistry, and mass spectrometry. *J Proteome Res, 4*(6), 2070-2080.

Loeb, S., Carter, H. B., Berndt, S. I., Ricker, W. and Schaeffer, E. M. (2011). Complications after prostate biopsy: Data from SEER-Medicare. *J Urol, 186*(5), 1830-1834.

Loo, D., Jones, A. and Hill, M. M. (2010). Lectin magnetic bead array for biomarker discovery. *J Proteome Res, 9*(10), 5496-5500.

López-Pedrera, C., Barbarroja, N. and Villalba, J. M. (2009). Novel biomarkers of atherosclerosis and cardiovascular risk in autoimmune diseases: Genomics and proteomics approaches. *Protemics Clin Appl, 3*(2), 213-225.

Lopez, M. F., Berggren, K., Chernokalskaya, E., Lazarev, A., Robinson, M. and Patton, W. F. (2000). A comparison of silver stain and SYPRO Ruby protein gel stain with respect to protein detection in two-dimensional gels and identification by peptide mass profiling. *Electrophoresis, 21*(17), 3673-3683.

Lovrien, R. E. and Matulis, D. (1997). *Selective precipitation of proteins*. New Jersey: John Wiley & Sons, Inc.

Lu, J. and Danielsen, M. (1996). A *Stu* I polymorphism in the human androgen receptor gene (AR). *Clin Genet, 49*(6), 323-324.

Luczak, M., Formanowicz, D., Pawliczak, E., Wanic-Kossowska, M., Wykretowicz, A. and Figlerowicz, M. (2011). Chronic kidney disease-related atherosclerosis - proteomic studies of blood plasma. *Proteome Sci, 9*(1), 25.

Luque-Garcia, J. L. and Neubert, T. A. (2009). On-membrane tryptic digestion of proteins for mass spectrometry analysis. *Methods Mol Biol, 536*, 331-341.

Mach, L., Scherf, W., Ammann, M., Poetsch, J., Bertsch, W., Marz, L. and Glossl, J. (1991). Purification and partial characterization of a novel lectin from elder (*Sambucus nigra* L.) fruit. *Biochem J, 278 (Pt 3)*, 667-671.

Maciel, C. M., Junqueira, M., Paschoal, M. E., Kawamura, M. T., Duarte, R. L., Carvalho Mda, G. and Domont, G. B. (2005). Differential proteomic serum pattern of low molecular weight proteins expressed by adenocarcinoma lung cancer patients. *J Exp Ther Oncol, 5*(1), 31-38.

Maciel, E. V., Araujo-Filho, V. S., Nakazawa, M., Gomes, Y. M., Coelho, L. C. and Correia, M. T. (2004). Mitogenic activity of *Cratylia mollis* lectin on human lymphocytes. *Biologicals, 32*(1), 57-60.

Madrid, J. F., Aparicio, R., Saez, F. J. and Hernandez, F. (2000). Lectin cytochemical characterization of the *N*- and *O*-linked oligosaccharides in the human rectum. *Histochem J, 32*(5), 281-289.

Madrid, J. F., Castells, M. T., Martinez-Menarguez, J. A., Aviles, M., Hernandez, F. and Ballesta, J. (1994). Subcellular characterization of glycoproteins in the principal cells of human gallbladder. A lectin cytochemical study. *Histochemistry, 101*(3), 195-204.

Mahmood, T. and Yang, P. C. (2012). Western blot: Technique, theory, and trouble shooting. *N Am J Med Sci, 4*(9), 429-434.

Maier, M., Peng, Y., Jiang, L., Seabrook, T. J., Carroll, M. C. and Lemere, C. A. (2008). Complement C3 deficiency leads to accelerated amyloid beta plaque deposition and neurodegeneration and modulation of the microglia/macrophage phenotype in amyloid precursor protein transgenic mice. *J Neurosci, 28*(25), 6333-6341.

Maiga, S. F., Kalopissis, A. D. and Chabert, M. (2014). Apolipoprotein A-II is a key regulatory factor of HDL metabolism as appears from studies with transgenic animals and clinical outcomes. *Biochimie, 96*, 56-66.

Majek, P., Reicheltova, Z., Suttnar, J., Maly, M., Oravec, M., Pecankova, K. and Dyr, J. (2011). Plasma proteome changes in cardiovascular disease patients: Novel isoforms of apolipoprotein A1. *J Transl Med, 9*(1), 84.

Mann, M. and Jensen, O. N. (2003). Proteomic analysis of post-translational modifications. *Nat Biotechnol, 21*(3), 255-261.

Marengo, E., Robotti, E., Antonucci, F., Cecconi, D., Campostrini, N. and Righetti, P. G. (2005). Numerical approaches for quantitative analysis of two-dimensional maps: A review of commercial software and home-made systems. *Proteomics, 5*(3), 654-666.

Matsuzaki, H., Kobayashi, H., Yagyu, T., Wakahara, K., Kondo, T., Kurita, N., Sekino, H., Inagaki, K., Suzuki, M., Kanayama, N. and Terao, T. (2004). Bikunin inhibits lipopolysaccharide-induced tumor necrosis factor alpha induction in macrophages. *Clin Diagn Lab Immunol, 11*(6), 1140-1147.

McNaughton-Collins, M., Fowler, F. J., Jr., Caubet, J. F., Bates, D. W., Lee, J. M., Hauser, A. and Barry, M. J. (2004). Psychological effects of a suspicious prostate cancer screening test followed by a benign biopsy result. *Am J Med, 117*(10), 719-725.

Mearini, L., Zucchi, A., Nunzi, E., Villirillo, T., Bini, V. and Porena, M. (2013). Low serum testosterone levels are predictive of prostate cancer. *World J Urol, 31*(2), 247-252.

Mechref, Y., Madera, M. and Novotny, M. V. (2007). Technical aspects of glycoprotein enrichment. In C. L. Nilsson (Ed.), *Lectin: Analytical Technologies* (pp. 267-298). Amsterdam: Elsevier.

Mechref, Y., Madera, M. and Novotny, M. V. (2008). Glycoprotein enrichment through lectin affinity techniques. In A. Posch (Ed.), *2D PAGE: Sample Preparation and Fractionation* (Vol. 424, pp. 373-396). New Jersey: Humana Press.

Mestecky, J., Tomana, M., Crowley-Nowick, P. A., Moldoveanu, Z., Julian, B. A. and Jackson, S. (1993). Defective galactosylation and clearance of IgA1 molecules as a possible etiopathogenic factor in IgA nephropathy. *Contrib Nephrol, 104*, 172-182.

Metcalfe, C., Patel, B., Evans, S., Ibrahim, F., Anson, K., Chinegwundoh, F., Corbishley, C., Dorling, D., Thomas, B., Gillatt, D., Kirby, R., Muir, G., Nargund, V., Popert, R., Persad, R. and Ben-Shlomo, Y. (2008). The risk of prostate cancer amongst South Asian men in southern England: The PROCESS cohort study. *BJU Int, 102*(10), 1407-1412.

Meyer, M. S., Penney, K. L., Stark, J. R., Schumacher, F. R., Sesso, H. D., Loda, M., Fiorentino, M., Finn, S., Flavin, R. J., Kurth, T., Price, A. L., Giovannucci, E. L., Fall, K., Stampfer, M. J., Ma, J. and Mucci, L. A. (2010). Genetic variation in RNASEL associated with prostate cancer risk and progression. *Carcinogenesis, 31*(9), 1597-1603.

Meyer, T. E., Coker, A. L., Sanderson, M. and Symanski, E. (2007). A case-control study of farming and prostate cancer in African-American and Caucasian men. *Occup Environ Med, 64*(3), 155-160.

Michalaki, V., Syrigos, K., Charles, P. and Waxman, J. (2004). Serum levels of IL-6 and TNF-alpha correlate with clinicopathological features and patient survival in patients with prostate cancer. *Br J Cancer*, *90*(12), 2312-2316.

Miguet, L., Bogumil, R., Decloquement, P., Herbrecht, R., Potier, N., Mauvieux, L. and Van Dorsselaer, A. (2006). Discovery and identification of potential biomarkers in a prospective study of chronic lymphoid malignancies using SELDI-ToF/MS. *J Proteome Res*, *5*(9), 2258-2269.

Mikolajczyk, S. D., Millar, L. S., Wang, T. J., Rittenhouse, H. G., Marks, L. S., Song, W., Wheeler, T. M. and Slawin, K. M. (2000). A precursor form of prostate-specific antigen is more highly elevated in prostate cancer compared with benign transition zone prostate tissue. *Cancer Res*, *60*(3), 756-759.

Miles, A. K., Matharoo-Ball, B., Li, G., Ahmad, M. and Rees, R. C. (2006). The identification of human tumour antigens: Current status and future developments. *Cancer Immunol Immunother*, *55*(8), 996-1003.

Miller, B. A., Chu, K. C., Hankey, B. F. and Ries, L. A. (2008). Cancer incidence and mortality patterns among specific Asian and Pacific Islander populations in the U.S. *Cancer Causes Control*, *19*(3), 227-256.

