DETERMINATION OF LYMPHOCYTE SUBSETS IN PATIENTS WITH MAJOR DEPRESSIVE DISORDER (MDD)

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2018
DETERMINATION OF LYMPHOCYTE SUBSETS
IN PATIENTS WITH MAJOR DEPRESSIVE DISORDER (MDD)

by

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Thesis submitted in fulfillment of the requirement for the Degree of Master of Sciences

January 2018
ACKNOWLEDGEMENT

In the name of Allah, the Most Generous and the Most Merciful. All praise be to Allah, for His blessing and guidance has helped me throughout this study.

First of all, I would like to express my utmost gratitude to my supervisor, Dr. Noor Suryani Mohd Ashari for her support, guidance and kind supervision throughout this study. I also would like to thank my co-supervisor, Dr. Mohd Azhar Mohd Yasin, my co-researchers, Dr. Che Maraina Che Husin, Dr. Rohimah Mohamud and Dr. Mohd Nazri Shafie for their assistance and great advices. Without their support, this study would not have been possible.

Special thanks to medical laboratory technologist, En. Jamaruddin Hassan for his time and effort in helping me with flow cytometric work. I also would like to thank nurses at Psychiatric Clinic, HUSM for their help with patients recruitment. Not to forget, my friends and staffs at Immunology Department for their support and sharing ideas together. I owe a lifetime gratitude to my parents and family for their great support and prayers in any situation or circumstances throughout this study.

Last but not least, a special thanks goes to Ministry of Higher Education for MyBrain15 scholarship. Finally, I would like to thanks all people that were involved directly or indirectly in this study. May Allah bless all of you.

Thank you.

Siti Nor Fairus Binti Mohamed Sanusi

P-UM0010/15 (R)
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LIST OF ABBREVIATIONS

α  level of significance

ACTH  adrenocorticotropic hormone

AIDS  acquired immunodeficiency syndrome

AR  allergic rhinitis

AVP  Vasopressin

β  Beta

BCR  B cell receptor

BDI  Beck Depression Inventory

CD  cluster of differentiation

CRH  corticotropin releasing hormone

DASS  Depression, Anxiety and Stress Scale

DSM-V  Diagnostic and Statistical Manual of Mental Disorders Fifth Edition

DCs  dendritic cells

HIV  human immunodeficiency virus

HPA  hypothalamic pituitary adrenal

HUSM  Hospital Universiti Sains Malaysia

IFN  interferon

IDO  indoleamine 2,3 dioxygenase

IL  interleukin

IQR  interquartile range

MADRS  Montgomery-Asberg Depression Rating Scale

MDD  major depressive disorder
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<td>major histocompatibility complex</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>n</td>
<td>number of sample</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NKCA</td>
<td>natural killer cell activity</td>
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<tr>
<td>PBS</td>
<td>phosphate buffer solution</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SPSS</td>
<td>statistical package for the social sciences</td>
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<tr>
<td>TCR</td>
<td>T cell receptors</td>
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<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>T_{C}</td>
<td>T cytotoxic</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>T_{H}</td>
<td>T helper</td>
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<tr>
<td>Tregs</td>
<td>regulatory T cells</td>
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<tr>
<td>µl</td>
<td>microliter</td>
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<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>%</td>
<td>percent</td>
</tr>
<tr>
<td>1-β</td>
<td>power of study</td>
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<tr>
<td>°C</td>
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<td>γ</td>
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ABSTRAK

mutlak subset limfosit antara pesakit MDD dengan tahap keterukan yang berbeza.
Hasil kajian menunjukkan bahawa mereka yang berkahwin, perokok, mempunyai tahap pendidikan yang rendah dan tinggal di kawasan luar bandar mempunyai risiko yang lebih tinggi untuk mendapat penyakit MDD. Berdasarkan BDI, simptom yang biasa dilaporkan oleh pesakit MDD adalah keletihan dan cepat marah. Walau bagaimanapun berdasarkan MADRS, pesakit MDD juga mempunyai ketegangan dalaman, masalah tumpuan, ketidakupayaan untuk merasa dan pemikiran pesimis. Kajian ini menunjukkan bahawa tiada perbezaan yang signifikan dalam peratusan dan bilangan mutlak sel CD4+ T (p=0.148; p=0.190), sel CD8+ T (p=0.316; p=0.783), sel CD16+CD56+ NK (p=0.731; p=0.530), dan sel CD19+ B (p=0.136; p=0.148) antara pesakit MDD dan individu sihat. Walau bagaimanapun, kami mendapati peratusan dan bilangan mutlak sel CD4+ CD25+ Tregs (p<0.001) dan sel CD4+ CD25+ Foxp3+ Treg (p=0.003; p=0.001) pada pesakit MDD lebih tinggi berbanding dengan individu sihat. Kajian ini juga menunjukkan tiada perbezaan yang signifikan dalam peratusan dan bilangan mutlak subset limfosit antara pesakit MDD biasa, sederhana dan teruk.
DETERMINATION OF LYMPHOCYTE SUBSETS IN PATIENTS WITH MAJOR DEPRESSIVE DISORDER (MDD)

ABSTRACT

Major depressive disorder (MDD) has been associated with dysregulation of immune system. While many studies on activation of innate immune response currently dominates the research area, the dysregulation in adaptive immune system especially in circulating lymphocyte subsets has rarely been explored. Some studies indicated that the severity of MDD is important with respect to the extent of the immune changes in MDD patients. The objective of this study was to determine the predisposing factors of MDD and the common symptoms in MDD patients based on Beck Depression Inventory (BDI) and Montgomery-Asberg Depression Rating Scale (MADRS), and to determine the percentage and absolute count of lymphocyte subsets in MDD patients and their comparison between different severity of the disease. This study involved 47 MDD patients recruited from Psychiatric Clinic, Hospital Universiti Sains Malaysia (HUSM) and 47 healthy controls. MDD patients were diagnosed according to Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (DSM-V) criteria. The symptoms and severity of MDD was assessed using BDI and MADRS scale. The percentage and absolute count of CD4+ T cells, CD8+ T cell, CD4+ CD25+ Tregs, CD4+ CD25+ Foxp3+ Tregs, CD16+ CD56+ NK cells and CD19+ B cells were determined by using immunophenotyping technique. Mann-Whitney test was used to compare the percentage and absolute count of lymphocyte subsets between MDD patients and healthy controls. Kruskal-Wallis test was used to compare the percentage and absolute count of lymphocyte subsets.
between different severity of MDD. The results showed that those married, smoker, possess lower educational level and living in rural area have higher risk for MDD. Based on BDI, the most common symptoms reported by our MDD patients were fatigue and irritability. While based on MADRS, MDD patients also have inner tension, concentration difficulties, inability to feel and pessimistic thought. This study showed that there were no significant differences in the percentage and absolute count of CD4⁺ T cells \( (p=0.148 ; \ p=0.190 ) \), CD8⁺ T cells \( (p=0.316 ; \ p=0.783) \), CD16⁺CD56⁺ NK cells \( (p=0.731 ; \ p=0.530) \), and CD19⁺ B cells \( (p=0.136 ; \ p=0.148) \) between MDD patients and healthy controls. However, we found a significantly higher percentage and absolute count of CD4⁺ CD25⁺ Tregs \( (p<0.001) \) and CD4⁺ CD25⁺ Foxp3⁺ Treg cells \( (p=0.003 ; \ p=0.001) \) in MDD patients compared with healthy controls. This study also showed that there were no significant differences in the percentage and absolute count of lymphocyte subsets between mild, moderate and severe MDD patients.
CHAPTER 1

INTRODUCTION

1.1 Major Depressive Disorder (MDD)

Major depressive disorder (MDD) is the most common mental disorder reported worldwide. MDD is characterised by the presence of psychological symptoms like sadness, loss of interest or pleasure, feelings of guilt or worthlessness and recurrent thoughts of death or suicide. These symptoms present together with somatic symptoms like sleep or appetite disturbance, significant weight loss, concentration difficulties, physical agitation or retardation and fatigue (Fava and Kendler, 2000). MDD can be long-lasting or recurrent and its condition significantly affects an individual's family and personal relationships, work or school life, and general health (Moussavi et al., 2007).

1.1.1 Epidemiology of MDD

Major depressive disorder is a mental disorder that can affect all people regardless of age, geographic location, demographics, or social position (Firdaus Mukhtar and Tian P.S. Oei, 2011b). According to the World Health Organization (WHO), an estimated 350 million people of all ages suffer from depression (World Health Organization, 2016). It is projected that MDD will be the second leading cause of worldwide disability by the year 2020 and major contributor to the overall
global burden of disease (World Health Organization, 2016). In 2014, about 6.7% of the U.S population aged 18 or older had MDD (National Institute of Mental Health, 2016b).

Across the Asia Pacific region, the rates of current or 1-month MDD ranged from 1.3 to 5.5%, while the rates of MDD in the previous year ranged from 1.7 to 6.7% (Chiu, 2004). The lifetime occurrence of MDD in any country is between 8 to 10% (Malaysian Psychiatric Association, 2006). MDD is predicted to affect about 2.3 million people in Malaysia and at some point in their lives (Firdaus Mukhtar and Tian P.S. Oei, 2011b). The prevalence of MDD in general population in Malaysia is about 8 to 12% regardless of the geographical differences of the study setting (Ng, 2014). While in the primary care population, the prevalence ranged from 6.7 to 14.4% (Firdaus Mukhtar and Tian P. S. Oei, 2011).

Epidemiologic studies consistently shown that women were twice as likely as men to be classified as having MDD (Van de Velde et al., 2010; Bromet et al., 2011). In the south-east Asian region (SEAR), 7 to 12% of men are at a lifetime risk of MDD, while the incidence of MDD among women is 20 to 25% (Khan et al., 2011). The mean age of onset for MDD is in the late 20s (Bromet et al., 2011). In Asia Pacific region, the rates of MDD are highest in those aged 25 to 44 (Chiu, 2004). MDD is more common in urban than in rural population (Wang, 2004) and the prevalence is higher in groups with lower socioeconomic status (Lorant et al., 2007; Meriam Omar Din and Noraini M. Noor, 2010; Shi et al., 2014).
1.1.2 Aetiology of MDD

Major depressive disorder is a multifactorial and heterogeneous group of disorder involving both environmental and biological factors. Childhood trauma and stressful life events such as separation, loss of someone, sexual abuse and poverty are the most common causes of MDD (Kendler et al., 2002; Shapero et al., 2014). Besides that, MDD was also associated with history of chronic illness and social life events such as drug abused and alcohol consumption. Previous study showed that people with chronic illness was 2.7 times at risk to develop MDD, while those with recent alcohol consumption or drug abuse were 39.1 times at risk (Aris et al., 2014). The study also reported that among those MDD patients presented with chronic illness, majority of them suffered from Hypertension and Diabetes Mellitus (Aris et al., 2014).

Genetic factors also play a crucial role in the aetiology of MDD. Evidence from twin studies suggested that MDD has a concordance of 40% to 50%, while family studies indicated that first-degree relatives of depressed individuals are about three times as likely to develop MDD as the general population (Tsuang and Faraone, 1990). It has been found that genetic polymorphisms of serotonin transporter gene (5-HTTLPR) are involved in the development of MDD. Caspi et al. (2003) reported that individuals with one or two copies of the short allele of serotonin transporter gene (5-HTTLPR) experienced more depressive symptoms and have higher rates of MDD in response to stressful life events than individual who is homozygous for the long allele (Caspi et al., 2003).
In addition, neuroimaging studies showed that MDD patients had increased volume of the lateral ventricles and adrenal gland and smaller volumes of the basal ganglia, thalamus, hippocampus, and frontal lobe compared to healthy controls (Koolschijn et al., 2009). In conclusion, the onset of MDD involved a combination of genetic, psychologic and environmental factors.

1.1.3 Pathophysiology of MDD

The pathophysiology of MDD has not been clearly defined. However, several biological mechanism with a possible role in the pathophysiology of MDD have been identified. One of the major theories of MDD pathogenesis is the monoamine deficiency theory (Hasler, 2010). This theory suggested that the underlying pathophysiological basis of depression is the depletion of the neurotransmitter serotonin, norepinephrine or dopamine in the central nervous system (Dunlop and Nemeroff, 2007; Hasler, 2010). Most of the serotonergic, noradrenergic and dopaminergic neurons are located in midbrain and brainstem nuclei which project to large areas of the entire brain. This monoaminergic systems are involved in the regulation of a broad range of brain functions, including mood, attention, reward processing, sleep, appetite, and cognition (Belmaker and Agam, 2008). The deficiency of the neurotransmitters produces a number of physiological and behavioural alterations that resemble the symptoms of MDD, including low mood, decreased appetite, sleep disturbance, poor concentration and psychomotor alteration (Delgado, 2000). Almost every compound that inhibits monoamine reuptake, leading to an increased concentration of monoamines in the synaptic cleft, has been proven to be a clinically effective antidepressant (Belmaker and Agam, 2008).
The hyperactivity of hypothalamic pituitary adrenal (HPA) axis is also involved in the pathophysiology of MDD (Vreeburg et al., 2009; Stetler and Miller, 2011). This hyperactivity is caused by malfunctioning of glucocorticoid receptors impairing the negative feedback circuit of the HPA-axis. Glucocorticoid receptor malfunction might also cause MDD via impaired neurogenesis and reduced hippocampus volumes (Manji et al., 2003; Pariante and Lightman, 2008). Corticotropin releasing hormone (CRH) and vasopressin (AVP) are released from the hypothalamus in response to the perception of psychological stress by cortical brain regions. These hormones induce the secretion of adrenocorticotropic hormone (ACTH) from the pituitary, which stimulates the adrenal gland to release glucocorticoid (cortisol in human) into plasma. Glucocorticoids then interact with their receptors in multiple target tissues including the HPA axis, where they are responsible for feedback inhibition on both CRH and AVP from the hypothalamus and directly on secretion of ACTH from pituitary (Vreeburg et al., 2009). The activated HPA axis not only regulates body peripheral functions such as metabolism and immunity but also has profound effects on the brain. For example, glucocorticoids regulate neuronal survival, neurogenesis, the sizes of complex anatomical structures such as the hippocampus, the acquisition of new memories and emotional appraisal of events (Vreeburg et al., 2009; Stetler and Miller, 2011).

Mounting data indicate that cytokine plays a major role in neuropsychiatric diseases, including MDD (Schiepers et al., 2005; Miller et al., 2009). Patients with MDD have been shown to exhibit increases in inflammatory cytokines including tumour necrosis factor (TNF)-α, interleukin (IL)-1 and interleukin (IL)-6 in the peripheral blood and cerebrospinal fluid (Miller et al., 2009; Dowlati et al., 2010). Studies have
demonstrated that the cytokines and pro-inflammatory cytokines can activate the HPA axis and impair the central serotonin system (Dantzer et al., 2008; Miller et al., 2013). These cytokine-induced changes in neurotransmitter and neuroendocrine function have, in turn, been correlated with the development of MDD (Dantzer et al., 2008; Anisman, 2009; Raison et al., 2009; Miller et al., 2013). Moreover, administration of cytokines including interferon (IFN)-α and cytokine inducers such as lipopolysaccharide (LPS) have been shown to lead to host behavioural changes that overlap with those seen in MDD patients (Reichenberg et al., 2001; Brydon et al., 2008). A number of cytokines and their signalling pathways have been shown to activate the enzyme indoleamine 2,3 dioxygenase (IDO), which in turn breaks down tryptophan, the precursor of serotonin into kynurenine (Schwarcz and Pellicciari, 2002; Dantzer et al., 2008). The breakdown of tryptophan is believed to contribute to reduced serotonin availability and cause depressive symptom (Neumeister et al., 2004)

1.1.4 Diagnostic criteria of MDD

Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (DSM-V) (American Psychiatric Association, 2013) is the current reference used by mental health professionals and psychiatrist to diagnose mental disorders. According to DSM-V, for a diagnosis of MDD at least five or more of the following symptoms must be present during the same 2-weeks period and it represents a change from previous functioning. At least one of the symptoms is either depressed mood or loss of interest or pleasure.
i. Depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g. feels sad or empty) or observation made by others (e.g. appears tearful). Note: In children and adolescents, can be irritable mood.

ii. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation made by others).

iii. Significant weight loss when not dieting or weight gain (e.g. a change of more than 5 percent of body weight in a month), or decrease or increase in appetite nearly every day. Note: In children, consider failure to make expected weight gains.

iv. Insomnia or hypersomnia nearly every day.

v. Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down).

vi. Fatigue or loss of energy nearly every day.

vii. Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick).

viii. Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others).

ix. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.

The symptoms must be accompanied by significant distress or impairment in social, occupational, or other important areas of functioning. The symptoms also are not due
to the direct physiological effects of a substance (e.g. drug abuse, medication) or medical condition (e.g. hypothyroidism) (American Psychiatric Association, 2013).

1.1.5 Assessment tools for MDD

Various assessment tools have been developed by medical practitioners to measure the depressive symptoms in patients. The use of rating scales is a well-established technique for assessing the severity of mental illness including MDD. In general, the rating scale can be divided into two groups: patient-rated scale and clinician-rated scale. Total score obtained from the rating scales provides an indication of the severity of MDD for a time period (Cusin et al., 2009; Uher et al., 2012). According to Uher et al. (2012), a complete assessment of depression should include both patient-rated scale and clinician-rated scale (Uher et al., 2012).

The commonly used patient-rated scales for MDD were Beck Depression Inventory (BDI), Patient Health Questionnaire (PHQ), Depression, Anxiety and Stress Scale (DASS), and Hospital Anxiety Depression Scale (HADS) (Firdaus Mukhtar and Tian P.S. Oei, 2011a). In Western studies, BDI has been established as the most commonly used outcome measure either in clinical setting or in research practice (Stahl, 2000). Previous studies also support the notion that BDI can be used as an instrument with confidence to measure the levels of depressive symptoms in Malaysian (Firdaus Mukhtar and Tian P.S. Oei, 2011a). BDI had been proven to have good psychometric properties with acceptable internal consistency and moderate concurrent validity (Schotte et al., 1997; Enns et al., 2000; Svanborg and Åsberg, 2001).
Beck Depression Inventory (BDI) was developed by J. Erbaugh based on the records of statements made by individuals with depressive disorders during psychotherapeutic sessions (Beck et al., 1961). BDI has 21-items that assess all DSM-V diagnostic symptoms of depression and additional symptoms (e.g. irritability). A large proportion of BDI items focus on the cognitive symptoms of depression, such as guilt, self-esteem, feeling disappointed in oneself, feeling of being punished, and pessimism (Uher et al., 2008). Each item is composed of four first-person statements graded by the degree of depression severity it typically represents and rated on a 4 point ordinal scale (0 to 3). BDI was originally designed to be read out to the patient by an interviewer, but has commonly been used as self-report questionnaire for literate patients. The total BDI score is calculated by summing the 21 items and the score can range from 0 to 63. Higher score reflects more severe depression. The advantages of BDI scale over clinician-rated scales are they may take less time, do not require trained personnel, and their administration and scoring process appear more standardized (Cusin et al., 2009).

