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Elucidation of Bipolar Disorder: A Convergent Approach using Genetics and Imaging

Name: Shareefa Dalvie (DLVSHA006)

Project Supervisor: Prof. Rajkumar S. Ramesar

Project Co-supervisors: Prof. Dan J. Stein and Dr Neil Horn

Division of Human Genetics

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University of Cape Town

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Table of Contents

| | |
|---|-----------|
| Acknowledgements | 2 |
| Plagiarism Declaration | 3 |
| List of Figures | 8 |
| List of Tables | 9 |
| Abbreviations | 10 |
| Abstract | 14 |
| Chapter 1: Introduction | 16 |
| 1.1 Features and Characteristics of Bipolar Disorder | 16 |
| 1.1.1 DSM-IV criteria for BPD | 18 |
| 1.2 Kraepelinian Dichotomy: BPD and Schizophrenia | 18 |
| 1.3 Techniques Used to Determine the Aetiology of BPD | 20 |
| 1.3.1 Mouse Models | 20 |
| 1.3.2 Human Gene Expression Data | 23 |
| 1.3.3 Human Blood Evidence | 24 |
| 1.3.4 Genetics and Family Studies | 25 |
| 1.3.5 Brain Imaging | 29 |
| 1.4 Convergent Functional Genomics | 32 |
| 1.5 Candidate Genes | 33 |
| 1.5.1 <i>KLF12</i> | 33 |
| 1.5.2 <i>ARNTL</i> | 33 |
| 1.5.3 <i>BDNF</i> | 35 |
| 1.5.4 <i>ALDH1a1</i> | 36 |

| | |
|---|-----------|
| 1.5.5 <i>A2PB1</i> | 37 |
| 1.5.6 <i>MBP</i> | 38 |
| 1.5.7 <i>AK3L1</i> | 39 |
| 1.5.8 <i>GSK3β</i> | 39 |
| 1.5.9 <i>ANK3</i> | 40 |
| 1.5.10 <i>CACNA1C</i> | 41 |
| 1.6 Aims and Objectives | 42 |
| Chapter 2: Materials and Methods | 44 |
| 2.1 BPD Cohort Description | 44 |
| 2.1.1 Cohort Description for Case-Control Analysis | 45 |
| 2.1.2 Cohort Description for Brain Imaging Analysis | 45 |
| 2.2 DNA Isolation and Quality Determination | 46 |
| 2.3 Gene and SNP Selection, Primer Design and Synthesis | 47 |
| 2.3.1. Gene and SNP selection | 47 |
| 2.3.2 External Primer Design | 48 |
| 2.4 Polymerase Chain Reaction (PCR) | 49 |
| 2.5 SNaPshot PCR | 51 |
| 2.5.1 SNaPshot PCR Internal Primer Design | 53 |
| 2.5.2 Sap and ExoI Clean-up of PCR Products | 55 |
| 2.5.3 SNaPshot PCR Conditions | 56 |
| 2.5.4 Post- SNaPshot SAP Treatment | 57 |
| 2.5.5 Capillary Electrophoresis on ABI PRISM | 58 |
| 2.5.6 Analysis of Results | 58 |
| 2.6 Genotype Validation | 58 |
| 2.6.1 PCR of DNA Samples for Cycle Sequencing | 59 |
| 2.6.2 Cycle Sequencing | 59 |
| 2.6.3 Ethanol Precipitation | 59 |
| 2.6.4 Capillary Electrophoresis on ABI Prism | 60 |

| | |
|--|-----------|
| 2.6.5 Analysis of Results | 61 |
| 2.7 Statistical Analysis | 61 |
| 2.7.1 Genotype Frequencies, Hardy-Weinberg Equilibrium Calculations and Logistic Regression with R | 61 |
| 2.7.2 M_{QLS} (adjusting for relatedness) and Pedstats | 62 |
| 2.8 Neuroimaging of Subset of Cohort | 62 |
| 2.8.1 Statistical Analysis of Neuroimaging Results | 63 |
| Chapter 3: Results | 64 |
| 3.1 BPD Cohort Description | 64 |
| 3.1.1 Cohort Description for Case-Control Analysis | 65 |
| 3.1.2 Cohort Description for Brain Imaging Analysis | 67 |
| 3.2 DNA Quality Determination | 67 |
| 3.3 Gene and SNP Selection, Primer Design and Synthesis | 68 |
| 3.4 Polymerase Chain Reaction (PCR) | 68 |
| 3.4.1 PCR Optimisation | 68 |
| 3.4.2 PCR Conditions for Candidate Genes | 69 |
| 3.5 SNaPshot PCR | 73 |
| 3.5.1 Sap and ExoI Clean-up of PCR Products | 73 |
| 3.5.2 Capillary Electrophoresis on ABI PRISM | 73 |
| 3.5.3 Analysis of Results | 74 |
| 3.6 Genotype Validation | 75 |
| 3.6.1 PCR of DNA Samples for Cycle Sequencing | 75 |
| 3.6.2 Cycle Sequencing | 75 |
| 3.6.3 Analysis of Results | 75 |
| 3.7 Statistical Analysis for Case Control Association | 77 |
| 3.7.1 Genotype Frequencies, Hardy Weinberg Equilibrium Calculations and Logistic Regression with R | 77 |

| | |
|--|------------|
| 3.7.2 M_{QLS} (adjusting for relatedness) and Pedstats | 80 |
| 3.8 Statistical Analysis of Neuro-imaging | 82 |
| Chapter 4: Discussion | 84 |
| 4.1 Future Directions | 91 |
| 4.2 Conclusions | 93 |
| Websites Referenced | 95 |
| References | 96 |
| Appendices | 121 |
| Appendix 1: Patient Consent Form | 121 |
| Appendix 2: Gentra Puregene™ DNA Isolation Protocol | 123 |
| Appendix 3: Reagents, Buffers and Solutions | 124 |
| Appendix 4: GeneScan -120 Liz size standard | 126 |
| Appendix 5: Ethanol Precipitation | 127 |
| Appendix 6: Annealing Temperatures (T_a) for Temperature Gradient Reactions | 128 |
| Appendix 7: Additional Temperature Gradient Gel Pictures | 129 |
| Appendix 8: Psychosis and relapse in bipolar disorder are related to GRM3, DAOA, and GRIN2B genotype. Dalvie et al. [2010] | 133 |

List of Figures

| | |
|---|----|
| Figure 1: An illustration of the SNaPshot PCR principle | 52 |
| Figure 2: Ethnic group distribution of BPD archive | 64 |
| Figure 3: Distribution of most prevalent phenotypes of BPD cohort | 65 |
| Figure 4: Number of males and females in case and control groups | 66 |
| Figure 5: Number of Caucasian and Mixed Ancestry individuals | 66 |
| Figure 6: Integrity gel of genomic DNA | 67 |
| Figure 7: <i>KLF12</i> rs9600160 (180bp) temperature gradient | 69 |
| Figure 8: Group A PCR Products for three primer pairs | 71 |
| Figure 9: Group B PCR Products for two primer pairs | 71 |
| Figure 10: Group C PCR Products for two primer pairs | 72 |
| Figure 11: Group D PCR Products for two primer pairs | 72 |
| Figure 12: Group E PCR Products for two primer pairs | 73 |
| Figure 13: SNaPshot results for Group A-B | 74 |
| Figure 14: SNaPshot results for Group C-D-E | 74 |
| Figure 15: PCR amplification for cycle sequencing reaction | 75 |
| Figure 16: Sequencing results for <i>BDNF</i> rs6265 | 76 |
| Figure 17: Sequencing results for <i>ANK3</i> rs9804190 | 76 |
| Figure 18: Genotype counts of cases and controls | 78 |
| Figure 19: Genotype counts of cases and controls | 79 |

List of Tables

| | |
|---|----|
| Table 1: Diagnostic criteria of mood episodes characteristic of BPD | 19 |
| Table 2: Genes and pathways implicated in human expression studies | 24 |
| Table 3: Summary of recent BPD Genome Wide Association Studies | 28 |
| Table 4: Candidate genes from CFG and GWAS and respective SNPs | 43 |
| Table 5: Selected candidate genes, respective SNPs and CFG scores | 48 |
| Table 6: External primer sequences for amplification of candidate gene regions | 50 |
| Table 7: PCR cycling conditions | 51 |
| Table 8: Dyes assigned to each of the ddNTPs | 52 |
| Table 9: SNaPshot internal primer sequences | 54 |
| Table 10: Conditions for Sap and ExoI purification | 55 |
| Table 11: Sap and ExoI DNA purification | 56 |
| Table 12: The components of the SNaPshot PCR reaction | 57 |
| Table 13: Conditions for SNaPshot PCR | 57 |
| Table 14: Components of the cycle sequencing reaction | 60 |
| Table 15: Cycle sequencing PCR conditions | 60 |
| Table 16: Optimal annealing temperature determined for each primer pair | 69 |
| Table 17: <i>R</i> logistic regression output for case-control data | 80 |
| Table 18: Pedigree structure from Pedstats output | 81 |
| Table 19: M_{QLS} Output for Case Control Association | 82 |
| Table 20: Kruskal Wallis and ANOVA values for each of the SNPs and imaging measures | 83 |

Abbreviations

| | |
|----------------|---|
| °C | Degrees Celsius |
| µl | Microlitre |
| µM | Micromolar |
| <i>A2BP1</i> | <i>Ataxin-2 Binding Protein 1</i> |
| ADHD | Attention Deficit Hyperactivity Disorder |
| AEI | Allelic Expression Imbalance |
| <i>AK3L1</i> | <i>Adenylate Kinase 3-Like 1</i> |
| <i>ALDH1A1</i> | <i>Aldehyde Dehydrogenase 1 Family, Member A1</i> |
| <i>ALDH1A2</i> | <i>Aldehyde Dehydrogenase 1 Family, Member A2</i> |
| AMP | Adenosine Monophosphate |
| <i>ANK2</i> | <i>Ankyrin-2</i> |
| <i>ANK3</i> | <i>Ankyrin-3</i> |
| ANOVA | Analysis of Variance |
| AOO | Age of Onset |
| <i>ARNTL</i> | <i>Aryl Hydrocarbon Nuclear Translocator-Like</i> |
| <i>BDNF</i> | <i>Brain Derived Neurotrophic Factor</i> |
| bp | Base pairs |
| BPD | Bipolar Disorder |
| BPDI | Bipolar Disorder type I |
| BPDII | Bipolar Disorder type II |
| BPDNOS | BPD not otherwise specified |
| <i>BRCA2</i> | <i>Breast Cancer 2</i> |

| | |
|-------------------|---|
| <i>CACNA1A</i> | <i>Calcium Channel, Voltage-Dependent, L type, Alpha 1A Subunit</i> |
| <i>CACNA1C</i> | <i>Calcium Channel, Voltage-Dependent, L type, Alpha 1C Subunit</i> |
| <i>CANB1</i> | <i>Calcium Channel, Voltage-Dependent, Beta-1</i> |
| <i>CACNB2</i> | <i>Calcium channel, voltage dependent, beta 2 subunit</i> |
| CFG | Convergent Functional Genomics |
| CNS | Central Nervous System |
| <i>Cry</i> | <i>Cryptochrome</i> |
| <i>DAOA</i> | <i>D-amino acid Oxidase Activator</i> |
| <i>DCTN5</i> | <i>Dynactin 5</i> |
| <i>DGKH</i> | <i>Diacylglycerol Kinase</i> |
| dH ₂ O | Distilled Water |
| dNTP | Deoxynucleotide Triphosphates |
| ddNTP | Dideoxynucleotide Triphosphates |
| DSM-IV | Diagnostic and Statistical Manual of Mental Disorders-IV |
| ExoI | Exonuclease I |
| EtBr | Ethidium Bromide |
| <i>GRIN2B</i> | <i>Ionotropic Glutamate Receptor, N-methyl D-aspartate 2B</i> |
| <i>GRM3</i> | <i>Glutamate Receptor, Metabotropic 3</i> |
| <i>GSK3β</i> | <i>Glycogen Synthase Kinase 3β</i> |
| GTP | Guanosine Triphosphate |
| GWAS | Genome-Wide Association Study |
| HCV | Hippocampal Volume |
| HWE | Hardy Weinberg Equilibrium |

| | |
|------------------------------|--|
| ICV | Intracranial Volume |
| KLF | Kruppel-like Factor |
| <i>KLF12</i> | <i>Kruppel-like Factor 12</i> |
| <i>MBP</i> | <i>Myelin Basic Protein</i> |
| MDD | Major Depressive Disorder |
| MDE | Major Depressive Episode |
| Met | Methionine |
| mm | Millimetres |
| MRI | Magnetic Resonance Imaging |
| MWM | Molecular Weight Marker |
| NCBI | National Centre for Biotechnology Information |
| <i>NDUFAB1</i> | <i>NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1</i> |
| ng | Nanogram |
| NMR | Nuclear Magnetic Resonance |
| <i>PALB2</i> | <i>Partner and Localizer of BRCA2</i> |
| PCR | Polymerase Chain Reaction |
| PD | Personality Disorder |
| <i>Per</i> | <i>Period</i> |
| <i>PER3</i> | <i>Period Homolog 3</i> |
| PTSD | Post traumatic Stress Disorder |
| <i>RARβ</i> | <i>Retinoic Acid Receptor β</i> |
| RBC | Red Blood Cell |
| <i>RXRβ</i> | <i>Retinoid X Receptor β</i> |

| | |
|------|--|
| SABP | Schizoaffective Disorder, Bipolar Type |
| SAP | Shrimp Alkaline Phosphatase |
| SCA | Spinocerebellar Ataxia |
| SCID | Structured Clinical Interview for the DSM-IV |
| SCZ | Schizophrenia |
| SNP | Single Nucleotide Polymorphism |
| Ta | Annealing Temperature |
| TBE | Tris Borate EDTA |
| UCT | University of Cape Town |
| Val | Valine |
| VBM | Voxel Based Morphometry |

University of Cape Town

Abstract

Introduction: Bipolar Disorder (BPD) is a severe, neuropsychiatric disorder which affects approximately 1% of the population. Due to the high heritability of BPD, genetic variations amongst affected individuals have been investigated. Recently, researchers have developed a method termed *Convergent Functional Genomics (CFG)* which aims to identify a prioritized candidate gene list, based on the results of previous studies, underlying the pathology of BPD and other psychiatric illnesses. In addition, neuro-imaging studies have revealed that BPD is associated with structural changes in the brain and that certain brain regional volumes are heritable.

Aims and Objectives: The aims were to determine whether variants, within ten selected candidate genes, have an association with BPD and whether any relationship exists between these variants and brain imaging volumes in subjects with BPD. The objectives were to i) select a list of BPD candidate genes, ii) identify a cohort of individuals from the BPD registry, iii) genotype the candidate genes using a polymerase chain reaction (PCR) based technology, iv) analyse the genotyping data with the appropriate statistical methods, and v) obtain brain imaging data and perform the appropriate statistical analysis

Materials and Methods: Candidate genes were selected based on the most significant findings of previous publications. Multiplex SNaPshot™ PCR was used to genotype 224 case and 202 control samples. Cases comprised of individuals in a South African cohort

of Mixed Ancestry and Caucasians, affected with BPD Type I. Magnetic resonance imaging (MRI) data was collected for a subset of the case group (n=22).

Results: Preliminary results suggest associations between SNPs in the candidate genes, *ankyrin3 (ANK3)*, *ataxin-2 binding protein1 (A2BP1)* and *myelin basic protein (MBP)*, with BPD. No significant associations were found between genotypes and brain imaging data.

Conclusions: These results suggest that BPD may have a common aetiology with neurodegenerative disorders. Polymorphisms within *ANK3*, *A2BP1* and *MBP* should be further investigated to determine the specific effects of these variants on the expression of the respective genes.

Chapter 1: Introduction

Bipolar Disorder (BPD) is a severe psychiatric illness which is characterized by extremes in mood and affects approximately 1% of the population. Much research has been undertaken to determine the underlying causes of this highly heterogeneous disorder. However, due to its high heritability, genetic factors have been thought to be a major contributor to the underlying aetiology. Recently, researchers have developed a *Convergent Functional Genomics* (CFG) approach which aims to derive a prioritized candidate gene list, based on the results of a wide range of previous studies, including brain imaging and animal models. The 113 CFG prioritized genes that have been identified comprise those involved in a wide range of central nervous system (CNS) function including neuronal maintenance, circadian rhythm regulation and transcriptional regulation [Le-Niculescu et al., 2008]. In addition, several other genes have been implicated through hypothesis-free genome-wide association studies (GWAS) [Stratton, 2007; Baum et al., 2008; Ferreira et al., 2008; Sklar et al., 2008; Zhang et al., 2008; Hattori et al., 2009; Scott et al., 2009; Smith et al., 2009]. Many of these genes overlap with those identified by the CFG approach and are ideal candidates to interrogate in studies investigating the aetiology of BPD.

1.1 Features and Characteristics of Bipolar Disorder

BPD is a severe, debilitating mental disorder affecting approximately 1% of the population [Merikangas et al., 2007]. This disorder is characterized by mood episodes ranging from an elevated mood to severe depression [Ogden et al., 2004]; onset usually occurs during early adulthood, and affects both males and females at equal frequencies

[Miller, 2006]. The affected individual may experience a decline in quality of life, productivity, and longevity [Thase and Denko, 2008] and psychotic features such as delusions and hallucinations may also be a feature [American Psychiatric Association. Task Force on DSM-IV, 1994]. Individuals with BPD have greater difficulty in sustaining interpersonal relationships and have trouble maintaining employment compared to those without mood disorders: a total of 54% of affected individuals were dismissed from their jobs compared to 29% of a matched unaffected group [Calabrese et al., 2003]. The same study showed that persons suffering with the disorder have difficulty abiding by the law; and have a significantly higher chance of being arrested for crimes. Violent behaviour is often observed during manic episodes [American Psychiatric Association. Task Force on DSM-IV, 1994]. In 1991, the total economic burden of BPD in the USA was estimated at US \$45 billion. This cost is attributed to medication, hospitalisation, misdiagnosis, and the estimated financial burden due to productivity loss [Hirschfeld and Vornik, 2005]. Between 10% and 20% of patients successfully commit suicide [Müller-Oerlinghausen et al., 2002; Bauer and Pfennig, 2005]. Currently, the diagnosis of BPD is based exclusively on clinical symptoms as the molecular pathology has yet to be discovered [Craddock and Sklar, 2009].

Based on different symptomatic criteria, there are several types of BPD distinguishable in the population: i) BPD type I (BPDI), ii) BPD type II (BPDII), iii) Cyclothymia, and iv) “BPD not otherwise specified” (BPDNOS) [American Psychiatric Association. Task Force on DSM-IV, 1994]. For the purposes of this study, a more detailed description of BPDI, the most severe type, is described below.

1.1.1 DSM-IV criteria for BPD

According to DSM-IV (Diagnostic and Statistical Manual of Mental Disorders-IV) criteria [American Psychiatric Association. Task Force on DSM-IV, 1994], BPD is a recurrent mood disorder, characterized by the presence of one or more manic or mixed episodes (see **Table 1** for diagnostic criteria of each mood episode). The occurrence of one or more major depressive episodes may also be a feature. The mania associated with BPD should not be due to Substance-Induced Mood Disorder or Schizoaffective Disorder. During severe manic episodes violent or abusive behavior may be experienced. On average, untreated BPD patients experience four episodes each decade. There are no differences in BPD prevalence across gender and ethnicity. However, it has been noted that males are more likely to experience a manic episode before a depressive episode and vice-versa for females. Most individuals return to an improved or functional state between episodes [American Psychiatric Association. Task Force on DSM-IV, 1994].

1.2 Kraepelinian Dichotomy: BPD and Schizophrenia

Less than a hundred years ago, Emil Kraepelin classified Schizophrenia (SCZ) and BPD as being two separate disease entities, which he termed dementia praecox and manic-depression, respectively. In other words, each disorder has a different underlying cause [Craddock and Owen, 2005; Greene, 2007]. However, these two disease entities share common clinical symptoms [Maier et al., 2005] and there exists an intermediate phenotype, termed Schizoaffective Disorder [Lichtenstein et al., 2009]. Schizoaffective Disorder is characterized by features of both SCZ and BPD [Craddock et al., 2009]. Family history research has shown the occurrence of SCZ and BPD in the same family [Wildenauer et al., 1999]. Relatives of BPD patients have an increased risk for SCZ, including adopted children whose biological parents have SCZ [Lichtenstein et al., 2009].

This suggests a genetic/biological overlap between the two groups of disorders [Maier et al., 2005]. There is a greater amount of genetic information available pertaining to the aetiology of SCZ than there is for BPD [McGuffin et al., 2003].

Table 1: Diagnostic criteria of mood episodes characteristic of BPD

| Episode | DSM-IV Criteria |
|--|---|
| <p>Major Depressive Episode (MDE)</p> | <p>a) Five or more of the following symptoms</p> <ul style="list-style-type: none"> • Depressed or irritable (children and adolescents) mood • Decreased interest or pleasure in almost all activities • Significant changes in body weight • Insomnia or hypersomnia • Psychomotor agitation • Fatigue and decrease in energy • Feelings of worthlessness and excessive guilt • Diminished concentration and increased indecisiveness • Increased thoughts of death and suicidal ideation <p>b) Symptoms do not meet criteria for mixed episode</p> <p>c) Symptoms cause functional impairment</p> <p>d) Symptoms not a result of physiological effect of substance</p> <p>e) Symptoms not as a result of bereavement and are present for longer than two months</p> |
| <p>Manic Episode</p> | <p>a) Period of abnormally elevated or irritable mood lasting at least one week</p> <p>b) Three or more of the following symptoms are present to a significant degree</p> <ul style="list-style-type: none"> • Inflated self-esteem or grandiosity • Decreased need for sleep • Talkative • Racing thoughts • Easily distractible • Increased goal-directed activity or psychomotor agitation • Excessive participation in pleasurable activities with potential for painful consequences <p>c) Symptoms do not meet criteria for mixed episode</p> <p>d) Symptoms cause functional impairment</p> <p>e) Symptoms not a result of physiological effect of substance</p> |

Table 1: Diagnostic criteria of mood episodes characteristic of BPD continued

| | |
|---------------------------------|--|
| <p>Mixed Episode</p> | <p>a) Criteria met for both Major Depressive Episode and Manic Episode nearly every day during a one week period b) Symptoms cause functional impairment c) Symptoms not a result of physiological effect of substance</p> |
| <p>Hypomanic Episode</p> | <p>a) Period of abnormally elevated or irritable mood lasting at least four days b) Three or more of the following symptoms are present to a significant degree</p> <ul style="list-style-type: none"> • Inflated self-esteem or grandiosity • Decreased need for sleep • Talkative • Racing thoughts • Easily distractible • Increased goal-directed activity or psychomotor agitation • Excessive participation in pleasurable activities with potential for painful consequences <p>c) Change in functioning d) Disturbance in mood observable by others e) Episode not severe enough to caused marked functional impairment f) Symptoms not a result of physiological effect of substance</p> |

[American Psychiatric Association (DSM-IV), 2005]

1.3 Techniques Used to Determine the Aetiology of BPD

1.3.1 Mouse Models

Mouse models have proven to be a reliable resource for geneticists. With the completion of the mouse genome sequence and the relative abundance of different mouse strains, the phenotypic effects of certain mutations are relatively easily observable [Seong et al., 2002]. Human disorders are modeled in animals via pharmacological or genetic

manipulation [Le-Niculescu et al., 2008]. In general, an animal model needs to meet the following three criteria in order for it to be considered effective:

(a) Face Validity- the model should have similar behavioural patterns as human disease.

(b) Construct Validity- the model should have the same or similar underlying pathological characteristics as the human disease.

(c) Predictive Validity- Symptoms of the animal model that are characteristic of human disease should improve with administration of medication [Jornada et al., 2009].

An animal model of BPD would help in the understanding of the aetiology as well as provide a means to test treatment options. However, due to the cyclical nature of BPD, the innovation of an animal model, which meets all three criteria, has proven to be elusive. There are models which mimic certain characteristics of the disorder, such as depression and mania, but none are able to provide a mechanism by which mood states are alternated [Machado-Vieira et al., 2004]. Each of the existing BPD models can be categorized as either pharmacological, nutritional, environmental, or genetic [Kato et al., 2007]. An example of each category is discussed below:

- i) **Pharmacological Model: Ouabain-** It has been shown that sodium-potassium-ATPase function is significantly reduced in individuals suffering from psychiatric disorders and that affected individuals appear to have increased intracellular levels of sodium and calcium [el-Mallakh et al., 1995], which suggests dysfunction of these ion transporters. Ouabain is an inhibitor

of sodium-potassium-ATPase and has been shown to induce a manic-like state in mice by increasing locomotor activity [el-Mallakh et al., 1995; Freitas et al., 2010].

- ii) Nutritional Model: Homocysteine-** Serum levels of homocysteine, a neurotoxin, has been found to be elevated in BPD patients. This is due to the effects of diet, inadequate vitamin B consumption, a decrease in physical exercise and an increase in smoking. In a mouse model, mild cognitive impairments were observed after chronic exposure to homocysteine [Levine et al., 2005].
- iii) Environmental Model: Learned Helplessness (LH)-** This mouse model is used to represent depression. This model, which is developed by treating the animal with electroshocks, displays symptoms of human depression and is unable to cope under difficult situations. The symptoms of this model are alleviated with the administration of antidepressants [Nakatani et al., 2004].
- iv) Genetic Model: Glucocorticoid Overexpression-** Glucocorticoid receptors have several functions including modulation of stress response and cognition. Overexpression of glucocorticoid receptors in the forebrain have shown an increase in anxiety-like and depressive symptoms in mice [Wei et al., 2004].

1.3.2 Human Gene Expression Data

Due to the nature of BPD, the brain would be the preferable tissue for gene expression studies. However, this may only be obtained from postmortem tissue which may present with a few disadvantages; for example, gene expression may be significantly altered due to death, tissue preservation methods [Matigian et al., 2007] or medication [Nakatani et al., 2006].

