Study of Oxytocin Receptor Gene Polymorphisms (rs53576, rs2254298) in Patients Suffering from Clinical Depression

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Dedicated To

My Parents

Mr. & Mrs. ARSHAD

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LIST OF ACRONYMS

OXTR	Oxytocin receptor
rs	Reference SNP
SNP	Single Nucleotide Polymorphism
$MgCl_2$	Magnesium Chloride
TAE	Tris Acetate EDTA
EDTA	Ethylene-diamine-tetraacetic Acid
SDS	Sodium Dodecyl Sulphate
µl	Micro-litre
dNTPs	Deoxyribonucleotide Triphosphates

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ABSTRACT

Depression is a chronic stress related disorder. Lifetime prevalence of depression in Pakistan is quite high as compared to other developing countries. Environmental factors and genetic predisposition enhance the risk of depression. One of the genes linked to depression is human oxytocin receptor gene (*OXTR*). The current study focused on two *OXTR* polymorphisms rs53576 and rs2254298. Blood of clinically depressed patients from Pakistan were collected and analyzed through Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP). The result showed no significant association of genotype distribution of one SNP, rs2254298, with clinical depression with p value 0.94 but AA genotype of second SNP, rs53576 (p= 0.04), suggests association with the risk of depression but allelic frequency resulted in no statistically significant association with depression (p= 0.7 and 0.4 respectively). *Insilico* analysis suggests an alteration in transcription factor binding pattern in these two OXTR SNPs. However, these results were obtained from a small sample size (82 patients vs 91 controls). Replication of these findings in larger population size from different ethnic groups is required to confirm these results.

This is the first report about *OXTR* gene polymorphisms and clinical depression from Pakistan.

CHAPTER 1

INTRODUCTION

Depression is a chronic stress-related disorder which has the potential to cause morbidity and mortality. According to Diagnostic and Statistical Manual of Mental Disorders (DSM) depression and anxiety are characterized as mood disorder. Depression is symptomatically identified as constant display of extreme sadness, worthlessness, hopelessness and irritable mood. Symptoms of anxiety disorder may include physical indications, like trembling, diarrhea, sweating, dizziness, muscle tension, faintness, palpitations, fear or feeling of unrest (Weiten, 2007). Major / Clinical depression is identified as an inability to carry out daily chores. It is marked by persistent feelings of sadness and despair along with loss of interest or pleasure in previous sources of joy. Disturbed sleep and appetite patterns, lack of energy, socially withdrawal, low self-esteem and self-blame, delusion of guilt and disease are some of the symptoms of major / clinical depression. Mostly major depression is accompanied by substance abuse or anxiety disorder. Patient showing mild and long-lasting symptoms of depression are diagnosed with dysthymic disorder. Such patients constantly complain and are extreme critical about any situation. According to World Health Organization (2012), depression is a serious concern when it becomes chronic, recurrent and interferes with normal work and social activities and is fourth major disease which has burdened the economy of the world.

Lifetime prevalence of depression in the world is high, ranging from 8-12% in most of the countries (Andrade, *et al.,* 2003). In comparison to other developing countries, Pakistan has

the highest rate of depression; about 25 % in general population (Khalily, 2011). This high percentage of depression in Pakistan may be due to poverty, illiteracy or poor education, unemployment, divorce, over population (Khalily, 2011; Husnain, *et al.,* 2000), physical, sexual or verbal abuse (Karmaliani, *et al.,* 2009) and act of terrorism (Asima, *et al.,* 2012). It is a dilemma that in teenagers, emotional stress, loneliness and low self-esteem are considered normal and remain untreated due to lack of awareness, which may serve as risk factors for depression later in life when exposed to hardship (Munira, 2012).

Pathophysiology of depression is complex and involves multiple factors. It may be triggered by genetics or environmental stimuli. Genomic variations in various genes regulating mood and social behavior such as serotonin, dopamine, and oxytocin etc may contribute towards depression. Environmental factors may include stressors in the form of physical, social, emotional trauma or lack of nutrition, etc. Whether the triggering agent is genetic or environment, in both cases, symptoms of depression occur mainly by alternating the activity of hypothalamic–pituitary–adrenal axis (HPA axis), neuro-degeneracy, immune activation, monoamine deficiency or altered plasticity in different regions of brain (Heim, *et al.,* 2008). HPA axis provides an important neuroendocrine stress response system by adapting to the change to maintain health but the constant exposure to stressors or major event of threat in early life can make HPA axis more sensitive to produce more corticotrophin releasing factor (CRF) and circulating glucocorticoids as a stress response. High responsiveness is further enhanced by disturbed negative feed-back mechanism (Korosi and Baram, 2008). Hyperactivity in HPA axis in turn reduces serotonin neurotransmitter (mood stabilizer) thus causing sad and gloomy mood. Depression also involves depletion of norepinephrine or dopamine in the central nervous system along with reduction in serotonin level which lead to less number of monoamine neurons in the

brain causing reduction in the volume of hippocampus (Herpfer, *et al.,* 2012). In addition, inflammatory cytokines such as IL-1, TNF and IL-6 (Farooq, *et al.,* 2012) along with glucocorticoids and secreted adrenal steroids during stress may induce neuron apoptosis and reduces neurogenesis (Sapolski, 2000).

To cope with stress, there is an important hormone called oxytocin (OT) having synthesis centers in brain regions: paraventricular (PVN) and supraoptic nuclei of hypothalamus. OT functions as centrally and peripherally as after synthesis it is transported to posterior pituitary as well as released in blood (Gimpl and Fahrenholz, 2001). Oxytocin has a well-known function in females for lactation and uterine contraction and a significant role in sperm release in males. But later researches have shown its role as a pro-social hormone. It is found to be responsible for coping with hardship and stress. It decreases the concentration of adrenocorticotropin (ACTH) and cortisol thus reducing the hyperactivity of (HPA) (Neumann, *et al.,* 2000; Heinrich, *et al.,* 2001). Moreover activity of oxytocin is mediated by serotinergic neurons in median raphe nucleus and adrenergic neurons. Thus, it increases the availability of serotonin (mood stabilizer) in body. Oxytocin also helps to cope with stress by increasing neurogenesis but still the exact mechanism of oxytocin in this regard is unknown (Leuner, *et al.,* 2012). Oxytocinergic system is not only involved in antidepressant activity (Heinrichs, *et al.,* 2003) but also associated with social interaction, social support, trust building, emotional empathy, memory, generosity, social recognition, pair bonding, parenting and well-being (Hurlemann, *et al.,* 2010; Zak, *et al.,* 2007).

Oxytocin gene (*OXT*) is present at chromosome 20p13 and its size is less than 1 kilobase. It mediates its activity through a receptor. Todate only one receptor is recognized i.e. oxytocin receptor (OXTR). The oxytocin receptor gene (OXTR) is located at chromosome 3p25.3 and has

a size of 19.2 kilobases. It has 3 introns and 4 exons. The translated protein is composed of 389 amino acid polypeptide and belongs to G-protein coupled receptor family (Rao, *et al.*, 1992).

Aims and Objectives:

Following are the salient objectives of this study.

- To determine genotype and allele frequency of two SNPs (rs53576, rs2254298) in oxytocin receptor gene in adult Pakistani individuals with clinical depression.
- To predict the possible role of these variants in receptor-function through *in-silico* analysis.

CHAPTER 2

REVIEW OF LITERATURE

Oxytocin (OT) is a small neuropeptide which was previously known as "maternal gland" due to its role in lactation and uterine contraction (Pedersen *et al.,* 1982), but in recent years its role as pro-social hormone is widely studied (Hurlemann *et al.,* 2010). Oxytocin and vasopressin, both hormones belong to same family of small neuropeptide. Both are involved in regulating the stress response. Vasopressin is responsible to induces stress while oxytocin acts as anxiolytic. A balance is to be maintained between these two regulatory hormones otherwise they may contribute in pathology of psychotic disorders including stress and depression (Neumann and Landgraf, 2012).

2.1 Oxytocin as Pro-social hormone

Oxytocin plays a very important role in regulating emotions and contributes in healthy interpersonal relationships (Hurlemann, *et al.,* 2010). Zak and his colleagues (2007) reported that nasal administration of 40 IU oxytocin help participants to make generous decisions by splitting their own money with some stranger. They reported that participants were 89% more eager to show generosity despite high risk of losing money as compared to the placebo control. Same results are supposedly expected when oxytocin is naturally produced in our body like, by touching, hugging and by sense of security. It was found that oxytocin reinforce generosity by inducing dopamine release in that part of brain which is related to reward (Liu and Wang, 2003). Further, functional magnetic resonance imaging (FMRI) showed that people who make more charity have increased activity in cingulate cortex , which was found to be dense with OT

receptors and neurons involved in dopamine release (Freedman *et al.,* 2000). Generosity is also associated with empathy, which is defined by understanding the needs of others by facial expressions and emotions. Empathy plays a key role in developing healthy social interaction and oxytocin was found to be associated with empathy (Barrazaa and Zak, 2009; Rodrigues *et al.,* 2009). This association was understood by studying autism, a disorder in which a person is severely socially impaired. He is unable to use language properly and lack emotional intelligence (Hollander *et al.,* 2006). Patients suffering from autism have low oxytocin activity as proved by voxel-based morphometry of people suffering from autism, showed decreased gray matter (containing cell bodies and dendrites) in hypothalamus consisting oxytocin neuron (Kurth *et al.,* 2011).