The commonly used clinician-rated scales in clinical studies were Hamilton Depression Rating Scale (HDRS), Montgomery-Asberg Depression Rating Scale (MADRS) and Quick Inventory of Depressive Symptomatology (QIDS) (Cusin et al., 2009; Uher et al., 2012). However, MADRS has been found to be internally consistent and discriminate levels of depression severity more accurately than other clinician-rating scales (Carmody et al., 2006; Bernstein et al., 2010). The inter-rater reliability of MADRS ranged from 0.89 to 0.97 (Montgomery and Asberg, 1979).
Montgomery–Åsberg Depression Rating Scale (MADRS) was develop in the late 1970. MADRS consist of 10 item assessing symptoms of depression that were found to be responsive to treatment (Montgomery and Asberg, 1979). It is commonly used in clinical practice and clinical studies for weekly administration. MADRS focused more toward psychological, as opposed to somatic aspects of depression (Cusin et al., 2009). Sad mood is assessed by two item that capture the observer perspective, and reported subjective experience respectively. The other eight items assess tension, sleep, appetite, concentration, lassitude (activity), inability to feel (anhedonia), pessimism, and suicidal thoughts. Each item is rated on a 7 point (0 to 6) ordinal scale. A total score is computed as the sum of the 10 items and the score range from 0 to 60. A higher total scores will reflect more severe depression (Müller et al., 2003). MADRS can be used by both psychiatrists and professionals without a specific or with minimal psychiatric training (Cusin et al., 2009).

1.1.6 Treatment of MDD

Several treatment approaches to MDD are currently available. These approaches include pharmacotherapy, psychotherapy, electroconvulsive treatment (ECT) and transcranial magnetic stimulation (TMS). Antidepressants drugs were used to reduce the symptoms of depression and help depressed people feel the way they did before they became depressed. Antidepressant drugs commonly used for the treatment of MDD are selective serotonin reuptake inhibitors (SSRIs) (e.g. Citalopram, Escitalopram, Fluoxetine, Paroxetine, Sertraline), serotonin-norepinephrine reuptake inhibitors (SNRIs) (e.g. Venlafaxine, Duloxetine), tricyclic antidepressants (e.g. Amitriptyline, Imipramine, Doxepin) and monoamine oxidase inhibitor (MAOIs) (e.g. phenelzine and tranylcypromine) (Valdivia and Rossy, 2004;
Halverson, 2016). Although choices of antidepressant drugs appear to be many, all current antidepressant drugs have essentially similar mechanism of action through the monoaminergic pathways (S. W. Tang et al., 2010).

Besides pharmacological treatment, MDD patients also have the alternative choice of psychotherapy treatments. Several studies stated that a combination of pharmacotherapy and psychotherapy provides the quickest and most sustained response in MDD patients (Trivedi Madhukar, 2008; Karyotaki et al., 2016). Psychotherapies normally used for adult with MDD including interpersonal psychotherapy (IPT), cognitive-behavioural therapy (CBT), problem-solving therapy (PST), behavioural activation (BA) and contingency management therapy (Valdivia and Rossy, 2004; Halverson, 2016).

Electroconvulsive treatment (ECT) and transcranial magnetic stimulation (TMS) be an option for those who have not responded to antidepressants. The treatments are known to impact the function of neurotransmitters in our brain and typically offers immediate relief of even severe depression (Halverson, 2016). The use of ECT and TMS are usually limited to MDD patients that are highly resistant to antidepressant treatment or have high risk of medical morbidity and mortality (Valdivia and Rossy, 2004; National Institute of Mental Health, 2016a). However, the use of ECT can give several side effect to patients like headache, muscle pain, nausea, fatigue and temporary loss of memory. In serious cases, ECT may cause cardiovascular complication and hypertension (Datto, 2000).
1.2 Human Immune System and Lymphocyte Subsets

1.2.1 Cell of immune system

Human immune system consists of complex network of cells, tissues and organs that work together to protect the body against foreign substances (antigen) such as bacteria, viruses, fungi and other parasites. The immune cells are known as white blood cells or leukocytes. Leukocytes can be divided into five different types based on their physical and functional characteristics. They are neutrophils, eosinophils, basophils, lymphocytes, and monocytes (Elgert, 2009). Lymphocytes are the smallest member of the leukocytes family that capable of complex biological response and activities. Lymphocytes are round cells with 6 to 15 µm in diameter and have a large nucleus and a coarse, dense cytoplasm. They represent 20-40% of the body’s leukocytes and are found circulating between blood and lymphoid tissues (Abbas et al., 2014).

All lymphocyte develop from pleuripotent haematopoietic stem cells originate in the bone marrow through a process called haematopoiesis. Pleuripotent haematopoietic stem cells (HSCs) are capable of giving rise to multiple cell lineages including erythrocytes, platelets, myeloid progenitor cells and lymphoid progenitors cells. Myeloid progenitor cells give rise to neutrophils, basophils, and eosinophils. They also give rise to monocytes that can further differentiate to macrophages or dendritic cells (DCs), depending upon exposure to different cytokine millieus. While, lymphoid progenitors cells develop into lymphocytes in the microenvironment of the primary lymphoid organ (thymus or bone marrow). The newly formed lymphocytes further mature and proliferate in these organs and finally give rise to various
lymphocyte subsets known as T lymphocyte, B lymphocytes or natural killer (NK) cells (Abbas et al., 2014). Although all lymphocytes are morphologically similar and rather remarkable in appearance, they are extremely heterogeneous in lineage, function, and phenotype (Elgert, 2009).

T lymphocytes and B lymphocytes express specific receptors for antigen and thus the key mediators of adaptive immunity. In contrast, NK cells do not express specific receptor for antigen and they play a crucial role in innate immunity (Elgert, 2009). These cells are often distinguished by surface protein that may be identified by panels of monoclonal antibodies. The standard nomenclature for these proteins is the “CD” (cluster of differentiation) with numerical designation (e.g. CD3, CD4, CD8, CD25, etc.), which is used to delineate surface protein that define a particular cell type or stage of cell differentiation (Figure 1.1) (Abbas and Lichtman, 2009).

Circulating lymphocytes play a key roles in maintaining immune homeostasis against invading pathogens and damaged host-derived cells that might otherwise destroy the immune balance. Many immune-related disorders such as autoimmune diseases, immunodeficiency syndromes, allergies, transplantation rejection, and leukemia are involved in alterations of lymphocyte subsets (Abul and Andrew, 2011). Recently, it has been reported that mental disorder like post-traumatic stress disorder, panic disorder and major depressive disorder were also associated with the alteration of various lymphocyte subsets (Li et al., 2010; Marazziti et al., 2010).
Figure 1.1. Lymphocyte subsets and cluster of differentiation
1.2.2 Total T cells (CD3+ T cells)

Lymphocytes that mature in the thymus after having originated in the foetal liver and adult bone marrow are known as T lymphocytes or T cells. Majority of T cells are small lymphocytes, possessing a large nucleus with very few intracytoplasmic organelles. T cells play a central role in cell mediated immunity and are functionally and phenotypically heterogeneous. T cells express T cell receptors (TCR) on the cell surface which can recognize a specific antigen. T cells that express αβ TCRs are considered part of the adaptive immune response and those expressing γδ TCRs are considered part of the innate immune response. αβ T cells can be divided into several subsets depending upon the co-receptor expressed and each have a distinct function. The main subsets of αβ T cells are T helper cells (CD4+ T cells) and T cytotoxic cells (CD8+ T cells) (Abbas et al., 2014).

In 1990s, a new subset of T cells were discovered known as regulatory T cells (Tregs). Tregs has been found to play a crucial role in immune regulation. T cell dysregulation is present in many diseases including autoimmune diseases, immunodeficiency diseases and also psychological stress-related diseases (Atanackovic et al., 2006; Yamanouchi et al., 2007; Kuhn et al., 2009; Hisamatsu et al., 2016). Through their neuroprotective and anti-inflammatory effects, T cells may play a pivotal role in both the development of MDD as well as its treatment (Miller, 2010).
1.2.3 T helper cells (CD4$^+$ T cells)

T helper (T$_H$) cells are a subset of T lymphocytes that express the surface protein CD4 and are also referred as CD4$^+$ T cells. The main function of CD4$^+$ T cells is to secrete cytokines for the activity of other immune cells (Zhu et al., 2009). CD4$^+$ T cells generally provide positive signals to other subsets, for example, they cooperate with B cells in the production of antibodies and in the maturation of T cytotoxic cells (Crotty, 2015). In addition, these cells also release cytokines that help macrophages to kill micro-organisms (Zhu and Paul, 2008).

T helper cells can be divided into three broad subsets, T$_H$1, T$_H$2, and T$_H$17. The distinction between the subsets is essentially phenotypic and is based on the profile of the cytokines they expressed (Zhu et al., 2009). T$_H$1 cells synthesize IL-2, IFN-γ, TNF-α and TNF-β (lymphotoxin). These cytokine activate CD8$^+$ T cells and NK cells. Once these cells have been activated, they kill host cells that have been infected with viruses or intracellular bacteria (Zhu et al., 2009).

T$_H$2 cells synthesize IL-4, IL-5, and IL-13. IL-4 and IL-13 influence B cells class switch to IgE and IgG in humans, while IL-5 activates eosinophils. T$_H$17 cells synthesize and secrete the IL-17 family of cytokines (particularly IL-17A anf IL-17F) and IL-22. These cytokine promote the inflammatory response at the mucosal sites. It has been found that, T$_H$17 cells and cytokines are involved in many autoimmune diseases including rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease (Bettelli et al., 2008; Hisamatsu et al., 2016). Loss of functional CD4$^+$ T cells leads to the symptomatic stage of infection known as the acquired immunodeficiency syndrome (AIDS) (Janeway et al., 1997).
1.2.4 T cytotoxic cells (CD8+ T cells)

T cytotoxic (T_c) cells are a subset of T lymphocytes that express the surface protein CD8 and referred as CD8+ T cells or cytotoxic T lymphocyte (CTL). CD8+ T cell express a dimeric co-receptor CD8, usually composed of one CD8α and one CD8β chain. CD8+ T cells are very important for immune defence against intracellular pathogens, including viruses and bacteria, and for tumour surveillance (Abbas and Lichtman, 2009). When a CD8+ T cell recognises its antigen and becomes activated, it has three major mechanisms to kill infected or malignant cells. The first is secretion of cytokines, primarily tumornecrosis factor (TNF)-α and interferon (IFN)-γ, which have anti-tumour and anti-viral microbial effects (Price et al., 1999).

The second mechanism is the production and release of cytotoxic granules. These granules, also found in NK cells, contain two families of proteins; perforin and granzymes. Perforin forms a pore in the membrane of the target cell. This pore allows the granzymes to enter the infected or malignant cell. Granzymes are serine proteases which cleave the proteins inside the cell, shutting down the production of viral proteins and ultimately resulting in apoptosis of the target cell. CD8+ T cells can contribute to protection against subsequent encounters with the same antigen (Stenger et al., 1998).

The third mechanism is the destruction of infected cells via Fas/Fas ligand interactions. The cell-surface Fas receptor (Fas) is a member of the tumour necrosis factor (TNF) and nerve growth factor (NGF) family of receptors (Schulze-Osthoff et al., 1998). Activated CD8+ T cells express Fas ligand (FasL) on the cell surface,
which binds to its receptor, Fas, on the surface of the target cell. This binding causes Fas molecules on the surface of the target cell to trimerise, which pulls together the signalling molecules. These signalling molecules result in the activation of the caspase cascade, which cause the apoptosis of the target cell (Hanabuchi et al., 1994; Waring and Müllbacher, 1999). Since CD8⁺ T cells can express both molecules, Fas/FasL interactions are a mechanism by which CD8⁺ T cells can kill each other, to eliminate immune effector cells during the contraction phase at the end of an immune response (Hanabuchi et al., 1994).

However, in addition to their critical role in immune defense against viruses, and tumors, CD8⁺ T cells can also contribute to an excessive immune response that leads to immunopathology, or immune-mediated damage. For example, self-antigens released from CD8⁺ T cell lysis during the process of killing virus-infected cells can be presented by antigen-presenting cells to CD4⁺ or CD8⁺ T cells, causing them autoreactive. Eventually, this might lead to damage at the site of inflammation or tissue containing self-antigen (Fujinami et al., 2006).
1.2.5 Natural killer cells (CD16+ CD56+ NK cells)

Natural killer (NK) cells are large granular lymphocytes comprising ≈15% of peripheral blood lymphocytes (Robertson and Ritz, 1990). NK cells develop from CD34+ hematopoietic progenitor cells (HPCs) within the microenvironment of the bone marrow. They are characterized phenotypically by the expression of CD16 and CD56 (Trinchieri, 1989). NK cells are the crucial component of innate immune system which display natural cytotoxic activity (Robertson and Ritz, 1990). They lack both immune memory and major histocompatibility complex (MHC) restriction, and are activated by the secretion of interleukin-2 (IL-2) and interferon-γ (IFN-γ) (Trinchieri, 1989).

NK cells express a functional heterodimeric interleukin-2 receptor (IL-2Rβγ), with intermediate affinity for IL-2 (Caligiuri et al., 1990). NK cells also express constitutively several receptors for monocyte-derived cytokines (monokines), including IL-1, IL-10, IL-12, IL-15 and IL-18 (Carson et al., 1995; Kunikata et al., 1998; Fehniger et al., 1999). Besides that, several other studies have identified human NK cells also expressed other surface molecule like CD43 (Aguado et al., 1999), CD55 and CD59 (Solomon et al., 1995), CD57 and HLA-DR (Sedlmayr et al., 1996).

NK cells play a major function in the elimination of compromised host cells, such as tumor or virus-infected cells. NK cell mediated killing involves exocytosis of cytoplasmic granules containing perforin and granzyme through a metabolically active process (Winkler et al., 1996). They also have the ability to kill target cells via death receptors like tumor necrosis factor (TNF)-related apoptosis-inducing ligand
(TRAIL) and Fas ligand (FasL) (Screpanti et al., 2001; Takeda et al., 2005). In addition, NK cells have the capacity to produce an early source of immunoregulatory cytokines. During the innate immune response to infection, monokines stimulate NK cells to produce immunoregulatory cytokines like IFN-γ, TNF-β and granulocyte macrophage-colony stimulating factor (GM-CSF) that are important to the early host defense against a variety of viral, bacterial, and parasitic pathogens (Scharton-Kersten and Sher, 1997; Biron et al., 1999).

NK cells also act as regulatory cells to influence various other cell types, such as dendritic cells (DCs), T cells, B cells and endothelial cells. For example, NK cells can kill immature DCs in humans and thereby influencing DC homeostasis. In addition, by means of IFN-γ and TNF, NK cells can promote the maturation of DCs which in turn activate the NK cells by means of IL-12 (Walzer et al., 2005). IL-2 can promotes NK cell proliferation, cytotoxicity and cytokine secretion. In the inflamed lymph node, NK cells can promote the priming T<sub>H</sub>1 cells by secreting IFN-γ (Martín-Fontecha et al., 2004; Morandi et al., 2006). Futhermore, NK cells can also kill activated T cells and suppress autoreactive B lymphocytes in vitro (Takeda and Dennert, 1993).

NK cells are unique as they have the ability to recognize stressed cells in the absence of antibodies and major histocompatibility complex (MHC), which allowing for a much faster immune reaction (Robertson and Ritz, 1990; Cooper et al., 2001). This role is important because harmful cells that are missing MHC class I markers cannot be detected and destroyed by other immune cells. In addition to their major functions in innate immune response, NK cells also play a role in adaptive immune response.
Numerous studies have demonstrated their ability to readily adjust to the immediate environment and formulate antigen-specific immunological memory which are fundamental for responding to secondary infections with the same antigen (Pyzik and Vidal, 2009; Rölle et al., 2013).

1.2.6 B cells (CD19⁺ B cells)

B cells also known as B lymphocytes are the effectors of humoral immunity. B cells are the only immunoglobulin (Ig) producing cells. They constitute approximately 15% of peripheral blood leukocytes and are found in the bone marrow, blood, lymphoid organs and lymph. B cells mature in the bone marrow (in mammals) or Bursa of Fabricus (in birds) (Cooper and Alder, 2006). Unlike T cells and NK cells, B cells express B cell receptor (BCRs) on their membrane. BCRs allow the B cell to bind a specific antigen, against which it will initiate an antibody response. Mature B cells differentiate into either plasma B cells or memory B cells (Elgert, 2009). During their development stages, B cells expressed several surface molecule such as CD19, CD20, CD21, CD22, CD23, CD24, CD40, CD72 and CD81. However, among all the surface molecule, only CD19 is expressed in all stages of B cell development including the mature B cell (but not the plasma cell) (LeBien and Tedder, 2008). So the expression of CD19 is a useful marker of all cells in the B-cell lineage up to the plasma cells. Besides that, B cells also express MHC class I and II molecules. The expression of MHC class II molecules allows them to present antigen to T helper cells (LeBien and Tedder, 2008).

The main function of B cells is antibody production. The antibodies are secreted by plasma B cell in large amount to assist in the destruction of microbes by binding to
them and making them easier targets for phagocytes and activation of the complement system. In addition to their essential role in humoral immunity, B cells also mediate or regulate many other functions essential for immune homeostasis. Of major importance, B cells are required for the initiation of T cell immune responses. The antigen-specific interactions between B and T cells may require the antigen to be first internalized by the BCR, processed, and then presented in an MHC-restricted manner to T cells (LeBien and Tedder, 2008). Previous studies also demonstrated that B cells are essential for optimal CD4 T cell activation during immune responses to low-dose foreign antigens and autoantigens (Bouaziz et al., 2007).

While critical for normal immune system development, B cells are also important for its maintenance. B cells can release immunomodulatory cytokines that can influence a variety of T cell, APC, and dendritic cells functions, regulate lymphoid tissue organization and neogenesis, regulate wound healing and transplanted tissue rejection, and influence tumor development. B cells can also function as cytokine-producing effector cells that influence T-cell differentiation (Harris et al., 2000). One phenotypically distinct subset, designated as B10 cells has been shown to uniquely regulate T cell mediated inflammatory responses through the production of IL-10 (Mizoguchi and Bhan, 2006).
1.2.7 Regulatory T cells (Tregs)

1.2.7 (a) Discovery of Tregs

Tregs is a newly discovered lymphocyte subsets compared to T helper, T cytotoxic, NK cells and B cells. In the early 1970s, Gershon and Kondon proposed that a specialized populations of T cells supressed the immune responses of other lymphocytes (Gershon and Kondo, 1970). The suppressor T cells, which were characterized by expression of the CD8 cell surface marker, have been intensively studied over the following years in various fields of immunology. However, because of the poor characterization of the cells and the lack of specific markers, the concept of suppressor T cells was largely abandoned by the end of the 1980s (Sakaguchi et al., 2007).

In mid-1990s, renewed interest in suppressor T cells emerged with the identification of CD4^+ T cells population which have the ability to downregulate T cell function. The suppressor T cells was named as regulatory T cells (Tregs). Tregs was observed to expressed CD25, the IL-2 receptors α chain, and are known as CD4^+CD25^+ Tregs. However, further studies shows that CD25 is not unique to Tregs only, as it was also expressed on activated effector CD4^+ T cells. This has made it difficult to isolate a pure Tregs subsets for functional studies (Sakaguchi et al., 2007).

