IDENTIFICATION OF CHEMICAL AND BIOLOGICAL MARKERS FOR SCHIZOPHRENIA

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By

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To my beloved Grandmama

ABSTRACT

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Chow Tze Jen

Schizophrenia is a debilitating mental disorder, which affects 1% of the world population. With the alarming high prevalence, this research aims to study the association of markers for schizophrenia. The potential of carotenoid antioxidant as a chemical marker, where imbalance in oxidative stress and antioxidant defense system was hypothesised in the pathogenesis of schizophrenia, was investigated. A total of 351 patients with schizophrenia from *Hospital Bahagia Ulu Kinta*, Malaysia and 247 healthy controls were recruited. Skin carotenoid levels were measured using Raman spectroscopy. Overall results showed significantly ($P < 0.000$) lower level of carotenoid in patients compared to controls. Higher mean carotenoid levels were found in females compared to males in both patients ($P = 0.005$) and controls ($P = 0.037$). Among patients, carotenoid level analysis across age, subtypes, antipsychotic drug treatment, and duration of illness showed no significant relationship with schizophrenia. Antipsychotics treatment was suggested to be associated with the higher oxidative stress seen in schizophrenic patients. For biological markers, acute phase proteins are hypothesised to be involved in the pathology of the disorder where immune abnormalities and the role of inflammatory markers have been widely described in schizophrenia. The

serum proteome of 20 patients and 20 controls was investigated using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry (MALDI-ToF/ToF MS). Five proteins were found to be significant different in patients compared to controls. Among these acute phase proteins, two apolipoprotein A-I isoforms: apolipoprotein A-I-1 (P = 0.004), apolipoprotein A-I-2 (P = 0.003), haptoglobin (P = 0.003), and apolipoprotein J (P = 0.003) were down-regulated in patients, while higher levels of apolipoprotein H (P = 0.003) was found in patients compared to controls. These findings support the hypothesis that the inflammatory response system is linked to the pathophysiology of schizophrenia. In conclusion, these analyses supported the hypothesis of oxidative stress and acute phase response in association with schizophrenia. Skin carotenoid, together with serum Hp, Apo A-I-1, Apo A-I-2, Apo H, and Apo J may serve as potential markers for schizophrenia, and may contribute to the understanding of the disease pathology.

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APPROVAL SHEET

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PERMISSION SHEET

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DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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LIST OF ABBREVIATIONS

2-DE	Two-Dimensional Gel Electrophoresis
ANOVA	Analysis of Variance
Apo	Apolipoprotein
APP	Acute Phase Protein
CNS	Central Nervous System
CSF	Cerebral Spinal Fluid
CSIRO	Commonwealth Scientific & Industrial Research Organisation
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders (fourth edition)
DTT	Dithiothreitol
FRAP	Ferric Reducing Ability of Plasma
HBUK	Hospital Bahagia Ulu Kinta
HDL	High Density Lipoprotein
Hp	Haptoglobin
IDL	Intermediate Density Lipoprotein
IEF	Isoelectric Focusing
kDa	Kilo Dalton
LCAT	Lecithin-Cholesterol Acyltransferase
LDL	Low Density Lipoprotein
MALDI-ToF/ToF-MS	Matrix-Assisted Laser Desorption Ionization Time-of-Flight/Time-of-Flight Mass Spectrometry
M.I.N.I.	Mini-International Neuropsychiatric Interview
MS	Mass Spectrometry
MW	Molecular Mass

n	Number of Subject (Sample size)
P	Probability
PAGE	Polyacrylamide Gel Electrophoresis
PMF	Peptide Mass Fingerprinting
r	Pearson Correlation Value
R^2	R-square Value for Equation
ROS	Reactive Oxygen Species
rpm	Revolutions per Minute
SDS	Sodium Dodecyl Sulphate
SPSS	Statistical Package for Social Sciences
TPTZ	2, 4, 6-tri(2-pyridyl)-s-triazine
TTR	Transthyretin
VLDL	Very Low Density Lipoprotein
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Significance of Schizophrenia

One in four people will suffer from a neurological disorder at a point in life, and schizophrenia affects approximately 1% of the world population (Numata *et al.*, 2008). The Global Burden of Disease study ranked schizophrenia among the top ten causes of disability worldwide. Statistics shown more than 50% of people with schizophrenia not receiving appropriate care, and 90% among them are in developing countries (World Health Organization, 2001).

National Mental Health Registry indicated that 7351 cases had been registered in Malaysia from year 2003 to 2005 (Aziz, 2008). There are no data concerning the burden of schizophrenia in Malaysia (Chee, 2009). However, mental disorder is responsible for 8.6% of the total Disability Adjusted Life Years and was ranked fourth as the leading cause of burden of disease. The assessment of Years Lived with Disability and non-fatal burden in Malaysia showed that 21% of the burden was contributed by mental disorders both in men and women (Ministry of Health Malaysia, 2004).

The economic burden to society is staggering. The Malaysian government has been subsidizing antipsychotics medications heavily and spend approximately 2.30 billion Malaysian Ringgit (0.74 billion US dollars) on total drug expenditure in 2005 (Chee, 2009). The pathogenesis of schizophrenia is still unknown, thus it is crucial to search for potential markers so as to facilitate prognosis and early treatment.

1.2 Schizophrenia

1.2.1 Background

The term 'schizophrenia' comes from the Greek roots *schizein* meaning 'to split' and *phren* meaning 'mind' (Scharfetter, 2001). Schizophrenia refers to a major psychiatric disorder that alters an individual's perception, thought, and behaviour (Ministry of Health Malaysia, 2009). It involves a complex array of neurophysiology, neurochemical, and psychological disturbance (Laviolette, 2007) that results from complex genetic interactions with environmental factors (Tsuang, 2000).

Clinical symptoms of schizophrenia usually begin in late adolescence or early adulthood and seldom occur after the age of 45 (National Institute of Mental Health, 2007). Symptoms are classified into three main categories, namely positive, negative and disorganised symptoms.

Positive symptoms, also known as active symptoms, are portrayed by psychosis, such as hallucinations, paranoid and delusions, with a 90% incidence in all patients (Barbato, 1998). In contrast, negative symptoms reflect a loss of normal psychomotor and social abilities, for instance: social isolation, incapable to experience emotions, and poverty of affect and speech (Cohen and Docherty, 2004). Negative symptoms are less obvious and often persist even after the resolution of positive symptoms (Ministry of Health Malaysia, 2009). Disorganised symptoms are usually related to difficulties in concentration and memory, characterised by chaotic speech, disorganised and slow thinking (Victor and Cuesta, 2001). Patients often experience overlapped symptoms and may develop their own unique combination of symptoms (Ministry of Health Malaysia, 2009)

There are five subtypes of schizophrenia, each portray different characteristics. The paranoid subtype is characterised by hallucination and delusion but thought disorder, disorganised behaviour, and affective flattening are absent from the symptoms. On the other hand, undifferentiated patients have psychotic symptoms but the criteria for paranoid, disorganised, and catatonic types are not met. For the disorganised subtype, both thought disorder and flat affect are present altogether, while the positive symptoms are present at low intensity in the residual subtype. Less frequently observed today, the catatonic subtype is characterised by psychomotor disturbances together with symptoms such as catatonic stupor and waxy flexibility (Bilder, 2006).

Population studies of schizophrenia in populations across the world have shown annual incidence rate of 15 per 100,000 populations and prevalence of 4.5 per 1000 population (Tandon *et al.*, 2008). Globally, mental illnesses are increasingly prevalent in developing countries due to poverty and demographic transition (WHO, 2001). In countries of South-East Asia, schizophrenia is a serious public health problem (Thara, 2001). The registered schizophrenia patients in Malaysia were predominantly Malays (54%), followed by Chinese (28%), Indians (9%), and others (9%), the ratio being consistent with Malaysian ethnic group distribution (Aziz, 2008).

On the issue of gender differences, incident rates are generally similar between the two genders (Piccinelli *et al.*, 1997), but when gender differences are detected, males scored a higher rate, especially in the younger age groups (McGrath *et al.*, 2004). Of note, more than 60% of schizophrenia cases in Malaysia were males (Aziz, 2008). Females tend to develop the disorder later in life, with approximate age of onset 3 to 6 years later than male (Thara, 2001). Although schizophrenia is one of the leading causes of disease burden for women aged 15 to 44 years (WHO, 2004), females tend to have a better course and outcome after treatment compared to males (Thara, 2001).

Besides that, it was also shown that age of onset for schizophrenia differs across countries and migration rate (Versola-Russo, 2006). Developing countries tend to have earlier onset than developed countries, while immigrants have earlier onset than non-immigrants. This may be due to differences in environment and social factors (Rabinowitz and Fennig, 2002).

1.2.2 Aetiology

The aetiology of schizophrenia is uncertain. There is strong evidence for genetic inheritance and reasons indicating stressful events (environmental factors) may provoke the disorder (Gelder *et al.*, 2006). The aetiology is also associated with factors such as oxidative stress (Othmen *et al.*, 2008), biochemical alterations, and immune abnormalities (Morera *et al.*, 2007).

Most presume that hereditary plays the most important role, and it is suggested that genetic factors account for approximately 80% of schizophrenia (Leboyer *et al.*, 2008). Family-based and twin-based studies showed that risk of developing schizophrenia is higher amongst those with family history. Monozygotic twin have the highest risk, followed by children with two affected parents, dizygotic twin, children, siblings, and lastly parents (Nasrallah and Smeltzer, 2003). A total of 21.6% of schizophrenic patients in Malaysia had a family history of mental illness (Aziz, 2008).

This proves that polygenic contributions are most likely and that multiple candidate genes might directly or indirectly contribute to schizophrenia. Many protein products of these genes (potential biomarkers) interact with each other to form complex networks, suggesting multigenic pathway that eventually leads to the disorder. The fact that most antipsychotic medications primarily target neurotransmission pathways and suppress neurotransmission activity suggests that the fundamental cause of schizophrenia might lie within those pathways (Rounsaville, 2007).

There are numerous environmental risk factors associated with schizophrenia, including: urbanisation (Pedersen and Mortensen, 2001), migration history (McGrath *et al.*, 2004), malnutrition (Tandon *et al.*, 2008), cannabis abusers (Zammit *et al.*, 2002), obstetric complications (Dalman *et al.*, 1999), prenatal infection (Koponen *et al.*, 2004), and paternal age (Sipos *et al.*, 2004). Despite many speculations on environmental risk factors, aetiology of the disorder remains poorly understood (Schwab and Wildenauer, 2008).

1.2.3 Diagnosis

Due to lack of reliable laboratory testing, diagnosis of schizophrenia is based on clinical interview, observation of patient behaviour and mental state, and patient's self reported experiences (Bertelsen, 2002). Diagnosis of mental disorders follows the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (Rounsaville, 2007).

The DSM-IV criteria focus on six main symptoms: (i) characteristic symptoms (delusion, hallucination, disorganised speech, grossly disorganised behaviour, and negative symptoms), (ii) social or occupational dysfunction, (iii) duration, (iv) schizoaffective and mood disorder exclusion, (v) substance/ general medical condition exclusion, and lastly (vi) relationship to a pervasive developmental disorder. When two or more characteristic symptoms present significantly for one month period, or present of the other five symptoms for duration of at least six months, is diagnose as schizophrenia.

However, Keefe and Fenton (2007) commented that the criteria did not carry sufficient information. Cognitive impairment is not listed as a requirement for schizophrenia diagnosis despite its role in the function and treatment of schizophrenia. The study conducted herein utilises the Mini-International Neuropsychiatric Interview (M.I.N.I.) (Sheehan *et al.*, 1998a), a structured psychiatric interview compatible with DSM-IV but more practical in clinical practices with improved diagnosis accuracy (Pinniti *et al.*, 2003).

As compared to DSM-IV and other diagnostic interviews, M.I.N.I. is preferred as screening tool for large-scale research due to the considerable shorter interview time and lower cost (Sheehan *et al.*, 1998b). The reliability of M.I.N.I. is gaining international acceptance and M.I.N.I is being translated into many languages such as Japanese (Otsubo *et al.*, 2005) and Moroccan Arabic (Kadri *et al.*, 2005).

1.2.4 Prevention and Treatment

Primary prevention is unlikely due to the fact that the cause of schizophrenia is still unknown. Research nowadays focuses on developing ways for early detection to increase the chance of early treatment. Nearly two-thirds of people with mental disorder never or discontinue to seek help from professional, resulting in relapse of the illness (Barbato, 1998). Up until year 2001, about one-third of patients recovered with possibility of relapse and 77% of them live without relapses (WHO, 2001). Approximately 75% patients

suffer some degree of disability due to schizophrenia (Ministry of Health Malaysia, 2009).

First-generation antipsychotic drugs such as haloperidol and chlorpromazine were included in the WHO essential drug list. They serve to block dopamine receptors in the brain and reduce positive symptoms of schizophrenia. Goldberg *et al.* (1977) reported that 20% of the patients taking typical drugs experienced complete remission. However, the benefits are limited by side-effects such as weight gain, tardive dyskinesia, cardiovascular and endocrine complications (Barbato, 1998; Chee, 2009).

Second-generation antipsychotic drugs, better known as atypical drugs, work to reduce positive symptoms and side-effects more effectively (Govitrapong *et al.*, 2000). Examples of the atypical drugs are clozapine and olanzapine. The side-effects of atypical drugs include weight gain, agranulocytosis, and seizures (McEvoy *et al.*, 2006). Although there are plenty of treatments available, many patients tend to resist treatment.

1.3 Markers

A marker is a characteristic that can be objectively measured and evaluated to indicate normal or disease state in the body (Biomarkers Definitions Working Group, 2001). Markers correlates with a disease state to predict disease development and allow early treatment (Starr and Taggard,

2001), they also facilitate the search of molecular targets for drug treatments to slow disease progression (Li *et al.*, 2002).

Sunderland *et al.* (2005) listed seven conditions that an ideal diagnostic marker should possess. These include: (1) sensitive enough to detect feature of the disease, (2) specific for the disease, (3) validated in post-mortem cases, (4) reliable in most testing environments, (5) non-invasive, (6) simple to apply and, (7) inexpensive. The marker may be the cause of the disease or it can be the effect that correlates to the disease. Types of markers used for clinical research includes chemical and biological markers.

1.3.1 Chemical Markers

With regards to human diseases, chemical markers are also referred to as biochemical markers. They are chemical compounds found in human body, in which abnormal quantities may indicate the presence of a disease. Most chemical markers are measured using analytical methods, such as mass spectrometry and gas chromatography (Dodd, 1996).

Various chemical compounds such as iron, copper (Tilson, 1982), and antioxidant (Maes *et al.*, 2000) act as chemical markers for specific diseases. Abnormally low iron in blood, for instance, hinders reproduction of red blood cells and is a sign of anaemia (Killip *et al.*, 2007). Activity of antioxidants

including superoxide dismutase, catalase, and carotenoid are often measured as indicators of oxidative stress (Nishino *et al.*, 2002; Gama *et al.*, 2008).

The imbalance in oxidative stress and body antioxidant defense system is hypothesised in human diseases (Mates *et al.*, 1999). Oxidative stress is the result of excess free radicals that are produced as part of the body response triggered by exogenous oxidants, for instance, cigarette smoke, inappropriate diet, and radiation (Packer, 2006). Free radicals tend to damage cellular components such as lipid, which is essential in nerve cell membrane function and would affect signal transmissions and brain functions (Block, 1999). Besides that, excessive psychological, biological, and social stress are also known to damage brain antioxidant defense and cause mental illness (Goncalves *et al.*, 2008).

Higher level of antioxidant could boost the antibody immune system by neutralising the damaging free radicals (Dimitrov *et al.*, 2008). Individual antioxidant level varies according to lifestyle, diet, and smoking habit. Deteriorating factors such as emotional stress, alcohol and drug abuse induce free radicals production in human brain (Hiramatsu, 2006; Tsuboi *et al.*, 2006).

Antioxidant level in blood and tissue of smokers tends to be lower than non-smokers. Combustion of the tar component in cigarettes actively reduced oxygen to hydrogen peroxide, causing oxidative stress (Duthie, 1999). Smoking psychiatric patients tend to develop more psychotic symptoms and require higher dose of antipsychotics compared to non-smoking patients

(Diehl *et al.*, 2009). In addition, maternal smoking during pregnancy has been associated with psychopathology (Thapar and Rutter, 2009).

The aetiology of diseases such as schizophrenia (Sarandol *et al.*, 2007; Othmen *et al.*, 2008), Alzheimer's disease, Parkinson's disease (Sofic *et al.*, 2002), cancer (Watters *et al.*, 2008), and diabetes (Webb and Falkowski, 2009) are associated with imbalance level of oxidative stress and antioxidant activity. It is inferred that antioxidants play a significant role to improve immune functions (Surh and Packer, 2005) and prevent diseases (Temple, 2000). People with high antioxidant level are known to have respectively lower oxidative stress (Block *et al.*, 2002). Thus, measuring antioxidant level can provide evidence of oxidative stress and diseases.

1.3.2 Biological Markers

Biological markers have been established in clinical diagnosis for a considerable time. Early markers such as blood group, is used as a basic marker in modern haematology to reflect genetic differences (Morgan and Watkins, 2000). It is determined by the differentiation of ABO gene to blood group antigen A, B, and O by specific glycosyltransferase activity (Harris *et al.*, 2005). Blood group is inherited and there is variation in blood group distribution in different ethnic populations (Chan, 1962; Whiteley, 2004). Thus blood grouping can be used to trace back the ancestral root of a certain population (Chiaroni *et al.,* 2004).

Blood grouping also serves to link different personality traits with specific blood groups (Wu *et al.*, 2005). Study suggested that approximately 40% variation in personality can be described by genes (Jang *et al.*, 1996). Blood group A and AB are often characterised as passive, while blood group B and O are associated with active natures (Rogers and Glendon, 2003).

Besides that, its easily identified phenotype renders the use of blood group as marker in diseases. For example, blood group O antigen binds more efficiently to *Helicobactor pylori* compared to other blood groups, with increasing susceptibility to the infection, thus can act as diagnostic marker for peptic ulcer (Mattos *et al.*, 2002).

However, there is controversy in research findings regarding the association of blood group with diseases. Elston *et al*. (1973) studied the linkage relationships of schizophrenia and ABO blood group amongst twins but found no significant association. On the other hand, blood group A was reported to present in significantly high frequency in schizophrenia (Irvine and Miyashita, 1965).

Through the advancement in clinical research, various genes and proteins are found to serve as diagnostic marker of diseases. Genetic markers act as tag for a group of closely linked genes associated to certain biological condition (Martin and Hine, 2000). Examples of genetic markers include: single nucleotide polymorphism and microsatellite. In schizophrenia, high-throughput screening, such as microarray analysis has discovered candidate

genes, but it is difficult to determine the gene expressions that correspond to changes in protein encoding (Vawter *et al.*, 2001).

On the basis of disorder, cascades of reaction might be involved where proteins were synthesised and degraded. The complex nature of schizophrenia requires contribution of proteomic analysis with phenotype approach that investigates protein expression and backtracks to the genes involved.

Example of protein markers can be observed in diabetic nephropathy, in which progression of microalbuminuria to macroalbuminuria signifies chronic kidney failure (Jain *et al.*, 2005). Similarly, progression of symptoms in schizophrenia could be assessed through increased serum neuro-steroid, dehydroepiandrosterone, which represent a marker for chronic distress and anxiety (Ritsner *et al.*, 2006). Studies also reported the role of protein marker in monitoring the effects of drug treatment. In schizophrenia, protein expression of 5-hydroxytryptamine receptor 2A, 5-hydroxytryptamine receptor 2C, and D3 dopamine receptor are used to monitor antipsychotic drug treatment (Reynolds *et al.*, 2005; Padin *et al.*, 2006).

1.4 Objectives of Study

With the alarming high prevalence of schizophrenia, there is a need to understand the aetiology of the disorder. The ambiguity of the situation can be solved by the exploring the relationship between schizophrenia with chemical

and biological markers, in hope to understand the aetiology of schizophrenia, facilitating early detection and appropriate treatment.

Thus, the objectives of this study are: (1) to study the association of carotenoid antioxidant and serum proteins that served as chemical and biological markers respectively with schizophrenia; and (2) to explore the relationship of schizophrenia with age, gender, schizophrenic subtype, and antipsychotic medications.

