Serum Level of Inflammatory Markers in Patients with Schizophrenia Attending a Tertiary Service in Addis Ababa, Ethiopia

A thesis submitted to the school of graduate studies of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Medical Biochemistry

Feyissa Challa (BSc, MLT)

August 2016

Addis Ababa, Ethiopia

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August 2016

This is to certify that the thesis prepared by Feyissa Challa Wake entitled***: " Serum Level of Inflammatory Markers in Patients with Schizophrenia patients at Attending a Tertiary Service in Addis Ababa, Ethiopia"*** and submitted in partial fulfillment of the requirements for the Degree of Master of Science (Medical Biochemistry) complies with regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the Examining Committee:

Examiner: Dr. Mistire Wolde Signature: _____ Date: _____

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_____

Chair of Department or Graduate Program Coordinator

Declaration

I declare that this research paper titled ***Serum Level of Inflammatory Markers in Patients with Schizophrenia patients at Attending a Tertiary Service in Addis Ababa, Ethiopia,*** is my original work and has nor presented for a degree in any other University, and that all sources of materials used for the research have been properly and suitable acknowledged.

Feyissa Challa

Signature_____

Date _____

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The study reported in this thesis was carried out at Amanuel Specialized Mental Hospital, at Department of Biochemistry Addis Ababa University and at the Department of Clinical Chemistry of the Ethiopia Public Health Institute (EPHI).

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Table of content

List of Tables

List of Figures

Abbreviations

AAU	Addis Ababa University
APA	American Psychiatry association
BMI	Body Mass Index
DSM	Diagnostic and Statistical Manual for Mental Disorders
ECLIA	Electrochemiluminescence immunoassay
EPHI	Ethiopia Public Health Institute
FMOH	Federal Ministry of Health
hsCRP	high sensitive C-reactive protein n
ICD	International classification of diseases
IL-6	Interleukin-6
LAMIC	low and middle-income countries
MGH	Massachusetts General Hospital
NCDs	Non-communicable diseases
PANSS	Positive and Negative syndrome scale
WHO	World Health Organization
NCDs	Non-communicable diseases

Abstract

Introduction: -*Accumulating evidence indicates that schizophrenia is accompanied by an activation of the immune/inflammatory system. But there is limited data from low and middle-income countries like Ethiopia. Inflammatory markers may even be more important in these settings where infectious conditions may have more prominent role in the causation and maintenance of schizophrenia.*

Objective: - *To assess the level of inflammatory markers in patients with schizophrenia*.

Materials and methods - *The level of inflammatory markers among patients with schizophrenia were compared with non-affected control groups. The study population consisted of a total 132 study participants, including 82 with schizophrenia and 50 control subjects. High sensitive C-reactive protein (hsCRP) and Interleukin-6(IL-6) were measured using Cobas e 411 and Cobas Intgera 400 Plus analysers at EPHI. Linear regression was used to adjust for confounders in estimating the level of inflammatory markers. The study was conducted from January 2015 to March 2016.*

Results: - *The levels of hsCRP were significantly increased in patients with schizophrenia when compared to control groups (B=; 95% CI=; p=0.003), and also there were significant differences in IL-6 were found in the patients with schizophrenia when compared control groups (B=; 95% CI=; p=0.002).Controlling for age, sex and body mass index, having a diagnostic schizophrenia was the most important factor associated with increased IL-6 and hsCRP.*

Conclusion: - *Higher levels of both hsCRP and IL-6 appear to be associated with schizophrenia, suggesting a role for the inflammatory process in the pathophysiology of schizophrenia. Further investigation using larger sample size are warranted. Clinical trials of interventions to decrease inflammation are worth considering.*

Key words: *- schizophrenia, Interleukin-6 (IL-6), high sensitive C - reactive protein (hsCRP) Inflammatory markers, Ethiopia.*

1. INTRODUCTION

Mental disorders are among the Non-communicable diseases (NCDs) responsible for a high proportion of the death and disability burden around the world. The global prevalence of mental disorder is increasing, with the greatest burden occurring in both developed and developing countries, and it is projected to increase over the next decades (WHO 2013).

Mental disorders or mental illness is a term that refers to a set of medical conditions that affect a person's thinking, felling mood, ability to relate to others and daily functioning. Sometimes referred to as mental health conditions or neuropsychiatric disorders, these conditions affect hundreds of millions of people worldwide. According to the WHO, one in four people-about 450 million people develop some kind of mental illness or disorders at some point in their lives. This is an overwhelming figure considering that mental health is not only essential for individual well-being but also essential for enhancing human development including economic growth and poverty reduction (Kessler *et al.,* 2011; WHO, 2011).

A mental disorder is often misunderstood. For centuries, it has been seen as either control by evil spirits, a moral weakness or punishment from a God. In Ethiopia, a cross-sectional survey indicated that most respondents attributed about the mental illness is more related to supernatural causes like spirit possession, evil eye, rather than the result of biomedical or psychosocial cause*s* (Deribew & Tamirat, 2005). As a result of this, affected person from mental disorders or family goes to traditional healers and seek from religion rather go to a health facility (Shibre *et al.,* 2008; Jacobsson, 2002).

Based on WHO report 450 million worldwide suffer from mental disorders in both developed and developing countries including 60 million from bi-polar disorder and 21 million from schizophrenia. By 2020, behavioral health disorders will increase 20 times which could make them the second leading causes of disability in the world. People with mental disorders experience extremely higher rates of disability and mortality (WHO, 2001). For example, persons with major depression and schizophrenia have a high chance of dying prematurely including in low and middle-income countries (LAMIC) from other health problems such as cancers, cardiovascular diseases, diabetes and HIV infection because this problem is often left unattended (Sicras-Mainar *et al.,* 2015; Teferra et al., 2011).

In Africa, even though the mental disorders are given low priority in health services, it is one of the public health problems with the highest mortality rate when compared to the general population. In this region, as in most LAMIC, mental health policies and interventions receive low priority and limited resources. Stigma also hampers progress. In a survey of 45 African countries, less than one-half reported having a dedicated mental health policy and only one-quarter reported having manuals available for the management and treatment of mental health disorders in the majority of primary health-care settings. Mental health services for young people, however, can play a key role in reducing the burden of mental health disorders and help control the rise of NCDs (Whiteford *et al.,* 2013) .

In Africa, a few data indicate that the prevalence of mental disorders in Kenya is 10% (Jenkins *et al*., 2012), common mental disorders among primary health clinics and traditional healers in urban Tanzania showed that the prevalence is 24 % and 48% respectively (Ngoma *et al.*, 2003) and in Ethiopia, mental illness (Table 1) is the leading non-communicable disorder in terms of

burden with depression and schizophrenia included in the top ten most burdensome conditions, out-ranking HIV/AIDS in Ethiopia (Federal Ministry of Health, FMOH, 2010)

Table 1. Prevalence of mental disorders in Ethiopia

Mental Illness	Prevalence / Incidence (%)
Bipolar disorder	0.5
Schizophrenia	0.5
Depression	5
Suicide (completed)	7.7/100000/year
Suicide attempt	3.2
Alcohol-problem drinking	2.2-3.7
Alcohol dependence	1.5
Cannabis abuse	1.5
Childhood mental illnesses	12-25
Epilepsy	1
Dementia	No Data

Source: *Ethiopia mental health Gap Action Programme (mhGAP) working group 2010 (FMOH)*

A report by the World Economic Forum and Harvard School of Public Health report said that the cumulative output loss of US$ 47 trillion over the next two decades. This loss represents 75% of global GDP in 2010 (US$ 63 trillion). It is recognized that mental disorder or illnesses are the public health problems both in developing as well as developed countries and also it is the most expensive disorders among non-communicable disease in terms of health care expenditure (Bloom *et al.,* 2012).

1.1 Schizophrenia

1.1.1 History

Schizophrenia was first described by Dr Emil Kraepelin in 1887, (Tölle, 2008) using the word dementia praecox. He categorized dementia praecox in to three subtype cataonia, hebephernia, and adds simple parapherania. But the word “schizophrenia” was coined in 1911 by Dr Paul Eugen Bleuler, a Swiss psychiatrist, derived from the Greek words for′Schiz-broken; “Phrenos”: soul or heart- "split" and "mind", when he gave lecture on the annual conference at German Berlin. In 1959, Dr Kurt Schneider, a German psychiatrist, defining features of schizophrenia especially First-rank symptoms (FRS) (American Psychiatric Association, 2013). Even after 100 years, Schizophrenia still remains an enigma and it is considered to be among the most common psychiatric disturbances worldwide (Heckers, 2011; McGlashan, 2011).

1.1.2 Diagnostic definition

There is no clear clinical pictures or signs for Schizophrenia. According to Diagnostic and Statistical Manual for Mental Disorders (DSM) (American Psychiatric Association, 2013) the disorder is defined by a set of symptoms which are described DSM. There are two categories of symptoms: The positive symptoms which are characterized by hallucinations, delusions, and disorganized speech; the negative symptoms have decreased emotional expression and lack of motivation symptoms (Holder & Wayhs, 2014). To fulfill the diagnostic criteria patients must have had a minimum set of characteristic psychotic symptoms for at least one month, as well as some symptoms of the disorder lasting for at least 6 months. A marked dysfunction must also be present.

Types of Schizophrenia

There are more than five subtypes of schizophrenia are catatonic, paranoid, disorganized, residual, and schizoaffective disorder. Many individuals have symptoms of more than one type.

Catatonic-type

Patients whose clinical presentation is dominated by profound changes in motor activity, negativism, and echolalia or echopraxia.

Paranoid-type

Patients who have a prominent preoccupation with a specific delusional system and who otherwise do not qualify as having the disorganized-type disease

Disorganized-type

In which disorganized speech and behavior are accompanied by a superficial or silly affect.

Residual-type

Negative symptomatology exists in the absence of delusions, hallucinations, or motor disturbance.

Schizoaffective disorder/ Schizophreniform disorder

Patients who meet the symptom requirements but not the duration requirements for schizophrenia, and schizoaffective disorder is used for those who manifest symptoms of schizophrenia and independent periods of mood disturbance. Prognosis depends not on symptom severity but on the response to antipsychotic medication. A permanent remission without recurrence does occasionally occur. About 10% of schizophrenic patients commit suicide (APA 2013).