Studies on the Scurfy disease in mice and immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) in humans led to the identification of a marker specific for Tregs, known as Foxp3 (forkhead box P3). Foxp3 is a transcription factor required for the development of Tregs in the thymus and has been
shown to induce non-regulatory T cells to acquire suppressive activity in the periphery (Hori et al., 2003). Therefore, Foxp3+ transcription factors is considered as the most reliable intracellular marker for functional Tregs to date and the Treg cells was known as CD4+ CD25+ Foxp3+ Treg cells.

1.2.7 (b) Characteristics of Tregs

Natural Tregs represent approximately 5-10% of the total CD4+ T cell population. In mice, CD4+ Tregs are homogenous population in which all CD4+ and CD25+ cells are Tregs. In human, the Treg are a heterogeneous population, in which not all CD25+ cells are Tregs. Studies by groups of scientists at Havard and the Royal Free and University College Medical School in London revealed that only those CD4+ T cells that expressed very high level of CD25+, representing approximately 2-3% of total CD4+ T cells were Treg (Baecher-Allan et al., 2005).

There are a number of Tregs subtypes with the best understood being those that express CD4, CD25, and Foxp3. Foxp3 is the transcription factor required for CD4+ CD25+ Treg cells development and function (Thompson and Powrie, 2004). Foxp3+ Treg cells use the αβTCR for antigen recognition, and have a broad repertoire, comparable in size to, but largely distinct in composition from conventional CD4+ T cells (Pacholczyk et al., 2006; Wong et al., 2007). It have been found that, when Tregs lose this transcription factor they become ‘ex-Tregs’ which are effector-like T cells that no longer serve to regulate immune function (Zhou et al., 2009). Loss of function or mutations in the human Foxp3 gene lead to IPEX syndrome, a severe multi-organ autoimmune and inflammatory disorder (Rudensky, 2011).
1.2.7 (c) Function and biological activities of Tregs

Regulatory T cells (Tregs) are known to play important roles in immune regulation. Tregs are specialized in suppressing the immune response to maintain tolerance to self-antigen and immune homeostasis (Maloy and Powrie, 2001; Sakaguchi et al., 2007). The activation of Treg cells is antigen-specific, which implies that suppressive activities of Treg cells are antigen-dependent (Corthay, 2009).

Tregs are capable of suppressing the immune response through various mechanism.Tregs could suppress the proliferation and/or cytokine production of T
effector cells (Piccirillo and Shevach, 2001; Li et al., 2010). Tregs can also inhibit inflammatory responses through the expression and release of inhibitory cytokines like IL-10, transforming growth factor beta (TGF-β), IL-35 (Bach, 2002; Workman et al., 2009), and regulation of antigen presenting cells (APC) (Beissert et al., 2006). Tregs also help to dampen monocyte activation, T\textsubscript{H}1, T\textsubscript{H}2, and T\textsubscript{H}17 cells (Carbone et al., 2014).

Besides that, Tregs have been found to exhibit neuroprotective functions as well (Liu et al., 2009; Miller, 2010). For example, in a murine model of human immunodeficiency virus (HIV), adoptive transfer of CD3 activated Tregs attenuated astrogliosis and microglia inflammation with concomitant decreased proinflammatory cytokines and increased brain derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (Liu et al., 2009). Moreover, in a rat stroke model, Tregs were found to enhance survival of progenitor cells in the subventricular zone (Ishibashi et al., 2009). Through these mechanisms, Tregs could suppress the development of many chronic inflammatory and autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis (Sakaguchi et al., 2008).
1.3 Lymphocyte Subsets in MDD

1.3.1 T cells in MDD

Meta-analytic studies have revealed that MDD was associated with the decreases in the percentage of T cells (Zorrilla et al., 2001; Irwin and Miller, 2007). In addition, in vivo measures of cell-mediated immune function including skin responses to commonly encountered antigens suggested decreased CD4+ T cells activity in depressed patients (Sephton et al., 2009). In a study involving HIV patients, depression have been associated with higher activated CD8+ T cells levels and viral load. Marazziti et al. (1992) demonstrated that patients with panic disorder also have lower CD4+ T cells in peripheral blood compared to normal controls (Marazziti et al., 1992).

Flow cytometric assessments have revealed that CD4+ T cells from depressed patients exhibit evidence of accelerated spontaneous apoptosis as well as increased expression of the receptor for Fas (CD95), which mediates apoptotic signaling by Fas-ligand (Ivanova et al., 2007; Szuster-Ciesielska et al., 2008). Increased T cell apoptosis has also been observed as a function of chronic stress in both humans and laboratory animals (Sakami et al., 2003; Shi et al., 2003).

Some studies suggested that, the increased T cell apoptosis in depression was caused by tryptophan depletion. A number of cytokines and cytokine signaling pathways have been shown to activate enzyme indoleamine 2,3 dioxygenase (IDO), which breaks down tryptophan into kynurenine (Schwarcz and Pellicciari, 2002; Dantzer et al., 2008). Tryptophan is an essential proliferative stimulus for effector T cells, and
in a tryptophan-deprived environment, T cells undergo apoptosis (Mellor et al., 2003).

Glucocorticoid have been shown to be increased in depressed patients. It has been found that glucocorticoids can inhibit T cells function and may caused reduced T cell responses in MDD. In addition, glucocorticoids have multiple effects on immune responses including inhibition of inflammation, mediation of cell trafficking and induction of apoptosis in multiple immune cell types including developing T cells in the thymus (Herold et al., 2006).

Besides that, T cell may be impaired in patients with depression through the disruption of T cell function by inflammatory cytokines, such as TNF-α, which, has been shown to be elevated in depressed patients (Miller et al., 2009). Both in vitro and in vivo studies have demonstrated that chronic exposure of T cells to TNF-α decreases T cell proliferation and cytokine production (Cope et al., 1997; Lee et al., 2008).

1.3.2 NK cells in MDD

Previous studies consistently reported that patients with MDD exhibit blunted natural killer cells activity (NKCA) compared with healthy control (Kronfol et al., 1989; Irwin et al., 1992). Locke et al. (1984) also have suggested that both stressful live events and depressive or anxiety symptoms may contribute toward NKCA suppression (Locke et al., 1984). However, some studies demonstrated that blunted NKCA was inversely related to severity of depression and appear to be a characteristic for major depression with melancholia rather than for simple major
depression (Maes et al., 1992b). In theory, blunted NKCA may be caused by either a decreased number of active NK cells in peripheral blood, or by a defective functioning of NK cells (e.g., postreceptor signalling, target cell recognition, target killing). Evans et al. (1992) found a positive correlation between the percentage of NK cells and NKCA. They concluded that the reduced NKCA in MDD may be caused by the lower availability of NK cells in the peripheral blood (Evans et al., 1992). However, a study on NK cells in MDD by Maes et al. (1994a) showed that there were no differences in the percentage and absolute count of NK cells between MDD patients and healthy controls (Maes et al., 1994a). Therefore, the pathophysiology underlying lower NKCA in depressed subject and the consequences of this phenomenon are still unidentifiable.

1.3.3 B cells in MDD

B cells play an important role in regulating the immune response in both physiological and pathological conditions. Dysregulation of B cell can lead to many chronic diseases like autoimmune diseases, cancer, and immunodeficiency diseases (Alizadeh et al., 2000; Mackay et al., 2006; Moir and Fauci, 2009). There have been some reports that MDD also may be accompanied by B cell proliferation or activation. Maes et al. (1992a) found that depressed patients exhibit a significantly increased number of CD19+ B and CD21+ B cells compared with normal controls (Maes et al., 1992a). Preliminary study by Maes et al., (1991a) also found that sera from MDD patients contain significantly higher antinuclear and antiphospholipid activities than those from normal controls (Maes et al., 1991). Therefore, it has been suggested that MDD are related with the existence of polyclonal B cell activation comparable to that seen in some autoimmune disorders (e.g., lupus erythematosus).
1.3.4 Tregs in MDD

Tregs deficiencies are associated with a variety of allergic, autoimmune and auto-inflammatory diseases (Carbone et al., 2014). Treg dysfunction have been shown to be involved in the pathogenesis of many autoimmune diseases like systemic lupus erythematosus, multiple sclerosis, and rheumatic diseases (Belkaid and Rouse, 2005; Baecher-Allan and Hafler, 2006). Previous studies clearly show that reduced cell number or impaired function of Tregs is correlated with increased autoimmunity (Kuhn et al., 2009) while it increase could benefit in treating autoimmune diseases (Hauben et al., 2008). Besides that, the number of Tregs were found to be decreased in allergic rhinitis patients and positively correlated with serum total IgE levels (Lee et al., 2007).

Recently, it has been reported that the number of Tregs is altered in a stress-related long-term disorders like post-traumatic stress disorder (Sommershof et al., 2009) and acute coronary syndrome (Cheng et al., 2008). Tregs also have been shown to be decreased in the peripheral blood of patients suffering from MDD (Li et al., 2010). It has been found that the decreased of Tregs number was positively correlated with the reduction of 5-HT level, which are known to play central roles in the pathogenesis of MDD. The reduction 5-HT level has been shown to diminish the activation potential of CD4⁺ Treg cells (Li et al., 2010).
1.4 RATIONALE OF THE STUDY

The dysregulation of immune system in MDD have been extensively studied. While activation of innate immune responses currently dominates the research landscape, early studies in depressed patients demonstrating that impairment in adaptive immune system in particular lymphocyte subsets, may warrant further consideration. Many studies demonstrated that MDD was associated with increased inflammatory cytokine such as TNF-α, IL-6, and IL-1. Intriguing data suggested that the dysregulation of immune system in MDD may not only reflected by the altered levels of inflammatory cytokines, but probably also by the changes in the level of various lymphocytes subsets.

To date, there were only few studies done on lymphocyte subsets in MDD and most of the studies were done more than a decade ago. Therefore, new data with better technologies are needed. Few studies for example NK cells showed contradictory result and thus new study needed to confirm the finding. Besides that, Treg cells, one of the most important T cell subsets, may also play a crucial role in MDD through downregulation of chronic inflammatory responses. Previous studies on Treg cells in MDD only measure natural CD4+ CD25+ Treg cells. This study will also focus on functional CD4+ CD25+ Foxp3+ Treg cells.

To the best of our knowledge, no study has been done regarding the lymphocyte subset and severity of MDD in Malaysia. The comparison of lymphocyte subsets between different severity of MDD are important as they might be considered as biomarkers of severe MDD. The positive findings of these lymphocyte subsets also are important to determine the pathophysiology of MDD. Decreases in the number of
the relevant T cell subset may directly contribute to the development and maintenance of MDD. While, enhancement of the relevant lymphocyte subset may represent an interesting and novel approach for the treatment of this disorder. Therefore, this study is undertaken to evaluate a wide range of lymphocyte subset in MDD patients, compare them with healthy controls and between different severity of MDD.
1.5 OBJECTIVES

1. General objective

To study the percentage and absolute count of lymphocyte subsets {T helper (CD4+ T cells), T cytotoxic (CD8+ T cells), NK cells (CD16+ CD56+ NK cells), B cells (CD19+ B cells), CD4+ CD25+ Treg cells and CD4+ CD25+ Foxp3+ Treg cells} and their comparison between different severity of MDD patients.

2. Specific objectives
   i. To study the predisposing factors of MDD and the common symptoms in MDD patients based on Beck Depression Inventory (BDI) and Montgomery-Asberg Depression Rating Scale (MADRS).
   ii. To compare the percentage and absolute count of lymphocyte subsets {T helper (CD4+ T cells), T cytotoxic (CD8+ T cells), NK cells (CD16+ CD56+ NK cells), B cells (CD19+ B cells), CD4+ CD25+ Treg cells and CD4+ CD25+ Foxp3+ Treg cells} between MDD patients and healthy controls.
   iii. To compare the percentage and absolute count of lymphocyte subsets {T helper (CD4+ T cells), T cytotoxic (CD8+ T cells), NK cells (CD16+ CD56+ NK cells), B cells (CD19+ B cells), CD4+ CD25+ Treg cells and CD4+ CD25+ Foxp3+ Treg cells} between different severity of MDD patients.
1.6 RESEARCH HYPOTHESIS

1. There is a difference in the percentage and absolute count of lymphocyte subsets {T helper (CD4$^+$ T cells), T cytotoxic (CD8$^+$ T cells), NK cells (CD16$^+$ CD56$^+$ NK cells), B cells (CD19$^+$ B cells), CD4$^+$ CD25$^+$ Treg cells and CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cells} between MDD patients and healthy controls.

2. There is a difference in the percentage and absolute count of lymphocyte subsets {T helper (CD4$^+$ T cells), T cytotoxic (CD8$^+$ T cells), NK cells (CD16$^+$ CD56$^+$ NK cells), B cells (CD19$^+$ B cells), CD4$^+$ CD25$^+$ Treg cells and CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cells} between mild, moderate and severe MDD patients.
CHAPTER 2

MATERIALS AND METHODS

2.1 Study Design

This is a case-control study which was conducted among major depressive disorder (MDD) patients and healthy controls.

2.2 Study Area

This study was conducted at Psychiatric Clinic, Hospital Universiti Sains Malaysia (HUSM) and Immunology Research Laboratory, Universiti Sains Malaysia (USM), Kelantan, Malaysia.

2.3 Source of Population

2.3.1 MDD patients

Subjects were recruited from psychiatric out-patient clinic, HUSM. All subjects diagnosed with major depressive disorder by the treating doctors were screened for eligibility. The diagnosis of all eligible subjects were confirmed by a researcher who is a psychiatrist according to DSM-V and all those who consented and met the inclusion and exclusion criteria were enrolled into this study. All participating subjects continued to receive standard pharmacological and non-pharmacological treatments for MDD from the treating doctors at the clinic.
2.3.2 **Healthy controls**

Control subjects were recruited from hospital employees and students that they were in good general health and had no history of MDD.

2.4 **Eligibility Criteria**

2.4.1 **Inclusion criteria**

2.4.1 (a) **Cases**

1. Meet criteria for MDD according to the DSM-V criteria
2. Aged between 18 to 65 years
3. Able to give written informed consent

2.4.1 (b) **Controls**

1. Never been diagnosed with serious psychiatric illnesses such as schizophrenia, substance abuse, suicidal or homicidal ideations or taking medications to treat depression or psychosis
2. Never been diagnosed with serious medical illnesses that may cause immunological disturbances such as autoimmune diseases, HIV/AIDS, allergy, neoplastic, endocrine diseases, diabetes mellitus, acute and chronic infections
3. Currently not taking medications that may cause immunological disturbances such as steroids or cytotoxic drugs
4. Aged between 18 to 65 years
2.4.2 Exclusion criteria

2.4.2 (a) Cases
1. Inability to understand the protocol or assessment questions
2. Having other serious psychiatric illnesses such as schizophrenia, substance abuse, suicidal or homicidal ideations
3. Having medical illnesses that may cause immunological disturbances such as autoimmune diseases, HIV/AIDS, allergy, neoplastic, endocrine diseases, diabetes mellitus, acute and chronic infections
4. Taking medications (e.g. steroids, cytotoxic drugs) that may cause immunological disturbances
5. Undergone major surgical operation in the past 6 months such as heart surgery, angioplasty, etc.
6. Pregnant

2.4.2 (b) Controls
1. Refused to give written informed consent
2. Inability to understand the protocol or assessment questions
3. Pregnant
2.5 Sample Size Calculation

Sample size (n) was calculated based on objective no. 2 by using Independent t-test.

Formula for Independent-t test:

\[ n = \frac{2\sigma^2}{\Delta^2} \left( Z_\alpha + Z_\beta \right)^2 \]

\[ = \frac{2\sigma^2}{\Delta^2} \left( 1.96 + 0.84 \right)^2 \]

\[ = \frac{2\sigma^2}{\Delta^2} \left( 7.84 \right) \]

Input:

Level of significance (\( \alpha \)) = 0.05

Confidence interval (1-\( \alpha \)) = 0.95

Type II Error (\( \beta \)) = 0.2

Power of the study (1-\( \beta \)) = 0.8

Ratio cases to control = 1:1

Standard deviation within group (\( \sigma \))

Difference in population means (\( \Delta \))

From literature:

1) Tregs

Based on Li et al. (2010):

\[ n = \frac{2(2.94)^2}{2^2} X 7.84 \]

\[ = \frac{34}{2} \]

\[ = \ 34 \]
2) CD4$^+$ T cells

Based on Schlatter et al. (2004);

$$n = \frac{2 \times (6.6)^2}{4^2} \times 7.84 = 43$$

3) CD8$^+$ T cells

Based on Schlatter et al. (2004);

$$n = \frac{2 \times (6.5)^2}{5^2} \times 7.84 = 27$$

4) CD19$^+$ B cells

Based on Ravindran et al. (1999);

$$n = \frac{2 \times (18)^2}{12^2} \times 7.84 = 35$$

5) CD16$^+$ CD56$^+$ NK cells

Based on Ravindran et al. (1999);

$$n = \frac{2 \times (16)^2}{11^2} \times 7.84 = 33$$

The biggest sample size from literature [Schlatter et al. (2004)] = 43

Sample size (n) = 43 + 10% dropout

= 47 per group
2.6 **Sampling Method**

All patients who fulfilled the inclusion and exclusion criteria were included in the study.

2.7 **Written Consent**

This study was approved by Research and Ethics Committee, Universiti Sains Malaysia. All eligible candidates were briefed about the study and a written informed consent form (Appendix A and Appendix B) was given to all participants before they participated in this study.

2.8 **Data Collection**

After getting the informed consent, subject’s demographic data such as full name, registration number, address, contact number, age, sex, race, occupation, marital status, educational level, smoking status, family history and medication were obtained by individual interview and patient’s medical record. All sociodemographic data were recorded in a study form (Appendix C and Appendix D).

2.9 **Assessment and Classification**

MDD was diagnosed according to the DSM-V criteria. The severity of MDD was assessed using Beck Depression Inventory (BDI) Scale and The Montgomery-Asberg Depression Rating Scale (MADRS) Scale. BDI questionnaire (Appendix E) was answered by patient at the time of recruitment. The total score was calculated by summing up the 21 items. The classification of BDI scale are shown in Table 2.1.
Table 2.1. The severity of MDD according to BDI classification

<table>
<thead>
<tr>
<th>Total Score</th>
<th>Severity of MDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 10</td>
<td>Normal</td>
</tr>
<tr>
<td>11 - 20</td>
<td>Mild depression</td>
</tr>
<tr>
<td>21 - 30</td>
<td>Moderate depression</td>
</tr>
<tr>
<td>31 - 63</td>
<td>Severe depression</td>
</tr>
</tbody>
</table>

MADRS questionnaire (Appendix F) was assessed by psychiatrist or clinician that treat the patient during the recruitment process. The total score was calculated by summing up the 10 items. The classification of MADRS scale are shown in Table 2.2.

