ELUCIDATING THE GENETIC AETIOLOGY OF BIPOLAR DISORDER

BY

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PLAGIARISM DECLARATION

I, Hannah-Ruth Engelbrecht, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. The referencing style used in this thesis is that of the *Journal of Affective Disorders*.

Signature: Signed by candidate

Date: 31 May 2018

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I think I'm crazy

I also think I like being this way

I don't care what others think or say they don't exist

physically I'll never be detached from them but they'll never tread my soul

I'm so fragile fickle small

yet I'm strong

even if they [throw] this vase on the ground shatter [it] and step the [glass] into the soil

it will not disintegrate

the pieces still exist

I know in time you'll

find them

pick them up

and keep them.

Maybe you'll even paste

them together

and put flowers in.

Sara Michéle Redelinghuys van Dyk, the artist known as Misjke

From *A Life Interrupted: The Story of Misjke*, by Stephanie Redelinghuys

(Redelinghuys, 2008)

NOTE TO THE READER

Dear Reader,

Please note that this thesis makes use of the *Journal of Affective Disorders* referencing style.

With regard to the inclusion of URLs: when referencing a URL in-text, I provide the URL in full in square brackets to allow those readers curious to seek further information the ability to follow the links immediately, rather than searching for a list of web-based references at the end of the document.

Numbers in text are referred to as one to ten, and numbers either smaller or larger than this are described by Hindu-Arabic symbols (eg. 194 or 0.1). Numbers between one and ten that are quoted along with a decimal point are also described by Hindu-Arabic symbols (eg. 5.5%).

Abbreviated gene symbols are italicised according to convention, while abbreviated protein symbols are not italicised. Full gene names and pathway names in text are italicised to simplify visual identification of the name in the body of text. Abbreviations are not included in section headings nor in figure or table headings to maintain the aesthetic flow of the document.

Best,

Hannah-Ruth Engelbrecht

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ABBREVIATIONS

ADHD	Attention Deficit Hyperactivity Disorder
ASW	African ancestry in SW USA
ANK2	Ankyrin 2
ANK3	Ankryin 3/Ankyrin G
APA	American Psychiatric Association
AQP8	Aquaporin 8
ASD	Autism Spectrum Disorder
BAM	Binary Alignment Map
BBMRI-NL	Netherlands Biobanking and Biomolecular Research Infrastructure
BBMRI-NL	Netherlands Biobanking and Biomolecular Research Infrastructure
BD (BD-I, BD-II, and BD-NOS)	Bipolar Disorder (Types I, II, and Not Otherwise Specified)
BDNF	Brain-Derived Neurotrophic Factor
Bed (file)	Binary Pedigree (File)
bp	Base Pairs
BQSR	Base Quality Score Recalibration
BSD	Bipolar Spectrum Disorder
BWA	Burrows-Wheeler Aligner
C	Caucasian
CACNA1C	Calcium Voltage-Gated Channel Subunit Alpha 1 C
CACNA1D	Calcium Voltage-Gated Channel Subunit Alpha 1 D
CAPN10	Calpain 10
CDX	Xishuangbanna in China
CBIO	Computational Biology Group

CI	Confidence Interval
CPGR	Centre for Proteomic and Genomic Research
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4
DALY	Disability Adjusted Life Year
DAVID	Database for Annotation, Visualization and Integrated Discovery
DDN	Dendrin
DGKH	Diacylglycerol Kinase Eta
DIGS	Diagnostic Interview for Genetic Studies
DNA	Deoxyribonucleic Acid
DPY19L3	Dpy-19 like C-mannosyltransferase 3
DSM	Diagnostic and Statistical Manual Of Mental Disorders
GBR	England and Scotland
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
ESN	Esan in Nigeria
EXOC3	Exocyst Complex Component 3
FBAT	Family-Based Association Test
FIN	Finnish
GATK	Genome Analysis ToolKit
GoNL	Genome of the Netherlands
GWAS(s)	Genome-Wide Association Study (Studies)
HGFAC	Hepatocyte Growth Factor Activator
HLA-G	Major Histocompatibility Complex Class I-G
HWE	Hardy-Weinberg Equilibrium
IBS	Iberians in Spain

ICD	International Classification of Diseases
ICTS	Information & Communication Technology Services
JPT	Japanese in Tokyo
KEGG	Kyoto Encyclopedia of Genes and Genomes
KIAA1009	Centrosomal Protein 162
KOBAS	KEGG Orthology Based Annotation System
LD	Linkage Disequilibrium
LWK	Luhya in Kenya
MA	Mixed Ancestry
MAD1L1	Mitotic Arrest Deficient 1-Like Protein 1
MAF	Minor Allele Frequency
MDD	Major Depressive Disorder
MDE	Major Depressive Episode
MIR2113-POU3F2	MicroRNA 2113- POU Class 3 Homeobox 2
µl	Microlitres
ml	Millilitres
MP1	Max Planck 1
mtDNA	Mitochondrial DNA
MYO5B	Myosin 5B
NCL	Nucleolin
NCKAP5	NCK associated protein 5
NGS	Next Generation Sequencing
NHS	National Health Service
NOS1	Nitric Oxide Synthase 1

NTRK2	Neurotrophic receptor tyrosine kinase 2
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
Ped (file)	Pedigree (file)
PGC	Psychiatric Genomics Consortium
PIK3C2A	Phosphatidylinositol-4-Phosphate 3-Kinase Catalytic Subunit Type 2 Alpha
PITPNM2	Phosphatidylinositol Transfer Protein Membrane Associated 2
PRSS55	Protease Serine 55
PUR	Puerto Ricans from Puerto Rico
RBC	Red Blood Cell
RDoc	Research Domain Criteria
SAM	Sequencing Alignment Map
SCID	Structured Clinical Interview for the DSM-IV
SCZ	Schizophrenia
SLC17A3	Solute Carrier Family 17 Member 3
SNP	Single Nucleotide Polymorphism
SNVs	Single Nucleotide Variants
CHS	Southern Han Chinese
SRA1	Steroid Receptor RNA Activator 1
STR	Short Tandem Repeat
THSD7A	Thrombospondin 7A
TMEM222	Transmembrane Protein 222
TRANK1	Tetratricopeptide Repeat and Ankyrin Repeat Containing 1

TSPAN8	Tetraspanin 8
UCT	University of Cape Town
VCF	Variant Call File
VQSR	Variant Quality Score Recalibration
WBCs	White Blood Cells
WebGestalt	WEB-based Gene SeT AnaLysis Toolkit
WES	Whole-Exome Sequencing
WGS	Whole-Genome Sequencing
WHO	World Health Organization
WWC1	WW and C2 Domain Containing 1
YLD	Years Lived with Disability
YLL	Years Lost to Premature Mortality
YRI	Yoruba in Nigeria

ABSTRACT

Introduction: Bipolar disorder (BD) is a debilitating mood disorder with substantial genetic contributions. However, while the existence of its heritability is well-established, the precise genetic components and mechanisms of BD remain unknown. This is a pilot study aimed at optimising the use of small-scale next generation sequencing (NGS) of family members affected with BD and extending the finding of variants to larger scale association studies, and an attempt at replicating associations from a large genome-wide association study.

Methods and Materials: This thesis describes the pathway analysis of whole-genome sequencing (WGS) data from four Afrikaner individuals to identify candidate variants for genotyping in a Family-Based Association Test (FBAT) of 621 individuals of Caucasian and Mixed Ancestry families from South Africa. This was followed by whole-exome sequencing (WES) of eight members of an Afrikaner family to identify rare, coding variation. These two approaches were used to identify both common and rare variation which may be involved in BD.

Results: FBAT indicated that variants in the genes *ACTN2* (rs4659702) and *ANK3* (rs10994318) are associated with BD in a combined group of both Mixed Ancestry and Caucasian individuals, $p = 0.0339$ and 0.0443, respectively. Furthermore, this study identified a variant in *ACTN2* (rs11355106) which was associated with a broad psychiatric phenotype in Mixed Ancestry families ($p = 0.0083$). WES revealed 168 exomic variants that were shared by five affected members of the family, one of which was rs142375896, a rare and potentially damaging missense variant in *SLC26A9*.

Conclusions: Pathway analysis of both WGS and WES data implicated that the burden of variation in affected individuals lies in regulatory networks, including the *regulation of the actin cytoskeleton* and *circadian entrainment* pathways. The association of *ANK3* (rs10994318) with BD in a European-ancestry cohort was replicated in a South African cohort comprising of Caucasian and Mixed Ancestry individuals, indicating that some risk variants for the disorder could be shared across populations. This thesis confirms the validity of relatively small-scale family-based studies for the study of complex disorders.

Artwork reprinted with the permission of the artist, Hanna Barczyk

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION TO BIPOLAR DISORDER

The co-manifestation of mania and melancholy has long been a source of fascination and discussion amongst biologists of many fields. Colloquially known as manic-depressive illness, bipolar disorder (BD) is the diagnosis given to individuals who present with episodic cycles of mania/hypomania and depression, interspersed with periods of euthymia (normal behaviour) (American Psychiatric Association, 2013). As shall be described in this chapter, BD is a debilitating condition that has a substantial effect on an individual's life; in a significant number of cases, sufferers commit suicide (Schaffer et al., 2015). The prevalence of BD also incurs great financial cost to many countries. In South Africa, a country with a poor record of care for mental health conditions, it is imperative to identify the biological mechanisms involved in BD in order to combat the stigma and discrimination surrounding such disorders, and to develop effective diagnostic and treatment capacity.

The *Diagnostic and Statistical Manual of Mental Disorders* (DSM) is the American Psychiatric Association's (APA's) clinical guide for the diagnosis of BD. In order to overcome some of the limitations imposed by the DSM classification of distinct disorders (Schildkrout, 2016), the most recent edition of this guide, the DSM-5™, recognises that BD must be viewed as "a bridge between the two diagnostic classes [schizophrenia spectrum, and other psychotic and depressive disorders] in terms of symptomatology, family history, and genetics" (American Psychiatric Association, p.p. 123, 2013), and indeed BD shares phenotypic and genetic features with other psychiatric disorders, such as schizophrenia (SCZ), and major depressive disorder (MDD) (American Psychiatric Association, 2013; Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Forstner et al., 2017). Manic episodes in BD are characterised by abnormally elevated mood, irritability, decreased need for sleep, and involvement in potentially dangerous or harmful behaviour. These symptoms must be present for most of every day for a minimum of one week, while any duration less than seven days, but more than four, constitutes a hypomanic episode. A depressive episode is characterised by a depressed mood, reduced interest in daily activities, disturbed sleep (either hypersomnia or insomnia), reduced focus, and possibly suicidal ideation (American Psychiatric Association, 2013). Table 1.1 provides the full symptomatology of these episode types.