1.1.3 Prevalence and Risk factor

Schizophrenia is arguably the most serious major psychiatric disorder affecting more than 21 million people worldwide. The WHO has identified schizophrenia as one of the ten leading global disability worldwide. In the United States of America, this disease affects about 2.4 million (1% of the general Population) (Keller *et al.,* 2013). Moreover, each year around 1.5 million people worldwide are diagnosed with schizophrenia (Fleischhacker *et al.,* 2014).The peak age of onset of first psychotic episodes is between 15-30 years but slightly older in females. The disease is equally distributed or prevalent among both men and women. But there is a difference in the onset of the course of the illness. Men have an early onset of schizophrenia when compared to women (Holder & Wayhs, 2014).

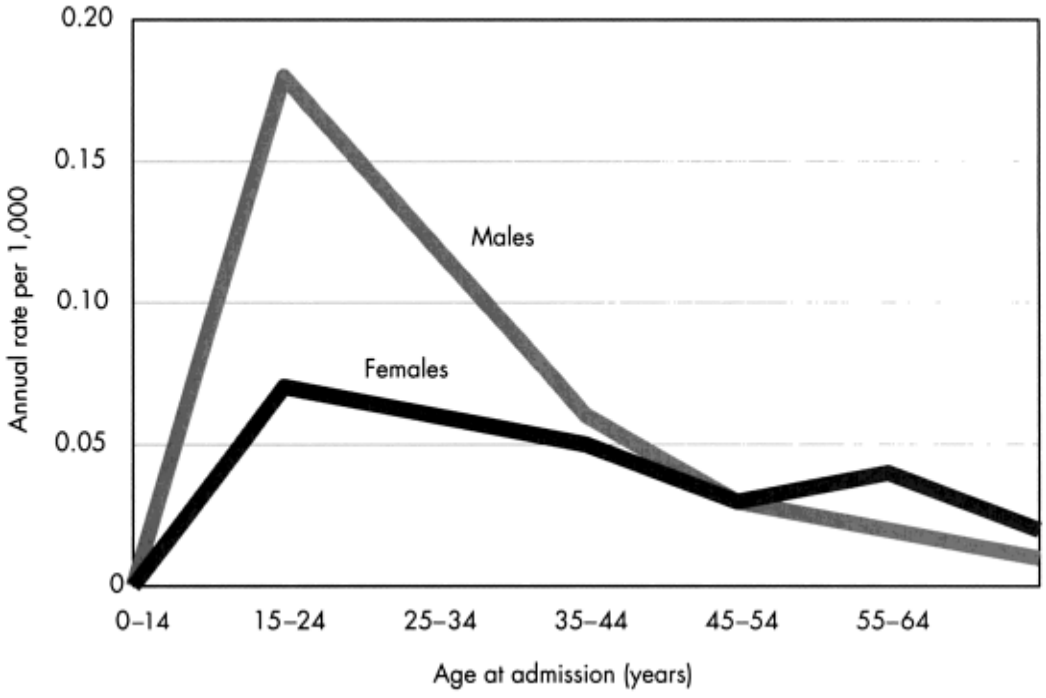


Figure 1. Age of onset of schizophrenia (WHO 1967)

An epidemiologic study indicates that several risk factors for schizophrenia including genetic susceptibility (Allen *et al.,* 2008), season of birth (McGrath & Welham 1999; Messias *et al.* ,2004), and increasing parental age (Sipos *et al.,* 2004; Wu *et al.,* 2012), and prenatal exposure to infection (Miller *et al.,* 2013). Despite some of the conflicting findings, prenatal infection and genetic susceptibility were considered relevant in the association of schizophrenia with inflammation. Inflammation is associated with the etiology of and pathophysiology of major psychiatric disorders such as bipolar disorders (Leboyer *et al.,* 2012), depression (Shelton & Miller 2011, Dantzer *et al.,* 2011) and schizophrenia (Muller *et al.,* 2012; Kirkpatrick & Miller 2013; Myint 2013). Genetic factors are involved in at least a subset of individuals who develop schizophrenia. Schizophrenia is observed in approximately 6.6% of all first-degree relatives of an affected proband. If both parents are affected, the risk for offspring is 40%.....References

Individuals with schizophrenia have high levels of medical comorbidity and cardiovascular risk factors. The major causes of mortality among schizophrenia patients are cardiovascular diseases (CVD) which associated with obesity, dyslipidemia, Impaired fasting glucose (Ryan *et al.,* 2003), and hypertension which is the result of lifestyle factors (poor nutrition, lack of exercise, alcohol consumption) and also antipsychotic medication especially clozapine.(Sicras-Mainar *et al.,* 2015). Due to these reason and other factors patients diagnosed with schizophrenia have a higher overall mortality rate which is estimated 20 years earlier than the general population (Tiihonen *et al.,* 2009) and also reported from a cohort study of people with schizophrenia in Ethiopia (Teferra *et al.,* 2011).

1.2 Cytokines

Cytokines are messengers that, besides hormones and neurotransmitters, represent the most important means of communication between human cells. The quantity and type of cytokine secreted depends on the type of cell type, the differentiation stage and the activation state of the cell. Cytokines are polypeptides with a molecular mass of 6-70 kDa which exert their effect via specific receptors both on the cell membrane and in the cell (Kumar & Clark, 2012).

A great number of cytokines have been discovered. As the knowledge and clinical practice about them has increased, they have become important markers for diagnostic purposes of different clinical conditions (Kumar & Clark 2012). Among the known cytokines, the determination of Tumor necrosis factor alpha (TNF-α), Interleukin 1β (IL-1β) and Interleukin -6 (IL-6) are three that have been widely investigated. Although all cells may secrete cytokines but monocytes and macrophages tend to produce more of them than any other cells (Drexhage *et al.,* 2010).

1.3 Inflammation

Inflammation is non-specific, first lines of defense in response to injury or infection (Kirkpatrick & Miller, 2013) and also the complex response which involves activation and recruitment of immune cells, and leads to increase blood supply and vascular permeability (Miller *et al.,* 2011). The inflammatory process also involves complement system in which a group of proteins are activated and kills bacteria and parasites. Cytokines are the key molecules that control or regulate inflammation process: they have also important roles in the immune system (Pepys & Hirschfield, 2003).

1.4 Inflammation and Schizophrenia

Cytokines, which encompass a family of soluble polypeptides, represent markers of prenatal infection and inflammatory conditions. Cytokines orchestrate the immune response to the presence of infections and other noxious insults and therefore play an essential role as part of the immune system. Hence, cytokine elevations may indicate exposure to a number of different types of infections during pregnancy (Suvisaari & Mantere, 2013).

A Large body of evidence suggesting that prenatal infections give rise to an increased risk of schizophrenia, some mixed evidence has suggested a link between certain infections in those who already have developed schizophrenia. Recent studies indicated that infection (measles, rubella, varicella-zoster, polio, Toxoplasmosis) during pregnancy in mother of the offspring leads to later development of schizophrenia (Kneeland & Fatemi, 2013). People with schizophrenia have increased blood concentrations of inflammatory cytokines like TNF-alpha (Kunz *et al.,* 2011), IL-6, (Stojanovic *et al.,* 2014) IL-2 and hsCRP (Lin *et al.,* 2013). In a series of studies conducted on animal models show that immune activation of the mother during the second trimester of pregnancy leads to developing schizophrenia-like symptoms. Increased IL-8 during pregnancy has been associated with schizophrenia in humans, especially at a time of neurodevelopment stage (Kneeland & Fatemi, 2013).

1.5 Inflammatory markers

There is a wide range of inflammatory molecules currently identified. Some of the inflammatory molecules (C-reactive protein and Interleukin-6) with the relation to schizophrenia described here.

1.5.1 C - reactive protein and Schizophrenia

C - reactive protein is the classical acute phase protein historically one of the first protein to recognized. Tillet and Francis first describe it in 1930 as substances in the serum of patients with acute inflammatory diseases (Tillett & Francis, 1930).

The CRP gene is located on the first chromosome (1q21-q23). It has a cyclic pentameric structure with calcium-dependent ligand binding. Five identical non-covalently associated protomers are situated symmetrically around a central pore. Each protomer consists of 206 amino acids residues with ligand binding site having a pocket with two ions of calcium. Calcium ions are required for ligand binding and stability of the molecule (Hirschfield & Pepys, 2003).

The name CRP is derived because of the ability to precipitate the somatic C-polysaccharide of Streptococcus pneumoniae (Pepys & Hirschfield, 2003). Initially, it was thought to be a pathogenic secretion because the elevated of serum CRP was found in myriad diseases. However, the finding of its hepatic synthesis discovered that it is a native protein. It was also investigated that hepatocytes were stimulated to produce CRP under the influence of inflammatory markers including IL-6 and tumor necrosis factor-α. It is well known that the level of CRP increases drastically in case of any of inflammation, and the elevated levels are usually observed in inflammatory conditions like rheumatic fever, cardiovascular diseases, and metabolic dysfunction. In addition to this, it is also known to be associated with cognitive impairment, depression, and schizophrenia (Singh & Chaudhuri, 2014b).

Evidence from animal studies shows that CRP per se, which is produced in the periphery, does not cross the blood-brain barrier under normal conditions; however, a high concentration of

peripheral CRP can increase the blood–brain barrier paracellular permeability by affecting the function of tight junctions (Campbell *et al.,* 2014). This allows pro-inflammatory cytokines and CRP itself to enter the central nervous system and ultimately turn the brain susceptible to CRP. Thus, increased CRP levels may explain at least in part the increased permeability of the blood–brain barrier that is assumed to have a role in psychiatric disorders, including schizophrenia. Once within the central nervous system, CRP may directly induce neuroinflammation. Glial cells provide critical support for neurons in the CNS and are comprised of three major cell types: astrocytes, oligodendrocytes, and microglia. Astrocytes provide metabolic support and play a key role in glutamatergic neurotransmission. Oligodendrocytes also provide metabolic support and produce myelin sheaths, which insulate the axons. Microglia is the chief immune effector cells in the brain. All three cell types have been implicated in the pathophysiology of schizophrenia. Evidence from a laboratory experiment, primary cell culture, CRP induces a pro-inflammatory state in microglia (Adami *et al.,* 2001).