Chapter 2 addressed the potential role of carotenoid antioxidant as a chemical marker for schizophrenia. This is followed by the proteomic analysis of candidate proteins for schizophrenia in Chapter 3. Lastly, Chapter 4 concluded the findings and discussed the recommendations for future study.

CHAPTER 2

ROLE OF CAROTENOID ANTIOXIDANT IN SCHIZOPHRENIA

2.1 Introduction

2.1.1 Antioxidant and Schizophrenia

Human brain has a very active aerobic metabolism and a relatively poor antioxidant defense (Strassnig *et al.*, 2005). The aerobic metabolism is generated by high neuronal activity, where a significant volume of oxygen is used to maintain neuronal membrane functions and subsequently, excessive reactive oxygen species (ROS) are produced (Kunz *et al.*, 2008).

In schizophrenia, excessive ROS may cause lipid peroxidation of the nerve cells (Sarandol *et al*., 2007), disrupting nerve cell membrane function and increasing oxidative stress in the brain (Block, 1999). It has been suggested that the pathogenesis of the disorder is associated with oxidative stress, which produces deleterious effects on signal transmissions as observed in the extra pyramidal symptoms of schizophrenia (Goncalves *et al.*, 2008).

Patients with schizophrenia have significant lower levels of bilirubin, uric acid, and major plasma antioxidants (Reddy *et al.*, 2003). They are shown to have increased oxidative stress compared to the general population (Prabakaran *et al.*, 2004). In addition, antipsychotics such as haloperidol have

oxidative capabilities that would further deplete antioxidant activities and resulting in neuronal degeneration (Sachdev *et al.*, 1999). There are controversies on the neurotoxicity of antipsychotics, but most study inclined towards the alteration of antioxidant activity and lipid peroxidation caused by antipsychotics (Othmen *et al.*, 2008; Schmidt *et al.*, 2008).

2.1.2 Role of Carotenoid as Antioxidant

Carotenoid can serve as a reliable indicator of overall antioxidant level in the human body (Zhao *et al.*, 2003; Svilaas *et al.*, 2004). They have unique natural colorants that are distributed in human tissue and can be detected as specific wavelengths when they are stimulated (Khachik *et al.*, 1999). Carotenoids not only inhibit free radicals, they are also efficient quenchers of the cancer-causing singlet oxygen. There are more than 600 carotenoids identified to date, but only 14 are found in human plasma and tissue. Among them are α-carotene, β-carotene, lutein, and zeaxanthin (Nishino *et al.*, 2002).

High plasma concentrations of carotene are associated with lower mortality in the elderly and a lower risk of cardiovascular disease and cancer (Hak *et al.*, 2003; Buijsse *et al.*, 2005). Besides carotene, phytoene was also suggested to have anti-carcinogenic activity (Nishino *et al.*, 2002). Important sources of carotenoid include: red, orange, yellow, and green vegetable and fruits (Stahl and Sies, 1999).

2.1.3 Measurement of Antioxidant Level

There are various ways of detecting antioxidants in the human body. Assays such as oxygen radical absorbance capacity and ferric reducing ability of plasma (FRAP) adopt simple chemical mechanism to quantify total antioxidant capacity. For instance, FRAP assay is based on the reduction of ferric (III) to ferrous (II) in the presence of antioxidants (Benzie and Strain, 1996).

In relation to diseases, most antioxidant studies measure antioxidant enzyme activity in red blood cell and plasma using techniques such as enzyme linked immunosorbent assay (Boots *et al.*, 2009) and high-performance liquid chromatography (Sarandol *et al.*, 2007). However, such methods are time-consuming, expensive, and impractical for a large sample size.

In the present study, carotenoid level was determined by a simple, rapid, and non-invasive technique using Raman spectroscopy (Smidt, 2005). This technique has been applied to measure the macular carotenoid level in patients with eye disease (Zhao *et al.*, 2003). It utilises a laser spectroscopic technique to detect the characteristic vibration energy level of carotenoids in human skin by the principle of reflect and scattered light.

Raman spectroscopy involves a blue, low-energy laser light source of approximately 490 nm directed to the human skin surface. When hit with the Raman resonance light source, the carotenoid species can reflect a set of

colour spectrum, emitting signals from 510 nm to 530 nm, thus providing a unique fingerprint (Smidt, 2005). The amount of reflective ray is quantified into skin carotenoid level and the intensity of the Raman peak is directly proportional to the concentration of carotenoids (Hata *et al.*, 2000).

A recent study demonstrated significant correlation between serum carotenoid and skin carotenoid level (Zidichouski *et al.*, 2004), attributing Raman spectroscopy to be more specific, cost effective, and may be used to develop a non-invasive, *in vivo* optical biopsy method to study oxidative biology (Bentz *et al.*, 2010).

2.2 Materials and Methods

2.2.1 Subject Assessment and Sampling

This study was approved by Medical Research and Ethics Committee, Ministry of Health Malaysia. Written consents were obtained from all subjects prior to participation in the study (Appendix C). A total of 351 patients with schizophrenia (male = 268, female = 83) and 247 controls (male = 189, female = 58) were recruited in this study. Age of patients (mean = 37.95 ± 7.53) and controls (mean = 30.57 ± 8.87) ranged from 18 to 50 years. For the analysis of age, subjects were grouped into three age groups: 18 to 30 years, 31 to 40 years, and 41 to 50 years.

Controls were recruited during blood donation campaign from church, Buddhist temple, Universiti Tunku Abdul Rahman, Jabatan Kimia Malaysia, and National Science Centre Malaysia. Controls were selected based on the following exclusion criteria: (i) no personal history of mental disorders, (ii) no family history of mental disorders, (iii) no history of major illness such as cancer and AIDS, (iv) not on drug therapy or substance abuse, and lastly (iv) non-smoker.

All patients were in-patients from Hospital Bahagia Ulu Kinta, Malaysia, where smoking is prohibited. Patients were assessed using Mini International Neuropsychiatric Interview (M.I.N.I.), English Version 5.0.0 (Sheehan *et al.*, 1998a) translated to patients' first language by treating psychiatrists. Patients were grouped into their respective subtype: paranoid (n = 156), undifferentiated (n = 108), disorganised (n = 78), residual (n = 7), and catatonic (n = 2).

In the analysis of schizophrenia subtype, residual and catatonic subjects were excluded from the analysis due to low sample size. For medication consumption, patients were divided into three categories depending on their medication: typical antipsychotics such as haloperidol and chlorpromazine; atypical antipsychotics such as olanzapine and clozapine; and other psychotropic drugs, such as diazepam and fluvoxamine.

Further analysis of depot injection compared patients that received intramuscular injection of antipsychotics with patients that did not receive any injections, regardless type of antipsychotics. In the analysis of duration of illness, patients were grouped into four duration periods: ill for less than 5 years, 5 to 10 years, between 10 to 20 years, and above 20 years.

2.2.2 Measurement of Carotenoid Score

Carotenoid levels of subjects were measured using a Pharmanex® BioPhotonic Scanner S2 (Pharmanex, U.S.A.) which utilises the concept of Raman spectroscopy. The scanner was first calibrated using *p*Cal® calibration standards (Pharmanex, U.S.A.) created from a silicone-based polymer to establish the carotenoid antioxidant parameter. Subjects were required to clean their palms before measurement and hold them to the scanner for two minutes. Individual skin carotenoid level was measured and complied for analysis.

2.2.3 Validation by Ferric Reducing Ability of Plasma (FRAP) Assay

As a validation to the skin carotenoid level obtained using Raman spectroscopy, FRAP assay was performed on 87 patients and 70 controls that were randomly chosen from the main study group. FRAP assay was chosen because of its relatively simple and inexpensive techniques using easily

assessable chemicals. This assay is based on the reduction of ferric (III) to ferrous (II) through antioxidants (Benzie and Strain, 1996).

Using a modified method adapted from Benzie and Strain (1996), total antioxidant level of the serum samples were determined using FRAP assay. Fresh FRAP solution (Appendix A) was prepared and kept at 37^{o}C prior to assay. Then, 10 µl of serum (diluted 100x with PBS buffer) were mixed with 300 µl of the FRAP solution in each well of the 96-well plate. The plate was kept in dark. After 10 minutes, the absorbance of the mixture was measured at 593 nm by using Infinite® 200 PRO Microplate Reader (Tecan, Germany). Trolox served as the positive control and FRAP solution as blank.

2.2.4 Statistical Analysis

Data analyses were perform using Statistical Package for Social Sciences version 16.0 for Windows (SPSS Inc, Chicago, USA). Levene's homogeneity test was used to test for equality of variances. Descriptive statistics were calculated for all variables and mean score was reported separately for each variable.

Carotenoid level differences between patients and controls were evaluated using an independent sample T-test. Pearson correlation and analysis of variance (ANOVA) were used to investigate the correlation between age and carotenoid level. Subjects were grouped into three age ranges:

18 to 30 years, 31 to 40 years, and 41 to 50 years. Other variables tested using ANOVA included gender, age, schizophrenia subtypes, patient medication, depot injection and duration of illness.

The correlation between antioxidant level evaluated by FRAP assay with carotenoid level measured by Raman spectroscopy was evaluated using Pearson's correlation test where *r*-value nearer to 1 was considered as a significant correlation.

2.3 Results

2.3.1 Skin Carotenoid Level

Each population in the study was homogenous ($P = 0.611$) for the equality of variances. Among patients with schizophrenia, 83.5% obtained carotenoid level less than 30,000, while 48% of healthy controls obtained carotenoid level more than 30,000. Significant ($P < 0.001$) lower carotenoid level was observed in patients as compared to controls, with a 12.5% difference. Females showed higher mean carotenoid levels than males for both patient ($P = 0.005$) and control ($P = 0.037$) groups (Table 2.1). Pearson's correlation showed no relationship between carotenoid level and age ($r = 0.168$, $P < 0.001$).

In subtype analysis (Table 2.2), the carotenoid level for paranoid, undifferentiated, and disorganised subtypes were not significantly different from each other. On the other hand, mean carotenoid level of patients with atypical antipsychotics treatment were found to be higher compared to those typical antipsychotics treatment and other psychotropic medications, but ANOVA did not show significant difference between mean carotenoid levels and types of medication ($P = 0.334$). Similarly, depot injection and duration of illness for patients were not significantly related with carotenoid level ($P = 0.418$).

Table 2.1: Mean carotenoid level for subject demographic characteristics

Characteristics	Mean Carotenoid Level			
	Number of subjects	Control	Number of subjects	Patient
Gender				
Male	189	24,900 ± 7,760	268	21,600 ± 7,340
Female	58	27,500 ± 9,220	83	24,300 ± 7,900
Age				
18 - 30 years	145	25,740 ± 6,800	61	23,100 ± 7,660
31 - 40 years	55	28,300 ± 7,920	144	22,200 ± 7,730
41 - 50 years	47	29,410 ± 7,970	146	22,000 ± 7,350

Table 2.2: Mean carotenoid level for clinical characteristics of patients

Variables	Number of subjects	Mean Carotenoid Level
Control subjects	247	25,500 ± 8,180
Schizophrenia subjects	351	22,300 ± 7,550
Subtypes		
Paranoid	156	21,400 ± 7,980
Undifferentiated	108	23,200 ± 7,140
Disorganised	78	22,300 ± 7,070
Medication		
Typical antipsychotics	130	21,950 ± 8,110
Atypical antipsychotics	125	23,230 ± 7,400
Other psychotropics	96	21,500 ± 6,700
Injection		
Intramuscular injection	218	22,220 ± 7,400
No injection	133	22,360 ± 7,810
Duration of illness		
Less than 5 years	79	22,180 ± 7,510
5 to 10 years	85	21,580 ± 7,240
Between 10 to 20 years	122	23,150 ± 7,770
More than 20 years	65	21,630 ± 7,580

2.3.2 Antioxidant Level Validation by FRAP Assay

Degree of colour change from straw colour to blue depends on the degree of reduction of ferric (III) to ferrous (II). The standard curve of FRAP assay with Trolox as the standard is shown in Figure 2.1. The correlation between antioxidant level evaluated by FRAP assay with carotenoid level measured by Raman spectroscopy was determined. The relationship was established as equation of y = 0.024x (R^2 = 0.688) for controls (Figure 2.2), and y = 0.027x (R^2 = 0.562) for patients (Figure 2.3). Analysis of Pearson's correlation showed strong significant positive correlation between FRAP antioxidant level and skin carotenoid level in controls (r = 0.830, P < 0.001) and patients (r = 0.811, P < 0.001).

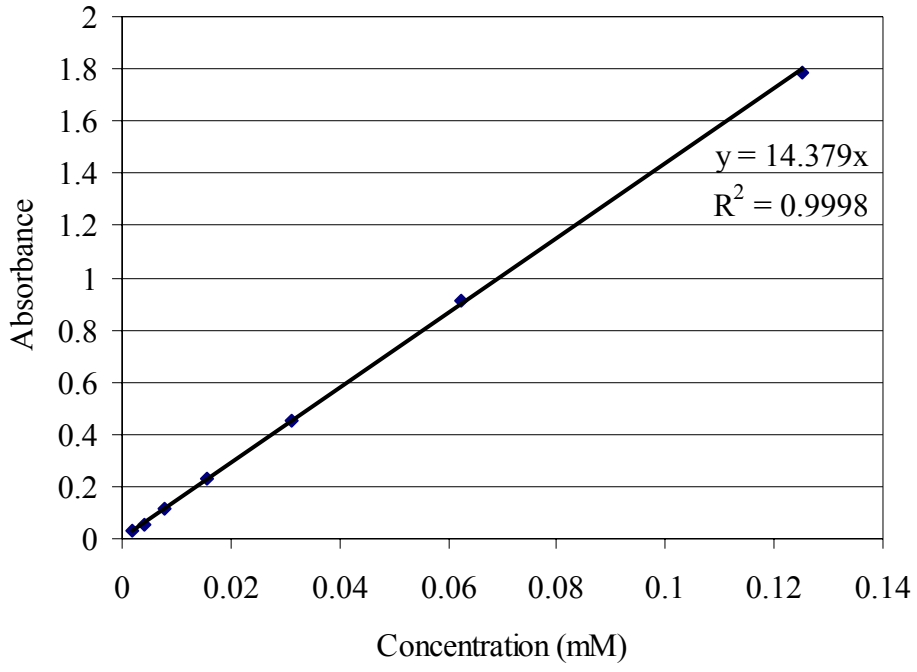

Figure 2.1: Standard curve of Trolox absorbance measured at 593nm

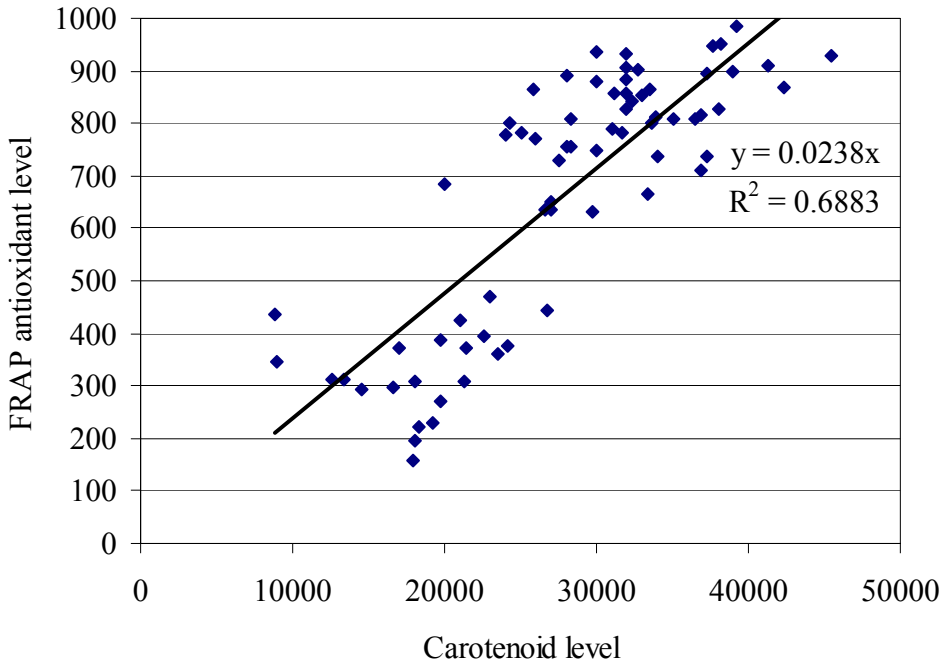

Figure 2.2: Relationship between FRAP antioxidant level with control skin carotenoid level

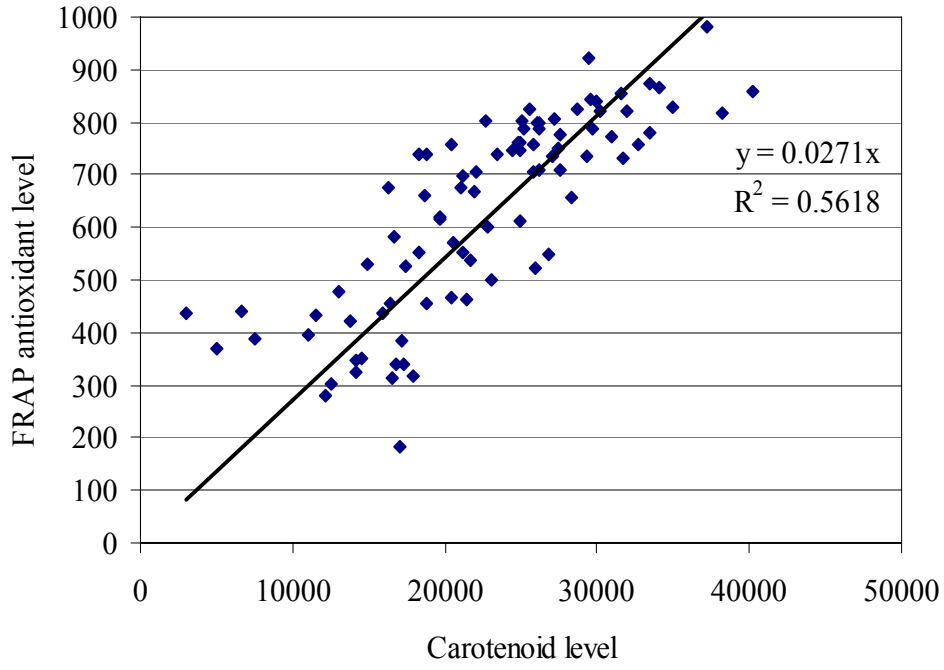

Figure 2.3: Relationship between FRAP antioxidant level with patient skin carotenoid level

2.4 Discussion

The total antioxidant level evaluated by FRAP assay correlates with carotenoid level measured by Raman spectroscopy. Results showed the trend of increasing serum antioxidant level with increasing skin carotenoid level. This positive correlation trend emphasised that carotenoid correspond to the total antioxidant capacity human serum (Svilaas *et al.*, 2004).

The main findings of the present study are: (i) schizophrenia was characterised by significantly lower antioxidant level compared to the controls; and (ii) treatment with antipsychotic drugs reduced the antioxidant levels. Recent studies on plasma antioxidant in patients with schizophrenia showed similar trend of reduced antioxidant level that is associated to high oxidative stress (Reddy *et al.*, 2003; Kunz *et al.*, 2008). However, it is important to investigate whether this trend is applied to certain schizophrenic subtypes. Gama *et al.* (2008) reported no difference in antioxidant level in different subtypes. Similarly, the present study found that carotenoid level did not significantly correlate with subtypes in patients, and thus suggests that subtype differentiation has no or equal effect on oxidative damage.

In comparison to female patients, carotenoid level of male patients was significantly lower. Nonetheless, a similar trend was observed in controls where female controls scored higher carotenoid level compared to male controls. These results could only explain that oxidative stress differs in

gender due to hormone differences in males and females (Zhang *et al.*, 2006), but does not demonstrate the affecting factor of gender in schizophrenia.

Aging and age-related diseases are often associated with high levels of free radicals and oxidative stress. In the process of brain aging, free radicals attack the lipid composition of brain cell membrane, resulting in abnormal signal transduction that is portrayed in symptoms of mental illness (Mazza *et al.*, 2007). An older person would have lower antioxidant level and more susceptible to diseases compared to a younger person (Buijsse *et al.*, 2005). However, the present study found that the oldest (aged 41 to 50 years) control group obtained higher carotenoid level than the younger patients and the youngest controls (aged 18 to 30 years). It appears that the dietary patterns of older adults have been found to be healthier than those of the younger generation (Commonwealth Scientific and Industrial Research Organisation, 1996).