Millioni, R., Miuzzo, M., Sbrignadello, S., Murphy, E., Puricelli, L., Tura, A., Bertacco, E., Rattazzi, M., Iori, E. and Tessari, P. (2010). Delta2D and Proteomweaver: Performance evaluation of two different approaches for 2-DE analysis. *Electrophoresis*, *31*(8), 1311-1317.

Millum, J., Wendler, D. and Emanuel, E. J. (2013). The 50th anniversary of the declaration of helsinki: Progress but many remaining challenges. *JAMA*, *310*(20), 2143-2144.

Misasi, R., Hozumi, I., Inuzuka, T., Capozzi, A., Mattei, V., Kuramoto, Y., Shimeno, H., Soeda, S., Azuma, N., Yamauchi, T. and Hiraiwa, M. (2009). Biochemistry and neurobiology of prosaposin: A potential therapeutic neuro-effector. *Cent Nerv Syst Agents Med Chem*, *9*(2), 119-131.

Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., Peterson, A., Noteboom, J., O'Briant, K. C., Allen, A., Lin, D. W., Urban, N., Drescher, C. W., Knudsen, B. S., Stirewalt, D. L., Gentleman, R., Vessella, R. L., Nelson, P. S., Martin, D. B. and Tewari, M. (2008). Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA*, *105*(30), 10513-10518.

Mohamed, E., Abdul-Rahman, P. S., Doustjalali, S. R., Chen, Y., Lim, B. K., Omar, S. O., Bustam, A. Z., Singh, V. A., Mohd-Taib, N. A., Yip, C. H. and Hashim, O. H. (2008). Lectin-based electrophoretic analysis of the expression of the 35 kDa inter-alpha-trypsin inhibitor heavy chain H4 fragment in sera of patients with five different malignancies. *Electrophoresis, 29*(12), 2645-2650.

Mohamed, E., Jayapalan, J. J., Abdul-Rahman, P. S., Omar, S. Z. and Hashim, O. H. (2013). Enhanced expression of a 35 kDa fragment of inter-alpha-trypsin inhibitor H4 in sera of healthy pregnant women and patients with hydatidiform mole. *Biomark Res, 1*(1), 19.

Molloy, M. P. and McDowell, M. T. (2005). Two-dimensional gel electrophoresis. In D. Garfin and S. Ahuja (Eds.), *Handbook of isoelectric focusing and proteomics* (Vol. 7, pp. 123-143). Amsterdam: Elsevier B. V.

Moreira, R. d. A., Ainouz, I. L., Oliveira, J. T. A. d. and Cavada, B. S. (1991). Plant lectins, chemical and biological aspects. *Memórias do Instituto Oswaldo Cruz, 86*, 211-218.

Mu, A. K.-W., Lim, B.-K., Hashim, O. H. and Shuib, A. S. (2012). Detection of differential levels of proteins in the urine of patients with endometrial cancer: Analysis using two-dimensional gel electrophoresis and *O*-glycan binding lectin. *Int J Mol Sci, 13*(8), 9489-9501.

Myers, R. B., Oelschlager, D. K., Weiss, H. L., Frost, A. R. and Grizzle, W. E. (2001). Fatty acid synthase: an early molecular marker of progression of prostatic adenocarcinoma to androgen independence. *J Urol, 165*(3), 1027-1032.

Myers, S. A. and Clements, J. A. (2001). Kallikrein 4 (KLK4), a new member of the human kallikrein gene family is up-regulated by estrogen and progesterone in the human endometrial cancer cell line, KLE. *J Clin Endocrinol Metab, 86*(5), 2323-2326.

Nakashima, M., Uemura, M., Yasui, K., Ozaki, H. S., Tabata, S. and Taen, A. (2000). An anterograde and retrograde tract-tracing study on the projections from the thalamic gustatory area in the rat: distribution of neurons projecting to the insular cortex and amygdaloid complex. *Neurosci Res, 36*(4), 297-309.

Nam, R. K., Saskin, R., Lee, Y., Liu, Y., Law, C., Klotz, L. H., Loblaw, D. A., Trachtenberg, J., Stanimirovic, A., Simor, A. E., Seth, A., Urbach, D. R. and Narod, S. A. (2010). Increasing hospital admission rates for urological complications after transrectal ultrasound guided prostate biopsy. *J Urol, 183*(3), 963-968.

Nam, R. K., Zhang, W. W., Trachtenberg, J., Seth, A., Klotz, L. H., Stanimirovic, A., Punnen, S., Venkateswaran, V., Toi, A., Loblaw, D. A., Sugar, L., Siminovitch, K. A. and Narod, S. A. (2009). Utility of incorporating genetic variants for the early detection of prostate cancer. *Clin Cancer Res, 15*(5), 1787-1793.

National Cancer Institute. (2007). SEER Cancer Statistics Review, 1975-2004. from http://seer.cancer.gov/csr/1975_2004/

Nelles, J. L., Hu, W. Y. and Prins, G. S. (2011). Estrogen action and prostate cancer. *Expert Rev Endocrinol Metab, 6*(3), 437-451.

Nishimura, H., Kakizaki, I., Muta, T., Sasaki, N., Pu, P. X., Yamashita, T. and Nagasawa, S. (1995). cDNA and deduced amino acid sequence of human PK-120, a plasma kallikrein-sensitive glycoprotein. *FEBS Letters, 357*(2), 207-211.

Nordahl, E. A., Rydengård, V., Mörgelin, M. and Schmidtchen, A. (2005). Domain 5 of high molecular weight kininogen is antibacterial. *J Biol Chem, 280*(41), 34832-34839.

O'Brien, J. S., Kretz, K. A., Dewji, N., Wenger, D. A., Esch, F. and Fluharty, A. L. (1988). Coding of two sphingolipid activator proteins (SAP-1 and SAP-2) by same genetic locus. *Science, 241*(4869), 1098-1101.

O'Brien, M. F., Cronin, A. M., Fearn, P. A., Smith, B., Stasi, J., Guillonneau, B., Scardino, P. T., Eastham, J. A., Vickers, A. J. and Lilja, H. (2009). Pretreatment prostate-specific antigen (PSA) velocity and doubling time are associated with outcome but neither improves prediction of outcome beyond pretreatment PSA alone in patients treated with radical prostatectomy. *J Clin Oncol, 27*(22), 3591-3597.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J Biol Chem, 250*(10), 4007-4021.

Oda, Y., Tatsumi, Y. and Aonuma, S. (1991). Mitogenic activity of *Tulipa gesneriana* lectins on mouse and human lymphocytes. *Chem Pharm Bull (Tokyo), 39*(12), 3350-3352.

Oesterling, J. E., Jacobsen, S. J. and Cooner, W. H. (1995). The use of age-specific reference ranges for serum prostate specific antigen in men 60 years old or older. *J Urol, 153*(4), 1160-1163.

Oesterling, J. E., Tekchandani, A. H., Martin, S. K., Bergstralh, E. J., Reichstein, E., Diamandis, E. P., Yemoto, C. and Stamey, T. A. (1996). The periurethral glands do not significantly influence the serum prostate specific antigen concentration. *J Urol, 155*(5), 1658-1660.

Oh, J., Pyo, J. H., Jo, E. H., Hwang, S. I., Kang, S. C., Jung, J. H., Park, E. K., Kim, S. Y., Choi, J. Y. and Lim, J. (2004). Establishment of a near-standard two-dimensional human urine proteomic map. *Proteomics, 4*(11), 3485-3497.

Ohnishi, M., Matsumoto, T., Nagashio, R., Kageyama, T., Utsuki, S., Oka, H., Okayasu, I. and Sato, Y. (2009). Proteomics of tumor-specific proteins in cerebrospinal fluid of patients with astrocytoma: Usefulness of gelsolin protein. *Pathol Int, 59*(11), 797-803.

Okotie, O. T., Roehl, K. A., Han, M., Loeb, S., Gashti, S. N. and Catalona, W. J. (2007). Characteristics of prostate cancer detected by digital rectal examination only. *Urology, 70*(6), 1117-1120.

Omar, Z. A. and Tamin, N. S. I. (2011). National cancer registry report: Malaysia cancer statistics - data and figure. Ministry of Health, Malaysia. *NCR Report 2007* (pp. 1-127). Putrajaya.

Ornellas, P., Ornellas, A. A., Chinello, C., Gianazza, E., Mainini, V., Cazzaniga, M., Pereira, D. A., Sandim, V., Cypriano, A. S., Koifman, L., Silva, P. C., Alves, G. and Magni, F. (2012). Downregulation of C3 and C4A/B complement factor fragments in plasma from patients with squamous cell carcinoma of the penis. *Int Braz J Urol, 38*(6), 739-749.

Ostrander, E. A. and Stanford, J. L. (2000). Genetics of prostate cancer: Too many loci, too few genes. *Am J Hum Genet, 67*(6), 1367-1375.

