IDENTIFICATION OF POTENTIAL PROTEIN BIOMARKERS IN SERA OF THE ELDERLY AND THEIR ASSOCIATION WITH FRAILTY STATUS

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Name of Candidate: Puvanesswaray d/o Ramakrishnan

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Field of Study: Frailty and proteomic

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ABSTRACT

Early identification of frailty is of increasing importance as it may reduce the risk of adverse outcomes in older people. Potential biomarkers of frailty may be detected using proteomic applications that analyse serum samples of older subjects who are stratified according to their frailty status. This study is aimed at identifying potential biomarkers of frailty associated with non-frail, pre-frail and frail status in older subjects. Frailty status was determined using the Frailty Index. Blood samples taken from participants recruited from University Malaya Medical Centre were analysed using proteomic techniques. Enhanced expression of alpha-1-antitrypsin (A1AT), alpha-1B-glycoprotein (A1BG), haptoglobin (HAP), hemopexin (HPX), kininogen (KNG1) and leucine-rich alpha-2-glycoprotein (LRG) and reduced expression of apolipoprotein E (APOE) and isoform 2 of clusterin (CLU) were detected in sera of frail elderly when samples were subjected to 2-Dimensional Electrophoresis (2-DE) and analysed by densitometry. In the pre-frail elderly, KNG and A1AT showed increased in abundance levels. In glycoprotein analysis utilising CGB-lectin affinity blotting, LRG levels were significantly reduced in pre-frail subjects while HAP levels were significantly higher in the frail elderly relative to control non-frail subjects when pooled serum samples were subjected to CMB-AP conjugated lectin in western blot analysis. Enzyme-linked immunosorbent assay analysis showed serum A1AT concentration levels that were significantly higher (degree of fold change <1.5) while APOE was reduced in frail older adults compared with the non-frail, consistent with proteomic findings. This study suggests an association between frailty and certain proteins with their levels identified at different frailty status. The identification of these potential biomarkers of frailty may

provides towards an understanding of frailty and identifies proteins which may contribute towards the early detection of frailty in older people.

Keywords: frailty, proteomics, lectin

PENGESANAN PENANDA BIOLOGI YANG BERPOTENSI DALAM SERUM WARGA TUA DAN HUBUNGANNYA DENGAN STATUS KELEMAHAN

ABSTRAK

Kelemahan atau 'frailty' dikalangan warga emas harus dikesan dengan lebih awal untuk mengelak keadaan kesihatan mereka terancam. Pengesanan awam kelemahan di kalangan warga emas adalah penting dan dengan menggunakan kaedah kajian proteomik menerusi analisa menggunakan serum mereka, ianya berkemungkinan mempunyai potensi untuk menentukan penanda biologi (biomarker) bagi keadaan ini. Kaedah ini semakin meningkat kepentingannya dan memberi pendekatan yang lebih mendalam dari segi perubahan dalam fisiologi berbanding dengan individu-individu sihat. Penyelidikan ini membahagikan golongan tua mengikut status kelemahan mereka iaitu; tidak lemah, separa lemah dan lemah, dan ianya diukur menggunakan formula pengiraan 'Frailty Index'. Sampel darah diperoleh daripada pesakit dari Pusat Perubatan Universiti Malaya. Ekspresi yang meningkat oleh alfa-1-antitripsin (A1AT), alfa-1B-glikoprotin (A1BG), haptoglobin (HAP), hemopeksin (HPX), kininogen (KNG1), dan leucine-rich alfa-2-glikoprotein (LRG) telah dikesan dalam serum individu tua yang lemah, pada masa yang sama, apolipoprotin E (APOE) dan isofom 2 klusterin (CLU) telah berkurangan dalam kadar ekspresi apabila sampel serum golongan tua yang lemah didedahkan kepada kaedah 2-DE dan analisis densitometri. Golongan tua separa lemah, menunjukkan peningkatan dalam ekspresi KNG dan A1AT. Walaubagaimanapun, dengan menggunakan aplikasi pemblotan keafinan lektin CGB, kami dapat mengesan kelimpahan protin LRG yang rendah dikalangan golongan tua yang separa lemah, sementara kelimpahan HAP adalah tinggi dalam kumpulan golongan tua yang lemah apabila dibandingkan dengan individu yang sihat daripada serum terkumpul dianalisa menggunakan lektin CMB pengikatan AP dalam 'western

blot'. Kaedah analisis 'ELISA' telah menunjukkan peningkatan kelimpahan A1AT dalam serum (gandaan peningkatan <1.5), sementara kelimpahan APOE adalah kurang dikalangan golongan tua lemah berbanding dengan individu-individu tua yang sihat. Hasil kajian ini menunjukkan sindrom kelemahan pada tahap berbeza dapat dikaitkan dengan tahap protin yang telah dikenal pasti yang mana mungkin boleh dipertimbangkan sebagai penanda yang berpotensi dalam mengesan dan mengawal masalah sindrom kelemahan dikalangan warga emas.

Katakunci: kelemahan, proteomik, lektin

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
° C	:	Celcius
µ	:	Micro
A	:	Ampere
ATP	:	Adenosine triphosphate
cf-DNA	:	plasma cell free DNA
CGA	:	Comperehensive Geriatric Assessment
cm	:	Centimetre
CRP	:	C-Reactive protein
DNA	:	Deoxyribonucleic acid
ELISA	:	Enzyme linked immunosorbent assay
g	:	Gram
Hb	:	Hemoglobin
hs-CRP	:	High sensitive C Reactive Protein
IgG	:	Immunoglobulin G
IgA	:	Immunoglobulin A
ICAM-1	:	Intercellular adhesive molecule-1
IGF-1	:	Insulin-like growth factor 1
IL-6	:	Interleukin -6
L	:	Litre
m	:	Mili
M	:	Molar
MCP-1	:	Monocyte chemoattractant protein-1

mRNA	:	messenger RNA
NT-proBNP	:	N-Terminal pro-B-type Natriuretic Peptide
RNA	:	Ribonucleic acid
V	:	Volt
v/v	:	volume/volume
w/v	:	Weight/volume
W	:	Watt

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 The Ageing Populations

The proportion of people over 60 years of age is growing faster than any other age group in almost every country around the world. This phenomenon is a result of both longer life expectancy and declining fertility rates (United Nations, 2015). This scenario can be seen as a success story of public health policies and improved personal hygiene, advances in medical knowledge and practices, considerably increased control over the environment, rising population income and standard of living (Timiras, 2007). In 2015, one in eight people worldwide was aged 60 years or over. By 2030, older persons are projected to account for one in six people globally. By the middle of twenty-first century, one in every five people will be aged 60 years or over.

The number of older persons is expected to grow fastest in Latin America and the Caribbean over the next 15 years with a projected 71% increase in the population aged 60 years or over, followed by Asia (66%), Africa (64%), Oceania (47%), Northern America (41%), and Europe (23%) (United Nations, 2015). This statistics report indicates an urgent need for health care professionals from all disciplines to identify appropriate measures and outcome for potential prevention and early intervention for healthy and successful ageing. Global efforts are required to understand and find cures or ways to prevent such age-related diseases as Alzheimer's and frailty and to implement existing knowledge about the prevention and treatment of heart disease, stroke, diabetes and cancer (National Institute of Health, 2011). Moreover, research on the changing relationship between health with age is crucial to understand physiological and molecular mechanism associated with the aging process.

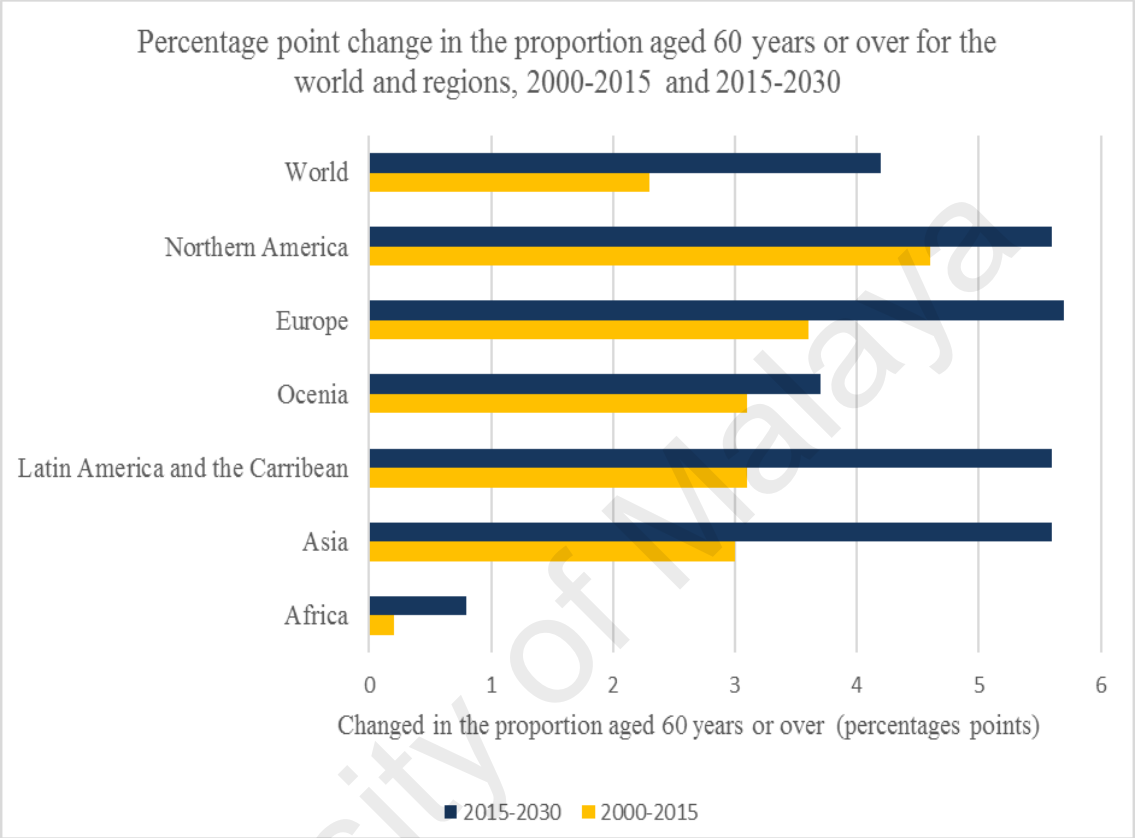


Figure 1.1: Percentage point change in the proportion age 60 years or over for the world and regions, 2000-2015 and 2015-2030 (United Nations, 2015)

1.2 Frailty: Geriatric Syndrome

There is a strong consensus among geriatricians and gerontologist that frailty is a clinical state of increased vulnerability and decreased ability to maintain homeostasis that is age related and centrally characterised by declines in functional reserves across multiple physiologic systems. This vulnerability is age related and also related to, but distinct from, disability and disease state (Jeffrey et al, 2009). Genetically identical animals that lived in the same environment showed apparent evident age-related differences in grooming, greying, and motor performance. Having said that, human

body are by far more complex and therefore, it is expected that ageing rates are heterogenous across the different populations (Gosney et al, 2009).

The prevalence of frailty is high, with estimates ranging from 10% to 25% of person aged 65 years and older, with as many as 30% to 45% of those aged 85 years and older identified as frail. Frail individuals are perceived to constitute those older adults at highest risk for a number adverse health outcomes, including disability, dependency, institutionalisations, falls, injuries, acute illness, hospitalisations, slow or incomplete recovery from illness and/or hospitalisation and mortality. One hallmark of frailty is the dysregulation of homeostatic or communications systems, at both the molecular and physiological level. These physiological systems are interrelated, affecting each other as well as the clinical presentation (Ahmed, Mandel, & Fain, 2007).

New evidence indicates that the risk of frailty is highly associated with multiple systems at abnormal level, significantly more than any one systems such as inflammation, coagulation, immune function and hormones. Theories of basic aging provide a molecular explanation for some of these changes. For example, increased oxidative stress generated in mitochondria is likely to set in motion many processes that can impair physiology. Furthermore, declines in energy production (ATP) can lead to less efficient signal transduction and transcription of inflammatory mediators, which are strongly associated with frailty. Studying the relationship between elements of different dysregulation systems and amongst it will help elucidate the age-related pathway to frailty and progressive disability.

1.3 Proteomics and Biomarkers

In recent years, there has been an enormous growth in the use of genome information in health science. Based on 2011 statistic report there were 87,308 experimentally identified post-translational modifications and 234 398 putative modifications on 530 264 proteins (Khoury, Baliban, & Floudas, 2011). Thus, genetic analysis alone does not give sufficient information to understand the cellular process. The new paradigm is that the genome gives rise to the transcriptome, which is then translated to produce the proteome as a set of proteins. (Twyman, 2014). The proteome gives an overall view about the proteins that result from the genome of cells, a tissue or an organism, and is therefore useful in evaluating disease presence, progression, and response to treatment. Hence, proteomics can be considered as a bridge between genomics and cellular behaviour (Van Eyk, 2008).

Proteomics is the systematic, large scale analysis of proteins which could provide a snapshot of the cell in action or under a defined set of conditions. Proteins are known as the central important macro molecules in the human body and diverse in nature that can be studied using a range of different methods depending on which properties are targeted. The correlation of the dynamic expression of a proteome and the physiological changes related to a healthy or diseased condition can help to support the understanding of disease mechanisms, design new ways for discovery and validation of disease model, find new diagnostics markers, identify potential therapeutic targets, optimise lead compound for clinical development, characterise drug effects and study protein toxicology (Kellner, 2000). Biomarker research is continuously expanding in the field of clinical proteomics with current advancement and combination of different proteomic based approaches which can be applied depending on the specific clinical context of use (Frantzi, Bhat, & Latosinska, 2014).

A biomarker is a biological feature of cell, tissue, or organism that corresponds to a particular physiological state. There are many different types of disease biomarker, including the presence of particular pathogenic entities, disease specific cytological or histological characteristics, gene or chromosome mutations, the appearance of specific transcripts or proteins, new post translational variants, or alterations in the level of mRNA or protein expression. Proteins are the advantageous biomarkers because the direct analysis of protein can reveal post translational modifications, that cannot be identified by DNA sequencing or mRNA profiling. Furthermore, protein biomarkers can be assayed in body fluids, among which serum or plasma is the most valuable because it is in contact with all parts of the body and its composition is influenced by secretions or leakage from cells that are damaged by disease (Twyman, 2014).

1.4 Lectin and post-translational modifications

The term 'lectin' was introduced in 1954 by Boyd is now used for the designation of a 'sugar binding protein of non-immune origin which agglutinates cells and/or precipitates glycoconjugates' (Bog, 1981). Lectins are which found abundantly in the plant and animal kingdom. Alterations in protein glycosylation are associated with many diseases; these may be useful markers of a disease state, reflecting changes in the environment in which most plasma membrane and secretory proteins are glycosylated (Durand & Seta, 2000). Both the carbohydrate structure and the concentration of glycoproteins may change in disease, thus could be utilised in providing diagnostic information. Examples of glycoproteins where such modifications have been described include the tumour products alpha-fetoprotein (AFP), chorionic gonadotropin (HCG), and alpha antitrypsin and the plasma proteins IgG, transferrin, α-1 acid glycoprotein, and fibrinogen. The extensive use of lectins to investigate glycosylation changes in development of disease is currently increasing our understanding of the biological and

pathological significance of glycosylation events and allowing the possibility of new diagnostic approaches (O. H. Hashim, Shuib, & Chua, 2001; Mestecky *et al.*, 1995; Mody, Joshi, & Chaney, 1995; Mohamed *et al.*, 2008)

Serum N-glycan was profiled as ageing biomarker in different age groups of healthy volunteers and patients by using DNA Sequencer Adapted-Fluorophore Assisted Carbohydrate Electrophoresis (DSA-FACE). This study implies that there is shift in human N-glycosylation status of serum proteins during ageing in 100 healthy volunteers, and this has been proposed as an ageing biomarker for healthy humans (Vanhooren *et al.*, 2010). Further research on glycoproteins expressions level will provide additional evidence on age related diseases such as frailty.

Several correlates or markers of frailty have been proposed in order to identify frailty more precisely. Although currently, no single marker could fully assess the complexity of frailty, there is growing evidence that certain contributing factors could facilitate a person's entry into the frailty state, or a define a pre-frailty state. These include biomarkers that have been implicated in its pathophysiology. The development of biomarkers for frailty will therefore be invaluable, but this is currently an under researched area. In order to understand frailty syndrome using a reliable and reproducible method, we initiated this study with the aim of:

Objective of study

1. Assessing frailty level using Frailty Index (FI) tool in order to group subjects into control, pre-frail, and frail based on standard cut off value.
2. To develop and compare profiles of proteins in serum of healthy geriatrics (non-frail), pre-frail and frail individuals using 2-Dimensional Electrophoresis (2-DE) analysis.

3. To analyse the difference in glycoproteome expression in serum of pooled healthy geriatrics (non-frail), pre-frail, and frail elderly using lectin detection method.
4. To identify and validate potential biomarkers of pre-frail and frail using serum sample.

CHAPTER 2: LITERATURE REVIEW

2.1 Ageing and Frailty

Among the challenges faced by the medical world in these early years of 21st century is the foreseen ageing of the world populations (United Nations, 2015), specifically the increase in the number of years spent in disability (Jagger, 2015). World Health Organisation (WHO) defines healthy ageing as a "process of developing and maintaining the functional ability that enables well-being in older age" (World Health Organization, 2015). The "healthy ageing" pathway corresponds to a lifelong process. After conception, the first and second parts of life appear to have the greatest influence on a person's functional trajectory which would able to shift towards a less or more positive living (Michel, Dreux, & Vacheron, 2016).

Ageing can be defined as the decline and deterioration of functional properties at the cellular, tissue and organ level which eventually yields a loss of homeostasis and decreased adaptability to internal and external stress. Ultimately, this conditions will lead to the increase in vulnerability towards disease and mortality (Fedarko, 2011). Ageing is a breakdown in maintenance of specific molecular structures and pathways, a loss of homeostasis, as well as a failure in homeodynamics (Lloyd, Aon, & Cortassa, 2001). Homeodynamics refers to biological systems that do not actively mandate stasis, but rather dynamically reorganise and reset points of balance in response to internal and external transformation, in order to maintain the systems functional capacity across time. Individuals are extremely heterogenous in the onset of the ageing process, the rate at which it progresses, and the extent to which it progresses. Alterations in the manifestations of ageing reflect differences in the functional capacity. Functional

capacity is a direct measure of the ability of cells, tissues, and organ systems to perform optimally and is influenced by both genotype and phenotype. Optimal cellular, organ and organism process reflect hemodynamic mechanisms/maintenance pathways such as, DNA repair, detection and clearance of defective proteins and lipids, clearance of defective organelles and cells, and defence against pathogens and injury (Fedarko, 2011).

Many of the characteristics of frailty also apply to the ageing process, hence it is equally important to study frailty as the world's population is preparing for a silver tsunami. Chronological age alone is only a rough proxy of a person's vulnerability to adverse outcomes. Some people appear to be frail at the age of 70 years, whereas others only reach this state in their 90s, in other words not all old people are frail (Bergman *et al.*, 2007). The first articles published on frailty was in the seventies, however it was not until the late nineties the actual trend in frailty research started to bloom (van Abellan Kan *et al.*, 2008).

Frailty is defined as a multifactorial syndrome that represents a reduction in physiological reserve(homeostasis) and in the ability to resist environmental stressors (Bergman *et al.*, 2007). Aristotle has described this syndrome as a period when the body's heat dissipates and is no longer able to provide energy and balance; the loss of inward heat depressed the spirit, caused illness, and decreased strength, and eventually human beings lost their passion for life and succumbed to death (Ahmed *et al.*, 2007). Frailty is a long established clinical expression that implies concern about an elderly person's susceptibility and outlook. Apparently small disturbance (e.g., a new drug, minor infection, or minor surgery) results in a striking and unequal change in health

state, it could be from independent to dependent, mobile to immobile and stability to falling.

Frailty is a disorder of several inter-related physiological systems. A gradual decrease in physiological reserve occurs with ageing but in frailty, this decrease is accelerated and homeostatic mechanism start to fail (Clegg, Young, Iliffe, Rikkert, & Rockwood, 2013). Frailty is not synonymous with disability or comorbidity, rather comorbidity is a risk factor for frailty and disability is a result of frailty (Hubbard & Jatoi, 2015). Thus, frailty appear to apply to distinct, but related, entities and should not be used interchangeably (Fried *et al.*, 2001a). The etiology of frailty can be outline as below (Bortz, 2002):

Genetic disorder

Errors in the genetic program can contribute to frailty either through primary muscle, bone, or neurologic malformation, or secondarily through many other entities, such as sickle cell anemia, mitochondrial DNA mutations which lead to decreased in energy level.

Disease and injuries

This causative element of frailty contributes a major part that could to provoke frailty. Toxins, infections, injuries, and malignancy all may provoke frailty.

Lifestyle

The greatest contributor to frailty is lifestyle. This include either insufficient or excessive calories, sedentary lifestyle which lead to diminished muscle strength and frailty.

Ageing

The effect of aging certainly has the potential to affect the development of muscle weakness through accumulation of metabolic debris, membrane stiffening, and DNA alteration.

It is quite clear from the etiology, that frailty (except for genetic factors), can be reversed through tailored interventions when frailty is identified at an early phase.

In relation to the decline in homeostatic reserve, three stages in the frailty process can be described: a pre-frail process, the frailty state and frailty complications (Ahmed *et al.*, 2007). The pre-frail process, which is clinically silent, corresponds to the state where physiological reserves are sufficient to allow the organism to respond sufficiently to any stressors such as acute disease, injury, with a chance of complete recovery. The frailty state is characterised by slow, incomplete recovery after any new acute disease, injury or stress, confirming that the available functional reserves are inadequate to allow a complete recovery (Lang, Michel, & Zekry, 2009).

The identification of frailty with an appropriate tool should be the first step taken. There is no consensus definition or official International Classification of Disease (ICD) diagnosis for frailty. A number of instruments have been developed to measure the

level of frailty as part of a stepwise assessment of vulnerability. These includes, Fried Criteria, Frailty Index (FI), Groningen Frailty Indicator, Frailty and Autonomy Scoring Instrument of Leuven (FRAIL), Edmonton Frail Scale, Frailty Staging System, British Frailty Index and other modified versions (De Lepeleire, Iliffe, Mann, & Degryse, 2009).

