Study of Arginine Vasopressin Receptor 1-A (*AVPR1A*) Promoter Polymorphisms and Pair Bonding Behavior in Human Males

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Dedicated To

My Parents

Mr. & Mrs. Haji Abdul Ghaffar

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LIST OF ACRONYMS

%	**Percentage**
°C	**Degree Centigrade**
AVPR1A	**Arginine vasopressin Receptor 1A**
AVP	**Arginine vasopressin**
bp	**Base Pair**
CT	**Cytosine-Thiamine**
DNA	**Deoxyribonucleic acid**
dNTP	**Deoxynucleotide Triphosphate**
EDTA	**Ethylenediaminetetraacetic acid**
GATA	**Guanine-Adenine-Thiamine-Adenine**
GT	**Guanine -Thiamine**
HCl	**Hydrochloric Acid**
KCl	**Potassium Chloride**
LINE	**Long Interspersed Nuclear Element**
mg	**Milligram**
mL	**Milliliter**
mM	**Millimolar**
mg/DL	**Milligram per Deci liter**
$MgCl_2$	**Magnesium Chloride**
OT	**Oxytocin**
PAGE	**Polyacrylamide Gel Electrophoresis**
PBS	**Phosphate Saline Buffer**
PCR	**Polymerase Chain Reaction**
Rpm	**Revolutions per minute**
RS	**Repeat Sequences**
SD	**Standard Deviation**
SDS	**Sodium Dodecyl Sulphate**

SEM	**Standard Error of Mean**
TAE	**Tris Acetate EDTA**
TBE	**Tris Borate EDTA**
TE	**Tris EDTA**
TT	**Thiamine-Thiamine**
UV	**Ultra violet**
V1aR	**vasopressin 1 a Receptor**
µg	**Micro Gram**
µL	**Micro Liter**

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Abstract

Behavioral genetics refers to the study of the behavior of an organism due to the effect of their genetic composition and the interaction of genetic makeup and environmental factors. Pair bond behavior has been the focus of many investigations in behavioral neurosciences and genetics. The nonapeptide, Arginine vasopressin (AVP) and its receptor (AVPR1A) have a role in the development of pair bond behavior. For this study, blood samples were taken from monogamous and polygamous males of different ethnic and language groups and their DNA was analyzed for microsatellite (GT) repeat expansion present in the promoter region of *AVPR1A* gene through PCR. Results showed the presence of expanded repeats in 100% individuals of the marital monogamous group, while shortened repeats were present in 41% individuals of the polygamous group. This is one of a kind study and needs a large group for validation of results in future along with identification of major/minor allele for this repeat locus in a general population.

INTRODUCTION

1.1 Behavioral Genetics

Behavioral genetics refers to the study of the behavior of an organism due to the effect on their genetic composition, the interaction of genetics and environmental factors. Until 1990, there were a huge margin in the fields of behavioral neurology i.e. systems neurosciences, behavior genetics, behavior ecology, and psychology of social behavior. To date, the latest findings on behavioral neurosciences and its genetics has filled up this gap up to adequate (Schorscher-Petcu *et al.*, 2009).

Focusing on neural response system, social neurosciences can be divided into either receptive or expressive processes. (Keverne *et al.*, 2004). The question of the elements of behavioral abilities and disabilities commonly refers to as the "nature-nurture" argument. Social neurosciences and genetics have a different perspective in vertebrate animal model, based on their molecular and cellular system or system neurosciences. The behavioral genetic research these days emphases on identifying specific genes that influence behavioral attitudes such as personality, intelligence and disorders i.e. depression, hyperactivity, autism and schizophrenia (Dulac *et al.*, 2003; Insel, 2010).

1.2 Social Behavior and Pair Bonding

Social behaviors i.e. aggression, sexual intercourse, social isolation have been topic of interest of many scientists in behavioral neurosciences and genetics. The neurological basis of pair bond behavior, an attachment between male and female or between same sexes in some cases, is largely unmapped. Due to the huge margin in this research area, the complexity of pair bond behavior can be explained by involving the genetic investigation on sensory

response, memory development, mode simulations, and more delicate aspects of behavior that unexplored or difficult to measure (Dulac *et al.*, 2003).

Social affiliation plays a cardinal role in the existence of an organism, which includes several aspects from persuading a mate, prenatal care, thriving in society and determining our success in the workplace. Abnormal social behavior, such as avoidance, rejection, lack of involvement and pathological trusting have been associated with different neurological or behavioral problems. Humans have also been evolved to rely on pair bond for their continuity. Therefore, various behavioral outcomes of an individual can be witnessed with or without social support. The presence of social support matters in many body functions like it plays its role in better cardiovascular system, reduced psychological stress and is also play its role in healthier immune system function of an individual (Uchino *et al.*, 1997), whereas person facing social isolation is more prone to depression and anxiety and two to three times more prone to death as compared to normal person suffering from same pathologies (Watanabe *et al.*, 2004). It has been suggested that evolutionary development of social brain has largely been influenced by pair-bonding, social attachment and communication (Walum *et al.*, 2008).

In most of the vertebrates, specific receptors in cortical regions of brain have been studied for the transfer of social information, either these are taken by pheromonal/olfactory, audio-vocal, somatosensory, or visual manifestations (Insel, 2010). Social/behavioral organization or patterns vary extensively from within species to between species. A hasty evolution lies in such a range of social behaviors and so the underlying neural substances. (Nair *et al.*, 2006).

1.3 Neuropeptides Involved in Social Behavior and Pair Bond Behavior

About 100 various neuropeptides have been reported in the mammalian brain to date; most of them are produced and secreted out from the hypothalamus as endocrine hormones with their peripheral effects. Neuropeptides usually work as carrier proteins, with a co-peptin that is a glycoprotein, (heptahelical guanine nucleotide-binding protein-coupled receptors (GPCRs) with no defined functionality and their action is very delayed. Among these neuropeptides, nona-peptides (peptide sequence consisting of nine amino acids) are the one of the oldest known neuropeptides having a genetic structure that includes a large precursor protein recognized as neurophysin (Insel, 2010; Koshimizu *et al.*, 2012). These neuropeptides, include oxytocin, arginine vasopressin, the adrenal glucocorticoid, and corticosterone which have been found to be involved in the neural regulation of partner preferences (Carter *et al.*, 1995). Two of these nonapeptides, Oxytocin (OT) and Arginine vasopressin (AVP) have been found expressed in the magnocellular neurons of paraventricular and supraoptic nuclei lobes in hypothalamic region. Oxytocin plays its function in uterine contraction and milk ejection whereas AVP takes part in blood pressure regulation and act as an anti-diuretic hormone. It also acts as a secretagogue of adrenocorticotropic hormone when released or administered peripherally (Nair *et al.*, 2006). Apart from acting as hormone they also play their role as neurotransmitters and neuromodulators in the brain. Oxytocin and agenine vassopressin producing neurons are projected towards diverse areas all over the brain. Targeted cells perceive their hormonal stimuli (mediated by AVP or OT) through three different AVP receptors i.e. $V1_a$, $V1_b$, and V_2, and OTR receptor (Koshimizu *et al.*, 2012). These receptors are found expressed in all over the limbic and autonomic nervous system (Barberis *et al.*, 1992). The anatomical distribution of these receptors propose their involvement in the regulation of social and pair bond behaviors(Witt *et al.*, 1992).

It has been hypothesized that OT and AVP also take part in regulating pair-bond formation. Central infusions of OT has been investigated for its involvement in pair bond formation in the female prairie vole (Cho *et al.*, 1999b; Insel *et al.*, 1995), whereas in male prairie voles, pair bond development was established by intravenous infusion of AVP, even in the absence of mating (Cho *et al.*, 1999a; Winslow *et al.*, 1993). Where the infusion of their antagonists prevented pair-bond formation in both. On another side, no pair bond formation was observed in male montane voles when they were injected with AVP peripherally (Young, 1999), despite of similar peptide distribution in both species (Wang *et al.*, 1996). Autoradiograpic analysis of distribution of these receptors in both species revealed that the brain receptor distribution patterns for the Oxytocin (Insel *et al.*, 1992) and arginine vasopressin receptor 1a (*AVPR1A*) are remarkably different between species (Fig 1.1) (Insel *et al.*, 1994).

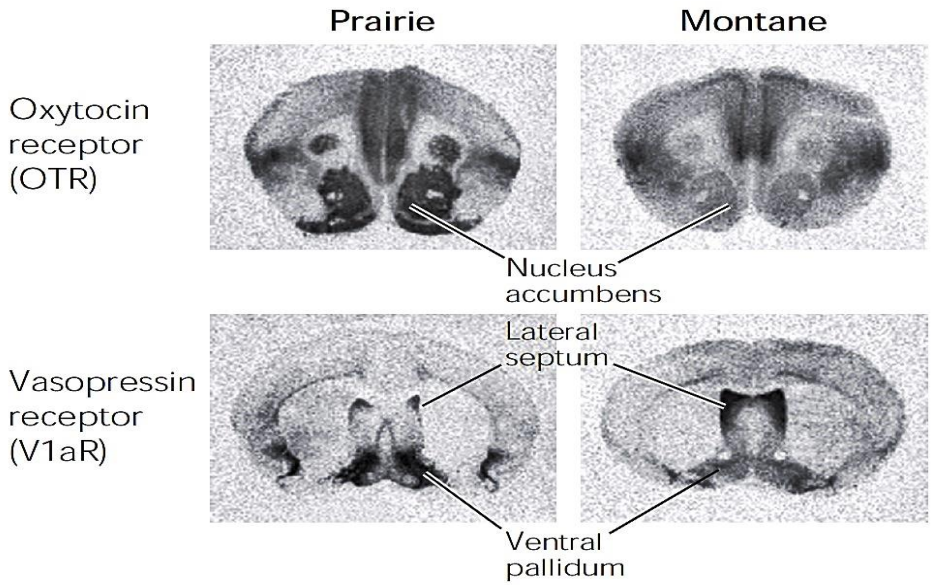

Figure 1.1.: OTR and V1aR receptor distribution patterns differ between prairie and montane voles (Nair *et al.*, 2006)

1.4 Genetic Influence of *AVPR1A* Receptor in Bair Bond Behavior

Arginine vasopressin exerts an imperative influence in boosting pair bond behavior in prairie voles. Polymorphic repeat variation in 5' flanking region of *AVPR1A* gene has been analyzed for its association with pair bond behavior in male microtine voles. However similar pattern of repeat variation and its association with pair bond behavior in human been has not yet been explored. An association of *AVPR1A* repeat polymorphism with the traits affiliation behavior in human males in twin couples and their children has been studies in Sweden, i.e. partner-partener bond, post marital problems, paternal affiliation and marital status. Results revealed ($(CT)_4$-TT-$(CT)_8$-$(GT)_{24}$) repeat polymorphism in in control region of *AVPR1A* gene in males is associated with their marital life eminence alleged by their wives. These findings reveal the influence of AVP on pair-bonding in male prairie voles may resemble with human males (Walum *et al.*, 2008). In another study by (Prichard *et al.*, 2007b), the polymorphism in the *AVPR1A* promotor region has been studied for its association with early age sexual intercourse exposure i.e. before 15 years. Males with the long/long $(TG)x(TC)_y$ repeat polymorphism in control region of *AVPR1A* were more likely to had sexual intercourse before their 15 years of age in comparison to those who had short/long genotypic allele. Similarly, females with the long/long (AGAT) repeat polymorphism in 5' flanking region of their *AVPR1A* gene, were more likely to had sexual intercourse before their 15 years of age than those with any other *AVPR1A* (AGAT) allele.