Palagi, P. M., Hernandez, P., Walther, D. and Appel, R. D. (2006). Proteome informatics I: Bioinformatics tools for processing experimental data. *Proteomics, 6*(20), 5435-5444.

Parekh, R. B., Dwek, R. A. and Rademacher, T. W. (1988a). Rheumatoid arthritis as a glycosylation disorder. *Rheumatology, 27*(Suppl 2), 162-169.

Parekh, R. B., Roitt, I. M., Isenberg, D. A., Dwek, R. A., Ansell, B. M. and Rademacher, T. W. (1988b). Galactosylation of IgG associated oligosaccharides: reduction in patients with adult and juvenile onset rheumatoid arthritis and relation to disease activity. *Lancet, 1*(8592), 966-969.

Partin, A. W., Alan, W. P., Robert, H. G., Marne, J. C., Don, V., John, Y., Jonathan, I. E. and Donald, S. C. (1993). Nuclear matrix protein patterns in human benign prostatic hyperplasia and prostate cancer. *Cancer Res, 53*, 744–746.

Partin, A. W., Criley, S. R., Subong, E. N., Zincke, H., Walsh, P. C. and Oesterling, J. E. (1996). Standard versus age-specific prostate specific antigen reference ranges among men with clinically localized prostate cancer: A pathological analysis. *J Urol, 155*(4), 1336-1339.

Pashayan, N., Duffy, S. W., Pharoah, P., Greenberg, D., Donovan, J., Martin, R. M., Hamdy, F. and Neal, D. E. (2009). Mean sojourn time, overdiagnosis, and reduction in advanced stage prostate cancer due to screening with PSA: Implications of sojourn time on screening. *Br J Cancer, 100*(7), 1198-1204.

Pawlik, T. M., Hawke, D. H., Liu, Y., Krishnamurthy, S., Fritsche, H., Hunt, K. K. and Kuerer, H. M. (2006). Proteomic analysis of nipple aspirate fluid from women with early-stage breast cancer using isotope-coded affinity tags and tandem mass spectrometry reveals differential expression of vitamin D binding protein. *BMC Cancer, 6*, 68.

Perez-Holanda, S., Blanco, I., Menendez, M. and Rodrigo, L. (2014). Serum concentration of alpha-1 antitrypsin is significantly higher in colorectal cancer patients than in healthy controls. *BMC Cancer, 14*(1), 355.

Perkins, D. N., Pappin, D. J. C., Creasy, D. M. and Cottrell, J. S. (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis, 20*(18), 3551-3567.

Petricoin, E., Wulfkuhle, J., Espina, V. and Liotta, L. A. (2004). Clinical proteomics: Revolutionizing disease detection and patient tailoring therapy. *J Proteome Res, 3*(2), 209-217.

Petricoin, E. F., 3rd, Ornstein, D. K., Paweletz, C. P., Ardekani, A., Hackett, P. S., Hitt, B. A., Velassco, A., Trucco, C., Wiegand, L., Wood, K., Simone, C. B., Levine, P. J., Linehan, W. M., Emmert-Buck, M. R., Steinberg, S. M., Kohn, E. C. and Liotta, L. A. (2002a). Serum proteomic patterns for detection of prostate cancer. *J Natl Cancer Inst, 94*(20), 1576-1578.

Petricoin, E. F., Ardekani, A. M., Hitt, B. A., Levine, P. J., Fusaro, V. A., Steinberg, S. M., Mills, G. B., Simone, C., Fishman, D. A., Kohn, E. C. and Liotta, L. A. (2002b). Use of proteomic patterns in serum to identify ovarian cancer. *Lancet, 359*(9306), 572-577.

Peumans, W. J. and Van Damme, E. J. (1995). Lectins as plant defense proteins. *Plant Physiol, 109*(2), 347-352.

Peumans, W. J. and van Damme, E. J. M. (1998). Plant lectins: Versatile proteins with important perspectives in biotechnology. *Biotechnol Genet Eng Rev, 15*, 199-225.

Peyromaure, M., Fulla, Y., Debre, B. and Dinh-Xuan, A. T. (2005). Pro PSA : A "pro cancer" form of PSA? *Med Hypotheses, 64*(1), 92-95.

Pieper, R., Su, Q., Gatlin, C. L., Huang, S. T., Anderson, N. L. and Steiner, S. (2003). Multi-component immunoaffinity subtraction chromatography: An innovative step towards a comprehensive survey of the human plasma proteome. *Proteomics, 3*(4), 422-432.

Pignata, S., Cannella, L., Leopardo, D., Bruni, G. S., Facchini, G. and Pisano, C. (2011). Follow-up with CA125 after primary therapy of advanced ovarian cancer: In favor of continuing to prescribe CA125 during follow-up. *Ann Oncol, 22*(Suppl 8), 40-44.

Pineiro, M., Andres, M., Iturralde, M., Carmona, S., Hirvonen, J., Pyorala, S., Heegaard, P. M., Tjornehoj, K., Lampreave, F., Pineiro, A. and Alava, M. A. (2004). ITIH4 (inter-alpha-trypsin inhibitor heavy chain 4) is a new acute-phase protein isolated from cattle during experimental infection. *Infect Immun, 72*(7), 3777-3782.

Pisitkun, T., Johnstone, R. and Knepper, M. A. (2006). Discovery of urinary biomarkers. *Mol Cell Proteomics, 5*(10), 1760-1771.

Pohleven, J., Štrukelj, B. and Kos, J. (2012). *Affinity chromatography of lectins.* S. Magdeldin (Ed.) *Affinity Chromatography.* Retrieved from http://www.intechopen.com/books/affinity-chromatography/carbohydrate-affinity-chromatography

Polito, L., Bortolotti, M., Pedrazzi, M. and Bolognesi, A. (2011). Immunotoxins and other conjugates containing Saporin-S6 for cancer therapy. *Toxins (Basel), 3*(6), 697-720.

Pollack, A., DeSilvio, M., Khor, L.-Y., Li, R., Al-Saleem, T. I., Hammond, M. E., Venkatesan, V., Lawton, C. A., Roach, M., Shipley, W. U., Hanks, G. E. and Sandler, H. M. (2004). Ki-67 staining is a strong predictor of distant metastasis and mortality for men with prostate cancer treated with radiotherapy plus androgen deprivation: Radiation therapy oncology group trial 92–02. *J Clin Oncol, 22*(11), 2133-2140.

Powell, A. E. and Leon, M. A. (1970). Reversible interaction of human lymphocytes with the mitogen concanavalin A. *Exp Cell Res, 62*(2–3), 315-325.

Presti, J. C., Jr. (2009). Repeat prostate biopsy - when, where, and how. *Urol Oncol, 27*(3), 312-314.

Principe, S., Kim, Y., Fontana, S., Ignatchenko, V., Nyalwidhe, J. O., Lance, R. S., Troyer, D. A., Alessandro, R., Semmes, O. J., Kislinger, T., Drake, R. R. and Medin, J. A. (2012). Identification of prostate-enriched proteins by in-depth proteomic analyses of expressed prostatic secretions in urine. *J Proteome Res, 11*(4), 2386-2396.

Prins, G. S., Birch, L., Habermann, H., Chang, W. Y., Tebeau, C., Putz, O. and Bieberich, C. (2001). Influence of neonatal estrogens on rat prostate development. *Reprod Fertil Dev, 13*(4), 241-252.

Puchades, M., Westman, A., Blennow, K. and Davidsson, P. (1999). Removal of sodium dodecyl sulfate from protein samples prior to matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun Mass Spectrom, 13*(5), 344-349.

Putzai, A., Croy, R. R. D., Grant, G. and Steward, J. C. (1983). *Seed lectins: Distribution, location and biological role.* New York: Academic Press.

Qu, Y., Adam, B.-L., Yasui, Y., Ward, M. D., Cazares, L. H., Schellhammer, P. F., Feng, Z., Semmes, O. J. and Wright, G. L. (2002). Boosted decision tree analysis of surface-enhanced laser desorption/ionization mass spectral serum profiles discriminates prostate cancer from noncancer patients. *Clin Chem, 48*(10), 1835-1843.

Raineri, M., Catalano, M. G., Hammond, G. L., Avvakumov, G. V., Frairia, R. and Fortunati, N. (2002). *O*-glycosylation of human sex hormone-binding globulin is essential for inhibition of estradiol-induced MCF-7 breast cancer cell proliferation. *Mol Cell Endocrinol, 189*(1-2), 135-143.

Rambaruth, N. D., Greenwell, P. and Dwek, M. V. (2012). The lectin *Helix pomatia* agglutinin recognizes *O*-GlcNAc containing glycoproteins in human breast cancer. *Glycobiology, 22*(6), 839-848.

Rehman, I., Evans, C. A., Glen, A., Cross, S. S., Eaton, C. L., Down, J., Pesce, G., Phillips, J. T., Yen, O. S., Thalmann, G. N., Wright, P. C. and Hamdy, F. C. (2012). iTRAQ Identification of Candidate Serum Biomarkers Associated with Metastatic Progression of Human Prostate Cancer. *PLoS ONE, 7*(2), e30885.