Evidence indicating that significance of neuroinflammation and immunogenetics in patients with schizophrenia. This neuroinflammation is characterized by an increased serum concentration of several pro-inflammatory cytokines (Zakharyan & Boyajyan, 2014). Increased serum concentrations of different inflammatory markers including IL-6 and CRP have been observed in schizophrenia patients (Singh & Chaudhuri, 2014a). Increased inflammatory markers both in serum and CSF levels, a suitable marker for the destruction of CNS tissue in the

context of different diseases including neurodegenerative disorder, were reported in schizophrenia patients with negative symptoms or a chronic duration figure 2.

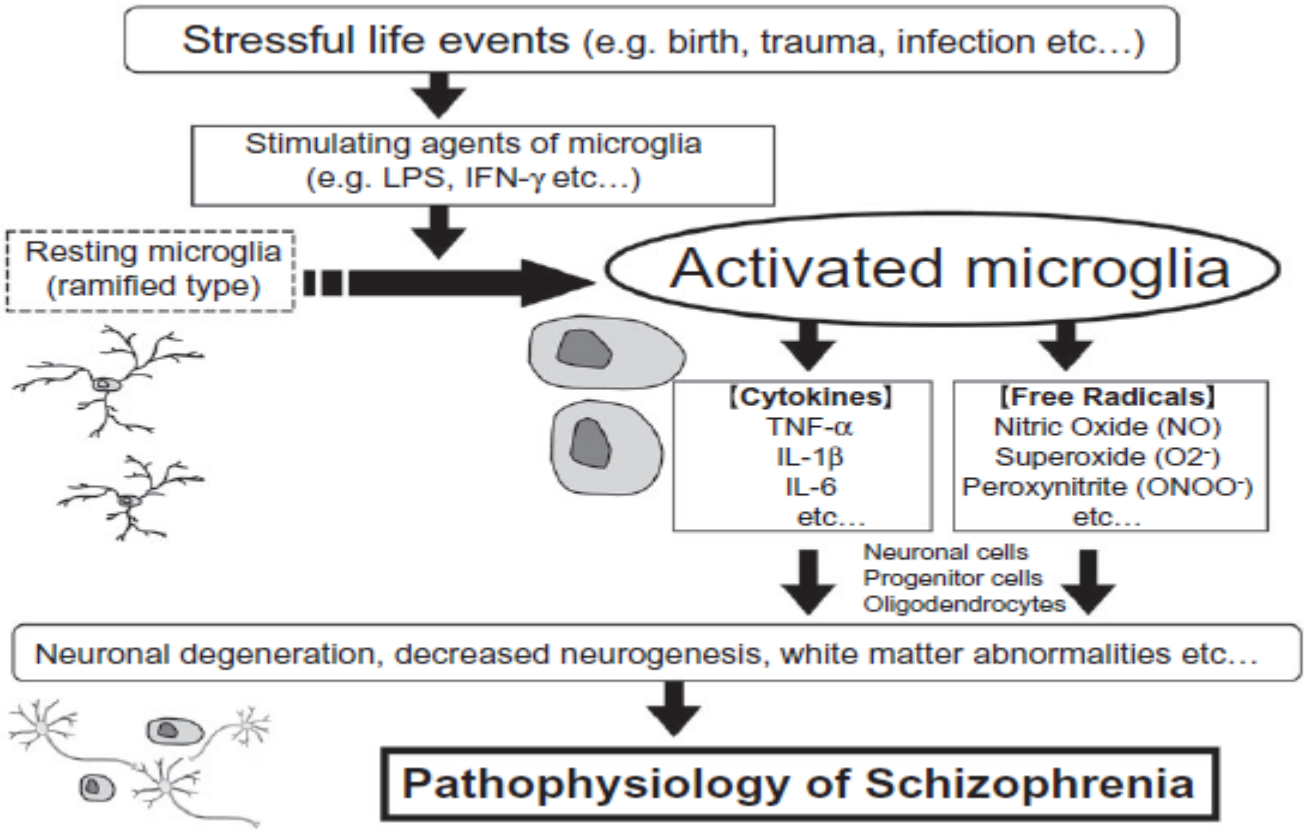


Figure 2. Microglia hypothesis of schizophrenia. IL, interleukin; TNF, tumor necrosis factor. Adapted from (Monji, Kato, & Kanba, 2009)

Numbers of efforts have been made previously to identify the inflammatory marker for schizophrenia but conflicting findings were documented. One of the nonspecific inflammatory markers and well known identified in patients with schizophrenia is CRP. In a study from Taiwanese population and Arab schizophrenic patients based on a small number of Asia and Middle East study indicated that increased high-sensitivity C-reactive protein levels in this group (Lin et al., 2013; Akanji et al., 2009). A study observed that patients with schizophrenia who were having both low BMI and under typical antipsychotic have also a high concentration

of serum CRP (Carrizo et al., 2008). Another meta-analysis done by Miller and his colleague's shows, there was a 28 % prevalence of an elevated CRP level in patients with schizophrenia and related disorders (Miller, Culpepper, & Rapaport, 2014). Various studies have reported the abnormalities of the CRP in the patients with schizophrenia. In contrast to this finding Hope *et .,al* and Fernandez-Egea et al., reports that there were no associations between the level of serum CRP and patient with schizophrenia (Fernandez-Egea *et al.,* 2009; Sigrun Hope *et al.,* 2009) .This inconsistency results may result from heterogeneity of studies participants, sample size, different methodology, and other confounding factors.

1.5.2 Interleukin-6 and Schizophrenia

Interleukin-6 is a pleiotropic one, earliest discovered and most investigated cytokines which act as both pro-inflammatory and anti-inflammatory. It is secreted by different cells including T-cells and macrophages and also produced by activated astrocytes and microglia cells in the central nervous system. IL-6 is not only involved in inflammation and infection responses but also in the regulation of metabolic, regenerative, and neural processes (Frydecka et al., 2013).

In schizophrenia the stimulation of epigenetic modification by IL-6 has been proposed as a mechanism in the pathology of schizophrenia through the hypermethylation and repression of the glutamic acid decarboxylase 67(GAD67) promoter (Kundakovic *et al.,* 2009). This hypermethylation may potentially lead to the decreased GAD67 levels seen in the brains of people with schizophrenia. GAD67 may be involved in the pathology of schizophrenia through its effect on GABA levels and on neural oscillations. Neural oscillations occur when inhibitory GABAergic neurons fire synchronously and cause inhibition of a multitude of target excitatory neurons at the same time, leading to a cycle of inhibition and disinhibition. These neural

oscillations are impaired in schizophrenia, and these alterations may be responsible for both positive and negative symptoms of schizophrenia (Uhlhaas & Singer, 2010) .

The interconnection between IL-6 and schizophrenia has been studied for long time and the finding suggested that high level of IL-6 among schizophrenia (Mauricio Kunz *et al.,* 2011; Kubistova *et al.,* 2012). Elevated high level of IL-6 was reported in chronic schizophrenic patients. A study conducted by Luo and co-investigators indicated using 160 patients with schizophrenia and 80 control groups shows that the chronic schizophrenia patients had significantly higher serum IL-6 levels than controls (Luo *et al.,* 2014).

Similarly, a study conducted in the southeast part of Iran by Shahraki *et.,al,* using 30 patients with schizophrenia and 20 health controls found that the serum IL-6 levels of the schizophrenic patients were significantly higher than those of the controls (5.28±1.1 and 2.54±0.32 pg/mL, respectively; P = 0.01) (Shahraki et al., 2016).

Inflammatory markers have been investigated in large studies, and have suggested elevated levels of some inflammatory markers reported in schizophrenia, although with somewhat inconsistent findings. No previous studies have investigated in Ethiopia whether elevated inflammatory markers are related to schizophrenia patients in Ethiopia.

Therefore, in our current study our aim was to investigate the level of inflammatory markers among patients with schizophrenia systematically established diagnosis of schizophrenia.

1.6 SIGNIFICANCE OF THE STUDY

An increasing number of clinical, epidemiological, and experimental studies have shown links between schizophrenia and inflammatory as indicated in different researchers conducted elsewhere. But, there is no data on the level of inflammatory markers in schizophrenia patients undertaken in Ethiopia.

Monitoring of these inflammatory markers and use of anti-inflammatory drugs enable to improve quality health of patients with schizophrenia. This research provide a baseline information that can be used as a reference for further study on this area.

Therefore, this study aimed to investigate the serum level of inflammatory markers among patients with schizophrenia.

1.7 HYPOTHESIS

We hypothesized that patients with schizophrenia will have increased concentrations of serum C-reactive protein and Interleukin-6 compared with non-affected control groups.

2. OBJECTIVE

2.1 General Objective:

To assess the level of inflammatory markers in patients with schizophrenia at Amanuel Specialized Mental Hospital, Addis Ababa Ethiopia

2.2 Specific Objectives

- To compare the level of inflammatory markers (hsCRP) between patients with schizophrenia and control groups.
- To compare the level of inflammatory markers (IL-6) between patients with schizophrenia and control groups.
- To evaluate additional factors, such as body mass index and age, responsible for the differences in level of inflammatory markers.
- To assess clinical factors, such illness severity and age of onset, that may contribute to increases in concentrations of inflammatory markers among patients with schizophrenia

Operational Definitions

Age of onset: Age of onset was defined as the age the schizophrenia patients when the first symptoms as reported by the patients family members and reviewing medicals records.

Body mass index: A standardized estimate of an individual's relative body fat calculated from his or her height and weight. It can be used to determine if study participants are at underweight (<18.50kg/m2), normal weight (18.50-24.99kg/m2), overweight/obese (>30kg/m2). It is equal to the weight, divided by the square of the height (BMI = weight / (height * height), weight in kilograms and height in meters).