Oxytocin not only increases the sense of empathy but also develop sense of security and trust. In a study with non-clinical individuals, administration of intranasal oxytocin showed an enhanced feeling of interpersonal trust. OT particularly enhances one's ability to endure social risks while interacting with people (Kosfeld *et al.,* 2005). Lischke and his co-workers (2012) showed that under threatening situation amygdala (dense with oxytocin receptor) activity varies with gender. It was found that threatening faces increase amygdala activity in males. While threatening scenes instead of faces were responsible for increasing amygdala activity in females. This reactivity difference based on gender may be due to hormones such as estrogen and progesterone. Domes *et al.,* (2007) further suggested that OT enhances one's ability to know intention of other person by facial expressions.

In addition to its pro-social function, oxytocin play important role in reproduction. It is essential for maintaining labor. Oxytocin receptors were found to be present in uterus on both myometrial and endometrial epithelial cells "where they mediate contraction and prostaglandin

release, respectively". Not only in females, but also in males, oxytocin receptor plays important role in male reproduction organ and help in sperm release (Bathgate *et al.*,, 1994).

2.2 Widely studied Oxytocin receptor SNPs

As oxytocin plays vital role in healthy social interactions, its availability in body is very important. Up till now only one oxytocin receptor (OXTR) is known. Activity of OXTR receptor may be influenced by single nucleotide polymorphism (SNPs) in its gene. There are many reported SNPs of this gene. One of them is rs237887 which was found to be associated with facial recognition. Oxytocin role in social recognition is conserved although perceiving senses differ, olfaction in rodents to visual/auditory memory in humans. The SNP rs237887 was not found to be associated with the capability of detecting the direction of eye stare (Skuse *et al.,* 2014), while rs9860869 was found to be associated with eye gaze (Dalton *et al.,* 2007). But patients suffering from autism were found to be unable to interpret emotional information from eye region but rely solely on the information from mouth and this is how they socially interact with people (Spezio *et al.,* 2006) and Wang along with his co-workers (2013) associated OXTR SNP rs53576 with autism spectrum disorders (ASDs). They reported that A-allele healthy male carriers have smaller volume of hypothalamus as compared to homozygous G alleles. It was also reported that volume was reduced in patients suffering from autism spectrum disorders than controls. It may be due to the functional disconnection in hypothalamus in A carriers. Local functional connectivity of brain test showed that there is significant role of rs53576 in hypothalamus. Distribution of genotype groups (AA/AG/GG) was not considered significantly different with regard to gender, age or year of education. rs53576 was also found to be linked with non-verbal intelligence. It was found that males with OXTR rs53576 A/A and T/T genotype in rs2228485 exhibited negative impact on overall non-verbal intelligence. Decreased non-verbal

intelligence was apparent in rs53576 but no link was found with social loneliness (Lucht *et al.,* 2009). OXTR SNP rs53576 was also found associated with social support seeking (Kim *et al.,* 2010), Sensitive parenting (Bakermans-Kranenburg and Ijzendoorn.2008) and pro-social temperaments. But rs2228485, located in the promoter region of OXTR gene was found to have no influence on emotional and social loneliness or intelligence (Lucht *et al.,* 2009).

2.3 Early Life Stress as predictor of depression

An important environmental factor which plays a critical role in developing depression in adulthood is early life stress. It is evident from animal models (Deussing, 2006) and human studies (Heim 2001) that early life stress (ELS) may trigger anxiety and depression like symptoms in adulthood. Experience of any trauma before puberty alters HPA axis by inducing long-lasting hyperactivity of corticotropin-releasing factor (CRF) and other neurotransmitters may result in increased stress responsiveness of HPA axis (Heim *et al.,* 2003) within the limits of genetic predisposition (Caspi 2003; Heim *et al.,* 2004). Early life stress may be in the form of death of a close relative, endure physical or emotional abuse, witness violence, poverty, dysfunctional relation between child and parent, surgeries, accident, natural disasters, war or act of terrorism (Heim *et al.,* 2003) parental substance and drug abuse, (Carpenter, 2004) kidnap etc. Studies suggested that not all cases of early life adversity, necessarily lead to depression but still it is an important contributor in developing symptoms of anxiety and depression. It has been shown that stresses in early life especially sexual abuse deregulate HPA axis in a long run (Weiss *et al.,* 1999). Moreover magnetic resonance imaging (MRI) observations showed that more than two adverse childhood events may cause small anterior cingulate cortex and caundate nuclei but does not have an apparent volume changes in amygdala and hippocampus (Cohen *et al.,* 2006).

Early life trauma increases the risk of microstructural integrity especially in white matter of brain (Paul *et al.,* 2008).

Depression affects both the gender but risk of developing depression in females is twice as compared to males due to wide range of fluctuations in female hormones: estrogen and progesterone during different phases of life (Seeman, 1997). One of the important hormones involved in stress coping pathway is oxytocin (Heinrichs *et al.,* 2003) whose activity and receptor binding ability is influenced by estrogen and progesterone. Studies show that estrogen stimulates hypothalamus to release oxytocin and encourage oxytocin to bind with its receptor in the amygdala, while progesterone reduces oxytocin release and binding. This suggestion is further supported by the study which showed that females exhibited more significant alteration in corpus callosum (Paul *et al.,* 2008).

2.4 Oxytocin as an antidepressant

Depression is a mood disorder and hypothalamic–pituitary–adrenal (HPA) axis plays a central role in stress response (Heim *et al.,* 2008). Abnormality in the regulation of this axis is found in different psychiatric disorders for example in melancholic depression, alcoholism, and eating disorders, there is found to be hyperactivity in this axis while reduced activity is associated with posttraumatic stress disorder, idiopathic pain syndromes, and chronic fatigue syndrome. But both are similar in producing high concentration of corticotrophin releasing hormone (CRH) (Ehlert *et al.,* 2001). In addition to increased sensitivity of HPA axis neural plasticity is also negatively affected in depressed patients. Studies have suggested that use of antidepressants regulate signal transduction pathway involving gene expression to promote neuronal plasticity. Moreover it is found that stress alters the structure of hippocampus

particularly CA3 pyramidal and granular cell layer. In granular cell layer cell proliferation is seen to be decreased and degeneration in apical dendrites of CA3 pyramidal neurons. Treatment with antidepressant is found to increase cell proliferation and inhibit atrophy of CA3 neuronal layer (Duman, 2002).

Oxytocin has therapeutic potential in treating mood disorders like anxiety and depression by targeting molecules and processes involved in pathophysiology of depression. Many studies have used oxytocin to check its activity as antidepressant in animals. In a study, mice were administered with oxytocin resulted in reduced immobility time in forced swim test (Cryan and Slattery, 2007) and resulted better in learned helplessness test (Nowakowska *et al.,* 2002), two of the known models for depression. Apart from these models anhedonia is another vital sign of depression. When oxytocin was subcutaneously injected in rats, the result showed that rats were more likely to reside the place of their preference and exhibit state of pleasure (Liberzon *et al.,* 1997). It is evident from above mentioned studies that oxytocin can be used as antidepressant. Javed and his co-workers (1999) suggested that oxytocin might carry out its antidepressant activity through serotonin receptors especially 5-HT1A- and 2A receptors or by renouncing resources by down regulating the activity in amygdala region in human brain (Kirsch, 2005).

2.5 Single Nucleotide polymorphism (SNPs) associated with depression:

OXTR single nucleotide polymorphism rs2254298 and rs53576 have been widely associated with depression in Caucasian population (Costa *et al.,* 2009; Thompson *et al.,* 2011; Saphire *et al.,* 2011; Mcquaid *et al.,* 2013). A study conducted on rs2254298 which suggested that mother having A allele have more chances to transmit depression in offspring. Children even with single A allele is at high risk to develop depression (Thompson *et al.,* 2014). Another study emphasized on the role of rs2254298 in sensitive parenting. It suggested that mothers having

genotype AA/AG showed depressed mood and such are more unlikely to take good care of their toddlers. While mother possessing homozygous G allele showed good care to their toddlers and were highly sensitive to babies cries and their needs (Bakermans-Kranenburg and IJzendoorn, 2008). This type of sensitive parenting is achieved by mind reading of children and ability increases by oxytocin as reported by Domes and his co-workers (2007) that 24 IU oxytocin increased the performance of "reading the mind in the eyes test". It is a test which predicts emotions and needs of other person by studying their region of eyes. This test forms the basis of checking empathy. Furman and his colleagues (2011) contradict the involvement of rs2254298 gene in depression. They say that females homozygous for G allele despite having increased gray matter had small both left and right amygdala volumes than that of carrying A allele. Volume differences based on genotype are also found in dorso-medial anterior cingulate cortex and posterior brain stem.

It was suggested that psychological resources such as self-esteem, optimism and mastery reduce the risk of depression and SNP rs53576 was found to be associated with these psychological resources in a study. Carriers of A allele are shown to have less optimism and self-esteem which are symptoms of depression (Saphire-Bernstein *et al.,* 2011). Healthy relationship between parent and children creates high optimism in children but it is partially moderated by adversities. Those reporting childhood adversities and poor parent-child relationships had less optimistic expectations (Korkeila *et al.,* 2004). Parents with low education level may contribute in making children less optimist. This pessimist attitude may be responsible in stress in teens in socioeconomic setup (Finkelstein *et al.,* 2007). Using Self-rating and Personal Health Questionnaire (PHQ) it was found that in Mardan, semi-urban village in KPK province of Pakistan had high depression rate due to illiteracy, big family, extreme financial crisis (Husain *et*

al., 2000). In addition emotional stress, loneliness and low self-esteem are considered normal in teenagers and remain untreated in Pakistan (Munira, 2012).

