Serum trace elements, antioxidant-vitamins, immunoglobulins, lipid peroxidation, cortisol, amino acid level in patients with Major Depressive Disorder and their correlation with disease status

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Abstract

Background:

Major Depressive Disorder (MDD) is a mental disorder characterized by a pervasive and persistent low mood which is accompanied by low self-esteem and loss of interest or pleasure in day to day activities that adversely affects a person's family, work and personal life. In Bangladesh 16.05% of adult population suffer from psychiatric illness of which 28.7% suffer from MDD. Currently this disease is diagnosed on the basis of patient's self-reported experiences, behavior reported by relatives or friends, and a mental status examination. There is no sufficient laboratory test for the diagnosis of MDD and it is expected that this investigation may be helpful for better diagnosis and management of MDD.

Methods:

Two hundred and forty seven patients with MDD were recruited from department of psychiatry, Bangabandhu Sheikh Mujib Medical University, Dhaka and 248 healthy volunteers were also recruited by matching with age, sex and socioeconomic status to the patient group with no previous history of any psychiatric disorders or any medical disease that may affect results. Analysis of the trace element has carried out by using flame atomic absorption spectroscopy using Varian SpectraAA 220. RP-HPLC was used for simultaneous determination of serum vitamin A and E concentrations. Concentration of vitamin C in serum was determined by spectrophotometric method. Serum concentrations of immunoglobulin A, G and M were determined by turbidimetry method using immunoglobulin kit. Modified method described by Satoh has been used to determine serum MDA. Serum cortisol level was measured by using ELISA kit. Serum levels of amino acids were measured by chromatographic methods (HPLC).

Results:

Our current study revealed that serum concentrations of Zn, Cu, Mn, Fe, Ca and Mg in MDD patients were 0.74±0.30, 0.86±0.41, 0.00056±0.00022, 1.24±0.30, 85.07±33.63 and 17.36±5.28 mg/L, while those were 1.01±0.19, 0.79±0.31, 0.00065±0.00032, 0.00132±0.00035, 104.33±11.13 and 21.24±3.03 mg/L in control subjects, respectively. Serum concentration of Zn, Mn, Ca and Mg decreased significantly in patient group (p<0.001, p=0.006, p<0.001 and

p<0.001, respectively). But the differences of the concentration of Cu and Fe between patient and control group were not significant (p=0.156 and p=0.056). Correlative analysis showed that serum Fe concentration has a significant correlation with age of patient (p=0.037). Serum Mg level has a direct correlation with Fe level (p=0.004) and Mg was inversely correlated with Cu (p<0.001) in the patient group. Serum levels of vitamin A, E and C were 2.05±1.16, 12.89±6.30 and 34.17±17.27 µmol/L in patients and the values were 2.33±0.92, 16.98±6.21 and 37.88±13.97 µmol/L for control subjects. Vitamin A, E and C levels decreased significantly in patients compared to the control subjects (p= 0.003, p<0.001 and p=0.009, respectively). Pearson's correlation coefficient suggested that there was a significant positive correlation between vitamin A and E. Mean serum concentrations of IgA, IgG and IgM in patients were found to be 209.07±104.93, 791.50±235.67 and 107.92±47.53 mg/dL while those were 195.34±92.16, 763.81±175.89 and 99.17±48.78 mg/dL in control subjects, respectively. There were no significant difference of serum IgA, IgG and IgM between patients and control subjects (p=0.404, p=0.407 and p=0.293). Mean serum concentration of MDA was 5.16±2.56 µmol/L for patients and 3.21±1.77 µmol/L for control subjects. Significantly elevated level of serum MDA was found in MDD patients (p<0.001). Mean serum concentration of cortisol was 19.32±5.38 µg/dL in patients and 17.38±6.32 µg/dL for control subjects. Statistically significant elevated level of cortisol was found in patient group (p=0.024). Serum levels of most of the amino acids were decreased in MDD patients compare to control subjects whereas serum glycine level showed opposite result. Serum level of methionine, phenylalanine, tryptophan, and tyrosine were 18.35±9.80, 68.77±12.55, 51.60±12.57 and 54.78±21.99 µmol/L in patients while those were 22.48±8.72, 74.73±11.44, 59.73±11.44 and 60.23±28.58 µmol/L in control subjects. Serum concentration of methionine, phenylalanine, tryptophan, and tyrosine decreased significantly in patient group (p<0.001, p=0.006, p<0.001, p<0.001 and p=0.018, respectively). Pearson's correlation coefficient supports that serum level of methionine in patients has a significant positive correlation with phenylalanine and tryptophan. Smoking habit of patients has a significant positive correlation with serum methionine, phenylalanine and tryptophan level.

Conclusion:

Our result indicates that serum concentrations of most of the trace elements, amino acids and anti-oxidant vitamins were lower in patients compare to healthy control. But the elevated levels of serum MDA and cortisol were found in patients than control subjects. Difference in serum concentrations of IgA, IgG and IgM was not significant between the groups.

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Declaration

Not any portion of this work referred to this thesis paper entitled **"Serum trace elements, antioxidant-vitamins, immunoglobulins, lipid peroxidation, cortisol, amino acid level in patients with Major Depressive Disorder and their correlation with disease status"** has been submitted for another degree or qualification of the University of Dhaka or any other University or any other institute of learning.

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Dedication

This work is dedicated to my parents, teachers, my wife and kids who always inspire me in every steps of my life for better outcomes.

List of Abbreviation

5-HTT	5-Hydroxy Tryptophan
5-HTTLPR	5-Hydroxy Tryptophan Linked Promoter Region
AD	Alzheimer's Disease
ADD	Attention Deficit Disorder
ADHAD	Attention Deficit Hyperactivity Disorder
AI	Adequate Intake
ALS	Amyotrophic Lateral Sclerosis
AMD	Age-related Macular Degeneration
ANOVA	Analysis of Variance
APA	American Psychiatric Association
AA	Amino Acid
BDNF	Brain Derived Neurotrophic Factor
BHT	Butylatedhydroxytolune
BMI	Body Mass Index
BSMMU	Bangabandhu Sheikh Mujib Medical University
CBT	Cognitive Behavioral Therapy
CED	Chronic Energy Deficiency
CHD	Coronary Heart Disease
CNS	Central Nervous System
CRH	Corticotrophin Releasing Hormone
CRP	Carbon Reactive Protein
CSF	Cerebrospinal Fluid
CYP	Cytochrome
dL	Deciliter
DSM	Diagnostic and Statistical Manual
EAR	Equivalent Activity of Retinol
ECG	Electrocardiogram
ELISA	Enzyme Linked Immunosorbent Assay
FA	Fatty Acid
FAAS	Flame Atomic Absorption Spectroscopy
FNB	Food and Nutrition Board

GAD	Generalized Anxiety Disorder
GSIS	Glucose Stimulated Insulin Secretion
GxE	Gene-environment Interaction
HPA	Hypothalamic Pituitary Axis
HPETE	Hydroperoxyeicosatetraenoic Acid
ICD	International Classification of Diseases
Ig	Immunoglobulin
IL-6	Interleukin-6
IPT	Interpersonal Psychotherapy
L	Liter
LDL	Low Density Lipoprotein
MAOI	Monoamine Oxidase Inhibitor
MDA	Malondialdehyde
MDD	Major Depressive Disorder
mg	Milligram
min	Minute
mL	Milliliter
MS	Multiple Sclerosis
NCCLS	National Committee for Clinical Laboratory Standard
NHANES	National Health and Nutrition Examination Survey
NMDA	N-methyl-D-aspartate
RDA	Recommended Daily Allowances
ROS	Reactive Oxygen Species
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
PG	Prostaglandin
SD	Standard Deviation
SNRI	Serotonin Norepinephrine Reuptake Inhibitor
SOD	Superoxide Dismutase
SSRI	Selective Serotonin Reuptake Inhibitor
TAOC	Total Antioxidant Capacity
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TCA	Tetracyclic Antidepressant

TMB	Tetramethylbenzidine
TNF-α	Tumor Necrosis Factor alpha
TRP	Tryptophan
UIL	Upper Intake Level
UV	Ultraviolet
WHO	World Health Organization
µmol	Micromole
µg	Microgram

CHAPTER ONE

INTRODUCTION

1. Introduction

Major Depressive Disorder (MDD) and its prevalence, etiology, pathophysiology, diagnosis, treatment options have been discussed in this part. Here reveals about the relationship of psychiatric disorders with few serum parameters e.g. trace elements, antioxidant vitamins, lipid peroxidation, immunoglobulins, cortisol and amino acids.

1.1 Major Depressive Disorder

Normal emotions of sadness and bereavement are linked with depression. But it persists after the external causes of these emotions dissipates and disproportionate to their cause. Sometimes without having external triggering factors classic severe states of depression may be happen. It is very difficult to make a clear difference between depressions with and those without psychosocial precipitating events (Wakefield et al., 2007). A depressed mood with sadness or irritability accompanied by several psychophysiological changes like disturbances in sleep, appetite, or sexual desire, constipation, loss of the ability to experience pleasure in personal and family life, crying, suicidal thoughts or plans, and slowing of speech and actions are the diagnostic criteria of MDD. Patient's work and family life hampered considerably by these changes that last at least 2 weeks. Lifetime prevalence of depression in USA is more than 12% in men and 20% in women (Kessler et al., 2003). Sometimes, severe depression is termed as melancholia or vital depression in a much narrower way (Van Praag et al., 1987).

Manic Episode consisting of hyperactivity, euphoria and increased pleasure seeking may appear in some MDD patients. There is an overlap of some pathogenetic mechanisms between major depression and manic episode. A distinct illness termed as bipolar disorder sometimes may be a history of mania (Belmaker, 2004). So depression is a mixed disorder with a highly irregular course with an inconsistent response to treatment and has no well-known mechanism.

In USA approximately 3.4% of people suffering from major depression are committed to suicide. People who are committed to suicide 60% of them had depression or another mood disorder (Barlow and Durand 2005).

1.1.1 Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5) criteria for MDD

A. Patients with MDD represent five or more of the following symptoms for a period of consecutive two weeks with a change from previous functioning along with at least one of the symptoms are either low mood or loss of interest or pleasure.
   1. Low mood most of the day or nearly every day (e.g., sadness, empty and hopeless)
   2. Noticeably diminished interest or pleasure in daily activities in most of the day or nearly every day.
   3. Significant change of body weight e.g., change is more than 5% in a month (decrease or increase in appetite).
   4. Change in sleeping behavior, insomnia or hypersomnia nearly every day.
   5. Change in activity like psychomotor agitation or retardation.
   6. Fatigue or loss of energy.
   7. Feelings of worthlessness or inappropriate guilt.
   8. Diminished ability to think or concentrate.
   9. Thoughts of death or suicide, or has suicide plan.

B. Social, occupational, personal or other areas of functioning have been impairment significantly by these symptoms

C. The episode is not related to any physiological matter or to another medical situation.

D. The incidence of the major depressive episode is not better explained by schizoaffective disorder, schizophrenia, delusional disorder, or other specified and unspecified schizophrenia spectrum and other psychotic disorders.

E. There has never been a manic episode or a hypomanic episode with MDD.

1.1.2 International Classification of Diseases, 10th Edition (ICD-10)

Depressive episode: According to ICD-10, depressive episodes are classified as mild, moderate or severe. Patients suffering from depression have low mood, reduction of energy and activity. Ability to enjoy interesting things and concentration on a particular subject are also reduced. A depressive person feels more fatigue even after giving minimum physical effort. Change in sleeping and appetite are common. Self-confidence is reduced even in the mild form and feelings of worthlessness or inappropriate guilt are often present. The sadness or low mood differ from

day to day which is insensitive to situations and may be supplemented by somatic symptoms. Loss of interest in daily activities, waking in the early morning, marked psychomotor retardation or agitation, change in appetite, weight loss or gain and loss of libido are the main symptoms of major depression. Based on the above symptoms depressive episode can be classified as mild, moderate and severe (WHO, 2016).

Mild depressive episode: Patients having two or three of the above symptoms are generally termed as mind depressive episode. In this case patients feel troubled in day to day activities but they perhaps are able to carry on with most activities.

Moderate depressive episode: Patients having four or more of the above symptoms are generally termed as moderate depressive episode. In this case patients feel great difficulty in current day to day regular activities.

Severe depressive episode without psychotic symptoms: An episode where most of the above symptoms are involved and patients feel decreased self-esteem, worthlessness or guilt. Suicidal ideas and plans are common with a number of somatic symptoms are generally present.

1.1.3 Prevalence of MDD

Approximately 4.3% population suffers from MDD globally (Vos et al., 2012). Twelve-month prevalence of MDD in men is 4.9% and for women the rate is 8.6%. Lifetime prevalence of 13.2% in men compared with 20.2% in women globally (Rustad et al., 2013). Lifetime occurrence is 3% in Japan and 17% in USA that differs extensively (Andrade et al., 2003). Prevalence of MDD for a year-long period is 3-5% in males and 8-10% in females among North Americans (Murphy et al., 2000). In Bangladesh prevalence of MDD is 4.6% and among all psychiatric patients the percentage is 28.7. Both mild to moderate and severe depression are more common in females and among singles (Firoz et al., 2006; Rahman et al., 2011). Different studies reveal that the chance of having major depression is twice in women than men but the reason is not clear and which factors are responsible for this are not known (Kuehner, 2003).

People generally experience their first depressive episode during forth decade of life and there may have a second smaller peak at the age 50 to 60 years. Some factors increase the chance of depressive episode such as stroke, Parkinson's disease or multiple sclerosis and during the first

year after childbirth (Rickards, 2003). Urban populations suffer depressive disorder more than the rural people.

1.1.4 Etiology of MDD

According to biopsychosocial model biological, psychological and social factors play an important role in developing depression (Fundamentals of mental health and mental illness by US Department of Health and Human Services, 1999). The diathesis–stress model postulates that depressive episode occurs due to preexisting vulnerability, or diathesis activated by stressful life events. The preexisting vulnerability can be either genetic (Caspi et al., 2003; Haeffel et al., 2008), implying an interaction between nature and nurture or schematic, resulting from childhood views of the world (Slavich, 2004). Depressive episode may be due to damage of cerebellum as is seen in cerebellar cognitive affective syndrome (Konarski et al., 2005; Schmahmann, 2004; Schmahmann et al., 2007).

Empirical support has been gained by these interactive models. A prospective approach on the study of depression has been taken by researchers from New Zealand and they documented that how depression develops among healthy people over time. They concluded that people with variation of serotonin transporter (5-HTT) gene may lead very stressful life which ultimately leading to experience depressive episode. To be precise, depressive episode may be related to such events, but people with one or two short alleles of the 5-HTT gene are more likely to have depression. (Caspi et al., 2003). Heritability of depression has been estimated in a Swedish study where they explained that the severity of depression is linked with the degree of genetic variation (Kendler et al., 2006). The genetic basis for MDD lies deep in the history of naturally selected adaptations which is proposed by evolutionary psychologists. Long-term drug use or abuse and withdrawal from certain sedative or hypnotic drugs are generally linked with major depression (Ashton, 2002; Schuckit et al., 1997).

1.1.4.1 Genetic and biological

Serotonin, norepinephrine and dopamine level in the synaptic cleft increases by most of the antidepressant medications. Some medications affect the monoamine receptors directly.

There is a hypothesis about serotonin that other neurotransmitter systems are regulated by it. These systems act in unusual and erratic ways when activity of serotonin is decreased (Barlow and Durand 2005). According to this hypothesis depression arises when low serotonin levels

allow low level of another monoamine neurotransmitter norepinephrine (Shah et al., 1999). Norepinephrine level directly increase by some antidepressants whereas others raise the levels of a third monoamine neurotransmitter, dopamine. According to monoamine hypothesis particular feature of depression develops due to deficiency of corresponding neurotransmitters. For example norepinephrine is related to alertness, energy, anxiety, attention and interest in life; serotonin is responsible for anxiety, obsessions and compulsions whereas dopamine for attention, motivation, pleasure, reward and interest (Nutt, 2008).

It is believed that effective antidepressants are capable to increase the level of available monoamines. According to psychiatric genetics, phenotypic variation in central monoamine function is slightly associated with vulnerability to depression. Despite all the above findings, monoamine deficiency is not the only cause of depression (Krishnan and Nestler, 2008). Multiple limitations of the monoamine hypothesis have been detected during last couple of decades and its clarifying insufficiency has been highlighted within the psychiatric community (Hirschfeld, 2000). Stressful life is a predictor for depressive episodes for some individuals but not for others this was explained by gene-environment interaction (GxE) hypothesis in 2003 depending on an allelic variation of the serotonin-transporter-linked promoter region (5-HTTLPR) (Caspi et al., 2003); Stressful life events are associated with depression but found no evidence for an association with the 5-HTTLPR genotype (Risch et al., 2009).

1.1.4.2 Psychological

Different types of personality and its development play an important role for the occurrence and persistence of depression (Raphael, 2000,) negative emotionality is a common precursor of depression (Morris et al., 2009). A person's typical style of managing situation may be correlated with his or her resilience or depression (Kaplan and Sadock, 2003). In addition, low self-esteem and distorted thinking are related to depressive episodes. A religious person is less likely to have depression as well as quicker to remit from it. (Dein, 2006; Mc Cullough and Larson, 1999; Moreira-Almeida et al., 2006). It is very difficult to identify which factors are directly related to depression and which are not but a depressed person who is capable to challenge his or her thinking patterns can improve mood and self-esteem quickly.

According to American psychiatrist Aaron T. Beck three concepts underlie depression- negative thoughts composed of cognitive errors about oneself, recurrent patterns of depressive thinking, and distorted information processing. Based on these principles he developed the structured

technique of Cognitive Behavioral Therapy (CBT) (Beck et al., 1987). Later a lot of research has been done to support the concept of distorted information processing in individuals with depression. These include attention and reward and punishment processing (Eshel and Roiser, 2010; Gotlib and Joormann, 2010). Another American psychologist Martin Seligman found the similarities of depression in humans with the learned helplessness in laboratory animals that remain in unpleasant situations but when they are able to escape, they don't do this as initially learned they had no control (Seligman, 1975).

Relationship between depressive disorder in life and the quality of the previous bond with parents or caregivers was proposed by English psychiatrist John Bowlby in 1960s. More precisely, the experiences of early loss, separation or rejection by parents or caregivers all may lead to develop depressive episode. Meanwhile a lot of research has been done to support the attachment by different scientists in different parts of the world (Ma, 2006).

Depressed individuals often blame themselves for their negativism and sometimes they don't want to take credit of positive outcomes for their activities (Barlow et al., 2005; Pinto and Francis, 1993). A Canadian social psychologist found that depressed individuals have negative beliefs about themselves based on their own experiences of life like failure, observing the failure of social models, a lack of social encouragement and they do not trust they can influence events what is happening or achieve personal goals in life (Bandura, 1998; Kanfer and Zeiss, 1983).

1.1.4.3 Social

Generally poverty and social isolation increases risk of mental health problems (Raphael, 2000). Child abuse is linked with increased risk of developing depressive episodes later life (May, 1994). A child learns from society how to become a social being during the years of development. This developing personality is distorted by the abuse of the child by the caregivers and creates a much greater risk for depression and many other devastating mental and emotional states. Family history of depression, severe marital clash or divorce, death of a parent, or other turbulences in parenting is additional risk factors (Raphael, 2000). Stressful life events are related with the onset of major depressive episodes (Kessler, 1997). There are many evidence about that the first episode of depression is more likely to be instantly after the stressful life events than the recurrent ones is consistent with the hypothesis that people may become

increasingly sensitized to life stress over succhessive recurrences of depression (Kaplan and Sadock, 2003; Monroe et al., 2007).

Stressful life events and social support are correlated with each other. Life become stressful due to the lack of social support which ultimately lead to depression, or the absence of social support may constitute a form of strain that leads to depression directly (Vilhjalmsson, 1993). Neighborhood social crime is a risk factor and neighborhood socioeconomic status with better amenities is a protective factor (Kim, 2008). Adverse work environment like demanding jobs with little scope for decision making are associated with depression (Bonde, 2008).

1.1.4.4 Evolutionary

According to the evolutionary theory major depression is hypothesized in some instances to increase a person's reproductive fitness. Depression is hereditarily combined into the human gene pool that is responsible for high heritability and occurrence of depression by suggesting that certain mechanisms of depression are adaptions (Panksepp et al., 2002) such as the behaviors connecting to attachment and social rank (Sloman et al., 2003). Relationships or resources can be regulated by adaptions that are explained as current behaviors though the result may be maladaptive in modern environments (Tooby and Cosmides, 2005).

From another point of view, a psychotherapist may explain depression is not as a biological illness or disorder but as a species-wide developed group of emotional programs that are mostly activated by a perception, almost always over-negative, a major decline in personal usefulness that is linked with guilt, shame or perceived rejection (Carey, 2005). The feelings of ineptness generated by such demotion can be prevented or recovery from this situation by the rapid support from friends and families. In a way similar to that in which physical pain has progressed to encumber actions that may cause further injury which is called psychic misery this may have developed to prevent quick and maladaptive reactions to stressful situations (Mashman, 1997).

1.1.4.5 Drug and alcohol use

Alcohol, sedatives and cannabis abuse is very high among the population suffering from mental disorder. A significant part of a psychiatric evaluation is differential diagnosis by which it is possible to identify whether mental disorder is substance associated or not or co-occurring (Mashman, 1997). Based on the fifth edition of diagnostic and statistical manual by American

society psychiatry mental illness cannot be diagnosed as depressive disorder which is produced by the direct physiological effects of a substance. When a syndrome like MDD is supposed to be produced directly by substance abuse or by an adverse reaction of any drug then it is diagnosed as substance induced mood disorder. Alcoholism or extreme alcohol intake considerably increases the risk of evolving depressive episodes (Falk et al., 2008; Boden and Fergusson, 2011). Alcohol and benzodiazepines are central nervous system depressants which are usually used to treat insomnia, anxiety and muscular spasms. Similarly alcohol and benzodiazepines accelerate the risk of evolving MDD due to their adverse or toxic effects. Sedative-hypnotic drugs decrease the levels of serotonin and norepinephrine (Ashton, 2002) or activation of immune regulated inflammatory pathways in the brain (Kelley and Dantzer, 2011). Long term use of benzodiazepines can cause or deteriorate depression (Semple et al., 2007) or depression may come as withdrawal syndrome of this group of drugs (Collier et al., 2003; Janicac et al., 2003). People recovering from alcoholism, 25% of them may experience anxiety and depression which can continue for up to two years (Johnson, 2011). Abuse of methamphetamine is also associated with depression (Marshall and Werb, 2010).

1.1.5 Pathophysiology

It is hypothesized that different biological mechanisms are involved in the pathophysiology of major depression and they have a role in the etiology and development of the disease (Veduijn et al., 2015). Pathophysiology of mood disorders is also associated with the central cholinergic system. Typically manic and depressive episodes are caused by an imbalance in central cholinergic neurotransmitter activity. According to neuropharmacological studies cholinergic effects of agonists and antagonists provided a considerable support for this hypothesis (Je Jeon et al., 2015).

Depression may be developed by an upregulating of inflammation due to decreased production of monoamines e.g. serotonin and increased production of tryptophan catabolites that are lethal for the brain (Moylan et al., 2013). Researchers found that that depressed subjects have significantly increased levels of the pro-inflammatory cytokine interleukin (IL)-6 in comparison with controls (Dowlati et al., 2010; Howren et al., 2009; Liu et al., 2012) and acute phase C-Reactive Protein (CRP) (Howren et al., 2009). Another study reveals that people with significantly higher level of Tumor Necrosis Factor alpha (TNF-α) experience more than three episodes (Moylan et al., 2013).

Many studies have been conducted to identify the cause of major depression particularly on hyperactivity of the Hypothalamic Pituitary Axis (HPA) (Stetler and Miller, 2011). This hyperactivity is apparently causes due to damage of negative response circuit of the HPA-axis by malfunctioning of glucocorticoid receptors. Glucocorticoid receptor error can cause depression by impaired neurogenesis and reduced hippocampus volumes (Manji et al., 2003; Pariante and Lightman, 2008). Cortisol levels may be considered as a risk factor for depression (Vrshek-Schallhorn et al., 2013) and time to repetition (Bockting et al., 2012) of depressive episode. This suggests that HPA malfunctioning is linked with MDD progression.

A third important probable pathophysiological mechanism of major depression is Brain Derived Neurotrophic Factor (BDNF). Low levels of BDNF are considered as a sign of reduced neurotropic growth that may cause depression (Maletic et al., 2007). From recent studies it was found that depressed patients have lower BDNF levels than controls (Molendijk et at., 2014). Another study found that drug-free patients with a short index episode had meaningfully higher BDNF levels compared with patients with a longer index episode (Birkenhäger et al., 2012).

It is found that low level of vitamin D is associated with depression. (Anglin et al., 2013). Vitamin D may be involved with the etiology or development of depression which is supported by different pathophysiological mechanisms (Cherniack et al., 2009; Ganji et al., 2010). Vitamin D is neuroprotective (Fernandes de Abreu et al., 2009) which reduce neurotoxic calcium levels in the brain (Kalueff a et al., 2004) but this is not supported by any study whether vitamin D is connected with the development of depression.

1.1.6 Treatment of MDD

MDD is a disease that is usually manageable; people who seek treatment 80% cases psychotherapy and medications are effective for them. Patients normally start to get significant benefits from treatment within 4-6 weeks after initiation (American Psychiatric Association, 2013).

However psychiatric medication is the most often prescribed therapy for major depression (Carson, 2000) but psychotherapy either alone or in combination with medication is very effective option (Callaway, 1972). Antidepressant medications don't consistently demonstrate their superiority over placebo or their benefit in treating depression is little. Similarly, substantial superiority over no-treatment has not been demonstrated by psychotherapy. Combination of both

psychotherapy and antidepressants can provide a slight advantage, but antidepressants alone or psychotherapy alone is not significantly different from other treatment options. Once MDD is accurately diagnosed then any treatment option is not generally effective than getting depressed patients involved in an active therapeutic program (Khan et al., 2012).

Patients who are under 18 years of age, psychotherapy is the treatment of choice for them. Depression due to substance abuse or other mental health problems, parents should be considered for psychotherapy parallel with the child and this help the child to get better treatment outcomes. (NICE, 2005).

1.1.6.1 Pharmacotherapy

Many classes of antidepressant medications have been used to treat major depression, for example tetracyclic antidepressant medication (e.g. maprotiline); Selective serotonin reuptake inhibitors (e.g. fluoxetine, sertraline, paroxetine, fluvoxamine, citalopram, and escitalopram); Serotonin norepinephrine reuptake inhibitors (e.g. venlafaxine, desvenlafaxine, and duloxetine); other antidepressant medications (e.g. bupropion, nefazodone, trazodone, and mirtazapine) and Monoamine oxidase inhibitors (e.g. phenelzine, tranylcypromine, isocarboxazid). There are no replicable or robust findings to establish a clinically meaningful difference of one medication over another. Antidepressant medications are usually comparable between classes and within classes of medications for their effectiveness. Treatment response rate of these medications were found in different clinical trials from 50% to 75% of patients with mild to moderate symptoms (Fournier et al., 2010; Khan et al., 2002; Kirsch et al., 2008).

However, antidepressant medications do vary in their likely to cause specific side effects such as adverse sexual effects, sedation, or weight gain. Thus, antidepressant medication selection should be based on the tolerability, safety, cost, patient preference and history of prior medication.

1.1.6.1.1 Selective Serotonin Reuptake Inhibitors (SSRIs)

SSRIs are effective in the treatment of MDD. It is demonstrated in many studies that the efficacy of SSRIs is superior to other antidepressant medications, mainly Tetracyclic Atidepressant (TCAs) (Anderson, 2000; Arroll et al., 2009; Cipriani et al., 2005; Macgillivray et al., 2003; Montgomery, 2000). Although a few analyses suggest that small advantages of serotonin

norepinephrine reuptake inhibitors (SNRIs) over SSRIs in rates of remission (Bauer et al., 2009). One meta-analysis suggests a slight superiority of escitalopram compared with other SSRIs and venlafaxine (Kennedy et al., 2006), and another found significantly greater efficacy for escitalopram, sertraline, venlafaxine, and mirtazapine as compared with duloxetine, fluoxetine, fluvoxamine, and paroxetine (Cipriani et al., 2009), but other studies show no differences in efficacy among individual SSRIs (Cipriani et al., 2005).

SSRIs have comparable tolerability overall, but the specific medications differ somewhat in their side effect profiles, which may guide selection of an agent for an individual patient. SSRIs commonly cause nausea, vomiting, and diarrhea (Edwards and Anderson, 1999). These adverse events are generally dose dependent and tend to dissipate over the first few weeks of treatment. In some patients, however, diarrhea persists. SSRIs sometimes precipitate or exacerbate restlessness, agitation, and sleep disturbances (Caley, 1997). Usually loss of erectile or ejaculatory function in men and loss of libido and anorgasmia in both genders may occur due to antidepressant medication; these side effects seem to be more common with SSRIs. SSRIs have also been associated with extrapyramidal side effects, including akathisia, dystonia, Parkinsonism, and tardive dyskinesia (Gerber and Lynd, 1998; Leo, 1996). The incidence of such side effects is very low with SSRIs but may be higher in older patients, especially those with Parkinson's disease. Weight gain, at times substantial, occurs in some patients taking SSRIs (Papakostas, 2007). Patients who take paroxetine have a higher incidence of weight gain than those who take other SSRIs (Fava et al., 2000). Fluoxetine causes an initial weight loss which tends to regularize over time with continued treatment (Michelson et al., 1999). Use of SSRIs has been associated with the rare development of a syndrome caused by an excess of central nervous system serotonergic activity. Features of serotonin syndrome include abdominal pain, diarrhea, flushing, tiredness, change in mental status, tremor and myoclonus, sweating, hyperthermia, rhabdomyolysis, renal failure and cardiovascular shock (Boyer and Shannon, 2005). Discontinuation emergent symptoms include both flu-like experiences such as nausea, headache, light-headedness, chills, and body aches, and neurological symptoms such as paresthesia, insomnia, and "electric shock-like" phenomena. These symptoms typically resolve without specific treatment over 1-2 weeks (Schatzberg et al., 2006).

1.1.6.1.2 Serotonin Norepinephrine Reuptake Inhibitors (SNRIs)

SNRIs were found superior to placebo in controlled studies and meta-analyses (Gartlehner, 2008; Thase et al., 2009). Clinically significant norepinephrine reuptake inhibition may not be achieved for the average patient at lower therapeutic doses, although desvenlafaxine has a much greater bioavailability, resulting in a lower effective dose. In individual studies, venlafaxine and duloxetine are generally as effective as SSRIs (Thase et al., 2007; Nemeroff et al., 2008). Relative to SSRIs, some analyses of pooled data sets have suggested a small advantage for SNRIs (Bauer et al., 2009), which might afford clinically modest benefits for patients with more severe depression (Thase et al., 2007) or for patients who have not responded to prior trials of SSRIs (103). However, other meta-analyses have shown equivalent efficacy for SSRIs and SNRIs (Gartlehner et al., 2008), whereas some have shown superiority of individual medications but no clear-cut medication class effects (Cipriani et al., 2009). Relative to TCAs, venlafaxine's efficacy is comparable (Bauer et al., 2009; Smith et al., 2002), whereas the more recently introduced duloxetine and desvenlafaxine have not been systematically compared with TCAs.

The most common side effects of the SNRIs are similar to those seen with SSRIs, including nausea and vomiting, sexual dysfunction, and activation; like the side effects seen with SSRIs, those with SNRIs can attenuate with continued use. The SNRIs also are more likely to be associated with side effects that reflect noradrenergic activity, including increased pulse rate, dilated pupils, dry mouth, excessive sweating, and constipation. Alternatively, in a patient with well-controlled depressive symptoms, it may be preferable to add an antihypertensive agent rather than risk a depressive relapse or recurrence with medication tapering.

1.1.6.1.3 Monoamine Oxidase Inhibitors (MAOIs)

MAOIs have similar efficacy to other antidepressants for outpatients with MDD and this group of medications is suitable for patients with MDD who didn't respond to safer and more easily used treatments (Quitkin et al., 1979; Thase et al., 1995). It is demonstrated in different studies that the effectiveness of MAOIs in patients who have not responded to other antidepressant medications, particularly TCAs (Thase et al., 1995). However, the efficacy of MAOIs relative to other approaches for treatment-resistant patients in current practice remains unclear, particularly for patients who have not responded to multiple sequential trials with SSRIs and SNRIs (McGrath et al., 2006). MAOIs have been shown to be particularly effective in treating

depressed patients with atypical features (Thase et al., 1995). There do not appear to be any significant differences in efficacy among the older MAOIs (Thase et al., 1995), although there are important individual differences in responsiveness and these medications are not interchangeable.

Different types of adverse effects may occur due to the use of MAOI. A hypertensive disaster can occur when a patient taking an MAOI ingests large amounts of tyramine or other vasoactive amines with foods or other medications (Rapaport, 2007). Serotonin syndrome is characterized by abdominal pain, diarrhea, tremor and myoclonus, flushing, sweating, hyperthermia, lethargy, mental status changes, rhabdomyolysis, renal failure, cardiovascular shock (Stahl et al., 2008; Sternbach, 1991). Orthostatic hypotension is usually seen during MAOI treatment. Use of MAOIs can be linked with the development of peripheral edema. Weight gain is commonly seen in patients treated with nonselective MAOIs (Amsterdam and Bodkin, 2006). Sexual side effects seen with MAOI therapy like anorgasmia, decreased libido and erectile or ejaculatory dysfunction (Clayton et al., 2007).

1.1.6.1.4 Tricyclic Antidepressants (TCAs)

TCAs are effective medications for MDD and have comparable efficacy to other classes of antidepressants, including SSRIs, SNRIs, and MAOIs (Anderson, 2000). TCAs may be predominantly effective in certain populations, such as in hospitalized patients (Anderson, 1998; Barbui and Hotopf, 2001). Conventional insight is that this benefit is explained by the superiority of TCAs over SSRIs among the subgroup of patients with melancholia or more severe depression because such a specific advantage has not been consistently recognized in studies for mild or moderate depression (Anderson, 2000; Barbui and Hotopf, 2001).

Side effects including cardiovascular arrhythmias can be challenging with TCA treatment (Miller et al., 1998). All TCAs have anti-muscarinic side effects. Tertiary amine tricyclic antidepressants produce the most anticholinergic side effects whereas the secondary amines desipramine and nortriptyline have less anti-muscarinic effects (Baldessarini, 2006). Tricyclic antidepressants also have affinity for histaminergic receptors thus produce varying degrees of sedation. In general, tertiary amines cause greater sedation while secondary amines cause less sedation (Baldessarini, 2006). Tricyclic antidepressants can cause weight gain possibly through their histaminergic properties or blockade of 5-HT_2 receptors (Deshmukh and Franco, 2003). The degree of weight gain seems to vary by agent and is often dose dependent. Opposite to weight

gain occurs by cessation of TCA therapy. Tricyclic antidepressants can cause myoclonus (Garvey and Tollefson, 1987). Use of TCAs has been related with an increased risk of falls in a number of studies and meta-analyses, and the relative risk of falling appears comparable to that with SSRI treatment (Hartikainen et al., 2007; Sterke et al., 2008; Thapa et al., 1998). A number of medications that inhibit, induce or are metabolized by hepatic microsomal enzymes can interact with TCAs (Nelson, 2003).

1.1.6.2 Psychotherapy

Time-limited psychotherapies have been supported by a considerable research for MDD. Although the number of studies is less compare to pharmacotherapies. Most research has focused on individual, in-person, outpatient treatment, need based and limitations of research methods. Nevertheless, research has also begun to explore psychotherapies in differing formats, including groups, over the telephone, and with computer assistance.

