Role OF miRNA in Regulation of α-Klotho Gene Expression and its Circulatory Levels in Schizophrenia

THESIS

Submitted to

All India Institute of Medical Sciences, Jodhpur

In partial fulfillment of the requirement for the degree of

DOCTOR OF MEDICINE (MD)

(BIOCHEMISTRY)

AIIMS, JODHPUR **DR. AMANDEEP BIRDI**

JUNE 2022

Role OF miRNA in Regulation of α-Klotho Gene Expression and its Circulatory Levels in Schizophrenia

THESIS

Submitted to

All India Institute of Medical Sciences, Jodhpur

In partial fulfillment of the requirement for the degree of

DOCTOR OF MEDICINE (MD)

(BIOCHEMISTRY)

AIIMS, JODHPUR **DR. AMANDEEP BIRDI**

JUNE 2022

All India Institute of Medical Sciences, Jodhpur

DECLARATION

I hereby declare that thesis entitled "**Role of miRNA in Regulation of α-Klotho Gene Expression and its Circulatory Levels 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 in full for any other degree of All India Institute of Medical Sciences or any other institution/ University.

Amandeep Birdi

Dr. Amandeep Birdi

Department of Biochemistry

AIIMS, Jodhpur

All India Institute of Medical Sciences, Jodhpur

CERTIFICATE

This is to certify that the thesis titled "**Role of miRNA in Regulation of α-Klotho Gene Expression and its Circulatory Levels in Schizophrenia**" is the bonafide work of **Dr. Amandeep Birdi** carried out under our guidance and supervision, in the Department of Biochemistry and the Department of Psychiatry, All India Institute of Medical Sciences, Jodhpur.

Guide

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

Co-Guides:

Dr. Praveen Sharma
Professor
Department of Biochemistry
AIIMS, Jodhpur

Dr. Mithu Banerjee
Additional Professor and Head
Department of Biochemistry
AIIMS, Jodhpur

Dr. Purvi Purohit
Additional Professor
Department of Biochemistry
AIIMS, Jodhpur

Dr. Naresh Nebhinani
Additional Professor and Head
Department of Psychiatry
AIIMS, Jodhpur

Dr. Prasenjit Mitra
Assistant Professor
Department of Biochemistry
AIIMS, Jodhpur

Dr. Tanu Gupta
Clinical Psychologist
Department of Psychiatry
AIIMS, Jodhpur

All India Institute of Medical Sciences, Jodhpur

CERTIFICATE

This is to certify that the thesis **"Role of miRNA in Regulation of α-Klotho Gene Expression and its Circulatory Levels in Schizophrenia"** is the bonafide work of **Dr. Amandeep Birdi**, in the **Department of Biochemistry,** All India Institute of Medical Sciences, Jodhpur.

Dr. Mithu Banerjee
Professor & Head
Department of Biochemistry
AIIMS, Jodhpur

ACKNOWLEDGEMENT

First and foremost, I would like to thank God Almighty for giving me the strength, knowledge, and ability to undertake this research study and to persevere and complete it satisfactorily. Without his blessings, this achievement would not have been possible.

I am deeply grateful and my proud privilege to express my deep sense of gratitude and sincere thanks to my guide ***Dr. Dharmveer Yadav****, Associate Professor, Department of Biochemistry, AIIMS, Jodhpur who provided me an opportunity to work under his guidance. His guidance was paramount in providing a well-rounded experience and knowledge. He has always been a source of encouragement and inspiration. I wish to express my deep in debt gratitude for devoting his valuable time out of his busy schedule and being concerned for completion of this project. I shall remain grateful to him forever.*

I express my sincere gratitude to my co guide ***Dr Naresh Nebhinani****, Additional professor, Department of psychiatry, AIIMS, Jodhpur,* ***Dr Mithu Bnaerjee****, Additional professor,* ***Dr. Purvi Purohit****, Additional Professor,* ***Dr. Prasenjit Mitra****, Assistant Professor, Department of Biochemistry AIIMS, Jodhpur and* ***Dr. Tanu Gupta****, Clinical Psychologist Department of Psychiatry, AIIMS, Jodhpur for their constant support, encouragement, guidance and help throughout my work.*

I owe a tremendous debt of gratitude to ***Dr Jaykaran Charan****, Department of Pharmacology, AIIMS, Jodhpur,* ***Dr. Sojit Tomo*** *and* ***Dr. Arun Sinha****,* ***Dr. Kiran Kumar****,* ***Dr. Pradeep Japa****, Senior Resident Department of Biochemistry AIIMS, Jodhpur for his mentorship and support during the course of work. Words were not sufficient to express my grateful thanks to him.*

I want to take moment to express my sincere thanks and gratitude to all the faculty of Department of Biochemistry, AIIMS, Jodhpur for guiding me, inspiring me and making what I am today.

I am also extremely thankful to my friend and my senior ***Dr Shruti Gupta****, and* ***Dr Sai Kiran****,* ***Dr. Abhilasha****,* ***Dr. Malavika*** *with whom I worked closely and puzzled over many similar problems. I am very grateful for their presence during my difficult times.*

*I am also deeply thankful to **Dr Goutam**, Senior resident, **Dr. Isha Kaur**, Department of Psychiatry for his cooperation during the period of work.*

*A special thanks is extended to and **Ms. Anupama Modi** PhD scholar, **Ms. Kavya Gauba**, M.Sc **Ms. Ashita** and **Mr. Manoj Khokhar**, Ph.D. students Department of Biochemistry for their cooperation. A special thanks to all my amazing juniors, **Dr Andy Star**, **Dr. Srividya**, **Dr. Jyoti** and **Dr Mehak Kakkar** for their selfless help during my day to day work. A special thanks my amazing lab Technician Mr. Shelendra Vashistha, Mr. Francis Massey and Mr. Devendra Yadav for their selfless help during my day-to-day work.*

I am thankful to Director, AIIMS, Jodhpur for thesis fund and permitting me to avail all the facilities available in the institute for conducting this study.

Most importantly I would like to thank my parents, Mr. Kishan Lal and Mrs. Avtar Kaur,and my wife Mrs. Baljit Kaur, my brother, Dr. Pankaj Birdi for their faith in me. Their support, encouragement and unconditional love were undeniably the bedrock upon which my life has been built.

SPECIAL ACKNOWLEDGEMENTS

My special acknowledgements go to all those people who made possible the difficult task of completing my MD thesis. My warm appreciation is due to all the biochemistry staff and technicians who cooperated in my long working hours.

At last, words are short to express my deep sense of gratitude to all the participants who willingly selflessly donated the blood for my experiments during my research endeavor

Dr Amandeep Birdi

DEDICATED TO MY PARENTS, MYWIFE, MY SON

AND

DEPARTMENT OF BIOCHEMISTRY

AIIMS, JODHPUR

ABBREVIATIONS

SZ	Schizophrenia
α- Klotho	Alpha Klotho
miR	Micro Rna
CSF	Cerebro Spinal Fluid
ChP	Choroid Plexus
DNA	Deoxy Ribonucleic Acid
RNA	Ribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
RT-PCR	Real Time Polymerase Chain Reaction
Rpm	Revolutions Per Minute
DEPC	Diethylpyrocarbonate
EDTA	Ethylenediaminetetraacetic Acid
RBC	Red Blood Cell
PBS	Phosphate Buffer Saline
cDNA	Complimentary Deoxyribonucleic Acid
mRNA	Messenger Ribonucleic Acid
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
RNase	Ribonuclease
Ct	Cycle Threshold
GAF	Global Assessment Of Functioning
PANSS	Positive And Negative Syndrome Scale
SD	Standard Deviation

SUMMARY

Background: SZ is a debilitating psychiatric disorder that can result in hallucinations, delusions and disordered thinking and behaviour. Approximately 1% of the world population is affected by this disorder. Cognitive impairments are a core feature of schizophrenia (SZ). Klotho is an anti-ageing protein with demonstrated cognitive-enhancing effects on the brain.

Aims & Objectives: This study aims to investigate the expression of α -Klotho gene & miR-339-5p in SZ, the differences in the levels of serum klotho between patients with SZ and healthy controls, as well as the relationship between klotho level and cognitive function in patients. Also, to find the association of miR-339-5p with Klotho protein levels with the cognitive scores in SZ.

Methods: In this study, a total of ninety subjects were recruited among them 60 were confirmed cases of SZ and 30 were healthy controls. Severity was assessed by using PANSS and GAF scores. A neuropsychological battery was performed to evaluate the cognitive function of participants. Serum Klotho protein levels were estimated by using sandwich ELISA. The expression of the Klotho gene and miRNA 339- 5p was assessed by using SYBR Green RT- qPCR assay.

Results: Results of this study showed that patients with SZ performed worse in the neurocognitive tests than the healthy controls. The levels of serum klotho were higher in cases as compared to the healthy controls with mean ± SD of cases and controls were 56.82 ± 8.62and 50.95 ± 15.73 respectively. In patients, serum klotho levels were positively correlated with cognitive function with a p-value <0.001. Expression of the Klotho gene was found to be upregulated and miR-339-5p was downregulated.

Conclusion: Collectively, these results indicate that anti-ageing protein klotho and miR-339-5p may be implicated in the pathogenesis of SZ. Klotho gene expression and Klotho protein levels were increased and miR-339-5p was decreased and were correlated with cognitive impairments, indicating that Klotho and miR- 339- 5p could be used as a predictor of functional outcomes in patients with SZ.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ANNEXURES

INTRODUCTION

Schizophrenia (SZ) is a severe chronic neuropsychiatric disorder that mostly exhibiting with hallucinations, delusions, disorganized speech, and behaviour. These symptoms may also be referred to as psychosis (1). Although these psychotic symptoms can be quite grave, and the presence and duration of negative symptoms correlate most strongly with poor outcomes. Disease onset is mostly in the age group of late teens to early twenties, and its prevalence is noted at 1% worldwide. According to the National mental health survey, 2015-2016 prevalence of SZ in India is 0.4%. The preponderance of probability of SZ is more in males. Although, the exact cause of SZ is not known but it can be due to interaction of the environmental and genetic factors (2,3). Over the past three decades, several studies have been undertaken to establish the role of genetic factors in this disorder. Initially, family, twin and adoption studies make available pieces of evidences towards the heritability of the disorder (4,5). Later, with the advancement of the Human Genome Project, multiple polygenic components including candidate genes and epigenetic modification contributing to the risk of SZ have been identified (6,7).

Cognitive dysfunction is a core feature of SZ. Deficits are moderate to severe across several domains. The importance of understanding and treating cognitive dysfunction is underscored by the relative lack of treatment success in most aspects of functional status. Many proteins have been identified and studied which are involved with cognitive dysfunction in humans and one such protein is α klotho. Klotho is a naturally occurring human protein discovered in 1997 which maps on chromosome 13. Since the discovery of klotho, two related paralogs, β-klotho, γ-klotho, have been identified as klotho family members (8). Klotho is also called α- Klotho to distinguish it from the other two members. Longitudinal studies on klotho mutant mice have demonstrated that cognitive decline in SZ is accompanied by a substantial decrease in Klotho expression in brain tissue (9). Both the serum and CSF α- Klotho protein levels were found to be negatively correlated with age.

Complications in studying this protein are due to difficulties in obtaining immunoprobes specific to different Klotho gene expression is mainly based on data on the localization of mRNA species encoding Klotho. In-situ hybridization and

immunohistochemistry have demonstrated the highest expression level of the Klotho gene in the ependyma of choroid plexus (10), where Klotho protein is localized in the apical membrane. Besides choroid plexus, mRNA encoding Klotho is revealed in many brain structures like Purkinje cells in the cerebellum, cell and nuclear membranes of brain cortex, hippocampus, olfactory bulb, striatum and medulla oblongata.

Klotho protein is also present in neurons and oligodendrocytes but absent in microglia and astrocytes. This protein is confined to torso and dendrites of hippocampal neurons, wherein the membrane form of Klotho found in pre and postsynaptic membranes (11). In choroid plexus cells, Klotho was found in the cell membrane, some amount was localized in the rough endoplasmic reticulum, and large amount was found near the nuclear membrane.

A microRNA (miR) is a small non coding RNA that is about 22 nucleotides in length and these are mostly single stranded formed by the action of Drosha and Dicer on RNA. These miR mostly bind to mRNA and forms an RNA silencing complex and causes a change in mRNA levels (12). As a result, these mRNA molecules are silenced, by one or more of the following processes: (1) degradation of the mRNA strand into pieces, (2) shortening of poly A tail of mRNA leads to less stable form, and (3) less efficient translation of the mRNA into proteins by ribosomes. Recent studies on cell line shown that the miR-339-5p and miR-556 were having a regulatory effect on the Klotho gene (13).

As cognitive dysfunction is a major symptom of SZ and many previous studies have shown that Klotho has a significant role in cognitive dysfunction in ageing but there are no human studies concerning Klotho and cognitive dysfunction in SZ.

However, there is a lack of data reporting such findings in clinical settings. Thus, the current study is proposed to evaluate the expression levels of the Klotho gene & its regulatory miR-339-5p in clinically diagnosed schizophrenic patients. To the best of our knowledge, there is no such study reported in the Asian SZ population. Due to scarcity of the data in the role of the miR in the regulation of Klotho gene expression and its circulatory levels in SZ in the Indian population, this study has been planned to fill this lacuna.

REVIEW OF LITERATURE

SZ is a chronic neuropsychiatric disorder which affects 1% of population worldwide and according to National mental health survey, its prevalence in India is 0.4% (13). This disorder shows many symptoms such as positive, negative and cognitive impairments. The most serious of these symptoms is cognitive impairment (14). Due to cognitive impairment, the patient is dependent on others for the whole life. Because of this the power of thinking is affected and the patient does not even know what is real and what is not (15). In 2019 total number of cases suffering from SZ was 20 million (15).

Onset:

This disease is more common in males and starts at an early age as compared to females. In males, this disease starts at their twenties and even earlier and in females it starts at twenties and mid-thirties (15,16). Many patients recovered completely from SZ but some patients remain affected due to the symptoms of this disease and most of those patients find it difficult to live life due to the cognitive changes(17–19). The cause of this disease is not yet clear, but still the context of genetic and environment is very important (15).

Pathogenesis:

Different causes which lead to SZ are still under investigation yet there is a dopamine model which is believed to be involved in the pathogenesis of SZ. According to this model, during severe psychotic phase there is an increased level of dopamine which leads to anomalous dopamine signaling and causing severe symptoms and also oxidative stress (20,21). But in case of cognitive symptoms, there is decreased expression of dopamine receptor D1 in frontal cortex of the brain causing memory impairment (22,23). And according to other studies, all these symptoms appear due to reduced expression of the glutamate NMDA (N-methyl D- aspartate) receptor in brain (24–26). Apart from dopamine model, another factor has been suggested in pathogenesis of SZ to be neuroinflammation.

Neuroinflammation:

In SZ, there is a neuroinflammation and due to this the levels of many cytokines get increased such as Interleukin 1, 6, 8 and tumor necrosis factor α (TNF- α) (27,28). These markers are usually increased in patients showing structural abnormalities in brain as seen in their computed tomography images or magnetic resonance imaging (29). The structural changes that were visible in the parts of brain in these patients were decreased in hippocampal and cortical volumes and enlargement of ventricles (30). Due to this neuroinflammation or imbalance of the immune system, there is an effect in the NMDA receptors because of which, many symptoms of SZ are seen (31). Apart from neuroinflammation, oxidative stress is also a culprit for SZ pathogenesis. Oxidative stress causes problems in many parts of the brain. Oxidative stress can lead to problem in differentiation as discussed below in oligodendrocytes.

Oligodendrocytes and myelination:

Oxidative stress is thought to cause trouble in maturation of oligodendrocytes from its precursor in SZ. The expression of oligodendrocyte gene is reduced so in case of SZ, mature oligodendrocytes are few in number. Also, there is defect in maturation of oligodendrocytes precursor in this disease and it leads to decreased number of mature oligodendrocytes in the brain. Some studies also reported dead oligodendrocytes in the brain of schizophrenic patients (32). Furthermore, decrease number of oligocytes leads to decreased myelination process in neurons which causes impairment in cognition (32–34). Some studies have reported that decreased levels of glutathione in this disease hampers tackling of oxidative stress and causing troubles in myelin formation (35). Now let's look at the symptomatology of this disease.

Symptomatology of SZ:

SZ is a neuropsychiatric disorder and is characterized by change in mood and behaviour and there is also change in organization, recognition and elucidation of sensory information in order to understand and explain what is already explained (36). The symptoms of this disease are divided into three parts i.e., positive, negative and cognitive symptoms.

Positive symptoms include delusion (a rigid and determined belief based on insufficient grounds not complaint to rational arguments), hallucination (perception in the absence of external stimulus that has qualities of real perception), and muddled thought and speech (37). Person suffering from SZ experiences that someone is putting thoughts in their mind or someone is talking about them, patients begin to suspect others and also patients had auditory perceptions and sometimes they also feel touch perception (38).

Negative symptoms include reduced response to emotions (39). It also includes flat faces, i.e. with no expression, patient didn't find words to express, reduced interest in the activities which are previously pleasurable, socially withdrawn, lack of judging power and do not maintain personal hygiene (39,40).

The third symptom due to which the patient has a lot of difficulty in day to day is cognitive decline. Because of it, you have to depend on others for your work. It is frequently seen in SZ patients even in early ages and adulthood (41,42). In this disease, patients Intelligent Quotient also reduced from 100 to 70-80 in SZ patients as demonstrated by many studies (43,44). Cognitive impairment is also divided into two parts i.e., social and nonsocial cognition (45).