The *International Classification of Diseases, 10th edition* (ICD-10) is the World Health Organization's (WHO) contribution to diagnostic literature for a variety of diseases and disorders. While the ICD classification of BD and its symptoms closely resembles those stated in the DSM, the ICD provides limited diagnostic criteria and does not stipulate any time frames for mood episodes (World Health Organization, 2004). Both the DSM and the ICD are written for the benefit of clinicians and researchers alike; however, both documents are flawed in their characterisation of psychiatric diseases as discrete

entities, and they both focus on symptomatology rather than disease pathology (Schildkrout, 2016). These shortcomings notwithstanding, there remains no clear and definitive alternative for the diagnosis of psychiatric diseases at this point in time. Genetic studies have not yet yielded any biomarker for psychiatric disorders, despite investigations of a variety of factors that include gene expression, neuroanatomy, and blood plasma protein levels for a variety of proteins (Sigitova et al., 2017). As such, the National Institute of Mental Health oversaw the formulation of the Research Domain Criteria (RDoC) framework for mental disorders [https://www.nimh.nih.gov/research-priorities/rdoc/index.shtml]. This framework identifies five domains of research which form a construct that aids understanding and research of the complex nature of mental disorders and human behaviour. These domains include negative valence systems, positive valence systems, systems for social processes, arousal/regulatory systems, and cognitive systems. Within each domain of research, psychological concepts that contribute to each domain are investigated using a variety of approaches, including molecular biology, physiology, and self-reporting psychological analysis.

The incidence of BD does not differ between the sexes (Miller, 2006) but it has been reported that depressive episodes are more common in females, while the age of onset of the disorder is earlier in males (Azorin et al., 2013; Nivoli et al., 2011). There are two principal sub-types of BD, BD-I and BD-II, which are seen as the more and less severe forms of the disorder, respectively. This distinction is principally based on whether the affected person experiences mania, in the case of BD-I, or hypomania, in the case of BD-II (American Psychiatric Association, 2013). The distinguishing feature between mania and hypomania is the duration of the episode. Both types of mania share many characteristics such as increased energy, a reduced need for sleep, increased irritability, and increased self-esteem, as illustrated in Table 1.1. However, there are minor differences between BD-I and BD-II phenotypes: depressive episodes are more frequent in BD-II than in BD-I, and the BD-II form of the disease is more subject to the effects of seasonality and rapid cycling, which, as the term suggests, is marked by frequent fluctuations between the manic and depressive phases. It has also been shown that the "irritable" symptom of mania seems more prevalent amongst individuals with BD-I than BD-II, and that BD-II persons may harbour greater suicidal ideation than individuals with BD-I (Baek et al., 2011). Investigations of BD cohorts have indicated that suicide rates in the BD-affected population may be as high as 42% (Slama et al., 2004). A meta-analysis of 3 275 completed suicides by Arsenault-Lapierre et al. (2004) indicated that approximately 88% of these individuals suffered from a mental disorder. Frequently comorbid diagnoses that accompany BD, such as substance abuse and anxiety disorders, may contribute to an individual's suicide risk (Kessler et al., 1999; Nock et al., 2010).

Table 1.1: DSM-5™ symptoms of mood episodes in bipolar disorder

Manic Episode	**Hypomanic Episode**	**Major Depressive Episode***
Abnormally elevated/irritable/expansive mood	Abnormally elevated/irritable/expansive mood	Depressed mood
Increased energy	Increased energy	Reduced interest and/or pleasure in activities
Increased goal-oriented activity	Increased goal-oriented activity	Decreased/increased appetite
Decreased need for sleep	Decreased need for sleep	Persistent fatigue/low energy
Talkative	Talkative	Hypersomnia / insomnia
Increased self-esteem	Increased self-esteem	Thoughts about death/suicidal ideation
Distractibility	Distractibility	Social impairment as a result of symptoms
Indulgence in potentially harmful behaviours	Indulgence in potentially harmful behaviours	The episode is not an effect of drugs/medication
Social/occupational functioning may be impaired		*Five or more of these symptoms that persist for a minimum of 2 weeks
Psychotic features may be present		
Hospitalisation may be required		
The episode is not an effect of drugs/medication		

Table adapted from the American Psychiatric Association, Diagnostic and Statistical Manual-5™, 2013

1.2 THE COSTS OF BIPOLAR DISORDER

1.2.1 THE ECONOMIC COST OF BIPOLAR DISORDER

BD has a considerable negative national economic impact globally. It has been reported that the UK incurred an annual cost of £2.10 billion, arising indirectly from the estimated 297 000 people with BD in the country (Das Gupta and Guest, 2002). The referenced study determined that a substantial portion of this cost arises indirectly as societal costs, which are the result of unemployment amongst those with BD, workplace absenteeism, and suicide. This indirect proportion of the national cost of BD was estimated at approximately £1.77 billion, with a further £199 million attributed to direct medical costs incurred through the National Health Service (NHS). This direct cost represents only 10% of the total economic burden of this disorder. The cost of BD to the NHS in the UK during the 2009/2010 period was £342 million (Young et al., 2011), alluding to an extrapolated indirect economic burden to the UK economy of approximately £3.04 billion. In the United States of America (USA), the cost of BD is estimated at $151 billion, with $120.3 billion attributed to indirect costs (Dilsaver, 2011). Data from the South African Stress and Health Study (SASH) states that the loss-of-income cost as a result of severe depression and anxiety disorders in South Africa amounted to approximately R50 billion (the approximate equivalent of $3.6 billion) (Lund et al., 2013).

These costs are an indication of the "life impact" of BD to the broader population, as well as an implication that BD remains an important public health consideration. The calculation of disability-adjusted life years (DALYs) is a measure to allow one to discern the impact of non-lethal disorders (Whiteford et al., 2015). DALYs are calculated as the sum of years lived with disability (YLD) and years lost to premature mortality (YLL). BD accounts for 5% (95% confidence interval [CI]:4.4-10.3) of all DALYs caused by mental and substance abuse disorders (Whiteford et al., 2015), illustrating the prospect for the escalation of the costs associated with this disorder.

In South Africa, the neglect of mentally ill patients in non-governmental organisations in Gauteng province has resulted in over 94 deaths due to a crude and cursory management of a sensitive patient population (Makgoba, 2017). This crisis highlights the imperative to investigate the biology of mental disorders, from the genetic to physiological levels, with the objectives of improved diagnosis, prognosis, and treatment. Ultimately, this may commence a reversal of the pervasive stigma against psychiatric disorders, perpetuated by those involved in the health care sector (Egbe et al., 2014), and at large.

1.2.2 THE SOCIAL COST OF BIPOLAR DISORDER

The epidemiology of disease is often measured according to lifetime prevalence, or projected lifetime risk. The former measure indicates the proportion of the population who have been afflicted up until the time of assessment, while the latter measure provides an indication of the proportion of the population who are likely to be afflicted during their lifespan (Kessler et al., 2007). The global

prevalence of bipolar spectrum disorder (BSD), which encompasses both forms of BD and includes sub-threshold forms of the disorder, is approximately 2.4-3.5% (Merikangas et al., 2011, 2007). The global lifetime prevalence of BD, including both BD-I and BD-II, according to international studies, is approximately 1-2.1% (Merikangas et al., 2011, 2007). However, these figures may not be an accurate reflection of the occurrence of mental disorders, and BD in particular. There may be consistent under-diagnosis of mood disorders in some countries, for example Canada. Pelletier et al. (2017) investigated the data available for research from the 2012 Canadian Community Health Survey – Mental Health, and 5.4% of all respondents reported symptoms which were consistent with a mood disorder. However, only 2.7% of these individuals had been professionally diagnosed (Pelletier et al., 2017). In addition, a study of patients in the UK with MDD investigated the number who were possibly misdiagnosed BD patients, and found that this may be the case in 3.3-21.6% of the original cohort of MDD patients (Smith et al., 2011). A similar study in Japan found that 25.4% of patients who had experienced a major depressive episode (MDE) suffered from BD rather than MDD (Inoue et al., 2015). A retrospective study of conversion from MDD to BD in Denmark noted that 8.7% and 7.7% of females and males, respectively, underwent diagnostic conversion (Musliner and Østergaard, 2018). The under-diagnosis may be related to a lack of recognition (by the BD subject) of past hypomanic/manic episodes. This may be due to the fact that individuals are more likely to take note of depressive episodes and the effects of these moods rather than manic episodes when describing potential mood disturbances to a health-care professional (Regeer et al., 2015).

Despite possible errors in measuring the prevalence of mental disorders, the estimates provided by the literature are sufficient to galvanise concern. The lifetime prevalence of mood disorders in South Africa, according to the SASH, is 9.8% (Herman et al., 2009). The WHO's World Mental Health survey found that the projected lifetime risk for any mood disorder in South Africa was 20%, and the same survey indicated that the projected lifetime risk for any mental disorder in South Africa, of which mood disorders are a subset, was 47.5% (Kessler et al., 2007). Clearly, there is a need to assess the impact of mental disorders on an individual and in the population, in order to assist understanding, diagnosis, and treatment.

Individuals afflicted with BD often experience several adverse symptoms which have a negative effect on their quality of life. Impaired prospective memory (Au et al., 2017) and cognitive function (Fakhry et al., 2013) are both features that have been noted in BD cases, and studies investigating emotional processing in BD have suggested that BD individuals have an altered ability to recognise the emotions portrayed by facial expressions; notably anger, fear, and disgust, even in the euthymic state (Bilderbeck et al., 2017; Iakimova et al., 2016). Such impairments may have an impact on quality of life. Those with BD have higher rates of divorce, as well as higher incidences of criminal activity, and occupational difficulties with higher rates of dismissal/retrenchment (Calabrese et al., 2003). A study of prison inmates in the USA indicated that prisoners with BD were at greater risk of recidivism than inmates

without a psychiatric disorder (Baillargeon et al., 2009). These "side-effects" of BD are a small indication of the difficulties experienced by those affected by it.

1.3 THE GENETICS OF BIPOLAR DISORDER

Heritability is defined by Visscher et al. (2008) as "a ratio of variances, specifically as the proportion of total variance in a population for a particular measurement, taken at a particular time or age, that is attributable to variation in additive genetic or total genetic values — termed the narrow-sense heritability (or just heritability, h^2) and the broad-sense heritability (H^2), respectively." To expand on this definition, heritability as a genetic concept is the variability in a phenotypic feature which can be attributed to genetic differences (H^2) rather than environmental variation, or to the interactions between genotypes, dominance effects, and epistatic influences between genotypes (h^2).

The heritability of BD is estimated at approximately 85% (95% CI: 73-93%) from data gathered by studies of both monozygotic and dizygotic twin pairs (McGuffin et al., 2003). A population-based study using Danish registries calculated that the heritability of BD was approximately 62% (95% CI: 58-65%) (Wray and Gottesman, 2012). Heritability from a Swedish cohort family study involving over 2 million nuclear families (identified using country registry data) indicated that BD heritability was 59% (Lichtenstein et al., 2009). Lichtenstein et al. (2009) also identified the risk of BD in first-degree relatives of a BD proband is higher than the expected risk of developing BD in people who do not have a BD relative – this is known as the relative risk. The relative risk of BD for a full sibling of a BD proband was calculated to be 7.90 (95% CI: 7.10-8.80). A meta-analysis of risk studies suggested that the risk of BD for first-degree relatives of BD probands was 10.54%, compared to 0.48% in first-degree relatives of control probands (Van Snellenberg and de Candia, 2009). A study of mood disorders in a Swiss population investigated the aggregation of BD, schizoaffective disorder, and MDD, and the co-aggregation of these disorders within families in which either a BD-affected, or an orthopaedic proband (to act as medical controls with no psychiatric disorder) had been identified (Vandeleur et al., 2013). While some co-aggregation of disorders within families was observed, this study further tested for the aggregation of specific psychiatric criteria within families, namely mania, psychosis, and major depressive episodes (MDEs). The results show that there is an aggregation of these specific characteristics within a family rather than an aggregation of disease type within families, which suggests that particular genetic mechanisms underlie each of the assessed psychiatric criteria.

