THE IDENTIFICATION OF CANDIDATE GENES USING cDNA MICROARRAY AND THE ANALYSIS OF TWO SNPs OF THE REELIN GENE IN A SOUTH AFRICAN AUTISTIC POPULATION

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Observing an autistic child endlessly biting his/her own hand, banging their head against a wall, compulsively spinning an object, or staring for hours at a piece of dust is terrifying because it is impossible to understand how his/her mind functions. He/she seems an alien in our everyday world. These past few years I embarked into the world of an autistic child reluctantly and fearfully. As I learned about their world I could look at these children through new eyes and told my friends and family what I observed. I have tried my best to make this thesis as informative as I could, including aspects surrounding the background and known causes of this disorder. From my own observation and research, it seems evident that autism is not a psychological malfunction but rather a neurological one. However, an enormous amount of research still needs to be undertaken in order to help autistic children and to understand their bewildering and often self-destructive behaviour.

ABSTRACT

Autism is a pervasive developmental disorder (PDD) that's incidence is approximately 1 in 158. It is four times more prevalent in males than females and is believed to be caused by both genetic and environmental factors. Research indicates that several genes are involved in autism and it is believed that these genes act together to produce autism. Many genes implicated in this disorder are involved with brain structure formation and brain functioning. Studies have identified the reelin (*RELN*) gene as necessary for proper formation of brain, which indicates that *RELN* abnormalities could contribute to the aetiology of several neurogenetic diseases such as schizophrenia, bipolar and autism. The aims of the study were (i) to genotype two SNPs (exonic rs3622691 and intronic rs736707) in the *RELN* gene using Taqman® SNP Genotyping assays to detect association with autism in three distinct South African (SA) ethnic groups (Black, Caucasian and Mixed), and (ii) to detect candidate genes that are over and under-expressed in the samples taken from a SA Caucasian autistic group and compare those with samples taken from a healthy Caucasian group using cDNA microarray. The Taqman® study indicated significant association for the intronic SNP, rs736707, with a p-value of 0.0009 in the total SA group. More so, the Mixed group displayed the highest significance amongst the ethnic groups, with a p-value of 0.00014. The microarray study yielded 21 genes with 95% significance in the Caucasian sample group. Most genes were hypothetical proteins and formed part of the FAM90A family. The *LOC83459* showed the highest level of expression in the autistic samples, while the *BTNL8* gene was shown to be highly suppressed in the control samples.

Keywords: ***Autism, Candidate genes, cDNA Microarray, Reelin gene, SNPs, Taqman®***

DECLARATION

I declare that "The identification of candidate genes using cDNA microarray and the analysis of two SNPs of the reelin gene in the South African autistic population" is my own work that has not been submitted for any degree or examination in any other university and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Hajirah Gameeldien December 2009

Signed:……

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INTRODUCTION

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1.1 BACKGROUND

In 1911 Eugen Bleuler, a Swiss psychiatrist, was the first person to use the term Autism (Ozand *et al*. 2003). However, the term he used applied to adult schizophrenia. Due to this, in the 1940's the medical community felt that children who had autism where schizophrenic. Autism was eventually discovered by psychologist Leo Kanner in 1943 (Wing 1993). Before Leo Kanner's classification, autistic children were classified as emotionally disturbed or mentally retarded. The common features amongst his patients included a profound lack of emotional contact with others, an intense wish for sameness in routines, muteness or abnormality of speech, fascination with manipulating objects and high levels of visio-spatial skills. In addition, he also noted that these children often did not demonstrate capabilities that showed that they were slow learners nor did they fit the patterns of emotionally disturbed children. Kanner's observations became the criteria for early studies of the prevalence of autism. Individuals with these features have the full triad of impairments and represent the most severely disabled end of the autism spectrum of disorders (ASD) known as Classic Autism. During the 1960's researchers had a better understanding of autism and identified autistic symptoms and treatment with more precision. Kanner's findings led to the formation of autism organizations in the 1960's and 1970's by the parents of children with autism (Wing 1993; Bailey *et al.* 1995).

1.1.1 What is autism?

Autism is a pervasive developmental disorder (PDD) and can also be termed as 'Classic Autism' (Bailey *et al.* 2005). This disorder affects some children from birth or infancy and leaves them unable to form normal social relationships or to develop normal communication skills (Rutter 2005). Its incidence is approximately 1 in 158 in the United States of America (Autism South Africa 2008). The statistics for South Africa are not yet known. Autistic males

outnumber females by 4 to 1 (Wing 1993) and there is no explanation as to why the 4:1 ratio exists. However, some scientists seem to think that autism could be X-linked. Therefore, since the female has XX chromosomes and the male XY chromosomes, the reason for this phenomena could be due to the protective X allele of the female; i.e. if autism is indeed X-linked and the X allele of the male is affected, then he would not have another X allele to act as a protective allele (Skuse 2000). The autistic child is unable to use language meaningfully or to process information from the environment. About half of all autistic children are mute, and those who do speak often only repeat mechanically what they have heard (Santangelo and Tsatsanis 2005). Other characteristics of autism include an uneven pattern of development, a fascination with mechanical objects, a ritualistic response to environmental stimuli, and a resistance to any change in the environment. Some autistic children have precocious ability, such as mathematical skill. At present, early diagnosis of autism is still rare, but scientists are currently working towards improving the diagnostic tools to achieve this goal (Fombonne 2003). The diagnosis of autism is only made if three types of behaviour are present. These behavioural types include; whether the child's social relationships and social development are abnormal, whether the child is failing to develop normal communication, and whether the child's interests and activities are restricted and repetitive rather than flexible and imaginative (LeCouteur *et al*. 1989)

Several studies have revealed abnormalities in different regions of the brain of an autistic individual. At present, various techniques are used to obtain these regions of the brain including Computer Axial Tomography (CAT) scans and Magnetic Resonance Imaging (MRI) scans *(fig. 1.1)*. The areas of abnormality noted include; abnormalities in the frontal lobes, which are the areas in the brain responsible for planning and control; abnormalities in the limbic system, which are the part of the brain responsible for emotional regulation; and

abnormalities in the brain stem and cerebellum, which governs motor coordination (Courchesne *et al*. 2001).

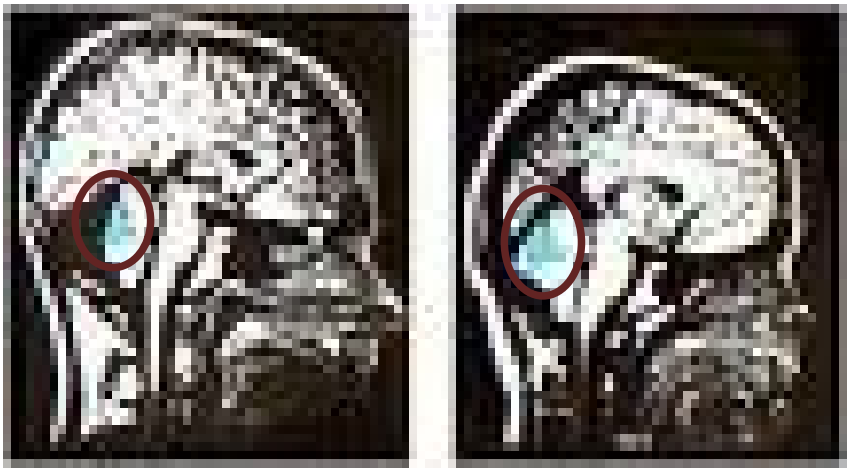


Fig. 1.1: In this MRI image, the person with autism (left) has a smaller cerebellum than the normal brain (right) [Pfizer 2001]

***Cerebellum region circled and indicated in blue**

1.2 WHAT CAUSES AUTISM?

It seems likely, given the research so far, that several factors combine to cause autism. However, no one really knows for sure what causes autism. Modern medical evidence suggests clear biological causes for autism such as genetic factors, viral infections, and birth or pregnancy complications; any of which may cause the subtle brain damage assumed to produce autism. Mothers of autistic children often report difficulties in pregnancy and labour. Other conditions such as viral infections, metabolic conditions and genetic abnormality are also closely related to autism. It is therefore believed that the causative agents of autism can be noted as either environmental or genetic, or consequently, a combination of both these factors (Santangelo and Tsatsanis 2005).

1.2.1 Environmental effects on autism

Exposure to a broad array of external factors and measurements of important physiologic factors are believed to be a causative part in autism. These factors include environmental exposures from chemicals used in industrial processes, consumer products, illnesses of the mother during pregnancy and of the child after birth, medications and vaccinations, as well as diet (Lawler *et al*. 2004). Clinicians and psychologists believe that by looking at children's medical history, environmental exposure to toxins, and many other aspects of their lives, both before and after birth, possible causes and contributing factors to autism may be evaluated. No clear picture has emerged from research into environmental contributors to autism, however, clues are available to support this theory (Santangelo and Tsatsanis 2005).

1.2.1.1 Pre and post-natal exposure

In utero, exposure to the rubella virus (the cause of German measles) can impair brain development of the fetus thus leading to varying degrees and combinations of blindness, deafness, birth defects, mental retardation as well as autism. Children with autism resulting from exposure to rubella virus may improve, worsen, or remain the same. Post-natal herpes simplex virus brain infections (encephalitis) may also result in an autism-like syndrome. Also, the morning-sickness drug, thalidomide, is best known for the tragic and severe limb defects that it caused in the offspring of some women who take it while pregnant (Desmond *et al*. 1967).

A 1994 Swedish study done by Stromland *et al.* reported that five out of a group of 100 children who had been exposed to the morning-sickness drug thalidomide *in utero* had developed autism. The risk was particularly increased when the exposure to the drug occurred early in pregnancy, that is, 20-24 days after conception. Subsequently, in an

autopsy examination of a person with autism, abnormalities in the brain stem and cranial nerves were detected. This kind of abnormality is best explained by altered brain development during early gestation, similar to the time of increased risk in people exposed to thalidomide (Aronson *et al*. 1997).

1.2.1.2 Chemical exposure

It was found that there were reports of chemical exposures that are potentially related to autism. Preconception parental occupational exposure to chemicals and maternal abuse of drugs have been linked to autism in offspring (Grandjean and Landrigan 2006). Maternal use of alcohol during pregnancy can result in fetal alcohol syndrome as well as intellectual and behavioral problems. Asperger syndrome and autism can coexist with other manifestations of fetal alcohol syndrome, suggesting that alcohol may be responsible for autism as a result of early gestational exposures (Harris *et al*. 1995).

1.2.1.3 Mercury and vaccine exposure

Vaccine-related concern stems from the use of thimerosal as a preservative in vaccine preparations. Thimerosal is an organic mercury compound, which metabolises to ethylmercury and thiosalicylate (Hessel 2003). It has been used in vaccines since the 1930s until it began to be phased out in the late 1990s. Concerns centered on the observation that organic mercury is a potent toxic agent in the developing brain and that children receiving a full set of recommended vaccinations were often exposed to mercury at levels that exceed a "safe" reference dose (Halsey and Goldman 2003).

However, thimerosal is not the largest source of mercury exposures in people, despite concerns on the administration of mercury-containing vaccines to pregnant women, infants, and children. Mercurials may be found in drugs for the eye, nose, ear, skin, and throat,

tooth pastes, antiseptics, disinfectants, contraceptives, in dental fillings and thermometers, and many other products. Recent studies have indicated that molecular mercury exposure can induce death, disorganization and/or damage to selected neurons in the brain similar to that seen in recent ASD brain pathology studies, and this alteration may likely produce the symptoms by which ASDs are diagnosed (Blaxil *et al.* 2003). Methyl mercury easily crosses the placenta and can thus impact normal fetal brain development. Therefore, mercury exposures from various sources must be considered when assessing a link between autism and mercury. Fortunately for the US and European countries, thimerosal-free vaccines are now available so parents are not faced with questioning the safety of vaccines for this reason (Geier *et al.* 2008).

1.2.2 Genetics as a cause of autism

Genes account for traits of growth, development, and physical characteristics. At conception a baby receives genes from both parents. There are about 100 000 genes in the body, each instructing cells how to grow and survive. Genes are made up of DNA and when an error occurs in any of these genes, a genetic disorder may result. Since genetic makeup also regulates brain development, several researchers have explored heredity as a cause of autism. Recent studies have pinpointed certain forms of genetic disorders that lead to symptoms of autism (Sykes and Lamb 2007).

1.2.2.1 Twin studies

Identical twins (known as monozygotic twins - MZ) come from the same fertilized egg and are therefore genetically identical. In contrast, non-identical (or dizygotic - DZ) twins develop from two separately fertilized eggs and are therefore not genetically identical. Twin studies are a design in behavior genetics that aid the study of individual differences by

highlighting the role of genetic causes on behaviour. Twins are invaluable for studying important questions because they disentangle the sharing of genes and environments. If we observe that children in a family are more similar than might be expected by chance, this may reflect shared genes inherited from parents. Twin studies compare the similarity of MZ twins who share 100% of their genes, to that of DZ twins, who share only 50% of their genes (Muhle *et al*. 2004). These studies have been performed for autism and indicated that if one MZ twin has autism then there's a 60% chance that the other will also be affected as compared to 0% in the case of DZ twins (*fig 1.2*). When broader autism phenotypes are included, for example communication skills and social disorder, the concordance rises to 60-92% in MZ twins and 0-10% in DZ twins. Thus, this evidence suggests that a genetic cause does exist for autism (Bailey *et al*. 1995).

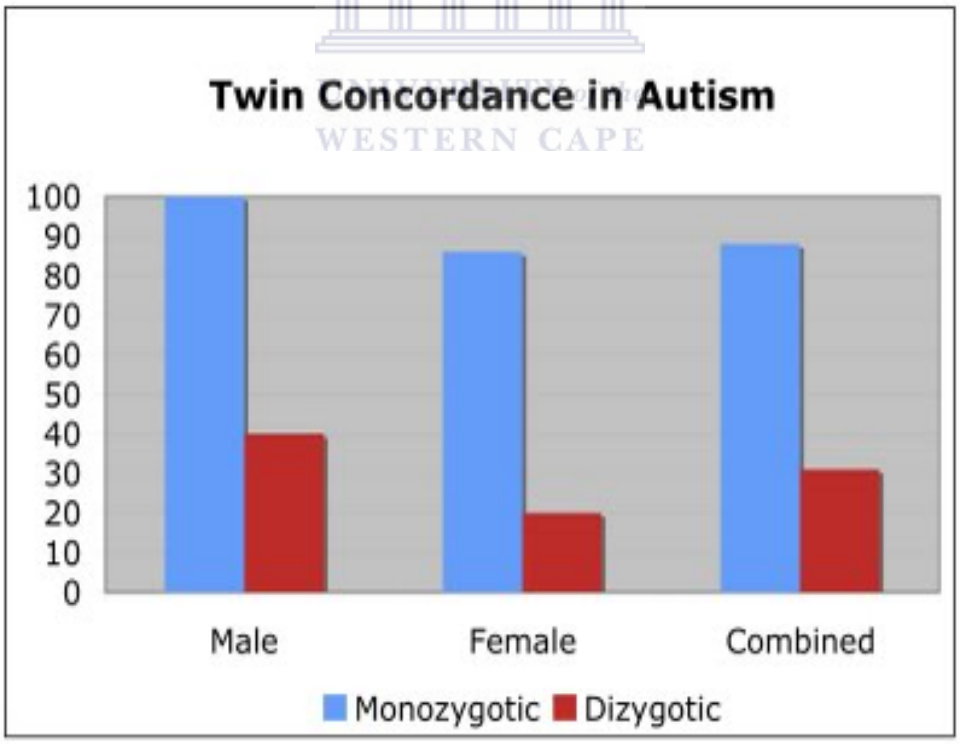


Fig. 1.2: Estimated heritability between MZ and DZ twins (Rosenberg *et al.* 2009)

1.2.2.2 Genetically inherited disorders

Several inherited disorders are associated with autistic-like behavior. Some of these disorders include Fragile X Syndrome, Tuberous Sclerosis Complex (TSC) as well as Phenylketonuria (PKU) [Muhle *et al*. 2004]. Fragile X only occurs in a small proportion of children with autism (under 10%); but this percentage nevertheless makes it the most common cause of autism yet identified. A child with Fragile X Syndrome is usually mentally handicapped and often has an unusual facial appearance (Zafeiriou *et al*. 2007).

Studies have also indicated that autism is quite common in children with TSC (Gutierrez *et al.* 1998). However, this condition is itself very rare and is an uncommon cause of autism. The features of TSC may include unusual skin pigmentation, a particular facial rash, and most tragically, the growth of tumours in the brain. Tuberous sclerosis may also give rise to a special form of epilepsy known as infantile spasms. This epilepsy has also been proposed as a cause of autism, so it is possible that the link between autism and TSC merely reflects the common association with infantile spasms (Deonna and Roulet 2006).

Another genetic condition which may lead to autism includes PKU, which is also an inherited condition involving an inability of the body to break down the naturally occurring chemical phenylalanine (Baieli *et al.* 2003). As a consequence there is a build-up of related toxins in the body, and these in turn may damage the brain (Waisbren *et al*. 2007). However, in many countries, newborn children are tested in the first week of life to identify PKU using a 'heel prick' blood test. Treatment starts immediately if PKU is identified in the newborn child. Therefore, autism due to PKU has become very rare (Therrell and Adams 2007).

1.2.2.3 Susceptibility genes

In spite of vigorous genetic studies, no single causative or susceptibility gene common in autism has been identified. Thus, multiple susceptibility genes in interaction are considered to account for the disorder. Since the identity and number of genes involved remain unknown, the wide phenotypic variability of autism likely reflects the interaction of multiple genes within an individual's genome and the existence of distinct genes and gene combinations among those affected (Folstein and Rosen-Sheidley 2001).

There are three main approaches to identifying chromosomal regions likely to contain relevant genes. These include: Whole genome screens that search for linkage of autism to shared genetic markers in populations of multiplex families (families with >1 affected family member), cytogenetic studies that may guide molecular studies by pointing to relevant inherited chromosomal abnormalities in affected individuals and their families, as well as evaluation of candidate genes known to affect brain development in these significantly linked regions (Bartlett *et al*. 2005).

Data from whole-genome screens in multiplex families suggest interactions of at least 10 genes in the causation of autism. Scientists have also discovered that a putative speech and language region at 7q31-q33 seems most strongly linked to autism, with linkages to multiple other loci under investigation. Cytogenetic abnormalities at the 15q11-q13 locus are fairly frequent in people with autism, and a chromosome 15 phenotype was described in individuals with chromosome 15 duplications (Ashley-Koch *et al.* 2006). Among other candidate genes known to be involved in autism are the neuroligin 3 (*NLGN3*), Forkhead box protein P2 *(FOXP2)* and the reelin (*RELN*) gene at 7q22 *(table 1.1)* [Bartlett *et al*. 2005].