Frailty has been measured using markers such as physical ability, self-reported health indicators and well-being, co-morbidity, physiological markers as well as psychological factors (Kamaruzzaman, Ploubidis, Fletcher, & Ebrahim, 2010). Even though there are different model or tools which correspond to varying trajectories of frailty in frailty identifications, ultimately it leads to adverse outcomes related to ageing. On top of that, this suggest that frailty is a multidimensional phenotype. The most widely used and validated frailty tool in gerontology is Frailty phenotype (Fried criteria) (Fried *et al.*, 2001b) and FI (K. Rockwood *et al*., 2005). According to Fried's, phenotypic definition of frailty as a geriatric syndrome, there are five phenotypic criteria: weakness as measured by low grip strength, slowness by slowed walking speed, low level of physical activity, low energy (self-reported) and unintentional weight loss. To be declared "frail" by these criteria, subjects had 3,4, or 5 components present, intermediate subjects with 1 or 2 of these criteria and subjects without any criteria classified as non-frail. The FI was developed by Rockwood and co-workers in 2007 based on comprehensive geriatric assessments by counting the number of deficits (things that individual have wrong with them) accumulated, including diseases, physical and cognitive impairments, psychosocial risk factors, laboratory measurements (Rockwood & Mitnitski, 2007). The total number of deficit (variable) that can be used in the FI is considered to be 80, with 30-70 items being typically calculated (X. J. Chen,

Mao, & Leng, 2014). FI does not define a syndrome, which is a collection of a specific symptoms and signs. Instead the FI can be considered as a state of variable, in that it characterizes the whole health of individuals. Compared to the Fried's criteria FI appears to be more sensitive predictor of adverse health outcomes, because of its more finely categorised risk scale and inclusion of deficits that possible have causal relationships with adverse clinical outcomes (Rockwood & Mitnitski, 2007).

2.1.1 Pathophysiologic factors

One hallmark of frailty is the dysregulation of homeostatic or communications systems, at both the molecular and physiological level. Decline in hormone dihydroepiandrosterones (DHEAs) level, increases in afternoon cortisol levels and in inflammatory and clotting markers, indicates the immune and neuroendocrine systems as likely candidates as the physiological source of this dysregulation. Theories of basic aging provide in part a molecular explanation for some of these changes. For example, increased oxidative stress generated in mitochondria is likely to set in motion many processes that can impair physiology. Further decline in energy production (ATP), can lead to less efficient signal transduction and protein translation in many cells, which in time, leads to alterations in biological systems. In addition, free radicals themselves damage mitochondrial DNA, which in turn will lead to even less efficient energy production and greater increases in oxidative stress. This process may lead to increasing DNA and protein damage, as well as direct transcription of inflammatory mediators, which are strongly associated with frailty. Through studying the relationship between and among elements of this paradigm (inflammatory, hormone, nutritional), we will better understand the age-related pathway to frailty and progressive disability (Halter, Jeffrey et al., 2003).

2.1.2 The Need to Identify Frailty

Identifying the underlying biological drivers of frailty and their relationship to age related traditional changes could enable targeted research into the causes of frailty, perhaps with the identification of a pre-frail population, and the possibility from this of developing preventative interventions (Martin & Brighton, 2008). The possibility of finding biomarkers of pre-frailty is being studied by several researchers as it would allow us to reach an early diagnosis of the risk of becoming frail. A consequence of this would be that frail patients would be much more effectively treated if treatment were established very early on or even before the onset of frailty (Vina *et al.*, 2016). Apart from that, it is equally important in studying frailty associations with biomarkers in populations with underlying chronic disease such as cancer, the outcome would be beneficial in terms of patient's management and further treatment plan. Tremendous advances in analytical platforms which is able to reveal the molecular aspect of underlying mechanism and pathway, will further improve understanding on biological basis of frailty and this could help in developing more effective interventional strategies that target specific physiologic systems and open a path for improvised geriatric care.

2.2 Biomarkers

A biological marker (biomarker) is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic interventions (Biomarkers Definitions Working, 2001). Biomarkers can be proteins, DNA, RNA, or even metabolites (Wan-Ibrahim, Singh, Hashim, & Abdul-Rahman, 2015). Among the valuable applications of biomarkers in disease detection and monitoring of health status includes, as a diagnostic tool for the identification of those patients with a disease or abnormal condition, tool for

staging of disease or classification of the extent of disease, indicator of disease prognosis and lastly for prediction and monitoring of clinical response to an intervention (Biomarkers Definitions Working, 2001).

In the past years, number of studies on frailty and putative biomarkers association were of great interest among clinicians and scientist working on the ageing research and a number of using different approach was taken. Longitudinal and prospective studies across country and region with large number of participants such as The Irish Longitudinal Study on Ageing (TILDA) (O'Halloran *et al.*, 2016), English Longitudinal Study of Ageing (ELSA) (Mekli, Marshall, Nazroo, Vanhoutte, & Pendleton, 2015), Invecchiare in Chianti , aging in the Chianti area (InCHIANTI) (Urpi-Sarda *et al.*, 2015), Toledo Study for Healthy Aging (TSHA) (Alvarez-Rios *et al.*, 2015), NewCastle 85+ (A. Mitnitski *et al.*, 2015) and ongoing study FRAILOMIC which is an international, large scale, multi-endpoint, community and clinic based research study funded by European commission with more than 75,000 subjects (Erusalimsky *et al.*, 2016), provide diverse information and this will increase the understanding on frailty trend in different geographic region. Several type of frailty biomarkers were identified so far based on different frailty measures (Ramakrishnan *et al.*, 2017). Collectively, the biomarkers were found to be associated with inflammation, markers of metabolic processes and clotting process, genetic and nutritional elements (Table 2.1).

Table 2.1: Examples on frailty association with identified potential biomarkers. Adapted from Ramakrishnan *et al.* (2017)

References	Biomarkers	Frailty Measure	Results
(Gale, Baylis, Cooper, & Sayer, 2013)	CRP, fibrinogen	Fried's criteria	Frail: ↑ CRP and ↑ fibrinogen ($p<0.001$) – In women populations.
(Mekli *et al.*, 2015)	Genes *IL-18, IL-12A, LRP, SELP*	Frailty Index	*IL-18, IL-12, LRP1, SELP* gene show significant genetic association with decreasing FI.
(Ronning *et al.*, 2010)	CRP, IL-6, TNF-α, D-dimer	Modified Fried's criteria. CGA	Frail: ↑ CRP (P=0.001), ↑ IL-6 (P<0.001), ↑ TNF (CGA) (P=0.001).

Table 2.1, continued'

References	Biomarkers	Frailty Measure	Results
(Shamsi *et al.*, 2012)	Plasma glycoproteins	Fried's criteria	Pre-frail: two-fold increase of haptoglobin, transferrin, and isoform of kininogen-1 variant. Pre-frail: two-fold or greater decrease of kininogen-1 variant isoform, hemopexin precursor, an isoform of fibrinogen, leucine-rich alpha-2-glycoprotein 1 and apolipoprotein E.
(Jylhava *et al.*, 2013)	cf-DNA, IL-6, IL-10, CRP, unmethylated cf-DNA, mt-DNA	Fried's criteria	↑ Frailty: ↑ cf-DNA (P=0.002), unmethylated cf-DNA (P=0.001), ↑mt-DNA (P=0.029). Frailty: ↑ CRP (P<0.001), ↑ IL-6 (P=0.004).

Table 2.1, continued'

References	Biomarkers	Frailty Measure	Results
(Sanchis *et al.*, 2015)	Inflammation, coagulation, hormonal dysregulation, nutrition, renal & heart dysfunction	Fried's criteria	Frail: ↓ Hb (P=0.001), ↓DHEA (P=0.006), ↓Vitamin D (P=0.004), ↓Albumin (P=0.007), ↓Zinc (P=0.03), ↑ Creatinine (P=0.001), ↑D Dimer (P=0.001), ↑Cystatin-C (P=0.0001), ↑NT-proBNP (P=0.0001).
(Brouwers *et al.*, 2015)	T/S ratio, IL-6, IGF-1, MCP-1, RANTES	Balducci score. LOFS (Leuven Oncogeriatric Frailty Score).	<u>Biomarkers vs chronological age</u> Mean telomere length P<0.0001, IL-6 P<0.0001, IGF-1 P<0.001, MCP-1 P<0.0001. <u>Balducci</u> Frailty: IL-6 (p=0.019) <u>LOFS</u> Frailty: IL-6 (p=0.013)

Table 2.1, continued'

References	Biomarkers	Frailty Measure	Results
(Lorenzi *et al.*, 2016)	HtrA1	Fried's criteria Modified Rockwood's Frailty index	<u>Fried's</u> Frail: HtrA1 ↑ 75.9 (67.4-85.6) (p<0.001). <u>Frailty Index</u> Frail: HtrA1 ↑ 72.2 (63.4-82.3) (p<0.001)
(W. J. Lee *et al.*, 2016)	sICAM-1, IL-6	Fried's criteria	sICAM-1 levels stepwise increased in robust, pre-frail and frail elderly p<0.001. Frail: Log IL-6 1.54(1.07-2.20).
(Zhu *et al.*, 2016)	hsCRP	Fried's criteria	Frailty: ↑ CRP 1.49 (1.05, 2.09) P=0.024 Overnight hospital admission: ↑ CRP 1.94 (1.08, 3.48) P=0.027.

2.3 Proteomics

2.3.1 Definition of proteomics

Proteins are one of the main structural and functional constituents of every living cell, and ever since the human genome was sequenced, several initiatiaves have focused on describing the proteomic landscape of human tissues, in order to translate the sequence information in the protein-coding genes into biologically relevant knowledge (Lindskog, 2016).

The term "proteomics" was first coined in 1995 and was defined as the large-scale characterisation of the entire protein-based gene expression of a cell line, tissue, or organism (Wilkins *et al.*, 1996). Proteomics is a tool to characterise protein structure, function, protein-protein interaction, and associated protein modifications (J. M. Lee, Han, Altwerger, & Kohn, 2011). Proteomics has been a rapidly growing field of research that can examine both the presence or absence of proteins and its translation modifications(Anderson & Anderson, 1996).

The introduction of 2 dimensional gel electrophoresis (2-DE) together with primary protein study in 1975 was the stepping stone into proteomics research (Klose, 1975). Proteomics has important clnical implications in providing direct information on druggable targets and phenotype characterisation because proteins are the molecules that are involved in many cellular functions for maintaning homeostasis in our body (Bin Goh & Wong, 2016). Additionally, proteomics offers complimentary information to genomics and transcriptomics that are essential for molecular level understanding of

complex biochemical processes. Although in many situations alterations in protein abundance and function can be attributed to altered gene expression, multiple posttranscriptional and post-translational mechanisms can affect protein abundance and function as well. Therefore, proteomes are often much more complex than the corresponding genomes (Z. R. Zhang, Wu, Stenoien, & Pasa-Tolic, 2014).

The principal aim of current proteomics research is to identify and characterise potential biomarkers by addressing two aspects: functional and expression proteomics. Functional proteomics deal with characterisation of proteins in organells and complexes, while the expression proteomics measure protein level fluctuations under certain conditions or parameters. Certainly, expression proteomics can serve as a powerful tool to detect changes in protein expressions in disease state or during treatment in response to drug therapy (Khalilpour, Kilic, Khalilpour, Alvarez, & Yazdi, 2017). Hence, comparative analysis of protein expression to identify aberrantly expressed proteins that may represent new markers in normal and disease state, is vital towards the understanding of pathological conditions and diagnosis.

2.3.2 Proteomics in frailty research

The proteome is seen as more closely linked to the phenotype compared to transcriptome; and it is more stable than the metabolome; and it is thus considered as the most promising *omics* in the ageing research. Collective quantification and characterisation of a pool of specific biological molecules that reflect the structure, function, and dynamics of an ongoing physiological state of all tissues will aid in comprehensive studies on the molecular and biological processes changing with age (da Costa, Rocha-Santos, & Duarte, 2016). Findings from the first application of high

throughput proteomics methodology to study the geriatric syndrome of frailty found seven glycoproteins to differ by at least two fold in pre-frail compared with non frail older adults. The glycoproteins includes haptoglobin, transferrin and fibrinogen which are related to the hematologic and inflammatory changes associated with frailty (Shamsi *et al.*, 2012). To confirm the pilot study, plasma levels of inflammatory glycoproteins, as well as of interleukin-6 was validated in a larger sample of community dwelling of older adults. The study reported higher levels of transferrin, fibrinogen, and interleukin-6 were associated with frailty status (Darvin *et al.*, 2014). In Malaysia, research in frailty and proteomic is very limited and to the best of our knowledge there has been no similar study or finding reported.

2.3.3 Techniques for proteomics analysis

In proteomics analyses; gel based approaches, including two-dimensional gel electrophoresis (2-DE) and differential in-gel-electrophoresis (DIGE), and gel-free mass spectrometry (MS) based approaches, e.g. isobaric tags for relative and absolute quantitation (iTRAQ) and liquid chromatography (LC), can be utilised separately or in a variety of combinations, (e.g. sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE and LC/MS), to separate proteins and peptides. Despite several limitations and laborious methods, 2-DE remains a powerful tool for high resolution separation of proteins in complex biological samples. 2D gels display thousands of protein species, their isoforms and post-translational modifications at the same time. In addition, 2D gels show the highest resolution on protein levels, display protein isoforms as well as post-translational modifications in the pattern through changes of the spot positions in the pI direction. They also possess a very wide separation range for both , isoelectric points and molecular sizes (Westermeier, 2016)

2-DE enables the visualisation of protein spots after staining, which greatly simplifies spots matching and downstream analysis. Furthermore, 2-DE can flexibly be integrated with different LC separation and MS analysis (e.g. 2DE-LC/MS) (Ning, Wu, & Wang, 2016). The basic workflow of a proteomics experiment start with separation of proteins, followed by the acquisition of protein structural information for the protein identification purpose and finally data utilisation. Current clinical proteomics is based on mass spectrometry (MS) tools, where different methods have been successfully implemented in analyses of blood proteome (Pietrowska & Widlak, 2012).

2.3.4 Separation techniques

Proteins and peptides are possibly the most studied class of molecules investigated by electrophoretic methods. These methods include: agarose and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) , 2-DE, capillary electrophoresis, isotachophoresis and others. Among these techniques is isoelectric focusing (IEF), where proteins are separated as they migrate through the pH gradient when electric field applied. In this way, proteins are condensed, or focused, into sharp bands in the pH gradient according to nature of their pI values. Second dimension separation is by protein mass or molecular weight (MW) using (SDS-PAGE). The proteins that are resolved in first dimensions are then separated by MW perpendicularly. The pores of the second dimension gel sieve proteins according to size in gradient based gel. Stains such as Coomassie blue, silver, SYPRO Ruby and Deep Purple can be used to visualise the resolved proteins in gels.

2.3.5 Protein identifications

Mass spectrometry (MS) has emerged as the most important tool to identify, characterise, and quantify proteins and their post-translational modifications (PTM) at high throughput and on a large scale (Z. R. Zhang *et al.*, 2014). Perhaps the greatest advantage of 2-DE and related IEF techniques is that they are complementary with MS. When used together, this approach is able to reduce proteome complexity with enough resolution so that single proteoforms can be analysed with significantly far depth compared to a standard shotgun approach (Pergande & Cologna, 2017). Fundamentally, MS measures the mass-to-charge ratio (*m/z*) of gas phase ions. MS consist of an ion source that converts analyte molecules into gas-phase ions, a mass analyser that separates ionised analytes on the basis of *m/z* ratio, and a detector that records the number of ions at each m/z value (Han, Aslanian, & Yates, 2008). There are two main technologies available for sample processing in MS quantitative proteomics analysis. These are electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI). ESI technology uses electric energy to change ions from a solution into gas phase, and after they obtain their charge, the ions travel through the analyser to the detector which can identify them according to their *(m/z)* ratio upon which the signals are recorded as a mass spectrum in a computer. MALDI MS technology on the other hand, uses a laser beam to irradiate a solid sample in an organic matrix. This causes the formation of protonated molecules. The ionised samples then travel through a mass spectrometer that in MALDI technology usually works with time of flight (TOF). This type of analysis eventually translates to the *m/z* of the sample (Licier, Miranda, & Serrano, 2016).

2.4 Lectin: Definitions and history

Lectins (from *lectus*, the past participle of *legere*, to select or choose) are defined as carbohydrate binding proteins other than enzyme or antibodies and exist in most living organism, ranging from viruses and bacteria to plants and animals. The beginings of lectinology dates back to 1888 when Hermann Stillmark described the agglutination properties of ricin; however, the modern age of lectinology started nearly 100 years later (Sharon & Lis, 2004). The initial pure lectin, concanavalin A (con A, from jack beans), was isolated in 1919 by Sumner, who also demonstrated its sugar specificity. Subsequently, lectins played a crucial role in elucidating the molecular basis for blood grouping specificity. Classical lectins consists two or more carbohydrate-binding sites; therefore, their interaction with sugars on the surface of erythrocytes results in the cross-linking of several blood cells and their subsequent precipitation. Both the agglutination and precipitation processes are inhibited by the carbohydrate for which the lectin is specific (Ambrosi, Cameron, & Davis, 2005). The main feature of these proteins are its selectivity to recognise and bind reversibly to specific mono- or oligosaccharides. Also, to be defined as lectins, carbohydrate -binding proteins have to display no enzymatic activity towards the recognised sugars and should not belong to the immunoglobulin family (Bellande, Bono, Savelli, Jamet, & Canut, 2017).

Lectins were first found and described in plants, but in over the years multiple lectins were isolated from microorganisms and also from animals (Sharon & Lis, 2004). Interestingly plant and animal lectins show no primary structural homology, yet they show similar preferential binding to carbohydrates (Ghazarian, Idoni, & Oppenheimer, 2011). Many primary and three dimensional structures of lectins have been elucidated in the past several years (de Oliveira *et al.*, 2008; Loris, 2002; Pratap *et al.*, 2002; Wah

et al., 2001) (M. Gabrielsen *et al.*, 2009). It was observed that the lectins from diverse sources lacked primary sequence similarity but shared similarities in their tertiary structures (Sharon & Lis, 2004).

2.4.1 Production of lectins

Lectins are can be found in a plethora of sources in nature. A large number of lectins or hemagglutinins have been purified from different organisms. On the other hand, lectins can also be purified by using recombinant techniques, for example, cloned cDNA and expressed in *Escherichia coli* strain in specific culture medium, can produce sizeable quantity of *Allium sativum* lectin . In animals, the yield of lectins are usually extremely low. Example of animal lectins are *Acropora millepora* (plasma fluid), *Aristichthys nobilis* (gills), *Bubalus bubalis* (heart tissue), *Holothuria scabra* (coelomic fluid), *Macoma birmanica* (foot muscles) and *Nemopilema nomurai* (jellyfish). Mass purification of animal lectins necessitates bulk quantities of raw materials which make it not feasible. The lectin content in some parts of plants are higher, example *Remusatia vivipara* tubers and *Astragalus mongholicus* roots. Apart from that, the source for lectins can be obtained from leaf *Allium sativum* (garlic), beans *Glycine max* (soybean), fruiting bodies *Polyporous squamosus* and from bark *Myracrodruon urundeuva* (aroeira preta) (Lam & Ng, 2011). Further examples of source of lectins and their specificities described in Table 2.2.

Table 2.2: List of examples of source of lectins and their specificities. Adapted from Manning *et al.* (2017)

Species	Abbreviation	Monosaccharide specificities	Potent glycan ligands
Canavalia ensiformis (jack bean)	ConA	Man/Glc	Glc*N*Aβ2Manα6(Glc*N*Acβ2Manα)Manβ Glc*N*Ac
Triticum vulgare (wheat)	WGA	Glc*N*Ac	Stem region of *N*-glycans, (Glc*N*Ac)
Griffonia simplicifolia isolectin-II	GSA-II	Glc*N*Ac	GlcNAcα3/4Gal, (Glc*N*Ac)
Phaseolus vulgaris erythroagglutinin (kidney bean)	PHA-E	unknown	Bisected complex-type-*N*-glycans: Galβ4G1c*N*Acβ2Manα6(Glc*N*Acβ2-Manα3)(Glc*N*Acβ4) Manβ4Glc*N*Ac
Phaseolus vulgaris leukoagglutinin (kidney bean)	PHA-L	unknown	Tetra- and tri-antennary N-glycans with β6-branching
Viscum album (mistletoe)	VAA	Gal	Galβ2(3)Gal, Galα3(4)Gal, Galβ3(4)Glc*N*Ac without/with α2,6-sialylation, Fucα2Gal
Limax flavus (slug)	LFA	Neu5Ac>Neu5Gc	Sialylated glycoconjugates

Table 2.2, continued'

Species	Abbreviation	Monosaccharide specificities	Potent glycan ligands
Artocarpus integrifolia (jackfruit)	Jacalin (JAC)	Gal/Gal*N*Ac	Galβ3Gal*N*Acα; sialylation of T/T antigens tolerated
Polyporus squamosus (polypore mushroom)	PSL	unknown	Neu5Acα6Galβ4Glc(*N*Ac) (over 300-fold more active than Lac*N*Ac, not reactive with free Neu5Ac); 6'-sulfation tolerated;6'-sialyl T not reactive.
Homo sapiens	CD22/Siglec-2	unknown	Neu5Acα6Galβ3(4)Glc*N*Ac; 9'-*O*-acetylation blocks binding
Arachis hypogaea (peanut)	PNA	Gal	Galβ3Gal*N*Acα/β
Maackia amurensis-I (leuko)agglutinin (Amur maackia)	MAA-I	unknown	Neu5Acα6Galβ4GIc*N*Ac/Glc; 3'-sulfation tolerated
Sambucus nigra-I (elderberry)	SNA-I	Gal/Gal*N*Ac	Neu5Acα6Gal/Gal*N*Ac; clustered T antigen

2.4.2 Applications of plant lectins

Lectin has very important role in biomedical research because of its diverse characteristics. Lectins isolated from *Moringa oleifera* seeds show specificity to blood group A and B lectin from *Erythrina velutina* seeds is A, B, and O blood group-specific. Lectins obtained from *Iris amara* is M blood group specific whilst *Vicia graminae* and *Bauhinia purpurea* lectins are N blood group specific (Hivrale & Ingale, 2013).

Since a set of cell surface proteins and lipids are glycosylated, they can function as lectin binding sites. Different cell types generally express glycoconjugates that differ in the glycosylation patterns, as with tumor cells compared with normal type. In this sense, lectins can interact differently with distinct cells and may act as carriers of drug specifically to targetted cells and tissues (Coelho *et al.*, 2017).

Besides that, the defensive effect of ricin (J.-Y. Lin, Tserng, Chen, Lin, & Tung, 1970), Con A (Shoham, Inbar, & Sachs, 1970) and *Griffonia simplicifolia* lectin (Eckhardt, Malone, & Goldstein, 1982) against tumour growth in experimental animals has been described. The shielding effect of ricin and abrin in humans againt tumour growth and in grouping with anticancer drugs has also been illustrated (H Lis & Sharon, 1986). Similarly, in lectin dependent cytotoxicity, *Gliffonia simplicifolia* hold the capacity to mediate carbohydrate explicit binding of mouse macrophages and tumour cells and to induce killing of the tumour cells by the macrophages (Hivrale & Ingale, 2013).

Efforts have also been made to synthesise lectin-monoclonal antibody conjugates that can specifically bind to target tumour cells and induce cytotoxic effects. In this system the lectin is the toxic entity and the antibody is a monoclonal tumour-specific antibody. The toxic lectins typically used are plant lectins such as ML-1 or the A-chain of ricin (Ghazarian *et al.*, 2011).

Lectins and glycan-binding antibodies, collectively known as glycan-binding proteins, can be used in histochemistry, the probing of electrophoretic gels, affinity chromatography, solid phase ELISA-type assays and microarray assays. For example, an immobilised antibody can capture a protein of interest, and the glycans on that protein may be probed using a variety of lectins. This information is essential because glycosylation state of a protein may be critically important to its function or perhaps associated with a particular condition (Haab, 2012).