Moreover, oxytocin receptor SNP rs53576 genotype moderates negative effects of social and economic stress of terror attacks which may later contribute in developing post-traumatic stress (PTS) symptoms and disturbed daily activities. Adverse social environment make carriers of A allele vulnerable for PTS without taking in account the financial stress. While carriers of homozygous G allele, experience increased PTS only if exposed to combine social and economic stress. Daily functioning is highly effected in females by traumatic social environment while male performance was differentially affected by social and economic stress (Thompson and Holman, 2013).

CHAPTER 3

PATIENTS AND METHODS

3.1. Ethical approval

To conduct this study ethical approval was obtained from Institutional Review Board (IRB) of ASAB- approval number IRB025 and Ethical Committee of Department of Mental Health, Benazir Bhutto Hospital (Rawalpindi General Hospital) located at Murree Road, Rawalpindi. Written informed consent was taken from patients or their guardians and unaffected controls who agreed to participate in this study (copies of sample consent form and IRB letter are given in Appendix-1).

3.2. Recruitment of patients and clinical evaluation

Patients suffering from depression were recruited only after diagnosis by psychiatrist of Benazir Bhutto Hospital. Blood (approximately 3ml) was extracted from 93 depressed patients visiting Benazir Bhutto Hospital and 110 random control subjects. Out of 93 patients, 55 were females and 38 were males. Blood was only drawn from those participants who had signed written informed consent form willingly for research purpose. Name, address, occupation, and other relevant family information of the participants were kept confidential. Special identification numbers were given to blood/DNA samples etc. to maintain the confidentiality of participants.

3.3. Inclusion and exclusion criteria

Following are the inclusion criteria for this study:

- Clinical depressed patients diagnosed by trained psychiatrist / medical specialists were recruited, after written informed consent.
- Patients, in past or currently, taking antidepressant medication for a significant period of time as suggested by psychiatrist.
- Both male and female were considered under study.
- Only one patient from families with multiple affected individuals was recruited.

Following are the exclusion criteria for the current study:

- Chronic diseases like hepatitis, diabetes, cancer etc
- Depressive episode due to bipolar disorder
- Psychotic disorders like schizophrenia.
- Drug induced depression or anxiety
- Anorexia nervosa.
- Substance/ drug abuse
- Current pregnancy
- Estrogen replacement therapy
- less than 6 months postpartum

3.4. Collection of blood sample

Blood samples (3-5ml) were drawn from effected and unaffected individual using 5ml syringes (BD 0.6 mm X 38mm, 23 G x 1 TW). The samples were then collected in 5ml ethylene-diamine-tetra-acetic acid (EDTA) tubes (BD vacutainer TM, Frankin Lakes, New Jersey, USA). EDTA tubes were properly labeled with the codes assign to the participant. The samples were immediately transported to genetic research laboratory at Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad, Pakistan and stored at 4°C until further processing which include DNA extraction and genetic analysis.

3.5. Extraction of human genomic DNA from whole blood

Genomic DNA was extracted following chloroform-phenol method from human blood sample. Four solutions were used for the extraction. Solution A was prepared using sucrose, tris and $MgCl_2$, autoclaved and then triton X100 was added. Solution B was autoclaved and all the solutions were refrigerated at 4°C. Composition of all four solutions is given in Table 3.1.

Phenol-chloroform method

Blood (0.7ml) was taken in a centrifuge tube with equal volume of solution. Centrifuge tube was kept at room temperature for 10 minutes after gently inverting the tube for few times. Supernatant was discarded after centrifugation at 13000 rpm for 1 minute in a centrifuge machine (Spectrafuge 24D Labnet, Edison, New jersey, USA) and nuclear pellet was re-suspended in 400 µl of solution A. Supernatant was discarded after centrifugation at 13000 rpm for 1 minute and nuclear pellet was suspended in 400µl of solution B, 12µl of 20% sodium

dodecyl sulfate (SDS) and 5µl of proteinase K (20mg/ml) and incubated at 37°C overnight. After 24 hours, 500µl of fresh mixture of equal volumes of solution C (Phenol, BDH, England) and solution D (1 volume of isoamyl alcohol and 24 volumes of chloroform) was added in samples and mixed thoroughly. The samples were centrifuged for 10 minutes at 13,000 rpm. As a result, upper layer in the form of aqueous phase was separated and was transferred to a fresh 1.5ml microcentrifuge tube. The same process was repeated after adding equal volume of solution D. Finally 55µl of 3M sodium acetate (pH 6) and equal volume of isopropanol or double volume of absolute alcohol (100% pure ethanol) was added to the isolated aqueous layer. Genomic DNA was precipitated after inverting sample tubes several times and centrifugation at 13000 rpm for 10 minutes. The DNA pellet was washed with 70% ethanol and dried in an incubator for 15-20 minutes set at 37°C. After evaporation of residual ethanol, DNA was dissolved in 100µl of TE [(Tris (pH 8.0, 10mM), EDTA (0.1mM)] buffer. DNA was stored at 4°C for further analysis.

Presence of DNA was confirmed in 1% agarose-TAE gel having 3µl ethidium (stock 1mg/ml), 3µl loading dye (3.9ml glycerol, 500µl 10% (w/v) SDS, 200µl 0.5M EDTA, 0.025g bromophenol blue, make final volume of 10ml by adding autoclaved distilled water). One kb ladder Gene Ruler (Thermo Scientific #SM0313) was used as a reference. Electrophoresis was done at 90 volts for 20-25 minutes. The gel was observed under (ultra-violet) UV trans-illuminator (Biometra, Goettingen, Germany) and photographed by using gel documentation software “Dolphin Doc” (Wealtec Dolphin Doc, Sparks, USA).

3.6. SNPs genotyping

SNPs rs53576 and rs2254298 were amplified using Polymerase Chain Reaction (PCR). Sequence of primers used was selected from the literature review (Costa *et al.,* 2009). Details of selected primers for PCR amplification is given in table 3.2.

The PCR was carried out in 0.2ml PCR tubes (Axygen, California, USA). The PCR reaction mixture was prepared by adding 1µl sample DNA (60 ng), 2µl dNTPs (2mM), 1µl of each forward and reverse primer (5pM), 0.5µl (one unit) Taq DNA polymerase (Invitrogen, USA), 2.5µl 10X buffer (100mM Tris-HCl, pH 8.3, 500mM KCl) and 2 µl $MgCl_2$ (25mM) (Invitrogen, USA) in 15µl PCR water to make final volume 25µl. The resulting mixture was vortexed and centrifuged for few seconds for thorough mixing. The cycling process was carried out on a Thermo-cycler (Applied Biosystem, Foster City, USA).

PCR conditions optimized for amplification were as follows:

a) One cycle for denaturing template DNA at 96°C for 5 minute.

b) Thirty five cycles consisting of denaturation of DNA into single strands at 94°C for 45 seconds, 45 seconds at 60°C for primers annealing to their complementary sequences on either side of the target sequence, 1 minute at 72°C for extension of complementary DNA strands from each primer.

c) Final extension for 5 minutes at 72°C.

The PCR products were observed in 2% agarose-TAE gel, electrophoresis for 30 minutes at 80 volts. 50bp ladder (Thermo Scientific, EU, Lithuania) was used as a reference.

3.7. Restriction Fragment Length Polymorphism (RFLP)

The SNPs located at restriction site can be identified by restriction digestion as any change at restriction site may alter digestion pattern of the PCR product by restriction enzyme. Restriction enzyme cuts DNA into fragments of different sizes which can be observed on agarose gel.

Both SNPs were found to have restriction site for specific restriction enzyme which cuts specifically and only at desired location (NEB cutter).

BamH1 enzyme (thermo scientific, EU, Lithuania) was used to genotype rs53576 SNP. Bam H1works best at 37° C. It recognizes GGATCC Sequence and cuts at G^G (Figure 3.1). Restriction Digestion was carried out in 0.2ml PCR tubes (Biologix, USA) by adding 1µl 10X buff BamH1, Lsp11091 Sgel (Thermo Scientific, EU, Lithuania) 0.5µl BamH1 enzyme, 5µl PCR product and 3.5µl PCR water to make a final volume of 10µl. The reaction was incubated at 37 °C for overnight.

Bsrl restriction enzyme (thermoscientific, EU, Lithuania) was used to genotype rs2254298 SNP. Bsrl enzyme works best at 65° C. It recognizes ACTGG Sequence (Figure 3.2). Restriction Digestion was carried out in 0.2ml PCR tubes (Biologix, USA) by adding 1µl 10X buff B (Thermo Scientific, EU, Lithuania) 0.5µl Bsrl (BseNI) enzyme, 5µl PCR product and 3.5µl PCR water to make a final volume of 10µl. The reaction was incubated at 65 °C for overnight. Expected fragment sizes (bp) after complete digestion is given in Table 3.3.

For gel electrophoresis, 8µl digested reaction was mixed with 3 µl loading dye and carefully loaded in the wells of 3% TAE-agarose gel. 50bp GeneRuler (Thermo Scientific. EU, Lithuania) was run to measure restricted fragments after restriction digestion. Electrophoresis was carried out at 70 Volts for 40 minutes in 1X TAE buffer.

3.8. Statistical Analysis

Statistical analysis was performed by using Graph pad Prism for windows (Version 5.01). Chi-squared Test was used for association analysis and Fisher's Exact Test was performed to further validate the results. Hardy Weinberg Equilibrium (HWE) Test was used to compare observed and expected genotype frequencies in controls to check random mating in selected

population sample. Haplotype distribution was performed, as both SNPs are located on the same chromosome 3, using Chi-squared Test.