Table 1.1: Candidate susceptibility genes in autism (www.genetests.org)

Gene Symbol	Chromosome Locus	Gene/Protein Function	References		Test Availability
			Pro [1]	Con [2]	
SLC25A12	2q24	Mitochondrial aspartate/glutamate carrier	Ramoz et al 2004		
C4B	6p21	Complement component	Odell et al 2005		
GLO1	6p21	Zinc metalloenzyme scavenges oxoaldehydes	Junaid et al 2004		Research only
GRIK2	6q21	Glutamate receptor 6 involved in neural development	Jamain et al 2002 , Shuang et al 2004		
HOXA1	7p15-p14.2	Homeobox gene involved in hindbrain development	Ingram et al 2000 , Conciatori et al 2004	Devlin et al 2002 , Li et al 2002 , Talebizadeh et al 2002 , Collins et al 2003 , Romano et al 2003 , Gallagher et al 2004	
RELN	7q22.1	Signaling protein involved in neuron migration	Persico et al 2001 , McCoy et al 2001, Zhang et al 2002	Krebs et al 2002 , Devlin et al 2004 , Li et al 2004	
WNT2	7q31	Signaling proteins involved in embryonic patterning, cell proliferation and cell determination	Wassink et al 2001 , Hutcheson et al 2004	McCoy et al 2002 , Li et al 2004	
FOXP2	7q31	Transcription factor involved in embryogenesis and neural functioning	Gong et al 2004 , Li et al 2005	Gauthier et al 2003 , Wassink, Piven et al 2002	
UBE2H	7q32	Ubiquitin-dependent proteolytic system enzyme	Vourc'h et al 2003		
EN2	7q36.2	Homeobox gene involved in midbrain and cerebellum development	Petit et al 1995 , Gharani et al 2004	Zhong et al 2003	
PTEN	10q23.31	Tumor suppressor	Butler et al 2005		
HRAS	11p15.5	Oncogene GTPase involved in cell division, differentiation and apoptosis	Herault et al 1995 , Comings et al 1996		
AVPR1A	12q14-q15	Arginine vasopressin receptor involved in social behavior	Kim, Young et al 2002 , Wassink et al 2004		
UBE3A	15q11-q13	Angelman syndrome causative gene encodes ubiquitin protein ligase	Nurmi et al 2001 , Jiang et al 2004	Nurmi, Dowd et al 2003	
ATP10C	15q11.2-q12	Phospholipid transporter involved in CNS signaling	Nurmi, Amin et al 2003	Kim, Herzing et al 2002	

GABRB3, *GABRA5*, *GABRG3*	15q11.2-q12	GABA receptor subunits	Cook et al 1998 ; Martin et al 2000 ; Nurmi et al 2001 ; Buxbaum et al 2002 ; Nurmi, Dowd et al 2003 ; Menold et al 2001 ; Shao et al 2003 ; McCauley et al 2004	Nurmi et al 2001 , Maestrini et al 1999 , Salmon et al 1999	
SLC6A4	17q11.1-q12	Serotonin transporter	Cook et al 1997 , Klauck et al 1997 , Yirmiya et al 2001 , Tordjman et al 2001 , Kim, Cox et al 2002 , Conroy et al 2004 , Mulder et al 2005	Persico et al 2000 , Persico et al 2002 , McCauley et al 2004	
NF1	17q11.2	Ras protein regulation	Mbarek et al 1999 , Marui et al 2004	Plank et al 2001	
HOXB1	17q21-q22	Homeobox gene involved in hindbrain development	Ingram et al 2000	Li et al 2002 , Romano et al 2003 , Gallagher et al 2004	
APOE2	19q13.2	Lipoprotein receptor involved in neuronal migration and lipid transport	Persico et al 2004		
ADA	20q13.12	Purine metabolism and immune response	Young et al 1999 , Kim, Young et al 2002		
NLGN3	Xq28 ?13	Neural synapse formation	Jamain et al 2003		Clinical Testing
NLGN4X	Xp22.33	Neural synapse formation	Jamain et al 2003 , Laumonnier et al 2004	Talebizadeh et al 2004 , Gauthier et al 2005 , Vincent et al 2004	
ARX	Xp22.13	Homeobox gene	Stromme et al 2002 , Turner et al 2002		Research only

*** List of gene names are in glossary section**
Based on a variety of analysis types including linkage, association, allele sharing, transmission disequilibrium, subset analysis using SNPs, coding polymorphisms, and specific mutations
1. Pro = study supporting candidate gene assignment
2. Con = study not supporting candidate gene assignment

1.3 SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs)

SNP's common variations that occur at a frequency in every 300 nucleotides, which means there are roughly 10 million SNPs in the human genome. These variations can be used to track inheritance in families. Each SNP represents a difference in a nucleotide. For example, a SNP may replace the nucleotide cytosine (C) with the nucleotide thymine (T) in a certain stretch of DNA. SNPs occur normally throughout a person's DNA. Most commonly, these

variations are found in the DNA between genes. They can act as biological markers, helping scientists locate genes that are associated with various diseases. When SNPs occur within a gene or in a regulatory region near a gene, they may play a more direct role in disease by affecting the gene's function (Aritoshi *et al.* 2004).

Most SNPs have no effect on health or development. However, some of these genetic differences have proven to be very important in the study of human health. Researchers have found SNPs that may help predict an individual's response to certain drugs, susceptibility to environmental factors such as toxins, and risk of developing particular diseases. SNPs can also be used to track the inheritance of disease genes within families. Future studies will work to identify SNPs associated with complex diseases such as heart disease, diabetes, and cancer (Shen *et al.* 2009).

Several types of genetics variations occur in humans; namely: insertions and deletions of repetitive DNA (e.g. Fragile X Syndrome, Huntington's Disease), smaller duplications and deletions of genes (e.g. lactase) and point mutations that result in single base pair substitutions or deletions (e.g. Sickle Cell Anaemia) [*fig. 1.3*]. Each of these types of variation requires different diagnostic tests. The earliest, common SNP diagnostics were Restriction Fragment Length Polymorphisms (RFLP) tests and Polymerase Chain Reaction (PCR). Since SNP's have become more understood, the methods for detecting them have been rapidly modified and improved (Fors *et al*. 2000). One such advanced method for SNP detection is known as Taqman® SNP Genotyping Assay (Morita *et al*. 2006)

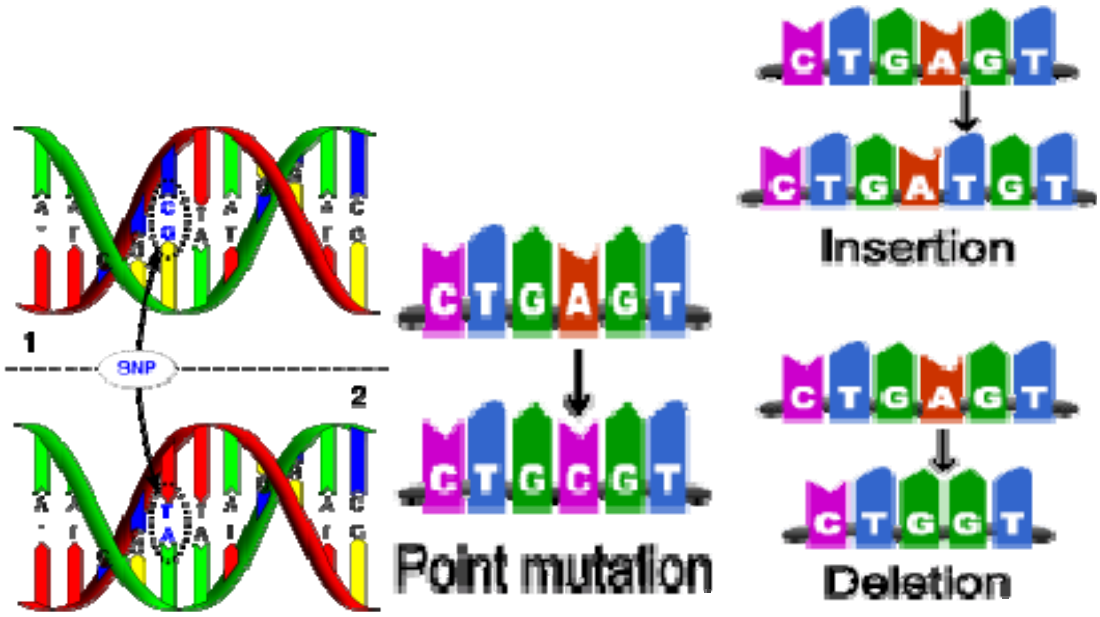


Fig. 1.3: Different forms of genetic variations (SNPS) (http://urgi.versailles.inra.fr/projects/GnpSNP/general_documentation.php)

1.3.1 SNPs associated with Autism

Turunen *et al.* 2008 reported that two SNPs (rs2056202 and rs2292813) within the Mitochondrial Aspartate/Glutamate Carrier SLC25A12 were shown to be strongly associated with autism. Family-based association analysis was performed in two samples with different phenotypic characteristics. The samples included 97 Finnish families with autism and 29 extended families with Asperger syndrome (AS). They detected an association at rs2292813 (FBAT, P=0.0018) in the autism samples but no association was found in the AS samples.

Another study done by Rehstrom *et al.* (2008) showed a significant linkage peak identified at 3q25-27 where a genome-wide linkage screen for ASD in Finnish families was performed. Eleven positional and functionally relevant candidate genes at 3q25-27 were tested for association with autistic disorder. Genotypes of 125 SNPs were determined in 97 families where at least one individual was affected with autism. The most significant association was observed using two non-synonymous SNPs in HTR3C; namely: rs6766410 and rs6807362.

Sequencing revealed no other variants in the coding region or splice sites of HTR3C. They proposed that HTR3C represents a novel candidate locus for ASDs and should be tested in other populations.

Vernes *et al.* 2008 hypothesized that neural pathways downstream of *FOXP2* gene influences more common phenotypes, such as specific language impairment. They tested for associations between SNPs in this gene and language deficits in a well-characterized set of 184 families affected with specific language impairment evident in autistic individuals. They found that *FOXP2* down-regulates the *CNTNAP2* gene, which is expressed in the developing human cortex. On analyzing *CNTNAP2* polymorphisms in children with typical specific language impairment, they detected significant associations with nonsense-word repetition, a heritable behavioural marker of this disorder at rs17236239. This region coincides with one associated with language delays in children with autism. They concluded that the *FOXP2-CNTNAP2* pathway provides a mechanistic link between distinct syndromes, such as autism, which involves disrupted language.

1.4 THE REELIN (*RELN*) GENE

The *RELN* gene consists of 65 exons spanning approximately 450 kb and is mapped at chromosome 7q22 (*fig. 1.4*). It is found in the spinal cord, brain, blood and other body organs and tissues. Reelin plays a pivotal role in the development of the cerebral cortex, cerebellum, hippocampus and several brainstem nuclei (Persico *et al.* 2001). One of the most distinct effects of the *RELN* deletion is abnormal formation of the cerebral cortex in reeler mice. Studies have identified *RELN* as necessary for proper formation of brain. This indicates that *RELN* abnormalities could contribute to the aetiology for several neurogenetic diseases. *RELN* protein levels are significantly reduced in multiple brain areas of patients with

schizophrenia and bipolar disorder. This suggests that persisting low *RELN* levels in the developed brain increases vulnerability to schizophrenia, thus inducing damage (Kim and Webster 2009). Similar abnormalities have been seen for *RELN* levels in autistic individuals. Blood levels of unprocessed reelin are significantly reduced in autistic twins, their fathers, their mothers, and their phenotypically normal siblings versus controls (Fatemi *et al.* 2002)

Serajee *et al.* 2006 reported that two SNPs in intron 59 (rs736707) and exon 22 (rs362691), located on the *RELN* gene, has shown significant association in individuals with autism. Their study was based on sample analysis of Caucasian individuals ascertained through the Autism Genetics Resource Exchange (AGRE). They suggested a role for *RELN* as a candidate gene in the aetiology of autism. My study was based on these two SNP's in order to detect whether they have a significant association in the South African autistic population.

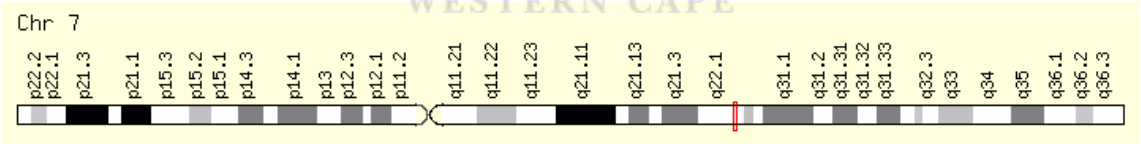


Fig. 1.4: *RELN* gene located on chromosome 7q22 (www.genecards.org)

1.5 WHAT IS MICROARRAY?

DNA microarray analysis is one of the fastest-growing technologies in the field of genetic research. It allows for the measurement of every gene that is present in a cell or tissue sample, thus indicating the biological processes occurring in a cell. By comparing gene expression profiles from two different cell types, one could detect what makes cells different from one another (Gibson 2003). Insights into disease have been one of the most beneficial results of microarray technology. The recognition of the reduction of gene expression during

a disease can point researchers in important directions. This is because genes that are lost during a disease are typically involved in a cellular function that directly or indirectly prevents the disease from occurring. Therefore, identification of these genes with a microarray brings about the potential for targeted therapies (Netterwald 2006).

1.5.1 Microarray techniques

1.5.1.1 Oligonucleotide arrays

Oligonucleotide arrays use small 25 base pair gene fragmented DNA to be spotted onto an array. Eleven to 16 copies of the DNA are placed onto the array. These DNA fragments, called probes, are selected in such a way that they possess little cross-reactivity with other genes. Hence, non-specific hybridization will be minimized. However, some non-specific hybridization is bound to occur, therefore, a second probe that is identical to the first (except for a mismatched base at its centre) is placed next to the first. This is called the Perfect Match/Mismatch (PM/MM) probe strategy. The arrays are printed using a combination of photolithography chemistry. This technique allows the probes to generate simultaneously and relatively quickly (Miller and Tang 2009).

Production begins with a quartz wafer. This wafer is washed with a blocking compound that can be removed by exposure to light. Then, a mask is used, which allows light to pass through areas where a specific nucleotide is needed. The quartz wafer is then washed with a solution of the desired nucleotide that is linked to the same blocking compound, causing the nucleotide to attach to the probes that were exposed to light, while the nucleotide-attached blocking compound ensures that all of the probes are protected again. The array is now ready for a new mask and the addition of a new nucleotide, a process that is repeated until all of the probes are complete (Primrose and Twyman 2006).

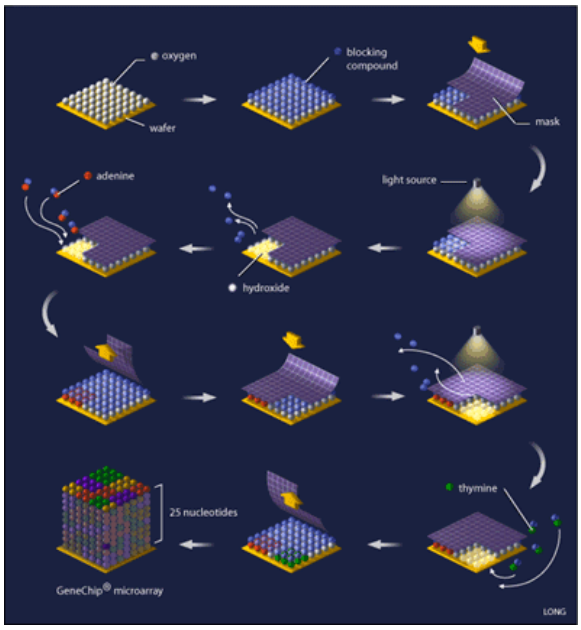


Fig. 1.5: Production of an Affymetrix Genechip (http://www.scq.ubc.ca/spot-your-genes-an-overview-of-the-microarray/)

Once the oligonucleotide array is constructed, it is ready for use in the laboratory. The array is generally used to quantify the amount of mRNA in a single sample or to compare two different samples hybridized to two separate arrays. Samples are prepared by extracting mRNA from a specific sample and turning it into back into DNA through a process that involves the reverse transcription of the mRNA into cDNA. This is followed by a labelling step during which the cDNA is then transcribed to cRNA while incorporating a label (e.g. biotin). Once labelled, the sample of cRNAs can be hybridized to the array and bound by the various oligonucleotide probes. A staining reaction is also performed in order to visualize the amount of hybridization (*fig. 1.6*) [Primrose and Twyman 2006; Leung and Cavalieri 2003].

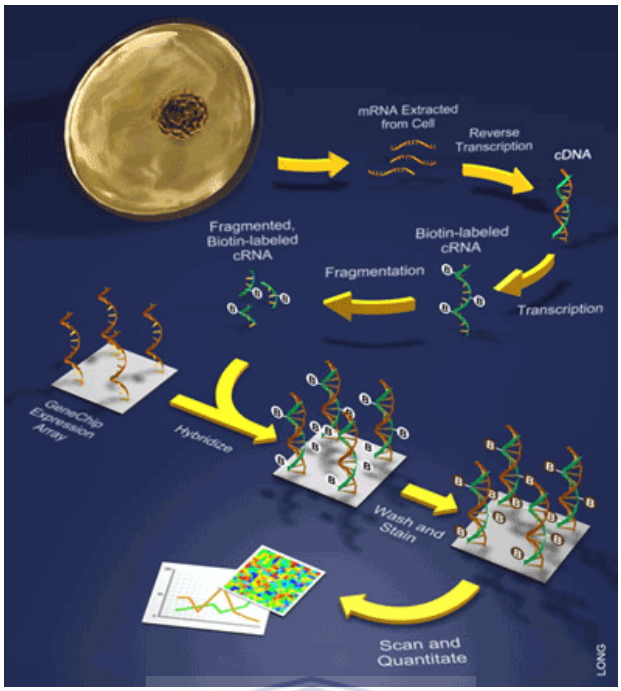
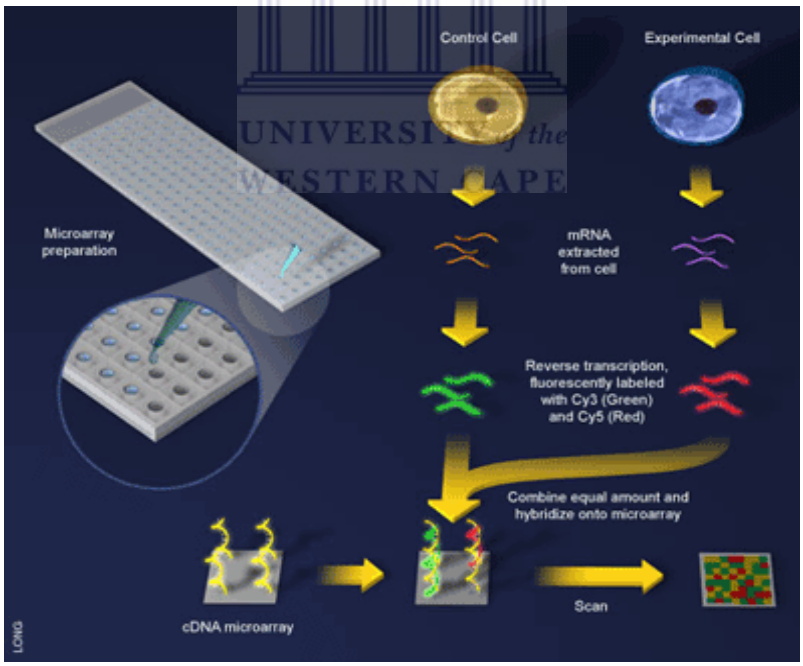


Fig. 1.6: The use of Oligonucleotide arrays. mRNA is extracted from cells and amplified through a process that labels the RNA for analysis. The sample is then applied to an array and any bound RNA stained (http://www.scq.ubc.ca/spot-your-genes-an-overview-of-the-microarray/)

1.5.1.2 cDNA arrays

A cDNA array uses the same principle as the oligonucleotide array. However, the probes are larger pieces of DNA that are complementary to the genes of interest. These are called cDNA. These cDNA probes needed for making the array can be generated from a commercially available cDNA library. Polymerase Chain Reaction (PCR) can also be used to amplify specific genes from genomic DNA to generate cDNA probes. Once the cDNA probes are prepared they can be mechanically spotted onto a glass slide. This normally occurs in duplicate, in order to serve as controls. An experiment using a cDNA microarray involves the preparation of two samples for hybridization to the array: namely; a control sample and an experimental sample (Leung and Cavalieri 2003). These samples are prepared the same way for the other array, with mRNA being extracted from cells and reverse transcribed into cDNA. There is, however, one exception; during reverse transcription, a fluorescent dye is

incorporated into the newly formed cDNA. A different dye is used to label different samples. For example, the control sample can be labelled with a green-fluorescing dye and the experimental sample labelled with a red-fluorescing dye. Since the samples are labelled with different colours, they can be combined and hybridized to the microarray at one time. The two samples will competitively bind to the probes on the array and the sample containing more gene expression for a particular probe will 'win'. That is, if there is more of an mRNA transcript in the experimental sample than in the control sample then more red dye will bind to the probe on the array and the spot will fluoresce red. If there is more control transcript, the reverse will happen and the spot will fluoresce green. When the two samples have the same amount of transcript, the dyes will cancel each other out and the spot will fluoresce yellow (*fig. 1.7*) [Sharlon *et al.* 1996].



<u>Fig. 1.7:</u> An example of a cDNA Microarray experiment. RNA is extracted from two different samples and converted into complementary DNA (cDNA), during which the DNA is labelled with florescent compounds. Differences in gene expression are revealed by fluorescent patterns on the array (http://www.scq.ubc.ca/spot-your-genes-an-overview-of-the-microarray/)

Table 1.2: Oligonucleotide Array vs cDNA Array

OLIGONUCLEOTIDE ARRAY	cDNA ARRAY
Greater hybridization specificity due to PM/MM probe design	*Eliminates the need for probe design*
More specific florescent detection	*Entire genome on the array is represented easily*
Useful for monitoring expression levels of a differentially sliced transcript	*Useful for analysis of gene expression on a global level*

1.5.2 Recent microarray studies in autism

Marshall *et al.* 2008 identified structural variation (copy number variation [CNV] including deletion and duplication, translocation, inversion) of chromosomes in some individuals with autism, but the full etiologic role is unknown. They performed genome-wide assessment for structural abnormalities in 427 unrelated ASD cases via single-nucleotide polymorphism microarrays. The microarray data revealed 277 unbalanced CNVs in 44% of ASD families that were not present in 500 controls.

Microarray analysis was also used to compare the mRNA expression profile in lymphoblastoid cells from males with autism due to a fragile X mutation (FMR1-FM), or a 15q11-q13 duplication [dup(15q)], and non-autistic controls. Gene expression profiles distinguished autism from controls and separated individuals with autism based on their genetic aetiology. Sixty-eight genes were identified that were dysregulated in the autistic samples with FMR1-FM and dup (15q) [Nishimura *et al.* 2007].

1.6 AIMS OF THE PROJECT

The aims were:

- To determine whether 2 SNPs in the *RELN* gene are associated with autism in a South African population using the Taqman® SNP Genotyping Assay.
- To detect candidate genes that are over and under-expressed in the samples taken from a South African Caucasian autistic group and compare those with samples taken from a healthy Caucasian group using cDNA microarray (pilot study).

