PROTEOMICS ANALYSIS OF SERA OF WOMEN WITH NORMAL PREGNANCY, HYDATIDIFORM MOLE AND CANCERS USING LECTIN- AND GEL-BASED APPROACHES

EMIDA BINTI MOHAMED

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
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EMIDA BINTI MOHAMED

THESIS SUBMITTED IN FULFILMENT

OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF MEDICINE

UNIVERSITY OF MALAYA

KUALA LUMPUR

2013

UNIVERSITY MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **EMIDA BINTI MOHAMED** (I.C/Passport No.: **690602-01-5915**)

Registration/Matric No: **MHA060004**

Name of Degree: **PhD**

Title of Project Paper/Research Report/Dissertation/Thesis:

PROTEOMICS ANALYSIS OF SERA OF WOMEN WITH NORMAL PREGNANCY, HYDATIDIFORM MOLE AND CANCERS USING LECTIN- AND GEL-BASED APPROACHES

Field of study: **PROTEOMICS OF SERUM PROTEINS**

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ABSTRACT

Previous study conducted by Abdul-Rahman *et al.* (2007) has highlighted the enhanced expression of a 35 kDa glycoprotein, termed lectin-detected protein 1 (LDP1), in sera of patients with endometrial adenocarcinoma (EACa). In this study, LDP1 was isolated from sera of patients with EACa using immobilised champedak-galactose-binding (CGB) lectin affinity chromatography. Subjecting LDP1 to mass spectrometry analysis, LDP1 was identified as the 35 kDa cleavage fragment of inter-α-trypsin-inhibitor heavy chain 4 (ITIH4f).

Subsequent to its identification, expression of ITIH4f in sera of patients with breast carcinoma (BrCa; n=10), epithelial ovarian carcinoma (EOCa; n=10), germ cell ovarian carcinoma (GOCa; n=10), nasopharyngeal carcinoma (NPCa; n=13) and osteosarcoma (OsSa; n=10) was investigated using 2-DE and HRP-CGB lectin approach. When compared to control sera, expression of ITIH4f was enhanced in sera of patients with BrCa, EOCa and GOCa, but not in patients with NPCa and OsSa. The data of this study, when taken together with reports of previous researchers, appeared to link the over-expression of ITIH4f with cancers associated with estrogen dysregulation. As such, the expression of ITIH4f is also likely to be enhanced in other diseases associated with increased levels of estrogen. Hence, its expression in women with normal pregnancy (NP; n=20), hydatidiform mole (HM; n=20) and menopausal women under the hormone replacement therapy (HRT; n=10) were studied. When compared to controls (n=20), it was demonstrated that the expression of ITIH4f was over-expressed in women with NP and HM, but was not altered in women with HRT. Hence, the expression of ITIH4f was not exclusive to cancers but was also enhanced in normal and benign conditions that are associated with estrogen dysregulation.

An attempt to establish the serum protein profiles of women with NP and HM was subsequently performed via 2-DE. Protein detection was either performed by silver staining or CGB lectin. Image analyses performed on profiles of pregnant women demonstrated that the expression of ITIH4f, α_1-antichymotrypsin (ACT) and leucine-rich-glycoprotein (LRG) was significantly enhanced in the women with NP. However, ITIH4f was the sole serum glycoprotein which appeared to be altered in sera of women with HM.

Comparative analysis performed on the silver-stained protein profiles demonstrated enhanced expression of ACT, LRG, clusterin (CLU) and zinc-α2- glycoprotein (ZAG) in sera of women with NP. In contrast, women with HM were characterized by the up-regulated expression of CLU and LRG. However, the LRG of women with HM appeared to be differentially glycosylated from that of women with NP and CLU in women with HM seemed to be more cationic, relative to than that of NP women. All proteins which showed differential expression were identified by mass spectrometry analysis. The aberrant expression of the identified proteins was subsequently validated using immunoblotting technique.

Finally, an attempt to simultaneously detect the differential expression of the respective proteins was performed using multiplex immunoassay. A multiplex immunoassay was developed to evaluate the expression of eight proteins but the development was only successful for three proteins- anti-thrombin III (ATR), CLU and ceruloplasmin (CLP). Nonetheless, the assay was capable of confirming the up-regulated expression of CLU and the unaltered expression of ATR and CLP.

ABSTRAK

Kajian terdahulu yang dijalankan oleh Abdul Rahman *et al*. pada tahun 2007 telah menonjolkan peningkatan pengekspresan suatu glikoprotein 35 kDa, yang dinamai protein yang dikesan lektin (LDP1), di dalam serum pesakit yang baru didiagnosis dengan adenokarsinoma endometrium (EACa). Protein LDP1 menunjukkan interaksi yang kuat dengan lektin pengikat galaktosa cempedak (CGB) berikat enzim walaupun sebelum ini ia tidak dikesan pada profil protein pesakit EACa yang diwarnai perak. Dalam hal ini, adalah agak menarik untuk mengenal pasti kelompok tompokan LDP1. Memandangkan LDP1 sebelum ini dikesan pada membran nitroselulosa, satu pendekatan alternatif telah diguna pakai untuk mengasingkannya. Dalam kajian ini, LDP1 telah diasingkan daripada campuran beberapa serum pesakit EACa menggunakan kromatografi afiniti jerapan lektin CGB. Dengan memisahkan glikoprotein terjerap-lektin menggunakan2-DE dan analisa spektometri jisim, LDP1 dikenalpasti sebagai serpihan 35 kDa rantai berat 4inter-α-perencat tripsin (ITIH4f).

Selepas pengenalpastiannya, pengekspresan serpihan 35 kDa ITIH4f di dalam serum pesakit karsinoma payudara (BrCa; n=10), karsinoma epitelium ovari (EOCa; n=10), karsinoma sel germa ovari (GOCa; n=10), karsinoma nasofaring (NPC; n=13) dan osteosarkoma (OsSa; n=10) telah disiasat menggunakan pendekatan 2-DE dan lektin CGB berikat enzim. Apabila dibandingkan dengan sera kawalan (n=20), pengekspresan serpihan 35 kDa ITIH4f didapati meningkat dengan ketara dalam serum pesakit BrCa, EOCa dan GOCa, tetapi tidak pada pesakit NPC dan OsSa. Data dari kajian ini, apabila diambil bersama dengan laporan penyelidik-penyelidik sebelumnya kelihatan menghubungkan peningkatan pengekspresan ITIH4f secara khusus dengan kanser yang berkait dengan disregulasi estrogen. Dalam kes yang sedemikian, walau bagaimanapun, pengekspresan

serpihan ini juga berkemungkinan tinggi berlaku dalam penyakit-penyakit lain yang berkaitan dengan peningkatan paras estrogen. Oleh itu, pengekspresan serpihan ITIH4f di dalam wanita hamil yang normal (NP; n=20), pengidap hydatidiform mol (HM; n=20) dan wanita menopaus yang menjalani terapi penggantian hormon (HRT; n=10) telah dikaji. Apabila dibandingkan dengan serum kawalan, pengekspresan serpihan ITIH4f didapati adalah tinggi di dalam kumpulan wanita NP dan HM, tetapi tidak berubah di dalam kumpulan wanita yang menjalani HRT. Oleh itu, data kajian ini mengambarkan bahawa pengekspresan serpihan 35 kDa ITIH4f adalah tidak eksklusif kepada kanser tetapi ia juga tinggi dalam keadaan normal dan benigna yang berkait dengan disregulasi estrogen.

Dalam bahagian kedua kajian ini, satu cubaan untuk mewujudkan profil serum protein wanita NP dan HM telah dilakukan melalui pendekatan 2-DE. Pengesanan protein dilaksanakan dengan menggunakan lektin CGB berikat enzim atau pun pewarnaan perak. Apabila serum protein yang dipisahkan menggunakan 2-DE dari dua kumpulan wanita hamil tersebut didedahkan kepada lektin CGB berikat enzim, profil yang terdiri daripada hanya protein O-terglikosilat telah diperolehi. Analisa imej yang dilakukan ke atas profil kedua-dua kumpulan wanita hamil menunjukkan perubahan berbeza pengekspresan O-glikoprotein, relatif kepada subjek kawalan. Keputusan yang diperoleh menunjukkan bahawa pengekspresan serpihan 35 kDa ITIH4f, α1-antikimotripsin (ACT) dan glikoprotein kaya leusina (LRG) telah dipertingkatkan dengan ketara dalam kumpulan wanita NP. Walau bagaimanapun, serpihan 35 kDa ITIH4f merupakan glikoprotein serum tunggal yang kelihatan berubahdengan ketara dalam serum wanita HM.

Sampel serum wanita NP dan HM kemudiannya dipisahkan menggunakan 2-DE, diikuti oleh analisa imej profil protein yang diwarnakan perak. Analisa perbandingan yang dilakukan ke atas profil protein serum menunjukkan peningkatan pengekspresan ACT,

LRG, klusterin (CLU) dan zink-α2-glikoprotein (ZAG) di dalam serum wanita NP. Sebaliknya, wanita HM telah dicirikan dengan peningkatan pengekspresan CLU dan LRG. Walau bagaimanapun, LRG wanita HM menunjukkan pengglikosilan berbeza berbanding wanita NP. Di samping itu, kelompok tompok protein CLU di dalam serum wanita HM kelihatan lebih kationik, relatif kepada wanita NP. Kesemua lima protein yang menunjukkan pengekspresan berbeza dalam kajian ini telah berjaya dikenalpasti dengan analisa spektrometri jisim. Perbezaan pengekspresan protein yang dikenal pasti di dalam serum wanita NP serta HM seterusnya divalidasikan menggunakan teknik imunoblot.

Dalam bahagian akhir kajian, satu cubaan untuk mengesan perbezaan pengekspresan panel protein secara serentak, bagi kumpulan wanita NP dan HM, telah dilakukan dengan menggunakan ujian imun multipleks. Satu ujian imun multipleks telah dibangunkan dengan tujuan untuk menilai pengekspresan lapan proteinberbeza tetapi hanya berjaya untuk menentukan tiga protein iaitu, anti-trombin III (ATR), CLU dan seruloplasmin (CLP). Namun itu, ujian tersebut mampu mengesahkan peningkatan pengekspresan CLU dan ketidakubahan pengekspresan ATR dan CLP di dalam kumpulan wanita yang dikaji.

ACKNOWLEDGEMENT

Alhamdulillah, praise be to Allah as it is with His grace that this thesis is finally completed. This dissertation was made possible with the help and assistance of many individuals.

I would like to express my deepest gratitude and sincere appreciation to my supervisor, Prof. Dr. Onn bin Haji Hashim for his invaluable support, encouragement and guidance in accomplishing this project. Heartiest thanks to my second supervisor, Prof. Dr. Siti Zawiah Omar for her kind encouragement and support in providing serum samples which were used in this work.

I would also like to thank the Ministry of Higher Education and UiTM for giving me the opportunity and scholarship for my PhD candidature.

Very special thanks for the considerable help and assistance from the entire staff at the Department of Molecular Medicine of Universiti Malaya. To my lab-mates, it was a privilege to work with all of you, and I appreciate the experience I have gained throughout my attachment in the lab. I will cherish our friendship always. Word of thanks are also extended to my dear friends in Faculty of Health Sciences, UiTm for all the support and encouragement given.

Above all, I shall remain forever grateful to my family; my parents, brothers and sisters for their love and never ending support that gave me the confidence and inspiration to keep going through the hardest of time.

Special wish for my husband and children for being my pillar of strength and for the love, understanding and patience given. To my husband, Mohamad Noor, and my four 'little angels': Shafiqah, Syahir, Shakirah and Shifa'- you make my days feel forever blessed.

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LIST OF SYMBOLS AND ABBREVIATIONS

A_{280}	- absorbance at 280 nm
A_{485}	- absorbance at 485 nm
AAT	- α_1-antitrypsin
ABG	- α_1-β-glycoprotein
ACCa	- adenocervical carcinoma
ACT	- α_1-antichymotrypsin
AHS	- α_2-HS-glycoprotein
ALB	- albumin
ATR	- anti-thrombin III
BSA	- bovine serum albumin
BRCa	- breast carcinoma
C	- control subjects
CGB	- champedak galactose binding
CLP	- ceruloplasmin
CLU	- clusterin
CMB	- champedak mannose binding
CNBr	- cyanogens bromide
2-DE	- two dimensional gel electrophoresis
Da	- dalton
DTT	- dithiothreitol
DVS	- divinylsulfone
EACa	- endometrial adenocarcinoma

ELISA	- enzyme-linked immunosorbent assay
EOCa	- epithelial ovarian carcinoma
et. al.	- et alia (and others)
g	- gram
Gal	- D-galactopyranose
GalNAc	- N-acetylgalactosamine
GTD	- gestational trophoblastic disease
GOCa	- germ cell ovarian carcinoma
HAP	- haptoglobin-β-chain
HM	- hydatidiform mole
HPX	- hemopexin
HRT	- hormone replacement therapy
i.e.	- id est (that is)
Ig	- immunoglobulin
ITIH	- inter-α-trypsin inhibitor heavy chain
kDa	- kilodalton(s)
KNG	- kinninogen
L	- litre
LDP1	- lectin-detected protein 1
LRG	- leucine rich glycoprotein
M	- molar
MALDI	- matrix assisted laser desorption ionization
ml	- milliliter
m/z	- mass/charge

Mr	- relative molecular mass
MS	- mass spectrometry
MS/MS	- tandem mass spectrometry
NP	- normal pregnant/ pregnancy
NPCa	- nasopharyngeal carcinoma
OsSa	- osteosarcoma
pI	- isoelectric point
PBS	- phosphate-buffered saline
rpm	- revolution per minute
SCC	- squamous cell carcinoma
SCCa	- squamous cell cervical carcinoma
SDS	- sodium dodecyl sulphate
SDS-PAGE	-sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SELDI	- surface-enhanced laser desorption/ionization
TEMED	- N,N,N',N'-tetramethylethylenediamine
V	- voltage
v/v	- volume over volume
w/v	- weight over volume
X g	- acceleration due to gravity
μg	- microgram(s)
μl	- microliter(s)
ZAG	- zinc-α_2-glycoprotein

CHAPTER 1:

INTRODUCTION

1.1 Benign Neoplasm

The term neoplasm describes any new and abnormal growth which involved progressive and uncontrolled cell multiplication. It usually forms a distinct mass of tissue called a tumor, which may be either benign or malignant. The growth tissue generally shows partial or complete lack of structural organization and functional coordination with the normal tissues and serves no physiological function. `Benign' implies a mild and non-progressive condition. The term benign neoplasm thus describes any tumor or abnormal growth which is non-malignant, treatable and lacks the ability to metastasize to other areas of the body. Benign neoplasms are very diverse. They are typically composed of cells which bear strong resemblance to normal cells in the organs from where they originated. Most of the benign conditions are named based on their organ of origin followed by the suffix "*-oma*" which refers to tumor in Latin. Common examples include lipomas and adenomas which refer to benign tumors of lipocytes and gland forming cells, respectively.

Even though benign tumors lack the invasive properties of cancer, they may still cause health problems. The negative effects are usually dependent on their anatomical location and tissue type. Among the pathological effects of some benign tumors are obstruction or compression of vital organs, cosmetic changes and over-production of certain hormones when involving tumors of the endocrine tissues. Surgery is usually the most effective approach for treatment of benign tumors.

1.1.1 Hydatidiform mole

Hydatidiform mole (HM) is the most common type of gestational trophoblastic disease (Alifrangis and Seckl, 2010; Batorfi *et al*., 2003). Gestational trophoblastic disease

(GTD) describes a spectrum of abnormal trophoblastic proliferation which originated from the placental trophoblastic epithelium of the uterus (Altieri *et al*., 2003). It is characterized by the production of irregular forms of human chorionic gonadotropin (HCG), which is a glycoprotein hormone produced by trophoblasts of the placenta. Examples of the abnormal forms of HCG include ßHCG, nicked HCG as well as dessialylated and hyperglycosylated forms of HCG (Altieri *et al*., 2003; Cole *et al*., 2001; Imamura *et al*., 1987). At present, under the WHO classification, GTD includes hydatidiform mole, invasive mole, choriocarcinoma and the rare placental site trophoblastic tumor (Alifrangis and Seckl, 2010; Seki *et al*., 2004; Li *et al*., 2002). The benign end of GTD is HM, whilst the malignant end is choriocarcinoma. HM can be further classified into complete and partial moles on the basis of its morphological, cytogenetic and clinical features (Jauniaux *et al*., 1999). Complete HM is characterized by hydrophobic degeneration of all villi and absence of embryo, cord and amniotic membranes. In contrast, partial HM is characterized by focal trophoblastic proliferation with a mixture of normal and edematous villi. An embryo, cord and amniotic fluid are usually present (Slim and Mehio, 2007).

The incidence of HM varies in different countries, being more common in South-East Asia (Ngan, 2003). Its prevalence also varies between ethnic groups (Slim and Mehio, 2007) and in Malaysia, the highest frequency being amongst the Chinese women (Loh *et al*., 2004). The higher rate of incidence in a particular ethnic group has been suggested to be attributed to genetic and various environmental factors such as food preferences (Slim and Mehio, 2007). HM results from an abnormal conception event and is characterized by the absence of a fetus with an inappropriate implacentation (Wihman *et al*., 1998). The classical clinical presentation used to be vaginal bleeding in the middle trimester of pregnancy. This may be accompanied by the passage of grape-like structures consisting of

edematous clusters of vesicles, 0.5 to 2.0 cm in size with varying amounts of hemorrhage and necrosis (Lage *et al*., 2003). More aggressive symptoms may include excessive uterine size, anemia, preeclampsia, hyperthyrodisim or hyperemesis. Nowadays, HM rarely presents with the classical symptoms due to the widespread use of obstetric ultrasound. Routine use of ultrasonography allowed for early diagnosis at 8.5 to 9.5 weeks of gestation, following incidence of vaginal bleeding. However, early diagnosis of this tumor is not always easy as in the initial stages it resembles normal pregnancy except for the higher level of HCG. Extremely marked elevation of maternal serum β-HCG for the fetus gestational age is suggestive but not diagnostic of HM. However, individual variation and other factors can cause problems in the clinical practice of β-HCG (Cole *et al.*, 2006; Khanlian *et al*., 2003). Hence, diagnosis of HM relies greatly on ultrasonogram and in cases of uncertainty, histological diagnosis will be performed (Ngan, 2003). The standard treatment for HM involved suction evacuation of the uterus and removal of the mole by curettage.

1.2 Cancer

1.2.1 Cancer as a disease

Cancer is opposite of benign conditions. It is a disease that begins in the cells and it encompass a broad range of different types, all of which involved unregulated cell growth. To date there are over 100 different known cancers that afflict human. Cancer is generally characterized by an uncontrolled cellular growth and development of malignant cells. These cellular activities lead to excessive proliferation and spreading of cells which will later form a tumor. The tumor can damage neighboring tissues or organs as it can invade

through the lymphatic system or the bloodstream (Hill and Tannock, 1998). The character of the cancerous cell fits its *Latin* meaning i.e, crab, due to its ability to 'crawl' and causing pain to any part of the body.

The diagnosis of cancer can be performed in a number of approaches involving the detection of certain signs and symptoms, physical examination, screening tests or via medical imaging. Once a possible cancer is detected further diagnosis will involve histological examination of tissue samples. Following diagnosis, management of patients usually involves treatment with chemotherapy, radiation therapy or surgery for removal of tumor cells. The chances of surviving the disease vary greatly by the type and location of the cancer and the extent of disease at the start of treatment.

1.2.2 Etiology of cancer

The question of the etiology of cancer has been the focus of medical scientists for many years. However, little is definitely known on the essential causes of a cancer due to the complex nature of this disease. At present, there are a number of predisposing factors that bear relation to the development of cancer. It has been suggested that 90-95% of cancer cases arise from environmental or non-genetic factors, whilst only 5-10% are due to genetic factors (Anand *et al*., 2008). Genetic defects have been implicated with various types of hereditary cancers (Knudson, 1985) with the most common being the breast and ovarian cancers. Alterations in the *BRCA1*and *BRCA2* genes are most closely associated with increased susceptibility to the two types of cancers (Scully, 2000). Likewise, mutations of the MLH1, MSH2 and MSH6 genes have been linked to increased risk of hereditary non-polyposis colorectal cancer (Wagner *et al*., 2003).

Environmental elements account for a substantial portion of the predisposing factors and are believed to contribute to the variation in the incidence of cancer (Brody and Rudel, 2003). Some of the environmental factors that have been associated with cancer include tobacco (Sasco *et al*., 2004; Hecht, 2003; Biesalski *et al*., 1998; Hackshaw *et al*., 1997), diet (Divisi *et al*., 2006; Abdulla and Gruber, 2000; Fraser, 1999), infection (Franco and Harper, 2005; Clifford *et al*., 2003; Kuper *et al*., 2000), radiation (Brody *et al*., 2007; Leszczynski *et al*., 2002) and environmental pollutants (Clapp *et al*., 2008; Cohen *et al*., 2000). In addition, hormonal factors have also been suggested as another causative agent for cancer (Brody and Rudel, 2003; Ames *et al*., 1995; Colditz *et al*., 1995), especially in cancers of the breast (Russo and Russo, 2006; Kaaks *et al*., 2005; Key *et al*., 2003), endometrium (Emons *et al*., 2000; Henderson *et al*., 1988) and prostate (Shaneyfelt *et al*., 2000; Gann *et al*., 1996) which involved the sex-hormones, as well as cancer of the thyroid which may be promoted by the growth hormones (Haymart *et al*., 2009; Soh *et al*., 1996). Factors such as obesity, alcohol consumption and lack of physical activity, all of which may affect hormone levels, are also believed to underlie the increased risk of certain cancers (Brody and Rudel, 2003). However, it is nearly impossible to prove the true cause of cancer in any individual, as most cancers resulted from interplay between genetic and environmental factors (Heston, 1965).

1.2.3 Female sex hormone-related cancers

Hormones are natural substances made by the glands and are carried in the blood circulation. There are many different types of hormones with each controlling or acting as messenger for particular cells or organs. Sex-hormones are produced mainly by the gonads, i.e. the ovaries and testis with small amounts produced by the adrenal glands and

other tissues such as the liver. There are three main classes of sex hormones, with androgens being considered as the male sex hormones while estrogens and progestogens are called the female sex hormones.

Estrogens, which are the dominant type of female sex hormone, are present at significantly higher levels in women of reproductive age. They are associated with ovulation and menstruation. In addition, they are also involved in promoting the development of the female secondary sexual characteristics, such as the breast, vagina and uterus. Disturbance in the female sex-hormone may result in a variety of disorders, with attention is usually focused on cancer. In light of their functions, the female sex hormone-related cancers generally encompass cancers of the breast, endometrium, cervix and ovary.

1.2.3.1 Cancer of the breast

Breast cancer is the most common cancer in women (Parkin *et al*., 2001). Incidence is highest in North America, Northern Europe and Australia and lowest in Asia and Africa. Nevertheless, according to the National Cancer Registry Report for 2007 (2011), breast cancer is the leading cancer affecting women in Malaysia. In 2007 alone, there were 3,242 female breast cancer cases diagnosed, which accounted for 18.1% of all cancer cases reported, and 32.1% of female cancers. Out of this, only 58% of cases were detected at stages I and II. The confounding factor could be in that at an early stage, the breast cancer usually does not cause any pain. In fact, when it first develops, there may be no symptoms at all. Only as the cancer grows, it causes changes such as a lump or thickening in/near the breast or in the underarm area or a change in the size or shape of the breast.

At present, mammogram is the best tool available for diagnosing breast cancer (Veronesi *et al*., 1995). Nevertheless, they have some limitations as a mammogram may miss some cancers (false negative) or may find abnormalities that turn out not to be cancer (false positive). Ultrasonography for visualization of breast tissues has also been developed and so far it has been used in symptomatic patients to distinguish benign lesions from malignant ones. The limitation of this method lies in its inability to detect tumors of less than 5 mm diameter and also microcalcifications, which are important indicators of early malignant lesions (Veronesi *et al*., 1995). Screening by detection of circulating tumor markers for breast cancer is less well established. The widely used markers in breast cancer are the cancer antigen 15-3 (CA 15-3) and carcinoembryonic antigen (CEA) (Duffy, 2006; Safi *et al*., 1991).

1.2.3.2 Cancer of the cervix

Cancer of the cervix is the third most common cancer among Malaysian women and the fifth most common cancer in the entire general population in this country (National Cancer Registry Report, 2011). Cervical cancer is divided into two major subtypes constituting squamous cell cervical carcinoma (SCCa) and adenocervical carcinoma (ACCa). Other less common types include the adenosquamous, clear cell and small cell carcinomas. In general, this form of cancer arises from cells at the junction between the primary columnar epithelium of the endocervix and the squamous epithelium of the ectocervix. SCCa, which is the most common form of cervical cancer, involved the squamous epithelial cells that line the cervix (Parkin *et al*., 2001), while ACCa is associated with changes in the glandular epithelial cells. Similar to the other types of cancers, the

early stage of cervical cancers may be completely asymptomatic. In more advanced stages, vaginal bleeding or a vaginal mass may indicate the presence of malignancy.

Cervical cytology screening is the common method for early detection of premalignant cervical lesion and it has been shown to be an effective screening approach (Kurman *et al*., 1994). However, confirmation of the diagnosis of cervical cancer requires a biopsy of the cervix for histological diagnosis. This is often performed during colposcopy, which is an internal vaginal examination to look for abnormalities in the cervix. The use of tumor markers further refines the diagnosis as well as the management and prognosis of cervical cancers. For SCCa, the squamous cell carcinoma (SCC) antigen is the marker of choice (De Bruijn *et al*., 1998; Brioschi *et al*., 1991), while cancer antigen 125 (CA 125) has been suggested to have possible utility in the diagnosis and monitoring of ACCA (Gadducci *et al*., 2009; Borras *et al*., 1995).

1.2.3.3 Cancers of the ovaries

Ovarian cancer is the fourth most common cancer in Malaysian females. It is a cancerous growth that occurs in one or both ovaries. Early ovarian cancer may not cause any symptoms. When symptoms do appear, ovarian cancer is often in a more advanced stage. Among the symptoms are pain or swelling in the abdomen, pain in the pelvis or gastrointestinal problems such as bloating and constipation. However, early diagnosis is difficult as the symptoms are non-specific.

At present, there are several types of ovarian cancers which are separated based on their distinct and pathological features i.e., epithelial, germ cell and sex cord stromal carcinomas (Trope *et al*., 1997). Most ovarian cancers are classified as epithelial ovarian

carcinoma (EOCa), which comprises approximately 80% of ovarian cancer cases (Morgan *et al*., 2011). In EOCa cases, development of cancerous cells begins in the lining of the ovary and the majority of EOCa cases are diagnosed in premenopausal women (median age range of 63 years). Germ cell ovarian cancer (GOCa), on the other hand, is associated with the egg producing cells within the body of the ovary. Unlike the EOCa, germ cell tumor occurs in young women of child-bearing age i.e., under 30 years of age and is highly curable (Ozols *et al*., 2001). Another type of ovarian cancer is the sex cord stromal cancer which is very rare. This cancer arises in the cells that releases steroid hormones and can occur in women of any age.

Diagnosis of ovarian cancer starts with a physical examination to check for any visible abnormalities of the uterus. Bimanual pelvic examination has been the most commonly used method for the detection of ovarian cancers. However, this technique is too insensitive and cannot reliably detect early disease (Neijt *et al*., 1995). The most effective screening method at present is a combination of serum CA 125 levels and transvaginal sonography. However, measurement of CA 125 alone is not reliable enough as it is not always present in ovarian cancer, and it may be present in benign ovarian conditions (Petricoin *et al*., 2002). Confirmation of diagnosis is thus performed by taking biopsies of the affected tissue and also by doing laparoscopy, colonoscopy and abdominal fluid aspiration.

1.2.3.4 Cancers of the endometrium

Endometrial cancer refers to several types of malignancies that arise from the endometrium or lining of the uterus. It is also referred to as womb cancer, cancer of the

corpus uteri and uterine cancer (Bull and Woolas, 2011). Tumors of the endometrium can be divided into two main subtypes i.e., endometrial adenocarcinoma (EACa) and uterine sarcomas. EACa is the major subtype and usually occurs in post-menopausal women. This cancer originates from the single layer of epithelial cells within the uterine lining (Amant *et al*., 2005). Other less common types of endometrial cancers are the papillary serous, clear cell, mucinous and squamous cell adenocarcinomas.

Cancer of the endometrium is easily recognizable due to its usual presentation of postmenopausal abnormal vaginal bleeding (Hubbard and Holcombe, 1990). However, in younger women, this symptom may lead to a delay in diagnosis of cancer as this symptom may be due to many other causes. Initial diagnosis of endometrial cancer is usually performed by doing a vaginal examination followed by an ultrasound scan of the uterus. For confirmation of endometrial cancer, biopsy is commonly performed for histological examination of the endometrial and endocervical tissue samples. To date, there has been no existing reliable biomarker for screening of endometrial malignancy (Banno *et al*., 2012). The only available biomarker that could aid in the diagnosis of endometrial cancer is CA 125. However, as mentioned earlier, the up-regulated level of this tumor marker is also seen in patients with ovarian cancer.

1.2.4 Non-sex hormone related cancers

1.2.4.1 Osteosarcoma

Osteosarcoma is the most common primary malignant bone cancer. Primary bone cancer refers to cancer that forms in cells of the bone. In contrast, secondary bone cancer refers to cancer which originates from a different place but metastasizes to the bones. For this type of sarcoma, the age-specific incidence curve peaks in the adolescent period of between 10 and 20 years old, and again in old age (Henderson *et al*., 1982). It was suggested that the incidence peak during adolescent is associated with the pattern of skeletal growth in which within these ages, there is rapid growth of the bone. Skeletal growth is highly influenced by a combination of factors including the stimulation by hormones, especially the pituitary growth hormones and the thyroid hormones (Henderson *et al*., 1982). This cancer often occurs in the long bones of the arms and legs, in particular at areas of rapid growth around the knees and shoulders. This type of cancer is often very aggressive with risk of spread to the lungs. The five-year survival rate is about 65%.

Patients with osteosarcoma may present with persistent pain, swelling or tenderness of a bone. They may have unexplained fracture of one or more bones, sometimes without noticeable trauma. The presenting radiologic finding on X-ray is often destruction of bone. Currently, diagnosis relies heavily on imaging techniques with the lack of available clinical tumor markers. Development in the field of imaging has contributed to effective diagnosis and management of osteosarcoma. These included computed tomography (CT) and magnetic resonance (MR) evaluation of the bone and soft tissue for the detection of metastatic disease as well as to facilitate surgical planning (Tan *et al*., 2006). A biopsy

sample of the tumor will be obtained either through a small needle (needle biopsy) or through a small incision (incisional biopsy) for histological investigation.

1.2.4.2 Nasopharyngeal carcinoma

The term nasopharyngeal carcinoma (NPCa) encompasses any carcinoma arising in the nasopharynx. The nasopharynx is a tubular space, situated at the base of the skull and it represents a transitional area between the nasal cavities and the oropharynx. This cancer is common in men particularly of Chinese ancestry (Huang, 1991). In Malaysia, NPCa is the fourth most common cancer among Malaysian and the third most common cancer among males (National Cancer Registry, 2011). NPCa is linked to three factors, which may play an inter-mingling role in its pathogenesis. Epstein-Barr virus infection, occupational exposure to wood dust and consumption of certain preservatives or salted foods, more specifically the 'Cantonese dried fish', has been suggested to be amongst the risk factors of NPC (Huang, 1991). To date, the etiology of NPCa has not been associated with any hormonal factors.

Symptoms that are common to NPCa include a lump or sore that does not heal, trouble speaking, breathing or swallowing, frequent headaches, pain or ringing in the ears. NPC is diagnosed by endoscopy using a nasopharyngoscope inserted through the nose, X-ray, CT scan, magnetic resonance imaging (MRI) and biopsy. To date, biopsy is the reliable test for confirmation of NPCa (Woo and Waldran, 1991). However, in some atypical cases, especially when the disease is submucosal, additional investigations are required. One of them is the detection of IgA antibodies to EB-virus specific antigens, which appeared to be helpful in the diagnosis of NPCa (Woo and Waldran, 1991). In

addition, the potential use of serum markers such as the circulating intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin, CYFRA 21-1 and C-erb B2 has also been reported (Lee *et al*., 2001; Yazici *et al*., 2001; Liu *et al*., 1999; Kuo *et al*., 1996).

1.3 Cancer and tumor markers: limitations

A tumor marker is a soluble substance produced by the tumor or in some cases, by the body in response to the diseased condition (Malati, 2007). They can also be defined as biological molecules found in blood, other body fluid or tissue, which signifies normal or abnormal process or condition or disease. Tumor markers may be present as intracellular substances in tissues or may be released into the circulation and appear in the serum of patients with cancer. Thus, based on their measurements in the blood or other body secretions, these markers can be used as an indicator of presence of cancer (Malati, 2007). These tumor markers may aid in the diagnosis, assessment of prognosis, selection of treatment, monitoring of progress during and after treatment, and/or be used as screening tests.

The need for specific and sensitive tumor markers for screening of cancer is critical as a timely diagnosis of cancer represents one of the most important ways of reducing cancer mortality. The success of treatment for cancer relies on the ability to detect it at its earliest stage (Wulfkuhle *et al*., 2003; Hutter and Sinha, 2001) whereby the tumor is still small enough to be completely removed surgically. Hence, finding sensitive and effective tumor markers is critical in the battle against cancer and has an important public health impact (Ardekani *et al*., 2002). At present, the diagnosis of cancer still relies greatly upon

histological and cytological investigations. Thus, patients had to be subjected to the invasive diagnostic procedure, which is not feasible for screening purposes. The ideal diagnostic choice would then be to use serum tumor markers due to its easy and inexpensive accessibility.

However, there are not many available tumor markers that are specific for the detection of cancer. Tumor markers such as CA 19-9, CA 125, CEA, SCC, HCG and PSA are currently used clinically as diagnostic clues. Although such tumor markers may have been in widespread use for many years, there still remain unresolved issues with regards to their optimal clinical use. This is due to the fact that these markers of cancers are also found to be expressed in other benign conditions as demonstrated in Table 1.1. In addition, many tumor markers, particularly CA125, CA 15-3, CA19-9 and CEA, are also raised in several other cancers. Owing to the 'low' diagnostic sensitivity and specificity, the biomarkers could not be applied in the screening of an asymptomatic population for the purpose of early detection. An ideal biomarker would require a specificity of greater than 99% to avoid the consequence of a high rate of false-positive results.

While some of these proteins have been examined individually as potential biomarkers and found to have 'low' sensitivity or specificity as individual tests, growing evidence indicates that these limitations may be overcome using panels of candidate tumor markers that together could better represent the patient's status. There is high probability of increasing the diagnostic power of the current tumor markers if supplemented by other tumor markers or aberrantly expressed proteins such as the acute-phase reactant proteins (Pang *et al*., 2010), instead of relying on single tumor marker as currently practiced. The concept of using more than one marker in a multiplexed format is not new. It has been used for many years for thyroid and Down's syndrome testing but has been limited in the

Table 1.1: Clinically available biomarkers for cancer

Tumor marker	Cancer	Other conditions in which marker may be raised	
		Cancer	Other
α fetoprotein (AFP)	Germ cell/testicular tumour; Hepatocellular carcinoma	Colorectal; gastric; lung; hepatobiliary; hepatocellular; pulmonary	Liver degeneration
Cancer antigen 125 (CA125)	Ovarian cancer	Breast; cervical; endometrial; hepatocellular; lung; non-Hodgkin's lymphoma; pancreas; peritoneal; uterus	Leiomyoma; acute hepatitis; acute/chronic pancreatitis; arthritis; osteoarthritis; rheumatoid arthritis; chronic liver disease
Cancer antigen 19-9 (CA19-9)	Pancreatic cancer	Colorectal; gastric; hepatocellular; oesophageal; ovarian	Acute/chronic pancreatitis; cholestasis; chronic liver disease; jaundice
Carcinoembryonic antigen (CEA)	Colorectal cancer	Breast; gastric; lung; mesothelioma; oesophageal; pancreatic	Chronic renal failure; Jaundice; Chronic liver diseases- Cirrhosis
Squamous cell carcinoma (SCC)	Cervical cancer	Head and neck; lung; esophagus; anal canal	Extensive liver disease
Prostate specific antigen (PSA)	Prostate cancer	None known	Acute urinary retention; benign prostatic hyperplasia

Data derived from Malati, 2007

use for the diagnosis of cancer (Whiteley, 2006). Recent studies conducted by several groups of researchers have shown that by using multiple tumor markers, they managed to develop a more accurate detection method for the early diagnosis of cancers of the breast (Li *et al*., 2002) and prostate (Laxman *et al*., 2008). Clearly, there is a need for more and better tumor markers with higher sensitivity and specificity to replace or complement the existing ones. This is where the emergence of proteomics technology could greatly facilitate the discovery of new markers for the development of highly accurate diagnostic approach for early diagnosis of cancer.

1.4 Pregnancy as a hormone-related condition

Pregnancy is the fertilization and development of one or more offspring, known as an embryo or fetus, in a woman's uterus. However, in a pregnancy, there can be multiple gestations as in twins or triplets and unfortunately, pregnancy could also be of abnormal form such as with ectopic and molar (hydatidiform mole) pregnancies. Pregnancy begins with implantation of the blastocyst, which is a developed form of the fertilized egg (zygote) to the uterine wall. This is the beginning of the embryonic stage which lasts for eight weeks. During this period, the embryonic cells differentiate to develop various basic body systems. By the end of this stage, early features of the eyes, mouth, ears and fingers could be seen. Once the embryonic stage is completed, the embryo enters the final stage where it becomes known as a fetus. The fetus then develops and grows in weight and length until the last week of pregnancy (Norwitz *et al*., 2001). A pregnancy usually occurs within 38 to 40 weeks after conception and is arbitrarily divided into three trimesters. The first twelve

weeks is considered as the first trimester, while the second and third trimesters cover the period of week 13 to 28 and week 29-40, respectively.

During the pregnancy period, a pregnant woman experiences several physiological changes. The various alterations are required to support fetal growth and development (Mansourian, 2010). The growth and development of the fetus requires several hormones, progesterone and estrogen in particular, to fulfill the cellular and molecular demand related to the pregnancy period (Bacq *et al*., 1996). These hormones are required to regulate endometrial differentiation and function, pregnancy recognition signaling, uterine receptivity for implantation and conceptus-uterine interactions (Spencer and Bazer 2004; Norwitz *et al*., 2001). During pregnancy, the serum estrogen and progesterone levels increase progressively and reach a maximum during the third trimester. Estrogen is produced by the ovaries, and later by the placenta, with the levels increasing steadily until birth. It plays important roles in the key physiological events essential to the development and maintenance of the fetus as well as protection of the mother. Estrogen is also implicated to the production of progesterone. Progesterone is one of the members of the progestogen group of hormones. It is produced first by the ovaries and followed by the placenta but at a much later time compared to the production of estrogen. Progesterone starts to be produced at the beginning of the second trimester and plays important role in maintenance of the pregnancy condition. It is involved in maintaining the uterine lining, function of the placenta and stimulates the growth of breast tissue.

1.5 PROTEOMICS

Currently, the search for new tumor markers is gaining momentum through the use of proteomics technologies (Wulfkuhle *et al*., 2003; Verma *et al*., 2001). Recent proteomics studies in cancer have identified proteins that were differentially regulated in cancer samples and have led to the discovery of several candidate biomarkers. This is further accelerated by the immense diagnostic potential of using serum or plasma. From a medical viewpoint, serum is the most informative proteome. Almost all cells in the body communicate with plasma directly or through extracellular or cerebrospinal fluids and may release at least part of their contents into plasma upon damage or death. Hence, any diseased state will produce some specific patterns of protein change in the body's working fluid (Anderson *et al*., 2004).