3.9. *In-silico* Analysis

We explored any alteration in siRNA binding pattern to the SNP site using siDirect (http://sidirect2.rnai.jp/). We also examined alternative splicing in SNPs using Alternative Splice Site Predictor (ASSP) program. Moreover, to examine the transcription factor binding pattern to the studied SNPs, PROMO-ALGGEN (Prediction of transcription factor binding sites) software was used.

Tables and Figures

Table 3.1. Composition of solutions used in Phenol-chloroform method of extraction of genomic DNA

Solution A	**Solution B**	**Solution C**	**Solution D**
• 0.32M Sucrose • 10mM tris (pH7.5) • 5mM $MgCl_2$ Autoclave and then add triton X-100 1% (v/v)	• 10mM tris (pH7.5) • 400mM NaCl • 2mM EDTA (pH8.0)	• 400µl phenol	• Chloroform: Isoamyl alcohol 24:1

Table 3.2. Properties of Primers for PCR amplification

Sr. No.	**SNP**	**Primer Sequence**	**Melting temp. (°C)**	**Annealing temp. (°C)**	**Product Size (bp)**
1.	**rs53576**	**Forward** 5’-GCCCACCATGCTCTCCACATC-3’	58°C	60°C	340
		Reverse 5’-GCTGGACTCAGGAGGAATAGG GAC-3’	61°C		
2.	**rs2254298**	**Forward** 5’TGAAAGCAGAGGTTGTGTGGAC AGG-3’	59°C	60°C	307
		Reverse 5’-AACGCCCACCCCAGTTTCTTC-3’	56°C		

Table 3.3. Restriction enzymes and fragment sizes

Sr. No.	**SNP**	**Product Size (bp)**	**Restriction Enzyme**	**Fragment Size (bp)**
1.	rs53576	340	BamH1	**GG:** 340 **GA:** 340 + 120 +220 **AA:** 120 + 220
2.	rs2254298	307	Bsr 1	**GG:** 163 +101 +34 + 9 **GA:** 163 +135 +101 +9 **AA:** 163 +135 +9

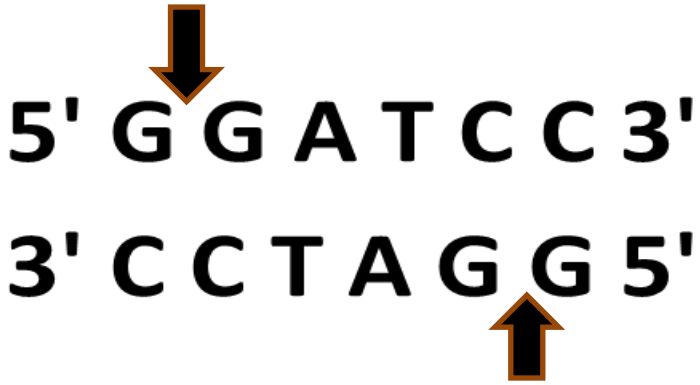

Figure 3.1. Restriction site for BamH1 endonuclease enzyme

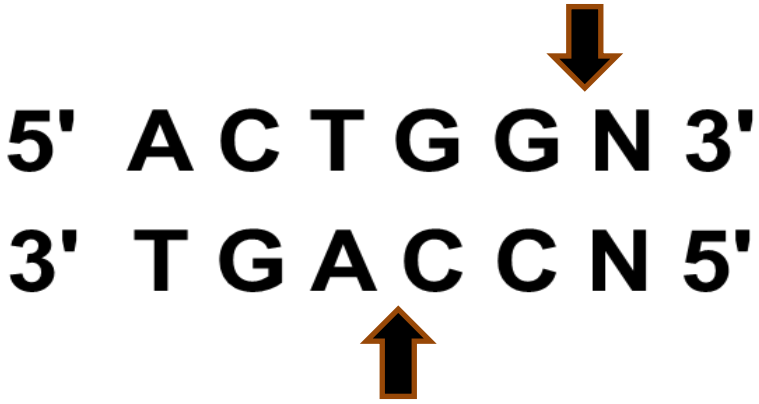

Figure 3.2. Restriction site for Bsrl endonuclease enzyme

CHAPTER 4

RESULTS

4.1. Study Samples

A total of 200 human blood samples were collected. Out of which, 93 had clinical depression and 107 were normal individuals having no past or present exposure to antidepressants. Only those patients were recruited who were diagnosed by a psychiatrist as depressed and were consuming antidepressant. In our study, 57% were given escitalopram (selective serotonin reuptake inhibitor), 15.4% fluoxetine (selective serotonin reuptake inhibitor), 2.3% prothiaden (tricyclic antidepressant), 7.1% paroxetine (selective serotonin reuptake inhibitor), 1.1% mirtazapine (atypical antidepressant with noradrenergic and specific serotonergic activity) and clomipramine (tricyclic antidepressant). 15.4% were given benzodiazepine (anti-anxiety and sedative) along with antidepressant (copy of Patient enrollment form is mentioned in Appendix-1).

Out of 93 patients, only 85 were genotyped, out of which 60% were females and 40% were males (Figure 4.1). In our study, the number of females was 0.6 times more as compared to number of males. This gender biasness was due to the fact that depression is more prevalent in females (Seeman, 1997).

The mean age was 39.47 ± 11.8. Control group had same age and gender. As polymorphism study varies with the population, in our study northern region of Pakistan was included. Our sample comprised mostly of Awans, Rajpoots, Pathans and Gujjars. There were 17.07% Awans, 15.8 % Rajpoots,7.31% Pathans, 6.09% Gujjars and Abbassis, 4.87% Sheikhs

and Mughals, 3.65% Bhattis and Quraishis, 2.43% Araains, Hazaras, Sattis and Suddozais, 1.21% Khawajas, Jatts, Rajas, Sudhans, Khattaks, Khokars, Sawatis, Gills, Chaddhars, Fadoons, Kiyanis, Manghs, Dhanyaals, Bashkaramis, Mirzas and Sardars.

Risk of depression may get influenced by genetic disposition along with environmental stress. In our study, 17.64% had a family history of depression, while 16.47% patients claimed to have no depression in their families. Data was not available for the remaining 65.88% suffering from depression. Environmental stressors which might have triggered sensitivity in the patients under study are demonstrated in figure 4.2. According to the information obtained from patients, loss or sickness of close family members has influenced high number of patients (19.1%), suffering from depression. Second highest environmental stressor was found to be family dispute (14.5 %) and social stress (13%). Very few patients reported that they were emotionally sensitive or had undergone emotional trauma (3.8 %).

4.2. PCR amplification

PCR amplification resulted in 340bp band size for rs53576 And 307bp band size for rs2254298 SNP. PCR products were run on 2% TAE-agarose gel along with 50bp ladder (Fig. 4.3 and 4.4)

4.3. Restriction Fragment Analysis

Both SNPs rs53576 and rs2254298 were digested by BamH1 and Bsrl respectively and run on 3% agarose gel.

In case of rs53576, band size of GG was 340 bp, AG was 340bp + 220bp + 340bp while AA was 220bp + 120bp obtained as expected. Figure 4.5 shows band sizes after treatment with restriction enzyme.

While in case of rs2254298, band sizes for GG genotype (163bp + 101bp), AG (163bp + 135bp + 101bp) while AA (163bp + 135bp) were observed as expected. Figure 4.6 shows band sizes after treatment with restriction enzyme Bsrl.

4.4. Statistical Analysis

Out of 93 patients, 70 individuals were genotyped for SNP rs53576 and 82 were genotyped for SNP rs2254298. Genotype and allele frequency was calculated using traditional counting method.

Hardy Weinberg Equilibrium (HWE) was calculated for the controls belonging to both SNPs (rs53576 & rs2254298). It was done with a purpose to check random mating in controls along with other factors. The observed and expected genotype frequencies are summarized in Table 4.1. Observed genotypes frequencies for polymorphism rs53576 and rs2254298 were not significantly different from the expected genotype frequencies and thus do not follow Hardy Weinberg Equilibrium. The reason might be the small number of controls.

Genotype (GG, GA and AA) distribution among individuals with depression and non-depressed healthy individuals was calculated using 2x3 Chi Squared Test. The test revealed that rs53576 (genotype AA) might be associated with clinical depression with p value 0.04329. But Chi-squared Test in rs2254298 did not show any association with vulnerability to clinical depression in our studied population with p value 0.94812. Genotype distribution among individuals with depression and non-depressed healthy individuals is given in Table 4.2.

When we analyzed genotypes based on presence or absence of A allele as done by Rodrigue *et al*., (2009), by combining rare genotypes like heterozygous (AG) and homozygous (AA) in a same group compared against homozygous (GG) and Chi-squared was applied, the Test suggested that AG/AA genotype for SNP rs53576 may have a role in vulnerability to depression with statistical significant association (p = 0.069). Chi-squared Test for rs53576 and rs2254298 is given in table 4.3.

To further find out whether specific allele is associated with the risk of depression, allele frequency analysis was done for both the SNPs using Chi Square Test, and odd ratios with 95% confidence interval were calculated. The statistical analysis does not show any significant drift between OXTR polymorphism of clinically depressed and healthy controls. The data is summarized in table 4.4. Moreover, Minor Allele Frequency (MAF) was calculated for both SNPs which are summarized in table 4.5.