Reis, S. E., Falcao, D. A. and Isaac, L. (2006). Clinical aspects and molecular basis of primary deficiencies of complement component C3 and its regulatory proteins factor I and factor H. *Scand J Immunol, 63*(3), 155-168.

Reisner, Y., Ravid, A. and Sharon, N. (1976). Use of soybean agglutinin for the separation of mouse B and T lymphocytes. *Biochem Biophys Res Commun, 72*(4), 1585-1591.

Reiter, R. E., Gu, Z., Watabe, T., Thomas, G., Szigeti, K., Davis, E., Wahl, M., Nisitani, S., Yamashiro, J., Le Beau, M. M., Loda, M. and Witte, O. N. (1998). Prostate stem cell antigen: A cell surface marker overexpressed in prostate cancer. *Proc Natl Acad Sci USA, 95*(4), 1735-1740.

Risbridger, G. P., Bianco, J. J., Ellem, S. J. and McPherson, S. J. (2003). Oestrogens and prostate cancer. *Endocr Relat Cancer, 10*(2), 187-191.

Rodriguez-Pineiro, A. M., Ayude, D., Rodriguez-Berrocal, F. J. and Paez de la Cadena, M. (2004). Concanavalin A chromatography coupled to two-dimensional gel electrophoresis improves protein expression studies of the serum proteome. *J Chromatogr B Analyt Technol Biomed Life Sci, 803*(2), 337-343.

Roeise, O., Sivertsen, S., Ruud, T. E., Bouma, B. N., Stadaas, J. O. and Aasen, A. O. (1990). Studies on components of the contact phase system in patients with advanced gastrointestinal cancer. *Cancer, 65*(6), 1355-1359.

Rook, G. A., al Attiyah, R. and Filley, E. (1991). New insights into the immunopathology of tuberculosis. *Pathobiology, 59*(3), 148-152.

Roop-ngam, P., Chaiyarit, S., Pongsakul, N. and Thongboonkerd, V. (2012). Isolation and characterizations of oxalate-binding proteins in the kidney. *Biochem Biophys Res Commun, 424*(3), 629-634.

Roque-Barreira, M. C. and Campos-Neto, A. (1985). Jacalin: An IgA-binding lectin. *J Immunol, 134*(3), 1740-1743.

Rosenfeld, R., Bangio, H., Gerwig, G. J., Rosenberg, R., Aloni, R., Cohen, Y., Amor, Y., Plaschkes, I., Kamerling, J. P. and Maya, R. B. (2007). A lectin array-based methodology for the analysis of protein glycosylation. *J Biochem Biophys Methods, 70*(3), 415-426.

Rosengren, A. T., Salmi, J. M., Aittokallio, T., Westerholm, J., Lahesmaa, R., Nyman, T. A. and Nevalainen, O. S. (2003). Comparison of PDQuest and Progenesis software packages in the analysis of two-dimensional electrophoresis gels. *Proteomics, 3*(10), 1936-1946.

Rudiger, H. (1988). Preparation of plant lectins. In H. Franz (Ed.), *Advances in Lectin Research* (Vol. 1, pp. 26-72). Berlin: VEB Verlag Volk und Gesundheit.

Rudiger, H. and Gabius, H. J. (2001). Plant lectins: Occurrence, biochemistry, functions and applications. *Glycoconj J, 18*(8), 589-613.

Ruffet, E., Paquet, N., Frutiger, S., Hughes, G. J. and Jaton, J. C. (1992). Structural and electron-microscopic studies of jacalin from jackfruit (*Artocarpus integrifolia*) show that this lectin is a 65 kDa tetramer. *Biochem J, 286 (Pt 1)*, 131-134.

Russo, J. and Russo, I. H. (2006). The role of estrogen in the initiation of breast cancer. *J Steroid Biochem Mol Biol, 102*(1-5), 89-96.

Rutkowski, M. J., Sughrue, M. E., Kane, A. J., Mills, S. A. and Parsa, A. T. (2010). Cancer and the complement cascade. *Mol Cancer Res, 8*(11), 1453-1465.

Rybicki, B. A., Neslund-Dudas, C., Nock, N. L., Schultz, L. R., Eklund, L., Rosbolt, J., Bock, C. H. and Monaghan, K. G. (2006). Prostate cancer risk from occupational exposure to polycyclic aromatic hydrocarbons interacting with the GSTP1 Ile105Val polymorphism. *Cancer Detect Prev, 30*(5), 412-422.

Rye, K. A., Wee, K., Curtiss, L. K., Bonnet, D. J. and Barter, P. J. (2003). Apolipoprotein A-II inhibits high density lipoprotein remodeling and lipid-poor apolipoprotein A-I formation. *J Biol Chem, 278*(25), 22530-22536.

Saguchi, K., Tobe, T., Hashimoto, K., Sano, Y., Nakano, Y., Miura, N. H. and Tomita, M. (1995). Cloning and characterization of cDNA for inter-alpha-trypsin inhibitor family heavy chain-related protein (IHRP), a novel human plasma glycoprotein. *J Biochem, 117*(1), 14-18.

Sali, A., Glaeser, R., Earnest, T. and Baumeister, W. (2003). From words to literature in structural proteomics. *Nature, 422*(6928), 216-225.

Salier, J. P., Rouet, P., Raguenez, G. and Daveau, M. (1996). The inter-alpha-inhibitor family: From structure to regulation. *Biochem J, 315 (Pt 1)*, 1-9.

Salinas, C. A., Koopmeiners, J. S., Kwon, E. M., FitzGerald, L., Lin, D. W., Ostrander, E. A., Feng, Z. and Stanford, J. L. (2009). Clinical utility of five genetic variants for predicting prostate cancer risk and mortality. *Prostate, 69*(4), 363-372.

Sanchez, J. C., Appel, R. D., Golaz, O., Pasquali, C., Ravier, F., Bairoch, A. and Hochstrasser, D. F. (1995). Inside SWISS-2DPAGE database. *Electrophoresis, 16*(7), 1131-1151.

Sancho, D., Mourao-Sa, D., Joffre, O. P., Schulz, O., Rogers, N. C., Pennington, D. J., Carlyle, J. R. and Reis e Sousa, C. (2008). Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. *J Clin Invest, 118*(6), 2098-2110.

Schaller, J., Akiyama, K., Tsuda, R., Hara, M., Marti, T. and Rickli, E. E. (1987). Isolation, characterization and amino-acid sequence of γ-seminoprotein, a glycoprotein from human seminal plasma. *Eur J Biochem, 170*(1-2), 111-120.

Scherp, P., Ku, G., Coleman, L. and Kheterpal, I. (2011). Gel-based and gel-free proteomic technologies. *Methods Mol Biol, 702*, 163-190.

Schröder, F. H., Hugosson, J., Roobol, M. J., Tammela, T. L. J., Ciatto, S., Nelen, V., Kwiatkowski, M., Lujan, M., Lilja, H., Zappa, M., Denis, L. J., Recker, F., Berenguer, A., Määttänen, L., Bangma, C. H., Aus, G., Villers, A., Rebillard, X., van der Kwast, T., Blijenberg, B. G., Moss, S. M., de Koning, H. J. and Auvinen, A. (2009). Screening and prostate-cancer mortality in a randomized European study. *N Engl J Med, 360*(13), 1320-1328.

Sengupta, S., Myers, R. P., Slezak, J. M., Bergstralh, E. J., Zincke, H. and Blute, M. L. (2005). Preoperative prostate specific antigen doubling time and velocity are strong and independent predictors of outcomes following radical prostatectomy. *J Urol, 174*(6), 2191-2196.

Seriramalu, R., Pang, W. W., Jayapalan, J. J., Mohamed, E., Abdul-Rahman, P. S., Bustam, A. Z., Khoo, A. S.-B. and Hashim, O. H. (2010). Application of champedak mannose-binding lectin in the glycoproteomic profiling of serum samples unmasks reduced expression of alpha-2 macroglobulin and complement factor B in patients with nasopharyngeal carcinoma. *Electrophoresis, 31*(14), 2388-2395.

Shan, S., Tanaka, H. and Shoyama, Y. (2001). Enzyme-linked immunosorbent assay for glycyrrhizin using anti-glycyrrhizin monoclonal antibody and an eastern blotting technique for glucuronides of glycyrrhetic acid. *Anal Chem, 73*(24), 5784-5790.

Sharon, N. and Lis, H. (1972). Lectins: Cell-agglutinating and sugar-specific proteins. *Science, 177*(4053), 949-959.

Sharon, N. and Lis, H. (1993). *Lectin* (2 ed.). Dordrecht: Kluwer Academic Publisher.

Shen, J., Wang, W., Wu, J., Feng, B., Chen, W., Wang, M., Tang, J., Wang, F., Cheng, F., Pu, L., Tang, Q., Wang, X. and Li, X. (2012). Comparative proteomic profiling of human bile reveals SSP411 as a novel biomarker of cholangiocarcinoma. *PLoS ONE, 7*(10), e47476.