1.4 IDENTIFYING THE GENETIC COMPONENTS OF BIPOLAR DISORDER

Douglas et al. (2016) illustrated the range of genes that had been associated with BD, an excerpt of which is provided in Figure 1.1. This ideogram highlighted the fact that the genes putatively involved in BD are numerous, as the manuscript lists 290 genes in total, and also that these genes are spread across the entire genome, not restricted to any particular locus. In addition, many of these genes have also been associated with SCZ and autism spectrum disorder (ASD). Previously published genome-

wide association studies (GWAS) had already indicated a shared genetic composition between BD and other psychiatric disorders, such as SCZ (Wang et al., 2010), attention-deficit hyperactivity disorder (ADHD), MDD, and ASD (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013). For complex disorders such as BD, it is accepted that there is a polygenic contribution of many variants, each with a small risk effect, to the development of this disorder (Craddock and Sklar, 2013). GWAS represent a useful method of investigating the common variants which may be involved in BD pathogenesis, as variants across the genome can be assessed for an association with a given phenotype; this removes the limits of *a priori* selection of candidate genes and variants from an extensive list. However, GWAS require large sample sizes in order to be effective at detecting association. One of the largest BD GWAS involved 9 747 cases and 14 278 controls (Mühleisen et al., 2014).

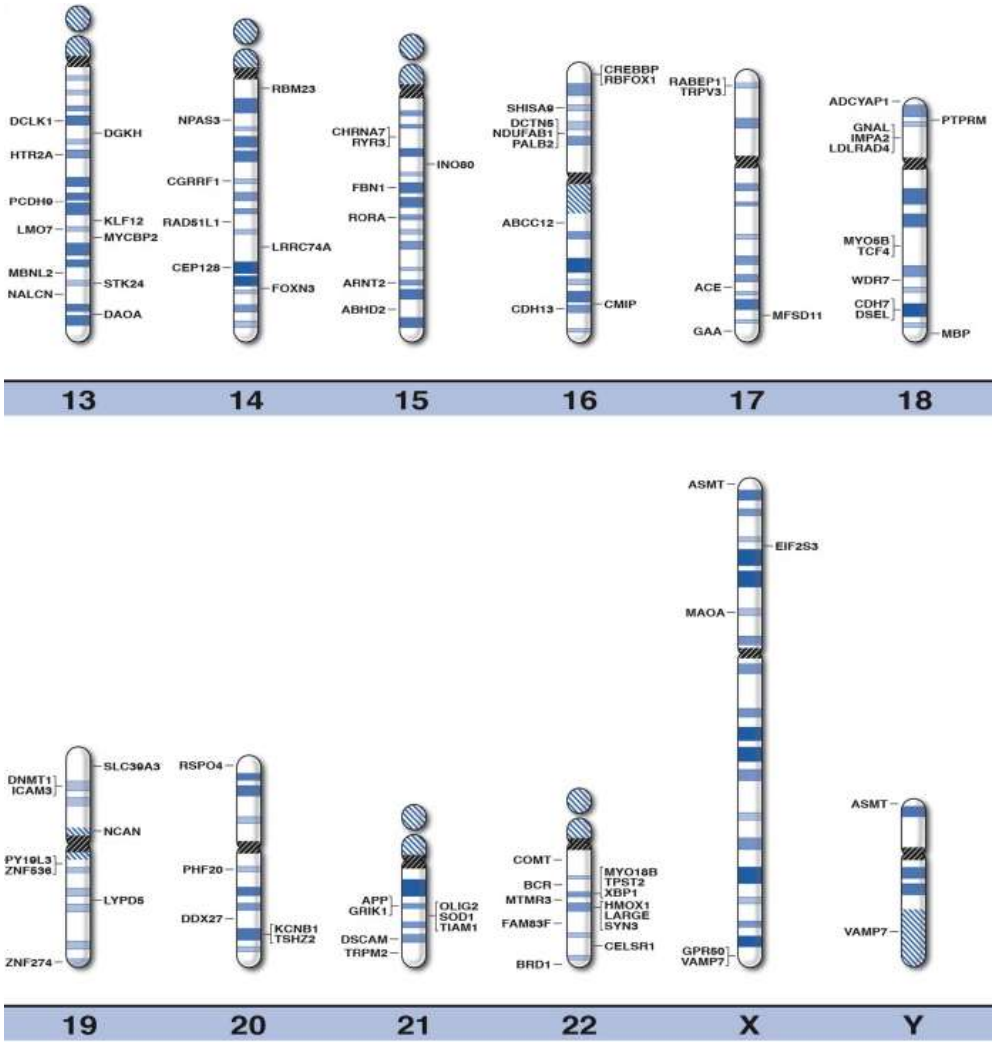


Figure 1.1: Ideogram excerpt of genes associated with bipolar disorder and their dispersal across chromosomes 13-22, X and Y (adapted from Douglas et al., 2016)

1.4.1 LINKAGE STUDIES

Linkage studies are used as a means of tracking disease-causing regions of the genome through generations by linking disease phenotypes to particular chromosomal regions. This is based on the principle that regions in close proximity on the genome will not be separated during meiosis, thus keeping the disease-causing locus in close proximity, or linkage disequilibrium, with other markers in the surrounding area (Pulst, 1999). Linkage disequilibrium (LD) refers to the occurrence of specific alleles in a particular genomic region at a higher frequency than would be expected if there was not a "link" between them (Pulst, 1999). As mentioned, a variety of regions from chromosomes 1 to 22 have been implicated, and a selection of these regions identified through linkage analysis is presented in Table 1.2. It is known that linkage analysis is only an effective method of analysis when the effect size of the implicated gene or variant is sufficiently large (Risch and Teng, 1998). It has been postulated that the limited success of linkage studies for BD is a result of the polygenicity of this disorder – there is lacking a single locus of sufficiently large effect size involved in BD to generate a result from a linkage-based study (Craddock and Sklar, 2013).

Table 1.2: Regions implicated in bipolar disorder through linkage studies

Study Authors	Genomic Regions	Number of Individuals Included
Detera-Wadleigh et al., 1999	1q, 4p, 7q, 12q, 18p, 21q and 22q	395
Badner & Gershon, 2002	13q and 22q	1228*
Liu et al., 2003	2p, 4q and 7q	1508
Segurado et al., 2003	9p, 10q and 14q	2437*
Macgregor et al., 2004	1q, 8q, and 9q	229*
McQueen et al., 2005	6q and 8q	5179

*Number of bipolar disorder cases included in the analysis

1.4.2 ASSOCIATION STUDIES

Association studies are the second conceptual step after linkage analysis for disease gene localisation. These studies are used to determine whether particular alleles of a variant are connected with a disorder, while taking into account the biological plausibility of the variant and its location and putative effect. The most pertinent difference between linkage and association studies is that linkage studies identify a physical locus which is inherited with the disease, while association studies create ostensible links between loci, which may be either causative, or in linkage disequilibrium with the causative locus, and a disorder (Hodge, 1993). Originally, association studies were confined to limited numbers of candidate polymorphisms or genes, but technology has developed to a point that enables association studies to be conducted on the whole genome (Marchini et al., 2004).

1.4.2.1 GENOME-WIDE ASSOCIATION STUDIES

GWAS investigate the association between a specified trait and a large number of variants across the genome. GWAS exploit the implicit LD between variants across the genome, and detected associations do not imply that the variant with the strongest statistical association is the causal variant, but can rather be in LD with the causal mutation (Visscher et al., 2017). The first GWAS of BD, using 461 BD-I probands, was published in 2008 (Baum et al., 2008). This GWAS was performed using over 550 000 SNPs and identified an association between BD-I and an intronic marker in *diacylglycerol kinase eta* (*DGKH*), a gene on chromosome 13q14.11. The authors concluded that this GWAS indicated the polygenicity of BD, and that the polygenic model presented the most logical explanation of the genetic origins of BD. Shortly thereafter, a second GWAS of 1 461 BD-I patients, and 372 193 SNPs (Sklar et al., 2008) identified the strongest associations with *myosin 5B* (*MYO5B*) and *tetraspanin 8* (*TSPAN8*). The 20 strongest SNP associations published by Sklar et al. (2008) did not include any variants from *DGKH*, reinforcing the polygenicity and complexity of BD. A more recent GWAS of 40 000 individuals of European descent identified four known variants in *tetratricopeptide repeat and ankyrin repeat containing 1* (*TRANK1*), *mitotic arrest deficient 1-like protein 1* (*MAD1L1*), *dendrin* (*DDN*), and *microRNA 2113- POU class 3 homeobox 2 region* (*MIR2113-POU3F2*), as well as two novel variants in the intronic region of *Erb-B2 receptor tyrosine kinase 2* (*ERBB2*), and within an intergenic region on chromosome 9p21.3 (Hou et al., 2016). Time has not refined the list of associated variants but rather has seen it increase, as exemplified more recently by Ikeda et al. (2017), who identified two novel loci in a Japanese cohort, which can be added to the growing list of putative BD-related variants. This list is currently in excess of 4 000 variants according to BDgene [http://bdgene.psych.ac.cn/index.do; accessed 30 March 2018]. Furthermore, popular associated polymorphisms, such as SNP rs6265 in *brain-derived neurotrophic factor* (*BDNF*), often called the "Val66Met" polymorphism, have been tested for association (Geller et al., 2004; Lohoff et al., 2005) , and found to be associated in some studies and to lack associations in others (Chen et al., 2014; González-Castro et al., 2015; Schosser et al., 2017; Wang et al., 2014).

1.4.2.2 FAMILY-BASED ASSOCIATION STUDIES

Family-based studies are, eponymously, genetic studies that focus on disease transmission in family groups, which can range from trios to multigenerational, complex, extended pedigrees. By 1998, it had been recognised that linkage analysis was limited in its ability to detect variants of small effect size in complex disorder aetiology (Risch and Teng, 1998). Family designs have several advantages over case-control methods, including their robustness to population stratification, their ability to control for heterogeneity, and the fact that rare variants may be enriched in family-based groups (Ott et al., 2011). Several methods have been described to assess both linkage and association within family settings, but one method which incorporates both components is the family-based association test (FBAT). The FBAT is an extension of the transmission disequilibrium test (TDT), and the TDT accommodates trios

alone. FBAT accommodates extended family structures, including unaffected siblings and individuals. In family studies, the alternate hypothesis (H_A) for the test always assumes both linkage and association between the marker used in the test, and the locus of disease susceptibility (Ott, 1989). Furthermore, FBAT allows the genetic inheritance model to be tested, whether it is additive, recessive, or dominant, and remains robust to population stratification like the TDT and other family-based designs (Laird & Lange, 2006). One means of conducting family-based studies is to investigate variants which have been detected in larger, case-control studies. Two such family-based studies of BD have identified significant variation, including the *BDNF* rs6265 polymorphism (Neves-Pereira et al., 2002), and *DGKH* rs9315885 (Ollila et al., 2009), which were originally identified using other methods.

1.4.3 NEXT-GENERATION SEQUENCING TO INVESTIGATE PSYCHIATRIC DISORDERS

Next-generation sequencing (NGS) is a group of evolving sequencing technologies which refer to a high throughput, massively parallel sequencing system. NGS is the second evolution of Sanger sequencing (Sanger et al., 1977). This involves dideoxynucleotide chain-termination sequencing by synthesis, using a single molecule at a time. This Nobel prize-winning method is still considered the gold standard of sequencing methods, as it is known for its accuracy and reliable results. However, this method is expensive on a large scale, and it is limited by read lengths of approximately 100bp. NGS was developed in the early 21st century as a more cost-efficient method of sequencing large numbers of sequences in parallel (Ansorge, 2009; Mardis, 2008). In the intervening years, the term "NGS" has expanded to include most branches of the "-omics" revolution. As NGS technologies improved, the feasibility of rapid large-scale investigations increased, and many research groups have attempted to elucidate the genome, exome, transcriptome, proteome, and methylome.