Duration of current episode: is a time in which the current episode was seen on the schizophrenia patients**.**

PANSS or the **Positive and Negative Syndrome Scale** is a medical scale used for measuring symptom severity of patients with schizophrenia.

The Diagnostic and Statistical Manual of Mental Disorders (DSM), published by the American **Psychiatric** Association (APA), offers a common language and standard criteria for the classification of mental disorders.

3. MATERIALS AND METHODS

3.1 Study Design

The study was nested within another study, which aims to evaluate the impact of minocycline, an anti-infective and anti-inflammatory drug. For the purposes of this thesis, inflammatory markers from the first 82 participants with schizophrenia in the original study were compared with non-affected control participants.

3.2 Study Area and Study Period

The study was conducted at Amanuel Specialized Mental Hospital, Department of Biochemistry, School of Medicine, College of Health Sciences, Addis Ababa University, and Ethiopia Public Health Institution (EPHI) Clinical Chemistry Laboratory, Addis Ababa Ethiopia, during the period of January 2015 to March 2016.

Amanuel Specialized Mental Hospital

Amanuel hospital is the main institution for the care of those with severe mental illness. Over 95% of the mental health budget of the country is channelled through Amanuel hospital. The hospital runs both inpatient and outpatient services. At the outpatient service over 150,000 persons with mental disorder, most with severe mental illness are seen every year. The inpatient unit has around 280 beds. Analysis of data from the inpatient unit indicates that most patients were admitted with schizophrenia (Fekadu *et al.,* 2007).

Ethiopia Public Health Institute (EPHI)

EPHI is the technical arm of the Federal Ministry of Health and the institute is mandated to conduct research on health and nutritional problems of the country, traditionally used traditional

medicines and medical practices along with modern drugs, and to provide high-tech referral diagnostic services.

National reference laboratory for Clinical Chemistry Laboratory, which is equipped with the state -of -art analyzers, responsible for the provision of high-level diagnostic laboratory testing services for patients referred to the institute and specimens referred from all regional and federal health facilities. In addition to this, the laboratory also participated in different research activities both biomedical and survey and surveillances.

3 .3 Source and Study population

3.3.1 Source population

The source population were patients with schizophrenia 18-65 years of age who were diagnosed with schizophrenia for another study at Amanuel Specialized Mental Hospital. For the control group, we used blood samples collected from health centers. They were confirmed to have no serious medical conditions, including severe mental disorders, at the time of the sample collection.

3.3.2 Study Population

The study population consisted of a total 132 study participant, including 82 with schizophrenia and 50 control subjects. The participants with schizophrenia were consecutive patients who consented to be part of the study.

Patients

The study subjects were patients with schizophrenia who had Primary Axis I diagnosis (DSM-IV) of schizophrenia, any subtype using a set of inclusion and exclusion criteria with the help of standardised diagnostic assessment by a psychiatrist. The diagnosis was according to International classification of Diseases (ICD) 10 classification system.

Controls

50 Healthy Volunteer controls were randomly selected from Addis Ababa Health Centers (wordea 5 Health Center, wordea 7 Health Center), which were within the catchment area of Amanuel hospital. Blood collection for control subjects were done during visiting health centers for HIV testing. All control groups were checked by a health professional for general medical check-up, including HIV testing and severe mental disorders.

3.4 Sampling Technique and Sample size determination

The sample size of different studies affected by different factors including effect size, parameters used, and practical constraints such as finance, subgroup analysis, and measurement used (Wu Suen *et al.,* 2014). Many studies which done using schizophrenia study subjects to assessing cytokines level in schizophrenia sample size varies from 20 to 90. In most of the study which published online power and sample size calculation not reported.

By considered the above conditions, for the patients with schizophrenia, we included 82 participants, all recent onset or recent relapse cases and attending Amanuel Hospital were consecutively approached and assessed. Once diagnosis confirmed and inclusion criteria fulfilled, participants were included in the study. For control groups 45 healthy controls included in study.

3.5 Study Variables

3.5.1 Dependent Variables

- hsCRP
- IL-6

3.5.2 Independent Variables

- Age
- Sex
- BMI
- PANSS
- Age of onset
- Duration of current episode
- Current episode

3.6 Inclusion and Exclusion criteria

3.6.1 Inclusion Criteria

Subjects who fulfilled the following criteria were involved in the study

For Patients with schizophrenia

- Age 18-65 years
- Primary Axis I diagnosis (DSM-IV) (American Psychiatric Association, 2013) of Schizophrenia, any subtype.
- Absence of serious general medical condition and neurological diseases
- Patients willing to give consent

For control Groups

- Age 18-65 years
- Patients willing to give consent
- Apparently Health subjects

3.6.2 Exclusion Criteria

Patients with the following conditions were excluded from the study

For Patients with Schizophrenia

- Age below 18
- Not diagnosed with Schizophrenic
- Patient not able to give consent (both guardians and patients self)

For control Group

- Patient not able to give consent
- Sign of ill
- Not willing to give consent

3.7 Method of Data Collection and Analysis

Questionnaire survey

The questionnaire was used to collect data on respondent's demographic, socioeconomic status, and clinical data (Annex 1).

Physical measurements

Body weight and height was measured by trained clinical Nurse in all study participants. Body weight was measured with weight scale and height was measured using standard height measurement scale. From both weight and height, Body mass Index (BMI) was calculated using the following formula:

Body weight (Kg) /Body height (m^2).

BMI is a ratio of body weight in kilograms to the square of body height in metres and is calculated according to above formula. Based on the BMI we categorized our study participant

into underweight, normal range, and overweight/obese, Table 2, as suggested by the WHO (WHO, 2004).

Table 2. The International Classification of adult underweight, overweight and obesity according to BMI

Classification	BMI(kg/m^2)
	Principal cut-off points
Underweight	**< 18.50**
Normal range	**18.50 - 24.99**
Overweight/Obese	**≥ 25.00**

Source: Adapted from WHO 1995

PANSS classification

PANSS was developed in order to provide a well-defined instrument to specifically assess both positive and negative symptoms of schizophrenia as well as general psychopathology (Kay *et al.,* 1987). PANSS is the most widely-used standardized instrument for assessing symptom severity in schizophrenia. It has been increasingly used in clinical practice and as an outcome measure in a multitude of treatment efficacy studies.

One drawback of the PANSS is instrument based on summary rating scores is the lack of a gold standard with which to interpret results.

In general the PANSS gives a total average symptom score, based on 30 items rated from one to seven (1=normal, not at all ill, 2=borderline mentally ill, 3=mildly ill, 4=moderately ill, 5=markedly ill, 6=severely ill, and 7=among the most extremely ill). The PANSS total score was the sum of the rating scores for 7 positive subscale items, 7 negative subscale items, and 16 general psychopathology subscale items from the PANSS panel. Based on this rate the PANSS has a range of 30–210. Higher scores indicate more severe symptoms. Based on the score

which range from 30-210 the PANSS was classified in to different parts which is mildly ill/moderately ill, markedly ill, and severely ill as indicated in table 3 (Leucht *et al.,* 2005).

Table 3. Classification of PANSS

Severity	PANSS
Mildly ill/ Moderately ill	Up to 75
Markedly ill	76-95
Severely ill	Above 96

3.8 Data Management and Statistical Analysis

Relevant data for this study were entered into the Statistical Packages for Social Sciences, version 20 (SPSS Inc., Chicago, IL, USA) by the candidate. Prior to analyses, the data entered were cross checked against the original paper data collection form. Statistical analyses was done using SPSS Version 20. Simple descriptive and comparative analyses were carried out initially. For more advanced analysis (Primarily linear regression), normality of the distribution of both hsCRP and IL-6 were assessed. hsCRP distribution was not normally distributed and thus was log-transformed.

Gender, age, and BMI; factors previously reported to be associated with hsCRP and IL-6, were considered confounders and adjusted for in a linear regression model (Khera *et al.,* 2005; Wener *et al.*, 2000). All value of $p<0.05$ were considered as significant for all comparison and the number of analysis were restricted to reduce the probability of chance association.

3.9 Quality Control and Quality Assurance

The data quality was started with appropriate sample collection. The blood sample collection were taken during the patients on the fasting time. The quality of the results was maintained by using standard operating procedures (SOPS) for analysis of IL-6 and hsCRP. Laboratory analysis was done by experienced Laboratory technologist. In addition to this the laboratory used two level of quality control materials (PreciControl CliniChem Multi 1 and 2, Roche 05 117 208 922, 05 117 291 922) during sample analysis. Clinical chemistry Laboratory of EPHI also participant in External Quality Assurance program, (EQA- Oneworld Accuracy) to maintain Good Laboratory Practice (GLP).

3.10 Ethical Consideration

Ethical Clearance was given by the Institutional Review Board (IRB) of Addis Ababa University, College of Health Sciences (Protocol number: 062/11/psy), Department of Biochemistry, Addis Ababa University, and appropriate consent was received from individual participants during the study time.

A consent form was prepared and detailed explanation the objectives of the study, risk and benefits, confidentiality of the study was explained to the study participants or family members.

Two approaches were used to take the consent process:-

1. For those who have capacity to consent participated if they have given informed consent
2. Those who lacked capacity to consent participated only if their guardian or next of kin gives informed permission for the patient to take part in the study.

Both clinical data and blood collection was performed by trained psychiatrist and other health professional using blood collection procedures of EPHI respectively.

3.11 Laboratory analysis

3.11.1 Specimen collection and storage

Experienced phlebotomist collected 4-5 mL of blood using red top test tube for laboratory testing based on SOPS for blood collection of EPHI. The collected blood using redtop test tubes were allowed to clot for 30 minutes and centrifuged at 4000 rpm for 5 minutes. The serum was separated into a sterile tube (Cryovial) using sterile Pasteur pipettes. Each serum specimen cryovial was labeled with the study participant identification/ lab serial number and all serums were stored at EPHI laboratory at -80°c until analysis done.