1.5.1 Definition of proteomics

The term *proteome* is an analogy to the term genome and was first introduced in 1994 by Marc Wilkins (Wilkins *et al*., 1995). Later, in 1995, Wasinger defined *proteome* as `the total protein complement encoded by a genome or by a particular cell or specific tissue type' (Wasinger *et al*., 1995). Proteins are the vital parts of a living organism as they are the main components of cellular physiological metabolic pathways. Hence, in order to study the cell metabolism or to identify possible disease-specific biomarkers or even possible drug targets, analyses on the proteome of the cell needs to be carried out.

Proteomics, on the other hand is defined as the systematic analysis of the protein expression of a proteome or more likely, a subproteome (Wilkins *et al*., 1995). It is also

defined as a large-scale characterization of the entire protein complement of a cell line, tissue or organism (Anderson and Anderson, 1996). However, Graves and Halstead later formulated a more extensive definition where proteomics could be seen as a large scale screening process that aims to profile the overall distribution of proteins in cells, characterize and identify proteins of interest and finally to elucidate their relationships and functional roles (Graves and Haystead, 2002). As such, proteomics at its simplest level is the study of protein expression in a proteome. It is a platform which attempts to understand the relative levels of protein within a mixture, characterize the proteins, compare variations in the expression levels between normal and disease states, study their interactions with other proteins and identify their functional roles.

1.5.2 Proteomics studies

Genomics and proteomics, in principle, hold promise for systematic routes for tumor marker discovery and as such have rapidly become popular. After genomics, proteomics is seen as the next step towards understanding complexities of biological systems. The development and progression of proteomics in the past decade have been substantially facilitated by the availability of developments in mass spectrometry (MS) and genomic information. Invention of mass spectrometry coupled with knowledge on the DNA sequence of human and other genomes has opened doors for proteomics by providing a sequence-based framework for protein identification and characterization.

However, proteomics is much more complicated than genomics mostly because while an organism's genome is more or less constant, the proteome is dynamic in nature. Gene expression analysis allows determination of transcript levels for many thousands of

genes, but this does not necessarily equate to the production of functional protein (Mischak *et al*., 2007). Production of protein differs from cell to cell and is highly influenced by the cell's current physiological state. As such, even though the complete human genome and many other important genomes have been successfully deciphered, direct correlation between genomic information and protein function is complicated.

There are numerous reports highlighting the disparity between mRNA transcript and protein expression levels (Varambally *et al*., 2005; Gygi *et al*., 1999). A possible reason for this phenomenon could be that even though mRNA were produced in abundance, they may be degraded rapidly or translated inefficiently, resulting in a small amount of protein. To add to these complexities, most mRNA transcripts are subjected to a wide variety of chemical modification subsequent to their translation. The post-translational modifications, such as phosphorylation, ubiquitination, methylation, acetylation, nitrosylation, oxidation and glycosylation, have the ability to change the functional activity of a protein. Some proteins even undergo all of these modifications, often in time-dependent combinations, aptly illustrating the potential complexity when studying protein structure and function (Jensen, 2006). In addition, a single gene may code for more than one protein due to alternative splicing of the mRNA (Graveley, 2001). Hence, genomic analysis alone is unable to provide adequate information on post-translational events that may often change in diseased conditions (Mischak *et al*., 2007). This problem may be directly addressed by proteomics, which can potentially capture dynamic changes in protein expression. In addition, as the proteome is far more extensive than the genome, it offers a richer source of potential tumor markers (Mischak *et al*., 2007). However, the advantages of proteomics also present enormous challenges, as the levels of complexity and dynamic range in body fluids are difficult to analyze and measure.

1.5.2.1 Areas of proteomics research

Currently, application of proteomics is rapidly growing with the scope of applications extending from microbiology to plant research, drug discovery, and clinical diagnostics. In the clinical field, proteomics can be divided in practice into three major areas: expression proteomics, functional proteomics and proteome related bioinformatics (Westermeier and Naven, 2002). Expression proteomics also known as structural proteomics is an important component of proteomics which deals with quantitative analysis and identification of large number of proteins encoded by genomes in a given cell type (Anderson and Seilhamer, 1997). The main focus of this approach is to search for aberrant differences in the expression of proteins between normal and diseased conditions and between different diseased states.

Functional proteomics, on the other hand, is targeted on obtaining information on protein function (Lee and Lee, 2004) and to look at complexing of proteins at the molecular level (Naylor and Kumar, 2003). The main goal of this type of study is to look into protein signaling, protein interactions and disease mechanism. In this area of research, it is important that the method of choice will maintain the activity and integrity of the particular protein or protein-protein complexes.

In the proteome related bioinformatics approach, bioinformatics is utilized to derive knowledge from computer analysis of biological data. Research in this area uses a large range of techniques including primary sequence alignment, protein 3D structure alignment, phylogenetic tree construction, prediction and classification of protein structure, prediction of RNA structure, prediction of protein function and expression data clustering.

1.5.2.2 Techniques of proteomics

Classically, research on protein science relied mostly on antibodies to a particular protein. With the introduction of proteomics, which requires separation and analysis of complex mixture containing as many as several thousand proteins, a number of technological platforms have been developed. The most commonly used laboratory techniques in proteomics are two-dimensional electrophoresis (2-DE) and mass spectrometry. Both techniques can be used in combination with more traditional techniques, including affinity column chromatography and western blotting. For a more quantitative determination of protein amount, technique such as enzyme-linked immunosorbent assay (ELISA) was and is still the gold-standard technique used.

Two-dimensional electrophoresis (2-DE) is a powerful gel-based separation technique already introduced in the early 1970s. The initial protein research projects using this method were carried out in 1975 by O'Farrell (1975), Klose (1975) and Scheele (1975) to map proteins from *Escherichia coli*, mouse and guinea pig, respectively. Since then, a number of modifications have been made to make gels more reproducible and more amenable to the higher-throughput use necessary for proteomics applications. The strength of this technique lies in its ability to separate and display thousands of proteins simultaneously in a single gel.

However, starting in the late 1990s, several companies began developing protein chips as an alternative to the gel-based 2-DE technique. These chips allow researchers to collect minute quantities of proteins that bind to specific molecules on their surface. Another advantage offered by this new technique is that the protein chips are often linked to the direct analysis of the protein or peptide such as the ProteinChip arrays in tandem with

Surface Enhance Laser Desorption/Ionization-Mass Spectrometry (SELDI-MS). Another emerging non-gel based proteomics approach that is currently applied by researchers is the Multi-dimensional Protein Identification Technology (MudPIT). This approach utilizes one- and two-dimensional liquid chromatography for the separation of peptides followed by on-line mass spectrometry analysis (2D LC-MS/MS).

The primary attributes in identifying protein include the protein's mass and its N- and C-terminal sequence tags. The first major technology to emerge for the identification of proteins was the sequencing of proteins using Edman N-terminal sequencing or Edman degradation (Edman, 1949). However, in today's modern proteomics workflow, mass spectrometry (MS) has assumed a key role for the analysis and identification of proteins. This is attributed by its sensitivity, accuracy, speed and capabilities to perform high throughput analysis (Biemann, 1988). Availability of database in turn allows protein information harvested from either Edman sequencing or MS to be used for protein identification.

The most comprehensive information about specific proteins is found in databases that store protein sequences. One of the first and probably the best known such database is SWISS-PROT, which was created in 1986. Other sequence-based protein databases include the Yeast Proteome Database and Human PSD. There are also a number of widely used pattern and profile databases that are used to reveal relationships among proteins based on the presence of particular groups of amino acids in the proteins' sequences. Some of the best known pattern and profile databases are: PROSITE, Pfam, PRINTS, and BLOCKS.

The value of protein quantitation for proteomics research is very substantial. The application of monoclonal antibodies had sparked discovery through close coupling of protein with simple quantitative monoclonal antibody-based assays such as the enzyme-linked immunosorbent assay (ELISA). However, the conventional immunoassay technique allows for the detection of single antigens or antibodies at a time. With the progression of proteomics, a multiplexed microsphere bead-based assay that utilizes the xMAP technology (Luminex Corporation, Austin, TX, USA) was invented with the aim of developing a more rapid, less time consuming and less sample demanding approach as compared to ELISA. This approach also permits analyses in a high-throughput manner from a single run.

Meanwhile, in determining proteins which are post-translationally modified, one way is by developing an antibody which is specific to a certain modification. This antibody can then be used to determine the set of proteins that have undergone the modification of interest. However, a more common way to determine post-translational modification is to subject the mixture of proteins to 2-DE which allows visualization of small differences in a protein. While for sugar modifications, such as glycosylation of proteins, certain lectins have been discovered which bind sugars. These lectin-binding properties offer alternative methods to investigate glycoprotein changes.

Most proteins function in collaboration with other proteins, and one goal of proteomics is to identify which proteins interact. This is especially useful in determining potential partners in cell signaling cascades. Several methods are available to probe these protein-protein interactions. The traditional technique applied in establishing protein-protein interaction was the yeast two-hybrid analysis. More recent techniques such as immunoaffinity chromatography, protein microarrays followed by mass spectrometry, dual polarization interferometry and experimental methods such as phage display and

computational methods have been used by researchers for this type of study. However, over the last decade, the use of surface plasmon resonance (SPR) technology with biosensor chip for interaction analysis has made it possible to look into the kinetics of protein-protein, protein-peptide and DNA-protein systems.

1.5.3 Application of proteomics in clinical research

One of the most promising developments to come from the study of human proteins has been the identification of potential tumor markers to monitor disease progression as well as discovery of new therapeutic targets and new drugs. Understanding the proteome, the protein's structure and function as well as the protein-protein interactions is critical for developing the most effective diagnostic techniques and disease treatments.

Tumor markers or biomarkers are important indicators as they could provide information about the physiological status of the cell and changes during a disease process (Miles *et al*., 2006). In cancer, the transformation of healthy into neoplastic cells brings about changes that can be followed distinctively at the protein level. These changes affect the cellular function of the cell and can range from alteration of protein expression level to differential protein modification and aberrant localization (Srinivas *et al*., 2001). Hence, the emergence of novel proteomics technologies allows for better understanding of the pathophysiology as well as etiology of various malignancies. These proteomics approaches also contribute to the discovery of potential biomarkers to several diseased conditions (Srinivas *et al*., 2002). Among the candidate tumor markers which have been identified using proteomics approaches are in the up-regulated expression of ceruloplasmin in sera of patients with nasopharyngeal carcinoma (Doustjalali *et al*., 2006) and the aberrant

expression of several secretory proteins, redox regulators and chaperonins in eutopic endometrium of women with endometriosis (Fowler *et al*., 2007).

At present, the basis of drug design is to find drugs with the ability to inactivate proteins (Ivanov *et al*., 2007; Archakov *et al*., 2003). This new approach relies on the genome and proteome information involved in a disease. One of the approaches that is widely applied is the structure-based drug design. In this approach, once the protein associated with a disease is identified, usually via 2-DE, the computer software will then use it as a target for development of new drugs. The computer simulation will attempt to fit millions of small molecules to the three-dimensional structure of the particular protein and rate the quality of the fit to various sites in the protein. The goal is either to enhance or disable the function of the protein, so as to interfere with its action. Example of this approach is in the identification of new drugs to target and inactivate the HIV-1 protease (Wlodawer and Vondrasek, 1998).

Application of proteomics techniques especially the 2-DE and mass spectrometry, have also been widely used for clinical microbiological research. The 2-DE-MS approach has been used to analyze global protein synthesis and provide new insights that complement genomic-based investigations. Other than providing a basic understanding of microbial gene expression, proteomics has also played a role in investigating the epidemiology and taxonomy of human microbial pathogens, identification of novel pathogenic mechanisms and analysis of drug resistance. Progress has been made with the use of these techniques to characterize and compare proteomes of pathogenic bacteria (Schmidt *et al*., 2004; Vandahl *et al*., 2001; Langen *et al*., 2000) and has recently found applications in the field of parasitology (Parodi-Talice *et al*., 2004; Drummelsmith *et al*.,

2003; Rout and Field, 2001) as more genome sequences of protozoan parasites become available.

1.5.3.1 Multiplex immunoassay

The development and application of a multiplex immunoassay that measures multiple proteins in complex matrices is becoming a significant tool for quantitative proteomic studies, diagnostic discovery and biomarker-drug development. Currently two broad categories of multiplex assay formats are available; direct labeling with single antibody experiment (Kingsmore and Patel, 2003) and sandwich immunoassay, in which dual antibodies are used (Schweitzer *et al*., 2002). In addition, a variety of microarray substrates (plastic microwells, nylon membranes, planar glass slides, gel-based arrays and beads) and signal generation strategies have been employed in the effort to optimize the performance of multiplex immunoassays (Perlee *et al*., 2004). Among the signaling platforms employed are the colorimetry, radioactivity, fluorescence, chemiluminescence, quantum dots, resonance light scattemide signal amplification, enzyme-linked assays and rolling circle amplification techniques. Each of these formats has distinct advantages and disadvantages relating broadly to their sensitivity, specificity, precision, throughput, ease of use and multiplexing power (Nielsen and Geierstanger, 2004; Haab, 2003; Kingsmore and Patel, 2003; Schweitzer and Kingsmore, 2002).

One of the multiplex immunoassay formats which is gaining popularity is the microsphere bead-based assay that utilizes the xMAP technology (Luminex Corporation, Austin, TX, USA). The multiplex suspension array is built around three core technologies. First is the family of fluorescently dyed microspheres, or beads which act as a solid support

that allows covalent coupling of antigens. Second is a flow cytometer with two lasers and associated optics to detect and measure the reactions based on the internal fluorescent ratio of the microspheres, and third is a high-speed digital signal processor to efficiently manage the fluorescent output. The Bio-plex multiplex suspension array system uses polystyrene microbeads which are internally colored with differing ratios of two spectrally distinct fluorophores. Each fluorophore can have any of ten possible levels of fluorescent intensity, thereby creating a family of 100 spectrally addressed bead sets. These dyed beads can be conjugated with different monoclonal antibodies, specific for a target protein or peptide. Hence, when these sets of antibody-conjugated beads are combined together in a single assay, it allows for simultaneous detection and quantitation of many target proteins in a single sample and a single run. This is possible as the array reader can identify each specific reaction based on the bead color and quantitates it, whilst the magnitude of the reaction is measured using fluorescently labeled reporter molecules which are also specific for each target protein.

At present, high throughput analysis of proteins has moved to a new level with the introduction of the multiplex immunoassay. With multiplexing, researchers today is given the opportunity to dramatically increase the amount of useful information from rare or volume-limited samples such as the rat serum (Rodriguez *et al*., 2009), human tear (VanDerMeid *et al*., 2012), aqueous humor (Takai *et al*., 2012) and even proteins from formalin-fixed paraffin embedded tissues (Strathmann *et al*., 2012) and frozen archived muscle biopsies (Baird and Montine, 2008). In addition, the convenience and consistency of this approach is complemented by the availability of commercially prepared cytokines, chemokines and growth factor assays. To date, there has been a lot of work carried out using these commercial assays especially for differential diagnosis purposes (Elomaa *et al*.,

2012; Gerritzen and Brandt, 2012; Guest *et al*., 2011; Hanly *et al*., 2010), for translating complex inter-relationships among proteins involved in signal transduction (Fauriat *et al*., 2010), as well as for evaluation on the safety and efficacy of candidate drugs (Ray *et al*., 2005). Aside from the commercially available beads, the feasibility of this immunoassay method has also allowed for in-house designed beads to be applied in different diagnostic areas including serodiagnosis and allergy as well as cancer diagnosis. Reactants attached to the beads can include antibodies (Schipper *et al*., 2010; Lindau-Shepard and Pass, 2010), peptides (van der Wal *et al*., 2012; Drummond *et al*. 2008) and antigenic proteins (Awan *et al*., 2012; Smits *et al*., 2012).

Hence, based on the robustness and high-throughput method offered by the immunoassay, it is of interest to develop an in-house multiplex assay targeted at several high-abundance serum proteins; α_1-antitrypsin (AAT), α_1-antichymotrypsin (ACT), α_2-HS-glycoprotein(AHS), anti-thrombin III (ATR), clusterin (CLU), ceruloplasmin (CLP), kininogen (KNG) and zinc-α-2-glycoprotein (ZAG). Selection of the respective proteins was made based on a recent report associating the differential patterns of these acute-phase proteins with several types of cancers (Pang *et al*., 2010). To date there is limited number of commercially available assays for serum proteins in the market and none are specific to the list of proteins stated earlier. Some of the protein assay kits which are available commercially are for ferritin, fibrinogen, procalcitonin, serum amyloid A, α-2-macroglobulin, C-reactive protein, haptoglobin and serum amyloid P (Bio-Plex Pro Human Acute Phase Assays, BioRad Laboratories, Inc., USA).

1.6 LECTINS

Currently, most serum proteome investigations are discovery driven, in which a large number of proteins are identified with the hope that one or more proteins are uniquely associated with a specific disease state. However, blood serum is a difficult sample to characterize on account of the large amount of its protein constituents as well as their vast differences of concentrations. Therefore, it might be difficult to use such profile in the search for novel tumor markers. Alternatively, by targeting at selective proteins, which shares specific characteristics, investigation could be narrowed down to a subset of the whole proteome. Hence, the development and application of techniques to fractionate the proteome has become an emerging field with one of the selection approaches currently used is by utilizing lectins.

1.6.1 Definition of lectin

The term lectin was first introduced by Boyd in 1954, which was coined from the Latin word *legere*, which means to pick-up or choose. Lectins by definition are multivalent proteins of non-immune origin that bind to sugars rather specifically, agglutinate cells and display no catalytic activity. The history of lectin was traced back to more than a century ago with the discovery of the actions of lectin by Herman Stillmark (1888). He discovered that the extracts of castor bean (*Rinucis communis*) and four other Euphorbiaceae plants agglutinated erythrocytes from rabbits, cats, dogs and horses. From there, lectins were regarded as phytohaemagglutines due to their plant origin and their capability to agglutinate erythrocytes. Later, the terminology was ruled out once it was discovered that other agents such as tannins and certain lipids or bivalent cations at high concentration could also trigger

agglutination of human and animal erythocytes (Rudiger, 1988). Furthermore, animals especially the invertebrates were also found to harbor lectins and not all lectins demonstrated agglutination activity towards erythocytes (Rudiger, 1981). Therefore, with the expansion in the field of lectin research, there was a need to determine the boundaries of the term lectin. Goldstein *et al*. (1980) proposed that lectin is a sugar-binding protein or glycoprotein of non-immune origin, which agglutinates cells and/or precipitates glycoconjugates. Later in 1983, Kocourek and Horejsi formulated a broader definition of lectin in which they are proteins of non-immunoglobulin nature capable of specific recognition and reversible binding to carbohydrate moieties of complex carbohydrates without altering the covalent structure of the recognized glysosyl ligands. A more simplified definition was later put forward by Barondes. It is his simple but concise definition of lectin as a carbohydrate-binding protein other than an enzyme or an antibody (Barondes, 1988) which many accept till today.

1.6.2 Source of lectin

The source of lectin is vast. Lectins have been found in plants, animals and even in microorganisms. Table 1.2 exhibits a list of commercially available lectins derived from the various sources. In the animal kingdom, lectins are widely dispersed among the phylas (Cohen, 1984). They have been successfully isolated from the hemolymph, tissue, serum, venom, egg and body fluid of some invertebrates (Kilpatrick, 2002; Cohen, 1984). In microrganisms, lectins have been detected on their cell surfaces or in their periplasm or cytoplasm (Giollant *et al*., 1993). However, the major contributor of lectin is from the plant kingdom. Lectin-containing plants include the mono- and dicotyledons, molds and

lichens, as demonstrated in Table 1.2. However, most frequently they have been detected in the *Leguminoseae* and *Euphorbiaceae* groups of plants.

Table 1.2: Examples of commercially available lectins, their origins and carbohydrate binding specificity(ies)

Source	**Common name**	**Taxonomic name**	**Carbohydrate specificity**
Plant	Jackfruit (jacalin)	*Artocarpusheterophyllus*	Gal
	Onion	*Allium cepa*	Man and/or Glc
	Jack bean (ConA)	*Canavaliaensiformis*	Man and/or Glc
	Watermelon	*Citrullus vulgaris*	Man and/or Glc
	Rice	*Oryza sativa*	GlcNAc
	Soy bean	*Glycine max*	GlcNAc
	Mushroom	*Psathyrellavelutina*	GlcNAc
	Asparagus pea	*Lotus tetragonolobus*	L-Fuc
	Wheat	*Triticum vulgaris*	Sialic acid
Microorganism	-	*Pseudomonas aeruginosa*	Gal
	Green algae	*Codium fragile*	GlcNAc
	Red marine algae	*Ptilota plumose*	Gal
Animal	Electric eel	*Electrophorus electricus*	Gal
	Japanese beetle	*Allomyrinadichotoma*	GlcNAc
	Garden snail	*Helix aspersa*	GlcNAc
	Saharan scorpion	*Biomhalariaglabrata*	Sialic acid

Data derived from Wu, 1988 and Doyle, 1994.

1.6.2.1 Plant lectin

Plant lectins are the largest and best categorised group of lectins with more than a thousand plant species have been reported to possess lectin. Most of the plant lectins are harboured in the seeds or more generally in their storage organs (Rudiger 1988). Nevertheless, they can also be found in other tissues such as the leaves and roots (Lis and Sharon, 1973). For instance, species like the *Aloe* plant stores their lectin in leaves vesicles (Suzuki *et al*., 1979), while garlic plant harbours lectin in its leave and root bulb (Smeets, 1997). However, not all plant lectins are stored. Hevein, lectin from rubber tree (*Hevea brasiliensis*) for example, is released in latex upon wounding of the bark (Giordani *et al*., 1999). Within the cell, lectins are localized in protein bodies, which are special cell organelles, derived from the cell vacuoles. It is also present in the cytoplasm and intercellular spaces (Rudiger and Gabius, 2001).

Purification of lectin was initially established using methods such as precipitation by salts, acids and organic solvents (Rudiger and Gabius, 2001). Of course, by using these approaches, the purity of the yielded lectin was not satisfactory, and as such, results obtained from its usage have to be interpreted with caution. With advancement in the area of lectinology, preparative chromatographic methods using ion exchangers, gel filtration media and affinity adsorbents further improved the purity of lectins (Rudiger and Gabius, 2001). At present, the common purification employed in isolating plant lectins is via means of immobilized affinity adsorbent in a one-step purification scheme. By selecting suitable glycoproteins or sugars as ligands, an affinity matrix presenting carbohydrate epitopes as ligands for the lectin can be devised. Examples are in the use of natural polysaccharides such as agarose or cyanogen bromide containing galactose (Gal) to isolate Gal-binding lectin such as the champedak galactose binding lectin (CGB) and ricin. For

eluting the immobilized lectin, the common method is by exploiting the sugar that acts as a competitive inhibitor. Alternatively, elution can be performed by lowering the pH of buffer with immediate neutralization of the eluant in cases whereby the lectin binds to oligosaccharides which are not readily accessible or are very expensive.

1.6.3 Binding specificity of lectin

The nature of lectin in which it exhibits considerable specificity in binding oligosaccharides and at the same time hold enormous structural diversity creates much debate on its basis of classification (Komath *et al*., 2006). At present, lectin could be classified using three approaches. The first method of classification is based on the specificities of the lectins. Based on this classification, lectins are categorised into six small groups according to their carbohydrate binding specificities i.e.; galactose (Gal), mannose and/or glucose (Man and/or Glc), N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), L-fucose (L-Fuc) and sialic acid (Wu and Sugii, 1991; Lis and Sharon, 1986).

In the second approach, lectins are categorized based on their structural similarities (Van Damme *et al*., 1998). The lectins are grouped based on the similarity of their molecular structure, sequence identity and specificity. Four families namely legume lectins, type-2 ribosome-inactivating proteins, chitin-binding lectins and monocot mannose binding lectins comprise the majority of currently known plant lectins. Aside from the four major groups, there are also smaller groups consisting of jacalin-related lectins, amarithin and Cucurbitaceae lectin which consisted of small numbers of lectins.

The last method of classification takes into account the structural features of the lectins. Therefore, they are categorized into three groups i.e., simple, mosaic and macromolecular assemblies (Lis and Sharon, 1998). Most plant lectins are classified as simple lectins. This type of lectin possesses small number of subunits, each with a carbohydrate recognition domain with possible additional domains for other types of ligands. In contrast, the mosaic types of lectins have multi-functional domains and only a single carbohydrate recognition domain. Lectins with macromolecular assemblies consist of filamentous proteins with several different subunits assembled together forming a defined complex (Ofek and Doyle, 1994).

1.6.4 Applications of lectin

The specificity of lectins in recognizing and interacting with carbohydrate moieties have allowed them to be utilized extensively in biomedical and biological studies. Lectins have found extensive applications for differentiating between cells, elucidation of the architecture and dynamics of cell surface carbohydrates, glycoconjugate purification and structural characterization. The reason behind the application of lectins across a broad spectrum is due to three reasons i.e., they are ubiquitously distributed in nature and they exhibit a wide repertoire of carbohydrate specificities as well as in the ease of their purification to homogeneity. It is therefore not surprising that lectins find extensive applications in many aspects of biology and medicine.

The earliest application of lectin was as a blood typing reagent and is still in wide use today. As many lectins display blood-group specificity, they provide an alternative to human sera in the field of transfusion medicine. Examples of useful lectins are the crude

extracts of *Lotus tetragonolobus* that agglutinate the type O erythrocytes (Renkonen, 1948), lectin extracted from *Phaseolus limensis* that specifically agglutinate type A erythrocytes (Boyd, 1949), lectin from *Dolichos biflorus* that agglutinate the type A1 erythrocytes (Bird, 1951) and lectin from *Ulex europaeus*, which agglutinates the type H erythrocytes (Cazal and Laularie, 1952). Other than blood typing, lectins are frequently used together with other parameters in the attempt to detect and classify polyagglutination of erythocytes (Judd, 1980). The principal lectins employed for this task are isolated from *Arachis hypogaea* (anti-T/Tk), *Dolichos biflorus* (anti-A1/Tn/Cad), *Salvia sclarea* (anti-Tn), *Salvia horminium* (anti-Tn + Cad) and *Glycine max* and *Bandeiraea simplicifolia* (anti-Tk) (Judd, 1980).

Another attractive property of many lectins is in their ability to induce mitosis. Several plant lectins have mitogenic characteristics on certain cells such as T and B cells, macrophages and neutrophils (Goldstein *et al*., 1980). Mitogenicity of lectins on T lymphocytes have been displayed by Con A (Powell and Leon, 1970) while pokeweed lectin had demonstrated mitogenic activity on both T and B cells (Janossy *et al*., 1976). Example from a more recent study was the finding of the mitogenecity of champedak mannose binding lectin on murine T cells by Lim *et al*. (1997). Based on their mitogenic activity, lectins had therefore opened up a new technique in the field of lymphocyte research. For example, Con A and PHA have been used to stimulate rat spleen cells to produce interleukin-2 (Gillis *et al*., 1980) while jacalin, another plant lectin, has been used to produce human γ-interferon from T-cell hybridoma (Crane *et al*., 1984). Lectins have also been used for the treatment of cancer via utilizing them in mitogenic lectin therapy, anti-tumor therapy and targeted therapy (Mody *et al*., 1995). Examples of these types of

lectins are abricin and ricin A which have been shown to be potent antitumor cytotoxins (Abdullaev and de Mejia, 1997).

Lectins have also been used in the separation of cells into biologically distinct subpopulations. One of the earliest usage of lectin was in the separation of mouse lymphocytes into B and T cells via soybean agglutinin (Reisner *et al*., 1976). Subsequently, the lectin was used to separate the cells responsible for graft-versus-host reaction from human marrow cells (Reisner, 1990). Another example is in the ability of peanut agglutinin to separate germ cells from somatic cells in mouse testis (Maekawa and Nishimune, 1985)

The ability of lectins to detect subtle variations in carbohydrate structures found on glycoproteins and glycolipids has paved way for the separation of closely related glycoproteins (Sharon, 1993). It is a known fact that the great variation of carbohydrate moieties on a protein has created difficulties in its purification. Thus, glycoproteins are among the most challenging of products to characterize because of the extreme and fine nature of the sample micro-heterogeneity. Due to the narrow specificity of the lectins, they have been successfully used for fractionation of this type of glycoproteins. An example is in the use of lentil affinity column that has been used to demonstrate the presence of L-fucose residue attached to the innermost GlcNAac residue (Kornfeld *et al*., 1981). In addition, the microheterogeneity of α_1-acid glycoprotein fucosylation has also been previously investigated using lectin from *Aleuria aurentia* (Liljeblad *et al*., 2001).

Lectins are also widely used in biomedical and clinical research areas. They have been used not only for purification but also for determining the structure of glycoprotein-derived oligosaccharides and glycopeptides. Characterization of glycans in some of the

altered glycoproteins, that manifest in clinical conditions, are of importance for understanding disease generation as well as finding candidate tumor markers for the diagnosis, prognosis and treatment of the pathological condition. Uses of lectins in the detection of glycosylation alteration associated with pathological manifestation of diseases were already reported. To date, they have been applied in several approaches, such as chromatography, array and blotting as well as probes for histological and cytological analyses (Haab *et al*., 2010; Zeng *et al*., 2010; Zhao *et al*., 2007).

The fact that sugars have an affinity for lectins, which is not as high as that for the carbohydrate-specific antibodies, is of immense practical utility in the purification of glycoproteins via affinity chromatography. If antibodies are immobilized as the affinity ligand, harsh conditions need to be employed to elute the glycoproteins that were adsorbed onto the matrix (Satish and Surolia, 2001). In doing so, there is probability of the isolated glycoprotein losing their biological activities. In contrast, when lectins are employed as the affinity ligand for purification of a glycoprotein, only mild conditions are needed for elution. Moreover, the task of isolating the lectins is not as arduous as raising carbohydrate-specific antibodies. As such, lectin affinity chromatography has been used to aid in the investigation of several diseased conditions. The use of champedak mannose-binding lectin affinity chromatography prior to 2-DE has successfully been employed in the search for aberrantly expressed serum glycoproteins in patients with nasopharyngeal carcinoma (Seriramalu *et al*., 2010). This approach has also made significant contribution to the isolation of several candidate tumor markers in patients with prostate cancer (Jayapalan *et al*., 2012; Tabares *et al*., 2006). Recently, the use of multiple lectin affinity chromatography in a serial fashion has been gaining popularity. Example is in the work of Zeng *et al*. (2011). Their group has reported on the use of a multi-lectin affinity

chromatography to study glycosylation changes in patients with breast cancer. Immobilization of lectin onto solid support could also be performed on magnetic beads as described by Lee (2006). In addition, various lectin conjugates, such as enzymes, biotin, fluorescent dyes or colloidal gold as well as radioactive labeled lectins have been utilized as specific and sensitive reagents for detection of glycoproteins (Mohamed *et al*., 2008 (this thesis); Abdul-Rahman *et al*., 2007; Abdul Rahman *et al*., 2002; Hashim *et al*., 2001; Li *et al*., 1993; Kijimoto-Ochiai *et al.*, 1985).

1.7 Champedak lectin: lectin of interest

The study on the champedak (*Artocarpus integer*) lectin started in the early nineties with the discovery of an IgA1-reactive and D-galactose-binding lectin from the seeds of our local champedak (Hashim *et al*., 1991). It was later discovered that this lectin was structurally and functionally similar to jacalin (Hashim *et al*., 1991 & 1992), which is also an IgA1-reactive and D-galactose-binding lectin isolated from the jackfruit (*Artocarpus heterophyllus*) seeds (Roque-Barreira and Campos-Neto, 1985).

Champedak galactose binding (CGB) lectin is a lectin that binds to the galactose residues of the O-linked oligosaccharide moietes. The CGB lectin comprises two types of non-covalently linked subunits with Mr of 13,000 and 16,000. And unlike the heterogeneous jacalins of distinctive origins, which were shown to have diverse specificity (Hashim *et al*., 1991; Kobayashi *et al*., 1988; Kondoh *et al*., 1987), the CGB lectin isolated from several clones demonstrated uniform reactivity (Hashim *et al*., 1993). Interaction of the CGB lectin with human immunoglobulins demonstrated reactivity with IgA1 but not with IgA2, IgD, IgG and IgM (Hashim *et al*., 1991). The lectin was also found to consume

complement and decrease the complement-mediated hemolytic activity of sensitized sheep red blood cells (Hashim *et al*., 1994). The use of CGB lectin to probe for glycoproteins has been performed using immunodiffusion, affinity chromatography and enzyme-conjugated lectin approaches. The lectin has been successfully used in the study on the etiopathogenesis of IgA nephropathy (Shuib *et al*., 1998; Hashim *et al*., 2001 a & b). This lectin has also been used to identify aberrantly expressed serum proteins, in relation to various cancers, by applying it to probe for the glycoproteins that had been separated by 2-DE and transferred onto nitrocellulose membranes (Jayapalan *et al*., 2012; Mu *et al*., 2012; Abdullah-Soheimi *et al*., 2010; Mohamed *et al*., 2008 (this thesis); Abdul-Rahman *et al*., 2007).

Apart from CGB lectin, champedak seeds also harbour a D-mannose-binding lectin, which was termed champedak mannose binding (CMB) lectin (formerly known as champedak lectin M). The lectin was discovered in 1997 by Lim *et al*. and was shown to have high specificity and affinity for the core-mannosyl residues of the N-glycans of glycoproteins. The isolated CMB lectin was later discovered to be structurally quite similar but not totally identical to artocarpin (Lim *et al*., 1997). Artocarpin is the CMB lectin's counterpart that was isolated from jackfruit (Miranda-Santos *et al*., 1991 a & b). Structural studies of the CMB lectin demonstrated it to be a 64 kDa protein (Lim *et al*. 1997). The lectin shows strong interaction with human IgE and IgM but displays weak interaction with IgA2 (Lim *et al*., 1997). It also possesses selective mitogenic activity towards T lymphocytes (Lim *et al*., 1998; Hashim *et al*., 1992). On the other hand, in the absence of T cells and macrophages, CMB lectin was incapable of activating the murine B cells for secretion of immunoglobulins (Lim *et al*., 1998). The CMB lectin has also been shown to selectively bind to a group of glycoproteins collectively known as the N-linked

glycoproteins (Hashim *et al*., 2001a). The use of enzyme-conjugated CMB lectin on 2-DE separated proteins of patients with congenital hypothyroidism has managed to demonstrate differences in the carbohydrate structure of haptoglobin ß-chain of the patients, relative to their control subjects (Yong *et al*., 2006). Meanwhile, recent proteomics investigation on serum of patients with nasopharyngeal carcinoma using CMB lectin affinity chromatography demonstrated reduced expression of α-2 macroglobulin and complement factor B in sera of patients (Seriramalu *et al*., 2010).

1.8 Research approaches and objectives of study

Previous report by Abdul-Rahman *et al.* has highlighted the enhanced expression of a protein, termed LDP1 that interacted with the CGB lectin probe (2007). This protein, which was not detected earlier in the silver-stained 2-DE gels, was found to be over-expressed in the sera of patients with endometrial adenocarcinoma (EACa) compared to control subjects. In this regard, it is of interest to identify LDP1. Since the protein was earlier detected on nitrocellulose membranes, an alternative approach was adopted to isolate LDP1, so as it may be identified by mass spectrometry. The pooled sera of EACa patients would instead be subjected to an immobilized CGB lectin affinity chromatography.

Following the identification of the LDP1, its expression in cancer and non-cancer conditions was assessed using the well-established enzyme conjugated-CGB lectin-probed 2-DE electrophoretic approach. Five groups of patients who were newly diagnosed with breast carcinoma (BrCa), epithelial ovarian carcinoma (EOCa), germ cell ovarian carcinoma (GOCa), nasopharyngeal carcinoma (NPCa) and osteosarcoma (OsSa) were subjected to analysis. Groups of women with normal pregnancy (NP) and hydatidiform

mole (HM) as well as menopausal women who were under the hormone replacement therapy (HRT) were also recruited for the determination of its expression level in non-cancer conditions.

In the second part of the study, an attempt was made to study the expression patterns of serum proteins in the sera of women with NP and HM. The study was performed via the gel-based 2-DE approach that was followed either by the enzyme-conjugated CGB lectin detection of O-glycosylated proteins or silver staining. Identities of all protein spots which showed differential expression were established by mass spectrometry and search of databases, and their aberrant expression was further validated using the immunoblotting technique. In the final part of the study, an attempt to simultaneously detect the differential expression of candidate protein tumor markers in the groups of women with NP and HM was performed using a multiplex immunoassay.

Objectives of the study

The present study was performed:

1. to identify the LDP1 in sera of women with EACa using immobilized CGB lectin affinity chromatography, followed by 2-DE and mass spectrometry analysis.

2. to analyze the expression of LDP1 in sera of patients with BrCa, EOCa, GOCa, NPCa and OsSa using the HRP-CGB lectin-probed 2-DE electrophoretic approach.

3. to analyze the expression of LDP1 in serum samples of women with NP, HM and HRT using the enzyme-conjugated CGB lectin-probed 2-DE electrophoretic approach.

4. to develop the O-glycosylated serum protein profiles of women with NP and HM as well as that of the normal non-pregnant women using the HRP-CGB lectin-probed 2-DE electrophoretic approach.

5. to develop the high abundance serum protein profiles of women with NP and HM as well as the control subjects using the 2-DE and silver staining of gels.

6. to analyze the differences in protein expression in the 2-DE profiles generated by lectin detection as well as silver staining by image analysis between two different groups of pregnant women and their control subjects.

7. to identify the aberrantly expressed proteins detected in the study using mass spectrometry analysis.

8. to validate the aberrant expression of the differentially expressed proteins using immunoblotting technique.

9. to develop a multiplex assay for the simultaneous determination of eight candidate serum proteins

10. to test the viability of the multiplex assay in detecting aberrantly expressed serum proteins in relation to the studied pregnancy conditions.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Champedak lectin

Previous study by Hashim *et al*, in 1993, had established that lectins obtained from seeds of six different cultivars of champedak (*Artocarpus integer*) did not vary in terms of their structure and glycoprotein binding specificity. Thus, in this study, champedak fruits that were used for isolation of champedak galactose binding (CGB) lectin were purchased from a local market.

2.2 Human Serum Samples

Sera were collected from newly diagnosed patients of the University of Malaya Medical Centre (UMMC) and University Malaya Specialist Centre (UMSC), Kuala Lumpur. Serum samples were collected from nine different groups of patients: sera from patients with endometrial adenocarcinoma (EACa; n=3), localized osteosarcoma (OsSa; n=10), nasopharyngeal carcinoma (NPCa; n=13), breast carcinoma (BrCa; n=10), germ cell ovarian carcinoma (GOCa; n=10), epithelial ovarian carcinoma (EOCa; n=10), women with normal pregnancy (NP; n=20), women with complete hydatidiform mole (HM; n=20) and menopausal women under hormone replacement therapy (HRT; n=10). Venous blood samples were taken from patients with cancer prior to surgery or treatment. Samples from the groups of women with NP and HM were collected from women in their first trimester of pregnancy while women with HRT were recruited from the Obstetrics and Gynecology Clinic, UMMC.