1.5 Hypothesis

Pair-bond behavior and its role in social attachment and communication have been thought as a key feature in the development of the social brain by an evolutionary process.

We hypothesize that microsatellite repeat polymorphisms present at the promoter region of *AVPR1A* may have an influence on marital pair bond behavior in human males, as it does in somewhat similar fashion in one species of prairie voles.

1.6 Aims and Objectives

The current study has been designed to investigate whether the size variation in the 5′ flanking region of *AVPR1A* is associated with the indicators of sexual pair-bonding behavior in human males; marital monogamy and marital polygamy. The study has been aimed to decipher role of genotypic variations (GT polymorphism) within 5′ flanking region of *AVPR1A* gene in marital monogamous / marital polygamous human males.

REVIEW OF LITERATURE

2.1 Growth of Behavioral Studies

The history of Behavioral Genetics has been derived from the individual histories of the psychology of individual differences, genetics, evolution, anthropology, sociology and some of their neighboring disciplines in multicolored (Gottesman, 2008; Lovejoy, 1981). Human society comprises of family, friends, life partner or spouses and they play a great role in generative support whereas social loneliness or any disruption in social life results in different psychological and behavioral problems (Lim *et al.*, 2006). In a recent analysis on the social organization of primates, it has been revealed that their organization is highly based upon bonded relationships and it is also advocated that it could be a particular demand for social brain development (Walum *et al.*, 2008). Study of the behavioral, ecological and social organization of primates enables us to understand mammalian social systems.

2.2 Animal Models and Social Cognition Studies

Rodent model has been evidenced an authentic model to explicate neurobiological mechanism involved in social behavior and to investigate for evidence at molecular and cellular level. Several rodent models have been studied for different neurological responses towards social discrimination stimuli, affiliation behavior, nonsocial or social avoidance. To substantiate this oxytocin knocked out mice, was designed to understand the context of social stimuli that how brain differentiate social stimuli from nonsocial. Vole species that behave either solitary and unrestrained or highly social and monogamous can be a good model to study neurological mechanisms playing their role in social bonding. Comparative studies in these studies led us finding out two neuropeptides vasopressin and oxytocin that are involved in regulation of communicating behavior and attachment (Young, 2002). Even though in a

general consensus, oxytocin plays its pro-social effects, there is no clear promise on how these effects are achieved. Research on human *OT* gene has been reviewed under three broad research visions i.e. attachment-trust, social memory, and fear reduction (Campbell, 2010).

2.3 Neuropeptides as Neuromodulator

In a rough estimate, about 100 neuropeptides have been identified in mammalian brain, most of them are synthesized and released from the hypothalamic region and act peripherally as an endocrine hormone. In usual these neuropeptides act via G-protein coupled receptor pathway (Insel, 2010). Rapid evolution in social behavior and substances that leads neural changes are thought to be responsible for wide variety of social organization within as well as between the species. Additionally, changes in such neuro substance may can also be arise by polymorphism which affects their expression which ultimately cause the change in social organization which can be perceived from generation to generation studies. There are many social behaviors, i.e. social recognition, communication, parental care, territorial aggression and social bonding are thought to be contributed by AVP and OT (Hammock *et al.*, 2006). The properties of these neuropeptides are thought to be specie-specific, that is entirely reliant on distribution of these two receptors in brain. Comparative studies on two vole species have exposed some of neurogenetic mechanism involved in social behavior. In prairie voles' male and female partners form long term monogamous pair bond and rear their offspring. While meadow voles in contrast, do not show monogamous pair bond behavior and only female bring-up their child. Neurogenetics studies revealed that difference in their social bond behavior is due to difference in density of oxytocin and vasopressin in ventral palladium of brain (Hammock *et al.*, 2006). The identification of AVPR and OTR in the brain and peripheral nervous system was based on the close similarities between their sites, ligand specificity and the kinetics of the hormonal binding. Structural and functional comparison of

AVP and OT receptors revealed that there is no difference between AVP and OT receptors expressed in the central and peripheral nervous system and the same receptors present in liver and uterus. Well characterization and difference in the binding sites of vasopressin and oxytocin receptors even if they are co-localized , they do not offer nonspecific binding opportunity to other components to neurophysin present in vasopressinergic and oxytocinergic fibers. (Barberis *et al.*, 1992).

2.4 Evolutionary Conservativeness of AVP and OT

In non-mammals, neuropeptides and social behavior have been investigated for their evolutionary conservation for social behavior. In pond snail (*Lymnaea stagnalis*), a single neuropeptide lys-conopressin has been examined for its selective expression in neuronal and gonadal nuclei. Lys-conopressin acts via seven-transmembrane G-protein coupled receptors and influence pair bond behavior in males (Van Kesteren *et al.*, 1995). In birds, these neuropeptides have been named as vasotocin and mesotocin. In finches, within species, differential distribution of mesotocin receptor in the lateral septum is associated with their flock size. Administration of mesotocin boost up pair bond formation whereas, its antagonist reduces (Goodson *et al.*, 2009). In bony fish, arginine vasotocin and isotocin have been studied for pair bond affiliation influence. The plainfin midshipman *(Porichthys notatus)*, a vocal teleost fish, their reproductive behavior is awakened by their specific voice known as grunts. Arginine vasotocin, regulates grunts in males whereas isotocin regulates the same in females (Goodson *et al.*, 2000).

2.5 Oxytocin and Vasopressin in Mammals

The principle functions of OT and AVP observed in non-mammal's vertebrates and invertebrates were same as have been observed in rodents. OT and AVP are manufactured in

paraventricular and superaoptic lobes of hypothalamus in brain, whereas AVP also expressed in the suprachiasmatic nuclei in the brain. As neurotransmitter, both peptides are supplied to the posterior hypothalamic lobe via large neurosecretory axons. Later they are transported to their common destination. OT is released in response to sexual stimulation, dilatation uterus, nursing, and in stress. OT receptors present in uterus and mammary glands are responsible of labor pain and milk ejection. Expression of these receptors has been sighted increased in response to the gonadal steroids secreted in the last stages of pregnancy. AVP is released in response to sexual stimulation, uterus dilatation, psychological stress, and dehydration. AVP V_2 receptor expressed in the kidney is responsible of conducting antidiuretic function of AVP, whereas $V1_a$ and $V1_b$ receptors in the vascular tree, adrenal gland, uterus, and other tissues, are responsible of conducting different functions on the stimuli. Neurons expressing OTR and AVPR in the hypothalamus, are projected centrally (Insel, 1997; Kendrick, 2004). AVP has also been reported for its dominant role in memory development and aggressiveness in behavior (Keverne *et al.*, 2004). In non-mammals, OT and AVP have been observed for their species specific response, while in some situations, it is gender specific, in most of them it was dependent on the secretion of gonadal steroids (Choleris *et al.*, 2003). In another study it has been explained that effects of OT and AVP are mediated via complex integrated network of neurons. Social perception processing has also been studied for being influenced by OT and AVP activities (Tobin *et al.*, 2010). AVP has been observed as a key neuropeptide molecule responsible for social and affiliative behavior in mammals. GATA and $(CT)_4$-TT-$(CT)_8$-$(GT)_n$ are the two microsatellite repeat sequences in upstream regions of *AVPR1A*, which have been reported for their involvement in behavioral regulation. These repeat sequences have been analyzed for several behavioral traits and nervous system disorders like autism. In the case of humans, the effect of these variations on the functionality of the brain

is largely unexplored. However, a strong association of these sequence variations has been reported with amygdala functions in humans (Meyer-Lindenberg *et al.*, 2009).

2.6 Oxytocin and Nurturing Behavior

In rats, maternal behavior begins after delivery of their fetus (Numan, 1988). Adult virgin females avoid or attack on unknown males. Centrally injected with OT, a virgin female has been reported for showing maternal care, i.e. building the nest and squatting over pups (Fahrbach *et al.*, 1984; Pedersen *et al.*, 1982). In contrast, antagonist of OT blocked the natural and postnatal maternal care in mice (Fahrbach *et al.*, 1985). Increased expression of OT receptors, effects on uterine and mammary tissues in rats (Insel, 1990). Maternal behavior has been the major focus for OT in mediating its action. An important role of AVP has also been reported in maternal affiliation in rats (Bosch *et al.*, 2008).

2.7 Arginine Vasopressin and Affiliation

To understand neurological basis underlying social and pair bond attachment microtine rodents are the best animals because of close resemblance with other related species. (Carter *et al.*, 1995; Lim *et al.*, 2005). Prairie voles and pine voles behave as monogamous voles live in excavation with their partner forming family while montane voles and meadow voles are abandoned species often living lonely. Such a specific difference in social organization in both groups is an open evidence very early life and its evolution. Prairie vole pups respond to social isolation if they are exposed to ultrasonic calls and increased corticosterone level in their blood circulation, whereas montane vole pups don't. (Shapiro *et al.*, 1990). Male prairie voles exhibited remarkable change in their behavior i.e. they exhibited selective partner preference, aggression towards intruders, paternal care (Carter *et al.*, 1995; Wang *et al.*, 1994). However, these traits were not observed in other vole's species. These observations

suggest that changes induced by therapy and are fundamental to the monogamous social organization in prairie voles reflecting pair bond affiliation for their mate. (Ross *et al.*, 2009).

2.8 Genetic Influence of Repeat Expansion

Tandem Repeats are found in both prokaryotes and eukaryotes; they are found genome wide comprising of different locus on different genes. Repeat expansion in coding/noncoding region can cause many biological problems from mild to swear. Repeat in coding region causes faulty proteins, while repeat expansion in noncoding region can cause changes in gene silencing, enhanced chromosomal brittleness and overall modulation of gene expression (Usdin, 2008).

Sequence analysis of the *avpr1a* loci in montane and prairie voles exposed that, two different expression patterns in the vole species can lead significant differences in their pair bond behavior. (i) The montane voles (polygamous vole) have only one *AVPR1A* loci and no LINE elements. While in prairie voles` genome, two copies of the *AVPR1A* gene were detected. One of those loci has a truncated long interspersed nuclear element (LINE) in 5' flanking region (Young *et al.*, 1999). As LINE sequences cause surrounding DNA to be duplicated and translocated to other regions of genome (Moran *et al.*, 1999). (ii) Difference in the *AVPR1A* locus due to the presence of di and tetra nucleotide repeats (microsatellite repeats) between 720-1,150bp upstream of coding region as shown in (Figure 2.1). These microsatellite repeats were more in prairie voles. It is thought that apart from LINE sequence duplication, the variation in these microsatellite repeat sequence can cause variation in the behavior in these voles (Nair *et al.*, 2006).