The simplified explanation of NGS technical methodology (for all except methylome and proteome sequencing) can be separated into two categories: polymerase chain reaction (PCR) amplification using multiple primer sets, and capture enrichment methods. The PCR-based method relies on many sets of primers that will amplify the desired regions of the genome – in WGS studies, for example, this would entail the entire genome, while the primers used in WES would amplify the exonic regions of DNA (Koboldt et al., 2013). Targeted NGS methodologies rely on a variety of capture methods, which are manufacturer-dependent, but the simplified theory involves capture probes which bind with high specificity to the DNA sequences of interest. These probes typically can be "pulled" from the solution, along with their captured sequence. Following the sequencing process, the reads are mapped to a reference genome in order to discover the variants that have been identified by observing regions that differ when compared to the reference. There is substantial effort in most pipelines to ensure that the base calling process is accurate, and there are many quality control steps during the data processing phase (Mardis, 2008). In the case of nucleic acids, these steps are used to ultimately minimise base-calling error. A simple quality control step, for example, is to ensure that all detected sexes match the

purported sex of each sequenced individual (Anderson et al., 2010). Other steps involve assessing the coverage and quality of each read, and the quality of the data is assessed both pre- and post-alignment.

NGS technologies such as WGS, WES, transcriptome sequencing, methylome sequencing, and proteome sequencing, allow researchers to investigate many levels of biological importance which may be involved in disease development, and facilitate the development of a more substantial map of what a disease may entail. NGS has been used to investigate the multiple levels at which biology may indicate the nature and cause of BD since 2014. Several publications, listed in Table 1.3, provide an indication of BD research that has been performed using NGS.

Technological advances in recent years have made NGS more financially viable in studies of human disease, and in this case of BD. WGS and WES can be used to detect rare variants which may have a role in BD, and both methods have been used to complement growing data (from clinical research of symptomatology, psychology, cognition, and brain imaging studies) regarding this disorder (Fiorentino et al., 2014; Georgi et al., 2014; Lescai et al., 2017). The first two NGS studies of BD were both published in 2014, by Georgi et al. (2014) and Fiorentino et al. (2014). The former group investigated the non-synonymous, rare (minor allele frequency [MAF] < 0.02), exonic variants present in the isolated Old Amish population in the USA. After the filtration process, 30 putative variants were identified in affected individuals. Of these, four were novel, and the others occurred in several genes across multiple chromosomes. Some of the implicated genes are *aquaporin 8* (*AQP8*), *transmembrane protein 222* (*TMEM222*), and *centrosomal protein 162* (*KIAA1009*). Fiorentino et al. (2014) restricted their approach by focusing on non-synonymous variants present in the candidate genes *ankryin 3* (*ANK3*) and *calcium voltage-gated channel subunit alpha 1C* (*CACNA1C*). After completing a Fisher's exact test to compare the MAFs of the variants in the study and those in the 1000 Genomes' European data set, only variants with significantly ($p < 0.05$) different allele frequencies relative to this reference data set were used for further analysis. In total, 13 variants remained after the filtering process, comprised of three single nucleotide polymorphisms (SNPs) in *ANK3*, and nine in *CACNA1C*. Of these 13 variants, five were novel.

In a family-based study using WGS, Ross et al. (2016) identified a rare variant in *calcium voltage-gated channel subunit alpha 1D* (*CACNA1D*) which segregated in seven affected family members. More recently, a BD WES study of an isolated Faroese population identified rare (MAF < 0.05) variants in *phosphatidylinositol transfer protein membrane associated 2* (*PITPNM2*) and *nitric oxide synthase 1* (*NOS1*), while the genes *nucleolin* (*NCL*) and *phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 alpha* (*PIK3C2A*) were implicated by gene-burden tests (a means of finding a gene burden score by collapsing the number of SNPs to a single representative gene and weighting the gene according to the number of SNPs therein to reduce multiple testing penalties and to increase the association signals to the genes under investigation).

Table 1.3: Next-generation sequencing and -omics investigations of bipolar disorder

Study Authors	Year	Title	Type of NGS*
Fiorentino et al., 2014	2014	Analysis of *ANK3* and *CACNA1C* variants identified in bipolar disorder whole genome sequence data	WGS
Georgi et al., 2014	2014	Genomic view of bipolar disorder revealed by whole genome sequencing in a genetic isolate	WGS
Ross et al., 2016	2016	A Rare Variant in CACNA1D Segregates with 7 Bipolar I Disorder Cases in a Large Pedigree	WGS
Bouwkamp et al., 2017	2016	A Balanced Translocation Disrupting BCL2L10 and PNLDC1 Segregates With Affective Psychosis	WGS
Goes et al., 2016	2016	Exome sequencing of familial bipolar disorder	WES
Monson et al., 2017	2017	Assessment of Whole-Exome Sequence Data in Attempted Suicide within a Bipolar Disorder Cohort	WES
Lescai et al., 2017	2017	Whole-exome sequencing of individuals from an isolated population implicates rare risk variants in bipolar disorder	WES
Kataoka et al., 2016	2016	Exome sequencing for bipolar disorder points to roles of de novo loss-of-function and protein-altering mutations.	WES
Akula et al., 2014	2014	RNA-sequencing of the brain transcriptome implicates dysregulation of neuroplasticity, circadian rhythms and GTPase binding in bipolar disorder	Transcriptome
Kohen, Dobra, Tracy, & Haugen, 2014	2014	Transcriptome profiling of human hippocampus dentate gyrus granule cells in mental illness	Transcriptome
Cruceanu et al., 2015	2015	Transcriptome Sequencing of the Anterior Cingulate in Bipolar Disorder: Dysregulation of G Protein-Coupled Receptors	Transcriptome

Table 1.3 continued: Next-generation sequencing and -omics investigations of bipolar disorder

Study Authors	Year	Title	Type of NGS*
Zhao et al., 2015	2015	Transcriptome sequencing and genome-wide association analyses reveal lysosomal function and actin skeleton remodeling in schizophrenia and bipolar disorder	Transcriptome
Vizlin-Hodzic et al., 2017	2017	Early onset of inflammation during ontogeny of bipolar disorder: the *NLRP2* inflammasome gene distinctly differentiates between patients and health controls in the transition between iPS cell and neural stem cell stages	Transcriptome
Dempster et al., 2011	2011	Disease-associated epigenetic changes in monozygotic twins discordant for schizophrenia and bipolar disorder	Methylome
Xiao et al., 2014	2014	The DNA Methylome and Transcriptome of Different Brain Regions in Schizophrenia and Bipolar Disorder	Methylome and Transcriptome
Walker et al., 2016	2016	DNA methylation in a Scottish family multiply affected by bipolar disorder and major depressive disorder	Methylome
Chen et al., 2015	2015	Comparative proteomic analysis of plasma from bipolar depression and depressive disorder: identification of proteins associated with immune regulatory	Proteome

*NGS indicates Next Generation Sequencing, WGS indicates Whole Genome Sequencing, and WES indicates Whole Exome Sequencing

Databases such as BDgene (Chang et al., 2013)and GWAS Catalog [https://www.ebi.ac.uk/gwas/] have been established to assist genetic studies involving complex disorders. BDgene currently (19 April 2018) lists 1 192 genes as associated with BD from published literature, across a variety of study methods, while the 961 SNPs in GWAS Catalog which have been associated with BD, obtained from GWAS studies, map to 729 unique genes [https://www.ebi.ac.uk/gwas/search?query=bipolar%20disorder#association; accessed 19 April 2018].

As indicated above, there are a large number of genes and variants associated with BD. Accordingly, methods to study this disorder have continued to evolve and develop. Traditional genetic methods such as linkage studies have met with limited success in the field of psychiatric genetics, while high-powered, large-scale association studies have yielded more promising results (Craddock and Forty, 2006).

1.4.4 PATHWAY-BASED ANALYSES

A limitation of genome-wide or whole-genome methods is that the wealth of data generated by large-scale experiments can obscure the biological insights within the results (Burkhardt, 2014). In the case of GWAS of complex disorders, when the results are associations across a seemingly disparate array of genes, it initially appears that this genome-wide method has rendered the biology of BD opaque. Finding the solutions to BD mechanisms requires integrated approaches using genetic/genomic information, and existing biological knowledge. In addition, the probable small effect sizes of the implicated loci suggest that such variants may not be detected with GWAS or NGS studies. One method, the convergent functional genomics approach, utilises a combination of gene expression data from animal models and linkage data from human studies in order to identify candidate genes (Ogden et al., 2004). This method uses gene ontology (GO) term analysis to create an idea of the broader biological mechanisms involved – in other words, the pathways at play. As hypothesised by Badano and Katsanis (2002), if multiple genes are associated with a disorder it is likely that these genes share a level of functional annotation. This holds true whether it is a cellular process or involvement in the same pathway, relative to genes that have not been implicated in association studies. The principal feature of pathway-based analysis (PBA) is a search for functional enrichment. For example, the method proposed by Shriner et al. (2008), Commonality of Functional Annotation, was designed in order to isolate a list of prioritised candidate genes from linkage studies for this purpose.

Pathway analysis can refer to a broad range of analyses that encapsulate expression data, gene list/gene set data, as well as methods that involve interacting proteins (Khatri et al., 2012). Many tools have been developed to annotate variants with biological information from various sources. ANNOVAR uses database information from the UCSC Genome Browser to annotate variants (Wang et al., 2010) while online programmes such as the KEGG Ontogeny Based Annotation System (KOBAS) integrates data from several pathway and disease databases (including KEGG PATHWAY, PID, Panther, Reactome, OMIM, KEGG DISEASE, NHGRI GWAS Catalog, and BioCyc) to give greater biological relevance

to any input list of genes (Xie et al., 2011). Aside from the potential disparity in pathway annotation sources, pathway analysis of BD has implicated many distinct pathways, using expression data as well as gene lists from association studies.

Pathway analysis of two diseases, BD and SCZ, from two locations, San Francisco (including Asian, African, Hispanic, and European individuals) and Taiwan (including only individuals of Han-Chinese ethnicity), identified distinct pathways in each ethnic population for BD (Bousman et al., 2010). In this study, pathway analysis was performed using the top 100 dysregulated genes identified by microarray assessment of peripheral blood samples. Two pathways which were identified in the top ten pathways in both the San Franciscan and Taiwanese BD cohorts were *integrin signalling* and the *protein ubiquitination* pathways. *Actin cytoskeleton signalling* was identified as significant in the Caucasian San Franciscan group. The *protein ubiquitination* pathway was also identified across the disorders BD, SCZ, and a "psychosis" phenotype of BD and SCZ sufferers with psychotic symptoms (Bousman et al., 2010). The fact that the research cohorts from each country have different implicated pathways indicates that pathway analysis may be sensitive to discrepancies between diagnostic methods, as the Diagnostic Interview for Genetic Studies (DIGS) was used to assess the San Franciscan disease groups, while the DSM-IV was used to diagnose the cases in Taiwan. The fact that looking at a specific phenotypic entity (psychosis) gave greater overlap between nationalities indicates that endophenotypes or specific clinical characteristics may prove more useful in identifying causative pathways involved in psychiatric diseases. Other studies have identified the *histone H3-K4 methylation*, *melanoma*, and *chromosomal synapsis* pathways (Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015); *myelin-related pathway* (Yu et al., 2014); *cation-channel activity*, *gated-channel activity*, and *metal ion transmembrane transporter activity* (Chuang et al., 2013); as well as *oxidative phosphorylation*, *mitochondrial dysfunction*, *eukaryotic initiation factor-2 signalling*, and *calcium signalling* (Föcking et al., 2016) in BD.