When psychotherapy is part of the treatment plan it must be integrated with psychiatric management or pharmacotherapy that is being provided. Nature and intensity of psychotherapy can be determined based on clinical considerations and other patient factors. Typically psychotherapy is given in an ambulatory setting while some psychotherapy might benefit depressed inpatients when given adequate lengths of stay and courses of treatment. Similar to pharmacotherapy, success of psychotherapy depends on the skill and training of the therapist. Patient factors, such as beliefs and attitudes toward psychotherapy, nature and length of depressive episodes and initial experiences also affect treatment outcome to psychotherapy. Progress or maintenance of depressive episodes is influenced by psychosocial stressors and psychological factors which are addressed in psychotherapy.

1.1.6.2.1 Cognitive Behavioral Therapy (CBT)

CBT combines cognitive psychotherapy with behavioral therapy and maintains that illogical beliefs and slanted attitudes toward self, environment and future perpetuate depressive affects and compromise functioning. The goal of CBT is to reduce depressive symptoms by challenging and retreating these beliefs and attitudes and inspiring patients to change their maladaptive prejudices and activities in real life (Beck et al., 1979). Cognitive-behavioral therapy is an effective treatment for MDD. CBT has generally surpassed control conditions in efficacy and has had equal efficacy compared with other empirically supported psychotherapies (Wampold et al.,

2002). Studies comparing the effectiveness of CBT with pharmacotherapy is methodologically challenging to conduct and results are inconsistent (Hollon et al., 2002; Parker et al., 2003). CBT is less effective for patients with more severe depressive symptoms.

Theoretical models drawn from behavior theory (Ferster, 1973) and social learning theory (Bandura, 1977) are used as behavior therapy for MDD. Behavioral activation is a newly articulated behavioral intervention with some positive preliminary results that merit further study (Cuijpers et al., 2007; Dobson et al., 2008). Specific behavior therapy techniques comprise activity scheduling (Lewinsohn et al., 1984; Lewinsohn and Clarke, 1984), social skills training (Bellack et al., 1983), self-control therapy (Rehm, 1979) and problem solving (Nezu, 1986). Behavior therapy includes graded homework, preparation of enjoyable activities, and reducing unpleasant activities (Martell et al., 2001).

1.1.6.2.2 Interpersonal Psychotherapy (IPT)

The main focus of IPT is on current life changes including losses, role disagreements and role changes, social isolation, deficits in social skills and other interpersonal factors that may interact with the development of the depressive episodes (Weissman et al., 2000; Weissman et al., 2007). The goal of IPT is to interfere by identifying the current trigger of the depressive episode, facilitating mourning in the case of bereavement, endorsing recognition of related affects, resolving role disagreements and role changes and building social skills to recover relationships and to gain needed social supports. MDD is regarded as a medical illness in IPT and the illness rather than the patient is blamed for the symptoms. Medical model of IPT makes it highly compatible with pharmacotherapy in combined treatment.

IPT is an effective treatment for MDD (Hollon et al., 2002; Markowitz and Weissman, 2008). It is demonstrated in different studies that efficacy of this treatment for primary care depressed patients and patients with more severe depression (Weissman et al., 2000). The efficacy of IPT has also been demonstrated for adolescents, pregnant women and geriatric patients (Weissman et al., 2000). It can also be used as a monthly maintenance therapy to prevent relapse (Frank et al., 1990; Frank et al., 2007; Reynolds et al., 1999).

1.1.6.2.3 Psychodynamic psychotherapy

Psychodynamic psychotherapy refers to a range of brief to long-term psychotherapeutic interventions (Bash, 1988; Gray, 1992). These interventions derive from psychodynamic theories about the etiology of psychological vulnerability, personality development and symptom formation as shaped by development and conflict occurring during the life cycle from earliest childhood onward (Blatt, 1998; Freud, 1917). Sometimes these theories focus on the conflicts related to guilt, shame, interpersonal relationships, management of anxiety and suppressed or intolerable desires. Developmental psychological deficits are produced by inadequacies or problems in the relationship between the child and emotional caregivers resulting in problems of self-esteem, sense of psychological cohesiveness and emotional self-regulation (Kohut, 1972; Rado, 1956).

Psychodynamic psychotherapy may be brief but usually has a longer duration than other psychotherapies and its aims extend beyond immediate symptom relief. Psychodynamic psychotherapy is therefore broader than most other psychotherapies encompassing both current and past problems in interpersonal relationships, self-esteem and developmental conflicts associated with anxiety, guilt, or shame. Time-limited structured psychodynamic psychotherapy may focus more on understanding the psychological basis of the presenting symptoms or on a selected underlying conflict. Sometimes the goal of psychodynamic psychotherapy is brief or extended to help the patient accept or adhere to necessary pharmacotherapy (Gray, 1996).

1.1.6.2.4 Problem solving therapy

Problem solving therapy is manually guided brief treatment lasting for six to twelve sessions. This can be given by nurses or social workers to prevent depression in elderly and medically ill patients. It has also been used to treat patients with relatively mild depressive symptoms. The approach combines elements of CBT and IPT. Some studies supported that this therapy moderately improves patients with mild depressive symptoms. Although problem solving therapy has not supported by enough studies for patients with MDD, it may have a role in targeted patient groups with mild depression (Alexopoulos et al., 2003, Arean et al., 2008; Robinson et al., 2008; Rovner and Casten, 2008).

1.1.6.2.5 Marital therapy and family therapy

Marital and family problems are common in the development of mood disorders and comprehensive treatment often demands assessing and addressing these problems. Marital and family problems may be the consequence of MDD but may also increase vulnerability to developing MDD or retard recovery from it (Keitner and Miller, 1990; Sargeant et al., 1990; Yager, 1992). A number of marital and family therapies are effective for the treatment of such depression. Techniques include behavioral approaches, problem-focused approaches (Ryan et al., 2005), and strategic marital therapy (Coyne et al., 1988; Leff et al., 2000). Family therapy has also been found to be helpful in the treatment of more severe forms of depression in combination with medications and hospitalization (Miller et al., 2005).

1.1.6.2.6 Group therapy

Group psychotherapy is widely practiced but this application to treat MDD is not enough supported by research. Specific types having some data to support their efficacy include CBT (Bright et al., 1999; Neimeyer and Feixas, 1990; Neimeyer et al., 1995) and IPT (Bolton et al., 2003; Klier etal., 2001; Yalom, 1995; Zlotnick et al., 2001). Relative effectiveness of psychotherapeutic approaches conducted in group format versus individual format has not involved patients with thoroughly defined MDD (McRoberts, 1998; Piper and Joyce, 1996; Smith, 1980; Toseland and Siporin, 1986). Mutual support group and group CBT were found to be equally effective in reducing depressive symptoms (Bright et al., 1999). Medication maintenance support groups may also offer benefits although patients with MDD are lacking. Such groups inform the patient and family members about prognosis and medication issues, providing a psychoeducational forum that contextualizes a chronic mental illness in a medical model. The efficacy of self-help groups led by lay members in the treatment of MDD has not been well studied (Lieberman and Borman, 1979). Higher proportion of depressed outpatients had remission following treatment in groups led by professionals than had remission following participation in groups led by nonprofessionals (Bright et al., 1999).

In general, group therapy has some evidence to support its use and the potential advantage of low cost as one or two therapists can treat a larger number of patients concurrently. This advantage needs to be weighed against the difficulties in accumulating the group, the lesser intensity of

focus patients receive relative to individual psychotherapy and possibly adverse effects from communications with other group members.

1.1.6.3 Psychotherapy plus antidepressant medication

Combined therapy provides superior and long term effect compared to antidepressants alone for MDD patients. Psychotherapy is an acceptable alternative for combined treatment in the acute phase as it is as effective as combined treatment in the long-term (Karyotaki et al., 2016). Predominantly large preservative effects have been observed in individual studies of patients with chronic depression, patients with severe recurrent depression and hospitalized patients (Schramm et al., 2007). Combined treatment might therefore be considered a treatment of first choice for patients with MDD with more severe, chronic, or complex presentations. Combined family therapy with pharmacotherapy has more benefits in post hospital care for depressed patients (Miller et al., 2005).

Dual treatment combines the unique advantages of each therapeutic modality. Pharmacotherapy may provide earlier symptomatic relief; psychotherapy produces broader and longer lasting improvement (Hollon et al., 2005). Psychotherapy can also be used to address issues that arise during pharmacotherapy, such as decreased adherence. Though, the advantage of regularly combining interventions may be modest for patients with less severe depressive symptoms (Thase et al., 1997). There are no empirical data from clinical trials for the selection of particular antidepressant medications and particular models of psychotherapeutic approaches for individuals who will receive the combination of both modalities. Results from a series of recent studies provide indirect evidence that for patients who have had only a partial response to pharmacotherapy, adding a course of CBT may be an effective strategy for preventing relapse (Hollon et al., 2005; Paykel et al., 1999).

1.2 Relationship between some parameters in serum level with psychiatry

A number of vital trace elements play an important role in different metabolic pathways. Copper (Cu), zinc (Zn), selenium (Se), manganese (Mn) and iron (Fe) are vital trace elements that have been studied in different psychiatric disorders. Though, the results of some studies on the status of trace elements in patients with psychiatric disorders are controversial.

Serum malondialdehyde (MDA) levels, serum vitamin E, vitamin C, total antioxidant capacity (TAOC), may have a correlation with the disease status in MDD patients. The central nervous

system and the immune system are closely related. Psychiatric illness is often associated with a deregulation of the immune response. Serotonin is synthesized from tryptophan and norepinephrine is synthesized from tyrosine, with the first step catalyzed by tyrosine hydroxylase. Both monoamine transmitters are stored in vesicles in the presynaptic neuron and released into the synaptic cleft, thereby affecting both presynaptic and postsynaptic neurons (Belmaker and Agam, 2008). So tryptophan, tyrosine and other amino acids might play an important role in the pathophysiology of psychiatric disorders.

Stress is perceived by the cortex of the brain and transmitted to the hypothalamus, where Corticotrophin Releasing Hormone (CRH) is released onto pituitary receptors. This stimulus results in the secretion of corticotrophin into plasma, stimulation of corticotrophin receptors in the adrenal cortex, and release of cortisol into the blood. Hypothalamic cortisol receptors respond by decreasing CRH production to maintain homeostasis. There is considerable evidence that cortisol and its central releasing factor, CRH, are involved in depression. Patients with depression may have elevated cortisol levels in plasma (Belmaker and Agam, 2008).

1.2.1 Trace elements and psychiatry

Serum Cu concentration was found expressively higher whereas Mn and Fe concentrations were lower in schizophrenic patients and Se and Zn concentrations remain normal level. According to above findings changes in vital trace elements Fe, Cu, and Mn might play a role in the pathogenesis of schizophrenia. However, findings from trace element levels in schizophrenia show a variety of outcomes that are tough to explain (Yanik et al., 2004). Pathophysiology and treatment of depressive disorder might be related with Zinc content in the body. Serum zinc level decreases during the depressive episodes of type I bipolar disorder and probably in depression at the late stage (Siwek et al., 2016). Though little is known about the trace element profile differences between Schizophrenia patients and healthy controls, one study suggests clear element profile differences between patients with schizophrenia and healthy controls, and reduced Zn level is confirmed in the schizophrenia patients (Cai et al., 2015).

The etiology and pathophysiology of schizophrenia still remain ambiguous. One study discovered the relations between schizophrenia risk factors and serum levels of ten important trace elements. The study showed that lower levels of Se, Cu and higher levels of Mn were found in schizophrenia patients (Liu et al., 2015). Serum manganese and calcium concentrations were

significantly higher in obsessive-compulsive disorder patients. Study showed a definite imbalance in inter-elemental relationships in obsessive-compulsive disorder patients compared to controls and therefore suggests a disturbance in the element homeostasis (Shohag et al., 2012). Decreased level of serum zinc in Panic Disorder (PD) patients may provide a prognostic tool for the diagnosis and treatment of this disease (Nahar et al., 2010). Data obtained from different inter-element relations in the Generalized Anxiety Disorder (GAD) patients and healthy volunteer strongly recommend that there is a trouble in the element homeostasis. So alterations in the serum trace element level in GAD patients happen independently and that may provide a prognostic tool for the diagnosis and treatment of this disease (Islam et al., 2013).

1.2.2 Antioxidant vitamins and psychiatry

Brain is predominantly susceptible to oxidative damage because of its high rate of oxygen consumption, plentiful lipid content, and relative scarcity of antioxidant enzymes compared with other organs. In fact, increased levels of proteins, lipids and oxidized nucleic acids have been described in the postmortem brains of patients with schizophrenia and bipolar disorders, and decreased antioxidant capacities have been described in blood samples obtained from patients with first-episode psychosis. Therefore, it is suggested that oxidative stress is a junction point for genetic and environmental vulnerabilities to not only neurodegenerative but also psychiatric disorders. In other words, oxidative stress potentially plays a central role in the path-mechanisms that integrate gene-environment interactions in neuropsychiatric disorders (Nunomura et al., 2014). Pathophysiology or inadequate dietary antioxidant intake is responsible for high level of oxidative stress and inflammation in subjects with depression. Depressed persons intake significantly low level of dietary antioxidants. However, dietary total antioxidant content depressed and normal person were not significantly different (Prohan et al., 2014). Association among life stress, physiologic response and chronic conditions is altered by nutritional status, with an emphasis on antioxidant vitamins that was proposed in a study (Tucker, 2005). Total antioxidant levels are significantly lower in GAD patients than healthy controls (Emhan et al., 2015). According to their findings, oxidative stress mechanism might have a role in GAD pathophysiology. In the future, total antioxidants might be used as a biologic marker in GAD etiology but more research is needed. Another study reveals that PD patients have considerably lower level of antioxidant vitamins than the healthy control subjects (Nahar et al., 2013).

1.2.3 Immunoglobulins and psychiatry

The central nervous system and the immune system are closely correlated. Psychiatric illness is often associated with a dysregulation of the immune response. There is a significant positive relationship between the stress-induced alterations of serum immunoglobulin A (IgA), immunoglobulin G (IgG) and immunoglobulin M (IgM). Research results propose that psychological stress is associated with the changed secretion of serum immunoglobulins, some acute phase proteins and complement factors (Maes et al., 1997). In another study serum levels of IgM, Ig G and Ig A were measured in psychiatric patients including bipolar depression, unipolar depression and schizophrenia and the comparative findings with healthy subjects reveal that a significantly high level of IgM concentration was found in all patient groups compared with the controls whereas IgM levels were more elevated in female patients than in male patients for the bipolar and unipolar groups. There were no significant differences for the other immunoglobulins (IgG, IgA) among the groups studied (Legros et al., 1985). Depressed patients had significantly higher percentages of circulating neutrophils, significantly lower percentages of circulating lymphocytes and significantly lower in vitro lymphocyte responses to mutagenic stimulation than normal controls (Kronfol and House, 1989). IgG level in PD patient was found significantly lower than that of the controls but the change in concentration of IgM and IgA were not significant. This finding may be considered as an important parameter for the diagnosis and treatment of the PD patients (Nahar et al., 2012). Serum concentration of IgA and IgM are significantly higher in conversion disorder patients compare to healthy control but the change of IgG is not significant. These findings suggest a possible diagnosis of conversion disorder as in serum level all the immunoglobulins were increased (Khanam et al., 2018). Serum IgM level of GAD patients was found significantly higher than that of the controls whereas IgG and IgA levels were insignificant. These results can be considered as a marker for the diagnosis and treatment of GAD patients (Islam et al., 2014).

1.2.4 Lipid peroxidation (MDA) and psychiatry

Inflammation, autoimmune tissue damage and prolonged psychological stress are the major causative factor for depression which leads to oxidative stress. Mood-regulating pathways in MDD may be affected by oxidative injury to lipids. A meta-analysis summarizes current knowledge regarding lipid peroxidation markers in clinical samples of MDD and the effects of antidepressant pharmacotherapy on those markers. Lipid peroxidation level was higher in

patients with MDD than healthy controls and which is associated with the severity of the disease. Antidepressant treatment can reduce lipid peroxidation in MDD patients. Increased lipid peroxidation was associated with MDD and may be normalized by antidepressants (Mazereeuw et al., 2015). Significant higher level of serum MDA concentration was found in the patients with MDD as compared to healthy controls. This study end up with the result that in the absence of known oxidative damage contributing mediators, the higher levels of MDA and lowered levels of antioxidants associate the high degree of oxidative stress in unipolar depression (Bajpai et al., 2014). MDA is a marker of lipid peroxidation which is elevated in schizophrenia. MDA level is significantly increased in schizophrenia cases compared to controls but this is not associated with the severity of the disease (Devanarayanan et al., 2015). Serum level of MDA significantly higher in schizophrenic patients compared to healthy controls. This finding put great emphasis on the weak antioxidant defense mechanisms and its role in the pathophysiology of schizophrenia (Reyazuddin et al., 2014). Serum levels of lipid peroxides and cortisol are significantly low in the aged women with MDD (De la Fuente et al., 1998). Another study reveals that PD patients have considerably higher level of MDA than the healthy control subjects (Nahar et al., 2013). MDD patients are associated with higher oxidative stress MDA levels when compared with healthy controls. This result suggest that oxidative stress plays a role in depression and that antidepressant activity may be mediated via improving oxidative stress/antioxidant function (Jiménez-Fernández et al., 2015).

1.2.5 Cortisol and psychiatry

Patients with depressive illness may have significantly elevated levels of free plasma cortisol which can be measured by variety biologically active parameters for cortisol. Hypercortisolemia don’t depends on the abnormalities in corticosteroid binding globulin and plasma unbound cortisol is confirmed by the elevated urinary of free cortisol level. Absence of physical effects for cortisol level elevation may not be present in patients with depression as the elevation is mild in those patients and high level of plasma cortisol don’t maintain throughout the day which is confirmed by direct measurements of free cortisol in plasma. The apparent difference between elevated total cortisol levels and the mild elevation of unbound plasma cortisol is best clarified by the substantial binding capacity of corticosteroid binding globulin. Minimum 25 micrograms/dl cortisol concentration in plasma is required to saturate the binding sites of corticosteroid binding globulin and after then free cortisol be measured in plasma (Schlechte and

Coffman, 1985). Hypercortisolemia during depression may develop due to another pathophysiological mechanisms relating to uneven basal hyper secretion of cortisol, linked with adrenal enlargement, possibly through splanchnic sympathetic stimulation of the adrenal cortex (Carroll et al., 2012). Cortisol is supposed to be a risk factor for stress and age related disorders, such as major depression and Alzheimer's Disease (AD). Pathophysiology of MDD may be associated with the elevated cortisol level in plasma. Association between high plasma cortisol and AD was found significantly (Zvěřová et al., 2013). Perioperative plasma cortisol concentrations may be elevated which is related with delirium after coronary artery bypass graft surgery. This may be a vital pathophysiological concern in the augmented risk of postoperative delirium seen in patients with a preoperative diagnosis of major depression (Kazmierski et al., 2013). Unbound plasma cortisol level is correlated with the CSF free cortisol values. Central Nervous System (CNS) and different tissues get exposure to physiologically active glucocorticoids in depressed patients. The presence of severe depressive symptoms which manifest a diurnal rhythm may be firm in part by extreme CNS exposure to glucocorticoids (Carroll et al., 1976).

1.2.6 Amino acid and psychiatry

Hypo-function of N-methyl-D-aspartate (NMDA) receptor may be related to the pathophysiology of schizophrenia. Endogenous l-serine-derived NMDA receptor co-agonists are D-3serine and glycine. The l-serine synthesis pathway could be involved in schizophrenia. Some research have done on this and found that in male schizophrenic patients l-serine synthesis system is altered that may cause schizophrenia (Ozeki et al., 2016). Controlling of seizure activity has been proven in many studies by the effects of a ketogenic diet. Although its mechanism of action remains indefinable in many regards. Antiepileptic effects of ketogenic diet may exert by influencing tryptophan (TRP) metabolism. Administration of fatty acid (FA) in brain undoubtedly amplified the seizure threshold and persuaded sedation. Then after, researcher confirmed that blocking TRP passageway into the brain eliminated these effects of FA but had no comparable outcome on the formation of ketone bodies. FAs are major components of a ketogenic diet and anticonvulsant properties of a ketogenic diet partly dependent on alterations in TRP metabolism (Maciejak et al., 2016).

Pathophysiology of depression depends on glutamatergic neurotransmission via NMDA receptor. Patients with depression have significantly higher serum levels of d-serine and l-serine than

those of healthy controls. On the other hand serum levels of glycine, glutamate and glutamine do not vary between two groups. Serine enantiomers may be peripheral biomarkers for depression, and that irregularity in the d-serine-l-serine-glycine cycle shows an important role in the pathophysiology of depression (Hashimoto et al., 2015).

Still effective methods for diagnosis of AD are inadequate. Altered levels of the NMDA receptor co-agonist d-serine have been connected with neurological disorders including schizophrenia and epilepsy. Nevertheless, whether d-serine levels are decontrolled in AD remains obscure. Elevated brain and CSF d-serine levels are linked with AD. CSF d-serine levels differentiated between non-demented and AD patients that might establish a novel biomarker for early diagnosis of AD (Madeira et al., 2015).

Risk of MDD is possibly due to deficiencies in micronutrients in the gluten-free diet. Serum concentrations of tyrosine, phenylalanine and tryptophan are lower in depressed patients. Although it's potential contrary effect, intake and serum levels of essential amino acids are not related to major depression (Van Hees et al., 2015). Glycine is a natural coagonist of the NMDA receptor and according to the hypo-NMDA hypothesis treatment with its high doses can improve symptomatology of schizophrenia. Elevated serum level of glycine may be involved in psychopathology of schizophrenia and cognitive functioning (Strzelecki and Rabe-Jabłońska, 2010). Glycine acts as an endogenous selective co-agonist at the glycine modulatory site of the NMDA receptor. Serum levels of glycine are significantly decreased in patients with schizophrenia in comparison to healthy controls. Some clinical trials shows that glycine can improve negative symptoms in schizophrenia when treated antipsychotics (Hons et al., 2010).

1.3 Objective and rationale this research

Objectives of the research:

A. Determination of the concentration of following parameters in patients suffering from MDD.

1. Trace elements (Mn , Cu, Zn, Se and Fe)
2. Antioxidant-vitamins (A, E, C)
3. Immunoglobulins (IgA, IgG, IgM)
4. MDA
5. Cortisol
6. Amino acid

B. Above determined concentration of six elements will be compared with that of control i.e. healthy volunteers and find out the correlation between these parameters in serum level and the disease status of MDD.

Rationale of the research:

Human brain is the control room of nervous system which sends output to the muscles by receiving input from the sensory organs. Human brain is bigger compare to body size than any other brains and has similar basic structure as other mammal brains. About 86 billion neurons are connected by trillions of synapses in human brain. That is why human beings are considered as the most evolved animal in the planet as they have the most evolved brain structure. They have the highest level of social affairs as their brain is most developed but this brain is mostly affected by several psychiatric illnesses. These psychiatric illnesses not only affect individual's activities but also have negative impact in the society.

In Bangladesh 16.05% of adult population suffer from psychiatric illness. The prevalence of MDD is 4.6%. Among all psychiatric patients 28.7% suffer from MDD. Both mild to moderate and severe depression are more common in females and among singles (Firoz et al., 2006). The most common time of onset is the third decade of life, with a later peak in forth decade and there is a second, smaller peak of incidence in sixth decade. Several neurological conditions such as stroke, Parkinson's disease, multiple sclerosis and during the first year after childbirth are the major risk factors of major depression (Rickards, 2003). Urban people suffer more in depressive disorders than rural population and the prevalence is higher in groups with stronger socioeconomic factors e.g. homelessness.

Patient's self-reported experiences, behavior described by families or friends and a mental status examination based on DSM criteria of American association of psychiatry are still used as the diagnostic tools for MDD. There is no laboratory test for this, though physicians usually demand tests for physical situations that may cause comparable symptoms. Some hypothesis indicates that trace elements, antioxidants-vitamins, immunoglobulins, MDA, cortisol and amino acids play an important role in some neuropsychiatric disorders, such as depression. The aim of this research is to measure the serum levels of these components and their correlation with the disease status in patients with major depression compared to healthy control subjects. It is expected that this investigation will be helpful for the management of MDD and it will highlight

on the regulatory effect of trace elements, antioxidants-vitamins, immunoglobulins, MDA, cortisol and amino acids on MDD, which will be also considered to be very important for the design of new drug molecules to treat this psychiatric disease.

CHAPTER TWO

MATERIALS AND METHODS

2 Materials and methods

2.1 Study design

Two hundred and forty seven MDD patients, age ranging from 18 to 60 years, were randomly recruited from the outpatient and inpatient Department of Psychiatry, Bangabandhu Sheikh Mujib Medical University (BSMMU) Hospital, Dhaka, Bangladesh. Specialist psychiatrist trained in the use of DSM-5 of Psychiatry Disorders (Text Version, 5th Edition), conducted the diagnosis and interview of the patients.

The control group included 248 healthy persons matching by age, sex and socioeconomic status to the patient group with no previous history of any psychiatric disorders or any medical disease that may affect the serum level of trace elements, immunoglobulins, antioxidant-vitamins, lipid peroxidation**,** cortisol and amino acids.

All patients were evaluated clinically by taking history and clinical examination, searching for other symptoms which are very much correlated to MDD that may mislead the research. Laboratory investigations including: 1) Complete blood count to exclude patients with anemia, leucopenia, leukocytosis, eosinophilia or any other blood related abnormality, 2) Thyroid function tests to exclude patients with increased levels of T3 and T4 in serum or to exclude patients with low serum T_3 and T_4 levels, 3) Renal function tests to identify blood urea and serum creatinine level to exclude patients with impaired renal function, 4) Liver function tests to exclude patients with impaired liver function, especially to identify patients with high liver enzymes or with reduced albumin levels or increased globulin levels and 5) Electrocardiogram (ECG) to exclude patients' heart problems and ECG, which uses an apparatus for recording electrical activity of the brain

Celiac disease is an autoimmune disorder by which gluten digestion is retarded. Neuropsychiatric symptoms of gluten may be manifested without any gastrointestinal symptoms. Some common complain of depression e.g. headaches, back pain, muscle aches and joint pain, chest pain, digestive problems, exhaustion and fatigue, sleeping problems, change in appetite or weight, dizziness or lightheadedness may be due to other pathological conditions which must need to exclude by differential diagnosis. Mentally retarded patients and who suffered from comorbid psychiatric disorders were also excluded from this study. The study subjects were briefed about the purpose of the study and written consent was taken from each of them. Each of

the subjects filled up a questionnaire form which contains personal information, socio-economic data, history of illness, family history etc. The forms of the patients who had no formal education were filled out with the help of an investigator. Study protocol and volunteer consent form was approved by the ethical review committee of Department of Psychiatry, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh.

2.1.1 Inclusion or exclusion criteria

a) Inclusion criteria

1. MDD cases attending the respective unit of BSMMU hospital
2. More than 18 years old
3. Both male and female were included in the study
4. Controls were matched regarding sex, age and socio-economic condition according to study population

b) Exclusion criteria

1. Non co-operative
2. Severe general medical condition
3. Children
5. More than 60 years old

2.1.2 DSM-5 (Diagnostic and Statistical Manual, 5th Edition) criteria of mental disorder

DSM-5 is the fifth edition of diagnostic and statistical manual of mental disorders of American Psychiatric Association (APA) published in 1994. This tool focuses on clinical, research and educational purposes. But highest priority has been given to provide a helpful guide to clinical practice. In this study patients and control subjects have been recruited based on DSM criteria.

2.1.3 Ethical issue

In a psychiatric study it would always be necessary to ask and discuss about some sensitive issues, regarding personal and family affairs. But extreme precaution would be taken not to break the limits of ethical issues and not to harm physically, psychologically socially and spiritually to the participants, during the course of thesis work. Points to preserve the ethical issues-

i. This study did not involve one's body organ or fetal tissues.

ii. Precaution was taken to ensure one's anonymity.

iii. Subject or key relatives were clearly informed about the scope and limitation of the study.

iv. A written or verbal consent was taken from the subject and / or from the key relatives if subjects are minor or unable to give reliable information (and for that purpose a consent form was supplied).

v. The patient and or relatives who were unwilling to participate were excluded from the study.

vi. Precautions were taken to maintain ones confidentiality of personal data and obscurity.

vii. No financial involvement of the client or respondents was encouraged.

viii. Basic human rights to refuse, or to accept were maintained.

ix. During the study period no medication was given to the patient for trial excepting therapeutic measures provide by a psychiatrist.

x. During the course of this study Precautions were taken about not to produce any environmental hazards or breach.

2.1.4 Volunteer consent form

I, the undersigned, authorize the researcher to consider me as a volunteer for this research work. I understand that I can change my mind at any time to withdraw myself as volunteer during this research work.

Volunteer consent to study treatment

Please tick as appropriate

Have you complete idea about the type, ultimate goal and methodology of the research?	Y	N
Are you aware that you don't have to face any physical, mental and social risk for this?	Y	N
There will be no chance to injury in any of your organs, are you aware of this?	Y	N
Have you got any idea about the outcome of this experiment?	Y	N
Have you decided intentionally to participate in this experiment?	Y	N
Do you think this experiment violate your human rights?	Y	N
Are you sure that all the information regarding you will be kept confidentially?	Y	N
No remuneration will be provided for this experiment, are you aware of this?	Y	N

After reading the above mentioned points, I am expressing my consent to participate in this experiment as a volunteer.

Volunteer signature: _____ Date: _____

[Please return the signed copy to the researcher and keep an extra copy for yourself]

Questionnaires

1. Identification

1.1 I.D Code:

1.2 Name:

1.3 Father's/ Husband's Name:

1.4 Sex: **Male** **Female** **1.5 Marital Status:**

1.6 Date of Birth (dd/mm/yy): **1.7 Age (yr):**

1.8 Mailing address

Ph

1.9 Permanent address

Ph

1.10 Religion

1.11 Nationality

2. Personal History:

2.1	**Area of residence**	**Rural**	**Urban**	**Sub-Urban**	**Others**
	Where have you spent your boyhood (1-15 y)?				
	Where have you spent at least ¾th or more of your life time?				

2.2	**Education Level**		**2.3**	**Occupation**		**2.4**	**Family expenses /month**	
	Illiterate			Professional/Managerial /Business				
	Can read only			Clerical		**2.5**	Impression about social class	
	Can write a letter			Technical			Rich	
	SSC or equivalent			Skilled worker			Upper Middle	
	HSC or equivalent			Unemployed/ Pensioner			Lower Middle	
	Graduate or higher			Housewife			Poor	
	Other			Others			Destitute	

2.3	**Smoking Habit**		**2.7a**	**Current smoker**	
	Never			Sticks/ Day	
	Ex-smoker > 6 months		**2.7b**	**Ex-smoker**	
	Current smoker			Sticks /day	

3. Previous history of psychiatric disorder: Y N

4. Family history of psychiatric disorder: Y N

5. Case: New Old

6. Biological Characteristics:

6.1 Height (cm): 6.2 Weight (kg):

6.3 Pulse/min: 6.4 Temperature:

6.5 BP (Sys/Dias):

7. Diagnostic criteria for MDD:

DSM-5 diagnostic criteria for MDD are as follows: Depressed mood or a loss of interest or pleasure in daily activities for more than two weeks. Mood represents a change from the person's baseline. Impaired function: social, occupational, educational. Specific symptoms, at least 5 of these 9, present nearly every day.

DSM-5 criteria for MDD	**Yes**	**No**
Depressed mood or irritable most of the day, nearly every day		
Decreased interest or pleasure in most activities, most of days		
Significant weight change (5%) or change in appetite		
Change in sleep: Insomnia or hypersomnia		
Change in activity: Psychomotor agitation or retardation		
Fatigue or loss of energy		
Guilt or worthlessness: Feelings of worthlessness or inappropriate guilt		
Concentration: diminished ability to think or concentrate		
Suicidality: Thoughts of death or suicide, or has suicide plan		

Principal investigator

Name: Signature: Date:

2.2 Blood sample collection

Before collecting blood sample, all the study subjects were briefed about the purpose and objective of the study and a written consent was taken from each of them.

2.2.1 Blood collection apparatus

1. Disposable syringe
2. Centrifugal tube
3. Cotton
4. Alcohol
5. Banded tape
6. Pipette
7. Refrigerator (-80 ^{o}C)

2.2.2 Collection of blood samples from MDD patients and control subjects

In total, 10 mL of venous blood was taken from each of the MDD patients and the healthy control subjects by plastic syringe built-in with a stainless steel needle. Then the blood sample was collected into a metal-free plastic tube. No glass material was used to prevent Al and Si contaminations. To eliminate metal contamination all the precautions were taken throughout the period of blood collection and storage as per the National Committee for Clinical Laboratory Standards criteria (Nahar et al., 2010; NCCLS, 1997)

2.2.3 Separation of serum from blood sample and processing

Blood samples were allowed to clot for half an hour and then centrifuged at 3000 rpm for 15 min. The required amount supernatant was collected and taken into six different Eppendorf tubes for analysis of different parameters by micropipette. Then all the Eppendorf tubes were marked properly and stored at -80 ^{o}C until the study day.

These samples were then analyzed for determining the serum level of trace elements (Zn, Cu, Fe, Mn, Ca and Mg), antioxidant-vitamins (A, E, and C), immunoglobulins (IgA, IgG and IgM), MDA, cortisol and amino acids as per required procedure and study protocol.

2.3 Statistical analysis

The SPSS software package (version 22.0) was used to analyze the data. Descriptive statistics were calculated for all the variables. Values were expressed as percentage and mean±SD. Comparison of all parameters of the MDD patients and healthy control subjects were performed by cross table variations and independent sample t-test and ANOVA. Pearson's correlation analysis was performed to find out the correlation of socioeconomic factors and disease with serum trace elements, anti-oxidant vitamins, immunoglobulins, lipid peroxidation, cortisol, and amino acid levels. The significance level was set at $p<0.05$ or 5%.

2.4 Socio-demographic data

Two hundred forty seven MDD patients who were diagnosed by consultant psychiatrists and two hundred forty eight healthy control subjects matched with age, sex and socioeconomic status were interviewed for his research. All the patients and control subjects were categorized based on socioeconomic conditions, biophysical characteristics and smoking habit. Socio economic data of MDD and control subjects have been shown in table 2.3.1. It was found that most of the patients were literate (87%) and nonsmoker (73%).

Average age for patients with depression was found 33.03±10.87 years which was very much similar to previous findings for other psychiatric disorder like somatization disorder, panic disorder, mania, schizophrenia and anxiety disorder (Karim et al., 2006; Baker et al., 2005; Bergquist et al., 1993)

Mean BMI for patient group and control subjects were found 22.82±2.53 and 23.15±3.01 kg/m2 respectively and the BMI difference is statistically insignificant. Eighty four percent patients had BMI in normal range and for control the percentage is 78%.

Average monthly family income for patient group was found 19.28±14.10 KBDT. Among all MDD patients 38% were very poor and 79% have family income within 25 KBDT per month. Only 7% patients have monthly family income above 40 KBDT. Among all MDD patients 15% were jobless which may correlate with their depression. Sometimes during and after student life people may have mild depression but this may develop major depression when they remain jobless for a long period.