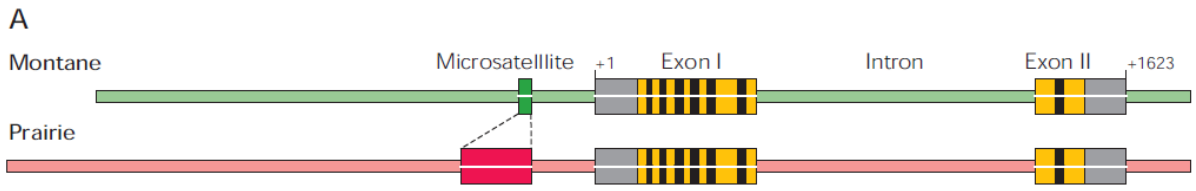

Figure 2.1: The genes for montane and prairie voles share a high degree of homology except for an expanded microsatellite sequence in the 5' flanking region of the prairie vole *Avpr1a* gene.

Genetic influence of infidelity in human is unclear so far, in a study conducted by (Cherkas *et al.*, 2004), it was proved that, number of sexual partners and infidelity influence on human heritable genetics in ratio 38% and 41% , the correlation between these two traits was up to 47% which is very strong.

MATERIALS AND METHODS

3.1 Ethical Approval

Approval to carry out this study was obtained from Institutional Review Board (IRB) of Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad, Pakistan (IRB#027- Appendex.1, 7.1). Written informed consent was obtained from every participant (Appendex.1, 7.2). Proforma to obtain information regarding marriage satisfaction was designed from supporting information Table S1 by (Walum, et al, 2008) and The Couples Satisfaction Index (CSI) by (Funk *et al.*, 2007), approved by local psychometry specialist after quality test and was presented to volunteer participants who agreed to participate in the present study (Appendix.1, 7.3).

3.2 Recruitment of Participants for Studies

In the current study, two groups of volunteer male participants were recruited from a random population of different language groups residing in Gujranwala Division of Punjab province of Pakistan. (Table. 3.1).

3.2.1 Group 1- Human males living their marital life with one female partner since last 10 years (Marital Monogamous human male) were included in this group and this group was categorized as a monogamous group (Group 1). The information was collected in one session that includes interviews, consent and blood collection for genetic analysis.

3.2.2 Group 2- Human males living their marital life with more than one life partners (Marital Polygamous human male) were included in this group and this group was categorized as polygamous group (Group 2). The information was collected in one session that includes interviews, consent and blood collection for genetic analysis.

A person who married again due to the death of his first wife and a person who has divorced his first wife and got married again were placed in group 2.

3.3 Exclusion Criteria

- Unmarried adults
- Males taking anxiolytic/antidepressant drugs or having any behavioral problems
- An individual with any chronic disease including HCV, HIV or cancer.
- An individual not willing to give written consent.

3.4 Collection of Blood Samples

Blood samples were collected from volunteer participants by 10mL syringes (0.8 X 38 mm 21G x 11/2) in standard potassium EDTA vacutainer tubes (BD, USA). The blood samples were processed further for extraction of genomic DNA at Healthcare Biotechnology Department in Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad, and were immediately processed.

3.5 Extraction of Human Genomic DNA from Whole Blood

Genomic DNA from venous blood was extracted in accordance with standard Phenol-chloroform method (Sambrook *et al.*, 2006). To extract genomic DNA, 0.75mL of venous blood was taken in a 1.5mL microcentrifuge tube (Biologix, USA) and mixed with the equal volume of solution A [10mM Tris of pH 7.5 (Invitrogen, USA), 0.32M Sucrose (USB Corporation, USA), 5mM $MgCl_2$ (USB Corporation, USA), 1% v/v Triton X-100 (Company)] and was kept at room temperature for 10-15 minutes. For thorough mixing, tubes were inverted several times and were centrifuged at 13,000 rpm for 1 minute in a centrifuge (Labnet Spectrafuge 24D, USA). Supernatant was discarded and the pellet was re-suspended in 400µL

of solution A and centrifuged once more as described above. In the next step, the nuclear pellet was re-suspended in 400µL of solution B [10mM Tris pH 7.5, 2mM EDTA of pH 8.0], 12µL of 20% SDS solution and 5µL of proteinase K (20mg/mL) and incubated at 65°C for three hours. Following three hours, 500µL of freshly mixed equivalent volumes of solution C (Phenol, BDH, England) and solution D (1 volume of isoamyl alcohol and 24 volumes of chloroform) were mixed in samples and inverted several times. After shaking, samples were centrifuged at 13000 rpm for 10 minutes. Thus resulted upper layer in the form of transparent aqueous phase was separated and it was transferred to another autoclaved 1.5 ml microcentrifuge tube and equal amount of solution D was added and centrifugation was done as previous. After the transfer of transparent aqueous layer second time into another autoclaved microcentrifuge tube, 500 µL absolute ethanol and 55µL of sodium acetate (3M, pH 6) were added. DNA was precipitated by inverting tubes several times and centrifugation was done at 13000 rpm for 10 minutes to get DNA pellet. The DNA pellet was washed with 70% ethanol and dried in the vacuum concentrator 5301 (Eppendorf, Germany) for 5-10 minutes. After vanishing of deposit ethanol, DNA was dissolved in suitable amount (150-200µL) of Tris EDTA [(Tris (pH 8.0, 10mM), EDTA (0.1mM)] buffer and was evaluated by Nanodrop-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) by taking optical density (OD) at 260 nm.

3.6 Primer Designing

Primers were designed aiming to study the variations in 5` promoter region of *AVPR1A* that includes GT dinucleotide repeat. For this purpose, upstream region of *AVPR1A* gene was taken using UCSC genome browser and with the help of primer designing software, MethPrimer, primers were designed to amplify this region (Li and Dahiya, 2002).

3.7 PCR Amplification of *AVPR1A* promoter region

Polymerase chain reaction was performed to amplify candidate region, in a 200µL tube (Biolgix 0.2mL PCR tubes, USA). The PCR reaction master mixture was prepared by adding 1µl sample DNA (40 ng), 2µl of dNTPs (2mM), 1.0µl of each forward and reverse primer (0.5µM), one unit of Taq DNA polymerase (Thermo-scientific, USA), 2.5µl 10X buffer (100mM Tris-HCl, pH 8.3, 500mM KCl) and 2.0µl $MgCl_2$ (25mM) (Thermoscientific USA) in appropriate amount of nuclease free water to make final volume 25µl. The resulting mixture was vortexed and centrifuged for few seconds for thorough mixing. The PCR tubes were placed in thermal cycler (T100™ Thermal Cycler Bio-Rad USA) using following standard conditions for amplification.

a) For initial denaturation of the template DNA, PCR machine was set at 96°C for 5 minute.

b) Then 30 cycles of amplicon synthesis were set each comprises of 3 further steps, denaturation of DNA into single strands at 96°C for 60 seconds, then primer annealing for 60 seconds at 57°C and 60 seconds at 72°C for extension of complementary DNA strands from each primer.

c) Final extension for 10 minutes at 72°C.

3.8 Agarose Gel Electrophoresis

The amplified products were resolved on 2% (2g of agarose in 100mL 1X Tris-Acetate EDTA TAE buffer) ethidium bromide stained agarose gel. For this purpose, 5 µl of amplified product was mixed with 2µl of tracking dye (0.25% bromophenol blue and 40% sucrose) and loaded into the wells. Electrophoresis was performed at 100 volts for 20-25 minutes and the

gel was placed on a UV Transilluminator (UV Transilluminator MD-25/HD-25, USA) and Dolphin-Doc Plus viewer for visualization.

3.9 Polyacrylamide Gel Electrophoresis (PAGE) by Ethidium Bromide Staining

Amplified product was resolved on 8% non-denaturing polyacrylamide gel. Eight (08) % PAGE solution was prepared by mixing 5mL 10X TBE (Tris0.89 M, Borate 0.89M, EDTA 0.02M), 17.5 l TEMED (N,N, N', N'-Tetra methylethylenediamine) (Sigma-Aldrich MO, USA), 350µl 10% Ammonium per sulphate (Sigma-Aldrich MO, USA), 13.5mL 30% acrylamide solution [29g acrylamide (MERCK, Germany) and 1g N, N' Methylene-bisacrylamide (BDH, England)] in 31.13mL nucleases free water to make final volume 50mL. Gel casting apparatus was made of two glass plates tightly packed by placing one horizontal and two vertical spacers of 1.5mm thickness at margins. Gel solution was poured in between the plates carefully by avoiding air bubbles and was allowed to polymerize for half an hour at room temperature. Gel plates were inserted in vertical gel electrophoresis apparatus (Whatman, Biometra, Germany) containing 1X TBE buffer. PCR products mixed with tracking dye were loaded into the wells and electrophoresis was performed at 100 volts for approximately 3 hours depending on the size of each amplicon. At last, ethidium bromide (10µg/mL) stained gels were placed on UV trans-illuminator for visualization and images were taken by Digital camera DC 290 (Kodak, Digital Sciences, USA) and analyzed to score size variation of alleles for group 1 and group 2 individuals. Association to known locus point was established based on the observation that in 41 individuals of group 2, characteristic double bands on PAGE resolution for repeat allele were observed.

Table 3.1: Sequences of primers for amplification of 5` promoter region of *AVPR1A* gene in Group-1 and Group-2

No	Primer Name	Sequence			Ta	Product Size
1	*AVPR1A*-F	5	TGTCAGACAAAACGCTGTTC	3	57	228 bp
2	*AVPR1A*-R	5	TGTGGCTTTAAAAGTTATCCAG	3	57	

RESULTS

4.1 Study Participants

In this study 197 blood specimens were collected from volunteer male participants having no chronic disease or disease history. Out of the 197 samples, 99 of them were polygamous who married more than one woman in their lives and the remaining 98 were those who married only one woman.

4.2 Demographic Characteristics

Group 1 donors were recruited from different regions of division Gujranwala, Punjab, Pakistan. The majority of contributors belonged to Punjabi and Urdu language group. Most of them belonged to age group 40 or above and mean CSI of this group was 59.4.

Participants of the group 2 were recruited from the same region as described above. The majority of men were from Punjabi, Urdu speaking while few of them were from Saraiki, Pushto, and Potohari speaking were also the part of this study. Most of them belonged to age group 40 or above and the mean couple's satisfaction index score (CSI) of this group was 53.7.

4.3 Genotyping of Promoter Region of *AVPR1A*

The two groups were subjected to genotyping using one primer set for promoter region that includes GT repeat element (Table 3.1). The analysis of promoter region was performed using standard PCR reaction and electrophoresis on 8% non-denaturing polyacrylamide gel as discussed in the previous chapter. The amplified PCR products were visualized by staining the gel with ethidium bromide and genotypes were assigned by visual inspection of the images, captured by digital camera DC 290 (Kodak, New York, USA). Amplified product size was assessed through 50 bp DNA ladder.