1.5 RESEARCH AIMS AND OBJECTIVES

The above review of genetic studies for BD indicates that NGS methods appear to be the most potent method of investigation, but also highlights the issues associated with the wealth of data that exists from decades of study. To contextualise this data, PBA remains a worthwhile method of observing the biological pattern and potentially burdened pathways that underlie BD manifestation.

1.5.1 AIM

The aim of this research is to elucidate the genetic aetiology of BD by establishing the biological context of variant data generated by NGS methods. This will be done by using two approaches. The first approach aims to investigate the role of common variation in BD via the pathway analysis of WGS data of four family members, and by genotyping identified variants from this analysis in a South African BD cohort. The second approach will be to investigate potential contribution of rare, putatively damaging

variation to BD within a single family, by performing WES on affected and unaffected members of the selected family.

1.5.2 RESEARCH OBJECTIVES

In order to fulfil this aim, the objectives of the study are to:

1) Perform pathway analysis on whole genome sequencing data from BD-affected family members.
2) Identify appropriate controls of European ancestry for the Afrikaner family using principal component analysis (PCA).
3) Identify suitable candidate genes and variants for genotyping purposes from the pathway analysis results.
4) Genotype family members and background controls for the identified BD candidate variants and complete a family-based association test.
5) Perform whole-exome sequencing of BD-affected family members and identify damaging variation.

CHAPTER 2: METHODS

2.1 BIPOLAR DISORDER RESEARCH GROUP

The Division of Human Genetics at the University of Cape Town (UCT) (hereafter referred to as the Division), commenced its investigation of BD in 1996 (REC Ref 081/1996, now updated to HREC Ref 091/2014) with the intention of recruiting families affected with BD-I, BD-II, and MDD. Participants were recruited from the following provinces of South Africa: Free State, Gauteng, the Eastern Cape, and the Western Cape. Recruitment to the project involved the completion of a Structured Clinical Interview for the DSM-IV (SCID), administered by a registered nurse and upon diagnosis of BD-I, confirmation by a psychiatrist. This project is linked as a sub-study to the aforementioned BD project, and was approved by the UCT Human Research Ethics Committee (HREC Ref 633/2016). Blood or saliva samples were collected with the informed consent of the participants as part of the larger study, an example of which is provided in Appendix A (Example Consent Form). DNA was obtained from the biological samples following an extraction process that comprised of: i) red blood cell lysis, ii) the lysis of other nuclear cells in the whole blood, iii) protein precipitation, iv) the precipitation of DNA, v) ethanol cleaning of the isolated DNA pellet, and vi) resuspension of this pellet in distilled H_2O. The full DNA extraction protocol can be found in Appendix B (DNA Extraction Protocol). The DNA samples were stored at -20^{o}C or -80^{o}C for short-term or long-term periods, respectively. For clarification, the sample code of individuals recruited for this investigation of BD (observed in later sections of this chapter) is BPD, and refers to BD, and not Borderline Personality Disorder.

2.2 FAMILY 30

In total, the Division recruited ~900 individuals from ~200 families for the larger BD project. One of the most thoroughly investigated families in this cohort is Family 30, in which psychiatric disorders, notably BD, are prevalent. The Division is custodian to DNA from 76 members of this family for molecular investigation. This family is of Afrikaner descent, which makes them suitable candidates for genetic analyses for several reasons, outlined below.

The Afrikaner ethnic population of South Africa, hereafter referred to as Afrikaners, derives from a relatively small number of Dutch immigrants who moved to South Africa from the mid-17th century to approximately the beginning of the 19th century, numbering around 4000 individuals according to genealogical data compiled by de Villiers & Pama (1966). This grouping of immigrants has been labelled as a founder population for several genetic disorders in the modern Afrikaner population, including Fanconi anaemia (Tipping et al., 2001), Beukes familial hip dysplasia (Cilliers and Beighton, 1990), and Huntington's disease (Hayden et al., 1980), amongst others (Botha and Beighton, 1983a, 1983b). The Afrikaner population is seen as a genetically isolated one, as this group migrated to remote

parts of South Africa and maintained relative separation from other populations through language and religion, with relative endogamy in the early period of the immigration (Abecasis et al., 2004; Karayiorgou et al., 2004). Population isolates are good candidates for genetic studies as individuals affected with certain disorders are expected to possess the same underlying genetic abnormality, inherited from the small number of founders (Peltonen et al., 2000), and may generally be exposed to similar environmental factors. Heese (1971) notes that the Afrikaners are not, however, genetically identical to the Dutch population from which it formed. This population also has genetic contributions from French, German, British, and Portuguese immigrants, as well as some contributions from non-European slave populations of the Western Cape Province of South Africa (hereafter referred to as the Cape). A study of a single Afrikaner's ancestry indicated that the identifiable heritage of this individual was approximately 37.45% Dutch, 27.38% German, 26.43% French, 0.24% Danish, 0.06% Norwegian, 0.47% Portuguese, 1.90% British, 1.9% Chinese, 0.27% Guinea, 1.68% Indian, 0.06% Madagascan, with a contribution of 2.17% attributed to the slave population of the Cape, according to genealogical records (Greeff, 2007). In order to confirm the apparent hereditary components, the author, Jaco Greeff, genotyped his own mtDNA and Y chromosome, as the archetypal Afrikaner individual, as well as the mtDNA of his father, a second Afrikaner individual. The author's mtDNA haplogroup was W, originating from Western Asia, while his father's mtDNA was haplogroup M, with origins in South/South East Asia or North Africa. The Y chromosome short tandem repeats (STRs) which were genotyped indicated the chromosome was classified as haplogroup R, with its own Asiatic origins. With this knowledge, members of Family 30 were selected for NGS analysis. This ancestry seems to concur with historical writings regarding the ancestry and history of South African populations (Marais, 1968). Thus, while the Afrikaners possess African and Asian components within their genome, the majority of their genetic constitution remains European.

2.3 WHOLE-GENOME SEQUENCING DATA

2.3.1 WHOLE-GENOME SEQUENCING DATA GENERATION

Previously, four BD-I affected members of Family 30 were selected for WGS, which was undertaken at the University of Southern California using an Illumina HiSeq 2000 system (Illumina Inc, San Diego, California, USA) (Dalvie, 2015). The Illumina WGS method involves cluster amplification of DNA fragments attached to a flow cell, which then undergo a proprietary non-reversible terminator sequencing process. Incorporated single nucleotides are detected using laser excitation as they are attached to the growing strand of DNA. The full detail of the WGS process can be found in Appendix C (WGS Protocol).

2.3.2 BIOINFORMATIC ANALYSIS OF WHOLE-GENOME SEQUENCING DATA

The appropriate bioinformatics analysis was applied to the raw fastq WGS data by the Computational Biology Group (CBIO) at UCT. The UCT ICTS High Performance Computing facility

[http://hpc.uct.ac.za] was used for all relevant computation processes. The workflow analysis of the CBIO group can be found in Appendix D (WGS Bioinformatics Protocol), but a précis is provided here:

1. Quality control

The programme FastQC [http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/] was used to assess the quality of the raw sequence data for further processing. The FastQC criteria and output can be found in Appendix D (WGS Bioinformatics Protocol).

2. Alignment to the reference genome

The sequence reads were aligned to the human genome reference hg19 sequence using the Burrows-Wheeler Aligner (BWA) (bwa-0.5.9) (Li and Durbin, 2009). The BWA-backtrack algorithm was applied as this facilitated alignment of the short sequence reads to the long reference sequence. This process created the sequencing alignment map (SAM) file used for downstream analysis.

3. Post-alignment processing and variant calling

Firstly, the SAM file was processed using SAMtools (samtools-0.1.17) (Li et al., 2009) in order to generate a binary alignment map (BAM) file for post-alignment processing. This involved the sorting and indexing of the BAM file. Picard (picard-tools-1.51) was used to assess the alignment summary and quality score distribution of the alignment files. Secondly, the sequence coverage (referring to the number of times a particular nucleotide is covered during the sequencing process), GC content, successfully-mapped-reads percentage, and read length was obtained using Qualimap (García-Alcalde et al., 2012). Thirdly, the Genome Analysis Toolkit (GATK) (gatk v1.2) was used to realign insertion/deletions (indels) by identifying the target intervals and realignment, using the *IndelRealigner* feature in GATK. The *FixMateInformation* feature of Picard was used to observe that the mate-pair information of all the reads was correct.

GATK was used to recalibrate the quality control scores from the aligned BAM file in order to overcome bias and error from the sequencer. This is done using base quality score recalibration (BQSR), which generates a model of covariation using the sequencing dataset and known variants to adjust the base-quality scores [http://gatkforums.broadinstitute.org/gatk/discussion/44/base-quality-score-recalibration-bqsr]. Variants were called using the GATK *UnifiedGenotyper*. Variant quality score recalibration (VQSR) was completed in order to calculate new, less biased quality scores for each variant using *VariantRecalibrator* and *ApplyRecalibration* from the GATK suite [http://gatkforums.broadinstitute.org/gatk/discussion/39/variant-quality-score-recalibration-vqsr].

The variant call file (VCF) was generated as a result of the above processes, and lists the variants called by the sequencer which are variant to the following reference genome resources: HapMap 3.3 (International HapMap Consortium, 2003), dbSNP 132, and the Omni 2.5 SNP human microarray.

2.4 PRINCIPAL COMPONENT ANALYSIS

PCA in genetics research can be utilised as a means of observing population variation that may be related to a historical geographic distribution (Cavalli-Sforza et al., 1993; Novembre and Stephens, 2008). In this study, PCA was performed using the package *SNPRelate* (Zheng et al., 2012) in RStudio version 1.1.383 (RStudio Team, 2016). WGS data from the four BD-affected Afrikaner individuals was compared to data from several populations from the Phase 3 release of 1000 Genomes data, and Dutch genotype data from 637 individuals from a GWAS performed by the Psychiatric Genomics Consortium (PGC) SCZ group (Dalvie, 2015). The 1000 Genomes VCFs were downloaded as chromosomal VCFs [ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20130502/] (Auton et al., 2015). The VCFs for chromosomes 1 to 22 were merged to create a single file for all samples, using PLINK version 1.9 in the binary ped format [http://zzz.bwh.harvard.edu/plink/] (Purcell et al., 2007).