Nonsocial is also called as neurocognition. It is the power of the person to get information and retain it. It comprises of perception of visual and auditory stimulus, storing information, sensing things, solving problem and processing information and speed of processing (46). The affected area in this disease is verbal and concentration (44).

Social cognition is the ability to interpret and understanding self and non self (45). Cognitive impairment usually does not show any response to antipsychotic drugs but early diagnosis can aid in its intervention and improves cognition. Now let us read about some genes that are involved in pathogenesis of this disease. Although lots of genes are under study but we are including few of them in this section.

Genes involved in SZ:

Different genes are involved in the pathogenesis of SZ such as BDNF, Notch and Klotho. Firstly, we are going to discuss little bit about BDNF (brain derived

neurotrophic factor). It is located on the short arm of chromosome 11. It shows its association with tyrosine kinase receptor family and bind with tyrosine kinase B for its action. Its binding to its receptor causes phosphorylation and gene expression of many phosphoproteins (47–49). Many studies were done on its polymorphism which is associated with SZ like valine and methionine single nucleotide polymorphism and valine and valine polymorphism which is involved in the structural changes in brain parts such as a decrease in the size of hippocampus causing memory and cognitive impairment (50,51).

Second gene which is involved in SZ is Notch. Notch is a transmembrane protein with larger extracellular and smaller intracellular part. Notch signaling is involved in neural development. Its mechanism of action is seen, when its ligand binds to its receptor and releases the intracellular part and that part enters the nucleus and leads to increase in expression of genes (52).

The third and the most important gene involved in pathogenesis and cognitive impairment in SZ is Klotho. We are focusing on this gene and its product protein in our study as we are also trying to find its association with cognition in SZ patients by measuring its expression and circulatory levels.

Klotho protein:

Klotho name was derived from Greek goddess Clotho, who is thought to be one of the three fates who spins the thread of human life (53). Klotho gene was first identified by Kuro-o et al in 1997 in animal study performed on mice. Findings of their study showed decreased life span and physiological changes of ageing due to the absence of Klotho gene in animals under research (54).

Klotho gene is located on the long arm of chromosome 13 (13q12) and encodes transmembrane protein (55). Klotho gene when undergoes transcription it forms primary transcript which have five Exons and four Introns. There is a presence of splice site after third exon, on which action of spliceosomes leads to formation of two mRNA by alternate splicing. One mRNA forms the transmembrane protein and other form soluble protein (55).

Klotho protein structure:

Klotho gene encodes single pass type I membrane protein which is having length of 1014 amino acids and is related to β glucuronidase (56). β glucuronidase are family of enzymes that catalyze the hydrolysis of complex carbohydrates like β glucuronic acid from mucopolysaccharides (57). Transmembrane proteins are proteins with extracellular part, membrane part and intra-cytoplasmic domain. The extracellular domain is the larger domain with two internal repeats of 450 amino acids in length (58). Klotho mostly exists on cell surface but can also circulate in body fluids by releasing from its surface (59).

Klotho protein is mainly expressed in brain and kidney. It is cleaved from its membrane part by metalloproteinases namely ADAM 10 and ADAM 17. After it gets detached from membrane part, it is secreted in cerebro spinal fluid (CSF), serum and urine. This detached part is known as soluble form of Klotho (60). ADAM is A Disintegrin and metalloproteinase Domain containing protein 10 and 17 and these are the member of ADAM family with two distinct domains that have adhesion and protease activity. Its protease activity cleaves extracellular domain of Klotho protein (61). There are three subfamilies of the protein namely α- Klotho, β- Klotho and γ- Klotho and when name is not specified then it is α- Klotho form (62). It has two more types like soluble Klotho as mentioned above that it is formed by activity of metalloproteinases and second is secreted form, it is mainly produced by alternate splicing of Klotho mRNA (primary transcript) (63).

Klotho and Central Nervous System

Choroid plexus (ChP), Oligodendrocytes and Myelin

The role of Klotho in the brain had been elucidated with the help of memory tests that the Klotho deficient mice were subjected to. A hippocampus-related cognitive function decline had been demonstrated in Klotho protein-deficient mice (63). Klotho in the brain had been observed to have a significant function in ChP and neurons (9,54,64). Klotho functions, which started appearing inutero, progressively increases till adulthood and then decline with age (65–67). Cells of the epithelial lining of the choroid and neurons of Purkinje cells of the cerebellum have shown the presence of

Klotho (54,66). Klotho present in the cells of ChP get shed into the CSF in presence of small quantities of calcium (68,69).

The expression of Klotho changes with the progression of age, and its primary effect has been in oligodendrocytes and myelin (67,69). The number of oligodendrocytes had demonstrated a reduction in Klotho deficient mice (11,70). There have been decreased myelin-forming processes observed at low Klotho protein concentrations in white matter tracts (70). Distinction and progression of oligodendrocytes required shed Klotho (70,71). Further, an increase in myelin formation occurs in Klotho overexpressing conditions. This shows that Klotho is important in oligodendrocytes and myelin formation (71).

Hippocampus and pituitary gland

Hippocampus, the central part of our brain related to cognitive functions like memory and learning ability. Short-term and long-term memories are associated with the hippocampal region. The functioning of hippocampus declines with increasing age and neurodegenerative diseases like Alzheimer's disease. In Klotho deficiency, there is increased oxidative stress in the hippocampus and increased markers of programmed cell death (63,72). An animal study in mutant mice with low Klotho protein levels demonstrated decreased memory and learning (cognition) in mice at the age of 6^{th} to 7^{th} week of life (70). Similarly, an increased expression of Klotho is associated with improved memory and learning with decreased oxidative stress (71,72). Klotho also helps in the formation of new neurons from a neural stem cell. As a result, Klotho protein deficiency leads to the formation of immature neurons, resulting in decreased cognition. An in vitro study has observed an increase in the proliferation of neurons with shed Klotho (73).

Although, Klotho does not show its expression in the hypothalamus-pituitary-adrenal axis, but its component i.e., shed Klotho, is circulated in the pituitary. Further, the presence of Klotho mRNA has been demonstrated in the pituitary gland (74). Klotho deficient mouse had demonstrated a decrease in the number of pituitary cells and reduced secretion of its hormones. This leads to reduced fertility, decrease growth, and atrophy of gonads (74). The shed Klotho has also been found to stimulate growth hormone secretion (75).

Klotho protein and SZ

SZ is characterized by redox imbalance leading to oxidative stress, resulting in the prefrontal cortex's dysfunction and dysconnectivity. This is attributed to decreased antioxidant enzymes glutathione peroxidase and superoxide dismutase in SZ patients (32,76–81). Morar et al. showed that increased levels of Klotho in heterozygous Klotho variants lead to improved cognition resulting in better learning and memory. The study also demonstrated the association between the heterozygous Klotho variant and an increase in the right prefrontal cortex volume, which usually decreases in size with ageing (82). SZ is also characterized by a reduction in myelin formation by oligodendrocytes leading to a more severe cognitive deficit. The maturation of oligodendrocytes from its precursor by neuronal activity is decreased in SZ (83–86).

The oxidative stress, in the brain of SZ patients, occurs mainly because of dopamine metabolism, which leads to both non enzymatic and enzymatic (monoamine oxidase) production of hydrogen peroxide in the brain. There is reduced Glutathione antioxidant and Klotho protein in genetically predisposed individuals, which leads to damage to the brain (87).

Klotho protein has been demonstrated to affect oxidative stress and oligodendrocyte maturation. The increased Klotho protein levels accelerate the development of oligodendrocytes from its precursor. This facilitates an increased formation of myelin leading to better cognition and behaviour. Further, by decreasing oxygen free radicals, Klotho protein prevents the development of oxidative stress (88–93). The effect of Klotho on oxidative stress and maturation of oligodendrocytes indicates the possible crucial role that Klotho may have in SZ.

Klotho Polymorphisms and Epigenetics

KL-VS is a common haplotype observed in the general population. The variant had been associated with a higher incidence of dementia in the elderly population (94). KL-VS homozygosity leads to decreased Klotho levels resulting in deranged intrinsic connectivity among the functional networks of the brain (95). However, KL-VS did not affect cognition and brain structure in early life development (96). KL-VS heterozygosity had also been found to be associated with reduced Alzheimer disease risk and β- amyloid burden in elderly individuals having apolipoprotein E4 (APOE4

is highest risk factor for Alzheimer disease). This advocates the inclusion of the KL-VS genotype in conjunction with the APOE genotype in Alzheimer disease prediction models (97,98).

Klotho polymorphisms had been associated with ageing-related outcomes and mortality in the elderly population (99). A recent meta-analysis by Zhu et al. had demonstrated an association between Klotho F352 V polymorphisms and longevity (100). Although individual studies have associated G-395 A with cognitive impairment, the meta-analysis did not find an association between G-395 A with cognitive impairment (100,101).

Klotho polymorphisms were also found to have a role in inflammatory outcomes in post-traumatic stress disorder (PTSD) patients. Klotho genotype (KL-VS SNPs) and methylation status at cg00129557 lead to reduced chromatin binding and expression resulting in individual differences in inflammatory outcomes in PTSD patients (102).

Wolf et al. demonstrated that the rs9315202 SNP in the Klotho gene showed increased peripheral inflammation and decreased white matter integrity, particularly in right-lateralized tracts connecting prefrontal to limbic regions. There is an alteration in Klotho gene expression via non-coding RNA (ncRNA) due to this SNP. This reduced Klotho gene expression leads to the weakened activity of synapses and neurocognitive loss, mainly in people more than 30 years of age (102).

Yin demonstrated that there is a decreased Klotho level in patients with unilateral ureteric occlusion due to TGF β which leads to renal fibrosis. In conditions like inflammation, there is an increase in tumor growth factor β, which leads to epigenetic changes in Klotho gene expression. TNF β leads to activation of DNA methyltransferase 1 and 3a causing hypermethylation of the Klotho gene promoter region, culminating in transcriptional inhibition. So Klotho protein formation is decreased, which can lead to neurocognitive loss (103).

Klotho protein is regulated by miRs either by gene silencing or degradation of mRNA or by altering its gene expression and it was stated that miR- 339- 5p is involved in decreasing expression level of Klotho protein in cancer cell line (13).

A miR is a small non coding RNA which is about 22 nucleotides in length and these are mostly single stranded formed by the action of Drosha and Dicer on RNA. These miR mostly bind to mRNA and forms RNA silencing complex and causes change in mRNA levels (104,105). As a result, these mRNA molecules are silenced, by one or more of the following processes: (1) degradation of the mRNA strand into pieces, (2) shortening of poly A tail of mRNA leads to less stable form, and (3) less efficient translation of the mRNA into proteins by ribosomes (105).

Role miR- 339-5p on Klotho gene expression and its circulatory levels:

miR-339 was found to be associated with klotho gene expression as demonstrated by Stephen J Mehi et al., they reported that this miR-339 decreases Klotho gene expression when it is upregulated and they also demonstrated that age related decrease in Klotho protein in cancer cells (13). So, this study was planned to find out the role of miR-339-5p in regulating the expression of Klotho gene and its circulating protein levels in SZ patients.

AIMS AND OBJECTIVES

Hypothesis:

Null hypothesis: There is no association of circulatory α-Klotho proteins and its regulatory miR-339-5p with cognitive functions in schizophrenic patients.

Alternate hypothesis: There may be an association between the expression of circulatory Klotho protein and its regulatory miR-339-5p with a change in cognitive functions in SZ.

Research Question:

- What is the expression level of α -klotho protein in SZ patients?
- What is the expression level of miR-339-5p in schizophrenic patients?
- Is there an association between α –klotho proteins and miR-339-5p in SZ patients?
- Is there an association of circulating α –klotho protein & miR-339-5p with the cognitive functions of schizophrenic patients?

AIMS AND OBJECTIVES:

AIMS: The study aims to find out the Role of miRNA in Regulation of α-Klotho Gene Expression and its Circulatory Levels in Schizophrenia.

Objectives:

- To study the expression of α -Klotho & miR-339-5p gene in SZ.
- To find out the possible association of miR-339-5p in the regulation of the Klotho gene in SZ.
- To correlate the circulating α -Klotho levels & miR-339-5p levels with the cognitive scores in SZ.

MATERIALS AND METHODS

Ethical considerations: This study conformed to the guidelines of the Institutional Ethics Committee (IEC) of All India Institute of Medical Sciences (AIIMS), Jodhpur. Informed written consent was taken from study participants and their family members to participate in the study. The study was verbally explained to the participants and adequate opportunities were given, for discussion of any of their queries. The subjects retained the right to withdraw consent at any stage of the study and complete confidentiality was maintained.

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

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

Study duration: The study was conducted over a period of two years (Jan 2020 to December 2021).

Study participants

Sample size calculation

For sample size calculation, existing literature for serum Klotho levels in SZ was referred. This information was used in the OpenEpi software to calculate sample size. The size with 80% power and 95% confidence interval came out to be thirty-six (36). We took a total of ninety (90) subjects, including sixty cases and thirty controls as part of our study.

Inclusion and Exclusion criteria

Inclusion Criteria:

For cases: All patients of age 18-60 years diagnosed with SZ and attending the OPD of the Department of Psychiatry in AIIMS, Jodhpur recruited in this study. The diagnosis of patients will be done as per ICD 10 (WHO 1992). The severity assessment is done by using PANSS.

For control: Age and sex-matched nonpsychiatric volunteers satisfying the exclusion criteria will be taken as healthy controls in the study.

Exclusion Criteria:

For cases: Comorbid psychiatric disorders such as Bipolar, MDD etc. Medical illnesses (e.g., autoimmune diseases, diabetes, HIV, endocrine disorders, hepatitis, cancer, or chronic infections) or medications (e.g., steroid medications, antioxidants, corticosteroids (oral, injected, inhaled and/or topical), immunotherapy, antibiotics that could affect the immune system, within previous 6 weeks, and females in lactation or in gestational period.

For controls: Medical illnesses (e.g., autoimmune diseases, diabetes, HIV, endocrine disorders, hepatitis, cancer, or chronic infections) or medications (e.g., steroid medications, antioxidants, corticosteroids (oral, injected, inhaled and/or topical), immunotherapy, antipsychotics, antibiotics that could affect the immune system, females in lactation or in gestational period

The healthy control group included healthy volunteers or the staff members who were matched to the patients with demographic variables of age, gender, and marital status. Their health status was confirmed by the provided information and general examination.

Severity of SZ assessed by PANSS and GAF:

The PANSS scale is the most widely used scale to measure the severity of symptoms in SZ patients. To assess the patient using PANSS, a clinical interview of approximately 45-minute is to be conducted. The patient is rated from 1 to 7 on 30 different symptoms based on the interview as well as the reports of family members. PANSS is a positive and negative syndrome scale, it is having positive syndrome scale which includes delusion, hallucination, conceptual disorganization, excitement, grandiosity, suspicion and hospitality. Its range is from 7 to 49, 7 being minimal and 49 more severe symptoms. Negative scale includes blunted affect, emotional withdrawal, poor rapport, passive/ apathetic social withdrawal, difficult in abstract thinking, lack of spontaneity and stereotyped thinking. It is also range from 7 to 49 score. General psychopathology scale has 16 items like somatic concern, anxiety,

tension, depression, uncooperativeness etc. minimum score of this scale is 16 and maximum is up to 112. Total PANSS score is from 30 to 210, 30 is lowest score with less severity and 210 is most severe case.

Neurocognitive tests:

Stroop color word test:

The Stroop test measures response inhibition that can be described as the ease with which a person can shift his or her perceptual set to conform to the changing demands of a situation and suppresses a habitual response in favor of an unusual one. Test consists of 3 pages: Word page (W), Color page (C), Color word page (CW). Each page has 100 items presented in 5 columns of 20 items. All the subjects were given 45 seconds to complete the items on each page. Before initiating the test, it was made sure that patient identifies all the colors used in the test and able to name them to rule out the possibility of color blindness. On word page, the subjects were asked to read the words down the columns starting with the first one. Similarly on the color page, the subjects were instructed to name as many colors within 45 seconds. For the color word page, the subjects were asked to name the color of the ink, in which the words are printed in and ignore the word. The numbers of items completed on three pages were obtained as their respective scores.

Interference score was obtained by subtracting color (C) scores from color word (CW) scores. Test-retest reliabilities for word, color and color-word were reported to be 0.86, 0.82 and 0 .73 respectively. Numerous studies (including Indian) have been published on the Stroop test because of its sensitivity to measure response inhibition (Spreen& Strauss., 1998).

Trail making test A & B:

The TMT is frequently used measure of cognitive functions. TMT Part A assesses the visual scanning and psychomotor speed. TMT B is known for the assessment of executive function (Attention shifting & cognitive flexibility).

PGI memory scale

The PGI-memory scale is one of the tests of PGI Battery of Brain Dysfunction (PGI-BBD) developed by Pershad and Verma (1990). The test material contains items for 10 subtests i.e. Remote Memory, Recent Memory, Mental Balance, Attention-Concentration, Delayed Recall, Immediate Recall, Verbal Retention for Similar Pairs, Verbal Retention for Dissimilar Pairs, Visual Retention and Recognition. It has been standardized with norms for adults between 20 and 45 years.