Shenkin, A. (2006). Serum prealbumin: Is it a marker of nutritional status or of risk of malnutrition? *Clin Chem, 52*(12), 2177-2179.

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem, 68*(5), 850-858.

Shibuya, N., Goldstein, I. J., Broekaert, W. F., Nsimba-Lubaki, M., Peeters, B. and Peumans, W. J. (1987). Fractionation of sialylated oligosaccharides, glycopeptides, and glycoproteins on immobilized elderberry (*Sambucus nigra* L.) bark lectin. *Arch Biochem Biophys, 254*(1), 1-8.

Shipley, M. T. (1985). Transport of molecules from nose to brain: Transneuronal anterograde and retrograde labeling in the rat olfactory system by wheat germ agglutinin-horseradish peroxidase applied to the nasal epithelium. *Brain Res Bull, 15*(2), 129-142.

Shirley, A. and Brewster, S. (2011). The digital rectal examination. *J Clin Examin, 11*, 1-12.

Shuib, A. S., Chua, C. T. and Hashim, O. H. (1998). Sera of IgA nephropathy patients contain a heterogeneous population of relatively cationic alpha-heavy chains. *Nephron, 78*(3), 290-295.

Silsirivanit, A., Araki, N., Wongkham, C., Vaeteewoottacharn, K., Pairojkul, C., Kuwahara, K., Narimatsu, Y., Sawaki, H., Narimatsu, H., Okada, S., Sakaguchi, N. and Wongkham, S. (2013). CA-S27: A novel Lewis a associated carbohydrate epitope is diagnostic and prognostic for cholangiocarcinoma. *Cancer Sci, 104*(10), 1278-1284.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal Biochem, 150*(1), 76-85.

Sokolowski, T., Peters, T., Perez, S. and Imberty, A. (1997). Conformational analysis of biantennary glycans and molecular modeling of their complexes with lentil lectin. *J Mol Graph Model, 15*(1), 37-42, 54.

Song, J., Patel, M., Rosenzweig, C. N., Chan-Li, Y., Sokoll, L. J., Fung, E. T., Choi-Miura, N. H., Goggins, M., Chan, D. W. and Zhang, Z. (2006). Quantification of fragments of human serum inter-alpha-trypsin inhibitor heavy chain 4 by a surface-enhanced laser desorption/ionization-based immunoassay. *Clin Chem, 52*(6), 1045-1053.

Sorace, J. M. and Zhan, M. (2003). A data review and reassessment of ovarian cancer serum proteomic profiling. *BMC Bioinformatics, 4*, 24.

Spicer, S. S. and Schulte, B. A. (1992). Diversity of cell glycoconjugates shown histochemically: A perspective. *J Histochem Cytochem, 40*(1), 1-38.

Sreekumar, A., Poisson, L. M., Rajendiran, T. M., Khan, A. P., Cao, Q., Yu, J., Laxman, B., Mehra, R., Lonigro, R. J., Li, Y., Nyati, M. K., Ahsan, A., Kalyana-Sundaram, S., Han, B., Cao, X., Byun, J., Omenn, G. S., Ghosh, D., Pennathur, S., Alexander, D. C., Berger, A., Shuster, J. R., Wei, J. T., Varambally, S., Beecher, C. and Chinnaiyan, A. M. (2009). Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature, 457*(7231), 910-914.

Srinivas, P. R., Kramer, B. S. and Srivastava, S. (2001). Trends in biomarker research for cancer detection. *Lancet Oncol, 2*(11), 698-704.

Srivastava, O. P. and Srivastava, K. (1998). Purification of gamma-crystallin from human lenses by acetone precipitation method. *Curr Eye Res, 17*(11), 1074-1081.

Sriyam, S., Sinchaikul, S., Tantipaiboonwong, P., Phutrakul, S. and Chen, S. T. (2008). Proteomic analysis of deglycosylated proteins in normal human serum using anhydrous hydrogen fluoride treatment. *Chiang Mai J Sci, 35*(2), 311-323.

Stanford, J. L., Feng, Z., Hamilton, A. S., Gilliland, F. D., Stephenson, R. A., Eley, J. W., Albertsen, P. C., Harlan, L. C. and Potosky, A. L. (2000). Urinary and sexual function after radical prostatectomy for clinically localized prostate cancer: The Prostate Cancer Outcomes Study. *JAMA, 283*(3), 354-360.

Stanford, J. L., Just, J. J., Gibbs, M., Wicklund, K. G., Neal, C. L., Blumenstein, B. A. and Ostrander, E. A. (1997). Polymorphic repeats in the androgen receptor gene: Molecular markers of prostate cancer risk. *Cancer Res, 57*(6), 1194-1198.

Stanley, P. (1981). Selection of specific wheat germ agglutinin-resistant (WgaR) phenotypes from Chinese hamster ovary cell populations containing numerous lecR genotypes. *Mol Cell Biol, 1*(8), 687-696.

Stanley, P., Sallustio, S., Krag, S. S. and Dunn, B. (1990). Lectin-resistant CHO cells: Selection of seven new mutants resistant to ricin. *Somat Cell Mol Genet, 16*(3), 211-223.

Steentoft, C., Vakhrushev, S. Y., Joshi, H. J., Kong, Y., Vester-Christensen, M. B., Schjoldager, K. T., Lavrsen, K., Dabelsteen, S., Pedersen, N. B., Marcos-Silva, L., Gupta, R., Bennett, E. P., Mandel, U., Brunak, S., Wandall, H. H., Levery, S. B. and Clausen, H. (2013). Precision mapping of the human *O*-GalNAc glycoproteome through SimpleCell technology. *EMBO J, 32*(10), 1478-1488.

Steinberg, G. D., Carter, B. S., Beaty, T. H., Childs, B. and Walsh, P. C. (1990). Family history and the risk of prostate cancer. *Prostate, 17*(4), 337-347.

Stenman, U.-H., Leinonen, J., Zhang, W.-M. and Finne, P. (1999). Prostate-specific antigen. *Semin Cancer Biol, 9*(2), 83-93.

Stillmark, H. (1888). *Ricin , a toxic enzyme from the seeds of Ricinus communis and some other Euphorbiaceae: Inaugural dissertation.* (PhD), Imperial Tartu Unievrsity, Estonia.

Suehara, Y., Kubota, D., Kikuta, K., Kaneko, K., Kawai, A. and Kondo, T. (2012). Discovery of biomarkers for osteosarcoma by proteomics approaches. *Sarcoma, 2012*, 425636.

Sundsten, T. and Ortsäter, H. (2009). Proteomics in diabetes research. *Mol Cell Endocrinol, 297*(1–2), 93-103.

Swaminathan, G. J., Leonidas, D. D., Savage, M. P., Ackerman, S. J. and Acharya, K. R. (1999). Selective recognition of mannose by the human eosinophil charcot-leyden crystal protein (Galectin-10): A crystallographic study at 1.8 Å Resolution. *Biochemistry, 38*(42), 13837-13843.

Tanaka, H., Shimazawa, M., Takata, M., Kaneko, H., Tsuruma, K., Ikeda, T., Warita, H., Aoki, M., Yamada, M., Takahashi, H., Hozumi, I., Minatsu, H., Inuzuka, T. and Hara, H. (2013). ITIH4 and Gpx3 are potential biomarkers for amyotrophic lateral sclerosis. *J Neurol, 260*(7), 1782-1797.

Tang, P., Du, W., Xie, K., Deng, X., Fu, J., Chen, H. and Yang, W. (2013). Transition zone PSA density improves the prostate cancer detection rate both in PSA 4.0-10.0 and 10.1-20.0 ng/ml in Chinese men. *Urol Oncol, 31*(6), 744-748.

Tavare, J. M., Fletcher, L. M. and Welsh, G. I. (2001). Using green fluorescent protein to study intracellular signalling. *J Endocrinol, 170*(2), 297-306.

Thadikkaran, L., Siegenthaler, M. A., Crettaz, D., Queloz, P. A., Schneider, P. and Tissot, J. D. (2005). Recent advances in blood-related proteomics. *Proteomics, 5*(12), 3019-3034.

Thomas, S., Thirumalapura, N., Crossley, E. C., Ismail, N. and Walker, D. H. (2009). Antigenic protein modifications in Ehrlichia. *Parasite Immunol, 31*(6), 296-303.

Thompson, I. M., Pauler, D. K., Goodman, P. J., Tangen, C. M., Lucia, M. S., Parnes, H. L., Minasian, L. M., Ford, L. G., Lippman, S. M., Crawford, E. D., Crowley, J. J. and Coltman, C. A., Jr. (2004). Prevalence of prostate cancer among men with a prostate-specific antigen level <or= 4.0 ng per milliliter. *N Engl J Med, 350*(22), 2239-2246.