All serum samples from carcinoma patients were confirmed histopathologically for stage 1 or 2 whilst for the OsSa serum samples, they were from patients showing no evidence of metastasis. For comparison, sera from normal healthy non-pregnant

individuals (n=20) were obtained from age matched volunteers (range of 21-45 years old) from the Department of Molecular Medicine, Faculty of Medicine, University of Malaya. Samples were obtained with patients' consent and approval, granted by the Ethical Committee (Institutional Review Board) of the Medical Centre in accordance to the ICH GCP guideline and the Declaration of Helsinki.

Blood samples were collected in fresh 1.5 ml plain blood collection tubes and were centrifuged at 3000g for 10 minutes (Centrifuge 5403, Eppendorf). Recovered serum were collected and stored in aliquots of 100 µl at -80° until used.

2.3 General Materials

The materials used during the course of this project and their respective suppliers are as listed.

2.3.1 Chemicals

All chemical used were of analytical grade and were supplied by Sigma Chemical Company, St. Louis, USA with exceptions of the following:

i. British Drug House Ltd. (BDH), Poole, England

Sodium thiosulphate-5-hydrate

Sodium dihydrogen phosphate

Sucrose

Glutaraldehyde

Formaldehyde

ii. BioRad Laboratories, Hercules, USA

Bis N,N'-methylene-bis-acrylamide

iii. ICN Biomedicals Inc., USA

Tris(hydroxymethyl)aminomethane (Tris)

Sodium dodecyl sulphate (SDS)

Glycine

Glycerol

iv. Merck, Darmstadt, West Germany

Potassium dihydrogen phosphate

Sodium acetate-trihydrate

Acetic acid

Hydrochloric acid

Tween 20

Methanol

v. Pharmacia Biotech,Uppsala, Sweden

N,N,N',N'-tetramethylethylenediamine

vi. Supelco, USA

Amberlite

2.3.2 Enzymes and substrates

i. Sigma Aldrich Company, St. Louis, USA

Activated horseradish peroxidase (HRP)

3,3'- Diaminobenzidine (DAB)

ii. Promega, Madison, WI, USA

Trypsin Gold, mass spectrometry grade

2.3.3 Chromatographic support

i. GE Health Biosciences, Uppsala, Sweden

Sepharose 4B

ii. Sigma Aldrich Company, St. Louis, USA

Cyanogen bromide-activated gel (CNBr)

2.3.4 Membrane

i. Schleicher and Schuell, Dassel, Germany

Nitrocellulose membrane

2.3.5 Materials for two-dimensional electrophoresis

i. GE Health Biosciences, Uppsala, Sweden

IEF immobiline dry strips 11 cm (pH 4-7)

IPG buffer(pH 4-7)

Drystrip cover fluid

Dithiothreitol (DTT)

Iodoacetamide

2.3.6 Materials for SELDI-MS

i. Ciphergen Biosystems Inc., Fremont, CA, USA

ProteinChip array NP20

Sinnapinic acid

2.3.7 Materials for MS/MS analysis

i. Sigma Aldrich Company, St. Louis, USA

α-cyano-4-hydroxycinnamic acid

ii. Millipore, MA, USA

ZipTip® pipette tips

2.3.8 Commercial kits and materials for Bioplex analysis

i. Pierce, Rockford Illinois, USA

N-hydroxysulfosuccinimide (S-NHS)

1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC)

ii. BioRad Laboratories Inc.,USA

Bio-Plex Amine Coupling Kit

Bio-Plex COOH Beads (COOH Beads 5, 12, 22, 34, 55, 62 and 91)

Bio-Plex Cytokine Reagent Kit

Bio-Plex Human Serum Diluent Kit

2.3.9 Serological Reagents

2.3.9.1Proteins and antibodies used in the Bioplex analysis

i. Abcam, Cambridge, USA

Human α_1-antitrypsin

Human α_2-HS-glycoprotein

Human ceruloplasmin

Human clusterin

Biotin conjugated goat anti-human α-1-antitrypsin

Biotin conjugated rabbit anti-human α-1-antichymotrypsin

Biotin conjugated rabbit anti-human ceruloplasmin

ii. Abnova, Taipei, Taiwan

Human kininogen

Human zinc α_2 -glycoprotein

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

Human α-1-antichymotrypsin

iv. RnD system, Minneapolis, USA

Human antithrombin III

Biotin conjugated goat anti-human antithrombin III

Biotin conjugated rabbit anti-human α_2-HS-glycoprotein

Biotin conjugated goat anti-human clusterin

Biotin conjugated rabbit anti-human kininogen

Biotin conjugated rabbit anti-human zinc α_2 -glycoprotein

2.3.9.2 Proteins and antibodies used in the immunoblotting assay

i. Abcam, Cambridge, USA

Rabbit anti-human α-1-antichymotrypsin

ii. Abnova, Taipei, Taiwan

Mouse anti-human leucine rich glycoprotein

Mouse anti-human zinc α_2 -glycoprotein

iii. Pierce, Rockford Illinois, USA

Peroxidase conjugated goat anti-rabbit IgG

Peroxidase conjugated rabbit anti-mouse IgG

METHODS

2.4 Purification of champedak lectin

2.4.1 Preparation of crude extracts of champedak seeds

Collection of crude extracts from champedak seeds were performed using the protocol established by Hashim *et al*. (1991).

Standard solutions

Phosphate-buffered saline (PBS)

NaCl	9.360 g
KCl	0.253 g
Na_2HPO_4	0.245 g
KH_2HPO_4	1.420 g

The solution was made up to 1 L with distilled water and the pH was adjusted to pH 7.2 using HCl. Solution was stored at room temperature. For preparation of PBS-azide, sodium azide was added at 0.1% (w/v) and stored at 4°C.

The champedak seeds were cleaned with distilled water and dried at 37°C for 24 hours. Dried seeds were ground to powder form using a blender and 150 g of the powdered seeds were suspended overnight in PBS at 10% (w/v) with constant stirring at 4°C. The suspension was later centrifuged at 8000 x g for 15 minutes (7000 r.p.m. for 20 minutes) also at 4°C using a Sorvall R2B centrifuge. Resulting supernatant was collected and subjected to ammonium sulphate precipitation to remove impurities thus resulting in a semi-pure extract. Ammonium sulphate was added gradually to 60% (w/v) saturation at 4°C and suspension was left to stir for 2 to 3 hours. Stirred solution was later centrifuged at 8000 x g for 15 minutes also at 4°C.

The resulting pellets were redissolved with cold PBS before being dialyzed extensively with PBS to remove the ammonium sulphate. After 4 changes of buffer, the crude extract of champedak seeds was stored at –20°C.

2.4.2 Preparation of sugar affinity columns

2.4.2.1 Activation of Sepharose 4B with Divinylsulfone

Activation of Sepharose 4B was performed using method of Hermanson *et al*. (1992). The use of Divinylsulfone (DVS) was to introduce reactive vinyl groups in the sepharose which enables immobilization of sugars via their hydroxyl groups.

In this step, 50 ml of settled Sepharose 4B was washed with 1 L of distilled water in a sintered glass funnel and suction-dried to a wet cake. The moist gel was transferred to a beaker and was suspended in 50 ml of 0.5 M sodium carbonate. Mixture was stirred slowly in a fume cabinet. 5 ml of DVS was added drop-wise over a period of 15 minutes followed

by another hour of continuous stirring at room temperature. The activated gel was washed extensively with distilled water until pH of the filtrate reached that of distilled water. At this point of time, the activated sepharose was ready for ligand coupling.

2.4.2.2 Coupling of sugar to DVS-activated Sepharose 4B

DVS activated gel was suspended in an equal volume of 20% (w/v) of D-galactose in 0.5 M sodium carbonate. The mixture was allowed to react overnight at room temperature with constant stirring. The sugar-coupled gel was washed successively with 2 L of distilled water and 2 L of 0.5 M sodium bicarbonate. Gel was later suspended in 50 ml of 0.5 M sodium bicarbonate containing 2.5 ml of 2-mercaptoethanol in a fume cabinet to block excess reactive vinyl groups. Suspension was left to stir for 2 hours at room temperature before washing with 2 L each of distilled water and PBS. The resulting galactose-coupled Sepharose 4B were then used to isolate the CGB lectin. Unused gel was stored in PBS azide at 4°C.

2.4.3 Purification of CGB lectin

CGB lectin was purified from the crude champedak seeds extract using the prepared galactose affinity column.

2.4.3.1 Isolation of CGB lectin

Fifty ml of settled galactose-Sepharose gel was suspended in 5 ml of crude extract. Suspension was left overnight at 4°C with gentle stirring to allow maximum coupling of CGB lectin to the immobilized galactose. The mixture was then loaded into a 100 cm polypropylene column and was allowed to pack at room temperature.

Once the column was packed, fractions of 3 ml were collected and absorbance was monitored at 280 nm using a Shimadzu UV-1201 spectrophotometer. The column was washed extensively with PBS until the absorbance reached baseline (A_{280} is less than 0.005) and leveled off. The wash fractions collected were unbound fractions which were free from CGB lectin. Bound CGB lectin was then eluted out with 0.8 M galactose in PBS and fractions with high absorbance were pooled together. After elution, the column was regenerated by extensive washing with PBS. Similar steps were repeated several times to obtain enough CGB lectin to be used in this study.

2.4.3.2 Purification of extracted CGB lectin

Collected bound fractions were pooled and dialysed against six changes of diluted PBS to remove sugar and salt. Resulting dialysate was freeze-dried using a freeze-drier (Virtis Research Equipment) to concentrate the CGB lectin. Aliquots of the lectin were then stored at –20°C.

2.5 Assessment of purified CGB lectin

2.5.1 Assessing purity of CGB lectin using SDS-PAGE

To access the purity of the isolated CGB lectin, SDS-PAGE was performed based on the discontinuous buffering system by Laemmli (1970). In this experiment, reduction of the CGB lectin was performed by addition of 2% (w/v) DTT.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method widely used in protein science to determine sample purity and molecular weight. In SDS-PAGE separation, migration is determined by molecular weight of polypeptides. SDS is an anionic detergent that denatures protein by wrapping around the polypeptide backbone conferring a negative charge to the polypeptides. When treated with SDS and a reducing agent such as dithiothreitol, the polypeptides become rods of negative charges and after separation and staining, the position of a protein along the lane gives a good approximation of its size. The band intensity is an indicator of the amount of protein present in the sample.

Standard solutions

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

Acrylamide	60.0 g
N,N'- methylenebisacrylamide	1.8 g
Deionised distilled water	topped up till 200 ml

The solution was deionised using amberlite MB-1, filtered and stored in a dark bottle at 4°C.

1.5M Tris-HCL, pH 8.8 (Solution B)

Tris base	36.2 g
Deionised distilled water	150.0 ml

The solution was corrected to pH 8.8 using HCL and was topped up till 200 ml. The solution was stored at 4°C.

10% (w/v) SDS (Solution C)

SDS	10.0 g
Deionised distilled water	100.0 ml

The solution was stored at room temperature.

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

Ammonium persulphate (APS)	2.0 g
Deionised distilled water	20.0 ml

The solution was kept in aliquots of 200 µl and stored at -20°C. Solution was thawed prior to use.

N,N,N',N'-tetramethylethylenediamine (TEMED) (Solution E)

0.5 M Tris-HCl, pH 6.8 (Solution F)

Tris base	6.1 g
Deionised distilled water	50.0 ml

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

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

0.5M Tris-HCl, pH 6.8	2.5 ml
Glycerol	2.0 ml
SDS	400.0 mg
Bromophenol blue	a few grains
DTT (added prior to use)	

Solution was topped up to 20 ml with deionised distilled water and stored at -20°C. Solution was thawed and added with 1% (w/v) of DTT prior to use.

Cathode buffer (25 mM Tris; glycine 198 mM; SDS 0.1% (w/v), pH 8.3)

Tris-HCl	1.2 g
Glycine	5.9 g
SDS	0.4 g

The buffer was made up to 1 L with distilled water.

Coomassie Blue Stain

Coomassie Blue Stain 10% (v/v) acetic acid;0.1% Coomassie Brilliant Blue

Staining solution was kept at room temperature and was re-used for up to 3 times.

Destaining solution

10% (v/v) acetic acid solution

2.5.1.1 Preparation of stacking and separating gel

The separating gel mixture was prepared according to Table 2.1 and was carefully pipetted into the glass plate sandwich. Subsequently, the gel was overlaid with a layer of distilled water to achieve an even gel surface. Once the gel polymerized, the overlay was removed and was replaced by the stacking gel. A comb was inserted into the stacking gel layer and gel was left to polymerize completely.

2.5.1.2 SDS-PAGE electrophoresis

Samples and sample buffer were mixed at a ratio of 1:1 and were boiled at 100°C for 5 minutes. 5 μl of molecular weight marker and 5 μl of each sample were filled into the wells. Electrophoresis was performed at 70 V until the blue dye front entered the separating gel. Voltage was then increased to 100 V and was stopped once the dye front was approximately 1 cm from the gel bottom. Gel was later stained using the Coomassie Blue method.

Table 2.1: Volumes of stock solutions for stacking and separating gels

Stock solution	Volumes of stock solutions for	
	Stacking gel	**18 % separating gel**
Solution A (ml)	0.65	12.00
Solution B (ml)	-	5.00
Solution C (μl)	50.00	200.00
Solution D (μl)*	25.00	100.00
Solution E (μl)*	5.00	6.60
Solution F (ml)	1.25	-
Distilled water (ml)	3.05	2.69
Total volume (ml)	5.03	20.00

*added prior to use

2.5.1.3 Staining with Coomassie Blue

Gel was soaked in Coomassie Blue staining solution and left overnight with continuous shaking. Staining solution was later replaced with destaining solution and left to shake until the gel background was clear. A piece of clean sponge or KimWipe tissue was placed on top of the solution to accelerate the destaining procedure. Gel was later rinsed in distilled water and scanned.

2.5.2 Assessing purity of CGB lectin using SELDI-MS

To substantiate data obtained from the SDS-PAGE analysis, purity of the CGB lectin was further evaluated using surface-enhanced laser desorption/ionization mass spectrometry (SELDI-MS). Analysis of the prepared CGB lectin by SELDI-MS was performed on pre-activated surfaces of NP20 array using the protocol recommended by the manufacturer reader (Bio-Rad Laboratories, California, USA).

2.5.2.1 Immobilization of CGB lectin

Prior to analysis, saturated matrix solution of sinnapinic acid (SPA) was dissolved in the matrix solution (50% aqueous ACN and 0.5% TFA). 1.0 µl of the SPA solution was applied onto each spot on the NP20 array and left to dry for 5 min, at room temperature. Once dried, 1 µl of 1 mg/ml CGB lectin at dilution of 1:50 was added onto the SPA spots. Finally, 1 µl of matrix was again applied onto the same spot thus, forming a sandwich preparation within the array spot. The array was left to dry completely for approximately 20 min at room temperature and was ready for analysis.

2.5.2.2 SELDI-MS analysis of CGB lectin

Mass analysis of the bound CGB lectin was performed using a ProteinChip SELDI reader (Bio-Rad Laboratories, California, USA). Optimization range was set between 10 to 60 kDa. MS time of flight spectra were generated by averaging 130 laser shots collected in 25kV Positive Ions mode. Initial arrays were read at three laser intensities before optimization at 1300 nJ. The protocol averaged 10 laser shots per pixel with a focus mass

of 15 kDa, a matrix attenuation of 1 kDa and a range of 0–20 kDa. Calibration was performed externally using the Proteinchip All-in-One Peptide Standard (Bio-Rad Laboratories, California, USA). Spectra obtained were analyzed using the ProteinChip SELDI reader (Bio-Rad Laboratories, California, USA).

2.5.3 Determination of CGB lectin concentration

Concentration of the purified lectin was estimated using a Protein Assay ESL kit (Roche, Germany). The protein assay kit utilizes a biuret-like reaction. Concentration estimation was performed according the manufacturer's instruction using the micro method. A standard curve was constructed based on the standard solution of bovine serum albumin (BSA). The BSA standard was diluted in PBS to achieve protein concentrations ranging from 25 to 800 μg/ml. CGB lectin sample was also serially diluted to ensure accurate estimation. Determination of the CGB lectin concentration was later made by referring to the BSA standard curve obtained.

2.5.4 Determination of CGB lectin binding activity using double diffusion test

Double diffusion test also known as Ouchterlony double immunodiffusion is a simple but rather dated assay for detecting antigen-specific antibodies. In the double diffusion test, both antigen and antibody are loaded into wells in a bed of agar and allowed to diffuse into the gel. The visual signature of reactivity between antigen and antibody is by formation of a thin white line called the precipitin line at their equivalence zone (Ouchterlony, 1949).

In this assay, 0.5% (w/v) of agarose was prepared in distilled water. Approximately 4 ml of warm agarose was pipetted onto a microscope slide placed on leveled surface. Several slides were prepared (assay was run in duplicate) and the coated slides were dried at 40°C. Precoating the slides provides an adhesive surface to support the analytical gel. Subsequently, 8 ml of buffered 1% (w/v) agarose in PBS-azide was pipetted onto each precoated slide and were allowed to solidify. Once the gel solidifies, holes were punched using a 7-well gel puncher (LKB 6808A, Sweden). Plugs of agar were removed using a Pasteur pipette attached to a water pump.

CGB lectin and all proteins used were prepared at 1 mg/ml in PBS. 20 μl of lectin was pipetted into the center well followed by 20 μl of proteins into the surrounding wells as shown in Figure 2.1. Gels were incubated overnight at room temperature in a humid chamber to develop. Unprecipitated excess proteins were removed from the gels by extensive dialysis against PBS. The precipitin lines were stained with Coomassie Blue for better visualization.

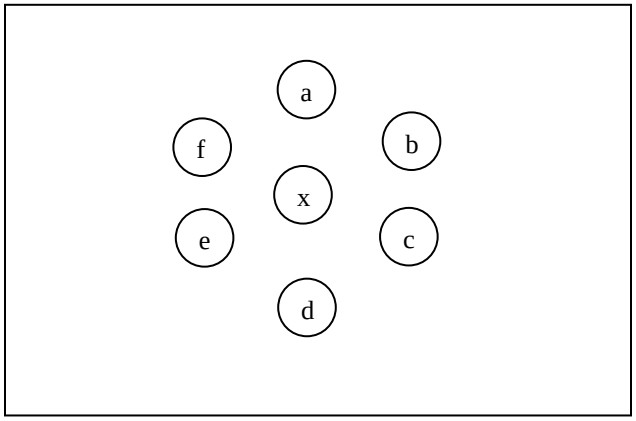

Figure 2.1: Double diffusion test of CGB lectin. Test was carried out on a 5% (w/v) agarose bed. 20 μl of the CGB lectin and glycoproteins were pipetted into their respective wells. Gel was then incubated overnight in a humid chamber.

x : CGB lectin

a : α_2-HS-glycoprotein (AHS)

b : IgA

c : IgA2

d : IgM

e : albumin

f : PBS

2.6 CGB lectin affinity chromatography

2.6.1 Preparation of CGB-CNBr activated gel

Cyanogen bromide (CNBr) activated gel was used in the preparation of the CGB lectin affinity column. 4 g of the activated gel was stirred overnight in ice-cold distilled water. Approximately 12 ml of rehydrated gel was then washed successively with 2 L of cold distilled water and 1 L of cold 0.1 M sodium bicarbonate, pH 8.5. The gel was later sucked into a moist cake and was incubated with 12 ml of the purified CGB lectin at a concentration of 4 mg/ml.

Incubation was carried at 4°C with gentle stirring for 20 hours. Later, the lectin immobilized gel was washed with 200 ml of 1.0 M sodium chloride and 200 ml of distilled water. The volume of filtrate collected was measured and its protein concentration was estimated based on the protocol in Section 2.5.3. The gel was then sucked dry and stirred in 100 ml of 1.0 M ethanolamine pH 9.0 for 1 hour followed by extensive washing with 1.0 M NaCl and distilled water.

2.7 Interaction of CGB lectin with intact human serum glycoproteins

2.7.1 CGB lectin column preparation

2.5 ml of settled CGB lectin-CNBr gel was packed in a minicolumn and was equilibrated with several washes of PBS.

2.7.2 Human serum sample preparation

50 μl of serum from each donor or patient were pooled according to their respective disorders.

2.7.3 Separation of CGB lectin-bound fractions

500 μl of sample was loaded into the CGB lectin column. Column was washed with PBS and fractions of 2 ml were collected. Absorbance was monitored at 280 nm using a Shimadzu UV-1201 spectrophotometer. Washing was done extensively with PBS and once the absorbance reached baseline, bound fractions were eluted with 0.1 M melibiose. Upon elution, bound fractions were pooled together.

2.7.4 Purification of CGB lectin-bound fractions

The affinity-separated fractions were dialysed against 6 changes of distilled water to remove both sugar and salt. The resulting dialysate were concentrated to 90% of the initial volume using a freeze-drier (Virtis Research Equipment). Aliquots of the fractions were stored at –20°C.

2.8 HRP conjugated CGB lectin

2.8.1 Conjugation of CGB lectin to HRP

Another application of CGB lectin in this study was to detect O-glycosylated serum proteins that were separated via 2-DE. The 2-DE separated human serum proteome was

electrophoretically transferred to nitrocellulose membranes which were then probed with enzyme conjugated lectin to form lectin-glycoprotein complexes. The complexes were then detected using colorimetric reaction of specific substrate towards the enzyme. In this study, CGB lectin was conjugated to horseradish peroxidase (HRP). The protocol for conjugation was adopted from that described in Hudson and Hay (1989).

Stock solutions

0.1 M Sodium periodate

Sodium periodate	21.4 mg

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

1mM Sodium acetate buffer, pH 4.4

Sodium acetate	8.2 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 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 crystal was dissolved in 1 ml of distilled water.

0.1 M Borate buffer, pH 7.4

Disodium tetraborate	9.54 g/250 ml
Boric acid	42.73 g/L

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

4 mg of HRP was dissolved in 1 ml of distilled water. Once dissolved, 200 µl of freshly prepared 0.1 M sodium periodate solution was added to the HRP solution. The solution was stirred for 20 minutes at room temperature after which it was dialyzed overnight against 0.1 mM sodium acetate buffer, pH 4.4 at 4°C. The pH of the recovered HRP dialysate was later raised to 9.5 by adding approximately 25 µl of 0.1 M sodium carbonate, pH 9.5. This was immediately followed by addition of 1 ml of CGB lectin at a concentration of 2 mg/ml. The mixture was left to stir at 4°C for 2 hours. Free enzyme

was reduced by addition of 100 µl of fresh sodium borohydride and left to stand for another 2 hours at 4°C. The HRP conjugated product was later dialyzed overnight against 2 L of 0.1 M sodium borate, pH 7.4, at 4°C with 2 changes of buffer. The HRP-CGB lectin coupled product was then diluted in equal volume with 60% glycerol in the same borate buffer and stored at 4°C.

2.9 Two-dimensional electrophoresis

Two-dimensional electrophoresis (2-DE) is a powerful gel-based proteomics approach for the analysis of complex protein mixtures extracted from cells or other biological samples. This technique sorts proteins according to two different properties in discrete steps. In the first dimensional electrophoresis, isoelectric focusing (IEF) will separate complex mixture of proteins according to their isoelectric points (pI). In the second dimensional run, SDS-polyacrylamide gel electrophoresis will further separate the proteins based on their molecular weights.

In this study, 2-DE was performed using the apparatus from Amersham Pharmacia Biotech. The first dimension separation was carried out using a Multiphor Flatbed electrophoresis system and Immobiline Drystrip (IPG) strips with pH 4-7. For the second step, strips were transferred the Hoefer SE 600 Ruby system for separation on gradient SDS-PAGE gels.

2.9.1 First dimensional electrophoresis

Standard solutions

Sample buffer

Urea	13.5 g
DTT	0.15 g
IPG buffer (pH 4-7)	0.5 ml
Triton X-100	0.13 ml

Mixture was made up to 25 ml with double distilled water and kept in 250 μl aliquots at –20°C.

Rehydration solution

Urea	12.0 g
IPG buffer (pH 4-7)	0.13 ml
Triton X-100	0.13 ml
DTT	

Mixture was made up to 25 ml with double distilled water. Solution was stored in 1250 μl aliquots at –20°C. Prior to use, DTT was added into the rehydration solution (0.2 g /1250 μl of rehydration solution).

2.9.1.1 Sample preparation

Samples were added to the sample buffer according to Table 2.2 and were left at room temperature an hour.

Table 2.2: Volume of sample and sample buffer for sample preparation

Sample	Volume of	
	sample (μl)	sample buffer (μl)
Serum	7	21
Bound fractions*	50	100

* Bound fractions from the CGB lectin affinity chromatography

2.9.1.2 Rehydration of immobiline dry strip

For an 11 cm IPG strip with pH of 4-7, a total volume of 200 μl solution was required per slot of the reswelling tray. Thus, mixture of sample and sample buffer was made up to 200 μl with the rehydration solution. Prior to use, the reswelling tray was treated with hot water and air-dried. 200 μl of mixture was loaded into each slot of the tray. The IPG strip was carefully lowered with the gel side facing downwards into the sample concoction. Subsequently, 1 ml of IPG cover fluid was pipetted onto the gel strip to minimize evaporation and to prevent urea crystallization. The rehydrated strips were left to stand overnight or for 18 hours at room temperature to ensure complete uptake of sample by the IPG strip.

2.9.1.3 First dimensional run

The setting up of apparatus and procedure for isoelectric focusing was conducted according to the protocol supplied by the manufacturer. 3 ml of dry cover fluid was pipetted on the center of the cooling plate. The drystrip tray was positioned on the cooling plate and 10 ml of dry cover fluid was poured on it. A drystrip aligner was placed inside the tray, on top of the dry cover fluid, to position the rehydrated IPG strips. Swelled IPG strips were then placed in the center of the drystrip aligner with the gel side facing upwards. Moistened electrode strips were placed across the cathodic and anodic ends of the aligned IPG strips and the electrodes were positioned on the electrode strips. Each IPG strip was overlaid with dry cover fluid to ensure good thermal contact. The temperature of the apparatus was maintained at 20°C using a thermostatic circulator. Running conditions were as stated below.

Table 2.3: Running conditions for the first dimension run

Phase	Voltage	mA	W	Time (hour)	V/ hr
1	300	5	5	0.30	-
2	3500	2	5	-	15 000

Once the first dimension run was completed, the focused strips can either be used immediately for the second dimension separation or can be stored individually in screw-cap tubes at –80°C.

2.9.2 Second dimensional electrophoresis

Standard solutions

SDS equilibration buffer

1.5 M Tris-HCl pH 8.8	6.7 ml
Glycerol	69.0 ml
Urea	72.07 g
SDS	4.0 g

Mixture was made up to 200 ml with double distilled water.

4 X Resolving buffer (1.5 M Tris-HCl, pH 8.8)

Tris-HCl	36.3 g

Solution was made up to 200 ml with double distilled water. HCl was used to adjust the pH to 8.8.

Monomer solution

30% acrylamide

0.8% N,N'- methylenebisacrylamide

The solution was deionised using amberlite MB-1, filtered and stored in a dark bottle.

Anode buffer (0.37 M Tris-HCl, pH 8.8)

Tris-HCl	136.2 g

Solution was made up to 3 L with double distilled water. HCl was used to adjust the pH.

Cathode buffer (25 mM Tris, 198 mM glycine, 0.1% SDS)

Tris-HCl	6.04 g
Glycine	29.73 g
SDS	2.0 g

Mixture was made up to 2 L with double distilled water and was prepared fresh.

2.9.2.1 Preparation of 8-18% gradient SDS-PAGE gel

A 1 mm thick gradient gel was prepared according to the recipe in Table 2.4. Gel was prepared using a gradient maker (model SG 30. Hoefer, USA) connected to a peristaltic pump and a gel caster. Casting procedure was done according to the manufacturer's manual. Both solutions were placed in the mixing chamber connected to the pump by a tube with a canula at one end. Mixed light and heavy solution was pumped into the glass plate sandwich at a constant rate of 2.5 ml/ min.

Table 2.4: Volumes of light and heavy solution for 8-18% gradient SDS-PAGE gel

Solution	**light solution (8 %)**	**heavy solution (18 %)**
4 X resolving buffer	3 ml	3 ml
Monomer	3.2 ml	7.2 ml
10 % SDS	120μl	120μl
Sucrose	-	1.8 g
Distilled water	5.64 ml	Made up to 12 ml
*TEMED	3.96μl	3.96μl
*10 % Ammonium persulphate	39.6μl	39.6μl

* Added just prior to use

2.9.2.2 Equilibration of IPG strips

Equilibration of IPG strips was carried out using SDS equilibration buffer in two separate steps. The first equilibration step required 1% (w/v) of DTT to be dissolved in SDS equilibration buffer. Once completely dissolved, 5 ml of solution was pipetted into each tube containing the IPG strips. Tubes were shaken gently for 15 minutes. The second equilibration step was similar to the first with the exception of using 4.5% (w/v) of iodoacetamide instead of DTT. Also added was a few grains of Bromophenol Blue.

2.9.2.3 Second dimensional run

Application of the equilibrated IPG strip onto the second dimension gel and setting up of the Hoefer SE 600 was carried out according to the procedures proposed by the manufacturer. Thermostatic circulator was set at 16°C. Electrophoresis was performed at constant current in two steps as shown below.

Table 2.5: Running conditions for the second dimension run

Phase	Voltage	mA	W	Time (minutes)
1	50	80	50	30
2	600	40mA/ gel	25 mA/ gel	-

Electrophoresis was stopped once the dye front was approximately 1 mm from the bottom of gel. After electrophoresis, gel was removed from its cassette in preparation for Western blotting (for HRP-CGB lectin probing) or silver staining. Prior to removal, gel was marked at the upper corner nearest to the pointed end of the IPG strip to identify the acidic end of the first-dimension separation. All samples were analyzed in duplicates.

2.10 Detection of 2-DE separated proteins using HRP-CGB lectin

2.10.1 Western blotting of 2-DE gels

To further analyze the isolated serum O-glycoprotein profiles, western blotting followed by incubation with HRP-CGB lectin solution was conducted. This involved a procedure of transferring the resolved serum proteins onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) using a semidry transfer system (Novablot Kit for the Multiphor II Electrophoresis System, GE Healthcare Biosciences, Uppsala, Sweden). The membranes were then probed with the HRP-CGB lectin to form lectin-glycoprotein complexes followed by detection using 3,3'-diaminobenzidine tetrahydrochloride (DAB).

Standard Solutions

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

Glycine	2.93 g
Tris	5.81 g
SDS	0.375 g
Methanol	200 ml

Chemicals were dissolved and made up to 1 L with double distilled water.

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

TBS	1 L
Tween-20	1 ml

A piece of nitrocellulose membrane and 6 pieces of filter paper was carefully cut according to the size of the 2-DE derived gel. The graphite anode plate was saturated with distilled water while 3 pieces of the filter paper as well as the nitrocellulose membrane were soaked with transfer buffer before being placed on the anode plate. The gel piece, which was also pre-equilibrated with the transfer buffer, was carefully placed on top of the membrane followed by another 3 pieces of filter paper that were already soaked in the transfer buffer. A roller was carefully rolled on the sandwich of filter papers, membrane and gel to remove any trapped air bubbles. The cathode tray was placed on top of the sandwich and transfer was performed for 2 hours at a constant current of 0.8 mA/cm^2.

2.10.2 HRP-CGB lectin blotting

The electroblotted membrane was subsequently blocked using 3% gelatin in TBST for 1 hour at room temperature with constant shaking. It was then washed trice with TBST for 10 minutes each. The washed blot was later incubated overnight with the prepared CGB-HRP in TBST at a concentration of approximately 1 µg/ml at 4°C. Washing step was repeated again before the membrane was developed using freshly prepared substrate solution, a solution containing 25 mg of DAB and 50 µl of 3% H_2O_2 in 50 ml TBS. The developed reaction was stopped by washing the membrane 3 times with double distilled water. Finally, the membrane was air dried before being scanned using the Image Master 2D-Platinum software, version 5.0 (GE Healthcare Biosciences).

2.10.3 Image analysis of HRP-CGB lectin detected blots

Captured images of the respective blots were then analyzed using Image Master 2D platinum software, version 5.0 (GE Healthcare Biosciences, Uppsala, Sweden). Automatic spot detection was performed with default parameters, whereas spot editing and removal of artifacts were done manually. Glycoprotein spots were analyzed in terms of percentage of volume contribution. The percentage volume contribution refers to the volume percentage of a protein taken against the total spot volume of all proteins in each gel. A minimum difference of two folds between differentially regulated proteins of controls and tests (represented by ratios of above 2.0 or below 0.5), was taken as the cutting point for the variation to be considered significant.

All values are presented as mean ± SEM (standard error mean). Data were analyzed by Student's *t*-test to determine differential expression between healthy and test samples. A false discovery rate (FDR) correction was applied to correct for multiple comparisons and finally a p value of less than 0.01 in all the tests was considered significant.

2.11 Detection of 2-DE separated proteins using silver staining

Silver staining was conducted according to the method by Heukeshoven and Dernick (1985) with some modifications.

Staining solutions

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

Ethanol	400 ml
Acetic acid	100 ml

Mixture was made up to 1 L with distilled water.

Incubation solution (30% (v/v) ethanol, 0.5 M sodium acetate, 8 mM sodium thio-sulphate, 0.13% (v/v) glutaraldehyde)

Ethanol	300 ml
Sodium acetate trihydrate	68 g
Sodium thiosulphate 5-hydrate	20 g

Solution was made up to 1 L with distilled water and 1.30 ml of glutaraldehyde was added prior to use.

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

Silver nitrate	1.0 g

Dissolved and made up to 1L with distilled water. Prior to use, 200 μl of formaldehyde was added.

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

Sodium carbonate	15.0 g

Dissolved and made up to 1L with distilled water. 100 μl of formaldehyde was added prior to use.

Stopping solution (40 mM EDTA-$Na_2.2H_2O$)

EDTA-$Na_2.2H_2O$	14.6 g

Dissolved and made up to 1L with distilled water.

Preserving solution (10% (v/v) glycerol)

Glycerol	100 ml

Made up to 1 L with distilled water.

2.11.1 Staining of gels

After completion of the second-dimension electrophoresis, gel slab was immersed in fixing solution for 30 minutes to precipitate the proteins and to allow SDS to diffuse out. Gel was later left in incubation solution for 30 minutes to overnight incubation. After incubation, the gel was washed three times with distilled water for 5 to 10 minutes each. Staining with silver stain was done for another 40 minutes. The gel was then rinsed with approximately 50 ml of developing solution before subjecting it to further incubation in fresh developing solution. Once protein spots were developed, gel was soaked in stopping solution for 30 minutes to block further color development. Stopping procedure was done twice followed by washing with distilled water. Incubation was carried out in a shaker as all steps in the staining technique required gentle shaking. Developed gel was stored in preserving solution at room temperature.

2.11.2 Image analysis of silver stained 2-DE gels

2-DE profiles of protein spots from sera of controls and women with NP and HM were performed using the Image Master 2D platinum software, version 5.0 (GE Healthcare Biosciences, Uppsala, Sweden). Automatic spot detection was performed with default parameters, whereas spot editing and removal of artifacts were done manually. Analysis was restricted to the protein spots with Mr of greater than 30,000 which were distinctively separated in the silver stained 2-DE gels. Albumin and serum polypeptides having idiotypic and/or allotypic variations (such as the heavy and light chains of all isotypes of immunoglobulins and the α-chains of haptoglobin) and the low Mr protein spots, which were not well resolved under the settings of our experiment, were not assessed.

Spots were analyzed in terms of percentage of volume contribution and all values are presented as mean ± SEM (standard error mean). The percentage volume contribution refers to the volume percentage of a protein taken against the total spot volume of all proteins in each gel. To determine the significance of the differential expression obtained from the comparative analysis conducted between healthy and test samples, a minimum difference of two folds, as represented by ratios of above 2.0 or below 0.5, was taken as the cutting point. In addition, when subjected to statistical analysis using the Student's *t*-test, the comparison should demonstrate a p value of less than 0.01 to be considered statistically significant.

2.12 Mass spectrometry analysis

Identification for aberrantly expressed proteins obtained from the comparative analysis between groups of test individuals with their control subjects were carried out using mass spectrometry (MS). Gel plugs containing the LDP1 protein was sent to the Biomolecular Research Facility, University of Newcastle, Callaghan, Australia for mass spectrometry analysis. As for the aberrantly expressed proteins detected in the serum samples of women with NP and HM, MS analyses were performed at the Proteomics Research Facility, Faculty of Medicine, University of Malaya.

For both MS analysis, 2-DE gel runs of serum samples were repeated. However, the resolved gels were stained with a different recipe of Commassie Blue stain. Following staining of the gels with Coomassie Blue, gel plugs containing protein spots of interest were excised from the gels. The gel pieces were then kept in HPLC grade water, in clean microfuge tubes. This was to ensure that the gel plugs remained hydrated until analysis is

carried out. The gel plugs were then either sent out for an out-sourced MS analysis or subjected to a series of procedures in preparation for an in-house MS analysis.

2.12.1 Coomassie Blue staining for MS analysis

Standard solution:

Staining solution

0.1% Coomassie Brilliant Blue (R250)

50% methanol

5% acetic acid glacial

Destaining solution

50% methanol

5% acetic acid glacial

Storage solution

5% acetic acid glacial

For each gel, 250 ml of each solution was prepared. All solutions were prepared in double distilled water. Gels were soaked in Coomassie staining solution either for 20-30 minutes or left overnight with continuous shaking. Staining solution was later replaced with destaining solution and left to shake until the gel background was clear. A piece of

clean sponge or KimWipe tissue was placed on top of the solution to accelerate the destaining procedure. All steps were carried out at room temperature.

2.12.2 Destaining of Coomassie-stained gel plugs

For identification of the proteins of interest, the selected clusters of protein were manually excised from the Commassie stained gels. The gel plugs were washed and destained by adding 100 µl of 25 mM ammonium bicarbonate/5% ACN to each piece of gel plug. Following a 15-minute incubation, the supernatant was removed and 100 µl of 25 mM ammonium bicarbonate/50% ACN was added. The washing and destaining steps were repeated twice.

2.12.3 Preparation of peptides

Following destaining of the gel pieces, the proteins in the gel plugs were reduced with 10 mM DTT in 100 mM ammonium bicarbonate solution for 30 minutes at 60ºC. After which alkylation was performed using 55 mM iodoacetamide in 100 mM ammonium bicarbonate for 20 minutes at room temperature and protected from light. After incubation, the gel pieces were washed thoroughly for 3 times with 50% ACN to dehydrate the gels. Plugs were then subjected to vacuum centrifugation (Speed Vac®) at room temperature to remove as much ACN as possible.

Once dried, the gel plugs were digested with 10 ng/µl trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate at 37°C for 18 hours. Extraction of the peptides from the gel plugs was carried out using 50% ACN and 100% ACN followed by

lyophilisation in a vacuum centrifuge. The lyophilised peptides were then reconstituted with 0.1% formic acid and purified using reversed phase ZipTip® pipette tips containing C18 resin (Millipore, MA, USA).

2.12.4 Tryptic peptide analysis

Equal amount of peptide sample and α-cyano-4-hydroxycinnamic acid (5 mg/ml) were mixed together and 0.7 µl of each mixture was spotted onto the Opti-ToF® 384 well MALDI plate insert (AB SCIEX, Foster City, California). The wells were then allowed to dry under ambient conditions and once dried the plate was loaded into the 4800 Plus MALDI ToF/ToF™ analyzer (AB SCIEX, Foster City, California). In this study, the MS and MS/MS spectra were calibrated using the mass standard kit (Applied Biosystems/ MDS Sciex, Toronto, Canada).