Bender visual motor gestalt test (BVMGT)

BVMGT is also a part of PGI-BBD developed by Pershad and Verma (1990). It consists of nine geometric designs (numbered A and 1-8) originally developed by Wertheimer to demonstrate the perceptual tendencies to organize visual stimuli into configural wholes. Each design is presented sequentially to the subject whose task is to reproduce them on a blank sheet of paper.

Methodology:

1. Enzyme linked Immunosorbent assay (ELISA) was used for Klotho protein levels in serum
2. Real Time- PCR (RT-PCR) was performed for Klotho gene expression and miR-339-5p expression levels.

1. **ELISA for Klotho levels**: ELISA test was used to quantify the circulatory Klotho protein levels in all the subjects recruited in this study by using Biogenuix Klotho ELISA Kit.

Reagents: Below table showing reagents provided with kit for ELISA.

Item	Specifications(96T)
ELISA-Microplate (Dismountable)	8×12
Lyophilized Standard	2vial
Sample/Standard Dilution Buffer	20ml
Biotin-labeled Antibody (Concentrated)	120ul 2
Antibody Dilution Buffer	10ml
HRP-Streptavidin Conjugate (SABC)	120ul
SABC Dilution Buffer	10ml
TMB Substrate	10ml
Stop Solution	10ml
Wash Buffer(25X)	30ml
Plate Sealer	5pieces

Principle

The Biogenuix ELISA kit was based on sandwich enzyme-linked immune-sorbent assay technology. Capture antibody was pre-coated onto 96-well plates. And the biotin conjugated antibody was used as detection antibodies. The standards, test samples and biotin conjugated detection antibody were added to the wells subsequently, and washed with wash buffer. HRP-Streptavidin was added and unbound conjugates were washed away with wash buffer. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the target amount of sample captured in plate. The Optical density (absorbance) was read at 450nm microplate reader, and then the concentration of target was calculated.

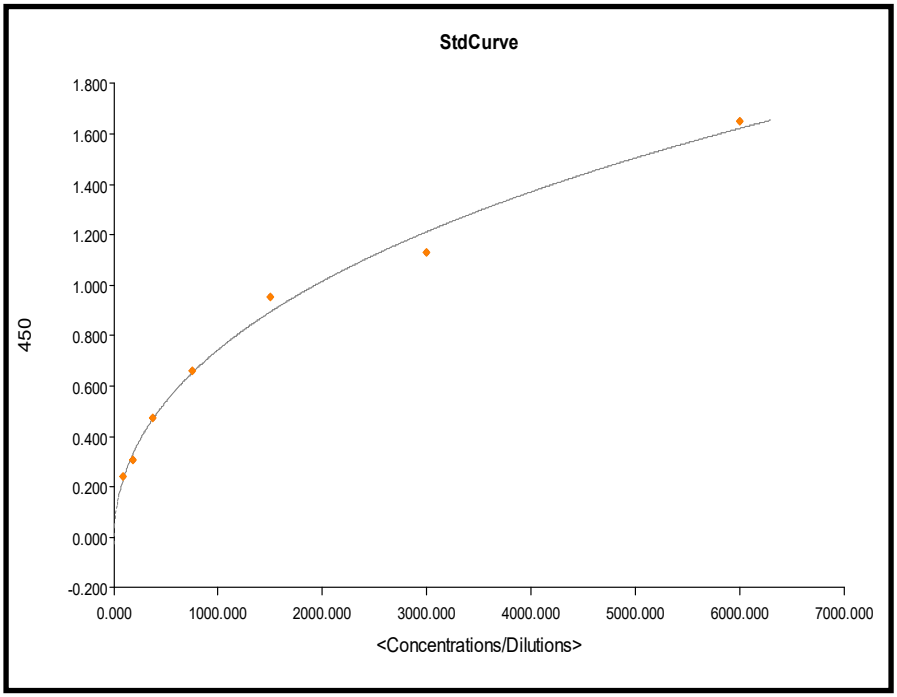
Procedure

The stored serum samples were removed from -80°C and were thawed. Meanwhile all the reagents and samples from the kit were brought to room temperature (20'C). After that 100 µl of each standard and sample (diluted) was added into appropriate wells. The wells were covered and incubated for 1 hour and 30 minutes at room temperature

with gentle shaking. After incubation the solution was discarded and washed 2 times with 1X wash buffer by filling each well with wash buffer (350 µl) using a multi-channel pipette or auto washer. After the last wash, the plates were removed and blotted against a clean lint free paper. 100 µl of 1X prepared biotinylated antibody was then added to each well and incubated for one hour at room temperature with gentle shake. The solutions were then discarded and the previously performed wash was repeated. This was followed by addition of 100 µl of 1X prepared biotinylated-HRP solution to each well and incubation of ELISA plate for 30 minutes at room temperature with gentle shaking. The solution was discarded and the wash step was repeated. Then, 100 µl of TMB substrate was added to each well and the plate was incubated in dark for 10- 20 minutes with gentle shaking. Finally, 50 µl of stop solution was added to each well and the plate was evaluated immediately after stopping the reaction.

Wash Buffer: We Diluted 30ml concentrated Wash Buffer into 750ml of distilled water. This makes the solution of wash buffer to 1X which was used for washing steps.

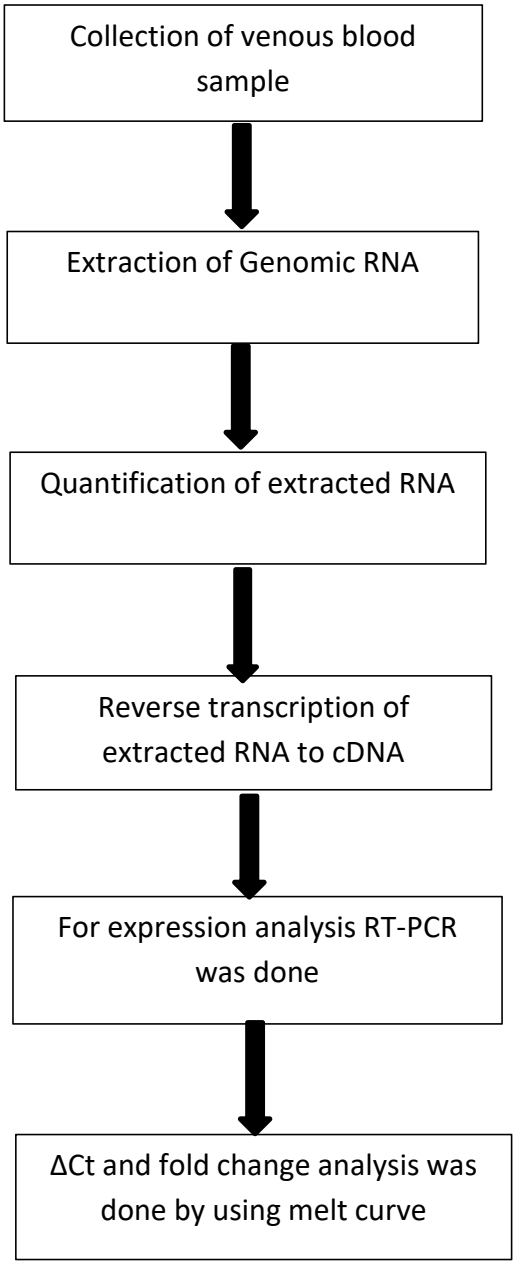
Calculation: Readings of the absorbance for standards and samples were taken by Eon BioTek multiplate ELISA reader (Serial No.14021320) and the standard curve (Shown below) was obtained from Gen 5 software (version 2.05.5).



2. Gene expression analysis:

Klotho gene and miR-339-5p expression

Schematic workflow for Klotho gene expression and miR-339-5p



2a. Isolation of RNA from whole blood venous samples:

Total RNA was isolated and separated from DNA and protein after extraction with a solution named TRIzol. TRIzol is an acidic reagent containing guanidinium thiocyanate, phenol and chloroform designed for the isolation of a variety of RNA species of large or small molecular size. This reagent maintains the integrity of RNA due to highly effective inhibitory RNase activity while disrupting cells and dissolving cell components during sample homogenization. The low pH of this reagent controls to separate RNA from DNA and protein, while a high pH can cause RNA and DNA to isolated together. The guanidinium salt serves as a chaotropic agent to denature proteins and the phenol is an organic compound also used to extract nucleic acid and proteins.

After solubilization and homogenization of samples in TRIzol, the RNA, DNA and protein are differentially extracted by the addition of a phase separation reagent (chloroform). The solution separates the RNA away from DNA and protein into different layers. An upper clear aqueous phase mainly contains RNA and the middle interphase and red lower phase contain DNA and protein respectively. Subsequently, the RNA in the upper aqueous phase is then collected by alcohol (isopropyl) based on precipitation. After incubation and centrifugation steps, the resulting white pellet is then washed in an ethanol solution, air dried and resuspended in the final DEPC treated water (Diethylpyrocarbonate).

Procedure

Venous blood sample was collected in EDTA tube for RNA isolation. For the isolation chilled RBC lysis buffer was added to it (in the ratio of 1:3 of for blood: RBC lysis buffer). The sample was inverted mixed and left undisturbed at room temperature for 15 minutes. The sample was then centrifuged at 1300 rpm for 15 min at room temperature. Supernatant formed after centrifugation was discarded and again 1 mL of lysis buffer was added and mixed and left undisturbed for 5 min at room temperature. This sample was transferred to the micro centrifuged tube and centrifuged at 3000 rpm for 2 minutes. The supernatant was discarded and 1 mL of PBS was added to dissolve the pellet, after which the tube was centrifuged at 3000 rpm for 2 minutes. Then TRIzol reagent was added and pellet formed in the previous

step was dissolved in it. Sample was incubated for 5 min after that 200 uL of chloroform was added to it. The contents were mixed by vigorous shaking and incubated on ice for 15 minutes. After incubation the tube was centrifuged at 12000 g for 15 minutes at 4°C. After centrifugation, upper aqueous layer was carefully removed and put in other micro centrifuge tube and was mixed with equal volume of isopropyl alcohol, then vigorous shaking was done and incubated on ice for 20 min. After incubation, centrifugation was done at 12000 g for 10 minutes. Supernatant formed was discarded and 1 mL of chilled 75% ethanol was added to the pellet and pellet was dissolved in ethanol. After vortex step centrifugation was done at 12000g for 5 minutes and this step was repeated for one more time. Finally, the pellet was air dried and was dissolved in DEPC treated water.

Quantification of total RNA in blood by microplate (ultraviolet spectrophotometer) reader:

Quantity and purity of total RNA were evaluated by measuring the absorbance at 260 nm for RNA concentration, 280 nm and 230 nm and A_{260}/A_{280} and A_{260}/A_{230} ratio for assessing RNA purity on a microplate reader (Eon BioTek).

Materials Required: Extracted RNA Samples, DEPC treated water, Micropipette (0.2-2µL), and microplate reader.

Procedure

Computer was switched on and machine reader (Eon BioTek). was started and allowed to warm to achieve room temperature. When ready, the nucleic acid estimation mode was selected. The platform was wiped with lint-free tissue to remove all the dirt. 1µL of DEPC treated water was used as 'blank'. 1µL of extracted RNA sample was taken and loaded on the platform to estimate the quantity and purity by measuring absorbance at 260nm and 280nm (before loading each sample, it was made sure that the platform is clean). The results were recorded. The yield of RNA in ng/µL and the A260/A280 ratio were considered indicators of RNA quantity and quality, respectively.

2b. cDNA conversion of RNA extracted by Reverse transcription PCR:

The previously extracted and stored human RNA from whole blood were taken out from -80°C and was thawed. Reverse transcription of human RNA was carried out using master cycler, bio rad iScript reverse transcription kit as per the manufacturer's instruction. The kit includes reaction mix, RNase – free water and reverse transcriptase. We used nuclease free water to prepare cDNA for RT-PCR which involves quantification of mature miR-339-5p and mRNA. Template RNA was added to each tube containing reverse transcription master mix such that the composition was as follows:

Table: Reverse transcription reaction components for cDNA conversion

Reagents	Volume
5x iScript Reaction mix	4µL
RNase free water	Variable
iScript reverse transcriptase enzyme mix	1µL
Template RNA	Variable
Total volume	10µL

The tube was then incubated at 37 °C for 60 minutes. After this, the tubes were incubated at 95 °C for 1 minutes to inactivate Reverse transcriptase and then placed it on ice.

Reaction set up for cDNA synthesis

Priming	5 min at 25˚C
Reverse transcription	20 min at 46˚C
RT inactivation	1 min at 95 ˚C
Optional Step	Hold at 4 ˚C

2c. Klotho gene expression by Real- time PCR:

To see the expression of Klotho gene in SZ patients, we performed RT- PCR test. Bio- Rad CFX96 Real time system and Bio- Rad CFX manager software was used for the real time expression analysis of Klotho gene using self-designed primer assay. Housekeeping gene used for mRNA was GAPDH. Table given below is showing primers used for RT-PCR for expression of Klotho gene.

Klotho gene	F: 5′-ACGAAGCTCTCAAAGCCCAC-3′ R: 5′-GCACTCAGTACACACGGTGA-3′
GAPDH housekeeping gene	F: GTCTCCTCTGACTTCAACAGCG R: ACCACCCTGTTGCTGTAGCCAA

While starting, we diluted the forward and reverse primers with nuclease free water as per the manufacturers protocol. Then from that mixture we took 10µL of primers and into it we added 90µL of nuclease free water. This mixture was used for RT- PCR as per standard protocol given by Bio- Rad kit insert and annealing temperature used was 60 ˚C.

Housekeeping gene:

Housekeeping gene also known as endogenous control, normalizer gene and reference genes. These genes show expression levels that are relatively constant and moderately abundant across tissues, cell types and treatment protocols. Such genes are currently the most accurate method to correct for potential biases that are caused by sample collection, variation in the amount of starting material, reverse transcription efficiency and nucleic acid preparation and quality.

The SYBR green PCR kit from Bio rad company was used with Klotho gene PCR assay as per manufacturer's instruction. The kit includes SYBR green PCR Master mix and RNase free water. All the reagents and cDNA samples were thawed at 4 °C after removing from – 80 °C and reaction master mix was prepared according to the following specifications:

Reaction set up for Real time PCR

Reagents	Volume
SYBR green PCR Master mix	5µL
Forward and Reverse primer	1µL each
RNase free water	Variable
Template cDNA	1µL
Total Volume	10µL

Master mix and cDNA was added to the wells of the PCR tubes. After thorough mixing, real time PCR reaction was carried out by placing the reaction strips in an

automated and temperature-controlled cycles of denaturation, annealing and elongation using a thermal cycler. The thermal cycle parameters were sets as follows:

Thermal cycler parameters shown in table below:

Initial Denaturation	30 sec at 95 ˚C
Denaturation	15 sec at 95 ˚C
Annealing	15- 30 sec at 60 ˚C
Cycles	40
Extension	30 sec at 72 ˚C
Melt curve analysis	2- 5 sec per step at 65 – 95 ˚C

2d. <u>miR 339-5p expression analysis by Real- Time PCR</u>

Bio- Rad CFX96 Real time system and Bio- Rad CFX manager software was used for the real time expression analysis of miR-339- 5p using self-designed primer assay. Housekeeping genes used for miR was RNU6.

Table for primer assay

miR- 339- 5p	F: GGGTCCCTGTCCTCCA R: TGCGTGTCGTGGAGTC
RNA U6 housekeeping gene	F: GCTTCGGCAGCACATATACTAAA R: CGCTTCACGAATTTGCGTGTCAT

The SYBR green PCR kit from Bio rad company was used with miR PCR assay primers as per manufacturer's instruction. The kit includes SYBR green PCR Master mix and RNase free water. All the reagents and cDNA samples were thawed at 4 °C after removing from – 80 °C and reaction master mix was prepared according to the following specifications:

Reaction set up for Real time PCR

Reagents	Volume
SYBR green PCR Master mix	5µL
Forward and reverse primer	1µL each
RNase free water	Variable
Template cDNA	1µL
Total Volume	10µL

The template DNA was dispensed into the individual wells of the PCR strips and then thoroughly mixed reaction mix was added into the cDNA containing wells of PCR strips. In order to detect miR levels in the samples, real time PCR reaction was carried out by placing the reaction strips in an automated and temperature-controlled cycles of denaturation, annealing and elongation using a thermal cycler. The thermal cycle parameters were sets as follows:

Thermal cycler parameters shown in table below:

Initial Denaturation	30 sec at 95 ˚C
Denaturation	2- 5 sec at 95 ˚C
Annealing	30 sec at 54 ˚C
Cycles	40
Melt curve analysis	2- 5 sec per step at 65 – 95 ˚C

Steps followed for double delta Ct analysis:

The average of the Ct value for the housekeeping gene and target gene being tested in all the samples was obtained through RT- PCR. The difference between tested experimental and housekeeping gene and healthy control and housekeeping gene were calculated, through which delta Ct values for the experimental and controls were respectively obtained. The difference between Ct value of experimental and Ct value of Control was used to calculate double delta (ΔΔ) Ct values. Since all the calculation there were made in the logarithmic vase 2 and every cycle there is twice as much DNA, therefore the value of $2^{-\Delta\Delta Ct}$ was calculated to get the fold change analysis of the gene in SZ cases as compared to normal controls.