Table: 2.3.1 Socio-demographic data of MDD patients and control subjects

Parameter	Patients (*n*=247)			Control (*n*=248)			*p* value
	n	%	Mean±SD	n	%	Mean±SD	
Education							
Illiterate	32	13		26	10		0.958
Can read only	47	19		51	21		
Secondary	31	13		35	14		
Higher secondary	63	26		67	27		
Graduate and above	74	30		69	28		
Occupation							
Service	22	9		21	8		0.673
Business	31	13		29	12		
Student	55	22		69	28		
Others	103	42		97	39		
Jobless	36	15		32	13		
Age in years							
18-24	58	23		54	22		0.575
25-34	78	32	33.03±10.87	79	32	33.55±9.56	
35-44	65	26		74	30		
45-60	46	19		41	17		
BMI (kg/m^2)							
Below 18.5 (CED)	23	9		25	10		0.193
18.5-25 (normal)	208	84	22.82±2.53	194	78	23.15±3.01	
Above 25 (obese)	16	6		29	12		
Monthly income in KBDT							
Below 10	94	38		59	24		0.413
10-25	101	41	19.28±14.10	97	39	20.33±14.32	
26-40	34	14		75	30		
Above 40	18	7		17	7		
Smoking habit							
Nonsmoker	180	73		190	77		0.352
Smoker	67	27		58	23		

Graphical presentation of socioeconomic status of MDD patients and control subjects

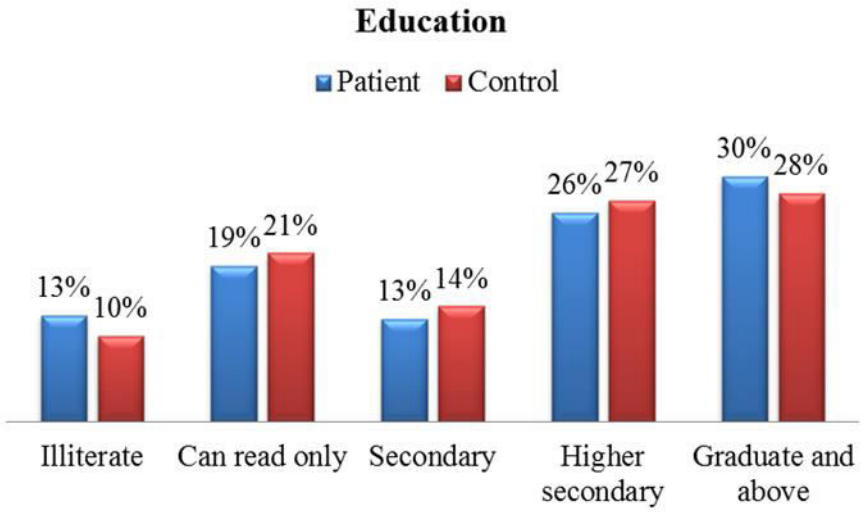

Figure: 2.3.1 Educational status of MDD patients and control subjects

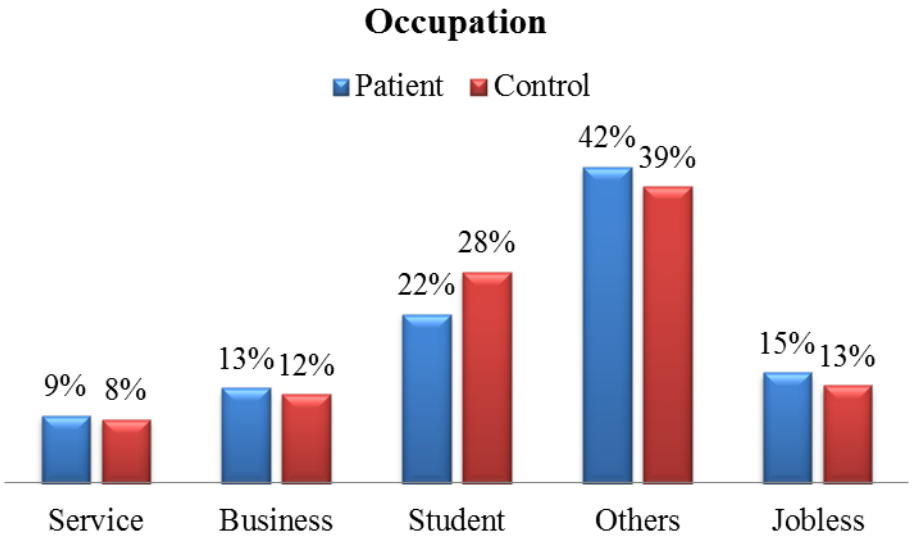

Figure: 2.3.2 Occupational status of MDD patients and control subjects

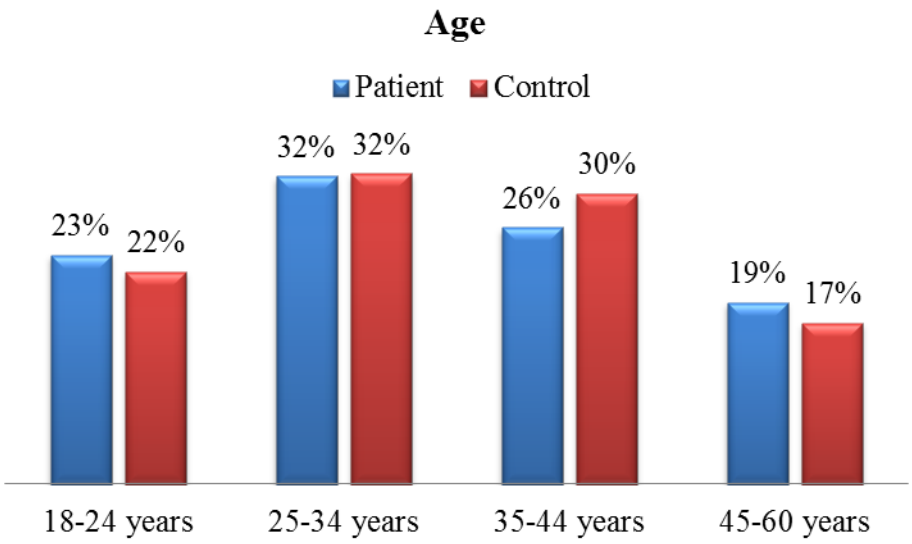

Figure: 2.3.3 Age distribution of MDD patients and control subjects

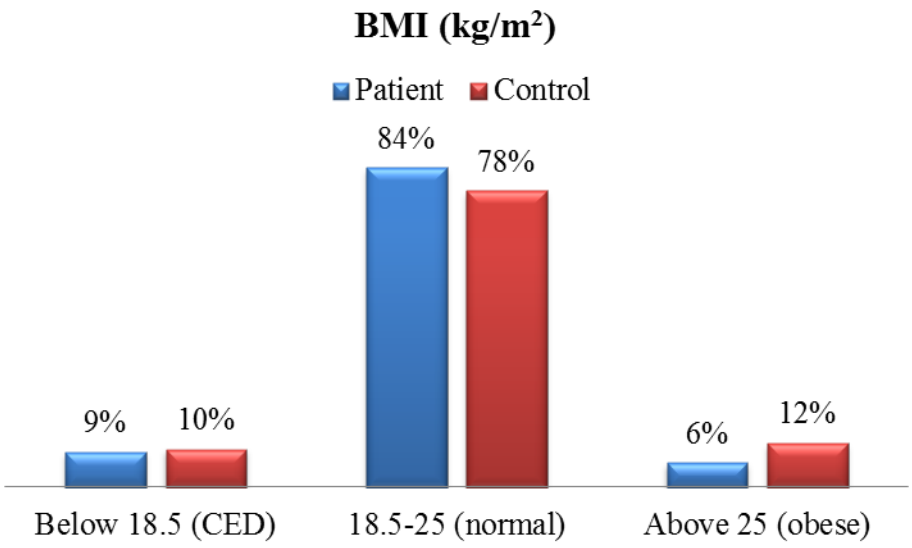

Figure: 2.3.4 BMI range of MDD patients and control subjects

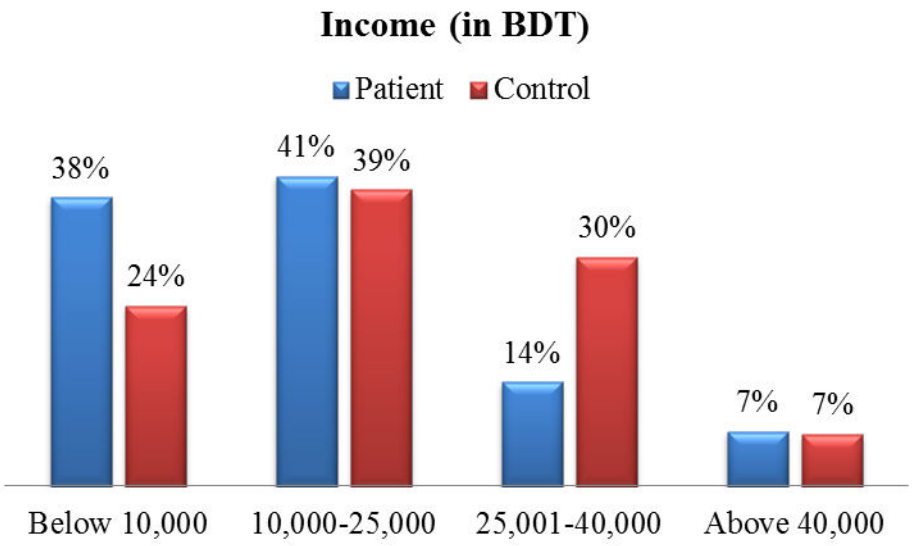

Figure: 2.3.5 Family income (BDT) of MDD patients and control subjects

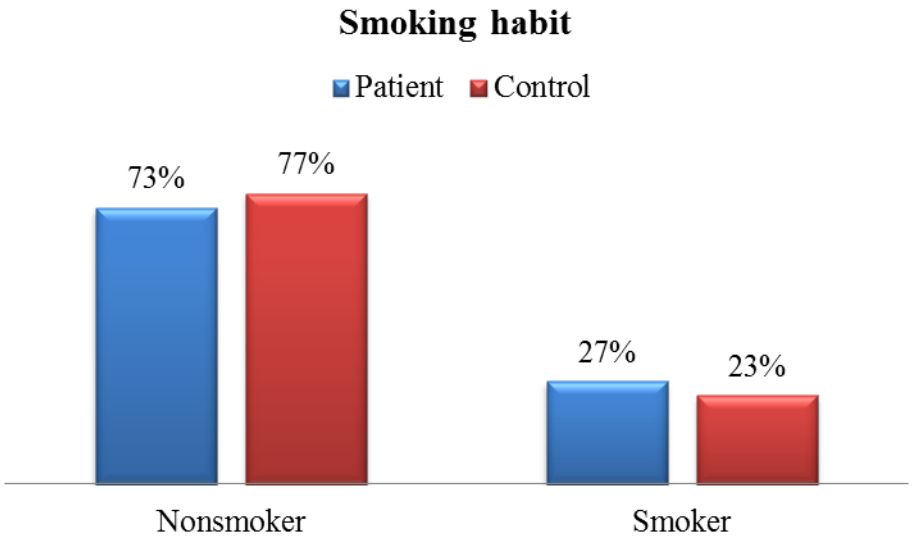

Figure: 2.3.6 Smoking habit of MDD patients and control subjects

CHAPTER THREE

DETERMINATION OF SERUM TRACE ELEMENTS, ANTIOXIDANT-VITAMINS, IMMUNOGLOBULINS, LIPID PEROXIDATION, CORTISOL AND AMINO ACIDS IN MDD PATIENTS AND CONTROL SUBJECTS

3.1 Determination of serum trace elements

3.1.1 Trace elements

Trace element is also termed as micronutrient. Very small amount of dietary trace element is required for proper growth, development and physiological functions. Exact needs of trace elements for human body vary with age, sex and physiological condition but commonly required trace elements are copper, zinc, manganese, iron, magnesium, iodine, selenium, cobalt and calcium.

3.1.1.1 Importance of trace elements in biological system

Trace elements are responsible for many metabolic and physiological functions in human body (Mertz, 1981). They play an important role for the synthesis and structural stabilization of both proteins and nucleic acids. Therefore, change of trace element levels and inter-elemental relationship may undesirably affect biological processes and are related with many diseases (Hegde et al., 2004). Micro-minerals or trace elements play a multipurpose function in human body ranging from providing antioxidant protection to developing immunity (Hossain et al., 2007). Various neuropsychiatric disorders such as schizophrenia, panic disorder may be developed due to alteration of these micro-mineral or trace elements in serum level (Hossain et al., 2007).

A large number of trace elements have been considered to be important for human nutritional requirement such as Fe, Zn, Cu, I_2, Mn, Mb, Cr and Co. Different diseases like breast, colon, prostate and lung cancers, leukemia, lupus, multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, attention deficit disorder**,** attention deficit hyperactivity disorder, bi-polar, heart and cardiovascular diseases, arthritis, diabetes, edema, arteriosclerosis, osteoporosis, herpes, influenza, many allergies and birth defects may occur due to the massive deficiency of trace elements in our daily food (Morowitz, 1992; Rhodes et al., 2005).

Currently, there are nine trace elements which are considered as nutritional requirement for human body e.g. Fe, Zn, Cu, Se, I_2, Mn, Mb, Cr and Co. Each of these elements contributes less than 0.01% to the total body weight. Although it is recognized that a nutritional requirement for additional micro-minerals can be confirmed and that in one case it is partly a question of semantics, it is also true that the evidence for a nutritional requirement for some trace elements

included in the "essential" list is still tenuous or limited. For example Co is included because an atom of this mineral is found in cyanocobalamine molecule. There is a wide range in apparent practical implications of these elements as nutrients in the likelihood and extent of clinical and/or public health importance of a nutrient deficiency or excess. There are also notable differences between the trace elements in the availability of biomarkers. Each of these factors influences the attention accorded to individual minerals (Krebs et al., 2003).

Essential components of enzymes may be present in trace elements that invite substrate molecules and enable their conversion to specific end products. In reduction or oxidation reaction has some donate or accept electrons which is important for the generation and utilization of metabolic energy. Fe is responsible for binding, transporting and releasing of oxygen in higher animals. Few biologically important molecules may get structural stability from some trace elements. Some trace elements control significant biological processes through facilitating the binding of molecules to receptor sites on cell membranes, changing the arrangement or ionic nature of membranes to prevent or permit specific molecules to enter or leave a cell and bringing gene expression ensuing in the formation of proteins involved in life processes.

Three dimensional structure of protein is maintained by the role of trace elements. Different types of enzymes and hormones are protein in nature, and trace elements play important role to maintain the integrity of protein structure. For example, superoxide dismutase, an oxidoreductase enzyme, is a metalloenzyme. It contains metal ions in its structure (Livesay, 2003). There are several forms of superoxide dismutase, including MnSOD, which contains a manganese ion and is located exclusively in the mitochondria, FeSOD which contains an iron ion and is generally found in some prokaryotes, and CuZnSOD, which is active in the cytoplasm of eukaryotic cells. In biological systems, copper is another rich trace element which is often found as a co-factor in proteins crossing over a wide range of function. Copper containing proteins can be found in electron transfer processes and in fibrinolytic pathways.

In human, three types of superoxide dismutase (SOD) are present. SOD_1 is cytoplasmic superoxide dismutase, SOD_2 is mitochondrial and SOD_3 is extracellular. SOD_1 is a dimer while SOD_2 and SOD_3 are tetramers. SOD_1 and SOD_3 contain Cu and Zn, while SOD_2 contain Mn in its reactive center (Cao et al., 2008).

Human superoxide dismutase

Crystallographic structure of the human cytoplasmic superoxide dismutase 1 (SOD_1)

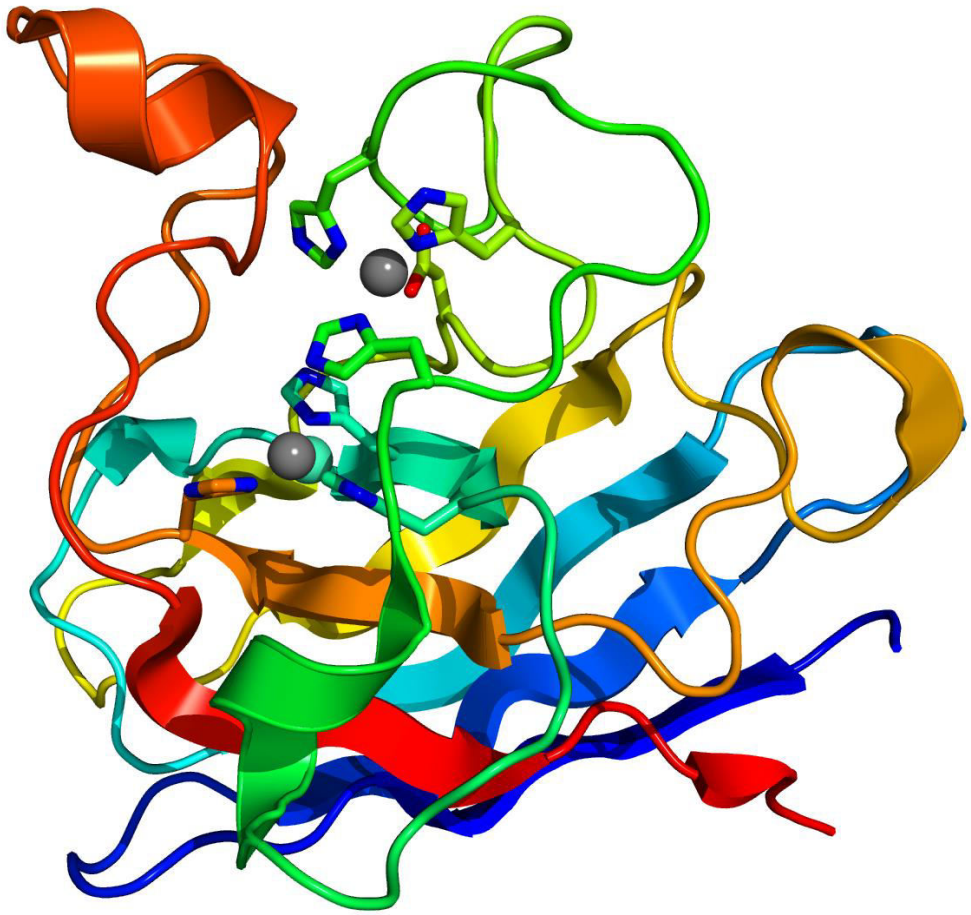

Figure: 3.1.1 Human SOD_1 dimer complexed with copper (blue-green sphere) and zinc (grey sphere)

Ribbon diagram of a human mitochondrial superoxide dismutase 2 (SOD_2)

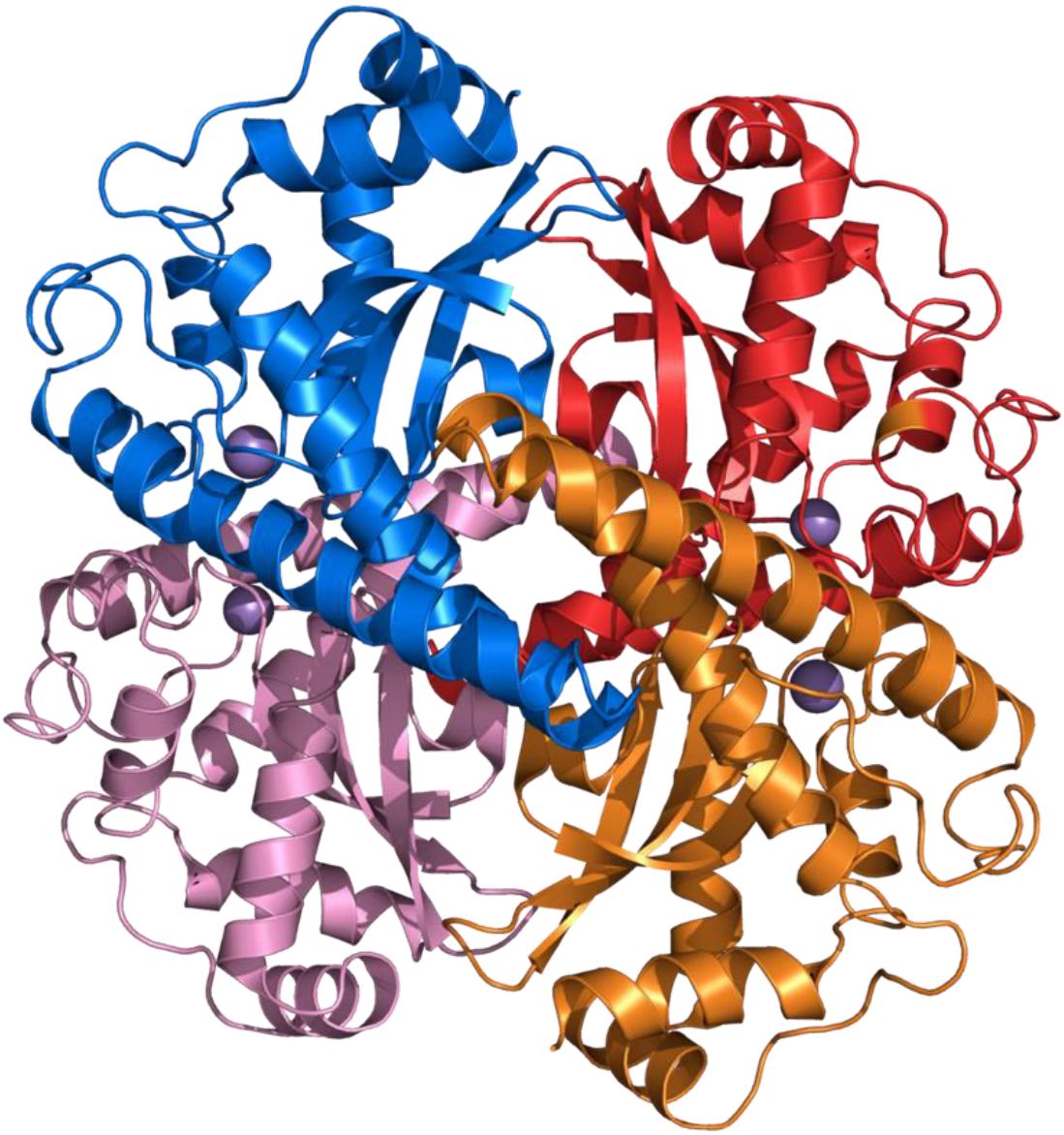

Figure: 3.1.2 Human SOD_2 tetramer. Manganese ions shown in violet

Crystallographic structure of the human extracellular superoxide dismutase 3 (SOD_3)

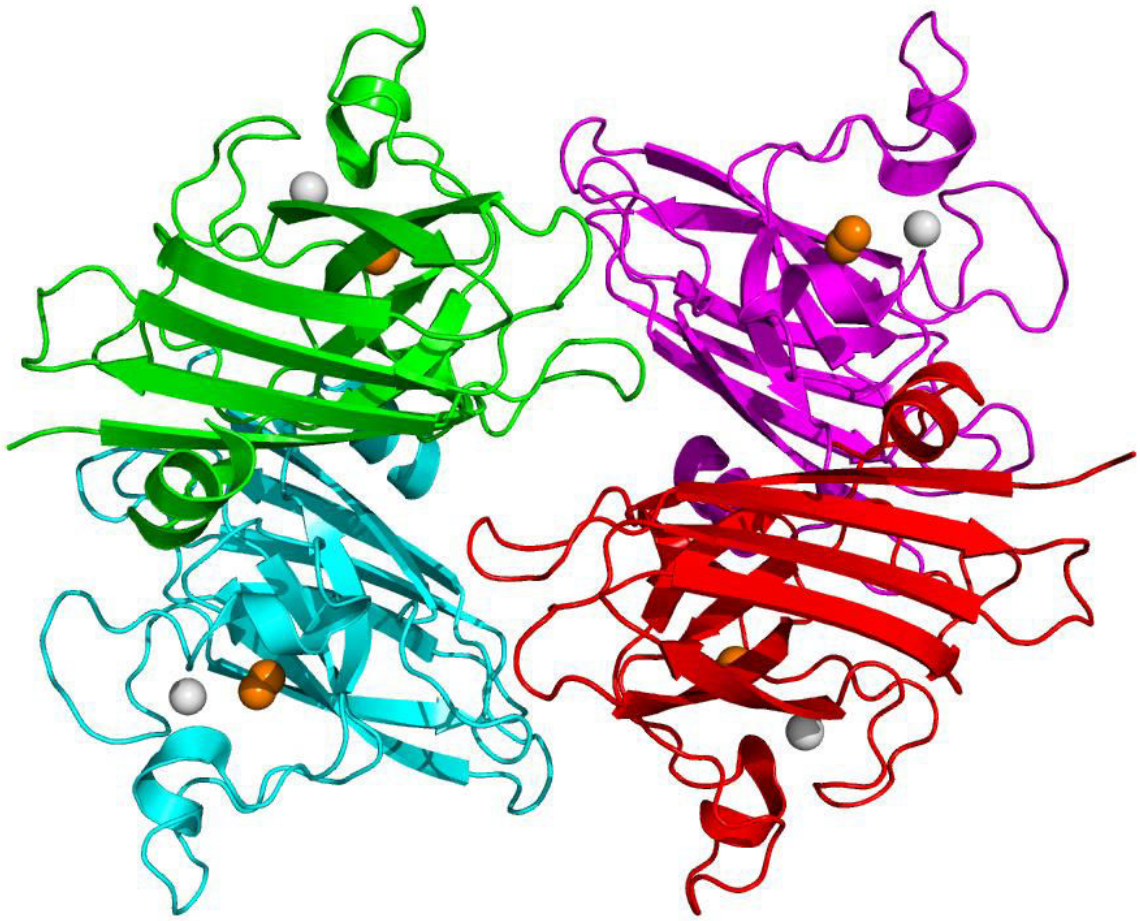

Figure: 3.1.3 Human SOD_3 tetramer complexed with copper and zinc cations (orange and grey spheres respectively)

3.1.1.2 Dietary requirements of trace elements

The human body needs several micro-minerals in trace amounts (mg) including iron, copper and zinc and some other micro-minerals are required in ultra-trace amounts (µg) including chromium, manganese, fluoride, iodide, cobalt, selenium, silicon, arsenic, boron, and vanadium. The above eighteen micro-minerals are considered as essential trace elements for human body but only four have been calculated in detail in different studies. So the recommended daily allowance (RDA) has been worked out for these four trace elements.

Table: 3.1.1 Recommended Daily Allowances (RDA) for iron, zinc, iodine and selenium

Age group	Iron (mg)	Zinc (mg)	Iodine (mg)	Selenium (μg)
Infants				
0 -6 months	6	5	40	10
7-12 months	10	5	50	15
Children				
1 – 3 years	10	10	70	20
4 - 7 years	10	10	90	20
8 – 11 years	10	10	120	30
Males				
12 – 14 years	12	15	150	40
15 – 18 years	12	15	150	50
19 – 24 years	10	15	150	70
25 – 50 years	10	15	150	70
51 + years	10	15	150	70
Females				
12 – 14 years	15	12	150	45
15 – 18 years	15	12	150	50
19 – 24 years	15	12	150	55
25 – 50 years	15	12	150	55
51 + years	10	12	150	55
Pregnancy	30	15	175	65
Lactation				
0 – 6 months	15	19	200	75
7 – 12 months	15	16	200	75

3.1.2 Determination of serum trace elements

The trace element (Zn, Cu, Mn, Fe, Ca and Mg) levels in serum samples were determined in both MDD patients and control subjects by using flame atomic absorption spectrometry (Spectra AA 220).

3.1.2.1 Flame Atomic Absorption Spectroscopy (FAAS)

It is an easy and fast method with high sensitivity mainly for elements like Cu and Cr, but difficulties may arise as a result of chemical and spectral interventions.

Radiation of a specific wavelength is chosen by using hollow cathode lamp through the sample is atomized. Concentration of the analyzed element is measured by the amount of absorbed radiation. The most courant gas mixtures used are air/acetylene and nitrous-oxide/acetylene. Background correction can be achieved with a deuterium lamp though several drawbacks subsequently occur. When absorbance becomes higher than 0.5 to 1 then the non-linearity of the standard curve is a major disadvantage of the AAS technique. The relative standard deviations are between 0.3 and 1% for absorbances of 0.1 to 0.2. Detection limits for flame AAS vary immensely from 1-5 ppb (e.g. Ca, Cd, and Cu) to more than 1000 ppb. Some elements like B, C, and Br cannot be measured at all by AAS.

Figure: 3.1.4 Flame Atomic Absorption Spectroscopy (Varian Spectra AA 210 VGP)

3.1.2.2 Determination of zinc

Zinc metal granules (99.99%; source: ABCR, Germany) was dissolved in hydrochloric acid to prepare the standard solutions in the concentration range of 0.5 to 8.0 mg/L. The serum samples were diluted by hydrochloric acid by a factor of 10. The standard solutions were run for every 10-test samples to confirm the test precision and quality. SpectrAA software package was used to calculate concentrations of Zn using calibration curve.

Instrument parameters: Wavelength: 213.9 nm; slit width: 0.7 nm; tube/site: pyro/platform

3.1.2.3 Determination of copper

Copper metal strip (99.99%; source: ABCR, Germany) was dissolved in nitric acid to prepare the standard solutions in the concentration range of 0.5 to 8.0 mg/L. The serum samples were diluted by nitric acid by a factor of 10. The standard solutions were run for every 10-test samples to confirm the test precision and quality. SpectrAA software package was used to calculate concentrations of Cu using calibration curve.

Instrument parameters: Wavelength: 327.4 nm; slit width: 0.1 nm; tube/site: pyro/platform

3.1.2.4 Determination of manganese

Standard manganese metal (99.97%; source: ABCR, Germany) was dissolved in nitric acid to prepare the standard solutions in the concentration range of 1.0 to 40.0 mg/L. The serum samples were diluted by nitric acid by a factor of 10. The standard solutions were run for every 10-test samples to confirm the test precision and quality. SpectrAA software package was used to calculate concentrations of Mn using calibration curve.

Instrument parameters: Wavelength: 279.8 nm; slit width: 0.7 nm; tube/site: pyro/platform

3.1.2.5 Determination of iron

Standard iron metal (99.95%; source: ABCR, Germany) was dissolved in nitric acid to prepare the standard solutions in the concentration range of 0.5 to 5.0 mg/L. The serum samples were diluted by nitric acid by a factor of 10. The standard solutions were run for every 10-test samples to confirm the test precision and quality. SpectrAA software package was used to calculate concentrations of Fe using calibration curve.

Instrument parameters: Wavelength: 248.3 nm; slit width: 0.2 nm; tube/site: pyro/platform

3.1.2.6 Determination of calcium

1.250 g of CaCO3 (analytical reagent grade), dried at 180 degrees C for 1 hour before weighing was suspended in deionized distilled water and dissolve cautiously with a minimum of dilute HCI to make the standard calcium solution. This was diluted to 1000 mL with deionized distilled water. 1 mL = 0.5 mg Ca (500 mg/L). The standard solutions were run for every 10-test samples to confirm the test precision and quality. SpectrAA software package was used to calculate concentrations of Ca using calibration curve.

Instrument parameters: Wavelength: 422.7 nm; slit width: 0.2 nm; tube/site: pyro/platform

3.1.2.7 Determination of magnesium

Standard 0.829 g of magnesium oxide, MgO (analytical reagent grade) was dissolved in 10 mL of redistilled HNO3 and dilute to 1 liter with deionized distilled water to make the standard magnesium solution. For dilution of each 10 mL volume of calibration standard and sample alike was added to 1.0 mL of the lanthanum chloride solution. The standard solutions were run for every 10-test samples to confirm the test precision and quality. SpectrAA software package was used to calculate concentrations of Mg using calibration curve**.**

Instrument parameters: Wavelength: 285.2 nm; slit width: 0.2 nm; tube/site: pyro/platform

3.1.3 Results

3.1.3.1 Serum trace element levels

Serum concentrations of trace elements (Fe, Mn, Zn, Cu, Ca and Mg) were analyzed in MDD patients and control subjects are showed in the table 3.1.2.

Analysis of serum trace elements indicated that the serum level of Zn (p<0.001), Mn (p=0.006), Ca (p<0.001) and Mg (p<0.001) decreased significantly whereas Cu has a tendency to increase and Fe was also decrease but not statistically significant (p=0.056) in MDD patients compare to control subjects (table: 3.1.2).

From these findings, it was observed that serum concentrations of all the trace elements significantly decreased compared to control subjects except Cu and Fe. These findings are supported to some extent by other research however some are not. Pathophysiology and treatment of depressive disorder might be related with Zn content in the body. Serum Zn level decreases during the depressive episodes of type I bipolar disorder and probably in depression at the late stage (Siwek et al., 2016). Excessive Cu and Zn levels may cause brain dysfunction (Nolan et al, 1983). It also alters the concentration of neurotransmitters. Cu level is generally higher in depressed patients than in normal individual (Narang et al, 1991).

Table: 3.1.2 Mean serum trace element concentration of MDD patients and control subjects

Trace element	Patient group			Control group			*p* value
	No.	%	Mean±SD	No.	%	Mean±SD	
Zn (mg/L)							
<0.50	20	8%	0.74±0.30	12	5%	1.01±0.19	
0.50-1.0	128	54%		141	59%		*p*<0.001
1.01-1.50	49	21%		56	24%		
>1.50	41	17%		13	5%		
Cu (mg/L)							
<0.60	64	27%	0.86±0.41	35	15%	0.79±0.31	*p*=0.156
0.60-1.20	129	54%		116	49%		
1.21-1.80	36	15%		62	26%		
>1.80	9	4%		11	5%		
Mn (µg/L)							
<0.30	0	0%	0.56±0.22	0	0%	0.65±0.32	*p*=0.006
0.31-0.50	206	87%		186	78%		
0.51-1.50	32	13%		32	13%		
>1.50	0	0%		6	3%		
Fe (mg/L)							
<0.80	0	0%	1.24±0.30	0	0%	1.32±0.35	*p*=0.056
0.80-1.60	174	73%		148	62%		
1.61-2.00	56	24%		64	27%		
>2.00	8	3%		12	5%		
Ca (mg/L)							
<60.00	25	11%	85.07±33.63	17	7%	104.33±11.13	*p*<0.001
60.00-100	94	39%		64	27%		
101-140	85	36%		115	48%		
>140	34	14%		28	12%		
Mg (mg/L)							
<15.00	57	24%	17.36±5.28	26	11%	21.24±3.03	*p*<0.001
15.00-20.00	132	55%		53	22%		
20.10-25.00	35	15%		124	52%		
>25.00	14	6%		21	9%		

* Correlation is significant at the 0.05 level (2-tailed).

Table: 3.1.3 Effect of socioeconomic factors on serum trace elements in MDD patients

		Fe	Mn	Zn	Cu	Ca	Mg
BMI	Pearson correlation	-.005	-.086	-.128	.022	-.050	.000
	Sig. (2-tailed)	.935	.193	.051	.739	.445	.998
Income	Pearson correlation	-.005	-.022	.009	.023	-.051	.036
	Sig. (2-tailed)	.944	.743	.896	.732	.438	.580
Age	Pearson correlation	-.137*	-.074	-.097	.031	-.009	.032
	Sig. (2-tailed)	.037	.261	.140	.637	.885	.625

* Correlation is significant at the 0.05 level (2-tailed).

Correlative analysis was also performed using the data of serum concentration of trace elements of MDD patients and socioeconomic factors. A significant correlation was observed between serum iron levels with age. No correlation was observed between serum trace element level with BMI and income.

Table: 3.1.4 Comparison of inter-element relations between patients and control subjects

Correlation parameters	Correlation coefficient (R)	
	Patient group	Control group
Mg and Ca	0.044	0.422*
Ca and Zn	-0.095	-0.220*
Mg and Fe	0.190**	0.023
Mn and Mg	0.020	-0.238*
Mg and Cu	-0.277**	0.314**

Value with a negative sign indicate an inverse correlation

*Correlation is significant at the 5% (0.05) level (2-tailed). ** at the 1% ($p<0.01$) level (2-tailed).

The correlation coefficient and the statistical confidence levels at which the correlations were determined are depicted in table 3.1.4. Mg was directly correlated with Fe and Mg was inversely correlated with Cu in the patient group. But in control group Mg is directly correlated with Ca and Cu but Ca and Mn were inversely correlated with Zn and Mg respectively.