In the Group 1, all 98 participants showed amplified product size between 300-350 bp (RS2) (Figures 4.4.1-4.4.11 and 4.6). For group 2 (marital polygamous human males) out of 99 participants, amplified product size in 41 individuals was between 200-250 bp (RS1), and in remaining 58 individuals amplified product size was between 300-350bp (Figures 4.3.1-4.3.11 and 4.5).

Table 4.1: Demographic Data of Group 2 Participants

Age Group	Number of Individuals	Ethnic/Language Group	Observed Amplicon Size
18-30	08	03 Urdu speaking, 04 Punjabi speaking 01, 01 Saraiki speaking	RS1 (200-250 bp) in 5 participants while RS2 (300-350 bp) in 3 participants
31-40	29	10 Urdu speaking, 15 Punjabi Speaking, 02 Saraiki speaking, 01 Pushto speaking	RS1(200-250 bp) in 16 participants while RS2 (300-350 bp) in 13 participants
41 or above	59	21 Urdu speaking, 36 Punjabi speaking, 01 Saraiki speaking, 01 Pushto speaking	RS1 (200-250 bp) in 18 participants while RS2 in 41participants

Table 4.2: Demographic Data of Group 1 Participants

Age Group	Number of Individuals	Ethnic/Language Group	Observed Amplicon Size
18-30	--	--	--
31-40	24	13 Urdu 11Punjabi Speaking	RS2 (300-350 bp)
41 or above	78	37 Urdu Speaking, 38 Punjabi speaking, 01 Pushto speaking	RS2 (300-350 bp)

4.4 Electropherograms of Participants of Group 2

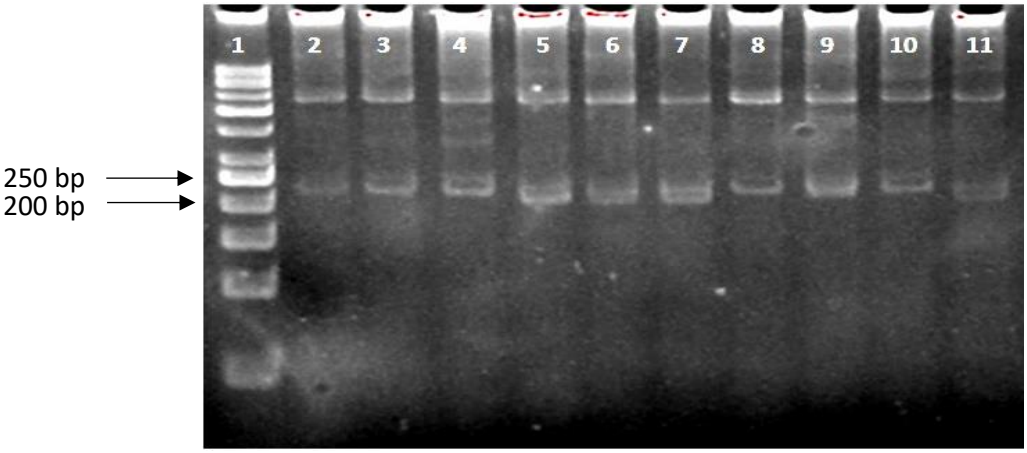

Figure 4.4.1: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 2 in participants from 2-11. Product size of 200-250 bp (RS1) was detected with the help of 50bp DNA ladder in lane 1.

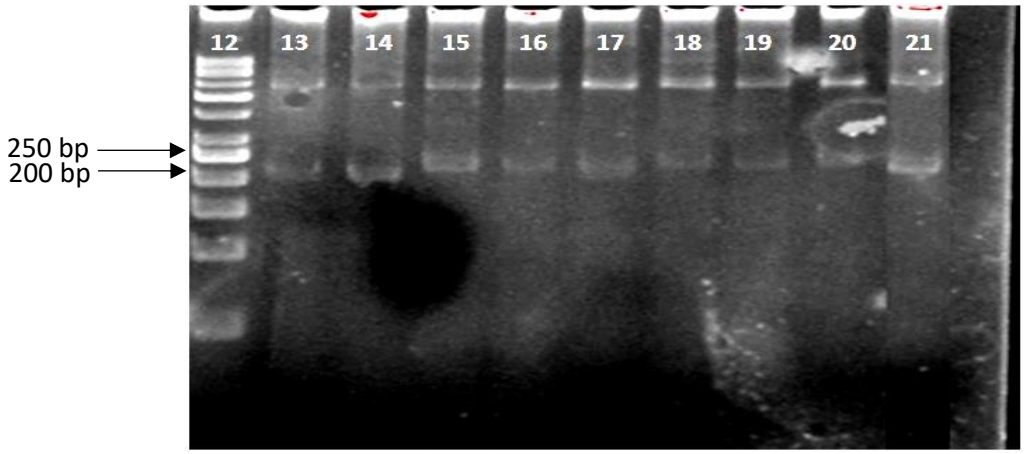

Figure 4.4.2: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 2 in participants from 13-21. Product size of 200-250 bp (RS1) was detected with the help of 50 bp DNA ladder in lane 12.

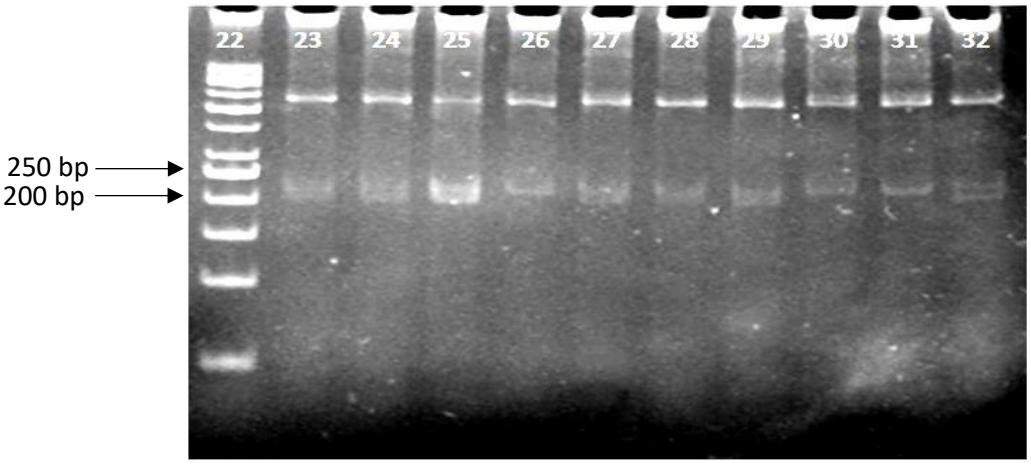

Figure4.4.3: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 2 in participants from 23-32. Product size of 200-250 bp (RS1) was detected with the help of 50 bp DNA ladder in lane 22.

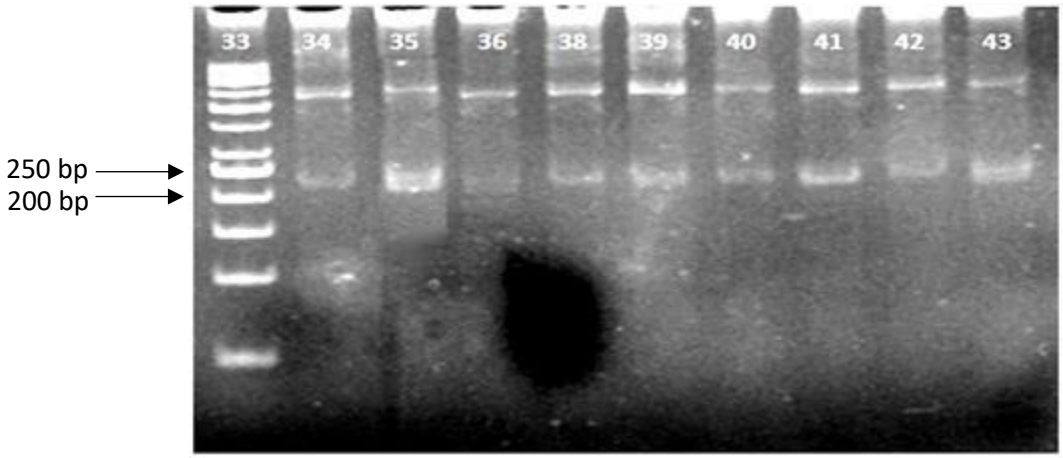

Figure 4.4.4: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 2 in participants from 34-43. Product size of 200-250 bp (RS1) was detected with the help of 50 bp DNA ladder in lane 33.

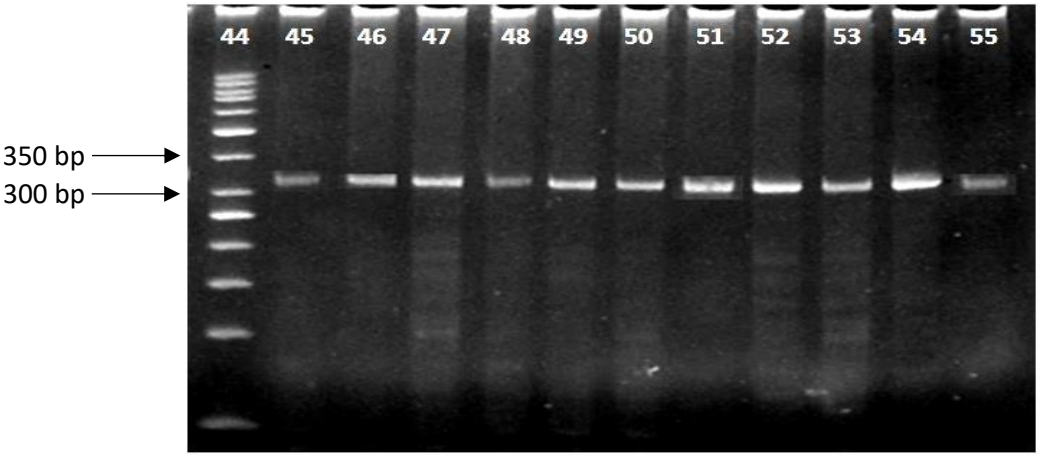

Figure 4.4.5: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 2 in participants from 45-55. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 44.