Statistical Analysis:

The data collected were tabulated and analyzed by Microsoft Excel 2016. Statistical tests were performed using SPSS 22.0 software. Descriptive statistics were carried out to determine mean, standard deviation (SD) and median (IQR). For. To assess the normal distribution of the data Shapiro Wilk Test was carried out. Student t-test was used to compare parametric data and the Mann Whitney U Test for non- parametric data. Pearson or Spearman test was performed to assess the correlation. Probability (p) value <0.05 was considered significant.

RESULTS

Study characteristics:

Demographic profile

Demographics were recorded for all the participants and for cases, clinical scores like GAF and PANSS were calculated with help of psychiatrist. Serum Klotho levels were measured using sandwich ELISA. Expression levels of Klotho gene and miR-339 -5p were done by RT-PCR.

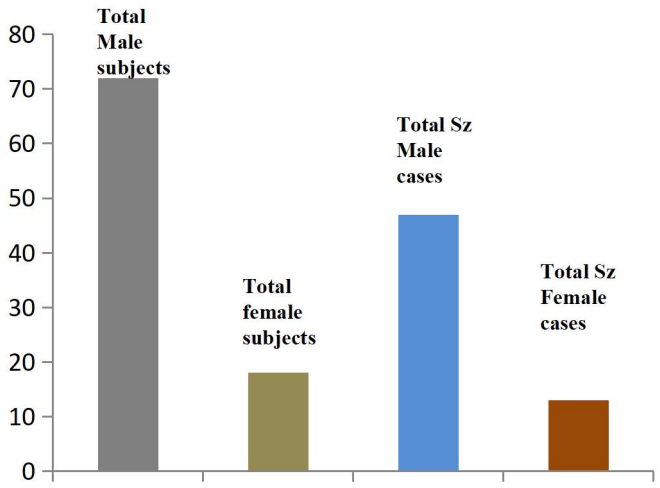


Figure 1: Gender- wise distribution of total study subjects and SZ cases.

In the present study, 90 subjects were recruited, among them there were 72 (Seventy-Two) males and 18 (eighteen) females. Further, the SZ group had 47 (forty-seven) males and 13 (thirteen) females (Figure 1). SZ patients were diagnosed as per ICD-10 criteria and the severity was assessed on the basis of GAF and PANSS scores. In cases, the mean and SD for age were 31.51 ± 12.0 years and that of controls were 37.46 ± 7.42 years. Figure 1 is showing number of subjects that were recruited in the study. Males and females were shown by different color bars, first bar is of total males second is of total females, third is total SZ male subjects and last bar represents female SZ subjects under study.

Table 1: Age and Gender distribution:

Parameters	Cases		Controls	
	n	Mean ± SD	n	Mean ± SD
Age (Years)	60	31.51 ±12.0 years	30	37.46 ±7.42 years
Sex (Age)				
M	47	30.80 ± 12.03	23	39.30 ± 6.83
F	13	34.07 ± 12.33	05	29.0 ± 2.3

n is total number of subjects

There were 47 males and 13 females among cases. The mean and SD of age of male cases were 30.80 ± 12.03 years and for female cases it was 34.07 ± 12.33 years. The mean ± SD of male control subjects was 39.30 ± 6.83 years and that of female controls were 29.0 ± 2.3 years (Table 1). Mostly the subjects were in the age group of 20 to 60 years in both the groups under study.

Table 2: Sociodemographic profiles in participants:

Parameters	Cases	Controls
Smokers	16	7
Non smokers	41	23
Education status	3 illitrate and rest matriculation to graduation	Matriculation to graduation
Occupation	Cases	Controls
Skilled	16%	50%
Semi- skilled	8%	50%
unemployed	76%	0

From Table 2 it is clear that, out of 60 cases, 16 were having history of tobacco smoking/ chewing and 41 were nonsmokers and among controls, 7 out of 30 were having tobacco smoking/ chewing history. To see the literacy, we gathered information about education status from all the subjects. Among cases, 15 were graduate, one post- graduate, 24 cases studied up to 10^{th} standard, 11 cases were

having education status of up to 12th standard and 3 were illiterate. In controls, 16 were having studies up to 12th standard, 9 were up to 10th standard and 5 were graduate among them. According to occupation, the number of skilled, semi-skilled and unemployed among cases were 16%, 8% and 76% respectively and among controls 50% were skilled and 50% were semi-skilled and there was no unemployed among controls.

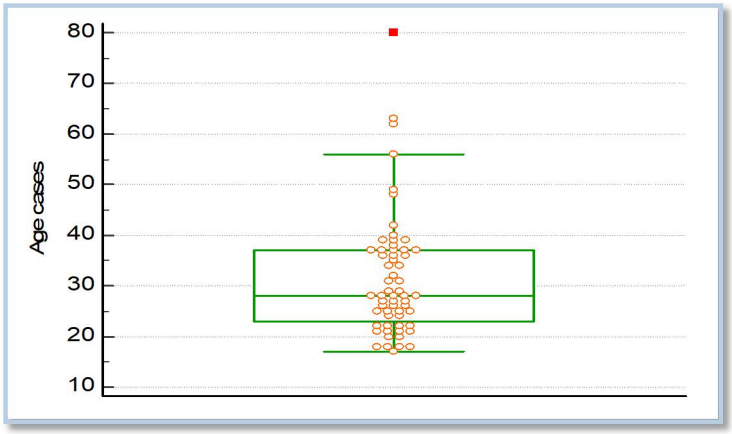


Figure 2: Age wise distribution of schizophrenia Cases.

Figure 2 is showing age wise distribution and accordingly, most cases that we recruited in this study were between 20 to 60 years and maximum in the range of 20 to 40 years as seen by the box plot diagram, most of the dots are clustered in the age between 20 and 40 years.

Table 3: Clinical measures in SZ patients:

Parameter	Mean	Median	Standard deviation
Age of onset (in years)	25.75	23.5	10.61
Duration of illness (in years)	5.91	4.0	5.39
Global assessment of functioning (GAF)	75.96	80.0	12.40
PANSS	38.73	34.0	10.99

Above, Table 3 is showing clinical measures in SZ patients. The Mean, SD and median of age of onset were 25.75, 10.61 and 23.5 respectively. In duration of illness, Mean, SD and median were 5.91, 5.39 and 4.0 respectively. As severity was assessed with the help of two parameters that is GAF and PANSS. The Mean, SD and median of GAF scores among cases were 75.96, 12.40 and 80.0 respectively. The Mean, SD and median of PANSS scores were 38.73, 10.99 and 34.0 respectively.

Table 4: Serum Klotho protein levels of study participants:

Parameters	Serum Klotho protein levels (pg/mL)			
	n	Mean ± SD	Median	p
Participants				
Cases	60	56.82 ± 18.62	52.87	
Controls	30	50.95 ± 15.73	48.23	0.09
Gender				
Cases M	47	58.38 ± 20.05	53.87	
F	13	51.05 ± 12.57	48.31	0.08
Gender				
Controls M	23	94.06 ± 16.86	45.44	
F	05	58.51 ± 8.67	56.39	0.47
Family History of SZ				
Present	4	48.78 ± 4.32	46.70	
Absent	56	59.00 ± 33.08	53.06	0.006*

* Significant with p value of < 0.05.

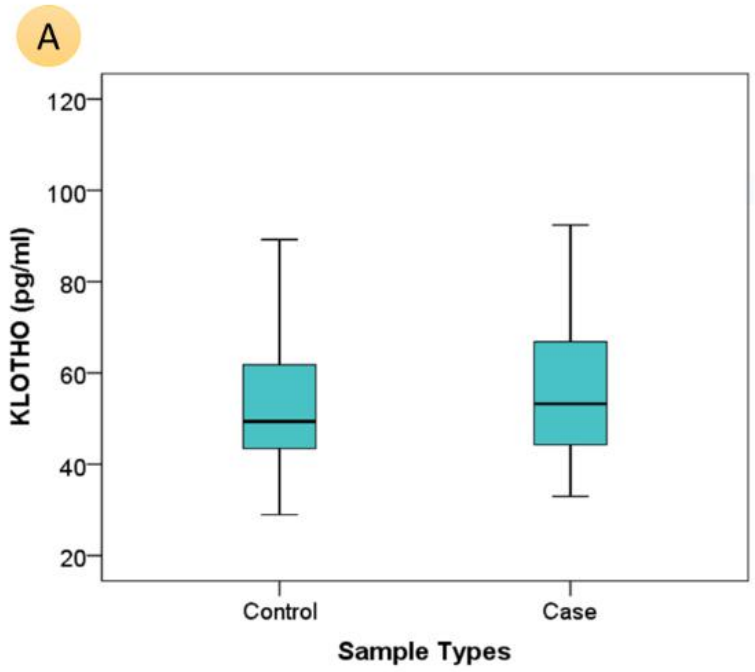


Figure 3: Serum Klotho protein levels (pg/mL) in cases and controls

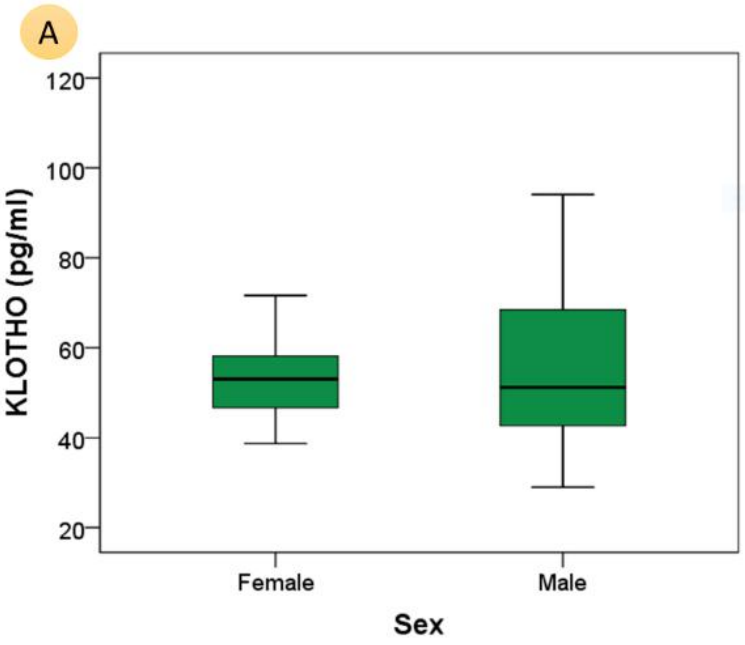


Figure 4: Serum Klotho protein levels in female and male cases

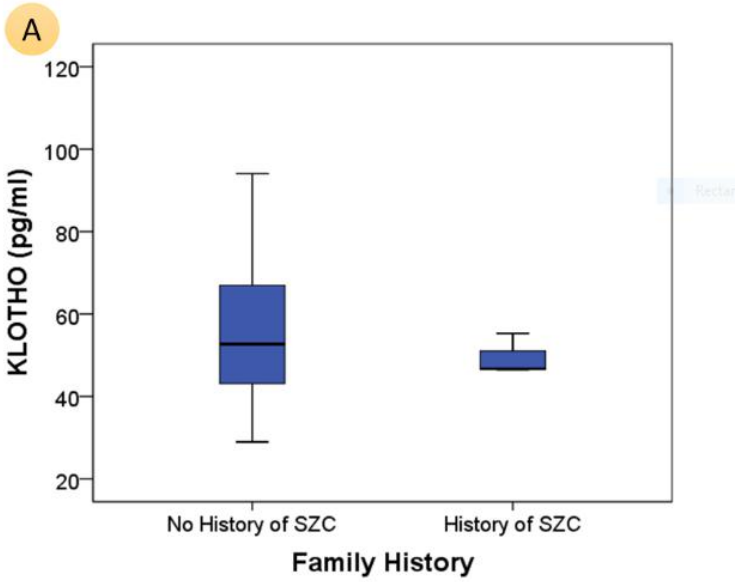


Figure 5: Correlation of Klotho protein levels with the family history

In SZ cases, serum Klotho protein levels were higher in comparison to healthy controls. Mean value of Klotho protein levels in cases came out as 56.82 pg/mL with standard deviation of 18.62 pg/mL and mean value of Klotho protein in healthy controls came out to as 50.95 pg/mL and standard deviation of 15.73 pg/mL. (Figure 3) Outliers were removed as per Tuckey's test from both cases and controls. The Mean ± SD and median of serum Klotho protein levels of male cases were 58.38 ± 20.05 pg/mL and 53.87 pg/mL respectively. (Figure 4) The Klotho protein levels in female cases were 51.05 ± 12.57 pg/mL and 48.31 pg/mL respectively. (Table 4) Differences between the mean of Klotho protein levels of the gender specific groups of cases and controls were not significant. The Mean ± SD and median of Klotho protein levels of control group males were 94.06 ± 16.86 pg/mL and 45.44 pg/mL and females were 58.51 ± 8.67 pg/mL and 56.39 pg/mL respectively. The Mean ± SD and median of Klotho protein levels in accordance with family history were 48.78 ± 4.32 pg/mL and 46.70 pg/mL and 59.00 ± 33.08 pg/mL and 53.06 pg/mL and is found to be highly significant with p value of 0.006*. (Table 4, Figure 5)

Table 5: Correlation analysis of Serum Klotho protein levels with age of cases and PANSS scores.

Parameter 1			Parameter 2	
			Klotho protein levels Mean ± SD	P value
Age	M	30.80 ± 12.03	58.38 ± 20.05 pg/mL	0.001*
	F	34.07 ± 12.33	51.05 ± 12.57 pg/mL	0.947
PANSS		38.73 ± 10.99	56.82 ± 18.62 pg/mL	0.01*

*Highly significant

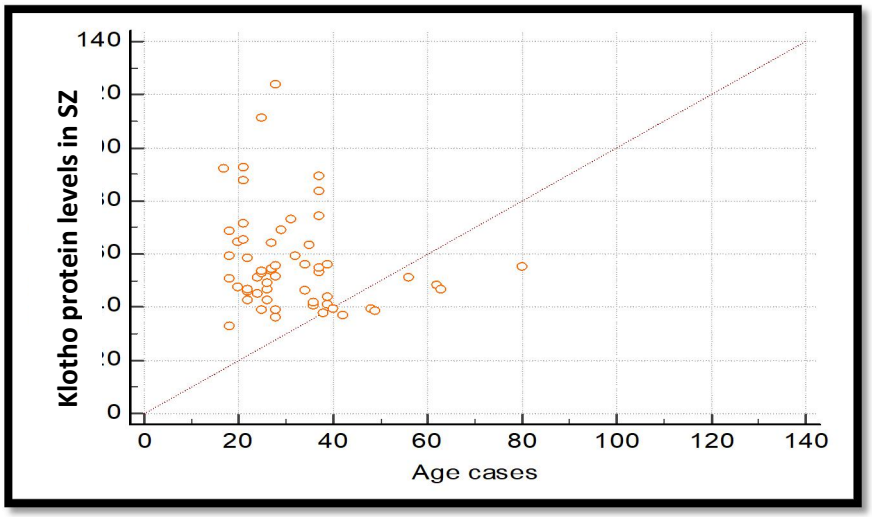


Figure 6: Correlation of Klotho protein levels and age of cases

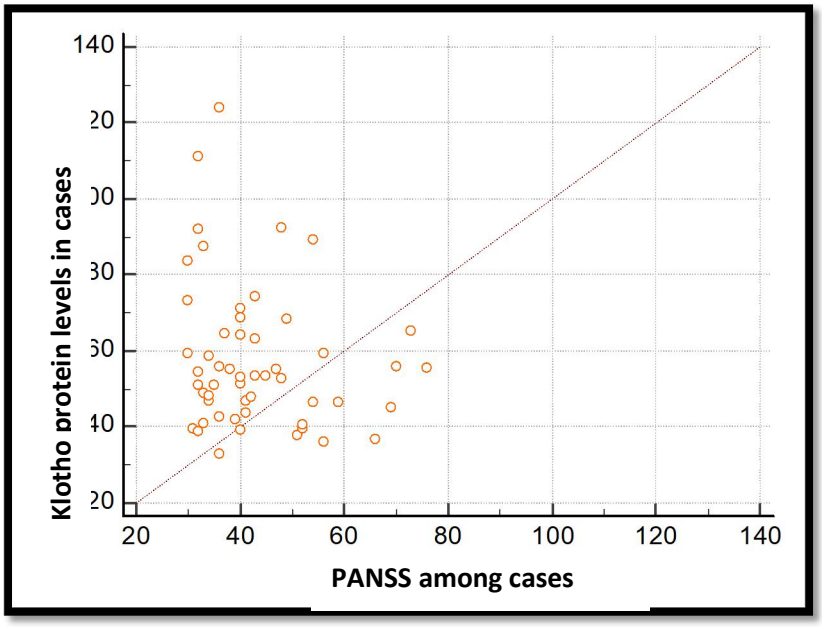


Figure 7: Correlation of Klotho protein levels and PANSS scores among cases

Table 5, is showing the correlation analysis of Klotho protein levels and age of cases and PANSS scores of cases and we found both the levels were highly significant with p value of 0.01. (Figure 6 and 7). The mean ± SD of total PANSS score was 38.73 ± 10.99 and when it was correlated with Klotho protein levels with mean and SD of 56.82 ± 18.62 pg/mL it was found to be highly significant with p value of 0.01. The mean ± SD of PANSS among male cases was 30.80 ± 12.03 and among female cases was 34.07 ± 12.33 respectively.