As both SNPs (rs53576 and rs2254298) are located at chromosome 3 in third intron of oxytocin receptor gene, we performed haplotype distribution to check the probability of inheriting these two SNPs together (Table 4.4). P values show that the probability of inheriting two SNPs on the chromosome 3 is not statistically significant. So it is suggested that these two SNPs of OXTR are not in linkage equilibrium.

4.5. *In-silico* Analysis

In rs2254298, we found that there was no change in siRNA binding pattern specifically to the SNP site when G/A. However we observed that siRNA binds 50bp upstream from the SNP site when A allele is present but that particular binding site is absent in G allele analysis. We found no alteration in splice site pattern when G is altered to A. Transcription factor binding

predictor showed binding of TFIID to both G/A but interferon regulatory factor -1 (IRF-1) and c-Ets-1 binds to the sequence ACTG**G**GAAA and TG**G**GAAA respectively, only when G allele is present.

In case of rs53576, we did not find any alteration in siRNA binding pattern. Moreover no change in splice site pattern was observed when G of rs53576 is altered to A allele. Retinoid X receptor-alpha (RXR-alpha) transcription factor binds to the sequence GG**G**TCCT when G allele is present but in case of A allele RXR-alpha does not bind.

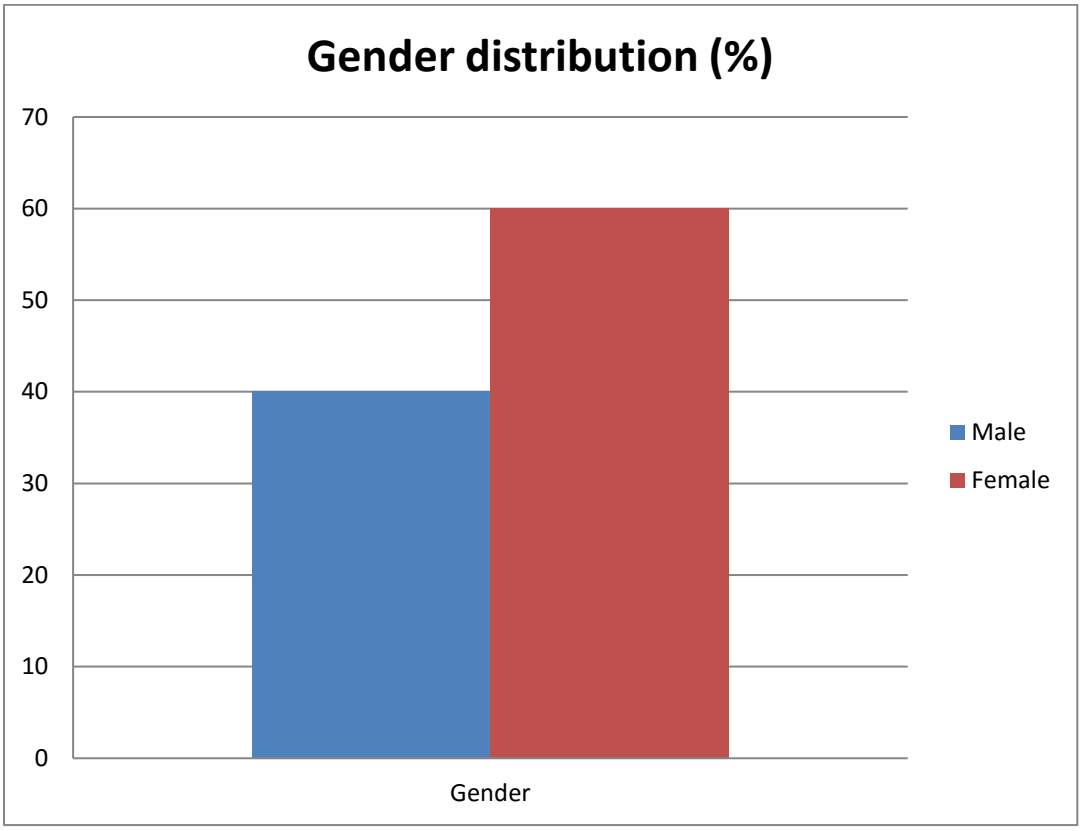

Figure 4.1. Gender distribution in studied sample. Female representation in our study is 0.3 times greater than males. (Females: 60%, Males: 40%)

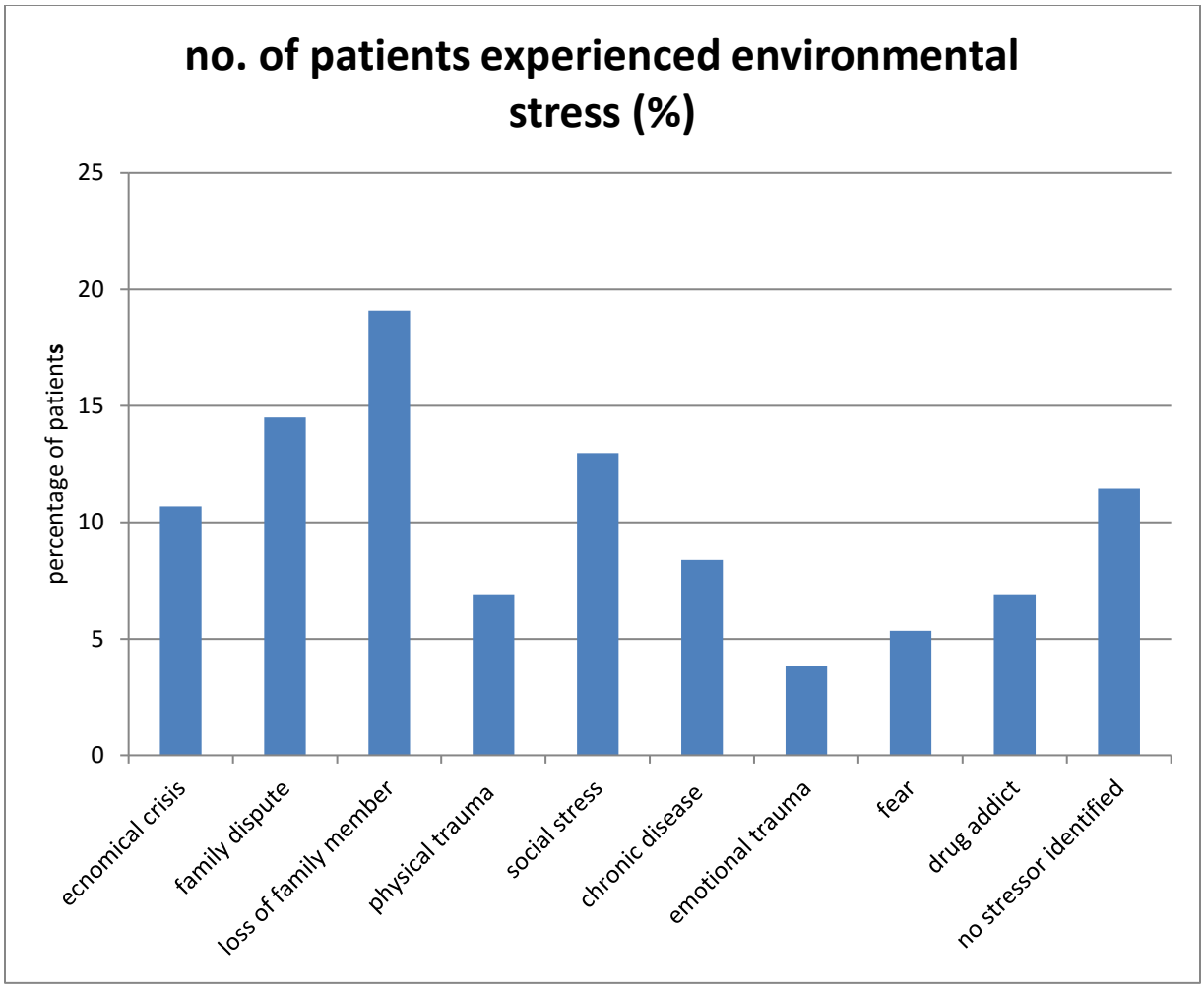

Figure 4.2. Types of environmental stressors in our studied sample. Loss or Sickness in family members was highly experienced environmental trauma in patient suffering from depression