3.11.2 Assays of laboratory variables

IL-6 was measured using cobas e 411 immunoassay analyzer which is a fully automatic run-oriented analyzer system for the determination of immunological tests using the electrochemiluminescence immunoassay "ECLIA"process and hsCRP was measured using Cobas Integra 400 Plus. Specific test principles of the two parameters are explained as follow.

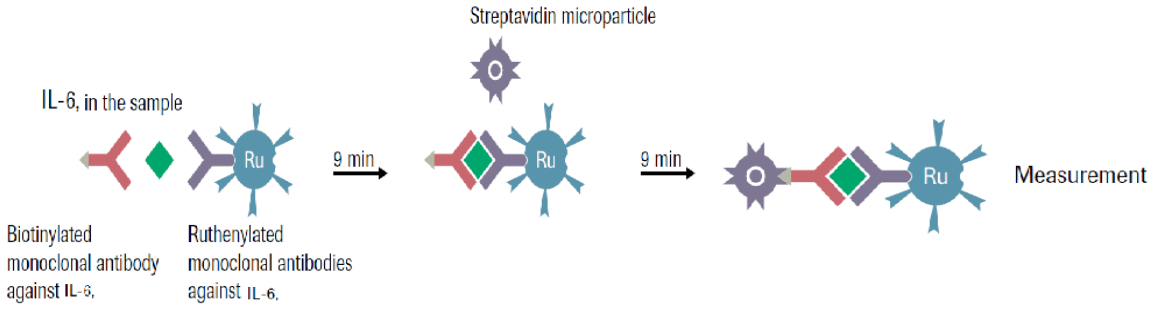
3.11.3 Interleukin-6 (IL-6) analysis

The samples for IL-6 were analyzed using Cobas e 411 (Roche Diagnostics GmbH, Mannheim, Germany) immunoassay analyzer which is fully automated run-oriented system for the determination of immunological tests using the Electrochemiluminescence immunoassay process. All components and reagents for this assay is integrated in on the analyzer. The sandwich principle was used in which 30µl of serum incubated with a biotinylated monoclonal

IL-6 specific antibody. After 18 minutes incubation time, IL-6 specific antibody labeled with a ruthenium complex (Tris(2,2'-bipyridyl)ruthenium(II)-Complex($Ru(bpy)^{2+}_3$) and streptavidin-coated microparticles, the antibodies form a sandwich complex with the antigen of the sample. The reaction mixtures was aspirated into the measuring cell where the microparticles magnetically captured onto the surfaces of the electrode. Unbound substances are then removed with Procell. Application of a voltage to the electrode to the electrode then induces chemiluminescent emission which was measured by a photomultiplier. Results were determined via a calibration curve which is instrument specifically generated by 2-point calibration and a master curve provided via the reagent barcode. The measuring range of IL-6 is 1.5-5000 pg/mL (defined by the lower detection limit and the maximum of the master curve). The precision was determined using Cobas e 411 reagent, samples and Controls, and the following results were found. 3.5 and 1.8 was found both PreciControl Mulitmarker level 1 and 2 respectively. The analytical sensitivity of the assay is 1.5 pg/ml. The normal value for IL-6 in a healthy individuals are expected to be <7 pg/ml Table 4. (Detail Procedures is explained at Annex 2)

Table 4. Product characteristics of IL-6

Assay	Elescys IL-6
Sample material	Serum, EDTA-Plasma
Sample volume	30ul
Assay time	18 min
Measuring Range	1.5-1500pg/ml
Analytical sensitivity	1.5 pg/ml
Traceability	WHO standard



IL-6= Interleukin-6

Figure 2. Test principle: one-step sandwich assay for measurement of IL-6 *(e-lab Doc .Roche.com)*

3.11.4 High sensitive C - reactive protein analysis (hsCRP)

HsCRP levels were measured by turbidimetric or immunonephelometry using Cobas Integra 400 Plus (Roche Diagnostics GmbH, Mannheim, Germany) clinical chemistry analyzer) is continuous and random-access analyzer with a consolidated test menu for routine clinical chemistry, specific proteins, drugs of abuse screening and therapeutic drug monitoring (TDM) and different measuring technologies.

Turbidimteric assay was used in which 6µl of serum was incubated with Reagent 1 (TRIS buffer with bovine serum albumin and immunoglobulins (mouse) and Reagent 2 (SR Latex particles coated with anti-CRP (mouse) in glycine buffer) for 98 seconds. The precipitate was determined turbidimetrically at 552 nm. The analyzer automatically calculates the analyte concentration (in mg/L) of each sample against standards. The measuring range of hsCRP is 0.1-20 mg/L (defined by the lower detection limit and the maximum of the master curve). The precision was determined using Cobas intgra 400 plus reagent, samples and Controls, and the

following results were found. 3.5 and 2.2 was found both control level 1 and 2 respectively. The analytical sensitivity of the assay is 0.1mg/L. The normal value for hsCRP in a healthy individuals are expected to be <1.0mg/L.

Table 5. Product characteristics of hsCRP

Assay	hsCRP
Sample material	Serum, EDTA-Plasma
Sample volume	2ul
Assay time	10 min
Measuring range	0.3 – 350 mg/L
Analytical sensitivity	0.3 mg/L
Traceability	WHO Standard

4. RESULTS

4.1 Demographic Data of the study Population

A total of 132 study participants were recruited, including 82 with schizophrenia and 50 control subjects. Among the participants with schizophrenia 79 (96.3%) were males and the remaining 3 (3.7%) were females. Among the control group 23 (46%) were males and 27 (54%) females control group.

The age ranged from 20 to 59, with mean age 35.1 (SD±9.7), and from 18 to 65 years with mean age of 28.8 (SD± 9.9) for schizophrenia and control subject respectively. The mean BMI was 22.8 (SD±17.9) for schizophrenia and 22.0 (±2.8) for the control group.

Table 6. Demographic data of the study population (Schizophrenia and Control Groups)

Study Subject	Schizophrenia N=82	Controls (n=50)
Age, Years (Mean and SD)	35.1(±9.7)	28.8(±9.9)
Gender N(%)		
Male	79(96.3)	23(46)
Female	3(3.7)	27(54)
BMI (Mean and SD)	22.8(±17.9)	22.0(±2.8)

There were no significant differences in BMI between the two groups (χ^2=4.46, df=2, p=0.087), but we found significant differences both in age (χ^2=13.04 df=3, p=0.004) and gender (χ^2=46.76, df=1, p=<0.001) between schizophrenia and control groups (Table 7). We have adjusted subsequent statistical analysis for the possible effect of these factors.

Table 7. Comparison of confounding factors (age, gender, and BMI) among schizophrenia and control groups.

Variables		Schizophrenia	Control group	d	χ^2 (*p*)
Gender	Male	79	23	1	46.76*(<0.001)*
	Female	3	27		
Age(Years)	18 – 24	12	20	3	13.04*(0.004)*
	25-34	32	19		
	35-44	24	6		
	45+	14	5		
BMI Category	Underweight	18	4	2	4.46*(0.087)*
	Normal	56	40		
	Overweight/obese	8	6		

The study population was grouped into three BMI categories: underweight (BMI <18.5), normal weight (BMI 18.5–24.99), overweight/Obese (BMI ≥25.0). Table 6 indicated that the mean BMI for schizophrenia participants were 22.8 with 56 (68.3 %) normal weight, 19 (23.2%) underweight, and seven (8.5%) overweight. Among control group mean BMI were 22.0 with 40 (80.0%) normal weight, four (8.0%) underweight, and remaining six (12.0%) were overweight.

Participants were classified by age groups 18–24 years, 25–34 years, 35–44 years and 45 years and above. This last category was used because of very small numbers in the 45 years and over age group. Based on this classification most of our study participants were young age group with 51(38.6%) of study participants categorized within 25-34 age groups.

Table 8. Socio-demographic data of the study population (Schizophrenia versus Controls) using Category Classification

Variables		Study participants		
		Schizophrenia	Control Groups	Total
		N(%)	N(%)	N(%)
Sex	Male	79(96.3%)	23(46.0%)	102(77.3%)
	Female	3(3.7%)	27 (54.0%)	30(22.7%)
Age	18 – 24	12(14.6)	20(40.0%)	32(24.3%)
	25-34	32(39.0)	19(38.0%)	51(38.6%)
	35-44	24(29.3)	6(12.0%)	30(22.7%)
	45+	14(17.1)	5(10.0%)	19(14.4%)
BMI	Underweight	19(23.2)	4(8.0%)	23(17.4%)
	Normal	56(68.3)	40(80.0%)	96(72.7%)
	Overweight	7(8.5)	6(12.0%)	13(9.9%)

4.2 Demographic and Clinical Characteristics of Schizophrenia group

A summary of the demographic and clinical characteristics features of the schizophrenic group were presented in Table 9.

Most participants were single (n=67/82, 81.7%), 7.3% of respondents were married and 6.1% and 4.9% of the study participants during the study period were widowed and divorced, respectively.

Concerning Ethnicity, Gurage accounted for over half (54.9%, n=45) of the study population. The other participants all accounted for under 20% each: Amhara (15.9%, n=13), Oromo (13.4%, n=11) followed by Tigray (9.8%, n=8) and other ethnic groups combined (6.1%, n=5)

In terms of schizophrenia subtypes, 31.7 % had paranoid schizophrenia sub types, 28% disorganized schizophrenia, 15.9% undifferentiated schizophrenia and 13.4% residual schizophrenia, with the rest (11%) with schizoaffective (Depressive and bipolar type), and catatonic schizophrenia subtype.

The living arrangement indicated that 76.8% of the study participants were living with Parental family, 12.2 % live with other relatives, 7.3% and 3.7 % and lives with marital family and live alone during the study period, respectively.

To assess tobacco use, patients with schizophrenia were asked about their current smoking status. Overall 15 (18.3%) of schizophrenia patients smoke a cigarette and 67 (81.7%) of schizophrenia patients were non-smokers.

Based on PANSS classification, 45.1% of schizophrenia patients were mildly ill, 19.5% were moderately ill and 35.4% were severely ill. Most were evaluated for a relapse episode (95.1%) with and 4.9% seen in the first episode.