CHAPTER 2

Materials and Methods

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2.1 ETHICAL CLEARANCE

Ethical clearance was obtained from the UWC ethics committee and permission to work with autistic individuals was obtained from the Western Cape Education department. Approval for blood sample collection for the microarray study was obtained from the principals of Vera school and Groote Schuur, Cape Town. Approval for DNA cheek cells for the Taqman® study was obtained from principals of various autistic and healthy schools in Cape Town, Gauteng and Kwazulu-Natal. Letters *(Appendix I and II)* and consent forms *(Appendix III*) were issued to the willing parents of the learners.

2.2 SAMPLE COLLECTION

Funding allowed for only one ethnic group to be used in this study, hence blood samples were collected from three high-functioning Caucasian males and three low-functioning Caucasian males affected with autism. Blood samples were also taken from three healthy Caucasian males, which were used as controls. All children were between the ages of 8 and 12 years old. The high-functioning and low-functioning status was based on the Griffiths test, which helps to pinpoint areas of strength and difficulty of their developmental ability (Benatti *et al.* 1986). The blood samples were drawn by three senior sisters and stored in an EDTA tube (BD-Plymouth, UK) at -80^{o}C.

DNA cheek samples were collected from autistic individuals from Cape Town, Gauteng and Kwazulu-Natal. Control samples were collected from ordinary primary schools in Cape Town. Samples were collected from 171 autistic individuals, 249 normal individuals and 11 families. Of the total of 420 samples collected 176 were Black (of African descent), 114 were Caucasian (white) and 128 were Coloured (of mixed ancestry). Individuals partaking in the study were swabbed by taking a sterile swab (Sterile Dacron Polyester Tipped Applicators,

South Africa) and rubbing it against the inside of their cheek for 1 minute and placing it into a labelled sterile 15ml grinder tube (Greiner Bio-One, Austria). This was done for both cheeks.

2.3 RNA ISOLATION FROM BLOOD

RNA for the microarray study was isolated using the High Pure RNA Isolation Kit (Roche, Switzerland). The protocol was as follows:

1 ml of Red Blood Cell Lysis Buffer was added to a sterile 1.5 ml microfuge tube. Thereafter, 500 µl of human blood sample was added to the tube and the solution was mixed by inversion. The microfuge tube containing the sample was then manually inverted periodically for 10 min. It was then centrifuged (Eppendorf Centrifuge 5415D, Germany) for 5 min at 2500rpm. Once the red, clear supernatant was carefully removed, 1 ml of red cell lysis buffer was added to the white pellet and resuspended by flicking the tube. The sample was then centrifuged (Eppendorf Centrifuge 5415D, Germany) for 3 min at 25000rpm. The supernatant was once again carefully removed and the white pellet was resuspended in 200 µl PBS. To the tube, 500 µl lysis/binding buffer was added and the sample was vortexed for 15 sec. The entire sample was then added to a high pure filter tube, which was then inserted into a collection tube. The tube assembly was then centrifuged (Eppendorf Centrifuge 5415D, Germany) for 15 sec at 500rpm. The filter tube was then removed from the collection tube and the flow through was discarded. The filter tube was once again inserted into the used collection tube and 90 µl of DNase incubation buffer, together with 10 µl of DNase1 mix, was added to the filter tube. The tube was then incubated for 15 min at room temperature.

Thereafter, 500 µl of Wash Buffer I was added to the filter tube and centrifuged (Eppendorf Centrifuge 5415D, Germany) for 15 sec at 500rpm. The flow through was then discarded and the filter tube was once again inserted into the used collection tube. The previous step was then repeated using Wash Buffer II. A further 200 µl of Wash Buffer II was added to the filter tube and centrifuged (Eppendorf Centrifuge 5415D, Germany) for 2 min at 13200rpm. The collection tube was then discarded and the filter tube was inserted into a sterile 1.5 ml microfuge tube. Thereafter, 50 µl of elution buffer was added to the filter tube and centrifuged (Eppendorf Centrifuge 5415D, Germany) for 1 min. The microfuge tube then contained the eluted RNA. The eluted RNA was stored at –80^{o}C before being used for cDNA synthesis.

2.4 DNA EXTRACTION FROM CHEEK CELLS

DNA was extracted from the swabs using the BuccalAmp™ DNA Extraction Kit (Epicentre, USA). The protocol was as follows:

A piece of the swab tip was cut using a sterile blade and placed into a 2 ml microfuge tube to which 250 µl of QuickExtract DNA extraction solution 1.0 was added. The tubes were then incubated at 65^{o}C for 1 min. They were then mixed by vortexing for 15 sec. The tubes were then incubated at 98^{o}C for 4 min. Thereafter they were vortexed once again for 15 sec. The swab tip was then removed with a sterilized tweezer and the tubes were stored at –20^{o}C.

2.5 QUANTITATION OF DNA

The concentration of each DNA sample was determined using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA).

2.6 TAQMAN® SNP GENOTYPING ASSAY

Genotypes were determined using the Taqman® SNP Validated Genotyping Assay (Applied Biosystems, USA). This is a real-time PCR method that accumulates amplified product during the exponential phase of the PCR cycle. Combining thermal cycling, fluorescence detection, and application-specific software it enables the cycle-by-cycle detection of the increase in the amount of nucleic acid sequences. Each SNP assay consists of two primers used for amplification as well as two minor groove binding (MGB) fluorescent probes used for allele detection. The two probes, vic and fam, detect the presence of alleles 1 and 2, respectively. Each probe contains a reporter dye at the 5' end and a quencher at the 3' end. When vic or fam probes fluoresce, this indicates homozygosity for the specific allele. When both probes fluoresce at the same time, it indicates heterozygosity for both alleles (***fig. 2.1***). Quantitative results are available directly after PCR with no additional purification or analysis steps (Shen *et al.* 2009). The Taqman® method has been shown to be very successful in the detection of SNPs. It has been used to characterize the APOE haplotypes in Alzheimer's disease (Livak 1999) and has been used to identify an association between the 5'UTR region and autism (Skaar *et al.* 2005).

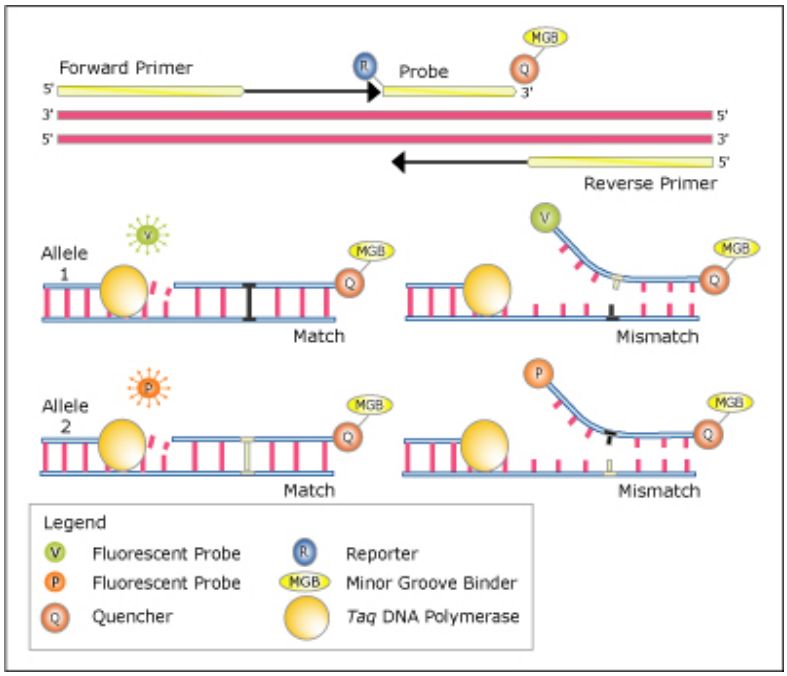

Fig. 2.1: Schematic diagram of the Taqman® SNP Genotyping Assay technique (http://www.servicexs.nl/dbres/?t=158)

2.6 SNP SELECTION AND PRIMER DESIGN

The exonic SNP (rs3622691) and intronic SNP (rs736707) on the *RELN* gene was identified from a previous experiment carried out by Serajee *et al.* (2006). The SNP sequence was blasted against the *Homo sapiens* genome using the BLAST tool at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were then manufactured by Applied Biosystems according to the specifications as indicated by the File Builder application. The Taqman® Custom Genotyping Assay probes (40x) were labelled VIC and FAM to the following sequences respectively:

Exon 22: 5'- TTCTTTGGGTGATTTCATCCTG -3' and 5'- CCGTCTCTGT TTGTATGTGCG - 3'

Intron 59: 5'- GCAGGGCTGACAGGTTACAC -3' and 5'-TGGTCTCCTC TATCAAAGTT GGC -3'

2.7 PCR PREPARATION

All samples were diluted with double distilled water (ddH20) to a final concentration of 20 ng/µl and then aliquoted individually into 96-deepwell plates (Applied Biosystems, USA). The first row of each 96 deep-well plate contained he negative control, which consisted of 50 µl ddh20. A PCR mastermix was prepared by adding 1650 µl of Taqman® Universal Genotyping Mastermix (Applied Biosystems, USA) to a microfuge tube. To the tube, 165 µl of 20x Taqman® SNP Custom Genotyping Assay (Applied Biosystems, USA) was added, along with 825 µl of SABAX water. The solution was then vortexed for 10 sec. The Microfuge epMotion 5070 automated pipetting system (Applied Biosystems, USA) was then used to transfer the samples and mastermix into the 384-well plate (Applied Biosystems, USA) yielding a final volume of 5 µl.

2.8 PCR AMPLIFICATION AND TAQMAN® READING

The Perkin Elmer 9700 PCR System (Applied Biosystems, USA) was used for PCR amplification. PCR parameters were as follows:

- Initial denaturation for 10 min at 95°C
- Denaturation for 15 sec at 92°C
- Annealing for 1 min at 60°C
- Extension for 1 min at 60°C

} 40 cycles

Results from the amplified PCR products were viewed using the Applied Biosystems 7900HT Real-Time PCR System.

2.9 MICROARRAY PREPARATION

10 µl of each sample analysed on the Agilent 2100 Bioanalyzer (Inqaba Biotech, South Africa) in order to detect whether the samples had the sufficient concentration of 10 ng/µl (*Appendix IV*). Samples that did not meet the specified requirements were optimized and re-sent to be analysed. Once all samples met the specified requirements, they were shipped to Nimblegen, Iceland where they were subjected to high throughput microarray analysis.

2.10 STATISTICAL ANALYSIS

2.10.1 Taqman® analysis

Allele frequencies for the A, G and C variants were calculated using the Hardy-Weinberg equation. The Fishers Exact test was performed to compare the number of alleles of the South African autistic group to the South African control group at a confidence interval of 95% (Esau *et al*. 2008) and the odds ratio was calculated using the standard 3-by-2 table method (http://ihg2.helmholtz-muenchen.de ihg/snps.html)

2.10.2 Microarray analysis

The microarray data were analysed using the ArrayStar3^{TM} software for gene expression analysis (DNASTAR inc, USA). The F-ANOVA test was used to compare gene expression values for each gene in the samples. The test was set to detect which genes were more than 95% significant in all samples.

CHAPTER 3

Results

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3.1 SNP DATA FOR AUTISTIC AND CONTROL SAMPLES

Genotyping results for SNPs in exon 22 (rs3622691) and intron 59 (rs736707) were obtained following PCR amplification and viewing with the Applied Biosystems 7900HT Real-Time PCR System. The data was displayed in graphical output files (*figs. 3.1* and *3.6)* for the autistic and control population, as well as the non-template controls.

3.1.1 Taqman® genotyping data for rs3622691

In the autistic group, 148 samples (62 Black, 43 Caucasian and 43 Coloured) were genotyped. In the control group, 203 samples (92 Black, 49 Caucasian and 62 Coloured) were genotyped. For the Taqman® SNP Assay, blue dots indicate homozygosity for allele G, red dots indicate homozygosity for allele C and green dots indicate heterozygosity for alleles CG (*fig. 3.1*). No amplification was observed in the non-template controls, thus ruling out the possibility of contamination. The frequencies of the case-control genotypes for the different population groups investigated in this study are provided in *figs. 3.2*, *3.3* and *table 3.1*. Genotype data was used to determine the G- and C- allele frequencies in the different study populations (*table 3.1; figs. 3.4* and *3.5*)

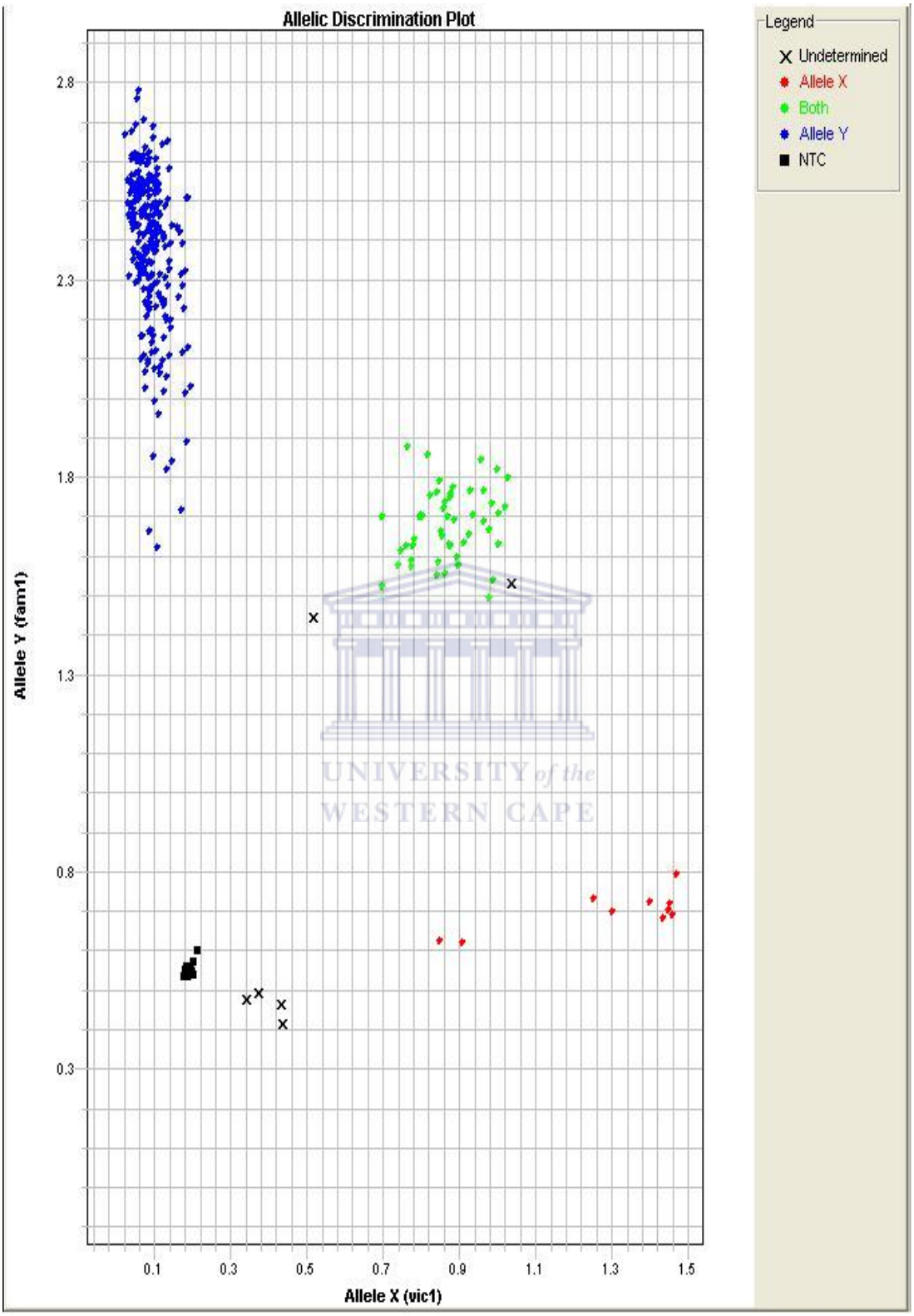

Fig. 3.1: Representation of Taqman® SNP Genotyping Assay data analysis for rs 3622691. Allele X=C and Allele Y=G. The red dots indicate homozygosity for allele C in both SNPs and the green dots indicates heterozygosity for alleles CG (rs3622691). The black cluster represents the non-template controls.

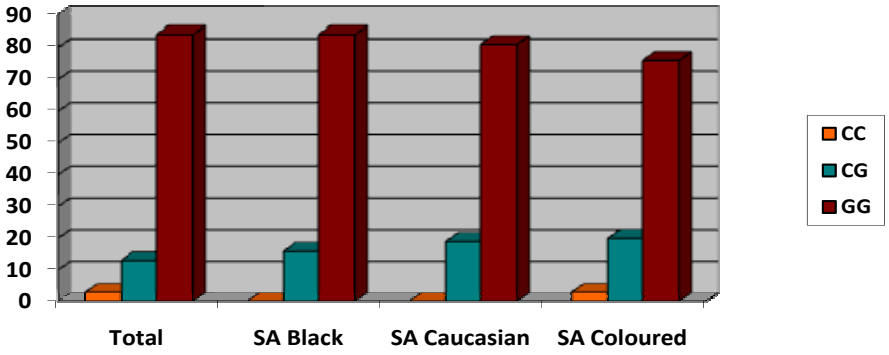

Fig. 3.2: Percentage of genotype distributions for rs3622691 on *RELN* in an autistic South African population.

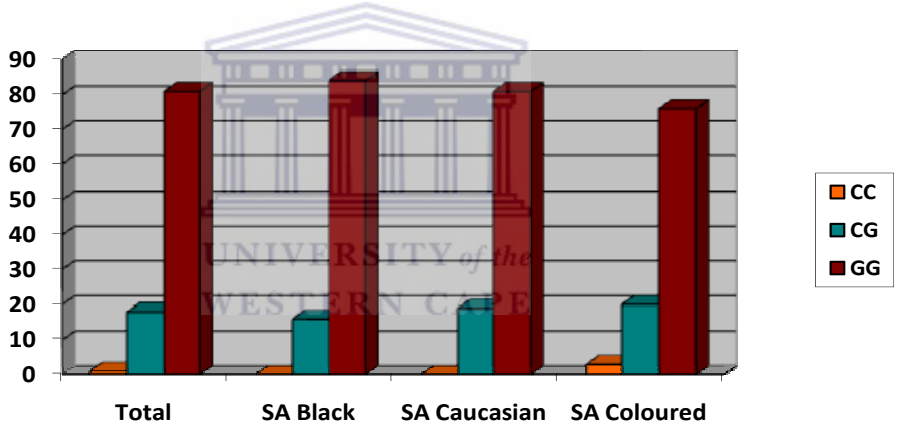

Fig. 3.3: Percentage of genotype distributions for rs3622691 on the *RELN* gene in a healthy South African population.

Table 3.1: Allelic distributions of rs3622691 on *RELN* in 3 distinct ethnic groups in the South African autistic and healthy population.

Ethnic Group	Number	Genotype (%)			P-value	Allele (%)		P-value
		G/G	G/C	C/C		C Allele	G Allele	
Total South African								
Autistic	148	124 (84)	19 (13)	5 (3)	0.11326	29 (10)	267 (90)	0.89535
Control	203	164 (81)	37 (18)	2 (1)		41 (10)	365 (90)	
OR (95% CI)		1	0.20 (0.04-1.16)	0.30 (0.06-1.59)				
SA Black								
Autistic	60	54 (90)	5 (8)	1 (2)	0.31662	7 (10)	64 (90)	0.50647
Control	96	81 (84)	15 (16)	0 (0)		15 (8)	177 (92)	
OR (95% CI)		1	0.50 (0.17-1.46)	4.49 (0.18-112.17)				
SA Caucasian								
Autistic	45	38 (84)	5 (11)	2 (4)		9 (10)	81 (90)	
Control	48	39 (81)	9 (19)	0 (0)		9 (9)	87 (91)	
OR (95% CI)		1	0.57 0.18-1.86	5.13 (0.24-110.36)	0.68334			0.88544
SA Coloured								
Autistic	43	32 (74)	9 (21)	2 (5)		13 (15)	73 (85)	
Control	59	45 (76)	12 (20)	2 (3)		16 (14)	102 (86)	
OR (95% CI)		1	1.06 (0.40-2.80)	1.406 (0.19-10.51)	0.82993			0.75317

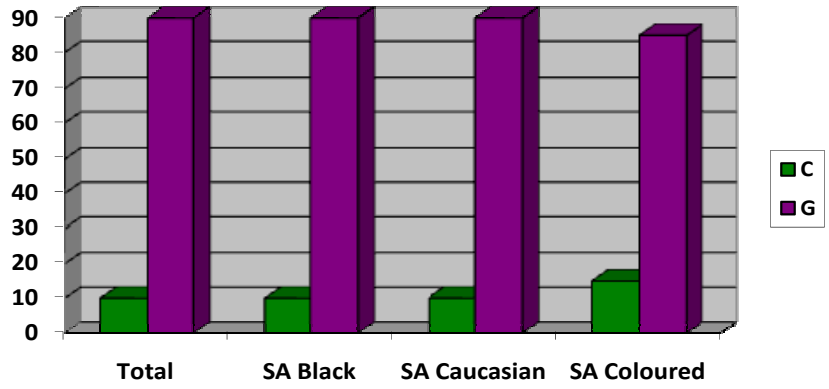

Fig. 3.4: Percentage of the C and G allelic distributions for rs3622691 on *RELN* in an autistic South African population.