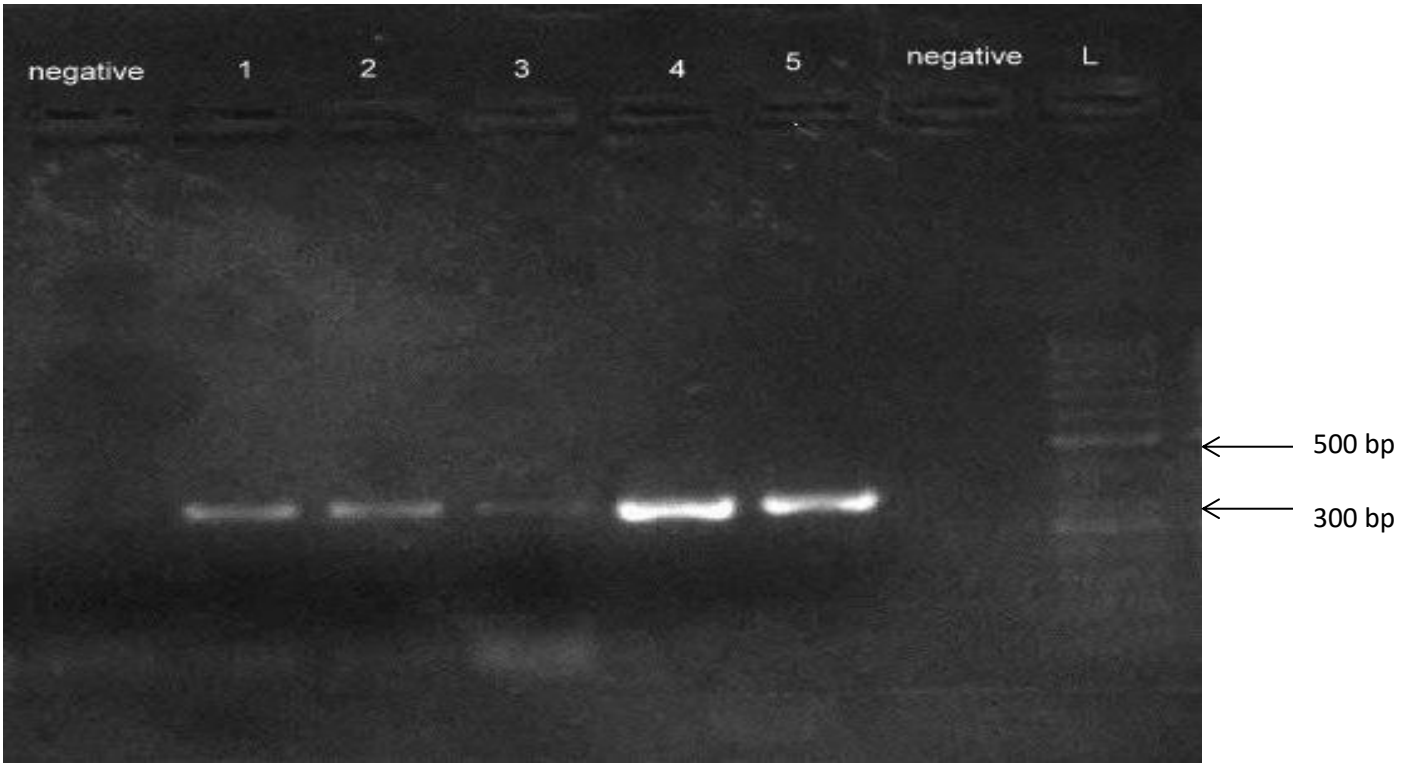

Figure 4.3. PCR amplification of SNP rs53576 run on 2% agarose gel. Size of rs53576 PCR product is 340 bp. Lane 1-5 represents amplicon of rs53576 (size 340bp). Two PCR negative controls were also run. "L" denotes 50bp ladder.

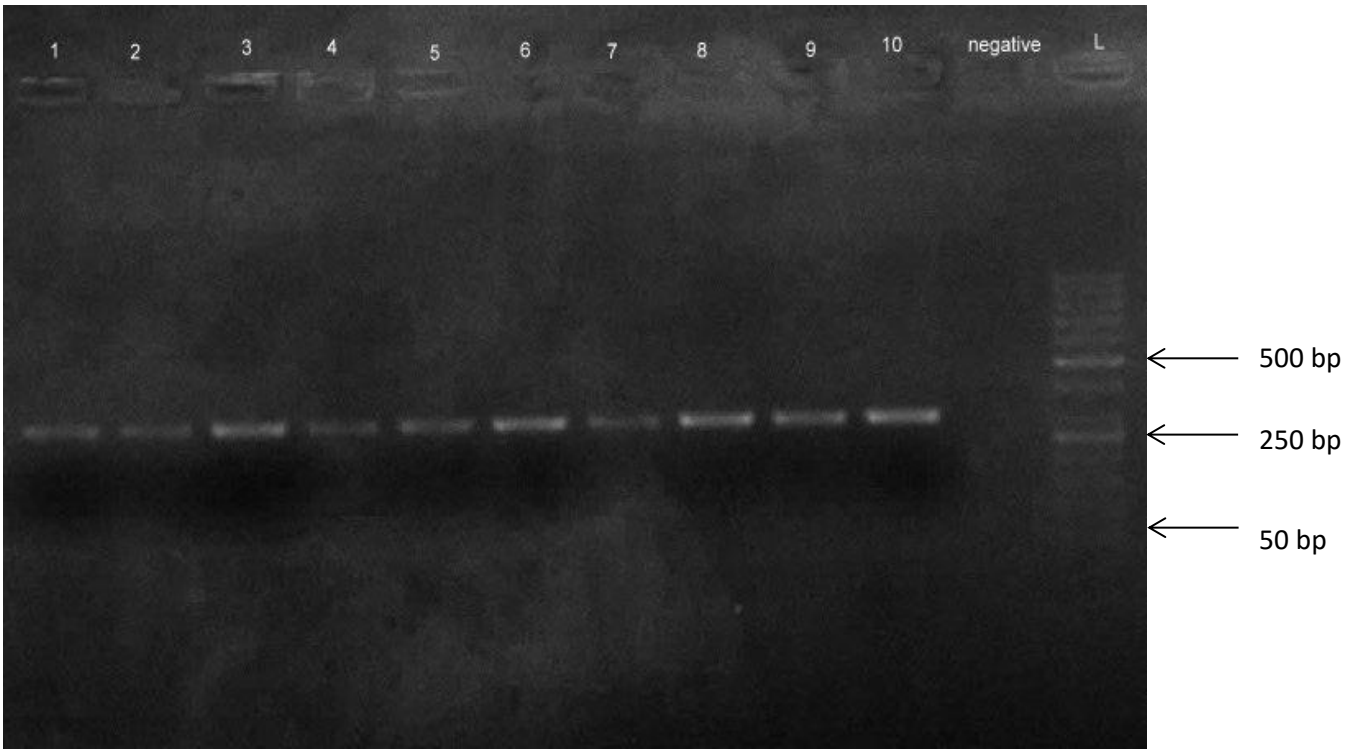

Figure 4.4. PCR amplification of SNP rs2254298 run on 2% agarose gel. Size of amplicon is 307 bp. Lane 1-10 represents amplicon of rs2254298 (size 307bp). PCR negative control was also run. “L” denotes 50bp ladder.

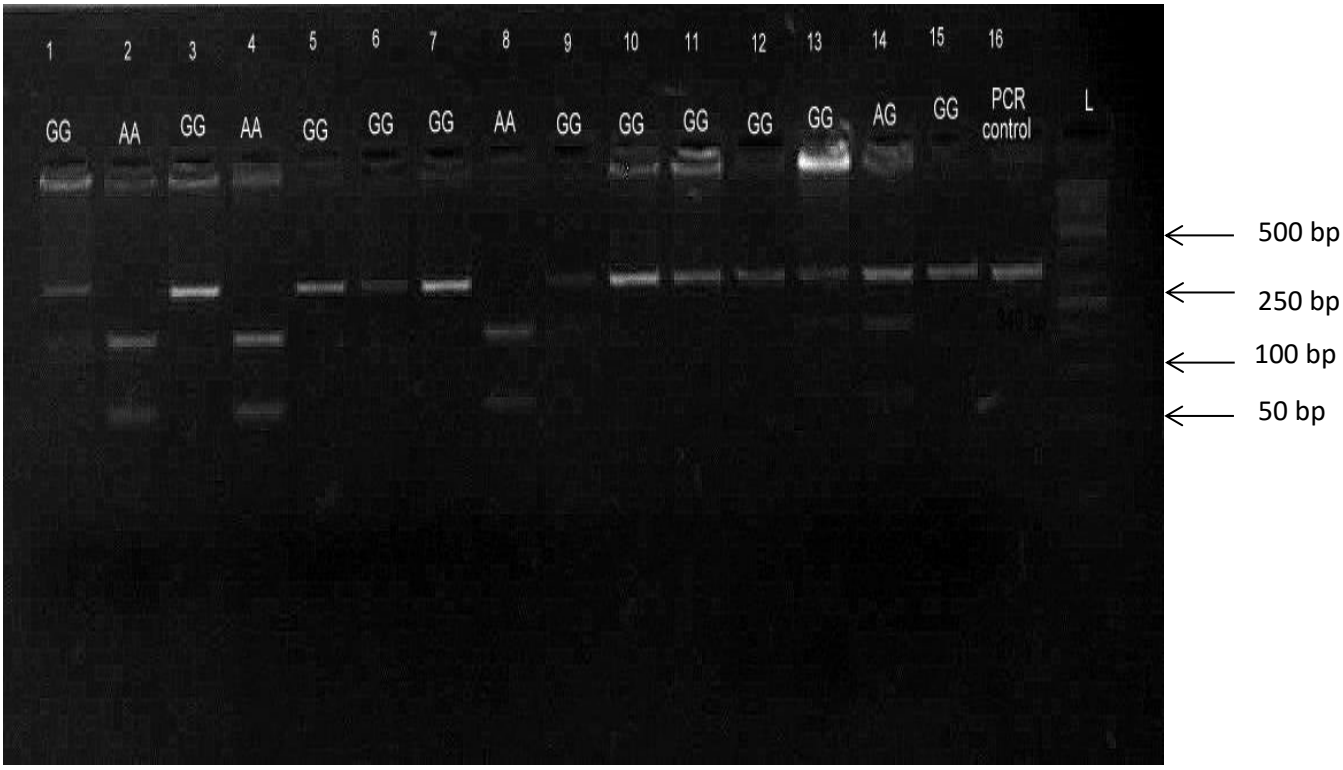

Figure 4.5. Restriction Fragment Length Polymorphism (RFLP) analysis of amplified PCR product of rs53576 subsequently digested by BamH1 and run on 3% agarose gel. Size of PCR control (340 bp), GG (340 bp), AG (340 bp, 220 bp and 120 bp), AA (220 bp and 120 bp). L denotes 50bp ladder, lane 1-15 depicts the result after digestion by enzyme. Lane 16 shows the amplified PCR product for rs53576 SNP