Nevertheless, Nakatani et al. [2006] performed a genome-wide expression study on seven postmortem BPD brains and seven control brains. They found 108 genes were differentially expressed in BPD brains and that these genes only had a minimal fold change. This is in keeping with the hypothesis that psychiatric illnesses are a manifestation of subtle changes in gene expression. Pathway analysis indicated that most of the genes could be classified as either forming part of the processes of cellular growth and proliferation, nervous system development, or cell death. In another expression study, also involving postmortem brains, pathways that were over-represented included the G-protein coupled receptor signaling pathway, stimulus response pathways, intracellular transport, and the ubiquitin cycle [Ryan et al., 2006]. **Table 2** lists some of the expression studies that have been performed and the major pathways that have been implicated in each. There appears to be no consensus of results amongst these expression studies. This could be due to different tissue “quality” in each of the studies which in turn has an effect on tissue pH levels. For example, energy metabolism and mitochondrial genes are typically down-regulated in low pH conditions [Nakatani et al., 2006].

Table 2: Genes and pathways implicated in human expression studies

| Study | Genes/Pathways implicated | Up- /Downregulated | Tissue type | Specific Region |
|------------------------|--|-----------------------|----------------|---|
| Tkachev et al. [2003] | Oligodendrocyte-related Myelin-related | ↓ ↓ | brain | Prefrontal Cortex |
| Iwamoto et al. [2004] | Channels, receptors, transporters Stress response | ↓ ↑ | brain | Prefrontal Cortex |
| Iwamoto et al. [2005] | Mitochondria-related | ↓ | brain | Prefrontal Cortex |
| Nakatani et al. [2006] | Cellular growth, proliferation Nervous system development Cell death | ↑ ↓ ↑ ↓ ↑ ↓ | brain | Prefrontal Cortex |
| Ryan et al. [2006] | G-protein coupled receptor signaling Response to stimulus Intracellular transport Ubiquitin cycle | ↑ ↑ ↓ ↓ | brain | Prefrontal and Orbitofro- ntal Cortex |

1.3.3 Human Blood Evidence

Currently, there are no available blood tests that can be used to determine the presence of a mood disorder in a patient. Diagnosis is strictly limited to clinical symptoms and patient self-report [Craddock and Sklar, 2009]. This presents with certain disadvantages to psychiatrists in obtaining an accurate diagnosis which is necessary for effective treatment and management. Therefore, discovery of appropriate peripheral tissue biomarkers for BPD would allow for greater accuracy in patient diagnosis. Unfortunately,

these types of studies have proven to be inconclusive and a definitive or other blood biomarker has yet to be discovered.

1.3.4 Genetics and Family Studies

Studies have shown that BPD has heritability estimates ranging from 59% to 85% [Lichtenstein et al., 2009; McGuffin et al., 2003]. First degree relatives and half-siblings of affected individuals are at a significantly increased risk (5-10%) of developing the disorder [Müller-Oerlinghausen et al., 2002; Lichtenstein et al., 2009]. Adoption studies have revealed that the biological relatives of a proband have a 31% higher risk of developing BPD than the relatives of control individuals. In a BPD twin study, it was shown that monozygotic and dizygotic twins have a 67% and 19% concordance, respectively [McGuffin et al., 2003]. Therefore, genetics is thought to hold a key to the understanding of BPD. However, efforts at elucidating the genetic cause of this disorder have been hampered by genetic heterogeneity, environmental factors and phenotypic variability that characterise BPD [Le-Niculescu et al., 2008]. As a result, researchers have adopted different approaches to determine the factors underlying the pathophysiology of BPD. One of these approaches involves the examination of intermediate phenotypes which are associated with the disorder under investigation but are easier to investigate genetically [Gottesman and Gould, 2003]. It is thought that discovering the genetic components of the more homogenous endophenotypes will aid in the understanding of genetically complex disorders like BPD. A particular trait needs to meet the following criteria to be considered as an endophenotype [Gottesman and Gould, 2003]:

- Is associated with a disorder in the population
- Is heritable
- Is evident in individuals whether the disorder is active or not
- Co-occurs with disease within families
- Manifests in non-affected family members of affected individuals at a higher frequency than in the general population

1.3.4.1 Linkage and Association Studies

The candidate approach investigates genes which have been reported to be linked to disease in previous research, or focuses on loci based on their purported role in the biology of the disorder. Linkage analysis involves the genotyping of genetic markers which are significantly associated with a disease phenotype. These approaches are more cost-effective than GWAS but require a hypothesis regarding the relationship of the gene to disease [Hirschhorn and Daly, 2005]. Regions on chromosomes 6, 7, 8, 10, 11, 12, 13, 15, 16, 17, and 22 have previously been reported to be linked to the BPDI phenotype [Ogden et al., 2004; Neves-Pereira et al., 2002; Sklar et al., 2002; Hattori et al., 2003; Chen et al., 2005; Schumacher et al., 2004; Fallin et al., 2005; Martucci et al., 2006; Avramopoulos et al., 2007].

1.3.4.2 Genome-Wide Association Studies

Over the last several years, researchers interested in mapping the genetic basis of several complex disorders have invested their resources in GWAS which do not require an *a priori* hypothesis. GWAS utilize high-throughput genotyping methods, on very large cohorts of subjects/patients and controls, to determine whether genetic variants, such as single nucleotide polymorphisms (SNPs) spread across the genome, have an association with disease status compared to equally large numbers of controls [Hirschhorn and Daly, 2005]. To date, eight GWAS have been performed on BPD cohorts; these are summarized in **Table 3**.

In 2007, The Wellcome Trust Case- Control Consortium (WTCCC) carried out a GWAS for seven common diseases, one of which was BPD [Stratton, 2007]. Their strongest signal of association for BPD was in the region of chromosome 16p12, which includes genes such as *PALB2* (*partner and localiser of BRCA2*), *NDUFAB1* (*NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1*), and *DCTN5* (*dynactin 5*) [Stratton, 2007]. The subsequent GWAS for BPD implicated genes such as *DGKH* (*diacylglycerol kinase*), *ANK3* (*ankyrin 3*), and *CACNA1C* (*Calcium Channel, Voltage-Dependent, L type, Alpha 1C Subunit*) [Baum et al., 2008; Ferreira et al., 2008]. However, the results from these studies have proven difficult to replicate in independent samples.

Table 3: Summary of recent BPD Genome-Wide Association Studies

| Study | No. of Samples | Phenotype | Ethnicity | Type of Polymorphism | Regions/Genes Implicated | Genotyping Platform |
|------------------------|---|--------------------|------------------|----------------------|---|--------------------------|
| Stratton, [2007] | 2000 cases 3000 controls | BPD | Caucasian | SNPs | 16p12 | Affymetrix GeneChip 500K |
| Baum et al. [2008] | 1233 cases 1439 controls | BPDI | Caucasian | SNPs | <i>DGKH</i> (13q14.11) | Illumina HumanHap550 |
| Sklar et al. [2008] | 1461 cases 2008 controls | BPDI | Caucasian | SNPs | <i>MYO5B</i> (18q21) <i>TSPAN8</i> (12q14.1) | Affymetrix GeneChip 500K |
| Ferreira et al. [2008] | 4387 cases 6209 controls | BPDI, BPDII, SABP* | Caucasian | SNPs | <i>ANK3</i> (10q21) <i>CACNA1C</i> (12p13.3) | Affymetrix GeneChip 500K |
| Zhang et al. [2008] | 1001 cases 1033 controls | BPDI, SABP | Caucasian | SNPs and CNVs | - | Affymetrix SNP 6.0 |
| Hattori et al. [2009] | 1st stage: 107 cases 107 controls | BPDI | Japanese | SNPs | <i>DEC1</i> (9q32), <i>IRF2</i> (4q34.1), <i>VEPH1</i> (3q24), <i>EXTL1</i> (1p36.1), <i>PCDH15</i> (10q21.1), <i>MYT1L</i> (2p25.3), <i>CSMD1</i> (8p23.2), <i>DYNC111</i> (7q21.3), <i>PCSK5</i> (9q21.3), <i>OLFM3</i> (1p22), <i>PLXNA2</i> (1q32.2), <i>KCNMB2</i> (3q26.2), <i>INTS9</i> (8p21.1), <i>AUTS2</i> (3q25), <i>NUP107</i> (12q15) | Affymetrix GeneChip 500K |
| | 2nd stage: 395 cases 409 controls | BPDI, BPDII | Japanese | | | Illumina Beadarray |
| Scott et al. [2009] | 2076 cases 1676 controls | BPDI | Caucasian | SNPs | 1p31.1, 3p21 (<i>ITIH1</i> , <i>GNL3</i> <i>NEK4</i> , <i>ITIH3</i>), 5q15(<i>MCTP1</i>), Xq27.1, <i>NAP5</i> | Illumina HumanHap550 |
| Smith et al. [2009] | 1001 cases 1033 controls | BPDI, SABP | Caucasian | SNPs | <i>DPY19L3</i> , <i>NTRK2</i> | Affymetrix SNP 6.0 |
| | 345 cases 670 controls | BPDI, SABP | African American | | | |

*Schizoaffective Disorder, Bipolar Type

1.3.5 Brain Imaging

Research within psychiatry has investigated the links between disease states, genotypes, psychological processes and brain structure and function. This effort has been facilitated by the development of recent neuro-imaging techniques [Dager et al., 2008]. Over the past 30 years, neuro-imaging studies have revealed that BPD is associated with structural changes in the brain [Vita et al., 2009]. However, different regions of the brain have been implicated and replication studies have proven to be inconclusive. Also, the causal reason, age of onset, and course of these brain abnormalities in affected individuals are still largely unknown [Vita et al., 2009]. Magnetic resonance imaging (structural-voxel based morphometry (VBM)) is a commonly used imaging technique that is discussed below in greater detail.

1.3.5.1 Magnetic Resonance Imaging (MRI)

This technique is able to provide a high resolution, three-dimensional view of soft tissues. The main advantage of this technique is its relative safety as it is non-invasive and does not involve any ionizing radiation [Leondes, 2005]. The MRI image is obtained by the use of a tomograph. The main constituent of this instrument is a large cylindrical coil which functions as a magnet. There are additional coils which form part of an electric circuit, whereby the electrical current within the magnet is able to produce a steady magnetic field. The subject is placed inside the cylindrical coil and exposed to the magnetic field [Hennel and Kryst-Widźgowska, 1997].

MRI relies on nuclear magnetic resonance (NMR), whereby protons and neutrons that constitute the nucleus of a molecule, have what is known as an angular momentum or spin [Leondes, 2005]. MRI involves the interaction between the nuclear spin of a proton within the nucleus of a hydrogen atom and the applied magnetic field. The human body consists mainly of lipids (~33%) and water (~70%) [Sheng and Huggins, 1979] hence, an abundance of hydrogen atoms in the brain [Leondes, 2005]. These “protons” once placed in a magnetic field, absorb radio waves (electromagnetic energy) at a certain frequency, and align themselves to the magnetic field by releasing the absorbed energy [Sajjad and Alam, 2007].

A meta-analysis of MRI studies for first episode BPD patients indicated that intracranial as well as white matter volumes were significantly lower in affected individuals compared to controls [Vita et al., 2009]. The authors propose that the apparent deficiency in white matter may indicate irregular neural development of the brain. Reduction in grey matter volumes has also often been found to be associated with BPD. Sassi et al. [2002] found that lithium treatment increased grey matter volumes compared to untreated patients. However, changes in grey matter volume could not be replicated in other studies [Vita et al., 2009; McDonald et al., 2005; Scherk et al., 2008]. These discrepancies in results could be due to the effects of medication and small sample sizes [van der Schot et al., 2009], suggesting a need for additional studies. Several studies have examined the relationship between hippocampal volumes and BPD [Blumberg et al., 2003; Rimol et al., 2010]. Findings from these studies suggest that individuals affected with BPD have reduced hippocampal volumes compared to controls. Similar to grey

matter volumes, treatment with lithium appeared to increase the hippocampal volumes of affected individuals compared to unmedicated individuals [Yucel et al., 2008].

1.3.5.2 Imaging Genetics

Certain brain volumes have been shown to be heritable [Goldman et al., 2008]. For example, twin studies have shown that frontal lobe structures and the hippocampus have heritabilities of 90-95% and 40-69%, respectively [Peper et al., 2007]. These findings indicate that genes may underlie the variation in brain structures seen across individuals.

van der Schot et al. [2009] examined the effects of genes and the environment on brain volumes in twin pairs concordant and discordant for BPD. They concluded that a decrease in white matter is associated with a genetic risk for developing BPD. This finding was also found in a previous twin study [Kieseppä et al., 2003]. Similarly, another twin study found that hippocampal volumes were smaller in the affected twin when compared to the unaffected co-twin [Noga et al., 2001]. It is thought that genes underlying BPD may be the cause for the observed decreases in white matter and hippocampal volumes. However, the biological mechanisms underlying these associations are still unknown.

Brain structural volumes and function have been investigated as endophenotypes of psychiatric illnesses [McDonald et al., 2004; Cannon and Keller, 2006; Glahn et al., 2007]. As endophenotypes are easier to investigate genetically [Gottesman and Gould, 2003], a

field which has received some attention of late is that of imaging genetics. Imaging genetics is a type of genetic association study in which genotypes are correlated with brain structure or function. This type of study is favoured as it gives a direct indication of the effect of underlying genetics on brain structure and function [de Geus et al., 2008] which may aid in the understanding of the genetics underlying complex psychiatric illnesses.

1.4 Convergent Functional Genomics

Due to the high heritability and familial pattern of inheritance of BPD, researchers have commonly focused on the genetics of this disorder. Many of these studies, as discussed previously, have focused on single gene approaches or expression studies which have, in turn, generated results implicating numerous genes. However, most of these findings could not be replicated particularly in those studies involving differing ethnic groups. With this abundance of data available it has become difficult to determine which finding would most likely be significant for follow-up studies. Therefore, researchers have focused their attention on a more comprehensive integrated approach to determine the genetic components of this and other psychiatric disorders. Le Niculescu et al. [2008] devised a gene prioritization method which they have termed CFG. CFG integrates data generated from GWAS together with results obtained from independent genomic investigations such as association or linkage studies, animal models and gene expression data. This approach aims to provide a means of prioritizing candidates for future hypothesis-driven studies [Le-Niculescu et al., 2008]. Using this approach, Le-Niculescu et al. [2008] generated an “optimal” candidate gene list for BPD.

1.5 Candidate Genes

All of the candidate genes and respective SNPs that have been investigated in the current study are described below and summarised in **Table 4**.

1.5.1 *KLF12*

This gene has not been functionally implicated in any psychiatric disorder but has been one of the genes that obtained the highest CFG score out of a total of 113 genes [Le-Niculescu et al., 2008]. *KLF12* is a member of a family of 16 kruppel-like factors (KLFs) that are DNA binding transcriptional regulators. Through their role as gene regulators, KLFs are involved in numerous cellular processes such as proliferation, apoptosis and development [Zhang et al., 2005]. *KLF12* is located on chromosome 13q22.1 and is specifically involved in the repression of the AP-2 alpha gene [Roth et al., 2000]. AP-2 genes have been implicated in the pathophysiology of cancers as they play a role in tumour progression [Roth et al., 2000]. With this, *KLF12* is also thought to play a role in the development of cancers. A recent study implicated *KLF12* in gastric cancers, and proposed this gene as a target for therapeutic intervention [Nakamura et al., 2009]. Baum et al. [2008] found that an intronic SNP (rs9600160) within *KLF12* was associated with BPD ($p < 0.05$).

1.5.2 *ARNTL*

ARNTL (also known as *BMAL1*), is located on chromosome 11p15.2 and encodes the aryl hydrocarbon nuclear translocator-like protein which forms a heterodimer with the protein CLOCK and is involved in circadian rhythm regulation [Wolting and McGlade, 1998; Hirota and Fukada, 2004]. The regulation of the circadian rhythm is accomplished

by the binding of the ARNTL/CLOCK complex to the E-box sequences of circadian genes such as *Period (Per)* and *Cryptochrome (Cry)*, thus promoting their transcription. Due to the cyclical nature of BPD, impairment in circadian rhythm patterns has often been implicated in the aetiology [Mansour et al., 2005]. Individuals undergoing a manic phase generally require less sleep or experience periods of insomnia, and those experiencing a depressive episode often have hypersomnia [Shi et al., 2008]. Sleep-deprived mice exhibit manic-like symptoms [Benedetti et al., 2008].

ARNTL has also been found to be up-regulated in post-mortem brains of BPDI patients [Nakatani et al., 2006]. In a comprehensive study of BPD, Mansour et al. [2006] used separate study designs to identify predisposing regions of the genome. In the sub-cohort study, involving a case-parent trio study design, they found a positive association between two SNPs in *ARNTL* (rs7107287 and rs895682) and BPDI. Both of these SNPs are located in intronic regions of the gene. The case-control study design also revealed an association between *ARNTL* and BPDI patients. However, the SNP with the significant finding was rs1481892 and not the SNPs previously associated in the a case-parent trio study design [Mansour et al., 2006]. Haplotype analysis revealed an association between polymorphisms within *ARNTL* and another circadian gene, *period homolog 3 (PER3)*, with BPD. However, after correction for multiple testing this association was no longer significant [Nievergelt et al., 2006].

1.5.3 *BDNF*

BDNF is located on chromosome 11p13, is 70 kb in size and has 11 exons [Sheikh et al., 2010]. This gene has often been suggested in the aetiology of psychiatric disorders, as the protein it encodes is involved in the growth and maintenance of neurons within the CNS. Various extraneous factors such as stress and certain types of drugs affect the expression of this gene [Duman, 2002]. Interestingly, although this family of neurotrophins appear to be ubiquitously expressed in vertebrate nervous systems, similar sequences have not been detected in either of the *C. elegans* or *D.melanogaster* genomes suggesting that particular neurotrophic factors have functions in higher order neural activities such as learning, memory and behavior [Chao, 2000]. Several studies have investigated whether variants within *BDNF* increase risk for developing BPD. Of particular interest is the Val66Met (rs6265) functional polymorphism due to a G/A substitution [McHughen et al., 2010], in the prodomain of the protein [Bath and Lee, 2006]. Although, the prodomain is cleaved from the mature protein, it is thought to be involved in the trafficking of BDNF. The Val66Met polymorphism has also been found on to be associated with BPD in several independent studies [Neves-Pereira et al., 2002; Sklar et al., 2002; Xu et al., 2010]. Most of these studies have found that BPD patients have a higher frequency of the valine (Val) allele. Subsequent studies did not show an association between the Val66Met polymorphism and the BPD phenotype [Green et al., 2006; Vinberg et al., 2009]. However, Green et al. [2006] noted that the Val allele was significantly over-transmitted in individuals who presented with rapid cycling BPD. The Val66Met polymorphism has been shown to have an association with the human brain motor system and short-term learning in healthy individuals [McHughen et al., 2010]. The methionine (Met) allele has been associated with poorer episodic memory and

hippocampal activation [Egan et al., 2003]. Also, individuals affected with BPD, possessing a Met allele, show a reduction in their temporal lobe grey matter volumes compared to those without a Met allele [McIntosh et al., 2007]. Similarly, Met carriers in a control population group, are more likely to have smaller hippocampal formations, [Pezawas et al., 2004; Bueller et al., 2006] as are individuals affected with major depression [Frodl et al., 2007].

1.5.4 *ALDH1a1*

ALDH1A1, on chromosome 9q21.13, encodes an enzyme which is part of the aldehyde dehydrogenase family of proteins which consists of 16 distinct enzymes [Duester, 2001]. Aldehyde dehydrogenases are involved in the conversion of vitamin A (retinol) to its active form, retinoic acid, which in turn functions as a regulator of gene expression and plays an important role in the modulation of apoptosis [Palha and Goodman, 2006]. Retinoic acid has been postulated to play a role in the development of SCZ [Goodman, 1998; LaMantia, 1999]. This is based on the observation that certain abnormalities that are characteristic of SCZ are also a feature of retinoid dysfunction. Some common characteristics include mental deficits and congenital abnormalities such as craniofacial and digital malformations. It has also been observed that several loci that have been previously linked to SCZ contain genes that are involved in the retinoic acid cascade. These include the genes *retinoid X receptor β* (*RXR β*) on chromosome 6p21.3 and the *retinoic acid receptor β* (*RAR β*) on chromosome 3p24.3 [Goodman, 1998]. Therefore, genes involved in the retinoic acid pathway are often investigated in relation to SCZ [Wan et al., 2009]. An association has previously been found between *ALDH1A2*

(*aldehyde dehydrogenase type 2*) and SCZ in a Chinese population group [Wan et al., 2009]. As BPD and SCZ are thought to have some genetic overlap [Maier et al., 2005], it would be reasonable to consider *ALDH1A1* as a possible candidate gene for BPD.

1.5.5 *A2BP1*

A2BP1 or *ataxin-2 binding protein 1*, predominantly expressed in both muscle and brain, encodes a protein which binds to the c-terminus of ataxin 2. A polyglutamine expansion in the gene *ataxin-2* has been implicated the aetiology of spinocerebellar ataxia (SCA), a severe hereditary neurodegenerative disorder [Shibata et al., 2000]. The exact function of the *A2BP1* gene is unknown [Ralsler et al., 2005], however, it is thought to play a role in the splicing of downstream targets [Lee et al., 2009]. A translocation which results in a deletion in exon1 of the *A2BP1* gene was previously observed in an individual with autism. This deletion resulted in decreased *A2BP1* expression in the affected individual compared to controls [Martin et al., 2007]. Autism is a neuro-developmental disorder which shares some behavioural features with BPD and SCZ. Based on results from GWAS, it has been suggested, that these three psychiatric disorders, although distinct, share some genetic overlap [Carroll and Owen, 2009]. Hamshere et al. [2009] reported a strong association between a SNP in *A2BP1* (rs4786811) and Schizoaffective Disorder. Also, two intronic SNPs within *A2BP1*, rs7204975 and rs10500336 have been found to be significantly associated (p-values= 0.001 and 0.018, respectively) with BPD in a German population group by the GWAS conducted by Baum et al. [2008].

1.5.6 MBP

Myelin basic protein or *MBP* encodes a protein which composes approximately 30% of the myelin membrane surrounding neuronal tissue [Chambers and Perrone-Bizzozero, 2004]. This gene is located on chromosome 18q22-q23 [Kamholz et al., 1987]. Myelination continues within the human brain, with the culmination of myelinogenesis occurring in the frontal and temporal lobes in late adolescence and early adulthood [Chambers and Perrone-Bizzozero, 2004]. Interestingly, myelination of these lobes coincide with the supposed time of disease onset in SCZ patients [Chambers and Perrone-Bizzozero, 2004] which is also the age of disease onset for some of the individuals affected with BPD [Miller, 2006]. It is therefore likely that a relationship between myelination and the development of such psychiatric disorders might exist [Chambers and Perrone-Bizzozero, 2004]. Genome-wide microarray studies involving the post-mortem brains of BPD and SCZ patients have revealed significant differential expression of genes involved in myelination [Tkachev et al., 2003; Hakak et al., 2001]. Five genes which are involved in the development and maintenance of the myelin membrane were found to be downregulated in SCZ brains when compared to controls. Thus, abnormalities in myelination may be an aetiological factor for BPD based on the purported genetic overlap between BPD and SCZ.

The GWAS conducted by Sklar et al. [2008] found an association between an intronic SNP (rs12967023) in *MBP* and individuals affected with BPD. This association has not been replicated in independent studies.

1.5.7 AK3L1

AK3L1 or *adenylate kinase 3-like 1* (also known as *AK3* and later renamed to *AK4*) encodes a member of the adenylate kinase family of enzymes. This family of kinases consists of five isozymes [Noma et al., 2001] and are primarily involved in the regulation of adenine and guanine bases [Yoneda et al., 1998]. The protein encoded by *AK3L1* has been shown to be localised to the mitochondrial matrix and is classified as a guanosine triphosphate: adenosine monophosphate (GTP:AMP) phosphotransferase which is involved with the phosphorylation of mitochondrial AMP (required for cellular respiration) [Noma et al., 2001; Dzeja and Terzic, 2009]. Expression studies have revealed that *AK3L1* is highly expressed in heart and skeletal muscle, liver and only weakly expressed in the brain [Noma et al., 2001]. *AK3L1*, on chromosome 1p31.3, has not previously been associated with any psychiatric disorder. However, the GWAS conducted by the WTCCC found that an intronic SNP within *AK3L1* (rs4916031) was strongly associated with BPD (p-value 9.8×10^{-5}) [Stratton, 2007].

1.5.8 GSK3 β

GSK3 β encodes glycogen synthase kinase 3 beta which is involved in the phosphorylation of serine and threonine. This protein is involved in the WNT signalling pathway [Cruceanu et al., 2009] which has an important function in cell fate and development [Logan and Nusse, 2004]. Expression of genes from the WNT signalling pathway has been previously found to be dysregulated in affected individuals with BPD [Matigian et al., 2007]. In a genome-wide expression study, *GSK3 β* was found to be downregulated in the post-mortem brains of individuals affected with BPD [Nakatani et al., 2006]. Similarly, *GSK3 β* mRNA is significantly downregulated in the prefrontal cortex of

teenagers who have committed suicide (which is the most severe consequence of BPD) compared to normal controls [Pandey et al., 2009]. Recently, it was noted that polymorphisms within *GSK3 β* (rs6438552) are associated with regional grey matter volumes in patients with major depressive disorder [Inkster et al., 2009]. *GSK3 β* is also an important regulator of the proteins involved in the circadian rhythm pathway [Sahar et al., 2010]. Lithium, the first line treatment for BPD, as well as the mood-stabiliser, sodium valproate, are thought to inhibit *GSK3 β* [Klein and Melton, 1996; Nishiguchi et al., 2006]. The -50 T/C polymorphism (rs334558), located in the promoter region of *GSK3 β* , has been found to have an association with response to lithium treatment for BPD and depression [Benedetti et al., 2005; Adli et al., 2007]. This polymorphism has also been associated with age of onset for BPD patients, which is an indicator of phenotype severity [Benedetti et al., 2004]. Therefore, *GSK3 β* seemed to be a reasonable candidate in the aetiology of BPD [Nishiguchi et al., 2006].