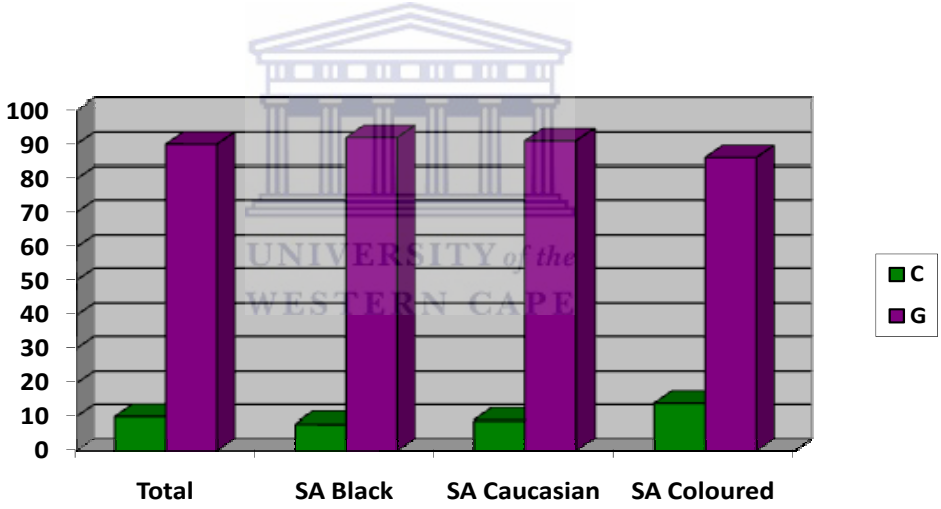

Fig. 3.5: Percentage of the C and G allelic distributions for rs3622691 on *RELN* in a healthy South African population.

3.1.2 Taqman® genotyping data for rs736707

In the autistic group, 140 samples (59 African, 39 Caucasian and 42 Coloured) were genotyped. In the control group, 216 samples (98 Black, 56 Caucasian and 62 Coloured) were genotyped. For the Taqman® SNP Assay, blue dots indicate homozygosity for allele G, red dots indicate homozygosity for allele A and green dots indicate heterozygosity for alleles AG (*fig 3.6*). No amplification was observed in the non-template controls, thus ruling out the possibility of contamination. The frequencies of the case-control genotypes for the different population groups investigated in this study are provided in *figs. 3.7*, *3.8* and *table 3.2*. Genotype data was used to determine the G- and C- allele frequencies in the different study populations (*table 3.2; figs. 3.9* and *3.10*).

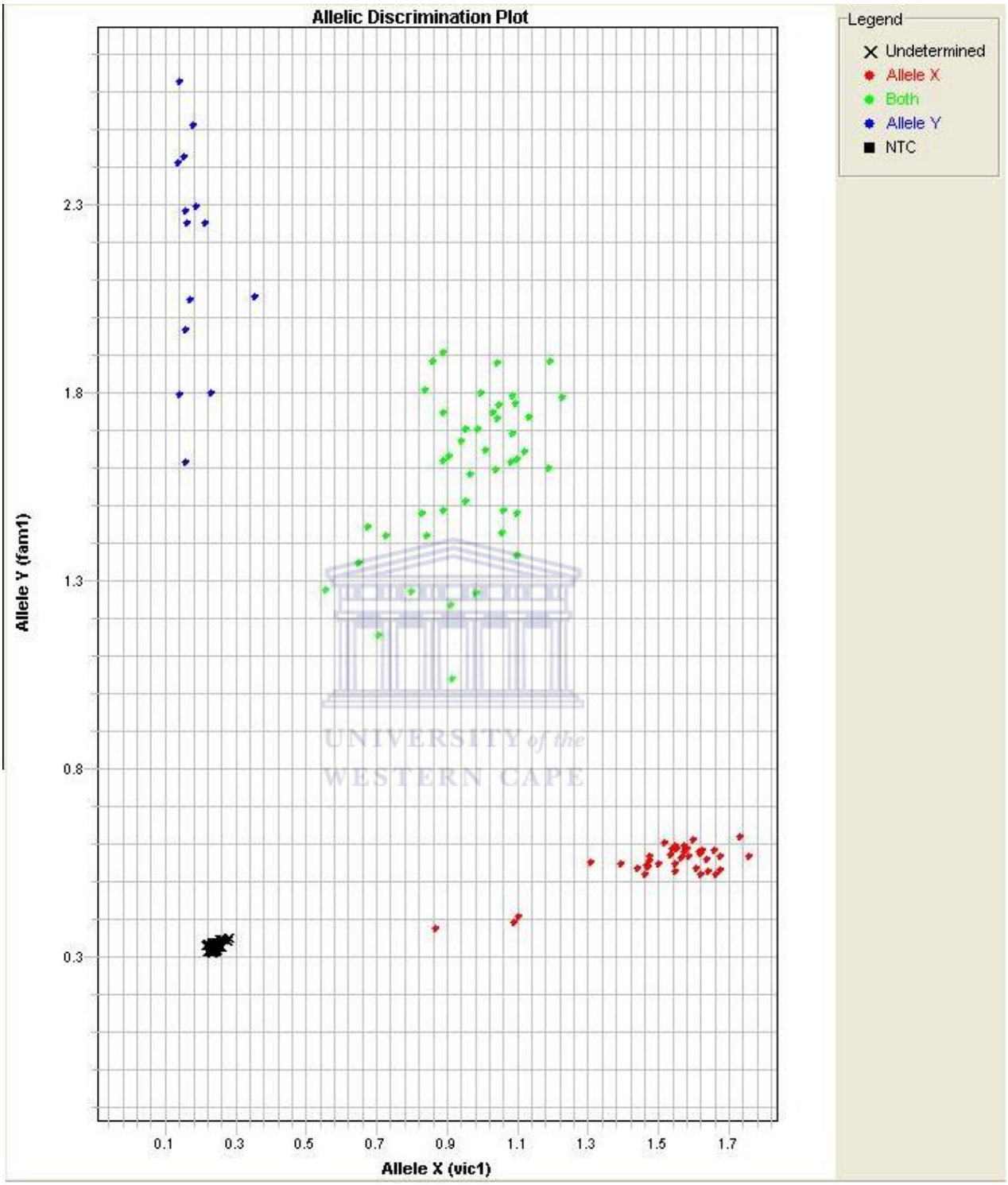

Fig. 3.6: Representation of Taqman® SNP Genotyping Assay data analysis for rs736707. Allele X=A and Allele Y=G. The red dots indicate homozygosity for allele A in both SNPs and the green dots indicates heterozygosity for alleles AG (rs736707). The black cluster represents the non-template controls.

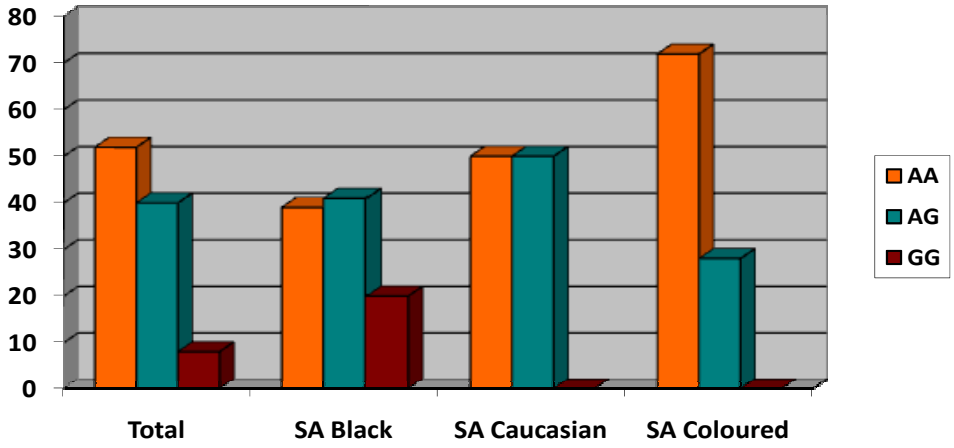

Fig 3.7: Percentage of genotype distributions for rs736707 on *RELN* in an autistic South African population.

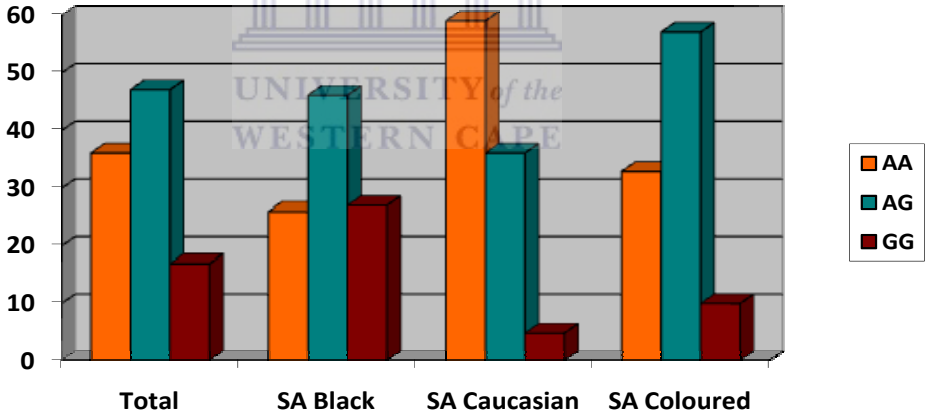

Fig 3.8: Percentage of genotype distributions for rs736707 on *RELN* in a healthy South African population.

Table 3.2: Allelic distributions of rs736707 on *RELN* in 3 distinct ethnic groups in the South African autistic and healthy population.

Ethnic Group	Number	Genotype (%)			P-value	Allele (%)		P-value
		A/A	A/G	G/G		A Allele	G Allele	
Total South African								
Autistic	140	73 (52)	56 (40)	11 (8)	0.00372	202 (72)	78 (28)	0.0009
Control	216	79 (36)	101 (47)	36 (17)		259 (60)	173 (40)	
OR (95% CI)		1	0.60 (0.38-0.95)	0.33 (0.16-0.70)			0.578 (0.42-0.80)	
SA African								
Autistic	56	22 (39)	23 (41)	11 (20)	0.09208	67 (60)	45 (40)	0.08004
Control	99	26 (26)	46(46)	27 (27)		98 (49)	100 (51)	
OR (95% CI)		1	0.60 (0.28-1.26)	0.49 (0.20-1.19)				
SA Caucasian								
Autistic	44	22 (50)	22 (50)	0 (0)	0.37300	66 (75)	22 (25)	0.76913
Control	56	33 (59)	20 (36)	3 (5)		86 (77)	26 (23)	
OR (95% CI)		1	1.650 (0.73-3.71)	0.213 (0.01-4.32)				
SA Coloured								
Autistic	40	29 (72)	11 (28)	0 (0)	0.00009	69 (86)	11 (14)	0.00014
Control	61	20 (33)	35 (57)	6 (10)		75 (61)	47 (39)	
OR (95% CI)		1	0.217 (0.09-0.53)	0.053 (0.003-1.0)			0.254 (0.12-0.53)	

Values in red indicate significant P-values. P < 0.001 indicates high significance.

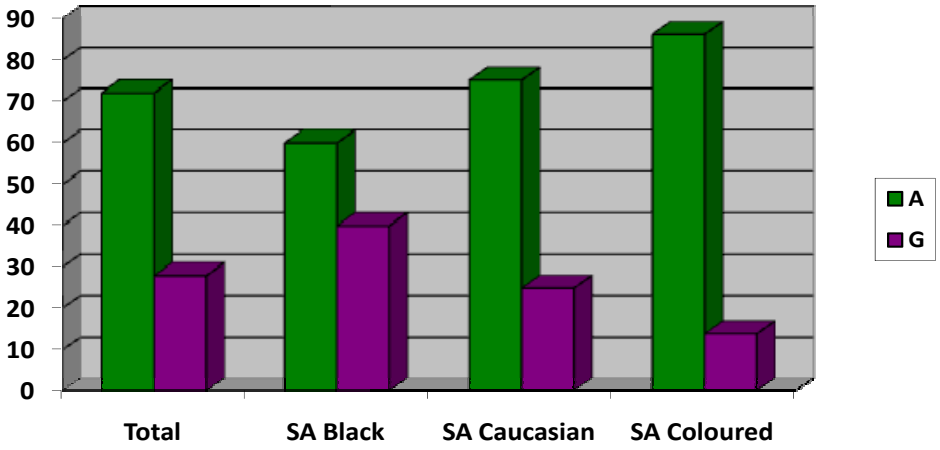

Fig 3.9: Percentage of the A and G allelic distributions for rs736707 on the *RELN* gene in an autistic South African population.

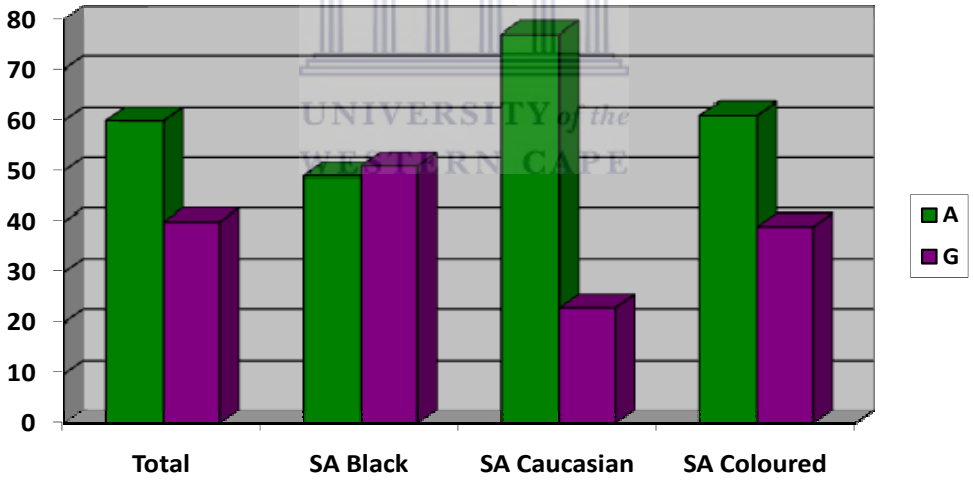

Fig 3.10: Percentage of the A and G allelic distributions for rs736707 on the *RELN* gene in a healthy South African population.

3.1.3 Genotyping analysis

3.1.3.1 rs3622691

In the total (autistic and control) South African (SA) population, more than 70% were homozygous for the GG genotype. The CC genotype was very low in all ethnic groups, with 3% in the total autistic sample group and 1% in the total control group (*table 3.1*). The heterozygous genotype CG was low, with 13% and 18% for the autistic and control groups respectively (*fig. 3.3* and *fig. 3.4*)

The SA total autistic population displayed the GG genotype at 84%, the CG genotype at 13% and the CC genotype at 3% (*table 3.1*). In the SA Black autistic group, 90% of individuals were homozygous for the GG genotype and 84% of the control sample was homozygous for the GG genotype. The heterozygous genotype CG was 8% for the autistic group and 16% for the control. Genotype CC was least prominent, displaying 2% for the autistic sample and 0% for the control sample. For allele frequency analysis in the SA Black autistic and control sample group, the minor C allele was observed in 10% and 8% of individuals, respectively (*table 3.1*).

For the SA Caucasian autistic group, 84% of individuals were homozygous for the GG genotype and 81% for the control group (*table 3.1*). The heterozygous CG genotype was 11% in the autistic group and 19% in the control group. The homozygous CC genotype displayed the lowest value for both samples, with 4% in the autistic sample and 0% in the control sample. Overall, the C allele was prevalent in the 10% of the autistic Caucasian group and 9% in the control group (*table 3.1*).

The SA Coloured population displayed the homozygous GG genotype in 74% of individuals in the autistic sample and 76% in the control sample (*table 3.1*). The heterozygous genotype CG was 21 for the autistic group and 20% for the control. The homozygous CC genotype was very low, indicating 5% for the autistic sample and 3% for the control sample. In contrast to the other groups screened in the present study, the SA mixed group displayed the highest numbers for the C allele, occurring with a frequency of 15% in the autistic group and 14% in the control group (*table 3.1*).

The Fishers exact test indicated no significant difference between the genotype and allele frequencies for the autistic and control groups, with all P-values being above 0.05.

3.1.3.2 rs736707

The SA total autistic population displayed the AA genotype at 52%, the AG genotype at 40% and the CC genotype at 8% (*table 3.2*). In the SA Black, 39% of the autistic group and 26% of the control sample were homozygous for the AA genotype. The heterozygous genotype AG frequency was 41% for the autistic group and 46% for the controls. The GG was observed in 20% of the SA Black autistic population, and in 27% of the control group (*table 3.2*). In the SA Black autistic group, 60% had the A allele as opposed to 40% who had the G allele. In the Black control group, 49% displayed the A allele and 51% displayed the G allele (*table 3.2*).

For the SA Caucasian autistic group, 50% of individuals were homozygous for the AA genotype, 50% were heterozygous for the AG genotype and 0% was homozygous for the GG genotype. For the control group 59% of individuals were homozygous for the AA genotype, 36% was heterozygous for the AG genotype and only 5% was homozygous for the GG

genotype (*table 3.2*). In the SA Caucasian autistic group, the A allele was observed in 75% of individuals and 25% carried the G allele. In the control group, 77% had the A allele and 23% the G allele (*table 3.2*)

The SA Coloured population displayed the homozygous AA genotype in 72% of individuals in the autistic sample and only 33% in the control samples. The heterozygous genotype AG was 28% for the autistic group and 57% for the control group. The homozygous GG genotype was low, observed in only 10% for the control sample and 0% for the autistic sample (*table 3.2*). The A allele was most prevalent in the SA Coloured autistic group, occurring at a frequency of 86%, whereas only 14% displayed the G allele. In the SA Coloured control group, 61% carried the A allele and 39% the G allele (*table 3.2*).

The odds ratio of 0.58 in the SA total group (*table 3.2*) indicates that individuals possessing the G allele are 42% less likely to develop autism. More so, the test also indicates that individuals who possess the G allele and form part of the SA Coloured population is 75% less likely to develop autism. This indicates that in the Coloured population the G allele acts as the protective allele while the A allele indicates predisposition to autism.

3.2 GENE EXPRESSION DATA

3.2.1 Expression analysis

All data was obtained using ArrayStar 3 software (DNASTAR inc, USA). The F-ANOVA test was set to output genes that were more than 95% significant. Twenty-one genes were found in all samples, most of which reside on chromosome 8. The output data indicated start and stop codons for each gene as well as the chromosome number and URL for retrieval of gene information. The list of genes names are in the 'notes' column of the table.