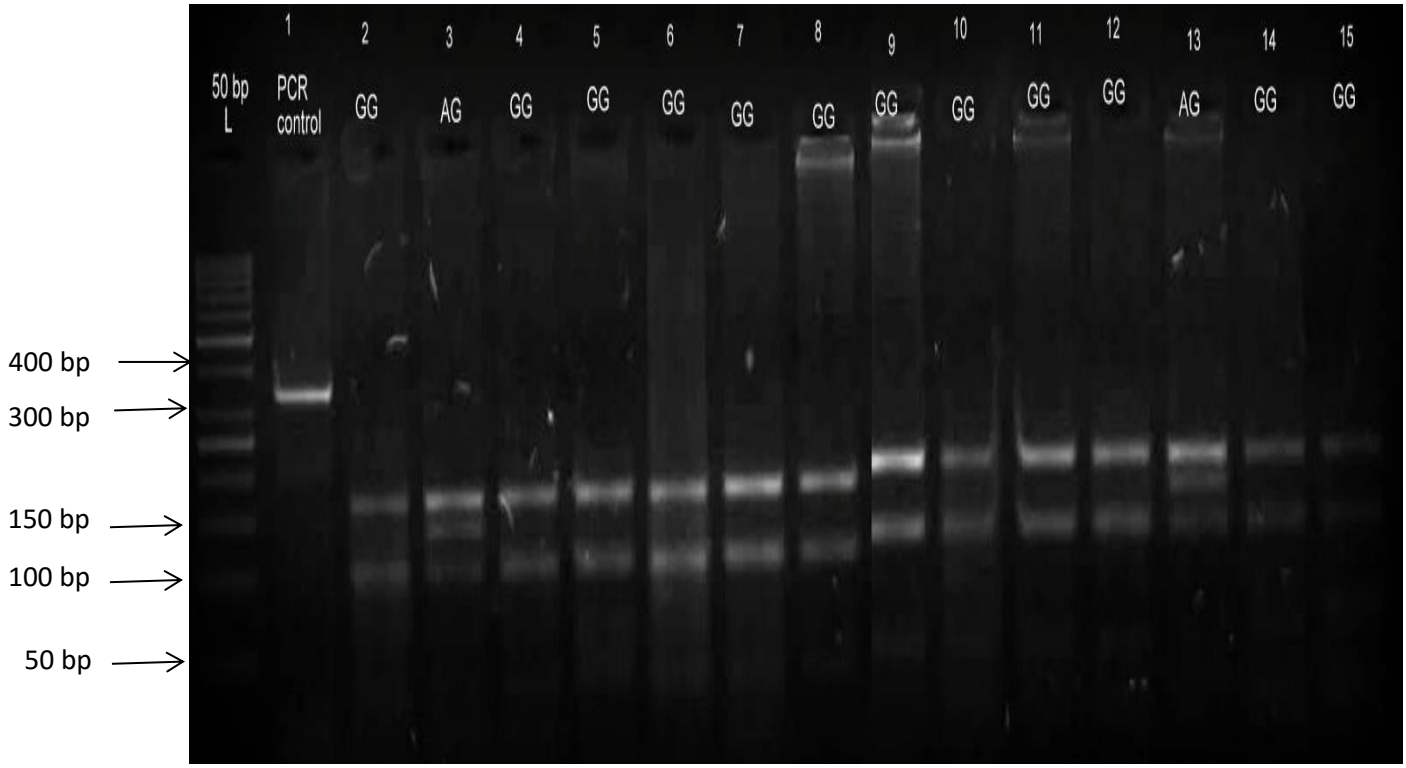

Figure 4.6. Restriction Fragment Length Polymorphism (RFLP) analysis of amplified PCR product of rs2254298 subsequently digested by Bsrl. Size of PCR control (307 bp), GG (163 bp and 101 bp), AG (163 bp, 135 bp and 101 bp). Here "L" denotes 50bp ladder, lane 1 shows undigested amplified PCR product of SNP rs2254298 and lane 2-16 indicates digested PCR product by Bsrl enzyme. Note: This representative gel does not show any individual with homozygous (AA) having 2 bands of sizes 163bp and 135bp.

Table 4.1.Hardy Weinberg Equilibrium calculations of study population for SNP rs53576 and rs2254298

Sr. No	**SNP**	**Observed Genotype Count**			**Expected Genotype Count**			**Chi squared**	**P value (<0.05)**
		GG	GA	AA	GG	GA	AA		
1.	rs53576	21 (28.3%)	44 (59.4%)	9 (12.1%)	24.99 (33.7%)	36.03 (48.7%)	12.99 (17.5%)	3.624	0.05
2.	rs2254298	75 (82.4%)	15 (16.4%)	1 (1.1%)	74.79 (82.1%)	15.41 (16.9%)	0.79 (0.87%)	0.065	0.7987

Table 4.2 OXTR SNPs genotype distribution among clinically depressed and healthy controls

Sr. no.	SNP id		Genotype Count (%)			X^2	P value (<0.05)
			GG	**AG**	**AA**		
1.	**rs53576**	**Cases** n=70	30 (42.8%)	27 (38.5%)	13 (18.5%)	6.2797	0.04329
		Controls n=74	21 (28.3%)	44 (59.4%)	9 (12.1%)		
2.	**rs2254298**	**Cases** n=82	66 (80.4%)	15 (18.2%)	1 (1.2%)	0.1065	0.94812
		Controls n=91	75 (82.4%)	15 (16.4%)	1 (1.09%)		

Table 4.3. OXTR SNPs rs53576 and rs2254298 Genotype (AG+AA and GG) distribution among clinically depressed and healthy control

Sr. no.	**SNP id**		**Genotype Count (%)**		**Chi Square**	**P value (<0.05)**
			AG + AA	**GG**		
1.	**rs53576**	**Cases** n=70	40 (57.14%)	30 (42.85%)	3.297	0.069
		Controls n=74	53 (71.62%)	21 (28.37%)		
2.	**rs2254298**	**Cases** n=82	16 (19.51%)	66 (80.48%)	0.107	0.744
		Controls n=91	16 (17.58%)	75 (82.41%)		

Table 4.4 Allele frequency distribution of OXTR SNPs rs53576 and rs2254298 among clinically depressed and healthy control

Sr. no.	**SNP id**		**Allele Count (%)**		**Chi Square**	**OR (95% CI))**	**P value (<0.05)**
			G	**A**			
1.	**rs53576**	**Cases** n=140	87 (62.14%)	53 (37.85%)	0.488	1.183 (0.718-1.952)	0.485
		Controls n=148	86 (58.10%)	62 (41.89%)			
2.	**rs2254298**	**Cases** n=164	147 (89.63%)	17 (10.36%)	0.102	0.891 (0.451-1.911)	0.749
		Controls n=182	165 (90.65%)	17 (9.34%)			

Table 4.5. Minor allele Frequency of rs53576 and rs2254298 in our studied sample

Sr. No.	SNP (ancestral/derived)	Minor Allele	MAF
1.	rs53576 (G/A)	A	0.4189
2.	rs2254298 (G/A)	A	0.0934

Table 4.6 Distribution of the OXTR SNPs (rs53576 & rs2254298) haplotypes 6930/9073 loci in clinically depressed patients and control subjects.

Sr. No	**Haplotype 6930/ 9073**	**Patients N=63**	**Control N=68**	**Chi square**	**P value**	**OR (95% CI)**
1.	G-G	74 (57.8%)	72 (52.1%)	Reference	___	___
2.	G-A	9 (7.03%)	7 (5.1%)	0.179	0.672	0.799 (0.252-2.505)
3.	A-G	41 (32.0%)	52 (37.6%)	0.991	0.320	1.304 (0.748-2.274)
4.	A-A	4 (3.1%)	7 (5.1%)	0.839	0.360	1.799 (0.447-7.692)

CHAPTER 5

DISCUSSION

In this study, we investigated a relation between oxytocin receptor polymorphism and risk of depression. For this purpose we selected widely studied SNPs rs53576 and rs2254298. We did not find any association of rs2254298 with the risk of depression. However genotype AA in rs53576 was shown to have effect on the vulnerability to depression (P = 0.04). But allelic frequency of rs53576 did not show any association with depression (P = 0.485).

Our results reported here, on one hand, are in contrast with the study conducted on Caucasian population which reported association of these two SNPs with depression (Costa *et al.*, 2009). Similarly Korkeila *et al* (2004), Bakermans and Marinus (2008), Saphire-Bernstein *et al* (2011), Krueger and colleagues (2012), Thompson and Holman (2013), Thompson *et al* (2014) and McInnis *et al* (2015) reported association of *OXTR* SNPs with increased vulnerability to depression.

But on the other hand, the results of our study are consistent with few literatures which showed no association of SNP rs53576 with depression. Further a meta-analysis shows no involvement of *OXTR* SNPs with close emotional relationship (Li *et al*., 2015). Apicella *et al* (2010) were unable to find any significant genetic association between *OXTR* gene and social behavior. No significant association was reported between rs2254298 and attachment security in depressed patients belonging to Caucasian population (Chen *et al*., 2011). In addition no significant associations were found in Caucasians *OXTR* gene polymorphism and psychotic disorder like depression (Haram *et al*., 2015).