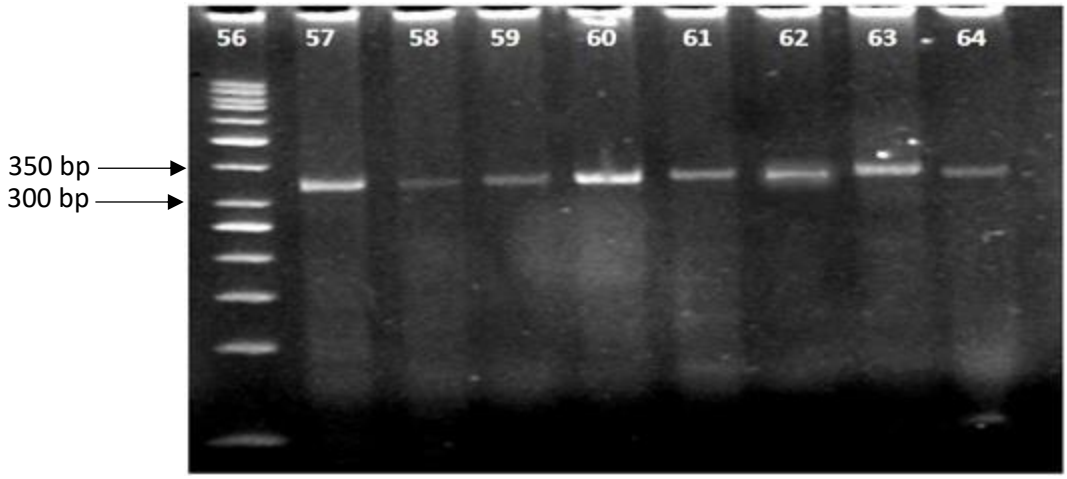

Figure 4.4.6: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 2 in participants from 57-64. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 56.

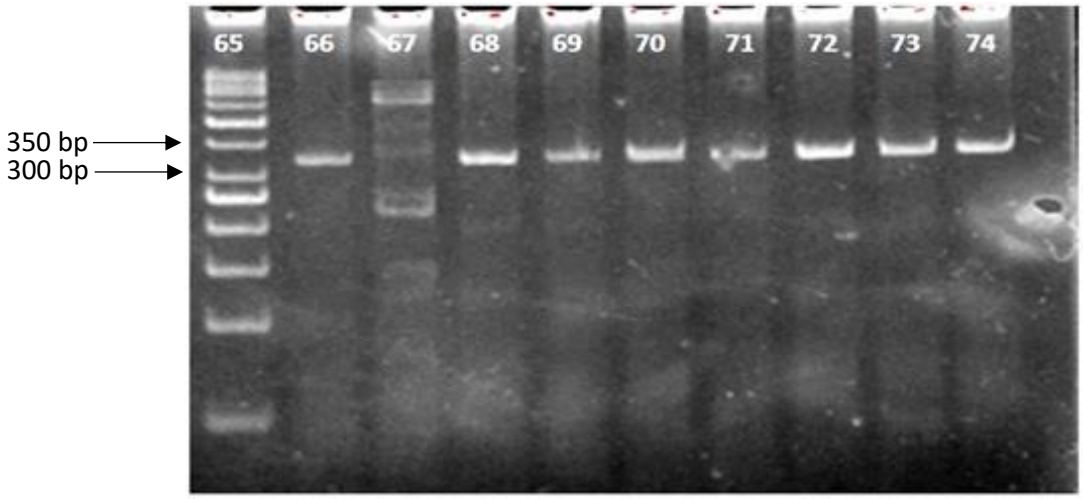

Figure 4.4.7: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 2 in participants from 66-74. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 65. In individual 67, which is heterozygous, RS1 allele (200-250 bp) was detected along with an unusual increased size of another allele (approx.-500 bp)

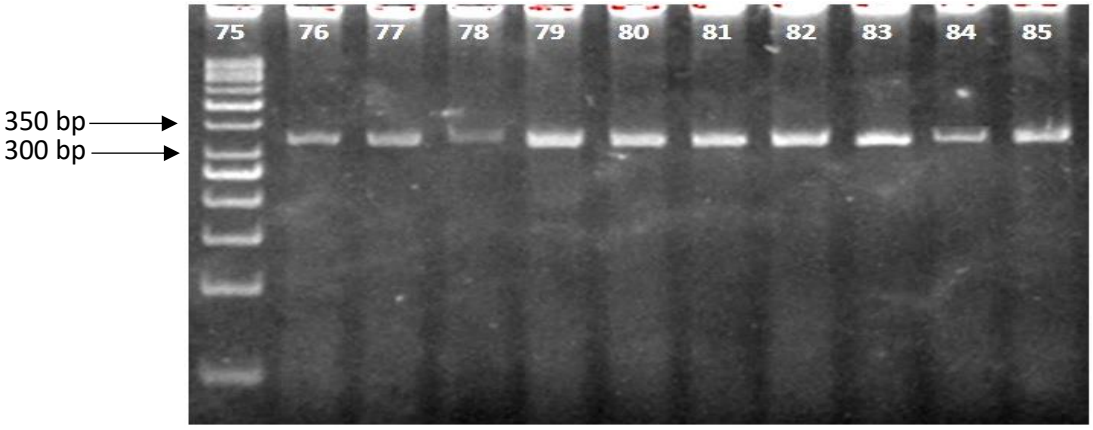

Figure 4.4.8: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 2 in participants from 76-85. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 75.

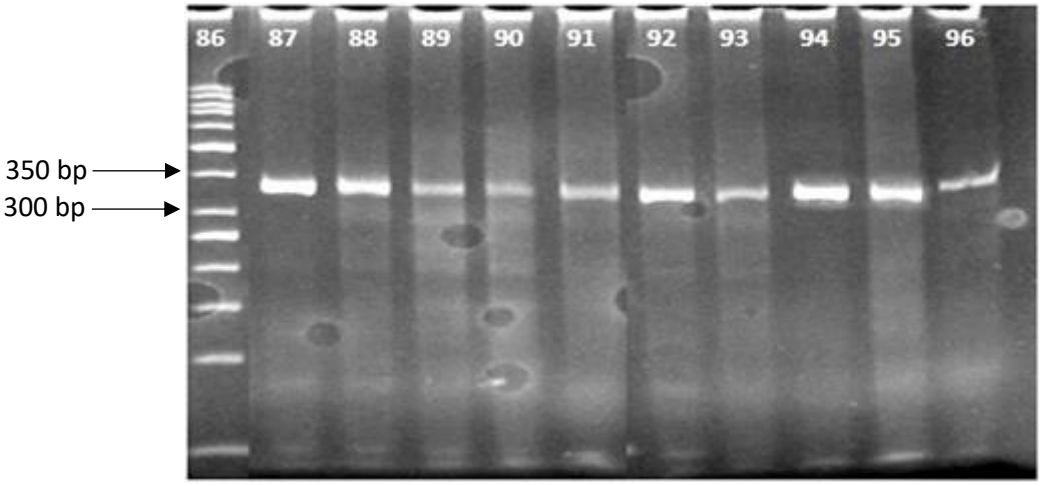

Figure 4.4.9: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 2 in participants from 87-96. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 86.

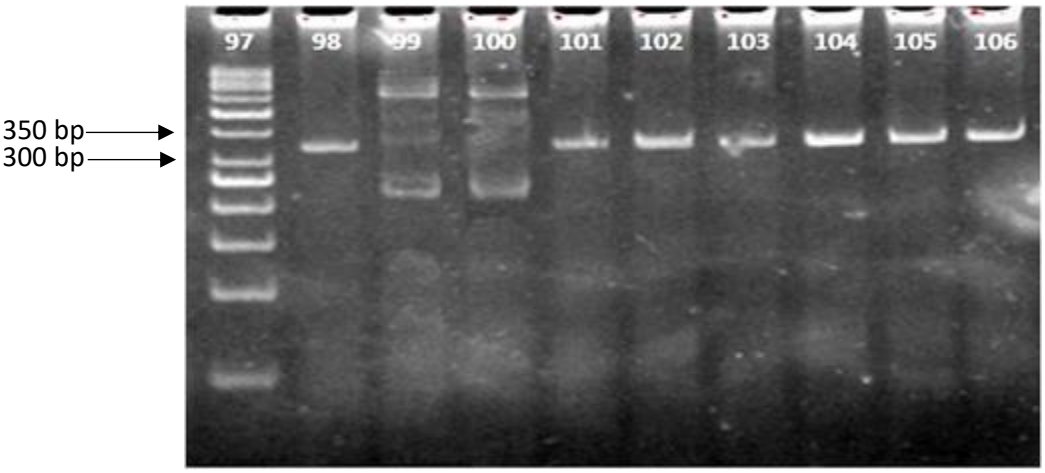

Figure 4.4.10: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 2 in participants from 98-106. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 97. In individual 99 and 100, which are heterozygous, RS1 allele (200-250 bp) was detected along with an unusual increased size of another allele (approx.-500 bp)

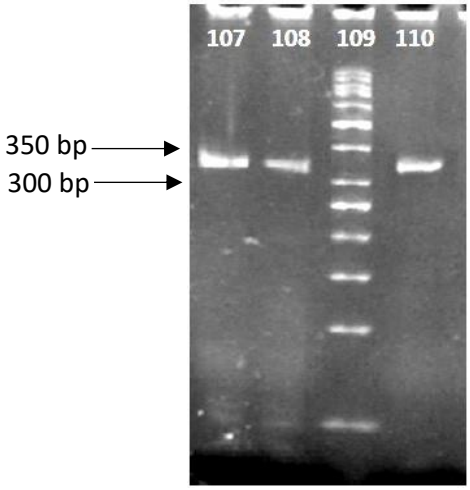

Figure 4.4.11: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 2 in participants from 107-110. Product size of 300-350bp (RS2) was detected with the help of50 bp DNA ladder in lane 109.

4.5 Electropherograms of Participants of Group 1

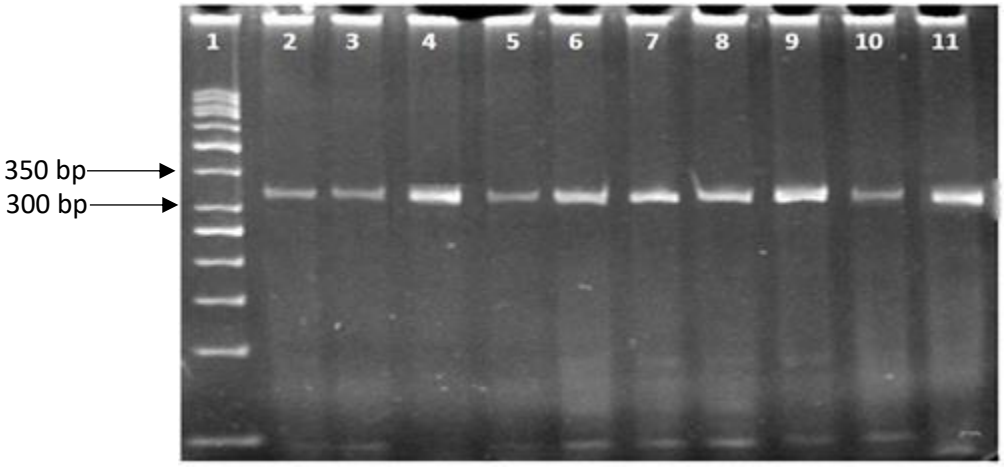

Figure 4.5.1: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 1 in participants from 2-11. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 1.