Thompson, P. A., Berbee, J. F., Rensen, P. C. and Kitchens, R. L. (2008). Apolipoprotein A-II augments monocyte responses to LPS by suppressing the inhibitory activity of LPS-binding protein. *Innate Immun, 14*(6), 365-374.

Thongboonkerd, V., McLeish, K. R., Arthur, J. M. and Klein, J. B. (2002). Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. *Kidney Int, 62*(4), 1461-1469.

Thorpe, A. and Neal, D. (2003). Benign prostatic hyperplasia. *Lancet, 361*(9366), 1359-1367.

Tomana, M., Schrohenloher, R. E., Koopman, W. J., Alarcon, G. S. and Paul, W. A. (1988). Abnormal glycosylation of serum IgG from patients with chronic inflammatory diseases. *Arthritis Rheum, 31*(3), 333-338.

Tomlins, S. A., Rhodes, D. R., Yu, J., Varambally, S., Mehra, R., Perner, S., Demichelis, F., Helgeson, B. E., Laxman, B., Morris, D. S., Cao, Q., Cao, X., Andren, O., Fall, K., Johnson, L., Wei, J. T., Shah, R. B., Al-Ahmadie, H., Eastham, J. A., Eggener, S. E., Fine, S. W., Hotakainen, K., Stenman, U. H., Tsodikov, A., Gerald, W. L., Lilja, H., Reuter, V. E., Kantoff, P. W., Scardino, P. T., Rubin, M. A., Bjartell, A. S. and Chinnaiyan, A. M. (2008). The role of SPINK1 in ETS rearrangement-negative prostate cancers. *Cancer Cell, 13*(6), 519-528.

Tomosugi, N., Kawabata, H., Wakatabe, R., Higuchi, M., Yamaya, H., Umehara, H. and Ishikawa, I. (2006). Detection of serum hepcidin in renal failure and inflammation by using ProteinChip System. *Blood, 108*(4), 1381-1387.

Topic, A., Ljujic, M., Petrovic-Stanojevic, N., Dopudja-Pantic, V. and Radojkovic, D. (2011). Phenotypes and serum level of alpha-1-antitrypsin in lung cancer. *J BUON, 16*(4), 672-676.

Törnblom, M., Norming, U., Adolfsson, J., Becker, C., Abrahamsson, P.-A., Lilja, H. and Gustafsson, O. (1999). Diagnostic value of percent free prostate-specific antigen: Retrospective analysis of a population-based screening study with emphasis on men with PSA levels less than 3.0 ng/mL. *Urology, 53*(5), 945-950.

Tountas, Y., Sparos, L., Theodoropoulos, C. and Trichopoulos, D. (1985). Alpha 1-antitrypsin and cancer of the pancreas. *Digestion, 31*(1), 37-40.

Tran, T. T., Nguyen, T. M. P., Nguyen, B. N. and Phan, C. V. (2008). Changes of serum glycoproteins in lung cancer patients. *J Proteomics Bioinform, 1*, 011-016.

Trinh, Q. D., Sun, M., Kim, S. P., Sammon, J., Kowalczyk, K. J., Friedman, A. A., Sukumar, S., Ravi, P., Muhletaler, F., Agarwal, P. K., Shariat, S. F., Hu, J. C., Menon, M. and Karakiewicz, P. I. (2014). The impact of hospital volume, residency, and fellowship training on perioperative outcomes after radical prostatectomy. *Urol Oncol, 32*(1), 29.e13-20.

Trougakos, I. P. and Gonos, E. S. (2002). Clusterin/Apolipoprotein J in human aging and cancer. *Int J Biochem Cell Biol, 34*(11), 1430-1448.

Trudel, D., Têtu, B., Grégoire, J., Plante, M., Renaud, M.-C., Bachvarov, D., Douville, P. and Bairati, I. (2012). Human epididymis protein 4 (HE4) and ovarian cancer prognosis. *Gynecol Oncol, 127*(3), 511-515.

True, L. D., Zhang, H., Ye, M., Huang, C. Y., Nelson, P. S., von Haller, P. D., Tjoelker, L. W., Kim, J. S., Qian, W. J., Smith, R. D., Ellis, W. J., Liebeskind, E. S. and Liu, A. Y. (2010). CD90/THY1 is overexpressed in prostate cancer-associated fibroblasts and could serve as a cancer biomarker. *Mod Pathol, 23*(10), 1346-1356.

Tsui, K. H., Tang, P., Lin, C. Y., Chang, P. L., Chang, C. H. and Yung, B. Y. (2010). Bikunin loss in urine as useful marker for bladder carcinoma. *J Urol, 183*(1), 339-344.

Tyagi, S., Raghvendra., Singh, U., Kalra, T., Munjal, K. and Vikas. (2010). Practical application of proteomics - a technique for large scale study of proteins: An overview. *Int J Pharm Sci Rev Res, 3*(1), 87-90.

Tyers, M. and Mann, M. (2003). From genomics to proteomics. *Nature, 422*(6928), 193-197.

Ueda, K., Fukase, Y., Katagiri, T., Ishikawa, N., Irie, S., Sato, T. A., Ito, H., Nakayama, H., Miyagi, Y., Tsuchiya, E., Kohno, N., Shiwa, M., Nakamura, Y. and Daigo, Y. (2009). Targeted serum glycoproteomics for the discovery of lung cancer-associated glycosylation disorders using lectin-coupled ProteinChip arrays. *Proteomics, 9*(8), 2182-2192.

Valadez-Vega, C., Alvarez-Manilla, G., Riveron-Negrete, L., Garcia-Carranca, A., Morales-Gonzalez, J. A., Zuniga-Perez, C., Madrigal-Santillan, E., Esquivel-Soto, J., Esquivel-Chirino, C., Villagomez-Ibarra, R., Bautista, M. and Morales-Gonzalez, A. (2011). Detection of cytotoxic activity of lectin on human colon adenocarcinoma (Sw480) and epithelial cervical carcinoma (C33-A). *Molecules, 16*(3), 2107-2118.

Varambally, S., Laxman, B., Mehra, R., Cao, Q., Dhanasekaran, S. M., Tomlins, S. A., Granger, J., Vellaichamy, A., Sreekumar, A., Yu, J., Gu, W., Shen, R., Ghosh, D., Wright, L. M., Kladney, R. D., Kuefer, R., Rubin, M. A., Fimmel, C. J. and Chinnaiyan, A. M. (2008). Golgi protein GOLM1 is a tissue and urine biomarker of prostate cancer. *Neoplasia, 10*(11), 1285-1294.

Varki, A., Etzler, M. E. and Cummings, R. D. (2009). Discovery and classification of glycan-binding proteins. In A. Varki, R. D. Cummings and J. D. Esko (Eds.), *Essentials of Glycobiology* (2 ed.). New York: Cold Spring Harbor Laboratory Press.

Varki, A., Freeze, H. H. and Manzi, A. E. (2001). Preparation and analysis of glycoconjugates. *Current Protocols in Molecular Biology*. New Jersey: John Wiley & Sons, Inc.

Vaughan, J. H., Jacox, R. F. and Gray, B. A. (1967). Light and heavy chain components of gamma globulins in urines of normal persons and patients with agammaglobulinemia. *J Clin Invest, 46*(2), 266-279.

Veenstra, T. D., Conrads, T. P., Hood, B. L., Avellino, A. M., Ellenbogen, R. G. and Morrison, R. S. (2005). Biomarkers: Mining the biofluid proteome. *Mol Cell Proteomics, 4*(4), 409-418.

Verma, M. and Srivastava, S. (2003). New cancer biomarkers deriving from NCI early detection research. *Recent Results Cancer Res, 163*, 72-84; discussion 264-266.

Versura, P., Maltarello, M. C., Cellini, M., Caramazza, R. and Laschi, R. (1986). Detection of mucus glycoconjugates in human conjunctiva by using the lectin-colloidal gold technique in TEM. II. A quantitative study in dry-eye patients. *Acta Ophthalmol (Copenh), 64*(4), 451-455.

Vickers, Cronin, A., Roobol, M., Savage, C., Peltola, M., Pettersson, K., Scardino, P. T., Schroder, F. and Lilja, H. (2010). Reducing unnecessary biopsy during prostate cancer screening using a four-kallikrein panel: An independent replication. *J Clin Oncol, 28*(15), 2493-2498.

Vickers, A. J., Till, C., Tangen, C. M., Lilja, H. and Thompson, I. M. (2011). An empirical evaluation of guidelines on prostate-specific antigen velocity in prostate cancer detection. *J Natl Cancer Inst, 103*(6), 462-469.

Vogel, T., Guo, N. H., Guy, R., Drezlich, N., Krutzsch, H. C., Blake, D. A., Panet, A. and Roberts, D. D. (1994). Apolipoprotein E: A potent inhibitor of endothelial and tumor cell proliferation. *J Cell Biochem, 54*(3), 299-308.