3.1.3.2 Comparison of mean serum trace element levels in patients and control subjects

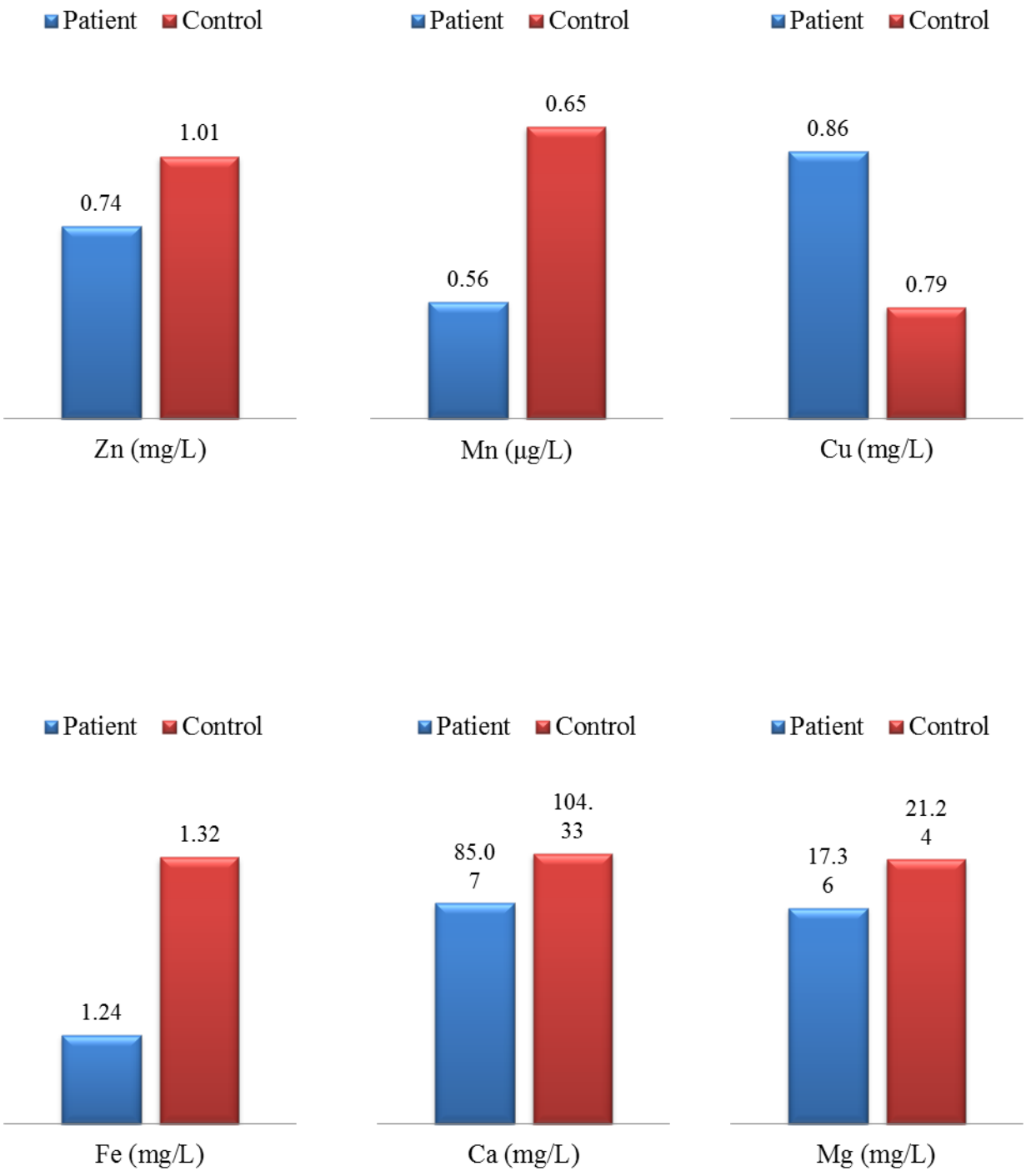

Figure: 3.1.5 Comparison of mean serum trace element levels in patients and control subjects

3.1.4 Discussion on trace element

Our current study showed that MDD patients have significantly low serum concentration of Zn (p<0.001), Mn (p=0.006), Ca (p<0.001) and Mg (p<0.001) than the healthy control subjects. On the other hand serum concentration of Cu has a tendency to increase and Fe has a tendency to decrease in MDD patients compared to control subjects. These findings are similar with some other previous findings in different studies. Depression, behavior and personality changes, apathy, irritability, and anxiety can be caused by low level of serum magnesium which is described in several studies (Wacker and Parisi, 1968). Maes et al. conducted three consecutive studies and found that serum zinc levels were significantly lower in depressed patients as compared to healthy matched controls (Maes et al., 1994). Psychiatric manifestations of zinc deficiency include behavioral disturbances, depression, and mental confusion. Lower zinc levels directly correlated with the severity of depression among MDD patients (Yanik et al., 2004). Though many studies support our result but further large scale study is needed to explore more in this field.

3.1.5 Conclusion

Our result indicates that most of the trace elements are significantly low in MDD patients compared to control subjects though the concentration of Cu has a tendency to increase and Fe has a tendency to decrease in MDD patients. So these findings suggest the possible involvement of depleted serum trace element in the pathogenesis of depression. Correlative analysis was used to find out correlation between serum trace elements and socioeconomic status of patients. It was found that there is no significant correlation with the trace elements with BMI and income of patient group. Only serum Fe concentration has a significant correlation with age of patient group (p=0.037). Correlative analysis was also performed to find out inter-elemental relationship in both patient and control groups. Mg was directly correlated with Fe (p=0.004) and Mg was inversely correlated with Cu (p<0.001) in the patient group. But in control group Mg is directly correlated with Cu (p=0.003). We thus recommend dietary supplementation to reduce the risk of depression which may require further study.

3.2 Determination of serum antioxidant vitamins

3.2.1 Antioxidants

Antioxidants protect cells damage by free radicals which are unstable molecules. This free radical damage may lead to cancer. Antioxidants interact with free radicals to stabilize these unstable molecules. Animal studies indicate that antioxidants may delay or perhaps stop the development of cancer. The most important antioxidants are β-carotene, lycopene, vitamin A, C and E.

3.2.1.1 Vitamin A (retinol)

Vitamin A, also known as retinol, is a fat-soluble vitamin important in vision and bone growth. In normal physiological system integration into vision pigments to regulatory transcription of a host of vital genes can be done by the help of vitamin A and its metabolites. Health depends on maintaining vitamin A level within a normal range, as either too little or too much of this vitamin lead to serious illness.

3.2.1.1.1 Chemistry

Retinol is the most functioning forms of vitamin A. All other forms are retinal (aldehyde form), retinoic acid (acid form) and retinyl esters (ester forms). These chemical compounds are collectively called retinoid and all possess biological activity as trans retinol is a common feature of their structure. A β-ionone ring and a polyunsaturated side chain, with alcohol or aldehyde, a carboxylic acid group or an ester group generally contain in the structure. Four isoprenoid units with a sequence of conjugated double bonds are the composition of side chain which may exist in trans or cis configuration.

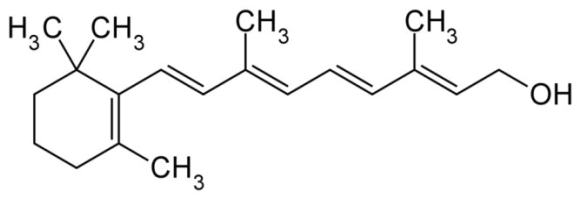

Figure: 3.2.1 Structure of the most common dietary form of vitamin A

3.2.1.1.2 Functions

Vitamin A metabolites can also affect some aspects of the adaptive immune response. Retinoic acid enhances cytotoxicity (Dennert and Lotan, 1978) and T-cell proliferation (Ertesvag et al., 2002). The latter probably mediated, at least in part, by enhancing Il-2 secretion and signalling in T cells. Retinoic acid can inhibit B-cell proliferation (Ballow et al., 1996; Blomhoff et al., 1992), although it has also been found to enhance B-cell activation under some conditions (Ertesvag et al., 2009; Saurer, 2007). Retinal is a necessary structural component of rhodopsin or visual purple, the light sensitive pigment within rod and cone cells of the retina.

Retinol plays an active role in prevention of xerophthalmia and blindness. Night blindness, xerophthalmia, and keratomalacia underline the role of this fat-soluble accessory vitamin, on both anatomic structure of the retina and its function (Wolf, 2001). Immunologically, low levels potentiate immune dysfunction, resulting in frequent infection and impaired mobilization of iron stores from tissues, thereby providing another mechanism for decreased red blood cell count (Gamble et al., 2001; Stephensen, 2001).

For proper diversity and maintenance many epithelial cells require vitamin A. Dysfunction of many epithelial cells like skin becomes keratinized and scaly, and mucus secretion is suppressed due to lack of vitamin A. Normal functioning of osteoblasts and osteoclasts is regulated by vitamin A (Bates, 1995). Adequate level of vitamin A is required for sperm production. Similarly, adequate availability of vitamin A is a prerequisite for normal reproductive cycles in females (Bates, 1995).

Vitamin A is essential for its key functions in

a. Vision
b. Gene transportation
c. Immune function
d. Embryonic development and reproduction
e. Bone metabolism
f. Haematopoiesis
g. Reducing risk for heart disease and cancer
h. Antioxidant activity

3.2.1.1.3 Daily requirement

The Third National Health and Nutrition Examination Survey (NHANES III) estimated that the median dietary intake of vitamin A is 744 to 811 μg/day for men and 530 to 716 μg/day for women using the new provitamin A carotenoid conversion factors for calculating the Equivalent Activity of Retinol (EAR). According to institute of medicine of the national academics, EAR for vitamin A is not set to meet the need of almost all (97 to 98%) individuals in a group. The EAR for vitamin A is based on a standard of satisfactory liver stores; thus, these data suggest that considerable proportions of adults have liver vitamin A stores that are less than required.

Table: 3.2.1 RDA for vitamin A (Institute of Medicine. Food and Nutrition Board, 2001)

Life stage group	Adequate Intake (μg/day)	Upper Intake Level (μg/day)
Infants		
0 -6 months	400	600
7-12 months	500	600
Children		
1 – 3 years	300	600
4 - 8 years	400	900
Males		
9 – 13 years	600	1700
14 – 18 years	900	2800
19 – 70+ years	900	3000
Females		
9 – 13 years	600	1700
14 – 18 years	700	2800
19 – 70+ years	700	3000
Pregnancy		
Below 19 years	750	2800
19 – 50+ years	770	3000
Lactation		
Below 19 years	1200	2800
19 – 50+ years	1300	3000

3.2.1.2 Vitamin E (α-tocopherol)

Vitamin E is found both in natural foods as well as dietary supplement. Vitamin E is a group of fat-soluble compounds with distinct antioxidant properties (Traber, 2006). Vitamin E from natural source exists in eight different chemical forms (alpha-, beta-, gamma-, and delta-tocotrienol and alpha-, beta-, gamma-, and delta-tocopherol) which have different types of biological activity (Traber, 2006). Human requirements generally fulfill by Alpha- (or α-) tocopherol form of vitamin E. Concentrations of this vitamin E (α-tocopherol) in serum depend on the liver as this organ gets the nutrient after various forms are absorbed from the small intestine.

3.2.1.2.1 Chemistry

Vitamin E from natural source includes two groups of very similar fat-soluble compounds, tocopherols and tocotrienols. The compounds of both groups are all derivatives of 6-chromanol. The first group derives from tocol, which carries a saturated isoprenoid C-16 side chain and three chiral centers with configuration -R at position 2, 4' and 8' (Fig. 3.2.2). The members of the second group have a triply unsaturated side chain at the positions 3', 7', and 11'. Within one group the members are designated a, b, g and d depending on the number and the position of the methyl groups attached to the aromatic ring (Stocker and Azzi, 2000).

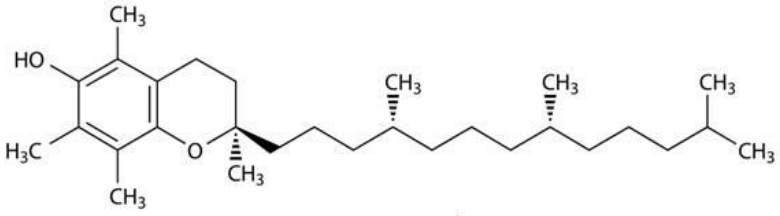

Figure: 3.2.2 Structure of naturally occurring components of vitamin E

3.2.1.2.2 Functions

Antioxidants properties of vitamin E protect cell damage by the effects of free radicals that contain an unshared electron. Cardiovascular disease and cancer may be developed by the free radical induced cell damage (Verhagen et al., 2006). Unshared electrons are highly reactive that react with oxygen to form reactive oxygen species (ROS). Our body normally forms ROS endogenously when it converts food to energy, and antioxidants might protect cells from the

damaging effects of this ROS. Our body is also unprotected to free radicals from environmental contacts, such as ultraviolet radiation from the sun, cigarette smoke and air pollution. Signaling mechanisms of cells are conducted by ROS which is produced through oxidation reaction of fat. This reaction can be inhibited by the fat-soluble antioxidant Vitamin E. Scientists are trying to limit free-radical production through other mechanisms. Vitamin E might help to prevent or postponement the chronic diseases related with free radicals.

Cell constituents can be protected by antioxidant nutrients like vitamin E from the damaging effects of free radicals that may lead to develop cancer, if unchecked (U.S. Department of Agriculture, Agricultural Research Service. 2011). Carcinogenic nitrosamines are formed in the stomach from nitrites in foods which is also blocked by Vitamin E thus protect against cancer by augmenting immune function (Weitberg and Corvese, 1997). Unfortunately, human trials and surveys that have objectives to correlate vitamin E intake with cancer occurrence have found that vitamin E is not helpful in most cases.

Significant vision loss in older people is due to age-related macular degeneration (AMD) and cataracts. The actual mechanism is usually unknown, but the cumulative effects of oxidative stress may play a vital role for this. If so, these conditions can be treated by nutrients with antioxidant functions, such as vitamin E. Coronary heart disease (CHD) may be prevented or delayed by vitamin E that comes from several sources. Crucial initiating step for atherosclerosis, oxidation of low-density lipoprotein (LDL) cholesterol is inhibited by vitamin E. It might also help to prevent the formation of blood clots that could lead to a heart attack or venous thromboembolism (Glynn et al., 2007).

The brain has a high oxygen intake rate with plentiful polyunsaturated fatty acids in the neuronal cell membranes. Researchers hypothesize that ingestion of sufficient or supplemental antioxidants like vitamin E might provide some protection if cumulative free-radical damage to neurons over time contributes to thinking deterioration and neurodegenerative diseases, such as Alzheimer's disease (Sano et al., 1997).

In addition to its activities as an antioxidant, *in vitro* studies of cells shows that vitamin E is involved in immune function such as cell signaling, regulation of gene expression, and other metabolic processes (Traber, 2006). Protein kinase C is an enzyme involved in cell differentiation and proliferation in platelets, smooth muscle cells and monocytes, activity of this enzyme is also inhibited by vitamin E. It also help to express the enzymes that suppress

arachidonic acid metabolism, thereby aggregate the release of prostacyclin from the endothelium, which, in turn, dilates blood vessels and inhibits platelet aggregation (Institute of Medicine, Food and Nutrition Board, 2000).

3.2.1.2.3 Daily requirement

Food and Nutrition Board (FNB) at the Institute of Medicine of the National Academies provides intake recommendations for vitamin E. RDA is the common term for a set of reference values used to assess and plan nutrient consumptions for healthy people. These values may vary by age and gender but the average daily level of intake is adequate to meet the nutrient necessities of almost all (97%–98%) healthy people. Adequate Intake (AI) is well-known when evidence is inadequate to develop an RDA and is set at a level presumed to confirm nutritional appropriateness and tolerable Upper Intake Level (UL) which is maximum daily intake not likely to cause adverse health effects (Institute of Medicine, Food and Nutrition Board, 2000). The FNB's recommends alpha-tocopherol alone as vitamin E which is the only form maintained in plasma. The FNB based these recommendations mainly on serum levels of the nutrient that provide satisfactory protection in a test measuring the survival of erythrocytes when exposed to hydrogen peroxide, a free radical. Recognizing "great suspicions" in these data, the FNB has suggested for investigation to identify other biomarkers for measuring vitamin E requirements.

Table: 3.2.2 RDA for vitamin E (Institute of Medicine, Food and Nutrition Board, 2000).

Life stage group	Adequate Intake (mg/day)	Upper Intake Level (mg/day)
Males		
0 – 6 months	4	-
7 – 12 months	5	-
1 – 3 years	6	200
4 – 8 years	7	300
9 – 13 years	11	600
14+ years	15	-
14 – 18 years	-	800
19+ years	-	1000
Females		
0 – 6 months	4	-
7 – 12 months	5	-

1 – 3 years	6	200
4 – 8 years	7	300
9 – 13 years	11	600
14+ years	15	-
14 – 18 years	-	800
19+ years	-	1000
Pregnancy		
14+ years	15	-
14 – 18 years	-	800
19+ years	-	1000
Lactation		
14+ years	19	-
14 – 18 years	-	800
19+ years	-	1000

3.2.1.3 Vitamin C (ascorbic acid)

Vitamin C or L-ascorbic acid is an essential nutrient for human body or some other animal species. Vitamin C describes several vitamers that have vitamin C activity in animals, including ascorbic acid and its salts, and some oxidized forms of the molecule like dehydroascorbic acid. Ascorbic acid and ascorbate both are naturally present in the body. When either of these is introduced into cells, the forms can convert to each other according to pH.

3.2.1.3.1 Chemistry

Vitamin C is a water soluble vitamin. It is originally the L-enantiomer of ascorbate; the reverse D-enantiomer has no physiological importance. Both forms are mirror images of the same molecular structure. When L-ascorbate plays its reducing role, which is a strong reducing agent, it is transformed to its oxidized form, L-dehydroascorbate. With the help of enzymes and glutathione L-dehydroascorbate can reduced back to active L-ascorbate in the body (Meister, 1994).

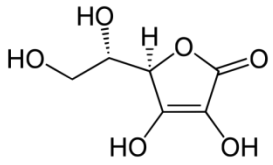

Figure: 3.2.3 Structure of vitamin C (ascorbic acid)

3.2.1.3.2 Functions

i) Antioxidant function

Vitamin C is a highly effective antioxidant for human body. It reduces the oxidative stress and act as a substrate for ascorbate peroxidase (Higdon, 2006). It also acts as an enzyme cofactor for the biosynthesis of many significant biochemicals and provides electrons to eight different enzymes as a donor (Levine et al., 2000).

- Three participate in collagen hydroxylation (Kivirikko et al., 1985; Peterkofsky, 1991; Prockop et al., 1995). These reactions add hydroxyl groups to the amino acids proline or lysine in the collagen molecule, thereby allowing the collagen molecule to assume its triple helix structure and making vitamin C essential to the development and maintenance of scar tissue, blood vessels, and cartilage (Mcgee, 2007).
- Two are necessary for synthesis of carnitine (Dunn et al., 1984; Rebouche, 1984). Carnitine is essential for the transport of fatty acids into mitochondria for ATP generation.
- The remaining three have the following functions:
    - Dopamine beta hydroxylase participates in the biosynthesis of norepinephrine from dopamine (Kaufmann, 1974).
    - Another enzyme adds amide groups to peptide hormones, greatly increasing their stability (Eipper et al., 1993).
    - One modulates tyrosine metabolism (Lindblad et al., 1970; Englard et al., 1986).

ii) Pro-oxidant

Ascorbic acid performs as an antioxidant as well as a pro-oxidant (Mcgregor and Biesalski , 2006). Reduction reactions of transition metals such as cupric ions (Cu^{2+}) to cuprous (Cu^{1+}) and ferric ions (Fe^{3+}) to ferrous (Fe^{2+}) are performed by the help of ascorbic acid during conversion from ascorbate to dehydroxyascorbate in vitro (Satoh and Sakagami, 1997). This reaction can produce superoxide and other ROS. Though, in the body, free transition elements are not likely to be present while copper and iron are attached with different proteins (Mcgregor and Biesalski, 2006).

iii) Neuroprotective functions

Though vitamin C is a vital antioxidant molecule in the brain, it has a number of other important functions. It participates as a cofactor in several enzyme reactions, including collagen production, catecholamine synthesis and regulation of HIF-1α. It is recognized that ascorbate is important for catecholamine biosynthesis in neural tissues, helping as a cofactor for dopamine β-hydroxylase in the transformation of dopamine to norepinephrine (Diliberto et al., 1980; Diliberto et al., 1981).

iv) Vitamin C in neurodegenerative disorders

Oxidative stress in the brain with a focus on neurodegenerative diseases has been extensively reviewed (Halliwell, 2006). Neurons have 10-fold higher rates of oxidative metabolism than supporting glia that's why they seem to be especially sensitive to ascorbate deficiency (Hediger, 2002; Wilson, 1997). When the ascorbate supply is low under conditions this neuronal sensitivity is most apparent. Enthusiasm for ascorbate as an antioxidant therapeutic approach has been explained by the involvement of reactive oxygen species in neurodegenerative disorders. It is complicated interactions with neurotransmitter systems as described above make it difficult to discern the specific mechanisms involved.

3.2.1.3.3 Daily requirements

The North American Dietary Reference Intake recommends 90-2000 milligrams daily requirements for healthy people (USRDA, 2000). Some other species that are unable to produce vitamin C require exogenous consumption of 20 to 80 times of this reference intake (Milton, 2003; Pauling, 1970). There is ongoing debate within the scientific community over the best dose schedule of vitamin C for sustaining ideal health in humans. It is usually established that a balanced diet without supplementation covers adequate vitamin C to check scurvy in an normal healthy adult but for those who are pregnant, under stress or smoke tobacco require slightly more (USRDA, 2000).

Table: 3.2.3 RDA of vitamin C in US (USRDA, 2000)

RDA for adult male	90 mg per day
RDA for adult female	75 mg per day
Tolerable UIL for adult male	2,000 mg per day
Tolerable UIL for adult female)	2,000 mg per day

Recommendations for vitamin C intake have been set by various national agencies:

- 40 milligrams per day: the United Kingdom's Food Standards Agency (COMA, 1991)
- 45 milligrams per day: the World Health Organization (WHO, 2004)
- 60 mg/day: Health Canada (2007)
- 60–95 milligrams per day: United States' National Academy of Sciences (USRDA, 2000).

3.2.2 Determination of serum vitamin A, E and C level

3.2.2.1 Simultaneous determination of serum vitamin A and E

Serum concentration of vitamin A and E were determined by a modified method as described by Bieri et al., (1979) using RP-HPLC method with UV detection.

3.2.2.1.1 Chemicals/reagents and instruments/glass wares

Table: 3.2.4 Chemicals and reagents for determination of serum vitamin A and E

Sl.	Chemicals and regents	Manufacturer
1.	Reference standard of vitamin A	Sigma Aldrich, USA
2.	Reference standard of vitamin E	Sigma Aldrich, USA
3.	Internal standard tocopherol acetate	Sigma Aldrich, USA
4.	HPLC grade methanol	Fisher Scientific, UK
5.	HPLC grade ethanol	Fisher Scientific, UK
6.	HPLC grade water	Fisher Scientific, UK

Table: 3.2.5 Instruments and glass wares for determination of serum vitamin A and E

Sl.	Instruments and glass wares	Manufacturer
1.	High Performance Liquid Chromatography (HPLC)	Shimadzu, Kyoto, Japan
2.	Spectrophotometric Detector (SPD-10Avp UV-VIS)	Shimadzu, Kyoto, Japan
3.	Column (Nucleosil C_{18}; 5 µ,4.6×250 mm)	Varian, CA, USA
4.	Micropipette (Pipetman)	Gilson, France
5.	Microcentrifuge machine (MIKRO 20)	Hettichi, Germany
6.	Centrifuge machine	Digi system, Taiwan
7.	Freeze (-80 ^{0}C)	Siemens, Germany
8.	Vortex mixer machine (Rotamixer-9590)	Hook & Tucker, England
9.	Eppendorf tube (1.5 mL)	Hamburg, Germany
10.	Pipette tips	ALA, NY, USA

3.2.2.1.2 Preparation of standard vitamin A

Vitamin A stock solution was prepared by dissolving appropriate amount of retinol in HPLC grade ethanol to have a concentration 10 µmol/L. The stock solution was further diluted with ethanol to prepare the solutions in the concentration range of 0.5, 0.1, 2.0, 3.0 and 4.0 µmol/L. Then these standards were analyzed by the validated HPLC method to construct the calibration curve.

3.2.2.1.3 Preparation of standard vitamin E

Stock solution of vitamin E was prepared by dissolving appropriate amount of α-tocopherol in ethanol to have a concentration 100.0 µmol/L. The stock solution was further diluted with ethanol to prepare the solutions in the concentration range of 10.0, 20.0, 30.0, 40.0 and 50.0 µmol/L. Then these standards were analyzed by the validated HPLC method to construct the calibration curve.

Table: 3.2.6 Extinction coefficient and wavelength of the standard

Standard	Extinction coefficient	Wavelength (nm)
α-tocopherol	75.8	292
Tocopheryl acetate	43.6	285
Trans-retinol	1780	325
Retinyl acetate	1510	328

3.2.2.1.4 Determination of retention time

To determine the retention times for all trans-retinol, retinyl acetate, α-tocopherol and tocopheryl acetate, the HPLC instrument was profiled as

Solvent flow rate : 1 mL/min
Elution monitored at : 291 nm
Detector set at : 1 attenuation

A 50µl of α-tocopherol (200 µg/dL) was injected into the HPLC instrument and retention time was recorded in chromatograph. Similarly, 50 µL of each of tocopheryl acetate (2 µg/dL), trans-retinol (20 µg/dL) and retinyl acetate (40 µg/dL) solutions were injected separately and their retention time was recorded. Finally, 50 µL of a mixture of all α-tocopherol, tocopheryl acetate, trans-retinol and retinyl acetate at a ratio of 1:1:1:1 was injected and the retention time was recorded.

3.2.2.1.5 Preparation of serum samples

The vitamins were extracted from the serum by liquid-liquid extraction method. 200 µL of serum was taken in an eppendorf and mixed with 250 µL of ethanol and 10µl internal standard (10 µg/ mL of tocopherol acetate). After brief mixing with the help of vortex mixer (15 seconds), 540 µL of hexane was added to it to extract the vitamins in the organic phase. Then it was vortexed for 5 min and then centrifuged at 12000 rpm for 10 min and hexane layer was collected. 20 µL of the supernatant was injected into HPLC. The concentrations of the vitamins were calculated against the calibration curve.

3.2.2.1.6 Instrumentation and chromatographic conditions

Shimadzu (Kyoto, Japan) HPLC system was used in quantification of serum retinol and α-tocopherol, which consisting of a SCL-10Avp system controller, two LC-8A pumps. The serum retinol and tocopherol data were acquired and processed using LC solution (Version 1.03 SP3, Shimadzu Corporation, Kyoto, Japan) software running under Windows XP on a Pentium PC. Ultraviolet detection was achieved with a SPD-10Avp UV-VIS detector (Shimadzu Corporation, Kyoto, Japan).

Mobile phase	Methanol and water (90:10)
Chromatographic condition	
Column	Nucleosil C_{18}; 5 µ , 4.6 x 250 mm
Temperature	Ambient
Flow rate	1.0 mL/min
Injection volume	20 µl
Detector	UV, 292 nm for both retinol and α-tocopherol (λ_{max})
Sensitivity	0.0005
Analysis time	15 min

3.2.2.2 Determination of serum vitamin C

Serum Vitamin C was measured by phenyl-hydrazine spectrophotometry method (Lowry et al., 1945).

3.2.2.2.1 Preparation of working solutions

Trichloroacetic acid solution	50 g of trichloroacetic acid dissolved in 1000 mL distilled water (5% TCA solution)
Acetic acid solution	20 mL of pure acetic acid diluted to 200 mL with distilled water (10% acetic acid solution)
Copper sulphate solution	0.6 g of copper sulphate dissolved in 100 mL distilled water (0.6% copper sulphate solution)
Sulphuric acid solution	68.5 mL of 95% sulphuric acid diluted to 100 mL with distilled water in an ice cold water bath (65% $H2SO_4$ solution and 25 mL diluted to 100 mL as above (9N H_2SO4 solution)
Dinitro phenyl hydrazine solution	2.2 g of 2,4-dinitro phenyl hydrazine dissolved in 100 mL 9N sulphuric acid solution (2.2% DPH solution)
Metaphosphoric acid solution	5 g anhydrous metaphosphoric acid dissolved in 100 mL acetic acid solution (5% metaphosphoric acid solution) and flittered before use
Thiourea solution	5 g thiourea dissolved in 100 mL distilled water (5% thiourea solution) and stored at 4-10 ºC
DTC solution	100 mL of 2, 4-dinitro phenyl hydrazine, 5 mL of thiourea and 5 mL of copper sulphate solutions were mixed at ratio of 20:11 for the preparation of 110 mL DTC solution. It was stored at 4-10 ºC and filtered before use

3.2.2.2.2 Preparation of standard solution

Standard ascorbic acid (100 mg) was heated at 105 °C for 60 min and stored in a desiccator for 16 h. Then 50 mg ascorbic acid was dissolved in 100 mL metaphosphoric acid solution (50 mg/dL) which was stored at 4 ^{o}C. The stock solution was further diluted with TCA or metaphosphoric acid solution (100 µg/mL) to prepare the solutions in the concentration range of 0.0, 5.0, 10.0, 15.0 and 20.0 µg/mL.

3.2.2.2.3 Procedure for serum analysis

A volume of 300 µL serum and 1.2 mL TCA solution was taken in a test tube, mixed well and centrifuged at 3000 rpm for 10 min. Clear supernatant of 960 µL was treated with 400 µL DTC solution and heated at 60 °C for 60 min in a water bath. Immediately after incubation, the sample was chilled in ice-cold water and 1.6 mL of 65% sulphuric acid solution was addedgradually. The procedure was repeated with 300 µL of working standard solution of ascorbic acid as well as with 300 µL of reagent blank. Absorbance of sample and standard were read against reagent blank at 520 nm in the spectrophotometer (UV-1201, UV-VIS, Spectrophotometer, Shimadzu Corporation, Japan).

3.2.3 Results

3.2.3.1 Serum antioxidant-vitamin levels

3.2.3.1.1 Serum level of vitamin A

3.2.3.1.1.1 Construction of standard curve of vitamin A

Calibration curve for vitamin A was found to be linear over the concentration range of 0.5 to 4 µmol/L (R^2 = 0.9932). Figure 3.2.4 represents the calibration curve of vitamin A.

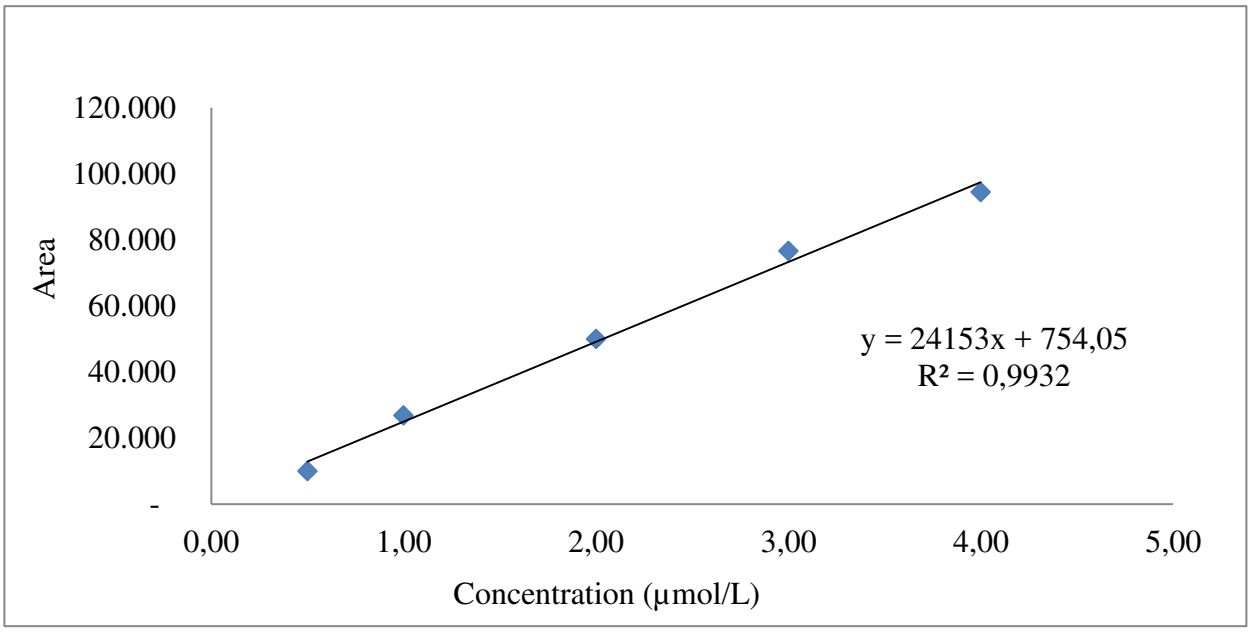

Figure 3.2.4 Standard curve of vitamin A

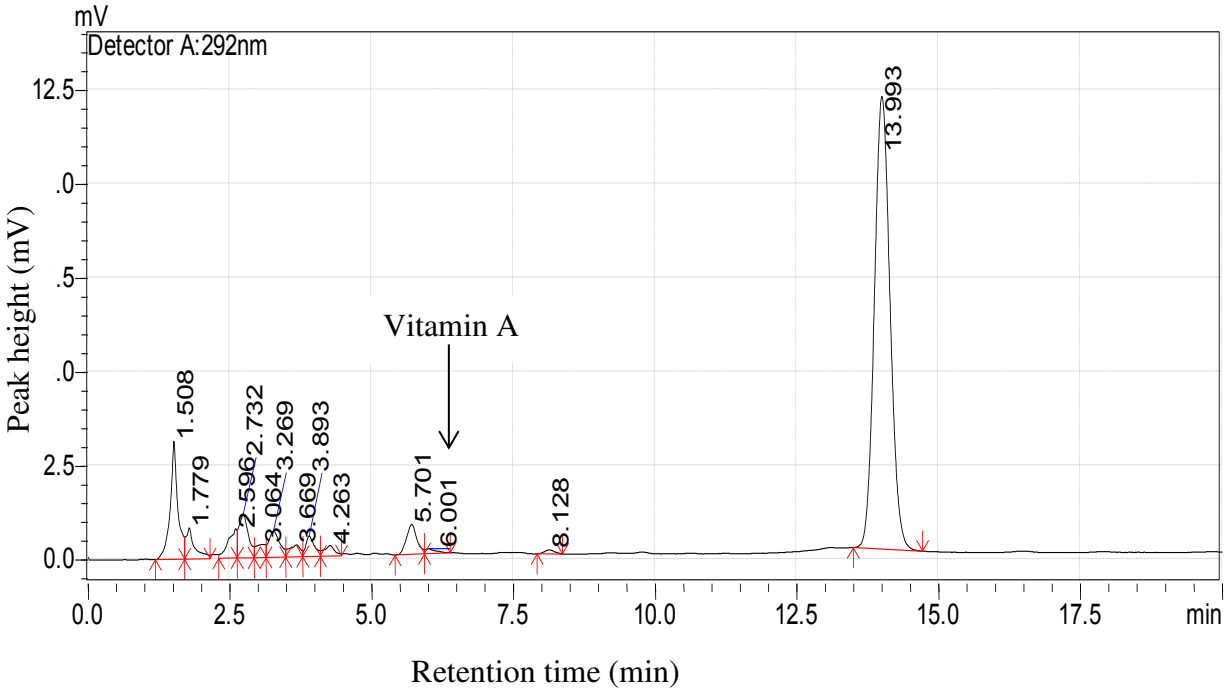

Figure: 3.2.5 HPLC chromatogram of standard (Std-2) vitamin A (retention time 5.701 minute). Condition: methanol: water, 90:10; flow rate, 1.0 mL/min, UV detector with 292 nm.