The glycan moieties covering cell surface are involved in many physiological and pathological processes related to cell. Disturbances in cell environment related to diseases frequently induce alteration in glycans, such as fucosylation, sialylation, abnormalities in glycan structure, and uncommon glycans. In this context, lectin abilities to bind carbohydrates are useful to investigate changes in the expression of glycans on cells or tissue surfaces. Histochemical analysis using conjugated lectins as potential markers for altered glycans may show differential binding patterns to normal and transformed tissues. Generally, lectin histochemistry uses peroxidase-conjugated lectin followed by addtion of diaminobenzidine (DAB) and hydrogen peroxidase for visualisation of binding. This method has been utilised as an approach for research,

such as in diagnosis, and prognosis of human diseases, which are signalised by altered cells in tissues, such as cancer (Coelho *et al.*, 2017).

2.4.3 Lectin of interest: *Artocarpus integer*

Artocarpus integer is a local fruit found commonly throughout Malaysia and a few other South East Asian countries such as Indonesia and Thailand. In Malaysia, the local name for this fruit is champedak. *A. integer* and jackfruit *(Artocarpus heterophyllus)* belong to a common series the Cauliflori and are closely related cytotaxonomically. Both species bear fruits with similar distinctive sweet aromatic smell and their inner part occupied with brown seeds covered with yellow fleshy pulp. On the contrary to jackfruit, the champedak fruit is elongated and smaller in size, contain more seeds and have a flesh that is softer and juicer.

2.4.4 Characteristics of *Artocarpus* lectins.

The seeds from *A.integer* contain two types of lectins: the D-galactose-binding lectin and the D-mannose-binding lectin. The IgA-reactive and D-galactose binding lectin, also named as the champedak galactose binding (CGB) lectin, was discovered by group of researchers from University of Malaya (Onn Haji Hashim, Ng, Gendeh, & Jaafar, 1991). Following that, the same research team have identified D-mannose binding lectin after six years (S. B. Lim, Kanthimathi, & Hashim, 1998). The CGB lectin comprises two distinct non-covalently linked subunits with apparent Mr of 13,300 and 16,000 (O. Hashim, Gendeh, & Jaafar, 1993), whereas the D-mannose binding lectin, also known as champedak mannose binding (CMB) lectin consists of monomeric

polypeptide subunits with Mr of 16,800. The structure of CMB is similar but not quite identical with the lectin from jackfruit due to the presence of disulphide linkages (Sing Bin Lim, Chua, & Hashim, 1997). The interaction of CMB lectin with IgE and IgM was strong, but not with IgA1, IgA2, IgD and IgG (Sing Bin Lim *et al.*, 1997). The CMB lectin demonstrated strong interaction with haptoglobin β chain, orosomucoid, α1-antitrypsin, α2-HS glycoprotein, tranferrin, hemopexin, α1B-glycoprotein, and the heavy chains of the immunoglobulins and α1β-glycoprotein (O. H. Hashim, Ahmad, & Shuib, 2001). CMB lectin was incapable of activating the murine B cells for the secretion of immunoglobulins in the absence of T cells and macrophages (Sing Bin Lim *et al.*, 1997).

2.4.5 Glycoproteins

There are two main types of glycosylation, namely, the N-glycosylation, where glycans are linked to asparagine residues in a consensus sequence N-X-S/T (X can be any amino acid, except proline) via an N-acetylglucosamine (N-GlcNAc) residue, and the O-glycosylation, where the glycans are attached to serine or threonine. It is estimated that over 50% of all human proteins are glycosylated, hence it become one of the major components of human plasma (Fanayan, Hincapie, & Hancock, 2012). Glycans are able to affect the physical properties of the proteins to which they are attached and provide lectin-recognition sites. Thus, the roles of glycosylation have been proved in protein folding in the endoplasmic reticulum, transport and secretion, anchoring of proteins and protease protection (Wormald & Dwek, 1999).

Serum N-glycans profile were identified using "DNA Sequencer Adapted-Fluorophore Assisted Carbohydrate Eletrophoresis" (DSA-FACE) to validate serum N-glycan ageing biomarker, GlycoAgeTest (NGA2F and NA2F glycans) in different age groups of healthy human volunteers and patients. Measuring the shift in serum N-glycan profile could be used to regularly evaluate the overall age-related health status. This not only facilitates the diagnosis of age-related disease such as dementia, but also the follow up different therapies in individuals not suffereing from accelerated ageing (V.Vanhooren *et al*, 2010).

CHAPTER 3: MATERIALS AND METHODS

3.1 Study subjects

Subjects were recruited with informed consent and approval by the Ethics Committee at the University of Malaya Medical Centre in accordance to ICH GCP guideline and the declaration of Helsinki. Elderly patients (n=137), were recruited from University of Malaya Medical Centre who were admitted in the Geriatric Ward. Control subjects (n=60) on the other hand, were recruited from the community, comprising local senior citizen group of the same range of age. Elderly subjects were interviewed about their general health, physical ability in carrying out daily activity, how arthritis is affecting their health, psychological problems (memory and depression) measuring blood pressure lying down and standing up, cardiovascular and respiratory symptoms and general physical evaluation including body mass index (BMI) and waist and hip circumference. Apart from that, information on metabolic conditions and subject health history were also obtained from available medical notes. Patients who were medically unstable, requiring continuous monitoring, bed fast prior to admission, reduced level of consciousness, acute gastrointestinal haemorrhage, acute kidney injury and cancer were excluded. Three ml of whole blood was obtained from each patient (taken when the patients were requested to do blood test in the ward) and normal volunteer. The blood was left at room temperature for 30 minutes, and then centrifuged for 20 minutes (Hettich Zentrifugen, Mikro 22) to separate the serum from packed blood cells. The separated sera was kept in aliquots of 100µl at -80° C until use.

Table 3.1: The list of health deficit variables included in the Frailty Index (FI) and how they were coded as deficit

	List of variables included in the FI	Score
1	Is your present state of health causing you problem with household chores?	Yes=1, No=0
2	Difficulty in carrying out activity on their own: Going up and downstairs?	Yes=1, Some Problem=0.5, Unable=0
3	Difficulty in carrying out activities on their own: Walking about/going out of the house?	Yes=1, Some Problem=0.5, Unable=0
4	Compared with your activity level 3 years ago, are you doing more, same or less?	Less=1, More/Same=0
5	Have you ever told by a doctor that you have or have had arthritis?	Yes=1, No=0
6	Have you had fall in the past 1 year?	Yes=1, No=0
7	Myocardial Infarction	Yes=1, No=0
8	Chest pain lasting more than 30 min?	Yes=0.5, No=0
9	Any discomfort in your chest?	Yes=0.5, No=0
10	Palpitations	Yes=0.5, No=0
11	Bronchitis, Emphysema	Yes=1, No=0
12	Do you get short of breath with other people of your own age on ground level?	Yes=1, No=0
13	Do you usually bring up phlegm (spit) from your chest first thing in the morning?	Yes=1, No=0
14	In the past 4 years, have you ever had a period of increased cough and phlegm lasting for 3 weeks or more?	Yes=1, No=0
15	Asthma	Yes=1, No=0
16	Your health over all: are you anxious or depressed?	Extremely=1, Moderately=0.5,Not Depressed=0
17	Compared to 5 years ago, how is your memory?	Worse/Much worse=1, Almost as good=0.5, Improved/same=0
18	Have you ever been told by a doctor that you have or have had depression?	Yes=1, No=0
19	Stroke	Yes=1, No=0

20	Hypertension	Yes=1, No=0
21	Diabetes Mellitus	Yes=1, No=0
22	Thyroid	Yes=1, No=0
23	Cancer	Yes=1, No=0
24	Peptic/Stomach Ulcer	Peptic Ulcer=1, Indigestion=0.5, No=0
25	Hearing Trouble	Yes=1, No=0
26	Eye sight trouble	Yes=1, No=0
27	Cataract	Yes=1, No=0
28	Glaucoma	Yes=1, No=0
29	Body Mass Index	<18.5 = 1, >30 =1
30	Waist Hip Ratio	Male >0.9 = 1, <0.9 = 0 Female >0.85 = 1, <0.85 = 0
31	Postural Hypotension	Systolic Drop>20mmHg=1 / Diastolic drop>10mmHg=1
32	Sinus Tachycardia	Heart Rate>100 =1

3.2 FI constructions

In this study, we used the 32 variables from the British Women's Heart and Health Study (BWHHS) (Kamaruzzaman *et al.*, 2010) for the construct of FI (Table 3.1). The variables gathered were patient's comorbidities, social activity, activity of daily living, physical measurement and general mood. The presence of a deficit in the patient was scored as 1 point and 0 points when no deficits is present. The FI was calculated as the total number of deficits present in the patient divided by the total number of variables (32) in the FI questionnaire. The frailty index ranged from 0 (representing non-frail status) to 1 (representing severely frail status). Patients were then stratified into 3 groups as non-frail, pre-frail and frail categories (Table 3.2)

Table 3.2: Frailty Index cut off value

	Categories	Cut off value
1	Non-Frail/Control	<0.08
2	Pre-Frail	0.08 – 0.24
3	Frail	≥0.25

$$\text{Frailty Index} = \frac{\text{Number of deficits present}}{\text{Total number of variables}} \quad \text{e.g.,} \quad \frac{15}{32} = 0.47. \text{ hence, frail}$$

Following the grouping, serum samples from 15 subjects from each group were selected and subjected to 2-DE analysis. Samples were run individually. Summary of workflow of this project is outlined in Figure 3.1.

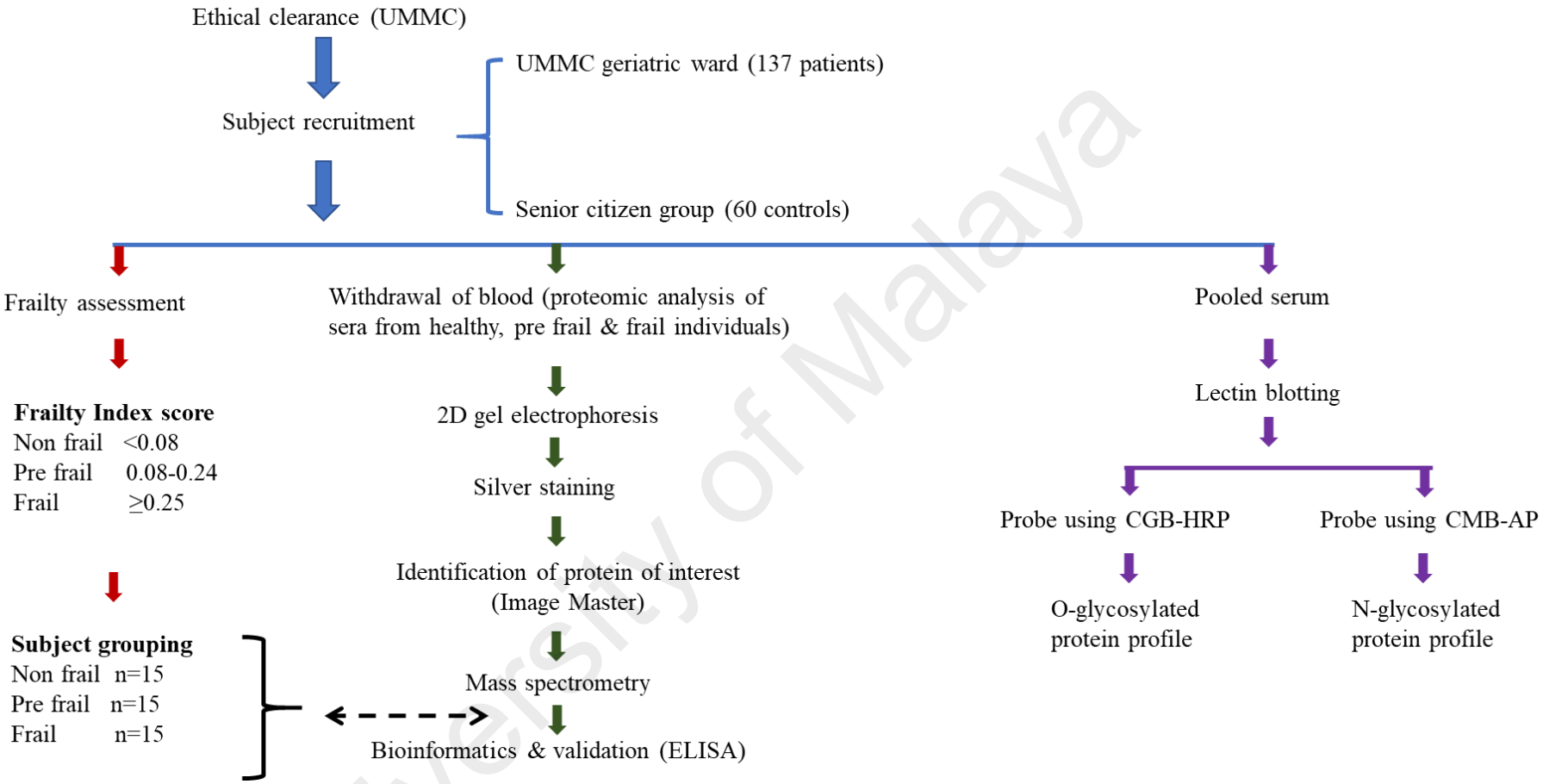


Figure 3.1: Summary of workflow

3.3 General Materials

The materials used in this project and their respective suppliers are as listed below.

3.3.1 Chemicals

All chemicals were of analytical grade and were purchased from supplier listed below:

I. *GE Healthcare Biosciences, Uppasala, Sweden*

Glycerol

N,N,N',N' -tetramethyl-ethylenediamene (TEMED)

Urea

Bis N, N' -methylene-bis-acrylamide (Bis-acrylamide)

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

Sodium thiosulphate-5-hydrate

III. *Merck, Germany*

Tween 20

Silver nitrate

Glycine

Potassium chloride

Tris(hydroxymethyl)aminomethane (Tris)

IV. *Bio-Rad Laboratories, Hercules, USA*

Ammonium persulfate (APS)

Sodium dodecyl sulphate (SDS)

3.3.2 Enzymes and substrates

I. *Roche Molecular Biochemicals, Mannheim, Germany*

Activated alkaline phosphatase (AP)

II. *Promega, Madison, USA*

Trypsin Gold, mass spectrometry grade

III. *Boehringer Manheim, Germany*

5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (NBT/BCPIP) tablet

3.3.3 Chromatographic materials

I. *GE Healthcare Biosciences, Uppsala, Sweden*

Sepharose 4B

3.3.4 Two-dimensional electrophoresis

I. *GE Healthcare Biosciences, Uppsala, Sweden*

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

IPG Buffer (pH 4-7)

Drystrip cover fluid

3.3.5 Mass spectrometry

I. *Millipore, MA, USA*

Zip Tip containing C_{18} reverse phase media

3.3.6 Commercial kits

I. Wuhan Fine Biological Technology, China

Human Alpha 1-Antitrypsin and Apolipoprotein E ELISA kit.

3.3.7 Lectins

Chempedak fruits (*Artocarpus integer*) were bought from a local market. Galactose and mannose-binding (CGB and CMB) lectins were extracted according to standard protocol.

3.4 Two-dimensional gel electrophoresis (2-DE)

3.4.1 First dimension electrophoresis

Standard solutions:

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

Urea	13.50 g
DTT	250.0 mg
IPG buffer 4-7	0.50 ml
Triton X-100	0.13 ml

All the chemicals were mixed and made up to 25 ml with distilled water. The solution was kept in aliquots of 250 µl and stored up to 2 months at -20° C.

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

Urea	12.00 g
IPG buffer 4-7	0.13 ml
Triton X-100	0.13 ml
A few grains of Orange G	

The solution was made up to 25 ml by addition of distilled water and kept in 1 ml aliquots at -20° C, prior to use. Twelve mM DTT was added to the dehydration solution.

3.4.1.1 Sample preparation

Serum sample (30 µg) was diluted with sample buffer at 3:1 ratio and it was left at room temperature for 30 minutes. The diluted sample was made up to 200 µl with rehydration solution followed by another 30 minutes incubation at room temperature. Then, 200 µl of the sample mixture was pipetted into the reservoir slot of the reswelling tray. The immobilised pH gradient (IPG) drystrip (11cm and 4-7L (linear) was carefully placed with the gel side down facing the solution. The IPG dry strip cover fluid was instantly pipetted onto the IPG strips to minimise evaporation and prevent urea crystallisation. The strips were allowed to rehydrate for 18 hours at room temperature to ensure complete uptake of the samples.

3.4.1.2 First dimension run

The temperature of the IPGphor electrophoresis Unit (IEF apparatus) was set at 18° C. Rehydrated IPG strips were removed from the reswelling tray and carefully placed on focusing tray with gel side facing up. The electrode was placed across the cathode and anode terminal. The focusing tray was positioned on the cooling plate and 10 ml of dry cover fluid was poured on the tray. Finally, cotton wick was placed on the terminal site of each strip to collect ionic sample contaminants and to prevent drying of the end of the IPG strips The purpose of dry cover fluid is to ensure good thermal contact. The running condition of the first dimension isoelectric focusing for the 11cm IPG strips pH 4-7 as follows:

Table 3.3: Isoelectric focusing protocol for 11cm IPG strip pH 4-7

Step	Volt	Time (hour)
Step and Hold	500	1
Gradient	1000	1
Gradient	8000	3.03
Step and Hold	8000	0.55

After the first dimension run was completed, the focused strips were either used immediately for the second-dimension separation or kept individually in screw-cap glass tubes at -80° C. The kept strips were used within 2 weeks.

3.4.2 Second dimension SDS-PAGE

Standard solution

Monomer solution

Acrylamide	60 g
N,N' -methylenebisacrylamide	1.8 g
Distilled water	Topped up till 200ml

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

APS 10%

APS	2 g
Distilled water	20 ml

APS was kept in aliquots of 0.2 ml and stored at -20° C. One tube was thawed and used for gel preparation. The remaining unused solution in the tube was discarded.

4 x Resolving gel buffer

Tris base	181.7 g
ddH_2O	750 ml
HCl	Adjust to pH 8.8
ddH_2O	Top Up to 1L

SDS 10%

SDS	10 g
Distilled water	100 ml

The solution was stored at room temperature.

SDS equilibration solution

Urea	144.14 g
Glycerol	138 ml
SDS	8.0 g

The solution was made up to 400 ml with distilled water.

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

Tris-HCl	136.2 g

The solution was made up to 3 L with distilled water. The pH was adjusted using HCl.

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

Tris-HCl	3.03 g
Glycine	14.8 g
SDS	1 g

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

Agarose Sealing Solution

Agarose	0.5 g
Cathode buffer	100 ml

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

3.4.2.1 Preparation of 8-18% gradient SDS-PAGE gel for second dimension electrophoresis

An 8-18% gradient was prepared using a gradient maker (SG 30, Hoefer, USA) connected to a peristaltic pump (EYELA Micro Tube Pump MP-3) and gel caster. Two types of gel solutions, light and heavy solutions were prepared as outlined in Table 3.4. Both light and heavy solutions were placed in the mixing chamber connected to the pump by tubing with a cannula in one end. The solutions were mixed and pumped into the glass plate sandwich at a constant rate of 2.5 ml/minute. The gel was overlaid with distilled water and was allowed to polymerise for a minimum of 4 hours.

Table 3.4: Volume of light and heavy solution for 8 – 18% gradient SDS-PAGE

Gradient Gel	8.0% (Light Solution)	18.0% (Heavy Solution)
Monomer (ml)	3.20	7.20
4× resolving solution (ml)	3.00	3.00
Sucrose (g)	-	1.8
10% SDS (µl)	120.00	120.00
Distilled water (ml)	5.64	5.64
10% APS (µl)	39.60	39.60
TEMED (µl)	3.96	3.96
Total volume (ml)	12.00	12.00

3.4.2.2 Equilibration and application of IPG strips

SDS equilibration buffer was used to equilibrate IPG strip in two steps. Firstly, the proteins in the serum sample was reduced by equilibrate the IPG strip in 1% (w/v) DTT. Each strip was equilibrated using 5 ml of DTT solution. The tubes were carefully placed on a rocking platform and shaken gently for 15 minutes. The second buffer containing 4.5 % (v/v) iodoacetamide in SDS equilibration solution was added into each strip after DTT equilibration buffer solution was discarded. The purpose of using iodoacetamide is to alkylate the protein mixture in serum sample. This step was carried out for 15 minutes. The surface of the 8-18% SDS gel was rinsed using freshly prepared cathode buffer after the overlaid double distilled water on the gel was removed. The IPG strip also was rinsed with the same buffer solution excessively and placed on top of the gel. The IPG strip was sealed in place using 0.5% agarose dissolved in cathode buffer.

3.4.2.3 Second dimension run

The temperature of the 2-DE tank (Hoefer SE 600 Ruby Electrophoresis System) was set at 16° C using a thermostatic circulator (Grant Instrument Ltd., Cambridge, UK). Electrophoresis was performed at constant current in two phases as shown in the table below:

Table 3.5: Electrophoresis phases for second dimension run of 2-DE

Phase	Voltage	mA	W	Time (minutes)
1	50	40/gel	25/gel	30
2	3500	40/gel	25/gel	90

Electrophoresis was stopped once the dye front was approximately 1 cm from the bottom of the gel. The gels were carefully removed from the cassette and subjected to silver staining as describe in the next section. A small diagonal cut was made at the upper corner nearest to the acidic end of the gel to assist identification of the orientation.

3.5 Silver staining of 2-DE gels

The 2-DE gels were developed by silver staining procedures according to the method developed by Heukeshoven and Dernick (1988) with some minor modifications.

Standard solutions

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

Ethanol	400 ml
Acetic Acid	100 ml

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

Sensitising solution ***(ethanol (30%, v/v), 0.5 sodium acetate, 8 mM sodium thiosulphate, glutaraldehyde (0.13%, v/v))***

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

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

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

Silver nitrate	1 g

Solution was made up to 1 L with distilled water. Formaldehyde (200 µl) was added prior to use.

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

Sodium carbonate	25 g

Solution was made up to 1 L with distilled water. Formaldehyde (100 µl) was added, prior to use.

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

EDTA-$Na_2.2H_2O$	14.6 g

Solution was made up to 1 L with distilled water.

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

Staining procedure

Once electrophoresis was completed, the gel was immersed immediately in the fixing solution and left overnight to allow the SDS to diffuse out and to precipitate the proteins. The fixing solution was removed and the gel was then incubated in sensitising solution for 40 minutes. Following that, the sensitising solution was removed and the gel was washed for three times with distilled water for about 5 minutes each. Silver solution was added and the staining was carried out for 40 minutes. Subsequently, the silver solution was removed and the gel was briefly rinsed with 50 ml of developing solution. Fresh developing solution was added for approximately 5 minutes or until the gel profile fully develops. Finally, the colour development was stopped by adding the

stopping solution. The gel was washed again with distilled water for 5 minutes with one change of water. The developed gel was stored in preserving solution at room temperature. Images of the developed gels were scanned using an image scanner and analysed using Image Master Platinum 7.0 software (GE Healthcare Biosciences, Uppsala, Sweden).