Table 3.3: Candidate genes identified from microarray data (ArrayStar 3 software, DNASTAR inc, USA)

Notes	SEQ_ID	GENE_INFO	ACCESSION	BUILD	FEAT_TYPE	CHROMOSOME	START	STOP	DESCRIPTION	SEQ_URL
BTNL8	AY358523	BLOCK1 /// AY358523|HG18|tr...	AY358523	HG18	transcript	chr5	180258734	1803105...	ExemplarFor 'AY358523'; gene...	http://www.ncbi.nlm.nih.gov/e...
LOC83459	BC090061	BLOCK1 /// BC090061|HG18|tr...	BC090061	HG18	transcript	chrM	1694	2458	ExemplarFor 'BC090061'; gene...	http://www.ncbi.nlm.nih.gov/e...
HNRNPA1	BC103707	BLOCK1 /// BC103707|HG18|tr...	BC103707	HG18	transcript	chr12	52960830	52965295	ExemplarFor 'BC103707'; gene...	http://www.ncbi.nlm.nih.gov/e...
LOC441426	NM_0010137...	BLOCK1 /// NM_001013727|H...	NM_001013727	HG18	transcript	chr9	43124806	43128858	ExemplarFor 'NM_001013727';...	http://www.ncbi.nlm.nih.gov/e...
LOC349196	NM_0010254...	BLOCK1 /// NM_001025473|H...	NM_001025473	HG18	transcript	chr8	7106572	7107007	ExemplarFor 'NM_001025473';...	http://www.ncbi.nlm.nih.gov/e...
DEFB4	NM_004942	BLOCK1 /// NM_004942|HG18|...	NM_004942	HG18	transcript	chr8	7789608	7791647	ExemplarFor 'NM_004942'; ge...	http://www.ncbi.nlm.nih.gov/e...
KIAA0738	NM_014719	BLOCK1 /// NM_014719|HG18|...	NM_014719	HG18	transcript	chr7	143180981	1432301...	ExemplarFor 'NM_014719'; ge...	http://www.ncbi.nlm.nih.gov/e...
FAM90A3	XM_372013	BLOCK1 /// XM_372013|HG18|...	XM_372013	HG18	transcript	chr8	7109457	7113024	ExemplarFor 'XM_372013'; gen...	http://www.ncbi.nlm.nih.gov/e...
FAM90A4	XM_496945	BLOCK1 /// XM_496945|HG18|...	XM_496945	HG18	transcript	chr8	7117143	7120646	ExemplarFor 'XM_496945'; gen...	http://www.ncbi.nlm.nih.gov/e...
FAM90A13	XM_496946	BLOCK1 /// XM_496946|HG18|...	XM_496946	HG18	transcript	chr8	7124701	7128268	ExemplarFor 'XM_496946'; gen...	http://www.ncbi.nlm.nih.gov/e...
FAM90A5	XM_496947	BLOCK1 /// XM_496947|HG18|...	XM_496947	HG18	transcript	chr8	7132323	7135890	ExemplarFor 'XM_496947'; gen...	http://www.ncbi.nlm.nih.gov/e...
FAM90A7	XM_496948	BLOCK1 /// XM_496948|HG18|...	XM_496948	HG18	transcript	chr8	7401075	7404644	ExemplarFor 'XM_496948'; gen...	http://www.ncbi.nlm.nih.gov/e...
FAM90A18	XM_496955	BLOCK1 /// XM_496955|HG18|...	XM_496955	HG18	transcript	chr8	7618022	7621589	ExemplarFor 'XM_496955'; gen...	http://www.ncbi.nlm.nih.gov/e...
FAM90A10	XM_496957	BLOCK1 /// XM_496957|HG18|...	XM_496957	HG18	transcript	chr8	7663908	7667476	ExemplarFor 'XM_496957'; gen...	http://www.ncbi.nlm.nih.gov/e...
LOC643368	XM_926698	BLOCK1 /// XM_926698|HG18|...	XM_926698	HG18	transcript	chr15	88007742	88008297	ExemplarFor 'XM_926698'; gen...	http://www.ncbi.nlm.nih.gov/e...
LOC6437...	XM_927069	BLOCK1 /// XM_927069|HG18|...	XM_927069	HG18	transcript	chr1	544631	555827	ExemplarFor 'XM_927069'; gen...	http://www.ncbi.nlm.nih.gov/e...
LOC645362	XM_928404	BLOCK1 /// XM_928404|HG18|...	XM_928404	HG18	transcript	chr8	7113178	7118027	ExemplarFor 'XM_928404'; gen...	http://www.ncbi.nlm.nih.gov/e...
LOC645489	XM_928514	BLOCK1 /// XM_928514|HG18|...	XM_928514	HG18	transcript	chr8	7391420	7393276	ExemplarFor 'XM_928514'; gen...	http://www.ncbi.nlm.nih.gov/e...
FAM90A14	XM_928665	BLOCK1 /// XM_928665|HG18|...	XM_928665	HG18	transcript	chr8	7610374	7613941	ExemplarFor 'XM_928665'; gen...	http://www.ncbi.nlm.nih.gov/e...
LOC645683	XM_928684	BLOCK1 /// XM_928684|HG18|...	XM_928684	HG18	transcript	chr8	7621743	7626618	ExemplarFor 'XM_928684'; gen...	http://www.ncbi.nlm.nih.gov/e...
LOC645681	XM_928690	BLOCK1 /// XM_928690|HG18|...	XM_928690	HG18	transcript	chr8	7629391	7634266	ExemplarFor 'XM_928690'; gen...	http://www.ncbi.nlm.nih.gov/e...

Several genes found belong to the primate-specific FAM90A gene family, which originated from multiple duplications and rearrangements (Bosch *et al.* 2007). The transcribed portions of all FAM90A genes share at least 93% nucleotide identity. Bosch *et al.* 2007 divided the FAM90A genes into subfamilies I and II based on differences in their upstream and 5-prime

UTR sequences. All the FAM90A genes found belong to subfamily II and are characterized by a 1.2-kb segment lacking repetitive elements at their 5-prime ends.

Most genes displayed were hypothetical proteins (all genes with prefix LOC). These hypothetical proteins existences have been predicted, but there is no experimental evidence that it is expressed in vivo. The usual scenario involving a hypothetical protein is in gene identification i.e. when the bioinformatic tool used for the gene identification finds a large open reading frame without an analog in the protein database, it returns "hypothetical protein" as an annotation remark. The *BTNL8* (butyrophilin-like 8) gene was also found, of which its function is still unknown (http://www.ncbi.nlm.nih.gov/).

The *DEFB4* (defensin, beta 4) gene forms part of a family of microbicidal and cytotoxic peptides made by neutrophils. Members of the defensin family are highly similar in protein sequence. This specific gene encodes defensin, beta 4, an antibiotic peptide which is locally regulated by inflammation (http://www.genecards.org/cgi-bin/carddisp.pl?gene=DEFB4).

The *HNRNPA1* gene belongs to the A/B subfamily of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs). The hnRNPs are RNA binding proteins and they complex with heterogeneous nuclear RNA (hnRNA). These proteins are associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA processing and other aspects of mRNA metabolism and transport (http://www.genecards.org/cgi-bin/carddisp.pl?gene=HNRNPA1).

3.2.2 Line graphs

Following the F-ANOVA testing, the twenty-one genes found were stipulated on line graphs using the ArrayStar3 software (DNASTAR inc, USA). Output data of twenty-one line graphs were achieved. These line graphs plots the expression levels for the selected genes over each experiment (sample), and then connects the data points with a line so that expression levels are shown relative to one another. Expression levels are plotted vertically along the Y-axis [log (base 2) scale], while the X-axis represents the sample. Each line represents a specific gene; the higher the position of the line graph, the greater the gene expression and conversely the lower the position, the less gene expression.

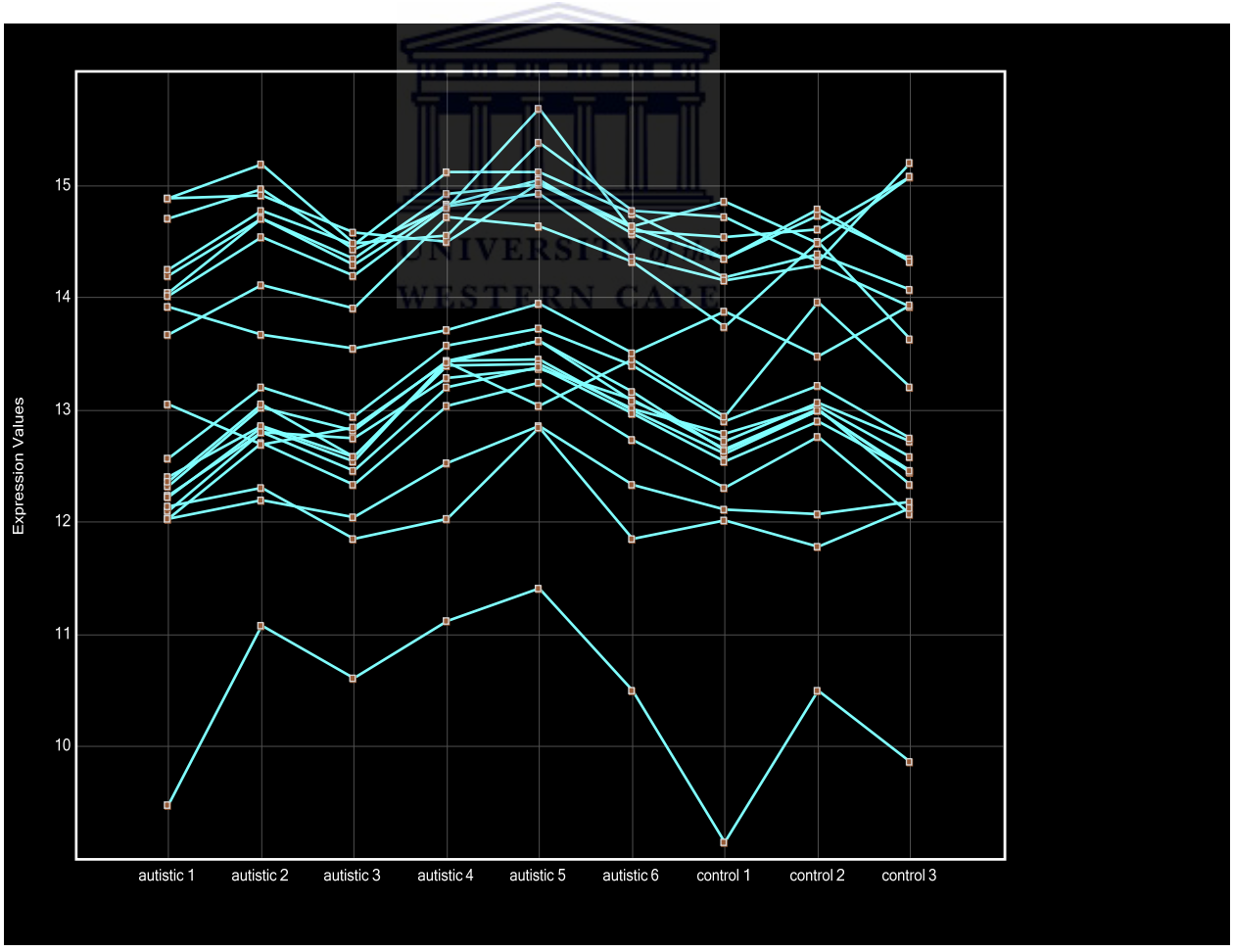

Fig. 3.11: Line graphs indicating all twenty-one gene expression levels in each sample (ArrayStar 3 software, DNASTAR inc, USA).

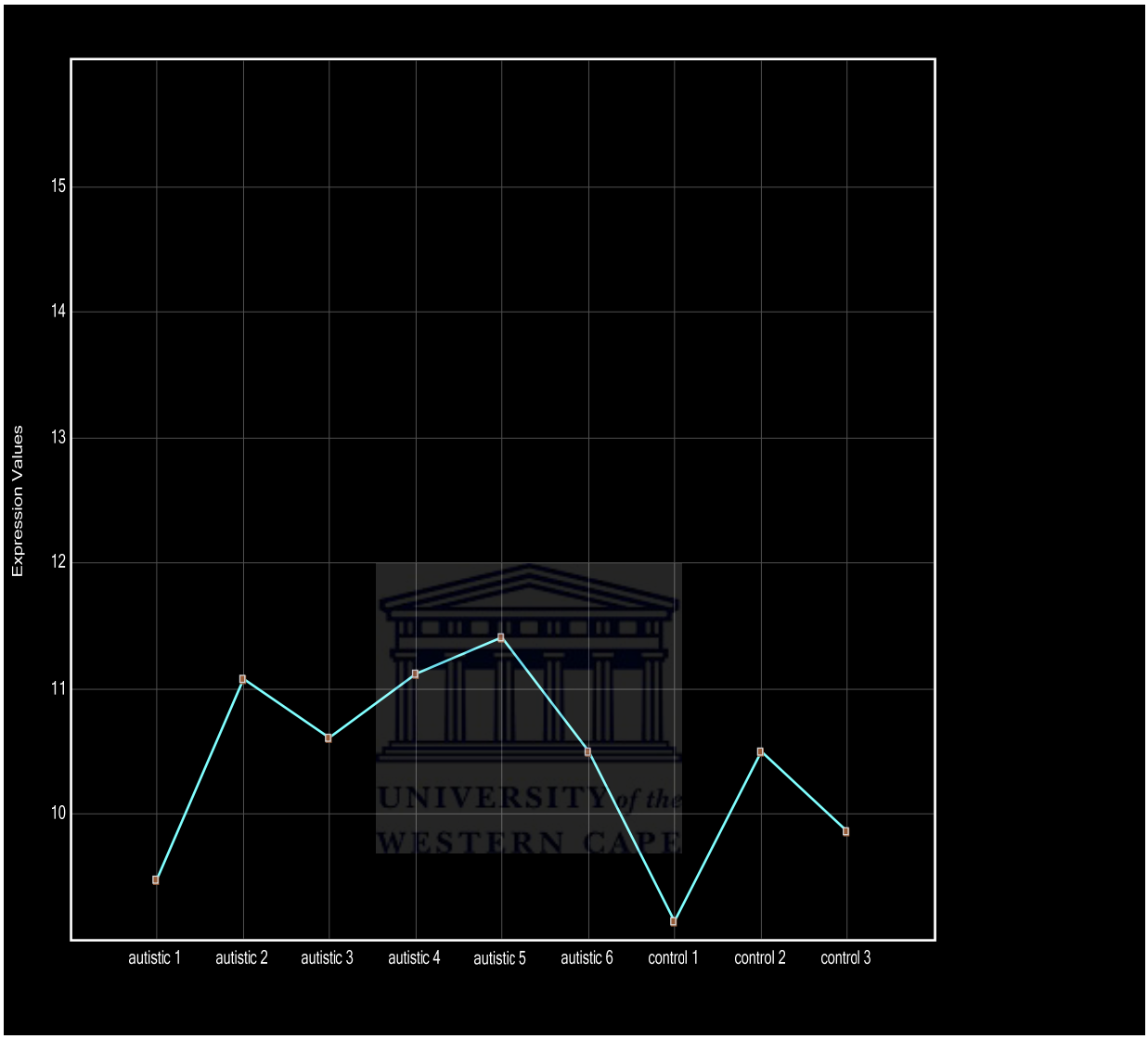

Fig. 3.12: Expression level of the BTNL8 gene in all samples (ArrayStar 3 software, DNASTAR inc, USA)

The line graph depicts the *BTNL8* gene expression level in all nine samples. It depicts autistic 5 having the highest level of expression for this gene. However, since this line graph is stipulated below an expression level of 12, the gene itself is not as expressed as the other genes found in the samples. Thus, the expression levels for all samples can be deemed as very low when compared to other genes.

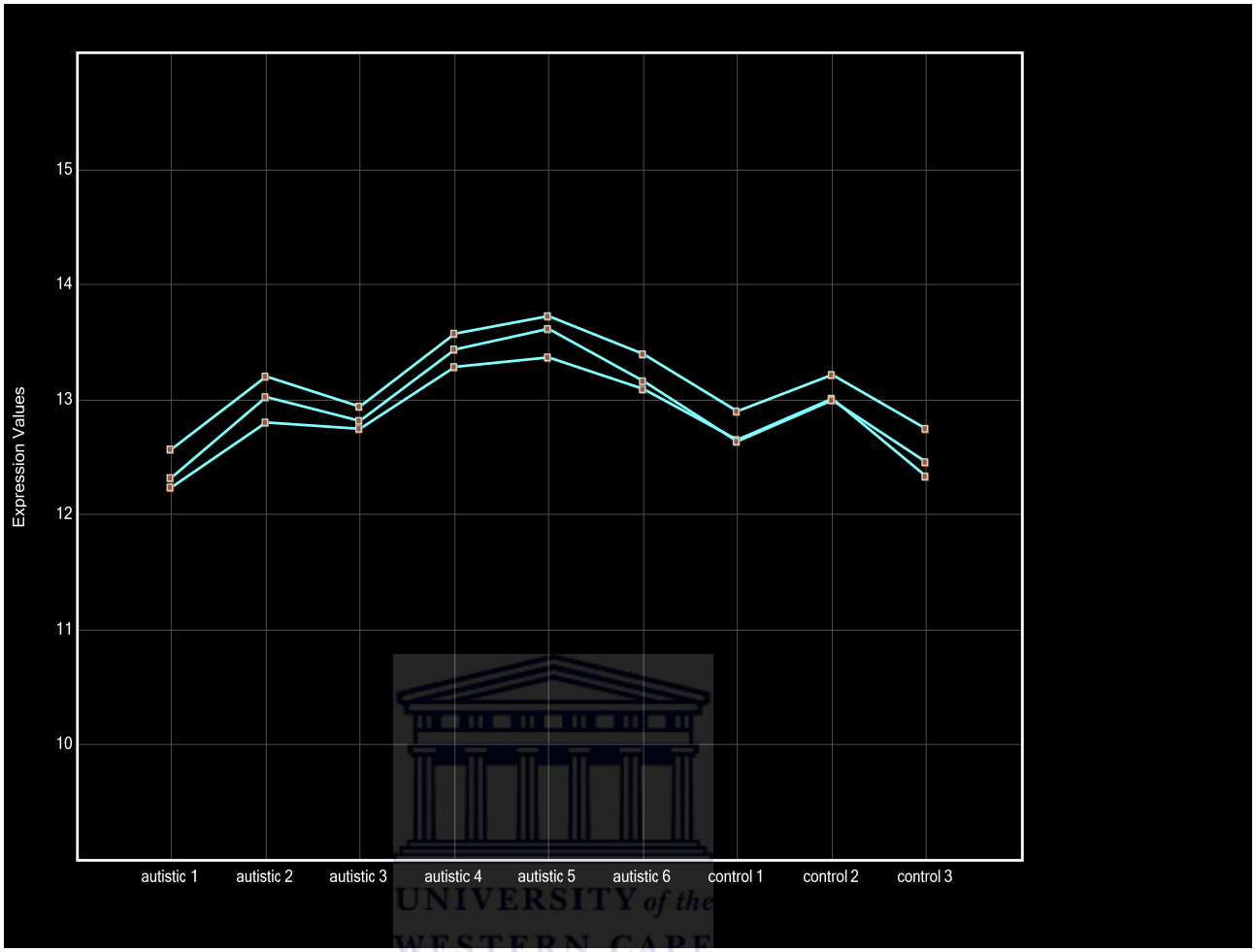

Fig. 3.13: The line graphs represent expression levels for *FAM90A14, FAM90A7* and *FAM90A4P* in all samples.
From top to bottom: *FAM90A14, FAM90A7, FAM90A4P* (ArrayStar 3 software, DNASTAR inc, USA).

The three line graphs depicts the epression levels of three genes in all nine samples. All three genes seems to be highly expressed in the samples with *FAM90A14* showing the highest level of expression. Once again, autistic 5 sample has the highest expression level for these genes, whereas autistic 1 and control 3 depicts the lowest level of expression for these genes.

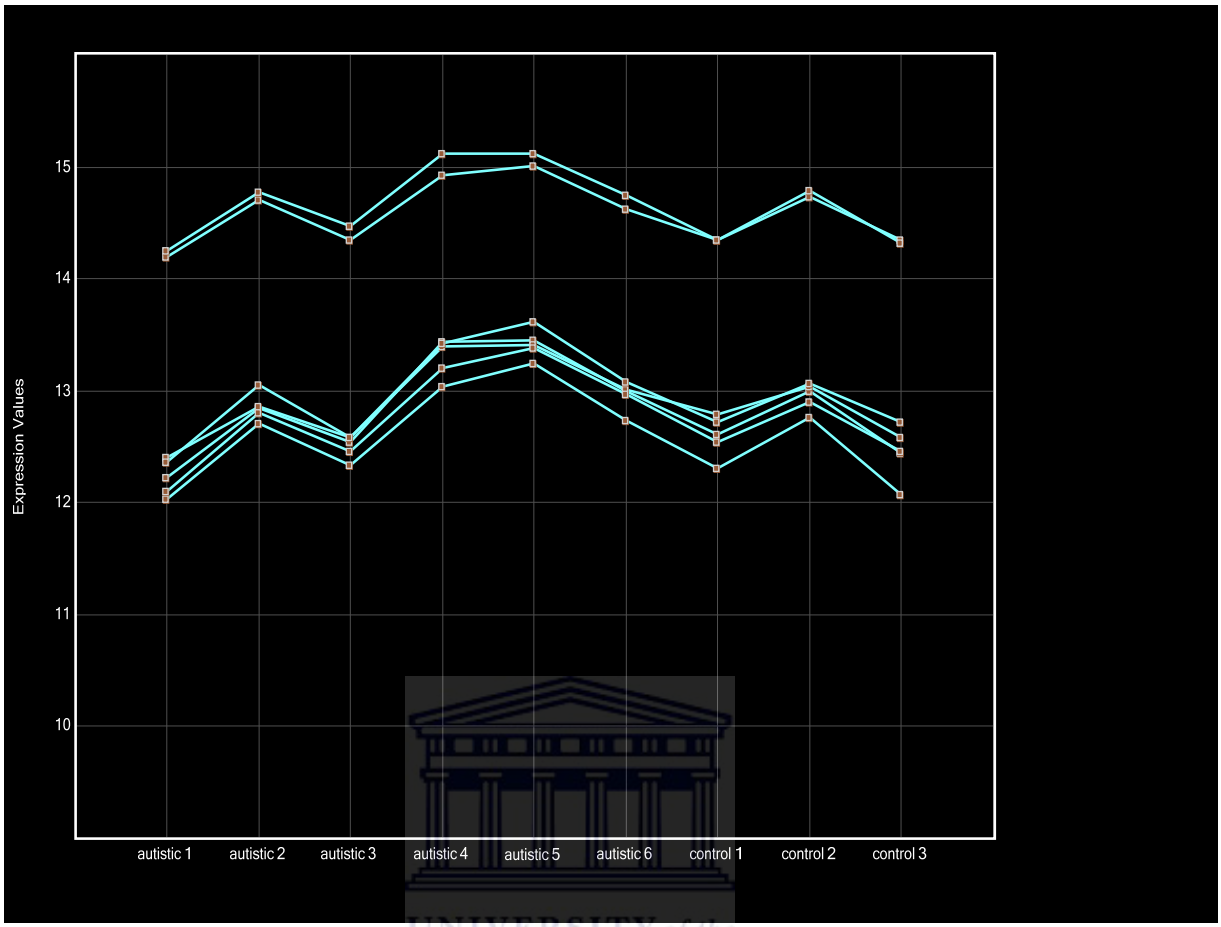

Fig. 3.14: The line graphs represent the expression levels of *LOC645681, LOC645685, FAM90A10, FAM90A13. FAM90A3, FAM90A18* and *FAM90A5* in all samples.
From top to bottom: *LOC645681, LOC645685, FAM90A10, FAM90A13. FAM90A3, FAM90A18, FAM90A5* (ArrayStar 3 software, DNASTAR inc, USA)

.