2.12.5 Database search

Data obtained from the MS/MS analysis was generated using the peaklist software 4000 Series Explorer (release version 3). The data was then exported for search using the MASCOT search engine (Matrix Science Ltd, London, UK; release version 2.2). Search was performed against all entries in the NCBI non redundant database (updated April 25, 2007, containing 192489 sequences). The following parameters were used in the MASCOT Ion Search: enzyme: trypsin; allowed missed cleavage-1; variable modification – 2; (i) carbamidomethylation of cysteine, and (ii) oxidation of methionine; MS precursor ion mass tolerance – 100 ppm; MS/MS fragment ion mass 2.2.4.

2.13 Immunodetection of aberrantly expressed proteins

The subsequent validation of the differentially expressed serum proteins in sera of women with NP and HM were performed by subjecting SDS-PAGE separated proteins to western blotting followed by immunodetection using antibodies.

Prior to the SDS-PAGE separation, serum samples were diluted to a 1/20 final dilution using double distilled water. Diluted samples were added to equal volume of SDS-PAGE sample buffer prior to boiling for 10 minutes. After boiling, 10 µl of each sample was loaded into a 12.5% SDS-PAGE gel. Alternatively, for serum proteins with high molecular weight, 7.5% gels were used. Electrophoresis was carried out as described in section 2.5.1.2. Upon completion of SDS-PAGE, blotting of gels was performed as described in section 2.10.1. Following protein transfer, blots were stained with Ponceau S so as the protein lanes became visible. The blots were then cut vertically into strips using a scissor. This was followed by decolorization of the stain using double distilled water.

2.13.1 Immunodetection using antibodies

Staining of blots with antibodies by an immunoperoxidase technique was performed as instructed by the manufacturers of the antibodies used in this part of the study. In the initial part of the work, the membrane strips were first blocked with either non-fat milk or BSA to saturate any unoccupied sites on the membranes. Strips were incubated with blocking buffer for 2 hours at room temperature with gentle agitation. Following blocking, the strips were then washed 3 times for 5 minutes per wash using their respective buffers and incubated with the desired antibodies overnight at 4°C. Prior to use, the antibodies were diluted with their respective blocking buffers into various concentrations as suggested

by the manufacturer. After incubation, strips were washed again in 3 changes (10 minutes each) of buffer. Subsequently, peroxidase conjugated goat anti-rabbit and rabbit anti-mouse secondary antibodies were diluted 1:100,000 and 1:200,000, respectively. Strips were incubated with the secondary antibody conjugates for 2 hours at room temperature with agitation followed by a washing step. After washing in 3 changes of buffer, the strips were developed with DAB substrate solution as described in section 2.10.2.

2.13.2 Image analysis of blots

To quantify the intensity of the resolved bands, stained strips were analyzed using the Image Master 2D-platinum software version 5.0 (GE Healthcare Biosciences, Uppsala, Sweden). Automatic spot detection was performed with default parameters, whereas spot editing and removal of artifacts were done manually.

All values are presented as mean ± SEM (standard error mean). Data were analyzed by Student's *t*-test to determine differential intensity between the bands of test and control samples. A false discovery rate (FDR) correction was applied to correct for multiple comparisons and a p value of less than 0.01 was considered significant.

2.14 Development of a multiplex assay for simultaneous detection of proteins

In the present part of the study, an attempt was made to develop a multiplexed immunoassay which allows for simultaneous quantification of multiple proteins in a single sample. Aside from evaluating the viability of the multiplex assay to simultaneously detect

the aberrantly expressed serum proteins, results derived from the assay would further corroborate the earlier findings of this study. Protocols employed in the preparation and validation of the multiplex immunoassay were as described by the manufacturer.

In the present study, an indirect competitive multiplex assay was developed to detect the differential regulation of the aberrantly expressed serum proteins. Unlike a customized bioplex immunoassay, whereby the beads are immobilized with antibodies to the protein of interest (section 1.5.3.1), the developed in-house design involved conjugation of the beads with the protein of interest. Hence, the detector antibodies were antibodies specific to the respective proteins on the beads. In this format, the protein conjugated microbeads were mixed with the serum sample in the microtiter wells. When the detector antibodies were added into the wells, immobilized proteins will compete with free serum proteins for binding sites on their corresponding detector antibodies. The serum protein-antibody complexes and any unbound proteins were then removed by the washing step. Hence, only the protein-conjugated bead-antibody complexes were detected and enumerated by the array reader.

2.14.1 Development of protein conjugated beads

In this study, Bio-Plex carboxylated (COOH) polystyrene beads numbered 5, 12, 22, 34, 55, 62, 79 and 92 with distinct emitting fluorescence patterns were chosen. Commercially available antibody and protein pairs that comprised of AAT, ACT, AHS, ATR, CLU, CLP, KNG and ZAG were purchased from commercial sources. The coupling process was performed following the procedures supplied in the Bio-Plex Amine Coupling Kit (BioRad Laboratories, Inc., USA). In the coupling step, proteins would be covalently

coupled on to the surface of the beads via carbodiimide reactions which involved the protein primary amino groups and the functional groups bound on the surface of the polystyrene beads.

2.14.1.1 Protein preparation

Prior to the coupling process, proteins must be free of sodium azide, BSA, glycine, Tris or any amine-containing additives and must be suspended in PBS, pH 7.4. Protein purification and buffer exchange were performed using Micro Bio-Spin™ 6 Tris chromatography columns (Bio-Rad Laboratories, Inc., USA). The protein of interest (20-75 µl) was carefully applied into the column, which was then spun for 5 minutes at 1000 xg. Purified protein sample, now in PBS, pH 7.4, was placed on ice and protein recovery was determined using the Protein Assay ESL kit (Roche, Germany) as stated in section 2.5.3.

2.14.1.2 Protein coupling

Optimal protein-coupling conditions must be established by determining the amount of protein that is required to promote optimal binding between the protein and the ligand on the beads. As such, each of the purified proteins was reconstituted in PBS, pH 7.4 to several concentrations ranging from 2-12 µg/ml. The beads stock solutions were vortexed for 30 seconds followed by sonication in a bath sonicator (Ultrasonik, NeyTech) for another 30 seconds. This step was to ensure that the beads were completely resuspended as single monodisperse particles. The microbeads are light-sensitive, thus,

throughout the process, beads were protected from light as much as possible by covering the tubes with aluminum foil. For a single coupling reaction, an aliquot of 1.25 x 10^6 beads was transferred into the coupling reaction tube. The beads were centrifuged at 14 000 x g for 4 minutes and the supernatant was carefully removed from the bead pellet. This was followed by a washing step using 100 µl of wash buffer. The beads were then resuspended in 80 µl of bead activation buffer and were sonicated until a homogeneous distribution of microspheres was observed.

Solutions of *N*-hydroxy-sulfosuccinimide (S-NHS) and 1-ethyl-3-(3-dimethlaminopropyl)-carbodiimide hydrochloride (EDC), both at 50 mg/ml, were prepared in bead activation buffer. Ten µl of each freshly-made solution was then added to the beads. Suspension was incubated for 20 minutes at room temperature with agitation to allow activation of the carboxyl groups on the beads. Then, 150 µl of PBS, pH 7.4 was added followed by centrifugation to remove the supernatant from the activated beads. The bead pellet was then resuspended with 100 µl of PBS, pH 7.4. Each of the protein samples, which were prepared into various concentrations, was added into the bead suspension. The volume of the mixture was adjusted to a final volume of 500 µl and was left on an agitator for 2 hours at room temperature or alternatively overnight at 4°C. After incubation, beads were washed with 500 µl PBS, pH 7.4. Further reaction was blocked using 250 µl of blocking buffer. The coupled beads were later enumerated with a haemacytometer and stored in the dark at 4°C.

2.14.1.3 Validation of coupling

Once the coupling reaction has been completed and the approximate numbers of protein-coupled beads have been counted, the functionality of the coupling reaction needs to be validated. In this step, the protein-coupled beads were reacted with its corresponding biotinylated antibody followed by activation of the biotin by streptavidin-PE (phycoerythrin). The intensity of the resulting fluorescent signal is directly proportional to the amount of coupled protein available on the surface of the beads.

Coupling efficiency was determined by incubating 5000 beads with 50 µl of its biotinylated antibody in staining buffer for 30 minutes. Fifty µl of diluted streptavidin-PE was added to the beads and mixture was incubated for another 10 minutes. This was followed by centrifugation to remove the supernatant and the resulting pellet was resuspended in 125 µl of storage buffer. Beads were vortexed at medium speed and 125 µl of sample was transferred to a single well of a 96-well 1.2 µm PVDF filter bottom microtiter plate (Millipore, Schwalbach, Germany). Beads were analyzed with the Bio-Plex array system (BioRad Laboratories Inc.,USA). A successful coupling would typically yield a fluorescent intensity signal greater than 2,000 median fluorescent intensity (MFI).

2.14.2 Determination of multiplex assay characteristic

The developed multiplex immunoassay was subjected to various tests to evaluate its performance. All evaluation assays were carried out at room temperature and protected from light as much as possible.

2.14.2.1 Evaluation of assay's specificity

In order to determine the specificity of the developed immunoassay, the degree of cross-reactivity between the various sets of beads was evaluated. The beads were put to challenge by the various biotinylated antibodies involved in the multiplex assay (Ling *et al*., 2007).

In this assay, a bead working solution was prepared by calculating the volumes required from the a single set of bead, so as in each well of the microtiter plate there would be approximately 5000 beads/50 µl of assay buffer. Next, 50 µl of the working bead solution was added into each well. This was followed by three times washing with 100 µl of wash buffer. All tests were run in duplicates and blank wells were devoid of any microbeads. Each of the biotinylated antibodies (detector antibodies) was dissolved in the Bio-plex detection antibody diluent to a final concentration of 2 µg/ml. The freshly prepared detector antibody solution was vortexed and 25 µl of the solution was added into each well. The microtiter plate was then sealed with a sealing tape before covering it with aluminum foil. The plate was vigorously mixed on a shaker at 1100 rpm for 30 seconds followed by 30 minutes incubation with shaking at 300 rpm on a microplate shaker (Eppendorf, Westbury, NY). Following incubation, the plate was washed three times with 100 µl of Bio-plex wash buffer.

For the detection step, 50 µl of 2 µg/ml streptavidin-PE, freshly prepared using the Bio-plex assay buffer, was added into each well. The plate was covered with a new sheet of sealing tape followed by aluminum foil before being placed on the shaker. Incubation was carried out for 10 minutes at room temperature with quick but vigorous shaking followed by gentle shaking at 300 rpm. At the end of the 10 minutes incubation, the buffer

was removed via vacuum filtration and plate was washed 3 times. This was followed by the addition of 125 µl of assay buffer into each well and plate was shaken at 1,100 rpm for 30 seconds to resuspend the beads. Finally, plate was placed on a calibrated Bio-Plex ™ array instrument (BioRad Laboratories Inc.,USA).

Percentage of cross-reactivity for a particular set of protein conjugated beads was calculated as the ratio of the fluorescence intensity in response to an antibody compared with the maximum intensity derived from the interaction of the protein on the beads to its corresponding detector antibody.

2.14.2.2 Optimization of sampel dilution

To determine the optimal serum dilution to be used in combination with the multiplexed immunoassay, serum samples were diluted with the Bio-Plex human serum diluent using dilution factors of 1/4, 1/50, 1/100 and 1/200. A working solution was prepared by mixing 1500 beads from each set of beads into 50 µl of assay buffer. Likewise, a cocktail of detector antibodies was also prepared by mixing each of the antibodies to a final concentration of 2 µg/ml.

Aliquots of 50 µl of bead mixture were pipetted into each well after which the beads were washed with 100 µl of wash buffer. Subsequently 50 µl of diluted serum samples were added into the wells and mixture were incubated for 30 minutes. 25 µl of freshly prepared detection antibody working solution was added into each well and this was followed by another 30 minutes of incubation. Beads were then washed and drained using a vacuum manifold. For detection, 50 µl of 2 µg/ml streptavidin-PE was added into each

well. Beads were then incubated for 10 minutes followed by 3 times of washing using the wash buffer. Finally, the beads were resuspended using 125 µl of assay buffer. The plate was later loaded into the Bio-Plex ™ array system.

2.14.2.3 Evaluation of assay's sensitivity

In this part of the study, the ability of the developed immunoassay to specifically detect differences in the concentration of serum proteins was investigated. The assay was run using the indirect competitive approach and the serum samples were spiked with different concentrations of proteins.

In the present study, each of the protein at initial concentrations of 2 µg/ml, was serially diluted to final concentrations of 0.2, 0.02, 0.002 and 0.0002 µg/ml. Test samples were prepared by mixing 45 µl of diluted serum with 5 µl of each serially diluted protein. The beads mixture was prepared as described earlier.

Aliquots of 50 µl of bead mixture were pipetted into each well after which the beads were washed with 100 µl of wash buffer. Subsequently 50 µl of test sample was added into the wells along with 25 µl of the detection antibody working solution. The mixture was then incubated with vigorous shaking for 30 seconds followed by gentle shaking at 700 rpm for 60 minutes. The incubation period is to allow free proteins i.e., serum proteins and the spiked proteins to compete with the bead conjugated proteins for binding sites on the detector antibodies. After the incubation period, the plate was placed on the vacuum manifold to remove buffer, serum sample and free protein-antibody complexes produced during the incubation. Wells were then washed 3 times with 100 µl of Bio-plex wash buffer as previously stated. For detection, 50 µl of streptavidin-PE (2 µg/ml) was added

into each well. Plate was then washed and beads were resuspended and analysed as described earlier.

2.14.2.4 Evaluation of assay's reproducibility

Reproducibility of the multiplex assay was assessed by determining the level of both intra- and inter-assay variation. Intra-assay variation within a plate was determined by assaying 10 control samples in duplicates. For each protein, the coefficient of variation (%CV) for each duplicate result was calculated and averaged (van Gageldonk *et al*., 2008).

Inter-assay variation (plate to plate variation) was assessed by testing 10 control samples in two separate assays. The %CV between each of the results for each protein was calculated and averaged.

2.14.3 Profiling of proteins using the competitive multiplex assay

A bead mix and serum samples were prepared as described in the earlier sections. 50 µl each of the prepared bead mix and diluted serum samples were added into the wells of the microtiter plate. Also added was 25 µl of the detector antibody mixture. This was followed by 30 minutes incubation on a plate shaker (700 rpm) at room temperature. All controls and samples were assayed in duplicates and blank wells were treated similarly to tests except for the absence of serum sample.

Following incubation, the plate was subsequently washed three times prior to addition of 50 µl of Streptavidin-PE as previously described. 125 µl of assay buffer was

then added to resuspend the beads. Finally, plate was placed on a calibrated Bio-Plex ™ instrument (BioRad Laboratories Inc.,USA) for analysis. Statistical analysis was performed by applying Student's *t*-test to the MFI values for each of the protein tested in the different groups of women studied. A p value of less than 0.01 was considered significant. All values were presented as mean ± SEM (standard error mean).

CHAPTER 3:

RESULTS

3.1 Identification of LDP1

Previous report by Abdul-Rahman *et al.* has highlighted the enhanced expression of a protein termed LDP1 that interacted with the CGB-HRP probe (2007). This protein, which was not detected earlier in the silver-stained 2-DE gels, was found to be over-expressed in the sera of patients with endometrial cancer (EACa) compared to control subjects. In the initial part of this study, an attempt was made to identify LDP1. Since the protein was earlier detected on nitrocellulose membranes, an alternative approach was adopted to isolate LDP1 and separate the protein by 2-DE so as it may be identified by mass spectrometry. Hence, pooled serum samples from EACa patients were subjected to CGB lectin affinity chromatography.

3.1.1 Isolation and purification of CGB lectin

In the present study, the CGB lectin affinity column was prepared initially by isolation and characterization of the lectin. The CGB lectin was purified by subjecting crude extracts of champedak (*Artocarpus integer*) seeds to galactose-Sepharose affinity separation. The CGB lectin's affinity towards galactose promotes its isolation from other lectins and compounds within the champedak seed extract.

Figure 3.1 demonstrates a typical elution pattern of the galactose affinity chromatography of the champedak seed extract. The column was first washed with PBS to ensure removal of all substances that did not bind to the galactose. The flow-through fractions are represented by the first peak of the elution profile. The galactose-bound CGB

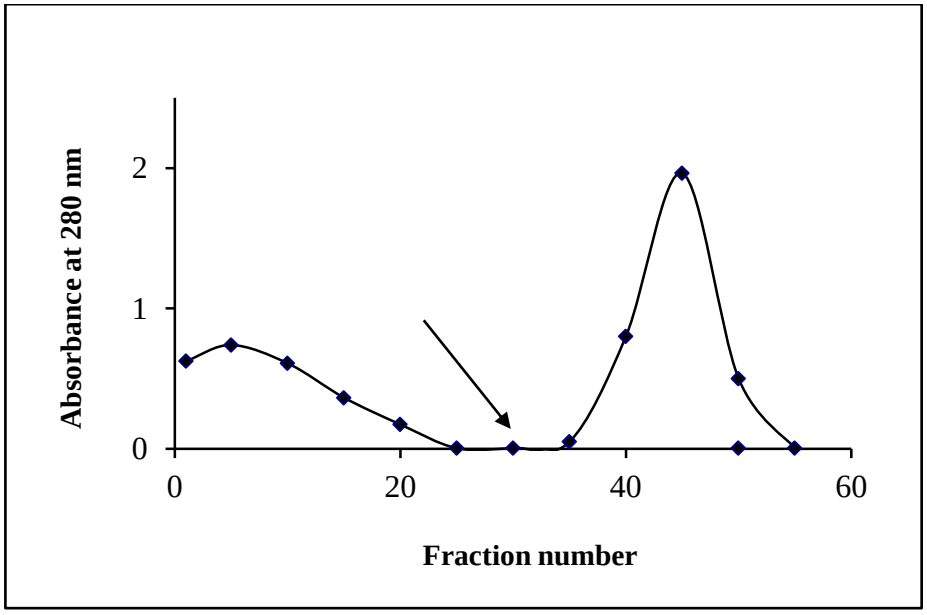

Figure 3.1: Elution profile of CGB lectin. A crude extract of the champedak seed was applied into a galactose-Sepharose affinity chromatography column. Fractions collected were monitored by measuring the absorbance at 280 nm. The column was washed extensively with PBS, pH 7.2 until the absorbance fell to less than 0.05. Bound CGB lectin was eluted using 0.8 M D-galactose in PBS until absorbance of the effluent returned to less than 0.05. Arrow denotes the point of application of the galactose solution.

lectin was then displaced from the column using 0.8 M D-galactose and appeared as the second peak in the elution profile.

3.1.2 Assessment of purified CGB lectin

The CGB lectin fractions collected from the galactose-Sepharose affinity chromatography were pooled, extensively dialysed and freeze-dried. To ensure that the CGB lectin obtained from the isolation process was highly purified, assessment for its purity and reactivity was performed.

3.1.2.1 Purity of CGB lectin

In order to assess its purity, the CGB lectin was subjected to SDS-PAGE electrophoresis. Figure 3.2 demonstrates the electrophoretic profile of the CGB lectin. The reduced lectin contained a protein which migrated as two highly resolved bands with relative molecular weights of approximately 16.5 and 14.0. These were the two typical bands of the CGB lectin as reported by other researchers (Abdul Rahman *et al*., 2002; Hashim *et al*., 1991). No other bands were detected in the SDS-PAGE profile of the isolated CGB lectin, thus, indicating that the prepared lectin was free from any contaminants.

To substantiate the data obtained from the SDS-PAGE analysis, purity of the CGB lectin was further evaluated using surface-enhanced laser desorption/ionization mass spectrometry (SELDI-MS). Analysis of the prepared CGB lectin by SELDI-MS was

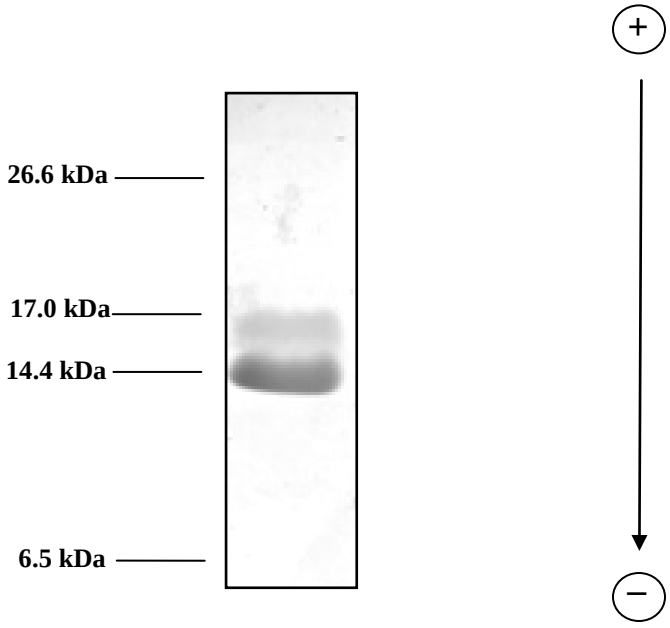

Figure 3.2: Analysis of CGB lectin by SDS-PAGE. The affinity purified CGB lectin was subjected to 18% SDS-PAGE electrophoresis and Coomassie-blue staining. Gel was calibrated with molecular weight markers for SDS-PAGE consisting of triosephosphateisomerase (26.6kDa), myoglobin (17.0 kDa), α-lactalbumin (14.4 kDa) and aprotinin (6.5 kDa). The lectin was resolved into two distinct bands. The arrow indicates the direction of sample migration.

performed on a ProteinChip NP20 array and resulted in a mass spectrum profile that is shown in Figure 3.3. Two pairs of successive sharp peaks of approximately 14 and 16 kDa were observed. These peaks correspond closely to the subunits of the CGB lectin and comparable with those that were previously established by Abdul-Rahman, (2007). The other pair of peaks was detected at the lower region, with masses of approximately 7 and 8 kDa, were the doubly charged subunits of the lectin. There was no other peak detected in the profile. And this reflects the high purity of the CGB lectin preparation.

3.1.2.2 Concentration of CGB lectin

The protein concentration of the CGB lectin prepared in this study was determined using the Protein Assay ESL kit (Roche, Germany). The overall yield of the concentrated CGB lectin was estimated to be approximately 6.34 mg/ml, which corresponds to 1.7 mg/g of dried champedak seed weight. Figure 3.4 demonstrates the calibration curve used to estimate the concentration of the purified lectin.

3.1.2.3 Specificity of CGB lectin

The binding specificity of the CGB lectin was assayed using the double diffusion test. In this study, interactions of the CGB lectin with various human serum proteins and isotypes of immunoglobulins were investigated. Figure 3.5 demonstrates the binding interaction of the purified CGB lectin towards selective proteins.

A positive reaction between the CGB lectin and a glycoprotein was reflected by formation of a precipitin line between the respective wells. Based on the observation made,

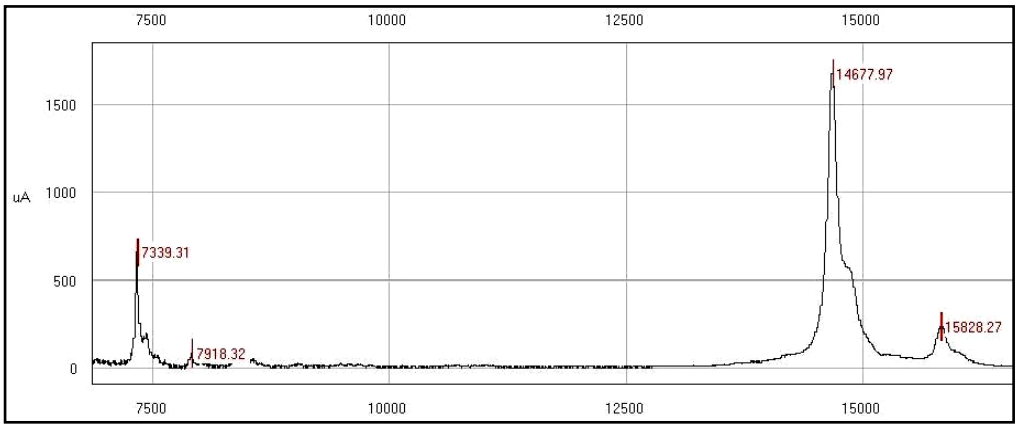

Figure 3.3: Analysis of CGB lectin by SELDI-MS. The prepared CGB lectin was analyzed using SELDI-MS. Four peaks were detected within the spectrum profile, at masses of approximately 7, 8, 14 and 16 kDa.

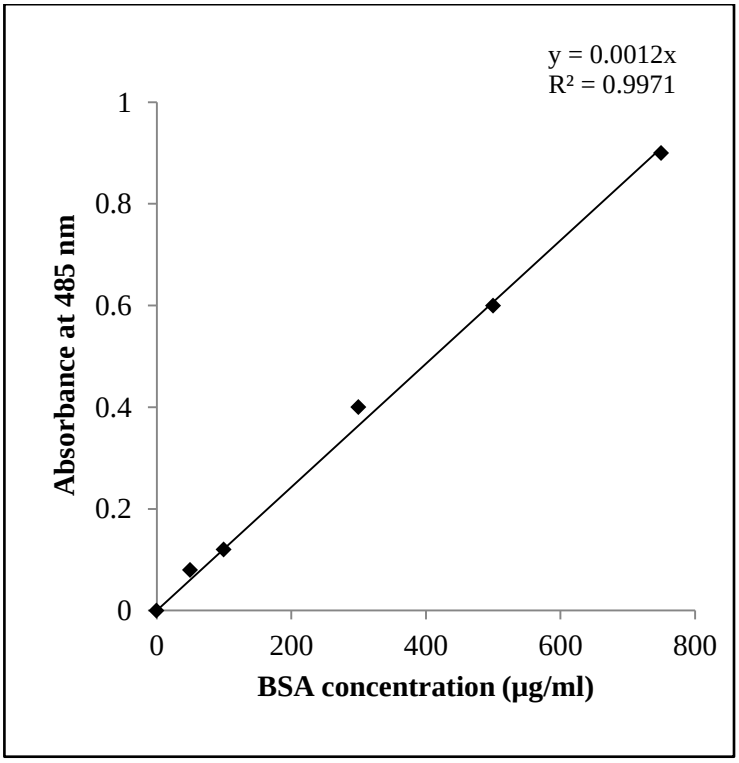

Figure 3.4: BSA standard curve. A typical standard curve was constructed based on standard solutions of BSA with concentrations of 0, 50, 100, 300, 500 and 750 µg/ml. Estimation of the CGB lectin was made by referring to this standard curve.

(a) (b)

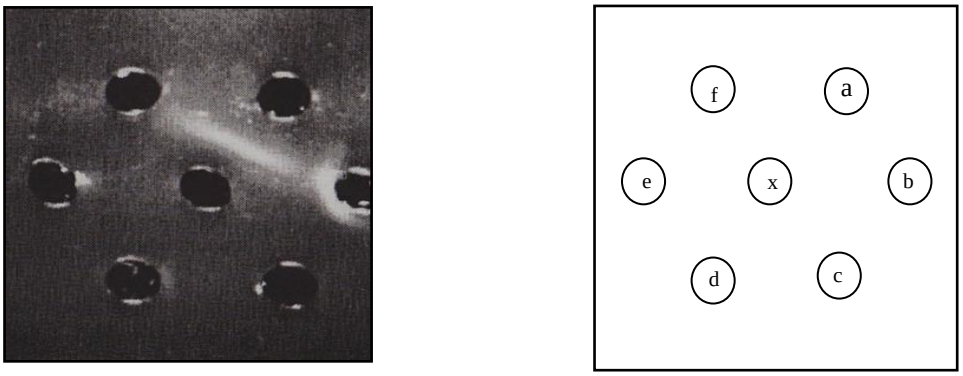

Figure 3.5: Interaction of CGB lectin with human proteins using double diffusion assay. The purified CGB lectin was tested with several human proteins and immunoglobulins. Positive reactivity is represented by formation of a precipitin line between adjacent wells. The center well (x) was filled with 20 µl of CGB lectin while the surrounding wells were filled with various proteins as listed below:

a : IgA1

b : AHS

c : IgA2

d : IgM

e : Albumin

f : PBS

positive reactivity was exhibited for wells containing IgA1 and α2-HS glycoprotein (AHS), which are known to have O-linked oligosaccharide structures. However, no precipitin lines were detected for the non-O-glycosylated IgA2 and IgM. Similarly, wells containing albumin and PBS, which served as negative controls, did not exhibit any reactivity with the CGB lectin.

3.1.3 CGB lectin affinity separation of O-glycosylated serum proteins

The highly purified CGB lectin was then used in the development of a CGB lectin affinity column, as previously described by Abdul Rahman *et al*., 2002. Subsequent to its development, the lectin column was used to isolate O-glycosylated proteins from serum samples of patients with EACa, which are known to contain substantial amount of LDP1.

The serum samples of EACa patients were pooled and applied into the CGB lectin affinity column. Bound O-glycoproteins were displaced from the lectin using 0.1 M melibiose in PBS. Figure 3.6 demonstrates the elution profile of the O-glycosylated serum proteins using the developed CGB lectin affinity chromatography column. Fractions from the first peak consisted of serum proteins which did not interact with the CGB lectin. The CGB lectin reactive serum proteins were then eluted in the fractions that form the second peak of the elution profile.

3.1.4 2-DE profile of lectin purified O-glycosylated serum proteins

The bound fractions from the CGB lectin affinity chromatography were pooled, dialyzed and freeze-dried. However, the pooled fractions obtained comprised a cocktail of

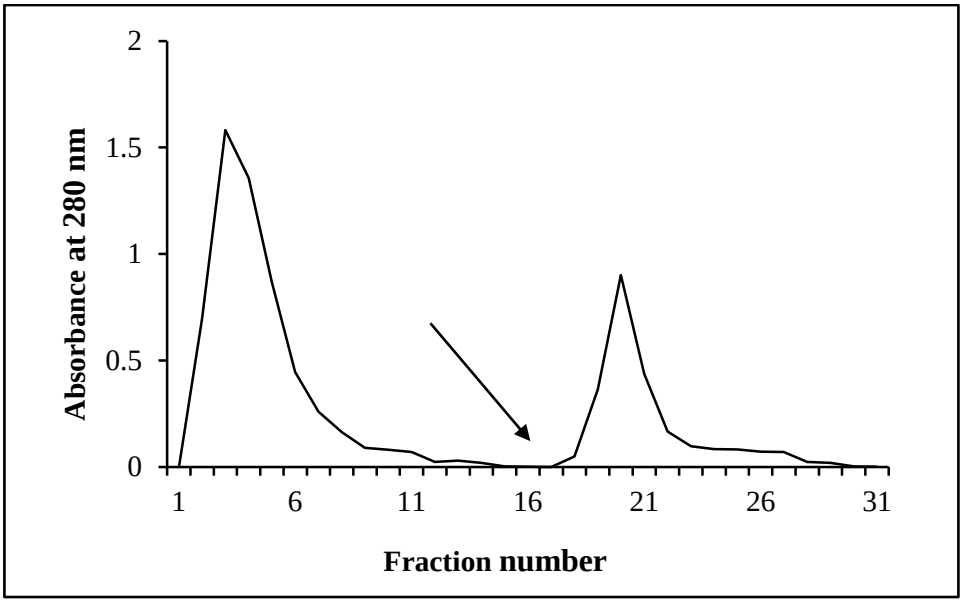

Figure 3.6: Isolation of O-glycosylated serum proteins. Pooled serum samples from three patients with EACa were subjected to CGB lectin affinity chromatography. Fractions collected from the column were monitored by measurement of absorbance at 280 nm. Column was washed extensively with PBS, pH 7.2 until the absorbance fell to less than 0.05. Arrow indicates start of elution of bound glycoproteins using 0.1 M melibiose in PBS. Elution was performed until absorbance of the effluent returned to less than 0.05.

CGB lectin reactive serum O-glycoproteins, including the LDP1 protein. To isolate the LDP1 protein spot cluster, the pooled concentrated protein mixture was subjected to 2-DE separation.

When the pooled lectin bound fractions were separated by 2-DE and stained with Coomassie Blue, several protein spots were resolved. Profiles obtained were found to be generally comparable to the HRP-CGB lectin blots previously generated by Abdul-Rahman *et al.* (2007) from sera of patients with EACa, except for presence of an intensely stained line of immunoglobulin light chains (Figure 3.7). Spots that were visually identified included IgA α-heavy chains, immunoglobulin light chains, α_1-antichymotrypsin (ACT), α_2-HS glycoprotein (AHS), hemopexin (HPX), cleaved high molecular weight kininogen light chain (KNG), leucine rich glycoprotein (LRG) and the LDP1 protein spot cluster.

3.1.5 MS/MS Identification of LDP1

To identify LDP1, its Coomassie-stained protein spot cluster was excised from the 2-DE gel prior to analysis by mass spectrometry. *The MS/MS results obtained from the Biomolecular Research Facility, University of New Castle, Australia were then exported for search using the MASCOT search engine. The information regarding its database search using the NCBInr database is summarized in Table 3.1, while the list of peptide sequences derived from the MS/MS analysis is presented in Table 3.2. Based on the MS/MS data and the MASCOT database search, the LDP1 protein spot cluster was identified as inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4).

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*Due to malfunction of the MS instrument at the University of Malaya during the period of time of the experiment, analysis was out-sourced.

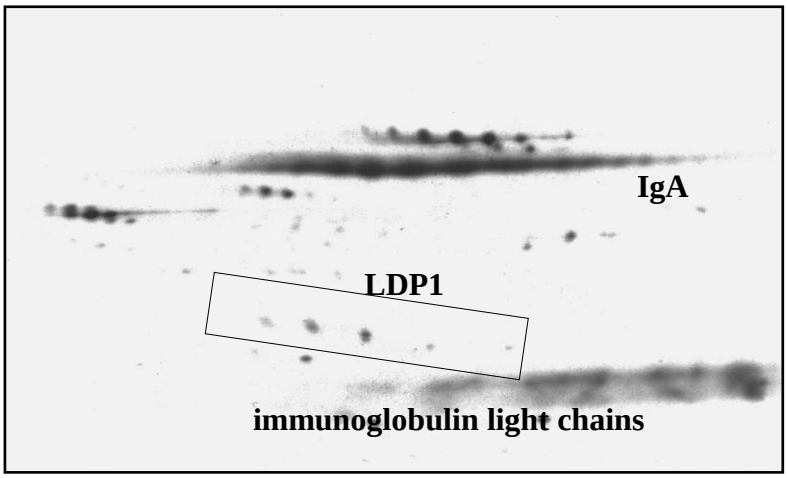


Figure 3.7: Profile of O-glycosylated serum proteins of pooled EACa patients. Bound fractions from the CGB lectin column was separated by 2-DE and stained with Coomassie Blue. The acidic side of the gel is to the left and relative molecular mass declines from the top.

Table 3.1: MS identification of LDP1 protein

Accession number [a)]	Protein matched	No. of peaks matched	MASCOT score	Sequence coverage [b)]
gi | 7770149	ITIH4	15	165	75.2%

[a)] Accession number is from the MASCOT database (www.matrixscience.com)
[b)] Percentage of sequence coverage was calculated against the ITIH4 fragment

Table 3.2: List of peptide sequences identified from MS/MS analysis of LDP1

Peptide sequence	Amino acid
R.LLLRDTDR.F	858-867
R.VQGNDHSATR.E	898-909
K.ETLFSVMPGLK.M	815-827
R.MNFRPGVLSSR.Q*	657-668
K.VTIGLLFWDGR.G	842-854
K.AGFSWIEVTFK.N	777-789
R.TLRVQGNDHSATR.E	894-909
K.WKETLFSVMPGLK.M	812-827
K.NPLVWVHASPEHVVVTR.N	788-806
R.RLDYQEGPPGVEISCWSVEL.-	909-930
R.QGPVNLLSDPEQGVEVTGQYER.E	752-776
K.TGLLLSDPDKVTIGLLFWDGR.G	831-854
R.QGPVNLLSDPEQGVEVTGQYEREK.A	752-778
R.VMNMKIEETTMTTQTPACPSCSR.S	709-733
R.FSSHVGGTLGQFYQEVLWGSPAASDDGR.R	865-895

Sequences of peptide were checked against the Swiss-Prot database for ITIH4 (Q14624). With exception of the sequence denoted by an asterisk, all other sequences correlate with the fragment region of ITIH4 (amino acids 696-930)

ITIH4 is a 120 kDa, single chain, plasma acute-phase glycoprotein. However, the LDP1 spot cluster detected in the 2-DE gel demonstrated relative molecular mass of approximately 35 kDa. Hence, this suggests that the LDP1 is only a cleavage fragment of ITIH4, and not the intact protein. This notion is further substantiated by the MS/MS derived amino acid sequences.

3.2 Prevalence of the ITIH4 fragment

In the preceding sections, the LDP1 has been identified as a fragment of ITIH4 (ITIH4f). Subsequent to its identification, it seemed interesting to study the expression of ITIH4f in other cancers. Hence, analysis of the expression of the fragment was extended to groups of patients with different types of malignancies.

3.2.1 Analysis of ITIH4f in sera of patients with different cancers

In the present work, the expression of ITIH4f in sera of patients with five different types of cancers was investigated. The study involved groups of patients with localized osteosarcoma (OsSa), nasopharyngeal carcinoma (NPCa), breast carcinoma (BrCa), germ cell ovarian carcinoma (GOCa) and epithelial ovarian carcinoma (EOCa). Serum proteins were initially separated by 2-DE before being transferred electrophoretically onto nitrocellulose (NC) membranes. Detection of the transferred ITIH4f along with the rest of the O-glycosylated serum proteins was performed using HRP-conjugated CGB lectin. Figure 3.8 demonstrates representative profiles of the CGB lectin detected blots from the five different types of malignancies.