Antipsychotic drugs serve to relieve schizophrenic symptoms but they would also produce distressing side effects and some patients may develop tardive dyskinesia (Liska, 2004; Parrott *et al.*, 2004). There is controversy in research findings regarding the effect of antipsychotics on lipid peroxidation and oxidative stress. Schmidt *et al.* (2008) reported treatments with antipsychotic drugs would induce lipid peroxidation and oxidative stress, thus lowering antioxidant level. Similarly, the present findings showed that regardless of the type of antipsychotics, patients under drug treatment have a significantly lower antioxidant level compared to controls.

This observation contradicted the findings of Singh *et al.* (2008), where olanzapine, an atypical antipsychotic drug can significantly reduce oxidative stress compared to haloperidol, a typical antipsychotic drug. In this study, the patients were prescribed a combination of drugs. Thus, the role of each antipsychotic drug in antioxidant reduction could not be concluded.

Intramuscular depot injection allows direct entry of antipsychotics into the bloodstream which promotes immediate effect of the drug but this might also cause more serious side effects (Liska, 2004). The present results indicated that carotenoid level of patients with and without injection were not statistically different, suggesting depot injection does not further increase oxidative stress in patients. Analysis in duration of illness did not differ significantly among patients, thus was excluded as contributing factor of reduced antioxidant level. Gender, age, and duration of illness were eliminated as factors reducing carotenoid level.

The strength of this study is studying large number of patients with schizophrenia and it is among one of the first research into measuring carotenoid level of those patients using a non-invasive technique. The limitations of this study are: (i) lifestyle and diet were not studied as factors reducing carotenoid level; (ii) patients were not assessed for co-morbid psychiatric disorders; and (iii) unable to recruit drug naïve patients. It would be beneficial to include drug naïve patients in order to understand the effects of antipsychotic treatments on carotenoid antioxidant levels.

2.5 Conclusion

Schizophrenia patients have significantly lower carotenoid level compared to healthy controls. This indicates higher level of oxidative stress in schizophrenia. Females obtained higher mean carotenoid level compared to males in both control and patient group. Among patients, there was no significant relationship between carotenoid level with age, schizophrenic subtypes, antipsychotic drug treatment, and duration of illness. Antipsychotic treatment and schizophrenia illness were suggested to be the possible reasons to the reduction of antioxidant level in schizophrenic patients.

CHAPTER 3

IDENTIFICATION OF BIOMARKERS IN SCHIZOPHRENIA BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

3.1 Two-dimensional Gel Electrophoresis and Biomarkers

Proteomics refers to the systematic analysis of all expressed proteins. Two-dimensional gel electrophoresis (2-DE) enables the characterisation of specific proteins expressed in a given tissue. Alterations in the protein concentration are detectable and reflect either altered expression of a gene, changes in protein turnover or post-translational modifications (Taurines *et al*., 2010). Another advantage of 2-DE is its capability of simultaneously analysing different isoforms of the same protein, differing either in isoelectric point or molecular weight (Schwarz and Bahn, 2008).

Two-DE is mostly used in clinical study to compare disease-affected tissue with unaffected tissue to detect proteins that are influenced by the disease process. For instance, 2-DE was reported to monitor cancer progression through comparative protein profiling of controls and patients (Greco *et al*., 2009).

In schizophrenia, the etiology is still far from being understood, hampered by the heterogeneity nature of the disorder (Schwarz and Bahn, 2008). Comparative proteome analysis using 2-DE can potentially and directly identify proteins that are involved in the pathogenesis of schizophrenia.

3.2 Acute Phase Protein (APP)

Acute phase proteins (APPs) are a class of proteins produced predominantly by the liver and serve as markers of inflammation, which plasma level alters in response to the inflammatory system (Hayat, 2006; Gaur *et al.*, 2008). In the event of stress, for instance, bacterial infection, local inflammation and injury, APPs are released into the plasma through stimulation by cytokines such as tumour necrosis factor and interlukins (Ruminy *et al.*, 2001).

There are various functions of APPs, most serving as inflammatory mediators and inhibitors, transport proteins, and promoters for bacterial phagocytosis (Whicher *et al.*, 1991; Wan *et al.*, 2007). The change in their plasma concentration varies considerably, where APPs whose concentration increased following inflammation are classified as positive APPs, and those with decreased concentration are negative APPs (Ruminy *et al.*, 2001).

In response to inflammation and infection, there is a profound alteration in lipid metabolism. APPs such as apolipoproteins will be released as part of the acute phase response. In the central nervous system (CNS), this process might impair the blood-brain barrier and cause damage to the brain (Yang *et al.*, 2006). In addition, in response to brain injury, microglia cells will be activated to remove the infectious agents, thus causing irreversible damage to the brain (Doorduin *et al.*, 2009).

Abnormalities in immune function and the importance of APPs as biomarker have been reported in diseases such as cancer (Chen *et al.*, 2009), depression, Alzheimer's disease (Harr *et al.*, 1996), and schizophrenia (Maes *et al.*, 1997; Morera *et al.*, 2007; Wan *et al.*, 2007). In the event of abnormal immune reactions, the production of APPs such as haptoglobin (Hp), apolipoproteins (Apo), and macroglobulin will activate the complement system (Greco *et al.*, 2009).

There are various possible causes for acute phase response in schizophrenia. For instance, psychological stress, accompanied by hormonal changes, may result in hepatic production of APPs (Corcoran *et al.*, 2003). Besides that, acute phase responses may be associated to the autoimmune aspect in schizophrenia (Eaton *et al.*, 2006). Previous research on APPs in schizophrenia mainly focused on haptoglobin and apolipoproteins such as Apo A-I and Apo A-IV (Table 3.1), although there is a lack of consistency in the degree of APP alteration in the disorder (Rothermundt *et al.*, 2001).

Table 3.1: Biomarkers for schizophrenia identified by proteomic analysis

Biomarker	Type of Sample	Observations	References
Apo A-I	Brain tissue	Decreased	Huang *et al.*, 2008
	Cerebral spinal fluid (CSF)	Decreased	Huang *et al.*, 2008
	Plasma	Decreased	Yang *et al*., 2006; La *et al.*, 2007; Huang *et al.*, 2008
Apo A-IV	CSF	Decreased	Jiang *et al*., 2003
	Plasma	Increased	Yang *et al*., 2006
Apo D	Brain tissue	Increased	Thomas *et al*., 2001
	Plasma	Increased	Mahadik *et al*., 2002
	Plasma	Decreased	Thomas *et al*., 2001
Apo E	Brain tissue	Increased	Dean *et al.*, 2003
	CSF	No association	Jiang *et al*., 2003
Haptoglobin	Plasma	Increased	Wong *et al.*, 1996; Maes *et al.*, 1997; Yang *et al*., 2006; Wan *et al.*, 2007
α1-antitrypsin	Plasma	Increased	Wong *et al*., 1996; Yang *et al*., 2006.
Transthyretin	CSF	No relationship	Huang *et al*., 2007
	Plasma	Decreased	Yang *et al*., 2006

3.2.1 Haptoglobin (Hp)

Hp is a free haemoglobin-binding protein where its low level is associated with haemolysis, allergy, and hepatocellular disorders (Sadrzadeh and Bozorgmehr, 2004). It is mainly synthesised in the liver, although studies also found Hp in brain tissue (Sanchez *et al.*, 2001; Jung *et al.*, 2008). The Hp gene consists of three common alleles: *Hp*1S, *Hp*1F, and *Hp*2, expressed as three phenotypes: Hp 1-1, Hp 2-1, and Hp 2-2 (Saha *et al.*, 1985). Individuals with Hp 1-1 have the highest plasma Hp concentration, followed by Hp 2-1 and Hp 2-2, with lowest plasma Hp concentration (Sadrzadeh and Bozorgmehr, 2004).

Hp has been shown to play the role as antioxidant (Sadrzadeh and Bozorgmehr, 2004). The iron in free haemoglobin enhances formation of ROS that lead to oxidative injury in the CNS as seen in schizophrenia (Kunz *et al.*, 2008; Othmen *et al.*, 2008). Hp forms dimer that binds with haemoglobin, which is then taken up by macrophages, removing it from circulation, thus preventing oxidative stress (Henderson *et al.*, 2009).

Besides that, Hp is able to inhibit lecithin-cholesterol acyltransferase (LCAT), which is involved in reverse cholesterol transport. LCAT removes cholesterol from peripheral tissues by transferring acyl chain from high density lipoprotein (HDL) to the cholesterol. This process is stimulated by the main HDL constituent, Apo A-I, but is inhibited by the binding of Hp to Apo A-I (Henderson *et al.*, 2009).

Hp has been associated with stroke (Brea *et al.*, 2009) and up-regulated levels of Hp is found in ovarian cancer (Ahmed *et al.*, 2004), pancreatic cancer (Firpo *et al.*, 2009), and thyroid carcinoma (Fan *et al.*, 2009). In neurodegenerative disease, decreased levels of Hp are reported in CSF of Alzheimer's disease patients (Jung *et al.*, 2008).

In contrast, Hp is characterised as a positive APP in schizophrenia, where its plasma expression increases during acute phase response (Wan *et al.*, 2007). In the early phase of the process, pro-inflammatory cytokine interlukin-6 secreted from macrophages and fibroblasts will act to stimulate the release of Hp into the bloodstream (Ruminy, 2001; Sanchez *et al.*, 2001).

3.2.2 Transthyretin (TTR)

TTR is a thyroid-binding protein produced by the liver and secreted into the plasma. In the brain, it is synthesised by choroid plexus and can be found in the CSF (Merched *et al.*, 1998; Lee *et al.*, 2009). TTR plays an important role in the transport of thyroxine and retinol across the blood-brain barrier, assuring availability of the hormones for brain development (Merched *et al.*, 1998; Sullivan *et al.*, 1999).

TTR is implicated in neurodegenerative diseases such as Alzheimer's disease (Merched *et al.*, 1998), depression (Sullivan *et al.*, 1999), and schizophrenia (Yang *et al.*, 2006). Studies found that reduced TTR in the brain

implies disrupted thyroid hormone feedback mechanisms in the hypothalamus. In light of this, and the fact that symptoms of hypothyroidism are often related to depression, low levels of TTR may be a risk factor for depression (Sullivan *et al.*, 1999). In Alzheimer's disease, TTR is known to inhibit beta amyloid protein formation, thus low concentration of TTR in the CSF is associated with the disease (Serot *et al.*, 1997).

Plasma TTR is known as a negative APP in schizophrenia, where it is down-regulated in patients during acute inflammation (Yang *et al.*, 2006), although plasma TTR level may not reflect TTR level in the CSF (Ruano *et al.*, 2007). TTR is linked to schizophrenia due to the fact that it lies in chromosome 18q11.2, a susceptible loci for the disease (Yang *et al.*, 2006). Besides that, TTR is found present in HDL and interacts with Apo A-I, which has been associated with risk for schizophrenia (Sousa *et al.*, 2000).

3.2.3 Apolipoprotein (Apo)

Apolipoprotein (Apo), also known as apoprotein, is the polypeptide outer shell of lipoproteins that interacts with lipids (Hornick, 2002). It is associated to lipoproteins such as: very low density lipoprotein (VLDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL), HDL, and chylomicron (Irshad and Dubey, 2005). During lipoprotein metabolism, Apo may dissociate from one lipoprotein and bind to another, thus changing their lipoprotein association and structural domains (Hornick, 2002).

The difference in structural domains renders various functions of Apo, including lipid transport and metabolism, as cofactor and activator for lipid enzymes such as LCAT and lipoprotein lipase, and inhibits thrombolysis. Based on the different characteristics, functions, and synthesis location, Apo is divided into different classes and subclasses, mainly Apo A, B, C, D, E, H, and J (Hornick, 2002; Irshad and Dubey, 2005).

The various forms of Apo are differently implicated in various diseases, although most of the Apos are related to cardiovascular diseases (Irshad and Dubey, 2005). For instance, Apo B is associated with ischemic heart disease, which is caused by formation of plaques due to elevated levels of LDL. Due to the fact that Apo B is the main protein constituent of LDL (Maury *et al.*, 1988), high levels of Apo B are reported as risk marker for ischemic heart disease (Benn *et al.*, 2007). Besides that, Apo C is also often found in cardiovascular diseases. Among the subclasses of Apo C (C-I, C-II, C-III, and C-IV), Apo C-III is the most-studied. It is associated to VLDL, which over-expression induces hypertriglyceridemia (Hornick, 2002). It has also been reported in diabetes mellitus (Singh *et al*., 2007) and coronary heart disease (Singh *et al*., 2008).

Apo is greatly implicated in the CNS due to its role in lipid metabolism and transport, which is essential in brain development and repair (Mahadik *et al.*, 2002, Jiang *et al.*, 2003). Apo mainly serves to supply lipids for neurons, and membrane phospholipid signal transduction (Thomas *et al.*, 2001). Besides that, Apo is also related to acute phase responses such as oxidation

and inflammation in the CNS (Irizarry, 2004), in which Apo A, D, E, H, and J are known as APP in various neurodegenerative diseases (Jiang *et al.*, 2003).

3.2.3.1 Apolipoprotein A (Apo A)

Apo A family consists of four subclasses: A-I, A-II, A-IV, and A-V, which are differentiated based on different chromosomal and synthesis location. They play significant role in different diseases. As such, Apo A-I and A-IV are emphasised in atherosclerosis and neurodegenerative diseases, and Apo A-II is mostly implicated in hepatic and colorectal cancer (Irshad and Dubey, 2005), while Apo A-V is associated with triglyceride-related cardiovascular and renal disease (Hirano *et al.*, 2007). Apo A-I and A-IV are most reported in schizophrenia (Yang *et al*., 2006).

Apo A-I is a lipid binding protein that serves as the major protein component of plasma HDL (Karathanasis, 1985). It is synthesised predominantly in the liver and intestine, although it has also been found in vitreous fluid (Simo *et al.*, 2008) and is characterised as a major lipoprotein in human CSF (Koch *et al.*, 2001; Huang *et al.*, 2008). There are two major isoforms of Apo A-I: Apo A-I-1 and A-I-2, that can be detected through isoelectric focusing. Apo A-I-1 is reported to be slightly more basic than Apo A-I-2. These isoforms may arise due to different carbohydrate moieties or differences in the primary structures (Menzel *et al.*, 1982; Menzel *et al.*, 1984).

Besides serving as the structural component of HDL, Apo A-I also activates LCAT (Ordovas, 2009) and is involved in the transport of cholesterol from peripheral tissues to the liver, a process known as reverse cholesterol transport (Rottman *et al.*, 1991). This mechanism involves binding of the alpha-helical segments of Apo A-I with plasma membrane, thus promoting lipid efflux to Apo A-I, which will then be transported to the liver for excretion (Gaus *et al.*, 2004).

In addition, Apo A-I is a potent scavenger of ROS, and may play a role in protecting the brain from oxidative stress (Robbesyn *et al.*, 2005). Accounting for approximately 70% of total HDL protein content (Shao *et al.*, 2005), Apo A-I is shown to be involved in an enzymatic reaction of the HDL during the mechanism to protect LDL against oxidative modifications (Mackness and Durrington, 1995).

A widely studied APP for schizophrenia, Apo A-I is encoded by the *APOAI* gene found on chromosome 11q23 (Arinami *et al.*, 1990), overlapping with schizophrenia susceptibility region 11q22.3-q24.1 (Lewis *et al.*, 2003). Thus, Apo A-I is often linked with the pathology of schizophrenia (La *et al.*, 2007; Huang *et al.*, 2008). Besides that, elevated levels of Apo A-I have been observed in atherosclerosis (Robbesyn *et al.*, 2005) and the retina of diabetes patients (Simo *et al.*, 2008). On the other hand, reduced expression of Apo A-I is reported to play a role in diseases such as ovarian cancer (Su *et al.*, 2010) and liver fibrosis of patients with hepatitis C (Ho *et al.*, 2010).

Unlike Apo A-I, Apo A-IV is produced primarily in the intestine (Ordovas, 2008). Although some are found associated with HDL, more that 95% of Apo A-IV is not associated with any major lipoproteins (Jiang *et al.*, 2003). However, Apo A-IV serves similar functions as do Apo A-I in activating LCAT and involve in reverse cholesterol transport (Ordovas, 2008).

Another APP associated with schizophrenia, human Apo A-IV has a common evolutionary origin with Apo A-I (Elshourbagy *et al.*, 1985). The gene of Apo A-IV is located 12 kilobases 3' to Apo A-I on chromosome 11 and that they are transcribed in the same direction (Karathanasis, 1985). The fact that Apo A-I and A-IV are closely linked together, and they both lie in the schizophrenia susceptibility gene region, suggests a possible association of Apo A-I and IV with schizophrenia (Yang *et al*., 2006).

Studies on the influence of Apo A-IV in schizophrenia yield different results, possibly due to different sample source. For instance, Yang and co-workers (2006) documented up-regulation of plasma Apo A-IV in schizophrenic patients to provide effective control of inflammatory damage to the CNS. On the other hand, decreased Apo A-IV level is found in the CSF of patients due to possible destruction of the degenerative and regenerative process in the CNS (Jiang *et al*., 2003).

3.2.3.2 Apolipoprotein D (Apo D)

Apo D is located in chromosome 3 and is expressed in the blood, intestine, liver and brain. It is classified as Apo due to its association with HDL and Apo A-I, but it shares little similarity with other members of Apos. Rather, it is a member of the lipocalin family involved in the transport of small hydrophobic ligands (Thomas *et al.*, 2001; Irshad and Dubey, 2005).

In the brain, Apo D plays a role in lipid transport, membrane phospholipid signal transduction, as well as lipid degeneration and regeneration (Thomas *et al.*, 2001). Besides that, it also acts as a positive APP in the CNS, where the up-regulation of the protein in the CSF reflects destruction in the blood-brain barrier (Reindl *et al.*, 2001). Apo D has been reported various neurodegenerative diseases such as Alzheimer's disease (Irshad and Dubey, 2005), multiple sclerosis (Reindl *et al.*, 2001), bipolar disorder, and schizophrenia (Thomas *et al.*, 2001; Mahadik *et al.*, 2002).

There are contradicting observations on the expression of Apo D in schizophrenia. Decreased plasma level of Apo D is found in patients, with relation to the ability of Apo D to bind with arachidonic acid and the fact that decreased arachidonic acid is reported in schizophrenia (Thomas *et al.*, 2001). In contrast, Mahadik *et al.* (2002) reported up-regulation of plasma Apo D in patients, which explains the need of lipid metabolism in the repair mechanism following oxidative injury.

3.2.3.3 Apolipoprotein E (Apo E)

Apo E is a secreted protein that is mainly synthesised by the liver, but has also been found in the brain, kidneys, and spleen (Li *et al.*, 2008). In the CNS, Apo E is known to be expressed by astrocytes and oligodendrocytes (Lee *et al.*, 2001). It is found on chromosome 19, and has three major isoforms: Apo E2, E3, and E4 (Ordovas, 2009). These isoforms influence plasma Apo E and cholesterol level. As such, the E4 isoform is often associated with lower Apo E (Irizarry, 2004) and higher cholesterol in the plasma (Ordovas, 2009).

Within the nervous system, Apo E is essential for lipoprotein metabolism, transport of cholesterol in neuronal membranes and immune response to injury such as T-cell proliferation, macrophage regulation, and oxidation (Lee *et al.*, 2001; Li *et al.*, 2008). During inflammation, Apo E acts as negative APP, where its level reduces greatly so as to conserve lipids for tissue repair (Li *et al.*, 2008). Apo E is strongly linked to Alzheimer's disease (Irizarry, 2004), although it has also been reported in schizophrenia (Liu *et al.*, 2003), renal disease, cancer, and atherosclerosis (Li *et al.*, 2008).

Different Apo E isoforms serve distinct role in Alzheimer's disease. Increased activity of Apo E4 is known as a risk factor for the disease, which is characterised by aggregation of the peptide beta-amyloid plaques (Irizarry, 2004; Filippini *et al.*, 2010). In contrast, Apo E2 isoform is shown to exhibit protections and delay the age of onset against the risk of Alzheimer's disease (Horsburgh *et al.*, 2000).