3.6 Purifications of champedak lectins

3.6.1 Standard Solutions

Phosphate buffer saline (PBS)

[170 mM NaCl, 3.4 mM KCl, 10.0 mM Na_2HPO_4, 1.8 mM KH_2HPO_4, pH 7.2]

NaCl	9.36 g
KCl	0.253 g
Na_2HPO_4	1.42 g
KH_2HPO_4	0.245 g

Solution was made up to 1 L with distilled water and pH was fixed to 7.2. The solution was kept at room temperature. To prepare PBS-Tween 20 (0.05%) (v/v) of Tween-20 was added and the buffer was stored at 4° C.

Tris buffered saline (TBS)

[100 mM Tris-HCl, NaCl (0.9%)]

Tris	12.11 g
NaCl	9.00 g

Tris was dissolved in 900 ml distilled water and the pH was fixed to 7.5 by addition of concentrated HCl. NaCl was then added and the solution was made up to 1 L using distilled water.

3.6.1.1 Extraction of crude lectin from seeds of champedak

The chempedak seeds were cleaned using distilled water and dried at 37° C. A blender was used to grind the seeds to a powdered form. The powdered seeds (eighty grams) were suspended in 800 ml of PBS pH 7.2 and stirred for 24 hours at 4° C and was centrifuged for 15 minutes using a Sorvall R2B centrifuge at 8000 × g. The supernatant was collected and subjected to 60% ammonium sulphate precipitation and left to stir for 2 to 3 hours at 4° C. Subsequently, the pellet containing the crude extract was collected and mixed in cold PBS and dialysed against several changes of PBS for 48 hours. The lectin was then stored at -20° C.

3.6.1.2 Preparation of galactose and mannose sepharose-4B column

The lectin from the crude extract was isolated using sugar affinity chromatography. The sugar affinity column was prepared by activation of Sepharose and coupling of sugar to the activated gel.

Activation of Sepharose 4B with Divinylsulfone (DVS)

Using a sintered glass funnel, one hundred ml of Sepharose 4B was washed with distilled water and suctioned to a wet cake and finally transferred into a 500 ml beaker. The moist gel was resuspended in 0.5 M of 100 ml sodium carbonate and stirred slowly. The gel was activated by slowly adding 10 ml of DVS to the suspension over a period of 15 minutes with continuous stirring in a fume cupboard. Following that, the gel was constantly stirred for another hour before the gel was washed extensively with water until the filtrate was no longer acidic. The Sepharose is now activated and ready for ligand coupling. The hydroxyl groups in the sugars immobilised by the reactive vinyl groups from DVS in the Sepharose.

Coupling of sugar to DVS-activated Sepharose 4B.

Using immobilised galactose and mannose columns, isolation of CGB-lectin and CMB-lectin was achieved respectively. The galactose and mannose column was prepared by adding 20% (w/v) of D-galactose or D-mannose in 0.5 M sodium carbonate suspended in an equal volume of 20 ml of DVS activated gel. The gel was washed consecutively with 2 litres of water and 0.5 M sodium bicarbonate after the mixture was stirred at room temperature for 24 hours. The gel was resuspended in 0.5 M sodium

bicarbonate containing 2 ml of β-mercaptoethanol and stirred in a fume hood for 2 hours. The galactose-coupled and mannose-coupled Sepharose 4B gels were filled up to 16 cm in height into polypropylene columns of 2.8 cm in diameter.

3.6.1.3 Purification of CGB- and CMB-lectins

CGB- and CMB-lectins were purified from the crude lectin extract (section 3.6.1.1) using the individual sugar affinity column.

(a) ***Isolation of CGB-lectin***

Before the isolation of CGB-lectin, the galactose column was pre-equilibrated with PBS buffer. Ten ml of crude extract was added into the column and washed with PBS. The absorbance was monitored at 280 nm and unbound fractions of 10 ml each were collected. The absorbance with peak fractions between 0.5-1.24 were pooled and kept separately for CMB-lectin affinity isolation. Once the absorbance fell to baseline (A280<0.005), CGB lectin fractions were eluted using 0.8 M galactose in PBS, pH 7.4. The high absorbance (A280 between 0.3-1.2) fractions were collected and dialysed against PBS. To concentrate the CGB-lectin, the dialysed bound fraction was freeze-dried and stored at -20° C.

(b) ***Isolation of CMB lectin***

The flow-through fraction saved from the CGB-lectin procedure was used to isolate the CMB-lectin. The fractions was put through mannose-Sepharose 4B and the similar procedure as described for the isolation of CGB-lectin was repeated. Solution of 0.8 M

D-Mannose in PBS was used for the elution of bound fraction containing CMB-lectin. The fraction was also dialysed against PBS and freeze-dried prior to storage.

3.7 Assessment of purified CGB- and CMB-lectins.

3.7.1 Determination of the concentrations of CGB- and CMB-lectins.

Using a commercial protein assay kit, based on the method of Bicinchoninic acid assay (BCA) from Fisher Scientific, the concentrations of the isolated lectins were determined. Bovine serum albumin (BSA) at 2 mg/ml was used as standard and the concentration estimation was performed according to the manufacturer's instruction

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

The two isolated lectins aliquots of CGB and CMB were subjected to an electrophoresis separation on an 18% SDS gel to evaluate the purity of the preparation. Coomassie Blue staining method was used to stain the gel to obtain the profiles of the lectins.

3.7.3 Standard solutions for SDS PAGE

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

Stock solution for SDS PAGE such as monomer, 10 % APS stocks and 10% SDS solution were prepared as described in section 3.4.2. Additional buffers and solutions were prepared as listed:

Solution A = Monomer

Solution B = 10 % APS

Solution C = 10 % SDS

Solution D = TEMED

Solution E = 4× resolving buffer (1.5 M Tris-HCl, pH 8.8)

Tris base	36.23 g
Deionised distilled water	150 ml

pH of the solution was adjusted to 8.8 using HCl and the solution was topped up till 200 ml

Solution F = 0.5 M Tris-HCl, pH 6.8

Tris	6.1 g
Deionised distilled water	50 ml

The solution was corrected to pH using HCl and topped up till 100 ml.

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

0.5M Tris-HCl, pH 6.8	2.5 ml
Glycerol	2 ml
SDS	400 mg
DTT	200 mg
Bromophenol blue	Few grains

All of the above was mixed and made up to 20 ml with deionised distilled water. The sample was mixed with sample buffer at a ratio of 3:1 prior to heating up for 10 minutes at 100° C.

Gel Electrophoresis Buffer: 25 mM Tris, 198 mM glycine, 0.1% (w/v) SDS, pH 8.3

Tris	1.22 g
Glycine	5.94 g
SDS	0.4 g

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

Preparation of stacking and separating gels

The 18 % separating gel mixture was prepared according to Table 3.4. The glass plate were assembled in sandwich style, and the separating gel mixture was carefully pipetted and following that overlaid with a layer of distilled water to become an even gel surface. The water overlay was discarded once the gel polymerised and replaced by

the stacking gel mixture. A gel comb was inserted into the stacking gel layer and the gel was allowed to polymerise fully.

Table 3.6: Volume of stock solution used to prepare the separating and stacking gels.

Stock Solution	Volume of stock solutions for	
	Separating gel (18%)	Stacking gel (4%)
Solution A (ml)	12.00	0.65
*Solution B (µl)	100.00	25.00
Solution C (µl)	200.00	50.00
*Solution D (µl)	6.60	5.00
Solution E (ml)	5.00	-
Solution F (ml)	-	1.25
Distilled water (ml)	2.69	3.05
Total volume (ml)	20.00	5.03

**Added prior to use*

3.7.3.1 SDS Electrophoresis

Sample buffer were mixed with samples at a ratio of 3:1 in a microcentrifuge tube and boiled at 100° C for 10 minutes. Respective sample was loaded into the wells alongside the broad and low range molecular weight standards in a separate well. At a constant voltage of 90 V, electrophoresis was performed and stopped once the blue dye was about 1 cm from the gel bottom. Hot Coomassie staining method was used to stain the gel.

Hot Coomassie staining protocol

Firstly, the Coomassie solution was heated up to 90° C in a microwave and poured into a wide container containing the gel and allow to shake on a rocking platform for 30 minutes. The gel was rinsed with 20 ml of destaining solution to remove excess stain solution, after the Coomassie solution was removed from the container. In order to fasten the destaining process, fresh destain solution was added to the container and a piece of Kim Wipe tissue paper was placed on the corner of the solution container and placed on rocking platform overnight. Next day, the gel was rinsed in distilled water and scanned.

Hot Coomassie blue stain

Coomassie stained was prepared by mixing 10 % (v/v) acetic acid and, 0.1 % (w/v) Coomassie Brilliant Blue (R250) in 100 ml of water. The stain solution was kept at room temperature and heated up to 90° C before use. The stain solution was re-used for up to 3 times.

Destaining solution

10 % (v/v) acetic acid solution.

3.7.4 Conjugation of CGB- and CMB-lectins to enzyme

The applications of lectins in this study were aim for the detection of human serum glycoproteins separated by 2-DE. The resolved human serum proteins were transferred

from 2-DE gel to nitrocellulose membranes electrophoretically. The enzyme conjugated lectins were then probed to form lectin-glycoprotein complexes and were detected using colorimetric reaction of specific substrates towards the enzyme.

In this study, CGB-lectin was conjugated to horseradish peroxidase (CGB-HRP) and CMB-lectin to alkaline phosphatase (CMB-AP), CGB-lectin binds mainly to galactosyl residues of glycoproteins hence incubation of CGB-HRP on 2-DE serum blots generate a profile of O-linked serum proteome. On the other hand, CMB-lectin bind predominantly to mannose residue of N-linked glycoproteins. Similarly, serum map of N-linked glycoproteins was developed by incubation of 2-DE serum transferred blots with CMB-AP solutions.

3.7.4.1 Conjugation of CGB-lectin to Horseradish Peroxidase

Stock solutions.

0.1 M Sodium periodate

Sodium periodate	21.39 mg

One ml of deionised distilled water was used to dissolve sodium salt. The solution was prepared fresh prior to use.

1 M Sodium acetate buffer, pH 4.4

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

Acetic acid solution was mixed with sodium acetate solution at a ratio of 1:2. Distilled water was used to dilute the solution 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 reached.

0.4 % (w/v) Sodium borohydride

Sodium borohydride	4.0 mg

One ml of distilled water was used to dissolve sodium borohydride crystal.

0.1M borate buffer, pH 7.4

Disodium tetraborate	9.54 g/ 250 ml
Boric acid	24.73g/ 4 L

Four L of boric acid was added with approximately 115 ml of borate solution until pH reaches 7.4.

Conjugation Protocol

One ml of distilled water was added to four mg of HRP to completely dissolved. The HRP solution was then added to freshly prepared two hundred µl of 0.1 M sodium periodate and a green coloured solution was obtained. The solution was then dialysed overnight against 0.1 mM sodium acetate buffer, pH 4.4 at 4° C after stirred for 20 minutes at room temperature. Sodium carbonate buffer of 0.1 M, pH 9.5 was added to the recovered HRP to raise the pH till 9.5. Immediately, one ml of CGB-lectin (2 mg/ml) was added into the mixture and left to stir for 2 hours at 4° C. Fresh sodium borohydride solution (4mg/ml) was added to reduce the free enzyme and allowed to stand for additional 2 hours at 4° C. The conjugated solution was dialysed overnight against 0.1 M sodium borate buffer pH 7.4 at 4° C. The CGB-HRP was then diluted in equal volume of 60% glycerol in borate buffer and stored at 4° C.

3.7.4.2 Conjugation of CMB-lectin to Alkaline Phosphatase

Solutions used

Solution 1: 1 M sodium carbonate/hydrogen carbonate buffer, pH 9.4

Na_2CO_3	10.6 g/ 100 ml
$NaHCO_3$	8.4 g/ 100 ml

Using Na2CO3, the pH of $NaHCO_3$ was adjusted to pH 9.4.

Solution 2: 100 mM sodium carbonate/hydrogen carbonate buffer, pH 9.8

Hundred ml of deionised distilled water was used to dilute 10 ml of solution 1.

Solution 3: 200 mM Sodium borohydride ($NaBH_4$)

One ml of cold deionised distilled water was added to 80 mg of $NaBH_4$ until completely dissolved.

Solution 4: 2 M triethanolamine solution, pH 8.0

Triethanolamine solution (2.66 ml) was diluted using 3 ml deionised distilled water. The pH of the solution was adjusted to 8.0 with 25% HCl and made up to 10 ml with deionised distilled water.

Solution 5: 1 M glycine, pH 7.0

Glycine	0.75 g

Six ml of deionised water was used to dissolve glycine and the pH was adjusted to 7.0 with 0.1M NaOH. The final solution was then made up to 10 ml with deionised distilled water.

Solution 6: Triethanolamine buffer, pH 7.6

[50 mM Triethanolamine, 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 10 mm Glycine and 0.1 % (w/v) sodium azide].

Triethanolamine	16.65 ml
NaCl	21.92 g
$MgCl_2$	0.51 g
$ZnCl_2$	34.0 g
Glycine	1.88 g
NaN_3	2.5 g

All the salts listed above was used to prepare the buffer by dissolving in 2.5 L of redistilled water.

Conjugation protocol

Hundred µl of activated alkaline phosphatase (AP) solution was mixed with 50 µl of concentrated CMB-lectin (7 mg/ml) and incubated overnight at 4° C. Conjugation reaction was stopped by adding 20 µl of 2 M triethanolamine solution, pH 8.0 followed by 40 µl sodium borohydride solution. The solution was incubated for 30 minutes at 4° C after well mixed. After 30 minutes, 5 µl of triethanolamine solution was added to the mixture and incubated for additional 2 hours at 4° C. The conjugate was stabilised by adding 10 µl of 1 M glycine solution pH 7.0. The conjugate solution was dialysed extensively against 4 changes of triethanolamine buffer in dialysing tube. After completion, the dialysed conjugate solution was added with bovine serum

albumin and sodium azide to the final concentration of 10 mg/ml and 1mg/ml separately. The CMB-AP conjugate was kept in aliquots of 15 µl and stored at -80° C.

3.8 Western Blotting

Principally, this semidry western blot involves transfer of resolved serum proteins from 2-DE gel into a nitrocellulose membrane. The corresponding lectin solution was then incubated with the nitrocellulose membrane to further analyse serum glycoproteins profiles.

Solutions used for western blot protocol

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

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

The chemicals were dissolved and made up to 1 L with distilled water.

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

TBS	1 litre
Tween-20	1 ml

Western blotting procedure

One piece of nitrocellulose membrane 0.45 µm and six pieces of filter paper was cut according to size of the gel. The filter paper and nitrocellulose membrane was soaked with transfer buffer and placed on the anode prior to graphite anode was saturated with distilled water. The 2-DE gel was pre-equilibrated with transfer buffer and was placed on top of the membrane followed by another three pieces of filter paper soaked with transfer buffer. Trapped air bubbles were removed by a test tube carefully rolled onto the layer of stacked filter paper, nitrocellulose and gel. The cathode tray was then placed on top of the sandwiched gel. The blot was performed under a constant current of 0.8 mA/cm2 for two hours.

3.8.1 Detection of glycoprotein spots on nitrocellulose blots incubated with CGB-HRP

TBS-T containing 3 % gelatin was used to block the nitrocellulose membrane for one hour on a shaker at room temperature. The blot membrane was then washed three times using TBS-T for ten minutes each time. It was then incubated for overnight with CGB-HRP in TBS-T at a concentration of approximately 1 µg/ml at 4° C. Freshly prepared substrate solution was used to develop the membrane, which contained 25 mg of DAB and 50 µl of 3 % H_2O_2 in 50 ml TBS. Finally, the development reaction was stopped by

washing the membrane two times with distilled water. The membrane was air dried completely and scanned.

3.8.2 Detection of glycoprotein spots on nitrocellulose blots incubated with CMB-AP

The detection of glycoprotein spot using CMB-AP lectin was done according to the method as described in section 3.7.1 with the exception that dilution of CMB-AP in TBS-T at 1: 10,000 was used in incubation procedure. The membrane was soaked in 10 ml solution, containing one NBT/BCIP tablet (Boehringer Manheim, Germany) dissolved in 10 ml distilled water for 15 minutes for colour development of the blot. The reaction was stopped by using water and the membrane was air dried briefly before being scanned.

3.8.3 Image analysis of blots and quantitative analysis of differential expression in normal individuals, pre-frail and frail subjects

Image Master 2D-Platinum version 6.0 (GE Healthcare Biosciences) was used to analyse the CGB-HRP and CMB-AP blots. In individual blot, the percentage of volume contribution refers to the volume of all proteins including the unresolved proteins/peptides.

3.9 Mass spectrometry analysis for the identification of proteins

3.9.1 Sample preparation

The protein spots were carefully excised from the silver-stained gels before the in-gel digestion and kept hydrated in clean microfuge tubes containing Mili-Q water. The gel plugs were destain using 15 mM $K_3[Fe(CN)_6]$ in 50 mM $Na_2S_2O_3.5H_2O$ until they were transparent and further reduced and alkylated using 10 Mm DTT in 100 mM NH_4HCO_3 and 55 mM iodoacetamide in 100 mM NH_4HCO_3. Using 50% ACN in 100 mM NH_4HCO_3 and 100% ACN the gel plugs was washed and dehydrated in vacuum centrifuge. The dried plugs were then incubated in 25 µl of 6ng/µl trypsin in 50 mM NH_4HCO_3 solution at 37° C for overnight. Eventually, using 50% and 100% ACN, the peptides was extracted and then were dried using a vacuum centrifuge for mass spectrometry analysis.

3.9.2 Mass spectrometry

The dehydrated peptides were reconstituted with 0.1 % formic acid and desalted using ZipTip containing C_{18} reverse-phase media (Milipore, MA, USA). Final volume of 10 µl of peptide were analysed on the mass spectrometry Q-TOF 6550 (Agilent, Santa Clara, CA, USA) coupled with HPLC-Chip for ion source. Two µl of sample volume were flushed into inner valve of the 40nl trap 75µmx150mm 5µm C-18SB-ZX (Series II) (Chip Id: G4240-62006). Mobile phase A consist of 0.1% formic acid in H_2O and mobile phase B consist of 0.1% formic acid in ACN. The elution gradient was delivered at 0.4 µL/min as follows: 5% B at start, 50% B at 11 min, 70% B at 15 min, 70% B at 18 min, 5% B at 19 min and stop time at 25 min. The parameters used for MS analysis under positive mode using MassHunter Workstation (version B.05.1, Agilent, Santa Clara, CA, USA) are; capillary voltage at 1800 V, fragmentor voltage was at 175

V, gas temperature 290° C and gas flow 11 (l/min), reference masses were 1221. 99063700 and 299.29445700 (m/z); MS data ranging from 200 m/z to 3000 m/z; MS scan rate 8.00 spectra/sec, MS/MS scan rate 4.00 spectra/sec. Acquired spectra were then searched with Spectrum Mill search engine against the Homo sapiens, UniProt protein database.

CHAPTER 4: RESULTS

4.1 Frailty Index

As explained in methodology section, 32 indicators that represented a multidimensional view of frailty incorporating its physical, physiological, psychological and social aspects in the form of a questionnaire set was completed by interviewing the study subjects. These included variables derived from self-reports of health status, diseases, symptoms and signs, social as well as lifestyle indicators. Following that, patients were stratified into 3 groups as non-frail, pre-frail and frail categories based on method explained in section 3.2. Among the 137 recruited subjects from geriatric ward, 25 were pre-frail and 112 was frail. Healthy control subjects (n=60) were recruited from community dwelling and their frailty status were non-frail.

Following the stratification of geriatric patients, blood sample was supposed to be collected for the next part of work. However, some difficulties was faced, as not all admitted patients agreed to provide blood for proteomic analysis even though they consented for the study. This may be due to the condition of the elderly patients who felt that they were physically weak and claimed to be anaemic. In this instance, the subjects was not forced or convinced to give additional amount of blood for the proteomics study although their blood was taken for routine test in the ward.

Table 4.1: Overall subjects demographic characteristics

	Control	Pre-frail	Frail
N (%)	60 (30.5)	25 (12.7)	112 (56.8)
Age (*mean, ±sd*)	66.9 (7.4)	77.8 (5.1)	79.1 (6.8)
Sex (n)			
Male	19	10	42
Female	41	15	70
Race (n)			
Malay	27	2	24
Chinese	25	14	57
Indian	9	9	31
Frailty score (*mean, ±sd*)	0.04 (0.05)	0.18 (0.04)	0.36 (0.11)

Table 4.2: Characteristics of study subjects included in proteomic analysis

Study subjects	Control	Pre-frail	Frail	Race	Gender	Age	FI score
1	x			Indian	Female	65	0.00
2	x			Indian	Female	64	0.03
3	x			Indian	Male	68	0.00
4	x			Indian	Male	68	0.06
5	x			Malay	Female	63	0.03
6	x			Malay	Female	69	0.03
7	x			Malay	Male	68	0.06
8	x			Malay	Male	71	0.00
9	x			Malay	Male	67	0.00
10	x			Malay	Male	69	0.03
11	x			Chinese	Female	64	0.03
12	x			Chinese	Female	65	0.03
13	x			Chinese	Male	71	0.06
14	x			Chinese	Male	66	0.06
15	x			Chinese	Male	65	0.03
16		x		Indian	Male	79	0.15
17		x		Indian	Male	73	0.24
18		x		Indian	Female	83	0.22
19		x		Indian	Male	69	0.24
20		x		Indian	Female	78	0.18

Table 4.2, continued'

Study subjects	Control	Pre-frail	Frail	Race	Gender	Age	FI score
21		x		Indian	Male	82	0.13
22		x		Indian	Male	69	0.23
23		x		Malay	Male	80	0.23
24		x		Malay	Male	80	0.15
25		x		Chinese	Female	73	0.12
26		x		Chinese	Female	83	0.14
27		x		Chinese	Female	88	0.20
28		x		Chinese	Male	80	0.18
29		x		Chinese	Male	70	0.20
30		x		Chinese	Male	82	0.21
31			x	Indian	Female	84	0.50
32			x	Indian	Male	68	0.45
33			x	Indian	Male	82	0.57
34			x	Indian	Female	78	0.36
35			x	Indian	Female	87	0.37
36			x	Chinese	Female	87	0.42
37			x	Chinese	Male	72	0.41
38			x	Chinese	Female	78	0.30
39			x	Chinese	Male	82	0.38
40			x	Chinese	Female	79	0.43
41			x	Malay	Female	70	0.25
42			x	Malay	Female	73	0.35
43			x	Malay	Male	74	0.26
44			x	Malay	Female	78	0.29
45			x	Malay	Male	71	0.38

4.2 Serum protein profiles generated by 2-dimensional gel electrophoresis (2-DE)

4.2.1 Serum protein profiles of non-frail healthy, pre-frail and frail subjects.

In this present study, 15 serum samples each from representative of normal healthy non-frail individuals, pre-frail and frail subjects were resolved using 2-DE and subjected to silver staining. Upon visualisation, a complex protein profile containing hundreds of spots were obtained. The profiles attained shows similar pattern to the standard SWISS ExPaSy plasma protein reference (Sanchez *et al.*, 1995). Initially, the identity of protein spots was identified through visual comparison between serum proteins profiles in this study with standard reference map. Among the proteins that were well resolved in the gels of healthy subjects were α1-antitrypsin (A1AT), albumin (ALB), haptoglobin (HAP), hemopexin (HPX), the heavy and light chains of IgA, IgG and IgM, and kininogen (KNG1). Apart from that, one unidentified protein spot cluster (UK1) that was not identified by comparison with the standard plasma reference profile were detected. Figure 4.1a shows the representative serum protein profile of a non-frail subject, whilst Figure 4.1b and 4.1c are representative for the pre-frail and frail subject respectively.