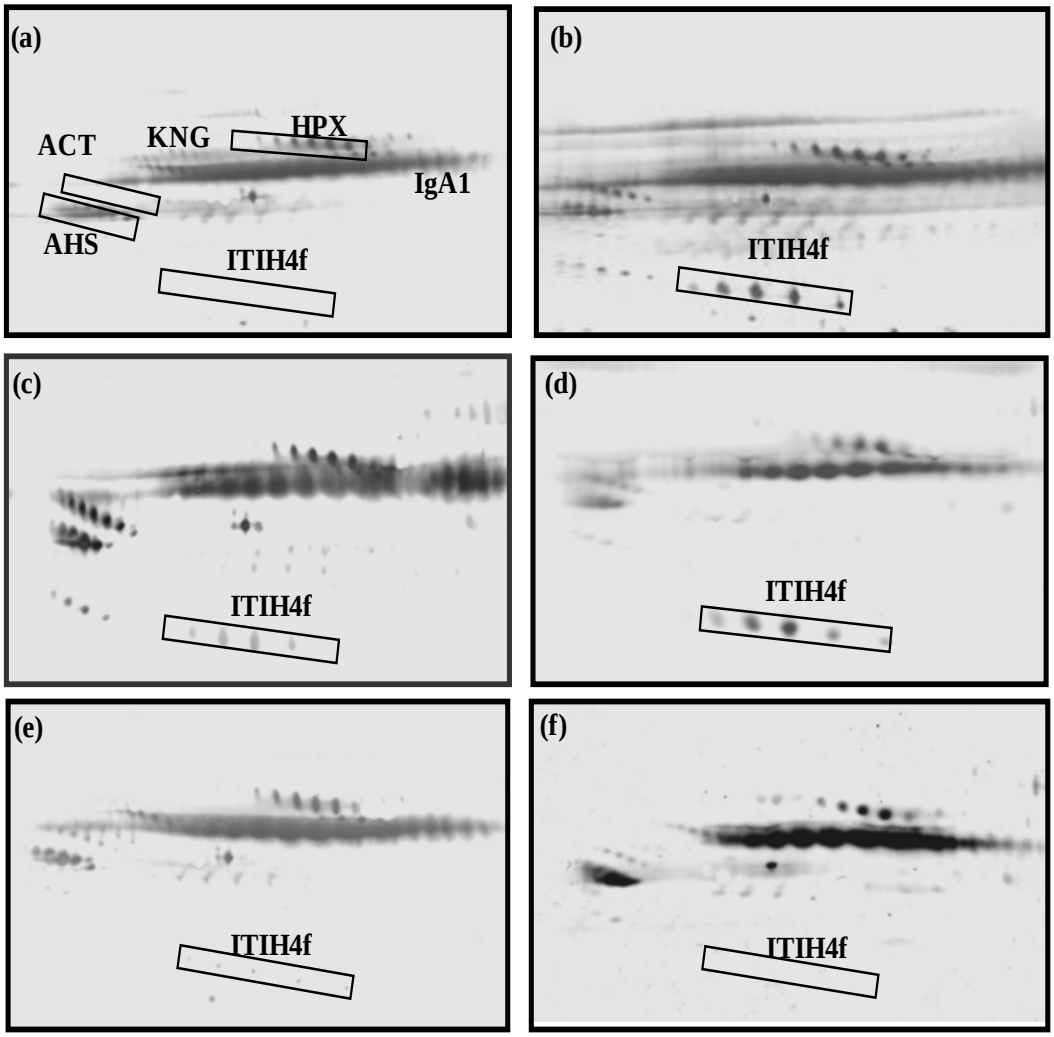


Figure 3.8: Separation and detection of ITIH4 fragment in five different groups of cancer patients. Unfractionated serum samples were subjected to 2-DE, blotting and detection with HRP-conjugated CGB lectin. Panels demonstrate typical profiles of (a) control subjects and patients with (b) BrCa , (c) EOCa, (d) GOCa, (e) NPCa and (f) OsSa. Up-regulated expression of the ITIH4 fragment (ITIH4f) is exhibited in profiles of patients with BrCa, EOCa and GOCa. For all panels, the acidic side of the blot is to the left and relative molecular mass declines from the top.

In general, all the resolved blots demonstrated specific differential expression of their O-glycosylated proteins. When analysis was focused on the expression of ITIH4f, significantly enhanced expression was detected in patients with BrCa, GOCa and EOCa, relative to the control subjects. However, the differential expression was not significantly detected in NPCa and OsSa patients. Figure 3.9 summarizes the results of the densitometry analysis which showed the over expression of ITIH4f in patients with BrCa (+10.9-fold; $p<0.0001$), EOCa (+4.4-fold; $p<0.0001$) and GOCa (+8.5-fold; $p<0.0001$) compared to the controls.

The question of why the enhanced expression of the fragment is selective to certain cancers is rather intriguing. One possibility is that the phenomenon may be hormonally correlated. BrCa, EOCa, GOCa, EACa are unlike NPCa and OsSa such that the former is generally associated with estrogen dysregulation. In such a case, however, the abundance of the particular fragment is also likely to be enhanced in other diseases associated with increased levels of estrogen.

3.2.2 Analysis of ITIH4f in sera of non-cancer patients

At this stage, the results of the present study appear to link the up-regulated expression of ITIH4f specifically with cancers that are associated with estrogen dysregulation. Therefore, it would be interesting to determine if the fragment is also over-expressed in other non-cancer conditions that are associated with enhanced levels of estrogen. This notion was then investigated in groups of women who were known to have high levels of plasma estrogen. Included in this study were women who were with a normal form of pregnancy (NP) and those associated with an abnormal pregnancy condition

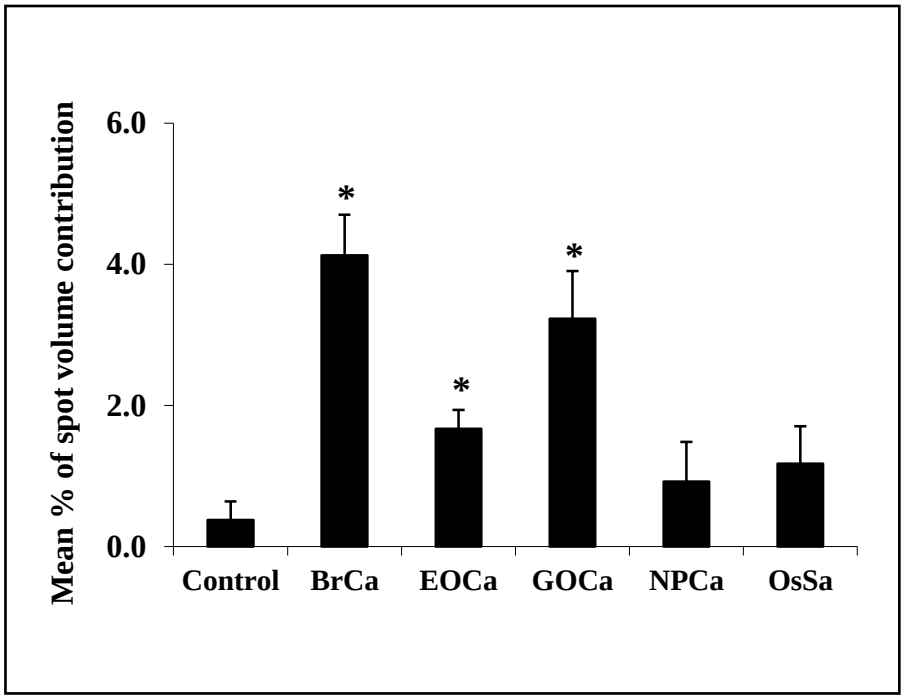


Figure 3.9: Relative expression of ITIH4 fragment in sera of five different groups of cancer patients. Analysis was performed on sera of healthy controls and patients with BrCa, EOCa, GOCa, NPCa and OsSa. Volume of ITIH4f was analyzed using Image Master 2D-Platinum software, version 5.0. All values are presented as mean ± SEM. Data were analyzed by Student's *t*-test and a p value of less than 0.01 was considered significant. Asterisks denote significantly different values as compared to controls.

referred to as hydatidiform mole (HM). To determine the influence of exogenous estrogen on the expression of ITIH4f, experiments were also performed on serum samples from menopausal women who were under the hormone replacement therapy (HRT).

In this part of the work, similar lectin-based electrophoretic approach was adopted. Representatives of the CGB lectin resolved NC membranes are shown in Figure 3.10. When densitometric analysis was performed on ITIH4f, up-regulated expression was observed for both groups of women with NP (+8.7-fold; $p=0.0002$) as well as HM (+5.6-fold; $p<0.0001$) compared to their non-pregnant controls. In contrast, the patients with HRT possess ITIH4f expression that was comparable to that of the control subjects. Figure 3.11 summarises the results of the densitometry analysis. Therefore, the data of this study suggest that ITIH4f was also over-expressed in normal and benign conditions that are associated with estrogen dysregulation.

3.3 Profiling of O-glycoproteins in sera of women with NP and HM

In the earlier sections, the enzyme conjugated CGB lectin was used to resolve the ITIH4f, as well as other O-glycosylated serum proteins that were separated by 2-DE. By using a similar approach, the aberrantly expressed high-abundance serum glycoproteins in sera of patients with BrCa (Doustjalali, 2004), EOCa (Chen, 2007), GOCa (Chen, 2007), EACa (Abdul-Rahman *et al*., 2007), NPCa (Doustjalali, 2004) and OsSa (Wan Ibrahim, personal communication, 2010) were also detected. However, the serum O-glycoproteins of women with NP and HM have not been previously profiled. Thus, an attempt was made to study their expression patterns in relation to the different types of pregnancies.

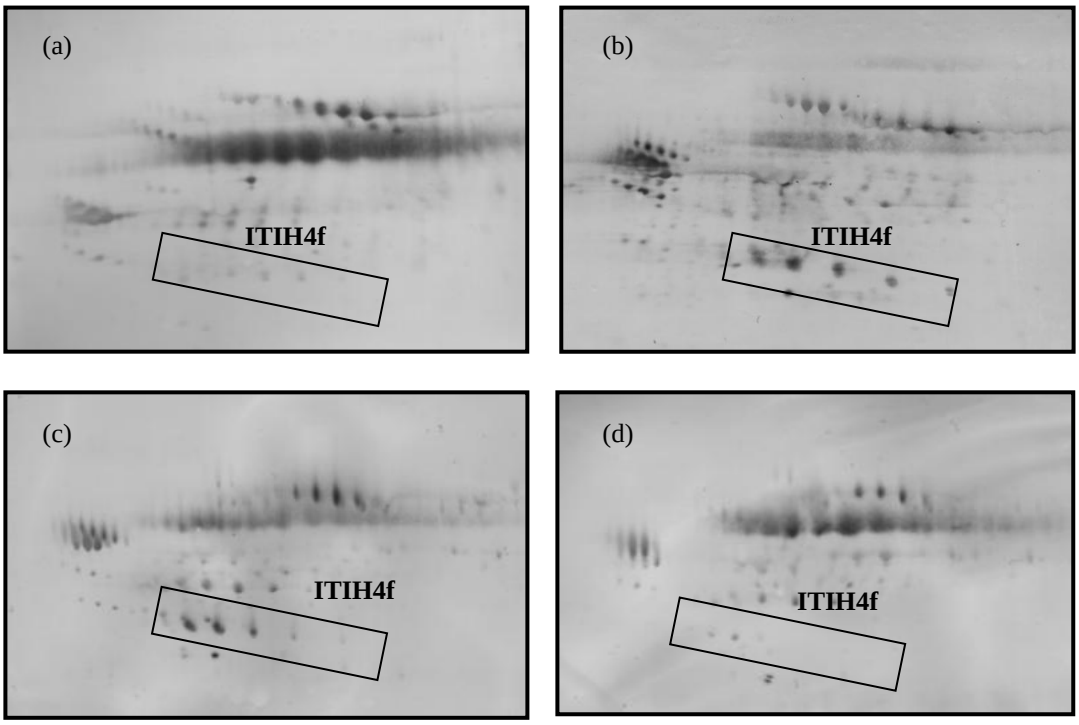


Figure 3.10: Separation and detection of ITIH4f in women with estrogen dysregulation. Unfractionated serum samples were subjected to 2-DE, blotting and detection with enzyme-conjugated CGB lectin. Panel (a) demonstrates a typical profile of healthy non-pregnant controls while panels (b), (c) and (d) are representative profiles of women with NP, HM and HRT, respectively. The cleavage fragment of ITIH4 appears to be over-expressed in profiles of patients with NP and HM. For all panels, the acidic side of the blot is to the left and relative molecular mass declines from the top.

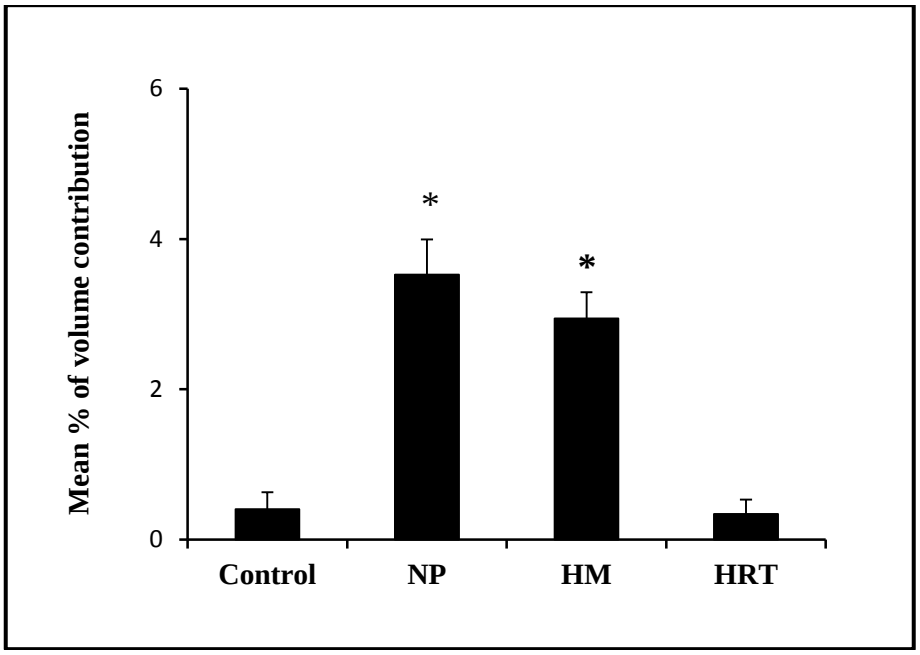


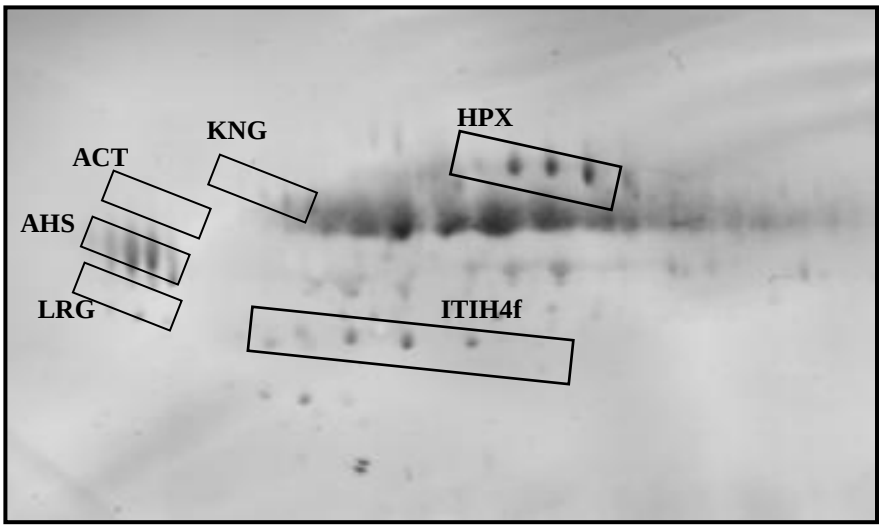
Figure 3.11: Relative expression of ITIH4 fragment in sera of women with estrogen dysregulation. Analysis was performed on sera of healthy controls and women with NP, HM and HRT. Volume of ITIH4f was analyzed using Image Master 2D-Platinum software, version 5.0. All values are presented as mean ± SEM. Data were analyzed by Student's *t*-test and a p value of less than 0.01 was considered significant. Asterisks denote significantly different values as compared to controls.

When the 2-DE separated serum proteins of the NP and HM patients and controls were exposed to HRP-conjugated CGB lectin, different profiles comprising just the O-glycosylated proteins were obtained. Figure 3.12 (a) demonstrates the representative profile of a normal female control while figures (b) and (c) represent typical examples of protein profiles of women with NP and HM, respectively. Resolved glycoprotein spots were initially identified through visual comparison with the HRP-CGB lectin detected profiles developed by previous researchers (Abdul-Rahman *et al*., 2007; Abdul Rahman *et al*., 2002). Spots that were visually identified included ACT, AHS, HPX, KNG, LRG and ITIH4f. Also noted was that, by using the lectin detection method, spots of HPX and KNG appeared to be well resolved in the absence of albumin streak that usually obscures their presence. Since the expression of ITIH4f had been studied in the preceding section, densitometry analysis was performed on the other O-glycoproteins resolved on the nitrocellulose membranes.

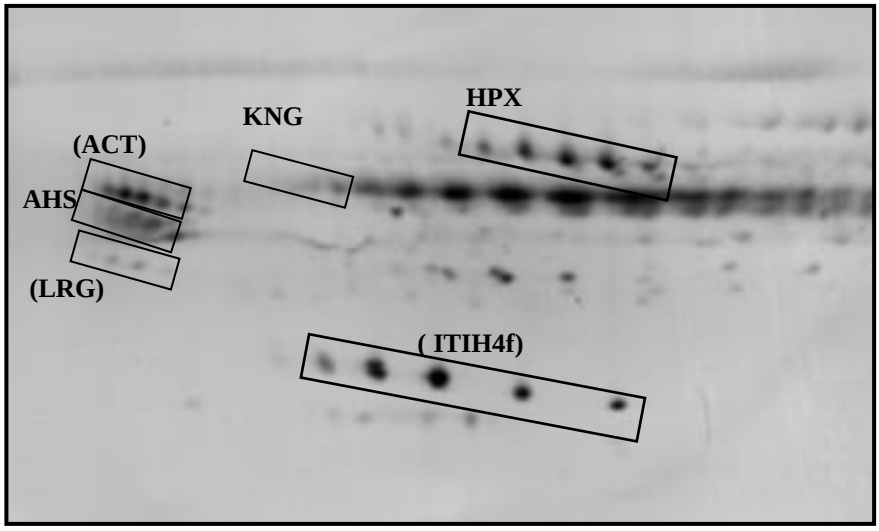
3.3.1 Profiling of O-glycoproteins in sera of women with NP

The present part of the study was aimed at the identification of O-glycosylated serum proteins that were aberrantly expressed in women with NP compared to non-pregnant controls. When the 2-DE separated O-glycoprotein profiles of NP women and controls were subjected to densitometry analysis, a striking difference in glycoprotein expression was observed. Figure 3.13 and Table 3.3 demonstrate the differential expression of the various glycoproteins released in the serum, in response to pregnancy. In addition to the aberrantly expressed ITIH4f, which was detected in the earlier section of the study, expression of ACT (+2.9-fold; p=0.0037) and LRG (+2.0-fold; p=0.0008) were also found to be significantly higher than their negative controls.

(a)



(b)



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(c)

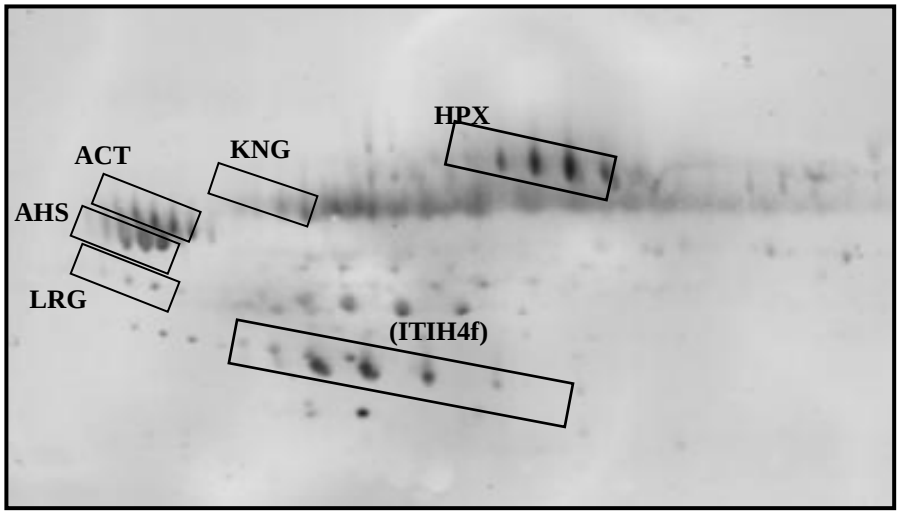


Figure 3.12: Typical CGB-lectin detected serum protein profiles of control individual and women with NP and HM. Whole serum samples of normal healthy females and women with NP and HM were subjected to 2-DE and HRP-CGB lectin detection. Panels (a), (b) and (c) are representative serum glycoprotein profiles of control subjects and women with NP and HM, respectively. The O-glycosylated protein spots resolved on the blots included ACT, AHS, CLU, HPX, KNG, LRG and ITIH4f. For all panels, the acidic side of the 2-DE blot is to the left and relative molecular mass declines from the top. Aberrantly expressed glycoproteins are marked in bracket.

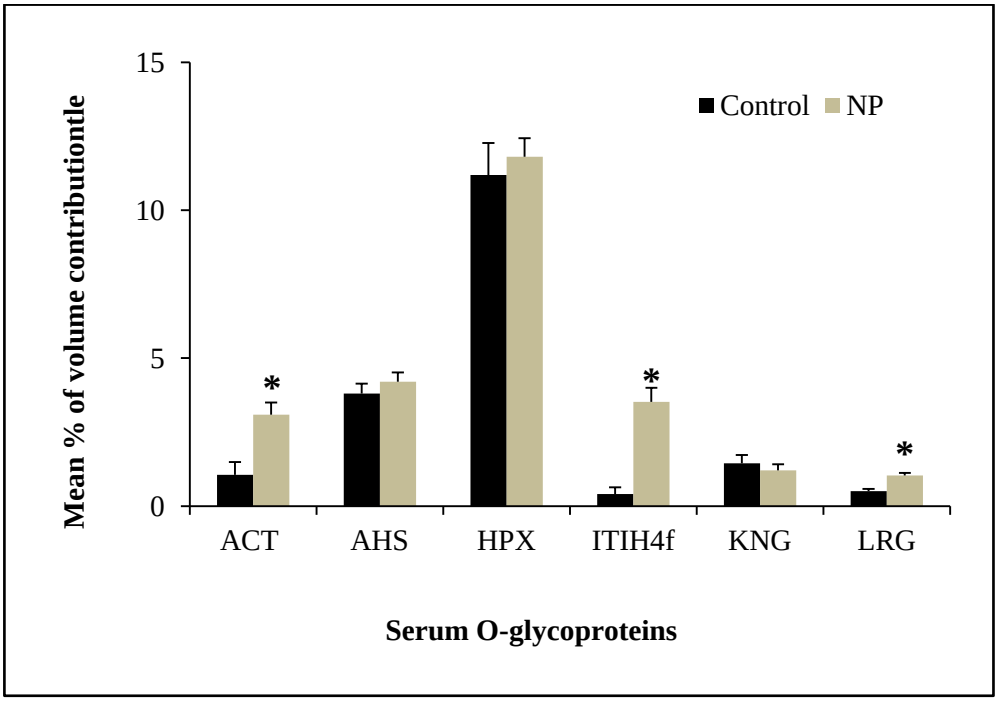


Figure 3.13: Mean percentage of volume contribution of O-glycoproteins of women with NP and control subjects. Volumes of O-glycosylated protein spots resolved in sera of women with NP and controls were analyzed using Image Master 2D-Platinum software, version 5.0. Analyses were restricted to high abundance serum proteins including ACT, AHS, HPX, KNG, LRG and ITIH4f. Asterisk denotes significantly different values when compared to that of control.

Table 3.3: Relative expression of O-glycoproteins in sera of women with NP

Serum proteins	Statistical analysis	
	***fold changes**	****p**
ACT ≠	+2.9231	0.0037
AHS	+1.1054	0.4326
HPX	+1.0556	0.0872
ITIH4f ≠	+8.6612	0.0002
KNG	–0.8351	0.5176
LRG	+2.0262	0.0009

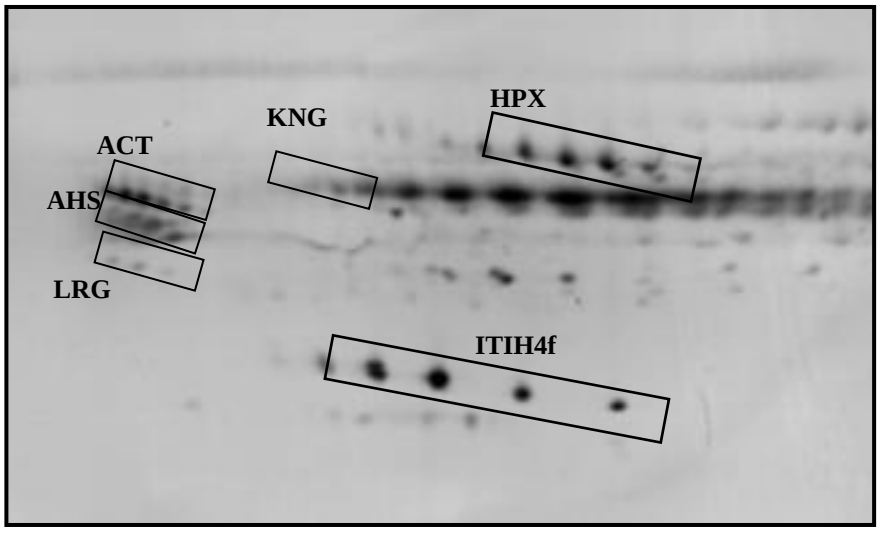
***fold changes** measures the degree of change in percentage volume of the respective glycoprotein in test individuals compared to controls. A minimum difference of two folds was taken as the cutting point for the variation to be considered significant

****p** Student *t*-test was used to analyze the significance of differences and $p<0.01$ was considered significant

+/– increased/decreased in expression level

≠ glycoprotein showing significant differential expression

(a)



(b)

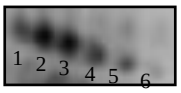
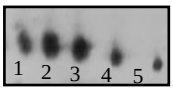
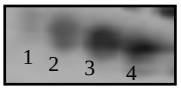
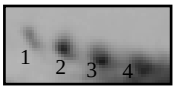
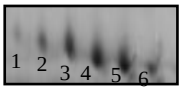
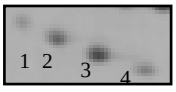
Protein	Isoforms	Protein	Isoforms
ACT		ITIH4f	
AHS		KNG	
HPX		LRG	

Figure 3.14: Profiles of isoforms of O-glycosylated proteins. Panel (a) demonstrates the position of the glycoproteins on a CGB lectin-detected blot. Panel (b) consisted of profiles of isoforms of the respective proteins. Isoforms were abbreviated using numbers in ascending order starting from the acidic side of the blot.

Aside from changes in a glycoprotein's abundance, alteration in serum constituents has also been linked to changes of the structures of its carbohydrate chains. And this may be reflected by alteration in the expression pattern of its isoforms (Sàez-Valero *et al*., 2001). Hence, in addition to the densitometry analysis performed on the lectin resolved glycoproteins, the study was also extended to the expression of well resolved isoforms of the protein clusters. For the purpose of this study, isoforms were abbreviated using numbers in ascending manner starting from the acidic to the basic sides of the nitrocellulose membrane (Figure 3.14).

When isoforms of individual glycoproteins in the O-glycosylated protein profiles of women with NP were compared to those of the controls, none of the isoforms demonstrated alteration in their relative distribution patterns. Table 3.4 demonstrates the summary of the volume analysis, which indicated high similarities in the relative distribution of the isoforms in all the six glycoproteins, between the two groups studied. However, glycoproteins which were found to be over-expressed, such as ITIH4f and ACT, demonstrated significant difference in terms of abundance of their isoforms, relative to that of the controls. Densitometry analysis performed on the isoforms of ITIH4f demonstrated that all of its isoforms were highly expressed, especially isoforms 3, 4 and 5 which were not detected in the membranes of the control subjects. As for ACT, marked increase of expression appeared to be contributed mainly by three of its isoforms, i.e., isoform 1 (+13.7-fold; $p<0.0001$), isoform 2 (+4.9- fold; $p=0.0009$) and isoform 3 (+3.8-fold; $p=0.0051$). Also observed was the over-expression of the other three isoforms of ACT, compared to those exhibited by the control women. However, the difference in abundance appeared to be not statistically significant.

Table 3.4: Relative expression of isoforms of O-glycoproteins in serum profiles of women with NP

Serum proteins	*Isoform number	Statistical analysis	
		**fold changes	***p
ACT	1≠	+13.7295	<0.0001
	2≠	+4.9689	0.0008
	3≠	+3.7505	0.0051
	4	+2.8239	0.0202
	5	+2.0120	0.1676
	6	+1.4461	0.4292
AHS	1	+1.8773	0.0156
	2	+1.5771	0.0032
	3	–0.9328	0.8082
	4	+1.3489	0.3314
HPX	1	+1.8034	0.0356
	2	+1.5621	0.0236
	3	+1.2823	0.2365
	4	+1.3671	0.1546
	5	+1.4115	0.1135
	6	+1.0190	0.9415
ITIH4f	1≠	+5.6882-fold	0.0047
	2≠	+5.9756-fold	0.0055
	3≠	#	0.0015
	4≠	#	0.0002
	5≠	#	0.0001

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Serum proteins	*Isoform number	Statistical analysis	
		**fold changes	***p
KNG	1	–0.4975	0.3428
	2	–0.6782	0.5340
	3	–0.6220	0.3133
	4	–0.6505	0.3422
LRG	1	+2.4478	0.0208
	2	+1.9562	0.0339
	3	+1.7304	0.0792
	4	+1.9544	0.0900

*Isoform number	numbering of the isoform starts from the acidic side of the glycoprotein (as shown in Figure 3.14)
**fold changes	measures the degree of change in percentage of volume contribution of each isoform over the total volume of its protein in test individuals as compared to controls. A minimum difference of two folds was taken as the cutting point for the variation to be considered significant
***p	Student *t*-test was used to analyze the significance of differences and $p<0.01$ was considered significant
+/–	increased/ decreased in expression level
#	not detected in serum protein profiles of negative controls
≠	isoform showing significant differential expression compared to that of the control

3.3.2 Profiling of O-glycoproteins in sera of women with HM

In the previous section, several O-glycosylated proteins from the sera of women with NP were found to be differentially expressed. Hence, a similar volume analysis was performed on the profiles of the O-glycoproteins of women with HM compared to that of non-pregnant subjects. Figure 3.11(c) demonstrates a typical representative of the 2-DE serum protein profiles of patients with HM. When the lectin detected membranes were analyzed, most of the O-glycoproteins in the sera of the HM patients appeared to exhibit comparable expression patterns to those of the control subjects (Figure 3.15). Out of the six O-glycoproteins analyzed, ITIH4f was the sole glycoprotein found to be significantly over-expressed, relative to the controls. The expression levels of the other O-glycosylated proteins including ACT, AHS, HPX, KNG and LRG were not significantly different between the HM patients and control women (Table 3.5).

In this study, the isoforms of the six O-glycosylated proteins detected in the membranes of HM patients were also subjected to densitometry and statistical analysis. Analysis performed on the isoforms of the glycoprotein clusters demonstrated that their expression patterns were generally similar to those of the controls (Table 3.6). As such, no significant alteration in the glycosylation pattern of the serum glycoproteins was detected. Observed from the densitometry analysis, however, was the isoforms of ITIH4f, which were notably different for their abundance but not for their relative isoform distribution, compared to those of the controls. As for the rest of the analyzed glycoproteins, none of them demonstrated significant difference in the levels of expression of their isoforms.

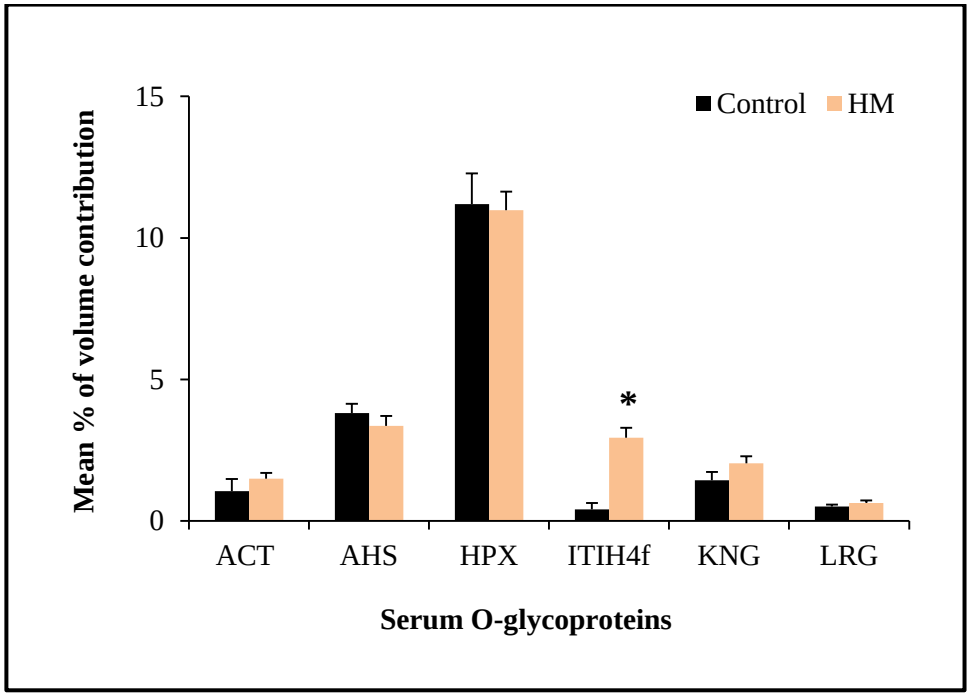

Figure 3.15: Mean percentage of volume contribution of O-glycoproteins of women with HM and control subjects. Volumes of O-glycosylated protein spots resolved in sera of control and women with HM were analyzed using Image Master 2D-Platinum software, version 5.0. Analyses were conducted on the high abundance serum proteins such as ACT, AHS, HPX, KNG, LRG and ITIH4f. Asterisk denotes significantly different values when compared to that of control.

Table 3.5: Relative expression of serum O-glycoproteins of women with HM

Serum proteins	Statistical analysis	
	***fold changes**	****p**
ACT	+1.4159	0.3012
AHS	–0.8839	0.3930
HPX	+1.2465	0.0872
ITIH4f≠	+5.6139	< 0.0001
KNG	+1.4081	0.4357
LRG	+1.2359	0.3552

*fold changes	measures the degree of change in percentage volume of the respective protein in test individuals compared to controls. A minimum difference of two folds was taken as the cutting point for the variation to be considered significant
**p	Student *t*-test was used to analyze the significance of differences and $p<0.01$ was considered significant.
+/–	increased/decreased in expression level
≠	glycoprotein showing significant differential expression compared to that of control

Table 3.6: Relative expression of isoforms of O-glycoproteins in serum profiles of women with HM

Serum proteins	***Isoform number**	**Statistical analysis**	
		****fold change**	*****p**
ACT	1	+3.2937	0.0159
	2	+2.8039	0.0196
	3	+2.6992	0.0213
	4	+1.4895	0.4304
	5	–0.5225	0.7641
	6	–0.4774	0.3069
AHS	1	+2.8636	0.1009
	2	–0.8725	0.5458
	3	–0.5663	0.0471
	4	–0.6700	0.1444
HPX	1	–0.9938	0.9812
	2	+1.4479	0.1352
	3	+1.0011	0.9957
	4	–0.9448	0.6188
	5	+1.1791	0.3408
	6	+0.8599	0.5155
ITIH4f	$1^{\neq}$	+4.9582	0.0025
	$2^{\neq}$	+4.5647	0.0032
	$3^{\neq}$	#	0.00015
	$4^{\neq}$	#	0.00026
	$5^{\neq}$	#	0.000038

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Serum proteins	*Isoform number	Statistical analysis	
		**fold change	***p
KNG	1	+1.0787	0.8638
	2	+1.0680	0.8927
	3	+1.0546	0.9459
	4	+1.1825	0.8439
LRG	1	+2.8636	0.3869
	2	+1.6871	0.1421
	3	–0.9492	0.8304
	4	+1.1942	0.5814

*Isoform number	numbering of the isoform starts from the acidic side of the glycoprotein (as demonstrated in Figure 3.14)
**fold changes	measures the degree of change in percentage of volume contribution of each isoform over the total volume of its protein in test individuals as compared to controls. A minimum difference of two folds was taken as the cutting point for the variation to be considered significant
***p values	Student *t*-test was used to analyze the significance of differences and $p<0.01$ was considered significant
+/–	increased/ decreased in expression level
#	not detected in serum protein profiles of negative controls
≠	isoform showing significant differential expression compared to that of the control

3.3.3 Comparison of the expression of serum O-glycoproteins between women with NP and those with HM

In this study, the expression of the O-glycoproteins in sera of women with NP and HM was investigated using a lectin-based electrophoretic approach. Figure 3.16

demonstrates the mean percentages of volume contribution of the glycoproteins resolved from serum samples of three groups of women. Densitometry and statistical analyses were performed on the six O-glycosylated serum proteins of women with NP and HM in order to identify disease-associated changes in the glycoproteins of women with HM. When the CGB lectin resolved glycoproteins were analyzed, the only significant difference detected was in the reduced expression of ACT (–0.5-fold; p=0.0023) in the serum samples of HM patients, relative to that of the NP women (Table 3.7). The expression levels of the other five O-glycosylated proteins, i.e., AHS, HPX, KNG, LRG and ITIH4f, appeared to be unaffected by the abnormal pregnancy condition of the women with HM.

When the isoforms of the glycoprotein clusters were analyzed, their distribution patterns were not significantly different between the HM patients and those with NP for most of the proteins (Table 3.8). In addition, their abundance was also not significantly different with exception of one isoform spot of ACT. The expression of isoform 5 of ACT appeared to be significantly reduced (–0.5-fold; p=0.0055) in the sera of women with HM as compared to those of NP women.

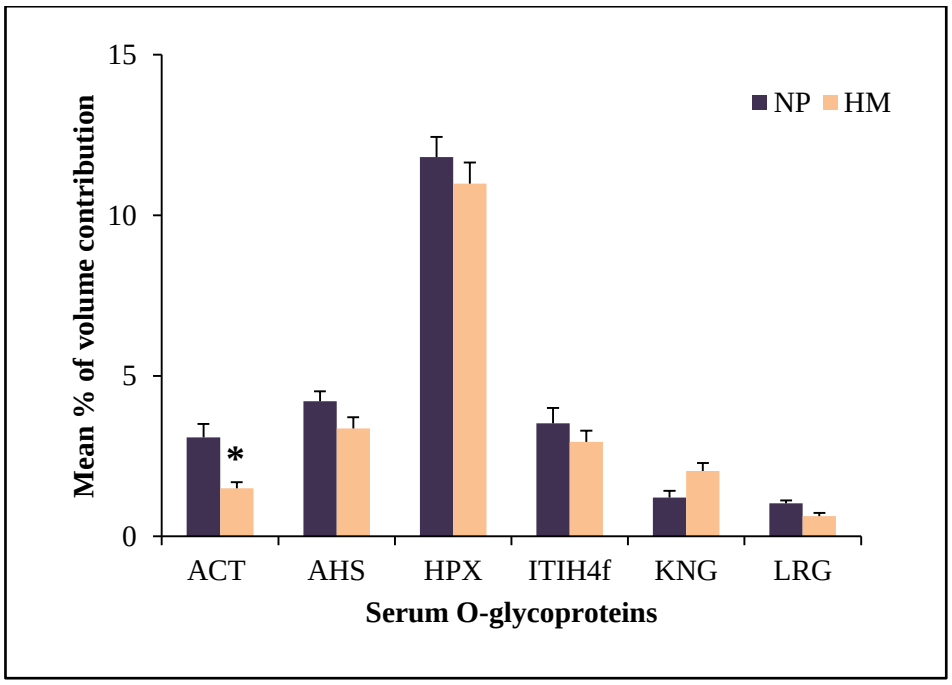

Figure 3.16: Mean percentage of volume contribution of O-glycoproteins of women with NP and HM. Volume of O-glycosylated protein spots resolved in sera of women with NP and HM were analyzed using Image Master 2D-Platinum software, version 5.0. Analyses were performed on the high abundance serum proteins resolved on the blots including ACT, AHS, HPX, KNG, LRG and ITIH4f. Asterisk denotes significantly different values when compared to that of control.