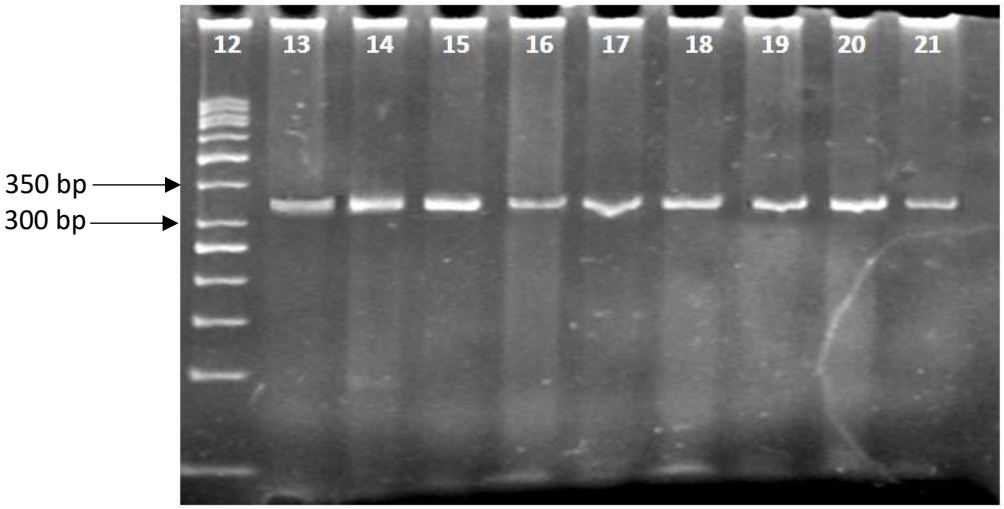

Figure 4.5.2: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 1 in participants from 13-21. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 12.

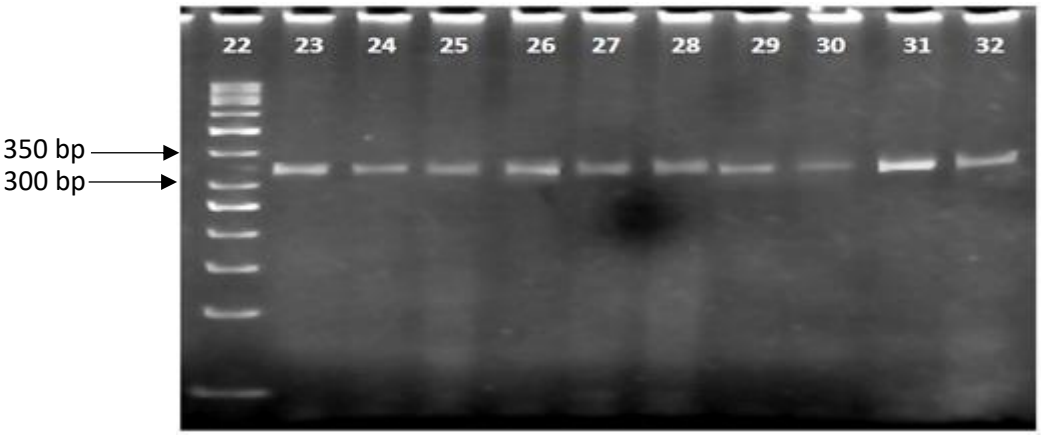

Figure 4.5.3: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 1 in participants from 23-32. Product size of 300-350 bp (RS2) was detected with the help of 50bp DNA ladder in lane 22.

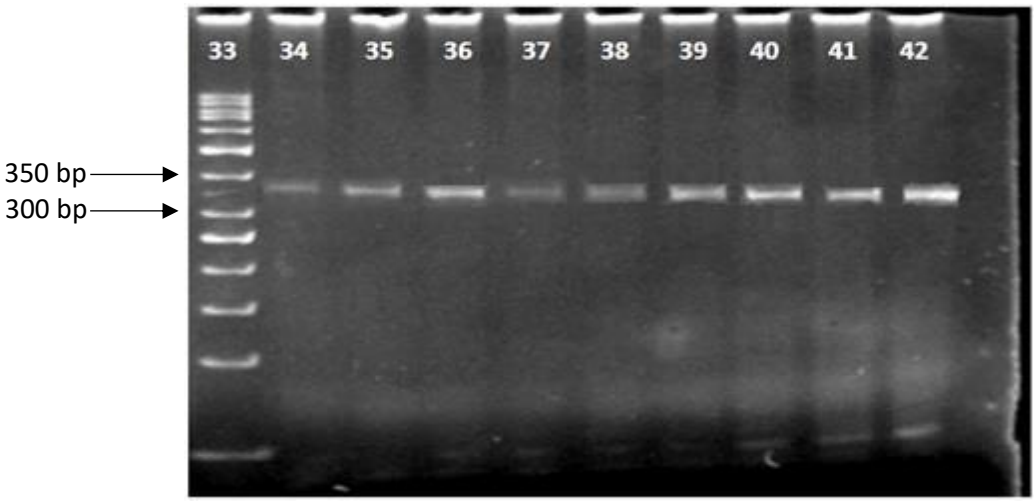

Figure 4.5.4: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 1 in participants from 34-42. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 33.

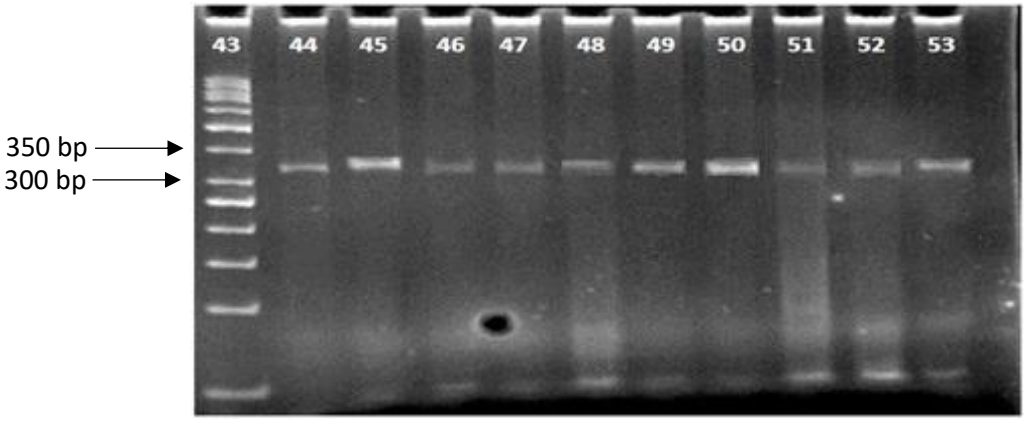

Figure 4.5.5: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 1 in participants from 44-53. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 43.

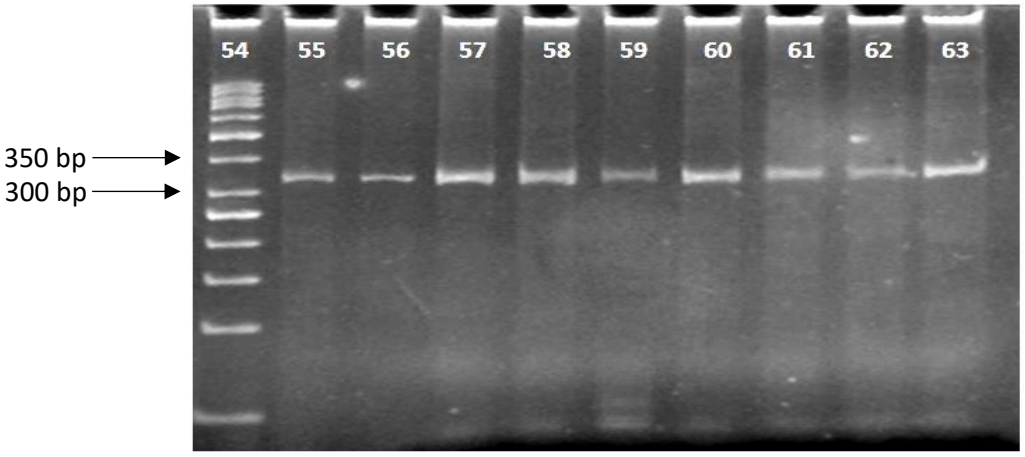

Figure 4.5.6: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 1 in participants from 55-63. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 54.

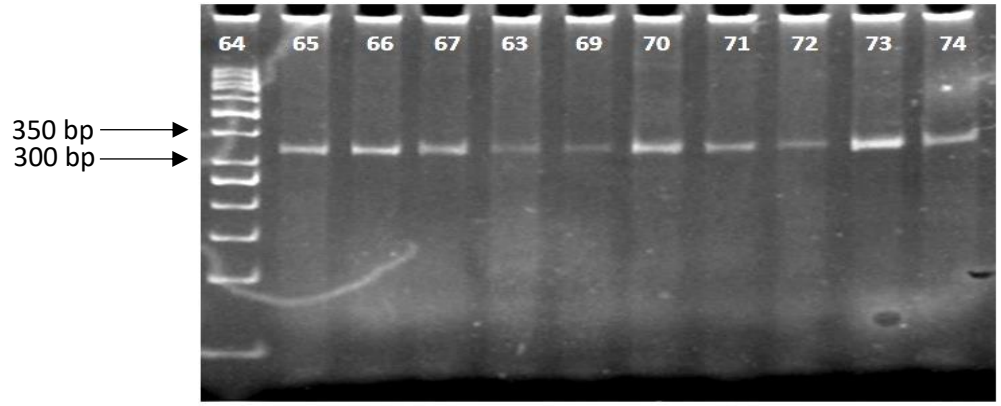

Figure 4.5.7: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 1 in participants from 64-74. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 64.

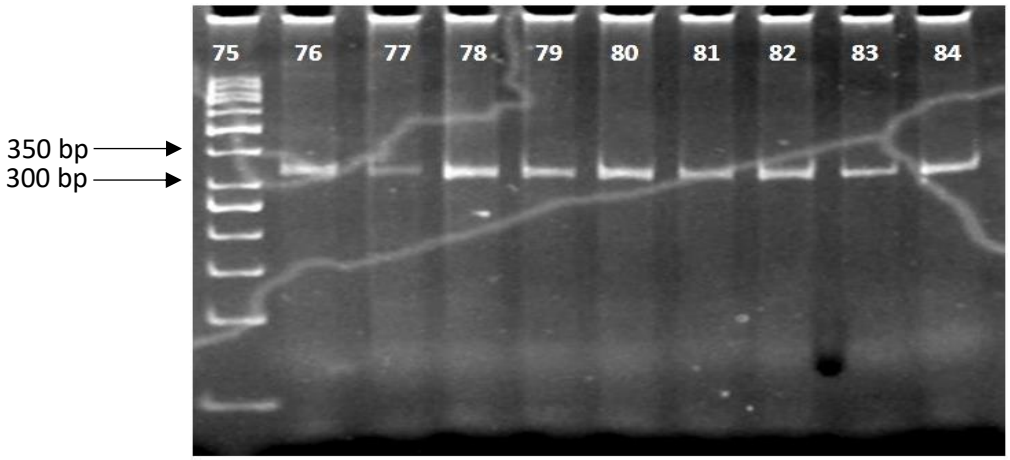

Figure 4.5.8: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 1 in participants from 75-84. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 75.