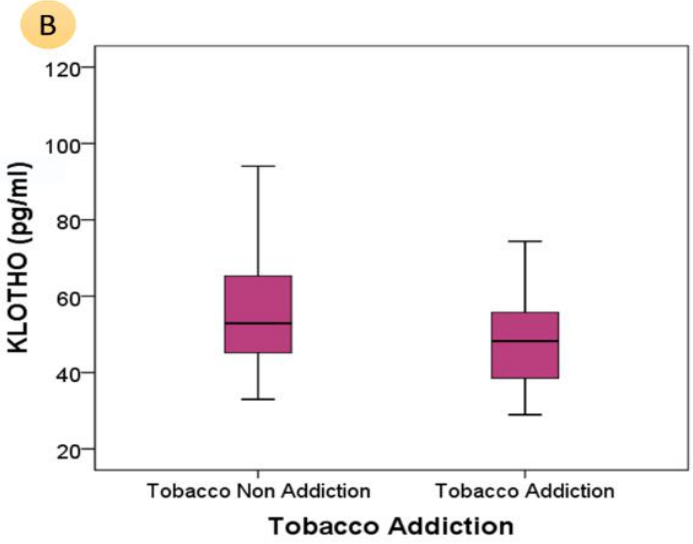


Figure 8: Comparison of Klotho protein among tobacco addict and non addicts

History of Tobacco consumption showing low Klotho protein levels in subjects those were taking tobacco in comparison to non addicts (Figure 8). We can clearly figure out from the above box plot that Klotho protein levels were higher in non-addict as compared to tobacco addict.

Klotho gene expression:

We analyzed the expression of Klotho gene and found that in blood, Klotho gene was upregulated. The average ΔCt value of cases was 9.56 and that of controls was 10.62. ΔΔ Ct values calculated among SZ cases and control population by subtracting ΔCt values of cases from Δ Ct values of healthy control population. The value came out to be -1.06. Fold change expression for the selected genes in SZ patients compared to healthy controls were calculated by the formula given by scientist Livaak i.e. 2 ^-ΔΔCt. After calculation we found FCE value as 2.08 which means that Klotho gene was two times upregulated in cases in comparison to healthy controls. Figure given below is showing Klotho gene expression among cases and controls and it is clearly seen that relative FCE is more in cases as compared to healthy controls. Figure 9 is showing relative fold change expression of Klotho gene among cases and controls. Figure 10 is showing amplification in normal and logarithmic scale during RT-PCR and figure 11 is showing melt curve analysis

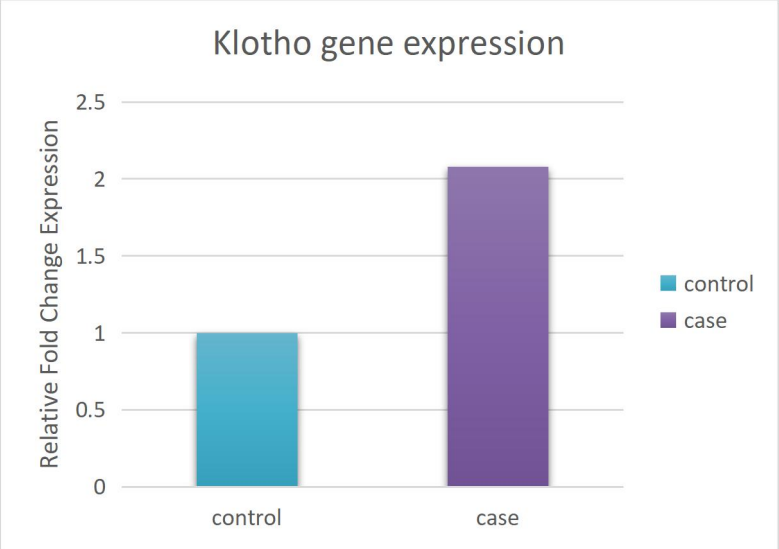


Figure 9: Relative FCE of Klotho gene among cases and controls.

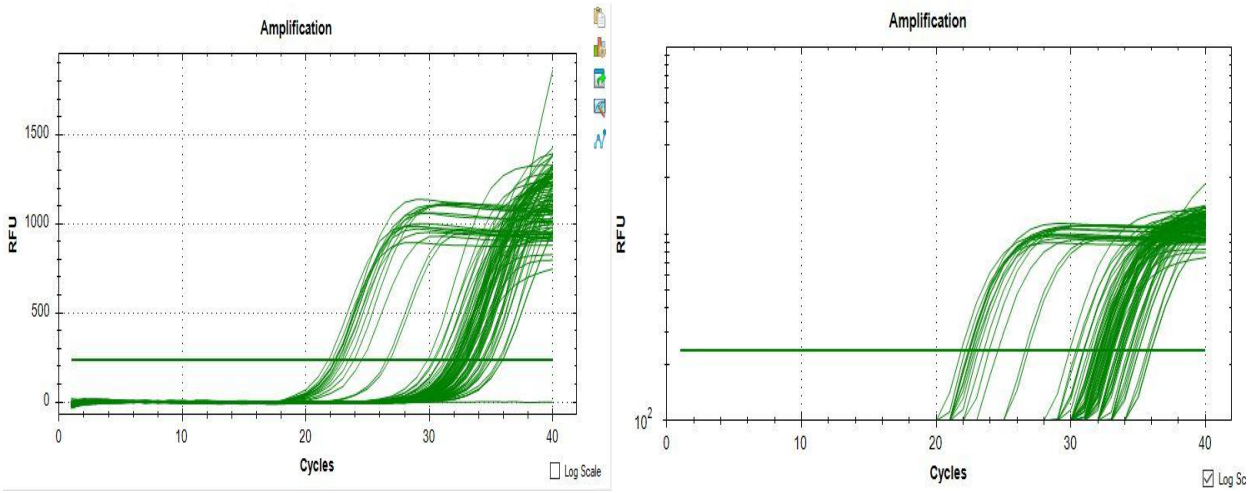


Figure 10: Showing Amplification and Log scale amplification in RT-PCR

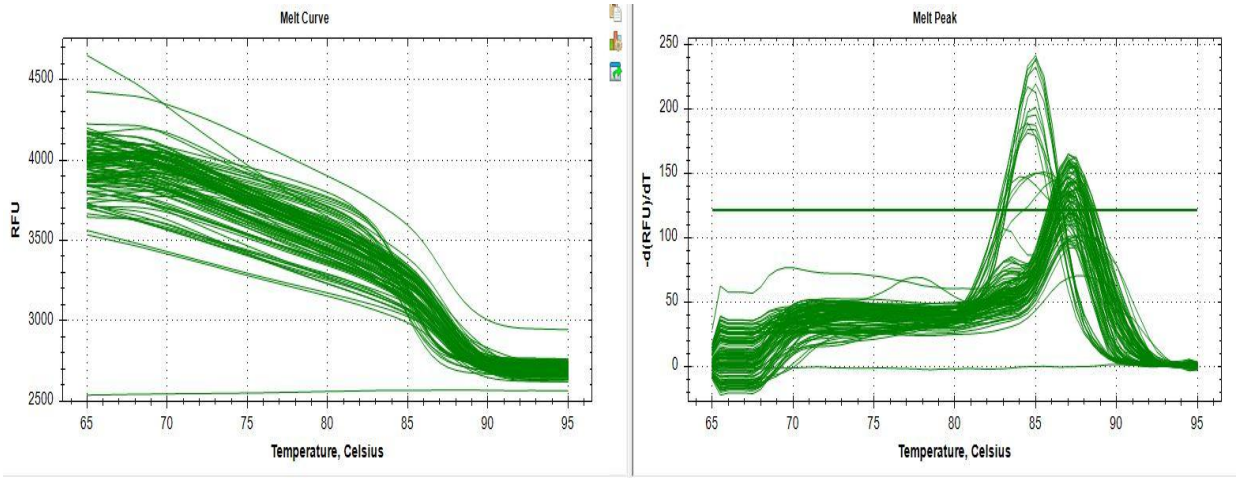


Figure 11: Showing melt curve analysis during RT-PCR

Correlation of cognition among cases and controls:

We performed different batteries of test to evaluate cognition in SZ cases and controls.

Table 6: Correlation of Stroop color word test with cases.

Parameter	P value	Rho	Mean± SD
Stroop word test Cases	0.036	-0.405	73.22 ± 25.80
Stroop color Cases	0.034	0.157	41.81± 17.51
Stroop color word Cases	0.434	0.260	29.9 ± 15.38
Stroop color test total Cases	<0.001	0.206	143.30 ± 53.88

Stroop color word test:

In this test, memory of SZ patients was tested by different parameters namely stroop color, word and color word. Correlation analysis was done between cases and this test for word test and the correlation is found to be significant with p value of 0.036 and Spearman's coefficient of rank correlation (rho) is -0.405. (Table 6)

Correlation of Stroop color and color word test and PANSS scores:

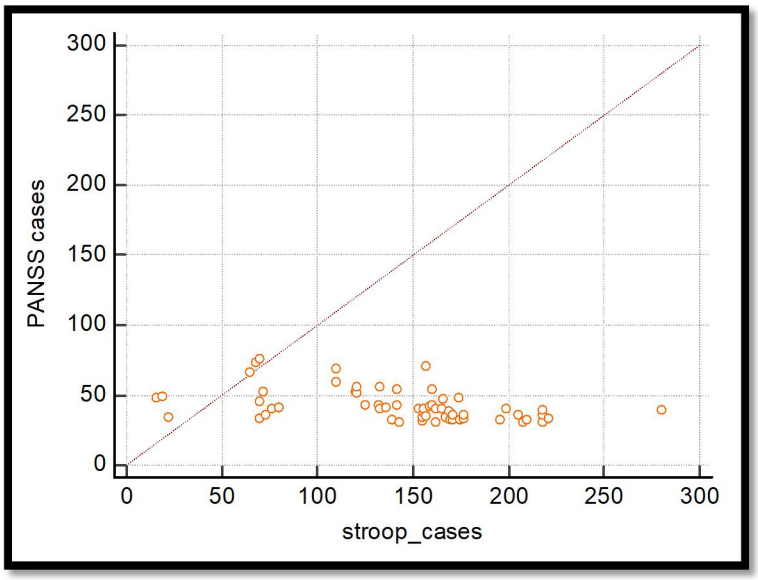


Figure 12: Scatter plot for Stroop test and PANSS scores among cases

Correlation of stroop color among SZ cases and stroop color test, this test was found significant with p value of 0.038 rho 0.157 and color word is not significant with p value and rho of 0.434 and 0. 260 respectively. Correlation of Stroop color test among cases and PANSS score showed the negative correlation with rho value -0.553 and is highly significant with p value of < 0.001 (Figure 12).

Trail making test A & B:

In this test, correlation found to be highly significant among SZ patients and Trail making test A and B with p value of 0.001. Further, we found significance in the errors made by cases as compared to controls. Errors were more in SZ group in comparison with controls. Similar results were found in Trail making test B in comparison to both groups.

PGI memory Scale

We performed this test and there was no significance in cases and controls with p value of 0.485 and rho value of 0.136 (Table 7).

Table 7: correlation of PGI memory scale among cases and controls

Parameter	P value	Rho
PGI Memory scale	0.485	0.136

Bender visual motor gestalt test (BVMGT)

There is highly significant correlation between SZ patients and controls with p value of 0.001 and correlation coefficient was 0.781 by students t test (Table 8).

Table 8: Comparison of BVMGT among cases and controls

Parameter	P value	Pearson's coefficient
Bender visual motor gestalt test	0.001	0.781

miR-339-5p expression analysis

Expression analysis for miR- 339- 5p was done by using RT- PCR with Bio Rad kit. RNA isolated from whole blood sample was checked for purity and concentration in microtitre plate reader and then stored at – 80 ^{o}C. Then RNA sample was thawed and cDNA was synthesized using Bio Rad cDNA synthesis kit and then stored at – 80 ^{o}C till PCR to be performed. Then cDNA is utilized for RT- PCR and expression levels of miR-339- 5p was assessed.

We analyzed the expression of miR-339-5p and found that in blood miR-339-5p was down regulated. We performed Correlation of Δ Ct values of SZ patients and healthy controls and it was negatively compared in significant manner with p value of 0.03 with rho value -0.380.

ΔΔ Ct values calculated among SZ cases and control population by subtracting ΔCt values of cases from Δ Ct values of healthy control population. The value came out to be 2.06. FCE for the selected genes in SZ patients compared to healthy controls were calculated by the formula given by scientist Livaak i.e. $2^{-\Delta\Delta Ct}$. After calculation we

found FCE value as 0.23 which means miR-339- 5p was downregulated in cases in comparison to healthy controls (Figure 13). On comparing ΔCt of cases and controls we found it highly significant with p value of 0.01 (Table 9).

Table 9: Comparison of Δ Ct among cases and controls.

Parameter	Cases	controls	p value	ΔΔ Ct	FCE
Δ Ct	12.76	10.7	0.01	2.06	0.23

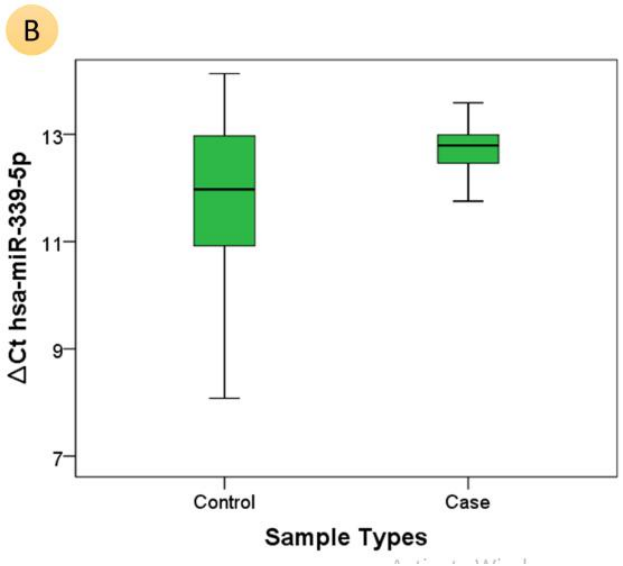


Figure 13: Comparison of Δ Ct between cases and controls.

Correlation of Klotho protein and miR 339-5p:

Correlation of miR-339-5p and Klotho protein levels showed negative association. This means that with increase in one variable other parameter decreases. Pearson's correlation showed value of -0.189. The median and interquartile ranges for Δ Ct were 12.80 and 12.60- 12.93 respectively and of Klotho proteins median and interquartile ranges were 52.70 and 47.35 – 55.70 respectively (Table 10).

Table 10: Correlation of Klotho protein and Δ Ct values of cases

Parameters	Median	Interquartile range
Δ Ct	12.80	12.60- 12.93
Klotho protein levels	52.70	47.32- 55.70

DISCUSSION

SZ is a severe psychiatric disorder with prevalence of 1 % worldwide and 0.4 % in India. It manifests as hallucination and delusions, which may also be referred to as psychosis.

In the present study, the Klotho gene expression and miR-339-5p was analyzed along with the serum Klotho protein levels. The serum Klotho protein levels were further correlated with demographic profiles and clinical measures of SZ in cases and controls.

Demographic profiles:

In the present study, we had included total of 90 study subjects. Among these 90 study subjects there were 72 males and 18 females. In SZ group, 60 cases were recruited who were diagnosed with SZ and their diagnosis was according to ICD- 10. Among this SZ cases, 47 were male cases and 13 were female subjects. The Mean age of male cases were 30.80 years and that of females were 34.07 years. And among controls 24 were males with Mean age of 39.30 years and 6 were females with mean age of 29 years. Furthermore, females with SZ were in the age group of 20 to 40 years and that of males were between 25 to 60 years (Figure 1).

Most of the subjects that were selected in this study were in the age group of 20 to 60 years and among them maximum number of cases were seen in the age between 20 to 40 years. This observation is in accordance with the previous study which was saying that this disease started at an early age between twenties and thirties (106) (Table 1). Our study results were also in line with the results reported by Zorkina et al, in which they stated that male SZ patients are more likely to get the disease in their early age (106). Our results are also aligned with the data in a number of previously published studies such as studies done by Abel et al, Cocchi et al, Cohen et al, all reported early onset of SZ and males were mostly affected at early onset of disease (4,107,108).

Socio demographic profiles:

According to the previous studies, education levels of SZ cases might contribute to the disease (5). As per those studies, the education can play a protective role in

neuropsychiatric disorders (5). Based on our findings, equal numbers of subjects were having education up to high school or less than high school levels (Table 2). It is in contrary to the study done by Christian et al who reported that those who have studied less have higher risk of SZ (109).

Apart from education, unemployment can be a major factor in the occurrence of SZ. As Christian et al reported that there is 20 times higher risk of SZ in unemployed persons (109). Similarly, in our study 24% of the cases were doing either skilled or semi-skilled works, and remaining were unemployed so it is in line with the study done by Christian et al. Now, coming to the substance abuse, 26% patients were having history of smoking or tobacco chewing and 28% were having history of alcohol abuse. So, our study results, does not support any casual association of smoking and SZ. This is also in accordance to the study done by Jianhua Chen et al. as they also reported no association of smoking and SZ (110). But to our contrary, there are few studies which reported that people who smoke tobacco have a 2-fold increased risk of incident of SZ or psychosis (111).