3.2.3.4 Apolipoprotein H (Apo H)

Apo H, also referred to as beta-2 glycoprotein 1, is a single chain plasma glycoprotein that is expressed in the liver (Irshad and Dubey, 2005). It can be found circulating as a free protein or associated to lipoproteins (Wang *et al.*, 2002). The Apo H gene has four common alleles and two autosomal codominant alleles: normal and deficiency (Kamboh *et al.*, 1988), attributing to ethnic-specific protein polymorphism (Sanghera *et al.*, 1997) and variation in individual Apo H level (Sepehrnia *et al.*, 1989).

The physiological role of Apo H remains speculative (Simo *et al.*, 2008), but it has been shown that Apo H has the preference to bind and neutralise negatively charged macromolecules in the bloodstream (Wang and Sui, 2002). This enables Apo H to inhibit the activation of blood coagulation and prothrombinase activity of platelets by covering the negatively charged surfaces necessary for both activities (Schousboe, 1985).

Apo H is involved in lipid metabolism, especially triglyceride metabolism, where it acts as the activator of lipoprotein lipase (Cassader *et al.*, 1997; Castro *et al*., 2010). It appears as antioxidant on LDL oxidation and inhibits cholesterol accumulation (Lin *et al*., 2001). Apo H also serves as a mandatory cofactor for binding anionic phospholipids to macrophages, thus involving in autoimmune diseases and inflammatory process (Hammel *et al.*, 2001). Besides that, it is able to clear apoptotic bodies from the circulation and triggers the complement system (Balasubramanian *et al*., 2005).

The abnormality of plasma Apo H level is commonly implicated in the development of hepatitis-B, atherosclerosis (Lin *et al.*, 2001; Nojima *et al.*, 2008), diabetes (Castro *et al*., 2010), and thrombosis-related diseases, particularly in anti-phospholipid syndrome, where Apo H is the target antigen of the specific autoantibody response (Greaves, 1999).

3.2.3.5 Apolipoprotein J (Apo J)

Apo J is a 70-80 kDa glycoprotein composed of two subunits that appears as two 35-40 kDa proteins under gel electrophoresis (De Silva *et al.*, 1990; Leskov *et al.*, 2003). It is encoded by the clusterin *CLU* gene and expressed in the liver, brain, and testes. Apo J can be found in nearly all body fluid including human plasma, CSF, urine, milk, and semen (Bertrand *et al.*, 1995; Jones and Jomary, 2002).

There are various names for Apo J due to its multifunctionality, among them are: clusterin, human serum protein-40,40, and complement lysis inhibitor (Jenne *et al.*, 1991). Apo J can be portrayed as different form in different cell compartments. As such, there are two types of Apo J: one expressed only in cytoplasm and nucleus; and the other secretory Apo J; in which the latter is being more extensively studied (Leskov *et al.*, 2003). Apo J is also part of HDL, particularly in a sub-fraction containing Apo A-I (De Silva *et al.*, 1990; Jenne *et al.*, 1991; Blatter *et al.*, 1993).

Apo J is involved in a variety of physiological processes, including cell adhesion, membrane recycling (Jones and Jomary, 2002), lipid metabolism (Irshad and Dubey, 2005), the complement system (Choi-Miura *et al*., 1992), and neuroprotection, due to its ability to cross the blood-brain barrier in the CNS (Calero *et al.*, 2000).

Apo J is usually expressed following tissue injury, stress, apoptosis, or neurodegeneration. During cellular stress, Apo J acts as anti-inflammatory and inhibits complement-mediated cell damage (Jones and Jomary, 2002). It also functions as an anti-apoptotic agent and protects the brain against oxidative stress (Calero *et al.*, 2000). It has the ability to complex with improperly folded or stressed proteins (Lee *et al.*, 2009) and is suggested as a membrane protectant against cytotoxic agents (Blatter *et al.*, 1993).

Over-expression of Apo J is mostly implicated in the pathogenesis of Alzheimer's disease (Lashley *et al.*, 2006; Lambert *et al.,* 2009) and brain injury following ischemia (Han *et al.*, 2001). However, there are variations in the expression of Apo J in different disorders. Kolialexi *et al.* (2008) reported down-regulation of Apo J in patients of Down syndrome, while Apo J is shown to have a positive association with panic disorder (Otowa *et al.*, 2009). Besides that, increased Apo J is also reported in liver fibrosis (Janig *et al.*, 2005), prostate cancer, ovarian cancer (Chen *et al.*, 2009), and coronary heart disease (Poulakou *et al*., 2008); while under-expression of Apo J is associated to rheumatoid arthritis (Takeuchi *et al.*, 2007).

3.3 Materials and Methods

3.3.1 Sampling

The subject assessment and sampling protocols are the same as discussed in section 2.2.1. A sub-sample of the 351 schizophrenic subjects were screened based on the following inclusion criteria: (i) age 30 - 45 years (patient mean age = 38.55 ± 5.33; control mean age = 37.00 ± 4.68), (ii) paranoid subtype, (iii) blood type O, (iv) no co-morbid medical illness, and (v) duration of schizophrenia more than 2 years. A total of 20 samples (10 males, 10 females) were identified. A control group of 20 samples matched with blood group and gender were also selected from the 247 controls.

3.3.2 Serum Collection

Approximately 10ml of fasting peripheral blood were collected from subjects into 10 ml vacutainers (Becton, Dickinson and Company, U.S.A.) for serum collection. All subjects were required to fast for at least 12 hours before the blood collection. The blood samples were spun down with 4000 rpm for 5 minutes at 4^{o}C. Serum was collected from the supernatant layer in 1.5 ml micro-centrifuge tubes and stored at -80^{o}C. Serum concentrations were quantified using Bradford assay.

3.3.3 Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis (2-DE) involved the following processes: first dimension separation by isoelectric focusing (IEF) performed using Ettan IPGphor 3 system (GE Healthcare Bio-Science, Sweden), followed by second dimension sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis (PAGE) using Hoefer SE 600 Ruby (GE Healthcare Bio-Science, Sweden).

For each sample, 50 μg of unfractionated whole human serum was rehydrated overnight at room temperature with rehydration buffer (Appendix A), dithiothreitol (DTT), and IPG buffer (GE Healthcare Bio-Science, Sweden) to a final volume of 250 μl on a 13 cm Immobiline DryStrip gels, pH 4 to 7 (GE Healthcare Bio-Science, Sweden).

The rehydrated sample was then subjected to IEF performed at 500V for 1 hour, ramped up to 1000V for 1 hour and then maintained at 8000V for 2 hours. The strip was equilibrated using SDS equilibration buffer (Appendix A) with DTT, followed by iodoacetamide for 15 min each. Second dimension was performed on 12.5% SDS-polyacrylamide gel (18 x 16 cm, 1 mm thick), with 10 mA for 15 minutes, followed by 30 mA for 3.5 hours. Gels were stained using PlusOne Silver Staining Kit (GE Healthcare Bio-Science, Sweden). Samples were analysed in duplicates.

In the spot analysis, LabScan ImageScanner III was used to capture images of the 2-DE gels while ImageMaster^{TM} 2D Platinum Software 7.0 (GE Healthcare Bio-Science, Sweden) was used for the analysis of gels. After spots detection and gels matching, differently expressed spots were identified. To eliminate possible variations due to differential protein staining, expression of proteins was evaluated in percentage of volume contribution (% vol), referring to the volume percentage of a protein against total spot volume of all proteins.

To fulfil the acceptable criteria for disease biomarkers in differential display techniques (Yang *et al.*, 2006), spots being selected were those that have expression levels of two-fold variances and were present in more than 50% of the sample population.

For peptide mass fingerprinting (PMF), a preparative gel was done and stained using Coomassie blue R-250, where spots were excised and prepared for trypsin digestion. Matrix-assisted laser desorption ionization time-of-flight/ time-of-flight mass spectrometry (MALDI-ToF/ToF MS) was outsourced to the Protein and Proteomics Centre in National University of Singapore.

The database search was done using MASCOT 1.9 search engine based on Matrix Science (www.matrixscience.com) where MASCOT scores greater than 82 are significant ($P < 0.05$) for the PMF search. Levels of proteins in gels were presented as mean % vol ± standard deviation. Differences between control and patient were assessed using one-way analysis of variance (ANOVA) where $P < 0.05$ was considered statistically significant.

3.3.4 Validation of Apo H by Western Blot

Western blot analysis was used as a validation to Apo H. This work was done with the help of a researcher from University Putra Malaysia. Serum samples of 4 patients and 4 controls were randomly chosen from the proteomic study group (section 3.2.1). Equal amounts of protein (20 μg) were used for each sample.

Protein sample were mixed with loading buffer (Appendix A) in 1:1 ratio and boiled for 3 minutes. Sample were then resolved by 10% SDS-PAGE and blotted onto a polyvinylidene fluoride membrane (Pierce, UK). The membrane were blocked for 1 hour at room temperature in blocking buffer (Appendix A) and incubated with goat polyclonal anti-human Apo H antibodies (Cat No: SC19179, Santa Cruz, USA) in (1:1000) for 1 hour. After several washes, the immunoblot was incubated in horse radish peroxidase-conjugated donkey anti-goat IgG solution (Cat No: SC2020, Santa Cruz, USA) (1:2000) for another 1 hour. The target proteins were detected using Enhanced Chemiluminescence substrate blotting reagent (Pierce, USA) according the manufacturer's instruction.

Images were captured using VersaDoc Imaging Device (BioRad, USA) and the band intensity was measured by Quantity One software. The statistical analysis was performed by using pair *t*-test for group comparison. The level of significant difference between the groups was accepted at $P < 0.05$.

3.4 Results

3.4.1 Proteomic Analysis

Human serum proteins were quantified and identified from 2-DE gels using the ImageMaster 2-D Platinum software and MALDI-ToF/ToF MS. An average of 774 protein spots was detected by the software on silver-stained gels. Figure 3.1 and 3.2 each demonstrate the representative 2-DE serum protein profile of the controls and patients respectively.

Five spots that were most significantly varied between 2-DE protein profiles of patient serum with those of controls were chosen for PMF analysis (Figure 3.3). The expression levels and fold-change of the five spots are summarised in Table 3.2. The protein spots were analysed using PMF method of MALDI-ToF/ToF MS. Table 3.3 lists the protein names, their Swiss-Prot accession numbers, MASCOT score, molecular mass (MW), pI values, protein amino acid sequence coverage by matching peptides, as well as the expectation level.

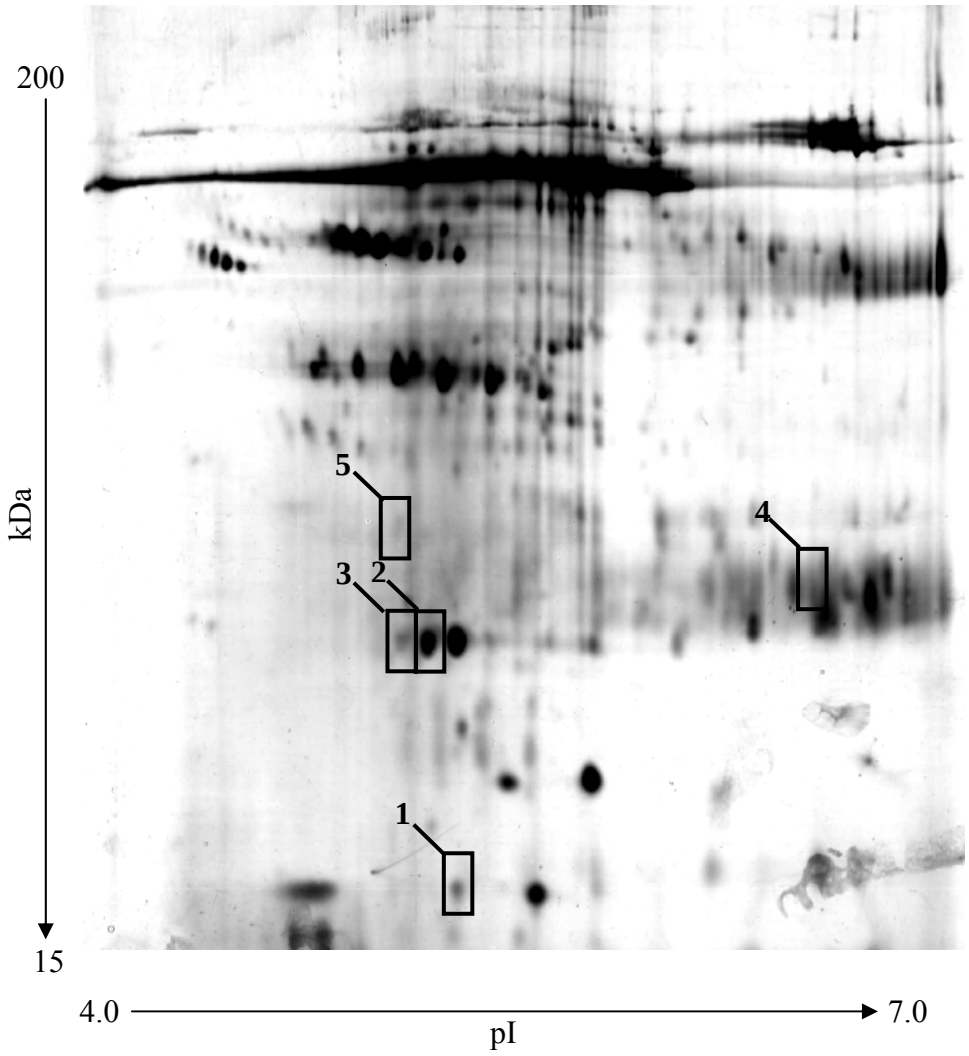

Figure 3.1: 2-DE map of serum proteins of controls. The gel was separated on 18 x 16 cm plate and silver-stained. The horizontal axis represents the IEF dimension, which stretches from pH 4 to 7. The vertical axis represents 12.5% SDS-PAGE gel. Boxes indicate differentially expressed proteins identified by MS

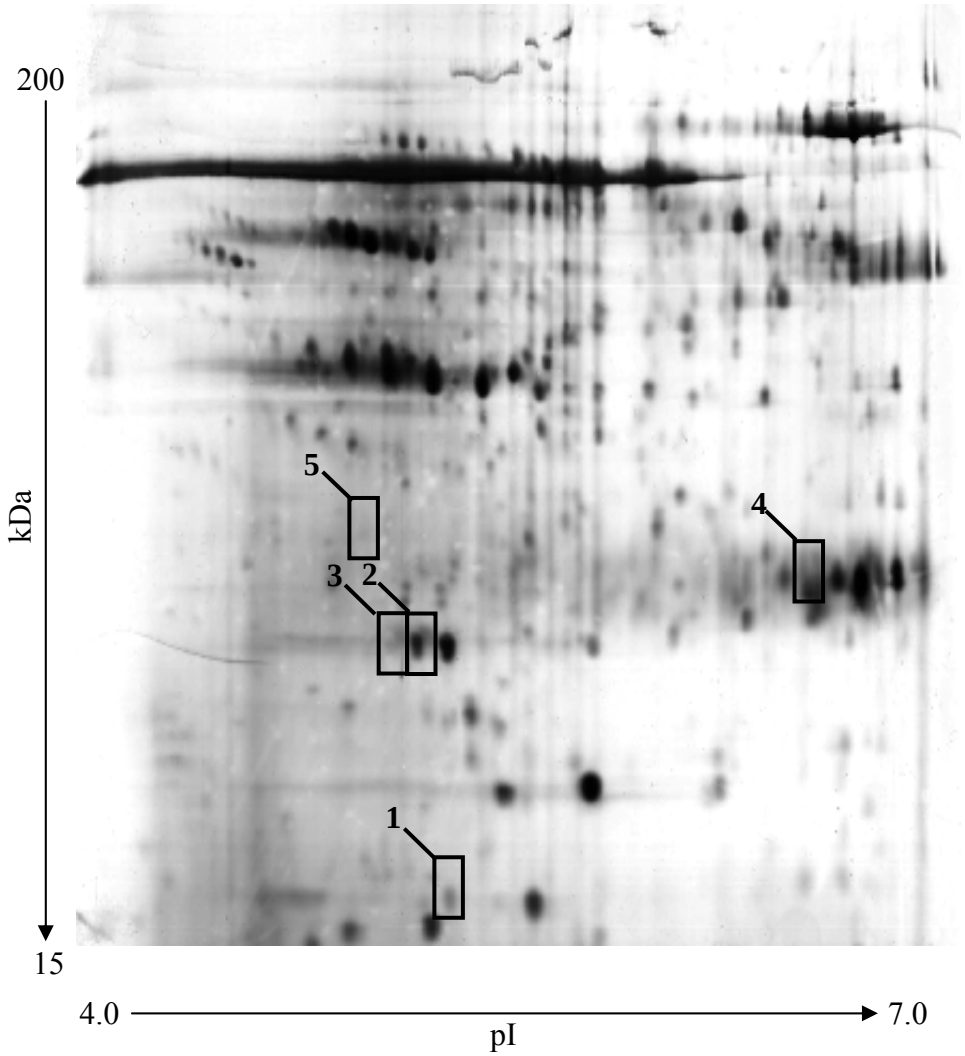

Figure 3.2: 2-DE map of serum proteins of patients. The gel was separated on 18 x 16 cm plate and silver-stained. The horizontal axis represents the IEF dimension, which stretches from pH 4 to 7. The vertical axis represents 12.5% SDS-PAGE gel. Boxes indicate differentially expressed proteins identified by MS

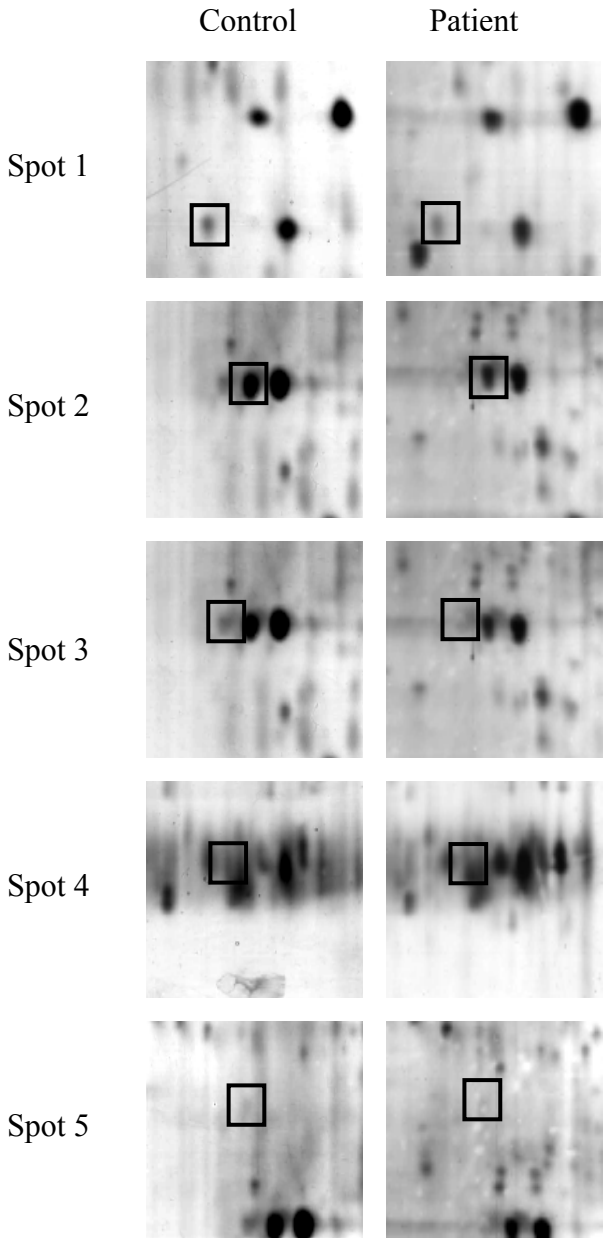

Figure 3.3 Enlarged panels show differences in serum protein patterns of controls and patients

Table 3.2: Differentially expressed spots in controls and schizophrenia patients (from Figure 3.1 and 3.2). Values are shown as mean concentration ± standard deviation

Spot	Protein name	Protein expression (% volume)			
		Control	Patient	Significance (*P*-value)	Fold change
1	Haptoglobin	0.91 ± 0.89	0.16 ± 0.13	0.003	5.69
2	Apolipoprotein A-I-1	2.51 ± 1.60	1.12 ± 0.55	0.004	2.24
3	Apolipoprotein A-I-2	1.00 ± 0.85	0.24 ± 0.29	0.003	4.17
4	Apolipoprotein H	< 0.01	1.00 ± 0.83	0.004	100
5	Apolipoprotein J	0.23 ± 0.22	< 0.01	0.003	23

Table 3.3: Mass spectroscopy identification of differentially expressed spots

Spot	Swiss-Prot accession no.	Protein name	MASCOT score	Mass (kDa)	pI value	Matched peptides	Coverage (%)	Expectation
1	P00738	Haptoglobin	391	38.72	6.14	9	10.00	7.30×10^{-33}
2	P02647	Apolipoprotein A-I-1	391	28.06	5.27	28	71.00	7.30×10^{-33}
3	P02647	Apolipoprotein A-I-2	197	28.06	5.17	22	65.00	1.80×10^{-13}
4	P02749	Apolipoprotein H	736	39.60	8.34	26	65.00	2.30×10^{-67}
5	P10909	Apolipoprotein J	48	37.00	5.74	6	21.00	1.30×10^{02}

The present study identified five proteins. Spot 1 was identified as Hp, while Spot 2 and spot 3 was determined to be Apo A-I-1 and Apo A-I-2 respectively, where both proteins are the major isoforms of Apo A-I (Menzel *et al.*, 1982). Spot 4 was identified as Apo H, and lastly Spot 5 had the highest possibility identified as Apo J.