The Phase 3 1000 Genomes dataset consisted of 26 world populations. Of these, the following 11 were selected for PCA, and are also shown in Table 2.1 below, with their designated 1000 Genomes acronym: England and Scotland (GBR), Finnish (FIN), Iberians in Spain (IBS), Esan in Nigeria (ESN), Yoruba in Nigeria (YRI), African ancestry in South West USA (ASW), Luhya in Kenya (LWK), Puerto Ricans from Puerto Rico (PUR), Southern Han Chinese (CHS), Xishuangbanna in China (CDX), and Japanese in Tokyo (JPT). These 11 populations were selected to represent a wide variety of populations from across the globe. This was done to broadly illustrate the similarity between European populations, and the genetic distinction between European populations and populations from other global regions, without using all 26 populations, which would make resolution difficult to visualise. The sample identifiers for all individuals from each of these population groups were obtained from the 1000 Genomes website [http://www.internationalgenome.org/data-portal/sample]. The individuals from the selected populations were extracted from the VCFs using PLINK version 1.9 [http://pngu.mgh.harvard.edu/purcell/plink/] [http://zzz.bwh.harvard.edu/plink/] (Purcell et al., 2007). All the data files generated by PLINK version 1.9 for PCA were in the binary ped file format. This makes use of bim, bed, and fam files to describe a single dataset of genotypes and individuals. The final PCA made use of 13 892 LD-pruned overlapping SNPs between the 1 074 samples from Family 30, Dutch individuals, and 1000 Genomes reference populations. The maximum allowed LD between any SNPs was 0.2 (using the r^2 value). The format of the genotype data from a German individual, MP1, from Suk et al. (2011) was not compatible with the format of the 1000 Genomes Project data, thus this individual was not included in this PCA. However, variants present in MP1, a psychiatrically healthy

male, were excluded from the composite VCF file of the variants in the BD-affected Family 30 individuals (as described in Section 2.5.2).

Table 2.1: The populations used in the principal components analysis from the 1000 Genomes dataset

1000 Genomes Population Group	1000 Genomes Population Group Abbreviation
England and Scotland	GBR
Finnish	FIN
Iberians in Spain	IBS
Esan in Nigeria	ESN
Yoruba in Nigeria	YRI
African ancestry in South-West USA	ASW
Luhya in Kenya	LWK
Puerto Ricans from Puerto Rico	PUR
Southern Han Chinese	CHS
Xishuangbanna in China	CDX
Japanese in Tokyo	JPT

2.5 THE BIPOLAR DISORDER CONTROLS

2.5.1 DUTCH CONTROLS

Given that the Afrikaner population is derived from the immigrant Dutch population and that PCA indicated that this group would be appropriate controls, Dutch genetic summary data of SNVs and small indels (<20bp) from the Genome of the Netherlands (GoNL) consortium, as part of the Netherlands Biobanking and Biomolecular Research Infrastructure (BBMRI-NL), was obtained on the 22nd of August 2014 [https://molgenis26.target.rug.nl/downloads/gonl_public/variants/release5] for 498 parents from 250 trios (comprised of a child, mother, and father). This data is from WGS of Dutch individuals who were involved in the efforts to characterise the genome of the Dutch population. The data used in this study was generated using the Illumina HiSeq2000 (13X coverage) (Boomsma et al., 2014; Francioli et al., 2014). The GATK *UnifiedGenotyper* v1.4 was used to call variants from the combined data of all the individuals.

2.5.2 GERMAN CONTROL

As explained above, the "Afrikaner genome" is not identical to its Dutch ancestor but remains largely European. The next largest genetic contribution to the Afrikaner genome is of German origin. Accordingly, the data of MP1, a male proband from the Northern Schleswig-Holstein region of Germany, involved in a population genetics research project [http://www.popgen.de/], was obtained. Medical assessment indicated that aside from treated arterial hypertension, MP1 was a healthy 51-year-

old individual with no history of severe diseases. DNA from MP1 was sequenced using the SOLiD platform, and the diBayes SNP caller [http://solidsoftwaretools.com/gf/project/dibayes] (Suk et al., 2011). The data was obtained in bed file format and downloaded from the link provided in the publication, "A comprehensively molecular haplotype-resolved genome of a European individual" by Suk et al. (2011) [https://owww.molgen.mpg.de/~genetic-variation/MaxPlanckOneData/Variants/]. While MP1 is a single individual and not representative of the entire German population, the genetic landscape of the German population is relatively homogeneous (Lao et al., 2008). At the time of analysis, it was not possible to obtain more German genomic data, nor an example of a French genome, although a larger group of German and French genomes would be more representative as controls.

2.6 COMPARISON OF CASES WITH CONTROLS

A total of 870 875 variants were shared (the same heterozygous or homozygous genotype) amongst the 4 affected individuals from Family 30. This list of variants was compared to the GoNL "control" dataset. The GoNL data contained 625 965 of these variants, leaving a remainder of 221 703 variants as present in the BD-affected data. These 221 703 variants were compared to the data from MP1. Data from MP1 contained 787 of the 221 703 variants in the BD-affected data, leaving 220 916 variants exclusively present in the BD-affected cases (Figure 2.1). The variants exclusively present in the BD-affected cases were annotated using ANNOVAR (Wang et al., 2010) using the hg19 build as a reference, in order to determine the function and genetic location of these 220 916 "case-only" variants.

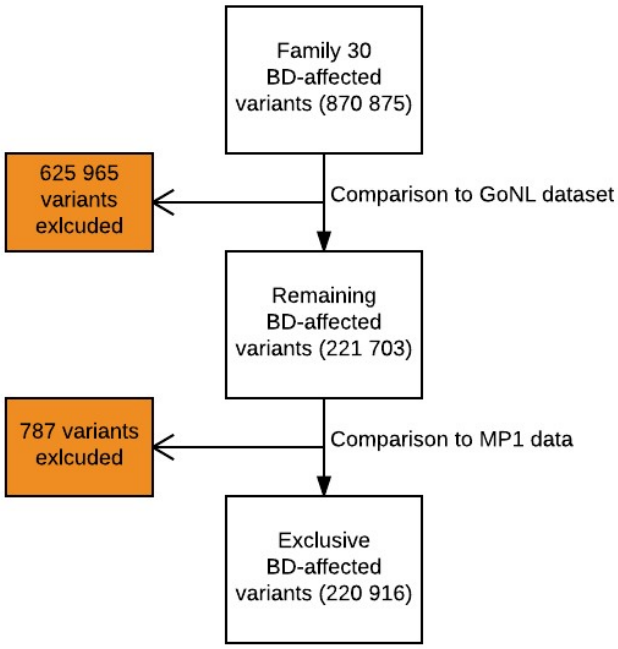


Figure 2.1: The process of filtering variants which are present in the bipolar disorder-affected family members when compared to Dutch and German "control" genomic data

2.7 PATHWAY ANALYSIS

Khatri et al. (2012) describe "knowledge base-driven pathway analysis" as an approach which relies on data stored in public repositories such as the Kyoto Encyclopedia of Genes and Genomes (KEGG). For this project, three free, publicly available pathway analysis programmes were used to further analyse and characterise the variants exclusively present in the BD-affected cases. The Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 [https://david.ncifcrf.gov/home.jsp] (Huang et al., 2009a, 2009b), WEB-based Gene SeT AnaLysis Toolkit (WebGestalt) [http://www.webgestalt.org/option.php] (Wang et al., 2013; Zhang, Kirov, & Snoddy, 2005), and KEGG Orthology Based Annotation System 3.0 (KOBAS 3.0) [http://kobas.cbi.pku.edu.cn/] (Xie et al., 2011) are all freely accessible web-based applications. These programmes accept gene lists as input, rather than GWAS association results and *p*-values, which is the requirement for other pathway programmes. Both KOBAS 3.0 and DAVID were updated in early 2017 from KOBAS 2.0 and DAVID version 6.7 respectively.

As mentioned above, the BD variants were annotated to genes using ANNOVAR. Intergenic variants were annotated to the nearest gene, and in this case, the furthest gene was approximately 2 008 965 bp away. The db2db feature of the Biological DataBase Network (bioDBnet) [https://biodbnet-abcc.ncifcrf.gov/] (Mudunuri et al., 2009) was used to convert the gene symbols from ANNOVAR to ENSEMBL identifiers for pathway analysis in order to ensure a uniform submission list across all pathway analysis programmes. A list of 8 775 of genes was included as input. KEGG pathway was selected as the pathway database of interest, as all three programmes made use of KEGG pathways for reference information.

2.8 PATHWAY AND VARIANT SELECTION

The most significant KEGG pathways identified from the DAVID, WebGestalt, and KOBAS 3.0 output were selected. Of these, the pathways that had previously been associated with BD or for which plausible hypotheses had been postulated, according to literature searches via PubMed, were chosen, and from this list three pathways were selected. Genes from these chosen pathways which were present in the initial input list of 14 593 genes from WGS (seen in the first block of Figure 2.2) were consolidated to form a single list. The BDgene database [http://bdgene.psych.ac.cn/index.do] provides a useful resource for BD investigators, as it is a curated database based of literature regarding BD and its overlap with MDD, and SCZ. BDgene currently lists 1 192 genes as associated with BD (Chang et al., 2013). The input gene list was compared to the list of associated genes from BDgene, and the genes present in both the input list and the list of genes from BDgene were kept. The variants present exclusively in the BD-affected cases from this pathway, which have previously been associated with BD according to BDgene, were analysed using the ENSEMBL Variant Effect Predictor (VEP)

[http://www.ensembl.org/Tools/VEP] (McLaren et al., 2016). The VEP can be used for annotation of coding and non-coding regions, and has greater functionality than either ANNOVAR or SNPEff, as it can identify regulatory features and transcription factor binding sites in non-coding regions, while the other two tools only identify the former. The variants from the VEP output were selected based on a MAF of 0.1-0.5, based on the common-disease common-variant hypothesis for BD (Becker, 2004; International Schizophrenia Consortium et al., 2009). The entire variant selection process is outlined in Figure 2.2 below.

2.9 GENOTYPING

Family samples from the BD research cohort from the Division were selected for genotyping using ThermoFisher OpenArray® plates (ThermoFisher Scientific, Carlsbad, California, USA). These families contain BD-affected and unaffected individuals, as well as members affected with schizoaffective disorder, cyclothymic disorder, and MDD. The OpenArray® plates are designed to user specifications. In this case, the variants in Table 2.2 were the final variants selected for the OpenArray® assays, based on the process described in Section 2.8, as well as the ability of ThermoFisher Scientific to design custom TaqMan® assays where possible. Variants were selected from the *actin cytoskeletal* and *focal adhesion* pathways from the analysis described above, and several other variants from the largest BD GWAS to date (Stahl et al., 2018) were included. At the time of variant selection, BDgene had only been updated to include papers published to 31 March 2016. The variants selected from this recent GWAS were included in order to assess recently associated variation. The source of each of the 17 selected variants are indicated in Table 2.2.

2.9.1 SAMPLE VERIFICATION AND PREPARATION

The selected DNA samples were assessed for starting concentration and quality using a Nanodrop® ND-100 spectrophotometer (ThermoFisher Scientific, Carlsbad, California, USA). The samples were diluted to 100ng/µl using distilled Sabax water (Adcock Ingram Critical Care (Pty) Ltd, Johannesburg, Gauteng, South Africa). The integrity of the diluted DNA samples was assessed using agarose gel electrophoresis.