Figure 4.1: Silver stained 2-DE protein profiles of normal, pre-frail and frail subjects

Unfractionated whole serum samples of normal elderly were subjected to 2-DE and silver staining. Figure 4.1a demonstrates the representative serum protein profiles of a non-frail healthy control. Clusters of protein spots that were well resolved included A1BG, A1AT, ACT, AHS, APOA, APOE, HPX, HAP, KNG1, LRG, VTDB and an unidentified protein spot termed UK1, was additionally detected. Panels (b) and (c) demonstrate typical serum protein profiles of the pre-frail and frail subjects respectively. For all panels, the acidic sides of the 2-DE gels are to the left and relative molecular mass declines from the top.

(a)

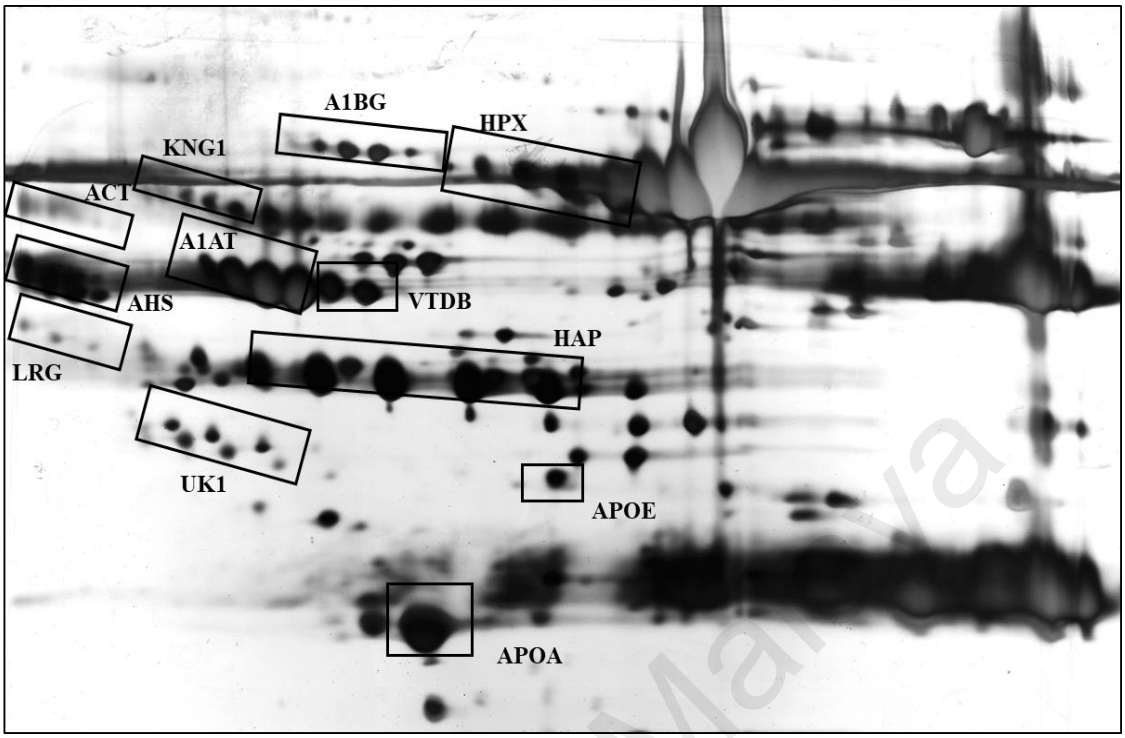
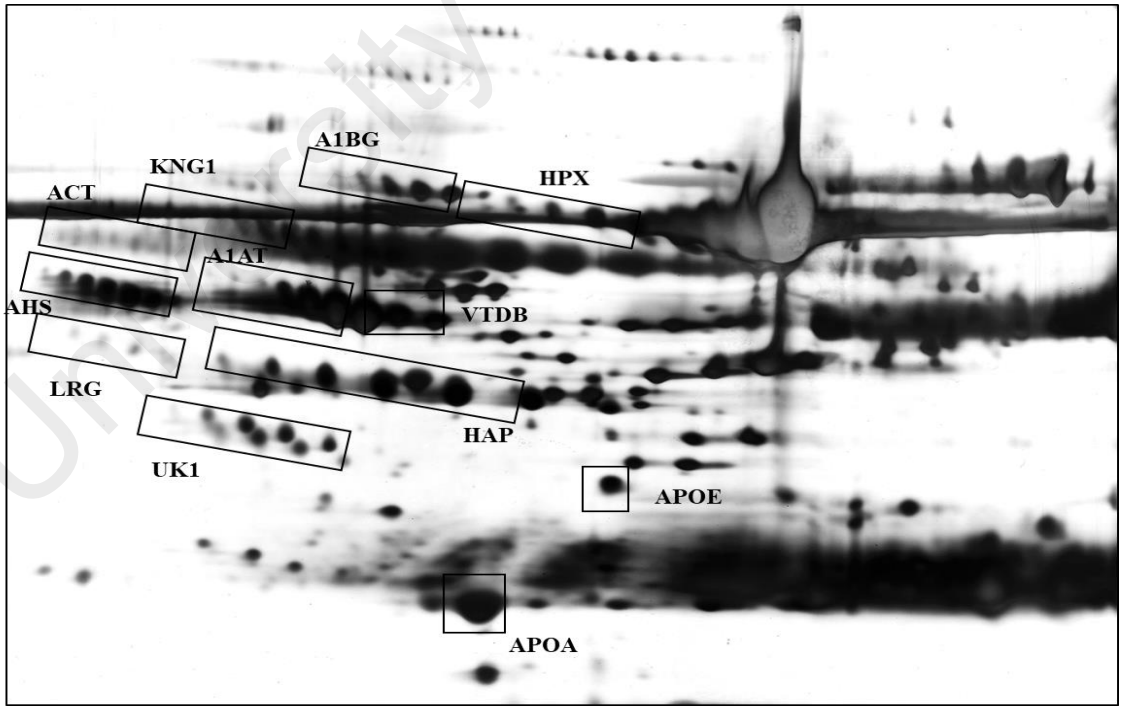
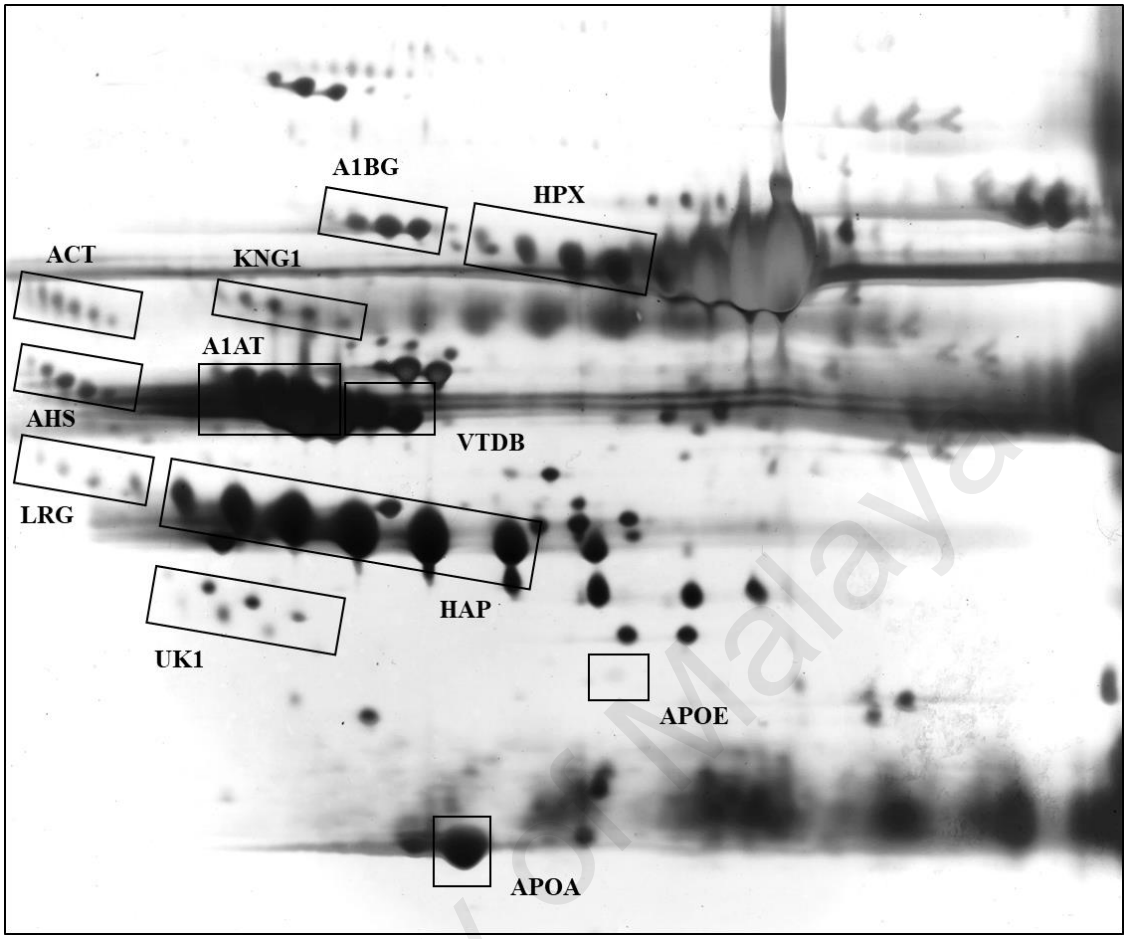


Figure 4.1: Silver stained 2-DE protein profiles of normal(a), pre-frail(b) and frail(c) subjects

(b)



(c)



4.2.2 Image analysis of 2-DE serum protein profiles

The silver stained 2-DE gels was scanned using an Imaging Densitometer GS690 from the Bio-Rad Laboratories, Hercules, USA. The digitised images were analysed using the Image Master Platinum 6.0 Software (GE Healthcare Biosciences, Uppsala, Sweden). The percentage of volume contribution refers to the spot volume of a protein expressed as a percentage of the total spot volume of all detected proteins. The Student T-test was used to analyse significance differences between normal subjects and patients. A p value of less than 0.05 ($p<0.05$) was considered significant.

Volume analysis of the 12 spot clusters in the 2-DE profiles of pre-frail and frail group, compared to that of control elderly group, demonstrated different altered expression of serum high abundance proteins in the two different frailty status subjects that were studied (Figure 4.2). Up regulated expression of KNG1 (1.56 fold, p=0.05) were significantly detected in pre-frail subjects when compared to the healthy controls. The up-regulated expression of A1AT (1.66 fold, p=0.00), HPX (1.55 fold, p=0.01), and downregulated expression of APOE (0.5 fold, p=0.01) and UK1 (0.61 fold, p=0.02) were significantly detected in the profiles of frail subjects. On the other hand, a slight upregulated expressions of A1AT (1.32 fold, p=0.05) were detected in pre-frail group whilst A1BG (1.31 fold, p=0.03), HAP (1.31 fold, p=0.01), KNG1 (1.36 fold, p=0.05) and LRG (1.42 fold, p=0.05) were detected in the frail group. The summary of volume analysis of the significantly detected spot cluster in the 2-DE profiles shown in Table 4.3.

Table 4.3: Summary of volume analysis of the significantly detected spot cluster in the 2-DE profiles

Protein	Pre-frail	Frail
A1AT	↑1.32 (p=0.05)	↑1.66 (p=0.00)
A1BG		↑1.31 (p= 0.03)
APOE		↓ 0.5 (p=0.01)
HAP		↑1.31 (p=0.01)
HPX		↑1.55 (p=0.01)
KNG1	↑1.56 (p=0.05)	↑1.36 (p=0.05)
LRG		↑1.42 (P=0.05)
UK1		↓0.61 (p=0.02)

Figure 4.2: Mean percentage of volume contribution of 2-DE resolved serum proteins for healthy, pre-frail and frail groups.

Volumes of protein spots were analysed and was restricted to twelve clusters of protein spots including A1BG, A1AT, ACT, AHS, APOA, APOE, HAP, KNG1, HPX, LRG, VTDB and UK1. Asterik denotes significantly different values.

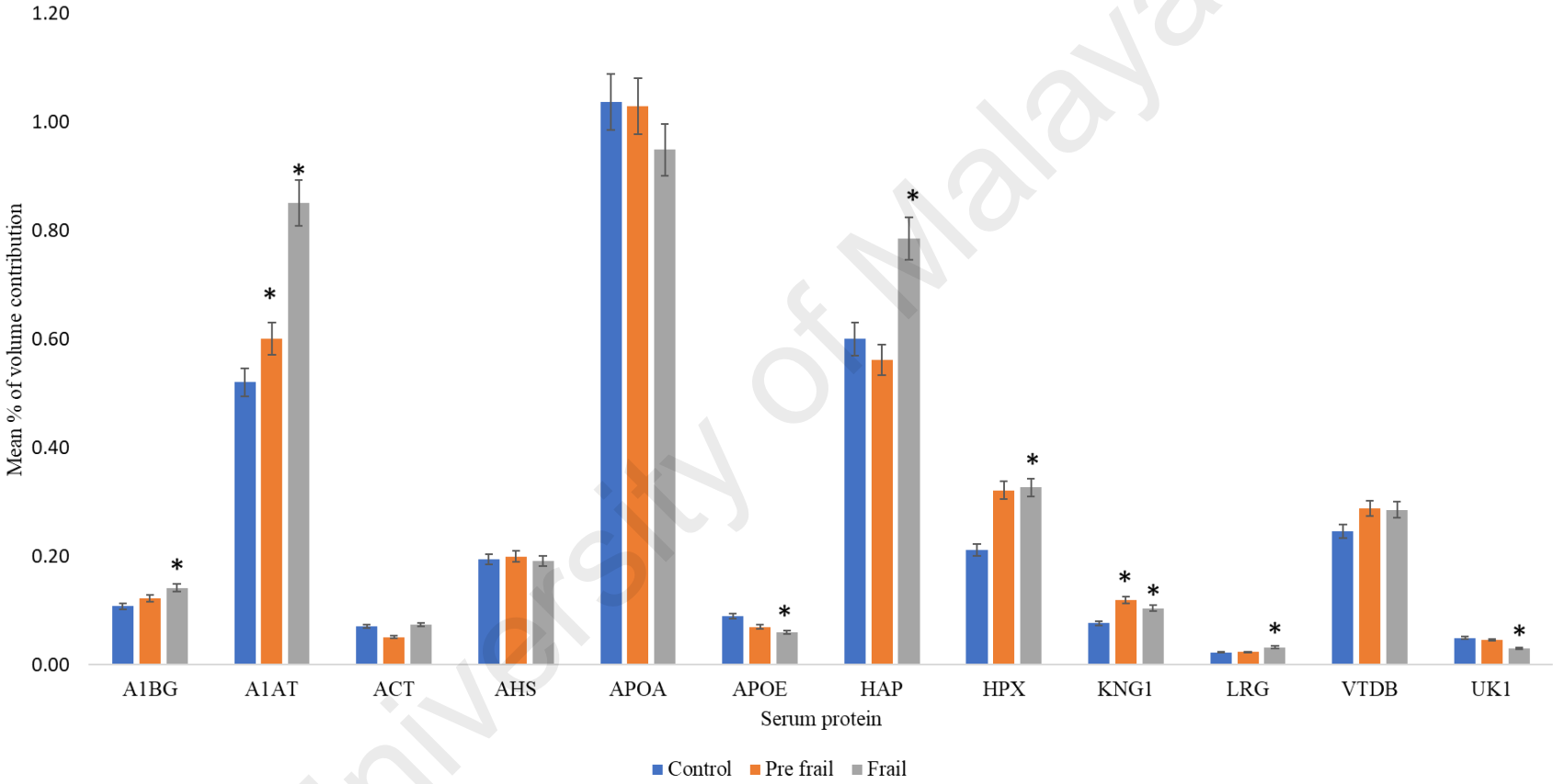


Figure 4.2: Mean percentage of volume contribution of 2-DE resolved serum proteins

4.2.3 Mass spectrometry analysis for the identification of serum proteins separated by 2-DE

Mass spectrometry analysis was performed for the confirmation of identity of proteins resolved in 2-DE gels which were differentially changed in abundance and showed significant statistical difference after image analysis. For mass spectrometry analysis of the respective protein clusters, the 2-DE gels were run in duplicate using respective serum samples of control, pre-frail and frail subjects. In silver staining step, a modified protocol was adopted according to the published method of Yan and co-workers (Yan *et al.*, 2000).

The gel plugs were analysed on the 1260 Infinity Nanoflow LC System (Agilent, Santa Clara, CA, USA) with the Mass Spectrometry is Accurate-Mass Quadrople Time-of-Flight (Q-TOF) 6550. Protein spots were identified using SPECTRUM MILL against *homo sapiens* entry in the Swiss-Prot database. Nine gel plugs were analysed and their identities are as shown in Table 4.2. The UK1 protein spot was successfully identified as isoform 2 of clusterin (CLU).

Table 4.2: Identifications of serum proteins by using mass spectrometry.

Resolved serum proteins/protein clusters as seen on Fig. 4.2 (a-c) were identified by using mass spectrometry analysis at the Proteomics Research Facility Centre at the Medical Biotechnology Laboratory, Faculty of Medicine, University of Malaya, Kuala Lumpur. The results obtained was filtered and validated using cut off score of 20. Scores lower than 20 was automatically removed from the data directory.

Table 4.4: Identifications of serum proteins by using mass spectrometry (searched against UniProt database)

Spot/Cluster ID	Total protein spectral intensity	Matched protein Name	Distinct summed MS/MS search score	Accession Nu (Swiss Prot)	Protein MW (Da)	Protein PI	Nu of peptide matched	Sequence coverage (%)
A1BG	4.74e+007	Alpha-1B-glycoprotein	301.35	P04217	54823.0	5.56	17	53.7
A1AT	7.84e+007	Alpha-1-antitrypsin	502.94	P01009	46906.8	5.37	28	61
ACT	1.67e+007	Alpha-1-antichymotrypsin	197.36	P01042	47821.1	5.33	12	20.8
APOE	3.13e+006	Apolipoprotein E	99.47	P02649	36267.7	5.65	6	19.8
HAP	2.79e+007	Haptoglobin	134.62	H0Y300	49846.8	6.18	7	16.2
HPX	2.20e+007	Hemopexin	273.11	P02790	52417.1	6.55	15	34.1
KNG1	1.31e+006	Kininogen	172.28	P01042	73040.1	6.34	11	17.2
LRG	1.33e+006	Leucine-rich alpha-2-glycoprotein	94.60	P02750	38405.4	6.46	6	17.5
UK1	1.17e+006	Isoform 2 of clusterin	66.35	P10909-2	58573.3	6.25	5	8.5

4.3 Purification and biochemical evaluation of champedak seed lectins.

4.3.1 Purification of champedak seed lectins: CGB- and CMB-lectins.

Preparation of crude lectin extract from champedak seeds was carried as outline in Section 3.6.1.1. To isolate the CGB-lectin as described in section 3.6.1.3a, 10 ml of crude extract was subjected to galactose Sepharose affinity chromatography. Thirty ml of the recovered flow through fraction from the column was then subjected to mannose-Sepharose affinity chromatography for extraction of CMB-lectin. The columns were washed extensively with PBS buffer, pH 7.2 to discard unbound substances. The flow-through fractions was observed for absorbance level at 280 nm until a baseline reading was reached. CGB-lectin bound to galactose column were eluted using 0.8 M galactose in PBS, pH 7.2. Sixty ml of the fractions was collected. On the other hand, 20 ml of the bound CMB-lectin in the mannose column was eluted using PBS buffer with 0.8 M D-Mannose as described in section 3.6.1.3b. Figures 4.3a and 4.3b show the elution profiles of CGB- and CMB-lectin by using corresponding sugar affinity columns.