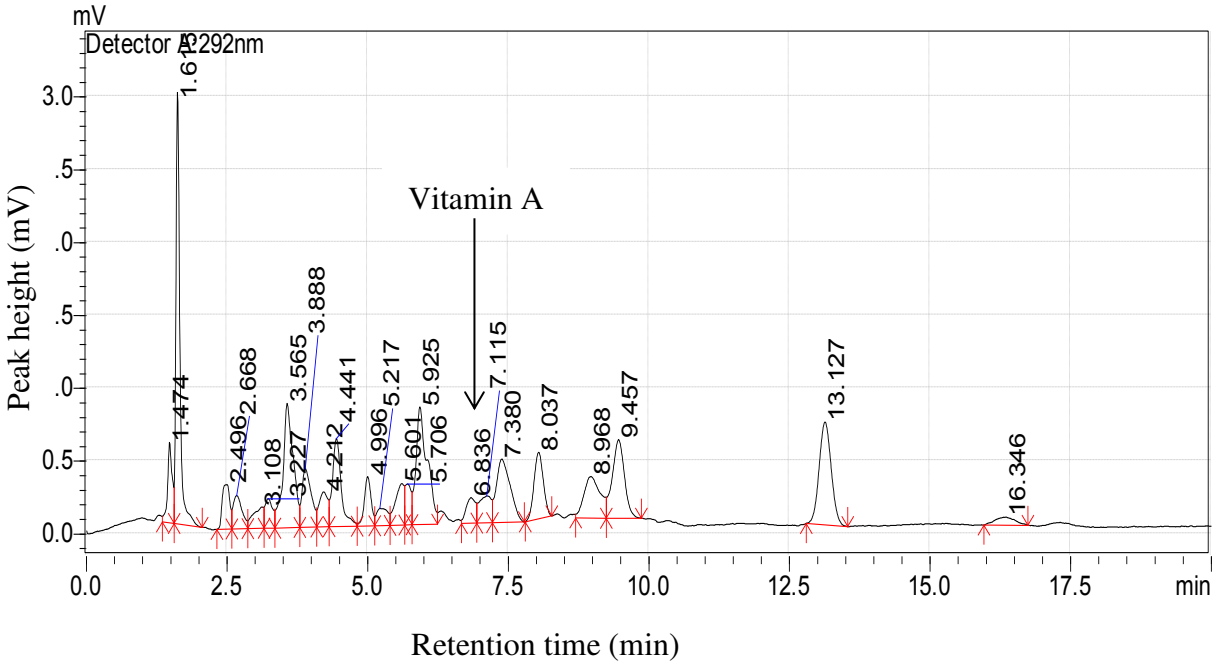

Figure: 3.2.6 HPLC chromatogram of serum (C-176) containing vitamin A (retention time 5.706 minute). Condition: methanol: water, 90:10; flow rate, 1.0 mL/min, UV detector with 292 nm.

3.2.3.1.2 Serum level of vitamin E

3.2.3.1.2.1 Construction of standard curve of vitamin E

Calibration curve for vitamin E was found to be linear over the concentration range of 10 to 50 µmol/L (R^2 = 0.9962). Figure 3.2.5 represents the calibration curve of vitamin E

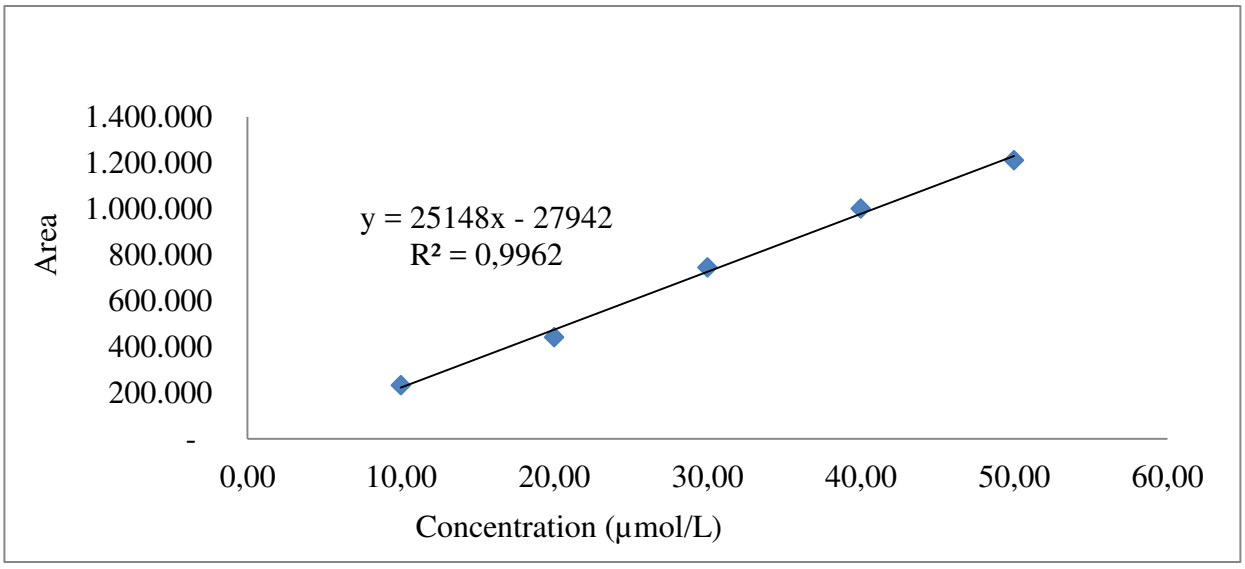

Figure: 3.2.7 Standard curve of vitamin E

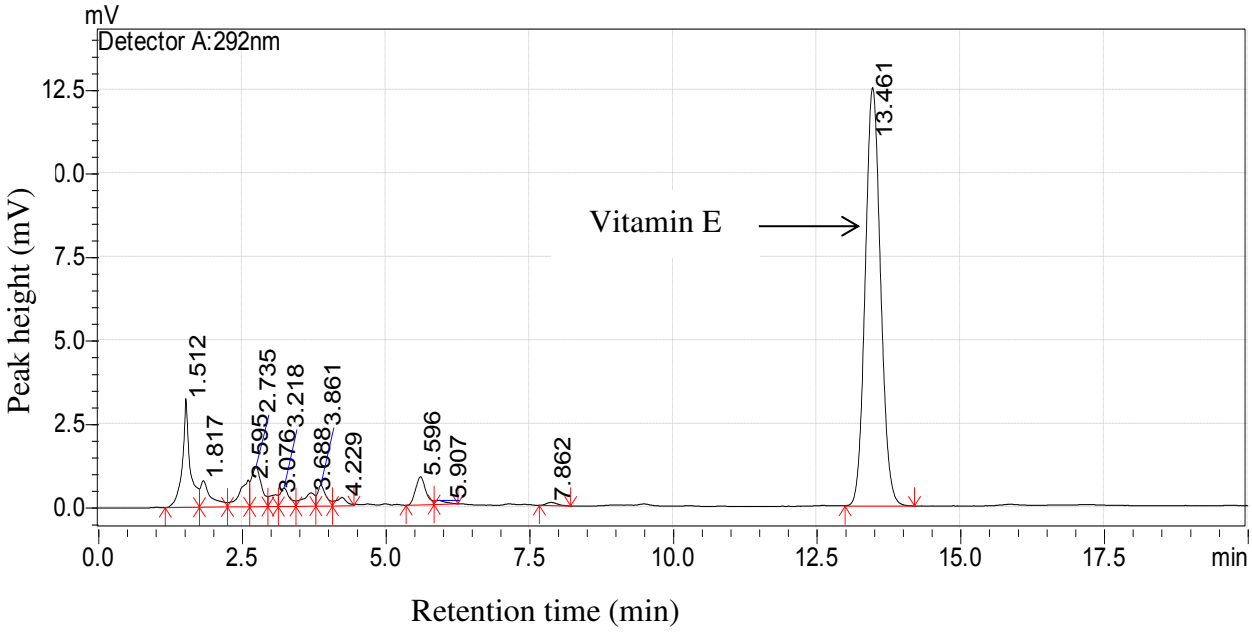

Figure: 3.2.8 HPLC chromatogram of standard (Std-1) vitamin E (retention time 13.461 minute). Condition: methanol: water, 90:10; flow rate, 1.0 mL/min, UV detector with 292 nm.

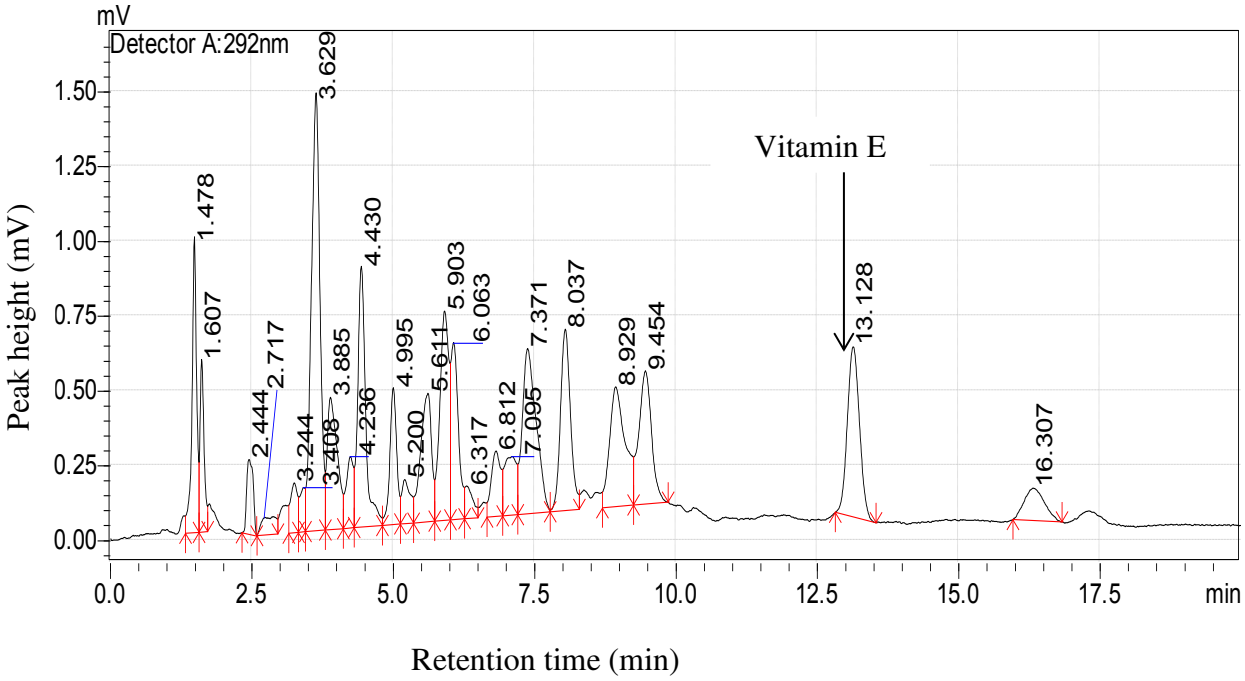

Figure: 3.2.9 HPLC chromatogram of serum (C-155) containing vitamin E (retention time 13.128 minute). Condition: methanol: water, 90:10; flow rate, 1.0 mL/min, UV detector with 292 nm.

3.2.3.1.3 Serum level of vitamin C

3.2.3.1.3.1 Construction of standard curve of vitamin C

Calibration curve for vitamin E was found to be linear over the concentration range of 0 to 25.0 µmol/L (R^2 = 0.9868). Figure 3.2.6 represents the calibration curve of vitamin C.

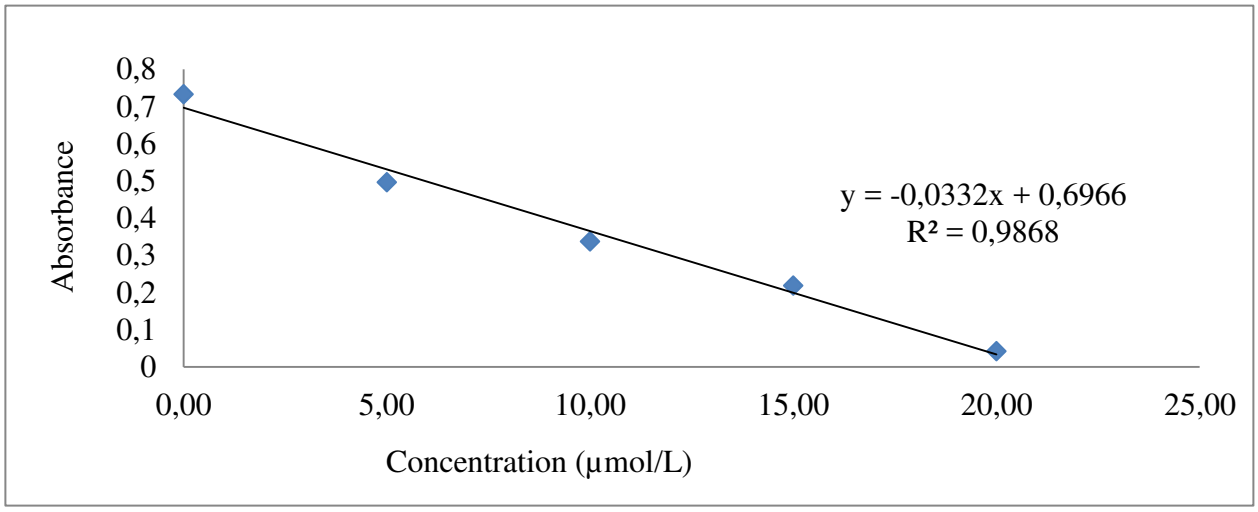

Figure: 3.2.10 Standard curve of vitamin C

3.2.3.2 Comparison of serum antioxidant levels in patients and control subjects

The mean serum concentrations of vitamin A, E, and C were analyzed in MDD patients and in normal control subjects.

Table: 3.2.7 Mean serum vitamin A, E & E levels in patients and control subjects

groups	Vitamin A (µmol/L)	Vitamin E (µmol/L)	Vitamin C (µmol/L)
MDD (*n*=247)	2.05±1.16	12.89±6.30	34.17±17.27
Control (*n*=248)	2.33±0.92	16.98±6.21	37.88±13.97
p value	*p*=0.003	*p*<0.001	*p*=0.009

The mean serum concentrations of vitamin A, E, and C were 2.05±1.16 (µmol/L), 12.89±6.30 (µmol/L) and 34.17±17.27 (µmol/L) for MDD group and 2.33±0.92 (µmol/L), 16.98±6.21 (µmol/L) and 37.88±13.97 (µmol/L) for healthy control group respectively. Sample t-test was used for statistical analysis. There was a statistically significant difference of antioxidant vitamins between the groups. At 5% level of significance ($p<0.05$) it had been found that the MDD patients had significantly low level of antioxidant vitamins like vitamin A ($p=0.003$), vitamin E ($p<0.001$) and vitamin C ($p=0.009$).

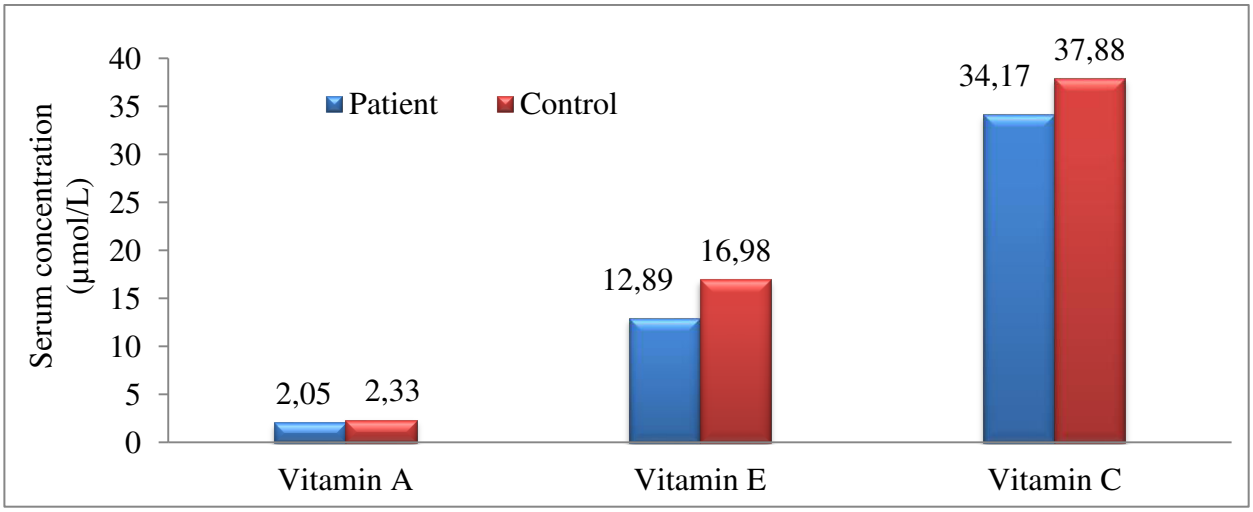

Figure: 3.2.11 Comparison of mean serum antioxidants in patients and control subjects

Table: 3.2.8 Pearson correlation of serum vitamin A level with vitamin E and C

		Vitamin A	Vitamin E	Vitamin C
Vitamin A	Pearson correlation	1	.926**	-.052
	Sig. (2-tailed)		p<0.001	.412
Vitamin E	Pearson correlation	.926**	1	-.073
	Sig. (2-tailed)	p<0.001		.251
Vitamin C	Pearson correlation	-.052	-.073	1
	Sig. (2-tailed)	.412	.251	

**. Correlation is significant at the 0.01 level (2-tailed).

3.2.4 Discussion on antioxidant vitamin

Antioxidant vitamins play an important role in the physiological process including neuroprotection, oxidative free radical production and immune-modulatory functions. Neurodegeneration processes are associated with oxidative stress and many studies suggest that some neurological disorders due to oxidative stress may be prevented or cured by antioxidant vitamin therapy (Rosário et al., 2016). Vitamins C and E provide synergistic neuroprotection in the jejunum (Tashima et al., 2015). Our present study shows that mean serum concentrations of vitamin A, E, and C were 2.05±1.16 (µmol/L), 12.89±6.30 (µmol/L) and 34.17±17.27 (µmol/L) for MDD group and 2.33±0.92 (µmol/L), 16.98±6.21 (µmol/L) and 37.88±13.97 (µmol/L) for healthy control group respectively. Similar to our study, serum levels of vitamin A, E and C were significantly low compared to healthy controls which have been found in various psychiatric disorders such as schizophrenia, PD, depression, anxiety disorders (Bremner and McCaffery, 2008; Islam et el., 2014; Kuloglu et al., 2002). Total antioxidant levels are significantly lower in GAD patients than healthy controls (Emhan et al., 2015). Another study reveals that PD patients have considerably lower level of antioxidant vitamins than the healthy control subjects (Nahar et al., 2013). Thus significant change in serum antioxidant vitamins level in MDD patients may contribute to the pathogenesis of disease and our findings may play a key role in the diagnosis and treatment of MDD patients. These findings will be established by further large scale study in this field.

3.2.5 Conclusion

This study revealed that serum levels of vitamin A, E and C decreased significantly in MDD patients compared to the control subjects (p=0.003, p<0.001 and p=0.009 respectively). These findings correlate the study findings of other psychiatric disorders. So the antioxidant enzymes and oxidative stress might have a pathological role in MDD patients. Thus these findings may play a key role in the diagnosis and treatment of MDD patients. Pearson correlation coefficient suggested that there was a significant positive correlation between vitamin A (Retinol) and vitamin E (α-Tocopherol). Same analysis reveals that none of the antioxidant vitamins in serum level have significant correlation with age, BMI, income, education and smoking habit of MDD patients.

3.3 Determination of serum immunoglobulins

3.3.1 Immunoglobulins

Plasma cells produce immunoglobulins in response to an immunogen. Immunoglobulins are generally termed as glycoprotein molecules which function as antibodies. The name of immunoglobulin is derived from the finding that when antibody comprising serum is placed in an electrical field they transfer with globular proteins.

3.3.1.1 Basic structure of immunoglobulins

In figure 3.3.1 the basic structure of the immunoglobulins is demonstrated. They all are built from the same basic units although different immunoglobulins can vary structurally.

A. Heavy and light chains

The basic unit of all immunoglobulins has a four chain structure. They are composed of two identical heavy chains (50-70 kD) and two identical light chains (23 kD).

B. Disulfide bonds

1. **Inter-chain disulfide bonds** - The light and heavy chains and the two heavy chains are held together by non-covalent interactions and inter-chain disulfide bonds. The number of inter-chain disulfide bonds differs among different immunoglobulin molecules.

2. **Intra-chain disulfide binds** - There are also intra-chain disulfide bonds within each of the polypeptide chains.

C. Variable and constant regions

When the amino acid arrangements of many distinct heavy chains and light chains were compared, it became clear that both the heavy and light chain might be separated into two regions based on variability in the amino acid arrangements. These are the:

1. Light chain - VL (110 amino acids) and CL (110 amino acids)

2. Heavy chain - VH (110 amino acids) and CH (330-440 amino acids)

D. Hinge region

The arms of the antibody molecule form a Y at this region. It is named the hinge region as there is some flexibility in the molecule at this point.

E. Domains

Three dimensional images of the immunoglobulin molecule show that it is folded into globular regions each of which comprises an intra-chain disulfide bond. These regions are called domains.

1. Light chain domains - VL and CL
2. Heavy chain domains - VH, CH_1 - CH_3 (or CH_4)

F. Oligosaccharides

In most immunoglobulins carbohydrates are attached to the CH_2 domain. However, in some cases carbohydrates may also be attached at other positions.

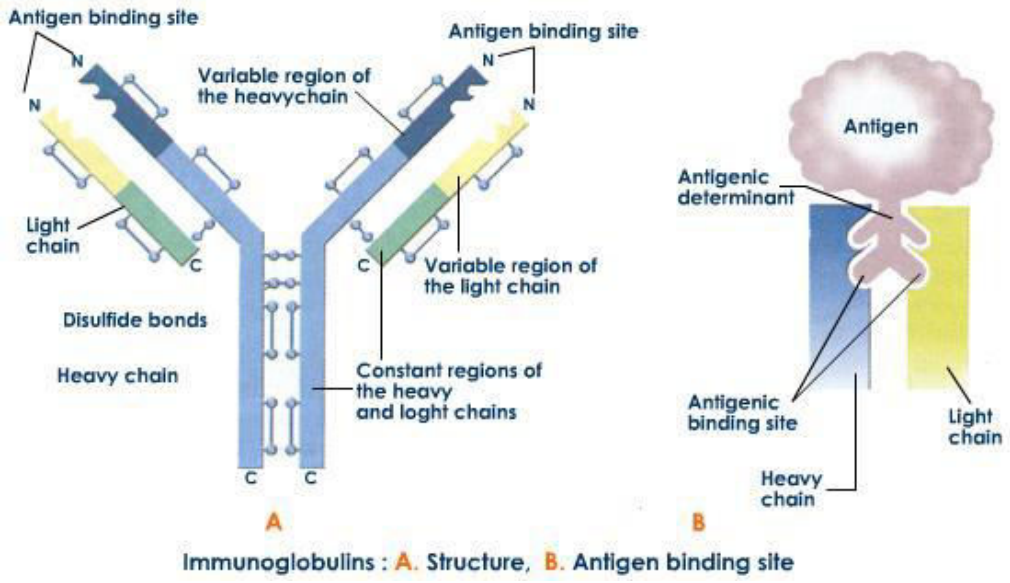

Figure: 3.3.1 General structure of immunoglobulin and antigen binding cleft

3.3.1.2 Classification and distribution of immunoglobulins

Immunoglobulin molecules consist of two kinds of polypeptide chains, heavy chains (H-chains) and light chains (L-chains). Based on differences in the amino acid sequences in the constant region of the heavy chains, there are five major types of immunoglobulins. Very similar heavy chain constant regions are common in all immunoglobulins within a given. By sequence studies or serological means (i.e. by the use of antibodies directed to these differences), these differences can be detected.

I) Immunoglobulin A (IgA) - Alpha heavy chains (IgA)
II) Immunoglobulin D (IgD) - Delta heavy chains (IgD)
III) Immunoglobulin E (IgE) - Epsilon heavy chains (IgE)
IV) Immunoglobulin G (IgG) -Gamma heavy chains (IgG) and
V) Immunoglobulin M (IgM) - Mu heavy chains (IgM)

3.3.1.3 Immunoglobulin subclasses

The classes of immunoglobulins can be divided into subclasses based on minor changes in the amino acid arrangements in the constant region of the heavy chains. All immunoglobulins within a subclass will have very similar heavy chain constant region amino acid sequences and these changes can only be detected by serological means (Janeway et al., 2001).

1. IgG subclasses

a) IgG1 - Gamma 1 heavy chains
b) IgG2 - Gamma 2 heavy chains
c) IgG3 - Gamma 3 heavy chains
d) IgG4 - Gamma 4 heavy chains

2. IgA subclasses

a) IgA1 - Alpha 1 heavy chains
b) IgA2 - Alpha 2 heavy chains

Based on the type of light chain immunoglobulins can also be divided. Light chain types are based on changes in the amino acid arrangement in the constant region of the light chain. By serological means these differences can also be detected.

1. Kappa light chains
2. Lambda light chains

3.3.1.4 Immunoglobulin subtypes

The light chain can also be divided into subtype based on the differences in the amino acid sequences in the constant region of the light chain.

1. Lambda subtype
    a. Lambda 1
    b. Lambda 2
    c. Lambda 3
    d. Lambda 4

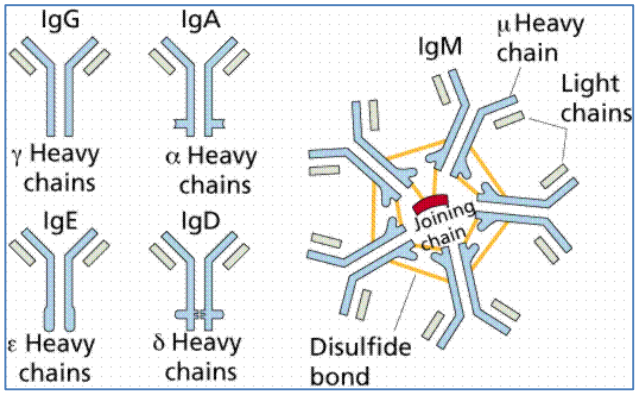

Figure: 3.3.2 General structures of the five major classes of secreted antibody (Liu and May, 2012)

Immunoglobulin A (IgA)

Structure - Serum IgA is a monomer but the secretory IgA is a dimer as presented in figure 3.3.3. When IgA exits as a dimer it contains J chain with it. When IgA is found in secretions it contains another protein with it which is called secretory piece or T piece; IgA is sometimes referred to as 11S immunoglobulin. Unlike the remainder of the IgA, which is made in the plasma cell, the secretory piece is made in epithelial cells and is added to the IgA as it passes into the secretions. The secretory piece helps IgA to be transported across mucosa and also protects it from degradation in the secretions.

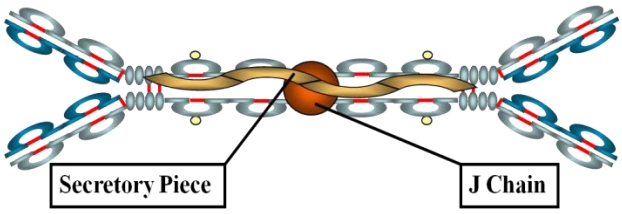

Figure: 3.3.3 Structures of immunoglobulin A (IgA)

Properties

a) IgA is the 2nd most common serum Ig.

b) IgA is the major class of Ig in secretions - tears, saliva, colostrum, mucus. Since it is found in secretions secretory IgA is important in local (mucosal) immunity.

c) Normally IgA does not fix complement, unless aggregated.

d) IgA can bind to some cells - PMN's and some lymphocytes

Immunoglobulin G (IgG)

Structure - The structures of the IgG subclasses are presented in figure 3.3.4. All IgG's are monomers (7'S immunoglobulin). The subclasses differ in the number of disulfide bonds and length of the hinge region.

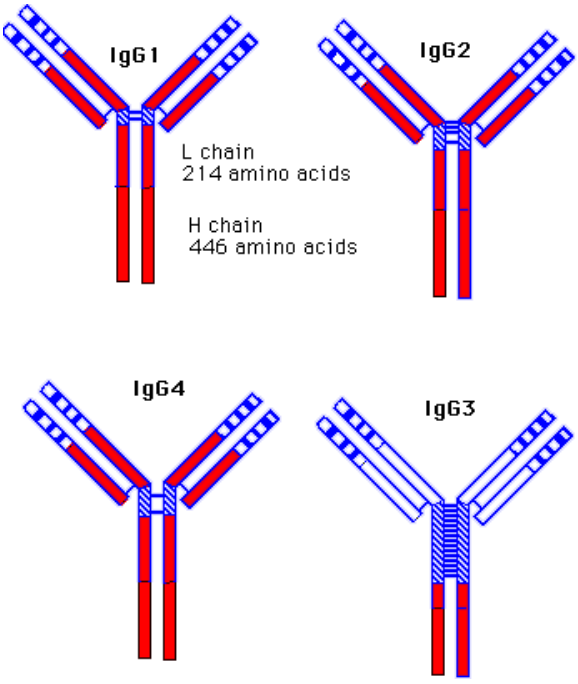

Figure: 3.3.4 Structures of immunoglobulin G (IgG)

Properties–IgG is the most useful immunoglobulin because it is capable of carrying out all of the functions of immunoglobulin molecules.

a) IgG is the major Ig in serum -75% of serum Ig is IgG

b) IgG is the major Ig in extra vascular spaces

c) Placental transfer - IgG is the only class of Ig that crosses the placenta. Transfer is mediated by receptor on placental cells. Not all subclasses can cross equally; IgG2 do not cross well.

d) Fixes complement - Not all subclasses fix equally well; IgG4 does not fix complement.

Immunoglobulin M (IgM)

Structure - The structure of IgM is presented in figure 3.3.5. IgM normally exists as a pentamer (19S immunoglobulin) but it can also exist as a monomer. In the pentameric form all heavy chains are identical and all light chains are identical. Thus, the valence is theoretically 10. IgM has an extra domain on the mu chain (CH_4) and it has another protein covalently bound via S-S bond called the J chain. This chain functions in polymerization of the molecule into a pentamer.

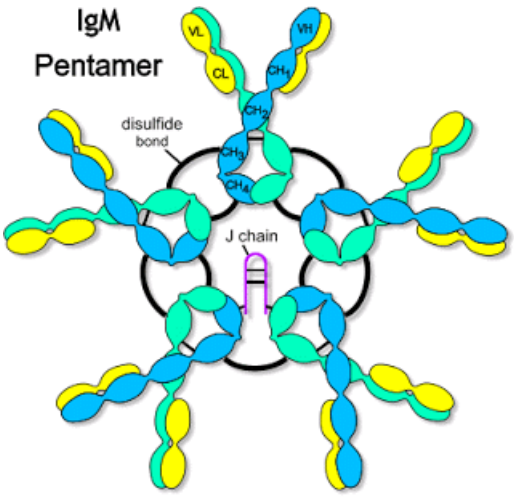

Figure: 3.3.5 Structures of immunoglobulin M (IgM)

Properties

a) IgM is the 3rd most common serum Ig.

b) IgM is the first Ig to be made by the fetus and the first Ig to be made by a virgin B cells when it is stimulated by antigen.

c) IgM is a good complement fixing Ig due to the consequence of its pentameric structure. Thus, IgM antibodies are very efficient in leading to the lysis of microorganisms.
d) IgM is also a good agglutinating Ig as a consequence of its structure. Thus, IgM antibodies are very good in clumping microorganisms for eventual elimination from the body.
e) IgM binds to some cells via Fc receptors.
f) Surface IgM exists as a monomer and lacks J chain but it has extra 20 amino acids at the C-terminal end to anchor it into the membrane

3.3.1.5 Functions of immunoglobulins

A. Antigen binding

Immunoglobulins bind precisely to one or a few closely related antigens. Each immunoglobulin actually binds to a specific antigenic factor. Antigen binding by antibodies is the key function of antibodies and can result in defense of the host. The number of antigenic factors that an individual antibody molecule can bind depends on the valence of antibody. The valence of all antibodies is at least two and in some cases more.

B. Effector functions

Antigen- antibody binding generally has no direct biological effect but the consequence of secondary "effector functions" of antibodies is significant. The immunoglobulins have a variety of these effector functions. A particular effector function of immunoglobulins requires the capacity of antibody bind to its antigen. Not every immunoglobulin will mediate all effector functions. Such effector functions include:

1. Fixation of complement - Biologically active molecules can be released by cell lysis.

2. Binding to various cell types - Immunoglobulins bind with receptors of phagocytic cells, lymphocytes, platelets, mast cells, and basophils. Some cell functions can be activated by this binding. Transfer of the immunoglobulin across the placenta is completed by binding with receptors on placental trophoblasts. Transfer maternal antibodies provide immunity to the fetus and newborn by this process.

3.3.2 Determination of serum immunoglobulins

3.3.2.1 Principle of the assay

The serum immunoglobulins were estimated by using a quantitative turbidimetric method. In this method samples containing IgA, IgG, IgM are mixed with the activation buffer and then with anti-human immunoglobulin reagent. These antibodies form insoluble complexes. These complexes cause an absorbance change, depended upon the immunoglobulin concentration of the patient sample that can be quantified by comparison from a calibrator of known immunoglobulin concentration (Dati et al., 1996; Hossain et al., 2007).

3.3.2.2 Materials and reagents

Material	Source
General protein calibrator, size: 1x2 ml	Chronolab, Switzerland
Anti-IgA	Chronolab, Switzerland
Anti-IgM	Chronolab, Switzerland
Anti-IgG	Chronolab, Switzerland
Saline water, sterile, non-pyrogenic	Opso saline, Bangladesh
Multiple well plate 96-well, flat bottom with lid, sterile (1.0x0.6 cm appox)	SARSTEDT, U.S.A
iMark microplate absorbance reader	BIO-RAD, Japan
Transferpette- 8 Dig. (0.5-10) µL, (5-50) µL	BRAND GMBH + CO KG, Germany

Reagents

Diluent (R1): Tris buffer 20 mmol/L, PEG 8000, pH 8.2; sodium azide 0.95 g/L

Antibody (R2): Goat serum, anti-human IgA, pH 7.5; sodium azide 0.95 g/L

3.3.2.3 Procedure

1. At first serum samples were centrifuged for 20 min at 3000 rpm.
2. The 20 µL of centrifuged serum was pipetted out. Then 80µl of normal saline (0.9% NaCl) was added to make the final volume 100 µL.
3. 10 µL of serum (diluted) was pipetted into microtitre plate according to plate design. Three separate plates were used for three immunoglobulins (IgA, IgG and IgM).
4. Then 150 µL of Tris Buffer was added to each of the serum-containing hole of the plates with the help of multichannel micropipette.
5. 0, 5, l0, 25, 50 and 75 µL of calibrator protein was also pipette into the microtitre plates.
6. After mixing well with the help of vortex mixer, the initial absorbance (A1) was taken.
7. Then 15µL of anti-Human immunoglobulin IgG, IgM and IgA were added to the each of the serum samples including the calibrator proteins and mixed briefly by vortex mixer.
8. Incubation period is 5 min for the reaction of antihuman immunoglobulin with the test serum and calibrator protein. After proper mixing, absorbance (A2) was taken at 630 nm for IgG and IgA and at 405 nm for IgM. Difference between the two absorbance (ΔA = A2 -A1) was used for calculation. The concentration of immunoglobulins was calculated against the calibration curves of each individual immunoglobulin.

Figure: 3.3.6 BIO-RAD iMark microplate absorbance reader

3.3.2.4 Design of microtitre plate

The microtitre plate has been designed for IgA, IgG and IgM separately in the following way for samples of both 247 patients and 248 controls subjects.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
B	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
C	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36
D	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	S47	S48
E	S49	S50	S51	S52	S53	S54	S55	S56	S57	S58	S59	S60
F	Std1	Std2	Std3	Std4	Std5	Std6	Std7	Blank	Blank	Blank	Blank	Blank
G	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
H	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24

Figure: 3.3.7 Separate microtitre plate has been designed for IgA, IgG and IgM

3.3.2.5 Concentration of calibrator standard

The parent calibration standard was used to prepare the calibrator standards of different concentrations mentioned in the table 3.3.1.

Table: 3.3.1 Concentration of general calibrator proteins

Concentration (mg/dL)		
IgA	**IgG**	**IgM**
0.0	0.0	0.0
31.7	46.5	12.6
63.4	93.0	25.2
158.5	232.5	63.0
317.0	464.9	189.0
634.0	697.4	252.0

3.3.3 Results

3.3.3.1 Serum level of IgA, IgG and IgM

3.3.3.2 Construction of calibration curve

Calibration curve for IgA was found to be linear over the concentration range of 0.0 to 634 mg/dL (R^2 = 0.8792). Figure 3.3.9 represents the calibration curve.