Hp, Apo A-I-1, Apo A-I-2, and Apo J displayed a significant decreased in expression levels in the serum of patients compared to controls. In contrast, Apo H was found to be significantly up-regulated in patients as compared to the controls. The other spots did not show significant quantitative alteration between controls and patients.

3.4.2 Western Blot Validation for Apo H

Of the five proteins identified, Apo H was chosen for western blot validation. This is because both Hp and Apo A-I are well-reported proteins in schizophrenia. Besides that, the current study is the first report of differential expression of Apo H in schizophrenia, thus it is important to validate the differential expression of Apo H observed in this study.

Western blot was done with anti-Apo H to a set of randomly selected patient (n = 4) and control serum samples (n = 4). A single specific band with molecular weight approximately 38 kDa was detected in membrane probed with anti-Apo H antibody in the serum tested.

In comparison, the expression level of Apo H in patients serum is significantly higher than controls ($P = 0.030$) as demonstrated in Figure 3.4. In order to further confirm the differential expression of Apo H, the same experiment was repeated with pooled sera of controls and patients. A similar trend of result was displayed in Figure 3.5, where patients showed significantly higher expression level of Apo H as compared to the controls ($P = 0.031$).

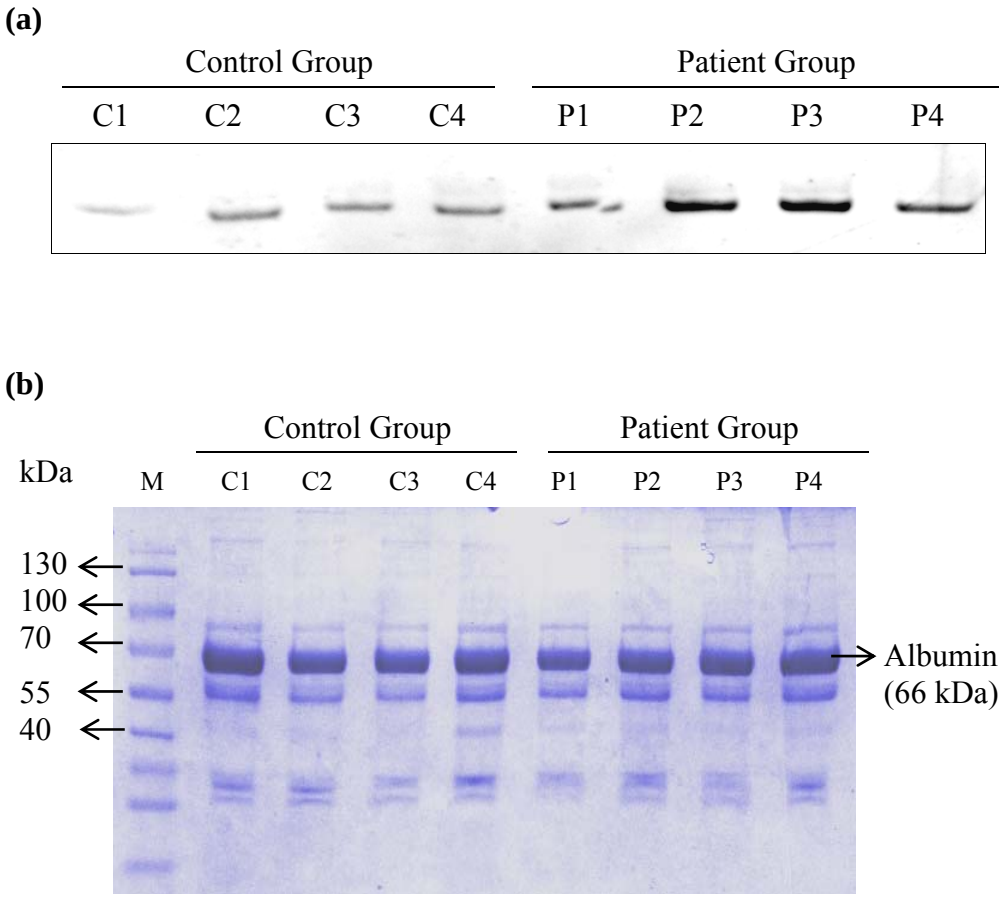

Figure 3.4: (a) Differential expression of Apo H in individual serum of controls and patients. The expression of Apo H was determined by Western blot probed with anti-Apo H, (b) Similar amount of 66 kDa band (albumin) in individual serum as loading control in Coomasie stained gel

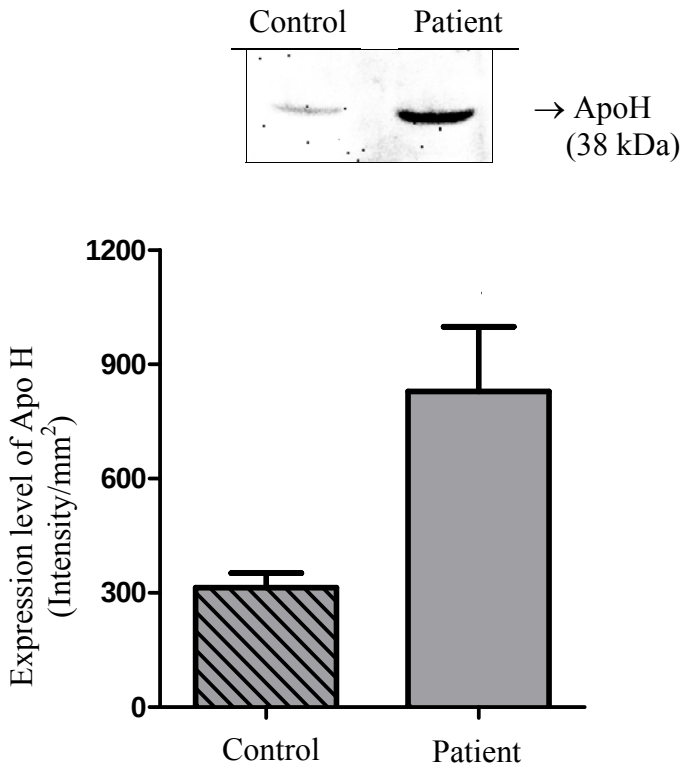

Figure 3.5: Differential expression of Apo H in pooled sera samples from control and patient group. The expression of Apo H was determined by Western blotting probed with anti-Apo H

3.5 Discussion

The role of APPs in schizophrenia is restricted to their function as non-specific markers of acute phase reaction. However, based on the immune-inflammation hypothesis, it has been shown that patients treated with the combination of antipsychotics and lipophilic anti-inflammatories would lead the down-regulation of immune response in the CNS (Muller *et al.*, 2002). Moreover, the human brain is rich in lipids; therefore, there is a possible role for these lipoproteins in supplying the needs of neurons for lipids.

To evaluate the role of APPs in schizophrenia, this study demonstrates the use of proteomic analysis of human serum in investigating the pathology of schizophrenia. In this study, the following observations were found: (i) the down-regulation of serum Hp, Apo A-I, and Apo J levels in patients; and (ii) drastic increase expression of Apo H in patients compared to controls.

3.5.1 Haptoglobin (Hp)

Hp is characterised as a positive APP, where its plasma expression increases during acute phase response (Wan *et al.*, 2007). Most of the investigations that studied the relationship between Hp and schizophrenia have found elevated levels of Hp in patients with schizophrenia (Wong *et al.*, 1996; Wan *et al.*, 2007). However, the present results showed a contradictory significant down-regulation of Hp in patients with schizophrenia.

Hp is characterised by three common phenotypes: Hp 1-1, Hp 2-1, and Hp 2-2. Individuals with Hp 1-1 reportedly have the highest plasma Hp concentration, Hp 2-1 individuals having intermediate levels and lastly Hp 2-2 individuals have the lowest plasma Hp concentration (Sadrzadeh and Bozorgmehr, 2004). It has been shown that Hp 2-2 is overrepresented in schizophrenia, affective psychoses and drug abuse (Maes *et al.*, 2001), thus suggesting that schizophrenic patients may have a lower Hp concentration as compared to their healthy counterparts. Although Hp phenotyping was not

performed in the present study, this finding may explain the low Hp level in patient serum as seen in the present study.

In addition, low individual antioxidant levels have been associated with schizophrenia (Chow *et al.*, 2010). The present sample population (sample size = 40 subjects) showed that patients (mean carotenoid level = 22,630) had significantly (P = 0.032) lower antioxidant level compared to controls (mean carotenoid level = 27,900), where carotenoid serves as indicator of overall antioxidant level in human (Zhao *et al.*, 2003; Svilaas *et al.*, 2004). Due to the fact that Hp has a mandatory role as an antioxidant (Henderson *et al.*, 2009), the reduced Hp level of patient may be associated to the low antioxidant level seen in schizophrenia (Sadrzadeh and Bozorgmehr, 2004).

Besides that, it was shown that antipsychotic drug treatments tend to lower the initially elevated plasma Hp in patients, where antipsychotic drug suppresses the synthesis of interleukin-6 and consequently Hp synthesis (Maes *et al.*, 1997). In view of the fact that all patients in the current study were medicated, antipsychotic drug treatment may be a factor influencing low Hp level in patients. Hp has also been found to bind with Apo A-I (Henderson *et al.*, 2009). Study showed that Apo A-I act as a negative APP in schizophrenia, where its expression decreases in times of acute phase response (Yang *et al.*, 2006). The low levels of Apo A-I observed in patients may cause the down-regulation of Hp reported in the present study.

3.5.2 Apolipoprotein A-I (Apo A-I)

Apo A-I is involved in lipoprotein degeneration and regeneration in the CNS (La *et al.*, 2007). As the major protein component of plasma HDL (Karathanasis, 1985) and the major lipoprotein in human CSF (Koch *et al.*, 2001; Huang *et al.*, 2008), Apo A-I serves to bind and supplying lipids to the needs of neurons.

The gene of Apo A-I is found on chromosome 11q23 (Arinami *et al.*, 1990), overlapping with schizophrenia susceptibility region 11q22.3-q24.1 (Lewis *et al.*, 2003). Evidence indicates that down-regulation of Apo A-I is linked with pathology of schizophrenia (La *et al.*, 2007; Huang *et al.*, 2008). Yang and co-workers (2006) reported significant down-regulation of Apo A-I in male schizophrenic patient but Apo A-I level is not associated with antipsychotic treatment. It is noteworthy that the present study observed a similar trend of significant decreased serum Apo A-I in schizophrenic patients, further confirming association of Apo A-I with the pathology of schizophrenia.

Besides overlapping with schizophrenia susceptibility chromosome 11, the gene for Apo A-I is also closely linked to the gene of Apo A-VI (Karathanasis, 1985), and that they both have a common evolutionary origin (Elshourbagy *et al.*, 1985). Studies indicate that Apo A-VI is involved in acute phase response and is well-associated to the pathology of schizophrenia (Jiang *et al.*, 2003; Yang *et al.*, 2006). This suggest that the closely linked

relationship between Apo A-I and Apo A-IV may contribute to the association of Apo A-I to schizophrenia.

Apo A-I serves to decrease cellular ROS and protects the brain from oxidative stress (Robbesyn *et al.*, 2005). The present study showed schizophrenia patients to have lower carotenoid antioxidant level as compared to controls, suggesting higher oxidative stress in patients (Chapter 2). Carotenoids are transported by HDL and were suggested to have a protective role for lipoproteins against oxidation (Tyssandier *et al*., 2002). Similarly, Apo A-I is shown to be involved in an enzymatic reaction to protect LDL against oxidative modifications (Mackness and Durrington, 1995). Besides that, studies suggested that both carotenoid and Apo A-I enhanced the up-regulation of LCAT (Aizawa and Inakuma, 2009; Ordovas, 2009). As such, the reduced Apo A-I observed in the current study is believed to be associated with the low carotenoid level reported in Chapter 2, thus suggesting association of Apo A-I to the high oxidative stress seen in schizophrenia.

3.5.3 Apolipoprotein H (Apo H)

Apo H gene is expressed when lipid is required for metabolic or proliferative processes (Ragusa *et al.,* 2006). In myocardial infarction, an inverse relationship was observed between levels of Apo H and the risk of the disease (Laat *et al.,* 2009), while Apo H was reported to be elevated in plasma of diabetic patients (Castro *et al.*, 2010).

For schizophrenia, the present study was the first to report a significant difference in the expression of Apo H in patient serum. The actual mechanism remains speculative; the observation might be due to the patient's inability to reduce Apo H under normal responses to inflammatory stimuli.

Validation study of Apo H was performed using western blot. As expected, a 38 kDa single band of Apo H was detected in the sample tested. Besides that, the result revealed an increase in the serum level of Apo H in patient serum compared to controls, which confirms the differential expression of Apo H previously reported.

Hammel and co-workers (2001) reported that Apo H is involved in autoimmune diseases and inflammatory processes. In the current study, high level of Apo H detected in schizophrenia may due to the activation of the complement system, where Apo H participates in the clearance of apoptotic bodies and triggering the complement system (Balasubramanian *et al*., 2005).

Plasma Apo H is characterised as trace protein in human plasma (Becker *et al*., 1969). The normal concentration of Apo H in human serum is approximately 20 mg/100ml, but there are few instances where quantitative variations can be observed (Sepehrnia *et al.*, 1989; Mehdi *et al.*, 1999). There are also reports of healthy individuals with no measurable amounts of Apo H and that their family had only half of the normal amount (Cleve, 1968; Becker *et al*., 1969) due to different synthesis rate influenced by the gene polymorphisms of Apo H (Mehdi *et al.*, 1999).

The concentration of Apo H is controlled by its two autosomal codominant alleles (Becker *et al*., 1969). Individuals with heterozygous-deficient alleles would have a lower Apo H concentration, while serum Apo H level in homozygous-deficient individuals is below the level of detection. It is reported that Apo H allele is ethnic-specific (Sanghera *et al.*, 1997), where the frequency of the deficiency allele was reported to be higher in Mongoloid and Black populations compared to Caucasians (Kamboh *et al.*, 1988).

Plasma Apo H levels are reportedly stable, not affected by long term storage, repeated freeze-thaw, or fasting status (Cleve, 1968; Mehdi *et al.*, 1999), thus eliminating any possibility of protein denaturation in any point of the experiment. As such, the trace concentration of Apo H in controls as seen in the present study may be related to the Asian sample population.

3.5.4 Apolipoprotein J (Apo J)

In the present study, mass spectrometry analysis of Spot 5 identified it as Apo J, although the MASCOT score was not significant. However, the present study took into consideration a few factors and decided to claim Spot 5 as Apo J. First of all, Apo J is a relatively small glycoprotein and during mass spectrometry analysis, it is being excised to short fragments, which were matched with protein database. Due to the reason that the percentage coverage of the fragments and the number of matching peptides were too low (Table 3.3), the MASCOT score obtained is inevitably low. Moreover, molecular

weight and pI value obtained from spot analysis of Spot 5 were similar to Apo J, thus the highest possible identity for Spot 5 is Apo J.

The present study observed significantly down-regulation of Apo J in schizophrenic patient serum as compared to controls. The possible explanations are: (i) Apo J acts as negative APP in schizophrenia, and (ii) suppression or rapid clearance of Apo J.

Research showed that Apo J is able to act as membrane protectant against cytotoxic agents (Blatter *et al.*, 1993) and inhibits complement-mediated cell damage in the complementary system (Choi-Miura *et al*., 1992; Jones and Jomary, 2002). The current results of the reduced concentration of Apo J in schizophrenia imply that Apo J have loss the protective role against complement-mediated cell damage in the brain, which is a characteristic in schizophrenia (Kolev *et al.*, 2010).

In addition, Apo J is associated to Apo A-I as part of the protein constituent of the HDL (De Silva *et al.*, 1990; Jenne *et al.*, 1991; Blatter *et al.*, 1993; Sousa *et al.*, 2000), thus it is suggested that the mechanism(s) of action of Apo J is similar to that of Apo A-I. Apo J may involve in lipid transport which is essential in the degeneration and regeneration of neuronal cell during brain injury.

Apo J is found to have a protective role by forming complexes with stress proteins. In amyloidoses, it stabilises TTR by forming complex with the tetrameric structure of TTR, thereby reducing the amount of monomeric form available for amyloid fibril formation (Lee *et al*., 2009). Plasma TTR has been shown to interact with HDL through binding with Apo A-I (Sousa *et al.*, 2000; Yang *et al.*, 2006). In light of this, and the fact that decreased level of TTR and Apo A-I have both been implicated in schizophrenia (Yang *et al*., 2006), the reduced level of Apo J in patients in the current study may also be associated to schizophrenia, where the disease mechanism may lie in the interaction between TTR, Apo A-I, and Apo J.

3.6 Conclusion

In conclusion, it was shown here that there are altered expressions of Hp, Apo A-I, Apo H, and Apo J in serum of schizophrenic patients. These findings suggest that serum Hp, Apo A-I, Apo H, and Apo J may be linked to schizophrenia and have the potential to become biomarkers for schizophrenia diagnosis. These potential biomarkers may contribute to identifying new biological pathways involved in the development of schizophrenia. The role of APPs in the pathogenesis of schizophrenia remains to be elucidated, but it is suggested to be related to the complement activation. The limitation of this study is that analysis on age, gender, and ethnicity was not done due to limited sample size, thus conclusions on the relationship of age, gender, and ethnicity with schizophrenia cannot be made.

CHAPTER 4

FINAL CONCLUSION

Oxidative stress and acute phase responses are hypothesised in the pathology of schizophrenia. High oxidative and psychological stress are often related with reduced antioxidant activities and acute inflammation. Besides that, in view of the fact that inflammation and immune abnormalities trigger APP level alteration in the acute phase response, identifying the APPs involved can be rewarding in understanding schizophrenia pathology.

The initial study of carotenoid antioxidant as chemical marker for schizophrenia was addressed. High oxidative stress seen in schizophrenia implicates impaired antioxidant defense system. Results showed that patients have lower carotenoid level as compared to controls, while females showed higher carotenoid level compared to males. None of the carotenoid antioxidant analyses of age, schizophrenic subtypes, antipsychotic drug treatment, and duration of illness were significantly associated with schizophrenia. Follow-up analysis using FRAP was encouraging and further supported carotenoid antioxidant as a risk factor for schizophrenia and suggested antioxidant defense system as a promising target for future studies.

In the study of biological markers, five APPs were identified to be differently expressed in schizophrenic patients as compared to controls. Down-regulation of serum Hp, Apo A-I-1, Apo A-I-2, and Apo J, together with the up-regulation of Apo H were found to be related with schizophrenia. These APPs may serve as potential biomarkers for schizophrenia. The present study also demonstrated APP and acute phase response to be associated with schizophrenia.

Taken together, these analyses supported the hypothesis of oxidative stress and acute phase response in association with schizophrenia. Oxidative and psychological stress lead to oxidative injury and inflammation in the CNS as seen in schizophrenia. Due to the reason that most APPs play the important role as antioxidant, carotenoid antioxidant and APPs are able to protect the brain from oxidative stress by decreasing cellular ROS. As such, low carotenoid antioxidant level in patients implies the impaired protective role of antioxidant against oxidative stress.