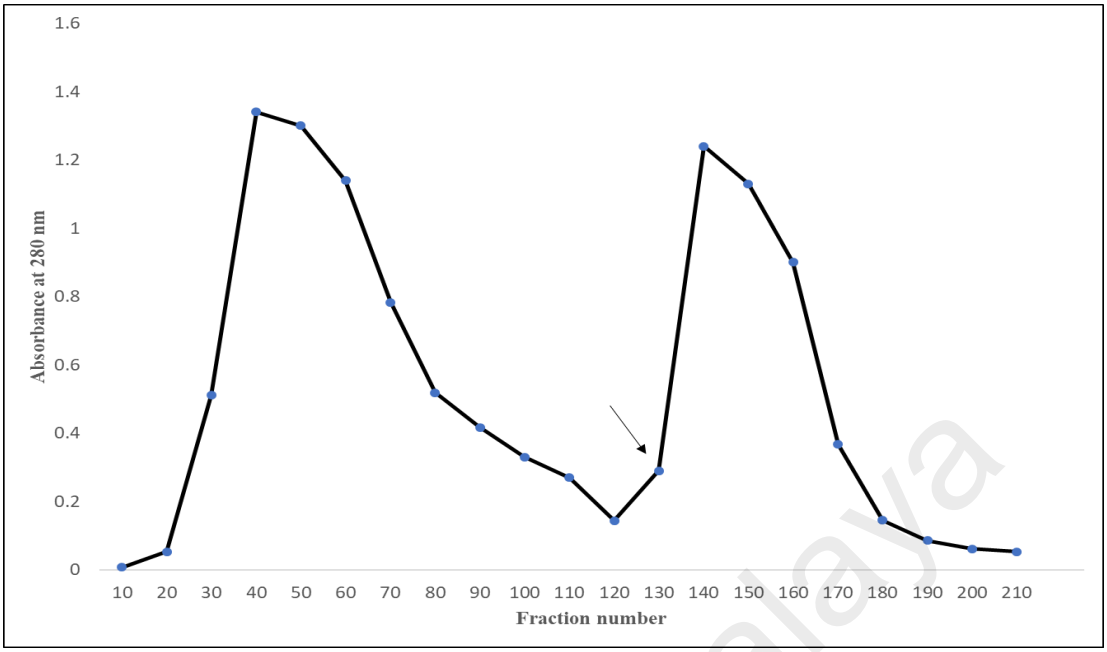


Figure 4.3 (a) Elution profile of CGB-lectin from galactose-Sepharose affinity chromatography

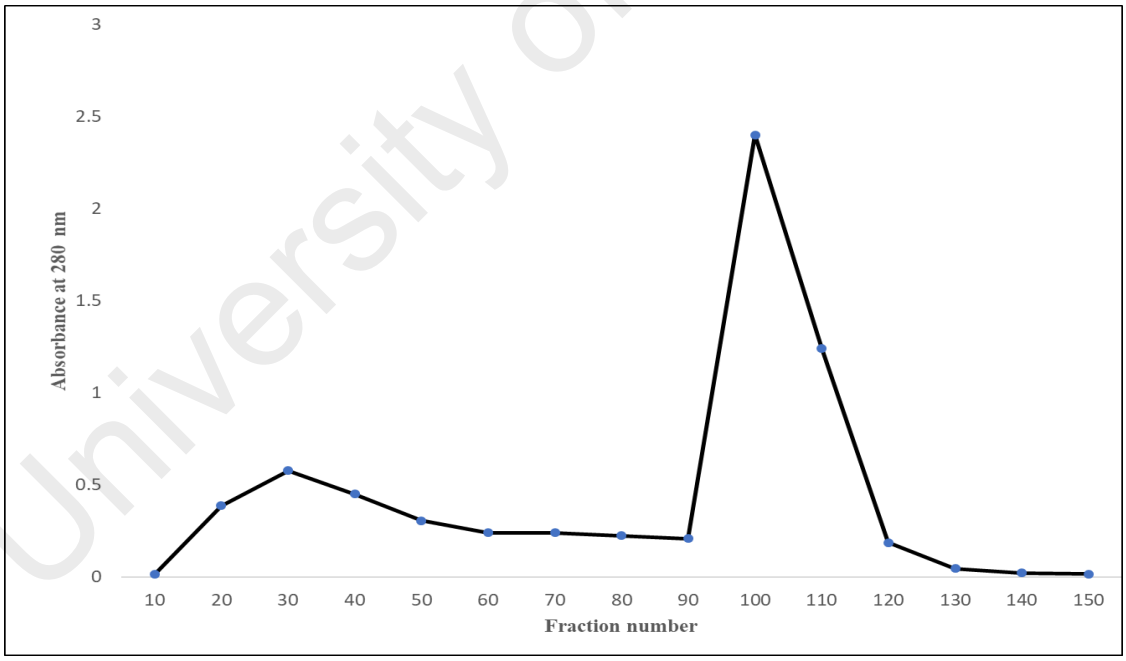


Figure 4.3(b): Elution profile of CMB-lectin from mannose-Sepharose affinity chromatography

4.3.1.1 Assessment of purified lectins

Biochemical analyses on the concentration and purity were carried to assess the quality of the isolated lectins before using the freeze-dried fraction of isolated CGB- and CMB-lectin. Assessments for concentration and purity were conducted as described in Sections 3.7.1 and 3.7.2 respectively.

4.3.1.2 Concentration of CGB- and CMB-lectins.

Concentration of the isolated CGB and CMB-lectins was estimated using a commercial BCA protein assay kit (Fisher Scientific). A standard calibration curve of absorbance values of several concentration of BSA was constructed. Standards and samples were prepared in triplicates. Based on the mathematical equation obtained from the standard curve, the concentration of CGB and CMB-lectin sample was at 1.47 mg/ml and 1.12 mg/ml respectively. The pooled freeze-dried fraction of CGB- and CMB-lectin contained a total of 5.27 mg and 9.34 mg of protein respectively

4.3.1.3 Purity of CGB- and CMB-lectin

The lectins isolated were subjected to SDS-PAGE electrophoresis using an 18% separating gel. Electrophoresis was performed under conditions described in Section 3.7.3.1 and the resolved gel was developed using Coomassie Blue. Figure 4.4 demonstrates the protein profiles obtained for both the lectins. The reduced CGB lectin was resolved into two bands with relative molecular weight, M_r of approximately 14,500 and 15,000 daltons (Da) (Lane 1). In contrast to the CGB-lectin, only single

band with M_r of approximately 15,000 Da was detected in the lanes resolved with CMB fractions.

Figure 4.4: SDS-PAGE analysis of the affinity purified CGB- and CMB-lectins

The purified CGB- and CMB-lectins was subjected to an 18% SDS-PAGE under reducing conditions and stained with Coomaisse Blue. CGB-lectin (lane 2) was electrophoretically resolved into two bands of approximately 14.5 and 15.0 kDa while CMB-lectin was resolved as a single band approximately 15 kDa (lane 3). The SDS-PAGE was run simultaneously with commercially available molecular weight markers (Spectra^{TM} Multicolor Broad Range Protein Ladder, Thermo Fisher) on lanes 1 and 4.

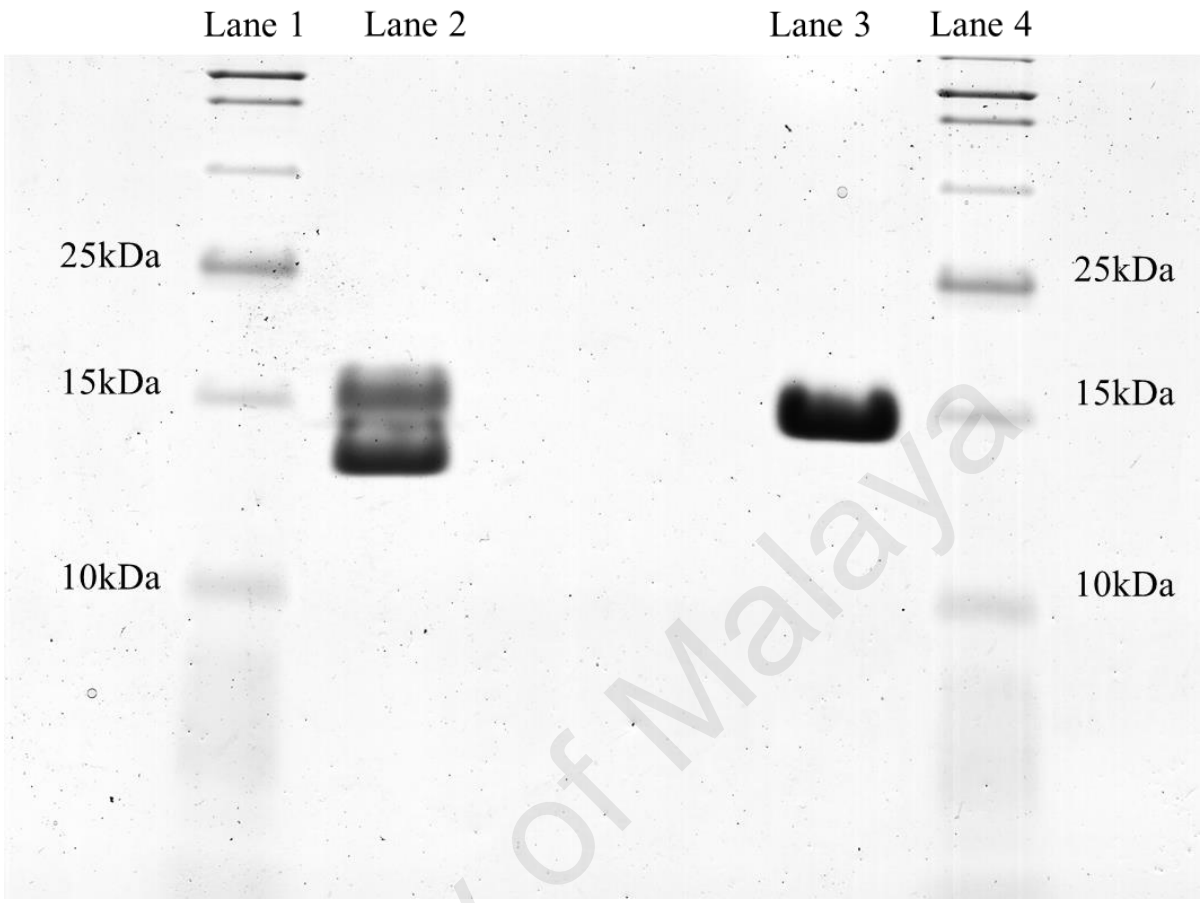


Figure 4.4: SDS-PAGE analysis of the affinity purified CGB- and CMB-lectin

4.3.2 2-DE serum glycoprotein profiling using lectin detection method

CGB- and CMB-lectin that were conjugated with enzyme as described in Sections 3.7.4.1 and 3.7.4.2 were utilised to detect serum glycoproteins resolved by 2-DE. Gels of resolved serum from controls, pre-frail and frail subjects were transferred onto nitrocellulose membrane via Western blotting technique as described in Section 3.8 and incubated with enzyme conjugated lectin solution.

CGB-HRP probed blots generated profiles of highly abundant serum O-linked glycoproteins while CMB-AP probed blots detected the presence of highly abundant N-linked glycoproteins.

4.3.2.1 CGB-HRP generated 2-DE serum glycoproteins profiles of healthy, pre-frail and frail elderly subjects.

When the pooled neat serum samples from healthy non-frail, pre-frail and frail subjects were subjected to enzyme-conjugated CGB-lectin, different profiles comprising only O-glycosylated peptides were obtained. (Figure 4.7a-c). The resolved profiles of glycoprotein spots were identified through visual comparison with the SWISS ExPASy plasma protein map and previously published work. Spots that were visually identified included ACT, AHS, AMBP, HPX, IgA, ITIH4, KNG1, LRG and VTDB. Spots of VTDB were observed only in the blots of serum of control and pre-frail subjects. Similarly, ACT spots were only detected on pre-frail CGB-lectin blots.

4.3.2.2 Image analysis of CGB-lectin generated 2-DE serum protein profiles

CGB-HRP probed blots generated profiles were subjected to densitometry analysis for percentage of volume contribution of a glycoprotein expressed (Figure 4.5). The student T-test was used to analyse the significance of differences between healthy elderly and patients. A *p* value of less than 0.05 ($p<0.05$) was considered significant. The image analysis detected AHS, HPX, and KNG1 in pre-frail and frail profiles which is not significantly expressed relative to control elderly whereas LRG (0.61 fold, $p=0.04$) levels were significantly reduced in pre-frail subjects. Under these conditions, VTDB expression in pre-frail subjects were decreased with fold change of 4.6 but not statistically, the p value was not significant.

Figure 4.5 (a-c): Glycoproteomic profiling of O-glycosylated pooled serum proteins retained by CGB-lectin affinity chromatography**.**

Pooled neat serum samples of controls (a), pre-frail (b), and frail (c), were subjected to 2-DE and CGB-lectin blotting. O-glycosylated serum proteins spot resolved included ACT, AHS, AMBP, HPX, IgA, ITIH4, KNG1, LRG, and VTDB.

(a)

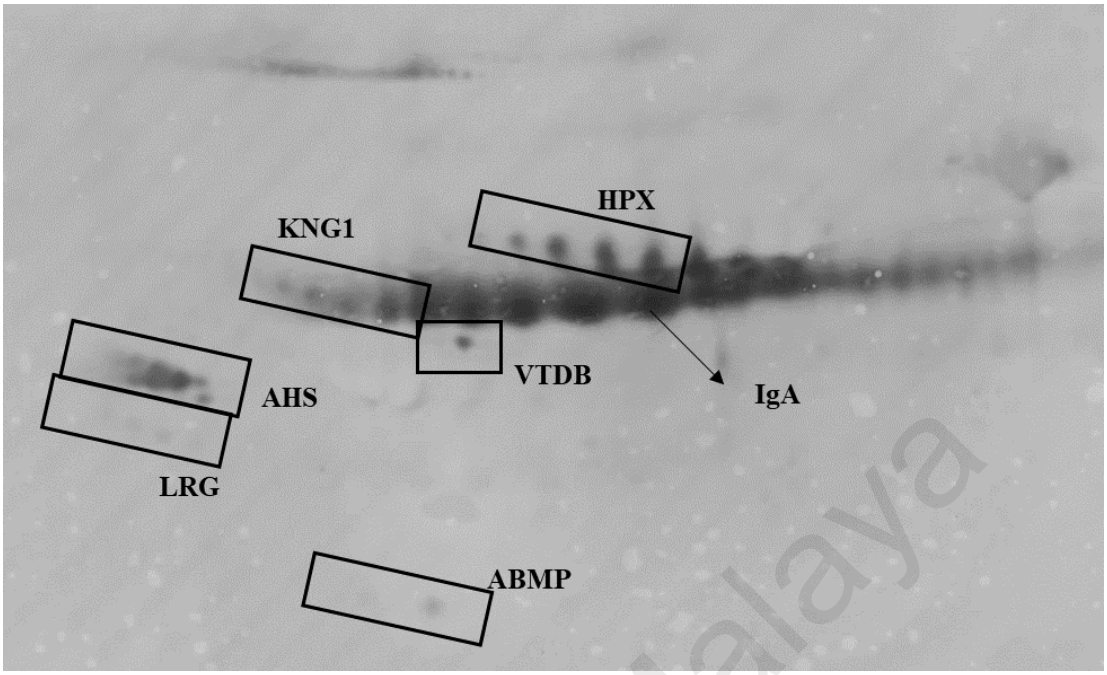
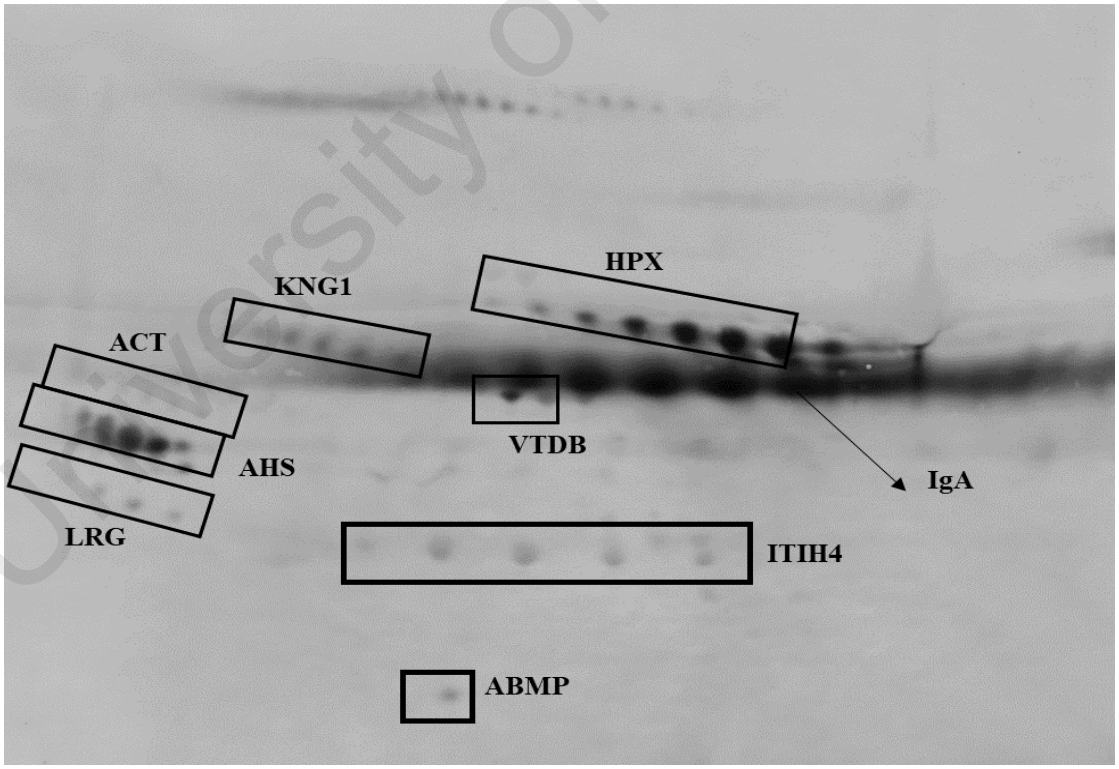


Figure 4.5 (a-c): Glycoproteomic profiling of O-glycosylated pooled serum proteins retained by CGB-lectin affinity chromatography.

(b)



(c)

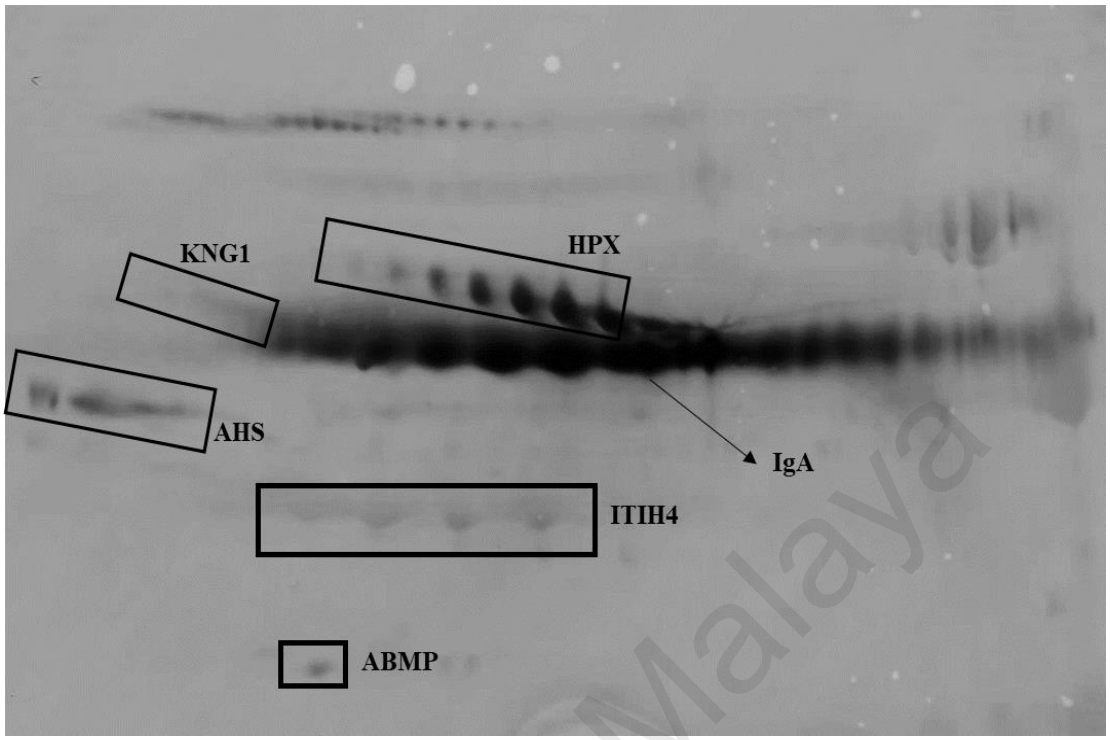


Figure 4.6: Mean percentage of volume contribution of CGB-lectin detected serum *O*-glycosylated proteins

Volumes of five protein spots that were detected by Image Master 2D-Platinum software version 7.0 was included in the analysis. The proteins are; AHS, HPX, KNG1, LRG, and VTDB. Asterik denotes significantly different values.

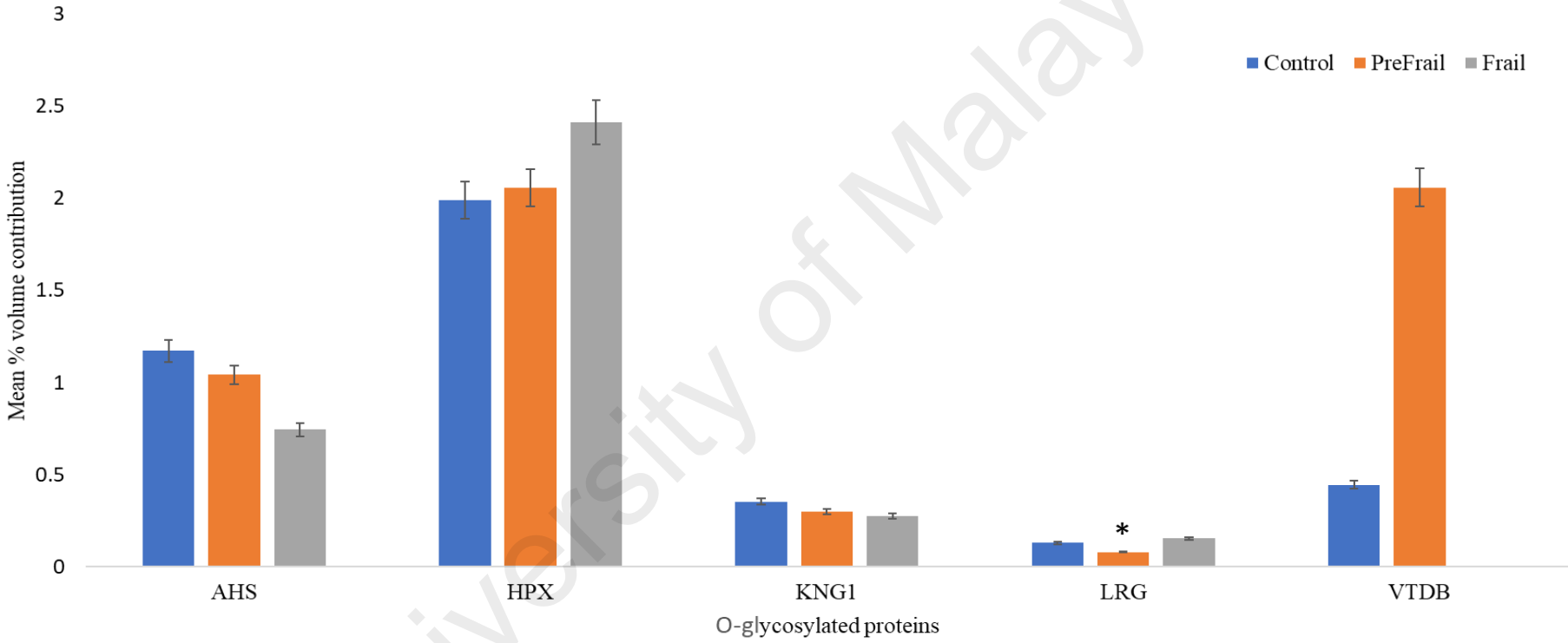


Figure 4.6: Mean percentage of volume contribution of CGB-lectin detected serum *O*-glycosylated proteins

4.3.2.3 CMB-AP generated 2-DE serum glycoproteins profiles of control, pre-frail and frail elderly subjects.

When 2-DE separated serum blots of control subjects were probed using CMB-AP conjugate, the high abundance N-glycosylated proteins that were detected included A1AT, A1BG, AHS, HAP, HPX, IgG, KNG1, LRG and VTDB. These protein spots were identified through comparison with the standard SWISS ExPASy plasma protein reference. Figure 4.7 (a-c) represents CMB-lectin probed pooled serum protein profiles of control, pre-frail and frail group that was developed using NBT/BCIP substrate.