Table 3.7: Relative expression of serum O-glycoproteins of women with HM aompared to women with NP

Serum proteins	Statistical analysis	
	*fold changes	**p
ACT≠	–0.4843	0.0023
AHS	–0.7996	0.0715

Serum proteins	*Isoform number	Statistical analysis	
HPX		–0.9293	0.3613
ITIH4f		–0.8346	0.3449
KNG		+1.6863	0.1305
LRG		–0.6099	0.0041

***fold changes**	measures the degree of change in percentage volume of the respective protein in test individuals compared to controls. A minimum difference of two folds was taken as the cutting point for the variation to be considered significant
****p* values**	Student *t*-test was used to analyze the significance of differences and $p<0.01$ was considered significant.
+/–	increased/decreased in expression level
≠	glycoprotein showing significant differential expression compared to that of control

Table 3.8: Relative expression of isoforms of O-glycoproteins in serum profiles of women with HM as compared to women with NP

Serum proteins	*Isoform number	Statistical analysis	
		**fold change	***p
ACT	1	–0.4584	0.0123
	2	–0.5643	0.0321
	3	–0.7197	0.1847
	4	–0.5274	0.0273
	5≠	–0.4613	0.0055
	6	–0.3301	0.0103
AHS	1	+1.5254	0.3855
	2	–0.5533	0.0023
	3	–0.6071	0.0659
	4	–0.4967	0.0299
HPX	1	–0.5511	0.0042
	2	–0.9269	0.7099
	3	–0.7807	0.1547
	4	–0.6911	0.0388
	5	–0.8353	0.2963
	6	–0.8439	0.4019
ITIH4f	1	–0.6938	0.6173
	2	–0.7639	0.3507
	3	–0.6876	0.2545
	4	–0.3775	0.1974
	5	–0.4977	0.0191

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KNG	1	+1.5399	0.4471
	2	+1.0062	0.9919
	3	+1.1110	0.8519
	4	+1.0729	0.9047
LRG	1	+1.0439	0.2979
	2	–0.8624	0.6284
	3	–0.5485	0.0435
	4	–0.6111	0.1547

*Isoform number	numbering of the isoform starts from the acidic side of the glycoprotein (refer to Figure 3.14)
**fold changes	measures the degree of change in percentage of volume contribution of each isoform over the total volume of its protein in test individuals as compared to controls. A minimum difference of two folds was taken as the cutting point for the variation to be considered significant
***p values	Student *t*-test was used to analyze the significance of differences and $p<0.01$ was considered significant
+/–	increased/ decreased in expression level
#	not detected in serum protein profiles of negative controls
≠	isoform showing significant differential expression compared to that of the control

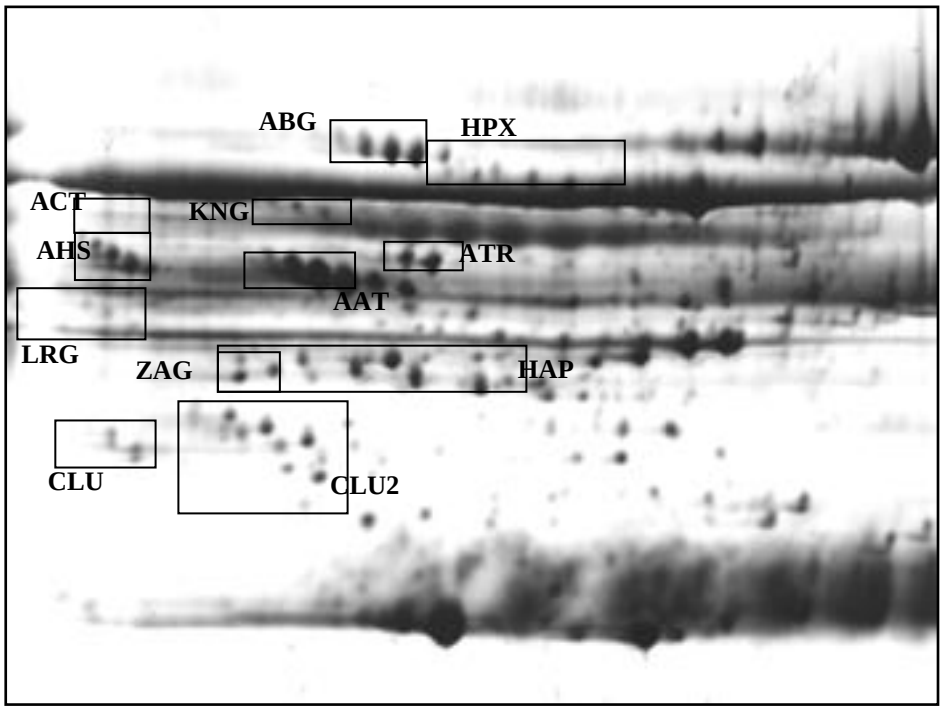
3.4 Profiling of serum proteins of women with NP and HM

When the 2-DE separated serum proteins of women with NP and HM as well as their non-pregnant controls were subjected to Western blotting and HRP-CGB lectin

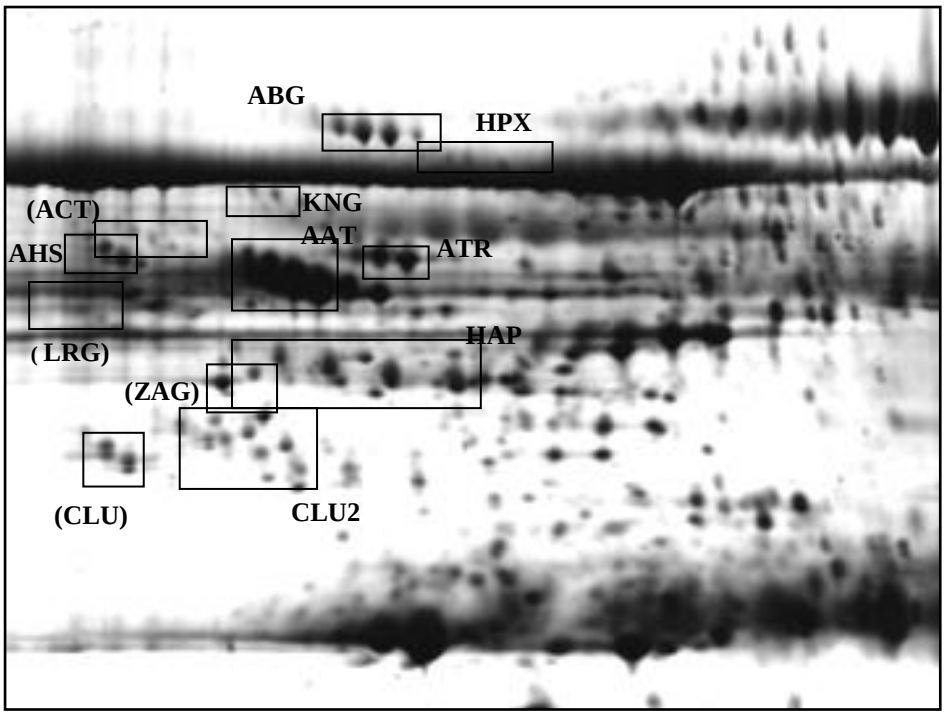
detection, different serum O-glycoprotein expression patterns were obtained. However, by using the CGB lectin electrophoretic approach, only serum proteins carrying the O-linked moieties would interact with the lectin and able to be detected. Hence, to complete the analysis on the serum protein profiles of the two groups of pregnant women, the expression of other high abundance serum proteins were studied using whole serum samples.

In the present part of the study, whole serum samples of women with NP and HM, along with their age-matched female controls, were subjected to 2-DE separation. Figure 3.17 demonstrates typical representative high resolution 2-DE profiles of serum proteins obtained from the non-pregnant controls (panel a) and women with NP (panel b) and HM (panel c). Obviously, more protein spots were detected in the present work as compared to the CGB lectin generated profiles. The silver-stained protein spots were initially identified by visual comparison with the standard SWISS ExPASy reference map (Sanchez *et al*., 1995). Among the high abundance serum proteins which were not resolved in the O-glycosylated protein profiles but were detected in the present gels were albumin (ALB), α1-antitrypsin (AAT), α1-ß-glycoprotein (ABG), antithrombin-III (ATR), clusterin (CLU and CLU2), haptoglobin (HAP), zinc-α2-glycoprotein (ZAG) and the heavy and light chains of immunoglobulins. However, in contrast to the serum O-glycoprotein profiles, the ITIH4f protein spot cluster was not seen. In addition, serum ALB was detected as a streak which obscured detection of other proteins of similar masses in this study mainly due to its high abundance.

(a)



(b)



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(c)

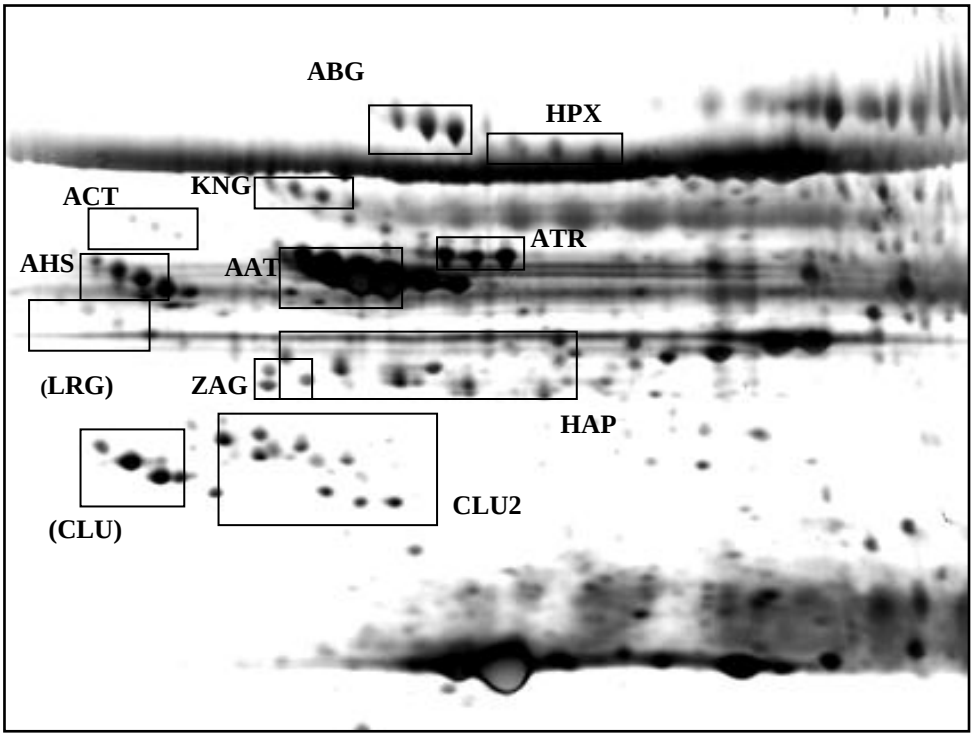


Figure 3.17: Typical serum protein profiles of control women and patients with NP and HM. Whole serum samples of non-pregnant females and pregnant patients were subjected to 2-DE and silver staining. Panel (a) demonstrates a typical serum protein profile of a healthy control individual. Clusters of protein spots clearly resolved include AAT, ABG, ACT, AHS, ALB, ATR, CLU, HAP, HPX, KNG, LRG and, ZAG. Panels (b) and (c) demonstrate typical profiles of women with NP and HM, respectively. The aberrantly expressed proteins are marked in brackets. For all panels, the acidic side of the 2-DE gel is to the left and relative molecular mass declines from the top.

Under the settings of the 2-DE experiments, ALB usually appears as a horizontal streak in the 2-DE profiles of serum samples. The immunoglobulin polypeptide chains, on

the other hand, are normally resolved as hundreds of spots, either in scattered- or train-like patterns. In addition, the idiotypic and/or allotypic variation of the immunoglobulin molecules lead to inconsistencies in the resolution of the polypeptide chains. Because of these reasons, the proteins were excluded from the analyses in this study. Hence, densitometry and statistical analysis were focused only on the twelve main clusters of well-resolved high abundance serum proteins.

3.4.1 Profiling of serum proteins of women with NP

In this study, whole serum samples from women with NP were subjected to 2-DE and followed by silver staining. The resulting silver stained serum protein profiles of the women with NP were then compared quantitatively to those obtained from control subjects. Analysis of the twelve proteins revealed presence of four proteins that were differentially expressed (Figure 3.18 and Table 3.9).

Among the serum proteins that were aberrantly expressed in the sera of women with NP relative to the non-pregnant controls were ACT, CLU, LRG and ZAG. In the present study, the expression of ACT (+4.3-fold; $p=0.0001$) and LRG +2.6-fold; $p<0.0001$) were confirmed to be enhanced as reported earlier from the analysis of the O-glycosylated serum protein profiles. In addition to these findings, CLU (+3.5-fold; $p=0.0009$) and ZAG (2.2-fold; $p<0.0001$) were also found to be highly expressed in the normal pregnant women compared to the levels in control subjects.

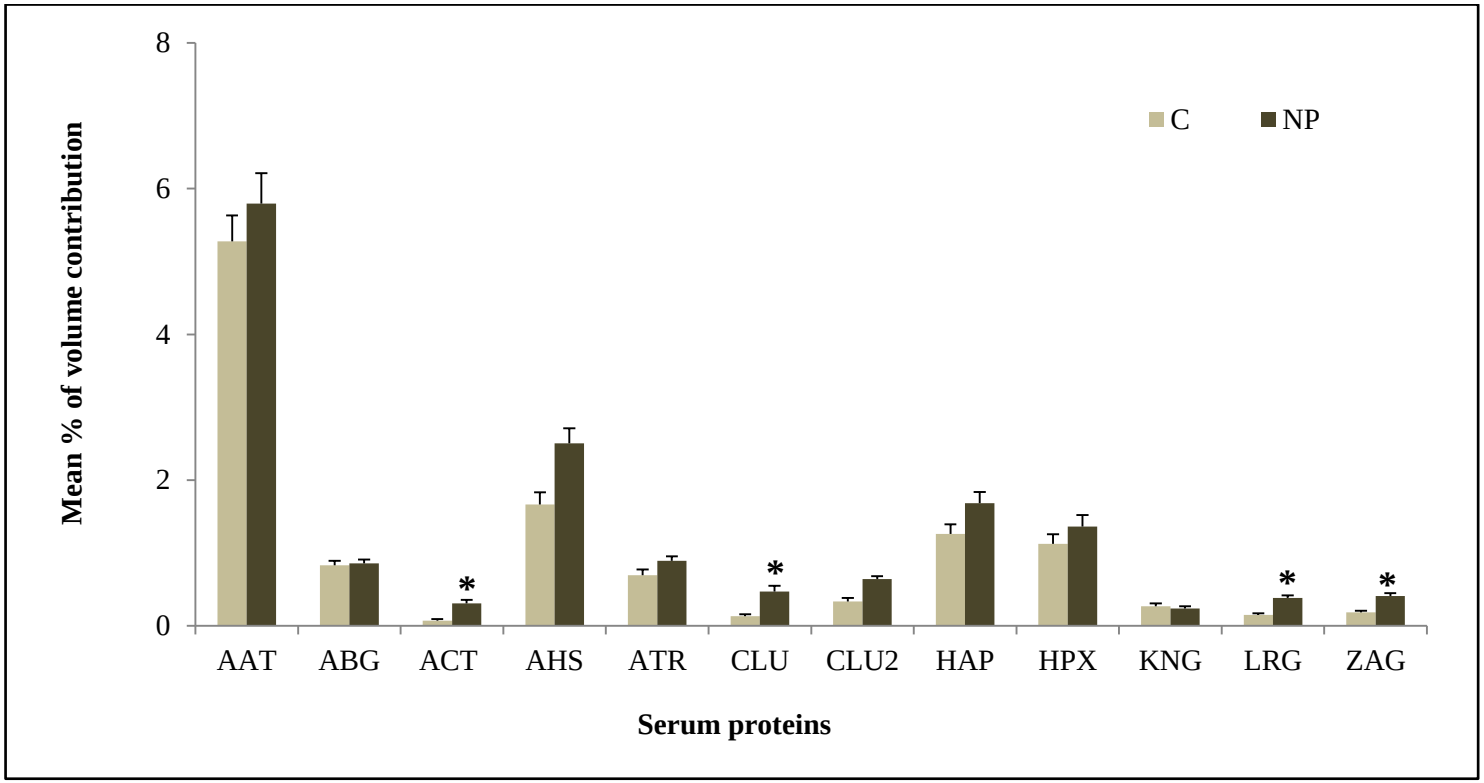


Figure 3.18: Analysis of serum protein expression of women with NP and control subjects. Silver stained 2-DE serum protein profiles were scanned using a densitometer and resulting protein spots were then analyzed using Image Master 2D platinum software (version 5.0). The percentage of contribution of each protein was calculated based on the volume of a selected protein over the total volume of all the analyzed proteins in the protein map. Asterisk denotes significantly different values when compared to controls.

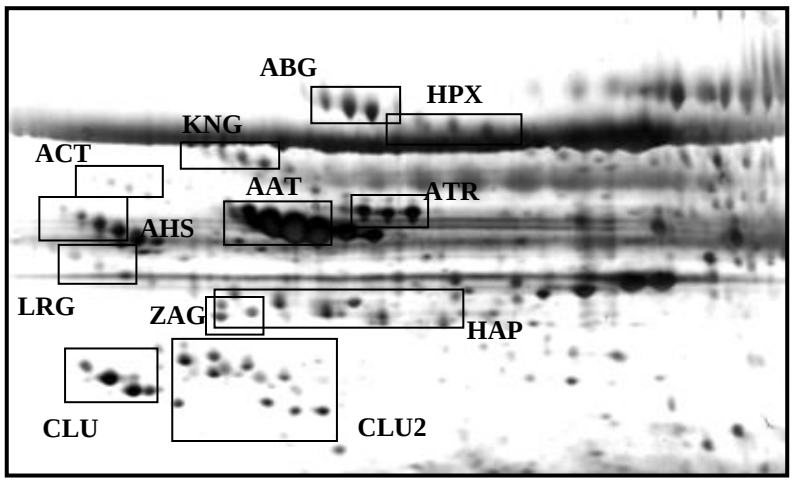
Table 3.9: Relative expression of serum proteins of women with NP

Serum proteins	Statistical analysis	
	***fold changes**	****p***
AAT	+1.0979	0.3746
ABG	+1.0333	0.7202
ACT≠	+4.2718	0.0001
AHS	+1.5035	0.0042
ATR	+1.2891	0.0451
CLU≠	+3.5095	0.0009
CLU2	+1.9061	<0.0001
HAP	+1.3346	0.0574
HPX	+1.2091	0.2852
KNG	–0.8812	0.5209
LRG≠	+2.5526	<0.0001
ZAG≠	+2.2139	<0.0001

*fold changes	measures the degree of change in intensities of proteins in patients compared to controls. A minimum difference of two folds was taken as the cutting point for the variation to be considered significant
**p values	Student t-test was used to analyze the significance of differences and $p<0.01$ between cohorts was considered significant.
+/-	increased/decreased in expression level
≠	protein showing significant differential expression

In this study, densitometry analysis was performed on the isoforms of the twelve high abundance serum proteins detected in the 2-DE protein profiles of women with NP. Figure 3.18 demonstrates the positions of the isoforms analyzed while Table 3.10 summarizes the result of the statistical analysis conducted between the groups of NP women and controls. Amongst the serum proteins that were analyzed, the isoform expression patterns of CLU and ZAG were significantly different compared to the patterns demonstrated by the control subjects. The abundance of CLU in the sera of women with NP seems to be attributed by the up-regulated expression of isoform 2 (+3.9-fold; $p=0.0038$) and isoform 3 (+3.4-fold: $p=0.0034$) of the CLU spot cluster. In the case of ZAG, the densitometry analysis performed on its isoform cluster demonstrated that isoform 1 (+2.5-fold; $p<0.0001$) was the sole isoform which was differentially expressed, as opposed to that of non-pregnant controls. As for the other ten serum proteins, including ACT and LRG, their isoforms demonstrated comparable expression pattern to that of the controls. Thus, the results of the isoform analysis performed on ACT and LRG were not in agreement with the earlier findings demonstrated by the respective proteins.

(a)



(b)

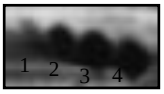
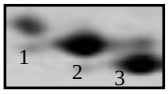
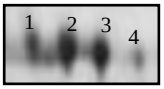
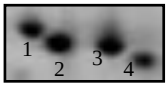
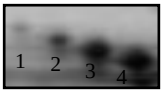
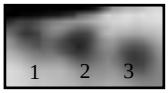
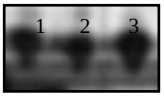
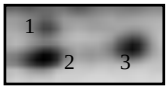
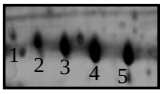
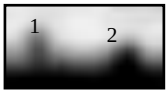
Protein	Isoforms	Protein	Isoforms
AAT		CLU	
ABG		CLU2	
AHS		KNG	
ATR		LRG	
ACT		ZAG	
HAP		HPX	

Figure 3.19: Profiles of isoforms of silver stained serum proteins. Panel (a) demonstrates the position of the proteins on a 2-DE silver stained gel. Panel (b) shows the isoform profiles of the respective proteins. Isoforms were abbreviated using numbers in ascending order starting from the acidic side of the blot.

Table 3.10: Analyses of isoforms of serum proteins in profiles of women with NP

Serum proteins	*Isoform number	Statistical analysis	
		**fold changes	***p
AAT	1	+1.3452	0.4801
	2	–0.9734	0.7651
	3	+1.0273	0.8782
	4	+1.0501	0.8934
ABG	1	+1.8353	0.0018
	2	+1.2175	0.1851
	3	+1.0109	0.9334
	4	–0.8186	0.0304
ACT	1	+1.5133	0.7932
	2	+3.5478	0.5954
	3	+5.6435	0.4261
	4	+2.732	0.5022
	5	+5.4133	0.3350
AHS	1	+1.8901	0.0469
	2	+1.8850	0.0566
	3	+1.7643	0.0178
	4	+1.2376	0.1654

Serum proteins *Isoform number Statistical analysis

		**fold changes	***p
ATR	1	–0.6193	0.0115
	2	+2.0130	0.0236
	3	+1.8797	0.0127
CLU	1	+1.3515	0.2854
	2≠	+3.9794	0.0039
	3≠	+3.4181	0.0034
CLU2	1	+1.8121	0.0164
	2	+1.9362	0.0157
	3	+1.8023	0.0113
	4	+1.6217	0.2718
HAP	1	+2.5060	0.0207
	2	+1.7609	0.0414
	3	+1.4684	0.1690
	4	+1.5695	0.1378
	5	+1.4875	0.3831
HPX	1	+1.2781	0.6341
	2	–0.7819	0.9784
KNG	1	+1.1375	0.6647
	2	–0.9341	0.8285
	3	–0.7844	0.4214

Serum proteins	*Isoform number	Statistical analysis	
		**fold changes	***p
LRG	1	+4.8425	0.0112
	2	+2.4774	0.0523
	3	+2.0559	0.0652
ZAG	$1^{\neq}$	+2.5812	<0.0001
	2	+1.5020	0.0177
	3	+3.0242	0.2109

*Isoform number	numbering of the isoform starts from the acidic side of the glycoprotein which is to the left of the diagram
**fold changes	measures the degree of change in percentage of volume contribution of each isoformover the total volume of its protein in patients as compared to controls. A minimum difference of two folds was taken as the cutting point for the variation to be considered significant
***p values	Student t-test was used to analyze the significance of differences and $p<0.01$ between groups was considered significant
+/–	increased/ decreased in expression level.
#	not detected in serum protein profiles of negative controls.
≠	isoform showing significant differential expression

3.4.2 Profiling of serum proteins of women with HM

Similar to the earlier profiling studies of the sera of women with NP, the whole serum samples of the patients with HM were subjected to 2-DE. The resulting silver stained protein profiles of patients with HM were then visually analyzed and compared to those of the control subjects. Figure 3.17(c) demonstrates a typical representative of the 2-DE serum protein profiles of patients with HM. When densitometry and statistical analyses were performed between the two groups, the protein profiles obtained from the sera of HM women appeared to be slightly different compared to the controls. Several proteins were found to be significantly altered as shown Figure 3.20 and Table 3.11. The expression of LRG, which was not significantly altered in the lectin derived profiles of patients with HM, was found to be up-regulated (+3.5-fold; $p<0.0001$) in the silver stained profiles. In addition, the expression of CLU was found to be significantly higher (+3.8-fold; $p=0.0013$) than that exhibited by the controls. The expression levels of AAT, ABG, ACT, AHS, ATR, CLU2, HAP, HPX, KNG and ZAG, on the other hand, appeared to be comparable to those of the control subjects.

Similarly the detected proteins were also analyzed for their relative distribution of isoform clusters. Table 3.12 demonstrates that the expression of isoforms of individual serum proteins of women with HM. The relative distribution of most of the protein isoforms of HM women was generally comparable with those of the non-pregnant controls. However, LRG demonstrated significantly altered expression although the relative distribution of its isoform clusters was maintained. The change in the abundance of LRG in sera of patients with HM seems to be attributed by the highly expressed isoform 1 (+5.3-

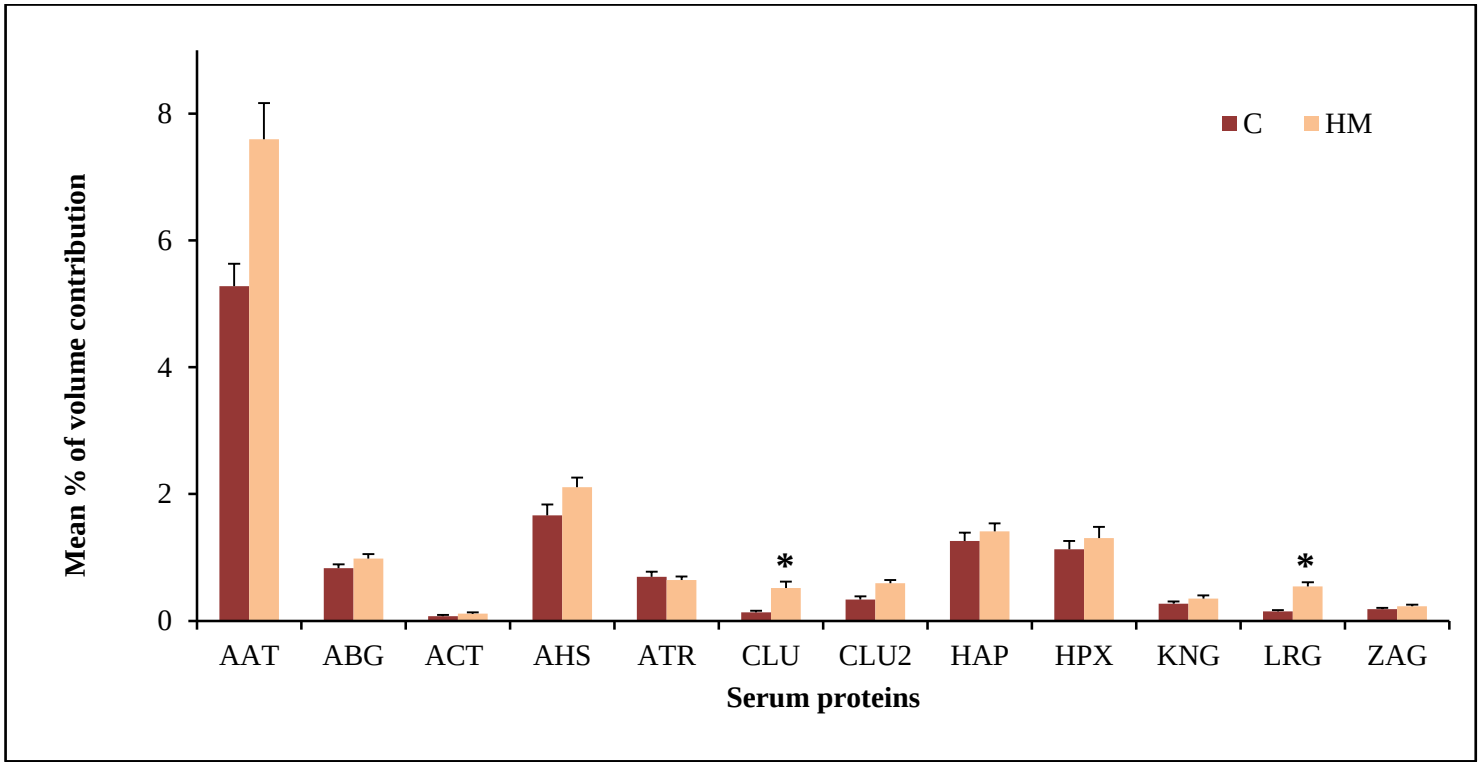


Figure 3.20: Analysis of serum protein expression of women with HM and control subjects. Silver stained protein profiles were scanned using a densitometer and resulting protein spot clusters were then analyzed using Image Master 2D platinum software (version 5.0). The percentage of contribution of each protein was calculated based on the volume of a selected protein over the total volume of all the analyzed proteins in the protein map. Asterisk denotes significantly different values when compared to controls.

fold; p=0.0009), isoform 2 (+3.9-fold; p=0.0007) and isoform 3 (+3.4-fold; p=0.0035) compared to that of non-pregnant controls. On the other hand, CLU was the sole protein showing altered distribution of its isoforms. The up-regulated expression of CLU appeared to be mainly contributed by the over-expression of its isoform 1 (+3.8-fold; p=0.0066) and isoform 2 (+5.2-fold; p=0.0029), relative to the others.

Table 3.11: Relative expression of serum proteins of women with HM

Serum proteins	**Statistical analysis**	
	fold changes	****p**
AAT	+1.4392	0.0020
ABG	+1.1798	0.1367
ACT	+1.5696	0.1869
AHS	+1.2673	0.0531
ATR	+1.0794	0.5974
CLU$^{\neq}$	+3.8695	0.0013
CLU2	+1.7639	0.0007
HAP	+1.1187	0.4246
HPX	+1.1568	0.4608
KNG	+1.2912	0.2730
LRG$^{\neq}$	+3.5991	<0.0001
ZAG	+0.4246	0.1836

*fold changes	measures the degree of change in intensities of proteins in patients compared to controls. A minimum difference of two folds was taken as the cutting point for the variation to be considered significant and is highlighted in red
**p values	Student t-test was used to analyze the significance of differences and an FDR-corrected $p<0.01$ between cohorts was considered significant.
+/-	increased/decreased in expression level
≠	isoform showing significant differential expression

Table 3.12: Analyses of isoforms of serum proteins in profiles of women with HM

Serum proteins	*Isoform number	Statistical analysis	
		**fold changes	***p
AAT	1	+1.4474	0.3971
	2	+1.044	0.8612
	3	+1.039	0.8331
	4	+1.4210	0.1409
ABG	1	+1.4951	0.5351
	2	–0.8547	0.5351
	3	+1.0877	0.6019
	4	+1.3510	0.0912
ACT	1	#	0.4291
	2	#	0.1245
	3	+4.5010	0.2011
	4	+1.2881	0.7319
	5	+1.7709	0.8364
AHS	1	+2.0490	0.0574
	2	+1.9185	0.0678
	3	+1.2197	0.5338
	4	–0.7625	0.8417

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Serum proteins	*Isoform number	Statistical analysis	
		**fold changes	***p
ATR	1	+1.2271	0.3597
	2	+1.1036	0.6087
	3	+1.4583	0.8435
CLU	1≠	+3.833	0.0066
	2≠	+5.2894	0.0029
	3	+1.7886	0.1710
CLU2	1	+1.4611	0.2046
	2	+1.8038	0.0738
	3	+1.5428	0.0799
	4	+1.5735	0.2474
HAP	1	+2.2706	0.0186
	2	+1.2439	0.4086
	3	+1.2277	0.4153
	4	+1.1709	0.4873
	5	+1.0900	0.8527
HPX	1	+1.5631	0.1327
	2	+1.0600	0.9100
KNG	1	+1.1302	0.7483
	2	–0.8832	0.3431
	3	–0.9304	0.4131

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Serum proteins	*Isoform number	Statistical analysis	
		**fold changes	***p
LRG	1≠	+5.3097	0.0009
	2≠	+3.9392	<0.0001
	3≠	+3.3536	0.0035
ZAG	1	+1.8012	0.0326
	2	+1.2694	0.1018
	3	–0.9995	0.9990

*Isoform number	numbering of the isoform starts from the acidic side of the glycoprotein which is to the left of the diagram
**fold changes	measures the degree of change in percentage of volume contribution of each isoformover the total volume of its protein in patients as compared to controls. A minimum difference of two folds was taken as the cutting point for the variation to be considered significant and is highlighted in red
****p* values	Student *t*-test was used to analyze the significance of differences andan FDR-corrected $p<0.01$between groups was considered significant
+/–	increased/ decreased in expression level.
#	not detected in serum protein profiles of negative controls.
≠	isoform showing significant differential expression

3.4.3 Comparison of protein expression in sera of women with NP and HM

In this section, the expression of serum proteins that were detected by silver staining of the 2-DE gels of women with HM was compared to those of women with NP. Figure 3.20 demonstrates the results of the volume analysis performed on the protein spot clusters of the studied groups. When subjected to statistical analysis, ACT was the sole protein found to be significantly down-regulated (–0.37-fold; p=0.0003) in the women with HM compared to the women with NP. The expression of the other eleven protein spot clusters in the 2-DE gels appeared to be not significantly different between the two groups of pregnant women as shown in Table 3.13. Subsequent analysis on the relative expression of isoforms of the protein clusters also demonstrated that none of the isoform of the analyzed proteins in the women with HM was differentially expressed, relative to those of NP women.

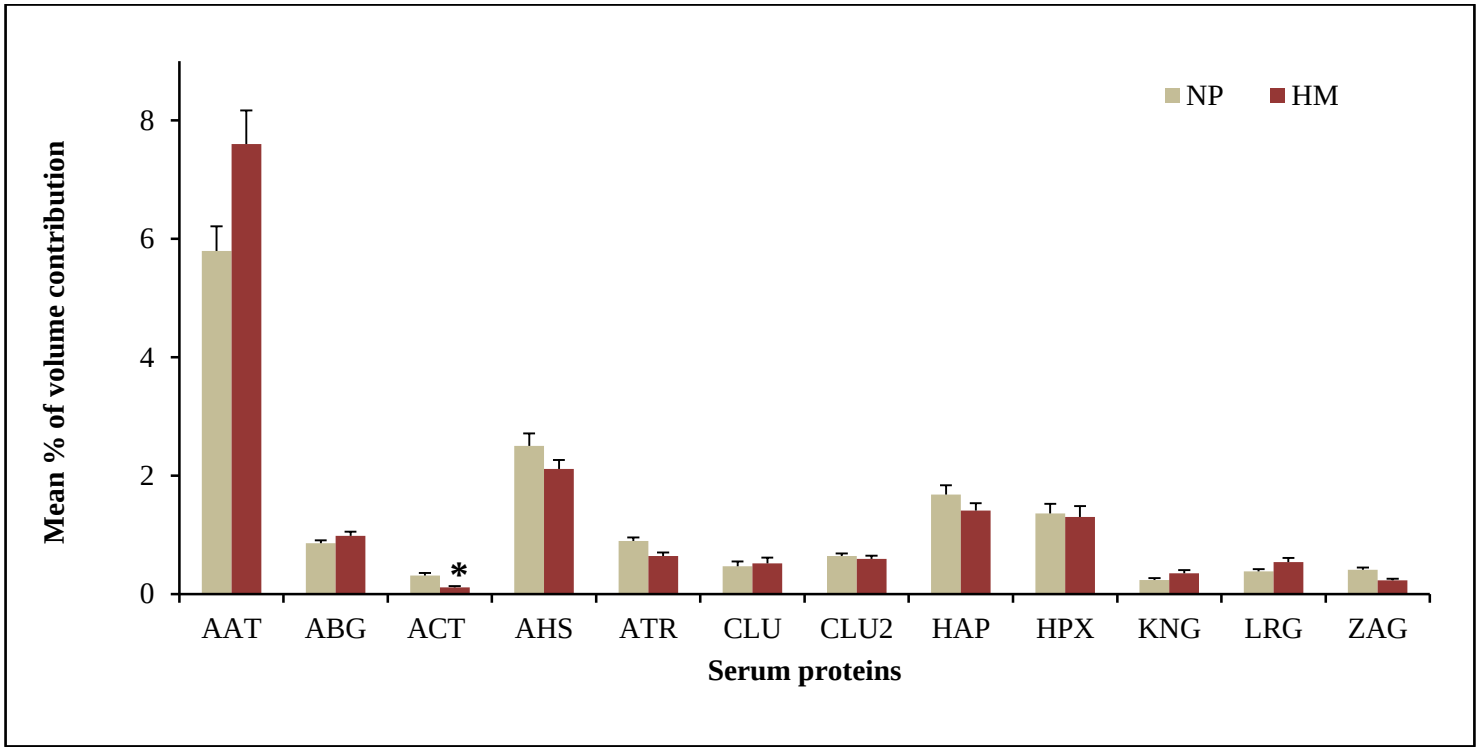

Figure 3.21: Analysis of serum protein expression of women with NP and HM. Silver stained 2-DE serum protein profiles were scanned using a densitometer and resulting protein spots were then analyzed using Image Master 2D platinum software (version 5.0). The percentage of contribution of each protein was calculated based on the volume of a selected protein over the total volume of all the analyzed proteins in the protein map. Asterisk denotes significantly different values when comparative analysis was performed between the two groups of pregnant women.

Table 3.13: Relative expression of serum proteins of women with HM compared to women with NP

Serum proteins	Statistical analysis	
	fold changes	**p
AAT	+1.3108	0.6866
ABG	+1.1418	0.1629
ACT≠	–0.3674	0.0003
AHS	–0.8429	0.1319
ATR	–0.7187	0.0035
CLU	+1.1026	0.7026
CLU2	–0.9254	0.4779
HAP	–0.8383	0.1847
HPX	–0.9567	0.8059
KNG	+1.4653	0.0767
LRG	+1.4099	0.0359
ZAG	–0.5639	0.0003

*fold changes	measures the degree of change in intensities of proteins in patients compared to controls. A minimum difference of two folds was taken as the cutting point for the variation to be considered significant and is highlighted in red
**p values	Student t-test was used to analyze the significance of differences and an FDR-corrected $p<0.01$ between cohorts was considered significant.
+/-	increased/decreased in expression level
≠	isoform showing significant differential expression

Table 3.14: Analyses of isoforms of serum proteins in profiles of women with HM compared to women with NP

Serum proteins	*Isoform number	Statistical analysis	
		**fold changes	***p
AAT	1	+1.1024	0.6281
	2	+1.0115	0.9671
	3	+1.3533	0.3669
	4	+1.0760	0.6939
ABG	1	–0.8974	0.7169
	2	–0.8229	0.4871
	3	+1.1211	0.5531
	4	+1.7111	0.0791
ACT	1	#	0.1907
	2	#	0.2383
	3	–0.7976	0.4788
	4	–0.4715	0.2747
	5	–0.3271	0.1461
AHS	1	+1.0841	0.8088
	2	+1.0177	0.9669
	3	–0.6913	0.3289
	4	–0.7625	0.3881

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Serum proteins	*Isoform number	Statistical analysis	
		**fold changes	***p
ATR	1	+1.9146	0.0049
	2	–0.5733	0.0094
	3	–0.5170	0.0119
CLU	1	+2.8358	0.1292
	2	+1.3292	0.3816
	3	–0.5233	0.0500
CLU2	1	–0.8663	0.4034
	2	–0.8108	0.6283
	3	–0.8559	0.5976
	4	–0.9703	0.9296
HAP	1	–0.9061	0.7876
	2	–0.7064	0.3304
	3	–0.8361	0.5719
	4	–0.7460	0.3049
	5	–0.7328	0.5849
HPX	1	+1.5139	0.8179
	2	+1.9991	0.5443
KNG	1	+1.0065	0.9865
	2	+1.0493	0.2883
	3	–0.8431	0.1057

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Serum proteins	*Isoform number	Statistical analysis	
		**fold changes	***p
LRG	1	+1.0965	0.8805
	2	+1.5900	0.1868
	3	+1.6312	0.2311
ZAG	1	–0.6979	0.1756
	2	–0.8452	0.3961
	3	–0.3305	0.3964

*Isoform number	numbering of the isoform starts from the acidic side of the glycoprotein which is to the left of the diagram
**fold changes	measures the degree of change in percentage of volume contribution of each isoformover the total volume of its protein in patients as compared to controls. A minimum difference of two folds was taken as the cutting point for the variation to be considered significant and is highlighted in red
****p* values	Student *t*-test was used to analyze the significance of differences andan FDR-corrected $p<0.01$between groups was considered significant
+/–	increased/ decreased in expression level.
#	not detected in serum protein profiles of negative controls.
≠	isoform showing significant differential expression

3.5 Identification of aberrantly expressed proteins

In the preceding sections, the serum proteins were generally identified based on visual comparison either with the already established CGB lectin resolved protein maps or the standard SWISS ExPASy plasma protein reference map. The aberrantly expressed protein spots which were visually identified in this study included ACT, CLU, LRG and ZAG. In the present study, the four protein spot clusters were subjected to mass spectrometry analysis for confirmation of their identities.