The present study represents evaluation of the relationship between schizophrenia with chemical and biological markers. The results would be particularly useful in contributing to the interpretation of schizophrenia pathology, thus facilitating early detection and treatment. Cross-validation of the relationship between schizophrenia with chemical and biological markers in terms of diet and antipsychotic drug dosage may be rewarding.

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APPENDIX A

SOLUTION RECIPE

Acetate buffer (300 mM, pH 3.6)

Sodium acetate trihydrate	3.1 g
Glacial acetic acid	16 ml

Agarose electrophoresis solution (1%)

Agarose	0.4 g
1 x TBE buffer	40 ml

Agarose sealing solution

1x SDS electrophoresis buffer	100 ml
Agarose	0.5 g
Bromophenol blue	200 µl

Ammonium persulphate (10%)

Ammonium persulphate	0.1 g
Distilled water	1 ml

Blocking buffer

BSA	0.3%
Tween-20 in pH 7.2 PBS	0.1%

Bromophenol blue stock solution (1%)

Bromophenol blue	100 mg
Tris-base	60 mg
Distilled water	10 ml

Comassie Blue Destaining Solution

Glacial acetic acid	100 ml
Methyl alcohol	50 ml
Distilled water	1 L

Coomassie Blue Staining Solution

Coomassie blue	1 g
Glacial acetic acid	100 ml
Methyl alcohol	500 ml
Distilled water	1 L

Ethanol (70%)

Absolute ethanol	70 ml
Distilled water	30 ml

Equilibration buffer

1.5 M Tris-HCl	10 ml
Urea	72.07 g
Glycerol (87% v/v)	69 ml
SDS	4 g
1% Bromophenol blue	400 µl
Distilled water	200 ml

Store at –20°C
Prior to use, add:
DTT (10 mg/ml)
Iodoacetamide (25 mg/ml)

Ferric Chloride (20 mM)

Ferric chloride hexahydrate	0.054 g
Distilled water	10 ml

FRAP solution

Solution I – Acetate buffer	100 ml
Solution II – TPTZ	10 ml
Solution III – Ferric chloride	10 ml
Distilled water	12 ml

Gel storage solution

4x Tris-HCl resolving buffer	50 ml
10% SDS	2 ml
Distilled water	200 ml

Loading buffer

Tris-HCl pH 6.8	0.5 M
Glycerol	10%
SDS	2%
Bromophenol blue	0.0002 %

Monomer solution

Acrylamide	60 g
N, N'- methylenebisacrylamide	1.6 g
Distilled water	200 ml

Filter through 0.45 µm filter and store at 4°C in dark

Rehydration buffer

Urea	12 g
CHAPS	0.5 g
1% Bromophenol blue	50 µl
Distilled water	25 ml

Store at –20°C. Prior to use, add:
DTT (to a final concentration of 20mM)
IPG buffer (to a final concentration of 0.5%, v/v)

SDS (10%)

SDS	10 g
Distilled water	100 ml

Filter solution through 0.45 μm filter and store at room temperature

SDS electrophoresis running buffer (10x)

Tris base	30.25 g
Glycine	144 g
SDS	10 g
Distilled water	1 L

SDS polyacrylamide gel (12.5%)

Monomer solution	41.7 ml
Tris –HCl resolving gel buffer	25 ml
10% SDS	1 ml
10% Ammonium persulphate	500 μl
TEMED	33 μl
Distilled water	31.8 ml

TBE buffer (10x, pH 8.0)

Tris-base	108 g
EDTA $(Na)_2$	9.3 g
Boric Acid	55 g
Distilled water	1 L

TBE buffer (1x, pH 8.0)

TBE buffer, 10x	100 ml
Distilled water	1 L

TPTZ (10 mM)

TPTZ	0.031 g
40 mM HCl	10 ml

Tris-EDTA (TE) buffer (1x, pH 8.0)

Tris-HCl	10 mM
EDTA	1 mM
Distilled water	1 L

Tris –HCl resolving gel buffer (1.5M, 4x, pH 8.8)

Tris base	181.7 g
Distilled water	1 L
HCl	adjust to pH 8.8

Filter solution through 0.45 μm filter and store at 4°C

Water-saturated butanol

Sec-butanol	50 ml
Distilled water	5 ml

APPENDIX B

MINI INTERNATIONAL NEUROPSYCHIATRIC INTERVIEW (M.I.N.I.)

M.I.N.I.

Mini International Neuropsychiatric Interview

English Version 5.0.0

DSM-IV

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PATIENT'S NAME :	_____	***PROTOCOL NUMBER :***	_____
DATE OF BIRTH :	_____	***Time Interview Began :***	_____
INTERVIEWER'S NAME :	_____	***Time Interview Ended :***	_____
DATE OF INTERVIEW :	_____	***TOTAL TIME :***	_____

M.I.N.I. 5.0.0 / English version / DSM-IV / current

MODULES	TIME FRAME	
A. MAJOR DEPRESSIVE EPISODE	Current (past 2 weeks) + Lifetime	
A'. MDE with melancholic features	Current (past 2 weeks)	Optional
B. DYSTHYMIA	Current (past 2 years)	
C. SUICIDALITY	Current (past month)	
D. (HYPO) MANIC EPISODE	Current + Lifetime	
E. PANIC DISORDER	Lifetime + current (past month)	
F. AGORAPHOBIA	Current	
G. SOCIAL PHOBIA	Current (past month)	
H. OBSESSIVE-COMPULSIVE DISORDER	Current (past month)	
I. POSTTRAUMATIC STRESS DISORDER	Current (past month)	Optional
J. ALCOHOL DEPENDENCE / ABUSE	Current (past 12 months)	
K. DRUG DEPENDENCE / ABUSE (Non-alcohol)	Current (past 12 months)	
L. PSYCHOTIC DISODERS	Lifetime + Current	
M. ANOREXIA NERVOSA	Current (past 3 months)	
N. BULIMIA NERVOSA	Current (past 3 months)	
O. GENERALIZED ANXIETY DISORDER	Current (past 3 months)	
P. ANTISOCIAL PERSONALITY DISORDER	Lifetime	Optional

GENERAL INSTRUCTIONS

The M.I.N.I. was designed as a brief structured interview for the major Axis I psychiatric disorders in DSM-IV and ICD-10. Validation and reliability studies have been done comparing the M.I.N.I. to the SCID-P and the CIDI. The results of these studies show that the M.I.N.I. has acceptably high validation and reliability scores, but can be administered in a much shorter period of time (mean 18.7 ± 11.6 min., median 15 min.) than the above referenced instruments. It can be used by clinicians, after a brief training session. Lay interviewers require more extensive training.

- **Interview :**

In order to keep the interview as brief as possible, inform the patient that you will conduct a clinical interview that is more structured than usual, with very precise questions about psychological problems which requires a yes or no answer.

- **General format :**

The M.I.N.I. is divided into **modules** identified by letters, each corresponding to a diagnostic category.

- At the beginning of each module (except for psychotic disorders module), **screening question(s)** corresponding to the main criteria of the disorder are presented in a **gray box**.
- At the end of each module, **diagnostic box(es)** permit(s) the clinician to indicate whether the diagnostic criteria are met.

- **Conventions :**

Sentences written in **«** normal font » should be read exactly as written to the patient in order to standardize the assessment of diagnostic criteria.

Sentences written in **«** CAPITALS » should not to be read to the patient. They are instructions for the interviewer to assist in the scoring of the diagnostic algorithms.

Sentences written in « **bold »** indicate the time frame being investigated. The interviewer should read them as often as necessary. Only symptoms occurring during the time frame indicated should be considered in scoring the responses.

Sentences (in parentheses) are clinical examples of the symptom .These may be read to the patient to clarify the question.

Answers with an arrow above them (➔) indicate that one of the criteria necessary for the diagnosis(es) is not met. In this case, the interviewer should go to the end of the module, to circle « **NO** » in all the diagnostic boxes and move to the next module.

When terms are separated by a *slash (/)*, the interviewer should read only those symptoms known to be present in the patient (for example, question A3).

- **Rating instructions:**

All questions read must be rated. The rating is done at the right of each question by circling either YES or NO.
The clinician should be sure that each dimension of the question is taken into account by the patient (i.e.: time frame, frequency, severity, « and/or » alternatives).

Symptoms better accounted for by an organic cause or by the use of alcohol or drugs should not be coded positive in the M.I.N.I.. The M.I.N.I. Plus has questions that investigate these issues.

For any questions, suggestions, need for a training session, or information about updates of the M.I.N.I., please contact :

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➔ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

A. MAJOR DEPRESSIVE EPISODE

A1	Have you been consistently depressed or down, most of the day, nearly every day, for the past two weeks ?	NO	YES	1
A2	In the past two weeks, have you been less interested in most things or less able to enjoy the things you used to enjoy most of the time ?	NO	YES	2
		➔		
	IS **A1** OR **A2** CODED **YES** ?	NO	YES	
A3	**Over the past two weeks, when you felt depressed and/or uninterested :**			
a	Was your appetite decreased or increased nearly every day or did your weight decrease or increase without trying intentionally ? (i.e., ± 5 % of body weight or ± 3,5 kg or ± 8 lbs., for a 70 kg / 120 lbs. person in a month) IF **YES** TO EITHER, CODE **YES**	NO	YES	3
b	Did you have trouble sleeping nearly every night (difficulty falling asleep, waking up in the middle of the night, early morning wakening, or sleeping excessively) ?	NO	YES	4
c	Did you talk or move more slowly than normal or were you fidgety, restless or having trouble sitting still, almost every day?	NO	YES	5
d	Did you feel tired or without energy, almost every day?	NO	YES	6
e	Did you feel worthless or guilty, almost every day?	NO	YES	7
f	Did you have difficulty concentrating or making decisions, almost every day?	NO	YES	8
g	Did you repeatedly consider hurting yourself, feel suicidal, or wish that you were dead ?	NO	YES	9
A4	ARE **3** OR MORE **A3** ANSWERS CODED **YES** ? (OR **4 A3** ANSWERS IF **A1** OR **A2** ARE CODED **NO**)	**NO**	**YES**	***MAJOR DEPRESSIVE EPISODE CURRENT***
	IF PATIENT MEETS CRITERIA FOR MAJOR DEPRESSIVE EPISODE CURRENT :			
A5a	During your lifetime, did you have other periods of two weeks or more when you felt depressed or uninterested in most things, and had most of the problems we just talked about ?	➔ NO	YES	10
b	Was there an interval of at least 2 months without depression and/or lost of interest between your current episode and your last episode of depression ?	NO	YES	11
	IS **A5b** CODED **YES** ?	**NO**	**YES**	***MAJOR DEPRESSIVE EPISODE PAST***

➔ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

A'. MAJOR DEPRESSIVE EPISODE WITH MELANCHOLIC FEATURES (optional)

IF THE PATIENT CODES POSITIVE FOR A MAJOR DEPRESSIVE EPISODE (**A4 = YES**), EXPLORE THE FOLLOWING :

A6 a	IS **A2** CODED **YES** ?	NO	YES	12
b	During the most severe period of the current depressive episode, did you lose your ability to respond to things that previously gave you pleasure, or cheered you up? **IF NO** : When something good happens does it fail to make you feel better, even temporarily ?	NO	YES	13
	➔ IS EITHER **A6a** OR **A6b** CODED **YES** ?	NO	YES	

Over the past two weeks period, when you felt depressed and uninterested :

A7 a	Did you feel depressed in a way that is different from the kind of feeling you experience when someone close to you dies ?	NO	YES	14
b	Did you fell regularly worse in the morning, almost every day ?	NO	YES	15
c	Did you wake up at least 2 hours before the usual time of awakening and have difficulty getting back to sleep, almost every day ?	NO	YES	16
e	IS **A3c** CODED **YES** ?	NO	YES	17
d	IS **A3a** CODED **YES** (ANOREXIA OR WEIGHT LOSS ONLY)?	NO	YES	18
f	Did you feel excessive guilt or out of proportion to the reality of the situation ?	NO	YES	19

ARE **3** OR MORE **A7** ANSWERS CODED **YES** ?

NO **YES**

MAJOR DEPRESSIVE EPISODE
With Melancholic Features
CURRENT

→ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

B. DYSTHYMIA

IF PATIENT'S SYMPTOMS CURRENTLY MEET CRITERIA FOR MAJOR DEPRESSIVE EPISODE, DO NOT EXPLORE THIS MODULE

B1	Have you felt sad, low or depressed most of the time for the last two years ?	→ NO	YES	20
B2	Was this period interrupted by your feeling OK for two months or more ?	NO	→ YES	21
B3	**During this period of feeling depressed most of the time :**			
a	Did your appetite change significantly ?	NO	YES	22
b	Did you have trouble sleeping or sleep excessively ?	NO	YES	23
c	Did you feel tired or without energy ?	NO	YES	24
d	Did you lose your self-confidence ?	NO	YES	25
e	Did you have trouble concentrating or making decisions ?	NO	YES	26
f	Did you feel hopeless ?	NO	YES	27
	ARE **2** OR MORE **B3** ANSWERS CODED **YES** ?	→ NO	YES	
B4	Did the symptoms of depression cause you significant distress or impair your ability to function at work, socially, or in some other important way ?	→ NO	YES	28
	IS **B4** CODED **YES** ?	**NO**	**YES**	
		DYSTHYMIA CURRENT		

➔ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

C. SUICIDALITY

In the past month did you :

C1	Think that you would be better off dead or wish you were dead ?	NO	YES	1
C2	Want to harm yourself ?	NO	YES	2
C3	Think about suicide ?	NO	YES	3
C4	Have a suicide plan ?	NO	YES	4
C5	Attempt suicide ?	NO	YES	5

In your lifetime

C6	Did you ever make a suicide attempt ?	NO	YES	6

IS AT LEAST **1** OF THE ABOVE CODED **YES** ?

IF YES, **SPECIFY** THE LEVEL OF SUICIDE RISK AS FOLLOWS :

C1 or C2 or C6 = YES : LOW
C3 or (C2 +C6) = YES : MODERATE
C4 or C5 or (C3 + C6) = YES : HIGH

NO	**YES**

SUICIDE RISK
CURRENT

LOW	☐
MODERATE	☐
HIGH	☐

➔ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

D. (HYPO) MANIC EPISODE

D1 a	Have you **ever** had a period of time when you were feeling "up" or "high" or so full of energy or full of yourself that you got into trouble, or that other people thought you were not your usual self ? (Do not consider times when you were intoxicated on drugs or alcohol) IF PATIENT IS PUZZLED OR UNCLEAR ABOUT WHAT YOU MEAN BY "UP" OR "HIGH", CLARIFY AS FOLLOW : By "up" or "high" I mean : having elated mood, increased energy, needing less sleep, having rapid thoughts, being full of ideas, having an increase in productivity, creativity, motivation or impulsive behavior.	NO	YES	1
	IF YES :			
b	Are you currently feeling "up" or "high" or full of energy ?	NO	YES	2
	:			
D2a	Have you ever been persistently irritable, for several days, so that you had arguments or verbal or physical fights, or shouted at people outside your family ? Have you or others noticed that you have been more irritable or over reacted, compared to other people, even in situations that you felt were justified ? (Do not consider times when you were intoxicated on drugs or alcohol)	NO	YES	3
	IF YES :			
b	Are you currently feeling persistently irritable ?	NO	YES	4
		➔		
	ARE **D1a** OR **D2a** CODED **YES** ?	NO	YES	

D3 IF D1B OR D2B = YES : EXPLORE ONLY **CURRENT** EPISODE
IF D1B AND D2B = NO : EXPLORE **THE MOST SYMPTOMATIC** PAST EPISODE

During the time(s) when you felt "high", full of energy or irritable did you :

a	Feel that you could do things others couldn't do, or that you were an especially important person ?	NO	YES	5
b	Need less sleep (e.g., feel rested after only a few hours sleep) ?	NO	YES	6
c	Talk too much without stopping, or so fast that people had difficulty understanding ?	NO	YES	7
d	Have thoughts racing?	NO	YES	8
e	Become easily distracted so that any little interruption could distract you ?	NO	YES	9
f	Become so active or physically restless that others were worried about you ?	NO	YES	10

➔ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

g Want so much to engage in pleasurable activities that you ignored the risks or consequences (e.g., spending sprees, reckless driving, or sexual indiscretions) ? NO YES 11

ARE **3** OR MORE **D3** ANSWERS CODED **YES** ➔
OR **4** IF **D1a = NO** (PAST EPISODE) OR **D1b = NO** (CURRENT EPISODE) ? NO YES

D4 Did these symptoms last at least a week **and** cause significant problems at home, at work, or at school,
or were you hospitalized for these problems? NO YES 12
IF YES TO EITHER, CODE YES

IS **D4** CODED **NO** ?

NO **YES**

HYPOMANIC EPISODE

IF YES, SPECIFY IF THE EPISODE EXPLORED IS CURRENT OR PAST

CURRENT
PAST

IS **D4** CODED **YES** ?

NO **YES**

MANIC EPISODE

IF YES, SPECIFY IF THE EPISODE EXPLORED IS CURRENT OR PAST

CURRENT
PAST

➔ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

E. PANIC DISORDER

E1	Have you, on more than one occasion, had spells or attacks when you **suddenly** felt anxious, frightened, uncomfortable or uneasy, even in situations where most people would not feel that way ? Did the spells peak within 10 minutes ? CODE YES ONLY IF THE SPELLS PEAK WITHIN 10 MINUTES	NO	YES	1
	IF **E1 = NO**, CIRCLE NO IN E5 AND SKIP TO F1			
E2	At any time in the past, did any of those spells or attacks come on unexpectedly or spontaneously, or occur in an unpredictable or unprovoked manner ? IF **E2 = NO**, CIRCLE NO IN E5 AND SKIP TO F1	NO	YES	2
E3	Have you ever had one such attack followed by a month or more of persistent fear of having another attack, or worries about the consequences of the attack ? IF **E3 = NO**, CIRCLE NO IN E5 AND SKIP TO F1	NO	YES	3
E4	**During the worst spell that you can remember :**			
a	Did you have skipping, racing or pounding of your heart ?	NO	YES	4
b	Did you have sweating or clammy hands ?	NO	YES	5
c	Were you trembling or shaking ?	NO	YES	6
d	Did you have shortness of breath or difficulty breathing ?	NO	YES	7
e	Did you have a choking sensation or a lump in your throat ?	NO	YES	8
f	Did you have chest pain, pressure or discomfort ?	NO	YES	9
g	Did you have nausea, stomach problems or sudden diarrhea ?	NO	YES	10
h	Did you feel dizzy, unsteady, lightheaded or faint ?	NO	YES	11
i	Did things around you feel strange, unreal, detached or unfamiliar, or did you feel outside of or detached from, part or all of your body ?	NO	YES	12
j	Did you fear that you were losing control or going crazy ?	NO	YES	13
k	Did you fear that you were dying ?	NO	YES	14
l	Did you have tingling or numbness in parts of your body ?	NO	YES	15
m	Did you have hot flashes or chills ?	NO	YES	16
E5	ARE **4** OR MORE **E4** ANSWERS CODED **YES** ? IF **E5 = NO**, SKIP TO E7	NO	YES ***Panic Disorder Life time***	
E6	In the past month, did you have such attacks repeatedly (2 or more) followed by persistant fear of having another attack ? IF **E6 = YES**, SKIP TO F1	NO	YES ***Panic Disorder Current***	17
E7	ARE **1**, **2** OR **3 E4** ANSWERS CODED **YES** ?	NO	YES ***Limited Symptom Attacks Lifetime***	18

➔ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

F. AGORAPHOBIA

F1 Do you feel anxious or particularly uneasy in places or situations from which escape might be difficult, and where help might not be available in case of panic attack, like being in a crowd, standing in a line (queue), when you are alone away from home or alone at home, or when crossing a bridge, traveling in a bus, train or car ? NO YES 19

IF **F1 = NO**, CIRCLE NO IN F2

F2 Do you fear these situations so much that you avoid them, or suffer through them, or need a companion to face them ? NO YES

Agoraphobia Current

IS **F2** (CURRENT AGORAPHOBIA) CODED **NO**
and
IS **E6** (CURRENT PANIC DISORDER) CODED **YES** ?