4.3.2.4 Image analysis of CMB-lectin generated 2-DE serum protein profiles

Image analysis of the resolved CMB-lectin probed 2-DE pooled serum protein blots were includes eight clusters of protein spots including A1AT, A1BG, AHS, HAP, HPX, KNG1, LRG, and VTDB. Figure 4.8 is the histogram for mean percentage of volume contribution of CMB-lectin detected serum *N*-glycosylated proteins. Densitometry analysis on blot profiles across three groups demonstrated that there was only HAP, that showed significant statistical difference. Expressions of HAP was significantly higher in the frail group (1.96 fold, p=0.02) relative to control elderly subjects. Apart from that, VTDB and KNG was expressed higher pre-frail group with fold change 1.5 and 2.0 respectively but were not significantly different. In frail elderly, A1AT and KNG proteins were aberrantly expressed with fold change 2.4 and 2.3 each in compared to their normal counterparts, however the difference was not significant. Although insignificant, this was in parallel with silver staining analysis, as significantly increased expression level of A1AT (1.66 fold, p=0.00) in frail individuals and KNG in both pre-frail (1.56 fold, p=0.04) and frail (1.36 fold, p=0.05) individuals. This could be due to

the limitation of using of pooled samples for lectin-blot analysis which may affect the quantitative analysis.

Figure 4.7 (a-c): Glycoproteomic profiling of N-glycosylated pooled serum proteins retained by CMB-lectin affinity chromatography.

Pooled unfractionated serum samples of controls (a), pre-frail (b), and frail (c), were subjected to 2-DE and CMB-lectin blotting. N-glycosylated serum proteins spot determined included A1AT, A1BG, AHS, HAP, HPX, KNG1, LRG and VTDB.

(a)

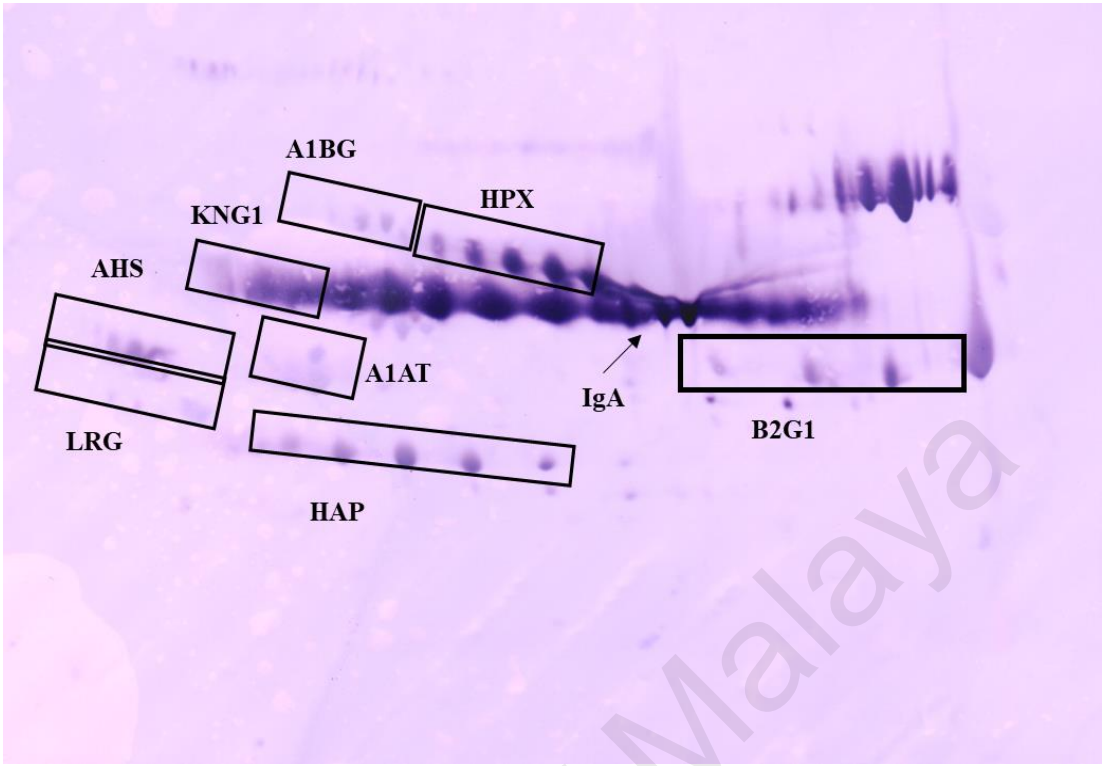
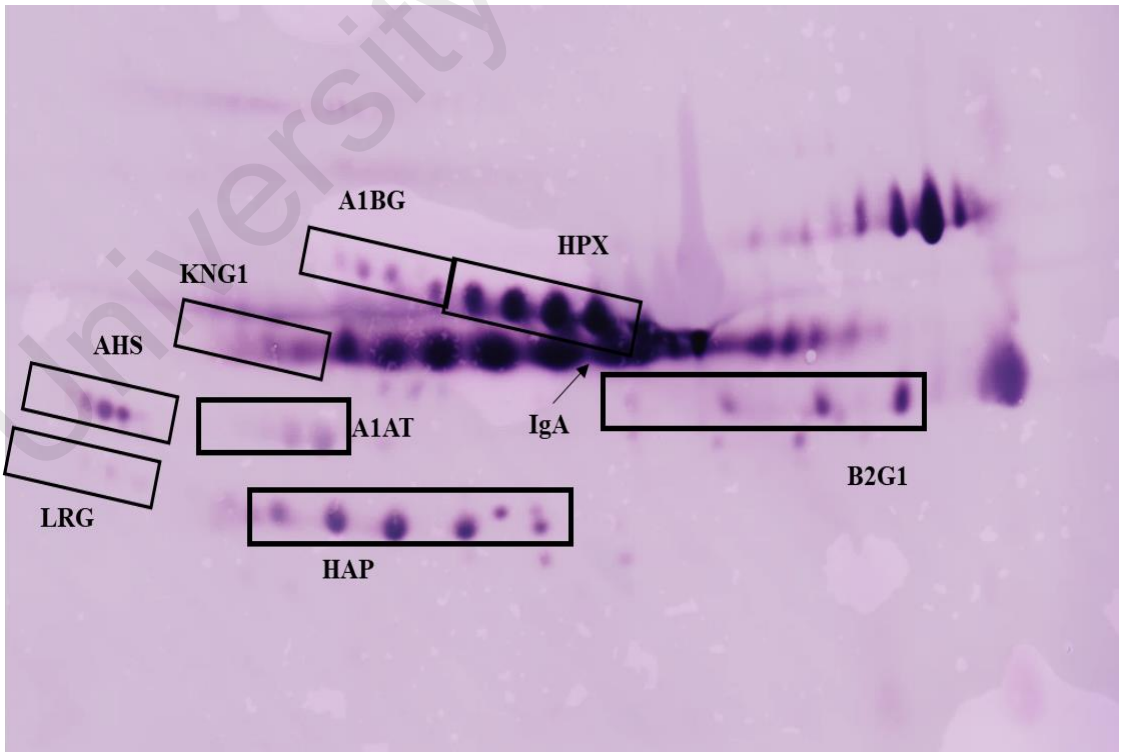


Figure 4.7 (a-c): Glycoproteomic profiling of N-glycosylated pooled serum proteins retained by CMB-lectin affinity chromatography.

(b)



(c)

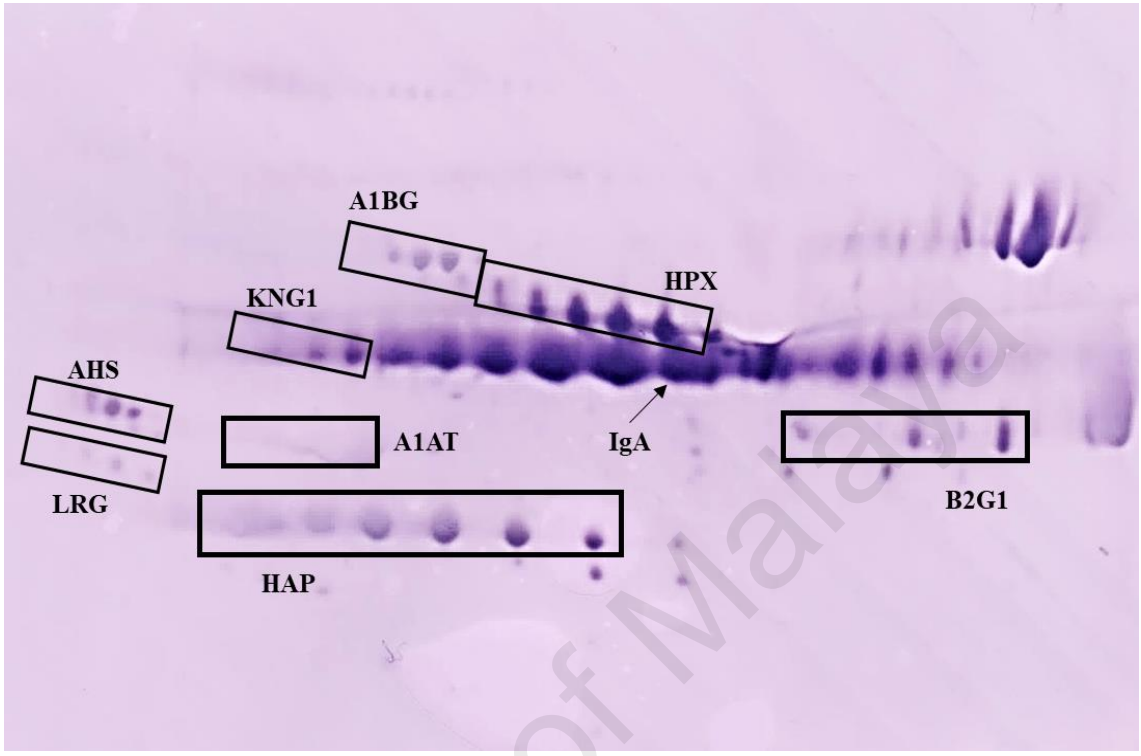


Figure 4.8: Mean percentage of volume contribution of CMB-lectin detected serum *N*-glycosylated proteins

Volumes of five protein spots including A1AT, A1BG, AHS, HAP, HPX, KNG1, LRG and VTDB were analysed. The volume of protein spot that were detected by Image Master 2D-Platinum software version 7.0 was included in the analysis. Asterik denotes significantly different values.

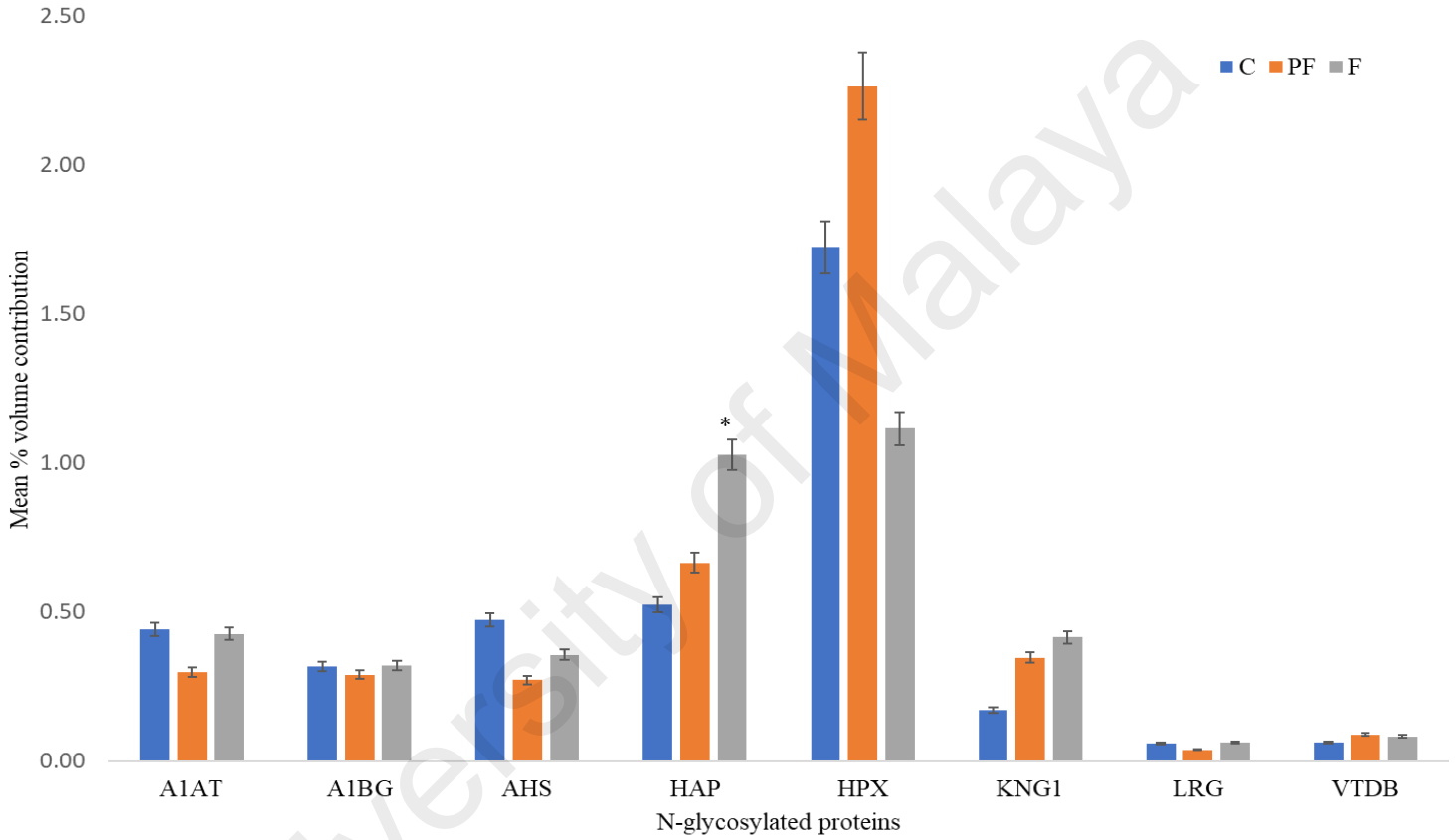


Figure 4.8: Mean percentage of volume contribution of CMB-lectin detected serum *N*-glycosylated proteins

4.4 Validations of serum protein expression by ELISA.

ELISA was performed according to step by step protocol described in the ELISA kit catalogue (Fine Test, Wuhan. China)., for the conformation of the differentially expressed known proteins according to frailty status. The assay was carried out using antisera against one protein which is significantly increased in fold change for both pre-frail and frail group and another that was reduced in expression level. Following the 2-DE result analysis, A1AT and APOE was appropriate for the further validation among other differentially expressed identified proteins.

4.4.1 Validation of A1AT

ELISA was performed in the presence of serum of control elderly (n=15), pre-frail (n=15) and frail (n=15) with the antisera of A1AT. The kit was based on sandwich enzyme-linked immune-sorbent assay technology. Anti-A1AT antibody was precoated onto 96-well plates (purchased) and the biotin conjugated anti-A1AT antibody was used as detection antibodies. The standards, test samples and biotin conjugated detection antibody were added to the wells subsequently and washed with wash buffer. HRP-streptavidin was added, and unbound conjugates was washed away with wash buffer. TMB substrate were used to visualise HRP enzymatic reaction. TMB was catalysed by HRP to produce a blue colour product that turned yellow after adding acidic stop solution. The density of yellow is proportional to the A1AT amount of sample captured in plate. The O.D. absorbance was read at 450nm in a microplate reader and then the concentration of A1AT was calculated. A1AT level for both groups were found to be significantly increased although higher level of A1AT was detected in the frail group

(1.30 fold, p=0.00) compared to pre-frail (1.16 fold, p=0.09) even though fold change less than 1.5. Figure 4.9 shows the analysis of A1AT expression by ELISA.

4.4.2 Validation of APOE

The validation method of APOE is similar to A1AT analysis (as explained in section 4.4.1). Anti APOE antibody was pre-coated onto 96-well plates (purchased) and the biotin conjugated anti-APOE antibody was used as detection antibodies. The density of yellow is proportional to the APOE amount of sample captured in plate. The O.D. absorbance at 450nm was read in a microplate reader and then the concentration of APOE was calculated. ELISA results showed lower level of APOE was significantly detected in frail group (1.43 fold, p=0.01). Figure 4.10: Analysis of APOE expression by ELISA.

Figure 4.9: Analysis of A1AT expression by ELISA

ELISA was performed using commercially available kit and analyses was performed in duplicate.

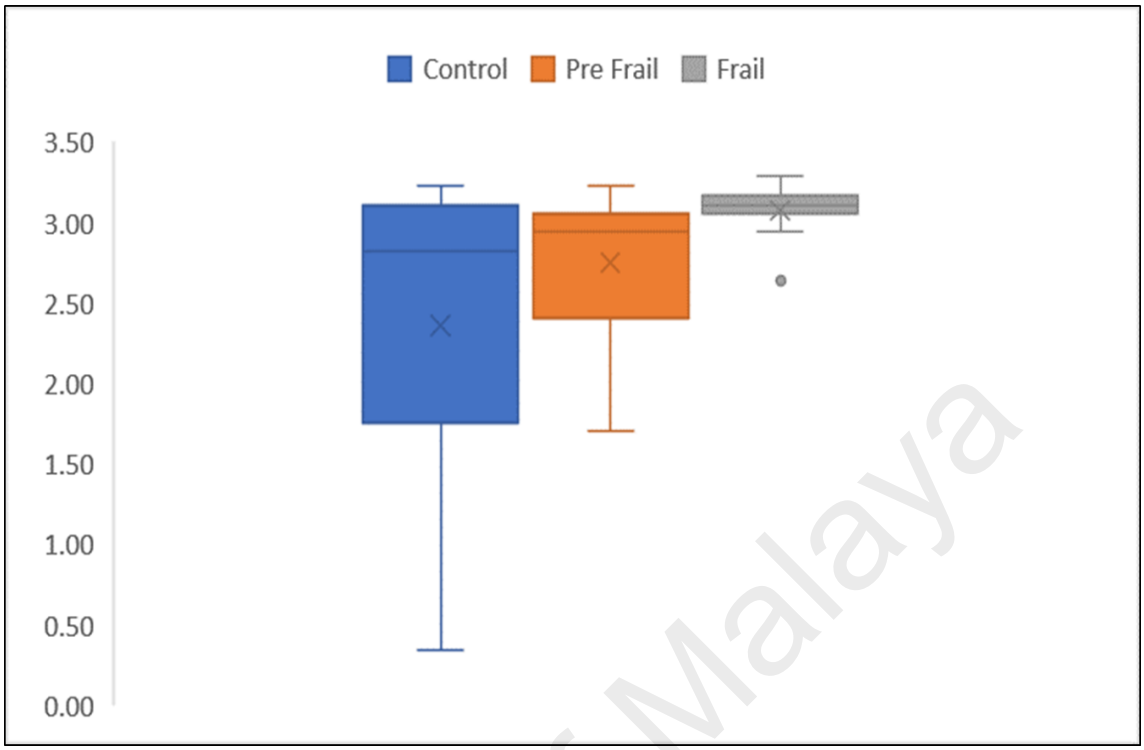


Figure 4.9: Analysis of A1AT expression by ELISA

Figure 4.10: Analysis of A1AT expression by ELISA

ELISA was performed using commercially available kit and analyses was performed in duplicate.

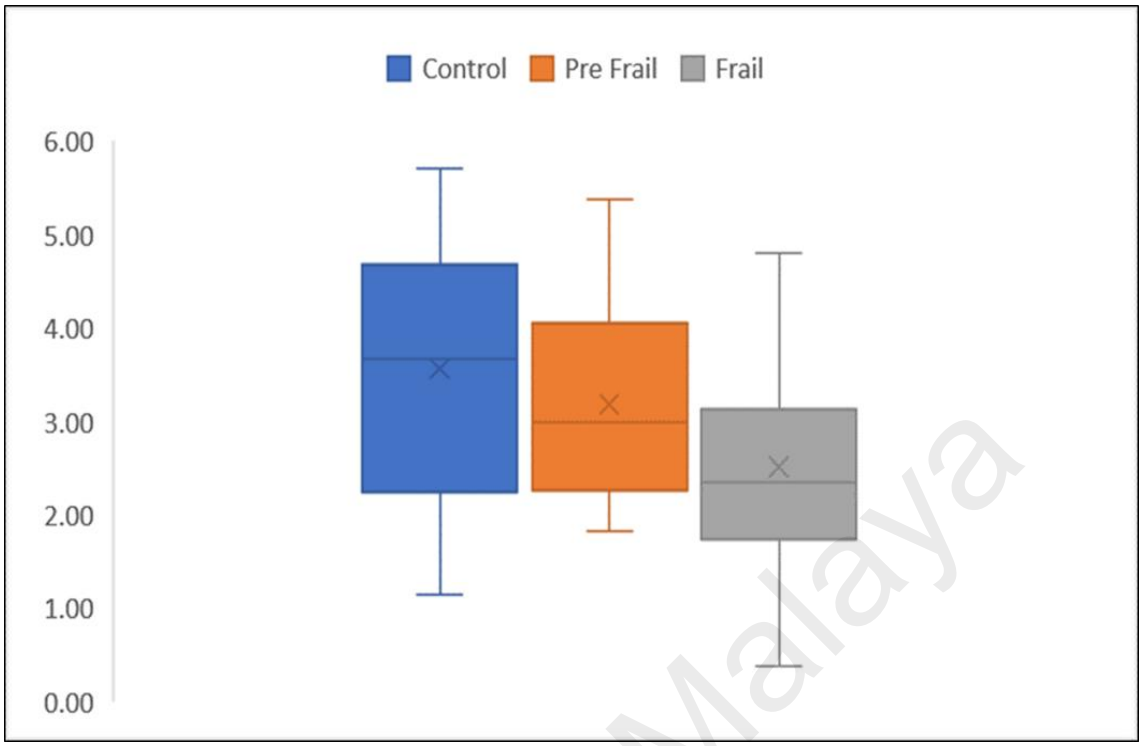


Figure 4.10: Analysis of APOE expression by ELISA

CHAPTER 5: DISCUSSION

In the past decade, research on frailty has evolved to focus more on the molecular pathways that lead to its development. This study explores the basis for its detection via a proteomic method. Frailty was first defined using the FI which is a multidimensional measure of frailty. In this study, frailty level among patients admitted in the geriatric ward of UMMC were assessed with variables that were easily available through patients' medical records and via interviews. This frailty measure was chosen as it closely reflects the comprehensive geriatric assessment for older people. This study was conducted on stable but frail older patients and compared to those who were healthy community dwellers. This was to enable establishment of comparable profiles of proteins expression in non-frail, pre-frail and frail older people.

In this study, we have used 2-DE technique to develop serum protein expression profiles of normal non-frail elderly, pre-frail and frail subjects. Using this method, unfractionated whole human serum was subjected to 2-DE without albumin depletion. Study conducted by Chen and co-workers by using unfractionated serum sample, highlighted the potential prognostic significance of the high abundance serum acute-phase proteins (APPs) in patients with epithelial ovarian carcinoma (EOC). The different altered stage correlative expression of CLU, LRG, ACT, HAP and A1AT in sera of patients with EOCa was identified (Y. Chen, Lim, & Hashim, 2009). In contrast, the removal of high abundant proteins, followed by 2-D DIGE proteomic analysis demonstrated a significant improvement in detection of serum proteins. This shows that there is improved coverage in terms of number of spots and the range of abundance that could be detected. However, albumin is a sticky protein which binds

low-molecular-weight molecules, including proteins and peptides which provides a rich source of candidate peptides and proteins with potential diverse tissue and cellular origins that may reflect important disease related information (Lowenthal *et al.*, 2005). Hence for this study, the use of neat unfractionated serum was chosen for 2-DE work.