Clinical Measures in SZ:

Among the numerous clinical characteristics used to diagnose the SZ spectrum disorders, the age of onset of illness is widely accepted to have high clinical and prognostic significance. Studies reported variable clinical outcomes like remission and relapse were influenced by age of onset. In this study, the mean, SD and median of age of onset of disease was 25.75 years, 10.61 years and 23.5years respectively (Table 3). This means that most of the cases were having an early onset of disease. Onset of SZ is typically between late teens and early 30s and in males its incidence starts around early to mid-twenties and in female's disease onset is between late twenties (112). In a recent meta-analysis by Immonen et al (2017) (113), it was found that the earlier the age of onset, more are the chances for remission. It was also found that the age of onset has no relation with the number of hospital admissions.

Now, in the confirmed cases of SZ severity was assessed by global assessment of functioning scale (GAF) and PANSS scores. GAF is a numerical scale to rate the social, occupational and psychological functioning of an individual. The range of the score is 1 to 100, 1 being severely impaired and 100 being extremely high functioning

(114). In this study, the mean and SD, for GAF score was 75.96 ± 12.40. (Table 3). So according to the results of our study, the GAF score of most of the patients were being under classification of some mild symptoms or some difficulty in social, occupation and school functioning (115). Similar results were found by Kohler et al (2016) in paranoid SZ patients and first-time SZ patients in Iranian population (115).

The PANSS scale is the most widely used scale to measure the severity of symptoms in SZ patients. To assess the patient using PANSS, a clinical interview of approximately 45-minute is to be conducted. Total PANSS score is from 30 to 210, 30 is lowest score with less severity and 210 is most severe case (116). In this study, the mean and SD of total PANSS score was 38.73± 10.99 among cases (Table 3). Total PANSS score of the present study indicated that the severity of the disease ranged from mild to moderate. The positive and negative scores conclude that most of the cases were presented with minimum to mild delusional symptoms with passive emotional and social withdrawal. General psychopathology score of the current study showed that the cases had impaired cognition and related features like poor attention and difficulty in thinking or reasoning. Kay et al (1987) (117) tested the PANSS scale on 101 adult SZ patients (20-68 years) and the mean scores of Positive scale, Negative scale and General psychopathology were 18.20, 21.01 and 37.74 respectively. PANSS score is a severity score and in SZ patients after starting antipsychotics there can be a reduction in 20- 25 % of PANSS score from the starting of treatment which is regarded as good response to treatment (118). So, this might be the reason for less severity in cases recruited in this study. As in this study the mean duration of illness was 5.91 ±5.39 years and they were on regular treatment and there was no drug naïve patient so this can influence our PANSS scores.

Klotho protein levels and variables:

In this study, comparison of the Klotho protein levels in cases and controls were done and also Klotho protein levels were correlated in relation to gender and family history of SZ. The mean level of Klotho protein was 56.82 pg/mL in cases and 50.95 pg/mL in controls (Table 4 &Figure 3). So, it is clear that in comparison to control population SZ cases have higher levels of Klotho protein. This finding is in accordance to the study done by Xiong JW et al in 2020 who reported that SZ cases have higher levels of Klotho protein as compared to healthy controls (118). Now,

gender wise correlation for the levels of Klotho protein in SZ group was assessed in which we found males were having mean levels of 58.38 pg/mL and females were having 51.05 pg/mL, which means it was higher in males as compared to female cases (Figure 4). In controls males, mean levels of Klotho protein was 94.06 pg/mL and that in females were 58.51 pg/mL which was also higher in males as compared to females. However, there is no study that reported gender- wise klotho levels in males and females but in contrary to our findings one study in acromegaly reported higher Klotho levels in females suffering from acromegaly as comparison to males (119). Further, to find its correlation with family history four subjects were having family history of SZ and 56 were not having any history of psychosis in family. The mean Klotho protein levels in cases with positive family history were 48.78 pg/mL and with negative family history were 59.0 pg/mL, it is clear that Klotho protein levels were higher with negative family history and is highly significant with p value of 0.006 (figure 5). This might be explained by the stress factor which family was suffering due to already having SZ patient in family as seen in study done by Parther AA et al, who demonstrated that chronic psychological stress on mothers or care givers of Autistic disorder suffering child have lower Klotho levels (120) (Table 4).

Correlation of Klotho protein with severity of SZ:

Now, we proceed forward to find the correlation between Klotho protein levels and PANSS in this study. The results showed that these two parameters were correlated negatively in a highly significant manner. This observation was in contrary to the previous study done by Esra et al. in which they did study on admitted patients and outpatient that came to their outpatient department and was in remission phase and their PANSS was higher in SZ patients and was positively correlated with Klotho protein levels (121) (Table 5).

Further, to correlate Klotho protein levels with age of SZ patients it was found that with advancing age Klotho protein levels were decreasing (Figure 6). It is in line with the previous studies in which they reported low levels of klotho protein in older adults ≥ 65 years (122). To see the effect of smoking in SZ patients we analyzed the Klotho protein levels in subjects addicted to tobacco and those who were not taking tobacco. The results showed that Klotho protein levels were lower in tobacco addict in comparison to non addicts (Figure 8). Our results are contradicting with the study

done by Kamizono Y et al (2018) (123) this study showed that Klotho protein levels were raised in patients that were addicts to tobacco smoking and when tobacco smoking cessation was done their Klotho protein levels were decreased but in our study Klotho protein levels were already in lower side as compared to healthy controls.

Klotho gene expression: We examined the expression level of Klotho gene in both the groups. From the results of this study, we found that the expression of Klotho gene was increased by two folds. Both the expression of Klotho gene and its product Klotho protein were found to be increased in this study. From this we can say that Klotho protein was also increased due to increased expression of Klotho gene. To the best of our knowledge this is the first study where both Klotho gene expression and circulatory Klotho protein levels were determined together in SZ patients.

Correlation of cognition among cases and controls:

We performed different batteries of cognitive test like Stroop color word test, Trail making A and B, PGI memory scale and Bender visual motor gestalt test. In Stroop color word test, we found that in word (stroop) test, mean value was 73.22 in cases and 91.44 in controls which means that controls performed better than cases in stroop word test and was significant between both groups with p value of 0.036. In Stroop color test, the mean value among cases was 41.81 and that in controls was 69.44 and this test was also better performed by controls but this test was not statistically significant. Same was with color word test. Total scores when saw statistically, it was found highly significant with p value of < 0.05 which means overall stroop color word test battery was better performed by controls in comparison to cases (Table 6). Our findings were similar to the study done by Jian- wen Xiong et al in 2020, they also demonstrated that stroop color word test was performed worse by cases as compared to control and was statistically significant (118).

Trail making test A and B was used to assess executive memory (Attention shifting and cognitive flexibility), this test was also better performed by controls in comparison to cases which was highly significant with p value of < 0.05. This observation is also in line with the study done by Jian- wen Xiong et al in 2020 in which their control patients performed Trail making test A better than cases (118).

PGI memory scale was also carried out among two groups and this scale had different questions which assess remote memory, recent memory, mental balance, attention visual retention and recognition. The result of this test showed that it was positively correlated among two groups but not statistically significant (Table 7). Our study replicates the finding of many previous studies that showed that SZ patients performed worse in cognitive domain in comparison to controls (124,125).

Bender visual motor gestalt test (BVMGT) was done on all the subjects under study, we found that this test was performed better by controls in comparison to cases and was highly significant with p value of <0.05. It was also positively correlated between SZ patients and controls (Table 8).

Correlation of Klotho gene with Klotho protein:

This study demonstrates that patients with SZ had higher serum levels of Klotho protein in relation to healthy controls and serum Klotho protein level was positively correlated with cognitive functions. In the present study, Klotho gene expression was also found to be upregulated by 2-fold. Klotho is a life span associated protein that displays cognitive- enhancing effect (16). Upregulation of Klotho gene is associated with higher levels of Klotho protein and better cognitive functions and longer lifespan, while lower Klotho gene expression is associated with shorter lifespan and deteriorating cognitive behavior (56,126,127). A study done by Morar et al. showed that heterozygous KL-VS carriers displayed worse performances than non-carriers (82).

In this study, we found that patients with SZ had higher levels of serum Klotho than did healthy controls. Given that the recruited patients in this study were on regular antipsychotic drug treatment.

Cognitive deficits are considered as a core feature of SZ (118). In consistent with the study done by Jian- wen Xiong et al in 2020, who demonstrated that SZ patients had worse performances in the cognitive test as compared to healthy control subjects (118). Several studies have demonstrated that Klotho exerts a cognitive- enhancing effect by regulating antioxidant defense and NMDAR functions in the brain (56,82). Mice with Klotho mutation displayed impaired cognitive function and increased lipid and DNA peroxide levels in the hippocampus (128). Treating these mice with a potent

antioxidant, alpha-tocopherol, improved cognitive function and reduced the accumulation of lipid peroxide and the number of apoptotic cells (129).

Alzheimer disease (AD) model mice reported that klotho upregulation in the brain reversed aging-associated memory deficits and oxidative stress damage (82,130). Here, we found that serum klotho protein levels were increased in patients with SZ, and Klotho levels were positively correlated with attention, working memory, verbal memory, and executive capacity in patients, indicating that Klotho dysregulation might contribute to the cognitive impairments in SZ.

Correlation of miRNA 339- 5p with Klotho gene expression and circulatory Klotho protein:

miRNA-339- 5p is shown to be associated with SZ. Moreau MP et al reported that miRNA expression levels were altered in SZ and mostly were underexpressed in SZ. They performed their study on around 435 miRNAs and miR- 339 was also in their study and they showed that this miR was also underexpressed (128). In our study we also found that miR- 339- 5p was underexpressed so this study is in line with study done by Moreau et al. who also reported similar findings. Expression of miRs are unique for genes in study and are found to be changed according to disease (129). Down regulation of miR means that the gene involved in the study might be altered or upregulated due to decreased action of miR.

Klotho protein levels were changed with advancing age i.e decreased with age and this may be due to miR expression changes however there is no study that could explain the changes in klotho protein with age (131–133). In this study we found significant negative correlation of Δ ct values between cases and controls. That means case group has higher Δ Ct than control patients (Table 9, 10).

If we look at two parameters that is Klotho gene expression with miR-339-5p we found that Klotho gene was upregulated and miR was down regulated. The results are in line with the study done by Mehi et al. who performed their study on cell lines and found that there is inverse relation between miR-339-5p with Klotho gene (13). So, it is clear from the result of the study that Klotho gene and miR-339-5 p was in inverse relation as miR-339-5p was downregulated and Klotho gene was upregulated.

Here, in this study we are correlating miR-339-5p expression with ELISA levels of serum Klotho protein and we found that there is a significant negative correlation of miR- 339-5p with Klotho protein levels. We found that miR339- 5p was downregulated and negatively correlated with Klotho protein levels that means with decreasing expression of miR- 339- 5p there is increased Klotho protein levels. Therefore, miR-339-5p expression was inversely related to the Klotho protein levels. Possible mechanism might be that Klotho mRNA is degraded or silenced by miR-339- 5p as in our study this miR was found to be down regulated so it is not affecting mRNA levels of Klotho (Table 11). Our result is aligned with the study done by Mehi et al, they demonstrated that miR-339 if overexpressed it leads to lower Klotho protein levels and they also said that Klotho gene undergoes methylation with advancing age and becomes inactive for expression and results in lower klotho protein levels (13).

CONCLUSION & SUMMARY

SZ is a debilitating psychiatric disorder that can result in hallucinations, delusions and disordered thinking and behaviour. Approximately 1% of the world population is affected by this disorder. The etio-pathogenesis of SZ is quite complex involving the interplay of several environmental and genetic factors. In the past three decades, numerous studies have tried to establish the role of genetic factors in this disorder.

This study was carried out in Department of Biochemistry in collaboration with the Department of Psychiatry of All India Institute of Medical Sciences, Jodhpur for a period of 1 year. The study was approved by the Ethics Committee of the institution. Study included 90 subjects who were recruited from the outpatient department (OPD) of Psychiatry after getting informed consent. Cases were defined as individuals who were diagnosed to be suffering from SZ as per ICD-10 criteria. Controls were defined as healthy individuals who were not suffering from any neuropsychiatric disorders. In this study, association of Klotho with cognitive changes and also association of miR339-5p with Klotho gene and Klotho protein circulatory levels was studied in SZ.

Socio demographic profiles like age, occupation, residence was collected in both cases and controls. For cases, clinical history like age of onset of disease, duration of the disease, family and smoking history was obtained.

Severity of SZ was assessed by GAF and PANSS with the help of psychiatrist. Severity score was correlated with the circulatory levels of Klotho protein. The correlation between Klotho protein levels and PANSS were showing highly significant negative correlation. Klotho protein circulatory levels were assessed with ELISA technique and we found serum Klotho protein levels were increased in SZ cases as compared to healthy controls. We also found higher Klotho protein levels in males as compared to females. While we compared Klotho protein levels with family history, we found lower Klotho protein levels in subjects having positive family

history of SZ. Its levels were also lower in tobacco addict patients. On correlating cognitive tests with Klotho protein, we found positive association of Klotho protein with cognitive performance in a highly significant manner in SZ patients.

In this study we also tested the expression levels miR-339-5p in both the groups and we found miR-339-5p is down regulated in SZ cases. In this study we found significant negative correlation of Δ ct values between cases and controls. We also performed miR-339-5p correlation with Klotho protein levels and we found negative correlation between these two variables. On correlating Klotho gene with miRNA-339-5p we found that there is inverse relation between these two parameters as Klotho gene expression was upregulated and that of miR- 339- 5p was down regulated.

To conclude, the result of this study discloses that serum klotho protein levels were increased and miR-339-5p is down regulated in patients with SZ when compared to healthy controls. The expression levels of Klotho gene were found to be upregulated. Which indicates that it is showing increased expression and its product was also increased. This indicates us that expression of miR- 339-5p is inversely related to serum levels of Klotho protein. Levels of serum klotho were positively associated with cognitive performance in SZ patients. So, these results indicate that anti-aging protein klotho and miR-339-5p may be implicated in the pathogenesis of SZ. The finding of this study provides better understanding of the molecular mechanisms underlying SZ-associated cognitive impairments. Furthermore, given that cognitive deficits are a core feature of SZ and the best predictor of long-term functional outcome for SZ patients, the finding that Klotho protein levels were increasing and miR-339-5p was decreasing were correlated with cognitive impairments, indicates that klotho and miR- 339- 5p could be used as a predictor of functional outcomes in patients with SZ.

FUTURE CONSIDERATIONS

To the best of our knowledge, this is the first study in SZ that evaluates Klotho gene expression, its serum levels and its regulatory miR-339-5p levels. Large cohort studies are warranted to see the role of Klotho protein and miR-339-5 p in SZ patients and to establish current results conclusively. There could be an interference from antipsychotic medications on the patients, therefore study conducted on drug naïve patients can provide better correlation of Klotho expression in newly diagnosed patients and its possible relation with the severity of the disease. Further confirmation from the site of disease such as inclusion of CSF sample can provide better clarity since Klotho is known to be highly expressed in choroid plexus. Additional assessment of Klotho function can be done by measurement of oxidative and neuroinflammatory stress markers (such as Malondialdehyde, Tumor necrosis factor alpha, Interleukins)

REFERENCES

1. Andreasen NC. Schizophrenia: the fundamental questions. Brain Res Brain Res Rev. 2000 Mar;31(2–3):106–12.

2. Hosak L. New findings in the genetics of schizophrenia. World J Psychiatry. 2013 Sep 22;3(3):57–61.

3. Do KQ. [Schizophrenia: genes, environment and neurodevelopment]. Rev Med Suisse. 2013 Sep 18;9(398):1672, 1674–7.

4. Cohen RZ, Gotowiec A, Seeman MV. Duration of pretreatment phases in schizophrenia: women and men. Can J Psychiatry Rev Can Psychiatr. 2000 Aug;45(6):544–7.

5. Wendt FR, Pathak GA, Lencz T, Krystal JH, Gelernter J, Polimanti R. Multivariate genome-wide analysis of education, socioeconomic status and brain phenome. Nat Hum Behav. 2021 Apr;5(4):482–96.

6. Singh S, Kumar A, Agarwal S, Phadke SR, Jaiswal Y. Genetic insight of schizophrenia: past and future perspectives. Gene. 2014 Feb 10;535(2):97–100.

7. Kinoshita M, Numata S, Tajima A, Shimodera S, Ono S, Imamura A, et al. DNA methylation signatures of peripheral leukocytes in schizophrenia. Neuromolecular Med. 2013 Mar;15(1):95–101.

8. Shiraki-Iida T, Aizawa H, Matsumura Y, Sekine S, Iida A, Anazawa H, et al. Structure of the mouse klotho gene and its two transcripts encoding membrane and secreted protein. FEBS Lett. 1998 Mar 6;424(1–2):6–10.

9. Nagai T, Yamada K, Kim H-C, Kim Y-S, Noda Y, Imura A, et al. Cognition impairment in the genetic model of aging klotho gene mutant mice: a role of oxidative stress. FASEB J Off Publ Fed Am Soc Exp Biol. 2003 Jan;17(1):50–2.

10. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, et al. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature. 1997 Nov 6;390(6655):45–51.

11. Duce JA, Podvin S, Hollander W, Kipling D, Rosene DL, Abraham CR. Gene profile analysis implicates Klotho as an important contributor to aging changes in brain white matter of the rhesus monkey. Glia. 2008 Jan 1;56(1):106–17.

12. MicroRNAs: Target Recognition and Regulatory Functions - ScienceDirect [Internet]. [cited 2021 Dec 20]. Available from: https://www.sciencedirect.com/science/article/pii/S0092867409000087

13. Mehi SJ, Maltare A, Abraham CR, King GD. MicroRNA-339 and microRNA-556 regulate Klotho expression in vitro. Age Dordr Neth. 2014 Feb;36(1):141–9.