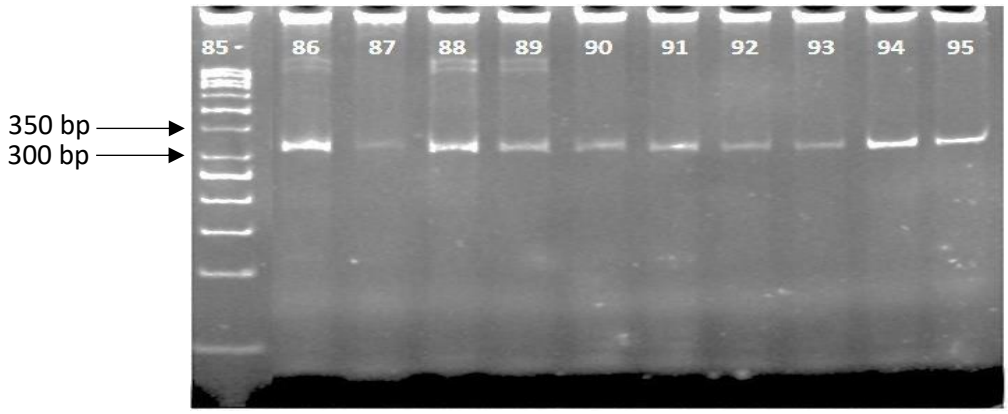

Figure 4.5.9: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 1 in participants from 86-95. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 85.

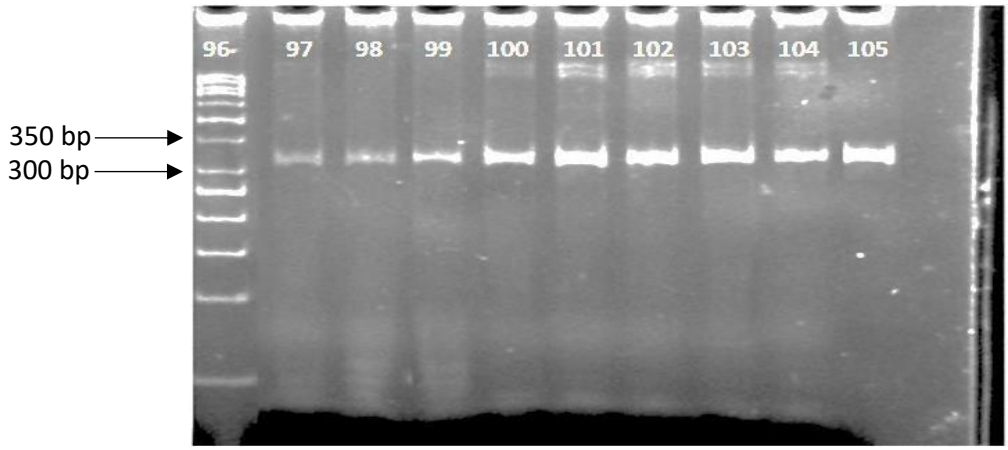

Figure 4.5.10: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 1 in participants from 97-105. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 96.

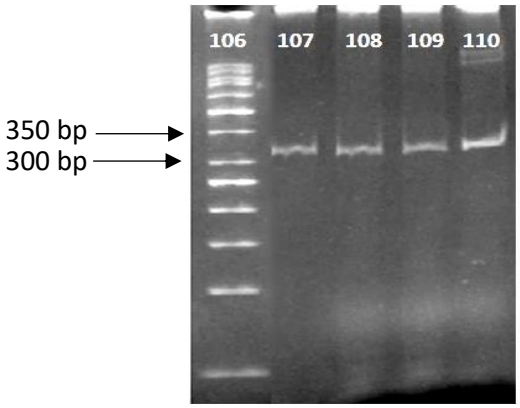

Figure 4.5.11: Electropherogram of GT repeat variation in *AVPR1A* receptor promoter region in group 1 in participants from 107-110. Product size of 300-350 bp (RS2) was detected with the help of 50 bp DNA ladder in lane 106.

4.6 Pi Chart Demonstration of RS1 and RS2 Allele Frequency in Group 2

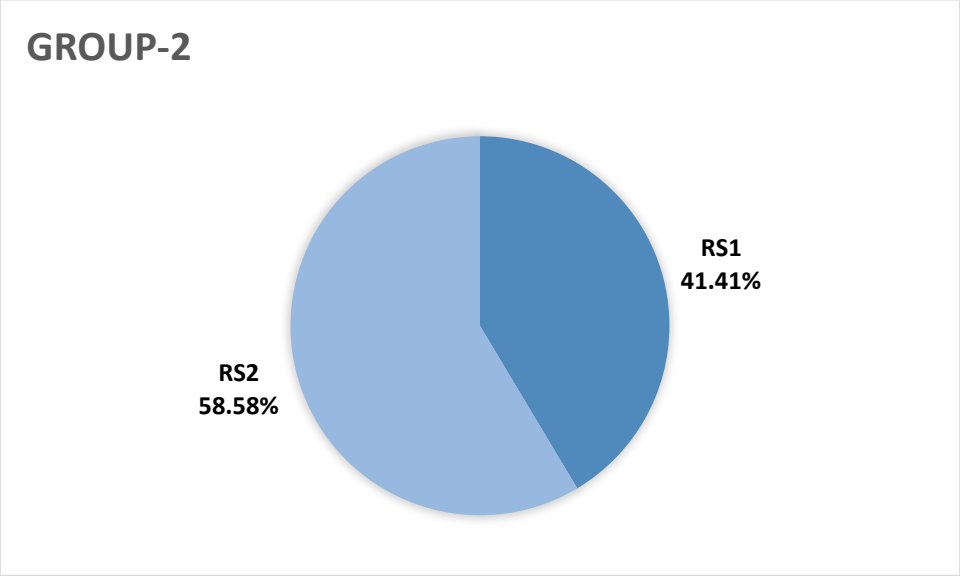

Figure 4.6.1: Pie chart graph illustrating participants of group 2 on the basis of observed band size. Region 41.41% showing allele RS1 while rest of the region showing RS2.

4.7 Pi Chart Demonstration of RS1 and RS2 Allele Frequency in Group 1

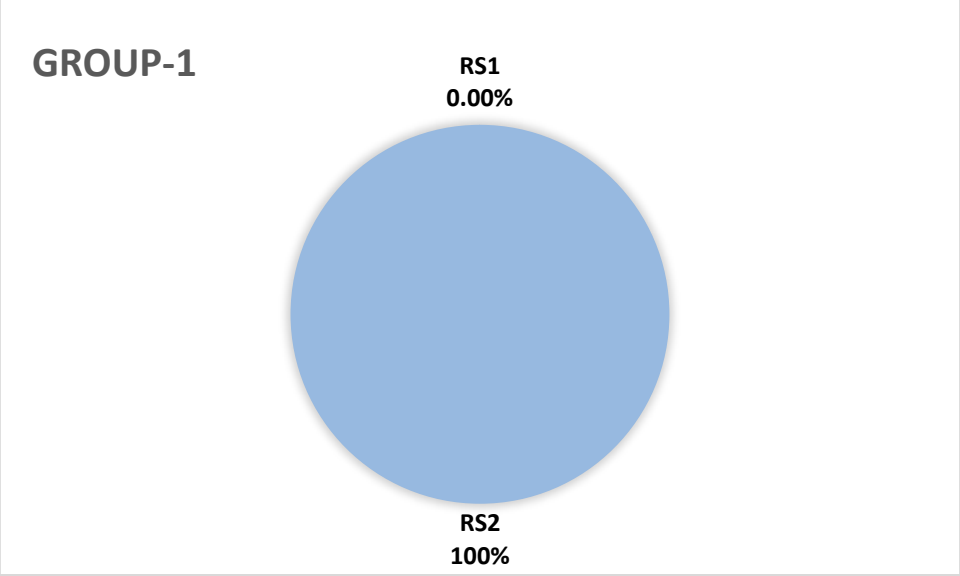

Figure 4.7.1: Pie chart graph illustrating participants of group 1 on the basis of observed band size RS1 allele size was observed in all participants.

4.8 Graphical Demonstration of CSI Scores of Both Groups

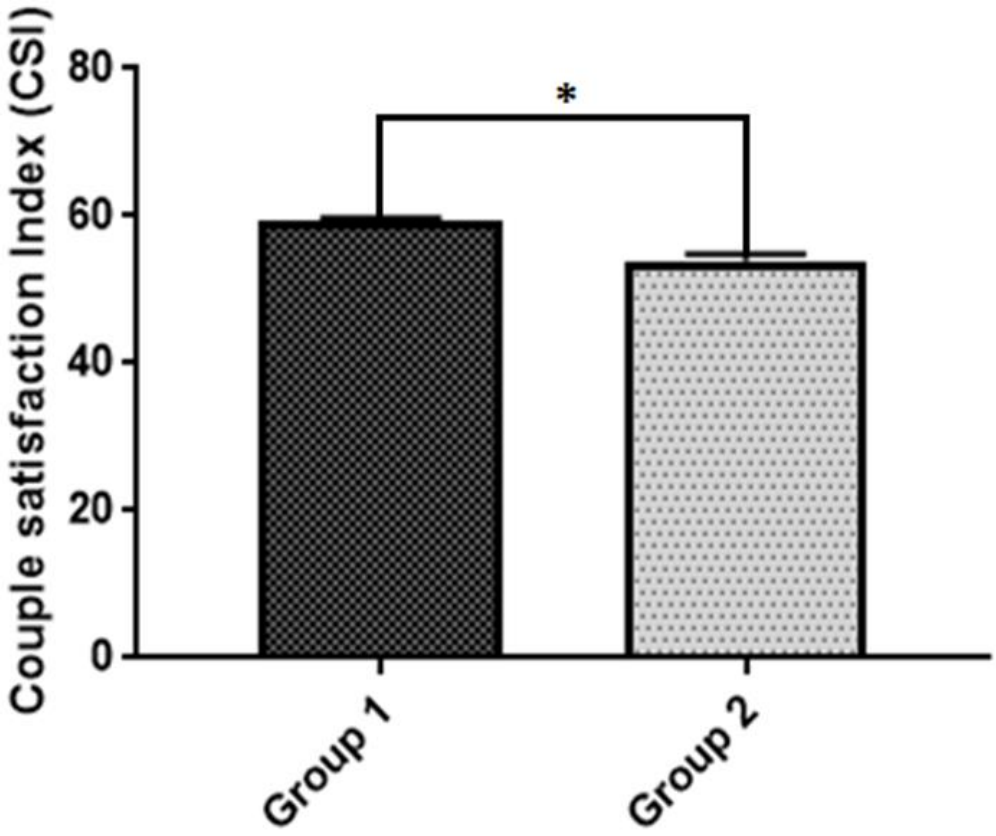

Figure 4.8.1: Couple satisfaction Index (CSI) score in group 1 participants in comparison to group 2 participants. Error bars represent standard error of mean (± SEM), ($p < 0.0001$) which shows significant outcomes.