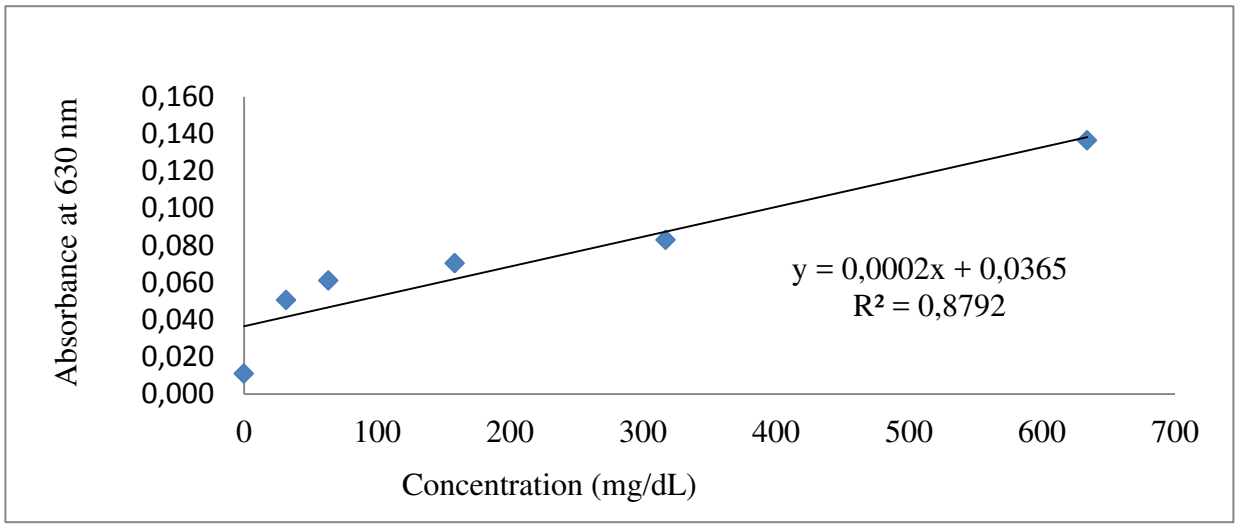

Figure 3.3.8 Standard curve of Immunoglobulin A (IgA)

Calibration curve for IgG was found to be linear over the concentration range of 0.0 to 697.3 mg/dL (R^2 = 0.9287). Figure 3.3.10 represents the calibration curve.

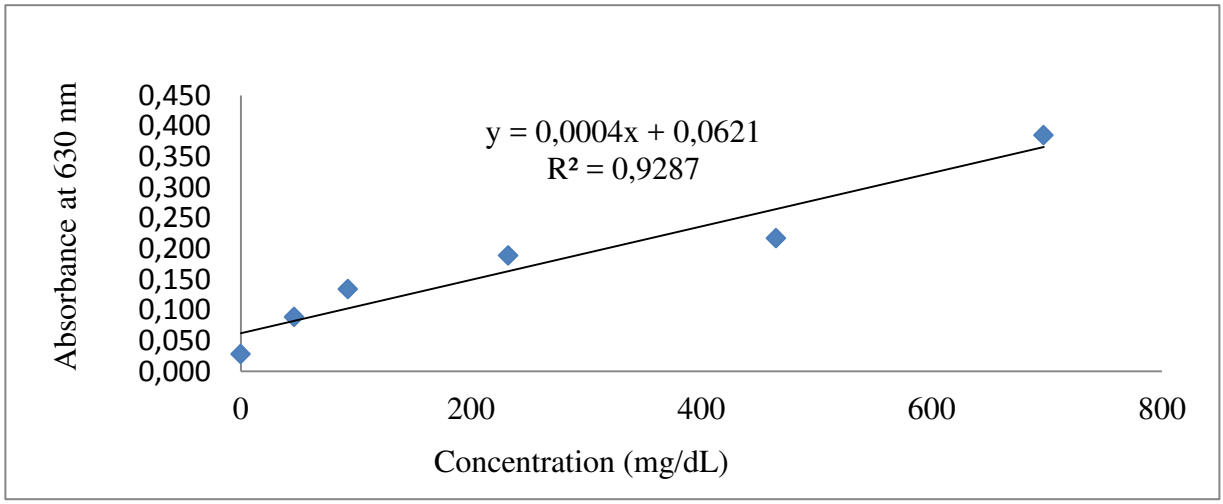

Figure 3.3.9 Standard curve of Immunoglobulin G (IgG)

Calibration curve for IgM was found to be linear over the concentration range of 0.0 to 252.0 mg/dl (R2 = 0.972). Figure 3.3.11 represents the calibration curve.

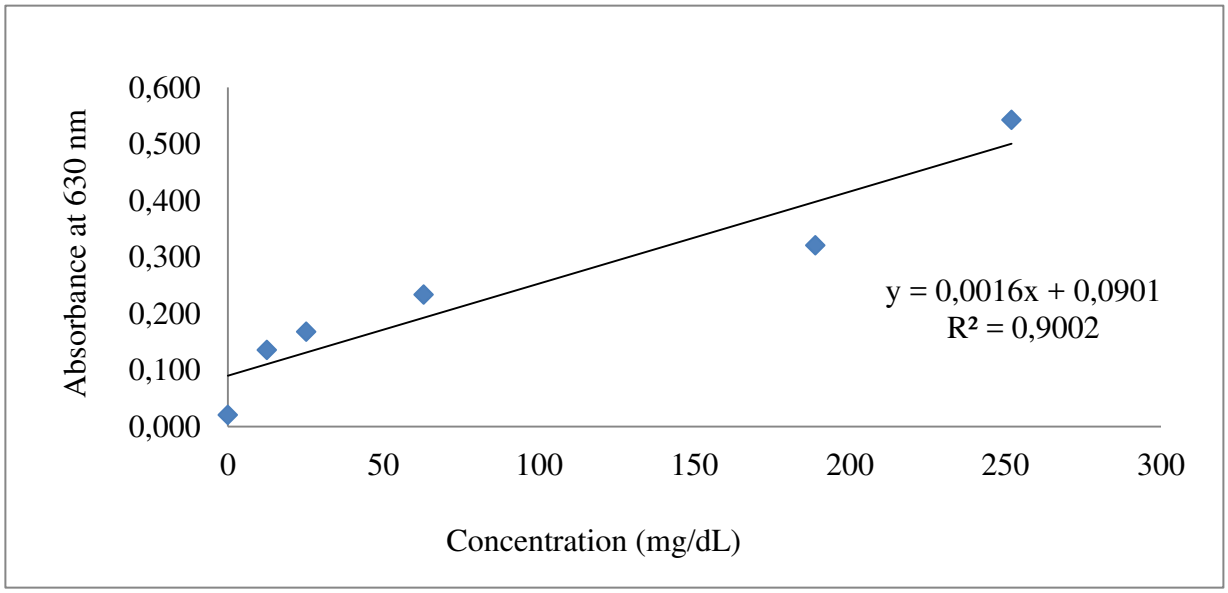

Figure 3.3.10 Standard curve of Immunoglobulin M (IgM)

3.3.3.3 Comparison of serum immunoglobulin levels in patients and control subjects

The mean serum concentrations of immunoglobulins (IgA, IgG and IgM) were analyzed in MDD patients and in normal control subjects.

IBM SPSS Statistics (Version 22, SPSS Inc. Chicago, USA) was used to analyze the immunoglobulins data. Descriptive statistics were used for all variables. Values were expressed as mean±SD. Comparisons of immunoglobulins for MDD patients to that of the healthy controls were performed by independent sample t-test and ANOVA analysis.

Table: 3.3.2 Serum immunoglobulin levels in patients and controls subjects

Groups	Immunoglobulin A (mg/dL)	Immunoglobulin G (mg/dL)	Immunoglobulin M (mg/dL)
MDD (*n*=247)	209.07±104.93	791.50±235.67	107.92±47.53
Control (*n*=248)	195.34±92.16	763.81±175.89	99.17±48.78
p value	0.404	0.407	0.293

3.3.4 Discussion on immunoglobulins

The mean serum immunoglobulin concentrations of IgA, IgG and IgM of MDD patients were found to be 209.07±104.93 (mg/dL), 791.50±235.67 (mg/dL), 107.92±47.53 (mg/dL) whereas the serum concentrations of IgA, IgG and IgM of control subjects were 195.34±92.16 (mg/dL), 763.81±175.89 (mg/dL), 99.17±48.78 (mg/dL) respectively. *p* value of serum IgA, IgG and IgM for the groups were found 0.404, 0.407 and 0.293 which indicate there were no significant difference of serum IgA, IgG and IgM between MDD patients and healthy control subjects. From this finding it reveals that there is a positive tendency to increase serum immunoglobulins in patient group compare to healthy control. Some earlier study results support this finding and some other not. There were no significant differences for the other immunoglobulins (IgG, IgA) among the groups studied (Legros et al., 1985). Research results propose that psychological stress is associated with the changed secretion of serum immunoglobulins, some acute phase proteins and complement factors (Maes et al., 1997). In another study serum levels of IgM, Ig G and Ig A were measured in psychiatric patients including bipolar depression, unipolar depression and schizophrenia and the comparative findings with healthy subjects reveal that a significantly high level of IgM concentration was found in all patient groups compared with the controls. Depressed patients had significantly higher percentages of circulating neutrophils, significantly lower percentages of circulating lymphocytes and significantly lower in vitro lymphocyte responses to mutagenic stimulation than normal controls (Kronfol and House, 1989).

3.3.5 Conclusion

There is a significant positive relationship between the stress-induced alterations of serum immunoglobulin A (IgA), immunoglobulin G (IgG) and immunoglobulin M (IgM) which is supported by many studies. The present study indicates that there were no significant difference of serum IgA, IgG and IgM levels between MDD patients and control subjects but a positive tendency to increase serum immunoglobulins in patient group compare to healthy control was observed. Thus, we recommend further research to explain the exact role of immunoglobulins in the pathogenesis of MDD.

3.4 Determination of serum lipid peroxidation (MDA)

3.4.1 Lipids for physiological functions

Lipids are classically divided into two groups- apolar and polar. Triglycerides (apolar), stored in various cells, but especially in adipose (fat) tissue, are usually the main form of energy storage in mammals (Frühbeck et al., 2001; Frayn, 1998). Polar lipids are structural components of cell membranes, where they participate in the formation of the permeability barrier of cells and subcellular organelles in the form of a lipid bilayer. Glycerol-based phospholipid is the major lipid type in almost all membranes (Vance E and Vance, 2002). Lipids may control the physiological state of a membrane organelle by modifying its biophysical aspects, such as the polarity and permeability. As signaling molecules lipids also play an important role in biology.

Lipids as signaling molecules: The main enzymes that generate lipid signaling mediators are lipoxygenase, which mediate hydroperoxyeicosatetraenoic acids (HPETEs), lipoxins, leukotrienes, or hepoxilins biosynthesis after oxidation of arachidonic acid (Massey and Nicolaou, 2011; Massey and Nicolaou, 2013), cyclooxygenase that produces prostaglandins [4], and cytochrome P-450 (CYP) which generates epoxyeicosatrienoic acids, leukotoxins, thromboxane, or prostacyclin (Massey and Nicolaou, 2011). G protein-coupled and nuclear receptors as well as some other receptors may help to activate lipid signaling process. Members of several different lipid categories have been identified as potent intracellular signal transduction molecules. Examples of signaling lipids include (i) phosphatidylinositol phosphates, diacylglycerol (DAG) and inositol phosphates (IPs). DAG is a physiological activator of protein kinase C (Giorgi et al., 2010; Jornayvaz and Shulman, 2012) and transcription factor called nuclear factor-kB (NF-kB), which promotes cell survival and proliferation. Diacylglycerol indirectly interacts with some other signaling molecules like small G proteins (Yang and Kazanietz, 2007). IPs are a highly charged family of lipid-derived metabolites, involved in signal transduction that results in activation of mTOR pathway which is an intracellular signaling pathway important in regulating the cell cycle (Baumann et al., 2013), and calcium-homeostasis (Conway and Miller, 2007; Fisher et al., 2002); (ii) sphingosine-1-phosphate, a sphingolipid derived from ceramide that is a potent messenger molecule involved in regulating calcium mobilization, migration, adhesion, and proliferation (Hannun and Obeid, 2008; Mattson, 2003; Takuwa et al., 2012) (iii) the prostaglandins, which are one type of fatty-acid derived eicosanoid involved in inflammation (Aoki T and Narumiya, 2012; Tang et al., 2012) and immunity

(Kalinski, 2012); (iv) phosphatidylserine, a phospholipid that have a vital role in a number of signaling pathways, includes kinases, small GTPases, and fusogenic proteins (Kay and Grinstein, 2013); (v) the steroid hormones such as estrogen, testosterone, and cortisol, which modulate a host of functions such as reproduction, metabolism, stress response, blood pressure, inflammation, and water and salt balance (Pluchino et al., 2013).

3.4.2 Lipids damage by free radicals

One of the consequences of uncontrolled oxidative stress (imbalance between the prooxidant and antioxidant levels in favor of prooxidants) is cells, tissues, and organs injury caused by oxidative damage. It has long been recognized that high levels of free radicals or Reactive Oxygen Species (ROS) can inflict direct damage to lipids. Mitochondria, plasma membrane, endoplasmic reticulum and peroxisomes are the primary sources of endogenous ROS production (Moldovan and Moldovan, 2004) through different mechanisms including enzymatic reactions or auto-oxidation of several compounds like catecholamine and hydroquinone. Different exogenous stimuli, such as the ionizing radiation, ultraviolet rays, tobacco smoke, pathogen infections, environmental toxins, and exposure to herbicide/insecticides, are sources of in vivo ROS production.

Hydroxyl radical (HO•) and hydroperoxyl (HO_2•) are the two most prevalent ROS that can affect the lipids profoundly. The hydroxyl radical (HO•) is a small, highly mobile, water-soluble, and chemically most reactive species of activated oxygen. This short-lived molecule can be produced from O_2 in cell metabolism and under a variety of stress conditions. A cell produces around 50 hydroxyl radicals every second. Each cell would generates 4 million hydroxyl radicals per day which can be neutralized or attack biomolecules (Lane, 2002). Hydroxyl radicals cause oxidative damage to cells because they unspecifically attack biomolecules (Halliwell and Gutteridge, 1984) located less than a few nanometres from its site of generation and are involved in cellular disorders such as neurodegeneration (Venero et al., 2003; Castellani et al., 2004), cardiovascular disease (Lipinski and Pretorius, 2012), and cancer (Dizdaroglu and Jaruga, 2012; Kanno et al., 2012). It is generally assumed that in biological systems is formed through redox cycling by Fenton reaction, where free iron (Fe^{2+}) reacts with hydrogen peroxide (H_2O_2) and the Haber-Weiss reaction that result in the production of Fe^{2+} when superoxide reacts with Fe^{3+}.

In addition to above described iron redox cycling system, a number of other transition-metal including Cu, Ni, Co may be accountable for the formation in living cells (figure 3.4.1).

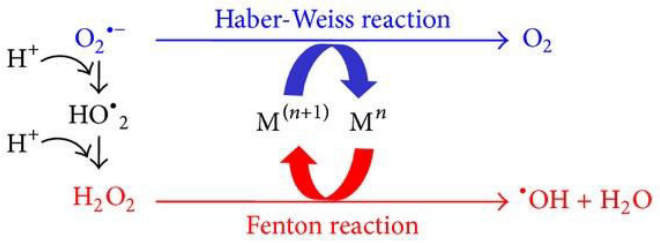

Figure 3.4.1: Fenton and Haber-Weiss reaction

Reduced form of transition-metals (Mn) reacts trough the Fenton reaction with hydrogen peroxide (H_2O_2), leading to the generation of (•OH) superoxide radical ($O_2^{\bullet-}$) can also react with oxidized form of transition metals ($M^{(n+1)}$) in the Haber-Weiss reaction leading to the production of M^n, which then again affects redox cycling.

The hydroperoxyl radical (HO_2•) plays an important role in the chemistry of lipid peroxidation. This protonated form of superoxide yields H_2O_2 which reacts with redox active metals for further generation of free radical through Fenton or Haber-Weiss reaction. There is a much stronger oxidant than superoxide anion-radical and could initiate the chain oxidation of polyunsaturated phospholipids, thus leading to impairment of membrane function (Bielski et al., 1983; Browne and Armstrong, 2000; Schneider et al., 2008).

3.4.3 Lipid peroxidation process

Lipid peroxidation is a process under which oxidants such as free radicals or nonradical species attack lipids comprising carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) that involve hydrogen abstraction from a carbon, with oxygen insertion resulting in lipid peroxyl radicals and hydroperoxides as described previously (Yin et al., 2011). Glycolipids, cholesterol (Ch) and phospholipids (PLs) are familiar targets of damaging and potentially lethal peroxidative modification. Lipids also can be oxidized by enzymes like lipoxygenases, cyclooxygenases, and cytochrome P450 (see above, lipid as signaling molecules). In response to membrane lipid peroxidation, and according to specific cellular metabolic circumstances and repair capacities, the cells may promote cell survival or induce cell death. The cells stimulate their maintenance and survival through constitutive antioxidant defense systems or signaling pathways activation under physiological or low lipid peroxidation rates that upregulate

antioxidants proteins resulting in an adaptive stress response. By contrast, under medium or high lipid peroxidation rates (toxic conditions) the extent of oxidative damage overwhelms repair ability, and the cells induce apoptosis or necrosis programmed cell death; both processes ultimately lead to molecular cell damage which may enable development of different pathological states and enhanced aging. The impact of lipids peroxidation in cell membrane and how these oxidative damages are involved in both major pathological conditions and physiological processes have been analyzed in different reviews (Fruhwirth et al., 2007; Kinnunen et al., 2012; Reis and Spickett, 2012; Volinsky and Kinnunen, 2013).

The overall process of lipid peroxidation consists of three steps: initiation, propagation, and termination (Girotti, 1998; Kanner et al., 1987; Yin et al., 2011). In the lipid peroxidation initiation step, prooxidants like hydroxyl radical abstract the allylic hydrogen forming the carbon-centered lipid radical (L•). In the propagation phase, lipid radical (L•) rapidly reacts with oxygen to form a lipid peroxy radical (LOO•) which abstracts a hydrogen from another lipid molecule generating a new L• (that continues the chain reaction) and lipid hydroperoxide (LOOH). In the termination reaction, antioxidants like vitamin E donate a hydrogen atom to the LOO• species and form a corresponding vitamin E radical that reacts with another LOO• forming nonradical products. Once lipid peroxidation is initiated, a propagation of chain reactions will take place until termination products are produced. Review with extensive information regarding the chemistry associated with each of these steps is available (Yin et al., 2011).

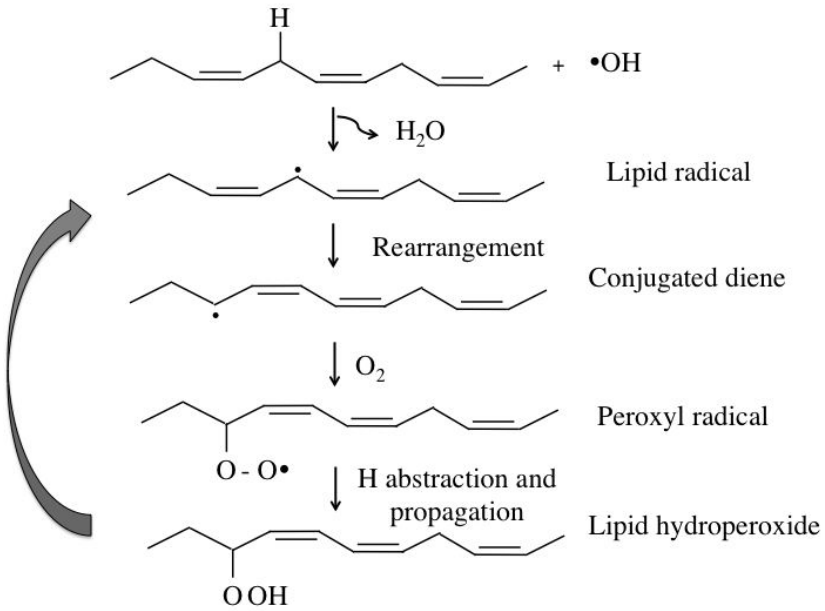

Figure: 3.4.2 Lipid peroxidation process

In Initiation, prooxidants abstract the allylic hydrogen forming the carbon-centered lipid radical; the carbon radical tends to be stabilized by a molecular rearrangement to form a conjugated diene (step 1). In the propagation phase, lipid radical rapidly reacts with oxygen to form a lipid peroxy radical (step 2) which abstracts a hydrogen from another lipid molecule generating a new lipid radical and lipid hydroperoxide (step 3). In the termination reaction, antioxidants donate a hydrogen atom to the lipid peroxy radical species resulting in the formation of nonradical products (step 4).

3.4.4 Lipid peroxidation products

MDA is an end-product generated by decomposition of arachidonic acid and larger PUFAs (Esterbauer et al., 1991) through enzymatic or nonenzymatic processes. MDA production by enzymatic processes is well known but its biological functions and its possible dose-dependent dual role have not been studied although MDA is more chemically stable and membrane-permeable than ROS and less toxic than 4-HNE and methylglyoxal (MG) (Esterbauer et al., 1991). So far, only few papers have reported that MDA may act as signaling messenger and regulating gene expression. Some recent research indicated that MDA acted as a signaling messenger and regulated islet glucose-stimulated insulin secretion (GSIS) mainly through Wnt pathway which is a signal transduction pathways made of proteins that pass signals into a cell through cell surface receptors. The moderately high MDA levels (5 and 10 μM) promoted islet GSIS, elevated ATP/ADP ratio and cytosolic Ca^{2+} level, and affected the gene expression and protein/activity production of the key regulators of GSIS (Wang et al., 2014); (ii) MDA induced collagen-gene expression by upregulating specificity protein-1 (Sp1) gene expression and Sp1 and Sp3 protein levels in hepatic stellate cells (García-Ruiz et al., 2002). Both Sp1 and Sp3 can interact with and recruit a large number of proteins including histone modifying enzymes, the transcription initiation complex and chromatin remodeling complexes, which strongly propose that Sp1 and Sp3 are vital transcription factors in the renovation chromatin and the regulation of gene expression (Li and Davie, 2010). On the other side, despite their potential therapeutic value nonenzymatic MDA production is poorly understood because MDA is thought to originate under stress situations and has high capability of reaction with multiple biomolecules such as proteins or DNA that leads to the formation of adducts (Blair, 2008; Łuczaj and Skrzydlewska, 2008; Zarkovic, et al., 2013), and extreme MDA production have been associated with different

pathological conditions (Garcia et al., 2013). In many literatures it indicated that identification of in vivo MDA production and its role in biology is very important.

MDA production by enzymatic processes:

During the biosynthesis of thromboxane A2, MDA can be produced in vivo as a byproduct through enzymatic processes (figure 3.4.3) (Hecker and Ullrich, 1989). TXA2 is a biologically active metabolite of arachidonic acid formed by the action of the thromboxane A2 synthase, on prostaglandin endoperoxide or prostaglandin H2 (PGH2) (Ekambaram et al., 2011; Ricciotti and FitzGerald, 2011). PGH2 previously is generated by the actions of cyclooxygenases on arachidonic acid (AA) (Ricciotti and FitzGerald, 2011; Yang and Chen, 2008).

MDA production by nonenzymatic processes:

A mixture of lipid hydroperoxides is formed during lipid peroxidation process. The peroxyl radical of the hydroperoxides with a cis-double bond homoallylic to the peroxyl group permits their facile cyclization by intramolecular radical addition to the double bond and the formation of a new radical. The intermediate free radicals formed after cyclization can cyclize again to form bicycle endoperoxides, structurally related to prostaglandins, and undergo cleavage to produce MDA. Through nonenzymatic oxygen radical-dependent reaction, AA is the main antecedent of bicyclic endoperoxide, which then undergoes further reactions with or without the participation of other compounds to form MDA (Figure 3.4.3) (Milne et al., 2008; Pryor et al., 1976)

However, it should be possible that other eicosanoids that can also be generated by nonenzymatic oxygen radical-dependent reaction (Brooks et al., 2008; Roberts II et al., 2005) may be precursor of bicyclic endoperoxide and MDA. Recent evaluation has talked the pathways for the nonenzymatic formation of MDA under specific conditions (Onyango and Baba, 2010).

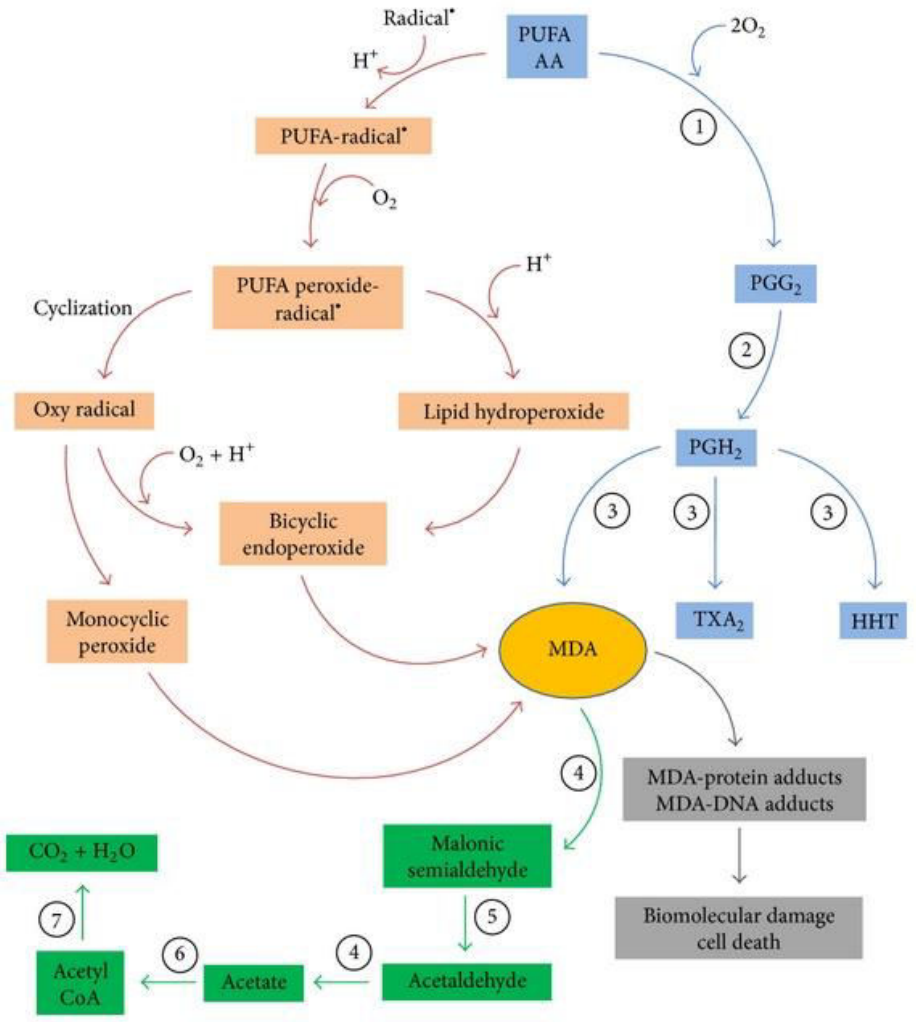

Figure: 3.4.3 MDA formation and metabolism

MDA can be generated in vivo by decomposition of arachidonic acid (AA) and larger PUFAs as a side product by enzymatic processes during the biosynthesis of thromboxane A2 (TXA2) and 12-l-hydroxy-5,8,10-heptadecatrienoic acid (HHT) (blue pathway), or through nonenzymatic processes by bicyclic endoperoxides produced during lipid peroxidation (red pathway). One formed MDA can be enzymatically metabolized (green pathway). Key enzymes involved in the formation and metabolism of MDA: cyclooxygenases (1), prostacyclin hydroperoxidase (2), thromboxane synthase (3), aldehyde dehydrogenase (4), decarboxylase (5), acetyl CoA synthase (6), and tricarboxylic acid cycle (7).

3.4.5 Role of MDA in human physiology

Lipids are important components of the cell membrane. Lipid peroxidation is concerned in the pathogenesis of various diseases and clinical situations including diabetes, adult respiratory distress syndrome, aspects of shock, premature birth disorder, Alzheimer's disease, Parkinson's disease, various chronic inflammatory conditions, pre-eclampsia and eclampsia, ischaemia, reperfusion mediated injury to organs which include the heart, brain and the intestine, atherosclerosis, organ injury which is associated with shock and inflammation, fibrosis, cancer, inflammatory liver injury, anthracycline induced cardiotoxicity, silicosis and pneumo-coniosis (Castranova and Vallyathan, 2000; Davì et al., 2005; Halliwell and Gutteridge, 1992; Yagi, 1987). It has been suggested that an increase in the free radicals may create neuronal deterioration through lipid peroxidation and a decrease in the glutathione peroxidase levels. The lipid peroxidation product, oxidative stress in cells is generally measured by free serum MDA. Lipid peroxidation, being a free radical reaction, it occurs when the hydroxyl radicals, possibly oxygen reacts with the unsaturated lipids of the bio-membranes, resulting in the generation of lipid hydroperoxide (ROOH), lipid peroxide radicals (ROO•) and disintegration products such as MDA (Aust and Svingen, 1982; Uchida et al., 1999). This aldehyde is a very poisonous molecule and it should be considered as more than just a marker of lipid peroxidation. Interaction of MDA with the DNA and proteins has been considered as hypothetically mutagenic and atherogenic.

3.4.6 Determination of serum MDA level

3.4.6.1 Preparation of working solution and spectrophotometric determination

Serum level of MDA usually measured as thiobarbituric acid reactive substances (TBARS) or lipid peroxides which are most widely produced biologically relevant free radical reaction (Samir and El-kholy, 1999). After mixing of 100 µL serum with 900 µL of 0.9% saline solution; 2 mL of freshly prepared thiobarbituric acid (TBA) reagent and 30 µL of 50 mM butylatedhydroxytolune (BHT) had been added. The mixture was then incubated at 60 ^{o}C for 15 min. For cooling the sample it was kept in ice for another 5 min. Then the samples were centrifuged at 5000 rpm for 10 min. The absorbance of the supernatant was measured spectrophotometrically at 535 nm using 1,1,3,3 tetraethoxypropane as standard.

3.4.7 Results

3.4.7.1 Serum level of MDA

3.4.7.1.1 Construction of standard curve of MDA

The standard curve had been constructed in the concentration range of 1 to 15 µmol/L. Thiobarbituric acid reactive substances (TBARS) were calculated as µmol/L (R^2 = 0.9918) in serum level. Figure 3.4.4 represents the calibration curve of MDA.

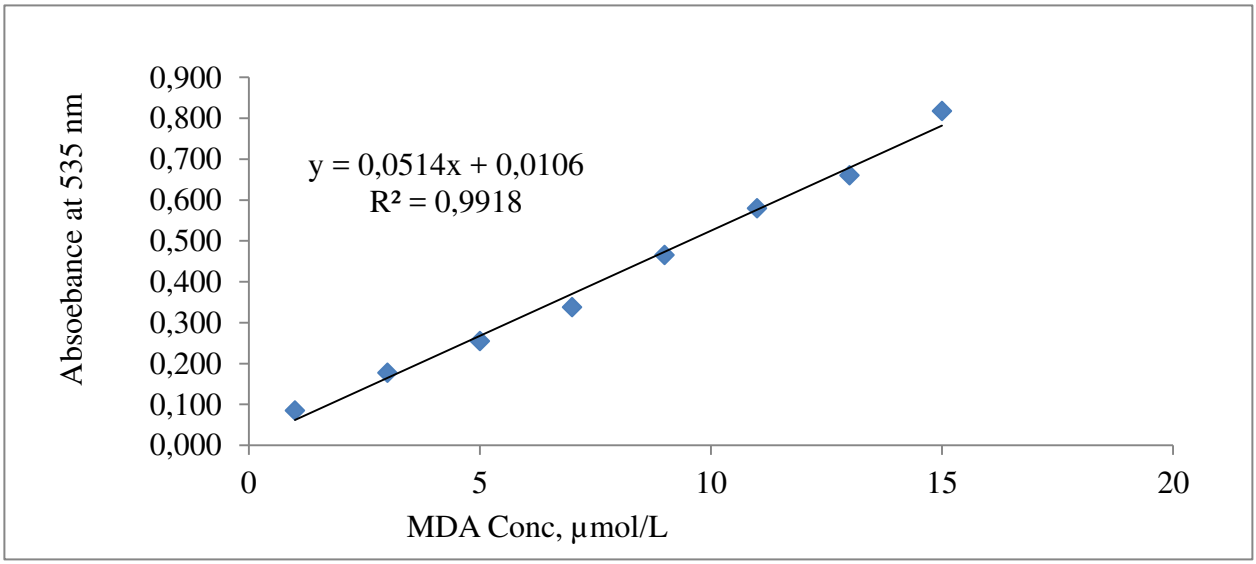

Figure 3.4.4 Standard curve of MDA

3.4.7.2 Comparison of serum MDA level in patients and controls subjects

The mean serum concentration of MDA was analyzed in MDD patients and in normal control subjects.

Table: 3.4.1 Mean serum MDA level in patients and control subjects

Groups	MDA (µmol/L)
MDD (*n*=247)	5.16±2.56
Control (*n*=248)	3.21±1.77
p value	*p*<0.001

The mean serum concentration of MDA was 5.16±2.56 (µmol/L) for MDD group and 3.21±1.77 (µmol/L) for healthy control group respectively. Sample t-test was used for statistical analysis. There was a statistically significant difference of MDA between the groups. MDD patients had significantly elevated level of serum MDA (p<0.001).

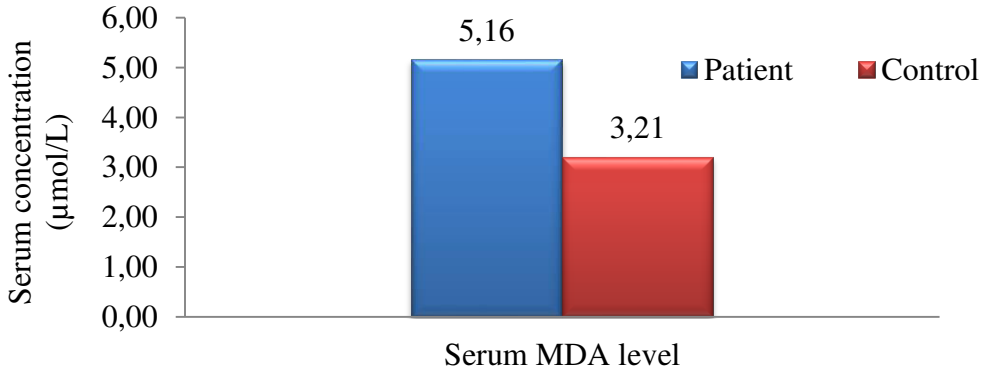

Figure: 3.4.5 Comparison of mean serum MDA level in patients and control subjects

Table: 3.4.2 Pearson correlation of serum MDA with age, BMI, education, income and smoking

		MDA	Age	BMI	Education	Income	Smoking
MDA	Pearson correlation	1	.060	.045	-.002	-.043	-.045
	Sig. (2-tailed)		.346	.481	.970	.503	.485
	N	247	247	247	247	247	247
Age	Pearson correlation	.060	1	.293**	-.315**	.004	.081
	Sig. (2-tailed)	.346		.000	.000	.950	.204
	N	247	247	247	247	247	247
BMI	Pearson correlation	.045	.293**	1	-.049	.129*	.037
	Sig. (2-tailed)	.481	.000		.439	.043	.564
	N	247	247	247	247	247	247
Education	Pearson correlation	-.002	-.315**	-.049	1	.305**	.102
	Sig. (2-tailed)	.970	.000	.439		.000	.109
	N	247	247	247	247	247	247
Income	Pearson correlation	-.043	.004	.129*	.305**	1	.028
	Sig. (2-tailed)	.503	.950	.043	.000		.662
	N	247	247	247	247	247	247
Smoking	Pearson correlation	-.045	.081	.037	.102	.028	1
	Sig. (2-tailed)	.485	.204	.564	.109	.662	
	N	247	247	247	247	247	247

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

Table: 3.4.3 Regression analysis using MDA as dependent variable and age, BMI, education, income and smoking as independent variable

Parameter	t	p
Age	0.957	0.340
BMI	0.546	0.585
Education	0.622	0.535
Income	-0.878	0.381
Smoking	-0.839	0.402

3.4.8 Discussion on MDA

Lipid peroxidation was determined by measuring the serum level of MDA content formed in the blood during oxidative stress. It had been found that the MDA content is significantly higher (p<0.001) in MDD patients when compared to healthy control. Some other previous studies support our current findings that the elevated MDA level was found in different psychiatric disorders. Increased lipid peroxidation was associated with MDD and may be normalized by antidepressants (Mazereeuw et al., 2015). Higher levels of MDA and lower levels of antioxidants associate the high degree of oxidative stress in unipolar depression (Bajpai et al., 2014). MDA level is significantly increased in schizophrenia cases compared to controls but this is not associated with the severity of the disease (Devanarayanan et al., 2015). Serum level of MDA significantly higher in schizophrenic patients compared to healthy controls. Serum level of MDA is significantly low in the aged women with major depressive disorder (De la Fuente et al., 1998). Another study reveals that panic disorder patients have considerably higher level of MDA than the healthy control subjects (Nahar et al., 2013). This result suggest that oxidative stress plays a role in depression and that antidepressant activity may be mediated via improving oxidative stress/antioxidant function (Jiménez-Fernández et al., 2015).