NO **YES**

PANIC DISORDER without Agoraphobia CURRENT

IS **F2** (CURRENT AGORAPHOBIA) CODED **YES**
and
IS **E6** (CURRENT PANIC DISORDER) CODED **YES** ?

NO **YES**

PANIC DISORDER with Agoraphobia CURRENT

IS **F2** (CURRENT AGORAPHOBIA) CODED **YES**
and
IS **E5** (PANIC DISORDER LIFETIME) CODED **NO** ?

NO **YES**

AGORAPHOBIA without history of Panic Disorder CURRENT

→ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

G. SOCIAL PHOBIA

G1	In the past month, were you fearful or embarrassed being watched, being the focus of attention, or fearful of being humiliated ? This includes situations like speaking in public, eating in public or with others, writing while someone watches, or being in social situations.	→ NO	YES	1
G2	Is this fear excessive or unreasonable ?	→ NO	YES	2
G3	Do you fear these situations so much that you avoid them or suffer through them ?	→ NO	YES	3
G4	Does this fear disrupt your normal work or social functioning or cause you significant distress ?	NO	YES	4

IS **G4** CODED **YES** ?

NO **YES**

SOCIAL PHOBIA
CURRENT

➔ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

H. OBSESSIVE-COMPULSIVE DISORDER

H1	In the past month, have you been bothered by recurrent thoughts, impulses or images that were unwanted, distasteful, inappropriate, intrusive or distressing ? (e.g., the idea that you were dirty, contaminated or had germs, **or** fear of contaminating others, **or** fear of harming someone even though you didn't want to, **or** fearing you would act on some impulse, **or** fear or superstitions that you would be responsible for things going wrong, **or** obsessions with sexual thoughts, images or impulses, **or** hoarding, collecting, **or** religious obsessions.) DO NOT INCLUDE SIMPLY EXCESSIVE WORRIES ABOUT REAL LIFE PROBLEMS. DO NOT INCLUDE OBSESSIONS DIRECTLY RELATED TO EATING DISORDERS, SEXUAL DEVIATIONS, PATHOLOGICAL GAMBLING, OR ALCOHOL OR DRUG ABUSE BECAUSE THE PATIENT MAY DERIVE PLEASURE FROM THE ACTIVITY AND MAY WANT TO RESIST IT ONLY BECAUSE OF ITS NEGATIVE CONSEQUENCES.	NO	YES	1
	IF **H1 = NO**, SKIP TO H4			
H2	Did they keep coming back into your mind even when you tried to ignore or get rid of them ?	NO	YES	2
	IF **H2 = NO**, SKIP TO H4			
H3	Do you think that these obsessions are the product of your own mind and that they are not imposed from the outside ?	NO	YES	3
H4	In the past month, did you do something repeatedly without being able to resist doing it, like washing or cleaning excessively, counting or checking things over and over, or repeating, collecting, arranging things, or other superstitious rituals ?	NO	YES	4
	ARE **H3** OR **H4** CODED **YES** ?	➔ NO	YES	
H5	Did you recognize that either these obsessive thoughts and / or these compulsive behaviors you can not resist doing them, were excessive or unreasonable ?	➔ NO	YES	5
H6	Did these obsessive thoughts and / or compulsive behaviors significantly interfere with your normal routine, occupational functioning, usual social activities, or relationships, or did they take more than one hour a day ?	NO	YES	6
	IS **H6** CODED **YES** ?	**NO**	**YES**	

OBSESSIVE-COMPULSIVE DISORDER CURRENT

→ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

I. POSTTRAUMATIC STRESS DISORDER (optional)

I1	Have you ever experienced or witnessed or had to deal with an extremely traumatic event that included actual or threatened death or serious injury to you or someone else ? EX OF TRAUMATIC EVENTS : SERIOUS ACCIDENT, SEXUAL OR PHYSICAL ASSAULT, A TERRORIST ATTACK, BEING HELD HOSTAGE, KIDNAPPING, HOLD-UP, FIRE, DISCOVERNG A BODY, UNEXPECTED DEATH,, WAR, NATURAL DISASTER...	→ NO	YES	1
I2	During the past month, have you re-experienced the event in a distressing way (i.e., dreams, intense recollections, flashbacks or physical reactions) ?	→ NO	YES	2

I3	**In the past month :**			
a	Have you avoided thinking about the event, or have you avoided things that remind you the event ?	NO	YES	3
b	Have you had trouble recalling some important part of what happened ?	NO	YES	4
c	Have you become less interested in hobbies or social activities ?	NO	YES	5
d	Have you felt detached or estranged from others ?	NO	YES	6
e	Have you noticed that your feelings are numbed ?	NO	YES	7
f	Have you felt that your life would be shortened because of this trauma ?	NO	YES	8
	ARE **3** OR MORE **I3** ANSWERS CODED **YES** ?	→ NO	YES	
I4	**In the past month :**			
a	Have you had difficulty sleeping ?	NO	YES	9
b	Were you especially irritable or did you have outbursts of anger ?	NO	YES	10
c	Have you had difficulty concentrating ?	NO	YES	11
d	Were you nervous or constantly on your guard ?	NO	YES	12
e	Were you easily startled ?	NO	YES	13
	ARE **2** OR MORE **I4** ANSWERS CODED **YES** ?	→ NO	YES	
I5	During the past month, have these problems significantly interfered with your work or social activities, or caused significant distress ?	NO	YES	14
	IS **I5** CODED **YES** ?	**NO**	**YES**	

POSTTRAUMATIC STRESS DISORDER CURRENT

→ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

J. ALCOHOL ABUSE AND DEPENDENCE

J1	In the past 12 months, have you had 3 or more alcoholic drinks within a 3 hour period on 3 or more occasions ?	→ NO	YES	1
J2	**In the past 12 months :**			
a	Did you need to drink more in order to get the same effect that you did when you first started drinking ?	NO	YES	2
b	When you cut down on drinking did your hands shake, did you sweat, or feel agitated ? Or, did you drink to avoid these symptoms or to avoid being hangover, e.g., "the shakes", sweating or agitation ? IF YES TO EITHER, CODE YES	NO	YES	3
c	During the times when you drank alcohol, did you end up drinking more than you planned when you started ?	NO	YES	4
d	Have you tried to reduce or stop drinking alcohol but failed ?	NO	YES	5
e	On the days that you drank, did you spend substantial time in obtaining alcohol, drinking, or in recovering from the effects of alcohol ?	NO	YES	6
f	Did you spend less time working, enjoying hobbies, or being with others because of your drinking ?	NO	YES	7
g	Have you continued to drink even though you knew that the drinking caused you health or mental problems ?	NO	YES	8

ARE **3** OR MORE **J2** ANSWERS CODED **YES** ?

NO	**YES**
ALCOHOL DEPENDENCE CURRENT	

DOES THE PATIENT CODES POSITIVES FOR ALCOHOL DEPENDENCE ? NO → YES

J3	**In the past 12 months :**			
a	Have you been intoxicated, high, or hangover more than once when you had other responsibilities at school, at work, or at home ? Did this cause any problems ? CODE YES ONLY IF THIS CAUSED PROBLEMS	NO	YES	9
b	Were you intoxicated in any situation where you were physically at risk, e.g., driving a car, riding a motor bike, using machinery, boating, etc. ?	NO	YES	10

➔ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

c Did you have any legal problems because of your drinking, e.g., an arrest or disorderly conduct ? NO YES 11

d Did you continue to drink even though your drinking caused problems with your family or other people ? NO YES 12

ARE **1** OR MORE **J3** ANSWERS CODED **YES** ?

NO **YES**

ALCOHOL ABUSE
CURRENT

AMPHETAMINE
CANNABIS
COCAINE
CODEINE
CRACK
DICONAL
ECSTASY
ETHER
FREEBASE
GASOLINE
GLUE
GRASS
HASHISH
HEROIN
LSD
MARIJUANA
MESCALINE
METHADONE
MORPHINE
OPIUM
PALFIUM
PCP
RITALIN
TEMGESIC
THC
TOLUENE
TRICHLORETHYLENE

➔ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

K. NON-ALCOHOL PSYCHOACTIVE SUBSTANCE USE DISORDERS

K1 Now I am going to show you (SHOW THE CARD OF SUBSTANCES) / to read to you a list (READ THE LIST BELOW) of street drugs or medicines. In the past 12 months, did you take any of these drugs, more than once, to get high, to feel better or to change your mood ?

➔ NO YES

CIRCLE EACH DRUG TAKEN :

Stimulants : amphetamines, « speed », crystal meth, « rush », Dexedrine, Ritalin, diet pills.
Cocaine : snorting, IV, freebase, crack, « speedball ».
Narcotics : heroin, morphine, dilaudid, opium, demerol, methadone, codeine, percodan, darvon.
Hallucinogens : LSD (« acid »), mescaline, peyote, PCP (« angel dust », « peace pill »), psilocybin, STP, « mushrooms », ecstasy, MDA, or MDMA.
Inhalants : « glue », ethyl chloride, nitrous oxide, (« laughing gas »), amyl or butyl nitrate (« poppers »).
Marijuana : hashish (« hash »), THC, « pot », « grass », « weed », « reefer ».
Tranquilizers : quaalude, Seconal (« reds »), Valium, Xanax, Librium, Ativan, Dalmane, Halcion, barbiturates, Miltown.
Miscellaneous : steroids, nonprescription sleep or diet pills. Any others ?

SPECIFY MOST USED DRUG(S) : _____

SPECIFY WHICH WILL BE EXPLORED IN CRITERIA BELOW **:**

- IF CONCURRENT OR SEQUENTIAL POLYSUBSTANCE USE :
  EACH DRUG (OR DRUG CLASS) USED INDIVIDUALLY
  MOST USED DRUG (OR DRUG CLASS) ONLY
- IF ONE DRUG (OR DRUG CLASS) USED :
  SINGLE DRUG (OR DRUG CLASS) ONLY

K2 **Considering your use of [NAME THE SELECTED DRUG / DRUG CLASS] in the past 12 months :**

a Have you found that you needed to use more of [NAME OF SELECTED DRUG / DRUG CLASS] to get the same effect than you did when you first started taking it ? NO YES 1

b When you reduced or stopped using [NAME OF SELECTED DRUG / DRUG CLASS] did you have withdrawal symptoms (aches, shaking, fever, weakness, diarrhea, nausea, sweating, heart pounding, difficulty sleeping, or feeling agitated, anxious, irritable or depressed) ?
Or did you use any drug(s) to keep yourself from getting sick (WITHDRAWAL SYMPTOMS) or so that you would feel better ?
IF **YES** TO EITHER, CODE **YES** NO YES 2

c Have you often found that when you used [NAME OF SELECTED DRUG / DRUG CLASS], you ended up taking more than you thought you would ? NO YES 3

d Have you tried to reduce or stop taking [NAME OF SELECTED DRUG / DRUG CLASS] but failed ? NO YES 4

e On the days that you used [NAME OF SELECTED DRUG / DRUG CLASS], did you spend substantial time (>2 hours), obtaining, using or recovering from the effects, or thinking about it ? NO YES 5

f Did you spend less time working, enjoying hobbies, or being with family or friends, because of your drug use ? NO YES 6

g Have you continued to use [NAME OF SELECTED DRUG / DRUG CLASS] even though it caused you health or mental problems? NO YES 7

ARE **3** OR MORE **K2** ANSWERS CODED **YES** ?

SPECIFY DRUG(S) : _____

NO	**YES**
DRUG(S) DEPENDENCE CURRENT	

➔

DOES PATIENT CODES POSITIVE FOR DRUG DEPENDENCE ? NO YES

K3 **In the past 12 months :**

a Have you been intoxicated, high, or hangover from [NAME OF SELECTED DRUG / DRUG CLASS], more than once when you had other responsibilities at school, at work, or at home ? Did this cause any problem ? (CODE YES ONLY IF THIS CAUSED PROBLEMS) NO YES 8

b Have you been high or intoxicated from [NAME OF SELECTED DRUG / DRUG CLASS] in any situation where you were physically at risk (e.g., driving a car, or a motorbike, using machinery, boating, etc.) ? NO YES 9

c Did you have any legal problems because of your [NAME OF SELECTED DRUG / DRUG CLASS] use, e.g., an arrest or disorderly conduct ? NO YES 10

d Did you continue to use [NAME OF SELECTED DRUG / DRUG CLASS] even though it caused problems with your family or other people ? NO YES 11

ARE **1** OR MORE **K3** ANSWERS CODED **YES** ?

SPECIFY DRUG(S) : _____

NO	**YES**
DRUG(S) ABUSE CURRENT	

L. PSYCHOTIC DISORDERS

ASK FOR AN EXAMPLE OF EACH QUESTION ANSWERED POSITIVELY. CODE YES ONLY IF THE EXAMPLES CLEARLY SHOW A DISTORTION OF THOUGHT OR OF PERCEPTION OR IF THEY ARE NOT CULTURALLY APPROPRIATE.
BEFORE CODING, INVESTIGATE WHETHER DELUSIONS QUALIFY AS « BIZARRE ».

DELUSIONS ARE BIZARRE IF : CLEARLY IMPLAUSIBLE, ABSURD, NOT UNDERSTANDABLE, AND CANNOT DERIVE FROM ORDINARY LIFE EXPERIENCE.

HALLUCINATIONS ARE RATED BIZARRE IF : A VOICE COMMENTS ON THE PERSON'S THOUGHTS OR BEHAVIOR, OR WHEN TWO OR MORE VOICES ARE CONVERSING WITH EACH OTHER.

Now I'm going to ask you about unusual experiences that some individuals may experience.

				BIZARRE	
L1 a	Have you ever believed that people were spying on you, or that someone was plotting against you, or trying to hurt you ?	NO	YES	YES	1
b	**IF YES** : Do you currently believe these things ?	NO	YES	YES → **L6a**	2
L2 a	Have you ever believed that someone was reading your mind or could hear your thoughts or that you could actually read or hear what another person was thinking ?	NO		YES	3
b	**IF YES** : Do you currently believe these things ?	NO		YES → **L6a**	4
L3 a	Have you ever believed that someone or some force outside of yourself put thoughts in your mind that were not your own, or made you act in a way that was not your usual self ?Have you ever felt that you were possessed	NO		YES	5
b	**IF YES** : Do you currently believe these things ?	NO		YES → **L6a**	6
L4 a	Have you ever believed that you were being sent special messages through the TV, radio or newspaper, or that a person you did not personally know was particularly interested in you ?	NO	YES	YES	7
b	**IF YES** : Do you currently believe these things ?	NO	YES	YES → **L6a**	8
L5 a	Have your relatives or friends ever considered any of your beliefs strange or out of reality ? ANY DELUSIONAL IDEAS NON EXPLORED IN QUESTIONS L1 TO L4, E.G., OF GRANDIOSITY, RUIN, GUILT, HYPOCONDRIASIS,...	NO	YES	YES	9
b	**IF YES** : Do they currently consider your beliefs strange ?	NO	YES	YES	10
L6 a	Have you ever heard things other people couldn't hear, such as voices ? HALLUCINATIONS ARE CODED « BIZARRE » ONLY IF PATIENT ANSWERS YES TO THE FOLLOWING : Did you hear a voice commenting on your thoughts or behavior, or did you hear two or more voices talking to each other ?	NO	YES	YES	11
b	**IF YES** : Have you heard these things in the past month ?	NO	YES	YES → **L8b**	12

L7 a Have you ever had visions when you were awake or have you ever seen things other people couldn't see ? NO YES 13
CODE YES ONLY IF THE VISIONS ARE CULTURALLY INAPPROPRIATE.

b **IF YES** : Have you seen these things in the past month? : NO YES 14

INTERVIEWER'S JUDGMENT :

L8 b IS THE PATIENT CURRENTLY EXHIBITING INCOHERENCE, DISORGANIZED SPEECH, OR MARKED LOOSENING OF ASSOCIATIONS ? NO YES 15

L9 b IS THE PATIENT CURRENTLY EXHIBITING DISORGANIZED OR CATATONIC BEHAVIOR ? NO YES 16

L10b ARE NEGATIVE SYMPTOMS OF SCHIZOPHRENIA, E.G. SIGNIFICANT AFFECTIVE FLATTENING, POVERTY OF SPEECH (ALOGIA) OR AN INABILITY TO INITIATE OR PERSIST IN GOAL DIRECTED ACTIVITIES (AVOLITION), PROMINENT DURING THE INTERVIEW ? NO YES 17

L11 FROM **L1 TO L10** :
- ARE **1 OR MORE** « **b** » QUESTIONS CODED **YES BIZARRE** ?

OR
- ARE **2** OR MORE « **b** » QUESTIONS CODED **YES** (RATHER THAN YES BIZARRE) ?

NO **YES**

PSYCHOTIC SYNDROME CURRENT

L12 FROM **L1 TO L7** :
- ARE **1** OR MORE « **a** » QUESTIONS CODED **YES BIZARRE** ?

OR
- ARE **2** OR MORE « **a** » QUESTIONS CODED **YES** (RATHER THAN YES BIZARRE**)** ?

(CHECK THAT THE 2 SYMPTOMS OCCURRED DURING THE SAME TIME PERIOD)

OR
- IS **L11** CODED **YES** ?

NO **YES**

PSYCHOTIC SYNDROME LIFETIME

L13a IF L12 IS CODED YES OR AT LEAST ONE YES FROM L1 TO L7 :

DOES THE PATIENT CODE POSITIVE FOR EITHER
MAJOR DEPRESSIVE EPISODE (CURRENT OR PAST)
OR MANIC EPISODE (CURRENT OR PAST) ? → NO YES

b You told me earlier that you had period(s) when you felt (depressed/ high/ persistently irritable).
Were the beliefs and experiences you just described (SYMPTOMS CODE YES FROM L1 TO L7) restricted exclusively to times when you were feeling depressed / high / irritable ? NO YES 18

IS **L13b** CODED **YES** ?

NO **YES**

MOOD DISORDER WITH PSYCHOTIC FEATURES CURRENT

➔ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

M. ANOREXIA NERVOSA

M1 a	How tall are you ?	|__|__|__|	Ft ☐ Ins ☐ Cm ☐	
b	What was your lowest weight in the past 3 months ?	|__|__|__|	Lbs. ☐ Kg ☐	
c	IS PATIENT'S WEIGHT LOWER THAN THE THRESHOLD CORRESPONDING TO HIS / HER HEIGHT ? SEE TABLE BELOW	➔ NO	YES	1

In the past 3 months :

M2	In spite of this low weight, have you tried not to gain weight ?	➔ NO	YES	2
M3	Have you feared gaining weight or becoming fat, even though you were underweight ?	➔ NO	YES	3
M4 a	Have you considered yourself fat or that part of your body was too fat ?	NO	YES	4
b	Has your body weight or shape greatly influenced how you felt about yourself ?	NO	YES	5
c	Have you thought that your current low body weight was normal or excessive ?	NO	YES	6
M5	ARE **1** OR MORE **M4** ANSWERS CODED **YES** ?	➔ NO	YES	
M6	FOR WOMEN ONLY : During the last 3 months, did you miss all your menstrual periods when they were expected to occur (when you were not pregnant) ?	➔ NO	YES	7

FOR WOMEN : ARE **M5** AND **M6** CODED **YES** ?
FOR MEN : IS **M5** CODED **YES** ?

NO **YES**

ANOREXIA NERVOSA CURRENT

TABLE HEIGHT / WEIGHT THRESHOLD (HEIGHT-WITHOUT SHOES ; WEIGHT-WITHOUT CLOTHING)

HEIGHT(cm)		140	145	150	155	160	165	170	175	180	185	190
WEIGHT (kg)	Females	37	38	39	41	43	45	47	50	52	54	57
	Males	41	43	45	47	49	51	52	54	56	58	61

THE WEIGHT THRESHOLDS ABOVE ARE CALCULATED AS A 15% REDUCTION BELOW THE NORMAL RANGE FOR THE PATIENT'S HEIGHT AND GENDER AS REQUIRED BY DSM-IV.

→ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

N. BULIMIA NERVOSA

N1	In the past three months, did you have eating binges or times when you ate a very large amount of food within a 2-hour period ?	→ NO	YES	8
N2	In the last three months, did you have eating binges as often as twice a week ?	→ NO	YES	9
N3	During these binges, did you feel that your eating was out of control ?	→ NO	YES	10
N4	Did you do anything to compensate for, or to prevent a weight gain from these binges, like vomiting, fasting, exercising or taking laxatives, enemas, diuretics (fluid pills), or other medications ?	→ NO	YES	11
N5	Does your body weight or shape greatly influence how you feel about yourself ?	→ NO	YES	12
N6	DOES THE PATIENT'S SYMPTOMS MEET CRITERIA FOR ANOREXIA NERVOSA ? IF **N6** = **NO**, SKIP TO **N8**	NO	YES	13
N7	Do these binges occur only when you are under _____kg/lbs.* ? * TAKE THE THRESHOLD WEIGHT FOR THIS PATIENT'S HEIGHT / WEIGHT TABLE IN THE ANOREXIA NERVOSA MODULE	NO	YES	14

IS **N5** CODED **YES** AND **N7** CODED **NO** (OR SKIPPED) ?

NO **YES**

BULIMIA NERVOSA
CURRENT

IS **N7** CODED **YES** ?

NO **YES**

ANOREXIA NERVOSA
Binge-Eating/Purging Type
CURRENT

➔ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

O. GENERALIZED ANXIETY DISORDER

O1 a	Have you worried excessively or been anxious about several things of day to day life, at work, at home, in your close circle over the past 6 months ? DO NOT CODE YES IF THE FOCUS OF THE ANXIETY IS CONFINED TO ANOTHER DISORDER EXPLORED PRIOR TO THIS POINT SUCH AS HAVING A PANIC ATTACK (PANIC DISORDER), BEING EMBARRASSED IN PUBLIC (SOCIAL PHOBIA), BEING CONTAMINATED (OCD), GAINING WEIGHT (ANOREXIA NERVOSA)...	➔ NO	YES	1
b	Are these worries present most days ?	➔ NO	YES	2
O2	Do you find it difficult to control the worries or do they interfere with your ability to focus on what you are doing ? FROM O3a TO O3f, CODE NO THE SYMPTOMS CONFINED TO FEATURES OF ANY DISORDER EXPLORED PRIOR TO THIS POINT	➔ NO	YES	2
O3	**When you were anxious over the past 6 months, did you, almost every day :**			
a	Feel restless, keyed up or on edge ?	NO	YES	3
b	Feel tense ?	NO	YES	4
c	Feel tired, weak or exhausted easily ?	NO	YES	5
d	Have difficulty concentrating or find your mind going blank ?	NO	YES	6
e	Feel irritable ?	NO	YES	7
f	Have difficulty sleeping (difficulty falling asleep, waking up in the middle of the night, early morning wakening or sleeping excessively) ?	NO	YES	8

ARE **3** OR MORE **O3** ANSWERS CODED **YES** ?

NO	**YES**
GENERALIZED ANXIETY DISORDER CURRENT	

➔ MEANS : **GO** TO THE DIAGNOSTIC BOX(ES) OF THIS MODULE, **CIRCLE NO** IN ALL OF THEM AND **MOVE** TO THE NEXT MODULE

P. ANTISOCIAL PERSONALITY DISORDER (optional)

P1 **Before you were 15 years old, did you :**

a	Repeatedly skip school or run away from home overnight ?	NO	YES	1
b	Repeatedly lie, cheat, « con » others, or steal ?	NO	YES	2
c	Start fights or bully, threaten, or intimidate others ?	NO	YES	3
d	Deliberately destroy things or start fires ?	NO	YES	4
e	Deliberately hurt animals or people ?	NO	YES	5
f	Force someone to have sex with you ?	NO	YES	6

➔

ARE **2** OR MORE **P1** ANSWERS CODED **YES** ? NO YES

P2 DO NOT CODE YES THE BEHAVIORS BELOW IF THEY ARE EXCLUSIVELY POLITICALLY OR RELIGIOUSLY MOTIVATED

Since you were 15 years old, have you :

a	Repeatedly behaved in a way that others would consider irresponsible, like failing to pay for things you owed, deliberately being impulsive or deliberately not working to support yourself ?	NO	YES	7
b	Done things that are illegal even if you didn't get caught (i.e., destroying property, shoplifting, stealing, selling drugs, or committing a felony) ?	NO	YES	8
c	Been in physical fights repeatedly (including physical fights with your spouse or children) ?	NO	YES	9
d	Often lied or « conned » other people to get money or pleasure, or lied just for fun ?	NO	YES	10
e	Exposed others to danger without caring ?	NO	YES	11
f	Felt no guilt after hurting, mistreating, lying to, or stealing from others, or after damaging property ?	NO	YES	12

ARE **3** OR MORE ITEMS FROM **P2** CODED **YES** ?

NO **YES**

ANTISOCIAL PERSONALITY DISORDER LIFETIME

REFERENCES

Lecrubier Y, Sheehan D, Weiller E, Amorim P, Bonora I, Sheehan K, Janavs J, Dunbar G. The Mini International Neuropsychiatric Interview (M.I.N.I.), a short diagnostic interview : Reliability and validity according to the CIDI. *European Psychiatry*, 1997 ; **12** : 224-231.

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Sheehan DV, Lecrubier Y, Harnett Sheehan K, Amorim P, Janavs J, Weiller E, Hergueta T, Baker R, Dunbar G. The Mini International Neuropsychiatric Interview (M.I.N.I.), : The development and validation of a structured diagnostic psychiatric interview. *Journal of Clinical Psychiatry*, 1998 ; 59 [suppl 20] : 22-33.

Amorim P, Lecrubier Y, Weiller E, Hergueta T, Sheehan D. DSM-III-R Psychotic disorders : procedural validity of the Mini International Neuropsychiatric Interview (M.I.N.I.). Concordance and causes for discordance with the CIDI. *European Psychiatry*, 1998 ; **13** : 26-34.

The M.I.N.I. was developed simultaneously into French and English. The French and English original versions of the M.I.N.I. for DSM-IV were translated and can be asked to the authors (see page 3). An ICD-10 version is also available into French, English and Danish.

Translations	**M.I.N.I. 4.4 or earlier versions**	**M.I.N.I. 5.0, M.I.N.I. Plus 5.0, M.I.N.I. screen 5.0**
Afrikaans		R. Emsley
Arabic		O. Osman, E. Al-Radi
Basque		In preparation
Bengali		H. Banerjee, A. Banerjee
Brazilian	P. Amorim	In preparation
Bulgarian		L.G. Hranov
Catalan		In preparation
Czech	P. Zvolsky	P. Zvolsky
Chinese		L. Caroll
Croatian		In preparation
Danish	P. Bech	P. Bech, T. Scütze
Dutch/Flemish	E. Griez, K. Schruers, T. Overbeek, K. Demyttenaere	I. van Vliet, H. Leroy, H. van Megen
Farsi/Persian		K. Khooshabi, A. Zomorodi
Finnish	M. Heikkinen, M. Lijeström, O. Tuominen	In preparation
German	I. van Denffer, M. Ackenheil, R. Dietz-Bauer	M. Ackenheil, G. Stotz, R. Dietz-Bauer
Gujarati		M. Patel, B. Patel
Greek	S. Beratis	T. Calligas, S. Beratis
Hebrew	J. Zohar, Y. Sasson	R. Barda, I. Levinson
Hindi		K. Batra, S. Gambir
Hungarian	I. Bitter, J. Balazs	I. Bitter, J. Balazs
Italian	P. Donda, E. Weiller, I. Bonora	L. Conti, P. Donda, A. Rossi, M. Piccinelli, M. Tansella, G. Cassano
Japanese		H. Watanabe
Latvian	V. Janavs, J. Janavs, I. Nagobads	V. Janavs, J. Janavs
Norwegian	G. Pedersen, S. Blomhoff	K. Leiknes, U. Malt, E. Malt
Polish	M. Masiak, E. Jasiak	M. Masiak, E. Jasiak
Portuguese	P. Amorim	P. Amorim, T. Guterres
Punjabi		S. Gambir
Romanian		O. Driga
Russian		A. Bystitsky, E. Selivra, M. Bystitsky
Serbian	I. Timotijevic	I. Timotijevic
Setswana		K. Ketlogetswe
Slovenian	M. Kocmur	M. Kocmur
Spanish	L. Ferrando, J. Bobes-Garcia, J. Gibert-Rahola	L. Ferrando, L. Franco-Alfonso, M. Soto, J. Bobes, O. Soto, L. Franco, J. Gibert
Swedish	M. Waern, S. Andersch, M. Humble	C. Allgulander, M. Waern, A. Brimse, M. Humble
Turkish	T. Örnek, A. Keskiner, I. Vahip	T. Örnek, A. Keskiner
Urdu		A. Taj, S. Gambir
Welsh		In preparation

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APPENDIX C1

SAMPLE OF CONSENT FORM – PATIENT

SUBJECT INFORMATION AND CONSENT

Research Title: Identification of Potential Chemical Markers, Biomarkers and Genetics marker for Schizophrenia (SZ).

Sponsor: Universiti Tunku Abdul Rahman

Project leader: Dato' Dr Suarn Singh a/l Jasmit Singh

Principal investigator: Dr. Cheah Yee Chuang

Co-investigators: Dr. Loh Han Chern, Dr. Woo Kwan Kit, Dr.Tang Pek Yee, Dr Rabaiah binti Mohd Salleh, Dr Bilbir Kaur, Dr Zulkifli Ghaus, Dr Loo Tak Wah, Dr Raziffah Abdul Rahman, Dr Satnam Kaur.

Institution address: Hospital Bahagia Ulu Kinta, 31250, Tanjung Rambutan, Perak.

The nature and purpose of the study

You understand that you are being asked to take part in a research that will involve a medical history, mental assessment using MINI scale and blood withdrawing of 20 cc. for chemical analysis in local laboratories. The blood sample for biochemical analysis will be disposed immediately after analysis, whereas the blood sample for biomarker and genetic study will be kept for two years before disposal.

The objectives of this research are to identify the chemical, biological and genetic markers in subjects with Schizophrenia (SZ). The secondary objectives are to:

a) To explore the relationship between markers and Schizophrenia on the following aspects: age, gender, age of onset, duration of illness, family history, subtypes (paranoid, disorganized, catatonic, undifferentiated and residual), medical history.
b) As a preliminary study for the development of SZ marker sensor development in the future.
c) To fabricate diagnosis kit for SZ measurement in the future.
d) To understand the complex inheritance of SZ.

Study description

If you decide to participate in this study, you will be one of 1000 subjects to be compared to a controlled group of 200 healthy volunteers. The maximum length of time of your participation in this study will be about 40 minutes. While participating in the study, you are expected to cooperate in answering the questionnaire and allow 20 cc of venous blood to be drawn for chemical analysis in local laboratories.

You understand that as a subject on a research study, you have certain responsibilities. Your responsibilities are to understand the protocol and give consent freely if you agree to do so.

Risk of participating in this study

Blood drawing may cause a small amount of pain. In addition, a temporary bruise or "black and blue mark" may develop.

Possible benefits of study participation
With identification of the chemical, biological, and genetic markers in patients with schizophrenia, you may achieve better understanding of the illness, early detection and with prompt intervention, and hopefully predict suitability of medication.

To obtain further information
Please ask your investigator if you do not understand or need more information or explanation about this research.

Voluntary participation
You understand that participation in this study is voluntary and that if you decide not to participate, you will experience no penalty or loss of benefits to which you would otherwise be entitled outside of this study. If you decide to participate, you may change your mind about being in the study, and may stop at any time. You understand that you must inform the doctor of this decision immediately. You understand that such a decision on your part will not influence the availability of future medical care or other benefits to which you are otherwise entitled outside of this study.

You understand that you or your legally acceptable representative will be informed in a timely manner of any new information that may affect your willingness to continue participation in this study. You will not receive any compensation for your participation in the trial. However, reimbursement on payment for transport to and from clinic for the visits scheduled in the study will be paid.

Confidentiality
All information, samples and specimens you have supplied will be kept confidential by the principal investigator and the research team. Your identity will not be made available to the public. If the result of research is published, your identity will be remained confidential fully. References will be recorded in code number form. However, you have no Intellectual Property Rights on the genetic outcome of the research. The outcome can be made available to the regulatory body and ethical committee of the Ministry of Health, Malaysia.

CONSENT TO PARTICIPATE IN THIS STUDY
I have read, or have had been read to me, in language understandable to me, the above information. The content and meaning of this information has been fully explained to me.

I have had time and opportunity to ask any questions that I have about the study and this form, and all my questions have been answered. I have read, or have been read to me, all pages of this consent form and the risks described. I voluntarily consent and offer to take part in this study. By signing this consent form, I certify that all information I have given, including my medical history, is true and correct to the best of my knowledge.
I understand that I will receive a copy of this signed consent form.

Printed name of subject	IC. No.
Signature of subject	Date
Thumbprint of subject (if applicable)	

Printed name of witness	IC. No.
Signature of witness	Date
Printed name of legally authorized representative (if applicable)	IC. No.
Signature of legally authorized representative	Date

Relationship to the subject: _____

*(The investigator, or person designated by the investigator to conduct the informed consent process, must sign **and** date form **at the same time as the subject**.)*

Printed name of person explaining consent	IC. No.
Signature of person explaining consent	Date

Appendix: Biodata and Medical History

Patient's initial:
ID:
Date of birth:
Gender:
Ethnic group:
Diagnosis by MINI:
Duration of illness:
No. of hospitalization:
Clinical Global Impression – Schizophrenia scale (CGI-S):
Brief assessment instrument – evaluates positive, negative, cognitive, depressive, and overall symptoms on the day of assessment on a seven-point scale.

1. Normal, not ill at all
2. Borderline ill
3. Mildly ill
4. Moderately ill
5. Markedly ill
6. Severely ill
7. Among most severely ill

Haro et al., Acta Psych Scand 2003; 107 (Suppl. 416): 16-23.

(a) Positive symptoms: ____
(b) Negative symptoms: ____

Comorbid substance abuse: Yes/No

1. Do you have any disease or physical problem?
   □ Yes, please state:_____
   (e.g. complicated birth history, perinatal infection/trauma, head injury, significant medical problems, e.g. HIV, Hepatitis, Hypertension, Diabetes Mellitus, Carcinoma, connective tissue disease)
   □ No
   □ Not sure

2. Do your family have any disease or physical problem?
   □ Yes, please state:_____
   □ No
   □ Not sure

3. Do you have any disease or mental problem?
   □ Yes, please state:_____
   □ No
   □ Not sure

4. Do your family have any disease or mental problem?
   □ Yes, please state:_____
   □ No
   □ Not sure

APPENDIX C2

SAMPLE OF CONSENT FORM - CONTROL

Research Consent Form

Universiti Tunku Abdul Rahman
FES Brain Science Research Group

Blood Draw Consent Form

Imprint Patient ID Number

BRIEF MEDICAL HISTORY

Particular of Volunteer:

Name: ……..… Age: …….. Ethnic: …….
NRIC: …… Blood Type: ……..……
Address: ……
Tel: (H) ……...… (HP) …….……..…….
Email address: …….

Please 'X' the answer:			
Have you ever had any of the following:		Yes	No
1	a serious illness or accident?		
2	an operation/investigative procedure?		
3	yellow jaundice or hepatitis?		
4	tuberculosis?		
5	malaria?		
6	a tattoo?		
7	a blood transfusion?		
8	contact with any infectious disease?		
9	heart disease?		
10	high blood pressure (>140/90 mmHg)?		
11	asthma?		
12	kidney disease?		
13	diabetes?		
14	a stomach ulcer?		
Do you or your family ever had any of the following:			
16	any cancer?		
17	contacted with any HIV carrier?		
18	psychiatric disease/mental problem?		

Blood Drawing Statement:
I certify that to the best of my knowledge all of my answers to the questions above are true. I voluntarily participate in this study (by FES Brain Science Research Group). Besides, I have read and signed the Blood Draw Consent Form at the next page.

Name: ……... Signature:…….……

Date: ……..

Research Consent Form

Universiti Tunku Abdul Rahman
FES Brain Science Research Group

Blood Draw Consent Form

Imprint Patient ID Number

DRAWING BLOOD FOR RESEARCH PURPOSES

PURPOSE: We would like permission to enroll you as a participant in a research study **to identify potential markers for Schizophrenia (SZ) and to develop SZ biosensor.**

PROCEDURE: **Sample language provided below for studies involving minimal risk and blood draw only from:**

1) **healthy adults weighing at least 40 kg for a blood draw in amounts not to exceed 20 cc;**
2) **other adults or children, considering their age, weight and health status, for a blood draw in amounts not to exceed 20 cc.**

by qualified medical personnel.

A brief medical history will be taken (you may choose not to complete it), and your temperature and heart rate will be measured. A small amount of blood will be taken by finger stick or by vein (needle stick). A needle will be inserted and up to 20cc (4 tsp) of blood will be withdrawn. We will make sure you are feeling well after your blood is drawn.

PAYMENT: You will not be paid for providing your blood sample. Your blood sample may be used to create new tests or treatments that could have commercial value. Researchers or the university may benefit financially if this happens. You will not be paid or benefit financially if this happens.

RISKS: Blood drawing may cause a small amount of pain. In addition, a temporary bruise or "black and blue mark" may develop. Very rarely, the vein in which the needle has been inserted may become inflamed or infected, which can be treated.

BENEFITS: Collection of your blood will not provide any direct benefit to you.

CONFIDENTALLY: Information obtained for this research study will be stored in the investigator's research files and will be identified only by a number. Your name or other information that could be used to identify you will not be recorded with or linked to the sample or health information that has been collected. This means that the blood samples and related health information cannot be linked back to you.

ALTERNATIVES: This research study does not involve treatment or diagnosis. The alternative to participation is not to participate.

STUDY CONTACTS: You can reach **Dr. Loh Han Chern (012-3124877) or Dr. Tang Pek Yee (016-2877304)** if you have questions.

SIGNATURE: I've read this consent form and understood the purpose of the research, the study procedures, possible risks and discomforts as well as potential benefit and alternatives. My signature below indicates my willingness to participate in this study.

Subject: Date:

Signature:

APPENDIX D

PUBLICATIONS

Journal Publications:

Chow, T. J., Loh, H. C., Tee, S. F. and Tang, P. Y. (2010). Evaluation of carotenoid level in schizophrenic patients using non-invasive measurement. *Asian Journal of Psychiatry*, 3(4), 190 - 193.

Publications in Conferences - Oral:

Loh, H. C. and **Chow, T. J.** (2010). *Novel Apolipoproteins as Biological Markers in Schizophrenia.* The 11th Biennial Australasian Schizophrenia Conference, ASC 2010 Molecules to Mind. September 22 - 24. Sydney: Sheraton on the Park.

Loh, H. C., **Chow, T. J.**, Tee, S. F. and Tang, P. Y. (2009). *Association of Carotenoid Antioxidant and Schizophrenia using Non-Invasive Measurement.* The 15th Biennial Winter Workshop in Psychoses. November 15 - 18. Barcelona: Hotel Fire Palace.

Chow, T. J., Tee, S. F., Loh, H. C., Tang, P. Y., Woo, K. K., Jasmit Singh, S. S., Cheah, Y. C., Salleh, R. M., Loo, T. W., Rahman, R. A., Harbhajan Singh, S. K., Abdullah, A. S. C., Narayanan, U. A., Chingara Singh, B. K. and Ghaus, Z. (2008). *Progress in Schizophrenia Markers Development.* The 7th Perak Mental Health Convention, Prioritising Mental Health - Enhancing Services through Partnership and Advocacy. October 10 - 11. Ipoh, Malaysia: Impiana Casuarina Hotel.

Publications in Conferences - Poster:

Loh, H. C. and **Chow, T. J.** (2010). *In vivo Quantification of Antioxidants Schizophrenic Patients.* The 12th Asian-Pacific Congress of Clinical Biochemistry. October 3 - 7. Seoul: COEX.

Loh, H. C. and **Chow, T. J.** (2010). *Apolipoprotein H as Novel Biological Marker in Schizophrenia.* The 12th Asian-Pacific Congress of Clinical Biochemistry. October 3 - 7. Seoul: COEX.

Loh, H. C. and **Chow, T. J.** (2010). *Non-Invasive Quantification of Carotenoid Antioxidant in Schizophrenic Patients.* The 11th Biennial Australasian Schizophrenia Conference, ASC 2010 Molecules to Mind. September 22 - 24. Sydney: Sheraton on the Park.

Chow, T. J., Tee, S. F., Loh, H. C. and Tang, P. Y. (2009). *Role of carotenoid level in schizophrenia.* The 9th World Congress of Biological Psychiatry. June 28 - July 2. France: Palais des Congrès de Paris.