The second approach that was adopted in this study involved the use of plant lectins as probes to examine the 2-DE serum protein profiles. To the best of our knowledge, the present study is the first to report the differentially expressed of serum proteins using CGB- and CMB-lectin in the sera of elderly control, pre-frail and frail subjects. Pooled serum samples of the study groups were subjected to lectin blotting. The main advantage of using plant lectin as probes to examine serum protein profiles is that only a selective group of the protein is detected as a result of the specific binding interaction of lectins to glycoproteins. Furthermore, the lectin approach provided an extra avenue to determine expression of additional high abundance serum proteins that were not detected or well resolved using the silver staining method. The CGB-lectin binds primarily to Gal and GalNAc residues thus able to detect O-glycosylated serum proteins (Rahman, 2002). On the other hand, CMB-lectin binds to the core-mannosyl residues that allows detection of N-glycosylated serum proteins (Sing Bin Lim *et al.*, 1997).

Our present study analysis of the 2-DE protein expression profiles across the three study groups show, the up-regulated expression of A1AT and KNG in pre-frail and frail groups whilst additionally, four other proteins A1BG, HAP, HPX, and LRG were significantly detected in the profiles of frail subjects when compared to the healthy controls. The downregulated expression of APOE and UK1 (which was later detected as CLU) were also significantly detected in the profiles of frail subjects. Consequently, A1AT and APOE was validated by using ELISA. Higher level of A1AT was detected

using ELISA in the pre-frail and frail group. ELISA assay performed using APOE antibody detected lower level of APOE in frail group. Both results of ELISA were comparable to the 2-DE silver stain results, although the magnitude of fold difference were lower than the densitometry analysis.

In our study, the majority of the observed protein fold change was less than two. This could probably be due to the age group of the study population. In this study, subjects were among the elderly individuals, where patients were compared with healthy elderly of the same age range. Therefore, the gap between the age group is not vast to show a comparable fold change. If the control subjects were from adult populations (18-60 years old), the changes of protein expression level from adult to elderly may be reflected in the profile with a higher or lower fold change. Additionally, our subjects were in-patients where they already have other underlying medical conditions involving inflammatory processes that may interrupt with protein expression level. Hence, the fold change considered as showing significant difference was set at 1.3 and above for this study.

In another experiment that was performed in 2010, Byerley investigated on development of serum profile of healthy ageing individuals, found differences of proteome profile across the three different age groups (20-34, 60-67, and ≥ 90) using 2-DE analysis. The group reported that haptoglobin was able to distinguish subjects into the three groups; although six other proteins were not successful. They suggested this serum protein spot may be important in the biological ageing process and that the proteomics approach can be used to identify potential protein or markers that important determinants of longevity. This finding might be useful in differentiating between frail and healthy subjects after further investigations in future (Byerley *et al.*, 2010).

The association between the aberrant expressions of proteins that were observed with the different frailty status of the study subjects could be linked to the protein's physiological role and maybe mediated via several mechanisms and pathways. The following section of discussion will discuss on the aberrant proteins that were observed in the 2-DE study and lectin blot analysis.

A1AT

One protein that was found upregulated in both the pre-frail and frail group is A1AT. A1AT is a protease inhibitor, an N-glycosylated serum glycoprotein and also immune response regulator (Marklova, Albahri, & Valis, 2012). The archetype of the serpin family is A1AT, the inhibitor presents at highest concentration in human plasma. The liver is widely recognised to represent the major source for the A1AT productions. During an inflammatory response, tissue concentrations of A1AT may increase as much as 11-folds as a result of local synthesis. Additionally, elevated A1AT secretion is mainly mediated by cytokines, such as IL-6, and TNF-α, which might induce AIAT expression by different magnitude (Sarabhai et al., 2017). Recently, findings in terms of increased level of A1AT in frail subjects of Taiwan population were reported, where they detected up-regulation of A1AT with the fold change of 1.48 ($p=0.02$) (C. H. Lin *et al.*, 2017). Similarly, in this study up-regulated expression of A1AT were observed in the pre-frail and frail subjects.

Previous investigations on frailty biomarkers identified inflammatory biomarkers were highly correlated with frailty status (Arts et al., 2015; Chang, Weiss, Xue, & Fried, 2012; Hutchins-Wiese et al., 2013; Jylhava et al., 2013; Lee et al., 2016; Tay, Lim,

Chan, Ye, & Chong, 2016). A pilot exploratory study recently reported, cytokines/chemokines predict frailty status by using both Fried's and Frailty Index significantly which further support systemic inflammation is involved in the biology of frailty (Lu et al., 2016). A1AT might induce neutrophil apoptosis by a pathway involving endoplasmic reticulum stress and TNF-α signalling (Sarabhai et al., 2017) and on the other hand, Shamsi et al. (2012) hypothesized, individuals who are frail would have increased inflammation and increased endoplasmic reticulum stress, leading to disruption in posttranslational modifications and subsequently frailty related alterations in expressed glycoproteins. Taken together, findings from this study with increased level of A1AT in frail subjects maybe associated with endoplasmic reticulum stress and inflammatory pathway which predispose towards frailty development.

KNG

KNG was another protein found to be upregulated in our silver stained 2DE studies. According to our findings, KNG was significantly increased in pre-frail (+1.56 fold) and frail (+1.36 fold) group. KNG are well known to be a large potential source of kinin in human plasma and as a cofactor in the contact activation of blood coagulation (Ishiguro, Higashiyama, Ohkubo, & Sasaki, 1987). These proteins are synthesised and secreted into the circulation by hepatocytes (Figueroa *et al.*, 1992). Identifications of this proteins is rather interesting, given that KNG is involved in coagulation responses, which has been shown in several prior studies to be altered in frailty (Reiner *et al.*, 2009) and among elderly women in the development of locomotor disability irrespective of lifestyle factors and underlying age related chronic disease (Nüesch *et al.*, 2012).

A study conducted in Taiwan by C. H. Lin *et al.* (2017) has also identified and characterised the biomarkers of non-frail and frail older adults through proteomics approach. Their sample were divided by the Chinese-Canadian Study of Health and Aging Clinical Frailty Scale (CSHA-CFS) into these two groups by the researchers. Using ultra-high-performance liquid chromatography-tandem mass spectrometry, they also found higher level of serum KNG (1.58 fold, p=0.005) in frail than non-frail subjects. This differences in fold level of KNG as compared to what was detected in our study could be due to the method used for proteomic analysis and/or the type of tool used to define frailty. 2-DE has some limitations that must be taken into account. Despite careful conduct of the procedure, there will be some degree of gel-to-gel and run-to-run variability in the expression of same protein profile (Chevalier, 2010) which may reduce the matching efficiency of the same spot in different gel. Since peptides can be more easily separated by liquid chromatography than proteins, a peptide based proteomic analysis can be performed faster and economical compared to complete gel-based analysis (Baggerman, Vierstraete, De Loof, & Schoofs, 2005).

There were only six subjects in category compared to our study and SDS-PAGE method was used to separate the proteins. The elderly in the study were measured for grip power and the 6-minute walk test for physical activity assessment. Other than that, the group also detected higher serum levels of angiotensinogen and antithrombin III in frail than non-frail subjects. They propose that the three proteins could be potential biomarkers for monitoring the development and progression of frailty in older adults.

HPX

HPX is a 60-kDa plasma glycoprotein consisting of single polypeptide chain and among the fourth most abundant group of plasma proteins after albumin, immunoglobins and the plasma proteases. Aside from being involved in plasma transportation of heme for iron metabolism, HPX also acts as a multifunctional agent in an important health-related processes such as iron homeostasis, antioxidant protection, bacteriostatic defence, nerve regeneration, and gene expression to promote cell survival (Delanghe & Langlois, 2001). Hemopexin also protects against peroxidative damage due to haemoglobin by binding heme released from methemoglobin or from oxyhemoglobin in the presence of H_2O_2 which is likely to be present in inflammation (Miller, Smith, Morgan, & Shaklai, 1996). Additionally, HPX has been shown to be one of the acute phase plasma proteins whose mRNA levels are increased by cytokines, and the synthesis of hemopexin increases in response to inflammation (Miller *et al.*, 1996).

Parallel to our findings, the aberrantly expressed HPX in frail subjects support that frailty is associated with inflammatory and hematologic changes. Meanwhile the protein was increased in level (1.32 fold, p=0.009) among frail subjects compared to control (C. H. Lin *et al.*, 2017), supporting our findings. In another study, that utilises lectin treated plasma sample, HPX was found to be downregulated in the sera of pre-frail subjects that was treated with Jacalin (Shamsi *et al.*, 2012). This may be attributed to glycosylation of HPX in pre-frail individuals.

HAP

HAP is an acute-phase response protein, acidic glycoprotein tetramer that is mainly secreted by the liver. Alterations of glycosylation of HAP have been reported in patients with many type of cancers, including pancreatic, hepatic, prostate, lung, breast, ovarian, colon and gastric cancer. The main function of this N-linked glycoprotein is, to bind and carry free haemoglobin for degradation in the liver and for iron recycling. It captures haemoglobin released from erythrocytes and restrains oxidative activity of haemoglobin to prevent kidney from damage when intravascular hemolysis occurs (S. Zhang, Shang, Li, Qin, & Liu, 2016). In this study, comparable HAP expression level were demonstrated using both silver and lectin analysis. Increase level of the protein was observed among frail elderly compared to healthy elderly subjects. In an earlier analysis of frailty study using Concanavalin A-treated plasma, also indicates an increased of HAP level among the pre-frail elderly (Shamsi *et al.*, 2012).

Conditions such as ageing and obesity are characterised by chronic systemic inflammation and by muscle wasting. Frail and disabled older adults have increased circulating levels of IL-6 and CRP (Walston, McBurnie, Newman, & et al., 2002). Continuous expression of inflammatory cytokines (IL-6, TNFα, IL-Ib) is harmful for muscle mass, because it activates signalling pathways that promote protein breakdown and suppress protein synthesis, causing atrophy of muscle cells (Bertaggia et al, 2014). As mentioned in the earlier section of discussion, HAP was also recently associated with aging and was identified using similar high-throughput 2-DE gel proteomics methodology (Byerley *et al.*, 2010).

A1BG

A1BG is mainly produced in the liver, and is secreted to plasma (Ishioka, Takahashi, & Putnam, 1986). It is a 474 amino acid polypeptide with an apparent mass of 63 kDa (including glycosylation). The protein consist of five repetitive domains that show high homology with known immunoglobulin heavy and light chain variable domains, making the protein part of the immunoglobulin superfamily (Clerc *et al.*, 2016). The overall role of the protein is still unknown, but it has been found to bind cysteine-rich secretory protein 3, and has been associated with breast, liver, pancreas and bladder cancer (Tian *et al.*, 2008; Yoon *et al.*, 2006).

In this study, A1BG was detected in the profiles of frail elderly with slight increase in expression level (1.31 fold, p=0.03) compared to healthy elderly in silver work. In another study, plasma treated with Concanavalin-A identified A1BG in pre-frail group but no changes was observed when compared with non-frail older adults (Shamsi *et al.*, 2012). Similarly, in our CMB lectin blot profile A1BG was detected in both pre-frail and frail group, however the changes are not significant compared to control elderly. So far, the literature search shows A1BG is not associate with frailty development or as a contributing factor. Furthermore, the role of these glycosylated protein remains to be determined and may likely has implications in ageing and frailty progressions as this protein identified in elderly protein profiles of serum and plasma.

LRG

LRG which is secreted by the liver in response to inflammation, are classified as acute-phase proteins. Low levels of LRG were observed in inflammatory arthritis patients who frequently had high levels of CRP. LRG is a secretory type 1 acute-phase proteins whose expression was upregulated by mediator of acute-phase response (Shirai, Hirano, Ohkura, Ikeda, & Inoue, 2009). Therefore, this suggests that, LRG expression could be related to CRP level as stated in two different studies that show high CRP level among frail women (Gale *et al.*, 2013) and correlation between physical frailty and increased level of CRP (Arts *et al.*, 2015).

Present study, demonstrate LRG expression level were increased among frail subjects (1.42 fold, p=0.05) compared to healthy elderly. Contrarily, densitometry blot image analysis of O-glycosylated proteins from our preliminary data determined LRG (0.62 fold, p=0.04) were reduced in pre-frail subjects. In a similar study conducted among community-dwelling elderly by Shamsi *et al.* (2012) utilising Wheat Germ Agglutinin and Jacalin-treated plasma demonstrated no difference in the expression level of LRG in pre-frail compared to non-frail older adults. The findings may differ due to the specific binding affinities of the different lectin used. Frailty is well known associated with inflammatory response in general, hence our findings could be related with other inflammatory markers as a potential mediator in the frailty pathway.

APOE

Protein products encoded by *APOE* gene play crucial role in lipid metabolism It is also associated with atherosclerosis, neuronal maintenance and repair, inflammation and possibly, immune response to infectious diseases. It is also involved in the development of several cardiovascular and neurodegenerative disorders (C.-C. Liu, Kanekiyo, Xu, & Bu, 2013)

APOE protein was found significantly downregulated (0.5 fold, p=0.03) in the silver stained 2-DE profiles pre-frail in our study. In another study, this protein was found decreased in pre-frail compared with non-frail adults when glycoproteins profiling was performed using Jacalin-lectin treated plasma sample in subjects from independent living retirement community (Shamsi *et al.*, 2012). A similar technique was utilised in our study, however in the O-linked glysoprotein profile of CGB-lectin, APOE was not detected. This may be due to the different binding affinities that the two lectin have even though structurally, CGB-lectin has a close homology to jacalin (Gabrielsen, Abdul-Rahman, Othman, Hashim, & Cogdell, 2014; Mads Gabrielsen *et al.*, 2009). Currently, there is limited literature that relates the role of APOE protein with frailty. However, in terms of the gene expression, a vast number of studies have been reported.

The *APOE* gene has three common allels, known as Ɛ2, Ɛ3, Ɛ4 which generate three homozygous (Ɛ2/Ɛ2, Ɛ3/Ɛ3 and Ɛ4/Ɛ4) and three heterozygous (Ɛ2/Ɛ3, Ɛ2/Ɛ4 and Ɛ3/Ɛ4) genotypes (G. D. Liu *et al.*, 2017). *APOE* gene, which is a major genetic risk factor for late onset Alzheimer disease (AD), is an important genetic biomarker for AD pathophysiology. It has been shown that *APOE* Ɛ4 is involved in β-amyloid deposition and formation of amyloid plaques (Ba *et al.*, 2016). Additionally, recent investigation by Bonham *et al.* (2016), reported that APOE Ɛ4 carrier status and dosage predict

progression to mild cognitive impairment and AD, this findings provide insights into preclinical AD and suggest that the Ɛ allele of *APOE* influences the earliest stages of the AD process.

Interestingly, cognitive frailty (CF) (heterogenous clinical syndrome found in elderly individuals that excludes those with AD and other types of dementia, and is characterised by concurrent physical frailty and potentially reversible cognitive impairment). The neuropathological processes overlap with those in individuals with AD and/or other neurodegenerative diseases, and the final outcomes of CF are AD or non-AD dementia (Ruan, D'Onofrio, Sancarlo, Greco, & Yu, 2016). Even though our study does not investigate on CF, but physical frailty and AD may share similar pathophysiological mechanisms. Certain AD-associated fluid biomarkers, such as oxidative stress and inflammatory markers, may also contribute to the screening of physical frailty in CF subjects (Ruan *et al.*, 2016).

Scientist have suggested that APOE gene should be considered a "frailty gene" that increases the age specific susceptibility to death. If the APOE gene really is a "frailty gene", it would be reasonable to expect Ɛ4 carriers to accumulate more age-related diseases and to demonstrate poor functional performance in advanced ages (Megale RZ. *et al*, 2016). This is in parallel with FI (A. B. Mitnitski, Mogilner, & Rockwood, 2001), which defined frailty as the proportion of accumulated deficits, which was shown to increase monotonically with chronological age.

CLU

CLU or known as Apolipoprotein J (ApoJ) is a multifunctional protein, capable of interacting with broad spectrum of molecules including itself, amyloid proteins, components of the complement membrane-attack complex or lipids. A classical secretion signal peptide allows clusterin to be released from producing cells, to remain bound at the surface of some cells, to circulate in extracellular compartments, and even to be internalised by non-producing cells (D. Michel, Chatelain, North, & Brun, 1997). Considering the increase in oxidative and proteotoxic stress during ageing, CLU expression levels have been found to increase in both cellular senescence and normal ageing of various tissue (Trougakos, 2013). Newer findings adding to this observation indicated plasma CLU is associated with incident dementia, Alzheimer Disease (AD) and stroke; however, age, or age-related factors, may interact with clusterin leading to contradictory associations among the young-old and the oldest-old (Weinstein *et al.*, 2016).

In this study, CLU was downregulated (0.61fold, p=0.02) in frail elderly compared to control subjects in 2-DE silver stain analysis. Our findings is contrary to recent study published, where increase CLU expression significantly protects against transactive response DNA-binding protein (TDP-43) mediated proteotoxicity to substantially enhance motor neuron survival, reduce locomotor deficits and extend lifestyle (Gregory *et al.*, 2017). Rationally, frail elderly are decrease in their functional ability and reduce in lifespan compared to healthy older adults, hence the lower expression of CLU is not able to reflect its protective effects.

Apart from physical frailty, CLU were among the plasma proteins which associated with disease severity and in progression of mild cognitive impairment (MCI)

and AD (Hye *et al.*, 2014), hence the early identification of AD may also be helpful in the screening of CF.

Lectin

Densitometry blot image analysis of O-glycosylated proteins from our preliminary data determined LRG (-1.61 fold, p=0.04) levels were significantly reduced in pre-frail subjects and VTDB expression in pre-frail subjects were decreased with fold change of 4.6 but not significant. In contrast, CMB-lectin blot identified, expressions of HAP were significantly higher in the frail group (1.96 fold, p=0.02) relative to control elderly subjects. Apart from that, VTDB and KNG was expressed higher in pre-frail group with fold change 1.5 and 2.0 respectively but were not significantly different. Similarly, in frail elderly, KNG and A1AT proteins were aberrantly expressed with fold change 2.4 and 2.3 each in compared to their normal counterparts, however the difference was not significant.

The detected protein using lectin approach has been discussed along with 2-DE silver work. VTDB which were detected only in CMB-lectin blot are discussed under this section.

Despite its insignificance association with frailty status, decreased fold change of VTDB, can be considered as potential frailty biomarker that could facilitate in determining progression from pre-frail to frail. VTDB may alter the biologic activity of 25-hydroxyvitamin D [25(OH)D]. Previous results indicate that the joint effect of serum 25(OH)D and VTDB levels is associated with risk of frailty, and serum VTDB levels effects 25(OH)D-frailty relationship in older men (Wang *et al.*, 2014). Similarly,

vitamin D insufficiency is common among alder adults and recent study show that VTDB was negatively correlated with age in female subjects indicating that age might be an independent factor affecting VTDB and 25(OH)D levels (Yousefzadeh, Shapses, & Wang, 2014). In supporting this, 25(OH)D levels were less frequently found in pre-frail and frail subjects (Pabst *et al.*, 2015) and significantly associated with frailty (Alvarez-Rios *et al.*, 2015).

Collectively, findings on lectin approach need to be carefully interpret in this study. Proteins that were significantly identified in using 2-DE silver work does not significantly detected in CGB-lectin blot. This may due to variability in the spatial distribution whereby the glycans are capable of branching and twisting (side chain modifications) with flexibility that pushes the boundaries for their molecular modelling (Marino, Bones, Kattla, & Rudd, 2010) which was reflected in protein abundance using lectin detection method. Apart from that, the choice of membrane for lection blot will affect protein binding capacity and chemical stability (O. H. Hashim, Jayapalan, & Lee, 2017). This may cause the protein bind loosely to the membrane and washed away during washing stage. Additionally, different from antibodies, lectins have comparatively weak affinity and low specificity towards binding in several different ligands. However due to other advantages, lectins are gaining more attentions as a potential powerful tool that may take great contributions to the dynamic development of glycomics (Dan, Liu, & Ng, 2016).

CHAPTER 6: CONCLUSION

The aim of this study is to provide novel and new insight into the identification of protein biomarkers and its association with the frailty status in older people. Comparative analysis between silver-stained 2-DE profiles of serum high abundance proteins demonstrated that different expression profiles were obtained when samples of pre-frail and frail patients were compared to non-frail elderly controls.

Image analysis evaluation of 2-DE profiles reveals upregulated expression level of proteins of A1AT and KNG1 in the pre-frail individuals as compared to the elderly control. Similarly, these proteins were also up-regulated in the frail group. In addition, up-regulated expression of A1BG, HAP, HPX and LRG; and downregulated expression of APOE and CLU were also significantly detected in the profiles of frail subjects when compared to normal elderly control. Identities of the aberrantly expressed proteins were confirmed through mass spectrometry analysis. By using ELISA, the altered level of A1AT and APOE in the analysed groups of the elderly were confirmed although the magnitude of fold changes detected were much lower compared to 2-DE analysis.

Profiles of pooled serum O-linked and N-linked glycoproteins for the study subjects were generated using enzyme conjugated CGB- and CMB-binding lectins. LRG was the only protein that was significantly reduced in pre-frail subjects in the CGB-HRP generated blots. Meanwhile, CMB-AP generated blot profiles across three groups demonstrated, expressions of HAP was significantly higher in the frail group relative to the control elderly subjects. Similarly, data obtained from the silver staining analysis, the expression of HAP was statistically higher, but fold change scale were lower.

Since, frailty is a syndrome and not a disease condition, it was difficult to make a clear conclusion about the protein biomarkers at this level of study. Findings from our proteomics study and preliminary data from CGB- and CMB-lectin probed blots may be useful as initial steps in the future research of frailty progression by looking at protein expression at early stage of the frailty trajectory. The present study found high correlation between frail elderly and A1AT level which is not reported before even though the expression level is also increased (lower than frail group) in pre-frail elderly. Larger numbers of subjects in each group could give statistically promising results along with using high throughput and sensitive identification method. As age increases, frailty is not static but dynamic, hence identifying frailty and its potential biomarkers could provide the possibility of reversing the frailty status of the elderly to the pre-frail or healthy phase of life.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Presentations

2014

1. Asia Pacific Geriatric Conference (Taipei, Taiwan) - **Oral**

   Frailty Assessment Among Geriatric In-Patient's

2015

2. 11th National Geriatric Conference (Kuala Lumpur) **– Poster**
   2-Dimensional Gel Electrophoretic Analyses of Serum Proteins from Pre Frail and Frail Subjects.

2016

3. Selangor Geriatric Conference (Faculty of Medicine, UiTM) – **Poster**

   Differential Expression of Serum Proteins in Pre Frail and Frail Subjects Using Gel-Based Proteomics Analysis

Publications

A systematic review of studies comparing potential biochemical biomarkers of frailty with frailty assessments. https://doi.org/10.1016/j.eurger.2017.07.010