3.4.9 Conclusion

It has been explored that MDD patients have high serum concentration of MDA than the healthy control. The present study suggests that alteration of serum MDA may contribute to the pathogenesis of MDD. This finding may have correlation between MDA level in MDD patients and the degree of the disorder.

Pearson correlation coefficient suggested that there were no significant correlation between serum MDA level in MDD patients and their socio-demographic status. Linear regression analysis also performed by using MDA as dependent variable and age, BMI, Education, income and smoking as independent variables but any significant relationship of MDA with independent variables was not established.

3.5 Determination of serum cortisol

3.5.1 Cortisol

A group of hormones named glucocorticoids generally known as cortisol. Cortisone, Cortisol and corticosterone all are belong to glucocorticoids. Metabolism in the cells and various stressors on the body both are regulated by these hormones. Cortisol is a steroid-based hormone which is produced from cholesterol. Adrenal cortex of the adrenal gland from where cortisol is produced (Jansen et al., 2015).

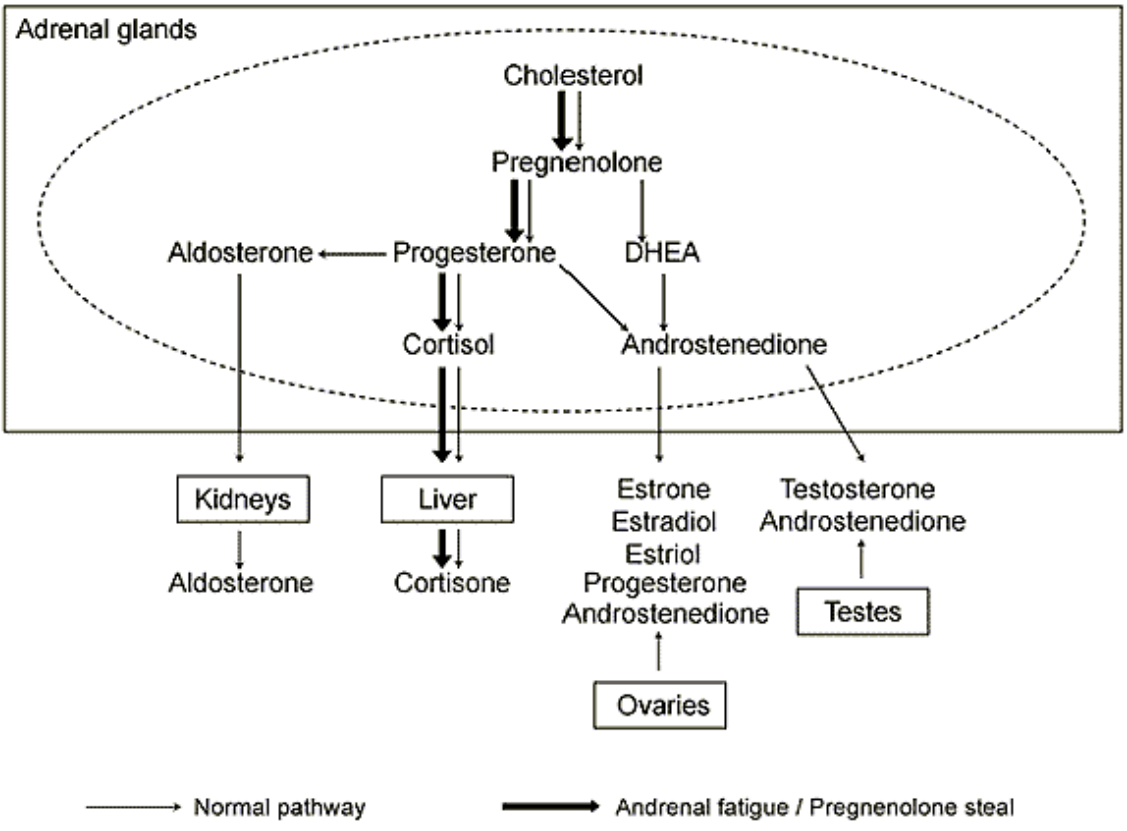

Figure: 3.5.1 Cortisol production in the adrenal cortex of the adrenal gland

3.5.1.1 Functions of cortisol in biological system

Cortisol is a powerful chemical like all steroid-based hormones. Steroid-based hormones enter into cells and modify the gene activity in the DNA which is a common mechanism of action for all steroid based hormones. Our foods habit and physical activity determine the amount of cortisol in our body. The maximum level of cortisol happens in the blood just after wake up in

the morning and the minimum level found in the evening just before sleep (Simmons et al., 2984).

The main function of cortisol is to aggravate the cell to produce glucose from proteins and fatty acids by gluconeogenesis process. Cortisol helps us to save glucose for the brain and driving the body to use fatty acids from stored fat for energy (Brown and Brown, 2003).

Cortisol also helps to breakdown of stored proteins into amino acids which ultimately utilized by our body for producing enzymes or repairing cells. Cortisol helps to distribute glucose and other nutrients as quickly as possible to the cells by increasing blood pressure as well as blood flow. Lastly, cortisol supports the body to produce resistance against stress and reduces the inflammatory response as well as overall immune response (Hoehn and Marieb, 2010).

Consequently, the amount of glucose, fatty acids and amino acids increase in the blood by stress which is promoted by cortisol. These are used to control many inflammatory diseases in the body such as rashes and allergies as well as more serious autoimmune conditions like rheumatoid arthritis.

3.5.1.2 Cortisol secretion in biological system

Deficiency of cortisol (hyposecretion): Addison's disease is caused by the damage of adrenal cortex which lead to decrease cortisol secretion. Functions of many systems in the body are affected by this low level of cortisol. Low level of blood glucose and sodium and high level of blood potassium generally found in persons affected by Addison's disease. They also have a tendency to lose weight. Low blood pressure and dehydration are also caused by Addison's disease. Corticosteroid replacement therapy is effective to treat cortisol deficiency in the body and to return cortisol to normal levels (NIDDK, 2014).

Excess Cortisol (hypersecretion): In response to stress, hypersecretion of cortisol is occurred that results in decreased inflammation and immune response. However, another more serious condition named Cushing's syndrome or disease that can cause hypersecretion of cortisol. This Cushing's syndrome may occur either by a tumor of the pituitary gland or adrenal cortex or excess doses of glucocorticoid drugs (NEMDIS, 2008).

Side effects of Cushing's syndrome are very serious such as water and salt retention, swelling, high blood pressure, deposits of excess fat in the abdomen and back of the neck and poor wound

healing. Perhaps the most severe side effect of Cushing's syndrome is the propensity to develop severe infections before showing any symptoms due to decreased immune response. Excess cortisol level in the blood may lead to cause additional stress. If this elevated level of cortisol in blood persists for a long period of time then it can cause cellular damage, depression, severe mood effects, weight gain and decreased neural function (Lado-Abeal et al., 1998).

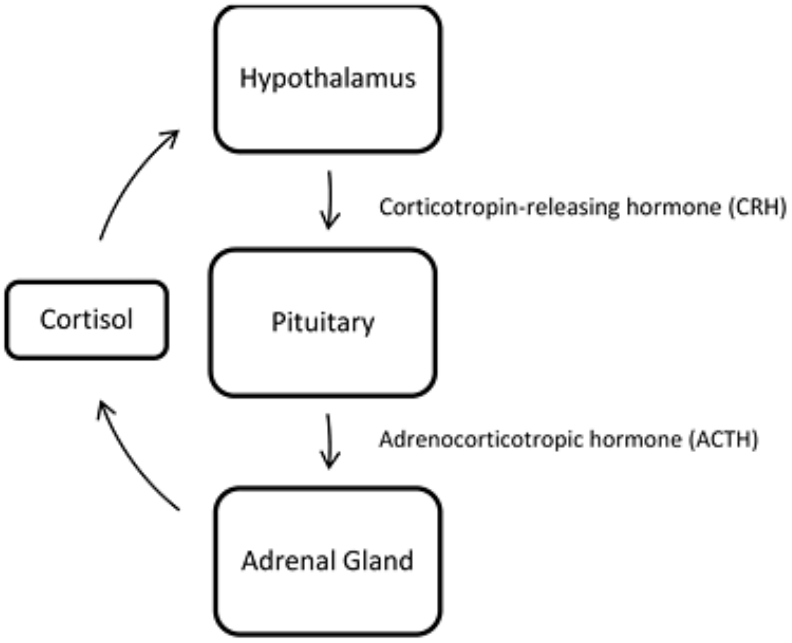

Figure: 3.5.2 Cortisol secretion in the adrenal cortex from hypothalamus

3.5.2 Determination of serum cortisol by Enzyme-Linked Immunosorbent Assay (ELISA) Kit

3.5.2.1 Principle of the assay

Enzyme-linked immunosorbent assay (ELISA) is based on the principle of competitive binding. Cortisol ELISA kit is a competitive inhibition enzyme immunoassay technique for the in vitro quantitative measurement of cortisol in serum, plasma, urine and other biological fluids. The concentration of cortisol in the sample is inversely proportional to the quantity of bound peroxidase conjugate. After adding the substrate solution, the intensity of color developed is inversely proportional to the concentration of cortisol in the donor sample. The microtiter wells are coated with a monoclonal antibody directed towards an antigenic site on the cortisol molecule. Endogenous cortisol of a donor sample competes with a cortisol-horseradish

peroxidase conjugate for binding to the coated antibody. After incubation the unbound conjugate is splashed off.

3.5.2.2 Materials and reagents

1. Microtiterwells, 12x8 (break apart) strips, 96 wells; wells coated with an anti-cortisol antibody (monoclonal).
2. Standard (standard 0-6), 7 vials, 1 mL, ready to use; concentrations: 0, 20, 50, 100, 200, 400, 800 ng/mL, thus corresponding to 0, 55.2, 138, 276, 552, 1104, 2,208 nmol/L (conversion factor: 1 ng/mL = 2.76 nmol/L). Contain 0.3% proclin as a preservative.
3. Enzyme conjugate, 1 vial, 25 mL, ready to use; cortisol conjugated to horseradish peroxidase; contains 0.3% proclin as a preservative.
4. Substrate solution, 1 vial, 14 mL, ready to use; tetramethylbenzidine (TMB).
5. Stop solution, 1 vial, 14 mL, ready to use; contains 0.5 M H_2SO4. Avoid contact with the stop solution. It may cause skin irritations and burns.
6. Wash solution, 1 vial, 30 mL (40x concentrated).

Equipment and material required but not included in kit

1. A microtiter plate calibrated reader (450±10 nm), (Bio-Rad iMark microtiter plate reader).
2. Calibrated variable precision micropipettes.
3. Absorbent paper.
4. Distilled water

3.5.2.3 Reagent preparation

All reagents and required number of strips were kept at room temperature prior to use.

Wash solution: 30 mL of concentrated wash solution was diluted with 1,170 mL deionized water to make the final volume of 1,200 mL. The diluted wash solution is stable for two weeks at room temperature.

3.5.2.4 Sample preparation

All serum samples of MDD patients and controls subjects were kept at room temperature prior to use. Serum samples were centrifuged for 20 min at 3,000 rpm. Now the sample is ready to use.

3.5.2.5 Design of microtitre plate

The microtitre plate has been designed for cortisol analysis in the following way for both 247 samples and 248 controls.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
B	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
C	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36
D	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	S47	S48
E	S49	S50	S51	S52	S53	S54	S55	S56	S57	S58	S59	S60
F	Std1	Std2	Std3	Std4	Std5	Std6	Std7	Blank	Blank	Blank	Blank	Blank
G	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
H	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24

Figure: 3.5.3 Microtitre plate for cortisol determination

3.5.2.6 Assay procedure

Prior to assay, reagents and samples were kept at room temperature. All reagents were gently mixed before use.

1. Desired numbers of microtiter wells were secured in the holder.
2. Twenty microliter of each patient, control and standard samples were dispensed into appropriate wells by disposable tips.
3. Then added 200 µL enzyme conjugate into each well.
4. The contents were mixed thoroughly for 10 seconds as proper mixing is important for accurate result.
5. Incubated for 60 min at room temperature without covering the plate.

6. Briskly shacked out the contents of the wells and bleached the wells three times with diluted wash solution (400 µL per well). Remaining droplets were eliminated by striking the wells abruptly with absorbent paper. The sensitivity and precision of this assay result is significantly depend the accurate performance of the washing procedure.
7. Then 100 µL of substrate solution was added to each well.
8. Again incubated for 15 min at room temperature.
9. Enzymatic reaction was stopped by adding 100 µL of stop solution to each well.
10. Reading was taken at 450±10 nm with a microtiter plate reader within 10 min after adding the stop solution.

BIO-RAD iMark Microplate Absorbance Reader

3.5.2.7 Calculation of results

1. Average absorbance value for each set of patient samples, control subjects and standards were calculated.
2. Standard curve has been made by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Corresponding concentration was determined from the standard curve by using the mean absorbance value for each sample.
4. The concentration of the samples was calculated directly from this standard curve.

3.5.3 Results

3.5.3.1 Serum cortisol level

3.5.3.1.1 Construction of standard curve

Calibration curve for cortisol was found to be linear over the concentration range of 0 µg/dL to 20 µg/dL (R^2 = 0.8426). Figure 3.5.4 represents the calibration curve of cortisol.

Standard	Absorbance (450 nm)
Standard 1 (0 µg/dL)	1.711
Standard 2 (2 µg/dL)	1.260
Standard 3 (5 µg/dL)	1.051
Standard 4 (10 µg/dL)	0.803
Standard 5 (20 µg/dL)	0.536

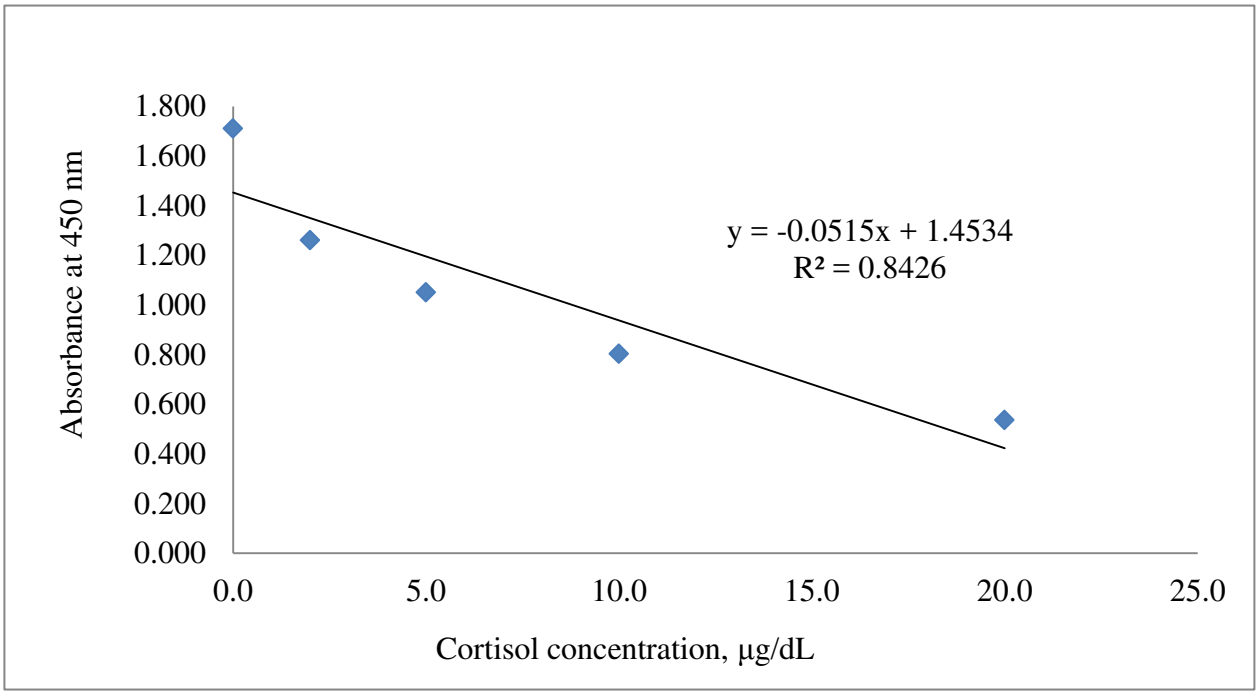

Figure 3.5.4 Standard curve of cortisol

3.5.3.1.2 Comparison of serum cortisol level in patients and control subjects

The mean serum concentration of cortisol was analyzed in MDD patients and in normal control subjects.

Table: 3.5.1 Mean serum cortisol level in patients and control subjects

Groups	Cortisol (µg/dL)
MDD (*n*=247)	19.32±5.38
Control (*n*=248)	17.38±6.32
p value	0.024

The mean serum concentration of cortisol was 19.32±5.38 µg/dL for MDD patients and 17.38±6.32 µg/dL for healthy control subjects respectively. Independent sample t-test was performed for statistical analysis. Statistically significant elevated cortisol was found in patient group ($p<0.05$).

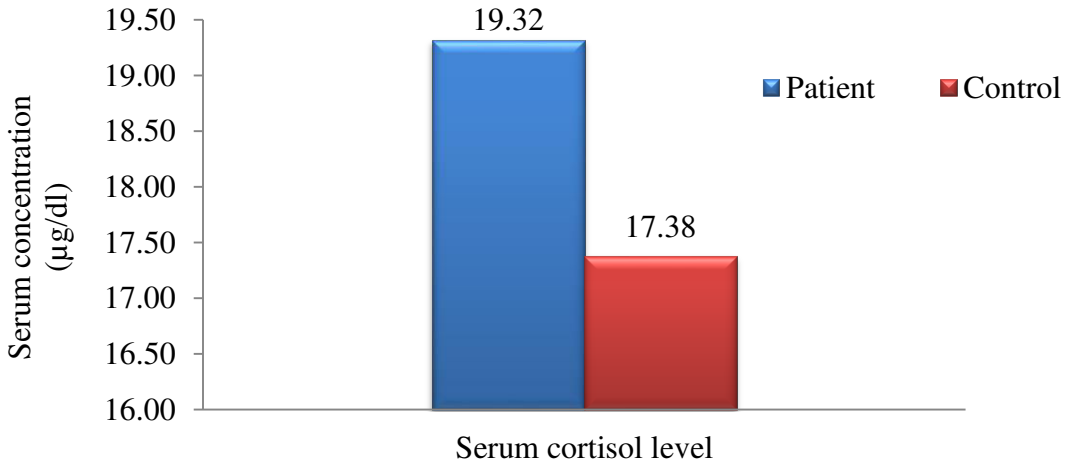

Figure: 3.5.5 Comparison of mean serum cortisol level in patients and control subjects

Table: 3.5.2 Pearson correlation of serum cortisol with age, BMI, education, income and smoking

		Cortisol	Age	BMI	Education	Income	Smoking
Cortisol	Pearson correlation	1	.009	.073	-.114	-.030	.080
	Sig. (2-tailed)		.906	.343	.137	.699	.301
	N	247	247	247	247	247	247
Age	Pearson correlation	.009	1	.293**	-.315**	.004	.081
	Sig. (2-tailed)	.906		.000	.000	.950	.204
	N	247	247	247	247	247	247
BMI	Pearson correlation	.073	.293**	1	-.049	.129*	.037
	Sig. (2-tailed)	.343	.000		.439	.043	.564
	N	247	247	247	247	247	247
Education	Pearson correlation	-.114	-.315**	-.049	1	.305**	.102
	Sig. (2-tailed)	.137	.000	.439		.000	.109
	N	247	247	247	247	247	247
Income	Pearson correlation	-.030	.004	.129*	.305**	1	.028
	Sig. (2-tailed)	.699	.950	.043	.000		.662
	N	247	247	247	247	247	247
Smoking	Pearson correlation	.080	.081	.037	.102	.028	1
	Sig. (2-tailed)	.301	.204	.564	.109	.662	
	N	247	247	247	247	247	247

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Table: 3.5.3 Regression analysis using cortisol as dependent variable and age, BMI, education, income and smoking as independent variable

Parameter	**t**	**p**
Age	-1.023	0.308
BMI	1.121	0.264
Education	-1.726	0.086
Income	-0.006	0.995
Smoking	1.201	0.231

3.5.4 Discussion on cortisol

Patients with depressive illness may have significantly elevated levels of free serum cortisol which can be measured by variety biologically active parameters for cortisol (Belmaker and Agam, 2008). In our present study, serum cortisol was determined by using solid phase enzyme-linked immunosorbent assay (ELISA) kit and we found the mean serum concentration of cortisol is 19.32±5.38 µg/dL for MDD patients and 17.38±6.32 µg/dL for healthy control subjects respectively. This finding was related with the results found by some earlier researchers, e.g. psychiatric patients had significantly elevated level of serum cortisol. Hypercortisolemia during depression may develop due to another pathophysiological mechanisms relating to uneven basal hyper secretion of cortisol, linked with adrenal enlargement, possibly through splanchnic sympathetic stimulation of the adrenal cortex (Carroll et al., 2012). Cortisol is supposed to be a risk factor for stress and age related disorders, such as major depression and Alzheimer's disease (AD). Pathophysiology of MDD may be associated with the elevated cortisol level in serum. Association between high serum cortisol and AD was found significantly (Zvěřová et al., 2013).

3.5.5 Conclusion

The present study suggests that patients with MDD have significantly higher (p=0.024) serum cortisol level compare to healthy control. This alteration of serum cortisol level may contribute to the pathogenesis of MDD. This finding may have a correlation between serum cortisol level in MDD patients and the degree of the disorder.

Pearson correlation coefficient suggested that there were no significant correlation between elevated serum cortisol level in MDD patients and their socio-demographic status. Linear regression analysis also performed by using serum cortisol as dependent variable and age, BMI, education, income and smoking as independent variables but any significant relationship of serum cortisol with independent variables was not established.

3.6 Determination of serum amino acids

3.6.1 Amino acids

Amino acids are organic compounds that bloc to build proteins. In human body twenty percent of component is protein which plays a vital role in almost all biological processes and amino acids are the structural units of it. Amino acids are formed by broken down of this protein. A large part of our cells, tissue and muscles are prepared by amino acids. Many essential biological functions such as giving cells their structure is carried out by amino acids (Escott-Stump, 2008). They also play an important role for transportation and the storage of nutrients. Functions of different organs, glands, tendons and arteries are influenced by amino acids. Moreover they considered as essential for healing wounds and restoring tissue, especially in the muscles, bones, skin and hair as well as for the elimination of all types of waste deposits formed in linking with the metabolism. Amino acids are considered as a source of energy in human body (Trumbo et al., 2012).

3.6.1.1 Types of amino acids

Amino acids are classified into three groups, essential nonessential and conditional.

Essential amino acids

Amino acids belongs to this group are not produced in our body. That's why we need to take it through food. Nine essential amino acids are histidine, threonine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, and valine.

Nonessential amino acids

Our body can produce this type of amino acids and we do not need to take it from food what we eat. That's why it is termed as "Nonessential" amino acid. Some nonessential amino acids are alanine, glutamic acid, asparagine and aspartic acid.

Conditional amino acids

Conditional amino acids are generally not required for our body except during stress and some other disease condition. Some conditional amino acids are arginine, glutamine, cysteine, tyrosine, ornithine, proline, glycine and serine.

3.6.1.2 Dietary requirement of amino acids

For defining the requirement of protein, first comes the requirements of essential amino acids. The desired amounts of the nine essential amino acids must be delivered in the food. Thirty percent requirement of methionine and tyrosine and 50% requirement of phenylalanine can be replaced by cysteine, so these amino acids should also be considered while determining the amounts of essential amino acids for human body. Extensive study was conducted during the period of 1950 to 1970 to determine the requirement of essential amino acids for infants, children, men, and women.

Table: 3.6.1 Estimates of amino acid requirements (WHO, 1985)

Amino Acids	Requirements, mg/kg per day, by age group			
	Infants, Age 3–4 months (Fomon and Filer Jr., 1967)	Children, Age ~2 years (Pineda et al., 1981)	Children, Age 10–12 years (Nakagawa et al., 1964)	Adult (FAO, 1973)
Histidine	28	?	?	8–12
Isoleucine	70	31	28	10
Leucine	161	73	42	14
Lysine	103	64	44	12
Methionine plus cystine	58	27	22	13
Phenylalanine + tyrosine	125	69	22	14
Threonine	87	37	28	7
Tryptophan	17	12.5	3.3	3.5
Valine	93	38	25	10
Total without histidine	714	352	214	84

Information on amino acid requirements for pregnant and lactating women is limited.

3.6.1.3 Functions of amino acids on human body

Amino acids are the building blocks of proteins and proteins are very essential macronutrients for body. Though some amino acids only make proteins but some others play a variety of roles, from auxiliary metabolism to shielding our heart. Our body can also use amino acids for energy when we are lack of carbohydrates and fats.

Build protein

Cells produce protein by following the instructions of DNA that defines the specific amino acids and the sequence in which they must attach to form the protein. DNA depends on another macromolecule; RNA to make the protein. RNA receives a replica of the code from DNA, leaves the cell, finds the amino acids and transports them back to the cell, where they bind into a chain. Each amino acid must be available at the time when it needed or the protein won't be synthesized. The chain twists and folds into a specialized shape when it is completed. Function of the protein depends on the final shape of each amino acid and this shape is controlled by individual chemical structure of amino acids (Creighton, 1993).

Synthesize neurotransmitters

Two well-known amino acids tryptophan and tyrosine produce neurotransmitters but some others can also do this. Tryptophan is used to synthesize serotonin, which controls our moods and makes the hormone melatonin. Tyrosine produces norepinephrine and adrenalin. Tyrosine and tryptophan compete with each other for entrance to our brain. When we take a large amount of carbohydrates, our brain gets more tryptophan and makes us sleepy. In the similar way a meal rich in protein increases the amount of tyrosine in our brain, which gives us more energy (Fernstrom, 1977).

Protect cardiovascular health

Nitric oxide is produced in our body by the amino acid arginine. It helps to keep the blood pressure low by relaxing muscles in our blood vessels. When nitric oxide produced in heart muscles, it controls contractions. It may also prevent atherosclerosis by inhibiting the progress of plaque in our arteries. Nitroglycerin is a medication in which nitric oxide is used as active ingredient to relieve angina or chest pain due to coronary heart disease (Deveaux et al., 2016).

Metabolism and other roles

Our body prefers to use carbohydrates and fats for energy, but amino acids are metabolized for energy during emergency condition. Three amino acids cysteine, glycine and glutamic acid combine to form glutathione, which is used as an antioxidant. The amino acid histidine synthesizes enzymes which is used to makes red blood cells and sustain healthy nerves. Thyroid hormones are produced by tyrosine, while methionine synthesizes S-adenosylmethionine (SAMe) which is essential for the metabolism of DNA and neurotransmitters (Yang et al., 2016).

3.6.1.4 Amino acid and MDD

Connection between nutritional deficiencies and physical illness is more prominent than nutrition and depression. Depression is usually thought as firmly biochemical based or emotionally rooted condition. Onset as well as severity and duration of depression may depend on nutritional status of patients. Many of the easily visible food patterns that lead to develop depression are the same as those that happen in the course of depression. These may include skipping meals, poor appetite and a leading desire for sweet foods. Nutritional factors are intertwined with human cognition, behavior, and emotions that is explained by nutritional neuroscience.

An interesting observation is that depressed people have nutrition which is far from adequate. Depressed person make poor food choices and picking foods that might really contribute to depression. There is link between low levels of serotonin and suicide which is suggested by many recent studies. It is concerned that low level of this neurotransmitter can lead to an overall insensitivity to future consequences like triggering risky, impulsive and aggressive behaviors which may end up with suicide, the ultimate act of inwardly directed impulsive aggression.

Depression is a disorder linked with major symptoms such as increased anxiety and sadness, depressed mood, loss of appetite and a loss of interest or pleasure in most of the daily activities. This disorder can lead to wide-ranging consequences if there is no timely therapeutic intervention. Depressed patients have a larger degree of suicidal tendency and therefore they are generally treated with antidepressants and/or psychotherapy (Brown et al., 1982). Deficiencies in neurotransmitters such as dopamine, serotonin, γ-aminobutyric acid (GABA) and noradrenaline are often allied with depression (Diehl and Gershon, 1992; Rush, 2007; Stockmeier, 1997; Van Praag, 1983). Tryptophan, phenylalanine, tyrosine, and methionine are the amino acids that often helpful in treating many mood disorders including depression which is reported in many studies

(Agnoli et al., 1976; Bourre, 2005; Firk and Markus, 2007; Leonard, 1997; McLean et al., 2004; Petty, 1995). Tryptophan, a precursor of serotonin, is frequently converted to serotonin when consumed alone on an empty stomach. Hence, tryptophan can induce tranquility and sleep. This suggests restoring serotonin levels lead to reduced depression caused by serotonin deficiencies (NIMH, 2000). Tyrosine and sometimes its precursor phenylalanine are altered into norepinephrine and dopamine (Hoes, 1982).

Pathophysiology of depression depends on glutamatergic neurotransmission via NMDA receptor. Patients with depression have significantly higher serum levels of d-serine and l-serine than those of healthy controls. On the other hand serum levels of glycine, glutamate and glutamine do not vary between two groups. Serine enantiomers may be peripheral biomarkers for depression, and that irregularity in the d-serine-l-serine-glycine cycle shows an important role in the pathophysiology of depression (Hashimoto et al., 2015).

3.6.2 Normal level of amino acids

Nutritional or metabolic status of individual person is expected to reflect by the total amino acid content in plasma and urine. Under defined experimental conditions; determination of each amino acid was done based on the retention time established for the specific amino acid. Calculation of amino acid concentration was done based on the area under the peak proven for a specified known concentration of amino acid. The described HPLC method was able to resolve and quantify most of the amino acids present in biological fluids within a total run period of 15 minutes.

Normal plasma level of different amino acids was found in adult Indian by Suresh BSV et al., 2002. The same value for Western populations was found in the research findings of Teerlink T et al., 1994. Comparison of normal level of different amino acids in adult Indian and Western populations have been shown in the table 3.6.5

Table: 3.6.2 Plasma amino acid profile of normal Indian and Western adult population

Amino Acids	Plasma concentration (µmol/L)	
	Indian range	Western range
Alanine	134–597	203-518
Arginine	26–123	50-126
Asparagine	8–83	36-71
Aspartate	5–43	0-9
Cysteine	245–332	268-300
Glutamate	8–99	12-98
Glycine	134–466	121-401
Histidine	18–155	48-121
Homocysteine	3.8–19	6-16
Isoleucine	38–94	42-98
Leucine	53–135	73-172
Lysine	54–240	107-244
Methionine	13–38	13-33
Ornithine	25–153	24-112
Phenylalanine	27–115	40-81
Proline	100-370	110-360
Serine	50–201	67-161
Taurine	21–212	38-197
Threonine	30–256	74-175
Tryptophan	32–85	24-72
Tyrosine	7–127	43-90
Valine	112–242	168-350

3.6.3 Determination of serum amino acid levels

3.6.3.1 Introduction

Amino acids were separated on a high performance anion-exchange column and directly detected them by integrated amperometry in the AAA-Direct ™ system (Clarke et al., 1999; Jandik et al., 1999). AAA-Direct system has benefits over pre and post column derivatization methods as sample preparation time and instrumentation complexity both are low. Precolumn derivatization is highly susceptible to interference from the sample matrix and complex sample matrices can reduce derivatization efficiency, causing high variability in amino acid recovery (Irvine, 1997).

3.6.3.2 Instrumentation

A Dionex Ultimate 3000 (Dionex, USA) analytical HPLC equipped with an autosampler and a column thermostat set at 40 °C was used. Separation was performed on a Acclaim RSLC 120 C_{18}, 2.2 µm, 100 mm x 2.1 mm analytical column (Dionex, USA) with a multi-step gradient elution at a flow of 722 µL/min. HPLC solvents contained dibasic sodium phosphate anhydrous 1.78 g, sodium tetraborate 3.81 g and sodium azide 0.0325 g dissolved in water as solvent A and acetonitrile, methanol and water at a ratio of 45:45:10 as solvent B. HPLC conditions are described in table 3.6.2; the total runtime was 15 min.

Table 3.6.3 HPLC conditions for amino acid determination

Column	Acclaim RSLC 120 C_{18}, 2.2 µm, 100 mm x 2.1 mm
Detector	338 nm
Injection volume	1µL
Flow rate	0.722mL/min
Column temperature	40 ^{0}C
Sample temperature	25 ^{0}C
Run time	15 min

3.6.3.3 Preparation of solvents and reagents

Preparation of mobile phase A	Dibasic sodium phosphate anhydrous 1.78 g, sodium tetraborate 3.81 g and sodium azide 0.0325 g were dissolved in water and finally made the volume 1000 mL. pH was adjusted at 7.8±0.01 with diluted phosphoric acid. The solution was filtered through a filter having pore size $\leq$ 0.2 µm and finally degased by an ultrasonic bath.
Preparation of mobile phase B	Acetonitrile, methanol and water were mixed at a ratio of 45:45:10. Filtered the solution through a filter having pore size not greater than 0.2 µm and finally degased by an ultrasonic bath.
Preparation of reagent A (0.1 M borate buffer: pH 9)	Sodium tetra borate 0.953 g was taken in a 25 mL volumetric flask and dissolve with water up to the mark. Then pH was adjusted at 9 by diluted hydrochloride solution.
Preparation of OPA solution (50 mg/mL)	0.50 g of o-phthalaldehyde was taken in a 10 mL volumetric flask and dissolve up to the mark with methanol.
Preparation of reagent C	0.025 g of 9-fluorenylmethoxycarbonyl chloride was taken in a 10 mL volumetric flask then dissolve and up to mark with acetonitrile.
Preparation of reagent B	0.8 mL 0.1 M borate buffer, 0.2 mL OPA solution and 20 µL MPA solutions were taken in a 2 mL vial with slotted septum.
Preparation of reagent D	0.5 mL of 85% phosphoric acid was taken in a 50 mL volumetric flask and dissolved up to mark with mobile phase A.

3.6.3.4 Preparation of standard amino acid solution

Ten milligram of 18 amino acid standard was taken in a 150 mL volumetric flask then 150 mL of 0.1 N hydrochloric acid was added to dissolve. Finally 0.1 N HCl was added to make the volume 250 mL and mixed well. Filtered the solution through a filter having pore size not greater than 0.2 µM and first 10 mL of filtrate was discarded.