DISCUSSION

Social interactions play an important role for the survival of an individual, which includes several aspects from persuading a mate, prenatal care, thriving in society and determining success in the workplace. Abnormal social behaviors, such as avoidance, rejection, lack of involvement and pathological trusting are associated with different neurological or behavioral problems (Uchino *et al.*, 1997). Social organization is perceived to be characterized by bonded relationships and suggested in a recent analysis that evolutionary development of the primate social brain may have been triggered by the particular demands for pair-bonding behavior (Dunbar *et al.*, 2007). Humans are also thought to be evolved to rely on social interactions and bonding for their survival, the presence or absence of social support can directly impact one's health (Nair *et al.*, 2006).

The neuropeptide arginine vasopressin (AVP), expressed in brain, has been studied for its mechanism of action through the receptor subtype AVPR1A which plays an important role in the regulation of pair-bonding behavior in rodent males. It has been verified by a series of elegant studies on closely related vole species, i.e., montane voles (Microtus montanus), meadow voles (Microtus pennsylvanicus), and prairie voles (Microtus ochrogaster) (Young et al., 2004). In prairie voles, which in contrast to other two (montane and meadow voles) are socially monogamous and highly social. Their pair-bond formation and related behavioral activities are facilitated by AVP and prevented by an AVPR1A antagonist (Cho et al., 1999a). In contrast to animals, the genetic influence of infidelity is little unclear in human. Finding of a study on human females who informed about their previous episodes of infidelity and a total number of sexual partner in their lifetime in secret (Cherkas et al., 2004), depicted the moderate influence of genetics on infidelity and number of partners. But the genetic correlation among these two traits was strong.

In the present study, two groups of human males, (Group 1: marital monogamous and Group2: marital polygamous) were evaluated for size variations in the promoter region of *AVPR1A* at the genetic level. For this purpose, we used questionnaire from supporting information Table S1 by (Walum *et al.*, 2008) and The Couples Satisfaction Index (CSI) by (Funk *et al.*, 2007). In our designed Performa, we included 24 items with a scale to measure one's satisfaction in a relationship. Performa has a variety of questions with different response scale and formats however according to the author; the scale can be shrunk according to study design and needs. We recruited participants on the basis of proforma into two groups, one who married more than once in their life due to various reasons and second who married once and have been living with their marital partner for more than 10 years. CSI score of our 99 participants of group 2 participants was 53.7 in average out of 77. But the genotypic comparison depicted that 43 males in group 2 had lesser GT repeat variation of size 200-250 bp while other 56 participants showed GT repeat variation of size 300-350 bp. While the same marker amplification in all (98) participants of group 1 showed GT repeat variation and amplicon size of 300-350 bp and the mean CSI score of these monogamous participants was 59.4. the CSI index score average also showed significant difference in both study groups from which we conclude that a person will be polygamous if he scores near average or below average in our study performa. The genotypic analysis of both groups found in half of group 2 reduced GT repeat expansion in their *AVPR1A* promotor regions while the same GT repeat sequences are found enlarged in all participants of group 1. However previously reported but not consistently replicated studies, association of *AVPR1A* repeat polymorphism has been associated between autism (Kim *et al.*, 2002; Yirmiya *et al.*, 2006), age at first sexual intercourse (Prichard *et al.*, 2007a), and altruism (Knafo *et al.*, 2008) which are enough to indicate that there may be an impact of repetitive sequences on human pair bond behavior. In the study conducted by (Hammock *et al.*, 2006) it is concluded that the distribution of *AVPR1A*

in brains of rodents prairie and montane voles determine their social life structure. Plasticity of microsatellite repeat sequences appeared to be raised from instability of 5' flanking region of *AVPR1A* gene. From this we can link this repeat polymorphism in rapid evolution of social behavior. Interestingly there are four microsatellite repeat markers lies in 5' flanking region of human *AVPR1A* gene, the sequence within and surrounding of these regions are different in nonhuman primates like chimpanzees weather these sequences in human could be responsible for variation in social and pair-bond behavior and they can play their role in expression of the gene are yet to be explored.

5.2 Conclusion

In this study, it was concluded that the GT repeat size variation is significantly reduced in polygamous human males in comparison to monogamous human males. The outcomes of our studies show adequate significance from which we can say that the GT repeat variation in *AVPR1A* promoter region can be responsible for human male infidelity of polygamous behavior. However, the population size was not enough to state its strong association in population, for this purpose we'll need larger group of participants and resources.

5.3 Future Perspectives

For future, similar studies may can be conducted with larger sample size and sequencing of this region in all participants may be carried out. Sequencing of this region in control participants must be done to determine the reference size of this region, this sequencing will also help us in determining major and minor alleles. Expression analysis of this gene due to variation can also be study by expressing this gene in different cell lines.

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https://genome.ucsc.edu/

http://www.urogene.org/methprimer/

APPENDIX-I

7.1 IRB Approval Form

ASAB

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

Ref.No:27/IRB- Date:20April, 2016

IRB APPROVAL LETTER

Project Title: Study of Arginine Vasopressin Receptor 1a (*AVPR1A*) Promoter Polymorphisms and Pair Bonding Behavior in Human Males

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 Arginine Vasopressin Receptor 1a (*AVPR1A*) Promoter Polymorphisms and Pair Bonding Behavior in Human Males"** 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
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7.2 Consent Form

INFORMED CONSENT FORM

Study of arginine vasopressin receptor 1a (AVPR1A) promoter polymorphisms and pair bonding behavior in human males

AIMS AND OBJECTIVES

To analyze microsatellite repeat polymorphism in marital monogamous and polygamous human males.

SAMPLE COLLECTION

5-10 ml blood sample will be collected from all participants (including both monogamous and polygamous individual males)

INFORMED CONSENT

You are being asked to participate in a research study to study genetic basis of the social behavior. You will be asked to donate 5-10ml 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 pair bonding and social interaction. Your identity in this study will be protected. You can withdraw 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. 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 تحقیقی منصوبہ آپ کے اندر وقوع پزیر ہونے والی جینیاتی بیرویشا کی وجہ سے وقوع ہونے والی سماجی رویوں میں تبدیلی معلوم کرنے کیلیے خالصتاً غیر تجارتی بنیادوں پر ترتیب دیا گیا ہے۔ آپکو ۱۰-۵ ملی لیٹر خون عطیہ کرنے کی درخواست کی جاتی ہے۔ خون دینے سے آپکو کسی قسم کا زخم یا درد نہیں ہو گا۔ آپ کی 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 جینیاتی تبدیلیوں کا مشاہدہ کرتے ہوئے سماجی رویےکو سمجھنے کی کوشش کریں گے۔ آپ اس تحقیق کے دوران کسی بھی وقت اپنی شمولیت سے دستبردار ہو سکتے ہیں۔ آپ کی شمولیت رضاکارانہ ہے۔ دوران تحقیق اگر ضروری ہوا تو آپکے رویئے سے متعلق مشاورت بھی کی جائے گی۔ حاصل شدہ سائنسی معلومات کا تبادلہ صرف تحقیق میں شامل سائنسدانوں کے مابین کیا جائے گا۔ تحقیق سے حاصل ہونےوالے نتائج کو سائنسی جریدے میں آپکی شناخت کو پوشیدہ رکھتے ہوے شائع 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 ہوں کہ جو کچھ بھی مجھ سے بیان کیا گیا ہے، میں اسے مکمل طور
پر سمجھ گیا ہوں۔ نیز میں اپنے خون کا نمونہ رضاکارانہ طور پر صرف تحقیق کے لئے بطور عطیہ دیتا/دیتی ہوں مجھے میرے تمام سوالات کے جواب مل گئے ہیں اور فی الوقت میرے ذہن میں کوئی اور سوالات نہیں۔

Signature/Thumb impression of the participant:_____

Name: _____ **Participant ID:** _____

Contact no._____

7.3 Questionnaire

PERFORMA

Study of arginine vasopressin receptor 1a (AVPR1A) promoter polymorphisms and pair bonding behavior in human males

Case no. _____ Sex_____

Cast/Ethnicity_____

Address_____

_____

Marital Status:	Married	☐	Separated	☐	Divorced	☐	
Type of Marriage:	Love	☐	Arranged	☐	Compromised	☐	
Age:	18-30	☐	30-40	☐	40 and above	☐	
Qualification:	Un-Ed	☐	Primary	☐	Middle	☐	
	Metric	☐	Inter	☐	Grad/above	☐	

Age at marriage: _____ Age at first sexual exposure_____

Number of spouse _____ No. of children_____

1. How much you were in love with your spouse before marriage?

Not at all	Don't know	Little	Adequately	Made for each other

2. (If in love) To what extent it was sustained after marriage?

Strongly Disagree	Moderately Disagree	Disagree	Don't know	Agree	Strongly Agree
0	1	2	3	4	5

3. Do you like to spend your spare time with your marital partner?

0	1	2	3	4	5

Reason if no._____

4. Do you prefer privacy of your matters with your marital partner?

0	1	2	3	4	5

Reason if no. _____

5. Do you feel comfortable while sharing feelings with your marital partner?

0	1	2	3	4	5

Reason if no: _____

6. Do you feel bonded when you are close to your marital partner?

0	1	2	3	4	5

Reason if no. _____

7. Do you have a need for your partner to exchange her thoughts?

0	1	2	3	4	5

Reason if no. _____

8. Do you feel comfortable/relaxed to be in your partner's company?

0	1	2	3	4	5

Reason if no: _____

9. Do you consider your partner's advice in making major decisions?

0	1	2	3	4	5

Reason if no. _____

10. Which one is important for a healthy relationship?

a). Physical interest b). Intellectual interest

11. Does your partner satisfy your sexual needs?

Yes_____ (1) No_____(0)

Reason if no. _____

12. How much do you think your partner fulfils your sexual needs?

0	1	2	3	4	5

Reason if no. _____

13. Do you depend only on your partner in this matter?

Yes_____(1) No_____(0)

Reason if no: _____

14. How much your partner physically attracts you?

0	1	2	3	4	5

Reason if no. _____

15. Do you ever feel physically withdrawn from your partner?

0	1	2	3	4	5

Reason if Yes: _____

16. When you are close to each other, which one of you is a good initiator of sexual activity?

You_____(1) Partner_____(0)

17. How many times do you feel aroused in a company of females?

0	1	2	3	4	5

18. Do you feel reluctant in a day to day communication with women other than your partner?

0	1	2	3	4	5

19. What is the first thing you notice in women in first interaction?

Answer_____

20. Do you feel like being in relationship with women other than your partner?

0	1	2	3	4	5

Reason if yes: _____

21. How much you wish to have another marital relationship?

0	1	2	3	4	5

Reason _____

22. How often do you discuss or have you considered divorce, separation, or terminating your relationship?

Not at all	Very little	Some times	Usually	Most of the time	All the time

Reason, if any. _____

23. In general, how often do you think that things between you and your partner are going unwell?

Not at all	Very little	Some times	Usually	Most of the time	All the time

23(a). What actions of your partner make you angry?

Answer_____

24. If divorced, main Reason for divorce :_____

_____

_____