Table 2.2. The severity of MDD according to MADRS classification

<table>
<thead>
<tr>
<th>Total Score</th>
<th>Severity of MDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 6</td>
<td>Normal</td>
</tr>
<tr>
<td>7 - 19</td>
<td>Mild depression</td>
</tr>
<tr>
<td>20 - 34</td>
<td>Moderate depression</td>
</tr>
<tr>
<td>35 - 60</td>
<td>Severe depression</td>
</tr>
</tbody>
</table>

For healthy controls, all eligible subjects were asked to complete Depression Anxiety Scoring System (DASS 21) questionnaire (Appendix G) to ensure that they were in good general health and had no depressive symptoms. DASS 21 is a quantitative measure of distress along three axes of depression, anxiety and stress. It is not a categorical measure of clinical diagnosis and was commonly use to measure the depression, anxiety and stress level in normal individual. The classification of DASS 21 scale are shown in Table 2.3. Control subjects with moderate, severe or extremely severe symptoms were excluded from this study.
Table 2.3. The classification of DASS 21 scale

<table>
<thead>
<tr>
<th></th>
<th>Depression</th>
<th>Anxiety</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0 – 4</td>
<td>0 - 3</td>
<td>0 - 7</td>
</tr>
<tr>
<td>Mild</td>
<td>5 – 6</td>
<td>4 - 5</td>
<td>8 - 9</td>
</tr>
<tr>
<td>Moderate</td>
<td>7 – 10</td>
<td>6 - 7</td>
<td>10 - 12</td>
</tr>
<tr>
<td>Severe</td>
<td>11 – 13</td>
<td>8 - 9</td>
<td>13 - 16</td>
</tr>
<tr>
<td>Extremely severe</td>
<td>≥ 14</td>
<td>≥ 10</td>
<td>≥ 17</td>
</tr>
</tbody>
</table>

2.10 Blood Collection

10 ml of peripheral blood was taken from each MDD patients and healthy controls. The blood was placed in BD Vacutainer® EDTA tube (Becton Dickinson, USA) and store at room temperature. The blood were processed within 4 hours of blood collection. About 500 µl of peripheral blood was separated from EDTA tube and placed in an appendorf tube. The blood was used for complete blood count analysis using SYSMEX XS-800i Hematology Automated Analyzer which was performed according to the manufacturer's instructions. The remaining 9.5 ml of the peripheral blood were used for immunophenotyping of lymphocyte subsets by using flow cytometry.
2.11 Immunophenotyping of Lymphocyte Subsets by Flow cytometry

2.11.1 Materials and apparatus

The list of kit and reagents used in the study were shown in Table 2.4. The list of apparatus were shown in Table 2.5.

Table 2.4. List of kit and reagents

<table>
<thead>
<tr>
<th>Kit and Reagent</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. BD Multitest IMK Kit</td>
<td></td>
</tr>
<tr>
<td>i. BD Multitest CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC</td>
<td>BD Biosciences, USA</td>
</tr>
<tr>
<td>ii. BD Multitest CD3FITC/CD16+CD56 PE/CD45 PerCP/CD19 APC</td>
<td></td>
</tr>
<tr>
<td>iii. BD Multitest IMK Kit lysing Solution</td>
<td></td>
</tr>
<tr>
<td>2. Human FoxP3 Staining Kit</td>
<td></td>
</tr>
<tr>
<td>i. FITC Mouse Anti-Human CD4</td>
<td>BD Biosciences, USA</td>
</tr>
<tr>
<td>ii. APC Mouse Anti-Human CD25</td>
<td></td>
</tr>
<tr>
<td>iii. PE Mouse Anti-Human Foxp3</td>
<td></td>
</tr>
<tr>
<td>iv. Human FoxP3 Buffer Set</td>
<td></td>
</tr>
<tr>
<td>3. Phosphate buffered saline (PBS)</td>
<td>Scimedx, USA</td>
</tr>
<tr>
<td>4. Lymphoprep</td>
<td>Axis-Shield, Norway</td>
</tr>
<tr>
<td>5. Deionized water</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.5. List of apparatus

<table>
<thead>
<tr>
<th>Materials and apparatus</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. Flow cytometer</td>
<td>Becton Dickinson, USA</td>
</tr>
<tr>
<td>ii. Centrifuge</td>
<td>Kubota, Japan</td>
</tr>
<tr>
<td>iii. Light microscope</td>
<td>Olympus, Japan</td>
</tr>
<tr>
<td>iv. Vortex</td>
<td>Scientific Industries, USA</td>
</tr>
<tr>
<td>v. Hemocytometer</td>
<td>Thomas Scientific, USA</td>
</tr>
<tr>
<td>vi. Pipette 1000 µl, 100 µl, 10 µl</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>vii. Pipette tips</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>viii. 15 ml falcon tubes</td>
<td>Becton Dickinson, USA</td>
</tr>
<tr>
<td>ix. 12x75 mm round bottom tubes</td>
<td>Becton Dickinson, USA</td>
</tr>
<tr>
<td>x. 1.5 ml appendorf tubes</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>xi. Duran bottle</td>
<td></td>
</tr>
<tr>
<td>xii. Beaker</td>
<td></td>
</tr>
<tr>
<td>xiii. Test tube rack</td>
<td></td>
</tr>
</tbody>
</table>
2.11.2 Immunophenotyping of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells

2.11.2 (a) Staining procedure for CD3, CD4, CD8, CD16, CD56 and CD19 monoclonal antibody

For each sample, two 12 x 75mm tubes were labelled with number 1 and 2. 20 µl of BD Multitest CD3/CD8/CD45/CD4 reagent was pipetted into the bottom of the tube labelled 1 and 20 µl of BD Multitest CD3/CD16+CD56/CD45/CD19 reagent was pipetted into the bottom of the tube labelled 2. Then, 50 µl of well-mixed, anticoagulated whole blood was pipetted into the bottom of each tube. The tubes were capped and vortexed gently. The mixtures were incubated for 15 minutes in the dark at room temperature (20°C – 25°C). After that, 450 µl of 1 X BD Multitest lysing solution was added to each tube. The tubes were capped and vortexed again. The mixtures were incubated for another 15 minutes in the dark at room temperature. The samples were run using flow cytometer by using appropriate setting.
2.11.2 (b) Flow cytometric analysis of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells.

The percentage of T cell (CD3\(^+\) T cells), T helper (CD4\(^+\) T cells), T cytotoxic (CD8\(^+\) T cells), NK cells (CD16\(^+\) CD56\(^+\) NK cells), and B cells (CD19\(^+\) B cells) were analysed by using FACSCanto\textsuperscript{TM} software. The flow cytometric gating strategy used in the study was shown in Figure 2.1. The absolute count of each lymphocyte subsets were calculated by multiplying their percentage with the lymphocyte count obtained from complete blood count analysis.

Figure 2.1. Gating strategy of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells. A) The gating of CD3\(^+\) T cells, CD4\(^+\) T cells and CD8\(^+\) T cells. B) The gating of CD3\(^+\) T cells, CD16\(^+\) CD56\(^+\) NK cells and CD19\(^+\) B cells.
2.11.3 Immunophenotyping of Tregs

2.11.3 (a) Isolation of peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation

PBMCs were isolated from peripheral blood by density gradient centrifugation technique. In brief, 3 ml of lymphoprep/ficoll was added into 15 ml falcon tube using pipette. 9 ml of the fresh blood samples was layered carefully over the lymphoprep using pasteur pipette. The sample was centrifuged at 500 x g for 30 minutes at 20°C in a swinging-bucket rotor without brake. After centrifugation, the second layer (buffy coat) was aspirated and transferred into new falcon tube using pipette. 8 to 10 ml of washing buffer (PBS) was added into the falcon tube and the mixture was centrifuged at 500 x g for 10 minutes at 20°C. The supernatant was removed completely. For removal of platelets, the cell pellet was resuspended in 10 ml of PBS and centrifuged at 300 x g for 10 minutes at 20°C. The supernatant was removed and the washing step was repeated again. The cell pellet was then resuspended in 1 ml of PBS. The number of cells was counted using hemocytometer and its concentration was adjusted to 1 x 10^7 cells/ml.
2.11.3 (b) Staining procedures for CD4, CD25, and Foxp3 monoclonal antibody

20 µl of FITC Mouse Anti-Human CD4 and 20 µl of APC Mouse Anti-Human CD25 were pipetted into the bottom of each 12 x 75 mm tube. 100 µl of PBMCs was added into the tube. The tube was vortexed and incubated on ice for 20 minutes, protected from light. At the end of incubation, 2 ml of washing buffer (PBS) was added. The cell suspension was centrifuged at 250 x g for 10 minutes. The supernatant was discarded and the cell pellet was dislodged by tapping the tube. 2 ml of 1 x Human Foxp3 buffer A was added drop by drop. The mixture was mixed well and incubated on ice for 10 minutes, protected from light. Then, the cell suspension
was centrifuged at 500 x g for 5 minutes. The supernatant was removed using pipette and the cell pellet was dislodged by tapping the tube. 500 µl of Foxp3 Buffer C was added into the tube to permeabilize the cells. The mixture was mixed well and incubated on ice for 30 minutes, protected from light. To wash the cell, 2 ml of PBS was added into the tube and centrifuged at 500 x g for 5 minutes. The washing step was repeated twice. 20 µl of PE Mouse Anti-Human Foxp3 was added into the tube. The mixture was mixed well and incubated on ice for another 30 minutes, protected from light. The washing step was repeated again. The cells were then resuspended in 300 µl of PBS and analysed immediately using flow cytometer.

2.11.3 (c) Flow cytometric analysis of Tregs

The number of cells binding to antibodies was acquired using flow cytometry with a FACSDiva™ software by using appropriate setting. Two types of Tregs were analysed in this study; CD4^+ CD25^+ Treg cells and CD4^+ CD25^+ Foxp3^+ Treg cells. The flow cytometric gating strategy used to identify CD4^+ CD25^+ Tregs and CD4^+ CD25^+ Foxp3^+ Tregs were shown in Figure 2.3. The absolute count of CD4^+ CD25^+ Tregs and CD4^+ CD25^+ Foxp3^+ Tregs subsets were obtained by multiplying their percentage with the lymphocyte count obtained from complete blood count analysis.
Figure 2.3. Gating strategy of CD4\(^+\) CD25\(^+\) Tregs and CD4\(^+\) CD25\(^+\) Foxp3\(^+\) Tregs A) Ten thousand events were acquired for each sample. Lymphocyte was analysed using forward scatter (FSC) and side scatter (SSC) gating strategy. B) The lymphocyte were further subgated with CD24 versus CD25 density plots. C4\(^+\) CD25\(^+\) Treg cells were selected from CD4\(^+\) versus CD25\(^+\) (double positive) plot. C) The boundary between positive and negative Foxp3 were gated by using Fluorescence minus-one (FMO) controls technique. D) CD4\(^+\) CD25\(^+\) Foxp3\(^+\) Treg cells were selected from C25\(^+\) versus Foxp3\(^+\) (double positive) plots.
2.12 Statistical Analysis

Data entry and statistical analysis were conducted using Statistical Package for Social Sciences (SPSS) version 22 (IBM Corp, Armonk, NY, USA). Descriptive statistics were applied to describe subjects characteristics including frequencies, percentages, means, and standard deviations. To identify the sociodemographic factors associated with MDD, Pearson chi-square and multiple logistic regression were utilized. The percentage and absolute count of lymphocyte subsets was deviated significantly from normal distribution by Kolmogorov-Smirnov test, so median and interquartile ranges (IQR) were calculated as measures of central tendency. Mann-Whitney test was used to compare the percentage and absolute count of lymphocytes subsets between MDD patients and healthy controls. Kruskal-Wallis test was used to compare the percentage and absolute count of lymphocyte subsets between different severity of MDD. The result was statistically significant if $p$ value less than 0.05 ($p<0.05$).
2.13 Flowchart of The Study

MDD Patients (n=47)  

Meets study criteria  
(Inclusion and exclusion criteria)

Verbal and written consent

Patients’ biodata and medical history were taken by psychiatrist

MDD was diagnosed according to DSM-V criterias

The severity of MDD patients were classified according to scoring system from BDI scale and MADRS scale

Blood withdrawal

Complete blood count

Enumeration of lymphocyte subsets  
(Immunophenotyping of lymphocyte subsets by flow cytometry)

Flow cytometric analysis

Data entry and statistical analysis

Report writing

Healthy Controls (n=47)

Controls completed DASS 21 questionnaire to exclude psychiatric illness

The severity of MDD patients were classified according to scoring system from BDI scale and MADRS scale
CHAPTER 3

RESULTS

3.1 Sociodemographic Characteristics of MDD Patients and Healthy Controls

The sociodemographic data of MDD patients and healthy control are shown in Table 3.1. A total of 47 MDD patients and 47 healthy control were recruited in the study within January 2015 to February 2016. The mean age of MDD patients was 39.7 (13.07) years old with 42.5% of them were in the age group of 25 to 65 years old. While for healthy control, the mean age was 28.0 (8.69) years old and 46.8% of them were in the age group of 18 to 24 years old. The mean age of onset of MDD was 36.26 (11.88) years old.

In MDD group, 29 (61.7%) were females and 18 (38.3%) were males, while in healthy control, 32 (68.1%) were females and 15 (31.9%) were males. Majority of MDD patients and healthy controls were Malays which accounted for 95.7% of study population, while the other 4.3% were Chinese. Almost 12.8% of MDD patient had family history of depression, while all healthy controls were in a good health condition and had no family history of depression.
<table>
<thead>
<tr>
<th></th>
<th>MDD (n=47)</th>
<th>Healthy Control (n=47)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean (SD)</td>
<td>39.7 (13.07)</td>
<td>28.0 (8.69)</td>
</tr>
<tr>
<td><strong>Age group, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-24</td>
<td>9 (19.2)</td>
<td>22 (46.8)</td>
</tr>
<tr>
<td>25-44</td>
<td>18 (38.3)</td>
<td>21 (44.7)</td>
</tr>
<tr>
<td>45-65</td>
<td>20 (42.5)</td>
<td>4 (8.5)</td>
</tr>
<tr>
<td><strong>Gender, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>18 (38.3)</td>
<td>15 (31.9)</td>
</tr>
<tr>
<td>Female</td>
<td>29 (61.7)</td>
<td>32 (68.1)</td>
</tr>
<tr>
<td><strong>Race, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malay</td>
<td>45 (95.7)</td>
<td>45 (95.7)</td>
</tr>
<tr>
<td>Chinese</td>
<td>2 (4.3)</td>
<td>2 (4.3)</td>
</tr>
<tr>
<td>Indian</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td><strong>Family history of MDD, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>6 (12.8)</td>
<td>0</td>
</tr>
<tr>
<td>No</td>
<td>41 (87.2)</td>
<td>47 (100)</td>
</tr>
<tr>
<td><strong>Age of onset of MDD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean (SD)</td>
<td>36.26 (11.88)</td>
<td></td>
</tr>
</tbody>
</table>
3.2 Predisposing Factors of MDD

The predisposing factors of MDD are shown in Table 3.2. Chi-square analysis showed that sociodemographic factors like marital status ($p=0.007$), smoking status ($p=0.027$), educational level ($p = 0.022$) and area of living ($p=0.036$) were significantly associated with MDD. Further analysis using multiple logistic regression revealed that, after being adjusted for OR, only marital status ($p=0.011$) was significantly associated with MDD, while smoking status ($p=0.150$) educational levels ($p=0.132$) and area of living ($p=0.174$) were not significantly associated.

However, the results showed that unmarried person were less likely to have MDD compared to those married with adjusted odds ratio of 0.31. Smoker were 5.16 at odds of having MDD as compared to non-smoker, while individual who are low educated were more likely to have MDD compared to those highly educated with adjusted odd ratio of 2.04. The result also showed those living in urban area were less likely to have MDD compared to those living in rural area with adjusted odd ratio of 0.48.
Table 3.2. Predisposing factors of MDD

<table>
<thead>
<tr>
<th></th>
<th>MDD n (%)</th>
<th>Control n (%)</th>
<th>Crude OR (95% CI)</th>
<th>( p )-value(^{a} )</th>
<th>Adjusted OR (95% CI)</th>
<th>( p )-value(^{b} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marital Status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmarried</td>
<td>19 (37.25)</td>
<td>32 (62.75)</td>
<td>0.32 (0.14,0.74)</td>
<td>0.007*</td>
<td>0.31 (0.13,0.77)</td>
<td>0.011*</td>
</tr>
<tr>
<td>Married</td>
<td>28 (65.12)</td>
<td>15 (34.88)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Smoking Status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>7 (87.5)</td>
<td>1 (12.5)</td>
<td>8.05 (0.95,68.26)</td>
<td>0.027*</td>
<td>5.16 (0.55,48.04)</td>
<td>0.150</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>40 (46.51)</td>
<td>46 (53.49)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Educational Level</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>26 (63.41)</td>
<td>15 (36.59)</td>
<td>2.64 (1.14,6.12)</td>
<td>0.022*</td>
<td>2.04 (0.81,5.14)</td>
<td>0.132</td>
</tr>
<tr>
<td>High</td>
<td>21 (39.62)</td>
<td>32 (60.38)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Area of Living</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban</td>
<td>30 (43.48)</td>
<td>39 (56.52)</td>
<td>0.36 (0.14,0.91)</td>
<td>0.036(^{a} )</td>
<td>0.48 (0.17,1.39)</td>
<td>0.179</td>
</tr>
<tr>
<td>Rural</td>
<td>17 (68.00)</td>
<td>8 (32.00)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\)Pearson Chi-square, \(^{b}\)Multiple Logistic Regression (Wald statistic), OR: odds ratio, CI: confidence interval

*Result was significant when \( p \textless 0.05 \)
3.3 Distribution of MDD Symptoms Among Patients

The distribution of MDD symptoms in patients were analysed using BDI and MADRS questionnaires.

3.3.1 MDD symptoms based on Beck Depression Inventory (BDI)

MDD symptoms in BDI were divided into two groups, somatic symptoms and cognitive symptoms. Based on BDI, the most common somatic symptom reported by MDD patients was fatigue (80.9%) (Figure 3.1), while the most common cognitive symptom was irritability (80.9%) (Figure 3.2).

Figure 3.1. The distribution of somatic symptoms in MDD patients based on BDI
Figure 3.2. The distribution of cognitive symptoms in MDD patients based on BDI
3.3.2 MDD symptoms based on Montgomery–Åsberg Depression Rating Scale (MADRS)

Based on MADRS, the most common symptoms in MDD patients were inner tension (100%), concentration difficulties (100%), inability to feel (100%), and pessimistic thought (100%) (Figure 3.3).

![Figure 3.3. The distribution of symptoms in MDD patients based on MADRS](image-url)
3.4 The Severity of MDD

Based on the total score obtained from BDI and MADRS questionnaire, MDD patients were classified into three severity group which is mild, moderate and severe. The severity of MDD patients were shown in Figure 3.4 and Figure 3.5.

3.4.1 The severity of MDD patients according to BDI scale

According to BDI scale, 28 (60%) patient were mild, 10 (21%) patients were moderate and 9 (19%) patients were severe MDD (Figure 3.4).

**Figure 3.4.** The severity of MDD patients according to BDI scale
3.4.2 The severity of MDD patients according to MADRS scale

According to MADRS scale, 10 (21%) patient were mild MDD, 24 (51%) patients were moderate and 13 (28%) patients were severe MDD (Figure 3.5).

![Figure 3.5. The severity of MDD patients according to MADRS scale](image-url)
3.5 Enumeration of Lymphocyte Subsets

3.5.1 Comparison of leukocyte between MDD patients and healthy controls

Table 3.3 shows the number leukocytes and its subsets (neutrophils, lymphocytes, monocytes, eosinophils and basophils) in MDD patients and healthy controls. The number of leukocytes and its subsets between the two groups were compared using Mann-Whitney test. The result showed that the median number of leukocytes in MDD patients was not significantly different from healthy controls. There were also no significant differences in median number of neutrophils, lymphocyte, monocytes, eosinophils and basophils between MDD patients and healthy controls.

Table 3.3. Comparison of leukocytes and its subsets between MDD patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>MDD (n=47)</th>
<th>Healthy control (n=47)</th>
<th>Z statistics</th>
<th>*p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Blood Cells (cell/µl)</td>
<td>7080 (1890)</td>
<td>6840 (2810)</td>
<td>-1.074</td>
<td>0.283</td>
</tr>
<tr>
<td>Neutrophils (cell/µl)</td>
<td>3810 (1480)</td>
<td>3450 (1700)</td>
<td>-1.044</td>
<td>0.297</td>
</tr>
<tr>
<td>Lymphocytes (cell/µl)</td>
<td>2500 (700)</td>
<td>2400 (1000)</td>
<td>-0.667</td>
<td>0.505</td>
</tr>
<tr>
<td>Monocytes (cell/µl)</td>
<td>470 (200)</td>
<td>450 (220)</td>
<td>-1.619</td>
<td>0.105</td>
</tr>
<tr>
<td>Eosinophils (cell/µl)</td>
<td>230 (240)</td>
<td>200 (210)</td>
<td>-0.980</td>
<td>0.327</td>
</tr>
<tr>
<td>Basophils (cell/µl)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>-2.686</td>
<td>0.070</td>
</tr>
</tbody>
</table>

*Result was considered significant when $p<0.05$ by using Mann-Whitney test
3.5.2 Comparison of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells between MDD patients and healthy controls

The percentage and absolute count of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells are shown in Table 3.4. The percentage and absolute count of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells between MDD patients and healthy control were compared using Mann-Whitney test. The result showed that there were no significant different in the median percentage and absolute count of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells between MDD patients and healthy controls.
Table 3.4. Comparison of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells between MDD patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>MDD (n=47)</th>
<th>Healthy control (n=47)</th>
<th>Z statistics</th>
<th>*p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total T cells (CD3⁺)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>67.14 (10.18)</td>
<td>66.92 (8.67)</td>
<td>-0.548</td>
<td>0.584</td>
</tr>
<tr>
<td>Absolute count (cells/µl)</td>
<td>1652 (517)</td>
<td>1696 (636)</td>
<td>-0.072</td>
<td>0.943</td>
</tr>
<tr>
<td><strong>T helper (CD3⁺ CD4⁺)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>36.47 (7.82)</td>
<td>34.61 (8.63)</td>
<td>-1.448</td>
<td>0.148</td>
</tr>
<tr>
<td>Absolute count (cells/µl)</td>
<td>931 (376)</td>
<td>836 (326)</td>
<td>-1.312</td>
<td>0.190</td>
</tr>
<tr>
<td><strong>T cytotoxic (CD3⁺ CD8⁺)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>27.12 (10.06)</td>
<td>27.65 (8.88)</td>
<td>-1.002</td>
<td>0.316</td>
</tr>
<tr>
<td>Absolute count (cells/µl)</td>
<td>688 (371)</td>
<td>681 (265)</td>
<td>-0.276</td>
<td>0.783</td>
</tr>
<tr>
<td><strong>NK cells (CD16⁺ CD56⁺)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>18.26 (10.82)</td>
<td>17.61 (9.15)</td>
<td>-0.344</td>
<td>0.731</td>
</tr>
<tr>
<td>Absolute count (cells/µl)</td>
<td>475 (241)</td>
<td>415 (244)</td>
<td>-0.628</td>
<td>0.530</td>
</tr>
<tr>
<td><strong>B cells (CD19⁺)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>15.36 (6.60)</td>
<td>13.54 (5.41)</td>
<td>-1.490</td>
<td>0.136</td>
</tr>
<tr>
<td>Absolute count (cells/µl)</td>
<td>377 (190)</td>
<td>345 (208)</td>
<td>-1.448</td>
<td>0.148</td>
</tr>
</tbody>
</table>

*Result was considered significant when p<0.05 by using Mann-Whitney test.
3.5.3 Comparison of Tregs between MDD patients and healthy controls

The percentage and absolute count of CD4⁺ CD25⁺ Tregs and CD4⁺ CD25⁺ Foxp3⁺ Tregs are shown in Table 3.5. The comparison of Tregs percentage and absolute count between MDD patients and healthy control showed a significantly higher percentage and absolute count of CD4⁺ CD25⁺ Tregs in the peripheral blood of MDD patients. Likewise, the study also demonstrated a significantly higher percentage and absolute count of CD4⁺ CD25⁺ Foxp3⁺ Tregs in MDD patients compared to healthy control.

Table 3.5. Comparison of Tregs between MDD patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>MDD (n=47)</th>
<th>Healthy control (n=47)</th>
<th>Z statistics</th>
<th>*p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (IQR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4⁺ CD25⁺ Tregs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>3.70 (3.30)</td>
<td>2.30 (2.50)</td>
<td>-4.574</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Absolute count (cells/µl)</td>
<td>95 (90)</td>
<td>53 (44)</td>
<td>-4.553</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD4⁺ CD25⁺ Foxp3⁺ Tregs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>1.55 (2.00)</td>
<td>1.10 (1.00)</td>
<td>-2.969</td>
<td>0.003</td>
</tr>
<tr>
<td>Absolute count (cells/µl)</td>
<td>39 (59)</td>
<td>28 (25)</td>
<td>-3.181</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*Result was considered significant when p<0.05 by using Mann-Whitney test
3.6 Comparison of Lymphocyte Subsets between Different Severity of MDD based on Beck Depression Inventory (BDI)

3.6.1 Comparison of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells between different severity of MDD based on BDI scale

The median percentage and absolute count of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells in mild, moderate and severe group are shown in Table 3.6. The comparison between the three groups was done using Kruskal-Wallis non parametric test. The highest median percentage and absolute count of total T cells was found in severe group, compared to moderate and mild MDD. However, there were no statistically significant difference between them. There were also no significant difference in the median percentage and absolute count of T helper and T cytotoxic cells between mild, moderate and severe MDD. The comparison using Kruskal-Wallis test showed highest percentage and absolute count of NK cells in mild group, followed by moderate and severe. However the differences between the three groups were not statistically significant. Likewise, the median percentage and absolute count of B cells between mild, moderate and severe group were also not significantly different.
Table 3.6. Comparison of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells between different severity of MDD based on BDI scale

<table>
<thead>
<tr>
<th></th>
<th>Severity of MDD</th>
<th>Median (IQR)</th>
<th>$\chi^2$ stat$^a$ (df)</th>
<th><em>p</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T cells (CD3$^+$)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>Mild</td>
<td>63.27 (9.91)</td>
<td>3.918 (2)</td>
<td>0.141</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>67.74 (5.11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>70.32 (14.94)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute counts (cells/µl)</td>
<td>Mild</td>
<td>1647 (521)</td>
<td>0.211 (2)</td>
<td>0.900</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>1625 (696)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>1733 (729)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>T helper (CD3$^+$ CD4$^+$)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>Mild</td>
<td>35.98 (8.74)</td>
<td>1.908 (2)</td>
<td>0.385</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>34.85 (9.05)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>38.29 (11.43)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute counts (cells/µl)</td>
<td>Mild</td>
<td>949 (381)</td>
<td>0.682 (2)</td>
<td>0.711</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>844 (403)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>895 (500)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>T cytotoxic (CD3$^+$ CD8$^+$)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>Mild</td>
<td>26.90 (6.52)</td>
<td>0.416 (2)</td>
<td>0.812</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>27.87 (4.32)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>28.58 (13.74)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute counts (cells/µl)</td>
<td>Mild</td>
<td>670 (322)</td>
<td>0.012 (2)</td>
<td>0.994</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>761 (336)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>714 (601)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NK cells (CD16$^+$ CD56$^+$)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>Mild</td>
<td>18.79 (9.58)</td>
<td>3.599 (2)</td>
<td>0.165</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>17.40 (12.81)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>13.72 (14.03)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute counts (cells/µl)</td>
<td>Mild</td>
<td>504 (265)</td>
<td>3.410 (2)</td>
<td>0.182</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>482 (286)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>357 (407)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B cells (CD19$^+$)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>Mild</td>
<td>15.44 (6.41)</td>
<td>0.877 (2)</td>
<td>0.645</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>13.49 (5.02)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>15.37 (9.09)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute counts (cells/µl)</td>
<td>Mild</td>
<td>407 (267)</td>
<td>2.315 (2)</td>
<td>0.314</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>369 (115)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>321 (200)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Result was considered significant when $p<0.05$ by using Kruskal-Wallis test
3.6.2 Comparison of Tregs between different severity of MDD based on BDI scale

The median percentage and absolute count of CD4⁺ CD25⁺ Tregs and CD4⁺ CD25⁺ Foxp3⁺ Tregs for mild, moderate and severe group are shown in Table 3.7. The result showed that there were no significant difference in the median percentage and absolute count of CD4⁺ CD25⁺ Tregs and CD4⁺ CD25⁺ Foxp3⁺ Tregs between mild, moderate and severe MDD.

Table 3.7. Comparison of Tregs between different severity of MDD based on BDI scale.

<table>
<thead>
<tr>
<th></th>
<th>Severity of MDD</th>
<th>Median (IQR)</th>
<th>χ² stata (df)</th>
<th>*p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4⁺ CD25⁺ Tregs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>Mild</td>
<td>3.95 (3.25)</td>
<td>1.290 (2)</td>
<td>0.525</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>3.25 (2.85)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>3.80 (4.60)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute counts</td>
<td>Mild</td>
<td>110 (87)</td>
<td>1.537 (2)</td>
<td>0.464</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>77 (62)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>95 (129)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD4⁺ CD25⁺ Foxp3⁺ Tregs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>Mild</td>
<td>1.62 (2.23)</td>
<td>0.616 (2)</td>
<td>0.735</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>1.54 (0.91)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>1.53 (1.95)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute counts</td>
<td>Mild</td>
<td>49 (58)</td>
<td>0.473 (2)</td>
<td>0.789</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>37 (29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>39 (72)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Result was considered significant when p<0.05 by using Kruskal-Wallis test
3.7 Comparison of Lymphocyte Subsets between Different Severity of MDD based on Montgomery–Åsberg Depression Rating Scale (MADRS)

3.7.1 Comparison of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells between different severity of MDD based on MADRS scale

The median percentage and absolute count of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells in mild, moderate and severe MDD are shown in Table 3.8. The comparison between the three groups was done using Kruskal-Wallis non parametric test. The highest median percentage and absolute count of total T cells was found in severe group, followed by moderate and mild MDD. However, the differences between the three groups were not statistically significant. Likewise, there were also no significant difference in the median percentage and absolute count of T helper and T cytotoxic cells between mild, moderate and severe MDD. The highest median percentage and absolute count of NK cells were found in mild group, followed by moderate and severe. However, the differences between the three groups were not statistically significant. There were also no significant difference in the median percentage and absolute count of B cells between mild, moderate and severe group.
Table 3.8. Comparison of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells between different severity of MDD based on MADRS scale

<table>
<thead>
<tr>
<th></th>
<th>Severity of MDD</th>
<th>Median (IQR)</th>
<th>$\chi^2$ stat&lt;sup&gt;a&lt;/sup&gt; (df)</th>
<th><em>p</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T cells (CD3&lt;sup&gt;+&lt;/sup&gt;)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>Mild</td>
<td>61.69 (13.54)</td>
<td>3.351 (2)</td>
<td>0.187</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>67.74 (10.69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>68.76 (9.90)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute counts (cells/µl)</td>
<td>Mild</td>
<td>1532 (501)</td>
<td>0.391(2)</td>
<td>0.823</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>1658 (695)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>1719 (512)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>T helper (CD3&lt;sup&gt;+&lt;/sup&gt; CD4&lt;sup&gt;+&lt;/sup&gt;)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>Mild</td>
<td>32.64 (11.02)</td>
<td>3.564 (2)</td>
<td>0.168</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>36.82 (8.82)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>37.85 (10.07)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute counts (cells/µl)</td>
<td>Mild</td>
<td>819 (369)</td>
<td>0.286 (2)</td>
<td>0.867</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>968 (480)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>895 (425)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>T cytotoxic (CD3&lt;sup&gt;+&lt;/sup&gt; CD8&lt;sup&gt;+&lt;/sup&gt;)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>Mild</td>
<td>26.30 (10.60)</td>
<td>0.538 (2)</td>
<td>0.764</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>27.05 (5.05)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>28.62 (13.89)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute counts (cells/µl)</td>
<td>Mild</td>
<td>606 (356)</td>
<td>0.019 (2)</td>
<td>0.991</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>719 (339)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>773 (476)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NK cells (CD16&lt;sup&gt;+&lt;/sup&gt; CD56&lt;sup&gt;+&lt;/sup&gt;)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>Mild</td>
<td>18.79 (5.36)</td>
<td>3.091 (2)</td>
<td>0.213</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>17.78 (12.34)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>13.72 (14.13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute counts (cells/µl)</td>
<td>Mild</td>
<td>525 (296)</td>
<td>3.618 (2)</td>
<td>0.164</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>484 (263)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>357 (336)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B cells (CD19&lt;sup&gt;+&lt;/sup&gt;)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>Mild</td>
<td>15.72 (6.96)</td>
<td>0.374 (2)</td>
<td>0.830</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>14.88 (4.57)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>15.70 (9.87)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute counts (cells/µl)</td>
<td>Mild</td>
<td>441 (192)</td>
<td>1.495 (2)</td>
<td>0.473</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>358 (276)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>345 (160)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Result was considered significant when $p<0.05$ by using Kruskal-Wallis test
3.7.2 Comparison of Tregs between different severity of MDD based on MADRS scale

The median percentage and absolute count of CD4$^+$ CD25$^+$ Tregs and CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs in mild, moderate and severe group are shown in Table 3.9. The highest median percentage and absolute count of CD4$^+$ CD25$^+$ Tregs was observed in severe group compared to moderate and mild MDD. However, there were no statistically significant different between them. There were also no significant difference in the median percentage and absolute count of CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs between mild, moderate and severe group.

Table 3.9. Comparison of Tregs between different severity of MDD based on MADRS scale

<table>
<thead>
<tr>
<th></th>
<th>Severity of MDD</th>
<th>Median (IQR)</th>
<th>$\chi^2$ stat* (df)</th>
<th>*p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em><em>CD4</em> CD25</em> Tregs**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>Mild</td>
<td>3.20 (1.18)</td>
<td>1.180 (2)</td>
<td>0.554</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>3.65 (4.48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>4.50 (4.85)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute counts</td>
<td>Mild</td>
<td>89 (42)</td>
<td>0.472 (2)</td>
<td>0.790</td>
</tr>
<tr>
<td>(cells/µl)</td>
<td>Moderate</td>
<td>91 (88)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>104 (130)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em><em>CD4</em> CD25</em> Foxp3 Tregs**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>Mild</td>
<td>1.33 (0.77)</td>
<td>2.859 (2)</td>
<td>0.239</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>1.65 (2.65)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>1.65 (2.19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute counts</td>
<td>Mild</td>
<td>33 (25)</td>
<td>1.831 (2)</td>
<td>0.400</td>
</tr>
<tr>
<td>(cells/µl)</td>
<td>Moderate</td>
<td>47 (56)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>39 (63)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Result was considered significant when $p<0.05$ by using Kruskal-Wallis test
MDD is a serious mental problem and it is expected to be the second leading contributor to the global burden of disease by the year 2020. MDD can cause severe impairment in social and physical functioning and is often a major precipitating factor in other mental illness and suicide. An increasing body of literature demonstrates the involvement of immune dysregulation in MDD (Grosse et al., 2015; Miller et al., 2009; Dantzer et al., 2008). Meta-analysis has shown that the pro-inflammatory cytokines like tumor necrosis factor (TNF), interleukin (IL)-6 and C-reactive protein (CRP) are consistently elevated in the serum of patients with MDD (Dowlati et al., 2010).

Immune dysregulation is, however, not only reflected by altered levels of inflammatory cytokines, but probably also by changes in the apportioning of various circulating lymphocytes subsets. A few studies reported altered level of circulating lymphocyte subsets in MDD patients, but the findings have not been entirely consistent. Some studies suggested that the inconsistent finding of immune variations in MDD patients might be the lesser severity of their illness (Schleifer et al., 1989; Maes, 1995). Therefore, this study was carried out to determine the percentage and absolute count of T helper cells (CD4^+ T cells), T cytotoxic cells (CD8^+ T cells), NK cells (CD16^+ CD56^+ NK cells), B cells (CD19^+ B cells), CD4^+ CD25^+ Treg cells and CD4^+ CD25^+ Foxp3^+ Treg cells in MDD patients and their comparison between
different severity of the disease. This study was conducted among 47 MDD patients recruited from out-patient psychiatric clinic, Hospital Universiti Sains Malaysia (HUSM) and 47 healthy controls.

4.1 Sociodemographic Characteristics of MDD Patients

In the present study, the mean age of healthy controls was 28.0 years, while the mean age of MDD patients was 39.7 years. The finding was in contrast with previous study done by Khan et al. (2011) in Penang, Malaysia which reported that the mean age of MDD patients was 45 (±17.8) years, with a majority aged over 50 year (Khan et al., 2011). The respondents age range from 18 to 65 years old. The rates of MDD was highest in those aged 45 to 65 years. Our result were in contrast with Chiu (2004) which reported that the highest rates of MDD in Asia Pacific region was in those aged 25 to 44 years (Chiu, 2004). However, some studies stated that the age group may varies with gender (Rait et al., 2009; Lee et al., 2016). Rait et al. (2009) reported the highest incidence rates for MDD was 25 to 44 year old for women and 44 to 65 year old for men (Rait et al., 2009). The findings were consistent with our study which also showed that the highest incident rate for men was 44 to 65 year old, while for women the incidence rate was highest in those aged 25 to 44 years. The mean age of onset for our MDD patients was 36.26 years. Based on Bromet et al. (2011), the mean age of onset for MDD was 25.7 years in the high-income and 24 years in low to middle-income countries (Bromet et al., 2011). In general, the mean age and age of onset of MDD may varies with study population across countries.
MDD patients consist of 61.7% females and 38.2% males. Epidemiologic studies had consistently shown that women have greater incidence rates of MDD than men with female to male ratio of 2:1 regardless of racial, ethnic background or economic status (Kessler et al., 2003; Bottomley et al., 2010; Van de Velde et al., 2010; Bromet et al., 2011; Khan et al., 2011). The cause of this sex differences remain unclear. However, several hypotheses have been put forward. Some researchers suggested that biological factors such as genetic different and hormonal changes may account for the sex difference (Kendler et al., 1993; Nolen-Hoeksema, 2001). Others hypothesized women may reported depressive symptoms more often than men, resulting in greater rates of MDD in women (Poutanen et al., 2009). While, some studies stated that psychosocial factors like relationship problems, lack of social support, adverse experiences in childhood, and life events may have a greater impact in women than men, thereby increasing the incidence rate of MDD (Piccinelli and Wilkinson, 2000; Accortt et al., 2008).

Majority of our MDD patients were Malays. However, a study on the prevalence of MDD in Selangor, Malaysia reported that the prevalence of MDD was highest among other ethnic groups (Iban, Kadazan, Orang Asli, Siam and other minorities group)(17.6%), followed by Chinese (13.8%), Malays (10.8%) and Indians (6.1%) (Maideen et al., 2014). The fact that MDD patients in this study population consists of mostly Malay (95.7%) merely reflects the racial distribution in Kelantans, where majority of the population are Malay ethnicity.

In this study, 12.8% of MDD patients had family history of depression. It has been found that people with a first-degree family member who has experienced depression
are 2 to 10 times more likely to develop MDD (Wallace et al., 2002; Goodwin and Jamison, 2007). Perris et al. (1982) hypothesized that patients without a family history of depression would be “less vulnerable from a genetic point of view and, consequently that such patients would require more, or more severe events to precipitate depression than the more vulnerable” patients with a family history of depression (Perris et al., 1982). In addition, Monroe et al. (2014) reported that patients with a positive family history of depression would have more lifetime episodes of depression than patients with a negative family history of depression (Monroe et al., 2014).

### 4.2 Predisposing Factors of MDD

Marital status has been found to be highly associated with the prevalence of MDD. This study showed that married person have higher risk for MDD compared to those unmarried. Few studies examine marital status differences in MDD in other countries showed different finding. A study in Kangwha Island, South Korea, reported higher risk of MDD in married person compared to unmarried (Lee, 1991), which is consistent with our study. However, survey studies in Western nations like Canada, Netherland and United States showed that unmarried persons were more likely to have MDD compared to married (Andrade et al., 2003). Stegenga et al. (2012) suggested that marital disruption increase the risk of MDD among married woman, while being unmarried was an important risk factor for MDD in men (Stegenga et al., 2012).

Our result showed that smoker were at increased risk of having MDD compared to non-smoker. Our results are in agreement with previous studies which also reported
increased odd ratio of depression in smokers (Hamalainen et al., 2001; Pasco et al., 2008). In a population based longitudinal Norwegian study, a dose-dependent relationship between smoking and depression was found, with the risk of the heaviest smokers (>20 cigarettes per day) being more than fourfold elevated compared to the risk of those who had never smoked (Klungsøy et al., 2006). Meanwhile, a population based retrospective Australian study (10 years) found almost a doubling of the risk for developing MDD among heavy smokers (>20 cigarettes per day) (Pasco et al., 2008). These findings showed that smoking is actually an important risk factor in the causal network leading to development of depression. Pomerleau and Pomerleau (1985) stated that nicotine use may increase vulnerability to depression as nicotine influences several neurochemical systems, such as acetylcholine and catecholamine systems (Pomerleau and Pomerleau, 1985), which may play an etiologic role in depression (Carmody et al., 2007). Furthermore, tobacco smoke generates free radicals, causing oxidation of proteins and other tissue damage (Ozguner et al., 2005), and depression has been characterized by elevated markers of oxidative stress which is positively correlated with the severity of the depression (Khanzode et al., 2003; Yanik et al., 2004)

Epidemiological studies of MDD support an inverse association between the prevalence of MDD and level of education (Lorant et al., 2003). Higher education was associated with lower risk of mood disorder in a pan-European study (Alonso et al., 2004) and in Holland those with the fewest years of education had the highest morbidity rates (Bijl et al., 1998). Coryell et al. (1992) also reported that men with no college education were more likely to develop MDD than men with college education (Coryell et al., 1992). The findings are in accordance with our study which
indicate that patients with lower educational level have higher risk for MDD compared to those with higher educational level. In some developing countries, educational level were recognized to be the key component that determining socio-economic position and income obtained in later life (Shi et al., 2014). People with lower educational level usually have lower socioeconomic position and faced economic hardship like financial strain, unemployment, deprivation and poverty. These living condition were associated with an increase in risk of MDD (Kessler et al., 2003; Lorant et al., 2007; Shi et al., 2014).

Urban versus rural residence is commonly cited as risk factor for MDD and it was identified to play an etiological role in these disorder (Wang, 2004). Rural area was defined as area with population less than 10,000 people, agriculture area, forest and water bodies. While urban area is characterized by higher population density of more than 10,000 people and vast human features in comparison to area surrounding it (Collins English Dictionary, 2013). Similar to Breslau et al. (2014) and Probst et al. (2006), our study showed that people living in rural area have higher risk for MDD compared to those living in urban area. Probst et al. (2006) suggested that people in rural areas were more likely to have MDD as they experience circumstances, conditions, and behaviors that challenge health and may increase the prevalence of depression. These include a greater likelihood of reporting fair or poor health, physical inactivity, and poverty (Probst et al., 2006).
4.3 Distribution of MDD Symptoms Among Patients

Major depressive disorder (MDD) is characterized by several diagnostic symptoms and feelings such as sadness, loss of interest or pleasure in usual activities, sleep and/or appetite disturbances, fatigue, irritability, suicidal thought and etc. (American Psychiatric Association, 2013). The proper identification of MDD symptoms is important for the accurate diagnosis of MDD, development of treatment strategies and measurement of outcome.

Somatic symptoms are very common in patients with MDD (Tylee and Gandhi, 2005; Demyttenaere et al., 2006). According to an analysis of data from the World Health Organization, 69% of patients in primary care settings meeting the DSM-IV criteria for MDD presented somatic symptoms as their primary reason for seeking medical care (Simon et al., 2001). Somatic symptoms in depressed patients are associated with more severe depression of longer duration and greater functional impairment (Fritzsche et al., 1999), poorer clinical outcome (McIntyre et al., 2006) and higher health-care costs (Gameroff and Olfson, 2006). Based on Beck Depression Inventory, the most common somatic symptoms reported by our MDD patients was fatigue. Fatigue has been considered a core feature of MDD by American Psychiatric Association (American Psychiatric Association, 2000). According to Baldwin and Papakostas (2005), fatigue is also one of the most common residual symptoms of a partially resolved depression. Fatigue symptoms can affect physical, cognitive, and emotional function, impair school and work performance, disturb social and family relationships, and increase healthcare utilization (Targum and Fava, 2011).
Previous studies show that in addition to somatic symptoms, cognitive symptoms also play a crucial role in MDD (Marvel and Paradiso, 2004; Naismith et al., 2007). Research indicates that verbal and visual short and long-term memory, executive functions, psychomotor skills and attention are all impaired in depressed patients (Austin et al., 2001; Castaneda et al., 2008; Hammar and Årdal, 2009; Marazziti et al., 2010). Increased cognitive dysfunction was often associated with greater symptom severity (Gonda et al., 2015). Based on BDI questionnaire, the most common cognitive symptoms reported by our MDD patients was irritability or psychomotor agitation. Irritability has been commonly found in clinical cases of adults with MDD and this symptom has been associated with greater overall severity, anxiety comorbidity, disability, and suicidality among depressed patients (Perlis et al., 2005; Perlis et al., 2009; Fava et al., 2010).

Based on MADRS questionnaire, all MDD patients reported inner tension, concentration difficulties, inability to feel, and pessimistic thought symptoms. According to a study done by Brown et al. (2016), inner tension and concentration disabilities symptoms in MADRS scale specify the presence of anxious distress or anxiety in MDD patients (Brown et al., 2016). The presence of anxiety symptoms in depressed patients was associated with more impaired functioning (Chan et al., 2012), worse quality of life (Chan et al., 2012), lower response rates and higher relapse (Flint and Rifat, 1997; Kennedy, 2008). Besides that, depressed patients with anxious symptom have also been associated with increased suicidality and overall severity of depression (Fava et al., 2006). This is consistent with our result from MADRS assessment which demonstrated that almost 93.6% of MDD patients have suicidal thought or tought of death. We observed that most severe MDD patients
have suicidal thought and conversely those patients with suicidal thought had worse symptoms of MDD. Previous study also reported that more than 90% of subject who commit suicide had previously been diagnosed with MDD with comorbid anxiety (Ibiloglu et al., 2016).

4.4 The Severity of MDD Patients

The severity of MDD patients were determined by using the total score obtained from BDI and MADRS questionnaire. Based on BDI scale, 28 patients were categorised as mild MDD, 10 patients were categorised as moderate and 9 patients were categorised as severe MDD. While based on MADRS scale, 10 patients were considered as mild, 24 patients were considered as moderate and 13 patients were considered as severe MDD.

The present study showed that the self-reported (BDI) and clinician-rated (MADRS) outcomes were not equivalent. MADRS scale demonstrated more moderate and severe patients, while BDI scale reported more mild patients. It has been found that patients often report fewer symptoms of depression than were observed by psychiatrist or surrounding people (Lyness et al., 1995; Hunt et al., 2003). For example, in this study only 31.9% of MDD patients reported suicidal thought, eventhough according to clinical judgement almost 93.6% of patients actually have this symptom. We also observed that MDD patients more readily to admit to fatigue and irritability symptoms than thoughts of hopelessness or suicide, even when these cognitive symptoms are actually present.
Besides that, many depressed patients believe that some symptoms are less socially acceptable, more stigmatizing, or riskier to report than the core symptoms. For example, it is easier for many medical patients to admit to sleep or appetite disturbances than loss of libido or self dislike. A number of studies have addressed the problem of self-report bias or underreporting are more exaggerated in men (Hunt et al., 2003) and patients with older age (Lyness et al., 1995).

This sort of reporting bias would result in lower total scores from patients who selectively report the symptoms than in those who are willing to report a broader range of symptoms. Consequently, it may seem that the patients are less severely depressed than they actually are and the total score obtained from self-reported scale would be less than the clinician-rated scale. However, according to Uher et al. (2012), self-report and clinician rating each provide unique information that is relevant to clinical prognosis. The self-report may provide information that is not accessible by clinician rating or vice versa (Uher et al., 2012). Therefore, an accurate and complete assessment of depression should include both patient-rated scale and clinician-rated scale.
4.5 Enumeration of Lymphocyte Subsets

4.5.1 Comparison of leukocytes between MDD patients and healthy controls

In the present study, we did not observed any significant differences in the total number of circulating leukocytes, neutrophils, eosinophils, basophils, monocytes and lymphocytes between MDD patients and healthy controls. The findings are in accordance with previous studies indicating no significant differences in total number of leukocytes and lymphocytes between MDD patients and healthy controls (Seide et al., 1996; Li et al., 2010). Li et al. (2010) suggested that the immune imbalance in MDD was associated with dysregulation among different subsets of lymphocytes, rather than the whole peripheral lymphocyte population (Li et al., 2010).

4.5.2 Comparison of total T cells, T helper cells, T cytotoxic cells, NK cells and B cells between MDD patients and healthy controls

The normal range of lymphocyte subsets in healthy individuals have been established by a number of studies from various population (Bisset et al., 2004; Wong et al., 2013; Choi et al., 2014). The median percentage and absolute count of lymphocytes, total T cells, T helper cell, T cytotoxic cells, NK cells and B cells in healthy controls are within the normal range of healthy adult of Asian population (Chng et al., 2004).

T cell may play a pivotal role in both the development and treatment of depression through their neuroprotective and anti-inflammatory effect (Miller, 2010). While
reduced T cell number and percentage has been reported in patient with MDD (Schleifer et al., 1984; Zorrilla et al., 2001; Miller, 2010), our study did not detect any significant differences in the percentage or absolute count of total T cell between MDD patients and healthy controls. However, our result was similar with previous study by Hosseini et al. (2007) which also did not find any significant difference in the absolute count of total T cells between MDD patients and healthy controls (Hosseini et al., 2007).

We also did not observed any significant differences in the percentage or absolute count of T helper cells between MDD patients and healthy controls. The finding was in contrast with previous studies by Zorrilla et al. (2001) and Miller (2010) which reported decreased absolute count and percentage of T helper cells in MDD patients compared to healthy controls. However, our finding are consistent with Robertson et al. (2005) who found no significant difference in the absolute count of T helper cells between MDD patients and healthy control (Robertson et al., 2005). Consistent with previous studies (Schlatter et al., 2004; Robertson et al., 2005; Başterzi et al., 2010), we found that the percentage and absolute count of T cytotoxic cells between MDD patients and healthy controls were not significantly different.

NK cells play important roles in immunological surveillance against viral infections and tumors. Natural killer cell counts and activity have been determined in MDD patients in a number of studies, but with divergent or even conflicting results. While some studies found decreased numbers of NK cells (Evans et al., 1992; Andreoli et al., 1993; Schleifer et al., 1996), other studies observed an increase in NK cells (Ravindran et al., 1995) and no differences in the percentage and absolute count of
NK cells between MDD patients and healthy controls (Maes et al., 1994a; Hosseini et al., 2007). Some studies also reported blunted natural killer cells activity (NKCA) in patients with MDD (Kronfol et al., 1989; Evans et al., 1992; Maes et al., 1992b). However, in this study, we found no significant difference either in the percentage or absolute count of NK cells between MDD patients and healthy controls.

B lymphocytes are the effectors of humoral immunity. They provide defence against pathogens through different functions including antibody production (LeBien and Tedder, 2008). We found that the percentage and absolute counts of CD19+ B cell in MDD patients did not differ from healthy controls. The investigation of B cell numbers also has yielded contradictory results. Maes et al. (1992a) found a significantly increased number of CD19+ B cells in depressed subject, Schleifer et al. (1984) detected lower B cell numbers during depression, while other authors were unable to find any alterations in B cell numbers during depression (Sengar et al., 1982; Darko et al., 1988; Evans et al., 1988; Hosseini et al., 2007).

The results concerning lymphocyte subsets in MDD patients were contradictory and more controversial. There are several factors that might influenced the results. One explanation for this could be that most of the previous studies determined the number of lymphocyte cells after separation of mononuclear cells, whereas we determined T cells, NK cells and B cells in the whole blood. Previous study showed that lower counts were obtained for CD3+, CD4+ and CD8+ T cells after mononuclear cell isolation (Levering et al., 2008). Furthermore, we assessed NK cells staining CD56+ and CD16+ antigen, while other groups determined NK cells by staining CD3− CD16+, CD56+, or CD16+ NK cells only.
Besides that, different flow cytometric technique also can influence the absolute count of lymphocyte subset. For example, different gating strategies and compensation during flow cytometric analysis might give different number of viable cells to researcher. Previous studies have shown that the “CD45-SSC” gating strategy yielded higher outcomes for CD3$, CD4$, and CD8$^+$ T cell counts, while lower results were obtained for NK cell counts using the “SSC-CD45-CD3” gating strategy (Levering et al., 2008).

It has been suggested that modulating factors such as the biological factors and environmental factors also might be responsible for the result variation. Biological rhythms of various type exert influences on lymphocyte subsets. For example, because of ultraradian (less than 24 hours) rhythms, there may be lower total numbers of lymphocytes in the peripheral blood at several time points during a 24 hours day. Hence, to minimize the effects of ultraradian or circadian rhythm on the lymphocyte subsets count, all blood samples were collected within 8.00 am to 12.00 pm.

The distribution of lymphocyte subsets in peripheral blood can also be altered by exercise. Exercise decreases the number of CD3$^+$ T cells and CD4$^+$ T cells in peripheral blood while increasing the percentage of CD16$^+$ NK cells. After cessation of vigorous exercise, the number of CD4$^+$ T cells return to normal within 120 minutes, whereas the NK cell numbers return to baseline only after 24 hours (Tvedeet al., 1993). Other studies suggested that the number of CD8$^+$ T cells also is increased by vigorous physical activity (Brahmi et al., 1985). Light to heavy cigarette smoking can also alter both total leukocytes count and the percentage of
lymphocytes. Among the subsets, lower percentage of CD4\(^+\) T cells with increased CD8\(^+\) T cells have been reported (Miller et al., 1982). Other studies have suggested that the number of NK cells is reduced in the blood of smoker (Tollerud et al., 1989).

In addition, environmental factor like pollutant also can cause variation in the lymphocyte subsets count. For example, exposure to persistent environmental pollutant such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) caused a significant decreased in the percentage of CD3\(^+\) T and CD4\(^+\) Tcells and an increased in the percentage of CD8\(^+\) T cells (Ciftci et al., 2011).

### 4.5.3 Comparison of Tregs between MDD patients and healthy controls

In the present study, we found that the percentage and absolute count of CD4\(^+\) CD25\(^+\) Tregs were significantly increased in MDD patients compared to healthy controls. Our finding was contradictory with Li et al. (2010) which reported decreased percentage and absolute count of CD4\(^+\) CD25\(^+\) Tregs in the peripheral blood of MDD patients (Li et al., 2010). However, Li et al. (2010) included patients that had not used antidepressant medication, while our MDD patients were on antidepressant treatment. Our finding was in accordance with Himmelrich et al. (2010) who described a similar increase of CD4\(^+\) CD25\(^+\) Treg cells in MDD patients during antidepressant treatment (Himmelrich et al., 2010).

Many studies addressing the number of Tregs in MDD reported CD4\(^+\) CD25\(^+\) Tregs only. Some studies suggested that measuring CD4\(^+\) CD25\(^+\) cells only may not provide a precise assessment of Tregs numbers (Ronaldson et al., 2015). This is because CD25 is the IL-2 receptors which are expressed across a continuum of cells,
and therefore is not unique to natural Tregs only (Brusko et al., 2005). For example, conventional T cells express CD25 when activated by T cell receptor (TCR) ligation (Workman et al., 2009). Hori et al. (2003) found that the development and function of natural CD4+ CD25+ Tregs required Foxp3 transcription factors (Hori et al., 2003). Continued Foxp3 expression in mature Tregs is needed to maintain the transcriptional and functional program established during Tregs cell development (Williams and Rudensky, 2007). According to Zhou et al. (2009), when Tregs lose this transcription factor they become ‘exTregs’ which are effector-like T cells that no longer serve to regulate immune function (Zhou et al., 2009). Hence, some studies conclude that Foxp3 is a specific marker for Tregs and measuring CD4+ CD25+ Foxp3+ Tregs are essential for the measurement of functional Tregs (Zhou et al., 2009; Ronaldson et al., 2015).

Therefore, to further confirm the number of Tregs in MDD, we also measured the percentage and absolute count of CD4+ CD25+ Foxp3+ Tregs. We found that the percentage and absolute count of CD4+ CD25+ Foxp3+ Tregs are lowered than the percentage and absolute count of CD4+ CD25+ Tregs in both study groups. Previous study reported that only those CD4+ T cells that expressed very high levels of CD25 will expressed Foxp3 transcription factors and they representing only ~2-3% of total CD4+ T cells, compared to natural CD4+ CD25+ Treg cells which represent ~5-10% of the total CD4+ T cell population (Hori et al., 2003; Baecher-Allan and Hafler, 2006). Hence, our finding showed that the number of functional Tregs are lower that the number of natural Treg.
We also found a significantly higher percentage and absolute count of CD4+ CD25+ Foxp3+ Tregs in the peripheral blood of MDD patients compared with healthy controls. Our result are in accordance with Grosse et al. (2015) who also found significant increased of CD4+ CD25+ Foxp3+ Tregs in MDD patients after antidepressant treatment. In another study, Zhang et al., (2013) also found that antidepressant desipramine upregulated CD4+ CD25+ Foxp3+ Tregs in allergic rhinitis (AR) patients, which were found down-regulated in established AR mice (Zhang et al., 2013).