1.5.9 ANK3

This gene, located on chromosome 10q21, encodes the protein ankyrin 3. Ankyrins are thought to be involved in the linking of membrane proteins to the cell cytoskeleton and are involved in cellular processes such as motility and proliferation [Kordeli et al., 1995]. *Ankyrin 2 (ANK2)* obtained a CFG score of 5 in the research conducted by Le-Niculescu et al. [2008]. *ANK3* is neural-tissue specific and is found at the Node of Ranvier where it is thought to play a role in maintenance of cell-adhesion molecules and ion channels [Kordeli et al., 1995]. The first study to implicate *ANK3* as having an association with BPD was the GWAS done by Baum et al., [2008]. The SNP that was implicated, in that instance,

was rs9804190 which is located in an intronic region of the gene. Subsequently, a meta-analysis of three GWAS found an association between a SNP within this gene (rs10994336) and BPD [Ferreira et al., 2008]. Furthermore, several other studies were able to replicate this finding in independent samples [Scott et al., 2009; Smith et al., 2009; Schulze et al., 2008].

1.5.10 CACNA1C

This gene encodes the alpha 1C subunit of the L-type voltage-gated calcium channel and is located on chromosome 12p13.3 [Lory et al., 1997]. Voltage-gated calcium channels are responsible for calcium influx, which in turn mediate cellular processes such as contraction, secretion, neurotransmission and gene expression. There are ten members of the voltage-gated calcium channel family, located in different cell types [Catterall et al., 2005]. *Calcium channel, voltage dependent, alpha 1a subunit (CACNA1A)* and *calcium channel, voltage dependent, beta 2 subunit (CACNB2)* had CFG scores of 6 and 5.5, respectively [Le-Niculescu et al., 2008]. *CACNA1C* has been shown to be associated with BPD in a meta-analysis involving 3 GWAS [Ferreira et al., 2008]. The particular SNP (rs1006737) which was associated in the study was also found to lead to an increased risk of developing major depression and SCZ [Green et al., 2009]. This SNP, located in an intron, has also been found to be a risk factor for developing a more severe depressive phenotype and insomnia [Casamassima et al., 2010]. In another study, rs1006737 was found to have an association with cerebral grey matter volume. Healthy individuals without any history of mental disorders, carrying the A allele of rs1006737 had larger grey matter volumes compared to individuals who were homozygous for the G-allele

[Kempton et al., 2009]. *CANB1* (*Calcium channel, voltage-dependent, beta-1 subunit*) was shown to be upregulated in post-mortem BPD brains [Nakatani et al., 2006]. The evidence therefore points to *CACNA1C* as a possible candidate gene for BPD.

1.6 Aims and Objectives

The first aim of the present investigation was to determine whether SNPs, within ten selected candidate genes, have an association with BPD. The second aim was to determine whether the genotypes of these variants were associated with brain imaging volumes. The objectives were the following:

- i) To select a list of candidate genes with very strong evidence of involvement in BPD
- ii) To identify a cohort of individuals from the BPD registry at the University of Cape Town (UCT)
- iii) To genotype the candidate genes using a PCR-based technology
- iv) To analyse the genotyping data with the appropriate statistical methods
- v) To obtain brain imaging data of a subset of the larger cohort and to perform the appropriate statistical analysis

Table 4: Candidate genes from CFG and GWAS and respective SNPs

| Gene | Chromosomal Region | CFG Score | SNP | SNP Location | Base Change | Variation Type |
|-------------------------------|---------------------------|------------------|------------|---------------------|--------------------|-----------------------|
| <i>KLF12</i> | 13q22.1 | 8 | rs9600160 | intron | G/A | Non-functional |
| <i>ARNTL</i> | 11p15.2 | 8 | rs7107287 | intron | G/T | Non-functional |
| <i>BDNF</i> | 11p14.1 | 8 | rs6265 | exon 2 | C/T | Missense |
| <i>ALDH1A1</i> | 9q21.13 | 8 | rs1888202 | intron | G/C | Non-functional |
| <i>A2BP1</i> | 16p13.3 | 7.5 | rs7204975 | intron | C/T | Non-functional |
| | | | rs10500336 | intron | C/T | Non-functional |
| <i>MBP</i> | 18q23 | 7.5 | rs12967023 | intron | C/T | Non-functional |
| <i>AK3L1</i> | 1p31.3 | 7 | rs4916031 | intron | G/A | Non-functional |
| <i>GSK3β</i> | 3q13.3 | 7 | rs334558 | promoter | C/T | Non-functional |
| <i>ANK3</i> | 10q21 | NA | rs9804190 | intron | C/T | Non-functional |
| <i>CACNA1C</i> | 12p13.3 | NA | rs1006737 | intron | G/A | Non-functional |

Chapter 2: Materials and Methods

2.1 BPD Cohort Description

Ethics approval for the “Genetics of Bipolar Disorder” research project was first granted by UCT’s Research Ethics Committee in 1996 (REC Ref 081/1996). The aim of the project was to build a BPD archive, predominantly consisting of families, for the purpose of investigating the genetic complexities of the disorder. Between 1997 and 1999, individuals were recruited to the project from Gauteng, Free State, Eastern Cape and Western Cape. The cases were diagnosed with the Structured Clinical Interview for the DSM-IV (SCID) applied by a qualified psychiatric research nurse and verified by a psychiatrist. As the name suggests, SCID is a semi-structured questionnaire which allows for the diagnosis of DSM-IV axis disorders. Criteria for inclusion included an index patient and an available first-degree relative, both with a BPDI or BPDII diagnosis. The BPDI diagnosis was subsequently confirmed by a qualified psychiatrist. Interaction with healthcare professionals, advocacy groups and genealogical assessment resulted in the further recruitment of family members with a diagnosis of BPDI, BPDII, or recurrent MDE.

More recently, participants have been recruited from the outpatient unit at Valkenburg Hospital, Observatory, in Cape Town. All individuals were recruited when stable (not experiencing a manic or depressive episode), and blood samples were obtained with informed consent (**Appendix 1**).

2.1.1 Cohort Description for Case-Control Analysis

For the current study, updated ethics approval was obtained in June 2009 (REC Ref 229/2009). The research cohort, a subset of the larger BPD cohort, consisted of: i) an experimental group consisting of 224 individuals specifically diagnosed with BPD (cases), and ii) 202 background population controls. The cases consisted of individuals of Mixed Ancestry and Caucasian ethnicity. A select number of cases were from the same family. These families were sampled for previous linkage studies. The inclusion of related cases in case control studies, if used appropriately, have been shown to increase study power as related individuals are thought to carry a higher frequency of disease risk alleles as compared to unrelated cases [Thornton and McPeck, 2007; Yoo et al., 2007]. The controls were selected from the Division of Human Genetics database. Most of these individuals were the unaffected spouses of individuals with other genetic disorders such as non-polyposis colorectal cancer and retinitis pigmentosa. All of the controls were unrelated and of unknown phenotype in terms of psychiatric disorders. The aim was to have a matched case-control design whereby controls were similar in terms of gender and ethnicity to case individuals. An advantage of having a matched case-control design is that a much smaller sample size is required to detect statistical significance [Mann, 2003] and that potential confounders such as gender and ethnicity are adjusted for [Niccolai et al., 2007]. A disadvantage is that if controls are too closely matched they do not represent the general population [Mann, 2003].

2.1.2 Cohort Description for Brain Imaging Analysis

The current study involved an investigation of a cohort of individuals affected with BPD, towards the identification of genes which may predispose to the condition. In addition, other modalities of neuropsychiatric investigations were undertaken as a means of

identifying potential endophenotypes which could be investigated genetically. In this regard, a subset of the BPD cohort was investigated by MRI brain imaging and genetics. The brain imaging analysis was done as part of the *“Neuroimaging the Effects of Mindfulness Training in Bipolar Disorder”* research project, carried out by the Department of Psychiatry (UCT). Separate ethics approval was obtained for this study (REC Ref 078/2009). For recruitment of participants, the study was advertised at the University of Cape Town, in newspapers and at specialized seminars. Participants were also recruited from the existing cohort and referrals from other health professionals. Imaging was performed for a subset (n=22) of the 224 BPDI case group.

2.2 DNA Isolation and Quality Determination

DNA was isolated, from blood samples, using the Genra Puregene™ DNA Isolation Kit (Genra Systems, Minneapolis, MN, USA). The protocol involved the following steps: the lysis of red blood cells (RBCs) and nucleated cells, protein precipitation, DNA precipitation, recovery, and rehydration. Following rehydration in DNA hydration solution, the isolated DNA was stored at either -20°C (for short-term storage) or -80°C (for long term storage). A more detailed protocol is included in **Appendix 2**.

The integrity and quality of the DNA samples were verified using two methods:

- (i) Spectrophotometric quantification
- (ii) Agarose gel electrophoresis

For quantification, the concentrations of undiluted, stock DNA samples were determined using a Nanodrop 1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA). Samples were diluted with Sabax distilled water (Adcock Ingram,

Johannesburg, SA) to a working concentration of 100ng/μl. For agarose gel electrophoresis, a volume containing 200ng of DNA together with loading buffer (Fermentas Life Sciences, Hanover, USA) was loaded onto a 1% agarose gel (**Appendix 3**). To estimate integrity of DNA fragments, the first lane of the gel was loaded with a 100bp molecular weight marker (MWM) (Fermentas Life Sciences, Hanover, USA) (**Appendix 3**). To enable DNA visualization, 30ng of ethidium bromide (EtBr) (Sigma, England), a DNA intercalating agent, was added to the agarose prior to solidification. Gel electrophoresis was carried out in a gel tank with 1x Tris Borate EDTA (TBE) (**Appendix 3**) electrophoretic buffer, at 160V for approximately 30 minutes. Thereafter, it was placed in the Uvipro Gold transilluminator (UVITec, Cambridge, UK) for the illumination and image capture of the DNA fragments.

2.3 Gene and SNP Selection, Primer Design and Synthesis

2.3.1. Gene and SNP selection

In 2008, Le-Niculescu et al., based on a CFG approach (see Chapter 1: Introduction, pg 32), constructed a prioritised candidate gene list for BPD. The top eight genes on this list were chosen to be genotyped in the current study. Each of these genes obtained a CFG score of seven and above (12 being the maximum). The CFG score is based on previous evidence, therefore SNPs were chosen based on their significant association to BPD in independent studies. The remaining two genes, *ANK3* and *CACNA1C* and their respective SNPs, were chosen based on their association to BPD in a GWAS [Baum et al., 2008; Ferreira et al., 2008]. **Table 5** contains all of the candidate genes, their respective SNPs and CFG scores.

Table 5: Selected candidate genes, respective SNPs and CFG scores

| Gene | SNP | CFG Score |
|-------------------------------|------------|-----------|
| <i>KLF12</i> | rs9600160 | 8 |
| <i>ARNTL</i> | rs7107287 | 8 |
| <i>BDNF</i> | rs6265 | 8 |
| <i>ALDH1A1</i> | rs1888202 | 8 |
| <i>A2BP1</i> | rs7204975 | 7.5 |
| | rs10500336 | |
| <i>MBP</i> | rs12967023 | 7.5 |
| <i>AK3L1</i> | rs4916031 | 7 |
| <i>GSK3β</i> | rs334558 | 7 |
| <i>ANK3</i> | rs9804190 | - |
| <i>CACNA1C</i> | rs1006737 | - |

2.3.2 External Primer Design

The sequences of each of the ten candidate genes (*KLF12*, *ARNTL*, *BDNF*, *ALDH1a1*, *A2BP1*, *MBP*, *AK3L1*, *GSK3 β* , *ANK3*, and *CACNA1C*) were obtained from NCBI (<http://www.ncbi.nlm.nih.gov>) and Ensembl (<http://www.ensembl.org/index.html>) databases. The sequence of each of the genes was then annotated to specify the location of the exons, coding sequence, start/stop codons and SNPs. This was accomplished using the *ANNOTV9* annotation programme [Rebello, 2006, Unpublished], which utilises the *Perl* scripting language [Wall, 1987].

Once the regions of interest were identified, suitable primers were designed to amplify these sequences. The web-based program, Primer3 (<http://fokker.wi.mit.edu/primer3/input.htm>), which searches for possible primer sequences from the nucleotide sequence, was used for this purpose. The selected primers were then analysed by Integrated DNA Technologies (IDT) oligo analyser

(<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer>), a program which predicts any secondary structures such as primer dimers and hairpins which may occur. Secondary structures greatly reduce the efficiency of the PCR reaction [Singh et al., 2000]. To ensure the primer only binds to the sequence of interest, the primer sequences were checked against a database, NCBI BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). All the primer sequences are contained in **Table 6**.

The oligonucleotides were synthesised using the Oligo 1000M DNA Synthesiser v4.20 (Beckman Instruments Inc., Department of Molecular and Cell Biology, UCT). The primer stock solutions were diluted with Sabax distilled water (dH₂O) (Adcock Ingram, Johannesburg, SA) to a working concentration of 20µM.

2.4 Polymerase Chain Reaction (PCR)

The target regions of interest in each of the candidate genes were amplified using PCR. To obtain the optimal annealing temperature (T_a), a temperature gradient was used to assay all of the primer pairs. The PCR reaction consists of deoxynucleotide triphosphates (dNTPs), Taq Polymerase, Taq Buffer, DNA, forward primer, reverse primer and distilled water (dH₂O). Based on the optimal T_a for each primer pair and amplicon size, certain primer sets were multiplexed together in one PCR reaction. Multiplexing of primers allows for the efficient usage of reagents and resources. A negative (water) control was included, which consisted of all the reaction components except DNA, to ensure that any product visualized was not due to contamination.

Table 6: External primer sequences for amplification of candidate gene regions using PCR

| Gene | Primer Direction | Primer Length | Primer Sequence (5'→3') | GC content (%) | Tm (°C) | Amplicon size (bp) |
|-----------------|------------------|---------------|--------------------------|----------------|---------|--------------------|
| <i>KLF12</i> | Foward | 18 | acgtagcagcaccagtct | 55.6 | 56.2 | 180 |
| | Reverse | 21 | gagagagtagcaagccaaatg | 47.6 | 53.4 | |
| <i>ARNTL</i> | Foward | 18 | gaagccatgagtctagca | 50 | 51.5 | 237 |
| | Reverse | 18 | gtagaacctggacagt | 50 | 51 | |
| <i>BDNF</i> | Foward | 24 | caagaggcttgacatcattggctg | 50 | 58.5 | 338 |
| | Reverse | 24 | ccgacatgtccactgcagtcttt | 50 | 59.6 | |
| <i>ALDH1A1</i> | Foward | 19 | tctatagttctgacccac | 47.4 | 50.2 | 394 |
| | Reverse | 18 | tttctcagctacgccatg | 50 | 52.3 | |
| <i>A2BP1(A)</i> | Foward | 17 | tctatagttctgacccac | 47.1 | 47.7 | 192 |
| | Reverse | 19 | tttctcagctacgccatg | 42.1 | 47.5 | |
| <i>A2BP1(B)</i> | Foward | 20 | cgtggtctcttctcttgttc | 50 | 52.8 | 250 |
| | Reverse | 20 | ggcacaacaggctatacaga | 50 | 54.5 | |
| <i>MBP</i> | Foward | 18 | cttggctcctcagtgaagcc | 55.6 | 52.9 | 170 |
| | Reverse | 19 | gaatcgtgtcctggttgtg | 52.6 | 53.6 | |
| <i>AK3L1</i> | Foward | 20 | cagactggcttgttacctc | 55 | 55.3 | 155 |
| | Reverse | 20 | ggatagaggttgagagctgg | 55 | 54.3 | |
| <i>GSK3β</i> | Foward | 22 | cgattcccagacgcctgttacg | 59.1 | 60 | 302 |
| | Reverse | 18 | tcaggaagtgtccgcgct | 61.1 | 59.4 | |
| <i>ANK3</i> | Foward | 18 | gccagtaacaatgaacc | 50 | 51.3 | 227 |
| | Reverse | 21 | gtcatatcctagtcccattc | 47.6 | 51.8 | |
| <i>CACNA1C</i> | Foward | 18 | gttcattcattctcagc | 50 | 50.5 | 220 |
| | Reverse | 18 | aatgtgtggaccagcaat | 44.4 | 52 | |

PCR was carried out on a PxE 0.2 Thermal cycler (ThermoHybaid, Middlesex, UK) at the conditions stipulated in **Table 7**. Once completed, the PCR products were maintained at a temperature of 4°C until required for downstream processes.

To determine whether amplification had occurred, the PCR products together with loading buffer were resolved on a 2% agarose gel (**Appendix 3**), run at 160V, for approximately 40 minutes. The current was set at the maximum value (approximately 110 MA). MWM (100bp) (**Appendix 3**) was added to the first lane of the gel. The gel was exposed to UV light in the Uvipro Gold transilluminator (UVITec, Cambridge, UK) and the resultant image captured.

Table 7: PCR cycling conditions

| Stage | Temperature (°C) | Duration | No. of Cycles |
|-------|------------------|------------|---------------|
| 1 | 94 | 5 minutes | 1 |
| 2 | 94 | 30 seconds | 30 |
| | Ta | 30 seconds | |
| | 72 | 30 seconds | |
| 3 | 72 | 7 minutes | 1 |

2.5 SNaPshot PCR

SNaPshot PCR is a SNP genotyping method and is an extension of the basic PCR process.

Internal primers were designed to bind 5' or 3' of the SNP of interest in each of the amplified candidate genes. The use of this technique requires SNaPshot™ multiplex ready reaction mix (Applied Biosystems, Warrington, UK) which contains fluorescently-labelled dideoxynucleotide triphosphates (ddNTPs), AmpliTaq polymerase, and reaction

buffer. The *Taq* polymerase catalyses the single base extension of the unlabelled primers by incorporating the complementary differentially-labelled ddNTP [ABI Prism SNaPshot™ Multiplex Kit Protocol, Applied Biosystems] (See **Figure 1**). The ddNTPs lack an acceptor 3' hydroxyl group. Therefore, the AmpliTaq polymerase is unable to add an additional base to the ddNTP and extension is terminated. The end results of this technique are peaks which represent the alleles at a particular SNP. **Table 8** indicates the dyes attached to each of the ddNTPs and the resulting peak colours.

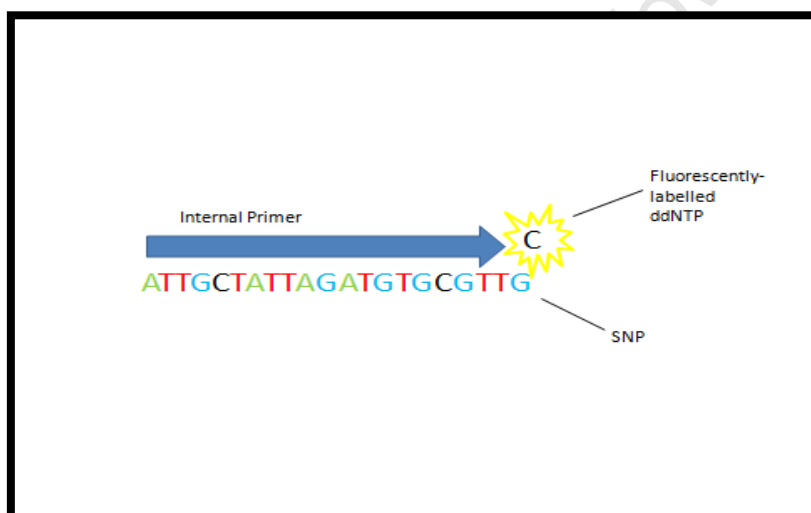


Figure 1: An illustration of the SNaPshot PCR principle.

Table 8: Dyes assigned to each of the ddNTPs

| ddNTP | Dye | Colour |
|--------------|---------|--------|
| Adenine (A) | dR6G | Green |
| Cytosine (C) | dTAMRA™ | Black |
| Guanine (G) | dR110 | Blue |
| Thymine (T) | dROX™ | Red |

2.5.1 SNaPshot PCR Internal Primer Design

The criteria for SNaPshot internal primers were the same as those stipulated for the external primers except that only one primer (either forward or reverse) was needed for SNaPshot PCR. The primers were designed by choosing consecutive bases immediately 5' or 3' of the SNP of interest. However, for the purposes of multiplexing, each of the primers needed to be of a different length to ensure that the appropriate alleles could be called for the respective gene and there would be no overlap in the SNaPshot product. Therefore, random tails of nucleotide repeats were attached to each of the primers. Another way of ensuring that primers could be multiplexed, even if two primers were the same length, was to ensure that those primers had different expected peak colours. A local BLAST search, using the sequence alignment program, BioEdit Sequence Alignment Editor 7.0.0 (Tom Hall, Isis Pharmaceuticals, Inc.), was done to ensure that the primers did not hybridise to one another. The sequences of the internal primers are displayed in **Table 9**.

Internal primers (25nmole) were synthesized by IDT® (<http://eu.idtdna.com/order/order.aspx>) and were received in a lyophilized form. To prevent contamination, each of the primers were desalted and quality was tested by mass spectrometry. Subsequently, the primers were resuspended with TE (10mM Tris pH8.0, 1mM EDTA) to a stock concentration of 100µM. Working solutions of 20µM primer concentration, in a total volume of 50µl, were made by the addition of Sabax dH₂O (Adcock Ingram, Johannesburg, SA).

2.5.2 Sap and ExoI Clean-up of PCR Products

Before SNaPshot PCR could be carried out, unincorporated dNTPS and primers from the initial PCR needed to be removed, to ensure that there was no interference with the genotyping process [ABI Prism SNaPshot™ Multiplex Kit Protocol, Applied Biosystems]. Shrimp alkaline phosphatase (SAP) (Promega, USA) allows for the dephosphorylation of the 5' phosphates of DNA. Exonuclease I (ExoI) (Biolabs, New England) catalyses the removal of bases from single-stranded DNA in a 3'-5' direction, promoting the degradation of the excess single-stranded primers (<http://www.neb.com/nebecomm/products/productm0293.asp>).

The purification process was carried out on an Applied Biosystems thermal cycler (Gene Amp® PCR System 9700) at the conditions listed in **Table 10** and using the reagents in **Table 11**. Firstly, a master mix was made up with the SAP, ExoI and dH₂O and aliquoted into each well of the segmented 96-well PCR Microplate (Axygen Scientific, California, USA). The PCR product was then aliquoted into each respective well. Wells were sealed with PCR cap strips (Axygen Scientific, California, USA).

Table 10: Conditions for Sap and ExoI purification

| Temperature (°C) | Duration |
|------------------|------------|
| 37 | 1 hour |
| 75 | 15 minutes |

Table 11: Sap and ExoI DNA purification

| Recommended | | | |
|------------------------|---------------|----------------------|---------------------|
| Reagents | Concentration | Amount | |
| | | Singleplex Reactions | Multiplex Reactions |
| SAP | 5 units | 1 unit | 1.5 units |
| ExoI | 2 units | 2 units | 2 units |
| PCR Product | - | 5 μ l | 5 μ l |
| dH₂O | - | 13.9 μ l | 13.4 μ l |
| Total Volume: | - | 20 μ l | 20 μ l |

2.5.3 SNaPshot PCR Conditions

Multiplex SNaPshot reactions, whereby an equal ratio of the respective primers were pooled, were carried out on 96-well PCR Microplates (Axygen Scientific, California, USA). For each SNaPshot reaction, a negative water control was also included. The control was included to ensure that the results obtained from the sample reactions were not a result of any form of contamination. This control contained all the components of the multiplex SNaPshot reaction but lacked any purified PCR sample. **Table 12** illustrates the volumes of the components of a SNaPshot reaction. The samples were loaded into a Applied Biosystems thermal cycler (Gene Amp® PCR System 9700) and run at the conditions defined in **Table 13**.

Table 12: The components of the SNaPshot PCR reaction

| Reagents | Reaction Volumes (μ l) | | |
|-------------------------|-----------------------------|---------------------|------------------------|
| | Singleplex Reactions | Multiplex Reactions | Negative Water Control |
| Reaction Mix | 1 | 1 | 1 |
| Internal Primers | 1 | 1 (pooled) | 1 |
| PCR Product | 1 | 5 | - |
| dH₂O | 7 | 3 | 8 |
| Total Volume: | 10 | 10 | 10 |

Table 13: Conditions for SNaPshot PCR

| Stage | Temperature ($^{\circ}$ C) | Duration | No. of Cycles |
|----------|-----------------------------|------------|---------------|
| 1 | 96 | 10 seconds | 25 |
| | 50 | 5 seconds | |
| | 60 | 30 seconds | |
| 2 | 4 | ∞ | |

2.5.4 Post- SNaPshot SAP Treatment

Before the samples were genotyped, all the unincorporated ddNTPs needed to be removed by the phosphatase action of SAP. If left untreated, the ddNTPs would co-migrate with the products of interest. Thus, the removal of the phosphatase groups of these nucleotides hindered the movement and prevented any interference [ABI Prism SNaPshot™ Multiplex Kit Protocol, Applied Biosystems].

One unit of SAP was added to the SNaPshot PCR products. The samples were then loaded into a Applied Biosystems thermal cycler (Gene Amp® PCR System 9700) at the conditions listed in **Table 10**.

2.5.5 Capillary Electrophoresis on ABI PRISM

Purified SNaPshot reactions were resolved on the ABI prism 3100 Genetic Analyzer (Applied Biosystems, CA, USA). A mixture of SNaPshot product, HiDi formamide (Applied Biosystems, Warrington, UK) and GeneScan-120 Liz size standard (Applied Biosystems, CA, USA) (**Appendix 4**) were loaded into a 96-well PCR Microplate (Axygen Scientific, California, USA). The HiDi functions to maintain the double-stranded DNA in a denatured state and the Liz allows for the sizing of the allele peaks. The dye set was set at E5, the analysis method at GeneScan-120 Liz and the run module at SNP36_POP4.

2.5.6 Analysis of Results

The genotyping results were analysed by the Genemapper 3.0 Genescan Software (Applied Biosystems, CA, USA). This software provides automated sizing of peaks and was used for the analysis of each of the SNPs. It generated a table with all the alleles and user-specified process-based quality values which relayed the integrity of the data. Bins which corresponded to a particular base-pair range for a specific allele were defined. Software algorithms determined whether peaks were representative of an allele at that position.

2.6 Genotype Validation

The results obtained from the SNaPshot assay were validated by cycle sequencing.

2.6.1 PCR of DNA Samples for Cycle Sequencing

A subset of the samples were chosen based on the results shown by SNaPshot PCR. These contained all possible genotypes (homozygous and heterozygous) for each of the 11 SNPs. PCR was carried out on the selected samples using the same cycling conditions as in **Table 7**. Amplified products were resolved on a 2% agarose gel (**Appendix 3**) together with loading buffer. MWM (**Appendix 3**) was added to the first lane of the gel. The gel was run for approximately 40 minutes and placed in the Uvipro Gold transilluminator (UVITec, Cambridge, UK) for visualisation and image capture purposes. PCR products of adequate band intensity were used for the sequencing reaction.

2.6.2 Cycle Sequencing

Cycle sequencing was done on amplified products using the reagents listed in **Table 14**. Cycle sequencing was carried out on an Applied Biosystems thermal cycler (Gene Amp® PCR System 9700) at the conditions displayed in **Table 15**. Most SNPs were sequenced using the forward primer. However, *ARNTL* rs7107287, *BDNF* rs6265 and *CACNA1C* rs1006737 were sequenced using the reverse primer as these SNPs were located towards the 5' end of the respective amplicons. Typically, peaks at the start (5'end) of a sequence are undistinguishable. Therefore, when sequenced with the forward primers, the *ARNTL* rs7107287, *BDNF* rs6265 and *CACNA1C* rs1006737 SNPs were not identifiable.

2.6.3 Ethanol Precipitation

The DNA was purified from the reaction mixture by precipitation with ethanol. This step removed any unincorporated ddNTPs which could potentially interfere with the start of the sequence and base-calling. The steps listed in **Appendix 5** were followed.

Table 14: Components of the cycle sequencing reaction

| Reagents | Recommended Concentration | Volume (μl) |
|-------------------------------|----------------------------------|-----------------------------------|
| BigDye Terminator Mix | 2.5x | 1 |
| Sequencing Buffer | 5x | 4 |
| Forward/Reverse Primer | - | 0.5 |
| PCR product | - | 2 |
| dH₂O | - | 12.5 |
| Total Volume | | 20 |

Table 15: Cycle sequencing PCR conditions

| Temperature (°C) | Duration | No. of Cycles |
|-----------------------------|-----------------|----------------------|
| 96 | 5 min | 25 |
| 96 | 30 sec | |
| 50 | 15 sec | |
| 60 | 4 min | |

2.6.4 Capillary Electrophoresis on ABI Prism

The sequencing samples were run on the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems, CA, USA). A volume of 5 μ l of the sequencing sample was added to 8 μ l HiDi Formamide (Applied Biosystems, Warrington, UK).

2.6.5 Analysis of Results

The sequences of each of the candidate genes were visualised in the BioEdit Sequence Alignment Editor version 7.0.0 (Tom Hall, Isis Pharmaceuticals, Inc.). This allowed for the identification of the genotype of the SNP of interest.

2.7 Statistical Analysis

2.7.1 Genotype Frequencies, Hardy-Weinberg Equilibrium Calculations and Logistic Regression with R

Genotype frequencies for all of the SNPs (cases and controls), Hardy Weinberg Equilibrium (HWE) calculations (controls only), and case-control association were determined using the statistical environment *R* (<http://www.R-project.org>). The method used to determine whether there was an association between disease status and genotype frequency, was logistic regression. This method fits a statistical model to a data set whereby the dependent variable is dichotomous and the independent variable is either continuous or discrete. In this instance, the dependent variable was disease affection status (i.e. case or control) and the independent variable were the three genotypes for a particular SNP. The logistic regression test computes the odds ratio which is defined as the probability of an event occurring over the probability of the event not occurring [Munro, 2001]. The z-test value is the test statistic and lower and upper confidence intervals are specified. As more than one statistical analysis was performed (disease status versus SNP), the Bonferroni correction was implemented for multiple testing, to avoid spurious significance. Therefore, the “corrected” significance level was set at the initial alpha value (0.05) divided by the number of tests performed.

2.7.2 M_{QLS} (adjusting for relatedness) and Pedstats

A number of the cases belonged to the same family. This presented both an advantage and challenge in the statistical analysis. The advantage is that affected individuals within the same family are expected to have a higher frequency of the disease causing allele which may be easier to detect than in unrelated cases. Therefore, the power of the association is increased as well as the effective sample size. The challenge is that the relatedness amongst cases needs to be adjusted for in the analysis [Thornton and McPeck, 2007]. The M_{QLS} method is able to perform basic case-control analysis with the addition of adjusting for related individuals. For this analysis, genotyping results of affected individuals were incorporated into a pedigree file to specify relationships within families. To ensure quality of pedigree data, i.e. whether there were any errors within the pedigree file, the program Pedstats was used [Wigginton and Abecasis, 2005].

2.8 Neuroimaging of Subset of Cohort

T1 weighted MRI scans were obtained using a high resolution Siemens Magnetom Allegra 3T scanner at the Cross Universities Brain Imaging Centre (<http://sun.ac.za/cubic>). Hippocampal and total intracranial volumes, from 22 individuals affected with BPDI, were obtained using the automated software package Freesurfer (version 5.0.0) [Fischl et al., 2002] (<http://surfer.nmr.mgh.harvard.edu/>) running at the Center for High Performance Computing in Cape Town (<http://chpc.ac.za>). Hippocampal volumes were normalised to total intracranial volume to correct for individual differences in anatomy.

2.8.1 Statistical Analysis of Neuroimaging Results

Analysis of variance (ANOVA) was used to determine whether there were any associations between imaging measures and genotypes. The null hypothesis for ANOVA is that the means between two or more groups are the same, therefore, the alternate hypothesis is that means between groups are significantly different. This statistical measure tests whether the “between group” variation is greater than the “within group” variation. If this is found to be true, it suggests that the means between groups are different. The F-value represents the ratio of the “between group” and “within group” variance. ANOVA is a fairly robust statistical test, requiring that the dependent variable be continuous (e.g. imaging measure) and the independent variables be categorical (e.g. SNP genotype). ANOVA has the added advantage of being able to include more than one independent variable in a single test. The use of ANOVA requires that three assumptions be met, namely, i) the dependent variable is normally distributed, ii) there is independence between groups, iii) and variances are equal (homogeneity of variance) [Munro, 2001]. The Shapiro-Wilk test was used to ensure a normal distribution for the following measures: left and right hemisphere hippocampal volumes and intracranial volumes. In instances where the data was non-normally distributed, the non-parametric Kruskal-Wallis test was utilised, which compares the mean ranks in each group [Munro, 2001]. The Bartlett’s Test was used to ensure that variances were equal across genotype groups.

Chapter 3: Results

3.1 BPD Cohort Description

To date, the BPD cohort consists of 919 individuals from 221 families. A total of 550 females and 366 males (three of unknown gender) have been recruited. The cohort consists of individuals from the Caucasian (British, Afrikaner, Portuguese, Greek, and Ashkenazi Jewish), Mixed Ancestry, Indian and African population groups. **Figure 2** represents the distribution of these ethnic groups within the cohort. The most prevalent diagnoses present in the archive includes mood disorders (BPDI, BPDII, MDE) (with BPDI being the primary diagnosis), Borderline Personality Disorder (PD), Post Traumatic Stress Disorder (PTSD) and Alcohol Abuse. **Figure 3** indicates the relative distribution of most of the diagnoses present in the BPD archive.

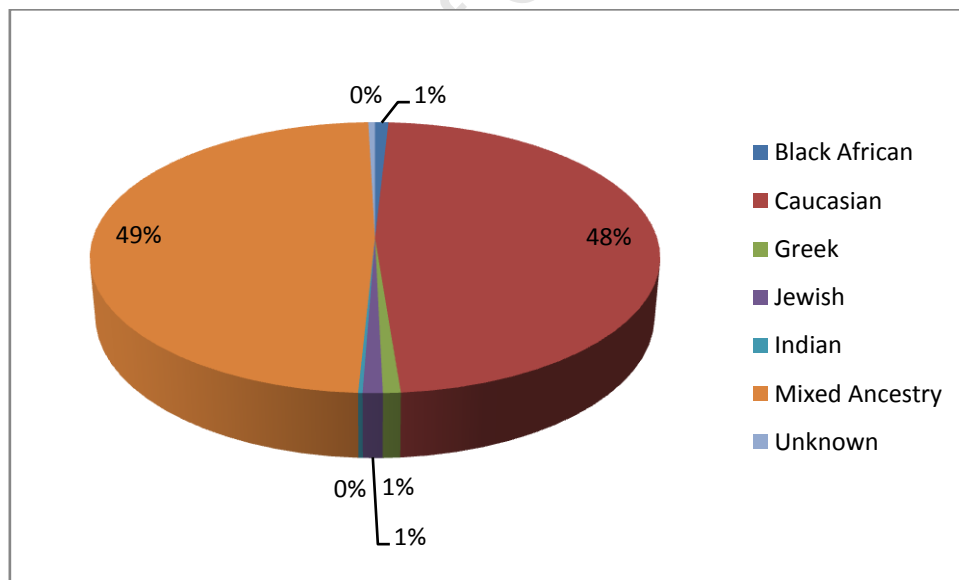


Figure 2: Ethnic group distribution of BPD archive

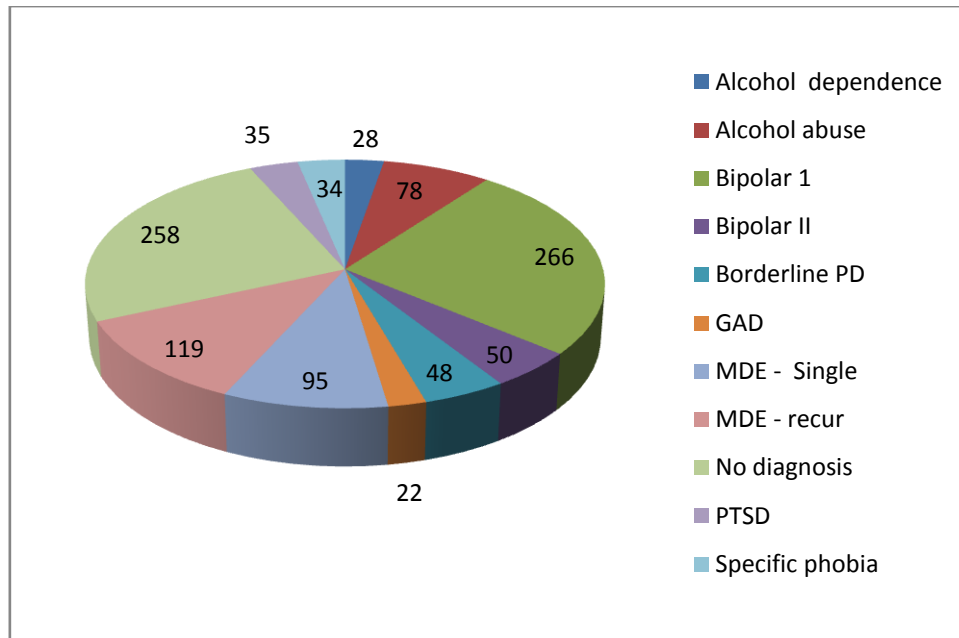


Figure 3: Distribution of most prevalent phenotypes of BPD cohort

3.1.1 Cohort Description for Case-Control Analysis

In terms of cohort size, the aim was to have an equal number of cases and controls, with the control samples similar to cases in terms of gender and ethnicity. However, the final cohort consisted of 224 cases affected with BPD and 202 background control samples. Within the case group, 93 individuals were derived from 35 BPD families and 131 were singletons (i.e. the only person to be represented in the cohort, from a particular family).

Figure 4 represents the numbers of females and males and **Figure 5** shows the number of individuals from each of the ethnic groups, within the study cohort. A proportion of controls were of unknown gender and age. There was more than two times the number of Caucasians as there were Mixed Ancestry individuals in the control group. Thus, controls were not similar to the case group in terms of gender and ethnicity. However, these variables were adjusted for in the statistical analysis.

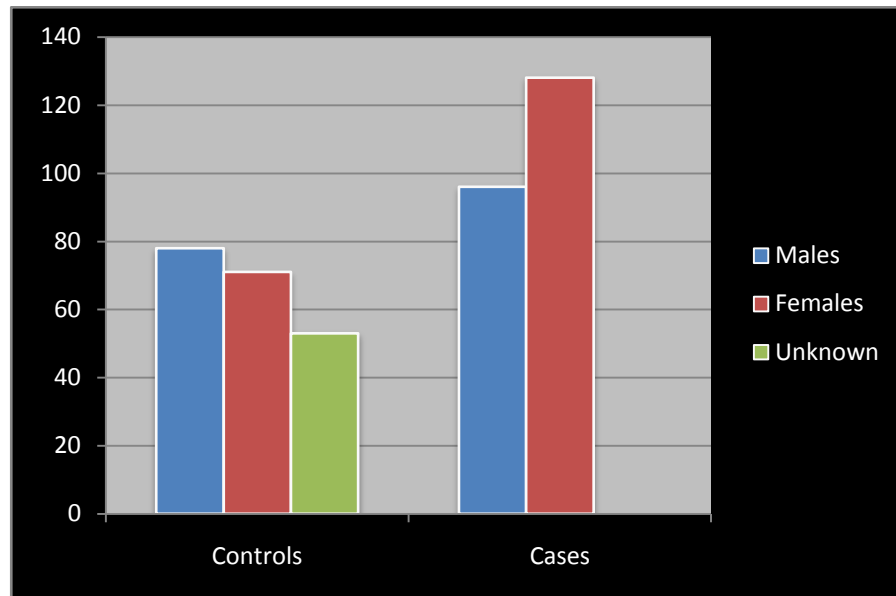


Figure 4: Number of males and females in case and control groups.

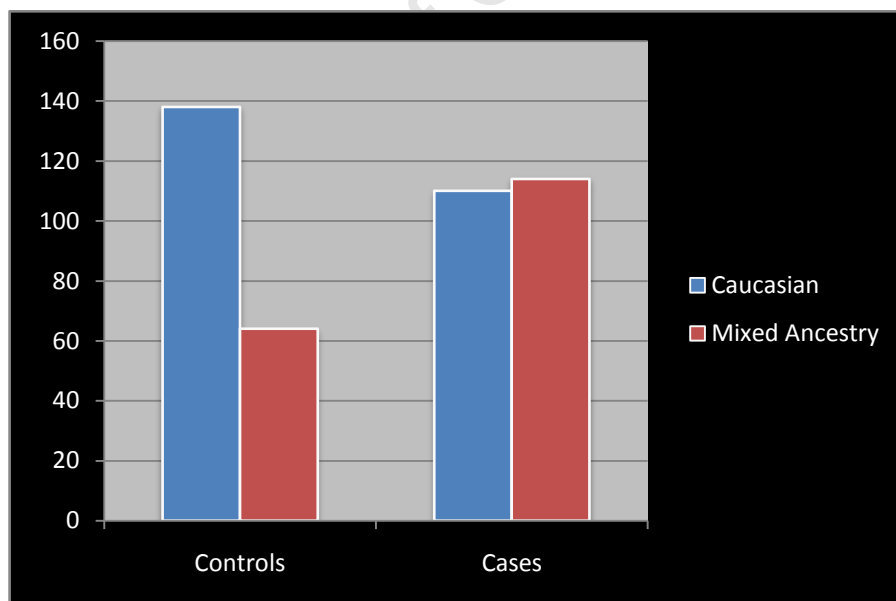


Figure 5: Number of Caucasian and Mixed Ancestry individuals in case and control groups.

3.1.2 Cohort Description for Brain Imaging Analysis

The imaging cohort consisted of 22 individuals affected with BPD, 20 of who were female and two were males. A total of 14 individuals were Caucasian and eight individuals were of Mixed Ancestry ethnicity.

3.2 DNA Quality Determination

To determine the quality of DNA, samples were quantified by means of spectrophotometry and agarose gel electrophoresis. Samples had to have a concentration of greater than 50ng/ μ l to be deemed satisfactory for further analysis. DNA quality was determined by resolution on a 1% agarose gel (**Appendix 3**). Bands which appeared thick and bright at the top of the lanes were indicative of non-degraded, good quality DNA. This was shown in lanes 4, 5, 7, 8, and 9 (**Figure 6**). Degraded DNA samples appeared as a smear, as can be seen in lanes 1, 2, 3 and 6 (**Figure 6**).

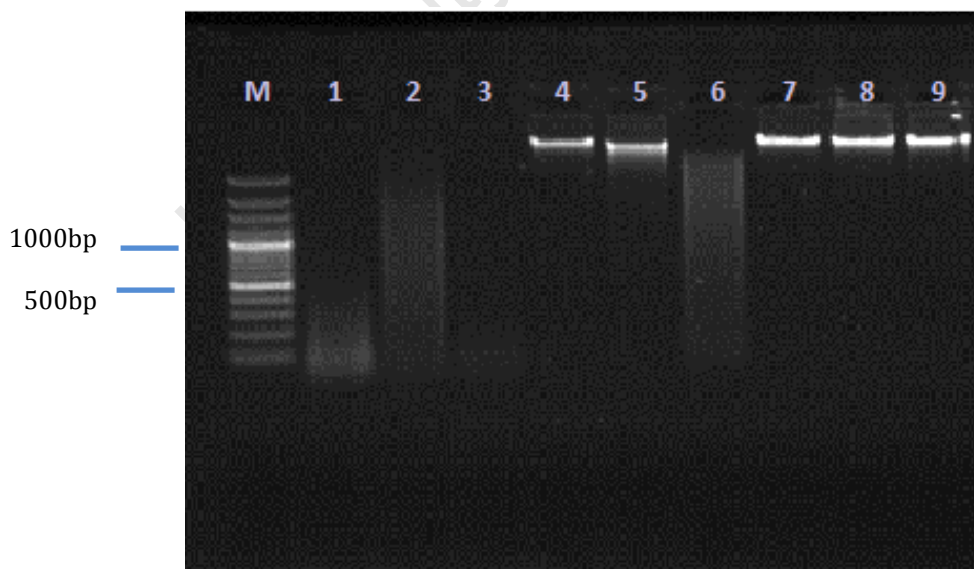


Figure 6: Integrity gel of genomic DNA. Lane M: 100bp MWM, Lanes 1-9: Genomic DNA from subjects in study.

3.3 Gene and SNP Selection, Primer Design and Synthesis

Candidate genes investigated in this study were selected based on results from the CFG method [Le-Niculescu et al., 2008] and the most significant findings in GWAS, related to neuropsychiatric disorders and BPD specifically. The specific SNPs that were finally chosen per gene were those that had been reported with significant results from previous GWAS and candidate-gene association studies. Primers were designed to produce a PCR product incorporating the specific (respective) SNP for each gene.

3.4 Polymerase Chain Reaction (PCR)

3.4.1 PCR Optimisation

The optimum T_a for each PCR reaction was determined by a temperature gradient, whereby 12 reactions were carried out at different temperatures ranging from 50°C to 62°C. The T_a for each of the 12 reactions is displayed in **Appendix 6**. A separate temperature gradient was carried out for each primer pair. **Figure 7** indicates the temperature gradient for *KLF12* rs9600160. Non-specificity is seen at lower temperatures (lanes 1-4) and little/no product at higher temperatures (lanes 11 and 12). The optimum annealing temperature for this reaction was chosen as approximately 58°C as there was ample product and an absence of non-specificity. Similarly, for the other primer pairs, an optimum T_a was chosen based on the best product band intensity and a lack of non-specific products (See **Appendix 7** for additional gel pictures). These values, for each primer pair, are displayed in **Table 16**.

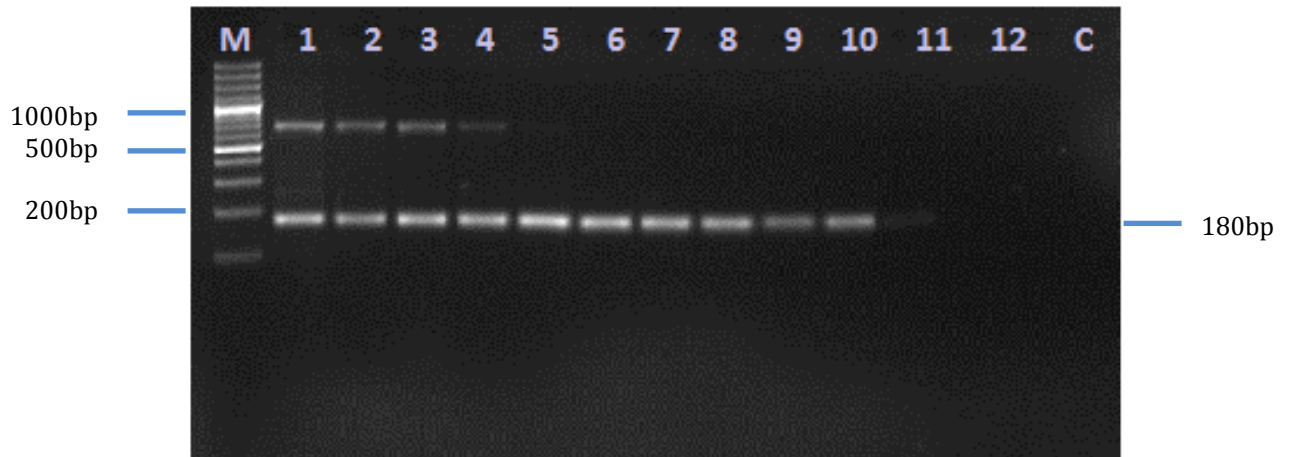


Figure 7: *KLF12* rs9600160 (180bp) Temperature Gradient. Lane M: 100bp MWM. Lanes 1-12: PCR products amplified at varying annealing temperatures as specified in Table 1. Lane C: water control.

Table 16: Optimal annealing temperature determined for each primer pair

| Primer | SNP | Approximate Optimum Ta (°C) |
|-----------------|------------|-----------------------------|
| <i>KLF12</i> | rs9600160 | 58 |
| <i>ARNTL</i> | rs7107287 | 57 |
| <i>BDNF</i> | rs6265 | 58 |
| <i>ALDH1A1</i> | rs1888202 | 57 |
| <i>A2BP1(A)</i> | rs7204975 | 53 |
| <i>A2BP1(B)</i> | rs10500336 | 53 |
| <i>MBP</i> | rs12967023 | 57 |
| <i>AK3L1</i> | rs4916031 | 57 |
| <i>GSK3β</i> | rs334558 | 62 |
| <i>ANK3</i> | rs9804190 | 57 |
| <i>CACNA1C</i> | rs1006737 | 62 |

3.4.2 PCR Conditions for Candidate Genes

Based on optimum Ta and PCR product size, each of the amplicons was amplified in one of five multiplex reactions. The amplified products were then resolved on a 2% agarose gel (Appendix 3) and stained with EtBr. The first lane of each gel (Figures 8-12) contained 100bp MWM which serves as a size standard. The last lane of each gel contains

the negative (water) control. None of the water controls had amplified product indicating that none of the PCR reactions were contaminated.

Group A consisted of three primer pairs, *ARNTL* rs7107287, *MBP* rs12967023, and *ALDH1A1* rs1888202 and had a Ta of 57°C (See **Figure 8**). Primer dimers formed at the bottom of each lane. The *GSK3β* rs334558 and *CACNA1C* rs1006737 primer pairs constituted Group B and had amplicon sizes of 302bp and 220bp, respectively (**Figure 9**). The Ta of this group was 62°C. Group C also consisted of two primer pairs, *KLF12* rs9600160 and *BDNF* rs6265. The Ta of this group was 58°C and had amplicon sizes of 180bp (*KLF12*) and 338bp (*BDNF*) (**Figure 10**). Groups, D and E, each consisted of two primer pairs. Group D was made up of the two *A2BPI* primer pairs, *A2BPI(A)* rs7204975 and *A2BPI(B)* rs10500336 (**Figure 11**). The amplicon sizes were 192bp and 250bp, respectively, and had a Ta of 53°C. The primer pairs *AK3L1* rs4916031 and *ANK3* rs9804190 formed part of Group E which had a Ta of 57°C (**Figure 12**). These had amplicon sizes of 155bp and 227bp, respectively. The primer pairs of Group C and Group E were originally placed into one multiplex reaction (due to similar optimal Ta). However, when these products were resolved on a gel, it was noted that amplification of *AK3L1* rs4916031 (155bp) was very faint and not easily distinguishable from the *KLF12* rs9600160 (180bp) fragment. Therefore, these four primer pairs were separated into two PCR reactions.

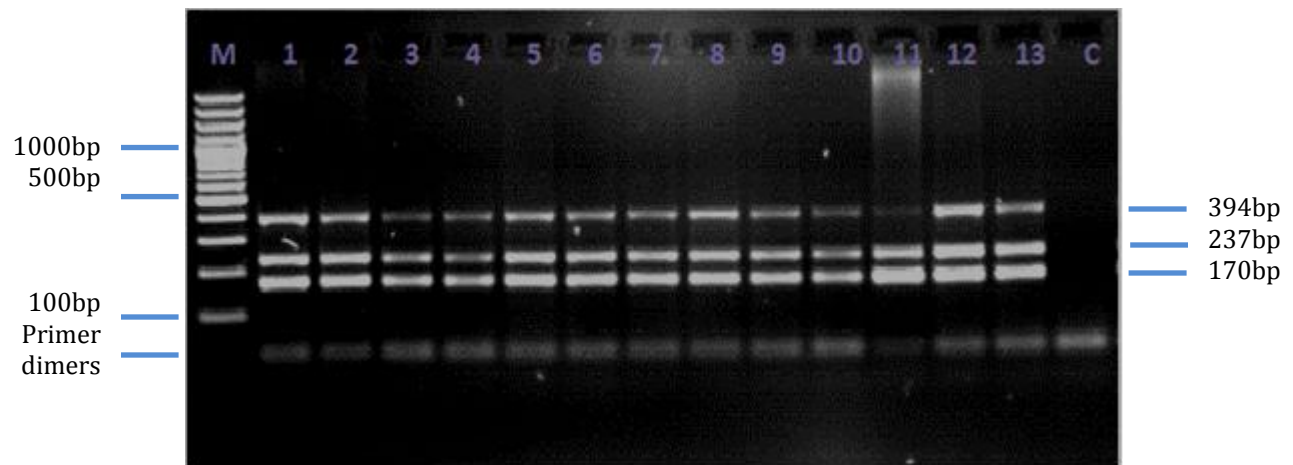


Figure 8: Group A PCR Products for three primer pairs- *MBP* rs12967023 (170bp), *ARNTL* rs7107287 (237bp), *ALDH1A1* rs1888202 (394bp). Lane M: 100bp MWM. Lane C: water control.

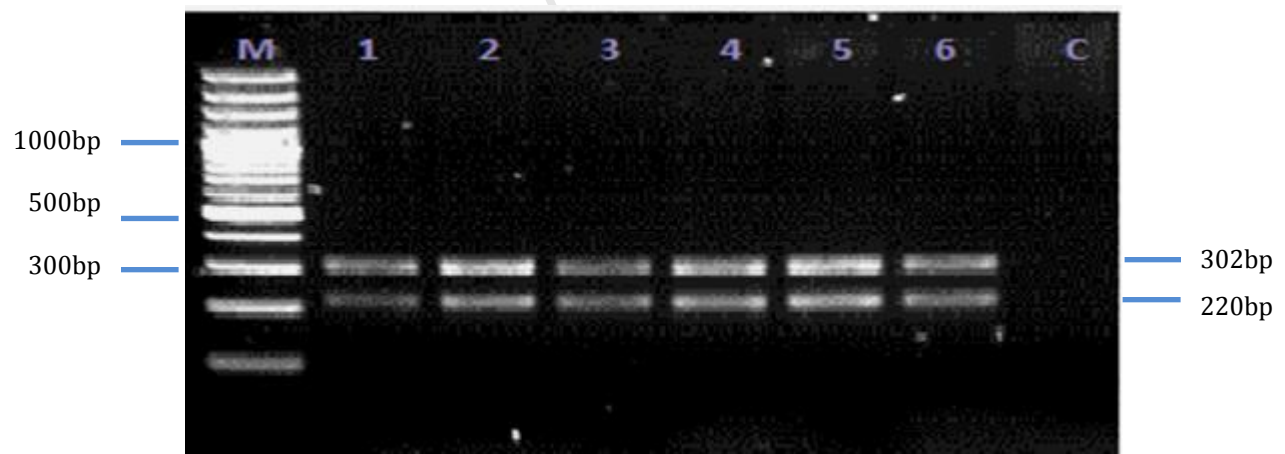


Figure 9: Group B PCR Products for two primer pairs- *CACNA1C* rs1006737 (220bp), *GSK3β* rs334558 (302bp). Lane M:100bp MWM. Lane C: water control.

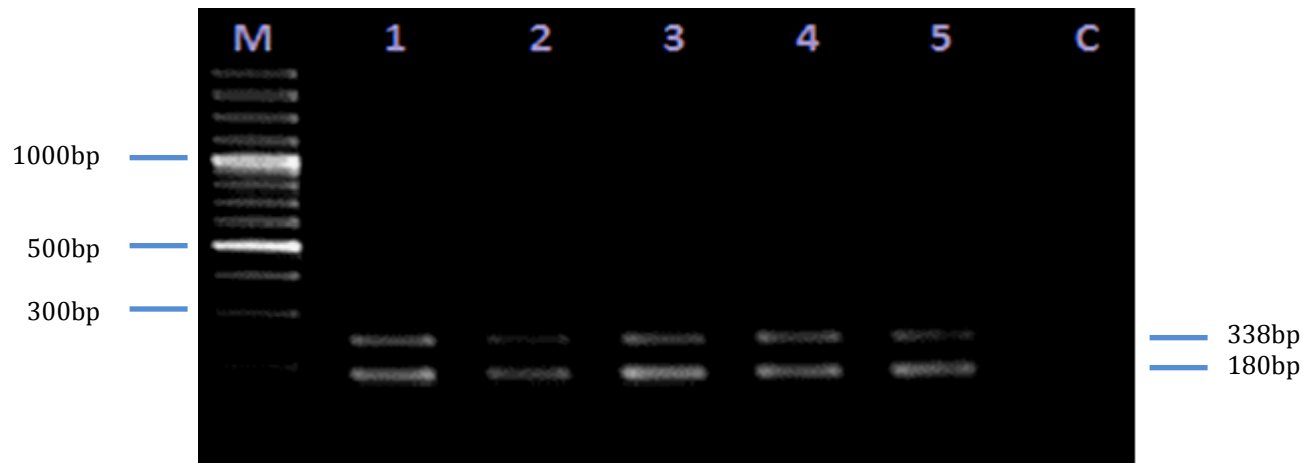


Figure 10: Group C PCR Products for two primer pairs- *KLF12* rs9600160 (180bp), *BDNF* rs6265 (338bp). Lane M: 100bp MWM. Lane C: water control.

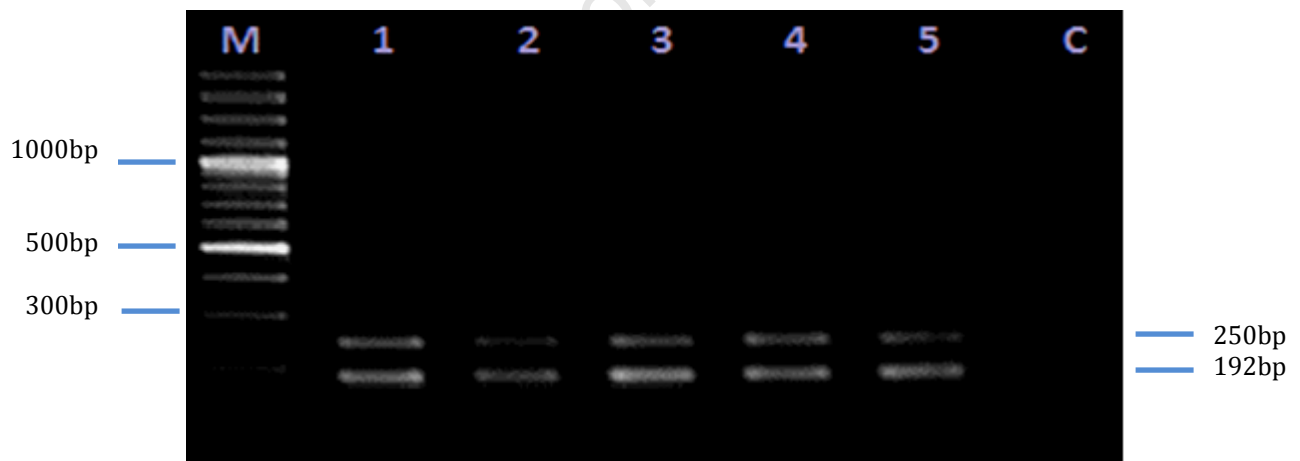


Figure 11: Group D PCR Products for two primer pairs- *A2BP1(A)* rs7204975 (192bp), *A2BP1(B)* rs10500336 (250bp). Lane M: 100bp MWM. Lane C: water control.



Figure 12: Group E PCR Products for two primer pairs- *AK3L1* rs4916031 (155bp), *ANK3* rs9804190 (227bp). Lane M: 100bp MWM. Lane C: water control.

3.5 SNaPshot PCR

3.5.1 Sap and ExoI Clean-up of PCR Products

PCR products were purified with SAP and ExoI. To allow for further multiplexing, which in turn saved reagents and time, product from Groups A and B were multiplexed in the same clean-up reaction i.e. an equal ratio of Group A and Group B PCR products were pooled. Similarly, Groups C, D, and E PCR products were multiplexed in the same clean-up reaction.

3.5.2 Capillary Electrophoresis on ABI PRISM

Due to multiplexing at the SAP and ExoI clean-up stage, genotyping was performed for two multiplexed reactions: Group A-B and Group C-D-E. Group A-B consisted of genotypes for five SNPs and Group C-D-E for six SNPs. Thus, the genotypes for 11 SNPs were incorporated into two SNaPshot reactions. SNaPshot reactions for Group A-B and Group C-D-E are represented by **Figures 13** and **14**, respectively. Homozygous genotypes are represented by a single, large peak and heterozygous genotypes by two,

overlapping or adjacent peaks, of different colour. G-alleles are blue, C-alleles are black, T-alleles are red and A-alleles are green in colour.

3.5.3 Analysis of Results

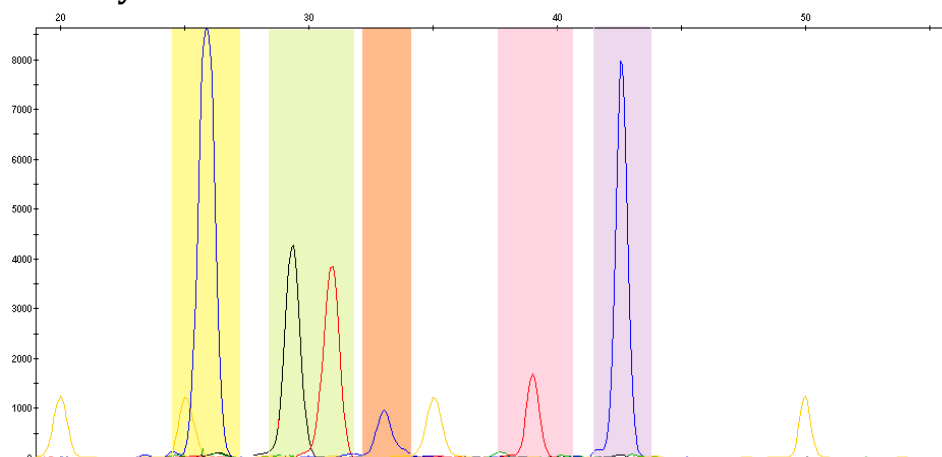


Figure 13: SNaPshot results for Group A-B. Yellow pane- *ARNTL* rs7107287 (genotype: GG), green pane- *MBP* rs12967023 (genotype: CT), red pane- *ALDH1A1* rs1888202 (genotype: GG), pink pane- *GSK3β* rs334558 (genotype: TT), purple pane- *CACNA1C* rs1006737 (genotype: GG). Y-axis represents fluorescent units. X-axis represents base position. Yellow peaks represent GeneScan-120 Liz size standard.

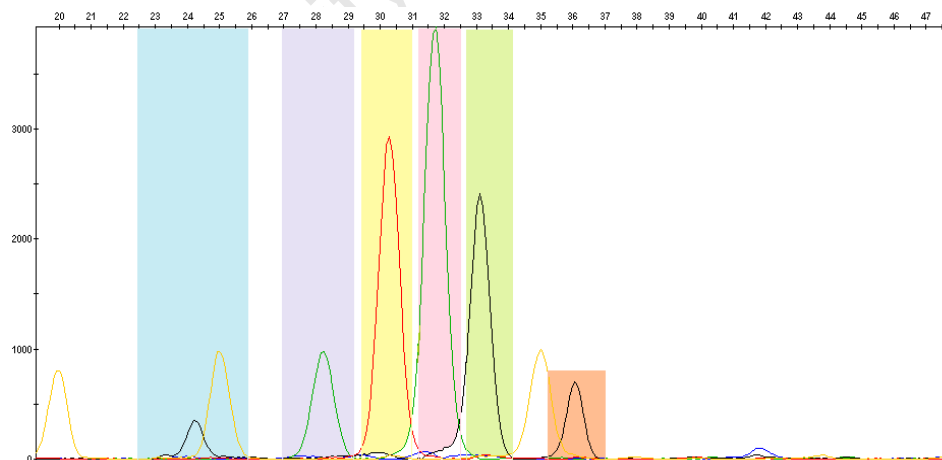


Figure 14: SNaPshot results for Group C-D-E. Blue pane- *ANK3* rs9804190 (genotype: CC), purple pane- *AK3L1* rs4916031 (genotype: AA), yellow pane- *A2BP1(B)* rs10500336 (genotype: TT), pink pane- *KLF12* rs9600160 (genotype: AA), green pane- *BDNF* rs6265 (genotype: CC), orange pane - *A2BP1(A)* rs7204975 (genotype CC). Y-axis represents fluorescent units. X-axis represents base position. Yellow peaks represent GeneScan-120 Liz size standard.

3.6 Genotype Validation

3.6.1 PCR of DNA Samples for Cycle Sequencing

Figure 15 illustrates *CACNA1C* rs1006737 PCR products (Lanes 1-3) used for sequencing. The first lane of the gel contained the 100bp MWM (**Appendix 3**) and the second lane the negative (water) control.

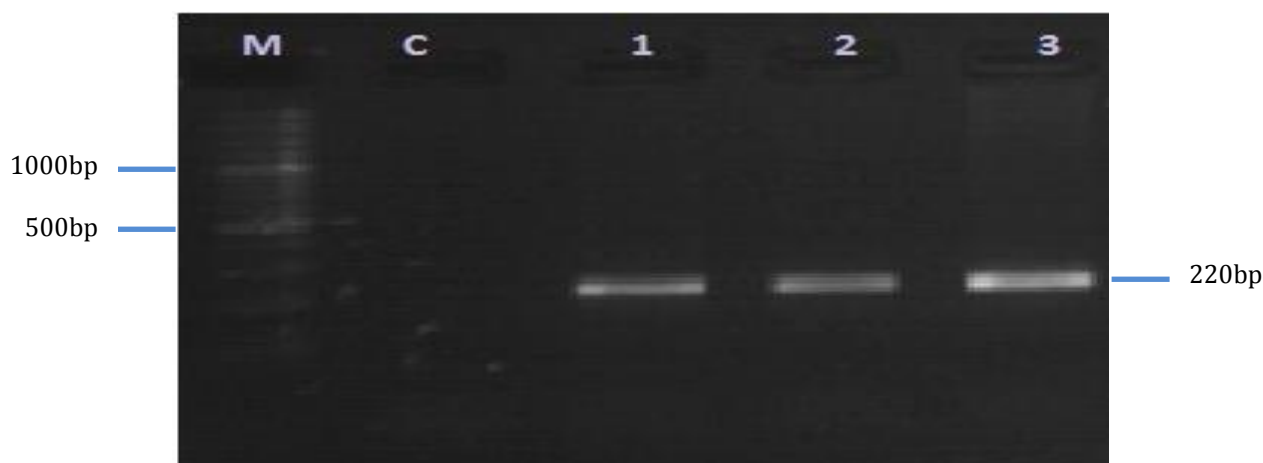


Figure 15: PCR amplification for cycle sequencing reaction. Lane M: 100bp MWM. Lane C: water control. Lanes 1-3: *CACNA1C* rs1006737 (220bp) PCR product.

3.6.2 Cycle Sequencing

In total, 32 samples were sequenced for validation of SNaPshot genotyping. These samples represented the genotypes for each of the 11 SNPs (*KLF12* rs9600160 had only two genotypes present in the sample population).

3.6.3 Analysis of Results

Sequencing results are represented by **Figures 16** and **17**. **Figure 16** represents a portion of the sequencing results of the *BDNF* rs6265 SNP. This particular sample was homozygous C for this SNP, which is represented as a blue peak in the electropherogram at position 250 (as indicated by the red arrow). **Figure 17** represents a heterozygous

sample (C/T) for the SNP *ANK3* rs9804190. This is seen by the blue and red peaks at position 142 (as indicated by the red arrow).

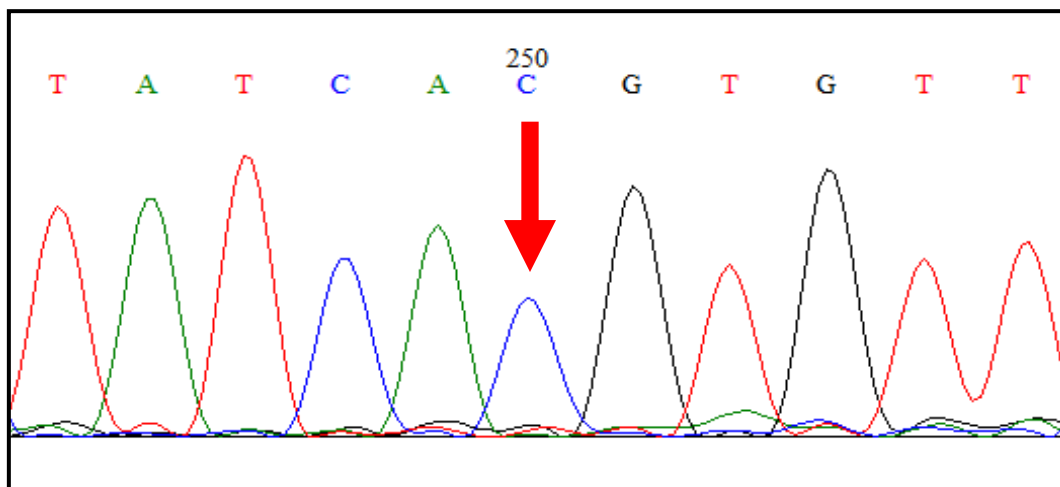


Figure 16: Sequencing results for *BDNF* rs6265. This particular sample was homozygous C (blue peak) which is seen at position 250 (as seen indicated by the red arrow).

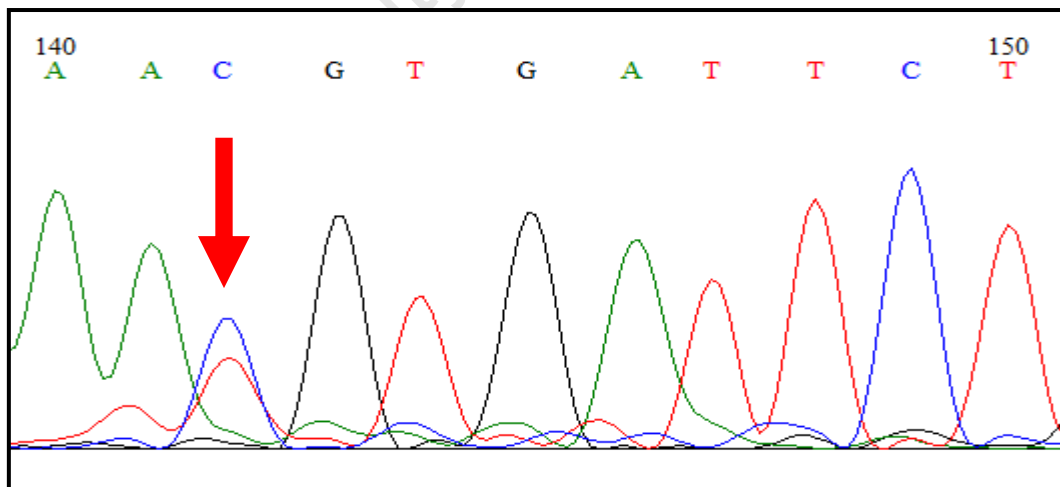
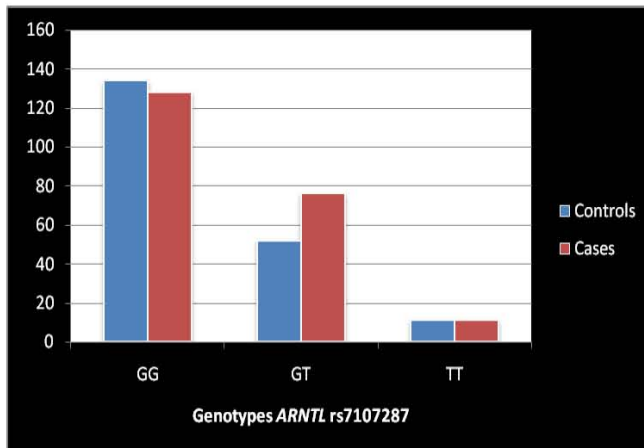


Figure 17: Sequencing results for *ANK3* rs9804190. This particular sample was heterozygous (C/T- blue and red peaks) seen at position 142 (as indicated by the red arrow).

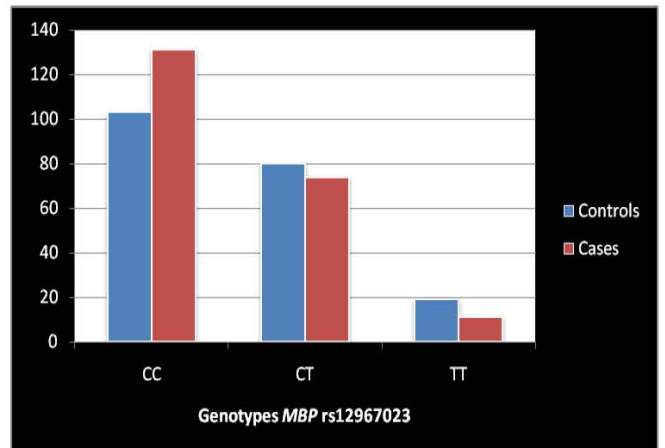
3.7 Statistical Analysis for Case Control Association

3.7.1 Genotype Frequencies, Hardy Weinberg Equilibrium Calculations and Logistic Regression with *R*

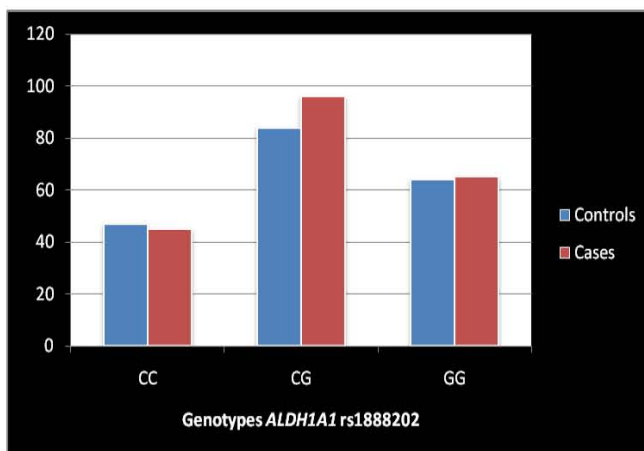
Genotype counts of each SNP, for cases (n=224) and controls (n=202), are displayed in the following graphical outputs **Figures 18** (A, B, C, D, E, and F) **and 19** (A, B, C, D, and E). *KLF12* (rs9600160) had no homozygous G individuals. It is evident from these figures, that there are no large differences in genotype frequencies between cases and controls. For control samples, none of the SNPs were found to be out of HWE. As a number of cases were related to one another, a random affected individual was selected for logistic regression analysis (logistic regression cannot adjust for relatedness). This resulted in a case cohort of 168 unrelated individuals. Based on the results from logistic regression, both gender (p-value 0.0151) and ethnicity (p-value 0.0107) had an association with disease affection status. Therefore, all subsequent association analyses were adjusted for gender and ethnicity. Case-control analysis showed that *ANK3* rs9804190 and *A2BP1* rs7204975 were the only SNPs to have obtained a p-value of less than 0.05 (**Table 17**), in a recessive genetic model. This suggests that having two copies of the C-allele of the *ANK3* rs9804190 increased the risk of being diagnosed with BPD by an odds ratio of 1.68. Also, being homozygous for the C-allele of *A2BP1* rs7204975, increased the risk of having BPD by an odds ratio of 1.77. Therefore, being homozygous C at both SNPs increases the risk for BPD nearly two fold. However, after adjusting for multiple testing using Bonferroni correction (new p-value cutoff 0.0038), these SNPs were no longer significantly associated with BPD.



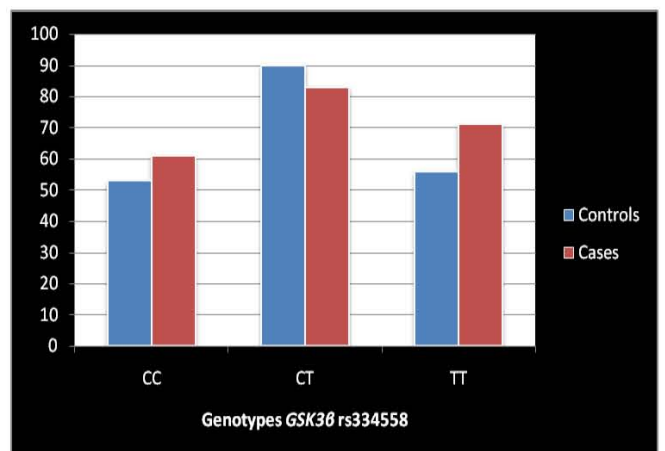
A



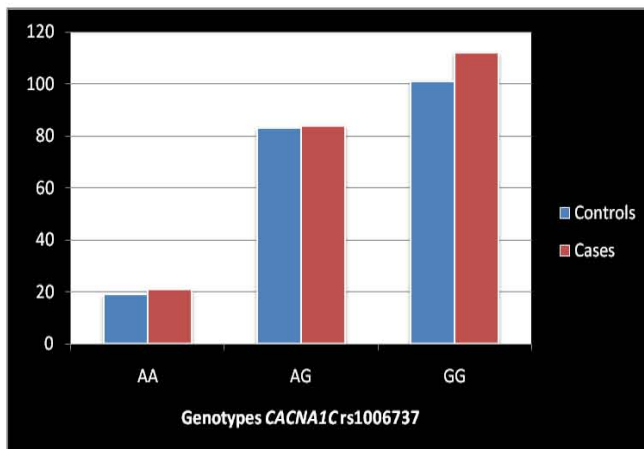
B



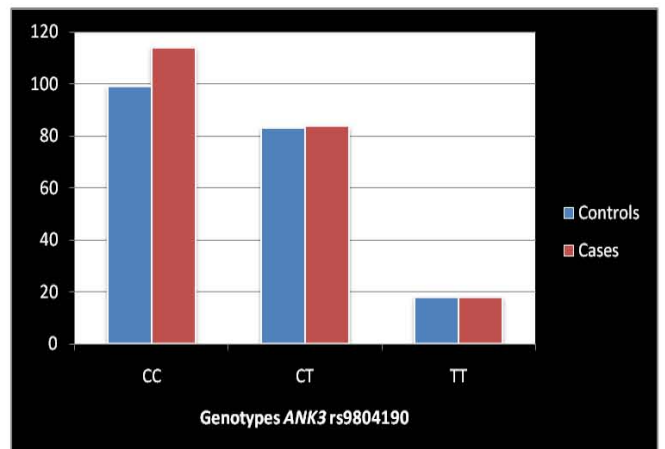
C



D



E



F

Figure 18: Genotype counts of cases and controls for the following SNPs A) *ARNTL* rs7107287, B) *MBP* rs12967023, C) *ALDH1A1* rs1888202, D) *GSK3β* rs334558, E) *CACNA1C* rs1006737, and F) *ANK3* rs9804190.

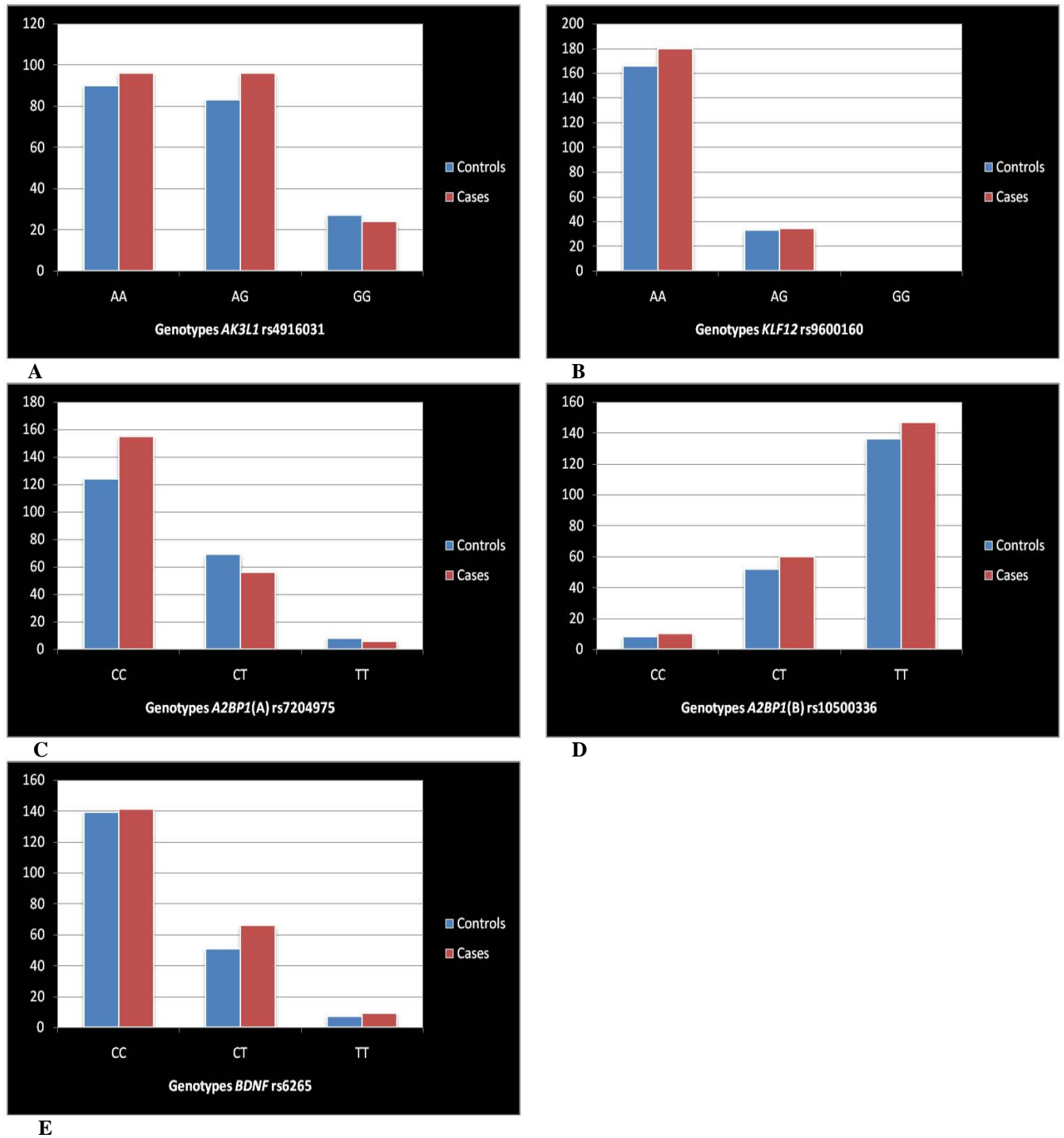


Figure 19: Genotype counts of cases and controls for the following SNPs of A) *AK3L1* rs4916031, B) *KLF12* rs9600160, C) *A2BP1(A)* rs7204975, D) *A2BP1(B)* rs10500336, and E) *BDNF* rs6265.

Table 17: R logistic regression output for case-control data using a recessive genetic model (adjusted for gender and ethnicity)

| Gene | SNP | Odds Ratio | Lower | Upper | Z-test | p-value |
|-----------------|------------|-------------------|--------------|--------------|---------------|----------------|
| <i>ARNTL</i> | rs7107287 | 1.21 | 0.77 | 1.91 | 0.82 | 0.4139 |
| <i>MBP</i> | rs12967023 | 0.89 | 0.55 | 1.45 | -0.45 | 0.6516 |
| <i>ALDH1A1</i> | rs1888202 | 1.44 | 0.81 | 2.56 | 1.25 | 0.2131 |
| <i>GSK3B</i> | rs334558 | 0.80 | 0.48 | 1.34 | -0.85 | 0.3979 |
| <i>CACNA1C</i> | rs1006737 | 1.19 | 0.57 | 2.51 | 0.47 | 0.6416 |
| <i>ANK3</i> | rs9804190 | 1.68 | 1.04 | 2.72 | 2.14 | 0.0324 |
| <i>AK3L1</i> | rs4916031 | 0.87 | 0.55 | 1.36 | -0.61 | 0.5437 |
| <i>A2BP1(B)</i> | rs10500336 | 0.97 | 0.31 | 3.02 | -0.04 | 0.9642 |
| <i>KLF12</i> | rs9600160 | 1.17 | 0.65 | 2.10 | 0.51 | 0.6101 |
| <i>BDNF</i> | rs6265 | 0.71 | 0.44 | 1.18 | -1.31 | 0.1893 |
| <i>A2BP1(A)</i> | rs7204975 | 1.77 | 1.09 | 2.89 | 2.30 | 0.0215 |

3.7.2 M_{QLS} (adjusting for relatedness) and Pedstats

M_{QLS} is a software package capable of analyzing case-control data where certain individuals within the study are related to one another. For this analysis, genotypes needed to be represented in a pedigree file which specified familial relationships between individuals (including unaffected, non-genotyped family members). Before M_{QLS} analysis could be performed, Pedstats a freely available program, was used to execute quality checks on pedigree data. For example, it was able to detect Mendelian inconsistencies whereby a mother was homozygous for one allele at a particular SNP and her offspring was homozygous for the other allele at that same SNP. In reality, this scenario is impossible as

the offspring would have to have inherited one of the alleles from the mother and either be homozygous for the “maternal” allele or be heterozygous at that position. This Mendelian inconsistency was due to genotype calling error and could therefore be rectified. Therefore, Pedstats was able to ensure that pedigree data based on genotypes were “error-free”. From the Pedstats results (**Table 18**), there were 593 individuals overall, in the pedigree file, with 370 families. There were 280 females and 258 males (the genders for a number of individuals were unknown).

From the M_{QLS} output (**Table 19**), it can be noted that only one of the SNPs, *MBP* rs12967023 obtained a significant p-value of less than 0.05. However, after Bonferroni correction for multiple testing, this SNP was no longer significant. It is noted that *A2BP1(A)* rs7204975 had a p-value approaching significance (p-value 0.0678).

No significant interactions were found, between *ANK3* rs9804190, *A2BP1(A)* rs7204975, and *MBP* rs12967023, after adjusting for Bonferroni Correction (results not shown).

Table 18: Pedigree structure from Pedstats output

| No. of individuals in Pedigree File | 593 |
|--|------------------------|
| No. of Founders | 385 |
| Gender Distribution | 280 Females, 258 Males |
| No. of Families | 370 |

Table 19: M_{QLS} Output for Case Control Association

| Gene | SNP | Chi-Squared Test Statistic | LOD | p-value |
|-------------------------------|------------|----------------------------|----------|---------------|
| <i>ARNTL</i> | rs7107287 | 1.85983 | 0.403857 | 0.1726 |
| <i>MBP</i> | rs12967023 | 4.84863 | 1.05287 | 0.0277 |
| <i>ALDH1A1</i> | rs1888202 | 0.075409 | 0.016375 | 0.7836 |
| <i>GSK3β</i> | rs334558 | 0.24238 | 0.052632 | 0.6225 |
| <i>CACNA1C</i> | rs1006737 | 0.242548 | 0.052669 | 0.6224 |
| <i>ANK3</i> | rs9804190 | 0.150696 | 0.032723 | 0.6979 |
| <i>AK3L1</i> | rs4916031 | 0.064394 | 0.013983 | 0.7997 |
| <i>A2BP1(B)</i> | rs10500336 | 0.450299 | 0.097781 | 0.5022 |
| <i>KLF12</i> | rs9600160 | 0.014813 | 0.003217 | 0.9031 |
| <i>BDNF</i> | rs6265 | 0.964785 | 0.2095 | 0.3260 |
| <i>A2BP1(A)</i> | rs7204975 | 3.33621 | 0.724448 | 0.0678 |

3.8 Statistical Analysis of Neuro-imaging

The following imaging data (reported as cubic millimeters (mm³)), were obtained for a small subset of affected individuals (n=22), of the larger cohort: right, left and total hippocampal volumes (HCV) and intracranial volumes (ICV). Each of the HCVs was divided by the ICV to account for inter-individual differences in brain volume. This accounted for differences in cranial volumes across gender and ethnicity. The Shapiro-Wilk test was used to determine whether imaging data were normally distributed. Results from this test indicated that only left HCV/ICV measure was not normally distributed (p-value 0.03). Therefore, the Kruskal-Wallis rank sum test, the non-parametric equivalent to ANOVA, was used to determine whether there was any difference in the median left HCV/ICV values among the genotype

groups. Bartlett's test was used to ensure that all variances were equal amongst the genotype groups. All calculations indicated non-significant p-values (not shown), therefore, all groups had equal variances. Results from **Table 20** (Kruskal Wallis chi-squared, F-values (ANOVA) and p-values) suggests that none of the genotypes have an association with left HCV/ICV, right HCV/ICV, and total HCV/ICV.

Table 20: Kruskal Wallis and ANOVA values for each of the SNPs and imaging measures, Left HCV, Right HCV and Total HCV

| Gene | SNP | Left HCV/ICV | | Right HCV/ICV | | Total HCV/ICV | |
|-------------------------------|------------|----------------|---------|---------------|---------|---------------|---------|
| | | KW chi-squared | p-value | F-value | p-value | F-value | p-value |
| <i>ARNTL</i> | rs7107287 | 1.0909 | 0.2963 | 0.996 | 0.3308 | 0.7478 | 0.398 |
| <i>MBP</i> | rs12967023 | 2.4304 | 0.2967 | 1.6437 | 0.2210 | 1.3163 | 0.2927 |
| <i>ALDH1A1</i> | rs1888202 | 2.3211 | 0.3133 | 1.1948 | 0.33 | 1.3019 | 0.3011 |
| <i>GSK3β</i> | rs334558 | 1.9813 | 0.3713 | 1.0793 | 0.3598 | 1.0866 | 0.3574 |
| <i>CACNA1C</i> | rs1006737 | 4.7621 | 0.09246 | 1.9667 | 0.1674 | 1.806 | 0.1914 |
| <i>ANK3</i> | rs9804190 | 2.7391 | 0.2542 | 1.0134 | 0.3828 | 1.1203 | 0.3479 |
| <i>AK3L1</i> | rs4916031 | 2.6 | 0.2725 | 1.8067 | 0.1927 | 1.623 | 0.2249 |
| <i>A2BP1(B)</i> | rs10500336 | 0.8475 | 0.6546 | 0.9849 | 0.3918 | 0.8857 | 0.4288 |
| <i>KLF12</i> | rs9600160 | 1.0435 | 0.307 | 2.3747 | 0.1390 | 1.5087 | 0.2336 |
| <i>BDNF</i> | rs6265 | 1.1938 | 0.2746 | 1.5175 | 0.2323 | 1.2198 | 0.2825 |
| <i>A2BP1(A)</i> | rs7204975 | 1.4522 | 0.4838 | 0.7756 | 0.4744 | 0.6812 | 0.5179 |

Chapter 4: Discussion

BPD is a severe disorder affecting the lives of millions of individuals worldwide. Currently, the exact aetiology of BPD is unknown, treatment options are limited and a definitive cure seems distant. Due to the high heritability of this disorder, genetics is thought to be a key factor in the discovery of the pathophysiology. However, to date, even with high through-put genomic technologies, this has proven to be challenging. Numerous genes have been implicated in the aetiology of BPD, but have subsequently been difficult to replicate in independent studies. Besides genetic research, brain imaging has also been utilized in an attempt to elucidate biological mechanisms of BPD causality or association. However, as with genetic research, findings have been inconsistent.

In the present study, ten genes were selected from a wide range of candidate genes which were prioritized through a convergent approach and GWAS. It was found that SNPs in the genes *ANK3*, *A2BP1* and *MBP* may play a role in the aetiology of BPD. In addition, due to the enabling multidisciplinary research environment here at UCT, it was possible to explore a dimension of brain imaging research of BPD subjects for whom genetic studies were also undertaken. In this pilot study of 22 affected individuals, none of the SNPs in ten genes tested were associated with MRI features.

Over the years, in efforts to determine the underlying genetics of BPD, linkage, association, and GWAS have implicated numerous chromosomal regions and genes. Generally, these original findings have proven difficult to replicate. These discrepancies

could be due, at least in part, to aetiological heterogeneity [Faraone and Tsuang, 2003]. The Division of Human Genetics at UCT has a well characterized BPD archive which serves as a rich resource for genetic studies of the disorder. This cohort is predominantly familial (total of 221 families), and limited to a few ethnic groups, which is surmised to reduce the aetiological heterogeneity. The offspring of individuals affected with BPD are at higher genetic risk for developing the disorder and offer a significant source of convergent information regarding the development of this disorder. Gene-environment interactions may also be assessed in this circumstance as these offspring may have been affected by mood episodes experienced by the affected parent [Chang et al., 2003]. Another way of attempting to reduce genetic and aetiological heterogeneity i.e. to have a more homogeneous study group, is to sample geographically and genetically isolated populations. These derived cohorts may be advantageous as they may have a higher prevalence of certain diseases, an endogamously-related probability of mapping recessive genes, and less migration [Peltonen et al., 2000]. In our cohort, the Afrikaner Caucasians and indigenous African Xhosa-speaking populations are relatively homogenous and genetically isolated, and may be ideal candidates for psychiatric genetic research. However, due to the small numbers of indigenous Africans and other isolated ethnic groups in our cohort, individuals from this population group were not included in the current study.

The first aim of this investigation was to determine whether variants in ten selected and previously associated candidate genes are risk factors for the development of BPD. Logistic regression is a statistical method commonly used in the analysis of case-control data. Using this statistical method, and indicating a recessive genetic model, only *ANK3* rs9804190 (OR 1.68) and *A2BP1(A)* (rs7204975) (OR 1.77) showed nominal

associations with BPD. Thus, individuals who were homozygous C at both SNPs had an increased risk for BPD. This suggests that BPD is a recessive disorder whereby individuals require two copies of the disease-predisposing allele to be affected. However, the mode of inheritance of BPD is thought to be non-Mendelian and a result of the interaction of numerous genes with the environment [Serretti and Mandelli, 2008]. After adjusting for multiple testing using the Bonferroni correction method, none of the SNPs were statistically significant. This situation may reflect i) that there are no genuine associations, or ii) that there is some association and this disappears upon correction for multiple testing. The Bonferroni correction decreases Type I error (the probability of rejecting the null hypothesis, when it is true). This method is often used as it is relatively simple to calculate and can be applied to most multiple-testing scenarios [Bender and Lange, 2001]. However, the Bonferroni correction has often been criticized as being overly conservative and increases the chances of Type II error (the probability of rejecting the alternate hypothesis, when it is true) [Perneger, 1998]. Thus, the debate continues as to whether correction for multiple-testing, particularly using the Bonferroni method, is necessary for genetic studies. In light of this, it is worth re-examining the biological role of the two candidates: *ANK3* and *A2BP1*.

ANK3 encodes a neurally expressed, structural protein that is thought to be involved in the maintenance of ion channels and cell adhesion molecules of axonal segments [Kordeli et al., 1995]. Four GWAS, in various population groups, have shown *ANK3* associated with BPD [Baum et al., 2008; Ferreira et al., 2008; Scott et al., 2009; Smith et al., 2009]. These findings were replicated in a meta-analysis study consisting of Caucasian subjects (n=1668 cases and 1604 controls) [Schulze et al., 2008]. The associated SNP is located within an intronic region of the gene and has no known

functional significance. *ANK3* is located on chromosome 10, in a region that was originally linked with late onset Alzheimer's disease [Myers et al., 2000]. Subsequently, a candidate gene association study detected a nominal association of this gene and late onset Alzheimer's disease [Morgan et al., 2008].

A2BP1 encodes a RNA-binding protein, which interacts with the gene *ataxin-2* which has been shown to underlie the aetiology of the neurodegenerative disorder, SCA type 2 [Shibata et al., 2000; Kiehl et al., 2001]. This gene was previously found to be associated with several neuropsychiatric disorders, including attention deficit hyperactivity disorder (ADHD) [Elia et al., 2009], autism [Martin et al., 2007], and Schizoaffective Disorder [Hamshere et al., 2009]. The *A2BP1* SNP, rs7204975, is located within an intron of the gene, and seemingly, has no known functional significance. However, it may be in linkage disequilibrium with the causal variant or it may play a role in the regulation of the gene. This SNP was found to be associated with BPD, in a German population group, by a previous GWAS (p-value 0.001) [Baum et al., 2008]. However, *A2BP1(B)* rs10500336 which had a p-value of 0.018 in the same GWAS, did not attain significance in the current study.

From the M_{QLS} analysis, only *MBP* rs12967023 showed a nominal association with a p-value of less than 0.05. However, after correction for multiple testing, this finding was no longer significant. The product encoded by *MBP* comprises 30% of the protein in CNS myelin. Myelin is found around the axons of neurons and increases the velocity of nerve impulses [Kamholz et al., 1987]. *MBP* expression has been shown to be reduced in SCZ postmortem brains [Tkachev et al., 2003] and a decreased intensity of myelin staining has been observed in BPD brain tissue [Regenold et al., 2007]. Similarly, a BPD twin

study has shown that an affected twin (n=24) has significantly reduced white matter volumes compared to the unaffected co-twin (n=15) and demographically matched control twins (n=27) [Kieseppä et al., 2003]. *MBP* has also been implicated in the recovery from neuronal insults. In animal models, autoimmune T-cells, activated against the CNS antigen MBP, reduce degeneration after optic nerve injury [Hauben et al., 2000]. Furthermore rats that have been immunized with MBP show more severe neuronal loss after CNS injury [Jones et al., 2004]. This suggests that MBP has a possible role in the progression of neurodegeneration. The *MBP* SNP, rs12967023, is located within an intron, therefore, the exact function is unknown. This SNP has however been previously associated with BPD (p-value 0.001) in a GWAS involving Caucasian subjects [Sklar et al., 2008].

Surprisingly, none of the other candidate genes (or at least the SNPs assayed) was found to be associated with BPD. *BDNF*, and particularly the Val66Met polymorphism, has often been found to be associated with BPD [Neves-Pereira et al., 2002; Sklar et al., 2002; Lohoff et al., 2005; Muller and De, 2006; Okada et al., 2006]. However, the results of the current study are in accordance with many studies that have not found an association between BPD and this neurotrophic factor [Nakata et al., 2003; Kunugi et al., 2004]. *CACNA1C* which has been strongly associated with BPD in a GWAS [Ferreira et al., 2008] and replicated in independent studies [Scott et al., 2009; Schulze et al., 2008] was not associated with BPD in the present study. The difference in results between the current and previous studies could be due to any one, or a combination, of several factors including the different ethnic groups under investigation, differences in study designs and/or sample numbers in the respective studies.

The final cohort consisted of 224 subjects affected with BPD and 202 background control individuals. The controls formed part of the background population and were not screened for any psychiatric illness. Thus, in accordance with the estimated prevalence of BPD, 1% of these controls may have been predisposed to BPD. The cases and controls were not “matched” with respect to gender and ethnicity. However, it is acceptable to adjust for potential confounders such as gender and ethnicity in the regression analysis [Bland and Altman, 1994].

An association was found between gender and BPD. This is not in accordance with previous findings, where it has been shown that BPD affects both males and females at equal frequencies [Merikangas et al., 2007; American Psychiatric Association. Task Force on DSM-IV, 1994]. However, it has been found that men, generally, have an earlier age of onset than females [Kennedy et al., 2005]. In the current study, differences in genotype frequencies between Caucasian and Mixed Ancestry individuals were observed. This finding does not agree with a previous U.S. study which found no difference in BPD prevalence amongst ethnic groups (n=9282 study subjects) [Merikangas et al., 2007]. However, different ethnic groups (viz. Hispanic, Caucasian, and African American) were investigated by Merikangas et al. [2007], compared to the current study which included Caucasians (British and Afrikaner ancestry) and individuals of Mixed Ancestry. The South African Mixed Ancestry group is an admixed population group consisting of individuals of Khoesan, Bantu-speaking African, European and Asian ethnicity [de Wit et al., 2010].

The second aim of the current study was to determine whether the endophenotypic hippocampal volumes have an association with any of the selected candidate genes. This

component of the project should be considered a pilot study as there were only 22 participants with both imaging and genotyping data available. Nonetheless, previous imaging genetic studies have had similar sample sizes [Bueller et al., 2006; Chepenik et al., 2008]. As with other similar studies, the brain area under investigation was the hippocampus, involved in long-term memory [Squire, 1992], stress responses [Sapolsky et al., 1984] and spatial navigation [Maguire et al., 2000]. In this study, no association was found between any of the genotypes and hippocampal volumes (left, right and total), corrected for total intracranial volumes. This finding could be expected, as the study was a pilot, testing the technologies and analytical capacities between the clinical phenotype, imaging endophenotypes and genetic data. From the outset, it was obvious that the small sample size and potential number of tests would render the study inadequately powered.

Previous studies have implicated the *BDNF* rs6265 (Val66Met) polymorphism with hippocampal volume. In healthy controls, the Met-allele was associated with reduced hippocampal volumes [Bueller et al., 2006; Chepenik et al., 2008]. These findings have also been shown for individuals affected with major depression (n=60 cases and 60 controls) [Frodl et al., 2007]. However, results from the current study suggest that there is no association between *BDNF* rs6265 and brain hippocampal and intracranial volumes of individuals affected with BPDI. This finding is similar to that shown by Dutt et al. [2009] where the *BDNF* rs6265 SNP has no association with hippocampal volumes of individuals diagnosed with psychosis (n= 128 cases, 194 unaffected relatives, and 61 controls).

4.1 Future Directions

Future studies should consider the following aspects: The functionality of the following SNPs should be investigated: *ANK3* rs9804190, *A2BP1* rs7204975 and *MBP* rs12967023. Statistical significance of genotype with diagnosis does not illustrate the biological mechanism for risk. Therefore, the underlying biology needs to be determined [Bigos et al., 2010]. The effect of the SNPs on expression of the gene can be investigated by the use of Allelic Expression Imbalance (AEI). This method relies on the differentiation of maternal and paternal alleles and to determine the relative mRNA expression of each [Buckland, 2006]. An individual needs to be heterozygous at the SNP of interest and any deviation from a 50:50 expression of each allele suggests an effect of the genetic variation [Quinn et al., 2010].

It is possible that other variants, within the candidate genes investigated, have an association with BPD. High throughput technologies, such as next generation re-sequencing, could be used to get a more dense coverage of variants, in a wide range of subjects, of a particular candidate gene. For example, exome sequencing has been able to identify the candidate gene and variants underlying the aetiology of a rare Mendelian disorder [Ng et al., 2009].

Subsequent to their original CFG publication, Le-Niculescu et al. [2008] did a follow-up study whereby they re-generated a prioritized BPD list using four instead of three GWAS results and included other, more recent lines of evidence [Patel et al., 2010]. The top three candidate genes were the same as those from the 2008 study. They also developed a genetic risk prediction score (GRPS) whereby individuals affected with BPD could be distinguished from controls. This score was based on the most significant panel

of SNPs from the CFG list (which included the *MBP* rs12967023 SNP), providing proof of principle whereby the candidate gene list could be used to predict risk for developing BPD at a population level [Patel et al., 2010]. Although it may not be used clinically yet, this predictive value is enormously important in dismantling large heterogeneous cohorts into more aetiologically homogeneous entities, towards identifying the underlying range of molecular pathologies.

Future work could also investigate alternate candidate genes. Interestingly, genes that have been previously implicated in the aetiology of cancer have also been shown to be significant for BPD. The WTCCC GWAS found a significant signal for the locus containing the *PALB2* gene [Stratton, 2007]. This finding was replicated in a subsequent study whereby both *PALB2* and breast cancer 2 (*BRCA2*) were found to be positively associated to BPD in a Scandinavian population group [Tesli et al., 2010].

Recently, work done by our group on a subset of the larger BPD cohort, have shown that genes involved in glutamatergic signaling have an association with BPD severity. The gene for *glutamate receptor, metabotropic 3 (GRM3)* was associated with a four-fold increased risk of having psychotic symptoms and the genes, *ionotropic glutamate receptor, N-methyl D-aspartate 2B (GRIN2B)* and *D-amino acid oxidase activator (DAOA)* interact to increase the risk of being admitted to hospital for mania [Dalvie et al., 2010] (**Appendix 8**). Therefore, these genes and others involved in glutamatergic signaling, should be investigated more extensively.

With regard to the imaging genetics, it is obvious that the technologies are at hand to carry out the work in this promising area. Apart from a significant increase in numbers of subjects, imaging data should be obtained from control individuals to determine whether hippocampal volumes have an association with BPD. Changes in hippocampal volumes have often been described in psychiatric illnesses such as borderline PD, PTSD, major depressive disorder (MDD) [Bremner et al., 2000] and BPD. Chepenik et al. [2009] have found that BPD-affected individuals have smaller hippocampal volumes compared to controls. However, results have varied across studies. Some studies have not found differences in hippocampal volumes between individuals affected with BPD and controls [Hajek et al., 2009].

Since the implicated candidate genes (i.e. *ANK3*, *A2BP1* and *MBP*) have been shown to have a relationship with neurodegeneration, it would be of interest to determine whether any of these genes have an association with any neurodegenerative disorders. The Division of Human Genetics has a long history of investigating neurodegenerative disorders such as SCA [Bryer et al., 2003; Greenberg et al., 2006] and Huntington's Disease [Greenberg et al., 1991; Squitieri et al., 1994; Bardien et al., 2007; Scholefield and Greenberg, 2007]. A cursory study may involve investigating neurodegenerative symptoms or disorders in individuals or families where the BPD phenotype is associated with these genes.

4.2 Conclusions

This study showed that intronic SNPs within *ANK3*, *A2BP1* and *MBP* may have an association with BPD and that polymorphisms within the ten investigated candidate genes do not show an association with endophenotypic measures of BPD. The three implicated candidate genes have an indirect relationship with neurodegenerative

disorders [Hauben et al., 2000; Myers et al., 2000; Shibata et al., 2000; Kiehl et al., 2001; Jones et al., 2004; Morgan et al., 2008]. Neurodegenerative disorders such as Parkinson's and Huntington's are characterized by a loss of neurons, BPD has often been shown to be accompanied by a decrease in neuronal plasticity as well as decreases in various regional volumes [Rajkowska, 2002]. This disorder has also been shown to be associated with minor cognitive impairments [Martinez-Aran et al., 2004], as well as an increased risk for developing dementia in the elderly [Nunes et al., 2007]. This may suggest that BPD could be considered to have features in common with the classical neurodegenerative disorders. Therefore, it can be postulated that the pathophysiology of BPD has a link with neurodegeneration.

The present study highlights the importance of examining complex phenotypes in a familial setting where the traditional view of comorbidities may be examined across individuals "at risk". Phenotypic studies in such high risk subjects and families continue to be important in future studies of complex psychiatric disorders, including BPD.

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University of Cape Town

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Appendices

Appendix 1: Patient Consent Form



REQUEST FOR MOLECULAR STUDIES (DNA)



Molecular Laboratory
Division of Human Genetics
IIDMM, LEVEL 3
UCT Medical School, Observatory 7925

Tel: (021) 406 6425 Fax: (021) 406 6826

Blood should be drawn in 2 plastic EDTA Tubes (Purple top) +/- 10ml each using a yellow barrel. Each tube should be inverted to mix and should be clearly labelled with the patient's name and DOB. Keep blood in fridge at 4°C until able to send to laboratory.

Please **DO NOT** send specimens on ice or frozen.

Please fill in all the information requested:

Surname: _____ First Name(s): _____

New Family: Yes No (If no, please fill in family name) Family name: _____

Medical Aid: _____ Medical Aid No: _____

Sex: M F Date of Birth: Year: _____ Month: _____ Day: _____

Number of children: _____

Ethnic Origin : (please indicate ancestry of both your mother and father) _____

Contact Address: _____ Town: _____ Fax: _____ Tel: _____

Referring Doctor/Sister: _____ Town: _____ Fax: _____ Tel: _____

Hospital or Address: _____ Town: _____ Fax: _____ Tel: _____

Reason for Referral (Clinical diagnosis):

Affected At Risk Carrier Spouse Query Unaffected

Becker Muscular Dys. Duchenne Muscular Dys Colonic Carcinoma

Fragile-X Syndrome Bipolar Disorder Huntington Disease

Retinitis Pigmentosa Spinocerebellar Ataxia Waardenberg Syndrome

Additional disorders (apparent or previously treated): _____

Additional family history _____

Clinical Details:

Physical disability Mental retardation Deafness Impaired vision Night blindness

Other: _____

Have samples from this patient been sent to a DNA lab before? (DELETE WHERE NOT APPLICABLE) YES / NO / Don't Know

If Yes, where: _____

For Laboratory use only:

DNA number: _____ Vol. Blood: _____ (ml) Other: _____

Date Received: Year: _____ Month: _____ Day: _____ Computer Index No: _____

CONSENT FOR DNA ANALYSIS AND STORAGE

- I, _____, request that an attempt be made using genetic material to assess the probability that: I / my child / my unborn child (DELETE WHERE NOT APPLICABLE) might have inherited a disease-causing mutation in the gene for: _____
- I understand that the genetic material for analysis is to be obtained from: blood cells/skin sample/other (specify) (DELETE WHERE NOT APPLICABLE) :
- I request that **no** portion of the sample be stored for later use. (MARK IF APPLICABLE)
Or
I request that a portion of the sample be stored indefinitely for (DELETE WHERE NOT APPLICABLE):
(a) possible re-analysis
(b) analysis for the benefit of members of my immediate family
(c) research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain confidential.
- The results of the analysis carried out on this sample of stored biological material will be made known to me, via my doctor, in accordance with the relevant protocol, if and when available.
In addition, I authorise that they may be made known to: (DELETE WHERE NOT APPLICABLE) :
other doctors involved in my care _____
the following family members: _____
other: _____
- I authorise / do not authorise my doctor(s) (DELETE WHERE NOT APPLICABLE) to provide relevant clinical details to the Division of Human Genetics, UCT.
- I have been informed that:
(a) there are risks and benefits associated with genetic analysis and storage of biological material and these have been explained to me.
(b) the analysis procedure is specific to the genetic condition mentioned above and cannot determine the complete genetic makeup of an individual.
(c) the genetics laboratory is under an obligation to respect medical confidentiality .
(d) genetic analysis may not be informative for some families or family members.
(e) even under the best conditions, current technology of this type is not perfect and could lead to incorrect results.
(f) where biological material is used for research purposes, there may be no direct benefit to me.
- I understand that I may withdraw my consent for any aspect of the above at any time without this affecting my future medical care.
- ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY:**

_____ DATE: _____

Patient signature _____ Witnessed consent _____

NOTE - PLEASE INSERT A FAMILY PEDIGREE DRAWING ON THE REVERSE OF THIS FORM

Appendix 2: DNA Consent Form

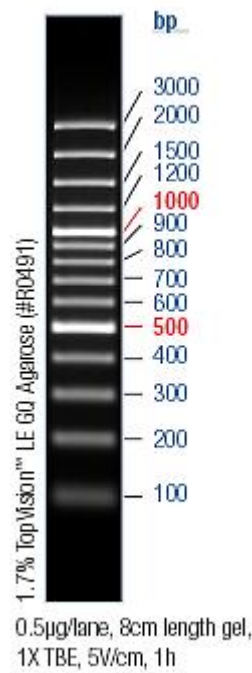
Appendix 2: Gentra Puregene™ DNA Isolation Protocol

- Added 900µl red blood cell lysis solution to microcentrifuge tube
- Added 500µl whole blood and mixed by inverting
- Incubated for one minute at room temperature
- Centrifuged for 20 seconds at 13 000rpm to pellet white blood cells. Discarded supernatant
- Added 300µl cell lysis solution and vortexed
- Incubated at 37°C for ten minutes
- Added 100µl protein precipitation solution and inverted
- Centrifuged for one minute at 13 000rpm
- Added supernatant to clean microcentrifuge tube with 300µl isopropanol
- Mixed by inversion until DNA was visible as threads or clumps
- Centrifuged for one minute at 13 000rpm
- Discarded supernatant
- Added 300µl 70% ethanol and inverted several times
- Centrifuged for one minute 13 000rpm
- Discarded supernatant
- Rehydrated with 50µl DNA hydration solution

Appendix 3: Reagents, Buffers and Solutions

Molecular weight marker- GeneRuler™ 100 bp Plus DNA Ladder (Fermentas Life Sciences, Hanover, USA)

- 25µl stock 100bp DNA ladder solution
- 225µl dH₂O
- 250µl loading dye



(Adapted from:
<http://www.fermentas.com/catalog/electrophoresis/generulers.htm>)

10x TBE Buffer (stock)

- 216g (0.89M) Tris (B&M Scientific cc, Cape Town, SA)
- 110g (0.89M) Boric Acid (MP Biomedicals Inc., USA)
- 14.8g (0.04M) EDTA (BDH Electron® Laboratory Supplies, UK)
- Made up to 2L with Sabax dH₂O (Adcock Ingram, Johannesburg, SA)

1x TBE Buffer (working stock)

- 1:10 dilution was made with stock TBE buffer with dH₂O

Loading Buffer(Fermentas Life Sciences, Hanover, USA)

1% Agarose Gel (100ml)

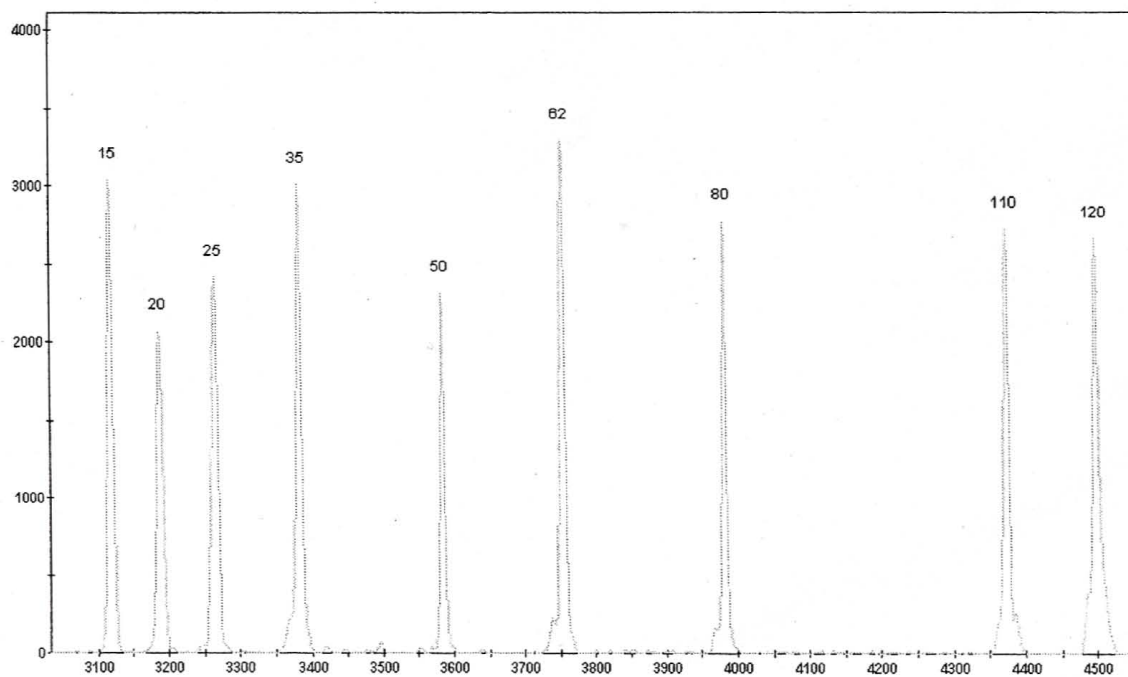
- 1g agarose (Whitehead Scientific, SA)
- 100ml 1X TBE (working stock)
- 6µl (5ng/ µl) EtBr (Sigma, England)

2% Agarose gel (100ml)

- 2g agarose (Whitehead Scientific, SA)
- 100ml 1X TBE (working stock)
- 6µl (5ng/ µl) EtBr (Sigma, England)

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Appendix 4: GeneScan -120 Liz size standard



Appendix 5: Ethanol Precipitation

- The sequencing reaction was transferred to a 1.5ml eppendorf tube
- Added 50µl absolute ethanol and 2µl sodium acetate (3mM, pH5.5)
- Solution was mixed and left at -20 °C for more than 1 hour
- Spun @ 10 000rpm for 10 minutes
- Supernatant was discarded
- Added 50µl of 70% ethanol, vortexed
- Spun @ 10 000rpm for 10 minutes
- Supernatant was discarded
- Air dried for approximately 1 hour to evaporate the ethanol
- The DNA was resuspended in 10µl dH₂O

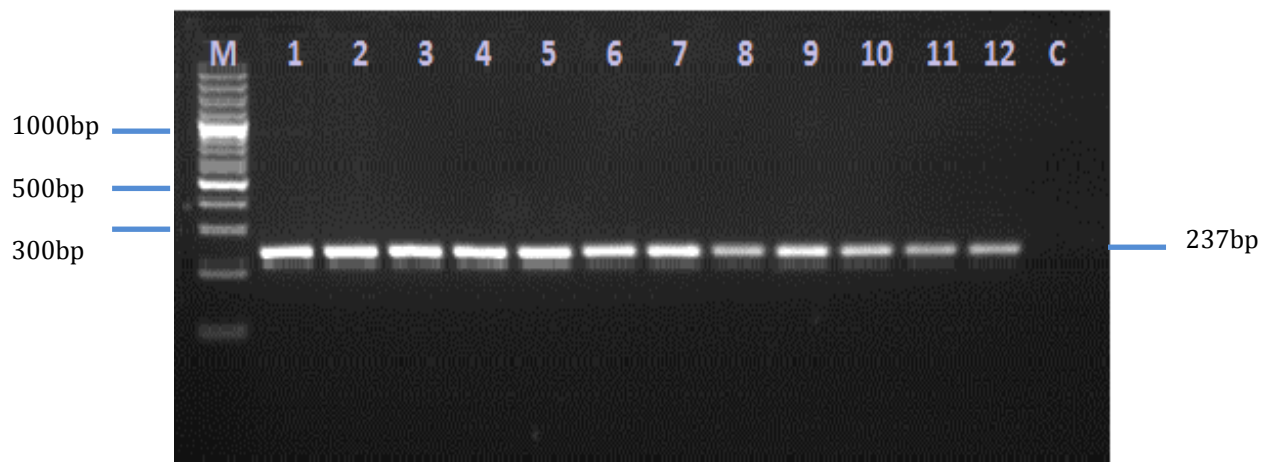
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Appendix 6: Annealing Temperatures (Ta) for Temperature Gradient Reactions

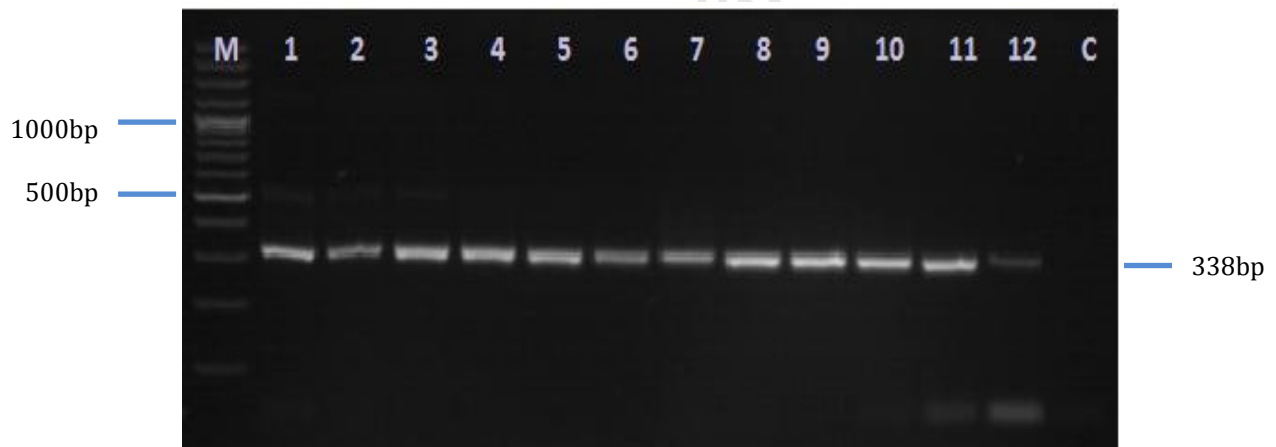
| Lane | Temperature (°C) |
|------|------------------|
| 1 | 50.0 |
| 2 | 50.7 |
| 3 | 51.3 |
| 4 | 52.9 |
| 5 | 54.7 |
| 6 | 56.4 |
| 7 | 57.2 |
| 8 | 58.7 |
| 9 | 60.1 |
| 10 | 61.3 |
| 11 | 61.6 |
| 12 | 62.0 |

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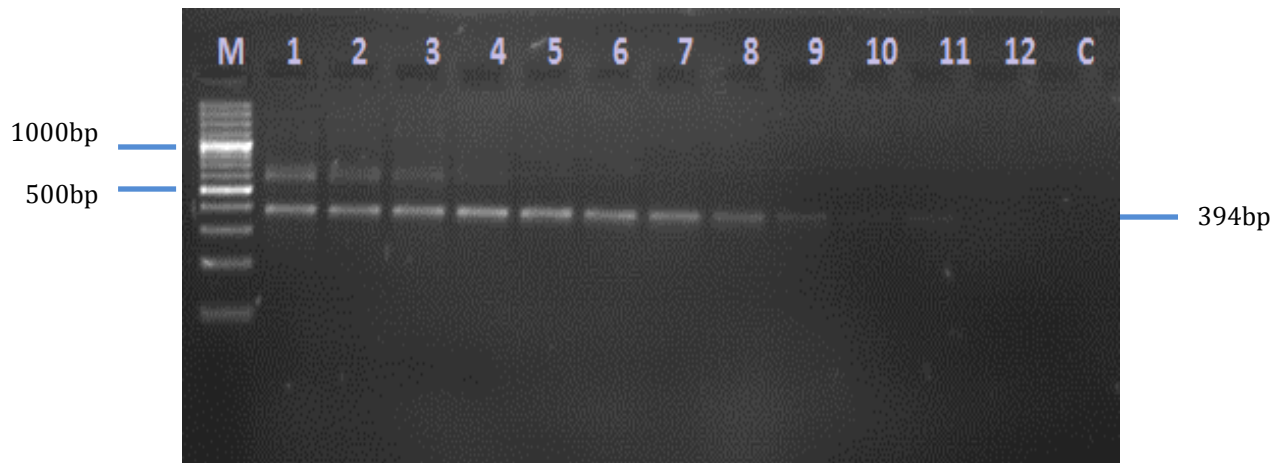
Appendix 7: Additional Temperature Gradient Gel Pictures for Candidate Gene SNPs



ARNTL (237bp) Temperature Gradient. Lane M: Molecular Weight Marker (MWM). Lanes 1-12: PCR products amplified at varying annealing temperatures as specified in Table 1. Lane C: water control.



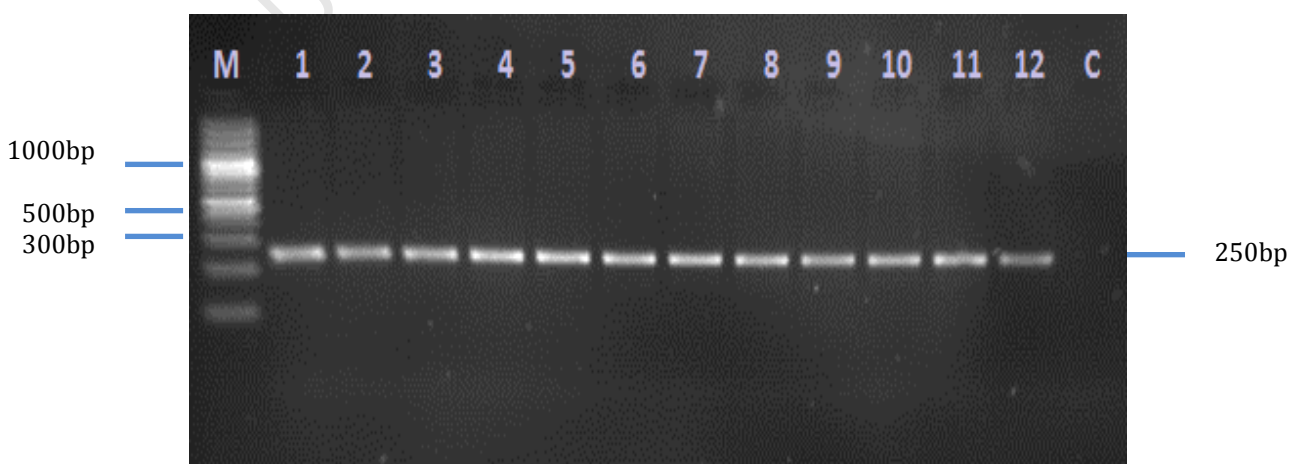
BDNF (338bp) Temperature Gradient. Lane M: Molecular Weight Marker (MWM). Lanes 1-12: PCR products amplified at varying annealing temperatures as specified in Table 1. Lane C: water control.



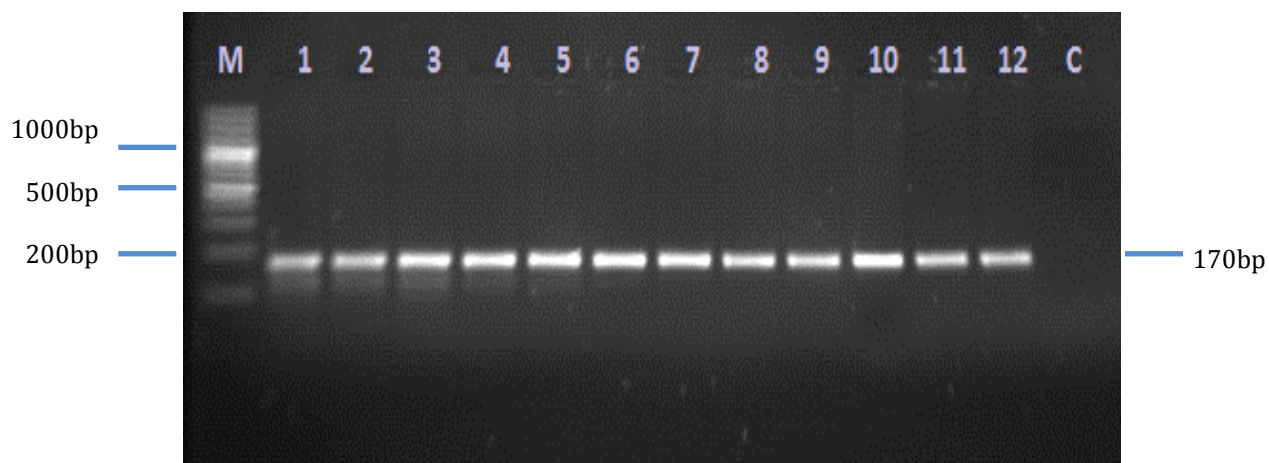
***ALDH1A1* (394bp) Temperature Gradient.** Lane M: Molecular Weight Marker (MWM). Lanes 1-12: PCR products amplified at varying annealing temperatures as specified in Table 1. Lane C: water control.



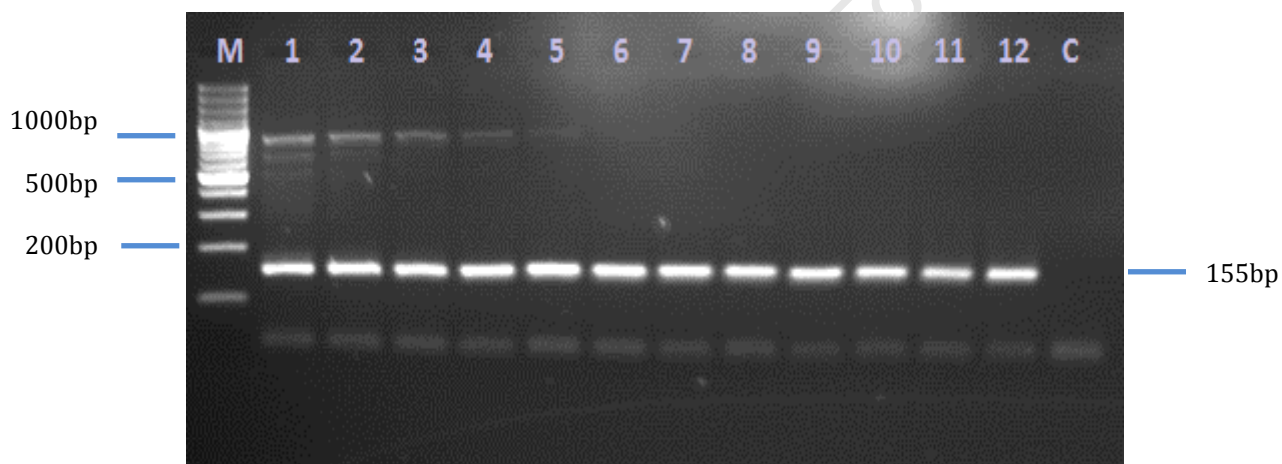
***A2BP1(A)* (192bp) Temperature Gradient.** Lane M: Molecular Weight Marker (MWM). Lanes 1-12: PCR products amplified at varying annealing temperatures as specified in Table 1. Lane C: water control.



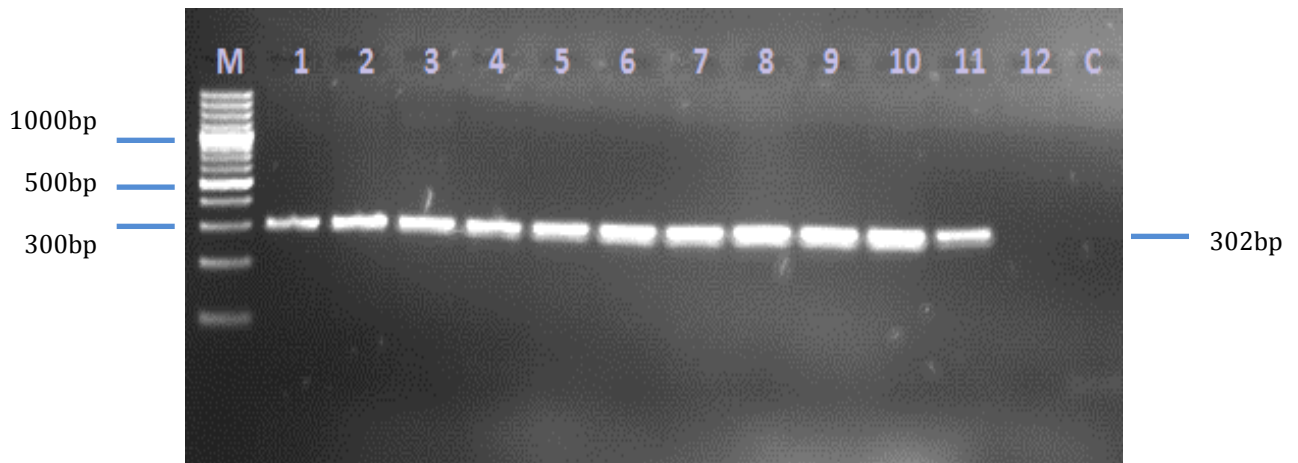
***A2BP1(B)* (250bp) Temperature Gradient.** Lane M: Molecular Weight Marker (MWM). Lanes 1-12: PCR products amplified at varying annealing temperatures as specified in Table 1. Lane C: water control.



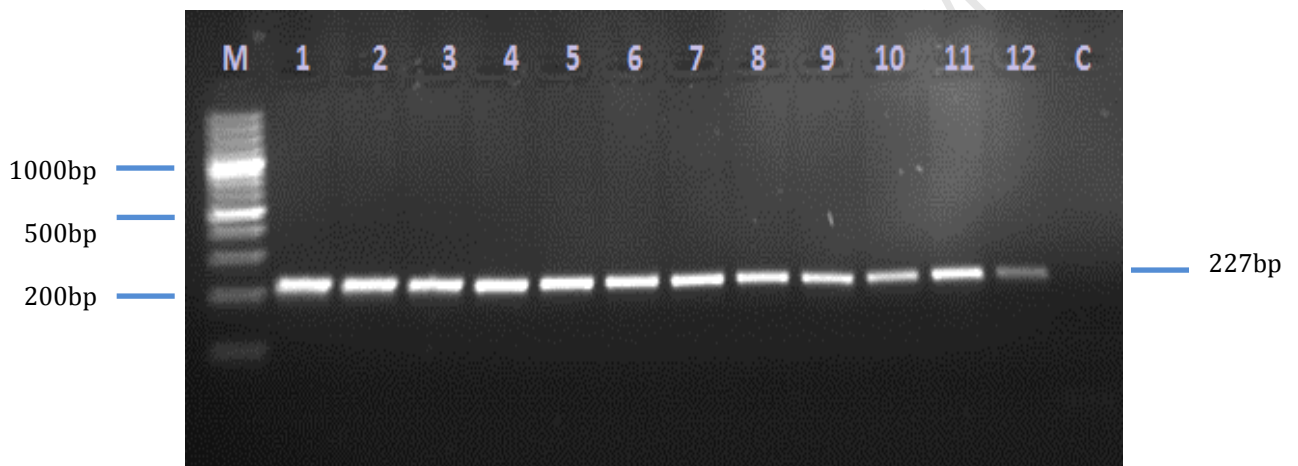
MBP (170bp) Temperature Gradient. Lane M: Molecular Weight Marker (MWM). Lanes 1-12: PCR products amplified at varying annealing temperatures as specified in Table 1. Lane C: water control.



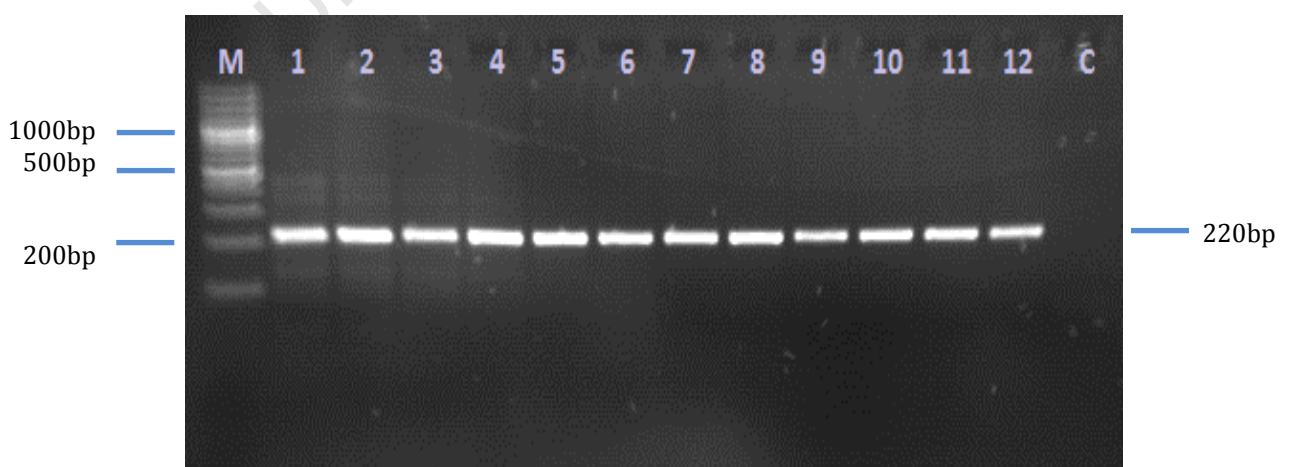
AK3L1 (155bp) Temperature Gradient. Lane M: Molecular Weight Marker (MWM). Lanes 1-12: PCR products amplified at varying annealing temperatures as specified in Table 1. Lane C: water control.



***GSK3β* (302bp) Temperature Gradient.** Lane M: Molecular Weight Marker (MWM). Lanes 1-12: PCR products amplified at varying annealing temperatures as specified in Table 1. Lane C: water control.



***ANK3* (227bp) Temperature Gradient.** Lane M: Molecular Weight Marker (MWM). Lanes 1-12: PCR products amplified at varying annealing temperatures as specified in Table 1. Lane C: water control.



***CACNA1C* (220bp) Temperature Gradient.** Lane M: Molecular Weight Marker (MWM). Lanes 1-12: PCR products amplified at varying annealing temperatures as specified in Table 1. Lane C: water control.

Appendix 8: Psychosis and relapse in bipolar disorder are related to GRM3, DAOA, and GRIN2B genotype. Dalvie et al. [2010].

Psychosis and relapse in bipolar disorder are related to GRM3, DAOA, and GRIN2B genotype

S Dalvie¹, N Horn², C Nossek¹, L van der Merwe^{3,4}, DJ Stein², R Ramesar¹

¹Division of Human Genetics, MRC/UCT Human Genetics Research Unit, Faculty of Health Sciences, University of Cape Town Medical School, Observatory, Cape Town, South Africa

²Department of Psychiatry and Mental Health, University of Cape Town Medical School, Observatory, Cape Town, South Africa

³Biostatistics Unit, Medical Research Council, Cape Town, South Africa

⁴Department of Statistics, University of Western Cape, Bellville, South Africa

Abstract

Objective: Dysfunction in glutamate signalling is thought to play a role in the pathophysiology of bipolar disorder (BD). There is evidence of associations between single nucleotide polymorphisms (SNPs) in GRM3, GRIN2B, and DAOA genes and the diagnosis of BD. In this pilot study, we investigated the frequency of SNP variants in these 3 genes within South African population groups, and assessed interactions between genes and phenotypes of BD disease severity. **Method:** Multiplex SNaPshot™ PCR was used to genotype 191 case and 188 control samples. Cases comprised of 191 individuals in a South African cohort of mixed ancestry and Caucasians, with BD Type 1. Phenotypes of BD disease severity were: age of onset, number of illness episodes, number of hospitalisations for depression or mania and history of psychotic symptoms. **Results:** There were no significant difference in SNP allele frequencies between cases and controls. In the case-only analysis, the GRM3 rs6465084 heterozygote was associated with a 4-fold increased risk of lifetime history of psychotic symptoms, and the specific variants within the gene pair, DAOA and GRIN2B, had a significant interaction with the number of hospitalisations for mania, with lowest admission rates associated with both pairs of ancestral alleles. **Conclusion:** In BD, variations in glutamatergic genes may influence phenotypes related to the severity of illness. Speculatively, newly derived genes associated with various evolutionary advantages, may also increase the risk for more severe BD. These preliminary findings deserve validation in a larger cohort.

Key Words: Manic-Depressive Psychosis; Glutamate; GRIN2B receptor; mGluR3; G72 protein; Human

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Introduction

The lifetime prevalence of bipolar disorder Type 1 (BD) is approximately 1% in the US and Europe, and likely equally high in Southern Africa.^{1,2} BD is associated with a high level of mortality (between 10-20% of individuals affected with BD successfully commit suicide) and morbidity (BD is estimated as one of the top ten causes of disability worldwide).³⁻⁵ An earlier age of disease onset, increased number of episodes and hospitalisations for mania and depression, and the presence of psychotic symptoms are markers of a more severe disease phenotype.⁶⁻⁸ Previous research has not examined associations

between phenotype severity and glutamate signalling in BD, although variants within brain derived neurotrophic factor (BDNF) may be associated with indices of severity in BD.⁹⁻¹¹

Glutamate signalling is involved in brain development and synaptic plasticity, both of which are modified in individuals affected with BD, and have been implicated in its aetiology.^{12,13} A range of psychotropics involved in the treatment of BD alter glutamatergic function; for example, lamotrigine inhibits the release of glutamate and lithium affects glutamate receptor function.¹⁴ It has also been observed that variation in the glutamate receptor encoding gene, GRIK4 (rs1954787) makes subjects with depressive episodes more likely to respond to citalopram.¹⁵ Fallin et al. found that the glutamatergic genes most likely to be associated with BD rather than schizophrenia (SZ) were GRM3 and GRIN2B, and replicated previous findings of strong associations between DAOA and BD diagnosis.¹⁶

Glutamate receptor, metabotropic 3 (GRM3) encodes subunit 3 of the Group II metabotropic glutamate receptors

Correspondence

Professor Rajkumar Ramesar
Division of Human Genetics, Faculty of Health Sciences,
University of Cape Town Medical School, Observatory, Cape Town,
South Africa, 7925
email: Raj.Ramesar@uct.ac.za

(mGluR). In glutamate receptor, metabotropic 2 (GRM2) and GRM3 knockout mice, Lyon et al. showed a compensatory up-regulation of remaining group II mGluRs and the N-methyl D-aspartate (NMDA) receptor subunit 2A (NR2A) expression, and down-regulation of glutamate transporter expression.¹⁷ Post-mortem studies have suggest altered GRM3 expression in BD.¹⁸ Crespi et al. identified GRM3 as one of 14 genes with significant evidence of selection in human populations.¹⁹ GRM3 modulates glutamate neurotransmission by regulating the excitatory amino acid transporter 2 (EEAT2). Egan et al. have demonstrated that decreased levels of EEAT2 are associated with a GRM3 SNP (rs6465084), which has a weak association with SZ.²⁰ Egan et al. have also demonstrated that individuals who are homozygous A for GRM3 rs6465084 have lower levels of N-acetylaspartate, an indirect indicator of synaptic function and glutamate levels and suggests that GRM3 rs6465084 has an effect on GRM3 transcript expression.²⁰

Inotropic glutamate receptor, N-methyl D-aspartate 2B (GRIN2B) encodes the NR2B subunit of the NMDA receptor. Mice with enhanced NR2B function show supernormal learning and memory function, while BD sufferers consistently show impairment in working memory.²¹ Associations have been observed between several polymorphisms in the GRIN2B gene and the 12p13.1-p12.3 region (containing the GRIN2B gene) and BD.^{16,22,23} Miyatake et al. have observed that the T allele of the GRIN2B rs1019385 SNP results in increased luciferase transporter activity in the presence of nerve growth factor (NGF).²⁴ The presence of a G allele had no effect on reporter activity, suggesting that GRIN2B rs1019385 may have a role in transcriptional control of the GRIN2B transcript.²⁴

The G72 gene product, D-amino-acid oxidase activator (DAOA), activates the peroxisomal protein D-amino-acid oxidase (DAO).²⁵ This protein (G30) degrades D-serine, which acts, similar to glycine, as a coactivator on the "glycine binding site" of the glutamatergic NMDA receptor. Hashimoto et al. showed that the percentage of D-serine in the total serine in the cerebrospinal fluid of drug naïve SZ patients was significantly lower than that of control samples.²⁶ Genetic variants of G72/G30 have been found to be associated with BD and SZ.^{16,27-32} In some populations, the at-risk haplotypes are shared between SZ and BD.³¹ The pathogenic mutations have not yet been identified but might be located in the vicinity of the G72/G30 gene complex or in the regulatory region.³⁰

Method

Subjects

The cohort comprised of two groups: a group of BD Type 1 affected individuals (cases) and the background population group (controls). Blood samples were previously obtained, with the appropriate written informed consent. This study was approved by the University of Cape Town Health Science Faculty Research Ethics Committee (081/96) and is in accordance with the guidelines of the Helsinki Declaration of 2000.

The Division of Human Genetics has a BD database consisting of 883 individuals comprising 185 families. For the purposes of this study, the cases consisted of 191 individuals specifically diagnosed with BD Type 1 with the Structured Clinical Interview for the DSM-IV (SCID). This group comprised of 87 (46%) males and 104 (54%) females of ages ranging from 22 to 91 years. Subjects included 103 (54%)

individuals of mixed ancestry and 88 (46%) Caucasian individuals. The cases included 95 individuals from 36 families that were related to at least one other case. The age of onset, number of depressive episodes, number of hospitalisations due to mania and depression of each of the cases were noted, as well as history of psychotic symptoms. The control group consisted of 188 unrelated individuals not diagnosed with any psychiatric disorder, and similar to the cases in terms of age, gender and ethnicity.

DNA Analysis

The following SNPs were chosen for genotyping based on previous association to either BD or SZ: GRM3 rs6465084 (intronic), GRIN2B rs1019385 (5' upstream), and DAOA rs701567 (intronic).¹⁻⁴ Gene sequences were obtained from the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) and Ensembl (<http://www.ensembl.org/index.html>) databases and the SNP locations were determined. DNA concentration and integrity was determined by spectrophotometric quantification and agarose gel electrophoresis, respectively. Polymerase chain reaction (PCR) was performed on genomic DNA, according to standard procedures, for each of the SNPs in the candidate genes. The polymorphisms were genotyped using the SNaPshot™ multiplex ready reaction mix (Applied Biosystems, Warrington, UK) and the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, CA, USA). Genotyping results were validated by cycle sequencing using BigDye Terminator Mix (Applied Biosystems, CA, USA) and the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, CA, USA).

Statistical Analysis

For the case-control analysis, one individual from each family was randomly selected, leaving 132 unrelated cases. For this analysis, logistic regression was used.

The case-only analyses involved all 191 cases, which were adjusted for relatedness. We used generalised linear models for all the association tests. For age of onset, we used a normal family with an identity link; for each of the three outcomes: (i) number of depressive episodes, (ii) hospitalisations due to mania and (iii) hospitalisations due to depression, we used the Poisson family with a log link and for dichotomous history of psychosis, a binomial family with logit link. There were only 4 GRM3 rs6465084 homozygous G cases. We therefore grouped them with the heterozygotes for the case-only analyses. Genotypes were coded as categories for the single-SNP analyses, and numerical (number of minor alleles) for testing interactions.

All of the six outcomes analysed (case-control, age of onset, number of depressive episodes, hospitalisations due to mania, hospitalisations due to depression, and history of psychotic events), were tested for an interaction between the three SNPs. When this was not significant, we tested all pairwise interactions between SNPs and where those were not significant, we tested the individual SNPs (main effects). All analyses were adjusted for gender, ethnicity and duration of disease as fixed effects and family-membership as random effect.

Statistical analyses were done with R, a language and environment for statistical computing and graphics, and R packages nlme and MASS (<http://www.R-project.org>).

Results

Case-control analysis (132 cases; 188 controls)

The differences in the genotype and allele frequencies between cases and controls were not significant. Neither were any SNP-interactions on case-control status significant (results not shown). The allele frequencies for each of the SNPs obtained from the cases and controls in the current study, are displayed in Table I.

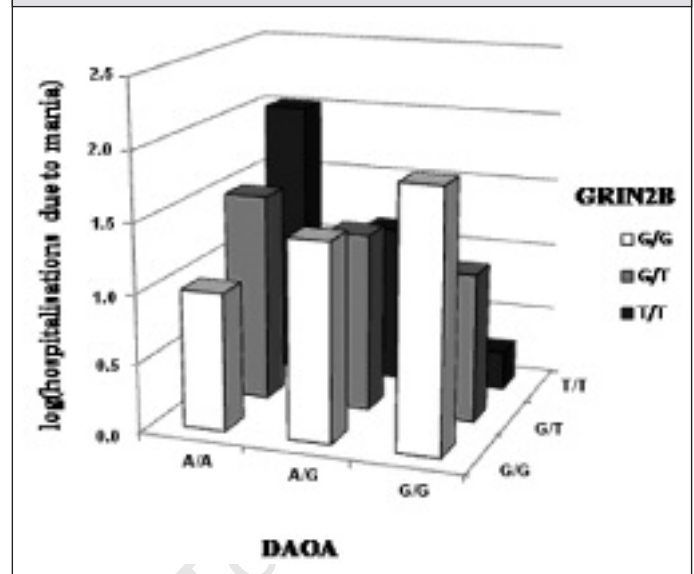
Case-only analysis (n=191)

All the results presented here were adjusted for gender, ethnic group, duration of disease and family-membership.

We did not find any significant association between the SNPs GRM3 rs6465084, GRIN2B rs1019385, DAOA rs701567 and main effects or interactions on age of onset (n=124), number of depressive episodes (n=130), or number of hospitalisations for depression (n=171). However, a significant association between GRM3 rs6465084 and history of psychosis (63% with a history and 37% without history of psychosis) was found. The GRM3 rs6465084 G-allele was found to increase the odds of having had a psychotic episode (OR=3.9; p=0.004) considerably. Even after adjusting for family-relatedness only, a significant association (OR=2.2; p=0.0137) was observed.

Reported number of hospitalisations for mania (n=167) varied between 0 and 11. The interaction between DAOA rs701567 and GRIN2B rs1019385 on number of hospitalisations for mania was significant (p=0.0108), and this interaction was independently significant after including other interactions in the model. The nature of the modelled interaction is illustrated in Figure 1 and can be described as follows: For individuals with the GRIN2B rs1019385 genotype G/G, their expected number of hospitalisations for mania increased with their number of DAOA rs701567 G alleles. For individuals with the GRIN2B rs1019385 genotype G/T, there were smaller decreases in number of hospitalizations and for those individuals with the T/T genotype, each DAOA rs701567 G allele resulted in larger decreases. Individuals who are homozygous A for DAOA rs701567 and homozygous T for GRIN2B rs1019385 had the largest number of hospitalisations, and those who are homozygous G for DAOA rs701567 in combination with being homozygous G for GRIN2B rs1019385, had the second largest. Those with DAOA rs701567 G/G and GRIN2B rs1019385 T/T had the least number of hospitalisations.

Figure 1: A three dimensional bar chart illustrating the modelled interaction of DAOA rs701567 and GRIN2B rs1019385 on the logarithm of the number of hospitalisations for mania.



Discussion

The first set of findings related to SNP frequency showed that all 3 SNPs examined had a similar distribution between Caucasian and mixed ancestry groups, and that BD diagnosis was not associated with any of the SNPs examined. The second set of findings, related to BD severity, was that the non-ancestral GRM3 G allele at rs6465084 carries a 4 times greater risk of developing psychosis in BD subjects, and that alleles at DAOA rs701567 and GRIN2B rs1019385 interact such that the risk of hospital admission is higher with a greater number of non-ancestral alleles.

This is the first time that allele frequencies in the candidate genes studies here have been described for the mixed ancestry population of South Africa. SNP frequencies were broadly similar to reported frequencies for Caucasian groups described elsewhere (NCBI <http://www.ncbi.nlm.nih.gov>).²² No difference in frequency was detected for the ancestral allele for GRM3 rs6465084 and of GRIN2B rs1019385 in individuals of both Caucasian and mixed ancestry. The A/G alleles of DAOA rs701567 were found to occur at almost equal

Table I: SNP Allele Frequencies For Each of the Candidate Genes

| Gene | SNP | Ethnic Group | No. of Cases | No. of Controls | Ancestral Allele Frequency Cases | Ancestral Allele Frequency Controls | p-value |
|--------|-----------|---------------------------|--------------|-----------------|----------------------------------|-------------------------------------|---------|
| GRM3 | rs6465084 | Caucasian, Mixed Ancestry | 132 | 188 | 0.75 | 0.77 | 0.5953 |
| | | Caucasian only | 69 | 89 | 0.73 | 0.78 | 0.3054 |
| | | Mixed Ancestry only | 63 | 99 | 0.77 | 0.76 | 0.7887 |
| GRIN2B | rs1019385 | Caucasian, Mixed Ancestry | 132 | 188 | 0.35 | 0.34 | 0.8268 |
| | | Caucasian only | 69 | 89 | 0.41 | 0.40 | 0.9181 |
| | | Mixed Ancestry only | 63 | 99 | 0.29 | 0.28 | 0.8304 |
| DAOA | rs701567 | Caucasian, Mixed Ancestry | 132 | 188 | 0.43 | 0.48 | 0.2000 |
| | | Caucasian only | 69 | 89 | 0.41 | 0.41 | 0.8961 |
| | | Mixed Ancestry only | 63 | 99 | 0.45 | 0.56 | 0.0725 |

frequencies in both population groups.

We did not demonstrate that any of the putative risk alleles were associated with BD diagnosis. This is consistent with the lack of consistently reproducible associations between individual genes and BD diagnosis even in large data sets.³³ There is increasing evidence that BD is a disorder where many genes of small effect contribute to risk of developing the disorder.²⁰

In this study, we observed that the G allele at SNP rs6465084 GRM3 carries a 4 times greater risk of developing psychosis in subjects with BD. This is unexpected as the A allele in this SNP has been weakly associated with SZ diagnosis— and might be expected to be a risk factor for psychosis in BD.²⁰ This finding by Egan et al., however, was not replicated by others.^{34,35} Egan et al. also reported an association between the A allele, impaired list learning and verbal fluency, and low prefrontal levels of glutamate transporter EEAT2 in normal subjects and people with SZ.²⁰ It is possible to speculate that the A allele confers a risk of a dysexecutive syndrome typical of certain types of SZ, while the G allele is associated with normal or enhanced executive function, consistent with the finding by Diller et al. that GRM3 is a gene which has evolved favourable new (derived) alleles.³⁶ The G allele may, however, together with other risk factors, be associated with more severe mania. Crespi et al. identified GRM3 as a gene with significant evidence of selection in human populations, and although not enough is known about this SNP in global populations or its function in normal or disease states, it may be one which differentiates Kraepelinian “dementia praecox” from “manic depression”.^{19,37}

The rs701567 SNP in DAOA was not associated with BD diagnosis in this study, contrary to the finding of Fallin et al.¹⁶ The G allele was not associated with any phenotype or severity measures; however there was a significant gene-gene interaction with GRIN2B rs1019385 SNP on the number of hospitalisations for mania. In the South African context, hospitalisation is most often required for emergency treatment of severe episodes. The combination of both ancestral alleles (G for DAOA and T for GRIN2B) conferred the lowest risk of admission while homozygotes with a derived allele/ancestral allele combination had the highest risk for hospitalisation. Homozygotes for both derived alleles had a relatively low risk of hospitalisation. DAOA activates DAO which determines synaptic glutamate and serine levels, while NMDA receptor function varies according to glutamate levels and GRIN2B genotype. Both DAOA and GRIN2B form part of the phosphoinositide-3 kinase/ AKT-signalling network (PI3K/AKT), involved in the regulation of cell proliferation, growth, development and apoptosis.^{38,39} We may speculate that heterozygotes have intermediate glutamate function and homozygotes an inflexible regulatory capacity for glutamate load or deficiency states, compromising normal neural development and plasticity.

A major limitation of this study was the relatively small sample size. To avoid this, recent studies have focussed on obtaining large consortia for genetic association tests. An example of this would be the Wellcome Trust Case Control Consortium which investigated the genetic basis of seven common diseases, including BD, using 2000 affected individuals for each disorder and 3000 shared controls. The outcome of this study was that modest genetic effects were able to be detected using larger sample sizes.⁴⁰

The most constraining limitation of the current study is the lack of information about the in vivo and in vitro function of the specific SNPs examined. Future work is needed to determine the functionality of glutamate SNPs in terms of gene expression and physiological correlates, and whether these link to phenotypes for BD. The interaction between DAOA rs701567 and GRIN2B rs1019385 may be indirect or direct and if direct could be examined with a protein interaction assay such as yeast-2-hybrid screen. Other SNPs in the genes we examined and other genes involved in glutamate metabolism including glutamate receptor, ionotropic, N-methyl D-aspartate 1 (GRIN1), glutamate receptor, metabotropic 1 (GRM1) and glutamate receptor, ionotropic, kainate 1 (GRIK1) and their interactions with BD phenotypes should also be explored. Another consideration for future work would be to have greater detail regarding cognitive phenotype. This could be helpful to test (post-hoc) hypotheses related to cognitive function in, for example, controls with GRM3 rs6465084 derived allele homozygotes.

Conclusion

To our knowledge this is the first study investigating these 3 glutamatergic SNPs and their interactions with BD diagnosis and phenotype. The study provided preliminary evidence that ancestral alleles in the 3 SNPs examined have a protective effect by decreasing the chances of having more severe phenotypes of BD, specifically psychosis and repeat hospital admission. It is possible to speculate that newly derived genes associated with various evolutionary advantages (including perhaps cognitive advantages), may also increase the risk for more severe bipolar disorder. However, findings from this pilot study warrant further research and validation.

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