For the identification of the ACT and LRG spots that were detected on the nitrocellulose membranes, a similar approach to that employed for the identification of the ITIH4f was performed. Pooled serum samples of women with NP were subjected to CGB lectin affinity chromatography prior to 2-DE separation. Subjecting the spots of interest to MS/MS analysis and NCBInr database search identified them as ACT and LRG. Table 3.15 (panel a) provides the information regarding the number of matching peaks, MASCOT score, sequence coverage and accession numbers as listed in the NCBInr database for each of the identified proteins.

In order to validate the identities of ACT, CLU, LRG and ZAG which showed significant statistical difference after analysis of their silver stained serum protein profiles, 2-DE run was repeated using pooled sera of women with NP. However, in this experiment, the 2-DE gel was stained with Coomassie Blue staining since it was the preferred method of staining for mass spectrometry analysis. Similarly, the peptide sequences obtained from the MS/MS analysis were searched against the NCBInr database and the results obtained from the MASCOT search gave positive identification for ACT, CLU, LRG and ZAG. Information regarding the number of matching peaks, MASCOT

score, sequence coverage and accession numbers as listed in the NCBInr database are shown in Table 3.11 (panel b).

Table 3.15: Mass spectrometric identification of aberrantly expressed serum proteins

Accession number*	Protein matched	No. of peaks matched	MASCOT score	Sequence coverage
P01011	ACT[(a)]	2	128	10%
P02750	LRG[(a)]	4	326	18%
P01011	ACT[(b)]	8	522	35%
P10909	CLU[(b)]	5	249	24%
P02750	LRG[(b)]	6	414	29%
P25311	ZAG[(b)]	4	284	13%

* Accession number is from the MASCOT database (www.matrixscience.com)
[(a)] Isolated from gels of samples subjected to CGB lectin affinity chromatography prior to 2-DE separation
[(b)]) Isolated from gels of whole serum samples subjected to 2-DE separation

3.6 Validation of aberrantly expressed serum proteins by immunoblotting

For confirmation of the aberrantly expressed proteins in the serum samples of women with NP and HM, an immunoblotting technique was employed. Western blotting was performed using commercially available antibodies against four of the differentially expressed serum proteins. In this study, proteins which were identified as significantly up-regulated in the sera of women with NP were ACT, CLU, LRG and ZAG. On the other

hand, CLU and LRG were the only proteins found to be abundantly expressed in the sera of women with HM.

The results obtained from the Western blotting and antibody detection of serum samples of the group of women with NP and HM and their non-pregnant controls are demonstrated in Figure 3.15a-d. Panel (a) of Figure 3.21 demonstrates a representative nitrocellulose membrane with the transferred SDS-PAGE separated proteins that were probed with anti-ACT. Following a reaction with anti-ACT, an intense band with a molecular weight of approximately 70 kDa was detected for each of three lanes which consisted of serum proteins from control, NP and HM women, respectively. When the resolved bands were subjected to densitometry analysis, the expression of ACT in the pooled sera sample of women with NP seemed to be significantly higher (1.7-fold; $p=0.005$) than that of the control women.

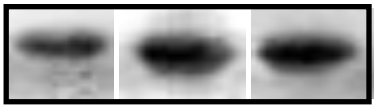
Panel (b) of Figure 3.21 represents an anti-CLU detected nitrocellulose membrane obtained from this study. When the HRP conjugated antibody towards CLU was interacted to the electrophoretically transferred serum proteins on the blot, an intensely resolved band with a relative molecular weight of approximately 60,000 was detected. Subsequent analysis performed on the bands resolved from the sera of control, NP and HM women managed to demonstrate the over-expression of CLU in the serum samples of women with NP (+2.2-fold; $p=0.0039$) as well as in women with HM (+2.7-fold; $p=0.0028$), relative to that of the controls.

Similarly, the expression of LRG was analysed by Western blotting using anti-LRG. Observed from the reaction was a band resolved with a molecular weight of approximately 38,000 [Figure 3.21 (c)]. From the analysis performed on the bands of the controls as well

(a)

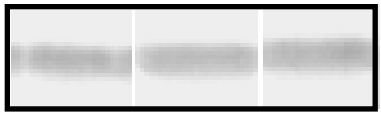
C NP HM

(b)



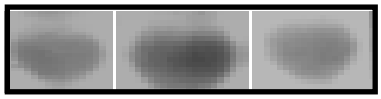
C NP HM

(c)



C NP HM

(d)



C NP HM

Figure 3.22: Representative Western blots for ACT, CLU, LRG and ZAG**.** Pooled serum samples of controls (C), women with NP (NP) and HM (HM) at concentrations of 4 µg/ml were separated on 12.5% SDS-PAGE gels. Resulting bands were transferred to electrophoretically onto nitrocellulose membrane. Immunodetection of the resolved protein bands were performed using their respective antibodies. Panels (a), (b), (c) and (d) demonstrate the immunoblots for ACT, CLU, LRG and ZAG, respectively.

as the women with NP and HM, the expression of LRG appeared to be altered in both samples of the NP (+1.5-fold; p=0.0096) as well as the HM (+1.5-fold; p=0.0094) women, relative to that of the control subjects.

When the immunoassay was performed using anti-ZAG, the antibody detected serum ZAG was resolved as a band with a molecular weight of 34,000. Higher levels of ZAG was significantly detected for women with NP (2.6-fold; p=0.0041) as compared to the controls [Figure 3.21 (d)].

3.7 Development of a multiplex immunoassay

The data of the present study demonstrates the different altered expression of serum proteins associated with the two different types of pregnancy. These findings were shown by subjecting serum samples to 2-DE followed by detection of the separated proteins either via silver stain or enzyme conjugated CGB lectin. These approaches, however, were suitable for the analysis of samples of small sizes. In cases of larger sample populations, an assay platform which allows for high through-put analysis appeared to be more feasible. Amongst the high through-put assay platforms that are available today is the multiplex immunoassay.

The multiplex immunoassay still maintains the traditional immunoassay principles which involved coupling of proteins with simple quantitative antibody-based assays. However, unlike other conventional immunoassay techniques, the multiplex assay is characterized by presence of multiple capture ligands which are immobilized in parallel. This permits simultaneous quantification of multiple serum proteins and at the same time,

eliminates multiple sample preparations. Therefore, at this point of the study, it seemed interesting to test the viability of the multiplex assay in detecting aberrantly expressed serum proteins in relation to the studied pregnancy conditions.

Hence, in the present work, an in-house competitive multiplex assay was designed using the four differentially expressed serum proteins, i.e. ACT, CLU, LRG and ZAG. Also included was four other target proteins comprising of AAT, AHS, ATR and ceruloplasmin (CLP), which acted as negative controls. In the prepared multiplex assay, a target protein was conjugated to the surface of a set of color coded microbeads. The different sets of protein-conjugated microbeads were then mixed together with the serum samples and the detector molecules. The detector molecules, which were biotin labeled antibodies, reacted with their own proteins irrespective of whether they are proteins circulating in the serum or proteins immobilized on the microbeads. Prior to detection, the antibody-serum protein complexes were removed from the reaction mixture, leaving the complexes on the beads to be enumerated by the array reader. The array reader then identifies each specific reaction based on the bead color and quantitates it.

3.7.1 Validation of conjugated beads functionality

In the initial part of the study, the target proteins were coupled to the surface of the microbeads. Once the coupling reaction has been completed, the functionality of the coupling reaction needs to be validated as described in section 2.14.1.3. A successful coupling typically yields a fluorescent intensity signal greater than 2000 median fluorescent intensity (MFI).

Out of the eight sets of protein conjugated microbeads prepared in this study, only three sets of microbeads ie. ATR, CLP and CLU demonstrated successful protein-bead coupling (Table 3.16). The other five proteins, however, were not successfully conjugated onto the beads, based on their low MFI values.

Table 3.16: Validation of microbeads analytical performance

Assay	Signal intensity (MFI) from different concentration of proteins					
	2 µg	**5 µg**	**7 µg**	**9 µg**	**10 µg**	**12 µg**
AAT	30	73	-	-	-	-
ACT	137.5	52	48.3	39.3	19.8	15
AHS	20.9	56	-	-	-	-
ATR	-	66.5	10783	-	-	-
CLP	-	6314.5	5160	5298.5	-	-
CLU	66	1745.8	-	-	3132.5	-
KNG	37	50	115	-	-	179
ZAG	-	93	25	-	-	-

(-) and signal intensity of less than 2000 MFI indicate unsuccessful conjugation

3.7.2 Evaluation on the specificity of the multiplex assay

Prior to multiplexing of the three sets of successfully coupled microbeads, each of the sets of beads was analyzed for their specificity. In general, to determine the specificity of the beads, the protein conjugated beads were challenged to the various detector antibodies involved in the multiplex assay. Specificity of the protein conjugated beads was

demonstrated by absence of any cross-reactivity between the beads and the detector antibodies of the other target proteins.

Table 3.17 shows the MFI signals and percentages of cross-reactivity of the assay performed. From the table, the three sets of beads appeared to display very low percentage of cross-reactivity (<1%) with antibodies directed against other proteins but exhibited strong reactivity with their own antibodies.

Table 3.17: Specificity of multiplex assay

Assay	Anti-ATR		Anti-CLP		Anti-CLU	
	Signal intensity (MFI)	**Cross-Reactivity (%)**	**Signal intensity (MFI)**	**Cross-Reactivity (%)**	**Signal intensity (MFI)**	**Cross-Reactivity (%)**
ATR	8165.3	100	5	0.13	3.5	0.22
CLP	42	0.51	3948.5	100	2	0.13
CLU	45.5	0.55	1	0.03	1555.8	100
Blank	4	-	2	-	4	-

Reactivity level of the beads to each antibody is demonstrated in terms of raw MFI values or represented as percentage of each MFI value over the MFI value generated from homologus protein-antibody interaction

3.7.3 Optimization of sample dilution

One of the essential issue that was addressed in the development of the multiplex assay was the selection of sample dilution factor. Optimal dilution factor needs to be predetermined to enable measurement of each protein constituent within the assayed samples. Therefore, pooled control sera were diluted using five different dilution factors.

In this study, the assay was conducted using a multiplex suspension of the three sets of microbeads and was run in a competitive assay manner. Results from the multiplex assay are summarized in Table 3.18. Based on the MFI values exhibited in the table, it appeared that diluting the serum samples using larger dilution factors (1/4 and 1/20) resulted in low MFI values. In contrast, higher signal intensities were obtained when smaller dilution factors were employed. From the MFI values exhibited in the Table 3.18, 1/50 to 1/200 dilution factors appeared to be the appropriate factors to use for the study. However, since the assay runs in a multiplex format, the dilution factor of choice should give optimal MFI values for all the target proteins involved. Hence, amongst the four ratios, 1:50 appeared to be the best ratio to be used in preparation of subsequent serum samples.

Table 3.18: MFI values in response to various dilutions of serum sample

Assay	**Signal intensity in response to samples with different dilution factors (MFI)**				
	1/4	**1/20**	**1/50**	**1/100**	**1/200**
ATR	31	74	1717.5	2336	2139
CLP	33	165.3	359.5	262	190
CLU	92.5	329.3	427.3	316.5	225.5

3.7.4 Assay sensitivity evaluation

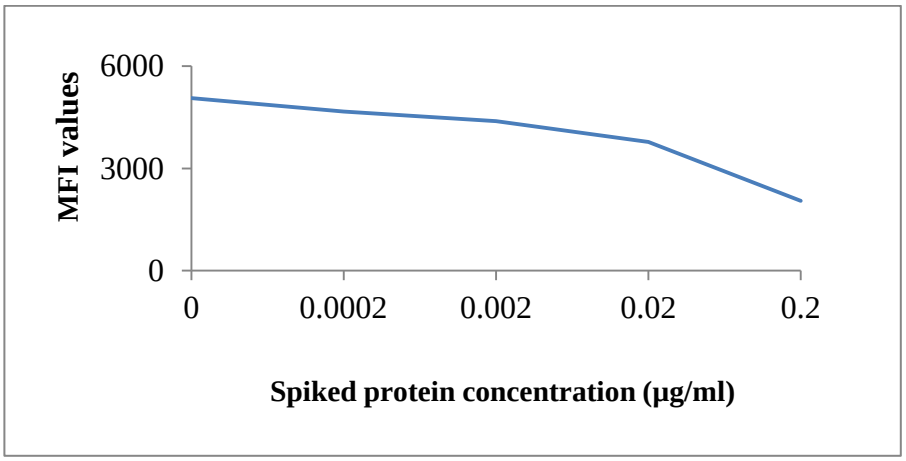
To evaluate the sensitivity of the developed multiplex immunoassay, serum samples spiked with different concentrations of proteins were measured in duplicates using the competitive immunoassay method. In the present study, the antibodies were incubated in the presence of their target proteins which were immobilized on the beads or within the serum samples. The immobilized proteins compete for the antibody binding sites with the serum proteins. Subsequent washing step will then remove any unbound antibodies as well as the serum antigen-antibody complexes. Hence, the higher the serum protein concentration, the weaker the signal intensity produced by the developed assay.

Figure 3.23 demonstrates the MFI values derived from the sensitivity test of the competitive multiplex assay. Based on the results, the developed multiplex assay was shown to be capable of recovering the spiked protein samples and thus have appropriate accuracy to be used to detect corresponding proteins in the sera.

3.7.5 Assay reproducibility evaluation

Reproducibility of an assay refers to the degree of agreement between measurements conducted on replicate samples due to differences in operator behaviour. The reproducibility of a multiplex assay is reflected by its intra- and inter-assays coefficient of variation (%CV). In this study, both the intra (within run) as well as inter (between run) variability were investigated using control samples and the results are shown in Table 3.19. Based on the %CV values obtained, the %CV for the intra-assay was found to range from

(a)



(b)

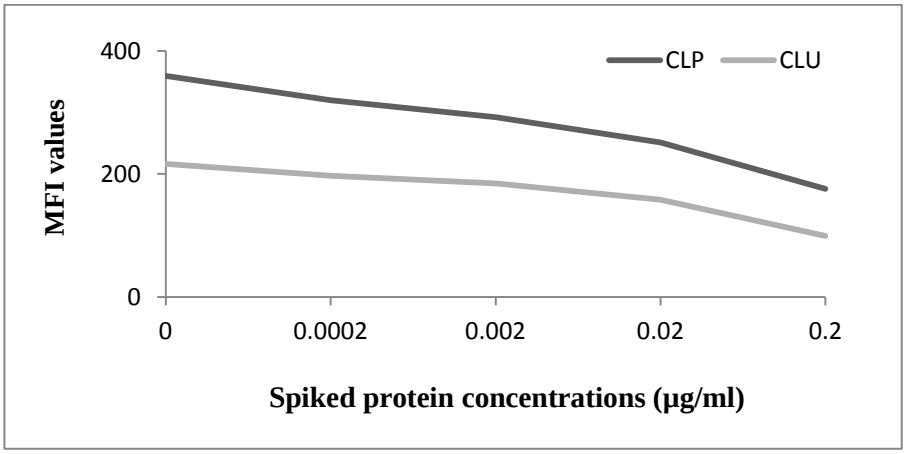


Figure 3.23: Analyses of MFI values derived from the multiplex assay sensitivity test. The sensitivity of the developed multiplex immunoassay was performed with a competition step. The MFI generated was from normal human sera spiked with ten-fold dilution of protein with initial concentration of 0.2 µg/ml. Panel (a) demonstrates the MFI values from response of various concentrations of ATR to the assay. Panel (b) demonstrates the MFI values obtained from response of various concentrations of CLU and CLP to the multiplex assay.

11.3 to 12.5% for the three target proteins. The inter-assay %CV which indicates the plate to plate variation ranged from 3.9 to 28.3%. And since the acceptable %CV for intra- and inter-assays should be <30% for the assay to be considered reproducible, the prepared multiplex assay appeared to be within acceptable limits.

Table 3.19: Reproducibility of the multiplex immunoassay

Assay	Intra-assay variation* Mean %CV	Inter-assay variation# Mean %CV
ATR	12.04	9.43
CLP	11.28	3.93
CLU	12.47	28.28

*Intra-assay variation indicates variation within plates
#Inter-assay variation indicates variation between assays

3.7.6 Protein profiling of serum proteins using competitive multiplex assay

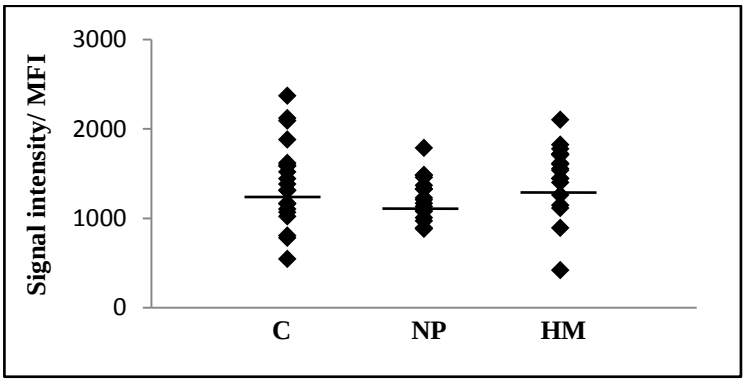
The developed multiplex assay was used to profile the expression level of ATR, CLP and CLU in serum of controls as well as women with NP and HM. For each serum sample, assay was performed in duplicates. The signal intensities obtained reflect the serum protein levels and was compared to those of controls for differences in their signal intensity.

Figure 3.24 demonstrates results of the competitive multiplex immunoassay performed with the protein conjugated microbeads. The MFI values of ATR for both cohorts of pregnant women were found to be comparable to that of controls. Statistical

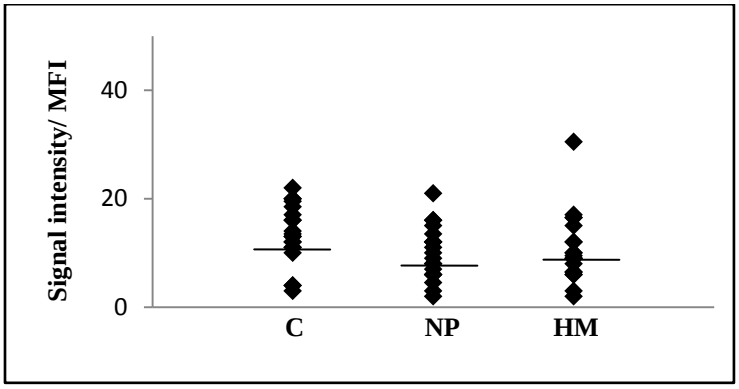
analysis conducted on the MFI values yielded no significant differences in sera of NP and HM women as opposed to their non-pregnant controls. When the MFI values of CLP from sera of women with NP and HM were compared with their non-pregnant controls, no significant differences were observed between the various groups to the controls.

While in the case of CLU, there is a trend of lower MFI values in both groups of pregnant women. Lower MFI values of CLU were significantly detected in women with NP (1.5-fold; $p<0.0001$) and HM (1.5-fold; $p<0.0001$), relative to the controls. Taking note that this assay runs in a competitive mode, the decrease in MFI values would be interpreted as an increase in CLU expression level. Thus, the over-expression of CLU demonstrated by the multiplex assay was in agreement with earlier results derived from analysis of 2-DE silver stained gels

(a)



(b)



(c)

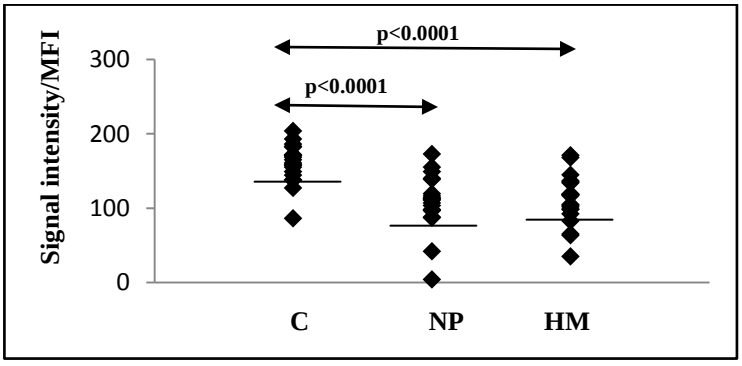


Figure 3.24: Analysis of serum protein expression by competitive multiplex assay. Multiplex immunoassay was performed using sera of women with NP (NP) and HM (HM) as well as control subjects (C). Panels (a), (b) and (c) demonstrate the results of the competitive multiplex assay for ATR, CLP and CLU, respectively.

CHAPTER 4:

DISCUSSION

4.1 Champedak galactose binding lectin

In 1991, Hashim *et al* had reported the discovery of a novel galactose binding lectin in the extract of the champedak (*Artocarpus integer*) seeds. The lectin, termed as CGB (champedak galactose binding) lectin, was found to possess a similar general structure, and to a certain extent binding specificity to jacalin, a lectin purified from the seeds of jackfruit (*Artocarpus heterophyllus*). The CGB lectin was observed to be capable of recognizing and binding to the O-linked Galß1-3GalNAc structure of IgA1 and C1 inhibitor molecules (Hashim *et al*., 1991, 1993 and 1994). Based on its unique reactivity towards the O-glycans, the CGB lectin has been extensively used to study the differential expression of serum O-glycoproteins in selective groups of patients with cancers as well as other diseases (Jayapalan *et al*., 2012; Mu *et al*., 2012; Abdullah-Soheimi *et al*., 2010; Mohamed *et al*., 2008 (this thesis); Abdul-Rahman *et al*., 2007; Hashim *et al*, 2001a; Hashim *et al*, 2001b; Shuib *et al*., 1998).

Amongst the several studies conducted using the CGB lectin, of interest was the work of Abdul-Rahman *et al*. (2007). In their work, they have highlighted the enhanced expression of a CGB lectin-detected 35 kDa protein, termed LDP1, in the serum samples of patients with endometrial cancer (EACa) compared to the controls. This protein was not detected when serum samples of the patients and control subjects were profiled using the conventional 2-DE approach which involved silver staining. In this regard, it is of interest to identify the LDP1. However, identification of a protein that has been electroblotted onto a nitrocellulose membrane and reacted with an enzyme conjugated lectin was not a straightforward task. There were several shortcomings associated with the direct use of such protein, including low accessibility of digestive enzymes and reagents to the membrane-trapped protein. In addition, presence of the HRP-CGB lectin and other

reagents that were used earlier in the development of the blots may also interfere with the performance of the mass spectrometer. Hence, in the present work, a different approach was adopted in the attempt to identify the protein of interest. O-glycosylated fractions containing the protein of interest, LDP1, was thus isolated by subjecting pooled serum samples of patients with EACa to a CGB lectin affinity chromatography followed by 2-DE.

4.1.1 Isolation and purification of CGB lectin

In order to use the CGB lectin to isolate and identify LDP1 in the pooled sera of patients with EACa, the lectin had to be prepared from the seeds of *Artocarpus integer*. Therefore, in the initial part of the study, a crude extract of the champedak seeds was subjected to affinity separation on immobilized galactose. The procedure for the isolation of the CGB lectin was essentially the same as that of Abdul Rahman *et al*. (2002). In both studies (Abdul Rahman *et al*. (2002) and ours), the CGB lectin was purified using a galactose-Sepharose affinity chromatography column, which was a modification from an initial protocol that was used to isolate similar lectin. Earlier, Hashim *et al*. (1991a and b) had purified their CGB lectin using an IgA1-Sepharose affinity column. However, using galactose as a ligand to capture the CGB lectin offered a more cost-effective method compared to using IgA1.

Similar to any extraction process of a plant lectin, the seeds must first be ground to the form of powder (Hermanson *et al*., 1992). Therefore, the champedak seeds was dried and ground prior to ammonium sulphate precipitation. Subsequent centrifugation and solubilisation of the seed precipitate yielded a yellowish supernatant containing a mixture of lectins and other compounds including the CGB lectin. To isolate the lectin of interest,

its carbohydrate-binding specificity towards galactose was exploited in the use of a galactose-affinity chromatography column. The prepared supernatant was passed slowly through a galactose-affinity column, allowing the CGB lectin to bind to the galactose ligands. Subsequently, the bound CGB lectin was desorbed with an elution buffer. The approach employed in this experiment involved displacement of the bound material by competition with a counter ligand. The counter ligand is either the same as the ligand itself or of similar structure so as it will effectively compete for the binding sites on the adsorbed molecules (Hermanson *et al*., 1992). Therefore, in our study, D-galactose was dissolved in PBS at a concentration of 0.8 M to break the CGB lectin-galactose interaction and elute the bound lectin off the column. Following displacement of the lectin from the column, the purified CGB lectin, which was still in the elution buffer, was dialysed against PBS and later pooled and freeze-dried.

4.1.2 Characterisation of CGB lectin

Prior to the application of the purified CGB lectin, it was important to ascertain that the lectin was of high quality. Hence, the prepared CGB lectin was analysed in terms of its purity and reactivity and compared with those reported from previous studies.

In the present study, the CGB lectin, when subjected to SDS-PAGE, was found to comprise two subunits. This is consistent with the earlier findings of Hashim *et al*. (1991). In their SDS-PAGE analysis of the purified CGB lectin, the lectin was demonstrated to consist of a combination of two types of non-covalently linked subunits with apparent molecular weights of 13.3 and 16 kDa. However, noted was a slight discrepancy in terms of the molecular weights of the resolved bands. In the present study, the CGB lectin

appeared to migrate into two bands with molecular weights of approximately 14 and 16.5 kDa. This minor molecular weight disparity may be attributed to the different percentage of polyacrylamide and/or differences in the chemicals and standards used during the run.

The mass of a protein could also be determined using mass spectrometry approaches such as the MALDI, ESI and SELDI. These techniques have been gaining popularity as they could accurately determine masses at the parts per million (ppm) levels (Dass, 2001). Therefore, to further corroborate the finding from the SDS-PAGE analysis, the molecular mass of the CGB lectin was also determined using SELDI-MS. SELDI mass spectrum profile obtained from this study showed two successive sharp peaks within the mass range of approximately 14 and 16 kDa. These peaks appeared to possess masses which correlate well with the masses of the two reported subunits of the CGB lectin. In 2002, Abdul Rahman *et al.* had sequenced these two subunits and reported that there exists 95% sequence homology for the larger subunit of the CGB lectin with the α1 subunits of jacalin. The larger subunit was thus designated as α1, while the smaller subunit was termed as ß1 (Abdul Rahman *et al*., 2002).

The presence of the smaller peaks with masses of 7 and 8 kDa in the SELDI mass spectrum profile, may be linked to the formation of doubly charged molecules of the respective subunits during ionisation of the target molecule (Merchant and Weinberger, 2000), and in this case, the CGB lectin. Since there was no other bands or peaks observed in the SELDI-MS analysis, the CGB lectin that was prepared in this study can be concluded to be of high purity.

In view of the high purity of the CGB lectin that was isolated in the present study, it was therefore possible to crystallize the lectin. As documented by Gabrielsen *et al*. (2009; this thesis), the highly purified solution of the CGB lectin managed to be crystallized at 293K in two space groups, $P2_1$ and $P2_12_12_1$. In addition, the resulting crystals were found to diffract to resolutions of 1.65 and 2.6Å, respectively. And since a protein crystal plays a pivotal part in the structural and functional studies of the respective protein, crystallographic study had been further conducted comparing the CGB lectin crystal with that of jacalin. The results of this study generally demonstrate that the CGB lectin is more specific in its binding to galactose than jacalin (Gabrielsen *et al*., personal communication).

Determination of the functional property of the CGB lectin was assessed by putting it to challenge with a panel of different types of human serum proteins and immunoglobulins. The panel that was chosen consisted of glycoproteins with established O- (such as IgA1 and AHS) and N-linked glycan moieties (such as IgA2 and IgM) as well as a non-glycosylated protein (albumin). Simple double diffusion test showed that the purified CGB lectin interacted strongly with AHS and IgA1 but were completely non-reactive with IgA2 and IgM. The reactivity of the CGB lectin to these glycoproteins had also been described previously (Abdul Rahman *et al*., 2002; Hashim *et al*., 1993). The preferential binding of the CGB lectin to AHS and IgA1 were not surprising as presence of O-glycan in the structures of these two glycoproteins had been documented (Van den Steen *et al*., 1998). And since IgA2 and IgM do not possess any O-glycans but harbor N-linked oligosaccharide moieties instead (Arnold *et al*., 2007; Field *et al*., 1994), they were unable to interact with the CGB lectin.

4.2 Identification of the LDP1

One of the primary requirements for protein identification using mass spectrometry approach is that the protein is of high purity. Hence, to identify the protein of interest, the LDP1 had to be isolated from a cocktail of proteins in the serum samples of patients with EACa. Based on the strong reactivity of the LDP1 to the CGB lectin (Abdul-Rahman *et al*., 2007), isolation of the protein was performed by subjecting pooled sera of patients with EACa to a CGB lectin affinity chromatography. Hence, the purified CGB lectin was used in the preparation of a CGB lectin affinity chromatography column.

The specific binding of CGB lectin to the Gal and GalNAc residues renders the affinity column capable of isolating serum proteins harbouring O-glycan moieties (Abdul Rahman *et al*., 2002). Therefore, when the pooled serum samples were loaded into the CGB lectin affinity column, serum O-glycoproteins including the LDP1 were bound to the lectin ligands. In the presence of 0.1 M melibiose which acted as a counter ligand, bound fractions consisting of CGB lectin reactive serum proteins were eluted out. Separation of the LDP1 protein from the mixture of CGB lectin-reactive proteins was subsequently performed by subjecting the bound fraction to 2-DE. Following electrophoresis, visualization of the separated proteins was performed using Coomassie stain. Based on reports by Abdul-Rahman *et al*. (2007) the LDP1 spots were located and excised prior to MS/MS analysis.

4.2.1 Identification of the LDP1 protein

When the LDP1 was subjected to MS/MS analysis and subsequent database search, the protein was initially identified as inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4).

The ITIH4, also previously denominated as sgp120, PK-120 and IHRP, is a member of the inter-alpha-trypsin inhibitor (ITI) family (Pineiro *et al*., 1999; Salier *et al*., 1996). This protein has been reported to be a 120 kDa (Gonzalez-Ramon *et al*., 1995) acute phase glycoprotein of hepatic origin (Nishimura *et al*., 1995). Interestingly, ITIH4 is the only member of the ITI family which harbors a kallikrein-released bradykinin-like domain on its C-terminal sequence (Nishimura *et al*., 1995) making it plasma kallikrein sensitive (Cai *et al*. 1998; Hashimoto *et al*.,1996; Saguchi *et al*.,1995). It has been reported that trace amount of plasma kallikrein will first cleave the ITIH4 to yield two fragments, *i.e*., the C-terminal 35 kDa and the N-terminal 85 kDa fragments (Saguchi *et al*., 1995). The 35 kDa ITIH4 fragment, which is O-glycosylated (Nishimura *et al*., 1995; Pu *et al*., 1994), is assumed to remain intact. However, the 85 kDa ITIH4 fragment is further cleaved to produce an N-terminal 57 kDa fragment and a putative 28 kDa fragment. The latter is believed to be further processed by protease(s) to generate smaller fragments (Song *et al*., 2006; Pu *et al*., 1994).

However, in the present study, the LDP1 spot cluster that was detected on the 2-DE gel appeared to be resolved at a much lower molecular weight region than 120 kDa. In the Coomassie stained 2-DE gel, the LDP1 appeared to demonstrate a relative molecular mass of approximately 35,000. Therefore, the serum LDP1 protein that was detected using the CGB lectin was not the intact ITIH4 but most likely a fragment derived from the kallikrein proteolytic activity onto the ITIH4 molecule. In addition, based on the MS/MS derived sequences, 14 out of the 15 peptide sequences appeared to be from the 35 kDa fragment region of ITIH4. As such, this strongly suggested that the LDP1 spot cluster detected by the CGB lectin was actually the 35 kDa cleavage fragment of ITIH4. This notion is further supported by the presence of three O-linked oligosaccharides at positions 696, 701 and 702

within the 35 kDa fragment (Nishimura *et al*, 1995; Pu *et al*., 1994) which explains its interaction to the CGB lectin.

4.3 Prevalence of the 35 kDa ITIH4f

In this study, the expression of the 35 kDa ITIH4 fragment (ITIH4f) was analyzed using the well established enzyme-conjugated CGB lectin-probed 2-DE electrophoretic approach. Five groups of patients with different cancers were subjected to analysis; OsSa, NPCa, BrCa, GOCa, and EOCa. Out of the five groups studied, only three of them demonstrated significant enhanced expression of the 35 kDa cleavage fragment. Using similar approach, Abdul-Rahman *et al*. (2007) have previously shown that the ITIH4f was only up-regulated in the sera of patients with EACa, whilst its expression in the groups of patients with SCCa and ACCa were comparable to the controls. Hence, the results from the present study when taken together with the previous findings of Abdul-Rahman *et al.* (2007) demonstrated that the 35 kDa ITIH4f appeared to be enhanced selectively in cancers like BrCa, EOCa, GOCa and EACa but not in NPCa, OsSa, ACCa and SCCa.

One of the differences between the groups of cancers showing up-regulated expression of their 35 kDa ITIH4f with those that did not is that the former is associated with estrogen dysregulation (Key *et al*., 2001; Henderson and Feigelson, 2000). The idea that endogenous sex steroid hormones can, under wrong circumstances, contribute to neoplastic transformation has been dwelled upon for a long time (Henderson *et al*., 1982). Besides clinical research, there have been a lot of cross-sectional as well as epidemiological studies (Greenwald and Dunn, 2009; Ganmaa and Sato, 2005) conducted to investigate the relationship of sex-steroids on the outcome, risk and control of cancer. To date, the best

studied sex-hormone is estrogen and it has been implicated in carcinogenesis of several different cancers (Greenwald and Dunn, 2009), being most evident in cancers of the breast (Key *et al*., 2002; Schairer *et al*., 2000), endometrium (Allen *et al*., 2008; Weiderpass *et al*., 1999) and ovary (Lukanova and Kaaks, 2005; Persson and Riman, 2000). Therefore, it was speculated that the 35 kDa ITIH4f was over-expressed in cancers under the influence of estrogen dysregulation. In such a case, however, there is a possibility that the abundance of the particular ITIH4f may not be restrictive to patients with cancer but may also be present in other diseases associated with the increased levels of estrogen.

To investigate the possibility that the 35 kDa ITIH4f may also be over-expressed in non-cancer conditions that are associated with enhanced levels of estrogen, the expression analyses was extended to include groups of non-cancer patients with similar hormonal dysregulation. In this study, two groups of pregnant women undergoing estrogen dysregulation were chosen. Both groups of subjects were with increased levels of estrogen, with the former representing a normal condition, while the latter a benign condition (Ahmed *et al*., 2000). Throughout a normal pregnancy, the maternal levels of circulating estrogens increases continuously as it is required to support fetal development (Takeyama *et al*., 2001; Loriaux *et al*., 1972). On the other hand, women with hydatidiform mole (HM) were reported to have plasma estrogen levels as high as those of normal pregnant controls (Hegab and Schindler, 2004).

In the present study, sera of women with NP and HM were subjected to similar CGB lectin-based electrophoretic approach. The expression of the ITIH4f was indeed prominently up-regulated in both groups of pregnant women as previously expected. Hence, these results generally indicate that the over-expression of the 35 kDa ITIH4f was

not exclusive towards estrogen-related cancers but also in other diseased conditions associated with estrogen dysregulation.

The marked difference in the expression of the 35 kDa ITIH4 cleavage fragment detected in the selective groups of subjects with estrogen-related conditions may be attributed by three possible reasons. One possibility is that the elevated levels of circulating kallikreins in the serum leads to increased cleavage of the ITIH4 protein. The second reason lies in the possibility that the ITIH4 protein is truly over-expressed in patients with increased levels of estrogen. Alternatively, a combination of the two said reasons may also contribute to the high abundance of the ITIH4f that was observed in the present study.

The idea that the abundance of the ITIH4 fragment is linked to the high amount of serum kallikreins is derived from previous reports demonstrating the over-expression of members of the kallikrein family in cancers of the breast, ovary and endometrium (Borgono and Diamandis, 2004; Myers and Clements, 2001; Diamandis *et al*, 2000). This is not surprising as kallikreins are known to be expressed in hormone-dependant tissues such as the breast and ovary (Diamandis and Yousef, 2002). In addition, the expression of these kallikrein genes have been shown, using cancer cell lines, to be regulated by steroid hormones including estrogen (Myers and Clements, 2001; Yousef *et al.*, 2001). As the ITIH4 protein is kallikrein-sensitive, there is likelihood that the over-expression of kallikrein may lead to increased cleavage of serum ITIH4 which in turn led to the enhanced liberation of its 35 kDa fragment (Mohamed *et al*., 2008; this thesis). In support of this correlation between the circulating serum kallikrein concentration and the abundance of the 35 kDa ITIH4f is a study conducted by Gangadharan *et al*. (2007). Based on their study, it

was demonstrated that the down-regulation of kallikrein in patients with hepatic cirrhosis resulted in low abundance of the ITIH4 fragments including the 35 kDa fragment.

Another possibility is that the expression of the ITIH4 may have been induced as part of an acute-phase response towards the studied conditions. ITIH4 has been shown to be an acute-phase protein (Gonzalez-Ramon *et al*., 1995). This notion is in line with several reports indicating the elevation of the ITIH4 protein in pregnancy conditions (Heitner *et al*., 2006; Geisert *et al*., 1998), as well as in conditions of acute stress both in human (Piniero *et al*., 1999) and cattle (Piniero *et al*., 2004). However, at present, no such finding has been reported with regard to cancers even though elevation in the level of the various ITIH4 fragments has been documented (Opstal-van Winden *et al*. 2012; van den Broek *et al*, 2010; Gast *et al*., 2009). At present, the mechanism underlying the increase in the intact ITIH4 protein as well as its function is not clear but it has been reported that members of the ITIH family, including the ITIH4, play important roles in stabilization of the extracellular matrix (Heitner *et al*., 2006; Bost *et al*., 1998; Salier *et al*., 1996). This role is attained via the interaction of the ITIH heavy chains with hyaluronic acid. The hyaluronic acid, which is a major component of the extracellular matrix, has been demonstrated to play modulatory roles in tissue scaffolding and remodeling activities (Bhanumathy *et al*., 2002), which are among the critical events of normal or pathological cellular morphogenesis in benign as well as cancerous conditions (West and Kumar, 1989). It has also been postulated that there may be a strong involvement of the ITIH4 protein with attachment of the placenta to the uterus during pregnancy (Geisert *et al*., 2003). In addition, numerous studies have also pointed out the role of the ITIH proteins with inhibition of apoptosis (Fujita *et al*., 2004; Mangnall *et al*., 2003). To further corroborate this suggestion is the fact that aside from the ITIH protein, estrogen also has a role in

regulating the extracellular matrix integrity in responsive organs such as the uterus and vagina (Cox and Helvering, 2006). Hence, when taken together, there is a likelihood of deregulation of the expression of the ITIH4 protein in patients with conditions associated with increased levels of estrogen.