Table 9. Clinical characteristics and other socio-demographic date for schizophrenia group

Socio-demographic characteristics		Number	Percent (%)
Ethnicity			
	Oromo	11	13.4
	Amhara	13	15.9
	Tigray	8	9.8
	Gurage	45	54.9
	Others	5	6.1
Marital Status	Single	67	81.7
	Married	6	7.3
	Divorced	4	4.9
	Widowed	5	6.1
	Cohabiting	0	-
Living Arrangement	Live alone	3	3.7
	Lives with Parental family	63	76.8
	Lives with marital family	6	7.3
	Live with other relatives	10	12.2
	Lives with friends	0	-
Schizophrenia subtype			
	Catatonic schizophrenia	3	3.7
	Disorganized schizophrenia	23	28.0
	Paranoid schizophrenia	26	31.7
	Residual schizophrenia	11	13.4
	Schizoaffective disorder, bipolar type	2	2.4
	Schizoaffective disorder, depressive type	4	4.9
	Undifferentiated schizophrenia	13	15.9
Current episode	First Episode	4	4.9
	Relapse Episode	78	95.1
Smoking Status	Yes	15	18.3
	No	67	81.7
PANSS	Mildly ill / Moderately ill	16	19.5
	Markedly ill	37	45.1
	Severely ill	29	35.4

Table 10 shows the course of illness in the schizophrenia group. According to this the mean ±SD of PANSS for schizophrenia participant were 89.2 (±20.2) with 37 the lowest and 147 the

highest PANSS score, The onset of the diseases was usually in young adulthood with the mean 22.88 years (SD±6.63) and the current episode varies a lot, but most episodes last with average of 13.8 months with 1 month was the lowest and 108 months the highest.

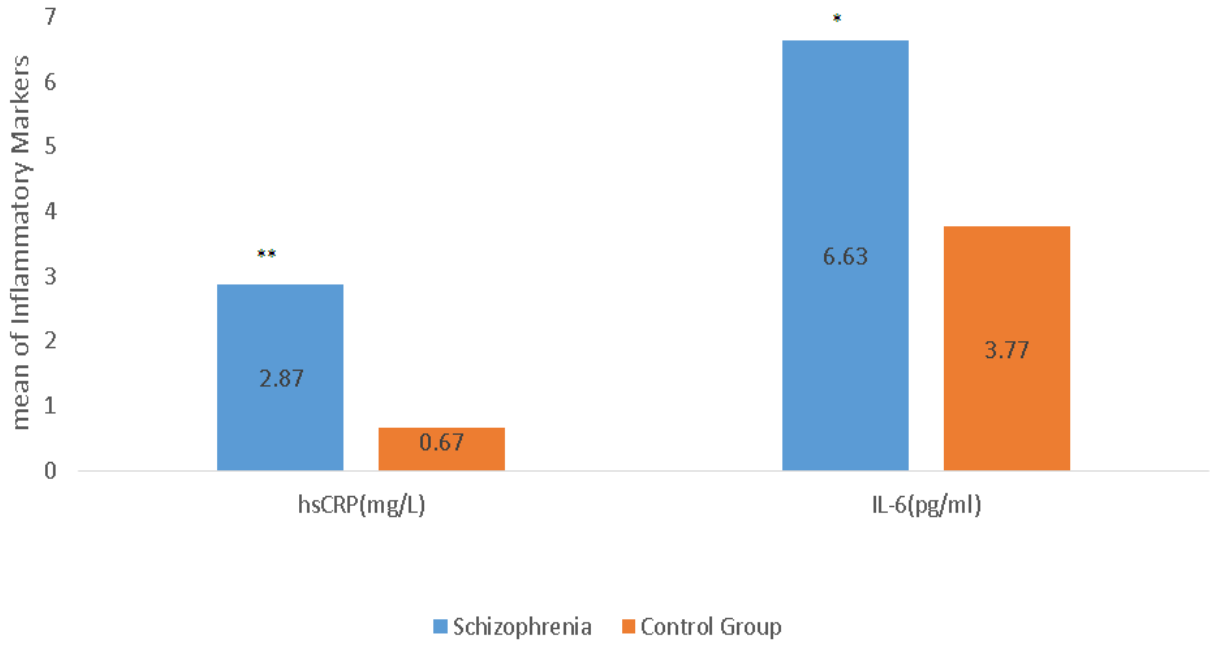
Table 10. Course of illness in schizophrenia group, data presented as mean and SD

Variables		Mean ± SD	Median (Range)
Course of illness	PANSS total score	89.2(±20.2)	86(37-147)
	Age of onset (Years)	22.88±6.63	22(7-40)
	Duration of current episode (months)	13.82±18.2	7(1-108)

4.3 Immunological findings

The prevalence of high value of hsCRP (value above cut off value 1mg/L) was higher (48.8% vs. 16.0%) among patients with schizophrenia when compared to control groups. Figure 3 shows that the mean value of hsCRP was 2.87mg/L (SD± 5.65) for schizophrenia and 0.67mg/L (SD± 0.60) for controls group.

The prevalence of high value of Serum IL-6 (proportion with value above cut off value 7pg/ml) was higher (42.7% vs. 20.0%) among schizophrenia patients when compared to control groups. The mean value of IL-6 was 6.63pg/ml (SD± 5.58) for schizophrenia and 3.77pg/ml (SD± 4.0) for controls group



* *P* =0.003, ** *P*=0.002

Figure 3. Mean serum level of hsCRP and IL-6 in schizophrenia and control group. P values derived after adjustment for gender, age, and BMI.

In order to investigate the association between inflammatory markers and diagnosis of schizophrenia, linear regression analyses were conducted. In these analyses, adjustments were applied, in which the covariates were age, gender, and BMI. The results of the linear regression analysis indicate that there was statistically significant differences of hsCRP (log-transformed) between patient with schizophrenia and control group (unadjusted: β = 0.37, 95% CI= (0.20, 0.54), P<0.001; adjusted: β =0.29, 95% CI= (0.10, 0.49), P=0.003).

There was association between hsCRP and gender, age, and BMI in our study. Unadjusted and adjusted model indicated that BMI remained significantly associated with high level of hsCRP in both model (Unadjusted β = -0.53 95% CI: -0.86, -0.21 *p* =0.001; Adjusted β -0.71 95% CI: -1.02 -0.41 *p* <0.001) in Underweight and also in normal weight (Unadjusted β -0.40 95% CI: -0.67 , -0.13 *p* =0.004; Adjusted β = -0.40 95% CI: -0.65 -0.15 *p* =0.002).

Table 11 presents also hsCRP significantly associated with male gender when unadjusted (β 0.21 95% CI: 0.01 0.42, *p*= 0.041) but there was no associations with male gender when adjusted (β 0.15 95% CI: -1.08 0.38, *p*= 0.206) for other factors. We found also significant associations of hsCRP with age, age group (18-24 years) when unadjusted (β = -0.45 95% *CI*: -0.72 -0.17 *p* =0.001) and when adjusted (β = -0.40 95% *CI*: -0.66 -0.14 *p* =0.003). There was also no association with all the rest age group except age group 25-34 years in adjusted model (adjusted β= -0.26 95% *CI* -0.49 -0.03 *p*=0.028).

Table 11. Factors associated with serum level of hsCRP

	Unadjusted hsCRP (log Transformed)				adjusted* hsCRP (log transformed)			
Factors	β	95% CI		P	β	95% CI		P
Schizophrenia	0.37	0.20	0.54	**<0.001**	0.29	0.10	0.49	**0.003**
Control	Ref.							
Gender								
Male	0.21	0.01	0.42	**0.041**	0.15	.-1.08	0.38	0.206
Female	Ref							
Age								
18-24 years	-0.45	-0.72	-0.17	**0.001**	-0.40	-0.66	-0.14	**0.003**
25-34 years	-0.23	-0.48	0.02	0.076	-0.26	-0.49	-0.03	**0.028**
35-44 years	-0.14	-0.42	0.14	0.313	-0.24	-0.48	0.01	0.062
45+ years	Ref.							
BMI								
Underweight	-0.53	-0.86	-0.21	**0.001**	-0.71..	-1.02	-0.41	**<0.001**
Normal	-0.40	-0.67	-0.13	**0.004**	-0.40	-0.65	-0.15	**0.002**
Overweight/Obese	Ref.							

* Adjusted for gender, age, and BMI

Abbreviations: hsCRP=high sensitive C-reactive protein; BMI= Body mass index

In linear regression analysis before and after controlling for age, gender, and BMI we found significant differences of IL-6 between schizophrenia and control groups in both model (Unadjusted β 2.86 95% CI: 1.11, 4.62 p =0.001; Adjusted β 3.60 95% CI: 1.35, 5.86 p = 0.002). For other factors we didn't find any association of IL-6 with gender in both model (Unadjusted β 1.16 95% CI: -0.94, 3.26 p =0.280; Adjusted β -0.92 95% CI: -3.52 1.68 p = 0.489), age (18-24years) (Unadjusted β -1.75 95% CI: -4.67, 1.17 *p* =0.241; Adjusted β -0.97 95% CI: -3.95, 2.01 *p* = 0.524), age (25-34years) (Unadjusted β -1.00 95% CI: -3.71,

1.72 p =0.471; Adjusted β -0.76 95% CI: -3.40, 1.89 p = 0.575), age (35-44 years) (Unadjusted β -1.46 95% CI: -4.42, 1.50 p =0.332; Adjusted β -1.76 95% CI: -4.62,1.10 p = 0.227), and BMI (Underweight) (Unadjusted β -0.58 95% CI: -4.04, 2.89 p =0.744; Adjusted β -1.20 95% CI: -4.69, 2.30 p = 0.503), BMI(normal) (Unadjusted β 0.27 95% CI: -2.63, 3.17 p =0.855; Adjusted β 0.38 95% CI: -2.50,3.26 p =0.797) Table 12.

Table 12: Factors associated with serum level of IL-6

	unadjusted IL-6			adjusted * IL-6		
Factors	β	95% CI	P	β	95% CI	P
Schizophrenia	2.86	1.11 4.62	**0.001**	3.60	1.35 5.86	**0.002**
Control	Ref.					
Gender						
Male	1.16	-0.94 3.26	0.280	-0.92	-3.52 1.68	0.489
Female	Ref.					
Age						
18-24 years	-1.75	-4.67 1.17	0.241	-0.97	-3.95 2.01	0.524
25-34 years	-1.00	-3.71 1.72	0.471	-0.76	-3.40 1.89	0.575
35-44 years	-1.46	-4.42 1.50	0.332	-1.76	-4.62 1.10	0.227
45+ years	Ref.					
BMI Underweight	-0.58	-4.04 2.89	0.744	-1.20	-4.69 2.30	0.503
Normal	0.27	-2.63 3.17	0.855	0.38	-2.50 3.26	0.797
Overweight/Obese	Ref.					

* Adjusted for gender, age, and BMI

Abbreviations: IL-6= Interleukin 6; BMI= Body mass index

We analyzed the relation between inflammatory markers and clinical characteristics such as the age of onset, duration of current episode, and PANSS score among schizophrenia groups. There were no associations between IL-6 and age of onset (p=0.840), duration of current episode (p=0.613), and PANSS (p=0.710) and smoking status (p=0.730). Also there was no association between hsCRP and age of onset (p=0.783), duration of current episode (p=0.868), PANSS (p=0.377), and smoking status (p=0.695) Table 13.

Table 13:- Associations between Clinical Characteristics of schizophrenia and inflammatory markers (hsCRP and IL-6).

Factors	IL-6				hsCRP log transformed			
	β	95% CI		P	β	95% CI		P
Age of onset of illness(Months)	0.02	-0.18	0.22	*0.840*	0.03	-0.02	0.02	*0.783*
Duration of current episode(Month)	0.02	-0.05	0.09	*0.613*	-0.0	-0.08	0.01	*0.868*
PANSS	-0.01	-0.07	0.05	*0.710*	-0.0	-0.01	0.03	*0.377*
Smoking	0.05	-0.23	0.33	*0.730*	0.06	-0.24	0.37	*0.695*

5. DISCUSSION

Among the 82 patients with schizophrenia, 40 (48.8 %) had a high CRP level above the cut of value which is 1 mg/L. Our results show significant differences in serum level of inflammatory markers in our study population. We found significant differences in elevated serum hsCRP (*p=0.003*) inflammatory markers between patient sufferings from schizophrenia than control groups.

Previous studies in schizophrenia have found increased levels of inflammatory markers. In a study of 200 patients with schizophrenia and 200 healthy controls done in Egypt found that they had higher levels of hsCRP than did matched controls (Fawzi *et al.,* 2011). A study conducted in Finland by (Suvisaari et al., 2011) in patients with medicated schizophrenia (n=45) show that serum hsCRP level increased than control groups. In another study conducted in Arab patients with schizophrenia using two age-matched groups of subjects (n= 207 schizophrenia patients and, n=165 health controls) shows that serum hsCRP level increased in clinically stable patients with schizophrenia. In this study, they used high hsCRP kits which are similar to our study. Our results also corroborated with the study carried out in India, which also found that plasma level of hsCRP increased in a patient with schizophrenia. In this study, they include 30 patients with schizophrenia and 15 health controls and they also found there was no association between the level plasma hsCRP and PANSS (Solanki et al. (2010). The elevated levels of CRP in schizophrenic patients were also observed in other studies. A cross-sectional study conducted by (Dickerson *et al.,* 2013) using large sample size (n= 295 patients with schizophrenia and n=192 controls group) indicated that patients with schizophrenia had significantly increased hsCRP than controls. These findings indicated that individuals with schizophrenia may be at

risk for the adverse health consequences associated with elevated hsCRP in the overall population.

Several studies have reported an elevated level of CRP. Lin *et al.,* 2013 in Taiwan population (n=36 patients with schizophrenia verses 36 health control) show that elevation of CRP level was seen in chronic schizophrenia under antipsychotic treatment. This study indicated that antipsychotic drug didn't alter the level of CRP.

A recent meta-analysis also showed the prevalence of an elevated CRP level in patients with schizophrenia and related disorders was 28% (Miller *et al.*, 2014). This study suggests that measurement of blood CRP levels may be useful to the clinical care of patients with schizophrenia and related disorders.

On the contrary, some of the studies do not find any significant association between the level of CRP and patients with schizophrenia. A study conducted by (Hope *et al.,* 2009; Fernandez-Egea *et al.,* 2009) indicated that there were no differences between a patient with schizophrenia and controls groups with serum level of hsCRP. The inconsistencies in this finding may be due to confounding factors like BMI. This is an important confounding factor since increased BMI is lead to increased CRP (Park *et al.*,2005). Another reason for inconsistencies results due to types of assay, biological specimen (serum, plasma vs Cerebrospinal fluid) and various phases of diseases conditions. Nowadays, several hsCRP assays have been developed with better sensitivity and precision to measure low concentrations of CRP than the classical CRP methods (Vodolazkaia *et al.,* 2011). Within the group of individuals with schizophrenia, the levels of hsCRP is associated with BMI ($p<0.001$, for underweight; $p=0.002$ for normal weight) and age (18-24 years, $p=0.003$; 25-34 years $p=0.028$), but not with Gender, PANSS total score, age of onset of illness, duration of current episode, and cigarette smoking.

Various studies report inconsistent results the relation between clinical phenotype and serum hsCRP. Our results supported with the recent study conducted San Diego, USA. In this study, they found that high CRP among patient with schizophrenia than control groups and hs-CRP levels were positively correlated with BMI. But these associations were not related to age, race, education, smoking status, or antipsychotic dosage (Joseph e*t al.*, 2015). A study conducted in Arab schizophrenic patients (Akanji et al.,2009), found that there were no significant associations of hsCRP levels with the current age of patients, gender (all males vs. all females), presence or absence of symptoms which as assessed by PANSS and age of onset of disease. Another study from India population also found the elevated of serum CRP among patients with schizophrenia, Akanji et al., but they didn't find any significant correlation between PANSS scores with CRP (Singh *et al.,* 2008).

But in contrary to our study, different studies have reported associations between the level of serum CRP and PANSS score. In a study from USA based on a small number of patients (n=26) with schizophrenia reported the elevated of serum CRP in schizophrenia patients who showed more severe clinical symptoms of schizophrenia as reflected by the PANSS total score (Fan *et al.,* 2007). Higher CRP level was found in a study conducted in male Egyptian antipsychotic-free patients and is positively correlated with the severity of the psychopathology as measured by PANSS (Fawzi *et al.,* 2011).

Among the 82 patients with schizophrenia, 35 (42.7 %) had a high IL-6 level above the cut of value which is 7 mg/L. Our results show significant differences in serum level of IL-6 (p=0.002) inflammatory markers among patient with schizophrenia when compared to control groups.

Within the group of individuals with schizophrenia, the levels of IL-6 is not associated with BMI (p=0.503 for underweight; p=0.797 for normal weight) and for all age groups table 12. In this study, there is also no associations between serum IL-6 and PANSS, the age of onset, duration of current episode, and cigarette smoking table1 3.

Elevated levels of IL-6 patients with schizophrenia were reported in numerous previous studies. A study conducted in Taiwanese population (Chin-Chuen and *et al.,* 2010) shows a significantly higher level of IL-6 (p=0.02) in patients with schizophrenia than healthy control subjects. In this study the major finding also, there was no significant differences were noted after 1 months of the first generation antipsychotic drug treatment. In another study which conducted in Serbia, using 43 versus 29 schizophrenia patients and health control respectively increased the concentration of IL-6 in schizophrenia reported in exacerbation and remission phase of schizophrenia indicating elevated IL-6 to be a state marker of exacerbation. In this study, no statistical significance was also found between levels of cytokines and age, BMI, sex, smoking status, antipsychotic treatment, and duration of illness (Dunjic-Kostic *et al.,* 2013). A study from Brazil population using 22 euthymic bipolar disorder patients, 53 patient with schizophrenia and 80 health controls were recruited. All study subjects were all non-smokers and non-obese. In this study, IL-6 levels were increased in schizophrenia patients when compared to controls (p<0.0001) and euthymic bipolar disorder patients (p<0.0001). These finding evidence a chronic activation in patients with schizophrenia (Kunz *et al.,* 2011).

Our result is also in line with those previous reports on the presence of increased inflammatory markers in patients with schizophrenia. A study conducted by Song *et al.,* using 96 normal weight schizophrenia and 22 overweight/obese schizophrenia compared with 60 health controls. In this study, they found that serum level of IL-6 in normal weight schizophrenia and

overweight/obese schizophrenia were significantly higher than control groups (Song *et al.,* 2013). High IL-6 also reported from China in chronic schizophrenia patients. In this study, chronic schizophrenia patients showed significantly altered level of IL-6 than controls group and also no significant association was found with gender, age, and smoking status (Luo *et al*., 2014).

In contrast to our finding, a study conducted by Hope *et al.,* 2015 found that there are no significant differences between schizophrenia, bipolar disorders, and health controls on the level of both IL-6 and hsCRP. In this study, one explanation for the lack of associations between schizophrenia and controls groups may be that value below detection was found in 64% for IL-6 and in 72% for hsCRP (*Hope et al.,* 2015). In this study, they used kits which have low sensitive which obtained from R&D Systems (Minneapolis, MN, USA).

There are considerable heterogeneity results among these studies with respect to age of onset, duration of current episode and total PANSS score. Serum Level of IL-6 had a significant positive correlation with duration of current episode reported in different studies (Ganguli *et al.,* 1994*,* Kim *et al.,* 2000*,)* and also a significant positive association between serum IL-6 and PANSS score was reported in two studies (García-Miss *et al.,* 2010; Dimitrov *et al.*, 2013). On the other hand, similar to this study, IL-6 not associated with duration of current episode (Dunjic-Kostic *et al.,* 2013*,* Song *et al.,* 2009) and the total PANSS score (Pae *et al.,* 2006). In another study, no statistical significance was found between levels of cytokines and sex, age, BMI, smoking habits, antipsychotic medication, and duration of treatment and duration of illness (Dunjic-Kostic *et al.,* 2013).