Many studies showed that MDD was associated with chronic inflammation as manifested by increased inflammatory cytokines including TNF-α, IL-1 and IL-6 in the peripheral blood (Miller, 2010; Liu et al., 2012). Since Tregs are the potent suppressor for chronic inflammation, the antidepressant medications might cause an upregulation of CD4+ CD25+ Foxp3+ Tregs to counter the exaggerated inflammatory response. Previous studies have shown that the inflammatory cytokines such as TNF-α, IL-6, and IL-1 decreased (Kubera et al., 2001; Basterzi et al., 2005; Piletz et al., 2009) and Tregs percentages were normalized and became similar to controls after successful antidepressant therapy (Grosse et al., 2015).

In addition, previous study found that prolonged exposure to stress in healthy people also caused an increased in Tregs percentage (Pukhalsky et al., 2008; Ronaldson et al., 2015). Pukhalsky et al. (2008) suggested that as inflammation increases after prolonged stress, Tregs could accumulate over time in order to counter this exaggerated inflammatory stress response. It is thus possible that in our study, the
antidepressants facilitate the upregulation of CD4\(^+\) CD25\(^+\) Foxp3\(^+\) Treg cells in order to counter the exaggerated inflammatory response in MDD patients.

4.6 The Comparison of Lymphocyte Subsets between Different Severity of MDD

Variation of circulating lymphocyte subsets have been reported in depression, however the findings were not consistent. Some studies suggested that these inconsistent finding of immune variations in MDD patients might be the lesser severity of their illness. Numerous studies have indicated that the severity of depression is important with respect to the extent of the immune changes in depressive patients (Schleifer et al., 1989; Maes, 1995). This impairment seems to increase with the severity of depression (Schleifer et al., 1989).

In the present study, we found that the level of lymphocyte subsets did not vary with the severity of illness. Our result showed that there were no significant differences in the percentage and absolute count of total T cells, T helper cells, T cytotoxic cell, NK cells, and B cells between mild, moderate and severe MDD. The similar result was found using both BDI and MADRS severity group. Our findings are in accordance with previous study by Hosseini et al. (2007) who also did not find any significant differences in total absolute count of NK cells, B cells and T cells among healthy controls, moderate and severe MDD patients. Maes et al. (1994b) found a significantly decreased NK cell number and activity in severely depressed subjects (Maes et al., 1994b). We observed a decreasing trend in median percentage and absolute count of NK cells with increasing severity of MDD using both BDI and MADRS scale, but the differences did not reach statistical significance. One
explanation for this could be due to the small sample size within each severity group especially in severe patients.

Miller (2010) hypothesized that Tregs may associated with the severity of MDD through downregulation of chronic inflammatory responses (Miller, 2010). However, our study showed that both percentage and absolute count of CD4+ CD25+ Tregs and CD4+ CD25+ Foxp3+ Tregs were not significantly different in mild, moderate and severe patients. This revealed that the increased in CD4+ CD25+ Tregs and CD4+ CD25+ Foxp3+ Tregs were not related with the severity of MDD. Our finding was supported by Himmerich et al. (2010) which also found that the level of CD4+ CD25+ Tregs in patients with moderate to severe depression did not differ from those with milder depression and the relative changes in severity score during antidepressant therapy did not significantly correlated with the relative changes or baseline level of CD4+ CD25+ Treg (Himmerich et al., 2010).
CHAPTER 5

CONCLUSION

5.1 Conclusion

This is the first study on lymphocyte subsets and severity of MDD done in Malaysia. This study found that sociodemographic factors like marital status, smoking status, educational level and area of living were significantly associated with MDD. The results showed that those married, smoker, possess lower educational level and living in rural area were at a higher risk for MDD. Among all symptoms, fatigue and irritability are the most common symptoms reported by our MDD patients. Based on clinical judgement, most of our MDD patients also have inner tension, concentration difficulties, inability to feel and pessimistic thought.

There were no significant differences in the percentage and absolute count of T helper cells (CD4+ T cells), T cytotoxic cells (CD8+ T cells), NK cells (CD16+ CD56+ NK cells), and B cells (CD19+ B cells) between MDD patients and healthy controls. However, we found that the percentage and absolute count of CD4+ CD25+ Tregs and CD4+ CD25+ Foxp3+ Tregs were significantly higher in MDD patients compared to healthy controls. Tregs are the potent suppressor for chronic inflammation. Therefore, the antidepressant medications might caused an upregulation of Tregs to counter the exaggerated inflammatory response in MDD patients.
The result also showed that the percentage and absolute count of T helper cells, T cytotoxic cells, NK cells, B cells, CD4^+ CD25^+ Treg cells and CD4^+ CD25^+ Foxp3^+ Treg cells were not significantly differences between mild, moderate and severe MDD patients. Hence, we can conclude that the percentage and absolute count of lymphocyte subsets in MDD patients were not related with the severity of the disease.

5.2 Limitations and Recommendations

This study has several limitations. First, the age of MDD group was not match with the control group. Mostly older people have health problem like high blood pressure, diabetes and increases stresses level that may affect the immunological parameters. Therefore, majority of healthy controls in this study were selected from a younger age group.

Second, this study has small number of severe patients. All MDD patients were recruited from outpatient psychiatric clinic. Hence, most of the patients were in mild or moderate state. Besides that, most patients with severe MDD have other chronic diseases like cancer, autoimmune disease and chronic infection. This is consistent with the reports that the prevalence of MDD in Malaysia was higher among adult with chronic diseases compared to those without any chronic disease (Aris et al., 2014; Maideen et al., 2014). Besides that, existing medical condition and chronic disease can cause a lot of stress to patients and as a result severe depression is often comorbid with other chronic diseases (Ng, 2014).
Third, this study is an explorative naturalistic study. MDD patients were treated with different kinds of antidepressant drugs according to their doctor’s choice and the individual dose was adjusted according to clinical judgment. The specific effects of different antidepressants cannot be excluded due to the low number of MDD patients. However, the possible effects of specific antidepressant have been calculated, but we did not find any significant influence on our patients.

At present, we can only speculate the antidepressants that exert the increasing of Tregs in our MDD patients. We cannot confirm its effect on the level of inflammatory cytokines in our patients since we only observed the level of lymphocyte subsets. Therefore, further in vitro studies regarding the effect of antidepressants on the number and functions of Treg cells in relation to anti-inflammatory response are warranted.
REFERENCES


subsets in patients with major depressive disorder: a flow cytometric analysis. 
*Progress in Neuro-Psychopharmacology and Biological Psychiatry, 34(1),70-75.*


Liu, Y., Ho, R. C.-M. & Mak, A. (2012). Interleukin (IL)-6, tumour necrosis factor alpha (TNF-α) and soluble interleukin-2 receptors (sIL-2R) are elevated in patients with major depressive disorder: a meta-analysis and meta-regression. *Journal of Affective Disorders, 139*(3),230-239.


to develop novel, improved therapeutics for difficult-to-treat depression. *Biological Psychiatry, 53*(8), 707-742.


Takeda, K. & Dennert, G. (1993). The development of autoimmunity in C57BL/6 lpr mice correlates with the disappearance of natural killer type 1-positive cells: evidence for their suppressive action on bone marrow stem cell proliferation, B cell immunoglobulin secretion, and autoimmune symptoms. *Journal of Experimental Medicine, 177*(1),155-164.


APPENDICES

APPENDIX A

BORANG KEIZINAN PESAKIT

MAKLUMAT KAJIAN UNTUK PESAKIT

Tajuk Kajian: Pengukuran tahap subset limfosit dan kaitannya dengan tahap keterukan penyakit kemurungan (major depressive disorder)

Nama Penyelidik: Dr. Noor Suryani Mohd Ashari (No MMC : 34465)
Dr. Mohd Azhar Mohd Yasin (No MMC : 34205)

Pengenalan


Tujuan Kajian

Kajian ini bertujuan untuk menentukan tahap subset limfositdi dalam darah untuk menentukan kaitannya dengan penyakit kemurungan

Kelayakan Penyertaan

Doktor yang bertanggungjawab dalam kajian ini atau salah seorang kakitangan kajian telah membincangkan kelayakan untuk menyertai kajian ini dengan anda. Adalah penting anda berterus terang dengan doktor dan kakitangan tersebut tentang sejarah kesihatan anda. Anda tidak seharusnya menyertai kajian ini sekiranya anda tidak memenuhi semua syarat kelayakan.

Keperluan untuk menyertai kajian ini adalah anda mengidap penyakit kemurungan

Anda tidak boleh menyertai kajian ini sekiranya anda mempunyai penyakit kekurangan sistem pertahanan badan seperti kanser, kencing manis ataupun HIV dan AIDS. Anda juga tidak boleh menyertai kajian ini jika anda mendapat jangkitan bakteria atau virus ataupun menjalani pembedahan dalam masa sebulan sebelum kajian ini

Risiko


Penyertaan di dalam kajian

Penyertaan anda dalam kajian ini adalah secara sukarela. Anda boleh menolak penyertaan dalam kajian ini tanpa sebarang hukuman. Penyertaan anda mungkin juga diberhentikan oleh doktor kajian sekiranya anda didapati tidak berkelayakan menyertai kajian ini.

Manfaat yang mungkin

Prosedur kajian akan diberikan kepada anda tanpa kos (percuma). Anda juga akan diberi saguhati untuk darah yang diambil bagi tujuan kajian. Kajian ini dijalankan untuk tujuan pengubatan di masa hadapan. Anda mungkin menerima maklumat tentang kesihatan anda daripada ujian makmal yang diakukan dalam kajian ini.

Kerahsiaan

**Pertanyaan**

Sekiranya anda mempunyai sebarang soalan mengenai prosedur kajian ini atau hak-hak anda, sila hubungi:

Dr Noor Suryani Mohd Ashari  
Pensyarah Perubatan  
No pendaftaran MMC : 34465  
Jabatan Imunologi  
Pusat Pengajian Sains Perubatan  
USM Kampus Kesihatan.  
Tel:09-7673000  
Sambungan USM: 6225.

Sekiranya anda mempunyai sebarang soalan berkaitan kelulusan Etika kajian ini, sila hubungi:

Puan Mazlita Zainal Abidin  
Setiausaha Jawatankuasa Etika Penyelidikan (Manusia) USM  
Pelantar Penyelidikan Sains Klinikal, USM Kampus Kesihatan.  
No. Tel: 09-7672355/7672352  
Email: iepem@kk.usm.my

**Tandatangan**

Untuk dimasukkan ke dalam kajian ini, anda mestilah menandatangani serta meletakkan tarikh di Lampiran 1.
Borang Keizinan Pesakit
(Halaman Tandatangan)

**Tajuk Kajian:** Pengukuran tahap subset limfosit dan kaitannya dengan tahap keterukan penyakit kemurungan (major depressive disorder)

Nama Penyelidik: Dr. Noor Suryani Mohd Ashari (No MMC : 34465)
Dr. Mohd Azhar Mohd Yasin (No MMC : 34205)

Untuk menyertai kajian ini, anda atau wakil sah anda mesti menandatangani mukasurat ini.

Dengan menandatangani muka surat ini, saya mengesahkan yang berikut:

- Saya telah membaca semua maklumat dalam Borang Maklumat dan Keizinan Pesakit ini termasuk apa-apa maklumat berkaitan risiko yang ada dalam kajian dan saya telah pun diberi masa yang mencukupi untuk mempertimbangkan maklumat tersebut.
- Semua soalan-soalan saya telah dijawab dengan memuaskan.
- Saya, secara sukarela, bersetuju menyertai kajian penyelidikan ini, mematuhi segala prosedur kajian dan memberi maklumat yang diperlukan kepada doktor, para jururawat dan juga kakitangan lain yang berkaitan apabila diminta.
- Saya boleh menamatkan penyertaan saya dalam kajian ini pada bila-bila masa.
- Saya telah pun menerima satu salinan Borang Maklumat dan Keizinan Pesakit untuk simpanan peribadi saya.

Nama Pesakit (Dicetak atau Ditaip)

Nama & Tandatangan Individu yang Mengendalikan Perbincangan Keizinan (Dicetak atau Ditaip)

Nama Saksi dan Tandatangan

---

LAMPIRAN 1
APPENDIX B

BORANG KEIZINAN KONTROL

MAKLUMAT KAJIAN UNTUK KONTROL

Tajuk Kajian: Pengukuran tahap subset limfosit dan kaitannya dengan tahap keterukan penyakit kemurungan (major depressive disorder)

Nama Penyelidik: Dr. Noor Suryani Mohd Ashari (No MMC : 34465)
 Dr. Mohd Azhar Mohd Yasin (No MMC : 34205)

Pengenalan


Tujuan Kajian

Kajian ini bertujuan untuk menentukan tahap subset limfositdi dalam darah untuk menentukan kaitannya dengan penyakit kemurungan

Kelayakan Penyertaan

Doktor yang bertanggungjawab dalam kajian ini atau salah seorang kakitangan kajian telah membincangkan kelayakan untuk menyertai kajian ini dengan anda. Adalah penting anda berterus terang dengan doktor dan kakitangan tersebut tentang sejarah kesihatan anda. Anda tidak seharusnya menyertai kajian ini sekiranya anda tidak memenuhi semua syarat kelayakan.

Keperluan untuk menyertai kajian ini adalah anda tidak mengidap penyakit psikiatri termasuk kemurungan. Anda tidak boleh menyertai kajian ini sekiranya anda mengandung, mempunyai penyakit kekurangan sistem pertahanan badan seperti kanser, kencing manis ataupun HIV dan AIDS. Anda juga tidak boleh menyertai kajian ini jika anda mendapat jangkitan bakteria atau virus ataupun menjalani pembedahan dalam masa sebulan sebelum kajian ini

Sekiranya anda bersetuju untuk menyertai kajian ini, anda diperlukan untuk diambil darah sebanyak 15 ml. Darah ini akan digunakan untuk menentukan subset limfositdi dalam darah.Dengan keputusan ujian yang diperolehi nanti kita akan menentukan paras subset limfositdi dalam darah untuk dibandingkan dengan paras subset limfositdi dalam pesakit kemurungan.Selepas ujian dilakukan, darah atau serum akan dilupuskan.
**Risiko**

Anda akan merasa sedikit sakit di tempat darah di ambil di tangan anda semasa prosedur pengambilan darah. Tiada risiko lain di dalam kajian ini. Jika apa-apa maklumat penting yang baru dijumpai semasa kajian ini yang mungkin mengubah persetujuan anda untuk terus menyertai kajian ini, anda akan diberitahu secepat mungkin.

**Penyertaan di dalam kajian**

Penyertaan anda dalam kajian ini adalah secara sukarela. Anda boleh menolak penyertaan dalam kajian ini tanpa sebarang hukuman. Penyertaan anda mungkin juga diberhentikan oleh doktor kajian sekiranya anda didapati tidak berkelayakan menyertai kajian ini.

**Manfaat yang mungkin**

Prosedur kajian akan diberikan kepada anda tanpa kos (percuma). Anda juga akan diberi saguhati untuk darah yang diambil bagi tujuan kajian. Kajian ini dijalankan untuk tujuan pengubatan di masa hadapan. Anda mungkin menerima maklumat tentang kesihatan anda daripada ujian makmal makmal yang dilakukan dalam kajian ini.

**Kerahsiaan**


**Pertanyaan**

Sekiranya anda mempunyai sebarang soalan mengenai prosedur kajian ini atau hak-hak anda, sila hubungi:

Dr Noor Suryani Mohd Ashari  
Pensyarah Perubatan  
No pendaftaran MMC : 34465  
Jabatan Imunologi  
Pusat Pengajian Sains Perubatan  
USM Kampus Kesihatan.  
Tel: 09 7673000 sambungan 6225.

Sekiranya anda mempunyai sebarang soalan berkaitan kelulusan Etika kajian ini, sila hubungi:  
Puan Mazlita Zainal Abidin  
Setiausaha Jawatankuasa Etika Penyelidikan (Manusia) USM  
Pelantar Penyelidikan Sains Klinikal, USM Kampus Kesihatan.  
No. Tel: 09-7672355/7672352  
Email: iepem@kk.usm.my

**Tandatangan**

Untuk dimasukkan ke dalam kajian ini, anda mestilah menandatangani serta meletakkan tarikh di lampiran 1.
Tajuk Kajian: Pengukuran tahap subset limfosit dan kaitannya dengan tahap keterukan penyakit kemurungan (major depressive disorder)

Nama Penyelidik: Dr. Noor Suryani Mohd Ashari (No MMC : 34465)
Dr. Mohd Azhar Mohd Yasin (No MMC : 34205)

Untuk menyertai kajian ini, anda atau wakil sah anda mesti menandatangani mukasurat ini. Dengan menandatangani mukasurat ini, saya mengesahkan yang berikut:

- Saya telah membaca semua maklumat dalam Borang Maklumat dan Keizinan ini termasuk apa-apa maklumat berkaitan risiko yang ada dalam kajian dan saya telah pun diberi masa yang mencukupi untuk mempertimbangkan maklumat tersebut.
- Semua soalan-soalan saya telah dijawab dengan memuaskan.
- Saya, secara sukarela, bersetuju menyertai kajian penyelidikan ini, mematuhi segala prosedur kajian dan memberi maklumat yang diperlukan kepada doktor, para jururawat dan juga kakitangan lain yang berkaitan apabila diminta.
- Saya boleh menamatkan penyertaan saya dalam kajian ini pada bila-bila masa.
- Saya telah pun menerima satu salinan Borang Maklumat dan Keizinan untuk simpanan peribadi saya.