These line graphs depict the expression levels of 7 genes, including five from the FAM90A family and two hypothetical proteins. The two hypothetical proteins indicate the highest level of expression, with autistic sample 4 and 5 showing the most expression taking place.

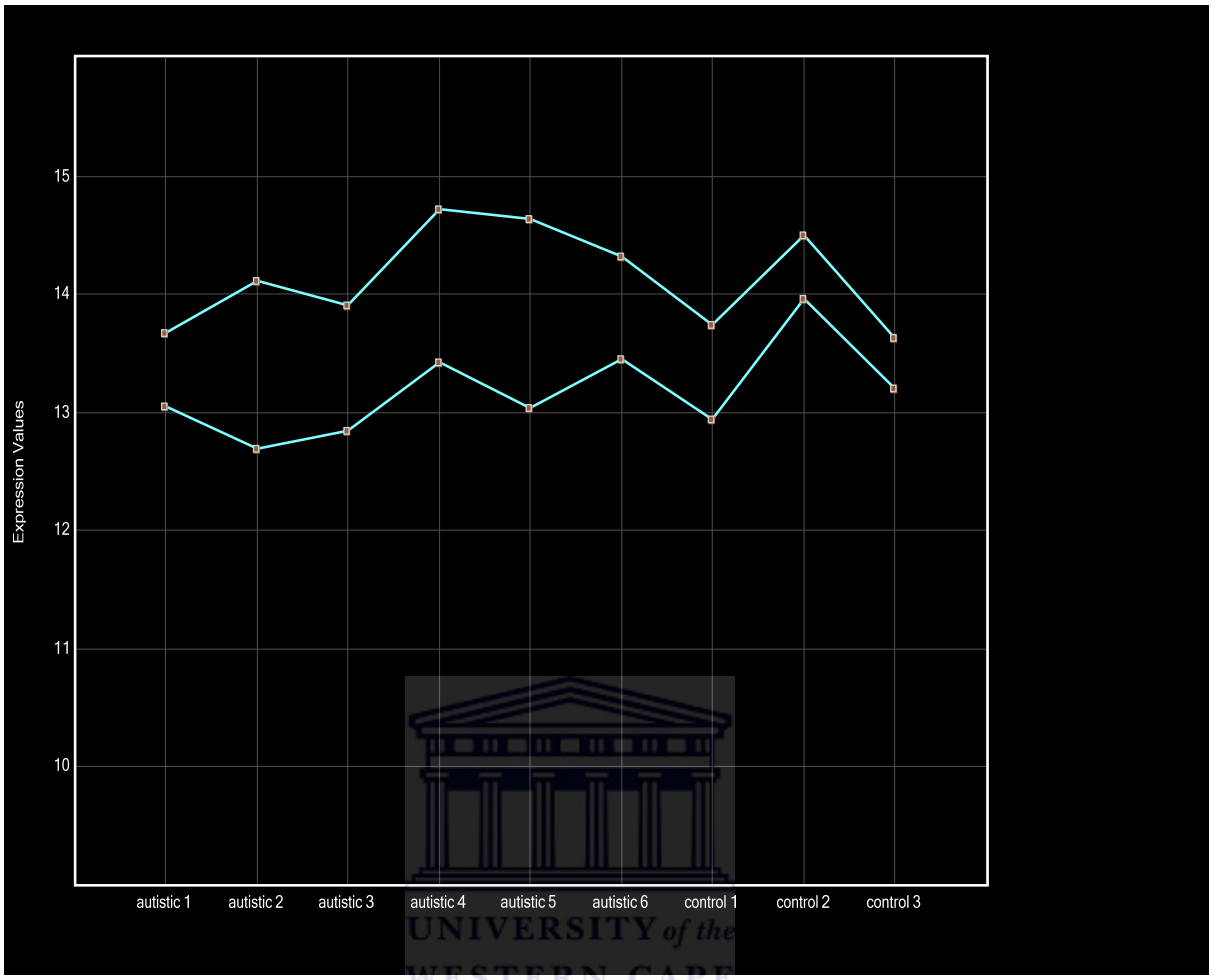

Fig. 3.15: The line graphs respresent the expression levels of LOC *349196* and *LOC 643368* in all samples. From top to bottom: *LOC 349196, LOC 643368* (ArrayStar 3 software, DNASTAR inc, USA)

These graphs represent two hypothetical proteins. In *LOC349196*, autistic 4 and 5 show the highest expression level for this gene while control 1 and 3 shows the lowest. However, in *LOC543368*, control 2 depicts the highest level of expression, whereas autistic samples 2 and 3 depict the lowest.

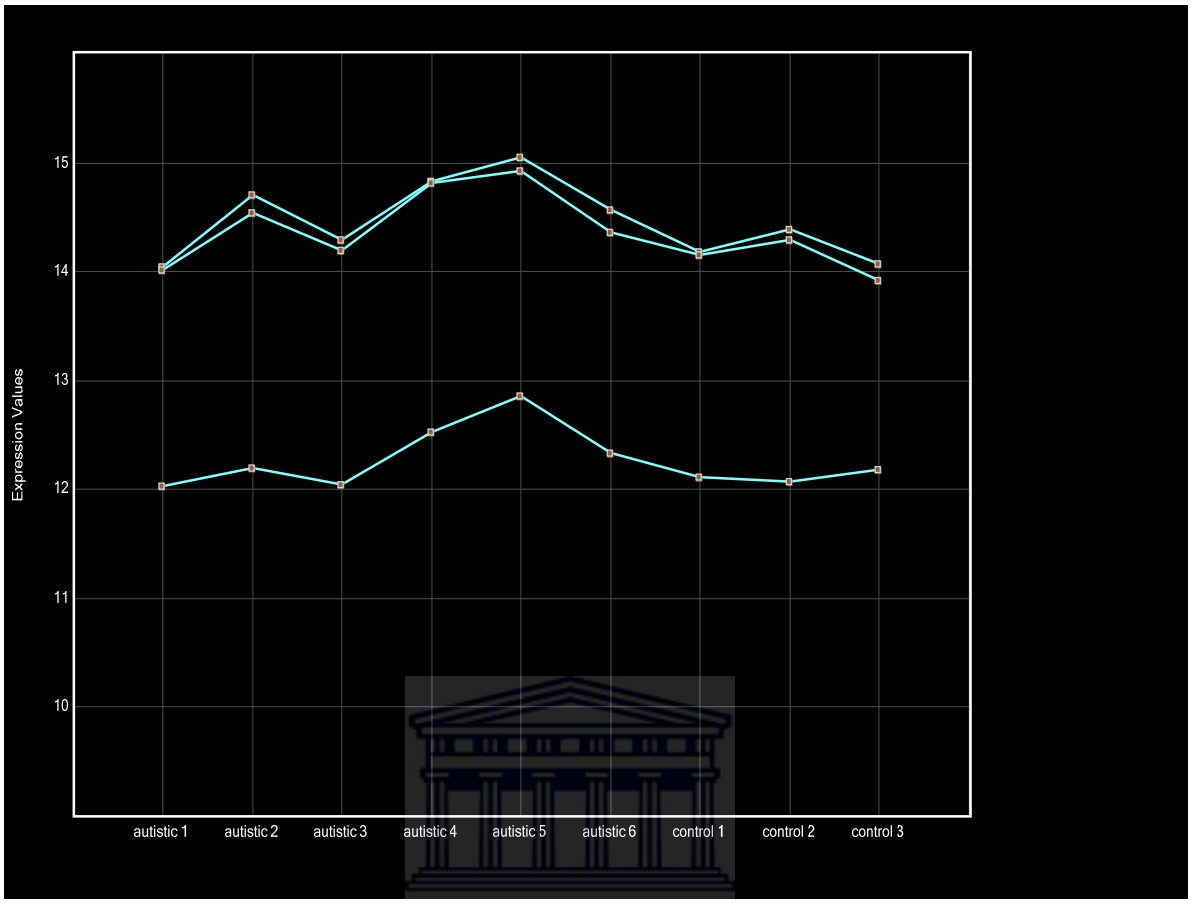

Fig. 3.16: The line graphs represent the expression levels for *LOC 645362, LOC645489* and *DEFB4* genes in all samples.
From top to bottom: *LOC 645362, LOC645489, DEFB4* (ArrayStar 3 software, DNASTAR inc, USA).

These three line graphs indicate two hypothetical proteins and the *DEFB4* gene, with the hypothetical proteins showing the most expression. These genes is shown to be highly expressed in samples autistic 4 and 5 as oppose to the control samples.

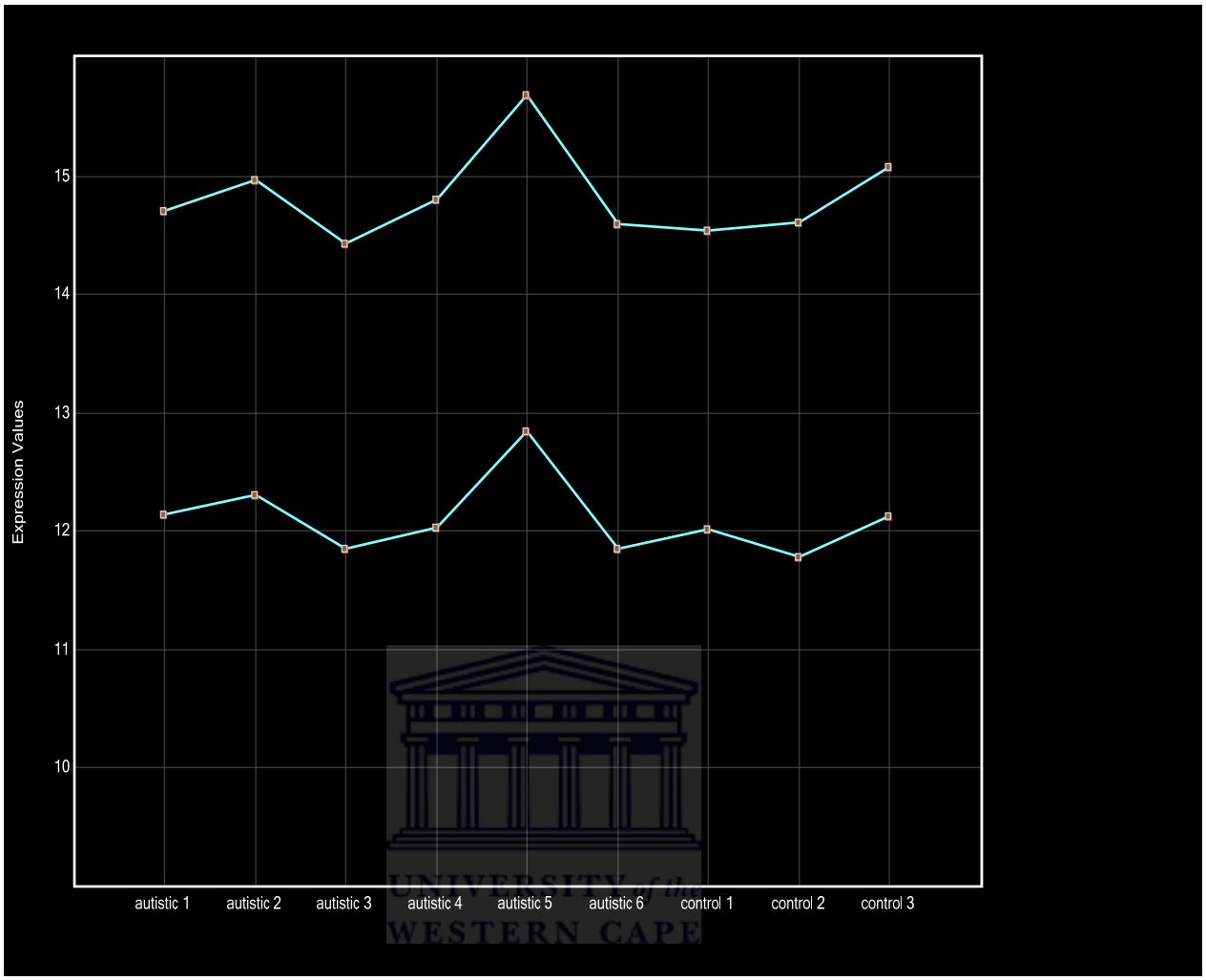

Fig. 3.17: The line graphs represent expression levels for *LOC83459* and *KIAA0738* in all samples. From top to bottom: *LOC83459, KIAA0738* (ArrayStar 3 software, DNASTAR inc, USA)

The two line graphs in *fig. 3.17* represent two hypothetical proteins, of which *LOC83459* is highly expressed. *LOC83459* is highly expressed in autistic 5 and control 3 and *KIAA0738* is highly expressed in autistic 2 and autistic 5.

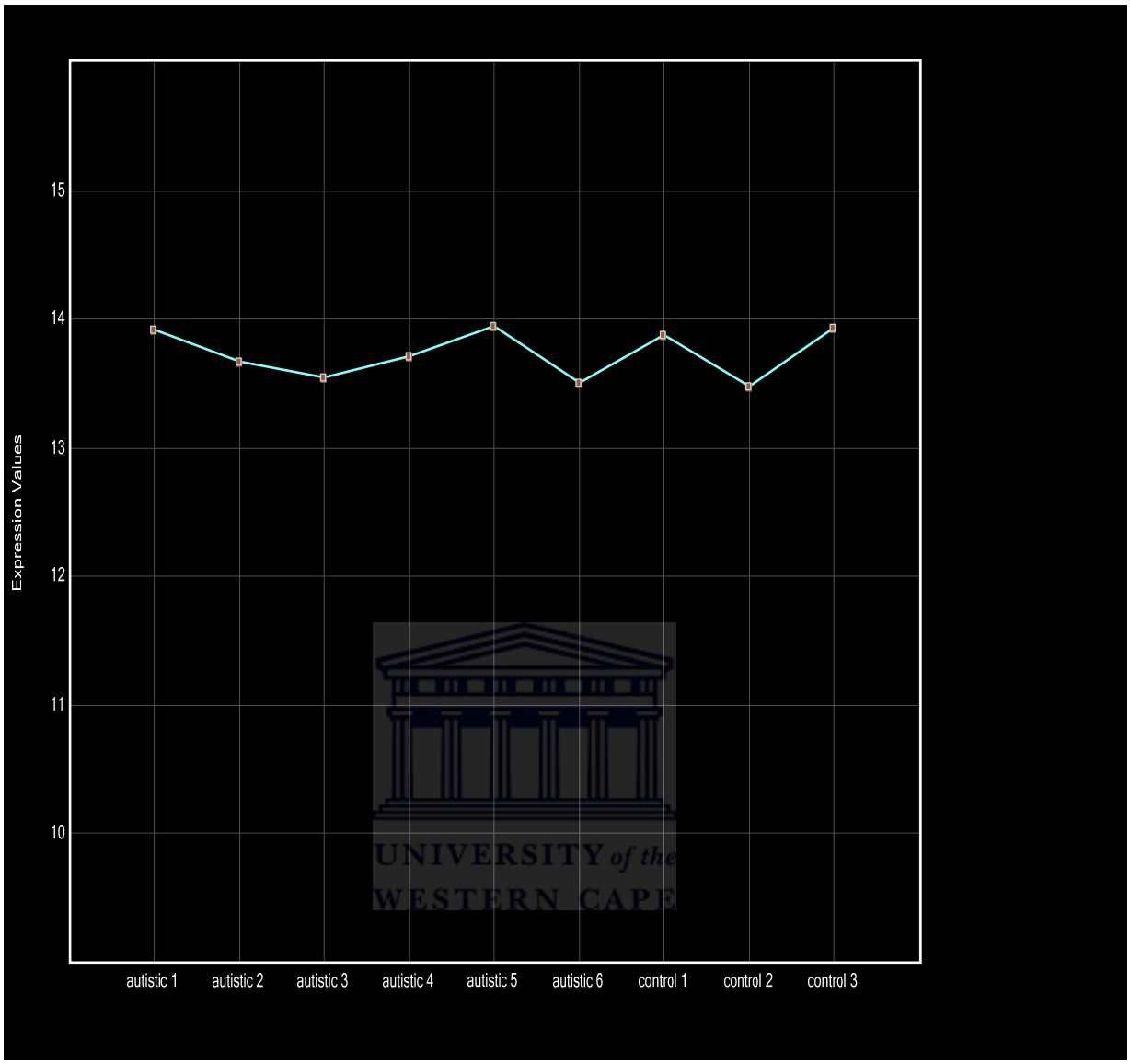

Fig. 3.18: The line graph repesents the epression level of the *HNRNPA1* gene in all samples (ArrayStar 3 software, DNASTAR inc, USA)

This graph indicates the expression level for the *HNRPA1* gene where the gene is highly expressed in autistic 1, autistic 5 and control 3. Little expression occurs in autistic 6 and control 2.

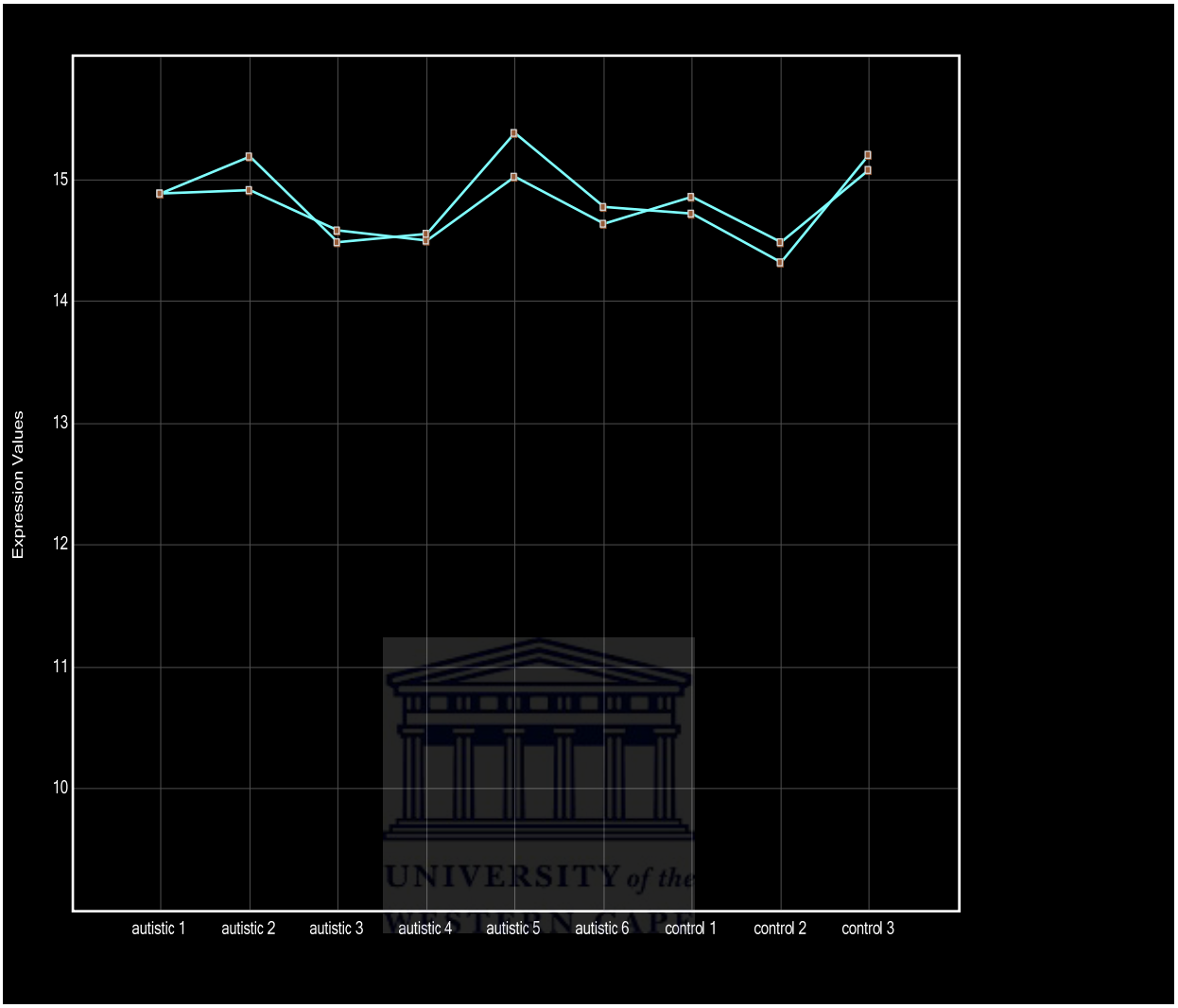

Fig. 3.19: The line graphs represent the expression levels of *LOC643788* and *LOC441426* in all samples. From top to bottom: *LOC643788, LOC441426* (ArrayStar 3 software, DNASTAR inc, USA)

These line graphs are indicative of genes that were very highly expressed in the samples. Autistic samples 2 and 5 shows the highest level of expression for these genes whereas autistic 4 and control 2 show the least.