Alternatively, the detection of the highly abundant 35 kDa ITIH4f may have resulted from the synergistic effect of both conditions of up-regulated expression of the ITIH4 protein and its proteolytic enzyme, kallikrein. As discussed earlier, the erratic secretion of estrogen is known to affect the expression of kallikrein genes (Borgono *et al*., 2004; Yousef and Diamandis, 2001) which resulted in the elevated levels of kallikreins in the serum. Mean while, the ITIH4 protein may also be highly expressed in serum of patients with estrogen dysregulation. Taken together, these two possibilities may lead to a high amount of the 35 kDa ITIH4f, product of the kallikrein fragmentation activity on the ITIH4 protein. However, all three possibilities warrant further studies to uncover the underlying mechanism connecting to the differential expression of the 35 kDa ITIH4 fragment to the estrogen-related cancers and diseased conditions.

In the present study, detection of the 35 kDa ITIH4f in serum samples using the HRP-conjugated CGB lectin is a simple bioanalytical approach that may be further exploited. ITIH4f is not possibly detected by silver staining of the 2-DE gels. The inability to detect the 35 kDa ITIH4f with the rest of the ITIH4-related fragments in serum samples that were subjected to the silver staining approach could be due to the considerably low concentration of the ITIH4 protein in the human serum ie. 55.1-176.6 µg/ml (Choi-Miura, 2001).

Based on the ability of the CGB lectin detection method, there is a potential in using it to assess the activity of kallikreins in clinical samples. This is in view of the fact that the 35 kDa ITIH4f is liberated through the enzymatic activity of kallikrein. Hence, the expression of the ITIH4 cleavage fragment may indicate the *in vivo* activity of circulating kallikreins. In addition, the approach may be incorporated in the assessment of the physiological relevance of the ITIH4 fragmentation. To the best of our knowledge, little is known on the expression of the 35 kDa cleavage fragment of the ITIH4. The peptide has only been detected in sera of patients with severe acute respiratory syndrome (Chen *et al*., 2004). Most reports concerning the ITIH4, in relation to cancer and diseased conditions, were focused on the 85 kDa proline-rich region and its proteolytic processing (van den Broek *et al*., 2010; Song *et al*., 2006; Fung *et al*., 2005). The question of why the 35 kDa ITIH4f did not get the same attention as its counterpart is rather intriguing. One factor that may have contributed to the lack of information on the 35 kDa cleavage fragment of the ITIH4 could be in the difficulty to detect the fragment. Unlike the 35 kDa fragment, the 85 kDa N-terminal region undergoes further proteolytic activities resulting in smaller fragments which made them easily identified by SELDI-TOF-MS, MALDI-TOF-MS or LC-MS. In contrast, the 35 kDa ITIH4 fragment remained intact making its identification and evaluation as potential disease markers using similar tools unfeasible. Therefore, at present, the enzyme-conjugated CGB lectin electrophoretic approach may appear to be the solution to the bottleneck detection issue. Currently, several studies have been conducted using this approach and the researchers have managed to detect the aberrant expression of the 35 kDa ITIH4f in urinary protein profiles of patients with cancers of the ovary (Mu *et al*., 2012; Abdullah-Soheimi *et al*., 2010) and prostate (Jayapalan *et al*., 2012; Jayapalan, personal communication, 2013).

4.4 Profiling of serum proteins of women with NP and HM by 2-DE

Human serum contains a wide variety of different proteins and peptides. Due to different diseases and physiological states, modifications in the production of specific proteins and enzymes can occur. This leads to alterations in the serum composition which could therefore serve as indicator of an individual's physical condition (van den Broek *et al*., 2008). Hence, the aberrantly expressed proteins and peptides could be utilized as valuable biomarkers, enabling diagnosis as well as prognosis of different diseased conditions.

In this study, attempts to profile the maternal serum proteins of women with NP and HM were performed using the gel- and lectin-based proteomic approach. Maternal serum samples at 11 weeks of gestation, on average, were initially subjected to 2-DE separation, followed by isolation of their O-glycoproteins using enzyme-conjugated CGB lectin as probes. Since O-glycosylated serum proteins have been strongly associated with several different types of cancers (Mohamed *et al*., 2008 (this thesis); Chen *et al*., 2008; Abdul-Rahman *et al*., 2007; Brockhausen, 2006) it would be interesting to determine if these O-glycoproteins were also aberrantly expressed in the groups of women with conditions that are considered to be benign and normal such as HM and NP.

When comparative analysis was performed on the serum O-glycoproteins resolved on the lectin-interacted membranes, different altered levels of several high-abundance proteins were detected in the groups of women with NP and HM, relative to their healthy non-pregnant controls. Three O-glycoproteins, including the 35 kDa ITIH4f, ACT and LRG, were differently expressed. In the two groups of pregnant women studied, the up-regulated expression of the 35 kDa ITIH4f was consistently observed. The 35 kDa ITIH4f

was also the sole O-glycoprotein that was found to be differentially expressed when comparison was made between the O-glycoprotein profiles of the women with HM and the controls. However, since its enhanced expression has been specifically discussed in the preceding section, the following discussion will only focus on the other two aberrantly expressed O-glycoproteins. In the case of the group of women with NP, the expression of ACT and LRG was found to be significantly higher compared to those of the control subjects. When the expression levels of the O-glycoproteins of the women with HM were compared to those generated from the sera of NP women, the only conspicuous difference was in the doubling of the levels of ACT in the sera of women with NP.

To corroborate our earlier findings from the enzyme-conjugated CGB lectin approach, analysis of the serum proteins of the three groups of women was further extended using a different detection method. In the second approach, whole serum samples were separated using the 2-DE technique and the resulting protein spots were then detected using silver staining. Earlier, several researchers have managed to develop high-resolution 2-DE serum protein expression profiles of patients diagnosed with early stages of cancers of the breast (Doustjalali *et al*., 2004), ovary (Chen *et al*., 2008), cervix (Abdul-Rahman *et al*., 2007), endometrium (Abdul-Rahman *et al*., 2007), nasopharynx (Doustjalali *et al*., 2006) and prostate (Jayapalan *et al*., 2012), as well as other diseased conditions such as congenital hypothyroidism (Yong *et al*., 2006) and IgA nephropathy (Shuib *et al*., 1998) by using a similar approach. In the present study, analysis of the silver-stained 2-DE gels of women with NP, HM and their normal subjects also managed to reveal the differential expression of several high abundance serum proteins. Some of the aberrantly expressed proteins were not detected earlier in the CGB lectin probed serum protein profiles as they were naturally

without any O-linked glycans, such as CLU (Stewart *et al*., 2007; Zhang *et al*., 2003) and ZAG (Hassan *et al*., 2008; Delker *et al*., 2004).

By subjecting the 2-DE serum protein profiles of women with NP and control subjects to densitometry analysis, the enhanced expression of ACT and LRG in the sera of NP women that was earlier detected using the CGB lectin was further confirmed. Added to the existing list of differentially regulated serum proteins of the women with NP was the over-expression of ZAG and CLU that was found in this part of the study. Similarly, the serum samples from the group of women with HM were also found to contain higher amounts of LRG and CLU, compared to those of the controls. When comparative analysis was performed on the profiles of women with NP and HM, the higher expression of ACT in the women with NP that was detected earlier in the lectin work, was also confirmed.

In the present study, confirmation on the identities of the four aberrantly expressed serum proteins was performed using mass spectrometry. The gel plugs containing the desired protein were subjected to MS/MS analysis followed by NCBInr database search, which positively identified the proteins as ACT, CLU, LRG and ZAG. Meanwhile, to validate the aberrant expression of the four serum proteins in the respective groups of women studied, an immunoblotting approach was employed. Using commercial antibodies to detect the expression of the electroblotted proteins, the altered levels of ACT, CLU, LRG and ZAG in the respective groups of pregnant women were confirmed, although the magnitude of deviation was generally much lower compared to the 2-DE experiments. This is indicative of the higher sensitivity of 2-DE compared to immunoblotting when used to evaluate the levels of serum proteins. Similar findings have been reported by previous researchers in their attempts to confirm the altered expression of serum proteins using competitive ELISA (Chen *et al*., 2008; Abdul-Rahman *et al*., 2007; Doustjalali *et al*., 2004

and 2006). By using the immunoblotting approach, the enhanced expression of ACT, CLU, LRG and ZAG, in the sera of women with NP, managed to be confirmed. Similarly, the high amounts of circulating CLU and LRG in the serum of women with HM were also shown.

In this study, the different altered expression of high abundance serum proteins in the maternal serum samples are thought to be the consequence of the various response of the body towards pregnancy. During pregnancy, the endometrium of the uterine responds to hormonal cues and growth factors to accommodate a developing fetus (Brown and Bundle, 1996). Thus, serum proteins which are sensitive to hormonal fluctuations, such as CLU (Brown *et al*., 1996) and fragmentation of the ITIH4 protein (Mohamed *et al*., 2008; this thesis), are at high risk of being differentially regulated.

The increased level of maternal serum CLU in association with pregnancy is well described. CLU, also known as apoJ, is an 80 kDa glycoprotein composed of two non-identical, 35 kDa disulfide-linked subunits generated *in vivo* by proteolytic cleavage of proapoJ (Scaltriti *et al*., 2004; Burkey *et al*., 1991). It was first characterized in the rat testis fluid as a sulphated secreted glycoprotein with a capacity to elicit clustering of cells (Blaschuk *et al*., 1983). The glycoprotein is found in most, if not all, biological fluids (Jordan-Starck *et al*., 1992) and based on its wide distribution, many physiological roles have been proposed for this protein. Several studies have reported on the involvement of CLU in apoptosis (Trougakos *et al*., 2005; Koch-Brandt and Morgans, 1996), sperm maturation (Rosenberg and Silkensen, 1995), lipid transport (Calero *et al*., 1999; de Silva *et al*., 1990), cell adhesion (Blaschuk *et al*., 1983; Fritz *et al*., 1983) and injury repair (Grima *et al*., 1990). There is also evidence on the association of CLU with tissue remodeling and cellular differentiation processes (French *et al*., 1996; Brown *et al*., 1995) which are

essential for embryonic development. At present, the exact role of CLU in remodelling of the uterus tissues is still unclear, even though there have been reports on the expression of the CLU gene in the uterus (Aronow *et al*., 1993). However, in 1993, French *et al*. had reported on the association between the expression of CLU gene with epithelial differentiation during murine embryogenesis. In addition, CLU has also been shown to play an active role in the implantation process of blastocyst onto the uterine tissue (Brown *et al*., 1996). Hence, the reported involvement of CLU in pregnancy may provide some explanation to its enhanced expression in the groups of women with NP and HM, as both conditions require similar implantation and cellular differentiation of the embryo regardless of whether the women were with normal and abnormal pregnancies.

Aside from being over-expressed during pregnancy, the increased expression of CLU has also been seen in other pathological conditions including aging, diabetes, neurodegenerative diseases, atherosclerosis, myocardial infarction and cancers (Trougakos and Gonos, 2009; Trougakos *et al*., 2005; Jones and Jomary, 2002; Rosenberg and Silkensen, 1995). Recently there have been numerous reports on the over-expression of CLU in patients with several human cancers, such as cancers of the prostate (Ouyang *et al*., 2001; Steinberg *et al*., 1997), breast (Doustjalali *et al*., 2004; Redondo *et al*., 2000), ovary (Chen *et al*., 2008; Hough *et al*., 2000), renal (Kang *et al*., 2004; Miyake *et al*., 2002), colon (Pucci *et al*., 2004; Chen *et al*., 2003) and endometrial (Abdul-Rahman *et al*., 2007; Vollmer, 2003).

Aside from CLU, high levels of maternal serum LRG were also detected in the two groups of pregnant women studied. LRG, which is a trace protein of the human plasma, was initially isolated by Haupt and Baudner in 1977. However, its amino acid sequence was determined in 1985 by Takahashi *et al*. in 1985. LRG was reported to contain 312

amino acid residues, out of which 66 were leucines. It also possesses eight repeating consensus sequence, termed leucine-rich repeat, which is commonly exhibited by members of the Leucine Rich Repeats family. The LRG polypeptide chain has been shown to contain an O-link oligosaccharide (Rodriquez-Pineiro *et al*., 2004), which explains its interaction to the CGB lectin.

The presence of leucine rich repeats in a polypeptide chain has been suggested to provide a versatile structural framework for the formation of protein-protein interactions (Kobe and Kajava, 2001). The respective peptide has also been shown to be involved in signal transduction as well as cell adhesion and development (Buchanan and Gay, 1996). However, relatively little is known on the function of LRG itself as not many studies have been carried out on this protein, especially in relation to pregnancy. At present, LRG has been proposed to be a membrane-associated glycoprotein (Eisenberg *et al*., 1984; Kaiser and Kezdy, 1983) and may be involved in the binding of extracellular matrix proteins (Saito *et al*., 2002) as well as the granulocytic differentiation of neutrophils (O'Donnell *et al*., 2002).

In 1996, Bini *et al*. had suggested LRG to be an acute phase protein as its level in plasma was found to be increased during the early stage of inflammation. This finding was further corroborated by the work of Shirai *et al*. (2009). Based on their observations, this glycoprotein was demonstrated to be a secretory type-1 acute-phase protein whose expression was up-regulated by the mediator of acute-phase response. Being an acute-phase protein, conditions such as inflammation, infection, trauma and cancer would trigger the over-expression of LRG (Gabay and Kushner, 1999; Heegaard *et al*., 1998). On the other hand, pregnancy is also accepted as a form of a tumor by some researchers. This notion of 'pseudo-malignant' condition is supported by the many similarities shared

between the conditions of pregnancy and cancer. Among the similarities are in the unique characteristics of their cellular components (Soundararajan and Rao, 2004; Quenby *et al*., 1998) as well as the dysregulated expression of several of their proteins (Fest *et al*., 2008; Toth *et al*., 2008; Jeschke *et al*., 2003). Hence, it is not surprising that elevated levels of LRG were detected in the sera of the two groups of pregnant women studied if that was the case.

To date, there have been very few studies in which the expression of LRG was evaluated. LRG was shown to be over-expressed in various diseases only recently, with the development of proteomic analysis. The aberrant expression of this glycoprotein has been reported in sera of patients with cancers of the ovary (Boylan *et al*., 2010; Chen *et al*., 2008), lung (Li *et al*., 2011; Okano *et al*., 2006) and pancreas (Kakisaka *et al*., 2007; Deng *et al*., 2007). In addition, the abundance of LRG has also been found in the cerebrospinal fluid of patients with idiopathic normal pressure hydrocephalus (Li *et al*., 2007).

Contrary to the expression of CLU and LRG, which were over-expressed in both types of normal and abnormal pregnancy conditions, the aberrant expression of ACT and ZAG appeared to be exclusive for women with normal pregnancy. In the present study, the finding of the up-regulated expression of serum ACT is compatible with the result of a previous study conducted by Chelbi *et al*. (2007) which showed elevated levels of ACT in the placenta of normal pregnant women. It is possible that higher maternal serum level of ACT is a consequence of a balancing mechanism to control trophoblast invasion of the uterus tissues. The development of pregnancy is highly dependent on the ability of the placenta's trophoblastic cells to invade the basement membrane of the uterus, especially in the first trimester of pregnancy. The trophoblast invasion on the other hand, is mediated by metalloproteases and serine proteases and its invasiveness is, in turn, controlled by protease

inhibitors such as ACT (Picken *et al*., 1990) and the tissue inhibitor of metalloproteases (TIM) and transforming growth factor-ß (TGF-ß) (Lala and Grahamm, 1990). However, the mechanism underlying the absence of such over-expression in the serum samples of women with HM is not known with certainty. A probable reason that may be considered is that the inhibitory activity of ACT is only for the strictly regulated normal pregnancies whereas the molar pregnancy is characterized by an uncontrolled excessive placental invasion by the molar tumor. This presumption is based on similar observation on PAI-1, also known as serpine E1, in placentas of normal and molar pregnancies (Floridon *et al*., 2000).

ACT, also known as serpina 3, is a member of the serine protease inhibitor family (Travis and Salvesen, 1983). As a protease inhibitor, ACT binds cognate proteases to form a serpine-protease complex, which is cleared from the circulation via the liver. Among the targeted serine proteases are pancreatic chymotrypsin, leukocyte cathepsin G, mast cell chymases, human glandular kallikrein 2, kallikrein 3 (PR3), pancreatic cationic elastase and an uncharacterised lung serum protease (Baker *et al*., 2007). Although the exact function of ACT *in vivo* remains to be determined, it has been implicated in a number of diseases including chronic obstructive pulmonary disease, Parkinson disease, Alzheimer's disease, stroke, cystic fibrosis, cerebral haemorrhage and multiple system atrophy (Baker *et al*., 2007). In addition, the aberrant expression of this protein has also been documented in several cancers. The increase in the expression of ACT has been reported in cancers of the breast (Mbeunkui *et al*., 2007; Doustjalali *et al*., 2004), lung (Zelvyte *et al*., 2004; Higashiyama *et al.*, 1995), gastrointestine (Wittekind *et al*., 1997; Bernacka *et al.*, 1988) and ovary (Chen *et al*., 2008). The significant increase of ACT in several cancers is not surprising. It is believed that tumorogenesis, similar to fetal development, is generally

associated with extensive tissue remodelling, which has been shown to be under the influence of proteinases. Hence, proteinase inhibitors, such as ACT, would be produced in abundance to protect tissues against the proteolytic activities of the proteinases (Zelvyte *et al*., 2004).

In the case of ZAG, its over-expression was also solely exhibited in the maternal sera of women with NP. However, there has been no report yet on the abundance of ZAG as well as its association with pregnancy. ZAG is a 41 kDa glycoprotein secreted by a variety of normal epithelia including those in the breast, prostate, liver as well as in the salivary, bronchial, gastrointestinal and sweat glands (Tada *et al*., 1991). Thus, it is secreted into different types of body fluids such as serum, semen, sweat, saliva, cerebrospinal fluid, milk, urine and amniotic fluid (Jain *et al*., 2005; Ding *et al*., 1990; Bundred *et al*., 1987; Burgi *et al*., 1989; Burgi and Schmid, 1961). ZAG was first identified in human serum and named for its tendency to precipitate zinc salts and for its electrophoretic mobility that was similar to plasma α2 globulins (Burgi and Schmid, 1961). The ZAG protein has been shown to consist of three isoforms based on the work of Jirka and Blanicky (1980), and this is consistent with the ZAG spot cluster that was detected by 2-DE in the present study.

Currently, the presence of ZAG is known to stimulate lipolysis, which is involved in depletion of fatty acids from the adipose tissues (Hassan *et al*., 2008; Hirai *et al*., 1998). However, due to its presence and different degree of expression in various tissues and body fluids, several other possible functions may be attributed to this protein. ZAG has been suggested to play a vital role in fertilization (Garcia-Ramirez *et al*., 2007; Ohkubo *et al*., 1990), immunoregulation (Willcox *et al*., 2002; West and Bjorkman, 2000) and cell

adhesion (Lei *et al*., 1999; Sanchez *et al*., 1999). Several studies have also suggested ZAG to be indirectly involved in deterring tumor progression (He *et al*., 2001).

Although the exact mechanism by which ZAG actively participates in tumor proliferation is not known, a lot of data exists on the over-expression of ZAG in different cancers. The concentration of ZAG has been reported to increase dramatically in cancers of the prostate (Bondar *et al*., 2007; Hale *et al*., 2001), breast (Diez-Itza *et al*., 1993; Bundred *et al*., 1987), oral cavity (Brysk *et al*., 1997), skin (Lei *et al*., 1997), endometrium and cervix (Abdul-Rahman *et al*., 2007). In addition, ZAG has also been considered as a marker for tumor differentiation (Lopez-Otin and Diamandis, 1998; Diez-Itza *et al*., 1993). Previous reports have demonstrated that higher ZAG level was detected in well-differentiated tumors relative to that in the moderately or poorly differentiated tumors of cancers of the prostate (Hale *et al*., 2001) and breast (Diez-Itza *et al*., 1993).

Changes in the patterns of glycosylation, which may occur during pregnancy (Mackiewicz and Mackiewicz, 1995; van Dijk *et al*., 1994), would most likely be reflected in the microheterogeneity of the isoforms of the glycoproteins (van Dijk *et al*., 1994; Mackiewicz *et al*., 1987). Hence, in the present study, densitometry analysis was performed on the isoforms of the protein clusters resolved in the 2-DE gels as well as blots of the groups of women studied. Comparative analysis between the profiles of women with NP and HM, relative to their normal subjects, revealed that all of the isoforms of the highly expressed 35 kDa ITIH4f, ACT, CLU and ZAG, although generally over-expressed, were similarly distributed in the silver stained gels as well as the CGB lectin detected blots. Hence, the abundance of the 35 kDa ITIH4f, ACT, CLU and ZAG in the women with NP and HM, appeared to be attributed by the overall increase in the expression levels of their isoforms and may not involve any alteration in their O-linked side chains.

Also detected in this study was the over-expression of LRG in the sera of women with HM, relative to the controls. The marked difference in the expression levels of LRG which was detected when using whole serum was quite intriguing since the disparity was not detected in the earlier studies using the HRP-conjugated CGB lectin. It is possible that the decreased binding of LRG to the Galß1-3GalNAc-reactive CGB lectin is due to the lack of O-glycans. A similar finding has been reported by Hashim *et al*. (2001) in their attempt to establish the pathogenesis of IgA nephropathy using the CGB lectin. In their work, the affinity of the CGB lectin to the serum IgA1 was significantly weaker in patients with IgA1 as compared to normal controls. The alteration in the glycosylation pattern of LRG, however, was independent of the rate of synthesis of LRG itself. This was reflected in the abundance of LRG in the silver stained profiles. In contrast, the expression of LRG in the group of women with NP appeared to be up-regulated in both profiles of the CGB lectin detected blots as well as the silver stained gels. Hence, this generally implies that the O-glycan moieties of LRG in women with NP were structurally different from those of women with HM.

In addition, analysis of the isoforms of CLU in the 2-DE silver stained gels of the pregnant women and their controls demonstrated different distribution pattern for the women with NP and HM. The enhanced expression of CLU in the women with NP was contributed by the over-expression of isoforms 2 and 3. In contrast, isoforms 1 and 2 were significantly up-regulated in the CLU protein cluster of the women with HM. The differential distribution of the volume of the isoforms generally implies that the CLU of HM women were more cationic as compared to those of NP women. This observed difference may be attributed by the lack of sialic acid content (Hashim *et al*., 2001; Shuib *et al*., 1998) in the CLU polypeptide chain of the women with HM as compared to that of the

women with NP. However, these findings, as well as those of LRG, warrant more in-depth analysis. Further investigation could be performed by subjecting the respective serum proteins to exoglycosidase digestion followed by analysis using either the high-performance liquid chromatography (Saldova *et al*., 2007) or the liquid chromatography (Heo *et al*., 2007). Alternatively, methods such as the lectin glycoarray (Qiu *et al*., 2008), isotopic glycosidase elution and labelling on lectin-column chromatography (IGEL) (Ueda *et al*., 2010) and Lectin-directed Tandem Labelling (LTL) (Shetty *et al*., 2012) could also be used to confirm the aberrant glycosylation of LRG as well as the sialylation changes of CLU detected in the present study.

Taken together, the present study demonstrated that the 35 kDa ITIH4f, ACT, CLU, LRG and ZAG were selectively over-expressed in the women with NP and HM, relative to their controls. In addition, there are possibilities that the oligosaccharide side-chains of CLU and LRG were structurally altered in the HM women. However, to use any of the aberrantly expressed proteins as individual candidate biomarker may not be practical as they are also elevated in different types of cancers. Nevertheless, the expression of the all potential candidate biomarkers when analyzed together appeared to demonstrate differently altered patterns and seemed to differ from those previously reported for cancers of the breast, nasopharynx, cervix, endometrium and prostate. This is illustrated in Figure 4.1, which summarizes the expression of selective high abundance serum proteins in the women with NP and HM that were investigated in the present study, relative to the earlier findings of previous researchers. The checkered diagram appears to highlight the distinctive patterns of the selective benign conditions of the pregnant women from those exhibited by the different cancers. For example, EOCa is associated with the over-expression of ACT,

Diseased conditions	Serum proteins														References
	AAT	**ABG**	**ACT**	**AHS**	**ATR**	**APO A2**	**C3f**	**CFB**	**CLU**	**CLP**	**HAP**	**KNG**	**LRG**	**ZAG**	
NP*			+						+				+	+	This thesis
HM*									+				+		This thesis
FDB*			+						+			–	+		Doustjalali *et al*., 2004
BrCa	-		+					+	+			–			Doustjalali *et al*., 2004
NPCa										+					Doustjalali *et al*., 2006
EACa	-	+			+				+			–	+		Abdul-Rahman *et al.*, 2007
SCCa	-	+			+							–		+	Abdul-Rahman *et al*., 2007
ACCa	-	+			+							–		+	Abdul-Rahman *et al*., 2007
GOCa	+			-					+	+	+		+		Chen *et al*., 2008
EOCa	+		+						+		+		+		Chen *et al*., 2008
PCA						+	+								Jayapalan *et al*., 2012

Figure 4.1: Aberrantly expressed serum proteins in several benign and cancer conditions. Checkered diagram of aberrantly expressed serum proteins in benign conditions (marked with asterisks) and cancers generated from analyses of whole sera subjected to 2-DE and silver staining. (+) indicates up-regulated expression and (-) indicates down-regulated expression of serum proteins in patients compared to healthy controls. FDB, fibrocystic disease of the breast; SCCa, squamous cell cervical carcinoma; GOCa, germ cell ovarian carcinoma; PCa, prostate cancer; APOA2, apolipoprotein AII, C3f, complement C3 ß chain fragment; CFB, complement factor B.

CLU, LRG, AAT and HAP, while NP is also associated with the enhanced levels of ACT, CLU and LRG but with increased level of ZAG.

Hence, the findings of the current study appear to provide additional evidence on the strength of assessing multiple biomarkers simultaneously as compared to the current usage of single biomarkers such as CA125 for ovarian cancer and PSA for prostate cancer. And since the profiles of the aberrantly expressed serum proteins demonstrated in Figure 4.1 were unique for each of the diseased conditions, the altered expression patterns may act as typical signatures or fingerprints to distinguish the different types of cancers, as suggested by Pang *et al*. (2010). However, although there is compelling evidence for the diagnostic and/or prognostic potential of the respective patterns, further validation using clinically acceptable sample sizes and involving other types as well as subtypes of cancers are required. These investigations may not be possible using the current gel-based proteomics technique, which is laborious and time-consuming but can perhaps be done using other state-of–the-art approaches such as the multiplex immunoassay, protein array or the targeted chip-based proteomic assays.

4.5 Development of a multiplex immunoassay

In the present study, an in-house multiplex assay was developed in an attempt to simultaneously detect altered expression of circulating serum proteins. Ideally, the developed multiplex assay would have the capacity to detect the levels of the four circulating serum proteins (ACT, CLU, LRG and ZAG) that had previously been shown to be differently altered in the groups of women with NP and HM. Also included in the

development of the multiplex assay were four other serum proteins (AAT, AHS, ATR and CLP) that had been shown to be differentially expressed in different types of cancers, relative to the controls (Chen *et al*., 2008; Abdul-Rahman *et al*., 2007; Doustjalali *et al*., 2004 and 2006). However, the development of multiplex assay in the present study was only successful for quantifying three serum proteins, i.e., CLU, CLP and ATR.

In order to develop the multiplex assay, serum proteins of interest were covalently coupled to carboxylated beads using carbodiimide reaction. In this study, the Bio-plex carboxylated beads were chosen due to the ease of their binding to any protein with molecular weights ranging from 6-150 kDa. However, the challenge in performing the coupling procedure was in establishing the best amount of protein that would promote optimal binding between a protein and the carboxylated beads. Thus, in the present study, six sets of coupling reactions were performed using various amounts of proteins, ranging from 2 to 12 µg for each of the protein used. Subsequent to the coupling reaction, the various sets of the protein-conjugated beads were subjected to a validation test to identify the set with successful coupling, which was reflected by a fluorescent intensity of greater than 2,000 MFI. Production of such signal intensity was demonstrated by the ATR-, CLU- and CLP-conjugated beads with values of 10,783.0, 3,132.5 and 6,314.5 MFI, respectively. Also noted was that the optimal amounts of proteins used in the conjugation reaction were apparently different for the different types of proteins used. This was demonstrated by the different amounts of proteins required in preparing the sets of ATR-, CLU- and CLP-coupled beads. The successful coupling of these proteins to the carboxylated beads were obtained when 7, 10 and 5 µg of the respective proteins were used.

In this study, attempts to develop the AAT-, ACT-, AHS-, KNG- and ZAG-coupled beads proved to be unsuccessful. This was demonstrated by their low fluorescent signal

intensities. However, the actual underlying cause, which leads to the unsuccessful development of the respective sets of protein-coupled beads was not able to be identified. Nevertheless, this could be due to two probable reasons. One possibility is that the proteins were not successfully conjugated onto the surfaces of the carboxylated beads due to the incorrect range in the amount of protein used in the coupling reactions. The search for an optimal amount of protein that would successfully couple a protein to the beads proved to be a difficult task and take quite a considerable amount of time. Similar difficulties have been earlier reported by van Gageldonk *et al*. (2008) in their attempt to develop an in-house multiplex assay for simultaneous determination of serum antibodies to *Bordetella pertussis*, *Corynebacreruim diptheriae* and *Clostridium tetani*. In the present study, it was observed that although the amount of proteins used in the conjugation process was in accordance to the range suggested by the manufacturer, optimal binding for each of the five proteins to their respective beads could not be established. In addition, there was no consistent correlation between the amount of protein used and the results of the coupling reaction. It was demonstrated that for some of the proteins, increasing the amount of protein or using the highest level of protein would not necessarily yield optimal binding reaction.

Alternatively, the unsuccessful development of the five sets of beads could also be attributed by the inability of the biotin-labeled antibodies to recognize their respective protein-coupled beads. There is a possibility that the protein may lose its three-dimensional structure when it was coupled or immobilized to the surface of the beads (Angenendt, 2005). These structural changes may result in the protein losing its antigenic recognition epitopes, thus making it no longer recognizable by the antibody that usually binds to it in its native form. In the same context, the failure in establishing a specific interaction between the antibody and the antigenic protein could also be due to stearic hindrance or masking of

the antigenic epitopes of the protein in its newly formed configuration, which is one of the common side-effects of the chemical reactions involved during the protein coupling procedure (MacBeath, 2002).

In an attempt to test the efficiency of the three sets of beads, which were successfully conjugated, to quantify the respective proteins simultaneously, the beads were then assembled into a single assay platform. Performance of the developed multiplex assay for the three proteins i.e., ATR, CLP and CLU, was evaluated in terms of its specificity, sensitivity and reproducibility. The specificity of each individual assay within the multiplex is of great importance in any multiplex immunoassay. This is due to the fact that multiple antibodies and various types of protein-conjugated beads are present in a single assay suspension, which may lead to non-specific binding of the protein-conjugated beads to the various antibodies. In addition, suspension arrays are also vulnerable to cross-reactivity between proteins as cross-linking may occur between the beads due to their ability to circulate freely in the assay fluid (Ling *et al*., 2007). Presence of cross-reactivity within the multiplex immunoassay would in turn, result in a decrease of the assay's sensitivity. In this study, the binding of the protein-conjugated beads to their respective antibodies were rather specific since the results from the specificity test demonstrated that the individual antibodies exhibited strong reactivity only to the beads carrying their specific antigenic proteins. In contrast, reactivity of the various antibodies was minimal on beads with other proteins. For example, when the ATR-conjugated beads were tested with anti-ATR, significant fluorescence signal was only demonstrated by the ATR beads (8,165.3 MFI), whilst the values from the CLP- and CLU-conjugated beads were merely 5 and 3.5 MFI, respectively. Hence, results obtained from the sensitivity test generally indicated that there was no significant cross-reactivity between the different antibodies and protein-

coupled beads used in the multiplex assay, and that the developed assay appeared to possess a sensitivity level within the desired working range.

In the present study, the sensitivity of the developed multiplex immunoassay for the detection of the three serum proteins was also determined using a method described by de Jager *et al*. (2009). In this sensitivity assay, serum samples were spiked with different concentrations of ATR, CLU and CLP, ranging from 0.0002 to 0.2 µg/ml. When analysis was performed, the assay was demonstrated to be sensitive enough to detect the small changes in the concentrations of the various proteins.

In addition, the reproducibility of the developed multiplex assay was determined by analyzing its coefficients of variations (%CV) between intra-assays as well as between inter-assays. The value of %CV reflects the degree of agreement between different measurements conducted on replicate specimens using the multiplex assay. In the present study, both the intra- and inter-assay variability was measured using the sera from control subjects. Based on the range of %CV obtained, variations observed from different wells within the same plate (intra-assay) or between different plates (inter-assay), were considered to be acceptable for all the three proteins. In this study, the %CV obtained for the intra-assays was <13% whilst that of the inter-assays was <29%. Findlay *et al*. (2000) have suggested that the acceptable %CV for the intra- and inter-assays of a multiplex immunoassay should be <30%. Hence, the assays performed using the developed multiplex immunoassay was shown to be reproducible based on a proposed standard operating procedure.

Besides subjecting the developed multiplex assay to a set of tests to evaluate its specificity, sensitivity and reproducibility, the optimal conditions required in running the

assay was also performed. This is due to the fact that an immunoassay performance is frequently related to the dilution at which samples were assayed (Drummond *et al*., 2008; Pfleger *et al*., 2008). When serum proteins are analyzed in cohorts of pathological and non-pathological subjects, it is expected that large dynamic variations of the proteins are present in the assay. As such, the dilution of a sample should preferably cover a concentration range, which includes highly down- and up-regulated expression levels of the proteins of interest. Hence, in order to determine the optimal dilution factor of serum samples that was capable of accommodating all the three sets of protein-conjugated beads, five dilutions of serum samples across a range of 1/4 to 1/200 were tested. Based on the results obtained, a dilution factor of 1/50 was chosen. This was based on the maximal signal intensities that were achieved when the serum samples were tested for CLU and CLP, although the signal intensity obtained for ATR was not quite optimal. Nevertheless, the concentration of serum ATR was still within quantifiable limit of the developed multiplex assay as fluorescence level detected was high (1,717.5 MFI) compared to the intensity emitted by the CLP- and CLU-conjugated beads (359.5 and 427.4 MFI, respectively).

Once validation on the performance of the developed assay was completed, the multiplex competitive immunoassay was used to profile the levels of ATR, CLP and CLU in the serum samples of patients with NP and HM as well as their control subjects. By using this technique, the up-regulated expression of CLU in the sera of women with NP and HM, relative to the controls, was confirmed. In addition, the levels of ATR and CLP in the serum samples of patients with NP and HM were also found to be comparable to those of the controls. This was based on the overall signal intensities from the sets of ATR- and CLP-conjugated beads, which were quite similar to those emitted from the serum samples of the controls.

The results of the multiplex assay that was developed in the present study were comparable to those that were earlier obtained using 2-DE. Hence, it is reasonable to conclude that the multiplex immunoassay developed in the present study had the ability to specifically measure the three different proteins simultaneously in small amounts of serum samples. Nevertheless, it needs to be further developed to include the other five proteins which were not successfully conjugated in the earlier part of this study.

The multiplex immunoassay that was partially developed in the present study offers several advantages that will probably make it an attractive assay platform in the future. The fact that it allows elimination of multiple samples processing, which greatly reduces the amount of time and labor, makes this technique operationally practical and feasible for routine diagnostic application. And most interestingly, the flexibility and robustness of this approach, makes it possible for continuous addition of target proteins or biomarkers to existing panels. This in turn will provide the opportunity to increase the diagnostic power of the immunoassay. In addition, the multiplex approach is also in line with the current trend of looking at multiple biomarkers in the diagnosis of different malignancies (Pang *et al*., 2010).

CHAPTER 5:

CONLUSIONS

A 35 kDa glycoprotein, whose abundance was previously demonstrated to be enhanced in the sera of patients with EACa, was isolated from the pooled sera of three EACa patients with the use of an immobilised CGB lectin affinity chromatography. Subjecting the isolated protein spot cluster to mass spectrometry analysis, the targeted glycoprotein was identified as the 35 kDa O-glycosylated fragment of the ITIH4 chain (ITIH4f). Subsequent to its identification, the expression of the 35 kDa ITIH4f in other cancers was investigated using the 2-DE and HRP-conjugated CGB lectin approach. When compared to the controls, the expression of the ITIH4 cleavage fragment was demonstrated to be significantly enhanced in sera of patients with BrCa, EOCa and GOCa, but not in patients with NPCa and OsSa. The data of the present study, when taken together with the reports of others (Abdul-Rahman *et al*., 2007; Chen *et al*., 2008) appeared to correlate the abundance of the 35 kDa ITIH4f specifically with cancers associated with estrogen dysregulation.

To determine if the fragment is also over-expressed in other non-cancer conditions with irregularities in the levels of estrogen, its expression in women with normal pregnancy (NP) and hydatidiform mole (HM) as well as menopausal women under the hormone replacement therapy (HRT) were studied. The 35 kDa ITIH4f was similarly over-expressed in the groups of NP and HM women, but was not differentially expressed in the group of women with HRT. Hence, it appeared that the expression of the 35 kDa ITIH4f was not exclusive to cancers but is also enhanced in normal and benign conditions that are associated with estrogen dysregulation.

Using the conventional 2-DE approach in combination with either the HRP-CGB lectin or silver staining to detect the resolved spots, an attempt to establish the serum protein profiles of women with NP and HM were performed. The aberrant expression of

the differentially expressed proteins was further validated using immunoblotting technique. Comparative analyses of the serum proteins in the profiles of the silver stained gels and lectin detected blots demonstrated simultaneous enhanced expression of the 35 kDa ITIH4f, ACT, CLU, LRG and ZAG in the women with NP, relative to the controls. In contrast, the women with HM were characterized only by the up-regulated expression of the 35 kDa ITIH4f, CLU and LRG. However, LRG of the HM women appeared to be differentially glycosylated from that of NP women. In addition, CLU of the women with HM seemed to be more cationic, relative to the CLU of the women with NP. The aberrant individual expression of ACT, CLU, LRG and ZAG which was detected in the present study, has also been previously reported in different types of malignancies. However, when taken together, the aberrantly expressed serum proteins formed profiles or "protein signatures" that were generally different from those of the different types of cancers. Hence, these findings provide an insight on the potential importance of using panels of biomarkers to further refine the diagnosis as well as prognosis of a particular diseased condition.

An attempt to simultaneously detect the differential expression of the panels of serum proteins was subsequently performed using a multiplex immunoassay that was developed in-house in the final part of the present study. However, this was only successful for the detection of ATR, CLU and CLP, whilst the other five targeted proteins were not quite successfully developed. Nonetheless, the assay was capable of detecting the enhanced expression of CLU and confirming the unaltered expression of ATR and CLP in sera of women with NP and HM. At this juncture, it is therefore reasonable to conclude that, when further refined to include other altered potential biomarkers that have been previously uncovered, the comprehensive multiplex assay may be possibly developed. Such an assay can be of value to confirm the hypothesis that, the serum proteins of interest

when analysed simultaneously, may ultimately be used as a “protein signature” in the diagnoses as well as prognoses of different types of cancers.

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