These inconsistencies result both in hsCRP and IL-6 may result from the heterogeneity of schizophrenia, the relatively small number of subjects, and potential confounding factors like BMI and smoking. Further prospective studies and replication of these findings are necessary.

Most studies done so far in relation to both hsCRP and IL-6 with schizophrenia are focused on western population and very few studies from other countries. To the best of our knowledge, ours is the first study to see the level of inflammatory markers among patient with schizophrenia.

In view of the apparent involvement of inflammatory processes, the use of compounds with primary anti-inflammatory properties has received increasing attention in the pharmacotherapy of schizophrenia. Importantly, recent clinical trials of anti-inflammatory add-on therapy in schizophrenia provide promising results by showing superior beneficial treatment effects when standard antipsychotics are co-administered with anti-inflammatory compounds.

Currently in Ethiopia, two randomized-control trials are underway in Addis Ababa. Investigators from AAU and Massachusetts General Hospital (MGH) are conducting whether supplementation of folate plus Vitamin B12 (Clinicaltrials.gov identifier: NCT01724476) and Minocycline (Clinicaltrials.gov identifier: NCT01809158.) will change in symptoms of schizophrenia as measured by the change in PANSS.

6. CONCLUSION

- In summary, this study is undertaken to see the level of inflammatory markers (hsCRP, IL-6) among patient with schizophrenia. To best our knowledge this is the first study conducted in Ethiopia.
- The investigations determined levels of inflammation in patients with schizophrenia after controlling confounding factors like age, BMI, and gender and in general, our results shows a significantly higher level of both hsCRP and IL-6 in patients with schizophrenia than in healthy control subjects.
- Our result suggests that disturbances of inflammatory markers may play a role in the pathophysiology of schizophrenia. A larger sample size and a longer period follow-up will be needed to confirm findings.

7. LIMITATIONS OF THE STUDY

- There are some limitations in this study.

- We have no data for control group concerning smoking status, future studies must include smoking habit in controls group.
- We didn't see the effect of anti-psychotic medications on the level of inflammatory markers, even though the increased level of both hsCRP and IL-6 not related to treatment with anti-psychotic drug. This is to be considered as an important limitation of these studies.
- Because our study was cross-sectional, we could not establish causal relations in a patient with schizophrenia. Future prospective studies recommended.

8. RECOMMENDATION

- Our sample size is too small, further studies are needed using large sample size for both schizophrenia and controls groups to better understand the level of inflammatory markers among schizophrenia patients.
- Inflammatory markers are may be affected by confounding factors like clozapine (second generation) and smoking. Those parameters must be taking into account during the investigation to see the effect of confounding factors.
- We recommend also randomized clinical trials to be conducted to see the effect of anti-inflammatory treatments on the level of inflammatory markers in patients with schizophrenia.

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Annex I

Thank you for your willingness to participate: your cooperation is very important to the success of the study. This is a questionnaire you are asked to fill out. Please answer the questions as frankly and accurately as possible. All information obtained in the study will be confidential

Please answer every questions in the questionnaire by marking ‘’x’’ in the space or filling the necessary information.

Demographic variable	Required details
Age	[ ][ ]
Weight	_____(kg)
Height	_____(m)
Sex	Male □ Female □
Marital status	Single □ Married □ Cohabiting □ Divorced □ Separated □
Living arrangement	Lives alone □ Lives with parental family □ Lives with marital family □ Lives with other relatives □ Lives with friends □
Ethnicity	Amhara □ Gurage □ Oromo □ Tigray □ Other (specify):

Clinical background	
Clinical diagnosis	Paranoid schizophrenia □ Disorganised schizophrenia □ Catatonic schizophrenia □ Undifferentiated schizophrenia □ Residual schizophrenia □ Schizoaffective disorder, bipolar type □ Schizoaffective disorder, depressive type □
Current episode	First episode □ Relapse episode □
Age of onset of illness	**[ ][ ](Months)**
Date of relapse/onset	**[ ][ ]:[ ][ ]:[ ][ ][ ][ ] (Date :month :year)**
Smoking status	□ Yes No □
Total PANSS score (After assessment)	_____
Duration of current episode (months)	**[ ][ ]**

Annex II
Standard operating procedures for IL-6

Purpose	This procedure provides instructions for performing IL-6 on the cobas e 411 Immunoassay system.

Abbreviations

IL-6 = Interleukin-6	EDTA-K_3 = Potassium Ethylene diamine tetra acetic acid
RT= Room Temperature	ECLIA= Electrochemiluminescence Immunoassay
Cal = Calibrator	

Materials

Reagents

IL-6 Reagent kits: Roche Cat., 05109442 190

M- Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 mL: Streptavidin-coated microparticles 0.72 mg/mL; preservative.

•**R1** Anti-IL-6~biotin (gray cap), 1 bottle, 9 mL: Biotinylated monoclonal anti-IL-6 antibody (mouse) 0.9 µg/mL; phosphate buffer 95 mmol/L, pH 7.3; preservative.

•**R2** Anti-IL-6 -Ab~ $Ru(bpy)^{2+}_3$, 9ml Monoclonal anti IL-6 antibody labeled with ruthenium complex 1.5 mg/l phosphate buffer 95mmol/l PH 7.3 (Black Cap)

Reagents preparation: ready for use.

Reagents stability and storage:

- Unopened at 2-8ºC up to the stated expiry date
- After opening at 2-8ºC -12 weeks
- On board - 8 weeks

Note: Store reagent kit **upright** in order to ensure complete availability of the micro particles during automatic mixing prior to use.

Supplies

1.Assay tips, Roche, 11706799
2. Assay cups, Roche, 11706802
3. wash water additives, Roche, 11930346
4. Procell, Roche , 11662988
5. Clean cell, Roche ,11662970
6.Syswash, Roche, 11930346
7.Gauze
8.70% of alcohol 9.Applicator stick

Equipment

- Elecsys cobas e 411 Analyzer
- Micro pipette(20-1000µl)
- Vortex
- Centrifuge
- Sample rack
- Pipettes of Different Volume

Sample

Sample type	Amount required	Transport and Storage	Stability
Serum	0.3mL	•Transport whole	•For seven days at

			blood at RT •Separate serum within 1 Hr. • store serum at 2-8°C or -20°C	2-8°c, •Three at -20°c.
	• **Plasma** treated with Heparin, EDTA-K_3, Sodium citrate or sodium fluoride/ Potassium oxalate	0.3mL	•Transport whole blood at RT •Separate serum within 1 Hr. • store serum at 2-8°C or -20°C	•For seven days at 2-8°c, •Three at -20°c.

Note: Serum should only be frozen once. Do not thaw and refreeze
Limitations: Gross hemolysis, lipemic and icterus specimen.

Sample retention: Specimens are discarded in accordance with EHNRI Specimen retention policy. This refers to both Specimens in the Primary and Secondary Containers.

Special Safety Precautions	• Use Universal safety precaution(wearing gloves lab coat and washing hands) when handling infectious materials • Refer to the National Health and Safety Guidelines for standard safety procedures

Calibration

Calibrator	Level	Stability	Frequency	Preparation (y/n)
IL-6 05109469190 Cal set, Roche, 11731483122	Cal1 Cal2	● Unopened up to expiry Date labeled on the Pack. •After opening ● 12 weeks at 2-8ºC ● Up to 5 hours on the analyzer at 20-25ºC	● After 1 month (28 days) when using the same reagent lot ● After Curative maintenance • When Quality control results indicate need for recalibration	No

Calibrator preparation:
Mix carefully, avoiding the formation of foam. Transfer into the empty labeled snap-cap bottles supplied. Due to Possible evaporation effects, not more than 5 calibration procedures per bottle set should be performed.
Note:
- Calibrators must be at room temperature before use
- Lot reagent calibration has to be done within 24 hours after registering the new reagent kit on the analyzer
- Write the opening date on the bottles.

Quality Control

Control	Level	Stability	Frequency	Preparation (y/n)
PreciControl	PCMulti	● Unopened up	● Daily	Yes

	<table><tr><td>Multimarker</td><td>marker 1 and 2</td><td>to expiry Date labeled on the Pack</td><td></td><td></td></tr></table> **Control preparation:** Add 3.0mL of distilled water using a volumetric pipette. Let it sit for 30 minutes and mix carefully, avoiding the formation of foam. ● Stability: the prepared Quality Control is stable for 1 days at RT, 2-8 ºC for 2 weeks and 1 month at -20ºC. ●Write the reconstitution date on the bottles. **Note**: • Controls must be kept at room temperature before use • Controls must be within range. If out of range, repeat the run. If still out of the range. Investigate for root cause. (Reagent, Calibration and QC Preparation….etc) • Solve the Problem, Document and rerun the Quality controls and Patient samples.

Procedure	Refer to how to order tests of *Elecsys 2010 Immunoassay system* CHEM.TP.005

Result Interpretation

Step	Action
1	Values below detection limit are reported as < 1.5 pg/mL.
2	Dilute values above the Analytical range (5000 pg/ml) using on board diluent. The recommended dilution is 1:10 **The concentration of the diluted sample must be 50pg/ml**. *If manual dilution, then multiply the result by the dilution factor*

Expected Values

Analyte	Reference Range		Analytical Range	Units
	Male	Female		
Adult	Up to 7pg/ml	Up to 7pg/ml	1.5- 5000	Pg/ml

Principle	IL-6 is measured using Elecsys 2010 immunoassay analyzer which is a fully automatic run-oriented analyzer system for the determination of immunological tests using the electrochemiluminescence immunoassay "ECLIA"process. All components and reagents for routine analysis are integrated in or on the analyzer.

Annex III. Information sheet for the Research and Informed consent of patients

Title <<Serum Level of Inflammatory Markers in Patients with Schizophrenia Attending a Tertiary Service in Addis Ababa, Ethiopia>>.

Since the study was conducted on already approved research project<< A double-blind randomized placebo-controlled trial of adjuvant therapy with minocycline for schizophrenia (The MINOS trial)>> in which the research objects included from the beginning, no need of preparing informed consent separately for this research.