3.2.3 Heat map

The heat map is another graphical form of analysing data using the ArrayStar3 software programme (DNASTAR inc, USA). The graphical output uses colour variants to indicate the level of expression of each gene within a sample. The columns represent the sample number and the rows represent the accession number of each gene. Each colour depicts the degree of expression. Blue indicates highly suppressed genes, yellow indicates mildly expressed genes, red indicates highly expressed genes and the light grey indicates missing values.

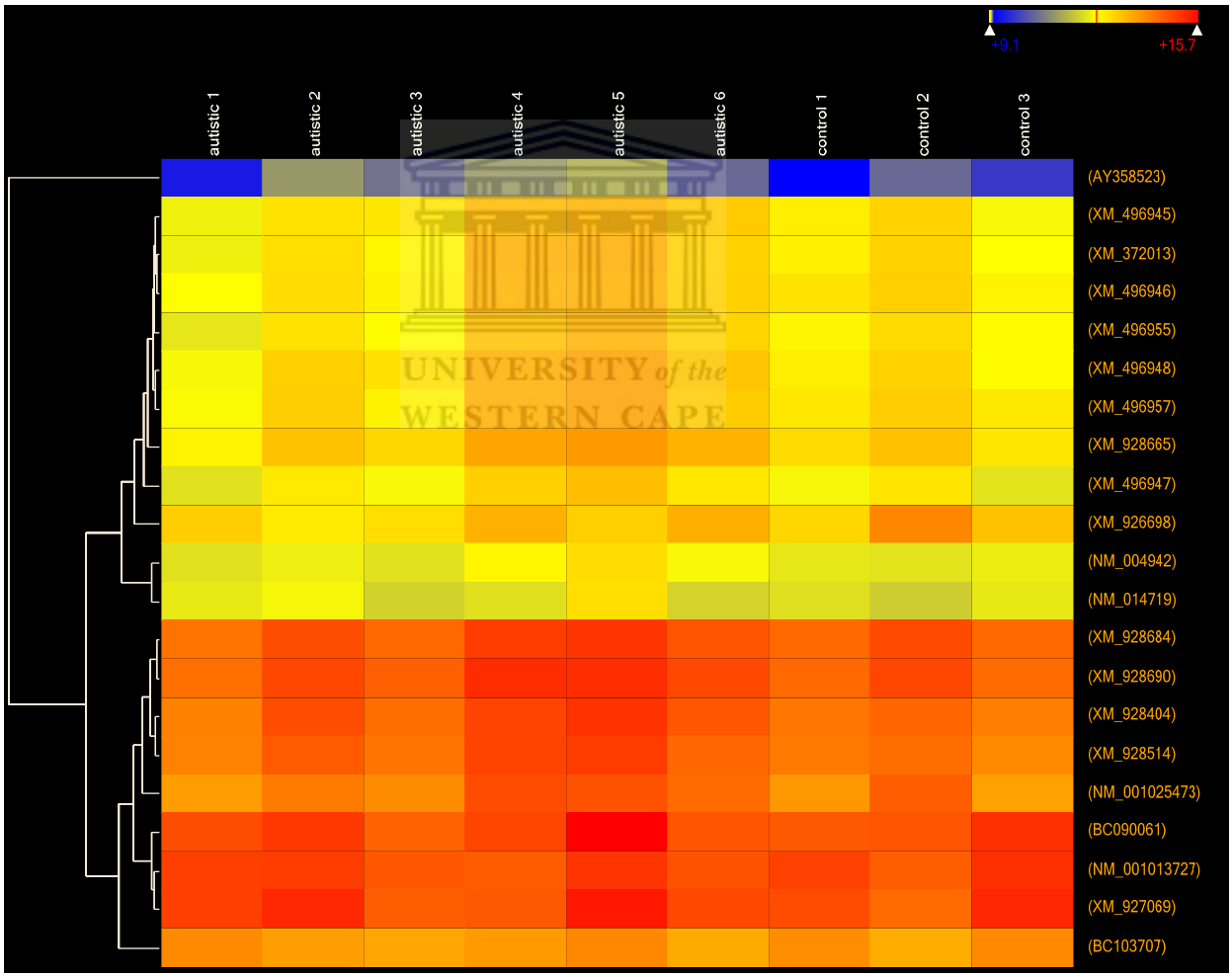

Fig. 3.20: The heat map uses colour to represent and compare expression levels of 21 genes in all samples (ArrayStar 3 software, DNASTAR inc, USA)

Table 3.4: The 21 genes that were expressed in the samples are listed below.

Number	Accession Number	Gene Name	Function
1	AY358523	BTNL8	Function unknown
2	XM_496945	FAM90A4	The functions of these genes are not specified. These genes belong to the sub-family II of the primate-specific FAM90A gene family, which originated from multiple duplications and rearrangements
3	XM_372013	FAM90A3	
4	XM_496946	FAM90A13	
5	XM_496955	FAM90A18	
6	XM_496948	FAM90A7	
7	XM_496957	FAM90A10	
8	XM_928665	FAM90A14	
9	XM_496947	FAM90A5	
10	XM_926698	LOC643368	Hypothetical protein
11	NM_004942	DEFB4	This gene forms part of a family of microbicidal and cytotoxic peptides made by neutrophils. This specific gene encodes defensin, beta 4, an antibiotic peptide which is locally regulated by inflammation
12	NM_014719	KIAAO738	Hypothetical protein
13	**XM_928684**	**LOC645683**	**Hypothetical protein**
14	**XM_928690**	**LOC645681**	**Hypothetical protein**
15	**XM_928404**	**LOC645362**	**Hypothetical protein**
16	**XM_928514**	**LOC645489**	**Hypothetical protein**
17	**NM_001025473**	**LOC349196**	**Hypothetical protein**
18	**BC 090061**	**LOC83459**	**Hypothetical protein**
19	**NM_001013727**	**LOC441426**	**Hypothetical protein**
20	**NM_927069**	**LOC643788**	**Hypothetical protein**
21	**BC 103707**	**HNRPA1**	**This gene is associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA processing and other aspects of mRNA metabolism and transport**

Text highlighted in blue indicate highly suppressed genes. Text highlighted in yellow indicate mildly expressed genes. Text in bold and highlighted in red indicate genes that were highly expressed.

The heat map indicates that the *BTNL8* gene is suppressed (not highly expressed) in autistic 1, autistic 3, autistic 6, control 1 and control 3. This finding supports the data shown in the line graph (*fig 3.12*).

Genes 2-12 (*table 3.4*) are mildy expressed in all the samples and genes 13-21 (*table 3.4*) are highly expressed in autistic samples when compared to controls. Hypothetical protein *LOC83459* and *LOC643788* is expressed the most in autistic 5 (*fig 3.20*).

CHAPTER 4

Discussion and Conclusion

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4.1 DISCUSSION

This SNP study was aimed at identifying the genotyping frequencies and allelic distributions of SNPs rs3622691 and rs736707 of the *RELN* gene in three major autistic and healthy ethnic groups in the SA population. The microarray study was aimed at identifying candidate genes using cDNA microarray, which are either up or down-regulated in the SA autistic population.

4.1.1 SNP analysis

When preparing DNA samples for the Taqman® SNP Genotyping assay, the dilution step was the most important as it was done manually and highly prone to error. Therefore, all dilutions were done carefully and under sterile conditions, in order to avoid contamination. The Taqman® protocol as well as the Eppendorf epMotion automated pipetting system had proven to be a very effective and time efficient. This system reduces the risk of human error and contamination. Non-template samples were used in order to detect the presence of any possible that might have occurred. In this experiment, the non-template controls indicated no contamination amongst the samples.

4.1.1.1 SNP in exon 22 (rs3622691)

The GG genotype was shown to be the most prominent amongst all ethnic groups in the autistic and healthy samples. The CC genotype occurred at the lowest frequency, indicating values between 0% and 5% in all autistic and healthy ethnic groups. The GG genotype showed to be higher in the SA Black and SA Caucasian autistic groups when compared to their controls. The same was seen for the CG allele in the Black and Caucasian sample group. However, the GG and CG genotypes seemed to be slightly lower in the SA Coloured group, as oppose to their control group (*table 3.1*).

The SA total population displayed same results for the distribution of A and G alleles in both the autistic and control groups, with the G allele being distributed amongst 90% of individuals and the C allele only 10%. Allele distribution for G was shown to be similar in all autistic and control ethnic groups, with differences of about 1% or 2%. Similarity occurs with the C allele as well, in all ethnic groups of autistic and control samples (*table 3.1*). For all of the samples that were genotyped, the Fishers exact test showed no significance of the SNP, rs3622691 in both the SA healthy and autistic population.

4.1.1.2 SNP in intron 59 (rs736707)

In the SA total autistic population, the AA genotype showed a higher frequency, present in more than 50% of sample group. The AG genotype was present in 40% of the groups with only 8% possessing the GG genotype. However, the total control population was predominant for the AG allele at 47%, with only 37% possessing the AA allele. The SA Black autistic and control group had similar values, with the AG genotype being predominant in both groups and the GG genotype being the least dominant (*table 3.2*). The AA and AG genotypes were equally shared amongst the SA Caucasian autistic group, indicating that 50% of individuals possessed the AA and AG genotypes respectively. The control showed to be more dominant for the AA group (*table 3.2*). It was evident that most of the Coloured population possessed the AA genotype (72%), with 0% of them possessing the GG genotype. Only 33% of the mixed control group possessed the AA genotype with most of them possessing the AG genotype (57%).

The A allele was dominant in all ethnic groups, with 72% of the total autistic population and 60 % of the total control group. When analyzing each ethnic group, it was evident that the A allele was predominant in the Coloured autistic group, with 86% possessing the A allele and

only 14% of the Coloured control group possessing the G allele (*table 3.2*). The Fishers exact test indicated a significant p-value of 0.0009, with an odds ratio of 0.58 in the total population group (*table 3.2*). This indicates that individuals possessing the G allele in the total sample group are 42% less likely to develop autism. The significantly higher frequency of the G allele in the controls suggests that this allele might be protective against developing autism. Taken together, these data indicate that those individuals in this sample group who possess the A allele would be more susceptible to autism than those who have the G alelle. This poses true for the Coloured autistic group, where 72% had the AA genotype and none had the GG genotype. The p-value was also highly significant for the Coloured group, where the odds ratio was 0.25. Hence, the individuals in the mixed autistic sample group were 75% less likely to develop autism had they had the G allele rather that the A allele.

These results are highly significant and indicate that the SNP rs736707 in intron 59 of the *RELN* gene is associated with autism in the Coloured autistic sample group. However, Serajee *et al.* 2006 found this SNP to be implicated in a Caucasian autistic population. South Africa's cultural heritage is much diversified, with Coloured groups having ancestors from various parts of the world. This could explain the allele differentiation of the Coloured group as opposed to the Caucasian and Black sample group.

4.1.2 Expression analysis

Since the microarray service was in Iceland (Nimblegen, Iceland), it was suggested that RNA or cDNA blood samples be shipped, as it would be more stable. Since blood is invasive, only a total of six autistic and three healthy samples could be used for this experiment. The first experimental attempt made was shipping cDNA blood samples, but the cDNA amount sent was not sufficient for analysis. The experiment had to be redone with changes to the protocol.

RNA samples were shipped on dry ice the second time after being analysed using the Bionanalyser (Inqaba Biotech, South Africa) to check the quality and quantity of RNA for microarray analysis.

Results, contained on a disk, was shipped back with documents which required the ArrayStar3 software (DNASTAR inc, USA) to work. The software uses the F-ANOVA for statistical analysis of more than three samples. Since nine samples were used in this study, the F-ANOVA test deemed sufficient. The test indicated a total of twenty-one genes (*table 3.3*) that had more than 95% significance in these samples. The data was stipulated on line graphs and a heat map.

4.1.2.1 Autistic candidate genes

The genes found were mostly hypothetical genes as well as genes belonging to the FAM90A family. The various line graphs indicated the expression levels of all genes in the nine samples, with the *LOC83459* (*fig 3.17*) gene showing the highest level of expression and the *BTNL8* (*fig 3.12*) gene showing the least. Sample number autistic 5 showed to have the highest expression level for most of the genes, with the *LOC83459* gene being highly expressed in this sample, as opposed to controls (*fig 3.21*). The heat map also indicated that the BTNL8 gene, for which there is no specified function, is suppressed in all samples (especially the control samples). The *DEFB4* gene and *HNRPA1* gene showed to be mildly expressed in all samples. All genes belonging to FAM90A family showed to be mildly expressed in most samples with higher expression taking place in autistic 4 and autistic 5.

4.2 CONCLUSION

In conclusion, I have shown that there was no significant association of rs3622691 of the *RELN* gene in the autistic SA population compared to the healthy SA population. However, there was a strong association of rs736707 of the *RELN* gene in the Coloured South African autistic population, indicating a highly significant p-value of 0.00014. It was also shown that the G allele serves as a protective allele in this specific population, whilst the A allele indicates predisposition to autism.

I have also identified 21 candidate genes, which are differentially expressed, using high throughput cDNA microarray technology. The *LOC83459* showed the highest level of expression in the autistic samples (especially autistic 5), while the *BTNL8* gene was shown to be highly suppressed in the control samples. Thus, I have met my aims and shown, on a small scale, that specific genes does indeed play a role in autism.

4.2.1 Future studies

Future studies could include the analysis of SNPs in candidate genes (*FOXP2, NLGN4X, EN2*) said to be involved in other autistic populations, as well as looking at their ancestral heritage and whether it plays a role in the aetiology of autism.

Also, candidate genes found using the microarray technique could be further analysed using RT-PCR. The shortcomings of the microarray study were that it was very costly, only a small sample group was used and the sample collection was invasive. Since samples collection were from a specific ethnic and age group, I suggest that future studies should include a larger sample group and different ethnicities; for example, repeating this microarray study using a larger sample size in a SA Coloured group and in a SA Black group.

Since several genes are said to be involved in autism (Bailey *et al.* 1995), the genes found in this study are merely a stepping stone in the right direction. More genetic studies still need to be done. Currently, an enormous amount of focus has been placed on genetics of autism and I foresee answers that will lead to the aetiology, understanding and treatment of this disorder.

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http://www.genecards.org/cgi-bin/carddisp.pl?gene=DEFB4
http://www.ncbi.nlm.nih.gov/
http://www.pfizer.com
http://www.scq.ubc.ca/spot-your-genes-an-overview-of-the-microarray/
https:// appliedbiosystems.com/
https:// www.genecards.org
https:// www.genetests.org

GLOSSARY

Allele frequency – *Allele frequency is the number of copies of a particular allele divided by the number of copies of all alleles at the genetic place (locus) in a population. It can be expressed for example as a percentage. In population genetics, allele frequencies are used to depict the amount of genetic diversity at the individual, population, and species level.*

Alzheimer's disease – *This is the most common form of dementia. It is an incurable, degenerative, and terminal disease that is unique for every individual. The earliest observable symptoms are often mistakenly thought to be 'age-related' concerns, or manifestations of stress.[6] In the early stages, the most commonly recognised symptom is memory loss, such as difficulty in remembering recently learned facts.*

Blood-brain barrier – *This is a membrane that controls the passage of substances from the blood into the central nervous system. It is a physical barrier between the local blood vessels and most parts of the central nervous system itself, and stops many substances from travelling across it.*

Candidate genes - *A candidate gene is a gene located in a chromosome region suspected of being involved in the expression of a trait such as a disease, whose protein product suggests that it could be the gene in question.*

CAT scan – *Computed Axial Tomography (CAT) scan is a medical imaging method employing tomography where digital geometry processing is used to generate a three-dimensional image of the internals of an object from a large series of two-dimensional X-ray images taken around a single axis of rotation.*

Cognition – *This term is used in several loosely related ways to refer to a faculty for the human-like processing of information, applying knowledge and changing preferences.*

Confidence interval - *In statistics, a confidence interval (CI) is a particular kind of interval estimate of a population parameter. Instead of estimating the parameter by a single value, an interval likely to include the parameter is given. Thus, confidence intervals are used to indicate the reliability of an estimate.*

Cytogenic studies - *Cytogenetics is the study of the structure of chromosome material. It includes routine analysis of G-Banded chromosomes, other cytogenetic banding techniques, as well as molecular cytogenetics such as fluorescent in situ hybridization (FISH) and comparative genomic hybridization (CGH).*

Exon - *An exon is a nucleic acid sequence that is represented in the mature form of an RNA molecule after either portions of a precursor RNA (introns) have been removed by cis-splicing or by two or more precursor RNA molecules have been ligated by trans-splicing.*

F-ANOVA test - *A F-test is any statistical test in which the test statistic has an F-distribution under the null hypothesis. The best know F-test is ANOVA, with the hypothesis that the means of several normally distributed populations, all having the same standard deviation, are equal.*

Fetal alcohol syndrome (FAS) - *FAS is a pattern of birth defects, learning, and behavioral problems affecting individuals whose mothers consumed alcohol during pregnancy.*

Fishers exact test - *This is a statistical significance test used in the analysis of contingency tables where sample sizes are small. It is named after its inventor, R. A. Fisher, and is one of a class of exact tests, so called because the significance of the deviation from a null hypothesis can be calculated exactly, rather than relying on an approximation that becomes exact in the limit as the sample size grows to infinity, as with many statistical tests.*

Fragile X Syndrome – *This is a genetic syndrome which results in a spectrum of characteristic physical, intellectual, emotional and behavioural features which range from severe to mild in manifestation.The syndrome is associated with the expansion of a single trinucleotide gene sequence (CGG) on the X chromosome, and results in a failure to express the FMR1 protein which is required for normal neural development.*

Genotype - *The genotype is the genetic constitution of a cell, an organism, or an individual (i.e. the specific allele makeup of the individual) usually with reference to a specific character under consideration*

Haplotype - *In genetics, a haplotype is a combination of alleles at multiple loci that are transmitted together on the same chromosome. Haplotype may refer to as few as one locus or to an entire chromosome depending on the number of recombination events that have occurred between a given set of loci. In a second meaning, haplotype is a set of single nucleotide polymorphisms (SNPs) on a single chromatid that are statistically associated.*

Hardy-Weinberg equation - *The Hardy–Weinberg principle states that both allele and genotype frequencies in a population remain constant—that is, they are in equilibrium—from generation to generation unless specific disturbing influences are introduced. Those disturbing influences include non-random mating, mutations, selection, limited population size, "overlapping generations", random genetic drift and gene flow.*

Heterozygous - *Having different alleles at one or more corresponding chromosomal loci.*

Homozygous - *Having the same alleles at a particular gene locus on homologous chromosomes.*

Huntington's Disease - *Huntington's disease, chorea, or disorder, is an incurable neurodegenerative genetic disorder that affects muscle coordination and some cognitive functions, typically becoming noticeable in middle age. It is the most common genetic cause of abnormal involuntary writhing movements called chorea.*

Intron - *An intron is a DNA region within a gene that is not translated into protein.*

MRI scan - *Magnetic resonance imaging (MRI) uses a strong magnetic field and radio waves to produce detailed pictures of the inside of your body. MRI scans can show muscles, joints, bone marrow, blood vessels, nerves and other structures within your body.*

Neurodevelopmental disorders – *Severe disabling conditions often associated with life-long impairment and are recognized to be the result of abnormalities in brain development due to both genetic and environmental/biological causes.*

Phenylketonura - *Phenylketonuria (PKU) is an autosomal recessive genetic disorder characterized by a deficiency in the hepatic enzyme phenylalanine hydroxylase.*

Polymerase Chain Reaction - *In molecular biology, the polymerase chain reaction (PCR) is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.*

Restriction Fragment Length Polymorphism - *In molecular biology, the term restriction fragment length polymorphism, or RFLP, refers to a difference between two or more samples of homologous DNA molecules arising from differing locations of restriction sites, and to a related laboratory technique by which these segments can be distinguished.*

Schizophrenia – *This is a psychiatric diagnosis that describes a mental illness characterized by impairments in the perception or expression of reality, most commonly manifesting as auditory hallucinations, paranoid or bizarre delusions or disorganized speech and thinking in the context of significant social or occupational dysfunction.*

Sickle Cell Anaemia – *This is a genetic life-long blood disorder characterized by red blood cells that assume an abnormal, rigid, sickle shape. Sickling decreases the cells' flexibility and results in a risk of various complications.*

Tourettes Syndrome – *This is an inherited neurological disorder with onset in childhood, characterized by the presence of multiple physical (motor) tics and at least one vocal (phonic) tic; these tics characteristically wax and wane. It is also defined as part of a spectrum of tic disorders, which includes transient and chronic tics.*

Tuberous Sclerosis Complex - *Tuberous sclerosis complex (TSC) is a rare, multi-system genetic disease that causes benign tumours to grow in the brain and on other vital organs such as the kidneys, heart, eyes, lungs, and skin. A combination of symptoms may include seizures, developmental delay, behavioural problems, skin abnormalities, lung and kidney disease.*

Full names of genes as depicted in *table 1.1*

GENE ID	GENE NAME
ADA	Adenosine deaminase
APOE2	Apolipoprotein E2
ARX	Aristaless related homeobox
ATP10C	ATPase class V type 10A
AVPR1A	Arginine vasopressin receptor 1A
C4B	Complement component 4B
EN2	Engrailed homeobox 2
GABRA5	Gamma-aminobutyric acid (GABA) A receptor, alpha 5.
GABRB3	Gamma-aminobutyric acid (GABA) A receptor, beta 3.
GABRG3	Gamma-aminobutyric acid receptor gamma 3 subunit
GLO1	Glyoxalase 1
GRIK2	Glutamate receptor, ionotropic kainate 2 Precursor
HOXA1	Homeobox A1
HOXB1	Homeobox B1
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog.
NF1	Neurofibromin 1
NLGN4X	Neuroligin 4, X-linked
PTEN	Phosphatase and tensin homolog
SLC25A12	Solute carrier family 25 (mitochondrial carrier, Aralar), member 12.
SLC6A4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4.
UBE2H	Ubiquitin-conjugating enzyme E2H
UBE3A	Ubiquitin protein ligase E3A
WNT2	Wingless-type MMTV integration site family member 2

Appendix I

University *of the* Western Cape

Private Bag X17 **BELLVILLE 7535** South Africa
Telephone: +27 21 959 2215 Telefax: +27 21 959 3505

UNIVERSITY *of the*
WESTERN CAPE

DEPARTMENT OF BIOTECHNOLOGY

24th August 2007

Alpha School
Palmerston Road
Woodstock

Dear Parents/Guardians

I am lecturing in the Biotechnology department at the University of the Western Cape. I have completed my doctoral studies in Human Genetics and I am pursuing a project which examines the genetics of Autism in a South African population. This is the first study of its kind done in South Africa. We have recently presented and published an article at the 2nd World Congress on Autism in South Africa (2006) and we are completing two more articles from results which we have generated in this year.