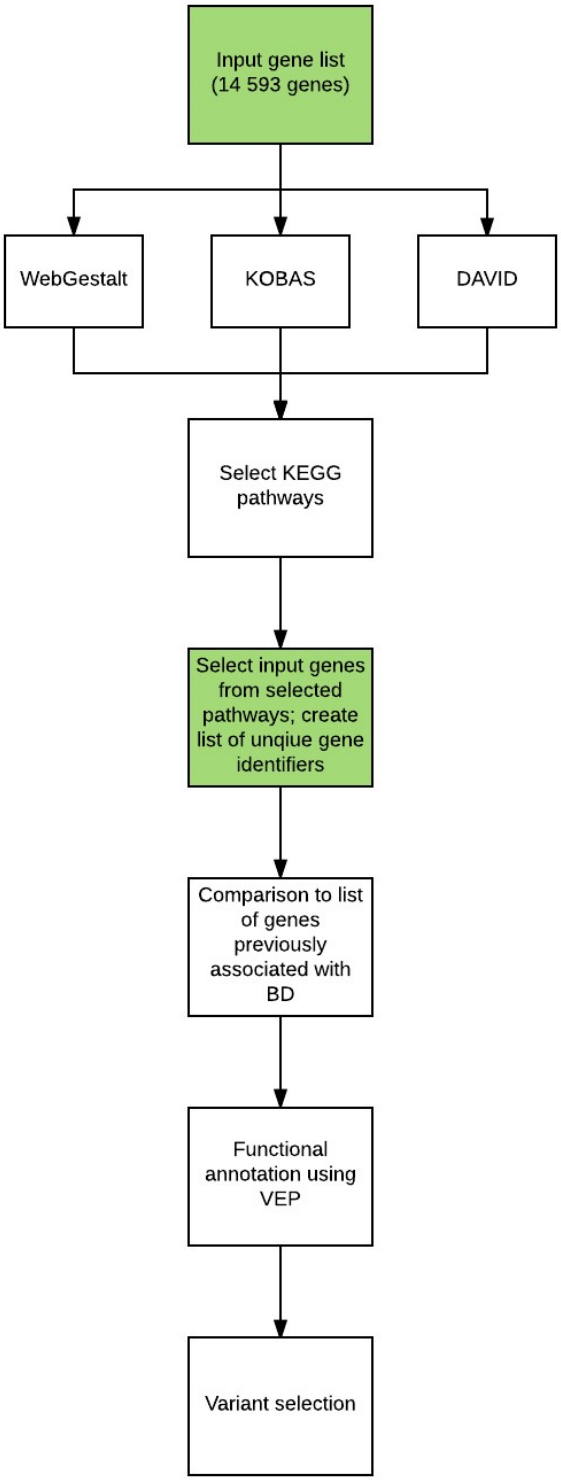


Figure 2.2: The process of performing pathway analysis and prioritising variants from the bipolar disorder-affected whole-genome sequencing data

Table 2.2: Variants selected for TaqMan® genotyping using the ThermoFisher Scientific OpenArray®

dbSNP identifier	Location	Gene	ThermoFisher Assay ID*	Source, Reason
rs4659702	1:236709945-236709945	*ACTN2*	C__32392083_10	WGS data, Actin pathway variant
rs819724	1:236713584-236713584	*ACTN2*	C__12008579_10	WGS data, Actin pathway variant
rs11355106	1:236736600	*ACTN2*	AN9HKFD	WGS data, Actin pathway variant
rs35798951	10:32962802-32962802	*ITGB1, AL365203.1*	AN7DRVF	WGS data, Actin pathway variant
rs10994318	10:60366098	*ANK3*	ANNKTXY	Stahl (2018), BD GWAS variant
rs855201	12:102484427	*IGF1*	C___7570411_10 (reverse complement)	WGS data, Focal adhesion pathway variant
rs10744560	12:2277933	*CACNA1C-IT3, CACNA1C*	C____346712_10	Stahl (2018), BD GWAS variant
rs7232062	18:23970609-23970609	*AC010754.1*	C__29014502_10 rs7232062_T_C ANT2AN4	WGS data, Focal adhesion pathway variant
rs9675777	18:3279953-3279953	*AP001025.3, MYL12B*	rs9675777 ANCE6DZ rs9675777_C_G ANYMKYU	WGS data, Focal adhesion pathway variant
rs1662809	18:3326781	*AP001025.3*	ANRWF3U	WGS data, Focal adhesion pathway variant
rs35195646	18:63236396-63236397	*BCL2*	ANAACT3	WGS data, Focal adhesion pathway variant
rs1064395	19:19250926	*AC138430.1, AC138430.2, HAPLN4, NCAN*	C___8931759_20	Stahl (2018), BD GWAS variant in LD with rs111444407
rs6130764	20:45121769	*WFDC12*	C__30439763_10	Stahl (2018), BD GWAS variant
rs133912	22:44127381	*PARVB*	C___2237909_10_	WGS data, Focal adhesion pathway variant
rs40502	5:68332444	regulatory region	C____977986_10	WGS data, Focal adhesion pathway variant
rs11761919	7:103529051-103529051	*RELN*	rs11761919_G_A ANZTFJR rs11761919_G_T AN2W94N	WGS data, Focal adhesion pathway variant
rs2177863	7:107007516	regulatory region	C__16148074_10	WGS data, Focal adhesion pathway variant

*All IDs that begin with C identify extant ThermoFisher TaqMan® probes, while IDs that begin with A denote custom probes designed for this study

2.9.1.1 AGAROSE GEL ELECTROPHORESIS TO ASSESS DNA INTEGRITY

Agarose gels were prepared to a final concentration of 1% agarose (weight/volume) using agarose (SeaKem® LE Agarose, Lonza, Basel, Switzerland) and X1 Tris-Borate-EDTA (TBE) buffer, prepared in the laboratory using the method described in Appendix E (TBE Buffer Preparation). SYBR® Safe (ThermoFisher Scientific, Carlsbad, California, USA) was used as the DNA gel staining agent for all integrity gels, and 4µg of Gene Ruler™ 100bp DNA Ladder Molecular Weight Marker (Fermentas Life Sciences, ThermoFisher Scientific, Carlsbad, California, USA) was used as the molecular weight marker to assess the size of the DNA fragments. All agarose gels underwent electrophoresis at 106V for 60 minutes and were visualised under UV light using the UVIPro Gold transillumintator (UVItec, Cambridge, UK). All resulting images were captured using the UVIPro software version 12.3.

2.9.1.2 SAMPLE PREPARATION FOR GENOTYPING

Following the assessment of the DNA integrity, 10µl (approximately 1 000ng) of each sample was transferred to a well of a 96-well plate. The 96-well plates were submitted to the Centre for Proteomic and Genomic Research (CPGR) and were kept at -20°C until genotyping commenced. The CPGR performed verification and normalisation of the sample concentrations.

2.9.2 GENOTYPING PROCESS WITH TAQMAN® OPENARRAY® REAL-TIME PCR PLATES

All genotyping was completed at the CPGR using TaqMan® OpenArray® Real-Time PCR Plates. The protocol used by the CPGR is included in the report, which is provided in Appendix F (CPGR OpenArray® Genotyping Protocol). The OpenArray® plates each contain 3 072 through holes, which have a hydrophilic core, and hydrophobic edges. This design keeps the reaction mixture suspended in the hydrophilic part of the through hole via surface tension, despite it lacking a solid base. The OpenArray® genotyping was performed using a QuantStudio™ 12K Flex Real-Time PCR System (ThermoFisher Scientific, Carlsbad, California, USA). TaqMan® probes can be used to genotype variation using real time-PCR detection of reporter fluorescence, which is not visible when the alternate allele is not present [http://www.thermofisher.com/za/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center/real-time-pcr-basics/how-taqman-assays-work.html].

2.10 FAMILY-BASED ASSOCIATION TEST

2.10.1 THE BIPOLAR DISORDER FAMILIES COHORT AND FILE CONSTRUCTION

The families selected for this analysis were chosen from the Division's BD research cohort based on the clinical records available for each family member, available familial relationship data, sufficient DNA of suitable integrity, and evidence of inherited familial affective disorders, particularly BD. The pedigree file was compiled according to general ped file standards, which include a pedigree identity

column, individual identity column, father identity column, mother identity column, individual's sex column, and a column for the affection status of the individual. These are followed by two columns per marker, one for each allele of each marker included. In order to include informative individuals for whom no genotype or clinical data was available but who were necessary to illustrate familial structure, a "0" serving to indicate an unknown affection status and/or absent genotypes. The programme PEDSTATS (Wigginton and Abecasis, 2005) was used to ensure that no pedigree errors were recorded in the ped file. PEDSTATS was also used to test whether the variants were in Hardy-Weinberg Equilibrium (HWE). A Pearson's Chi-square test was performed in R to assess the potential significance of the relative number of males versus females in the cohort.

A map file was constructed which contained 14 lines, one for each marker, and five columns as follows: the variant identity, the chromosome number of the variant, the locus start position, the locus end position, and a column to indicate whether the variant is X-linked or not. As none of the variants used in this study were on the X chromosome, all the values in this column were "0". The 14 lines in the map file were in the same order as the variant genotypes in the pedigree file.

2.10.2 MIXED ANCESTRY POPULATION OF THE WESTERN CAPE

The Mixed Ancestry population of the Western Cape of South Africa are a distinct cultural collection of individuals with its origins formed from several notable populations present in South Africa from the mid-17th century. This population arose from the miscegenation of European settlers, principally Dutch colonialists, who arrived from 1652 along the west coast of the country, with the local San and Khoikhoi populations as well as slaves imported from eastern countries such as Mozambique and Madagascar, Indonesia, and Bengal (Marais, 1968; Venter, 1974). Recent sequencing studies of Mixed Ancestry individuals have confirmed the presence of European, Bantu, Khoisan and South Asian components of the Mixed Ancestry genome (Choudhury et al., 2017). The majority of the individuals in the Division's research cohort, and the majority of the individuals genotyped for FBAT were Mixed Ancestry, and thus the ancestral sources of the genomic regions that contain the variants under investigation are not known, but may be attributed to the ancestral populations.

2.11 FAMILY-BASED ASSOCIATION TESTING

The map and pedigree files were loaded to the FBAT programme using Linux terminal FBAT commands, as per the FBAT manual. A short example is provided in Figure 2.3. The command in line three of Figure 2.3, model [r/d/a], was used to specify the genetic model as either recessive, dominant, or additive. An additive model is the default in FBAT software. In order to change the offset (μ) to the disease prevalence to maximise the FBAT, line four provides the example command. In this case, the population prevalence of the disease was obtained from global epidemiological studies (Merikangas et al., 2011, 2007) and the SASH (Herman et al., 2009). The command in line five of Figure 2.3 is used

to perform the FBAT. FBAT was performed on the research group using additive, recessive, and dominant models, both with and without offsets. The research group in the ped file was then split to contain either Caucasian or Mixed Ancestry individuals in order to observe possible associations that may exist in each of these particular ethnic groups but not in the other. Correction for multiple testing was applied using the Bonferroni method. The statistical significance threshold after this correction was 0.0036.

```
>> load map /path/to/map_file.txt
>> load /path/to/ped_file.ped
>> model [r/d/a]
>> offset [prevalence]
>> fbat
```

Figure 2.3: Basic commands to load pedigree and map files to FBAT, and to run the FBAT analysis

2.12 *IN SILICO* PREDICTION OF FUNCTIONAL EFFECTS OF ASSOCIATED VARIATION

Non-coding intronic and intergenic variants identified as significantly associated with any psychiatric phenotype by FBAT were further assessed *in silico* for putative functional effects on transcription factor binding using ConSite [http://consite.genereg.net/cgi-bin/consite] (Sandelin et al., 2004). The genomic sequence without the variant, as well as the sequence with the variant allele present is provided as input to ConSite, in order to determine whether any transcription factor binding sites are disturbed by the presence of the variant allele. All genomic sequences were obtained from NCBI [https://www.ncbi.nlm.nih.gov/].