Wagenlehner, F. M., van Oostrum, E., Tenke, P., Tandogdu, Z., Cek, M., Grabe, M., Wullt, B., Pickard, R., Naber, K. G., Pilatz, A., Weidner, W. and Bjerklund-Johansen, T. E. (2013). Infective complications after prostate biopsy: outcome of the Global Prevalence Study of Infections in Urology (GPIU) 2010 and 2011, a prospective multinational multicentre prostate biopsy study. *Eur Urol, 63*(3), 521-527.

Walport, M. J. (2003). *The complement system* (5 ed.). New York: Oxford University Press.

Wang, J., Cai, Y., Ren, C. and Ittmann, M. (2006). Expression of variant TMPRSS2/ERG fusion messenger RNAs is associated with aggressive prostate cancer. *Cancer Res, 66*(17), 8347-8351.

Wang, M. C., Papsidero, L. D., Kuriyama, M., Valenzuela, L. A., Murphy, G. P. and Chu, T. M. (1981). Prostate antigen: A new potential marker for prostatic cancer. *Prostate, 2*(1), 89-96.

Wang, Y.-C., Nakagawa, M., Garitaonandia, I., Slavin, I., Altun, G., Lacharite, R. M., Nazor, K. L., Tran, H. T., Lynch, C. L., Leonardo, T. R., Liu, Y., Peterson, S. E., Laurent, L. C., Yamanaka, S. and Loring, J. F. (2011). Specific lectin biomarkers for isolation of human pluripotent stem cells identified through array-based glycomic analysis. *Cell Res, 21*(11), 1551-1563.

Wang, Z., Wang, C., Huang, X., Shen, Y., Shen, J. and Ying, K. (2012). Differential proteome profiling of pleural effusions from lung cancer and benign inflammatory disease patients. *Biochim Biophys Acta, 1824*(4), 692-700.

Ward, D. G., Suggett, N., Cheng, Y., Wei, W., Johnson, H., Billingham, L. J., Ismail, T., Wakelam, M. J., Johnson, P. J. and Martin, A. (2006). Identification of serum biomarkers for colon cancer by proteomic analysis. *Br J Cancer, 94*(12), 1898-1905.

Warwas, M., Gerber, J. and Pietkiewicz, A. (1986). Haptoglobin and proteinase inhibitors in the blood serum of women with inflammatory, benign and neoplastic lesions of the ovary. *Neoplasma, 33*(1), 79-84.

Wasinger, V. C., Cordwell, S. J., Cerpa-Poljak, A., Yan, J. X., Gooley, A. A., Wilkins, M. R., Duncan, M. W., Harris, R., Williams, K. L. and Humphery-Smith, I. (1995). Progress with gene-product mapping of the Mollicutes: *Mycoplasma genitalium*. *Electrophoresis, 16*(1), 1090-1094.

Weiss, J. M., Andersson, P. L., Lamoree, M. H., Leonards, P. E., van Leeuwen, S. P. and Hamers, T. (2009a). Competitive binding of poly- and perfluorinated compounds to the thyroid hormone transport protein transthyretin. *Toxicol Sci, 109*(2), 206-216.

Weiss, W., Weiland, F. and Görg, A. (2009b). Protein detection and quantitation technologies for gel-based proteome analysis. In J. Reinders and A. Sickmann (Eds.), *Proteomics: Methods and protocol* (Vol. 564). New York: Humana Press.

Welch, C. L., Bretschger, S., Wen, P.-Z., Mehrabian, M., Latib, N., Fruchart-Najib, J., Fruchart, J. C., Myrick, C. and Lusis, A. J. (2004). Novel QTLs for HDL levels identified in mice by controlling for Apoa2 allelic effects: Confirmation of a chromosome 6 locus in a congenic strain. *Physiol Genomics, 17*(1), 48-59.

Whitesel, J. A., Donohue, R. E., Mani, J. H., Mohr, S., Scanavino, D. J., Augspurger, R. R., Biber, R. J., Fauver, H. E., Wettlaufer, J. N. and Pfister, R. R. (1984). Acid phosphatase: Its influence on the management of carcinoma of the prostate. *J Urol, 131*(1), 70-72.

Wiggins, R. C., Kshrisagar, B., Kelsch, R. C. and Wilson, B. S. (1985). Fragmentation and polymeric complexes of albumin in human urine. *Clin Chim Acta, 149*(2-3), 155-163.

Wiley, R. G., Blessing, W. W. and Reis, D. J. (1982). Suicide transport: destruction of neurons by retrograde transport of ricin, abrin and modeccin. *Science, 216*(4548), 889-890.

Wilkins, M. R., Sanchez, J. C., Gooley, A. A., Appel, R. D., Humphery-Smith, I., Hochstrasser, D. F. and Williams, K. L. (1996). Progress with proteome projects: Why all proteins expressed by a genome should be identified and how to do it. *Biotechnol Genet Eng Rev, 13*, 19-50.

Wilt, T. J., MacDonald, R., Rutks, I., Shamliyan, T. A., Taylor, B. C. and Kane, R. L. (2008). Systematic review: Comparative effectiveness and harms of treatments for clinically localized prostate cancer. *Ann Intern Med, 148*(6), 435-448.

Wolf, A. M. D., Wender, R. C., Etzioni, R. B., Thompson, I. M., D'Amico, A. V., Volk, R. J., Brooks, D. D., Dash, C., Guessous, I., Andrews, K., DeSantis, C. and Smith, R. A. (2010). American Cancer Society guideline for the early detection of prostate cancer: Update 2010. *CA Cancer J Clin, 60*(2), 70-98.

Wolk, A. (2005). Diet, lifestyle and risk of prostate cancer. *Acta Oncol, 44*(3), 277-281.

Wolters, D. A., Washburn, M. P. and Yates, J. R. (2001). An automated multidimensional protein identification technology for shotgun proteomics. *Anal Chem, 73*(23), 5683-5690.

Wong, C.-H. (2005). Protein glycosylation: New challenges and opportunities. *J Org Chem, 70*(11), 4219-4225.

Wu, Y., Sun, L., Zou, W., Xu, J., Liu, H., Wang, W., Yun, X. and Gu, J. (2012). Prosaposin, a regulator of estrogen receptor alpha, promotes breast cancer growth. *Cancer Sci, 103*(10), 1820-1825.

Wulfkuhle, J. D., Liotta, L. A. and Petricoin, E. F. (2003). Proteomic applications for the early detection of cancer. *Nat Rev Cancer, 3*(4), 267-275.

Xiao, Z., Adam, B. L., Cazares, L. H., Clements, M. A., Davis, J. W., Schellhammer, P. F., Dalmasso, E. A. and Wright, G. L., Jr. (2001). Quantitation of serum prostate-specific membrane antigen by a novel protein biochip immunoassay discriminates benign from malignant prostate disease. *Cancer Res, 61*(16), 6029-6033.

Yakovleva, M. E., Moran, A. P., Safina, G. R., Wadstrom, T. and Danielsson, B. (2011). Lectin typing of *Campylobacter jejuni* using a novel quartz crystal microbalance technique. *Anal Chim Acta, 694*(1-2), 1-5.

Yan, J. X., Wait, R., Berkelman, T., Harry, R. A., Westbrook, J. A., Wheeler, C. H. and Dunn, M. J. (2000). A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. *Electrophoresis, 21*(17), 3666-3672.

Yanagisawa, K., Shyr, Y., Xu, B. J., Massion, P. P., Larsen, P. H., White, B. C., Roberts, J. R., Edgerton, M., Gonzalez, A., Nadaf, S., Moore, J. H., Caprioli, R. M. and Carbone, D. P. (2003). Proteomic patterns of tumour subsets in non-small-cell lung cancer. *Lancet, 362*(9382), 433-439.

Yang, P., Bamlet, W. R., Sun, Z., Ebbert, J. O., Aubry, M. C., Krowka, M. J., Taylor, W. R., Marks, R. S., Deschamps, C., Swensen, S. J., Wieben, E. D., Cunningham, J. M., Melton, L. J. and de Andrade, M. (2005). Alpha1-antitrypsin and neutrophil elastase imbalance and lung cancer risk. *Chest, 128*(1), 445-452.

Yao, X., Freas, A., Ramirez, J., Demirev, P. A. and Fenselau, C. (2001). Proteolytic 18O labeling for comparative proteomics: Model studies with two serotypes of adenovirus. *Anal Chem, 73*(13), 2836-2842.

Yong, P.-H., Junit, S. M., Harun, F. and Hashim, O. H. (2006). Patients with congenital hypothyroidism demonstrate different altered expression of plasma fibrinogen and haptoglobin polypeptide chains. *Clin Biochem, 39*(2), 126-132.

Yoshihara, Y., Mizuno, T., Nakahira, M., Kawasaki, M., Watanabe, Y., Kagamiyama, H., Jishage, K., Ueda, O., Suzuki, H., Tabuchi, K., Sawamoto, K., Okano, H., Noda, T. and Mori, K. (1999). A genetic approach to visualization of multisynaptic neural pathways using plant lectin transgene. *Neuron, 22*(1), 33-41.