The success of this study is as a result of the participants and their families. One shortcoming of the project was that the sample size of individuals with Autism was too small and therefore, to overcome this obstacle we are trying to increase our sample size by collecting as many samples as possible.

These samples are biological material which will be obtained from your child by collecting cheek cells from the inside of his/her mouth, using a clean sterile swab. It is a painless and non-intrusive procedure. The whole procedure will only take a minute and will be done by properly trained individuals under hygienic conditions in their respective classroom in the presence of their teacher.

The project has been approved by the principal, UWC Ethics Committee and the Western Cape Education Department. Being the parent/guardian of the child, I seek your permission for the participation of your child in this study.

Dr Arieff

……

Dr Zainunisha Arieff
Dept of Biotechnology
Email: zarieff@uwc.ac.za
Tel: (021) 9592214/5

Appendix II

Private Bag X17, Belville, 7535
South Africa
Tel: +27 (0) 21 959 2215
Fax: +27 (0) 21 959 3505
Website: www.uwc.ac.za

FACULTY OF NATURAL SCIENCES

Department of Biotechnology

September 2008

Dear Parent/Guardian

I am a human genetics Masters student, under the direct supervision of Dr. Z Arieff, at the University of the Western Cape. A few of my colleagues and I have been to Vera this year and last year to collect cheek cells from your child, which you have consented for. This year, my study involves collecting samples from six children and analysing their DNA by looking at which genes are over and under expressed. The results from this study will indicate new genes involved in the pathology of Autism. This is a very powerful study and has not been undertaken in South Africa, and we anticipate that the results from this study will aid the understanding of genetics as a cause of Autism.

In order to do this study, I need blood samples from six boys at Vera school. I've been working closely with Dr Schlegal. She and Jana Forrester suggested we ask your permission for your child to be involved in our study. Dr Schlegal, together with a nurse, will take the blood sample.

The project has been approved by the principal and the UWC Ethics Committee. If you need any further information about this project, please do not hesitate to contact me telephonically or via email.

Yours in anticipation

Hajirah Gameeldien
(021) 959 2503
hajirahg@gmail.com

……
Dr. Zainuneesha Arieff

……
Dr. Birgit Schlegal

Appendix III

Clearly print child's: Name ……..
Date of birth ……

Please indicate with a cross on the slip, the option which you have chosen.

Option 1: I hereby give permission for my child to be included in this research. ☐

Option 2: I hereby give permission for my child to be included in this research and would like more information on the project. ☐

Option 3 I do not give permission for participation in this research. ☐

If you have chosen option 1, please read and fill in the form below:

1. I, _____, consent to the use of my/ daughter's/son's genetic material in the study outlined above.

2. I understand that the genetic material for analysis is to be obtained from a blood sample.

3. I understand that the sample has been assigned a unique identification number and that there will be no link between my child's name and the unique identification number.

4 Since the sample has been collected anonymously it cannot be withdrawn from the study.

5 The sample will be stored indefinitely.

6 The results of the project will be published in a scientific journal.

7. The analysis procedure only provides information on variable genetic elements on specific chromosomes and cannot determine the complete genetic makeup of an individual.

8. At no stage will the sample provided be used for anything other than the analysis of biomolecules involved in autism.

9. **ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY:**

Dr Arieff

Parent's/Guardian's signature: _____

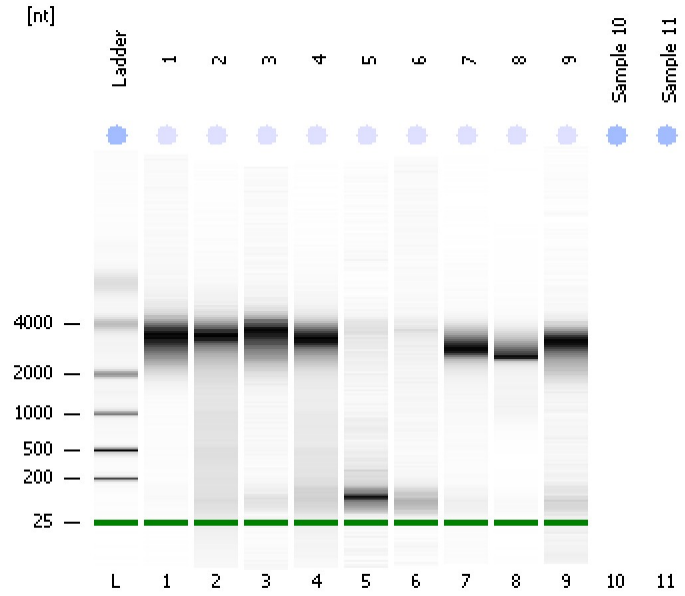
Dr Zainunisha Arieff
Dept of Biotechnology
Email: zarieff@uwc.ac.za
Tel: (021) 9592214/5

Microarray Project 2009

Assay Class: EukaryoteTotal RNA Pico
Data Path: T:\...9-09-08\hajirah2_EukaryoteTotal RNA Pico_2009-09-08_001.xad

Created: 2009/09/08 02:19:20 PM
Modified: 2009/09/08 02:39:18 PM

Electrophoresis File Run Summary



Instrument Information:

Instrument Name:	DE54700643	Firmware:	C.01.055
Serial#:	DE54700643	Type:	G2939A

Assay Information:

Assay Origin Path:	C:\Program Files\Agilent\2100 bioanalyzer\2100 expert\assays\RNA\Eukaryote Total RNA Pico Series II.xsy
Title:	Eukaryote Total RNA Pico Series II
Version:	2.5
Assay Comments:	Copyright © 2003-2006 Agilent Technologies

Chip Information:

Chip Lot:

Reagent Kit Lot:

Chip Comments:

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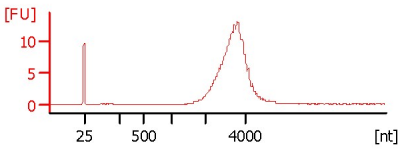
RIN: 7.60



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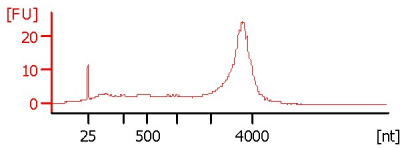

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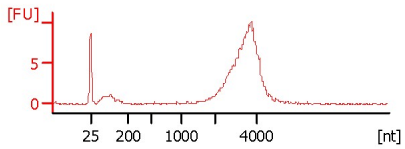
RIN:9



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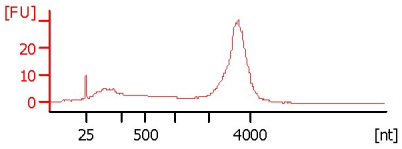

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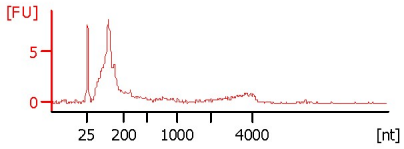
RIN: 2.60



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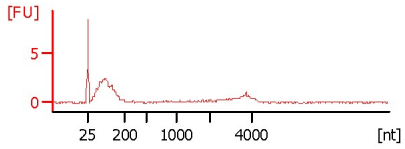

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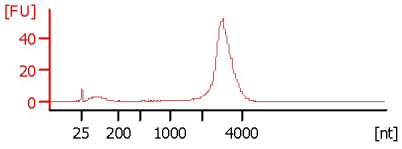

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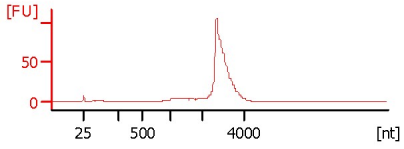

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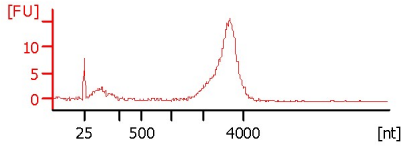


Assay Class: EukaryoteTotal RNA Pico
Data Path: T:\...9-09-08\hajirah2_EukaryoteTotal RNA Pico_2009-09-08_001.xad

Created: 2009/09/08 02:19:20 PM
Modified: 2009/09/08 02:39:18 PM

Electrophoresis File Run Summary (Chip Summary)

Sample Name	Sample Comment	Status	Observation	Result Label	Result Color
1		✔		RIN: 7.60	
2		✔		RIN:7	
3		✔		RIN:9	
4		✔		RIN: 8.50	
5		✔		RIN: 2.60	
6		✔		RIN: 2.40	
7		✔		RIN:7	
8		✔		RIN: 6.90	
9		✔		RIN: 7.90	
Sample 10				All Other Samples	
Sample 11				All Other Samples	

Chip Lot # **Reagent Kit Lot #**

Chip Comments :

Assay Class: EukaryoteTotal RNA Pico
Data Path: T:\...9-09-08\hajirah2_EukaryoteTotal RNA Pico_2009-09-08_001.xad

Created: 2009/09/08 02:19:20 PM
Modified: 2009/09/08 02:39:18 PM

Electrophoresis Assay Details

General Analysis Settings

Number of available sample and ladder wells (max.) : 12
Minimum visible range [s] : 17
Maximum visible range [s] : 70
Start analysis time range [s] : 18
End analysis time range [s] : 69
Ladder Concentration [pg/µl] : 1000
Lower Marker Concentration [pg/µl] : 0
Upper Marker Concentration [pg/µl] : 0
Used lower marker for quantitation
Standard curve fit is Logarithmic
Show data aligned to lower marker

Integrator Settings

Integration start time [s] : 18
Integration end time [s] : 69
Slope Threshold : 0.6
Height Threshold [FU] : 0.5
Area Threshold : 0.2
Width Threshold [s] : 0.5
Baseline Plateau [s] : 6

Filter Settings

Filter width [s] : 0.5
Polynomial order : 4

Ladder

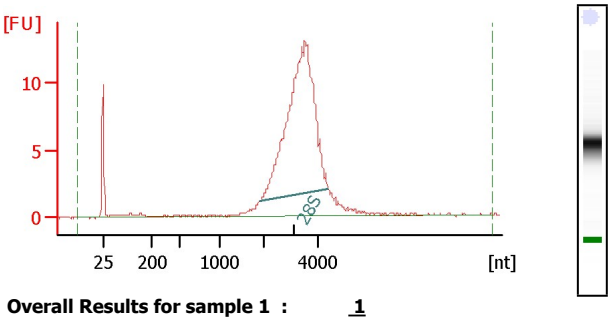
Ladder Peak	Size
1	25
2	200
3	500
4	1000
5	2000
6	4000

Assay Class: EukaryoteTotal RNA Pico
Data Path: T:\...9-09-08\hajirah2_EukaryoteTotal RNA Pico_2009-09-08_001.xad

Created: 2009/09/08 02:19:20 PM
Modified: 2009/09/08 02:39:18 PM

Electropherogram Summary

1



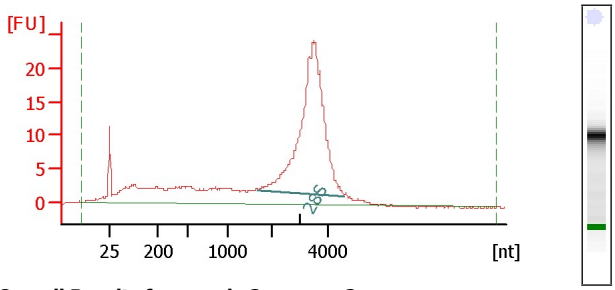
Overall Results for sample 1 : 1

RNA Area:	131.2
RNA Concentration:	1,061 pg/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	7.6 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 7.60

Fragment table for sample 1 : 1

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
28S	1,935	4,366	88.0	67.1

2



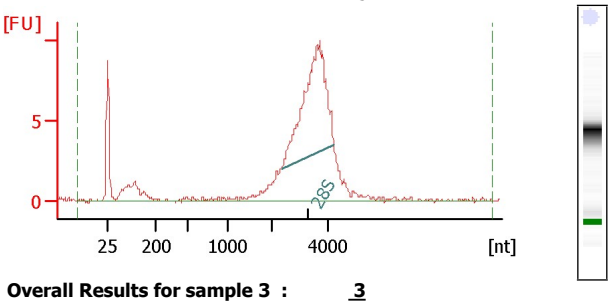
Overall Results for sample 2 : 2

RNA Area:	323.9
RNA Concentration:	2,620 pg/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	7.0 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN:7

Fragment table for sample 2 : 2

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
28S	1,694	4,623	152.9	47.2

3



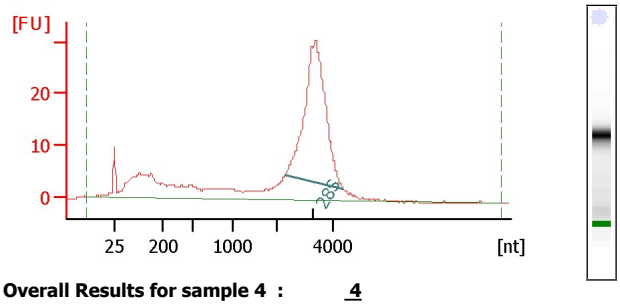
Overall Results for sample 3 : 3

RNA Area:	109.4
RNA Concentration:	885 pg/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	9.0 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN:9

Fragment table for sample 3 : 3

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
28S	2,347	4,215	42.3	38.7

4



Overall Results for sample 4 : 4

RNA Area:	384.6
RNA Concentration:	3,111 pg/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	8.5 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.50

Fragment table for sample 4 : 4

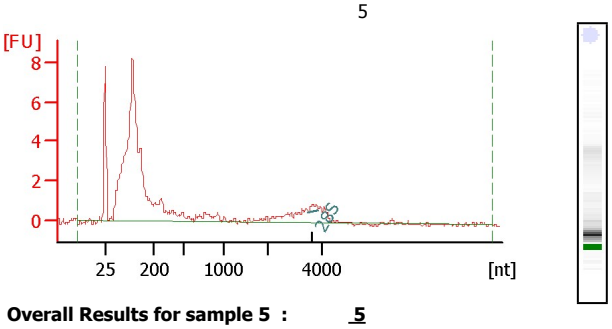
Name	Start Size [nt]	End Size [nt]	Area	% of total Area
28S	2,323	4,395	165.8	43.1

Assay Class: EukaryoteTotal RNA Pico
Data Path: T:\...9-09-08\hajirah2_EukaryoteTotal RNA Pico_2009-09-08_001.xad

Created: 2009/09/08 02:19:20 PM
Modified: 2009/09/08 02:39:18 PM

Electropherogram Summary Continued ...

5



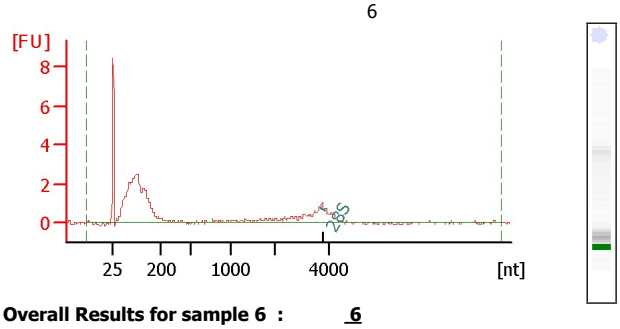
Overall Results for sample 5 : 5

RNA Area:	78.1
RNA Concentration:	632 pg/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	2.6 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 2.60

Fragment table for sample 5 : 5

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
28S	3,512	3,864	0.5	0.7

6



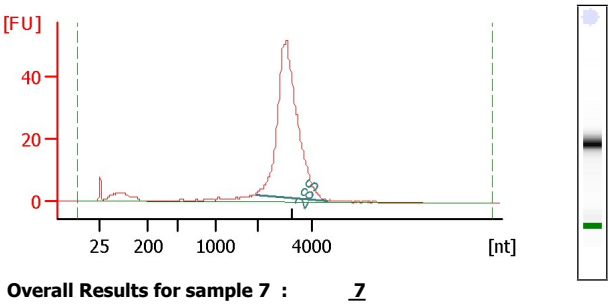
Overall Results for sample 6 : 6

RNA Area:	33.7
RNA Concentration:	273 pg/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	2.4 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 2.40

Fragment table for sample 6 : 6

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
28S	3,698	3,806	0.1	0.4

7



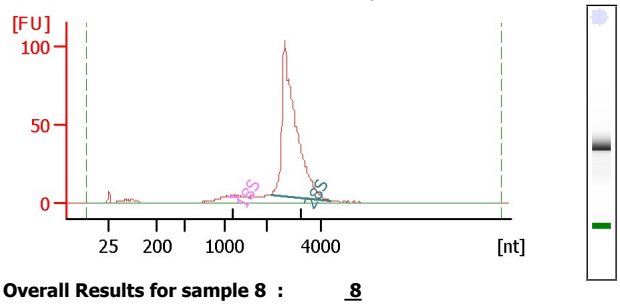
Overall Results for sample 7 : 7

RNA Area:	401.1
RNA Concentration:	3,244 pg/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	7.0 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN:7

Fragment table for sample 7 : 7

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
28S	1,925	4,603	307.9	76.8

8



Overall Results for sample 8 : 8

RNA Area:	601.2
RNA Concentration:	4,863 pg/µl
rRNA Ratio [28s / 18s]:	339.3
RNA Integrity Number (RIN):	6.9 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.90

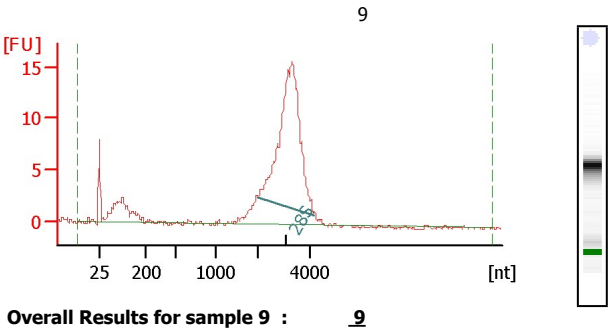
Fragment table for sample 8 : 8

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,120	1,328	1.2	0.2
28S	2,160	4,434	423.2	70.4

Assay Class: EukaryoteTotal RNA Pico
Data Path: T:\...9-09-08\hajirah2_EukaryoteTotal RNA Pico_2009-09-08_001.xad

Created: 2009/09/08 02:19:20 PM
Modified: 2009/09/08 02:39:18 PM

Electropherogram Summary Continued ...



Overall Results for sample 9 : 9

RNA Area:	152.0
RNA Concentration:	1,230 pg/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	7.9 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 7.90

Fragment table for sample 9 : 9

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
28S	2,064	4,123	85.5	56.3

Assay Class: EukaryoteTotal RNA Pico
Data Path: T:\...9-09-08\hajirah2_EukaryoteTotal RNA Pico_2009-09-08_001.xad

Created: 2009/09/08 02:19:20 PM
Modified: 2009/09/08 02:39:18 PM

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