3.6.3.5 Serum sample preparation

The samples were thawed at room temperature on the day of analysis and centrifuged at 1000 x g for 5 min. 100 µL of serum sample was spiked with 5 µL of 25 µmol/L of homoserine used as an internal standard and 300 µL of methanol was added then centrifuged at 1200 x g for another 5 min. For derivatization 10 µL of supernatant was used.

3.6.3.6 Procedure for serum analysis

A Dionex Ultimate 3000 (Dionex, USA) analytical HPLC equipped with an autosampler was used. The auto injector rack contained four reagent and two buffer solutions. The samples (10 µL) were loaded on to sample vials. Derivatization was performed by setting gradient program for elution and injection programing. The gradient profile is described in table 3.6.3 and the injection programing is described in table 3.6.4.

Table: 3.6.4 Gradient program for elution in Dionex Ultimate 3000 analytical HPLC

Time (min)	Mobile phase A (%v/v)	Mobile phase B (%v/v)
0.000	95	5
0.210	95	5
7.210	47	53
7.800	0	100
9.420	0	100
9.530	95	5
12.00	95	5

Table: 3.6.5 Injection programing for Dionex Ultimate 3000 analytical HPLC

1	Draw water (10 µL; UdpDraw)
2	Draw air (1 µL; UdpDraw)
3	Draw 0.1 M borate buffer, pH 9.0 (5 µL; UdpDraw)
4	Draw sample (1 µL; UdpDraw)
5	Draw air (6 µL; UdpDraw)
6	Mix the needle 3x (6 µL; UdpMoveSyringe Load and Unload)
7	Wait 15 seconds (UdpMixWait)
8	Needle wash (100 µL; UdpMixNeddleWash)
9	Draw OPA/MPA reagent with borate buffer, pH 9.0 (10 µL; UdpDraw)
10	Draw air (7 µL; UdpDraw)
11	Mix the needle 6x (7 µL; UdpMoveSyringe Load and Unload)
12	Wait 12 seconds (UdpMixWait)
13	Needle wash (100 µL; UdpMixNeddleWash)
14	Draw FMOC solution (1 µL; UdpDraw)
15	Draw air (8 µL; UdpDraw)
16	Mix the needle 6x (8 µL; UdpMoveSyringe Load and Unload)
17	Needle wash (100 µL; UdpMixNeddleWash)
18	Wait 15 seconds (UdpMixWait)
19	Draw injection diluent (3 µL; UdpDraw)
20	Draw air (11 µL; UdpDraw)
21	Mix the needle 4x (11 µL; UdpMoveSyringe Load and Unload)
22	Draw water (10 µL; UdpDraw)
23	Inject (total derivatization mixture was injected; UdpInjectMarker)

3.6.3.7 Calculation

Calculation for the amount of all serum amino acids was done in g/100 mL by using following equation.

Serum amino acid level (µg/L) = (As/Astd) x Cstd x (100 mL/Vs) x 100 mL

Here,

As = Peak are of respective amino acid in sample solution

Astd = Peak are of respective amino acid in standard solution

Vs = Volume of sample (mL)

Cstd = Concentration of respective amino acid in standard solution

Then convert serum level of each amino acid from g/100 mL to µmol/L unit.

3.6.4 Results

3.6.4.1 Serum amino acid levels

3.6.4.2 Comparison of serum amino acid levels in patients and control subjects

Analysis of Variance (ANOVA) and independent sample t-test were used for the examination of group mean difference and standard deviation.

Table: 3.6.6 Comparison of mean amino acid level in MDD patients and control subjects

Amino Acids	Mean±Standard Deviation (µmol/L)		*p* Value
	Patients	Control	
Alanine	194.66±66.05	197.90±66.46	0.587
Arginine HCl	70.04±25.39	72.39±29.84	0.347
Aspartic acid	19.36±11.03	20.82±11.32	0.149
Cysteine	270.57±27.49	274.19±28.79	0.152
Glutamic acid	45.38±15.43	47.57±23.98	0.228
Glycine	219.85±70.99	209.19±66.50	0.085
Histidine HCl	63.70±19.21	66.99±25.77	0.108
Isoleucine	55.60±12.57	55.73±11.44	0.904
Leucine	65.60±12.55	66.73±11.44	0.296
Lysine HCl	124.85±49.17	123.70±49.70	0.794
Methionine	18.35±9.80	22.48±8.72	<0.001
Phenylalanine	68.77±12.55	74.73±11.44	<0.001
Proline	174.86±55.45	184.58±66.79	0.079
Serine	109.60±40.47	110.13±41.36	0.885
Threonine	130.27±58.63	135.95±56.17	0.272
Tryptophan	51.60±12.57	59.73±11.44	<0.001
Tyrosine	54.78±21.99	60.23±28.58	0.018
Valine	130.76±30.09	132.89±36.36	0.478

Table: 3.6.7 Pearson correlation of methionine with phenylalanine, tryptophan and tyrosine

		Methionine	Phenylalanine	Tryptophan	Tyrosine
Methionine	Pearson correlation	1	0.970**	0.970**	-0.089
	Sig. (2-tailed)		<0.001	<0.001	0.163
Phenylalanine	Pearson correlation	0.970**	1	1.000**	-0.112
	Sig. (2-tailed)	<0.001		<0.001	0.080
Tryptophan	Pearson Correlation	0.970**	1.000**	1	-0.119
	Sig. (2-tailed)	<0.001	<0.001		0.062
Tyrosine	Pearson correlation	-0.089	-0.112	-0.119	1
	Sig. (2-tailed)	0.163	0.080	0.062	

**. Correlation is significant at the 0.05 level (2-tailed).

Table: 3.6.8 Pearson correlation of tryptophan with age, BMI, education, income and smoking

		Tryptophan	Age	BMI	Education	Income	Smoking
Tryptophan	Pearson correlation	1	-.053	.009	.055	-.041	.148*
	Sig. (2-tailed)		.410	.892	.388	.523	.020
	N	247	247	247	247	247	247
Age	Pearson correlation	-.053	1	.293*	-.315*	.004	.081
	Sig. (2-tailed)	.410		.000	.000	.950	.204
	N	247	247	247	247	247	247
BMI	Pearson correlation	.009	.293*	1	-.049	.129*	.037
	Sig. (2-tailed)	.892	.000		.439	.043	.564
	N	247	247	247	247	247	247
Education	Pearson correlation	.055	-.315*	-.049	1	.305*	.102
	Sig. (2-tailed)	.388	.000	.439		.000	.109
	N	247	247	247	247	247	247
Income	Pearson correlation	-.041	.004	.129*	.305*	1	.028
	Sig. (2-tailed)	.523	.950	.043	.000		.662
	N	247	247	247	247	247	247
Smoking	Pearson correlation	.148*	.081	.037	.102	.028	1
	Sig. (2-tailed)	.020	.204	.564	.109	.662	
	N	247	247	247	247	247	247

* Correlation is significant at the 0.05 level (2-tailed).

Different studies showed that MDD may be accompanied by the alteration of serum excitatory amino acids level (Maes et al., 1998). Elevated serum level of glycine may be involved in

psychopathology of schizophrenia and cognitive functioning (Strzelecki and Rabe-Jabłońska, 2010). Risk of major depressive disorder is possibly due to deficiencies in micronutrients in the gluten-free diet. Serum concentrations of tyrosine, phenylalanine and tryptophan are lower in depressed patients (Van Hees et al., 2015).

In our study, there is significant difference between the levels of most of the amino acids in both MDD patients and healthy controls. At 5% level of significance only four (methionine, p value <0.001; phenylalanine, p value <0.001; tryptophan, p value <0.001; tyrosine, p value 0.018) out of eighteen amino acid levels were significantly decreased in MDD patients compare to controls.

Chromatogram of amino acid standard-1

Sample Name:	**Std-1**	*Injection Volume:*	**5.0**
Vial Number:	**BA1**	*Channel:*	**UV_VIS_1**
Sample Type:	**Standard**	*Wavelength:*	**338.0**
Recording Time:	**12/20/2015 16:28**	*Sample Weight:*	**1.0000**

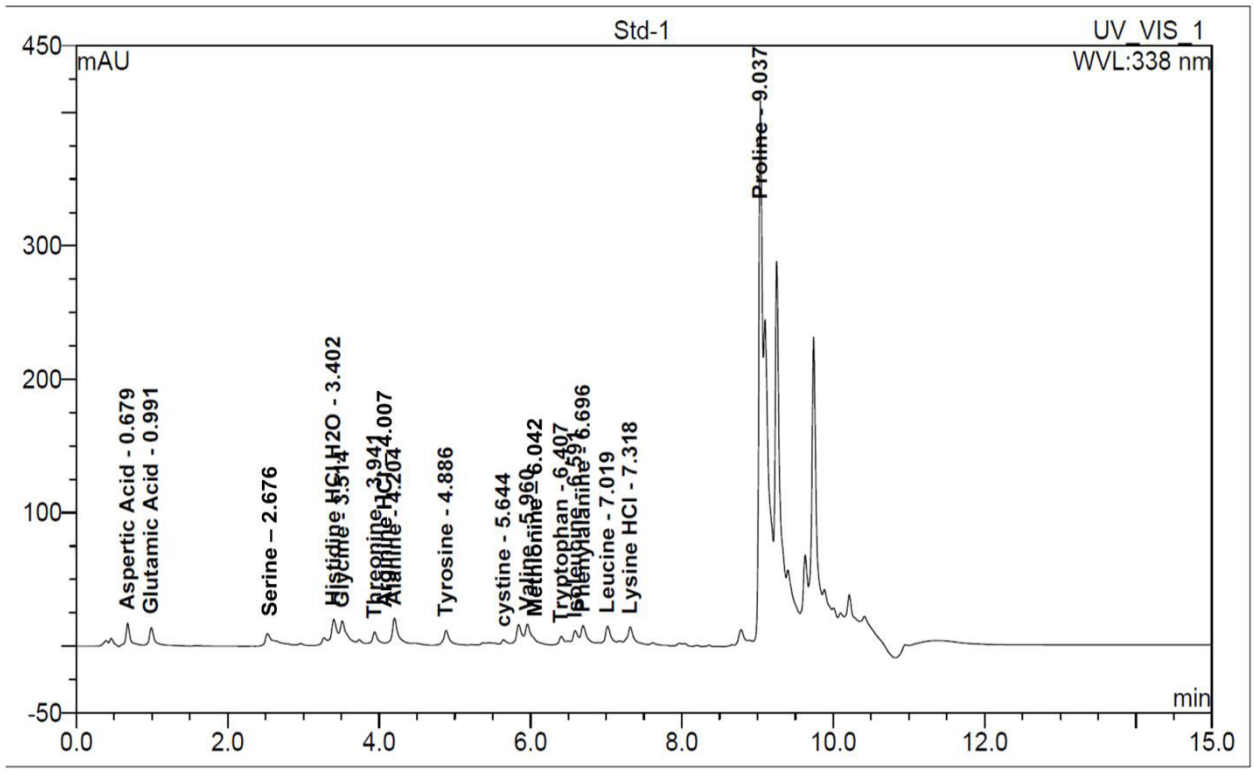

Figure: 3.6.1 Chromatogram of amino acid standard-1

Chromatogram of amino acid standard-2

Sample Name:	**Std-2**	*Injection Volume:*	**5.0**
Vial Number:	**BA1**	*Channel:*	**UV_VIS_1**
Sample Type:	**Standard**	*Wavelength:*	**338.0**
Recording Time:	**12/20/2015 16:50**	*Sample Weight:*	**1.0000**

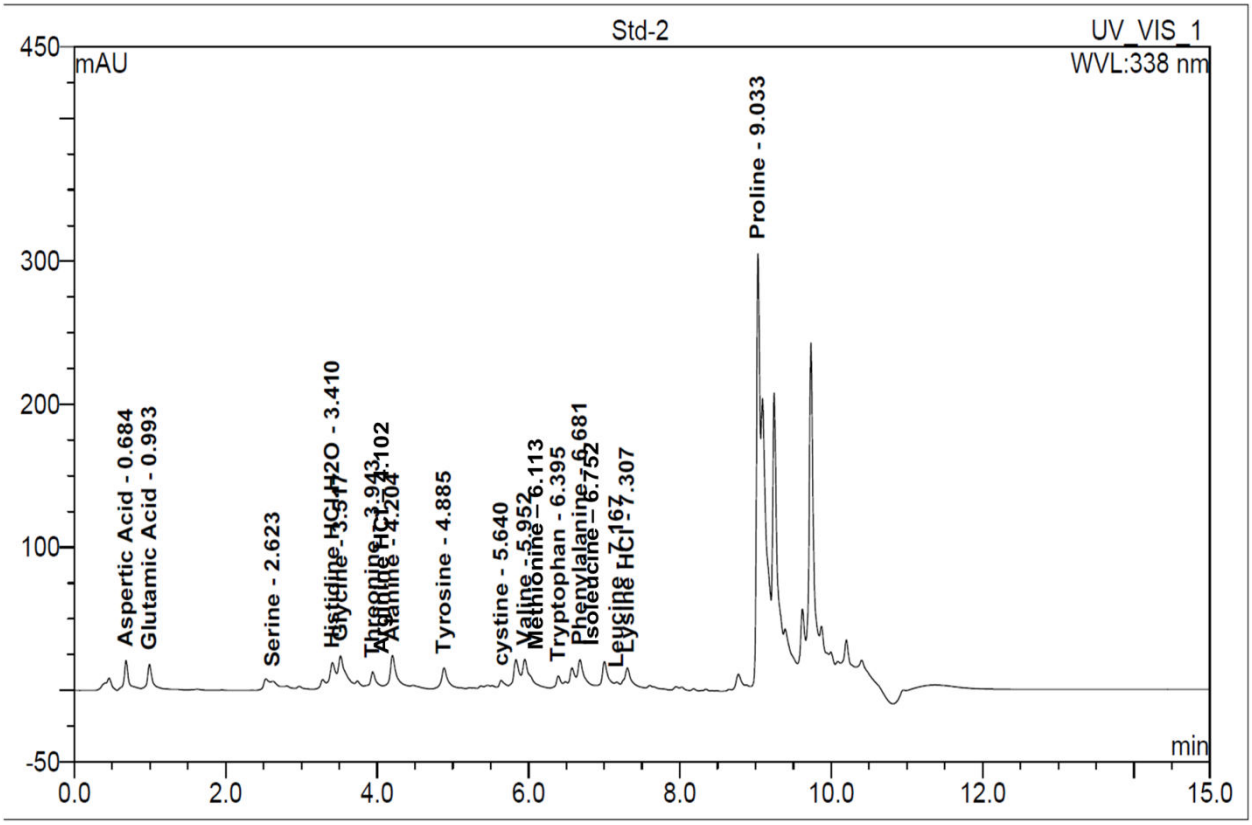

Figure: 3.6.2 Chromatogram of amino acid standard-2

Overlap chromatogram of amino acid standard-1 and standard-2

Sample Name:	**Std-2 and 1**	*Injection Volume:*	**5.0**
Vial Number:	**BA1**	*Channel:*	**UV_VIS_1**
Sample Type:	**Standard**	*Wavelength:*	**338.0**
Recording Time:	**12/20/2015 16:50**	*Sample Weight:*	**1.0000**

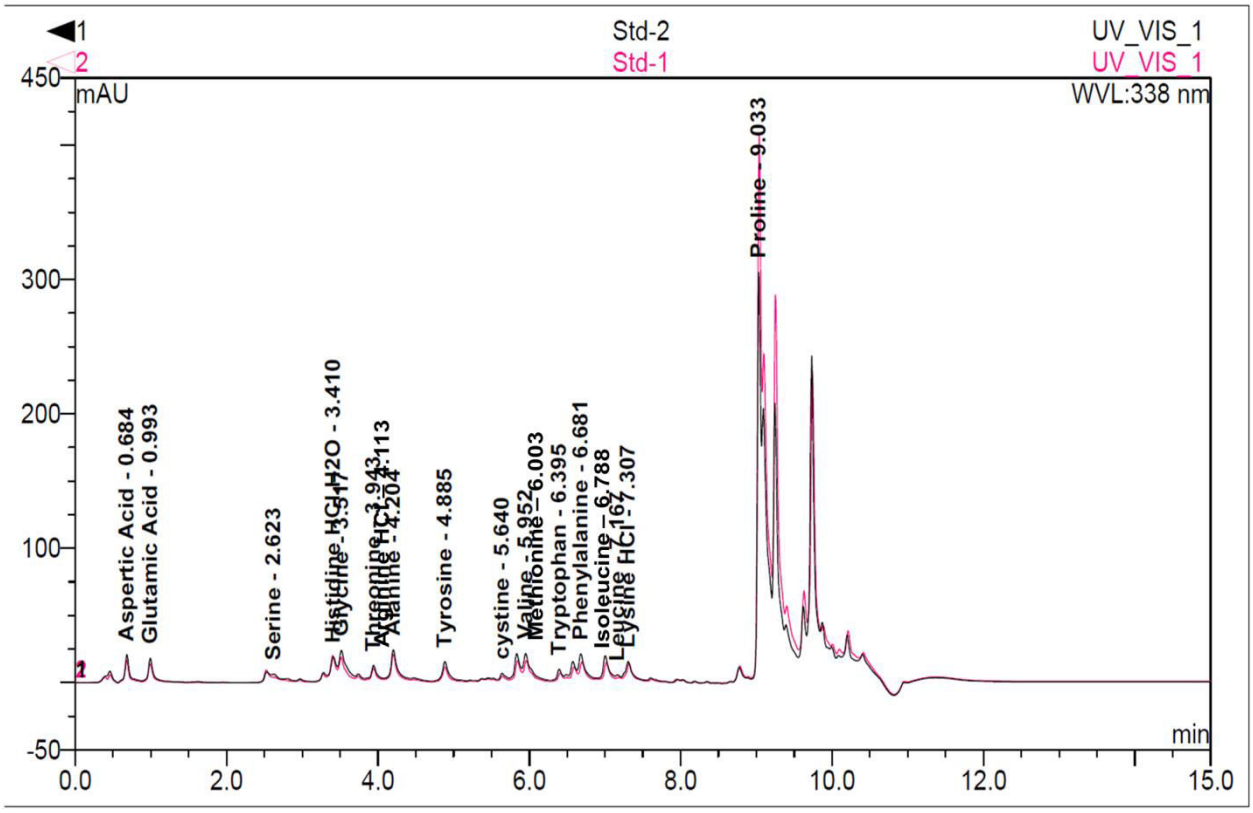

Figure: 3.6.3 Overlap chromatogram of amino acid standard-1 and standard-2

Chromatogram of control sample-1

Sample Name:	**C-1**	*Injection Volume:*	**5.0**
Vial Number:	**BE1**	*Channel:*	**UV_VIS_1**
Sample Type:	**Unknown**	*Wavelength:*	**338.0**
Recording Time:	**12/20/2015 23:00**	*Sample Weight:*	**1.0000**

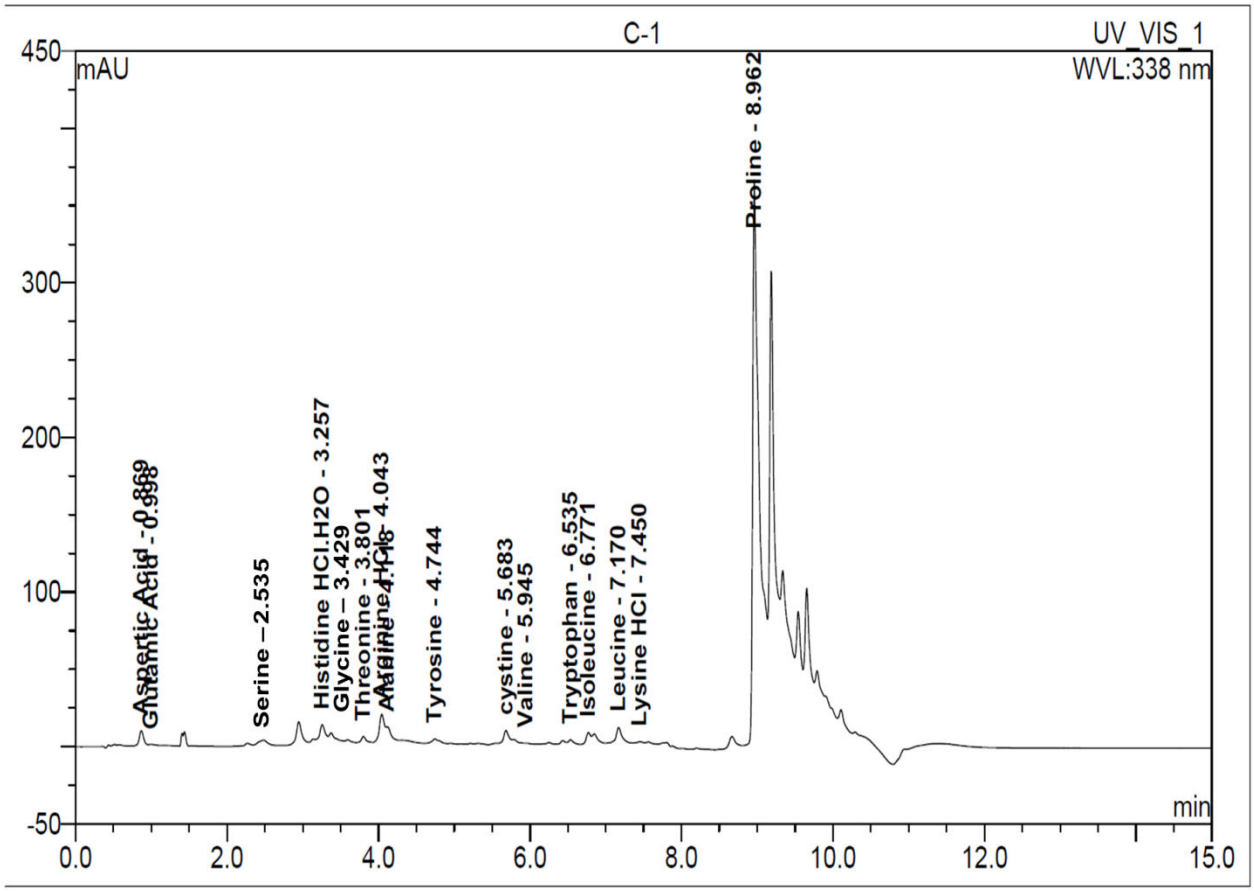

Figure: 3.6.4 Chromatogram of control sample-1

Chromatogram of patient sample-1

Sample Name:	P-1	Injection Volume:	5.0
Vial Number:	BC2	Channel:	UV_VIS_1
Sample Type:	Unknown	Wavelength:	338.0
Recording Time:	12/20/2015 17:33	Sample Weight:	1.0000

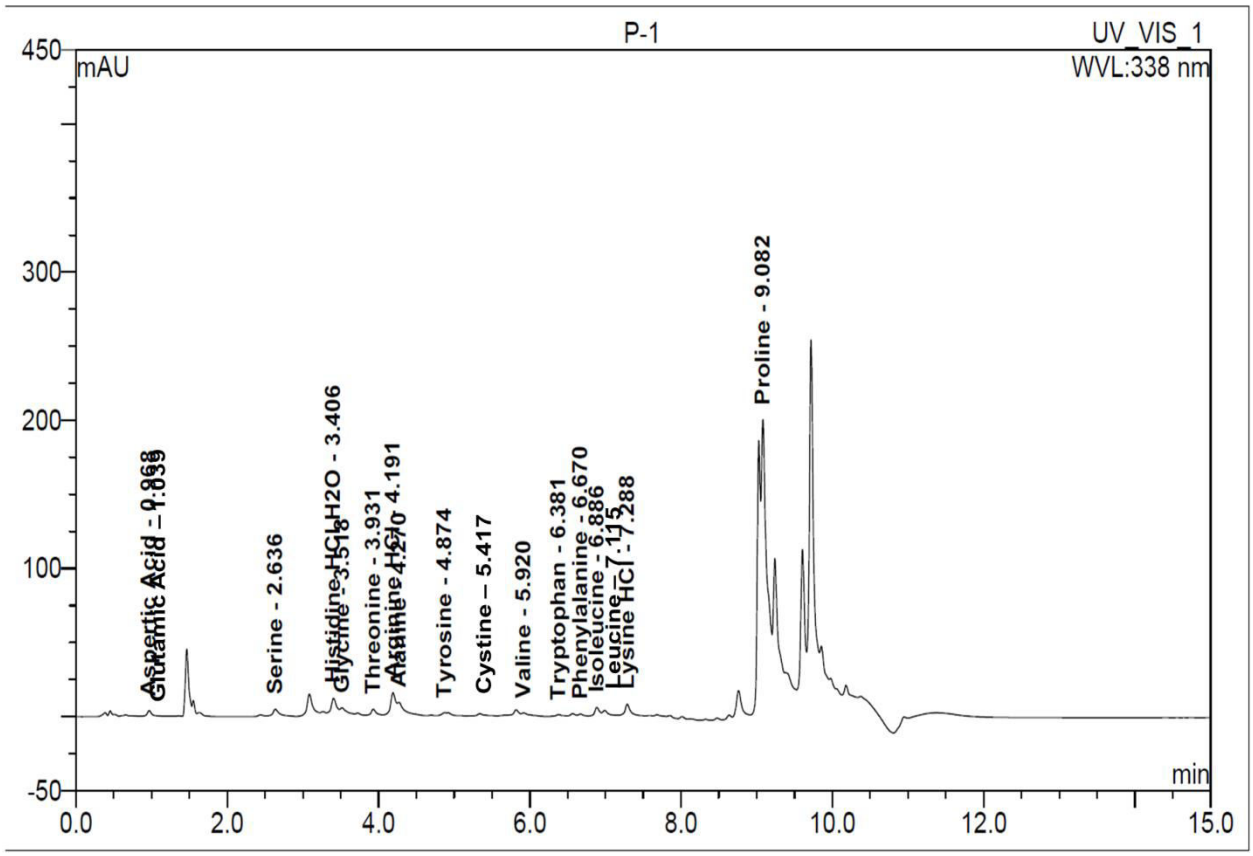

Figure: 3.6.5 Chromatogram of patient sample-1

3.6.5 Discussion on amino acid

Neurotransmitters are chemical substances in the brain that play a vital role in communication message through chemical signals between different nerve cells. Brain synthesizes these neurotransmitters from certain amino acids. Serotonin is synthesized from tryptophan and norepinephrine is synthesized from tyrosine, with the first step catalyzed by tyrosine hydroxylase. Both of these excitatory neurotransmitters regulate different functions in physiological systems like appetite, sleep, mood and behavior. Alteration of these neurotransmitter concentrations can cause different mental disorders. Deficiency of serotonin and norepinephrine are associated with depression (Belmaker and Agam, 2008).

Amino acid determination in our present study was performed on the basis of established retention time for individual amino acid under experimental conditions. In this study, serum levels of eighteen different types of amino acids have been measured in MDD patients and healthy controls. Here we reveal that mean serum level for most of the amino acids were decreased in MDD patients than the control subjects except glycine but the changes were not significant. Elevated level of serum glycine was found in MDD patients which was insignificant compare to control. Serum concentration of methionine, phenylalanine, tryptophan, and tyrosine were found significantly low in MDD patients compare to controls. Some previous study results support our current findings. Patients with MDD have significantly low level of glutamic acid in cerebral spinal fluid (Wu JL et al., 2016). The serum levels of aspartic acid and glycine were significantly lower with MDD patients which may serve as a clinical trait-marker for MDD (Lu YR et al., 2014). Decreased serum level of aspartic acid and glycine may serve as a clinical biomarker for MDD (Fu XY et al., 2012). Serum levels of glutamate, glutamine, glycine and taurine were significantly increased in the depressed patients compared to the controls (Mitani H et al., 2006). So our current findings may play a vital role in the diagnosis and management of MDD.

3.6.6 Conclusion

Serum levels of most of the amino acids have a tendency to decrease among MDD patients than the control subjects except glycine. But the elevated level of serum glycine was found in MDD patients which was not significant compare to control. Serum concentration of methionine, phenylalanine, tryptophan, and tyrosine were found significantly low in MDD patients compare to controls which may play a vital role in the diagnosis and management of MDD.

Pearson correlation coefficient supports that serum level of methionine in MDD patients has a significant positive correlation with phenylalanine and tryptophan. The same analysis found that smoking habit of MDD patients has a significant positive correlation with serum levels of methionine, phenylalanine and tryptophan.

CHAPTER FOUR

SUMMARY

4. Summary

Major Depressive Disorder is a mental disorder characterized by a universal and persistent low mood which is associated with low self-esteem and loss of interest or pleasure in day to day activities. Person's family, work and personal life is unfavorably affected by this disabling disorder. Normal emotions of sadness and sorrow are linked with depression if it persist after the external cause of these emotions dissipates and disproportionate to their cause. Sometimes without having external triggering factors classic severe states of depression may be happen. That's why it is very difficult to make a clear distinction between depression with and those without psychosocial precipitating events (Wakefield et al., 2007). A depressed mood with sadness or irritability is supplemented by several psychophysiological changes. Patient's work and family life hampered considerably by these changes that last at least two weeks. Lifetime prevalence of depression in USA is more than 12% in men and 20% in women (Kessler et al., 2003). Sometimes severe depression is termed as melancholia or vital depression in a much narrower way (Van Praag et al., 1987).

Manic episode consisting of hyperactivity, euphoria and increased pleasure seeking may appear in some MDD patients. There is an overlap of some pathogenetic mechanisms between major depression and Manic Episode. A distinct illness termed as bipolar disorder sometimes may be a history of mania (Belmaker, 2004). So depression is a mixed disorder with a highly irregular course with an inconsistent response to treatment and has no well-known mechanism. MDD is a biologically heterogeneous condition. The present study explicates the relationship among the different biological components like trace elements, anti-oxidant vitamins, immunoglobulins, malondialdehyde, cortisol and amino acids with MDD patients.

Trace elements regulate a number of cellular metabolic reactions and some of them also take part in etiology of several neurological disorders. Different research activities have shown the neurological effect on Mn, Zn, Cu, Fe and Se in a variety of neuropsychiatric disorders. Dopaminergic function has been affected by low level of Fe. Cu is important trace element because it an essential oxygen carrying blood component. It also plays a major role in developing chronic mental illness. High level of Zn is neurotoxic and higher level of Mn targets tissue. Lower level of Mn has been found in schizophrenic patient. Study shows that there is a significant deficiency of Mg was found in depression.

In this present study it has been observed that MDD patients have significantly low serum concentration of Zn, Mn, Ca and Mg than the healthy control which suggests the possible involvement of depleted serum trace element in the pathogenesis of depression. On the other hand serum concentration of Cu has a tendency to increase and Fe has a tendency to decrease in MDD patients compare to control subjects.

Alteration of serum level of vitamin A, vitamin C and vitamin E has been found in different neuropsychiatric disorders like depression, bipolar disorder, schizophrenia, OCD, oxidative stress is also involved in the etiology of this type of psychiatric disorders. The activation of immune cells, lipid peroxidation and oxidative stress are the main source of free radicals. Free radicals cause neuronal damage that plays an important role in the pathology of schizophrenia and depression.

The present study reveals that serum levels of vitamin A, E and C decreased significantly in MDD patients compared to the control subjects. These findings correlate the study findings of other psychiatric disorders. So the antioxidant enzymes and oxidative stress might have a pathological role in MDD patients. Thus these findings may play a key role in the diagnosis and treatment of MDD patients.

In some psychiatric disorders immunoglobulin levels have been changed. Depression and anxiety can alter the immunoglobulin levels. It has been reported that serum concentrations of IgA, IgG and IgM have been increased in schizophrenic patients. Also a small number of patients with MDD and chronic schizophrenia found to have low level of IgA concentrations. Patients with mania found only increased level of IgA. Different research studies show the involvement of immunoglobulins in different psychiatric disorder like schizophrenia, somatization, mania, depression and anxiety.

The present study indicates that there were no significant difference of serum IgA, IgG and IgM between MDD patients and healthy control subjects. From that finding it also revealed that there is a positive tendency to increase serum immunoglobulins in patient group compare to healthy control. Thus, we recommend further research to explain the exact role of immunoglobulins in the pathogenesis of MDD.

Prolonged psychological stress is a major causative factor for depression which leads to oxidative stress. Mood-regulating pathways in MDD may be affected by oxidative injury to lipids. MDA level was higher in patients with MDD than healthy controls and which is

associated with the severity of the disease. Antidepressant treatment can reduce MDA level in MDD patients (Mazereeuw et al., 2015). Serum level of MDA significantly higher in schizophrenic patients compared to healthy controls. Another study reveals that panic disorder patients have considerably higher level of MDA than the healthy control subjects (Nahar et al., 2013). This result proposed that oxidative stress plays an important role for causing depression and antidepressant activity may be arbitrated by improving oxidative stress or antioxidant function (Jiménez-Fernández et al., 2015).

It has been explored that MDD patients have high serum concentration of MDA than the healthy control. The present study suggests that alteration of serum MDA may contribute to the pathogenesis of MDD. This finding may have correlation between MDA level in MDD patients and the degree of the disorder.

Patients with depression may have significantly elevated levels of free serum cortisol. Cortisol is supposed to be a risk factor for stress and age related disorders, such as major depression and Alzheimer's disease. Pathophysiology of MDD may be associated with the elevated cortisol level in serum. Association between high serum cortisol and AD was found significantly (Zvěřová et al., 2013). Perioperative serum cortisol concentrations may be elevated which is related with delirium after coronary artery bypass graft surgery. This may be a vital pathophysiological concern in the augmented risk of postoperative delirium seen in patients with a preoperative diagnosis of major depression (Kazmierski et al., 2013).

The present study suggests that patients with MDD have significantly higher serum cortisol level compare to healthy control. This alteration of serum cortisol level may contribute to the pathogenesis of MDD. This finding may have a correlation between serum cortisol level in MDD patients and the degree of the disorder.

Neurotransmitters are chemical substances in the brain that play a vital role in communication message through chemical signals between different nerve cells. Brain synthesizes these neurotransmitters from certain amino acids. Serotonin is synthesized from tryptophan and norepinephrine is synthesized from tyrosine, with the first step catalyzed by tyrosine hydroxylase. Both of these excitatory neurotransmitters regulate different functions in physiological systems like appetite, sleep, mood and behavior. Alteration of these neurotransmitter concentrations can cause different mental disorders. Deficiency of serotonin and norepinephrine are associated with depression (Belmaker and Agam, 2008). Patients with

MDD have significantly low level of glutamic acid in cerebral spinal fluid (Wu JL et al., 2016). The serum levels of aspartic acid and glycine were significantly lower with MDD patients which may serve as a clinical trait-marker for MDD (Lu YR et al., 2014). Decreased serum level of aspartic acid and glycine may serve as a clinical biomarker for MDD (Fu XY et al., 2012). Plasma levels of glutamate, glutamine, glycine and taurine were significantly increased in the depressed patients compared to the controls (Mitani H et al., 2006).

In this study, serum levels of eighteen different types of amino acids have been measured in MDD patients and healthy controls. Here we reveal that mean serum level for most of the amino acids were decreased in MDD patients than the control subjects except glycine but the changes were not significant. Elevated level of serum glycine was found in MDD patients which was insignificant compare to control. Serum concentration of methionine, phenylalanine, tryptophan, and tyrosine were found significantly low in MDD patients compare to controls which may play a vital role in the diagnosis and management of MDD.

The objective of this study was to compare the serum levels of trace elements, antioxidant vitamins, immunoglobulins, MDA, cortisol and amino acids in MDD patients with control subjects. Then find out the correlation between these parameters in serum level and the disease status of MDD. Our findings strongly indicate that serum concentrations of most of the trace elements, amino acids and anti-oxidant vitamins were lower in MDD patients compared to healthy control. But elevated level of serum MDA and cortisol were found in patient group than control. There were no significant difference of serum IgA, IgG and IgM concentrations between MDD patients and healthy control subjects. Further large scale study is required to reveal more about the disease and establish our findings.

CHAPTER FIVE

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