14. Murthy RS. National Mental Health Survey of India 2015–2016. Indian J Psychiatry. 2017;59(1):21–6.

15. Schizophrenia [Internet]. [cited 2021 Dec 20]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4940219/

16. Schizophrenia is a cognitive illness: time for a change in focus - PubMed [Internet]. [cited 2021 Dec 20]. Available from: https://pubmed.ncbi.nlm.nih.gov/23925787/

17. American Psychiatric Association, American Psychiatric Association, editors. Diagnostic and statistical manual of mental disorders: DSM-5. 5th ed. Washington, D.C: American Psychiatric Association; 2013. 947 p.

18. Schizophrenia Fact sheet [Internet]. www.who.int; 2019. Available from: https://www.who.int/news-room/fact-sheets/detail/schizophrenia

19. Schizophrenia.docx - Schizophrenia Submitted to Fulfill One of English Course Subject Requirements Lectured by Dra Nurhasanah M.Ed Created by Verena |

Course Hero [Internet]. [cited 2021 Dec 20]. Available from: https://www.coursehero.com/file/60607166/Schizophreniadocx/

20. Fusar-Poli P, Meyer-Lindenberg A. Striatal presynaptic dopamine in schizophrenia, part II: meta-analysis of [(18)F/(11)C]-DOPA PET studies. Schizophr Bull. 2013 Jan;39(1):33–42.

21. Howes OD, Kambeitz J, Kim E, Stahl D, Slifstein M, Abi-Dargham A, et al. The nature of dopamine dysfunction in schizophrenia and what this means for treatment. Arch Gen Psychiatry. 2012 Aug;69(8):776–86.

22. Novel Dopamine Therapeutics for Cognitive Deficits in Schizophrenia - PubMed [Internet]. [cited 2021 Dec 28]. Available from: https://pubmed.ncbi.nlm.nih.gov/26946382/

23. Maia TV, Frank MJ. An Integrative Perspective on the Role of Dopamine in Schizophrenia. Biol Psychiatry. 2017 Jan 1;81(1):52–66.

24. Pratt J, Dawson N, Morris BJ, Grent-'t-Jong T, Roux F, Uhlhaas PJ. Thalamo-cortical communication, glutamatergic neurotransmission and neural oscillations: A unique window into the origins of ScZ? Schizophr Res. 2017 Feb;180:4–12.

25. Catts VS, Lai YL, Weickert CS, Weickert TW, Catts SV. A quantitative review of the postmortem evidence for decreased cortical N-methyl-D-aspartate receptor expression levels in schizophrenia: How can we link molecular abnormalities to mismatch negativity deficits? Biol Psychol. 2016 Apr;116:57–67.

26. Michie PT, Malmierca MS, Harms L, Todd J. The neurobiology of MMN and implications for schizophrenia. Biol Psychol. 2016 Apr;116:90–7.

27. Markers of inflammation and stress distinguish subsets of individuals with schizophrenia and bipolar disorder [Internet]. [cited 2021 Dec 28]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3944638/

28. Rao JS, Kim H-W, Harry GJ, Rapoport SI, Reese EA. Increased neuroinflammatory and arachidonic acid cascade markers, and reduced synaptic

proteins, in the postmortem frontal cortex from schizophrenia patients. Schizophr Res. 2013 Jun;147(1):10.1016/j.schres.2013.02.017.

29. Allen AJ, Griss ME, Folley BS, Hawkins KA, Pearlson GD. Endophenotypes in schizophrenia: a selective review. Schizophr Res. 2009 Apr;109(1–3):24–37.

30. Harrison PJ. The neuropathology of schizophrenia. A critical review of the data and their interpretation. Brain J Neurol. 1999 Apr;122 (Pt 4):593–624.

31. Dalmau J, Lancaster E, Martinez-Hernandez E, Rosenfeld MR, Balice-Gordon R. Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis. Lancet Neurol. 2011 Jan;10(1):63–74.

32. Hakak Y, Walker JR, Li C, Wong WH, Davis KL, Buxbaum JD, et al. Genome-wide expression analysis reveals dysregulation of myelination-related genes in chronic schizophrenia. Proc Natl Acad Sci U S A. 2001 Apr 10;98(8):4746–51.

33. Katsel P, Davis KL, Haroutunian V. Variations in myelin and oligodendrocyte-related gene expression across multiple brain regions in schizophrenia: a gene ontology study. Schizophr Res. 2005 Nov 15;79(2–3):157–73.

34. Uranova NA, Vostrikov VM, Orlovskaya DD, Rachmanova VI. Oligodendroglial density in the prefrontal cortex in schizophrenia and mood disorders: a study from the Stanley Neuropathology Consortium. Schizophr Res. 2004 Apr 1;67(2–3):269–75.

35. French HM, Reid M, Mamontov P, Simmons RA, Grinspan JB. Oxidative stress disrupts oligodendrocyte maturation. J Neurosci Res. 2009 Nov 1;87(14):3076–87.

36. Wayback Machine [Internet]. 2014 [cited 2021 Dec 28]. Available from: https://web.archive.org/web/20140420070946/http://www.nice.org.uk/nicemedia/live/14382/66534/66534.pdf

37. RAISE Questions and Answers [Internet]. National Institute of Mental Health (NIMH). [cited 2021 Dec 28]. Available from: https://www.nimh.nih.gov/health/topics/schizophrenia/raise/raise-questions-and-answers

38. Császár N, Kapócs G, Bókkon I. A possible key role of vision in the development of schizophrenia. Rev Neurosci. 2019 May 27;30(4):359–79.

39. Carson, V.B. (2000) Mental Health Nursing The Nurse-Patient Journey. W.B. Saunders, Philadelphia, 423. - References - Scientific Research Publishing [Internet]. [cited 2021 Dec 28]. Available from: https://www.scirp.org/(S(i43dyn45teexjx455qlt3d2q))/reference/ReferencesPapers.aspx?ReferenceID=1702901

40. Adida M, Azorin J-M, Belzeaux R, Fakra E. [Negative symptoms: clinical and psychometric aspects]. L'Encephale. 2015 Dec;41(6 Suppl 1):6S15-17.

41. Longitudinal Studies of Cognition in First Episode Psychosis: A Systematic Review of the Literature - Vasilis P. Bozikas, Christina Andreou, 2011 [Internet]. [cited 2021 Dec 28]. Available from: https://journals.sagepub.com/doi/10.3109/00048674.2010.541418

42. Shah JN, Qureshi SU, Jawaid A, Schulz PE. Is there evidence for late cognitive decline in chronic schizophrenia? Psychiatr Q. 2012 Jun;83(2):127–44.

43. Auditory dysfunction in schizophrenia: integrating clinical and basic features - PubMed [Internet]. [cited 2021 Dec 28]. Available from: https://pubmed.ncbi.nlm.nih.gov/26289573/

44. Megreya AM. Face perception in schizophrenia: a specific deficit. Cognit Neuropsychiatry. 2016 Jan 2;21(1):60–72.

45. Murante T, Cohen CI. Cognitive Functioning in Older Adults With Schizophrenia. FOCUS. 2017 Jan 1;15(1):26–34.

46. Nonsocial and social cognition in schizophrenia: current evidence and future directions - Green - 2019 - World Psychiatry - Wiley Online Library [Internet]. [cited 2021 Dec 28]. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/wps.20624

47. Hanson IM, Seawright A, van Heyningen V. The human BDNF gene maps between FSHB and HVBS1 at the boundary of 11p13-p14. Genomics. 1992 Aug;13 (4):1331–3.

48. Trk receptors: mediators of neurotrophin action - PubMed [Internet]. [cited 2021 Dec 28]. Available from: https://pubmed.ncbi.nlm.nih.gov/11399424/

49. Neurotrophic factors and their receptors - PubMed [Internet]. [cited 2021 Dec 28]. Available from: https://pubmed.ncbi.nlm.nih.gov/7612265/

50. Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, et al. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell. 2003 Jan 24;112(2):257–69.

51. Smith GN, Thornton AE, Lang DJ, Macewan GW, Ehmann TS, Kopala LC, et al. Hippocampal volume and the brain-derived neurotrophic factor Val66Met polymorphism in first episode psychosis. Schizophr Res. 2012 Feb;134(2–3):253–9.

52. p300 Acts as a Transcriptional Coactivator for Mammalian Notch-1 | Molecular and Cellular Biology [Internet]. [cited 2021 Dec 28]. Available from: https://journals.asm.org/doi/10.1128/MCB.21.22.7761-7774.2001

53. Klotho protein: Its role in aging and central nervous system pathology | SpringerLink [Internet]. [cited 2021 Dec 28]. Available from: https://link.springer.com/ article/ 10.1134/S0006297917090024

54. Mutation of the mouse klotho gene leads to a syndrome resembling ageing | Nature [Internet]. [cited 2021 Dec 28]. Available from: https://www.nature.com/articles/ 36285

55. Secreted and Transmembrane αKlotho Isoforms Have Different Spatio-Temporal Profiles in the Brain during Aging and Alzheimer's Disease Progression [Internet]. [cited 2021 Dec 28]. Available from: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0143623

56. Association of human aging with a functional variant of klotho | PNAS [Internet]. [cited 2021 Dec 28]. Available from: https://www.pnas.org/content/99/2/856

57. McCarter JD, Stephen Withers G. Mechanisms of enzymatic glycoside hydrolysis. Curr Opin Struct Biol. 1994 Jan 1;4(6):885–92.

58. Henrissat B, Davies G. Structural and sequence-based classification of glycoside hydrolases. Curr Opin Struct Biol. 1997 Oct 1;7(5):637–44.

59. Anti-aging protein alpha Klotho's molecular structure revealed [Internet]. ScienceDaily. [cited 2021 Dec 28]. Available from: https://www.sciencedaily.com/ releases/2018/03/180329190845.htm

60. The Organ Handling of Soluble Klotho in Humans - FullText - Kidney and Blood Pressure Research 2019, Vol. 44, No. 4 - Karger Publishers [Internet]. [cited 2021 Dec 28]. Available from: https://www.karger.com/Article/Fulltext/501316

61. Gooz M. ADAM-17: the enzyme that does it all. Crit Rev Biochem Mol Biol. 2010 Apr;45(2):146–69.

62. FGF19 subfamily members: FGF19 and FGF21 | SpringerLink [Internet]. [cited 2021 Dec 28]. Available from: https://link.springer.com/article/10.1007/s13105-019-00675-7

63. Secreted αKlotho isoform protects against age-dependent memory deficits | Molecular Psychiatry [Internet]. [cited 2021 Dec 28]. Available from: https://www.nature.com/articles/mp2017211

64. Clinton SM, Glover ME, Maltare A, Laszczyk AM, Mehi SJ, Simmons RK, et al. Expression of klotho mRNA and protein in rat brain parenchyma from early postnatal development into adulthood. Brain Res. 2013 Aug 21;1527:1–14.

65. Li S-A, Watanabe M, Yamada H, Nagai A, Kinuta M, Takei K. Immunohistochemical localization of Klotho protein in brain, kidney, and reproductive organs of mice. Cell Struct Funct. 2004 Dec;29(4):91–9.

66. Xiao N, Zhang Y, Zheng Q, Gu J. Klotho is a serum factor related to human aging. Chin Med J (Engl). 2004 May;117(5):742–7.

67. German DC, Khobahy I, Pastor J, Kuro-O M, Liu X. Nuclear localization of Klotho in brain: an anti-aging protein. Neurobiol Aging. 2012 Jul;33(7):1483.e25-30.

68. King GD, Rosene DL, Abraham CR. Promoter methylation and age-related downregulation of Klotho in rhesus monkey. Age Dordr Neth. 2012 Dec;34(6):1405–19.

69. Development and functions of the choroid plexus–cerebrospinal fluid system | Nature Reviews Neuroscience [Internet]. [cited 2021 Dec 28]. Available from: https://www.nature.com/articles/nrn3921

70. Laszczyk AM, Fox-Quick S, Vo HT, Nettles D, Pugh PC, Overstreet-Wadiche L, et al. Klotho regulates postnatal neurogenesis and protects against age-related spatial memory loss. Neurobiol Aging. 2017 Nov;59:41–54.

71. Chen C-D, Li H, Liang J, Hixson K, Zeldich E, Abraham CR. The anti-aging and tumor suppressor protein Klotho enhances differentiation of a human oligodendrocytic hybrid cell line. J Mol Neurosci MN. 2015 Jan;55(1):76–90.

72. The Antiaging Protein Klotho Enhances Oligodendrocyte Maturation and Myelination of the CNS | Journal of Neuroscience [Internet]. [cited 2021 Dec 28]. Available from: https://www.jneurosci.org/content/33/5/1927

73. Life Extension Factor Klotho Prevents Mortality and Enhances Cognition in hAPP Transgenic Mice | Journal of Neuroscience [Internet]. [cited 2021 Dec 28]. Available from: https://www.jneurosci.org/content/35/6/2358

74. Impaired Regulation of Gonadotropins Leads to the Atrophy of the Female Reproductive System in klotho-Deficient Mice | Endocrinology | Oxford Academic [Internet]. [cited 2021 Dec 28]. Available from: https://academic.oup.com/endo /article/147/1/120/2500138

75. The aging suppressor klotho: a potential regulator of growth hormone secretion | American Journal of Physiology-Endocrinology and Metabolism [Internet]. [cited 2021 Dec 28]. Available from: https://journals.physiology.org/ doi/full/10.1152/ajpendo.00090.2014

76. Reyazuddin M, Azmi SA, Islam N, Rizvi A. Oxidative stress and level of antioxidant enzymes in drug-naive schizophrenics. Indian J Psychiatry. 2014 Oct;56(4):344–9.

77. Zhang XY, Chen D-C, Tan Y-L, Tan S-P, Wang Z-R, Yang F-D, et al. The interplay between BDNF and oxidative stress in chronic schizophrenia. Psychoneu roendocrinology. 2015 Jan;51:201–8.

78. First-episode psychosis is associated with oxidative stress: Effects of short-term antipsychotic treatment - Sarandol - 2015 - Psychiatry and Clinical Neurosciences - Wiley Online Library [Internet]. [cited 2021 Dec 28]. Available from: https://onlinelibrary.wiley.com/doi/10.1111/pcn.12333

79. Analysis of oxidative stress expressed by urinary level of biopyrrins and 8-hydroxydeoxyguanosine in patients with chronic schizophrenia - PubMed [Internet]. [cited 2021 Dec 28]. Available from: https://pubmed. ncbi.nlm. nih.gov/25975810/

80. Dietrich-Muszalska A, Kwiatkowska A. Generation of superoxide anion radicals and platelet glutathione peroxidase activity in patients with schizophrenia. Neuropsychiatr Dis Treat. 2014;10:703–9.

81. Oxidative stress in schizophrenia: a case-control study on the effects on social cognition and neurocognition | BMC Psychiatry | Full Text [Internet]. [cited 2021 Dec 28]. Available from: https://bmcpsychiatry.biomedcentral.com/ articles/10.1186/s12888-014-0268-x

82. Morar B, Badcock JC, Phillips M, Almeida OP, Jablensky A. The longevity gene Klotho is differentially associated with cognition in subtypes of schizophrenia. Schizophr Res. 2018 Mar;193:348–53.

83. Biology of Oligodendrocyte and Myelin in the Mammalian Central Nervous System | Physiological Reviews [Internet]. [cited 2021 Dec 28]. Available from: https://journals.physiology.org/doi/full/10.1152/physrev.2001.81.2.871

84. NG2-Positive Oligodendrocyte Progenitor Cells in Adult Human Brain and Multiple Sclerosis Lesions | Journal of Neuroscience [Internet]. [cited 2021 Dec 28]. Available from: https://www.jneurosci.org/content/20/17/6404

85. PDGFRA/NG2 glia generate myelinating oligodendrocytes and piriform projection neurons in adult mice | Nature Neuroscience [Internet]. [cited 2021 Dec 28]. Available from: https://www.nature.com/articles/nn.2220

86. Synaptic vesicle release regulates myelin sheath number of individual oligodendrocytes in vivo | Nature Neuroscience [Internet]. [cited 2021 Dec 28]. Available from: https://www.nature.com/articles/nn.3991

87. Gibson EM, Purger D, Mount CW, Goldstein AK, Lin GL, Wood LS, et al. Neuronal activity promotes oligodendrogenesis and adaptive myelination in the mammalian brain. Science. 2014 May 2;344(6183):1252304.

88. Bošković M, Vovk T, Kores Plesničar B, Grabnar I. Oxidative stress in schizophrenia. Curr Neuropharmacol. 2011 Jun;9(2):301–12.

89. Asai O, Nakatani K, Tanaka T, Sakan H, Imura A, Yoshimoto S, et al. Decreased renal α-Klotho expression in early diabetic nephropathy in humans and mice and its possible role in urinary calcium excretion. Kidney Int. 2012 Mar 2;81(6):539–47.

90. Choi BH, Kim CG, Lim Y, Lee YH, Shin SY. Transcriptional activation of the human Klotho gene by epidermal growth factor in HEK293 cells; role of Egr-1. Gene. 2010 Jan 15;450(1–2):121–7.