Pattern of genotype frequency distribution in rs53576 in our study is similar to that of Germany representing Caucasian population (GG: 47.75%, AG: 39.45%, AA: 12.8%). But genotype frequency in this study is contradictory with respect to Asian population like China (GG: 8.91%, AG: 44.55%, AA: 46.53%) and Koria (GG: 16.42%, AG: 41.04%, AA: 42.54%). Frequency of AA is less in our sample because Caucasians have rather less A allele with reference to rs53576 (Costa *et al.,* 2009). Further research is required to confirm these results.

In our study minor allele frequency of rs53576 (A) and rs2254298 (A) are in accordance with reported minor allele frequency in Swedish population (Apicella *et al*., 2010) but is different from Asian populations. Moreover *Insilico* anaylsis has shown that G allele of rs53576 provides binding site for RXR- alpha transcript which is absent in case of A allele. The reason for the population which associate A allele to depression may be that normally RXR-alpha (Retinoid X receptor-alpha) is a transcriptor factor that regulates genes which encode Thyroid Hormone receptor. But in case of A allele RXR-alpha is underrepresented which may cause hypothyroidism, a condition which induces similar symptoms to that of depression. RXR-alpha also interacts with serotonin but it's interaction with oxytocin as a risk factor in depression is still unknown. But it is clear that lack of RXR-alpha in mice causes depressive symptoms like anhedonia, despair and lack of motor activity (Krzyzosiak *et al*., 2010). We found under-representation of c-Ets-1 transcription faction in rs2254298 A allele. According to Cesari and his colleagues (2004) Ets-1 play might play a role in synaptic plasticity (the ability to learn and adapt in a mature brain. Dwivedi and his colleagues (2001) suggested that Ets might involve in pathophysiology of depression. He stated that Ets phosphorylation was decreased in the brain of depressed suicidal individual. But its role in oxytocin pathway is still unclear. There is a need to study oxytocin and its pathway in a molecular level to completely understand the mechanism of

social and emotional behavior, only then we will be able to understand role of oxytocin as a risk factor for depression.

It should be taken as caution to relate *OXTR* polymorphism with symptoms of depression. Depression is a multifactorial disorder, taking into account environmental and genetic variations. We cannot say that environmental factors included in our study are solely responsible for depression in patients under study because there are multiple genes which may contribute towards the symptoms of depression e.g. polymorphism in Serotonin Transporter (5-HTTLPR) gene (Luo and Shihui, 2014), Brain Derived Neurotrophic Factor (BDNF), Tryptophan Hydroxylase (TPH2) gene present a risk factor for depression (Duncan *et al.*, 2009). Not only may this, but polymorphisms in oxytocin and methylation in *OXTR* also have some contribution towards clinical depression (Reine *et al*., 2015).

There are several limitations of our study which should be taken into account. First, the sample size was not sufficient enough to completely represent the general population of Pakistan. Secondly, a complex disease like depression cannot be genetically represented by relatively small sample size. Thirdly, depression is multifactorial and cannot be solely viewed by two or three polymorphisms. Fourthly, the observed genotype frequencies in control group for rs2254298 SNP analysis did not follow Hardy Weinberg Equilibrium. Fifth, females are underrepresented in control group, which may cause gender biasness.

Conclusion

Depression is a chronic stress-related disorder which has the potential to cause mortality and morbidity. In conclusion, the present study demonstrates no association of rs53576 and rs2254298 OXTR SNPs with clinically depressed patients in our studied sample. However, these results were obtained from a moderate sized sample population. Replication of these findings in larger population with diverse ethnic groups is required to further validate these results. In addition, association of *OXTR* SNPs with depression should be used as a caution because there are contradictory results in history of literature.

Moreover, *insilico* analysis suggests that no alteration in siRNA or alternative splice site pattern was observed when allele G is altered to allele A. Further analysis using more sensitive and accurate tools is required to confirm these findings.

Future Perspective

The limitations in our study should be addressed in future. Moreover these results should be replicated in larger population. Further studies should be carried out to understand the mechanism or pathway through which oxytocin influences social or emotional behavior and leads to depression-like symptoms.

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ELECTRONIC DATA-BASE INFORMATION

- **NCBI (**http://www.ncbi.nlm.nih.gov/gene/5021**)**
- **NEB cutter (**http://nc2.neb.com/NEBcutter2/)
- **SiDirect (**http://sidirect2.rnai.jp/)
- **PROMO-ALGGEN** (Prediction of transcription factor binding sites) (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3)

APPENDIX-1

IRB Approval Letter

ASAB

ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES
NATIONAL UNIVERSITY OF SCIENCES & TECHNOLOGY

Ref.No:25IRB- Date:20April, 2016

IRB APPROVAL LETTER

Project Title: Study of Oxytocin Receptor Gene Polymorphisms (Rs53576, Rs2254298) in Patients Suffering From Clinical Depression

Name of Principal Investigator I: Dr. M. Jawad Hassan

Name of Principal Investigator II: _

Field and Subfield of Project: Biosciences

Duration: 12 Month

Name of the Department: Healthcare Biotechnology, ASAB, NUST

The aforesaid project has been reviewed by Institutional Review Board (IRB) Committee, ASAB, keeping in view the following selection criteria:

- Qualification, Expertise and Scientific Caliber of the Principal Investigators
- Proposed Goals of the Study
- Subject Selection
- Selection Criteria of Subjects
- Informed Consent Process
- Potential Problems
- Research Design and Methods
- Potential Benefits of the Study
- Risks of the Study
- Management of Risks
- Assessment of Risk
- Confidentiality
- Conflict of Interest

The committee thus **APPROVES** the project on **"Study of Oxytocin Receptor Gene Polymorphisms (Rs53576, Rs2254298) In Patients Suffering From Clinical Depression"** on the scales and criterion set by IRB.

Dr. Najam us Sahar Zaidi
Member, IRB
ASAB, NUST

Dr. Muhammad Tahir
Member, IRB
ASAB, NUST

Dr. Hajra Sadia
HoD Research, Head of IRB
ASAB, NUST

Atta-Ur-Rahman School of Applied Biosciences, National University of Sciences & Technology, H-12, Islamabad, Pakistan.
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INFORMED CONSENT FORM

Study of oxytocin receptor gene polymorphisms (rs53576, rs2254298) in patients suffering from clinical depression.

AIMS AND OBJECTIVES
To identify and analyse disease associated gene variants in patients suffering from Clinical depression
SAMPLE COLLECTION
5ml blood sample will be collected from all participants

INFORMED CONSENT

You are being asked to participate in a research study to find the genetic risk of the disease. You will be asked to donate 5ml of blood. This will not cause any physical injury. Your samples will be preserved in the laboratory and will be tested for genetic polymorphism of specific genes involved in the disease. Your identity in this study will be protected. You can terminate your participation at any time in the course of this study. This research project will be carried out solely on a non commercial basis. Your participation is voluntarily. Further, if it becomes necessary, in your interest, counselling will be provided to you. The scientific information will only be shared among the collaborating scientists. The results of the study if novel or of medical interest will be published in scientific journals without disclosing your identity.

اجازت نامہ
مجوزہ تحقیقي منصوبہ آپ میں پائی جانے والي بیماري کي وجوہات معلوم کرنے کے لیئے خالصتا غیر تجارتي بنیادوں پر ترتیب دیا گیا ہے. آپ سے ۵ ملي لیٹر خون کے عطیے کي درخواست کي جاتي ہے. خون دینے سے آپ کو کسي قسم کا درد ي زخم نہیں ہو گا. آپ کي شناخت کو مکمل طور پر صیغۂ راز میں رکھا جائے گا. آپ کا دیا ہوا عطیہ تجربہ گاہ میں محفوظ رکھا جائے گا اور ہم ان خاص جینیاتي تبدیلیوں کا مشاہدہ کرتے ہوئے آپ کي بیماري کو سمجھنے کي کوشش کریں گے. جس سے آپ اور آپ جیسے دوسرے مریضوں کا بہتر علاج ممکن ہو سکے گا. آپ اس تحقیق کے دوران کسي بھي وقت اپني شمولیت سے دستبردار ہو سکتے ہیں. آپ کي شمولیت رضاکارانہ ہے. دوران تحقیق اگر ضروري ہوا تو آپ کو بیماري سے متعلق مشاورت بھي کي جائے گي. حاصل شدہ سائنسي معلومات کا تبادلہ صرف تحقیق میں شامل سائنسدانوں کے مابین کیا جائے گا.

I hereby confirm that I fully understand what has been stated above. I voluntarily donate blood sample from myself / and from my family for research purposes only.

میں تصدیق کرتا/ کرتي ہوں کہ جو کچھ بھي مجھ سے بیان کیا گیا ہے، میں اسے مکمل طور
پر سمجھ گیا/گي ہوں. نیز میں اپنے خون کا نمونہ رضاکارانہ طور پر صرف تحقیق کے لئے بطور عطیہ دیتا/دیتي ہوں مجھے میرے تمام سوالات کے جواب مل گئے ہیں اور في الوقت میرے ذہن میں کوئی اور سوالات نہیں.

Signature/Thumb impression of the patient: ----------

Name: ---------- **Participant ID**----------

Patient Enrollment Form

Patient's ID: ----------

Patient's name: ----------

Father's name: ----------

Age: ----------

Gender: Male/Female

Marital status: single/ married/ divorced

Annual income: ----------

Occupation: ----------

Cast/language: ----------

Address: ----------

Any trauma, family dispute, accident, natural disaster or chronic illness in early years of life: yes/ no

If yes, then describe the event

Is the tragic incident occurred in your close family members Yes/No

Have you witnessed the incident in public/ media Yes/No

Is the above mentioned incident, your personal experience Yes/No

Medication diagnosed:

Researcher name:

Signature: