A study of TNF-α and blood metals profile in Schizophrenia

THESIS

Submitted to

All India Institute of Medical Sciences, Jodhpur

In partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE (M.Sc.)

(BIOCHEMISTRY)

JUNE, 2023 **Ms. ARTI RAY**

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DECLARATION

I hereby, declare that the thesis entitled "**A study of TNF-α and blood metals profile in Schizophrenia**" embodies the original work carried out by the undersigned.

I further state that no part of the thesis has been submitted either in part or full for any other degree from All India Institute of Medical Sciences or any other institution/ University.

Arti Ray

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CERTIFICATE

This is to certify that the thesis titled "**A study of TNF-α and blood metals profile in schizophrenia**" is the bonafide work of **Ms. Arti Ray** carried out under our guidance and supervision in the Department of Biochemistry, All India Institute of Medical Sciences, Jodhpur.

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Arti Ray

ABBREVIATIONS

SZ	Schizophrenia
TNF-α	Tumor Necrosis Factor Alpha
ELISA	Enzyme-Linked Immunosorbent Assay
ICP-OES	Inductively Coupled Plasma - Optical Emission Spectrometry
AAS	Atomic Absorption Spectrophotometer
No.	Number
PSI	Pound Per Square Inch
TNFR	Tumor Necrosis Factor Receptor
NMDAR	N-Methyl-D-Aspartate Receptor
PANSS	Positive And Negative Syndrome Scale
FEDN	First Episode Drug Naïve
ROS	Reactive Oxygen Species
GPx	Glutathione Peroxidase
SOD	Superoxide Dismutase Enzymes
CNS	Central Nervous System
p38	p38 Mitogen-Activated Protein Kinases
JNK3	c-Jun N-terminal Kinases 3
HRP	Horseradish Peroxidase
TMB	3,3′,5,5′-Tetramethylbenzidine
BBB	Blood-Brain Barrier
5HT2A	5-Hydroxy Tryptamine
CB1 Receptor	Cannabinoid Type 1 Receptor
GABA	Gamma-Aminobutyric Acid
MAO	Monoamine Oxidase
SELENBP1	Selenium Binding Protein 1
LOD	Limit Of Detection
LOQ	Limit Of Quantification

SUMMARY

Background: SZ is a chronic neuropsychiatric disorder with a prevalence of 1% worldwide. Neuroinflammation is a critical feature of SZ, mediated by cytokines, including TNF-α. Heavy metals may be linked to an increased risk of SZ because they can harm the body's biological functions. In addition, altering trace element levels is crucial to the onset and severity of SZ.

Aims and Objectives: The present study aimed to assess serum TNF-α levels and blood metals in cases and controls. A further correlation was evaluated between TNF-α and blood metals with PANSS score and TNF- α with blood metals concentration.

Methods: In this study, 80 participants were recruited, among them, 40 were SZ patients, and 40 were healthy controls. Severity was assessed by using the PANSS score. Serum TNF-α levels were estimated using sandwich ELISA, and blood metal profiles were estimated by inductively coupled plasma optical emission spectrometry (ICP-OES) and atomic absorption spectrophotometer (AAS).

Results: According to findings, SZ patients had significantly higher Iron (Fe), Copper (Cu), Magnesium (Mg), selenium (Se), Lead (Pb), and Cadmium (Cd) concentrations and lower Zn concentrations than controls (p-value < 0.05). TNF-α positively correlates with Cu levels (rho 0.234; p-value 0.041). Concentrations of (Cu, Se, and Cd) were associated with the risk of SZ with the OR (95% CI) of 0.93 (0.91-0.96), 0.80 (0.68-0.93) and 0.007 (0.001-0.80).

Conclusion: The obtained results suggested immune system impairments, neuroinflammation and alteration of blood metals in SZ patients. Our study indicates that compared to healthy controls, SZ patients have higher serum levels of Fe, Cu, and Se and lower serum levels of Zn. In addition, there was a correlation between the levels of Cu and TNF-α in SZ patients. The high Cu and Fe level affects neurotransmission and cause lipid peroxidation by increasing free radicals. High Se increases SELENBP1 gene expression Heavy metals (Pb and Cd) affect neurotransmitters and their function after binding with divalent metal ion transporters by increasing oxidative stress.

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Introduction

Schizophrenia (SZ) is a chronic mental disorder that affects an individual's thinking, perception, and behaviour. According to World Health Organization's (WHO) estimated data (2022), SZ touches approx. 24 million or 1 in 300 or 0.32% population globally. The typical onset of SZ is in late adolescence or early 20s, with a slightly later onset in females (1,2) with an average age of 18 and 25 for males and females (3). Prevalence and severity are higher in males than in females (4). Early onset and chronic course of SZ make it a debilitating disorder that impacts the quality of life. This disability is due to hallucinations, delusions, i.e., positive symptoms, or cognitive impairment (5). Due to its poor recovery rates, SZ can be considered one of the major contributors to increasing healthcare costs. SZ has been found to increase the burden on family members and society (6). It is estimated that most patients with SZ relapse within five years of the first episode, and many are not fully recovered (7).

SZ is a highly heterogeneous disorder with unknown etiology. There are several hypotheses about the development of SZ. One of the most common hypotheses claims that there is an imbalance in the neurotransmitter dopamine, serotonin, and gamma-aminobutyric acid (GABA). This imbalance results in psychiatric symptoms (8). It is assumed that the cause of SZ lies between genes and the environment (9). SZ is associated with genetic factors such as small nucleotide polymorphisms (SNPs), copy number variations (CNV), and altered gene expression. In addition, epigenetic changes are reported as a factor in SZ development. The estimated hereditary burden of SZ is approximately 80%. Genome-wide association studies have resolved hundreds of loci, numerous of which contain multiple genes that are particularly associated with SZ (10). It is estimated that when both parents are affected, the chances of SZ development are 40%, which is increased to 46 % in the case of monozygotic twins. Several assumed environmental risk factors include prenatal factors; rubella, influenza, toxoplasmosis, maternal cytokines malnutrition, and stress; in addition to that, perinatal factors and post-natal factors such as obstetric complications, low birth weight, hypoxia, jaundice, childhood head injury after birth, drug abuse, family history, and social isolation (11,12) are responsible for SZ development.

Evidence suggests that immune system activation and systemic inflammation can lead to neuroinflammation (13,14). There are various ways to mediate this inflammation to reach the brain: excitation of the vague nerve through carrier transport across the blood-brain barrier (BBB), activation of BBB endothelial cells, perivascular macrophages and through circumventricular organs other than BBB (15). In expansion, other studies point out that

cytokines-mediated neuroinflammation may have a role in the activation of psychotic symptoms (16). Cytokines are signaling molecules that stimulate an immune response by controlling their proliferation and activation. Microglial, macrophages, B-lymphocytes as well as T lymphocytes are the cells that produce cytokines. Microglia are the local innate immune cells of the central nervous system. Under certain pathological situations, microglia can induce neuroinflammation, which further causes neurodegeneration (17). In neurodegenerative processes, chronically activated microglia discharge pro-inflammatory cytokines, including tumour necrosis factor α (TNF-α) (18).

TNF-α is a cytokine of the TNF superfamily containing several transmembrane proteins and a homologous TNF domain. An imbalance in the levels of TNF-α may be correlated with SZ psychopathology. TNF α shows its effect after binding with the receptors. TNF signaling is regulated by two receptors: TNF receptor-1 (TNFR1) and TNF receptor-2 (TNFR2) (19,20).

According to epidemiological findings, urbanization has increased the risk for SZ, possibly due to exposure to toxic environmental metals. Arsenic (As), Cadmium (Cd), Chromium (Cd), Lead (Pb), Mercury (Hg), and Aluminum (Al) are known toxic heavy metals. Chronic exposure to these heavy metals alters several biological functions, such as abnormal immune responses and neuropathological conditions (21). For example, metals like As stimulated neurotoxicity by the change in composition and increased cytoskeletal protein phosphorylation, which leads to the breakdown of the cytoskeletal framework of cells and causes neurodegenerative disease. All toxic heavy metals (As, Cd, Pb, Al) increase oxidative stress, followed by DNA damage through deletion, base pair mutation, or DNA blocking channels for calcium, lipid peroxidation, and protein modification. These metals have a role in neurological disorders and chronic inflammation due to their ability to do neurobehavioral alterations and impair dopamine receptors (22,23). Altered serum trace elements may cause the onset and stimulation of positive and negative symptoms or cognitive impairments. Trace elements, also called micronutrients, are those the body requires in low amounts. Trace elements such as Iron (Fe), Copper (Cu), Zinc (Zn), Selenium (Se), and Magnesium (Mg) are needed for proper growth and development. They are essential in scavenger of reactive oxygen species (ROS) due to their antioxidant activities. Fluctuation in the levels of trace elements is also observed in several psychiatric disorders, including SZ (24).

To the best of our knowledge, there is no combined Indian study on trace elements, heavy metals, and pro-inflammatory marker tumour necrosis factor-alpha (TNF-α) in SZ. Moreover, the study results on different populations are quite controversial. In addition, the diagnostic and prognostic role of trace elements and heavy metals in people with SZ has not yet been established. Therefore, to fill the lacuna, this study is being undertaken to estimate the levels of TNF-α and metals profile in SZ patients and to find out the association with the severity of the disease if it exists.

Review of Literature

SZ is a chronic, complex and heterogeneous neurodevelopmental disorder. According to the National Institute of Mental Health (NIMH) estimated data, SZ is one of the 15 causes of disability in the country, with an incidence of 1.5 per 10,000 population (6). The onset is variable among the sex, with early twenty in males and late thirty in females (25). SZ can be challenging to diagnose due to often lack of symptoms that other mental health conditions may have (26,27).

SZ is characterized by suspiciousness, hallucinations, delusion, and behavioural changes (positive symptoms). Hallucination can occur in any form: touch, smell, or vision; however, auditory hallucinations are the most common. Positive symptoms often cause a relapse of the disease. The prodromal phase begins with negative symptoms like lack of emotion, motivation, depression, and flat affect. People with SZ frequently suffer from cognitive impairments such as working memory and attention deficits (25,28).

SZ is thought to be caused by different factors, from genetics and environment to neurodevelopmental issues, immune system activity and inflammation, and many more (28). To explain SZ, there are many hypotheses:

1. Neurodevelopmental hypothesis

The neurodevelopmental hypothesis assumes brain pathologies emerge during brain development in intrauterine life (29). For example, MRI studies of first-episode schizophrenic patients have morphologic brain deformities such as ventricular enlargement and decreased hippocampal volume (30). The disconnection hypothesis states that impaired synaptic plasticity, and synaptic efficacy, particularly in brain areas responsible for learning and memory, participate in SZ pathophysiology (31). While dual hit theory states, two hits are needed to activate the clinical phenotype of SZ (6,32).

2. Neurochemical hypothesis

There are three neurochemical hypotheses related to psychosis: first, dopamine hyperactivity at D2 (dopamine type 2 receptors) in the mesolimbic pathway. Second, N-methyl-D-aspartate receptor (NMDAR) hypofunctions on the prefrontal cortex's GABAergic interneurons. Third, serotonin hyperactivity of 5-hydroxy-tryptamine or serotonin (5-HT2A) receptors on

glutamate neurons in the cerebral cortex. All three tracks can lead to a common path to over-activate the mesolimbic dopamine pathway (33-39).

The cannabinoid hypothesis suggests that disturbance in the endocannabinoid system may be responsible for SZ. The disruption involves increased activation of the cannabinoid type 1 receptor (CB1 receptor) on GABAergic interneurons (40,41).

3. Neuroinflammation hypothesis

Neuroinflammation means inflammation of nervous tissue. The over-activation of microglial cells is a hallmark of neuroinflammation in SZ (42). The vulnerability-stress model states that following neuroinflammation and microglial activation in pregnancy, either genetically or due to infection, can change glutamatergic, dopaminergic, serotonergic, and non-adrenergic systems. At a later age, re-exposure to stress increases cytokines further and gives SZ symptoms (43).

Tumor necrosis factor alpha (TNF- α):

TNF-α is one of the most common pro-inflammatory cytokines with beneficial and destructive properties for the brain (44). In the brain, TNF-α is produced by neurons, astrocytes, and microglia and has a significant role in acute phase inflammation by introducing inflammatory cytokine signaling cascades (45). Balotsev et al. found no significant difference in the median (IQR) of cases 3.23 (0.03-1.13) pg/ml and controls 3.38 (0.04-0.35) pg/ml in their study (46). A study was done by Lin et al. on 103 (51 FEDN and 52 chronic) patients and 114 healthy controls found that TNF-α levels in FEDN patients were significantly higher than in healthy controls and chronic patients. They observed no significant difference in the TNF-α level in FEDN patients with SZ before and after treatment (47). In the Zhu et al. study, they took 69 first episode drug naïve patients (FEDN), 87 chronic patients and 61 healthy controls and reported that TNF-α serum concentrations were higher in chronic patients mean and standard deviation (SD), (19.3 ± 11.3) than both healthy controls and FEDN patients. They also found that TNF-α was moderately positive correlations with the PANSS negative score in the chronic patients ($r=0.964$, p-value < 0.001) but not in FEDN patients with SZ (48). The previous study investigated pro-inflammatory and anti-inflammatory cytokines alterations depending on the disease stage (49). Boerrigter et al.

showed that decrease in the anti-inflammatory (interleukin) IL-2 mRNA and an increase in serum pro-inflammatory cytokines, TNFα in people with SZ compared to healthy controls indicating a role of moderate chronic inflammation in SZ ($p < 0.001$) (50). However, a meta-analysis by Khandaker et al. suggested normal TNF-α in episodes of remission after antipsychotic treatment (51). A meta-analysis by Upthegrove et al. suggested high TNF-α levels in acute and FEDN patients of SZ and patients with major depressive disorder. TNF-α has been suggested as a trait marker of neuroinflammation. It is confirmed that TNF-α was raised in acute illness and stable 'outpatients' with chronic SZ compared to controls and remained so after treatment (52).

4. Environmental factors

Environmental pollutants can disrupt brain development and causes neuropsychiatric disorders, whereas some essential elements have a role in neurological development via several immune and metabolic processes (53,54). Excess toxic elements without crucial features might be linked to SZ (21).

Pb, Al, As, Hg and Cd are considered heavy metals in polluted environments. The high concentration of these toxic elements can cause oxidative stress. Oxidative stress is a condition where there is an imbalance between the formation of free radicals and the body's antioxidant capacity. It is known that most heavy metal act via reactive oxygen species (ROS) (55). ROS are derived from oxygen—free radicals, reactive molecules, and ions. Environmental stressors such as ionizing radiation, pollutants, and heavy metals can also make them. ROS have been linked to several diseases, including SZ (56-58). Trace elements are constituents of enzymes involved in antioxidant mechanisms (55).

Trace elements:

Elements are divided into two categories: Abundant and trace elements, according to their amount in the human body. Abundant elements form covalent bonds and are essential constituents of tissues such as carbon, hydrogen, oxygen and nitrogen. Trace elements comprise less than 0.01% of total body weight. Some trace elements are essential for humans because they cannot be synthesized or replaced by other metals in the body. Other nonessential trace elements can be toxic if levels exceed certain thresholds (59,60).

Trace elements are essential components of biological tissues and molecules (61). For example, Se is a component of the active site of glutathione peroxidase (GPx) (55). The levels of many essential trace elements in the blood are related to human health (62). Few studies showed alteration in these levels with SZ and other neuropsychiatric disorders, but some have also observed negative associations (63).

Iron (Fe):

Fe plays a vital role in storing and delivering oxygen to cells, forming hemoglobin in red blood cells, catalase in the mitochondria, peroxidase, and cytochrome oxidase. Fe is vital for the adequate functioning of the brain, where it plays roles in neurotransmitter syntheses, such as dopamine and serotonin, synaptic plasticity development, and myelination formation (64–70). Increased levels of Fe cause neuronal death and cellular toxicity by increasing oxidative stress and lipid peroxidation (71,72). Kim. et al. investigated that latent Fe deficiency is significantly associated with adverse symptoms in antipsychotic SZ patients, and its imbalance alters dopaminergic activity that might be linked to negative symptoms in SZ (73). A study by Curz et al. found higher Fe with mean and SD (632 ± 279 μg/l) in SZ patients as compared to healthy controls (421 ± 121 μg/l) ($p < 0.05$) (74). Cao et al. study found higher concentrations of Fe in patients with a median (IQR) of 1.31 (1.09–1.51) ng/ml than healthy controls with 1.16 (0.96–1.41) ng/ml. (75). However, Chen et al. reported antipsychotic drugs to decrease serum Fe levels in cases. They also found that decreased Fe and Zn levels were associated with an increased risk of SZ. (76). Lin et al. investigated a significant difference between SZ and the control group (77). Sussulini et al. investigated that antipsychotic treatment (risperidone, olanzapine, and quetiapine) only increases Fe levels in good responders (78).

Copper (Cu):

Cu is the third most abundant transition element in the human body. It involves proper Fe metabolism, myelination, neurotransmitter synthesis, antioxidant defence, and signaling pathways. It is a component of several enzymes, such as tyrosine hydroxylase (involved in dopamine and norepinephrine production), superoxide dismutase, and cytochrome c oxidase. Cu also affects synaptic transmission by binding to GABA, NMDA receptors, and voltage-gated calcium channels. Cu toxicity is primarily associated with free radical-induced

oxidative damage (64,79–82). In Coa et al. study they took 105 patients (who had not taken any antipsychotic medication for a minimum of 1 month before hospitalization) and 106 age and sex-matched healthy controls. They showed no significant difference in median (IQR) level when comparing serum Cu in first episode SZ cases 0.81 (0.71,0.88) ng/ml and recurrent SZ cases 0.86 (0.77-1.04) ng/ml to healthy control 0.83 (0.71,0. 95) (75). A study done by Wolf et al. investigated significantly elevated serum Cu levels and circulating ceruloplasmin among people with SZ compared to controls. (83). Chen et al. study reported that serum copper was lower after antipsychotic medication (clozapine and aripiprazole) in cases than in healthy controls (76).

Zinc (Zn):

Zinc maintains protein structure by acting as a cofactor for several enzymes, such as dopamine b-hydroxylase and monoamine oxidase (MAO). Zn is crucial for adult neurogenesis and proper hippocampal functioning by inhibiting the release of glutamate. It can prevent neurotransmission at synapses between neurons by blocking calcium channels and inhibiting GABA receptors within neurons (84–87). Zn is an essential cofactor for superoxide dismutase (SOD). Increased levels of Zn in the brain lead to its accumulation inside the central nervous system (CNS). Deficiencies in Zn during development can lead to mental retardation and altered learning ability (88). Curz et al. investigated lower serum Zn levels in SZ patients using antipsychotics (valproate, carbamazepine, lithium and mood stabilizers) against healthy control (74). Cao et al. examined patients (without antipsychotic treatment for a minimum of one month before hospitalization) and showed no significant difference in serum Zn level between cases and controls (75). A study by Chen et al. showed a significant decrease in serum Zn levels in patients using antipsychotic drugs; the concentration of Zn significantly decreased after 3 weeks of treatment than before treatments ($P < 0.05$). There was no association between Zn levels and olanzapine treatment (76). A meta-analysis showed that SZ patients tend to present lower Zn and Fe serum concentrations and excessive Cu amounts than healthy controls (89). Ma et al. showed a negative correlation between Zn and PANSS total scores in SZ patients (54).

Magnesium (Mg):

Magnesium is a mineral that plays a crucial role in over 300 enzymes. These enzymes control neuromuscular conduction and prevent neuronal overstimulation by blocking NMDA receptors. Hypermagnesemia can cause neuromuscular dysfunctions (90–92). Mg has a

protective role in the nervous system, which helps to prevent excessive excitation and potential excitotoxicity (24). Ruljancic et al. did not find any differences in serum Mg levels between the three groups: patients with attempted suicide, patients without suicidal behaviour, and the control group but high Mg in platelets in patients with attempted suicide (93). Chen et al. found that Mg levels in SZ patients were higher compared to healthy control groups ($p < 0.01$); however after antipsychotic treatment (risperidone, clozapine, Olanzapine, aripiprazole, quetiapine, perphenazine, sulpiride) Mg, serum levels was decreased (76). Ma et al. found a negative correlation between Mg and PANSS total scores. They also investigated higher serum Mg in cases than healthy controls with mean and SD of (55.52 ± 15.27) and (32.04 ± 11.40) in their study. Higher Mg concentration increases the risk of SZ (54). Li et al. showed elevated serum Mg in cases than controls with mean and SD of (55.22 ± 15.27) mg/l and (33.04 ± 11.40) mg/l (94).

Selenium (Se):

Selenium is a trace element involved in antioxidant reactions, including glutathione peroxidase and thioredoxin reductase. Se is necessary for the proper development of GABAergic interneurons; It is essential for the upkeep of dopamine pathways. A high level of Se in the blood might be poisonous and result in selenosis, manifested as irritability and neurological damage (64). Curz et al. found that mean and SD values for Se were lower in patients (31.8 ± 3.1) μg/ml against healthy controls (37.6 ± 5.5) μg/ml regardless of antipsychotic medication (74). Cai et al. investigated significantly reduced Se in SZ drug naïve patients compared to healthy controls (63). Previous studies showed higher concentration of serum Se might be a protective factor for SZ (94). In a previous study, serum Se in SZ patients before the Se supplement was 64.11 μg/l. However, after the Se Weifang supplement for 1 month, serum Se was improved in patients (94). Previous studies found an increased Se binding protein 1 (SELENBP1) gene expression in patients than controls; however, it is unclear whether increased expression of SELENBP1 is related to a higher incidence of psychosis (95,96). Ma et al. showed a negative correlation between Se with PANSS total scores. They also reported that a lower-level Se odd ratio (OR 0.954; 95% CI: 0.937–0.972) was associated with an elevated risk of SZ (54). Previous studies reported lower levels of Cu (≤ 0.97 μg/ml) and Se (≤ 72 ng/ml) for which the OR were 20.95 (95% CI: 1.38-318.14) and 16.83 (95% CI: 2.13 – 133.11) associated with an increased risk of SZ (97).

Heavy metals:

Heavy metals are naturally occurring elements with a higher density than water by five times (98). After being exposed to industrialization, they can enter the body through food or water (99,100). The role of trace and heavy metals in SZ is seen in antioxidant and neurotransmitter dysfunction. Changes in trace element concentrations and increases in heavy metal concentrations can lead to redox dysregulation (99,101).

Aluminum (Al):

Naturally, Aluminum is present in air, water and soil. It shows neurotoxicity in its cation (Al^{3+}) form. Al is a toxic nonessential metal with no biological role (102,103). Al accumulation in the brain can cause memory loss and other neurological problems. It enters the brain through transferrin and accumulates in the transferrin receptors-rich region. Excessive exposure to Al might induce inflammatory responses due to the increased expression of nuclear factor kappa B (NF-κB) and TNF-α. It might impair hippocampal calcium signaling pathways responsible for learning and memory (104). Liu et al. found higher Al levels before treatment in poor responders compared to suitable responders' patients. They also found that a lower level of Al was associated with the risk of SZ (97). Sussulini et al. took 56 plasma samples from SZ patients and analyzed them before and after treatment. They found that after 6 weeks of treatment, SZ patients show higher levels of plasma Al. They also investigated the decreased level of Al in serum is associated with an increased risk for SZ (78).

Arsenic (As):

Arsenic, a harmful heavy metal, is one of the main risk factors for human health. Arsenic exists as a metalloid (As0), inorganic (As^{3+} and As^{5+}), organic, and arsine (AsH_3). Among all forms, arsine gas has the highest toxic effect (99,105,106). The As-induced neurotoxicity mechanism includes mitochondrial dysfunction, lipid peroxidation, and apoptosis. Arsenic toxicosis results in the activation of p38 mitogen-activated protein kinase and c-Jun N-terminal kinase 3 (JNK3) pathways or increased activity of caspase-9, an apoptosis initiator caspase (107). A previous study reported that TNF-α were elevated in rural women's plasma when exposed to As (108). A meta-analysis investigated significantly higher TNF-α concentrations in As exposed individuals compared to the control group (109). The previous

study investigated serum As level was significantly lower in cases with the median (IQR) value 0.593 (0.253–0.984) ng/ml compared to controls 0.767 (0.325-1.80) ng/ml (22). In the previous study, serum, Se in SZ patients before Se supplement mean and SD was 31.43 ± 6.38 μg/l. However, after supplementing with Se Wei Kang for 3 months, serum As was decreased in patients at 9.84 ± 2.43 (p-value < 0.001) (94).

Lead (Pb)

Lead (Pb^{2+}) affects human health, and it can be absorbed through the skin but is chiefly absorbed through the respiratory and digestive systems. Pb exposure can cause neurological, respiratory, and urinary disorders due to immune modulation, oxidative stress, and inflammation mechanisms (99). It can cause toxicity by generating reactive oxygen species and direct depletion of antioxidant reserves. Pb can make enzymes nonfunctional by binding to their sulfhydryl groups (110). Children are more likely than adults to suffer from these effects and contributing to 6 lakh cases of intellectually disabled children annually worldwide (111). Kulkarni et al. reported that maternal late pregnancy lead was related to deficits in the mental development index (MDI) of children at 6 months. Mothers having less dietary iron intake during pregnancy can transfer to their developing fetuses and cause abnormalities like neurodevelopmental disorders (112). A longitudinal cohort study by Reuben et al. observed that lead exposure in childhood was significantly associated with more significant psychopathology across the life course and with complex personality traits in adulthood (113). Ma et al. suggested Pb levels were significantly higher in the drug naïve patients compared to the control group with median (IQR) of 0.62 (0.48–0.85) ng/ml and 0.546 (0.38-0.72) ng/ml. They also showed that the Pb level was associated with the risk of SZ, OR: 0.60 (95% CI: 0.04-0.79) (22). One Indian study by Sharma et al. found that Pb was significantly higher in battery workers SZ patients with mean and SD (35.04 ± 13.39) μg/dl against controls (5.54 ± 1.75) μg/dl. They further investigated those battery workers exposed to Pb greater than 10 years have increased blood Pb levels have decreased hemoglobin levels in the body they all become mental disorders (114). A study done by Karim et al. reported decreased Pb levels in blood with a mean value of (0.94 ± 0.19) mg/l against controls (1.25 ± 1.60) mg/l (119).

Cadmium (Cd):

Cadmium is a toxic heavy metal with no significant biological role in the human organism. It enables the induction of oxidative stress. Along with that, it activates various gene expressions, such as metallothioneins. Chronic exposure might lead to neuropsychological dysfunctions, including cognitive delay (115–118). A study done by Ganiyu et al. also investigated higher blood Cd levels in drug naïve SZ patients as well as patients on antipsychotics with mean and SD (59.73 ± 2.89, 53.50 ± 4.14) than healthy controls (119). Li. et al. showed a decrease in serum Cd levels in patients with SZ than the control group (94). Ma et al. found no significant difference between drug naïve patients and controls with mean and SD values of 0.756 (0.619–0.886) ng/ml and 0.768 (0.655–0.898) ng/ml. In addition, they found that Cd concentration was not associated with the risk of SZ (p-value 0.50) (22). A previous animal study found that Cd exposure significantly increased plasma levels of TNF-α than normal rats. A negative correlation was found between TNF-α and actions of catalase, SOD and antioxidant capacity in rats that were exposed to Cd (120).

Aims and Objectives

HYPOTHESIS:

Schizophrenia is associated with altered levels of pro-inflammatory markers (TNF-α), trace elements (Fe, Cu, Zn, Mg, Se) and heavy metals (Cd, As, Pb, and Al).

RESEARCH QUESTION:

Do altered levels of TNF-α and blood metals profile show an association with Schizophrenia?

AIM:

To evaluate circulatory levels of TNF-α and blood metals profile in SZ and to find the association between them and PANSS score.

OBJECTIVES:

- ✓ To estimate and compare circulatory levels of TNF-α in the serum of controls and SZ patients
- ✓ To estimate and compare blood metals profile in controls and SZ patients.
- ✓ To find out the association between TNF-α, blood metals profile and PANSS score.

Materials and Methods

Ethical considerations:

The Institutional Ethics Committee of All India Institute of Medical Sciences, Jodhpur, approved this study. Study participants were asked to give informed consent, and their family members were also informed about the study. Subjects were given adequate time to discuss any questions they might have had about the research study. A complete description of all procedures was given beforehand, and subjects signed consent forms before carrying out any procedures. No invasive procedures were done during this study, and all specimens collected were kept in secure facilities.

Study setting: The study was carried out at AIIMS, Jodhpur, involving the Departments of Biochemistry and Psychiatry.

Study design: Cross-sectional case-control design was adopted for this study.

Study duration: The study was conducted over 1-year (December 2021 to December 2022).

Study participants

Sample size calculation

For sample size calculation, existing literature for serum TNF-α levels in SZ was considered (48). That information was used in the OpenEpi software to calculate the sample size. The sample size was calculated by comparing two means with 95% power and a 95% confidence interval. As part of our study, we took a total of ninety (80) participants, including 40 cases and 40 controls.

Inclusion and Exclusion criteria

Inclusion Criteria:

For cases:

- All patients with 18-60 years diagnosed with SZ and attending the OPD of the Department of Psychiatry in AIIMS, Jodhpur.

- The diagnosis of patients will be made as per ICD 10 (WHO 1992)
- The severity assessment is done by using PANSS.

For control:

- Non-psychiatric volunteers or staff members were used as healthy controls in the study. The provided information and general examination established their health status.

Exclusion Criteria:

For cases:

- Comorbid psychiatric disorders, for example, Bipolar and MDD (Major depressive disorder).
- Medical illnesses (e.g., autoimmune diseases, diabetes, HIV, endocrine disorders, hepatitis, cancer, or chronic infections).
- Medications (e.g., steroid medications, antioxidants, corticosteroids (oral, injected, inhaled, or topical), immunotherapy, antibiotics that could affect the immune system, psychotropic medication use (including antidepressants, mood stabilizers, anti-anxiety medications or antipsychotics) within previous 6 weeks.
- In addition, females in lactation or the gestational period were excluded.

For controls:

- The immune system disorders include autoimmune diseases, diabetes, HIV/AIDS, endocrine disorders, hepatitis, and cancer.
- Medications that may affect the immune system include steroid medications (oral, injected, and inhaled), corticosteroids (topical or oral), immunotherapy, and antibiotics that could affect the immune system were excluded.
- Females in pregnancy or lactation were also excluded.

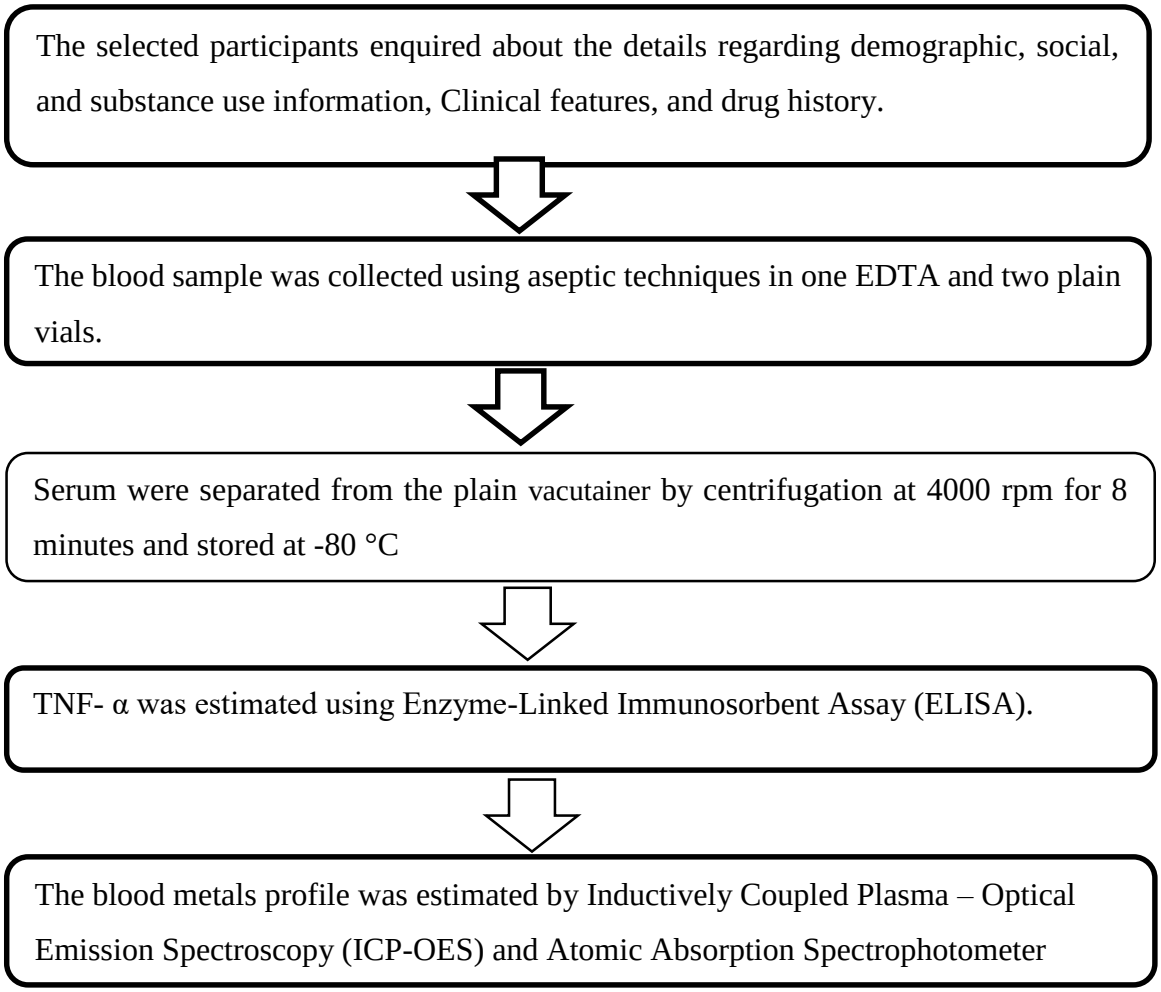
Severity assessment for patients:

The positive and negative syndrome scale (PANSS) was used to measure the severity of symptoms in SZ patients. 45–50 minutes clinical interview is conducted for every patient, and based on the discussion and reports from family members, the patient is rated from 1 to 7 on 30 different symptoms. The positive syndrome scale includes delusion, hallucination, conceptual disorganization, excitement, grandiosity, suspicion, and hospitality.

The negative syndrome scale includes Blunted affect (difficulty in expressing emotions). Emotional withdrawal (less social expression). Poor rapport (difficulties in establishing connections with others), Passive/apathetic social withdrawal (not displaying interest in others during conversations), Difficult abstract thinking (problems in understanding complex ideas or concepts), lack of spontaneity (no sense of adventure or willingness to take risks), Stereotyped thinking (not only isolated but also repetitive and rigid). PANSS range is from 7 to 49 points, 7 being minimal and 49 more severe symptoms.

The General Psychopathology Scale contains 16 items, with a minimum score of 16 and a maximum score of 112. The total PANSS score is from 30 to 210, 30 is the lowest and 210 is the highest score in the most severe case.

Methodology



ELISA for TNF-α:

ELISA test was performed to quantify the serum TNF- α levels in all the subjects recruited in this study by using Fine Test Human TNF- α ELISA Kit.

Reagents and consumables:

- ELISA-Microplate
- Standards
- Sample/Standard Dilution Buffer
- Biotin-labeled Antibody
- Antibody Dilution Buffer
- HRP-Streptavidin Conjugate (SABC)
- SABC Dilution Buffer

- TMB Substrate
- Stop Solution
- Wash Buffer
- Plate Sealer

Principle

The Fine Test kit was based on sandwich ELISA technology. First, the capture antibody was pre-coated onto 96-well plates. Then the biotin-conjugated antibody was used as a detection antibody. The standards, test samples, and biotin-conjugated detection antibodies were added to the wells subsequently and washed with wash buffer. After that, HRP -Streptavidin was added, and unbound conjugates were washed away with a wash buffer. TMB substrates were used to visualize HRP enzymatic reaction. HRP catalyzed TMB to produce a blue colour product that changed into yellow after adding an acidic stop solution. The density of yellow is proportional to the target amount of sample captured in the plate. After reading O.D. absorbance at 450nm in a microplate reader, the target concentration can be calculated using calibration curves from spectrophotometer readings.

Reagent Preparation

1. **Wash Buffer Preparation**

   To dilute the 30ml concentrated Wash Buffer into 750ml with distilled water, we added a volume of 1X distilled water. This made the Wash Buffer solution 1X, used for washing steps.

2. **Standards:**

a) We added 1 ml sample dilution Buffer into one standard tube and labelled it as zero tube, then we incubated it at room temperature for 10 minutes and mixed it properly.
b) We labeled 7 Eppendorf tubes with 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank respectively. Further, we added 0.2ml of the Sample Dilution Buffer into each tube and added 0.2ml of the above Standard solution (from zero tubes) into the 1st tube and mixed them.
c) Then we transfer 0.2ml from the 1st tube to the 2nd tube and mix them thoroughly. Transfer 0.2ml from the 2nd tube to the 3rd tube and mix them thoroughly, and so on.
d) We used a sample dilution buffer as blank control.

3. Preparation of Biotin-labeled Antibody Working Solution:

For this, we dilute Biotin-detection Antibodies with Antibody Dilution Buffer at 1:100 and mix them thoroughly.

4. Preparation of HRP-Streptavidin Conjugate (SABC) Working Solution:

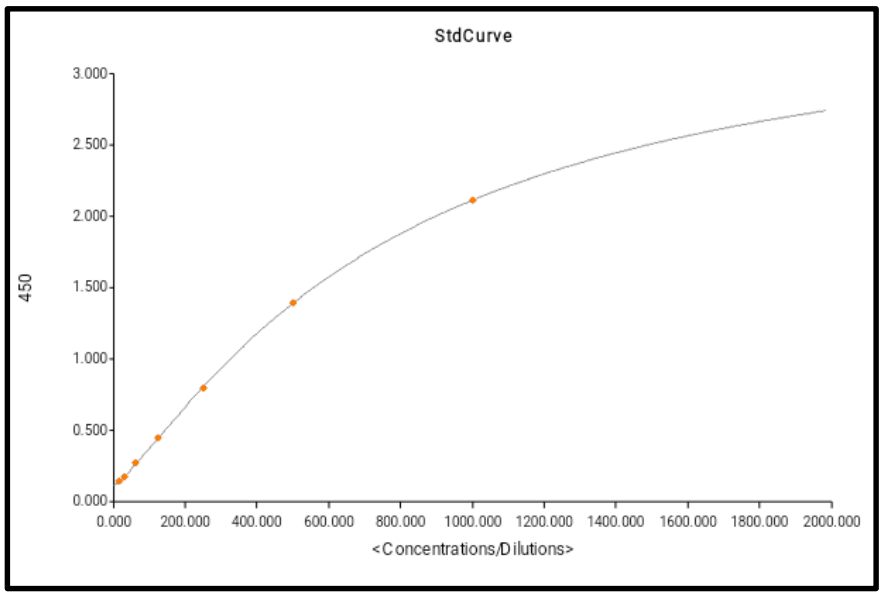
For this, we dilute the SABC with SABC Dilution Buffer at 1:100 and mix them thoroughly.

Procedure

- The serum samples were taken out from -80°C and waited until thawed.
- Meanwhile, we brought all the reagents from the kit to room temperature (20°C).
- After the wash, the plate was prepared by adding 100 µl of each standard and sample (diluted) into appropriate wells.
- We covered the plate with provided sealer and incubated it for 90 minutes at 37°C with gentle shaking.
- After incubation, the solution was discarded. Then, we washed the well two times with 1X wash buffer by filling each well with wash buffer (350 µl) using a multichannel pipette or auto washer.
- After adding 100 µl of 1X prepared HRP-Streptavidin Conjugate solution to each well, the plate was incubated for 30 minutes at 37°C with gentle shaking.
- We discard the solution and repeat the wash step five times.
- Then, we added 90 µl of TMB substrate to each well, and the plate was incubated in the dark for 10- 20 minutes at room temperature.
- Finally, we added 50 µl of stop solution to each well and evaluated the plate immediately after stopping the reaction.

Calculation:

Eon Bio Tek's multiple ELISA reader (Serial No.14021320) was used to take absorbance readings for standards and samples. The standard curve shown below was obtained from Gen 5 software (version 2.05.5).



TNF-α ELISA standard curve

Estimation of metals level by inductively coupled plasma-optical emission spectrometry

Principle

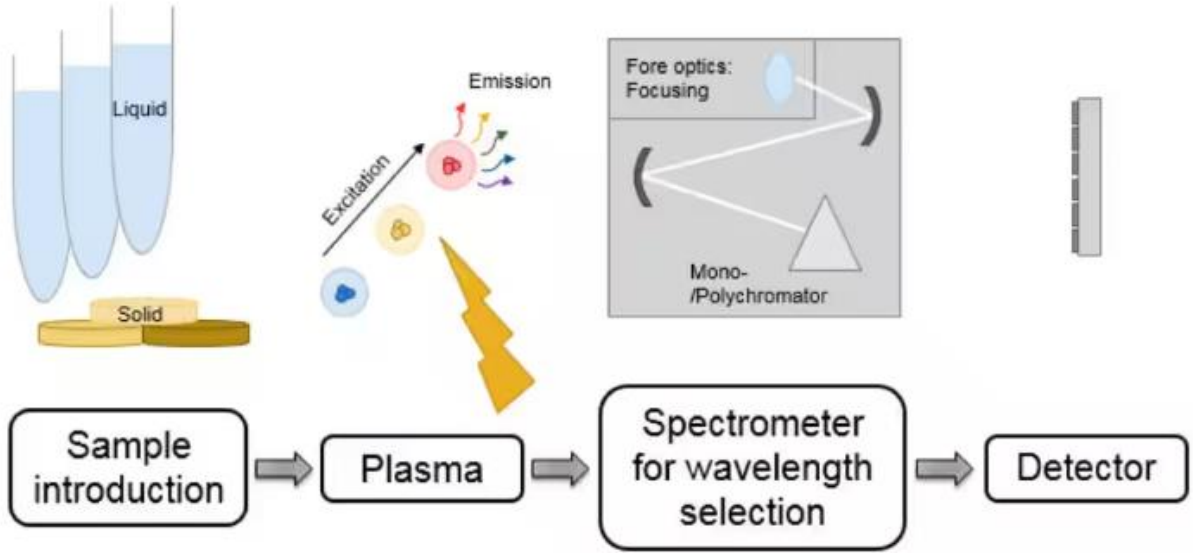
One of the optical emission spectrometry methods is Inductively Coupled Plasma (ICP-OES). A sample's atoms are excited and move into a higher energy state when plasma energy comes from the outside. However, the atom is unstable in this state and emits emission rays to return to its lower energy state. The photon-like wavelengths of the emission rays are measured. The position of photon rays determines the element type, and the intensity of the rays determines the quantity of each element.

Atoms in ICP-OES are excited by plasma, a source of energy. First, the torch coil receives argon gas, and the work coil at the torch tube's tip receives high-frequency electric current for plasma generation. This high-frequency current creates an electromagnetic field in the torch tube. Plasma is produced, and argon gas is ionized as a result. This energy is utilized in the excitation-emission of the sample's atom due to the plasma's high electron density and temperature of about 10,000 K. Through the narrow tube in the torch tube's centre; the sample solution enters the plasma in an atomized state.

Instrumentation

ICP-OES instrument has four essential components:

- Sample introduction system,
- Excitation source (plasma),
- Spectrometer (for wavelength selection),
- and Detector



Principle of ICP-OES

(121)

Consumables and instruments

- ICP-OES, Prodigy 7, Teledyne Leeman Labs, (USA).
- MARS6 micro digester system
- Coolant
- Argon and Nitrogen gas
- Trace elements standards (Merck, 1000 ppm)
- HNO_3 (65% Sigma Aldrich)
- H_2O_2 (30 % Molychem)
- Milli-Q water (18 Ω)
- Teflon PFA (Perfluoro alkoxy) coated microwave digestion vessel

- Polypropylene tubes 15 ml and 50 ml
- ClinChek whole serum controls (lot No. 8880)

Standard preparation, absorption wavelength, LOD and LOQ

Aqueous standards were prepared from 1000 mg/l of single-element standards (Merck, Germany). For standards preparation, 18 Ω Milli-Q water was used.

Elements	Standard	Concentration (mg/l)	Wavelength	Mode	LOD	LOQ
Iron Copper Zinc			259.94 324.75 206.20	Axial Axial Axial	9.7 0.87 2.7	29.39 2.93 8.18
	1	0.25				
	2	0.5				
	3	1				
	4	2				
Magnesium	1	5	279.55	Radial	0.4	1.21
	2	10				
	3	20				
	4	40				

Source settings for ICP-OES	
RF power (W)	1000
Coolant (L/min)	15
Auxiliary gas flow rate (L/min)	0.5
Nebulizer gas flow rate (PSI)	25
Replicates	2
Sample uptake rate (ml/min)	1
Sample uptake delay (s)	15

Quality control

- For quality control, we used the commercial reference material "ClinCheck controls for trace elements in serum (lot no. 8880) to check the accuracy of the results.
- Lyophilized bottle reconstructed as same as provided kit insert.
- It is processed the same as the sample.

Procedure

- Firstly, before digestion, we vortexed all the samples properly to make a homogeneous matrix.
- Each sample was put into a 15 ml precleaned and trace element-free Teflon PFA-coated digestion vessel.
- We put 500µl of each serum sample into an acid-washed microwave digestion vessel, followed by adding 1000µl HNO_3 (65% Sigma Aldrich) in each vessel.
- Then, we allowed the samples for 30 minutes for reaction, i.e., pre-reaction time in the clean hood.
- After completion of pre-reaction time, we added 1000 µl of non-stabilized 30 % H_2O_2 (Molychem) solution.
- After sealing the Teflon vessels, we placed them into the microwave digester (model MARS6 of CEM, USA).
- After digestion of the samples, 2500 µl of Milli-Q (18 Ω) water was added.
- Reagent blanks were prepared the same as the sample, but we added Milli-Q (18 Ω) water instead of the sample.
- Serum trace concentrations were measured using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), Prodigy 7 (Teledyne Leeman Labs, USA).

Estimation of metals level by Atomic absorption spectrophotometer (AAS)

Principle

The idea behind AAS is that atoms and ions can only absorb light at a specific wavelength. The atom absorbs the energy (light) when this particular wavelength of light is provided—the atom's electrons transition from the ground state to the excited state. The element's concentration in the sample can be determined by measuring the amount of light absorbed.

The Beer-Lambert law outlines the relationship between the element's concentration and light absorption. The Beer-Lambert law states that the number of atoms excited from the ground state directly correlates with the amount of light absorbed.

Graphite furnace AAS (GFAAS)

Liquid samples are directly injected into a tiny graphite-coated graphite tube in GFAAS, which can be heated to vaporize and atomize the analyte. In an electrothermal atomizer system, the graphite furnace can reach temperatures of 3000°C. Free ground state atoms are produced when chemical bonds in a sample held in a graphite tube are broken by the thermal energy provided by the heated graphite furnace.

Hollow cathode lamps provide specific elemental light directed through the cuvette's center to enable measurements during atomization. After that, ground-state atoms can absorb light energy and reach an excited state. As the concentration of the chosen element increases, so does the light energy absorb.

Consumables and instruments:

- GFAAS ICE 3400 (Thermo Fisher, USA)
- Argon gas
- Pb standard (1000 mg/l)
- Se standard (1000 mg/l)
- As standard (1000 mg/l)
- Al standard (1000 mg/l)
- Cd standard (1000 mg/l)
- 65% HNO_3 (Sigma Aldrich)
- Diammonium Hydrogen phosphate (Sigma Aldrich)
- Palladium matrix modifier (Sigma Aldrich)
- ClinChek whole blood and serum controls (Lot No. 8840, 8880)
- Polypropylene cups (2 ml and 20 ml)

Procedure for Pb estimation

1. To estimate Pb in blood, 10 µl of blood sample was diluted with a matrix modifier 900 µL (containing 0.5% TritonX- 100, 0.1 % diammonium hydrogen phosphate, and 0.2% HNO_3).
2. After thoroughly mixing the sample, 20 µl was introduced into the cuvette and subjected to proper atomization and ashing temperature.

Procedure for Se estimation

1. 100 µl of serum sample was diluted with a 900 µl mixture of matrix modifier (containing 0.5% Triton X- 100, Palladium matrix modifier, and 0.2 % HNO_3) in 1: 10 dilutions.
2. After adequately mixing the sample, 20 µl was introduced into the cuvette and subjected to proper atomization and ashing temperature.

Procedure for As estimation

1. 100 µl of blood sample was diluted with 900 µl of a mixture of matrix modifier (containing 0.5% Triton X- 100, Palladium matrix modifier, and 0.2 % HNO_3) in 1: 10 dilutions.
2. After adequately mixing the sample, 20 µl was introduced into the cuvette and subjected to appropriate atomization and ashing temperature.

Procedure for Cd estimation

1. 100 µl of blood sample was diluted with 900 µl of 0.2% HNO_3.
2. After adequately mixing the sample, 20 µl was introduced into the cuvette and subjected to appropriate atomization and ashing temperature.

Procedure for Al estimation

1. 100 µl of serum sample was diluted with 900 µl of 0.2% HNO_3.
2. After adequately mixing the sample, 20 µl was introduced into the cuvette and subjected to appropriate atomization and ashing temperature.

Optimized Experimental Conditions

Values					
Parameter	**Pb**	**Se**	**As**	**Al**	**Cd**
Wavelength (nm)	283.3	196	193.7	309.3	288.8
Bandpass (nm)	1.0	0.5	0.5	0.5	0.5
Lamp current (mA)	90	80	90	80	50
Measurement	Peak height	Peak height	Peak height	Peak height	Peak height
Background correction	Zeeman	Zeeman	Zeeman	Zeeman	Zeeman
Replicates	2	2	2	2	2
Standard Unit	µg/dl	µg/dl	µg/dl	µg/dl	µg/dl
Sample Unit	µg/dl	µg/dl	µg/dl	µg/dl	µg/dl
Sample Volume	20µl	20 µl	20 µl	20 µl	20 µl

Furnace temperature for Pb

Standards (µg/l)	**Temp °C**	**Ramp °C/s**	**Hold (sec.)**	**Internal gas (L/min)**
2.5	130	10	2	0.2
5	200	10	30	0.2
10	600	150	10	0.2
20	2000	0	3	Off
40	2450	0	3	0.2

Furnace temperature for Se

Standards (µg/l)	Temp °C	Ramp °C/s	Hold (sec.)	Internal gas (L/min)
25	100	10	30	0.2
50	1100	150	20	0.2
100	2200	0	3	0.2
200	2500	0	3	Off

Furnace temperature for As

Standards (µg/l)	Temp °C	Ramp °C/s	Hold (sec.)	Internal gas (L/min)
3	100	10	2	0.2
6	1200	150	30	0.2
12	2600	0	10	0.2
24	2700	0	3	Off

Furnace temperature for Al

Standards (µg/l)	Temp °C	Ramp °C/s	Hold	Internal gas (L/min)
5	100	10	2	0.2
10	1500	150	30	0.2
20	2300	0	10	Off
40	2500	0	3	0.2

Furnace temperature for Cd

Standards (µg/l)	Temp °C	Ramp °C/s	Hold (sec.)	Internal gas (L/min)
0.125	130	10	20	0.2
0.250	200	10	30	0.2
0.50	600	150	10	0.2
1	2000	0	3	0.2
2	2450	0	3	Off

Quality Control

All equipment, including glassware, sample cups, and stoppers, were washed with 5% HNO_3 and later rinsed with distilled ionized water to prevent contamination. We ran the sample in duplicates for precision. To check quality control, ClinChek Whole blood control levels I, II, III (Lot No. 8840), containing (54.5, 219, 425) µg/l of Pb, (1.23, 2.888, 6.32) µg/l of Cd and (5.42, 9.97, 19.4) µg/l of As and ClinChek serum control levels, I and II (Lot No.8880), containing (55.9 and 112) µg/l of Se and (11.3, 53.8) µg/l of Al were used.

Calibration/ Standard curve

The solution containing (2.5, 5, 10. 20) µg/l of Pb, (25, 50,100,200) µg/l of Se, (3, 6, 12. 2) µg/l of As, and (0.125, 0.25, 0.5, 1, 2) µg/l of Cd and similarly (5, 10, 20, 40) µg/l of Al were used for the calibration curve. Standards were prepared by serial dilution from their stock solution (1000 mg/l). The absorbance of the standard solution after correction for the blank measure and plotted against the corresponding concentrations. Acceptable for calibration Curve was > 95%.

Statistical Analysis:

Data analysis was performed using SPSS 19.0 and Jamovi software. Descriptive statistic was used in the form of number, percentages and median (IQR). Normal distribution was assessed by using the Shapiro-Wilk test. Case and control were compared by chi-square test for nominal variables. The Mann-Whitney U test was used for blood metals and TNF-α levels comparison in patients and controls (continuous variables). Spearmen's correlation was done to identify the correlation between pro-inflammatory markers TNF- α and blood metal profile with PANSS scale (for the severity of SZ), and p-value < 0.05 was considered statistically significant. Univariate odd ratio (OR) was evaluated using the unconditional Logistic regression model.

Results

Description of participant

In the present study, 40 Schizophrenia (SZ) patients and 40 healthy controls were recruited. SZ patients were diagnosed as per ICD-10 Criteria and evaluated the severity based on positive and negative syndrome scale (PANSS) scores.

Table 1. Demographic information of participants

Characteristics	**Cases (n=40) No., (%)**	**Controls (n=40) No., (%)**	**p**
Age (years)			< .001
18-28	12 (15)	19 (23.8)	
29-39	9 (11.3)	19 (23.8)	
40-50	12 (15)	2 (2.5)	
51-60	7 (8.8)	0	
Gender			0.012
Male	24 (30)	34 (42.5)	
Female	16 (20)	6 (7.5)	
BMI (kg/m^2)			0.257
Normal	18 (22.5)	26 (32.5)	
Overweight	9 (11.3)	5 (6.3)	
Obesity	7 (8.3)	7 (8.8)	
Underweight	6 (7.5)	2 (2.5)	
Marital status			0.491
Married	26 (32.5)	23 (28.7)	
Unmarried	14 (17.5)	17 (21.3)	
Family type			0.178
Joint	15 (18.8)	21 (26.3)	
Nuclear	25 (31.3)	19 (23.8)	

Comparison between cases and controls using the chi-square test. ($p < 0.05$) are significant.

Demographic information

The distributions of demographic information of the patients and controls are presented in (Table 1). Characteristics of patients and control are shown as numbers and percentages. There were 22 female participants and 58 male participants overall. There were 24 (30%) male cases and 16 (20%) female cases, respectively. In controls, there were 34 (42.5%) and 6 (7.5%) controls. Cases and controls were further divided into age subgroups: 18-28, 29-39, 40-49, and 50-60.

The number of cases was 12 (15%), 9 (11.3%), 12 (15%), and 7 (8.8%) and the number of controls was 19 (23.8%), 19 (23.8%), 2 (2.5%) and 0 those were lies in the above age subgroups. When comparing the case to the control group, age and gender showed statistically significant differences (p-values .001 and 0.012, respectively). Using a BMI calculator, the body mass index (BMI) was divided into four subgroups: normal, overweight, obese, and underweight. 18 (22.5 %), 7 (8.3%), 9 (11.3 %), and 6 (7.5 %) were the cases, while 26 (32.5 %), 5 (6.3 %), 7 (8.8%), and 2 (2.5 %) were the controls who fell in above BMI criteria. There were 26 (32.5%) married people in the case group, while 14.5% were unmarried. 23 (28.7%) people in the control group were married, while 21.3% were unmarried. The majority of participants in the case group came from nuclear families. The p-values were insignificant for BMI, marital status, and family type.

Table 2. Social and substance use information of participants

Characteristics	Cases (n=40) No., (%)	Controls (n=40) No., (%)	p
Locality			
Rural	20 (25)	22 (27.5)	0.654
Urban	20 (25)	18 (22.5)	
Religion			
Hindu	35 (43.8)	35 (43.8)	0.574
Islam	5 (6.3)	4 (5)	
Sikhism	0	1 (1.3)	
Employment status			
Employed	22 (27.5)	40 (50)	< .001
Unemployed	18 (22.5)	0	
Smoking			
Yes	14 (17.5)	7 (8.8)	0.075
No	26 (32.5)	33 (41.3)	
Alcohol consumption			
Yes	1 (1.3)	7 (8.8)	0.025
No	39 (48.8)	33 (41.3)	
Family history			
Yes	7 (8.8)	0	0.006
No	33 (41.3)	40 (50)	

Comparison between cases and controls using the chi-square test. ($p < 0.05$) are significant.

Table 2; represents social and substance use information about the participants. Patients came from both urban and rural areas in equal numbers. Case and control came primarily from Hindu families in religion. 18 (22.5%) of the 40 patients were unemployed. There were no unemployed participants in the control group. 14 (17.5%) of the 40 people in the case group were smokers. 1 (1.3%) of the cases consumed alcohol. 7 (8.8 %) of the patients in the case group had SZ disorder in their families. There were significant differences between subjects

and healthy controls (p-values .001, 0.025, and 0.006) in alcohol consumption, family history, and employment status.

Table 3. Drug history of patients

Medication	Cases (n=40) No., (%)	Controls (n=40) No., (%)
Olanzapine	13 (16.3)	-
Clonazepam	21 (26.6)	-
Risperidone	21 (26.6)	-
Clozapine	5 (6.3)	-
Lorazepam	5 (6.3)	-
Amisulpride	7 (8.8)	-
Aripiprazole	6 (7.5)	-
THP (trihexyphenidyl)	10 (12.6)	-
Sodium valproate	3 (3.8)	-
Fluoxetine	6 (7.5)	-
Alprazolam	1 (1.3)	-
Lithium	1 (1.3)	-
Chlorpromazine	2 (2.5)	-
Haloperidol	1 (1.3)	-

Data of patients are shown in numbers and percentages.

Table 3. Shows the drug history among the case group. The maximum number of patients who used clonazepam and risperidone was 21 (26.9%). Olanzapine was used by 13 patients (or 16.3%). SZ patients used the remaining medications, including clozapine, lorazepam, amisulpride, THP (Trihexyphenidyl), sodium valproate, fluoxetine, alprazolam, lithium, chlorpromazine, haloperidol, in proportions of 5 (6.3%), 5 (6.3%), 7 (8.85%), 6 (7.5%), 10 (12.6%), 3 (3.8%), 6 (7.5%), 1 (1.3%), 2 (2.5%), 1 (1.3%).

Table 4. Clinical features of patients

Clinical features **Cases (n=40)**	**Median (IQR)**
Age of illness (years)	28.5 (22.75-32-25)
Duration of illness (years)	6.5 (2.75-15.25)
PANSS positive score	12.5 (7.75-20)
PANSS negative score	19 (12.75-24.25)
PANSS general score	27 (18.75-37.5)
PANSS total score	58.5 (43.25-78.25)

IQR, interquartile range in years and numbers

Table 4; shows that the age and duration of illness for cases were 28.5 (22.75-32-25) and 6.5 (2.75-15.25) years, respectively, in the median (IQR). The PANSS score was used to determine the severity of the patient by the guidelines. The median (IQR) for PANSS positive symptoms was 12.5 (7.75-20). Similarly, 19 (12.75-24.25), 27 (18.75-37.5), and 58.5 (43.25-78.25) median (IQR) for negative, general, and total PANSS scores.

Table 5. Comparison of TNF-α in serum of cases and controls

Cases (n=40) **Controls (n=40)**	**Median (IQR) pg/dl**	**p**
Cases	40.15 (18.11-126.94)	0.38
Controls	24.03 (19.58-68.30)	

Comparison of mean and median between case and controls using Mann-Whitney U test

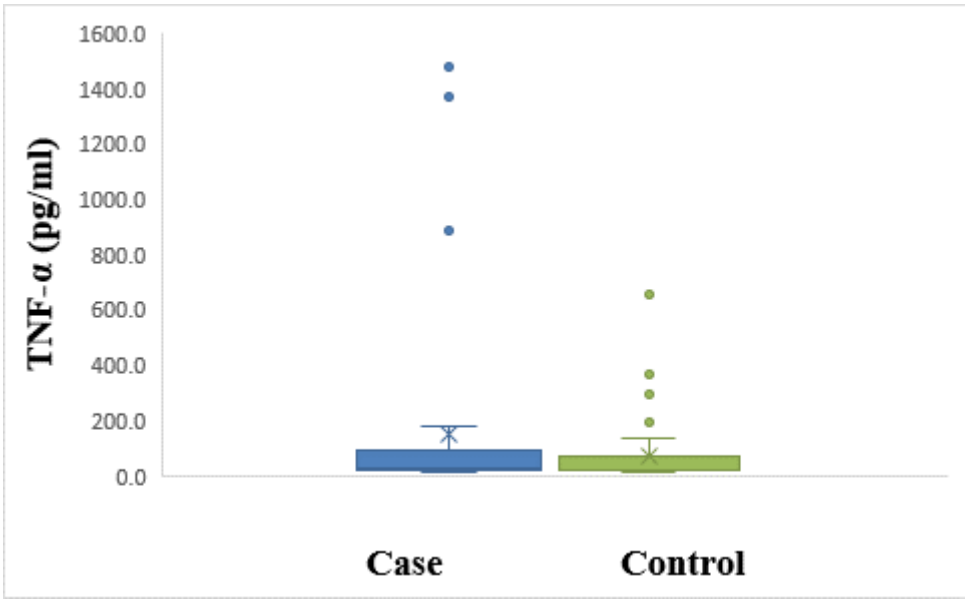


Figure1. Serum TNF-α level in case and control group

(Figure 1 &Table 5) shows that SZ patients had higher serum levels of the pro-inflammatory cytokine TNF-α than healthy controls who did not have SZ. The median (IQR) was 40.15 (18.11-126.94) pg/dl for cases and 24.03 (19.58-68.30) pg/dl for healthy controls. The p-value was insignificant when comparing serum TNF-α in the case and control groups.

Table 6. Blood metals levels comparison in participants

Metals	Median (IQR) µg/dl		p
	Cases (n=40)	Controls (n=40)	
Iron	133.13 (101.78-176.16)	108.60 (77.55-137.63)	0.038
Copper	143.43 (127.31-160.96)	97.30 (78.25-127.74)	< .001
Zinc	123.25 (103.85-144.73)	133.59 (110.53-163.44)	0.338
Magnesium	1649.5 (1557.5-1782)	1550 (1427-1821.5)	0.511
Selenium	6.71 (3.56-8.3)	3.31 (1.76-5.05)	< .001
Aluminum	6.25 (4.45-8.2)	6.34 (4.87-8.72)	0.436
Lead	1.11 (0.59-1.26)	0.75 (0.46-1.06)	0.015
Cadmium	0.9 (0.4-1.72)	0.11 (0.04-0.18)	< .001
Arsenic	1.01 (0.68-1.81)	0.89 (0.61-1.11)	0.262

Comparison of the median (IQR) µg/dl between cases and controls by Mann-Whitney U test. ($p < 0.05$) are significant.

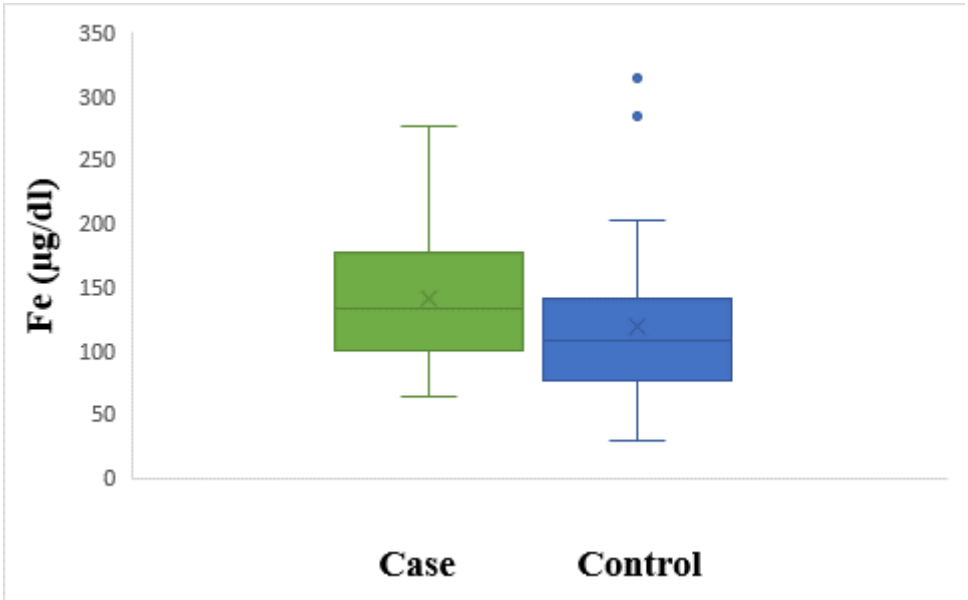


Figure 2. Serum Iron levels in case and control group

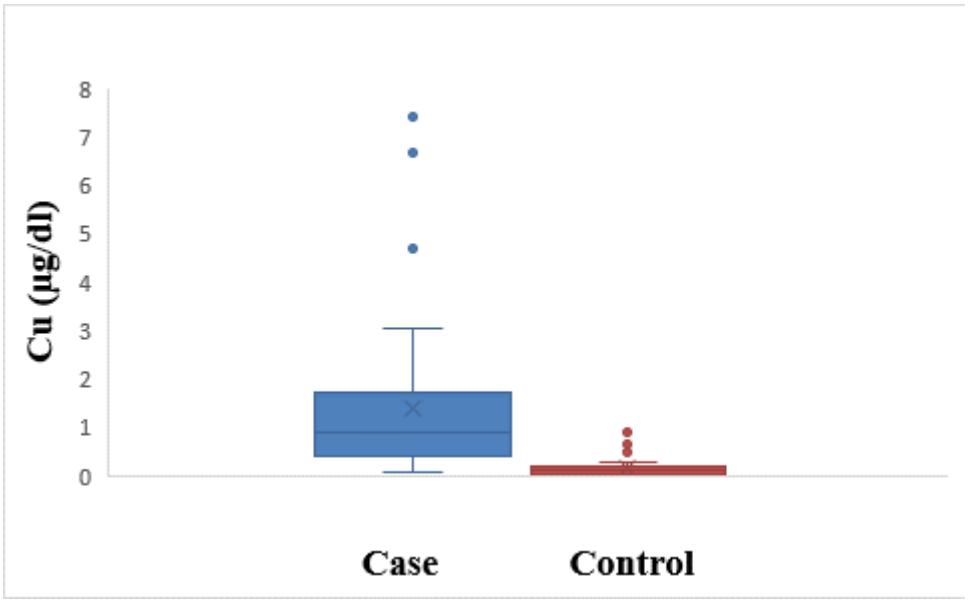


Figure 3. Serum Copper levels in case and control group

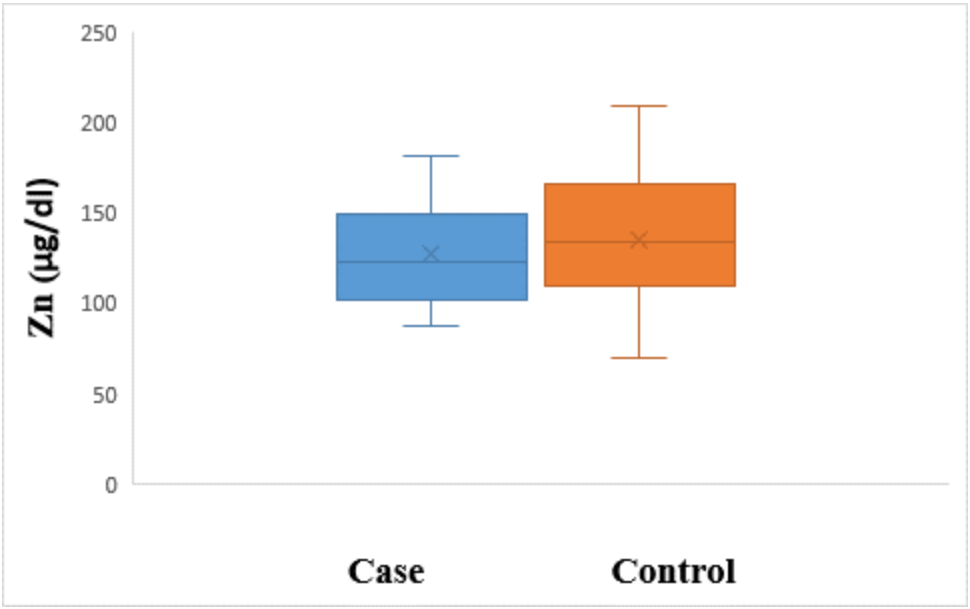


Figure 4. Serum Zinc levels in case and control group

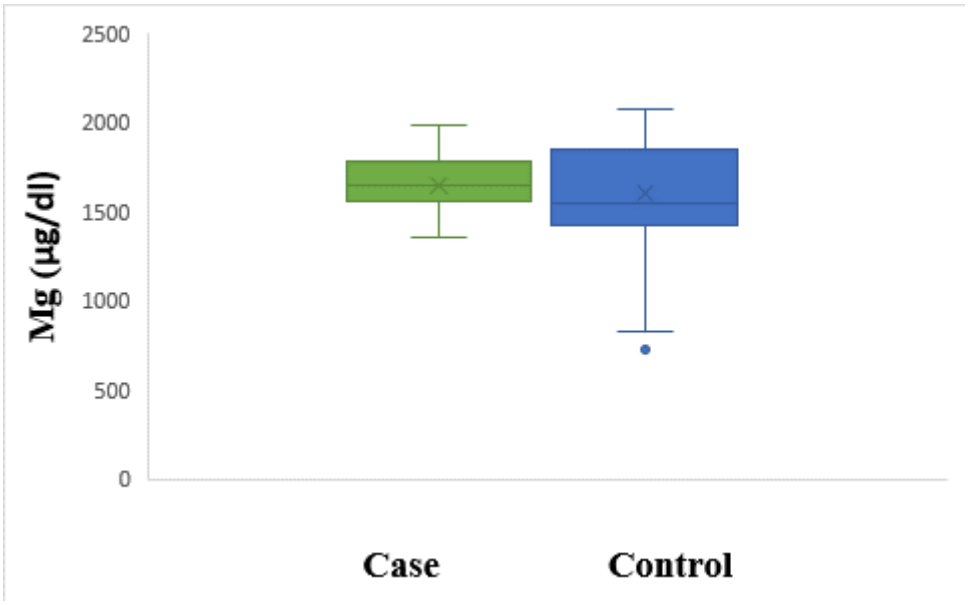


Figure 5. Serum Magnesium levels in case and control group

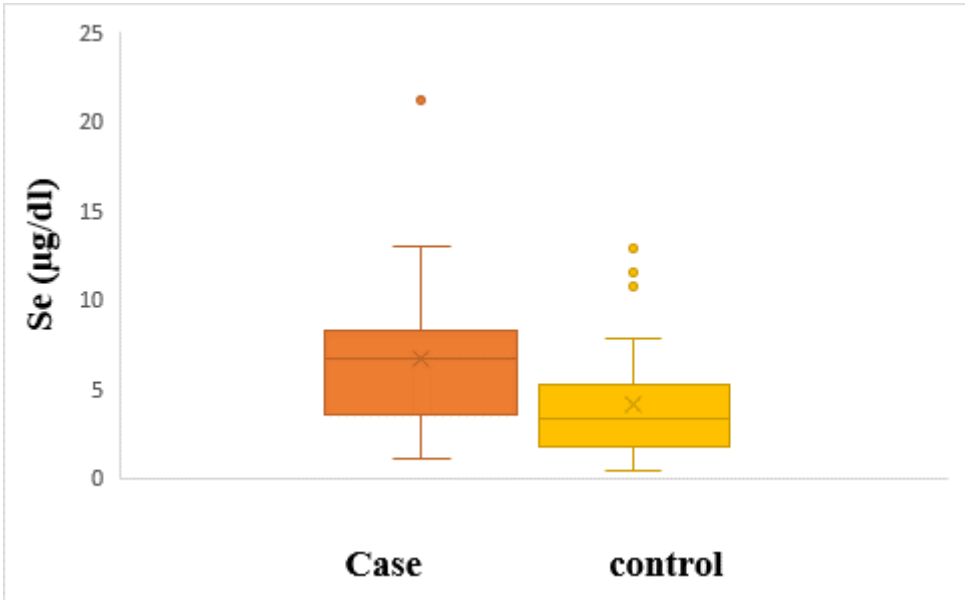


Figure 6. Serum Selenium levels in case and control group

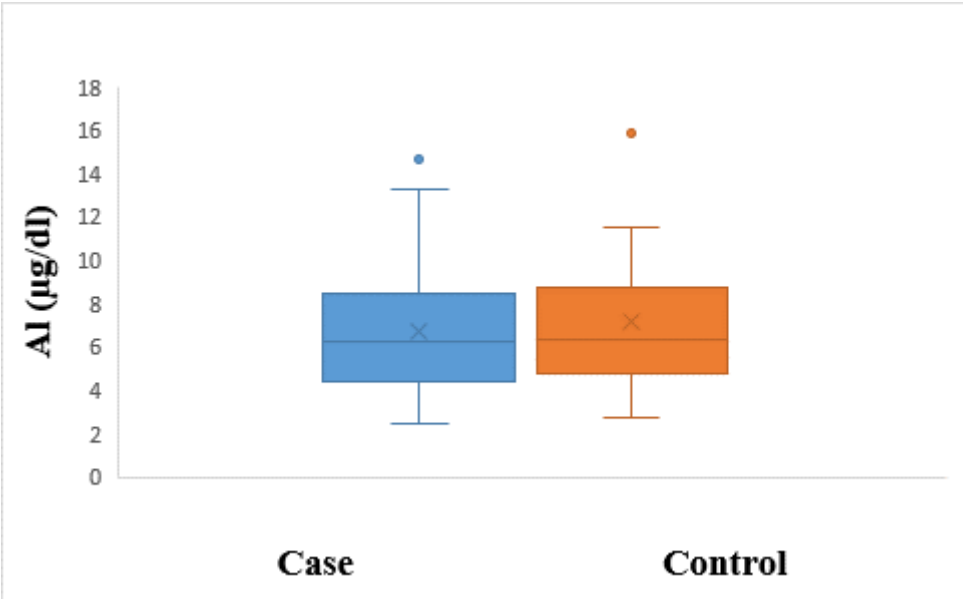


Figure 7. Serum Aluminum levels in case and control group

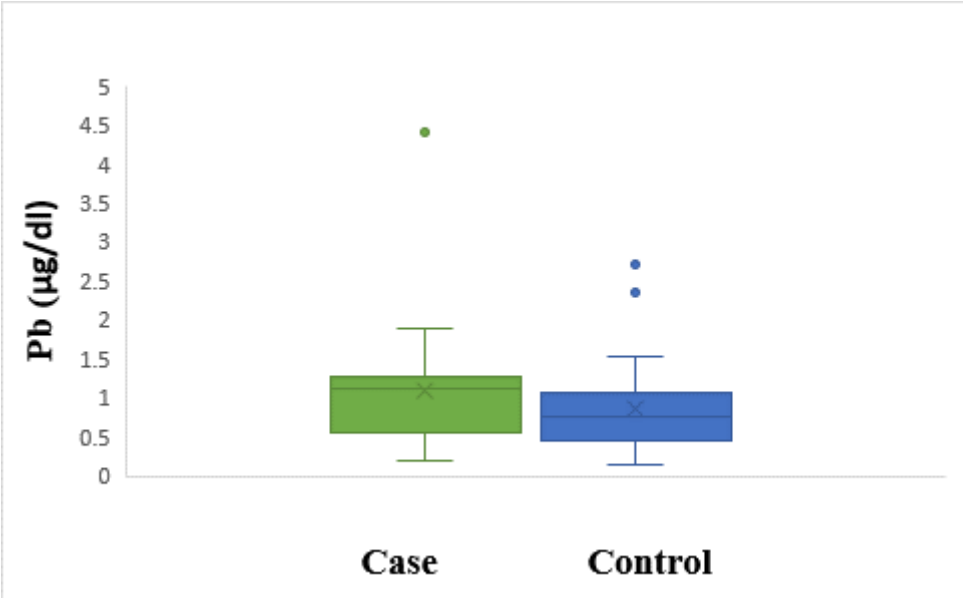


Figure 8. Blood Lead levels in case and control group

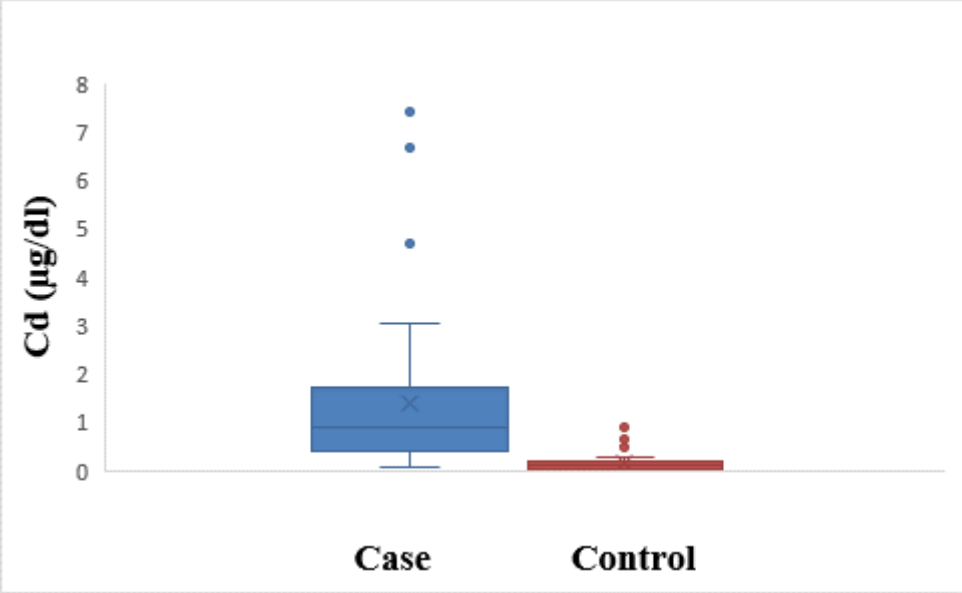


Figure 9. Blood Cadmium levels in case and control group

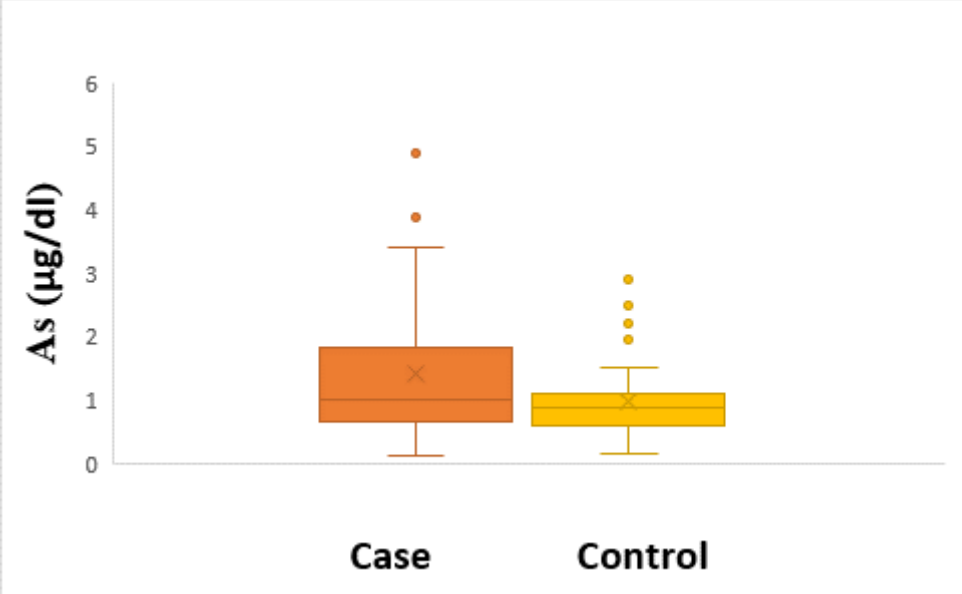


Figure 10. Blood Arsenic levels in case and control group

Table 6; represent the comparison of blood metals level in the case and the healthy controls. The median (IQR) for Fe level in cases came out as 133.13 (101.78-176.16) µg/dl than healthy controls 108.60 (77.55-137.63) µg/dl (Table 6, Figure 2). Cu level in serum was higher in the case group with a median (IQR) of 143.43 (127.31-160.96) µg/dl compared to the control

group with a median (IQR) of 97.30 (78.25-127.74) µg/dl (Table 6, Figure 3). Serum Zn levels were lower in the case group with a median (IQR) of 123.25 (103.85-144.73) µg/dl when comparing the median (IQR) of the control group 133.59 (110.53-163.44) µg/dl (Table 6, Figure 4). Serum Mg levels were higher in the case group, with a median (IQR) of 1649.5 (1557.5-1782) µg/dl, compared to the control group, with a median (IQR) of 1550 (1427-1821.5) µg/dl (Table 6, Figure 5). Se levels in serum were higher in the case group with a median (IQR) of 6.71 (3.56-8.3 µg/dl when compared to the median (IQR) of the control group 3.31 (1.76-5.05) µg/dl. (Table 6, Figure 6). Al levels in serum were lower in the case group with a median (IQR) of 6.25 (4.45-8.2) µg/dl compared to the control group 6.34 (4.87-8.72) µg/dl (Table 6, Figure 7). Similarly, the median (IQR) for blood Pb and Cd levels were increased in the case group with 1.11 (0.59-1.26) µg/dl and 0.9 (0.4-1.72) µg/dl compared to the control group 0.75 (0.46-1.06) µg/dl and 0.11 (0.04-0.18) µg/dl (Table 6, Figure 8 and 9). Blood As levels were higher in the case group with a median (IQR) of 1.01 (0.68-1.81) µg/dl as compared to the control group 0.89 (0.61-1.11) µg/dl. (Table 6, Figure 10). (Fe, Cu, Se, Pb, and Cd) was found to be significant (p-value 0.038, <.001, <.001, and .001).

Table 7: Correlation between TNF-α levels and PANSS score in SZ patients

PANSS score	**Spearman's rho**	**p**
PANSS positive	0.244	0.130
PANSS negative	0.197	0.223
PANSS general	0.198	0.221
PANSS total	0.265	0.098

TNF-α and PANSS score correlation was calculated using Spearman (rho)

Table 7; describe the correlation between TNF-α levels and PANSS score in patients. The serum TNF-α levels showed a weak positive correlation with the PANSS positive and total scores. However, the correlation was statistically insignificant.

Table 8. Correlation between blood metals and PANSS score in SZ patients

Metals (µg/dl)	**PANSS positive score**		**PANSS negative score**		**PANSS general score**		**PANSS total score**	
	rho	p	rho	p	rho	p	Rho	p
Iron	0.055	0.747	0.038	0.825	-0.082	0.628	0.041	0.811
Copper	0.151	0.353	0.23	0.154	0.299	0.061	0.269	0.094
Zinc	0.011	0.945	0.106	0.517	0.123	0.451	0.098	0.548
Magnesium	0.165	0.309	0.241	0.134	0.135	0.406	0.215	0.182
Selenium	0.062	0.705	0.023	0.886	0.125	0.443	0.089	0.586
Aluminum	-0.172	0.289	-0.278	0.082	-0.184	0.256	-0.208	0.197
Lead	0.232	0.151	-0.103	0.528	-0.162	0.318	-0.185	0.252
Cadmium	-0.069	0.674	-0.276	0.085	-0.095	0.560	-0.172	0.288
Arsenic	-0.057	0.726	-0.034	0.837	0.008	0.961	-0.038	0.185

Correlation between blood metals levels and PANSS scores in patients using Spearman (rho)

Table 8; Shows the correlation assay between blood metals levels and PANSS of SZ patients. A p-value < 0.05 was considered significant. From the table, none of the metals (Fe, Cu, Zn, Mg, Se, Al, Pb, Cd and As) showed a significant correlation with PANSS scores ($p > 0.05$).

Table 9. Correlation between TNF-α levels and blood metals profile in SZ patients

Metals (µg/dl)	Spearman's rho	p
Iron	0.067	0.566
Copper	0.234*	0.041
Zinc	-0.122	0.284
Magnesium	0.064	0.580
Selenium	-0.116	0.307
Aluminum	0.062	0.591
Lead	-0.01	0.931
Cadmium	0.001	0.991
Arsenic	0.042	0.710

Spearman correlation (rho) of TNF-α and blood metals levels in patients (Note* $p < 0.05$) are significant.

Table 9; Shows the correlation assay between serum TNF-α level and blood metals profile. Only Copper showed a significant positive correlation with TNF-α (rho = 0.234; p = 0.04). In comparison, other metals (Fe, Mg, Al, Pb, Cd and As) did not significantly correlate with TNF-α.

Table 10. Association between metals profile and SZ risk (OR)

Metals (µg/dl)	Univariate OR (95% CI)	p
Iron	0.99 (0.98-1.00)	0.89
Copper	0.93 (0.91-0.96)	0.00
Zinc	1.00 (0.99-1.02)	0.30
Magnesium	0.99 (0.99-1.00)	0.36
Selenium	0.80 (0.68-0.93)	0.004
Aluminum	1.06 (0.91-1.22)	0.43
Lead	0.56 (2.52-1.24)	0.15
Cadmium	0.007 (0.001-0.80)	0.00
Arsenic	0.56 (0.32-0.98)	0.44

Association between metals and SZ risk using an unconditional Logistic regression model. ($p < 0.05$) are significant.

Odd ratio (OR) was used to measure the strength of the association between risks of SZ and metals profile. Evaluation based on unconditional logistic regression models demonstrated that the concentrations of (Cu, Se, and Cd) were associated with the risk of SZ. The OR and 95% confidence interval (95% CI) were 0.93 (0.91-0.96), 0.80 (0.68-0.93), and 0.007 (0.001-0.80) with p-value (0.00, 0.004, and 0.00). However, the concentrations of (Fe, Zn, Mg, Al, Pb, and As) were not associated with the risk of SZ. (Table 10).

Table 11. Association of TNF-α and SZ risk (OR)

Pro-inflammatory cytokine	**Univariate OR (95% CI)**	**p**
TNF-α (pg/dl)	0.99 (0.96-1.00)	0.98

Association between TNF-α and SZ risk using an unconditional Logistic regression model.

The strength of the association between TNF-α and SZ risks was measured using the odds ratio (OR). TNF- had an odd ratio and 95% confidence interval (CI) of 0.99 (0.96-1.00). The outcome, however, was insignificant.

Discussion

Schizophrenia is a severe mental disorder with a worldwide prevalence of 1%. This disorder affects an individual's thinking, perception, and behaviour. The typical onset of SZ is in late adolescence or early 20s, with a slightly later onset in females. The aim of the present study is to assess serum TNF-α and blood metals levels in cases and controls. A further correlation was evaluated between TNF- α with PANSS, blood metals and PANSS score.

Demographic information

In the present study, 80 participants were enrolled. Among 80 participants, there were 58 males and 22 females. Of 80 participants, 40 cases were diagnosed with SZ according to ICD-10 criteria. Forty apparent healthy individuals without any known history of SZ were taken as controls. The age of the participants was in the range of 18-60 years. Demographic information such as age, gender, employment status, alcohol consumption and family history were compared between case and controls with significant differences ($p < .001$, $0.012 < .001$, 0.025 and 0.006 respectively) (Table 1 & 2). A maximum number of cases were between the age group of 20 and 40 years. Our study results are in line with previous results reported by Zorkina et al. and Cohen et al that said that this disease onset is started before 30 years of age and males are more affected at the early onset of the disease (122,123).

Serum TNF-α level

The median (IQR) level of TNF-α was 40.15 (18.11-126.94) pg/ml in cases, 24.03 (19.58-68.30) pg/ml in controls ($p = 0.38$). Therefore, compared to the control population, SZ cases have higher levels of TNF-α (Table 5 & Figure 1). This finding is in accordance with previous studies done by Lin et al. who showed that TNF-α levels in FEDN patients were significantly higher than in healthy controls and chronic patients. In another study, Zhu et al. also found similar results. These studies suggested that a high TNF-α could be attributed to chronic inflammation, cytokines alterations, and increased oxidative stress in patients (47,48). Inconsistent with our findings, Balotsev et al. reported no significant difference in TNF-α levels in cases and control group (46).

Correlation between TNF-α with the severity of SZ

Furthermore, we carried out a correlation analysis. We reported a positive correlation between TNF-α and the PANSS positive score ($rho = 0.244$; $p = 0.13$) in our study (Table 7). A study done by Zhu et al. found a positive correlation between TNF-α and the negative score in

PANSS, and also they mentioned no significant correlation between the positive and general PANSS scores (48). Another study done by Lin et al. showed that psychotic symptoms (PANSS total score, positive subscale score, negative subscale score, and general psychopathology subscale score) did not significantly correlate with TNF-α levels in FEDN patients before or after treatment. After adjusting for age, sex, education and TNF-α levels in chronic patients showed a significant negative correlation with the PANSS total scores and the PANSS general psychopathology subscale scores.

Blood metal levels

Trace elements:

In our study, we evaluated the blood metal concentration in both groups. We found median (IQR) for Iron was 133.13 (101.78-176.16) µg/dl for cases and 108.60 (77.55-137.63) µg/dl for healthy controls ($p = 0.038$). The result showed that serum Fe was significantly higher in the cases as compared to the healthy controls (Table 6 & Figure 2). Cao et al. in their study found high concentrations of Fe in patients than in healthy controls, these high Fe may enhance oxidative stress and subsequent neural death. Neurotoxic oxygen radicals produced via the Fenton reaction may be responsible for such high Fe in their study (75). Some studies also reported high levels of Fe in the brain are associated with neurodegenerative diseases like Alzheimer's (124,125). Contrary to our findings antipsychotic drugs (78,126,127) and an imbalanced diet induce alterations in serum trace element levels in schizophrenic patients (128).

A statistically significantly higher level of serum Copper was found in cases than controls with median (IQR) of 143.43 (127.31-160.96) µg/dl and 133.59 (110.53-163.44) µg/dl, ($p < .001$) in our study (Table 6 & Figure 3). This finding is in line with the study done by Wolf et al., in which they showed elevated ceruloplasmin, was correlated with high Cu levels. Cu toxicity may lead to a decreased dopamine as Cu and dopamine are linked to various biochemical pathways in dopamine production and compensation increased number of dopamine receptors seen in a certain area of the brain is seen in SZ patients may be responsible for such high Cu (83). A previous study suggested that Cu can undergo a redox cycle and promote the formation of ROS (80). A study by Olatunbosun et al. indicated significantly increased serum Cu levels among male and female patients compared to the control group (129) and drug naïve patients (130). However, some previous studies

investigated a decreased level of Cu in cases than controls (76,126,131). In Cao et al. study no significant difference was found in serum Cu levels in people with SZ than controls (75)

On estimating selenium we found that Selenium levels were significantly elevated in patients 6.71 (3.56-8.3) µg/dl than in controls 3.31 (1.76-5.05) µg/dl, ($p < .001$) (Table 6 & Figure 6). In another study done by Kanazawa et al., they found increased Se binding protein 1 (SELENBP1) gene expression in SZ patients than in healthy controls. A high level of Se increases Se-binding protein 1 gene expression (95,96). High Se in food from soil caused by increased air pollution was responsible for a high of this element in the Karim et al. study (119). In conversely, other studies showed lower selenium in patients with SZ compared to the control group (74,94,126).

Zinc level in the present study was found to be lower in patients 123.25 (103.85-144.73) µg/dl as compared to the healthy controls with median (IQR) of 133.59 (110.53-163.44) µg/dl, ($p = 0.33$) (Table 6 & Figure 4). This finding was similar to other previous studies (74,127,131). Curz et al also found lower Zn levels in their cases on antipsychotic treatment indicating that SZ patients might undergo oxidative stress processes regardless of drug treatment in their study. Zn deficiency occurs usually due to insufficient Zn intake and absorption or loss of Zn from the body or increased requirement of the body, which was the reason for the low Zn level in their study (74). While in Cao et al., study they did not find any significant difference between cases and controls (75).

The present study reported higher levels of Magnesium 1649.5 (1557.5-1782) µg/dl for patients as compared to healthy controls 1550 (1427-1821.5) µg/dl, ($p = 0.51$) (Table 6 & Figure 5). These study results are in line with some previous studies (130,132). Ordak et al. also suggested that an increased level of serum Mg might be due to the use of Mg aspartate as a treatment (132). Moreover, Jenn et al. also found hypermagnesemia in SZ patients as many patients receive Mg oxide in their study also they found elevated Mg which acts as a protective factor in female SZ patients (133). Previous studies also investigated higher Mg levels are related to more severe psychopathology after metal exposure (134). In contrast with our findings, previous studies showed a decrease in serum Mg in cases than the control group (135,136). Ruljancic et al. did not find any difference in serum Mg in three groups: patients with attempted suicide, patients without suicidal behaviour, and the control group (93).

Heavy metals:

We noted a significantly higher level of Pb in cases 1.11 (0.59-1.26) µg/dl than in healthy controls 0.75 (0.46-1.06) µg/dl, (p = 0.015) (Table 6 & Figure 8). The results of our study were consistent with the previous studies (114,126). Kulkarni et al. reported, maternal late pregnancy Pb was associated with deficits in the mental development index (MDI) of children at 6 months. Pb causes neurotoxicity in the developing brain. High Pb disrupts and can decrease the number of synapses and interfere with the release of neurotransmitters, which can be a possible reason for their study (112). A high level of Pb might be a reason behind the increased oxidative stress, and inflammation and contribute to eventual psychopathology (22). In contrast with our findings, Karim et al. reported a decreased level of Pb in the case group than in controls (119).

Blood Cadmium level in this study was significantly elevated in patients with SZ than in healthy controls. The median (IQR) was 0.9 (0.4-1.72) µg/dl for cases and 0.11 (0.04-0.18) µg/dl for controls (Table 6 & figure 9). Ganiyu et al. also investigated higher blood Cd levels in drug naïve SZ patients as well as patients on antipsychotics than healthy controls. The high Cd level may displace metallothionein thereby causing Cd-induced Zn deficiency in their study just like in our study (119). However, our findings are inconsistent with other studies done by Li et al. where they found a decrease in blood Cd levels in cases than controls (94) and no significant difference in blood Cd levels was seen between cases and controls in the study done by Ma et al. (22).

Further, we found serum Aluminum levels in cases were lower as compared to controls with median (IQR) of 6.25 (4.45-8.2) µg/dl and 6.34 (4.87-8.72) µg/dl, (p = 0.436) (Table 6 & figure 7). Our findings are similar to a previous study done by Liu et al. they found that those individuals with a lower serum concentration of Al are associated with an increased risk of schizophrenia. They also found that treatment-responsive SZ patients had lower levels of Al than those poor responders to treatment (97). However, Sussulini et al. study reported elevated Al levels before treatment in poor responders as compared to good responder patients for treatment (78).

The present study reported a higher level of blood arsenic in SZ patients than in healthy controls with median (IQR) of 1.01 (0.68-1.81) µg/dl and 0.89 (0.61-1.11) µg/dl (p = 0.26) (Table 6 & Figure 10). This finding is in accordance with a previous study (108). Some

studies found that increased As levels lead to increased oxidative stress, followed by DNA damage through deletion, base pair mutation, or DNA blocking channels for calcium, lipid peroxidation, and protein modification (21,22). These metals have a role in neurological disorders and chronic inflammation. On contrary, Zhang et al. and Ma et al. found lower arsenic levels after the treatment of Se (109, 22). Li et al found in their study that before the supplementation of Se, As levels were higher but after the Se supplementation they also found that As levels improved (94).

Correlation of blood metals, TNF-α and PANSS (severity of SZ)

Blood metals and PANSS score

To identify the relationship between metals and PANSS (severity), we further assessed the correlation between blood metals and PANSS score. From Table 8, we found no significant correlation between blood metals concentration and PANSS (severity of SZ) ($p > 0.05$). In contrast, a study by Ma et al. reported that Zn, Se and Mg were negatively correlated with PANSS total scores (54).

Blood metals profile and TNF-α

We investigated a significant positive correlation between TNF-α and serum Cu level (rho = 0.234; $p = 0.041$), Table 9. To date correlation between TNF-α and Cu in SZ has not been explored.

Association of blood metals and TNF-α with odd ratio (SZ risk)

We calculated the odd ratio to identify the association of SZ risk with TNF-α and blood metals which came out as OR of 0.99 (95 % CI: 0.96-1.00; $p = 0.98$) as shown in Table 10. In contrast to our findings, Balõtšev et al. investigated, increased concentration of TNF-α was associated with lower SZ risk (OR 0.29) (45).

In the case of metals (Table 11), the present study reported that Cu, Se, and Cd were associated with the risk of SZ in univariate analysis. The (OR = 0.93; 95 % CI: 0.91-0.96, $p = 0.00$), (OR = 0.80; 95 % CI: 0.68-0.93, $p = 0.004$) and OR = 0.007; 95% CI: 0.001-0.80, $p = 0.00$) respectively. However, the concentrations of Fe, Zn, Mg, Al, Pb, and As were not associated with the risk of SZ which is evident from the insignificant odds ratio. In our study this findings was insignificant after multivariate analysis.

In another study, they reported that lower levels of Cu and Se were associated with an increased risk of SZ (97). Ma et al. study did not reported an association of Cd with SZ risk. However, like our result, they also found Pb and As have no associations with SZ risk (22).

Conclusion and Summary

Schizophrenia is a heterogeneous mental disorder that affects an individual's thinking, perception, and behaviour. According to World Health Organization's (WHO) estimated data (2022), SZ touches approx. 24 million population globally, which makes up 1% total worldwide population.

This study was conducted in the Department of Biochemistry in association with the Department of Psychiatry of All India Institute of Medical Sciences, Jodhpur, with 1-year study duration. The Ethics Committee of the institution approved the study. This study included 80 participants, of which 40 patients were enrolled from the outpatient department of Psychiatry based on ICD-10 Criteria, and 40 were healthy control. All participants enrolled only after taking informed consent. Healthy controls are individuals without SZ or any other neuropsychiatric disorders. This study compares the TNF-α in the serum of cases and controls and the blood metals profile in patients and controls. In addition, we find out the association between TNF-α, blood metals profile and PANSS score. In this study, we also find the risk of association between SZ and TNF-α and blood metals.

Demographic information was in age, gender, BMI, marital status, and family type. Social and substance use information such as locality, religion, employment status, and substance use like smoking or alcohol consumption were obtained. In addition, clinical features such as illness duration and age were obtained. In the patient group, the PANSS score, with the help of a psychiatrist, assessed severity. Serum TNF-α analyzed by ELISA showed a difference in the case and controls. However, the p-value was not significant. Serum Fe, Cu, Se, Pb, and Cd levels showed significant differences in the case and control groups. We further correlated TNF-α and blood metals with the PANSS score. We found that there was no significant correlation between PANSS with TNF-α, and also no correlation between PANSS with blood metals level. The Cu was significantly positively correlated with TNF-α.

In conclusion, the obtained results suggested immune system impairments, neuroinflammation and alteration of blood metals in SZ patients. Our study indicates that compared to healthy controls, SZ patients have higher serum levels of Fe, Cu, and Se and lower serum levels of Zn. In addition, there was a correlation between the levels of Cu and TNF-α in SZ patients. The high Cu and Fe level affects neurotransmission and causes lipid peroxidation by increasing free radicals. Heavy metals Pb and Cd affect neurotransmitters and their function after binding with divalent metal ion transporters by increasing oxidative stress.

Limitation and Future Considerations

The first limitation is that the present study was on a small sample population. The second limitation, all SZ patients were on antipsychotics none of them was drug naïve. The third limitation is that only participants blood samples were assessed in our study.

To the best of our knowledge, this is the first Indian study on SZ that estimate serum TNF-α and blood metals profile and their correlation with the severity of SZ. The small sample size and non-drug naïve patients were limitations of our study. Therefore, validations are needed using large cohorts of newly diagnosed drug naïve SZ patients. The current study's finding can be strengthened by further estimating the correlation between TNF-α and Cu levels, which can present a clear picture. The same research conducted on drug naïve patients using the CSF, blood, urine and tissue simultaneously on a large cohort can provide a better conclusion since neuroinflammation and affected neurocircuit are known in SZ patients. Additional assessment of TNF-α can be done by expressing TNF-α receptors and other cytokines.

References

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Annexures

INSTITUTIONAL ETHICS CERTIFICATE

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर
All India Institute of Medical Sciences, Jodhpur
संस्थागत नैतिकता समिति
Institutional Ethics Committee

Chairman

Dr. F.S.K. Barar
Basic Medical Scientist

Members

Justice N N Mathur
Legal Expert

Dr. Varsha Sharma
Social Scientist

Mr. B.S. Yadav
Lay Person

Dr. K.R. Haldiya
Clinician

Dr. Arvind Mathur
Clinician

Dr. Kuldeep Singh
Clinician

Dr. Pradeep Kumar Bhatia
Clinician

Dr. Pankaj Bhardwaj
Clinician

Dr. Surajit Ghatak
Basic Medical Scientist

Dr. Vijaya Lakshmi Nag
Basic Medical Scientist

Dr. Sneha Ambwani
Basic Medical Scientist

Dr. Abhinav Dixit
Basic Medical Scientist

Dr. Tanuj Kanchan
Basic Medical Scientist

Dr. Praveen Sharma
Member Secretary

No. AIIMS/IEC/2022/4008 Date: 22.03.2022

Certificate Reference Number: AIIMS/IEC/2022/3944

To,
Dr. Dharmveer Yadav
Associate Professor
Department of Biochemistry
AIIMS, Jodhpur

Subject: A study of TNF-alpha and blood metalprofile in schizophrenia.

Dear Dr. Yadav,

This has reference to your above mentioned **M.Sc Thesis protocol of Arti Ray**. The project was discussed in the Ethics Committee online meeting held on 21.03.2022 and the following members of the Ethics Committee attended the meeting:

1. Dr. F.S.K Barar, Former Professor & Head, Pharmacology, SMS Medical College	Chairman
2. Justice N.N. Mathur, Former Vice Chancellor, National Law University, Jodhpur	Member
3. Dr. Varsha Sharma, Former Principal Konoria Girls PG College, Jaipur	Member
4. Mr. B.S. Yadav, Principal, Delhi Public School, Jodhpur	Member
5. Dr. K.R. Haldiya, Former Scientist F, DMRC, Jodhpur	Member
6. Dr. Arvind Mathur, Former Principal, Dr. S.N. Medical College, Jodhpur	Member
7. Dr. Surajit Ghatak, Professor & Head, Dept. of Anatomy, AIIMS, Jodhpur	Member
8. Dr. Sneha Ambwai, Professor & Head, Dept. of Pharmacology, AIIMS, Jodhpur	Member
9. Dr. Kuldeep Singh, Professor & Head, Dept. of Paediatrics, AIIMS, Jodhpur	Member
10. Dr. Pradeep Bhatia, Professor & Head, Dept. of Anaesthesiology, AIIMS, Jodhpur	Member
11. Dr. Abhinav Dixit, Professor, Dept. of Physiology, AIIMS, Jodhpur	Member
12. Dr. Tanuj Kanchan, Professor, Dept. of Forensic Medicine, AIIMS, Jodhpur	Member
13. Dr. Pankaj Bhardwaj, Additional Professor, Dept. of CM&FM, AIIMS, Jodhpur	Member
14. Dr. Praveen Sharma, Professor, Dept. of Biochemistry, AIIMS, Jodhpur	Member Secretary

The Project has been approved with effect from 21.03.2022 subject to the following conditions:

- The approval is valid for the period of the conduct of study according to this protocol under the responsibility of Guide: Dr. Dharmveer Yadav, Co-Guide: Dr. Praveen Sharma, Dr. Mithu Banerjee, Dr. Shailja Sharma, Dr. Naresh Nebhinani, Dr. Navratan Suthar, Dr. Vikas Chandra Janu.
- No significant changes to the research protocol could be made and implemented without prior consent of the IEC and any changes/deviations from the protocol which increase the risk for the subjects should be submitted to the IEC and approved by it prior to implementation.
- It is confirmed that the Ethics Committee of AIIMS, Jodhpur is composed of and functions as per ICH GCP and other applications regulatory guidelines.
- It is hereby confirmed that neither you nor any of the study team members have participated in the voting/decision making procedures of the committee.
- The study progress report should be made available to the IEC for review annually.

With Warm regards.

Yours Sincerely,

Dr. Praveen Sharma
Member Secretary

Member secretary
Institutional Ethics Committee
AIIMS,Jodhpur

Basni Phase-2, Jodhpur, Rajasthan-342005; **Website:** www.aiimsjodhpur.edu.in; **Phone:** 0291-2740741 Extn. 3109
E-mail : ethicscommittee@aiimsjodhpur.edu.in; ethicscommitteeaiimsjdh@gmail.com

INFORMED CONSENT

DEPARTMENT OF BIOCHEMISTRY

ALL INDIA INSTITUTE OF MEDICAL SCIENCES, JODHPUR

Name: Age/Gender:

Phone No:

Address:

AUTHORISATION:

I feel free to accept or refuse to participate in this study.

I have had a choice to ask questions, and all of my questions were answered to my satisfaction.

I have been given the information on the survey concerning its nature, purpose and duration, as well as the procedures involved in the study, including any known or expected inconvenience, and I accept the same.

By signing this form, I give my free and informed consent to participate in this study as outlined in the information sheet and this consent form. I understand that I can withdraw from the study at any time. I have not given up my legal rights by signing up for this form.

I have been assured that the blood drawn for genotyping will not be used for any other investigations except it.

Hence, I hereby give my willful consent for my inclusion in this study which the Department of Biochemistry is conducting, at All India Institute of Medical Sciences, Jodhpur, by Arti Ray.

(Signature of the investigator)

(Signature of the participant /relative/family members) (Thumb impression)

सूचित सहमति

जैव रसायन विभाग

अखिल भारतीय इंस्टीट्यूट ऑफ मेडिकल साइंसेज, जोधपुर

नाम: उम्र और लिंग:

फोन नंबर:

पता:

प्राधिकरण:

मैं इस अध्ययन में भाग लेने के लिए स्वीकार करने या इनकार करने के लिए स्वतंत्र महसूस करता हूं। मेरे पास सवाल पूछने का एक विकल्प है और मेरे सभी सवालों का जवाब मेरी संतुष्टि के लिए दिया गया था।

मुझे इसकी प्रकृति, उद्देश्य और अवधि के साथ –साथ अध्ययन में शामिल प्रक्रियाओं से संबंधित सर्वेक्षण के बारे में जानकारी दी गई है, जिसमें किसी भी ज्ञात या अपेक्षित असुविधा शामिल हैं और मैं इसे स्वीकार करता हूं।

इस फॉर्म पर हस्ताक्षर करके मैं इस अध्ययन में भाग लेने के लिए अपनी स्वतंत्र और सूचित सहमति देता हूं, जैसा कि सूचना पत्र और इस सहमति फॉर्म में उल्लिखित है। मैं समझता हूं कि मैं किसी भी समय अध्ययन से हटने के लिए स्वतंत्र हूं। इस फॉर्म पर हस्ताक्षर करके मैंने अपने कानूनी अधिकारों को नहीं दिया है।

मुझे इस तथ्य का आश्वासन दिया गया है कि TNF-α के उद्देश्य के लिए खींचा गया रक्त किसी अन्य जांच के लिए इसका उपयोग नहीं किया जाएगा।

इसलिए, मैं इस अध्ययन में अपने समावेश के लिए अपनी विलफुल सहमति देता हूं, जो कि बायोकेमिस्ट्री विभाग, अखिल भारतीय चिकित्सा विज्ञान संस्थान, जोधपुर द्वारा आरती राय द्वारा आयोजित किया जा रहा है।

(अन्वेषक का हस्ताक्षर)

(प्रतिभागी/ परिवार /रिस्तेदार का हस्ताक्षर) (अंगूठे का निशान)

CASE RECORD FORM

DEMOGRAPHIC DATA

OPD Registration No. _____

Age: _____ Years: **Sex:** M/F **Education Years: _____**

Contact No.: _____

Marital Status: 1) Single 2) Married

Occupation: 1) Professional 2) Clerical, Shop Owner/Farm 3) Skill Worker
4) Semi-Skilled/Unskilled Worker 5) Unemployed
6) House Wife/House Hold 7) Retired 8) Student
9) Not Known

Monthly income per capita: _____INR

Religion: 1) Hindu 2) Islam 3) Sikhism 4) Christianity 5) Others 6) Not Known

Family Type: 1) Nuclear 2) Extended 3) Joint 4) Others 5) Not Known

Locality: 1) Urban 2) Rural 3) Town/suburban

General Examination

Height: _____ **BMI: _____** **Pulse: _____**

Weight: _____ **BP: _____**

Clinical Profile Sheet

Control/case:

Psychiatric diagnosis:

Age at onset: _____(Years)

Duration of illness: _____(Months)

Current Reaction:

Substance: Yes / No **Type of substance:**

Family history of psychotic disorders, including SZ: Yes / No

केस रिकार्ड फॉर्म

जनसांख्यिकीय डेटा

ओपीडी पंजीकरण संख्या _____

आयु: _____ वर्ष लिंग : M / F शिक्षा वर्ष: _____

संपर्क नंबर।: _____

वैवाहिक स्थिति: 1) अविवाहित 2) विवाहित

व्यवसाय: 1) पेशेवर 2) लिपिक, दुकान मालिक/खेत 3) कौशल कार्यकर्ता

4) अर्धकुशल / अकुशल श्रमिक 5) बेरोजगार 6) गृहिणी/घरेलू

7) सेवानिवृत्त 8) छात्र 9) ज्ञात नहीं

प्रति व्यक्ति मासिक आय: _____INR

धर्म: 1) हिंदू 2) इस्लाम 3) सिख धर्म 4) ईसाई धर्म

5) अन्य 6) ज्ञात नहीं

परिवार का प्रकार: 1) एकल 2) विस्तारित 3) संयुक्त

4) अन्य 5) ज्ञात नहीं

स्थान : 1) शहरी 2) ग्रामीण 3) नगर / उपनगरीय

सामान्य परीक्षा

ऊंचाई: _____ BMI: _____ पल्स: _____

वजन: _____ बीपी: _____

क्लीनिकल प्रोफ़ाइल शीट

नियंत्रण/मामला:

मनोरोग निदान:

शुरुआत में आयु: _____ (वर्ष)

बीमारी की अवधि: _____ (महीने)

वर्तमान प्रतिक्रिया:

पदार्थ: हाँ / कोई प्रकार का पदार्थ नहीं:

सिज़ोफ्रेनिया सहित मानसिक विकारों का पारिवारिक इतिहास: हाँ / नहीं

Positive and Negative Syndrome Scale (PANSS)

1=Absent, 2=Minimal, 3=Mild, 4=Moderate, 5=Moderately severe, 6=Severe, 7=Extreme

Total Positive Subscale Score								
P1	Delusions	1	2	3	4	5	6	7
P2	Conceptual disorganisation	1	2	3	4	5	6	7
P3	Hallucinatory behaviour	1	2	3	4	5	6	7
P4	Excitement	1	2	3	4	5	6	7
P5	Grandiosity	1	2	3	4	5	6	7
P6	Suspiciousness/Persecution	1	2	3	4	5	6	7
P7	Hostility	1	2	3	4	5	6	7
Total Negative Subscale Score								
N1	Blunted affect	1	2	3	4	5	6	7
N2	Emotional withdrawal	1	2	3	4	5	6	7
N3	Poor Rapport	1	2	3	4	5	6	7
N4	Passive/apathetic social withdrawal	1	2	3	4	5	6	7
N5	Difficulty in abstract thinking	1	2	3	4	5	6	7
N6	Lack of spontaneity &flow of conversation	1	2	3	4	5	6	7
N7	Stereotyped thinking	1	2	3	4	5	6	7
Total General Psychopathology Subscale Score								
GP1	Somatic concern	1	2	3	4	5	6	7
GP2	Anxiety	1	2	3	4	5	6	7
GP3	Guilt feeling	1	2	3	4	5	6	7
GP4	Tension	1	2	3	4	5	6	7
GP5	Mannerisms& posturing	1	2	3	4	5	6	7
GP6	Depression	1	2	3	4	5	6	7
GP7	Motor retardation	1	2	3	4	5	6	7
GP8	Uncooperativeness	1	2	3	4	5	6	7
GP9	Unusual thought content	1	2	3	4	5	6	7
GP10	Disorientation	1	2	3	4	5	6	7
GP11	Poor attention	1	2	3	4	5	6	7
GP12	Lack of judgement & insight	1	2	3	4	5	6	7
GP13	Disturbance of volition	1	2	3	4	5	6	7
GP14	Poor impulse control	1	2	3	4	5	6	7
GP15	Preoccupation	1	2	3	4	5	6	7
GP16	Active social avoidance	1	2	3	4	5	6	7
Total PANSS Score								