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APPENDIX C

STUDY FORM FOR PATIENT

LYMPHOCYTE SUBSETS AND THEIR ASSOCIATION WITH THE SEVERITY OF PATIENTS WITH MAJOR DEPRESSIVE ILLNESS (MDD)

Sample no : _____________________________________
Registration no : _____________________________________
Address : _____________________________________

Tel no : _____________________________________
Age : ____________  Sex: ____________
Race : _____________________________________

Occupation : _____________________________________
Income : _____________________________________
Marital status : _____________________________________

Education level :  □ UPSR  □ SRP/PMR
     □ SPM/STPM  □ Degree
     □ Master/PHD

Active Smoking :  □ Yes, times/day
    □ No

Passive Smoking :  □ Yes, state the smoker: ____________
   □ No

Age of onset of illness : _____________________________________
Family history of depression : _____________________________________
Medication (duration) : _____________________________________
### DIAGNOSIS

#### Pre-treatment:
- □ Normal
- □ Mild depression
- □ Moderate depression
- □ Severe depression
- □ Very severe depression

#### Post-treatment:
- □ Normal
- □ Mild depression
- □ Moderate depression
- □ Severe depression
- □ Very severe depression

#### a) BDI

#### b) MADRS

---

**Nota:** Jika terjadi sesuatu yang tidak diingini, rujuk pesakit ke pakar psikiatri secepat mungkin
APPENDIX D

STUDY FORM FOR CONTROL

LYMPHOCYTE SUBSETS AND THEIR ASSOCIATION WITH THE SEVERITY OF PATIENTS WITH MAJOR DEPRESSIVE ILLNESS (MDD)

Sample no : ________________________________
Registration no : ________________________________
Address : ______________________________________
Tel no : ______________________________________
Age : ___________ Sex: ______________
Race : ______________________________________
Occupation : ______________________________________
Income : ______________________________________
Marital status : ______________________________________

Education level : ☐ UPSR ☐ SRP/PMR
☐ SPM/STPM ☐ Degree
☐ Master/PHD

Active Smoking : ☐ Yes , times / day
No

Passive Smoking : ☐ Yes , state the smoker: _________
☐ No

History of any diseases: ☐ Yes , state the disease : _________
☐ No

Nota: Jika terjadi sesuatu yang tidak diingini, rujuk pesakit ke pakar psikiatri secepat mungkin.
APPENDIX E

HOSPITAL UNIVERSITI SAINS MALAYSIA
16150 KUBANG KERIAN, KELANTAN
Tel: 609-7663000 samb 4300/3385/3387

BECK DEPRESSION INVENTORY

| 1.0 | Saya tidak merasa sedih |
| 1. | Saya rasa sedih |
| 2. | Saya rasa sedih sepanjang masa dan tidak dapat menghilangkan kesedihan saya. |
| 3. | Saya rasa terlalu sedih dan tidak dapat menghilangkan kesedihan saya lagi. |

| 2.0 | Masa depan saya tidak nampak muram. |
| 1. | Masa depan saya nampak muram. |
| 2. | Masa depan saya nampak gelap. |
| 3. | Masa depan saya nampak terlalu gelap dan tidak apa-apa harapan lagi. |

| 3.0 | Saya tidak ada perasaan gagal. |
| 1. | Saya rasa saya lebih kerap gagal berbanding dengan orang lain. |
| 2. | Apabila saya menoleh ke belakang, hidup saya penuh dengan kegagalan. |
| 3. | Saya rasa saya sudah gagal di dalam segala-galanya yang telah saya lakukan. |

| 4.0 | Saya dapat banyak kepuasan atas apa yang biasa saya lakukan. |
| 1. | Saya tidak gembira dengan apa yang biasa saya lakukan. |
| 2. | Saya rasa tidak dapat kepuasan sebenar dengan apa yang saya lakukan lagi |
| 3. | Saya rasa tidak puas dan bosan dengan semua perkara. |

| 5.0 | Saya tidak merasa bersalah. |
| 1. | Saya kerap kali rasa bersalah. |
| 2. | Saya rasa bersalah kebanyakan masa. |
| 3. | Saya bencan diri sendiri. |

| 6.0 | Saya tidak rasa yang saya dihukum. |
| 1. | Saya rasa saya mungkin dihukum. |
| 2. | Saya menjangka bahwa saya akan dihukum. |
| 3. | Saya rasa saya sedang dihukum. |

| 7.0 | Saya sukaakan diri saya. |
| 1. | Saya tidak sukaakan diri saya. |
| 2. | Saya rasa meluat / menyampa terhadap diri sendiri. |
| 3. | Saya bencan diri saya sendiri. |

| 8.0 | Saya rasa tidak sebegitu teruk seperti orang lain. |
| 1. | Saya mengkritik diri sendiri bagi setiap kelemahan atau kesilapan. |
| 2. | Saya menuduh diri saya sepanjang masa atas setiap kesilapan saya. |
| 3. | Saya menuduh diri saya atas sebarang kejadian buruk yang berlaku. |

| 9.0 | Saya tiada perasaan bunuh diri. |
| 1. | Saya ada perasaan bunuh diri, tapi saya tidak akan melakukannya. |
| 2. | Saya ada keinginan untuk bunuh diri. |
| 3. | Saya akan bunuh diri jika ada peluang. |

| 10.0 | Saya tiada lebih kerap menangis. |
| 1. | Saya menangis lebih daripada dulu. |
| 2. | Kini, saya menangis lebih daripada dulu. |
| 3. | Dulu saya selalu menangis, tetapi sekarang saya tidak dapat menangis walaupun saya ingin menangis. |

| 11.0 | Saya tiada rasa lebih jengkel dari dulu. |
| 1. | Saya mudah jengkel / marah dari dulu. |
| 2. | Saya jengkel setiap masa. |
| 3. | Sekarang saya tidak lagi merasa marah terhadap perkara-perkara yang biasa membuat saya marah dulu. |

| 12.0 | Saya tiada hilang minat terhadap orang-orang lain. |
| 1. | Saya kurang berminat terhadap orang-orang lain berbanding dengan masa dulu. |
| 2. | Saya hilang banyak minat terhadap orang lain. |
| 3. | Saya tiada minat langsung terhadap orang lain. |

| 13.0 | Saya boleh membuat keputusan-keputusan seperti biasa. |
| 1. | Saya lebih kerap mengelak dari membuat keputusan. |
| 2. | Saya dapat lebih susah membuat keputusan sekarang berbanding dengan dulu. |
| 3. | Saya tidak dapat membuat apa-apa keputusan pun sekarang. |

<p>| 14.0 | Saya tidak rasa wajah saya lebih buruk dari dulu. |
| 1. | Saya bimbang bahawa wajah saya semakin tua dan tidak menarik lagi. |
| 2. | Saya rasa ada perubahan kekal pada wajah saya, yang membuat saya tidak menarik. |</p>
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</table>
| 15. | 0. Saya boleh bekerja saperti dahulu.  
1. Saya memerlukan usaha yang lebih untuk membuat sesuatu.  
2. Saya perlu memaksa diri saya untuk melakukan sesuatu.  
3. Saya tidak dapat melakukan apa-apa kerja lagi. | 16. | 0. Saya boleh tidur saperti biasa.  
1. Saya tidak dapat tidur nyenyak saperti biasa.  
2. Saya bangun 1-2 jam lebih awal dari biasa dan susah hendak tidur balik.  
3. Saya bangun beberapa jam lebih awal dari biasa dan tidak dapat tidur semula langsung. |
| 17. | 0. Saya tidak rasa lebih leih dari dulu.  
1. Saya lebih mudah leih berbanding dengan dulu.  
2. Saya merasa leih apabila hendak membuat apa-apa pun.  
3. Saya sugang leih dan tidak dapat buat sebarang apa pun. | 18. | 0. Seler saya saperti biasa.  
1. Seler saya kurang baik dari biasa.  
2. Seler saya banyak menurun sekarang.  
3. Saya tiada seler makan langsung. |
| 19. | 0. Saya tiada banyak kekurangan berat badan.  
1. Berat badan saya hilang lebih daripada 5 paun.  
2. Berat badan saya hilang lebih daripada 10 paun.  
1. Saya bimbangkan tentang kesihatan saya saperti sakit perut dan sembelit.  
2. Saya sugang bimbang tentang kesihatan saya sehingga susah untuk memikirkan perkara-perkara lain.  
3. Saya sangat bimbangkan tentang kesihatan dan tidak dapat langsung memikirkan perkara-perkara lain. |
| 21. | 0. Saya tiada perubahan dalam nafsu seks saya.  
1. Saya kurang bermimpi terhadap seks sekarang berbanding dengan dulu.  
2. Saya banyak kekurangan minat terhadap seks sekarang.  
3. Saya hilang nafsu seks sama sekali. |   |   |

**SCORE:**

0 – 10 *Normal*

11 – 20 *Mild depression*

21 – 30 *Moderate depression*

31 – 40 *Severe depression*

41 – 63 *Very severe depression*
**APPENDIX F**

**Montgomery-Asberg Depression Scale (MADRS)**

Instructions: The ratings should be based on a clinical interview moving from broadly phrased questions about symptoms to more detailed ones which allow for a precise rating of severity. The rater must decide whether the rating lies on the defined scale steps (0, 2, 4, 6) or between them (1, 3, 5). It is important to remember that it is only rare occasions that a depressed patient is encountered who cannot be rated on the items in the scale. If definite answers cannot be elicited from the patient, all relevant clues as well as information from other sources should be used as a basis for the rating in line with customary clinical practice. This scale may be used for any time interval between ratings, but it weekly or otherwise, but this must be recorded.

1. **Apparent Sadness**
   Representing despondency, grief and despair (more than just ordinary sadness), and despair. Rate on depth and intensity to brighten up.
   - 0 No sadness
   - 2 Looks dispirited but does not brighten up without difficulty.
   - 4 Appears sad and unhappy most of the time.
   - 5 Looks miserable all the time. Extremely despondent.

2. **Reported Sadness**
   Representing reports of depressed mood, regardless of whether it is reflected in appearance or not. Includes low spirits, despondency or feeling of being beyond help without hope. Rate according to intensity, duration and the extent to which the mood is reported to be influenced by events.
   - 0 Occasional sadness in keeping with the circumstances.
   - 2 Sad or low but brightens up without difficulty.
   - 4 Pervasive feelings of sadness or gloominess. The mood is still influenced by external circumstances.
   - 5 Continuous or unvarying sadness, misery or despondency.

3. **Irritable Tension**
   Representing feelings of ill-defined discomfort, edginess, inner turmoil mounting to either panic, dread or anguish. Rate according to intensity, frequency, duration and the extent of remissiveness called for.
   - 0 Placid. Only reflecting inner tension.
   - 1 Occasional feelings of edginess and ill-defined discomfort.
   - 3 Continuous feelings of inner tension or intermittent panic which the patient can only mingle with some difficulty.
   - 5 Uncontrollably broad or anguish. Overwhelming panic.

4. **Reduced Sleep**
   Representing the experience of reduced duration or depth of sleep compared to the subject's own normal pattern when well.
   - 0 Sleeps as usual.
   - 2 Slight difficulty dropping off to sleep or slightly reduced light or light sleep.
   - 4 Sleep reduced or broken by at least two hours.
   - 6 Less than two or three hours sleep.

5. **Reduced Appetite**
   Representing the feeling of loss of appetite compared with when well. Rate by loss of desire for food or the need to force oneself to eat.
   - 0 Normal or increased appetite.
   - 2 Slightly reduced appetite.
   - 3 No appetite. Food is tasteless.
   - 5 Needs persuasion to eat.

6. **Concentration Difficulties**
   Representing difficulties in collecting one's thoughts mounting to incapacitating lack of concentration. Rate according to intensity, frequency, and degree of incapacity produced.
   - 0 No difficulties in concentrating.
   - 1 Occasional difficulties in collecting one's thoughts.
   - 3 Difficulties in concentrating and sustaining thought which reduces ability to read or hold a conversation.
   - 5 Unable to read or converse without great initiative.

7. **Lesssitude**
   Representing a difficulty getting started or slowness initiating and performing everyday activities.
   - 0 Hardly any difficulty in getting started. No sluggishness.
   - 1 Difficulties in starting activities.
   - 3 Difficulties in starting simple mundane activities which are carried out with effort.
   - 5 Complete lesssitude. Unable to do anything without help.

8. **Inability to Feel**
   Representing the subjective experience of reduced interest in the surroundings, or activities that normally give pleasure. The ability to react to surrounding emotion to circumstances or people is reduced.
   - 0 Normal interest in the surroundings and in other people.
   - 1 Reduced ability to enjoy usual interest.
   - 3 Loss of interest in surroundings: Loss of feelings for friends and acquaintances.
   - 5 The experience of being emotionally paralysed, inability to feel anger, grief or pleasure and a complete or even painful failure to feel for close relatives and friends.

9. **Pessimistic Thoughts**
   Representing thoughts of guilt, inferiority, self-reproach, self-scrutiny, remorse and ruin.
   - 0 No pessimistic thoughts.
   - 1 Fluctuating ideas of failure, self-reproach or self-depreciation.
   - 3 Persistent self-accusations, or definite but still rational ideas of guilt or sin. Increasingly pessimistic about the future.
   - 5 Delusions of ruin, remorse or unremorseful sin. Self-accusations which are absurd and unshakeable.

10. **Suicidal Thoughts**
    Representing the feeling that life is not worth living, that a natural death would be welcome; suicidal thoughts, and the preparations for suicide. Suicidal attempts should not in themselves influence the rating.
    - 0 Enjoy. We or takes it as it comes.
    - 1 Weary of life. Only floating suicidal thoughts.
    - 3 Probably better off dead. Suicidal thoughts are common, and suicide is considered as a possible solution, but without specific plans or intention.
    - 5 Explicit plans for suicide when there is an opportunity. Active preparations for suicide.

Total Score: ________

Date: _______________
Please read each statement and circle a number 0, 1, 2 or 3 which indicates how much the statement applied to you over the past week. There are no right or wrong answers. Do not spend too much time on any statement.

**The rating scale is as follows:**

0  Did not apply to me at all  
1  Applied to me to some degree, or some of the time  
2  Applied to me to a considerable degree, or a good part of time  
3  Applied to me very much, or most of the time

<table>
<thead>
<tr>
<th></th>
<th>Statement</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>1</td>
<td>I found it hard to wind down</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td>I was aware of dryness of my mouth</td>
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<td></td>
<td></td>
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<tr>
<td>3</td>
<td>I couldn’t seem to experience any positive feeling at all</td>
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<td></td>
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<td>4</td>
<td>I experienced breathing difficulty (eg, excessively rapid breathing,</td>
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<tr>
<td></td>
<td>breathlessness in the absence of physical exertion)</td>
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<tr>
<td>5</td>
<td>I found it difficult to work up the initiative to do things</td>
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<td>6</td>
<td>I tended to over-react to situations</td>
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<td>7</td>
<td>I experienced trembling (eg, in the hands)</td>
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<td>8</td>
<td>I felt that I was using a lot of nervous energy</td>
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<tr>
<td>9</td>
<td>I was worried about situations in which I might panic and make a</td>
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<td></td>
<td>fool of myself</td>
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<tr>
<td>10</td>
<td>I felt that I had nothing to look forward to</td>
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<td>11</td>
<td>I found myself getting agitated</td>
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<td>12</td>
<td>I found it difficult to relax</td>
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<td>13</td>
<td>I felt down-hearted and blue</td>
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<td>14</td>
<td>I was intolerant of anything that kept me from getting on with what I</td>
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<td></td>
<td>was doing</td>
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<td>15</td>
<td>I felt I was close to panic</td>
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<td>16</td>
<td>I was unable to become enthusiastic about anything</td>
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<td>17</td>
<td>I felt I wasn't worth much as a person</td>
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<td>18</td>
<td>I felt that I was rather touchy</td>
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<td>19</td>
<td>I was aware of the action of my heart in the absence of physical</td>
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<td></td>
<td>exertion (eg, sense of heart rate increase, heart missing a beat)</td>
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<tr>
<td>20</td>
<td>I felt scared without any good reason</td>
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<tr>
<td>21</td>
<td>I felt that life was meaningless</td>
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</table>
DASS Severity Ratings

The DASS is a quantitative measure of distress along the 3 axes of depression, anxiety and stress. It is not a categorical measure of clinical diagnoses.

Emotional syndromes like depression and anxiety are intrinsically dimensional - they vary along a continuum of severity (independent of the specific diagnosis). Hence the selection of a single cut-off score to represent clinical severity is necessarily arbitrary. A scale such as the DASS can lead to a useful assessment of disturbance, for example individuals who may fall short of a clinical cut-off for a specific diagnosis can be correctly recognised as experiencing considerable symptoms and as being at high risk of further problems.

However for clinical purposes it can be helpful to have 'labels' to characterise degree of severity relative to the population. Thus the following cut-off scores have been developed for defining mild/moderate/severe/extremely severe scores for each DASS scale.

Note: the severity labels are used to describe the full range of scores in the population, so ‘mild’ for example means that the person is above the population mean but probably still way below the typical severity of someone seeking help (ie it does not mean a mild level of disorder.

The individual DASS scores do not define appropriate interventions. They should be used in conjunction with all clinical information available to you in determining appropriate treatment for any individual.

1Symptoms of psychological arousal
2The more cognitive, subjective symptoms of anxiety

<table>
<thead>
<tr>
<th>DASS 21 SCORE</th>
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<tbody>
<tr>
<td>DEPRESSION</td>
</tr>
<tr>
<td>SCORE</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Normal</td>
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<tr>
<td>Mild</td>
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<tr>
<td>Moderate</td>
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<tr>
<td>Severe</td>
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<tr>
<td>Extremely Severe</td>
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</tbody>
</table>
APPENDIX H

13th November 2014

Dr. Noor Suryani Mohd Ashari
Department of Immunology
School of Medical Sciences
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.

JEPEM Code : USM/JEPEM/1403122
Protocol Title : Lymphocyte Subsets and their Association with the Severity of Patients with Major Depressive Disorder (MDD).

Dear Dr.,

We wish to inform you that your study protocol has been reviewed and is hereby granted approval for implementation by the Jawatankuasa Etika Penyelidikan Manusia Universiti Sains Malaysia (JEPEM-USM). Your study has been assigned study protocol code USM/JEPEM/1403122, which should be used for all communication to the JEPEM-USM related to this study. This ethical clearance is valid from November 2014 until October 2015.

The following documents have been approved for use in the study.

1. Research Proposal

In addition to the abovementioned documents, the following technical document was included in the review on which this approval was based:

1. Patient Information Sheet and Consent Form (Malay version)
2. Questionnaires
3. Study Form

Attached document is the list of members of JEPEM-USM present during the full board meeting reviewing your protocol.

While the study is in progress, we request you to submit to us the following documents:

1. Progress report using the JEPEM-USM FORM 3(B)2014: Continuing Review Application Form every 1 years from date of approval (NOTE: In view of active ethical clearance, this report is mandatory even if the study has not started or is still awaiting release of funds.)
2. Any changes in the protocol, especially those that may adversely affect the safety of the participants during the conduct of the trial including changes in personnel, must be submitted or reported using JEPEM-USM FORM 3(A) 2014: Study Protocol Amendment Submission Form.
3. Revisions in the informed consent form using the JEPEM-USM FORM 3(A) 2014: Study Protocol Amendment Submission Form.
4. Reports of adverse events (if any) including from other study sites (national, international) using the JEPEM-USM FORM 3(G) 2014: Adverse Events Report.
5. Notice of early termination of the study and reasons for such using JEPEM-USM FORM 3(E) 2014.
6. Any event which may have ethical significance.
7. Any information which is needed by the JEPEM-USM to do ongoing review.
LIST OF PUBLICATIONS AND PRESENTATIONS

Original articles:


2. Siti Nor Fairus Mohamed Sanusi, Mohd Azhar Mohd Yasin, Che Maraina Che Hussin, Rohimah Mohamud, Mohd Nazri Shafei, Noor Suryani Mohd Ashari. Level of Regulatory T Cells in Major Depressive Disorder (MDD) and Their Association with Disease Severity. *Journal of Postgraduate Medicine*. In review.
Poster presentations:

1. **Title**: Predisposing Factors of Major Depressive Disorder (MDD)
   **Authors**: Siti Nor Fairus Mohamed Sanusi, Mohd Azhar Mohd Yasin, Noor Suryani Mohd Ashari
   **Venue**: International Scientific Conference (INASCON) 2016
   **Date**: 16-17 May 2016

2. **Title**: The Level of Regulatory T Cells in Major Depressive Disorder (MDD) Patients and Their Association with The Severity of The Disease
   **Authors**: Siti Nor Fairus Mohamed Sanusi, Mohd Azhar Mohd Yasin, Noor Suryani Mohd Ashari
   **Venue**: Joint Congress of APAAACI and APAPARI 2016
   **Date**: 17-20 October 2016