Yoshikawa, H., Tegoshi, T. and Yoshida, Y. (1987). Detection of surface carbohydrates on *Pneumocystis carinii* by fluorescein-conjugated lectins. *Parasitol Res, 74*(1), 43-49.

Yousef, G. M. and Diamandis, E. P. (2001). The new human tissue kallikrein gene family: Structure, function and association to disease. *Endocr Rev, 22*(2), 184-204.

Yu, L., Shen, J., Mannoor, K., Guarnera, M. and Jiang, F. (2014). Identification of ENO1 As a potential sputum biomarker for early-stage lung cancer by shotgun proteomics. *Clin Lung Cancer,* 10.1016/j.cllc.2014.05.003.

Yurkovetsky, Z. R., Linkov, F. Y., D, E. M. and Lokshin, A. E. (2006). Multiple biomarker panels for early detection of ovarian cancer. *Future Oncol, 2*(6), 733-741.

Zaki, M. E., Amr, K. S. and Abdel-Hamid, M. (2013). APOA2 polymorphism in relation to obesity and lipid metabolism. *Cholesterol, 2013*, 5.

Zaki, M. E., Amr, K. S. and Abdel-Hamid, M. (2014). Evaluating the association of APOA2 polymorphism with insulin resistance in adolescents. *Meta Gene, 2*(0), 366-373.

Zehentner, B. K., Secrist, H., Zhang, X., Hayes, D. C., Ostenson, R., Goodman, G., Xu, J., Kiviat, M., Kiviat, N., Persing, D. H. and Houghton, R. L. (2006). Detection of alpha-methylacyl-coenzyme-A racemase transcripts in blood and urine samples of prostate cancer patients. *Mol Diagn Ther, 10*(6), 397-403.

Zeng, Z., Hincapie, M., Pitteri, S. J., Hanash, S., Schalkwijk, J., Hogan, J. M., Wang, H. and Hancock, W. S. (2011). A proteomics platform combining depletion, multi-lectin affinity chromatography (M-LAC), and isoelectric focusing to study the breast cancer proteome. *Anal Chem, 83*(12), 4845-4854.

Zhang, J. C., Claffey, K., Sakthivel, R., Darzynkiewicz, Z., Shaw, D. E., Leal, J., Wang, Y. C., Lu, F. M. and McCrae, K. R. (2000). Two-chain high molecular weight kininogen induces endothelial cell apoptosis and inhibits angiogenesis: partial activity within domain 5. *FASEB J, 14*(15), 2589-2600.

Zhang, S.-D. and Gant, T. W. (2005). Effect of pooling samples on the efficiency of comparative studies using microarrays. *Bioinformatics, 21*(24), 4378-4383.

Zhang, Z., Bast, R. C., Jr., Yu, Y., Li, J., Sokoll, L. J., Rai, A. J., Rosenzweig, J. M., Cameron, B., Wang, Y. Y., Meng, X. Y., Berchuck, A., Van Haaften-Day, C., Hacker, N. F., de Bruijn, H. W., van der Zee, A. G., Jacobs, I. J., Fung, E. T. and Chan, D. W. (2004). Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Res, 64*(16), 5882-5890.

Zheng, S. L., Sun, J., Wiklund, F., Smith, S., Stattin, P., Li, G., Adami, H.-O., Hsu, F.-C., Zhu, Y., Bälter, K., Kader, A. K., Turner, A. R., Liu, W., Bleecker, E. R., Meyers, D. A., Duggan, D., Carpten, J. D., Chang, B.-L., Isaacs, W. B., Xu, J. and Grönberg, H. (2008). Cumulative association of five genetic variants with prostate cancer. *N Engl J Med, 358*(9), 910-919.

Zhong, S., Goldberg, I. J., Bruce, C., Rubin, E., Breslow, J. L. and Tall, A. (1994). Human ApoA-II inhibits the hydrolysis of HDL triglyceride and the decrease of HDL size induced by hypertriglyceridemia and cholesteryl ester transfer protein in transgenic mice. *J Clin Invest, 94*(6), 2457-2467.

Zhu, B., Li, Y., Li, M., Yang, X., Qiu, B., Gao, Q., Liu, J. and Liu, M. (2013). Dynamic proteome analysis of spinal cord injury after ischemia-reperfusion in rabbits by two-dimensional difference gel electrophoresis. *Spinal Cord, 51*(8), 610-615.

APPENDIX I: LIST OF PUBLICATION, IP & PRESENTATION

Publications

Jayapalan, J. J., Ng, K. L., Shuib, A. S., Razack, A. H., Hashim, O. H., Urine of patients with early prostate cancer contains lower levels of light chain fragments of inter-alpha-trypsin inhibitor and saposin B but increased expression of an inter-alpha-trypsin inhibitor heavy chain 4 fragment. *Electrophoresis* 2013, 34 (11), 1663-9.

Jayapalan, J. J., Ng, K. L., Razack, A. H., Hashim, O. H., Identification of potential complementary serum biomarkers to differentiate prostate cancer from benign prostatic hyperplasia using gel- and lectin-based proteomic analyses. *Electrophoresis* 2012, 33 (12), 1855-62.

Intellectual properties

Use of indices relating to levels of serum and urinary fragment peptides of inter-alpha trypsin inhibitor light and heavy chain h4 for diagnosis and/or discrimination of prostate cancer, **PI 2014701798, 2014 (National)** and **PCT/MY2015/000021 (International)** – Patent pending.

Presentations

Jayapalan, J. J. *Identification of potential serum and urine biomarkers for patients with prostate cancer using lectin-based proteomic analyses.* 2015, Annamalai University, India. (Oral presentation).

Jayapalan, J. J., Ng, K. L., Razack, A. H. A., Hashim, O. H. Serum and urinary inter-alpha trypsin inhibitor peptides as biomarkers for prostate cancer. 19th World Congress on Oncology, Athens. Int. J. Mol. Med., 2014. 34:S36-S36. (Proceeding).

Jayapalan, J. J., *Proteomics analysis of sera and urine from patients with prostate cancer.* Proteomics Symposium 2013, F.O.M Research Week, Faculty of Medicine, University of Malaya. (Oral presentation).

Jayapalan, J. J., Abdul Rahman, P. S., Ng, K. L., Razack, A. H., Hashim, O. H. Lectin-based glycoproteomic profiling of serum samples from patients with prostate cancer. 31st Congress of the Société Internationale d'Urologie, Berlin, Germany. Urology, 2011; 78: S3A. (Proceeding).

Jayapalan, J. J., *Lectin-based proteomic and prostate cancer.* National Urology Research Seminar 2011, Clinical Auditorium, Faculty of Medicine, University of Malaya. (Oral presentation).

Jayapalan, J. J., Abdul Rahman, P. S., Ng, K. L., Razack, A. H., Hashim, O. H. Lectin-based glycoproteomic profiling of serum samples from patients with prostate cancer. 19th Malaysian Urological Conference, Kuching, Malaysia, 2010. (Poster presentation).

APPENDIX II: MEDICAL ETHIC COMMITTEE APPROVAL LETTER

PUSAT PERUBATAN UNIVERSITI MALAYA
ALAMAT: LEMBAH PANTAI, 59100 KUALA LUMPUR, MALAYSIA
TELEFON: 03-79564422, 03-79574422 KEBEL: UNIHOS, KUALA LUMPUR
FAX NO: 6-03-79545682

NAME OF ETHICS COMMITTEE/IRB: Medical Ethics Committee, University Malaya Medical Centre ADDRESS: LEMBAH PANTAI 59100 KUALA LUMPUR	ETHICS COMMITTEE/IRB REFERENCE NUMBER: 435.18
PROTOCOL NO: TITLE: Lectin Based Approaches In Biomedical And Proteomics Studies.	
PRINCIPAL INVESTIGATOR: Prof. Dr. Onn Hashim TELEPHONE: KOMTEL:	SPONSOR:

The following item [✓] have been received and reviewed in connection with the above study to be conducted by the above investigator.

[✓] Borang Permohonan Penyelidikan	Ver date: 7 March 2005
[ ] StudyProtocol	Ver date:
[ ] Investigator's Brochure	Ver date:
[✓] Patient Information Sheet	Ver date:
[ ] Consent Form	Ver date:
[ ] Advertisement/Payment & Compensation to Subjects	Ver date:
[✓] Investigator(s) CV's (Prof. Dr. Onn Hashim)	

and have been [✓]

[✓] Approved
[ ] Conditionally approved (identify item and specify modification below or in accompanying letter)
[ ] Rejected (identify item and specify reasons below or in accompanying letter)

Comments:

Date of approval: 20th April 2005

c.c Ketua
Jabatan Perubatan Molekul

Timbalan Dekan (Penyelidikan)
Fakulti Perubatan, Universiti Malaya

Setiausaha
Jawatankuasa Penyelidikan Pusat Perubatan
Fakulti Perubatan, Universiti Malaya

PROF. LOOI LAI MENG
Chairman
Medical Ethics Committee