91. Forster RE, Jurutka PW, Hsieh J-C, Haussler CA, Lowmiller CL, Kaneko I, et al. Vitamin D receptor controls expression of the anti-aging klotho gene in mouse and human renal cells. Biochem Biophys Res Commun. 2011 Oct 28;414(3):557–62.

92. Zhang R, Zheng F. PPAR-gamma and aging: one link through klotho? Kidney Int. 2008 Sep;74(6):702–4.

93. Cellular mechanisms underlying acquired epilepsy: The calcium hypothesis of the induction and maintainance of epilepsy [Internet]. [cited 2021 Dec 28]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2819430/

94. Longevity Klotho gene polymorphism and the risk of dementia in older men - PubMed [Internet]. [cited 2021 Dec 28]. Available from: https://pubmed.ncbi.nlm.nih.gov/28539162/

95. Yokoyama JS, Marx G, Brown JA, Bonham LW, Wang D, Coppola G, et al. Systemic klotho is associated with KLOTHO variation and predicts intrinsic cortical connectivity in healthy human aging. Brain Imaging Behav. 2017 Apr;11(2):391–400.

96. de Vries CF, Staff RT, Noble KG, Muetzel RL, Vernooij MW, White T, et al. Klotho gene polymorphism, brain structure and cognition in early-life development. Brain Imaging Behav. 2020 Feb;14(1):213–25.

97. Association of Klotho-VS Heterozygosity With Risk of Alzheimer Disease in Individuals Who Carry APOE4 | Dementia and Cognitive Impairment | JAMA Neurology | JAMA Network [Internet]. [cited 2021 Dec 28]. Available from: https://jamanetwork.com/journals/jamaneurology/fullarticle/2763599

98. Erickson CM, Schultz SA, Oh JM, Darst BF, Ma Y, Norton D, et al. KLOTHO heterozygosity attenuates APOE4-related amyloid burden in preclinical AD. Neurology. 2019 Apr 16;92(16):e1878–89.

99. KLOTHO polymorphisms and age-related outcomes in community-dwelling older subjects: The São Paulo Ageing & Health (SPAH) Study | Scientific Reports [Internet]. [cited 2021 Dec 28]. Available from: https://www.nature.com/articles/s41598-020-65441-y

100. Zhu Z, Xia W, Cui Y, Zeng F, Li Y, Yang Z, et al. Klotho gene polymorphisms are associated with healthy aging and longevity: Evidence from a meta-analysis. Mech Ageing Dev. 2019 Mar;178:33–40.

101. Hao Q, Ding X, Gao L, Yang M, Dong B. G-395A polymorphism in the promoter region of the KLOTHO gene associates with reduced cognitive impairment among the oldest old. Age Dordr Neth. 2016 Feb;38(1):7.

102. Study finds PTSD interacts with klotho gene, may cause premature aging in the brain -- ScienceDaily [Internet]. [cited 2021 Dec 28]. Available from: https://www.sciencedaily. com/releases/2020/10/201026114208.htm

103. Yin S, Zhang Q, Yang J, Lin W, Li Y, Chen F, et al. TGFβ-incurred epigenetic aberrations of miRNA and DNA methyltransferase suppress Klotho and potentiate renal fibrosis. Biochim Biophys Acta Mol Cell Res. 2017 Jul;1864(7):1207–16.

104. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009 Jan 23;136(2):215–33.

105. Metazoan MicroRNAs - PubMed [Internet]. [cited 2021 Dec 28]. Available from: https://pubmed.ncbi.nlm.nih.gov/29570994/

106. Sex differences in social functioning of patients with schizophrenia depending on the age of onset and severity of the disease - Zorkina - 2021 - Early Intervention in Psychiatry - Wiley Online Library [Internet]. [cited 2021 Dec 28]. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/eip.13063

107. Abel KM, Drake R, Goldstein JM. Sex differences in schizophrenia. Int Rev Psychiatry Abingdon Engl. 2010;22(5):417–28.

108. Cocchi A, Lora A, Meneghelli A, La Greca E, Pisano A, Cascio MT, et al. Sex differences in first-episode psychosis and in people at ultra-high risk. Psychiatry Res. 2014 Feb 28;215(2):314–22.

109. The association between early-onset schizophrenia with employment, income, education, and cohabitation status: nationwide study with 35 years of follow-up | SpringerLink [Internet]. [cited 2021 Dec 28]. Available from: https://link.springer.com/article/10.1007/s00127-019-01756-0

110. Chen J, Chen R, Xiang S, Li N, Gao C, Wu C, et al. Cigarette smoking and schizophrenia: Mendelian randomisation study. Br J Psychiatry J Ment Sci. 2021 Feb;218(2):98–103.

111. Evidence of a Causal Relationship Between Smoking Tobacco and Schizophrenia Spectrum Disorders [Internet]. [cited 2021 Dec 28]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6255982/

112. American Psychiatric Association, American Psychiatric Association, editors. Diagnostic and statistical manual of mental disorders: DSM-5. 5th ed. Washington, D.C: American Psychiatric Association; 2013. 947 p.

113. Immonen J, Jääskeläinen E, Korpela H, Miettunen J. Age at onset and the outcomes of schizophrenia: A systematic review and meta-analysis. Early Interv Psychiatry. 2017 Dec;11(6):453–60.

114. GAF.pdf [Internet]. [cited 2021 Dec 28]. Available from: https://www.albany.edu/counseling_center/docs/GAF.pdf

115. Gold LH. DSM-5 and the assessment of functioning: the World Health Organization Disability Assessment Schedule 2.0 (WHODAS 2.0). J Am Acad Psychiatry Law. 2014;42(2):173–81.

116. Kay SR, Fiszbein A, Opler LA. The positive and negative syndrome scale (PANSS) for schizophrenia. Schizophr Bull. 1987;13(2):261–76.

117. Leucht S, Kane JM, Kissling W, Hamann J, Etschel E, Engel RR. What does the PANSS mean? Schizophr Res. 2005 Nov 15;79(2–3):231–8.

118. Xiong J, Zhan J, Luo T, Chen H, Wan Q, Wang Y, et al. Increased Plasma Level of Longevity Protein Klotho as a Potential Indicator of Cognitive Function Preservation in Patients With Schizophrenia. Front Neurosci. 2020;14:610.

119. Gender dependence of serum soluble Klotho in acromegaly - Sze - 2014 - Clinical Endocrinology - Wiley Online Library [Internet]. [cited 2021 Dec 28]. Available from: https://onlinelibrary.wiley.com/doi/10.1111/cen.12385

120. Prather AA, Epel ES, Arenander J, Broestl L, Garay BI, Wang D, et al. Longevity factor klotho and chronic psychological stress. Transl Psychiatry. 2015 Jun;5(6):e585–e585.

121. Klotho, vitamin D and homocysteine levels during acute episode and remission periods in schizophrenia patients: Nordic Journal of Psychiatry: Vol 73, No 3 [Internet]. [cited 2021 Dec 28]. Available from: https://www.tandfonline.com/doi/abs/10.1080/08039488.2019.1582697?journalCode=ipsc20

122. Semba RD, Cappola AR, Sun K, Bandinelli S, Dalal M, Crasto C, et al. Plasma klotho and mortality risk in older community-dwelling adults. J Gerontol A Biol Sci Med Sci. 2011 Jul;66(7):794–800.

123. Impact of cigarette smoking cessation on plasma α-klotho lev... : Medicine [Internet]. [cited 2021 Dec 28]. Available from: https://journals.lww.com/md-journal/Fulltext/2018/08310/Impact_of_cigarette_smoking_cessation_on_plasma.21.aspx

124. Sharma T, Antonova L. Cognitive function in schizophrenia. Deficits, functional consequences, and future treatment. Psychiatr Clin North Am. 2003 Mar;26(1):25–40.

125. Krishnadas R, Moore BP, Nayak A, Patel RR. Relationship of cognitive function in patients with schizophrenia in remission to disability: a cross-sectional study in an Indian sample. Ann Gen Psychiatry. 2007 Jul 30;6(1):19.

126. Vita A, Barlati S. Recovery from schizophrenia: is it possible? Curr Opin Psychiatry. 2018 May;31(3):246–55.

127. Abraham CR, Mullen PC, Tucker-Zhou T, Chen CD, Zeldich E. Klotho Is a Neuroprotective and Cognition-Enhancing Protein. Vitam Horm. 2016;101:215–38.

128. Schizophrenia - The Lancet [Internet]. [cited 2021 Dec 28]. Available from: https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(15)01121-6/fulltext

129. Zhou H-J, Zeng C-Y, Yang T-T, Long F-Y, Kuang X, Du J-R. Lentivirus-mediated klotho up-regulation improves aging-related memory deficits and oxidative stress in senescence-accelerated mouse prone-8 mice. Life Sci. 2018 May 1;200:56–62.

130. Association of human aging with a functional variant of klotho | PNAS [Internet]. [cited 2021 Dec 28]. Available from: https://www.pnas.org/content/99/2/856

131. Moreau MP, Bruse SE, David-Rus R, Buyske S, Brzustowicz LM. Altered microRNA expression profiles in postmortem brain samples from individuals with schizophrenia and bipolar disorder. Biol Psychiatry. 2011 Jan 15;69(2):188–93.

132. Yamamoto M, Clark JD, Pastor JV, Gurnani P, Nandi A, Kurosu H, et al. Regulation of oxidative stress by the anti-aging hormone klotho. J Biol Chem. 2005 Nov 11;280(45):38029–34.

133. Melatonin attenuates memory impairment induced by Klotho gene deficiency via interactive signaling between MT2 receptor, ERK, and Nrf2-related antioxidant potential - PubMed [Internet]. [cited 2021 Dec 28]. Available from: https://pubmed.ncbi.nlm.nih.gov/25550330/

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

No. AIIMS/IEC/2020/2023 **Date: 01/01/2020**

ETHICAL CLEARANCE CERTIFICATE

Certificate Reference Number: **AIIMS/IEC/2019-20/942**

Project title: **"Role of miRNA in Regulation of α-Klotho Gene Expression and its Circulatory Levels in Schizophrenia"**

Nature of Project:	**Research Project**
Submitted as:	**M.D. Dissertation**
Student Name:	**Dr. Amandeep Birdi**
Guide:	**Dr.Dharmveer Yadav**
Co-Guide:	**Dr.Praveen Sharma, Dr.Naresh Nebhinani, Dr.Purvi Purohit, Dr.Mithu Banerjee, Dr.Prasenjit Mitra & Dr. Tanu Gupta**

This is to inform that members of Institutional Ethics Committee (Annexure attached) met on **23-12-2019** and after through consideration accorded its approval on above project. Further, should any other methodology be used, would require separate authorization.

The investigator may therefore commence the research from the date of this certificate, using the reference number indicated above.

Please note that the AIIMS IEC must be informed immediately of:

- Any material change in the conditions or undertakings mentioned in the document.
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The Principal Investigator must report to the AIIMS IEC in the prescribed format, where applicable, bi-annually, and at the end of the project, in respect of ethical compliance.

AIIMS IEC retains the right to withdraw or amend this if:

- Any unethical principle or practices are revealed or suspected
- Relevant information has been withheld or misrepresented

AIIMS IEC shall have an access to any information or data at any time during the course or after completion of the project.

On behalf of Ethics Committee, I wish you success in your research.

Dr. Praveen Sharma
Member secretary
Institutional Ethics Committee
AIIMS,Jodhpur

Enclose:
1. Annexure 1

Basni Phase-2, Jodhpur, Rajasthan-342005, **Website:** www.aiimsjodhpur.edu.in, **Phone:** 0291-2740741 Extn. 3109
Email: ethicscommittee@aiimsjodhpur.edu.in

INFORMED CONSENT

DEPARTMENT OF BIOCHEMISTRY

ALL INDIA INSTITUTE OF MEDICAL SCIENCES, JODHPUR

Name: Age/Gender:

Phone No:

Address:

AUTHORIZATION:

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 take part in this study as outlined in the information sheet and this consent form. I understand that I am free to withdraw from the study at any given time.By signing up this form I have not given up my legal rights.

I have been assured of the fact that the blood drawn for the purpose of 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 is being conducted by the Department of Biochemistry, All India Institute of Medical Sciences, Jodhpur by Dr. Amandeep Birdi

(Signature of the investigator) (signature of the participant / Thumb impression)

Witness 1: Witness 2:

ऑल इंडिया इस्टीटयुट ऑफ मेडिकल साईसिस
जोधपूर , राजस्थान
रोगी सुचना पत्रक

प्रधान शोधकर्ता – अमनदीप बिरदी

अस्पताल - अखिल भारतीय आयुविज्ञान संस्थान , जोधपूर राजस्थान

टेलीफोन - 8146215821

प्रोजेक्ट का शीर्षक - अल्फा -क्लोथो जीन अभिव्यक्ति के विनियमन में सूक्ष्म

की भूमिका और यह सिज़ोफ्रेनिया में परिसंचरण स्तर है: एक पायलट अध्ययन ।

अध्ययन का उद्देश - - अल्फा - क्लोथो जीन अभिव्यक्ति के विनियमन में सूक्ष्म

की भूमिका और यह सिज़ोफ्रेनिया में परिसंचरण स्तर है: एक पायलट अध्ययन ।

आपका हस्ताक्षर का मतलब है ?
पृष्ठ पर हस्ताक्षर का अर्थ है कि आप अध्ययन मे शामिल होने के लिए सहमत है।आपके अपने रेकॉर्ड के साथ रखने के लिए इस रोगी सूचनापत्र की एक प्रतिलिपि प्रदान की जाएगी।

रोगी के सापेक्ष शब्द
मेरे पास सूचना पत्र है और मुझे अध्ययन प्रतिक्रया के लाभ और हानि को बताया गया है।मुझे अध्ययन के बारे मे प्रश्न पुछने का मौका दिया गया है । मैं / मेरा रिश्तेदार अध्यपन मे भाग लेने के लिए तैयार है।

रोगी हस्ताक्षर **रोगी सापेक्ष हस्ताक्षर**

दिनांक

जांचकर्ता शब्द

अध्ययन के उद्देश्य, प्रक्रियाओं, लाभ और हानि रोगी/रोगी के रिश्तेदारों को विस्तार में समझाया गया है। अध्ययन के बारे में सभी जानकारी का खुलासा किया गया है और प्रशन के लिए पर्याप्त अवसर दिया गया है ।

प्रधान जांचकर्ता हस्ताक्षर

साक्षी हस्ताक्षर

दिनांक

आगे की पूछताछ के लिए कृपया निम्नलिखित पते पर संपर्क करें :

अमनदीप बिरदी
छात्र
जैव रसायन विभाग
एम्स जोधपुर
मोबाइल नंबर 8146215821

Patient Information Sheet

Name: Age: Sex:

Address: Education:

Occupation: Socio-Economic Status:

Contact:

PERSONAL HISTORY:

Marital status:

Monthly income:

Smoking History:

GENERAL EXAMINATION:

Height _____ Weight_____

BMI _____ Pulse_____ BP_____

Duration of illness:

Family history of any psychiatric disorder:

Positive and Negative Syndrome Scale (PANSS)

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

P1	Delusions	1	2	3	4	5	6	7
P2	Conceptual disorganization	1	2	3	4	5	6	7
P3	Hallucinatory behavior	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 Positive 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 Negative 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 General Psychopathology Subscale Score								
Total PANSS Score								

ASSESSMENT OFCOGNTIVE FUNCTIONS

PGI memory scale

The PGI-memory scale is one of the tests of PGI Battery of BrainDysfunction (PGI-BBD) developed by Pershad and Verma (1990). The test material contains items for 10 subtests i.e Remote Memory,Recent Memory, Mental Balance, Attention-Concentration, Delayed Recall,Immediate Recall, Verbal Retention for Similar Pairs, Verbal Retention forDissimilar Pairs, Visual Retention and Recognition. It has been standardized with norms for adults between 20 and 45 years.

Bender visual motor gestalt test (BVMGT)

BVMGT is also a part of PGI-BBD developed by Pershan and Verma (1990). It consists of nine geometric designs (numbered A and 1-8) originally developed by Wertheimer to demonstrate the perceptual tendencies to organize visual stimuli into configural wholes .Each design is presented sequentially to the subject whose task is to reproduce them on a blank sheet of paper.

Trail Making Test (TMT A & B)

The TMT is frequently used measure of cognitive functions. TMT Part A assesses the visual scanning and psychomotor speed. TMT B is known for the assessment of executive function (Attention shifting & cognitive flexibility).

StroopColor Word Test

The Stroop test measures response inhibition that can be described as the ease with which a person can shift his or her perceptual set to conform to the changing demands of a situation and suppresses a habitual response in favor of an unusual one. Test consists of 3 pages: Word page (W), Color page(C), Color word page (CW). Each page has 100 items presented in 5 columns of 20 items. All the subjects were given 45 seconds to complete the items on each page. Before initiating the test, it was made sure that child identifies all the colors used in the test and able to name them to rule out the possibility of color blindness. On word page, the subjects were asked to read the words down the columns starting with the first one. Similarly on the color page, the subjects were instructed to name as many colors within 45 seconds. For the color word page, the subjects were asked to name the color of the ink, in which the words are printed in and ignore the word. The numbers of items completed on three pages were obtained as their respective scores. Interference score was obtained by subtracting color (C) scores from color word (CW) scores. Test-retest reliabilities for word, color and color-word were reported to be 0.86, 0.82 and 0 .73 respectively. Numerous studies (including Indian) have been published on the Stroop test because of its sensitivity to measure response inhibition (Spreen& Strauss., 1998).