2.13 WHOLE-EXOME SEQUENCING

In order to investigate rare, putatively damaging exomic variation that may be involved in BD, WES was performed on eight members of Family 30 using Ion Torrent semiconductor methodology (Rothberg et al., 2011), as WGS did not indicate the presence of any variation in exons. These eight individuals included a nuclear family of four (BPD 30.27, BPD 30.28, BPD 30.29, and BPD 30.30), allowing examination of allele transmission between generations, and three affected siblings (BPD 30.8, BPD 30.10, and BPD 30.50), which facilitated analysis of variants in one generation. The siblings are the maternal cousins to the nuclear family, thus family-specific, and potentially Afrikaner-specific variation could be assessed. An unaffected cousin, BPD 30.5, was included based on her lack of any psychiatric disorder during continuous assessment over several years. Four of these family members (listed in in Table 2.3 below) were also analysed by WGS (see Section 2.3). With the Ion Torrent method, unlike the Illumina system, there is no visual detection of the nucleotide, and nucleotide

incorporation into a growing strand is detected based on pH changes after exposure to a known nucleotide solution (either dATP, dTTP, dGTP, or dCTP). The dNTPs are released consecutively in a particular order. Unfortunately, WES only became available after the TaqMan® OpenArray® Real-Time PCR Plates had been ordered, and thus no variation detected using WES could be included in FBAT analysis.

2.13.1 EXOME PREPARATION

2.13.1.1 DNA QUANTIFICATION

The 100ng/µl sample DNA dilutions were quantified using the RNAse P assay outlined in the manufacturer's protocol (ThermoFisher Scientific MAN0007732) using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA). This assay makes use of a real-time PCR to compare the rate of amplification of the sample DNA relative to a known quantity and concentration of *ribonuclease P* (*RNAse P*) DNA.

Table 2.3: Type of next generation sequencing performed on members of Family 30

Individual ID	Whole-Genome Sequencing	Whole-Exome Sequencing	Affection Status (BD-type)
BPD 30.5	No	Yes	Unaffected (no diagnosis)
BPD 30.8	No	Yes	Affected (BD-I)
BPD 30.10	Yes	Yes	Affected (BD-I)
BPD 30.27	No	Yes	Unaffected (single MDE)
BPD 30.28	No	Yes	Unaffected (no diagnosis)
BPD 30.29	Yes	Yes	Affected (BD-I)
BPD 30.30	Yes	Yes	Affected (BD-I)
BPD 30.50	Yes	Yes	Affected (BD-I)

2.13.1.2 LIBRARY PREPARATION AND ENRICHMENT

The exome libraries for each sample were prepared with the Ion Ampliseq™ Exome RDY Library Preparation kit, according to the protocol provided by the manufacturer (ThermoFisher Scientific MAN0010084). This was performed using the SimpliAmp™ Thermal Cycler (Applied Biosystems, Waltham, Massachusetts, USA) for all reactions. Library enrichment was completed by means of capture probes attached to magnetic beads and these captured probes were amplified as described in the manufacturer's protocol (ThermoFisher Scientific MAN0010967).

2.13.1.3 TEMPLATING, SEQUENCING PROCEDURE, ALIGNMENT, AND VARIANT CALLING

The Ion PI™ chip was used for WES of each individual. The templating and preparation of the PI WES chips (ThermoFisher Scientific, Carlsbad, California, USA) was completed with the Ion Chef™ (ThermoFisher Scientific, Carlsbad, California, USA), according to the manufacturer's instructions (ThermoFisher Scientific MAN0010967). Sequencing of the chip's contents was performed using an Ion Proton™ (ThermoFisher Scientific, Carlsbad, California, USA) according to the manufacturer's instructions. Alignment was completed using the Ion Torrent™ Suite on the Ion Torrent™ server, and samples were aligned to the hg19 reference genome. This process generated bam files which were uploaded to Ion Reporter™ version 5.6 for further analysis. Variant calling was undertaken by selecting the AmpliSeq Exome HiQ (Germline) workflow for the HiSeq sequencing kit. Ion Reporter™ analysis facilitates visualisation of data, and both VCF and tsv files of the sample variation can be downloaded.

2.13.2 WHOLE-EXOME SEQUENCING ANALYSIS

2.13.2.1 VARIATION PRESENT IN AFFECTED INDIVIDUALS

The pedigree in Figure 2.4, generated using PhenoTips® (Girdea et al., 2013), illustrates the individuals from a family that were selected for WES in their family structural context. There is one nuclear family which comprises of a father (BPD 30.29), mother (BPD 30.27), one affected son (BPD 30.30), and one unaffected daughter (BPD 30.28). The second group of individuals selected for WES are the maternal cousins of individual BPD 30.29, and this group comprised three siblings (BPD 30.10, BPD 30.8, and BPD 30.50), and their paternal cousin (BPD 30.5). The variants which were exclusively present, either as heterozygous or homozygous SNPs, in all affected individuals given in Table 2.3 above, and not in the unaffected individuals, were determined by first analysing the nuclear family as an isolated group, followed by the siblings and their paternal cousin as an isolated group.

The variation present in the affected father and son (BPD 30.29 and BPD 30.30) was observed using the "Visualize" function in the web-based Ion Reporter™ software. This overlap which was not present in either the unaffected mother or daughter (BPD 30.27 and BPD 30.28) was retained. These variants were then uploaded to the web-based version of ANNOVAR, wANNOVAR (Wang et al., 2010; Yang & Wang, 2015). Similarly, the variants which were present in each of the three siblings (BPD 30.10, BPD 30.8, and BPD 30.50) were observed using the "Visualize" function in Ion Reporter™ software package. The variation which was also present in the unaffected paternal cousin (BPD 30.5) was excluded, and the file of variation was also uploaded to wANNOVAR for annotation. The next step was to determine the variation which occurred in all affected individuals. Figure 2.5 illustrates this graphically. The wANNOVAR output generated dbSNP identifiers which could be used to determine the overlapping variation between all affected individuals.

2.13.2.2 FILTERING OF VARIANTS IN AFFECTED INDIVIDUALS

The variation of the affected individuals was annotated with dbSNP identifiers as well as prediction scores for several programmes, 1000 Genomes MAF, ExAC frequencies, and other information (for example, OMIM associations), by wANNOVAR. Scores from prediction tools such as Sorting Intolerant From Tolerant (SIFT) and Polymorphism Phenotyping version 2 (PolyPhen-2), which can be used to predict the potential pathogenicity of variants (Adzhubei et al., 2013; Kumar et al., 2009), are included in the standard wANNOVAR output. Both SIFT and PolyPhen-2 were considered when identifying pathogenic variants in the affected individuals. The database BDgene was used once again in order to determine whether any of the genes or variants identified by WES had been previously associated with BD.

2.13.2.3 PATHWAY ANALYSIS OF EXOMIC VARIATION SHARED BY AFFECTED INDIVIDUALS

In order to determine whether broader genomic variation and exomic variation indicated similar pathways involved in BD, pathway analysis of WES data was completed using KOBAS 3.0 [http://kobas.cbi.pku.edu.cn/] (Xie et al., 2011). A single program was used for simplicity, given the similarity of results for KEGG-annotated pathways identified by DAVID, KOBAS 3.0, and WebGestalt. KOBAS 3.0 was used as previously described (Section 2.7).

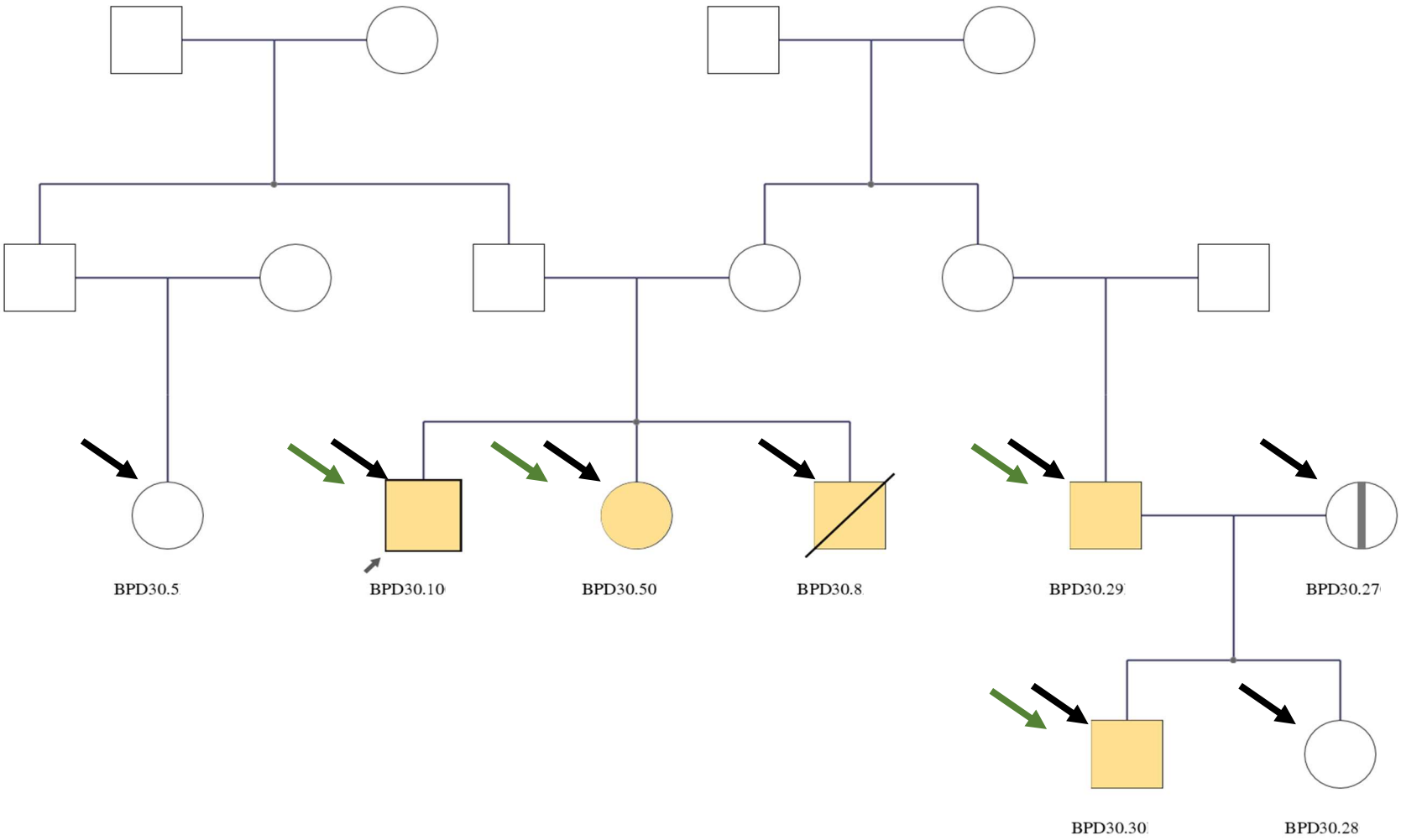


Figure 2.4: Pedigree of Family 30 individuals selected for whole-exome sequencing

The individuals for whom WES was performed are indicated by a large black arrow. Shaded individuals indicate affected persons, while a vertical bisecting line indicates a case of a single MDE. Squares represent males, while circles represent females. A diagonal line through a square or circle indicates a deceased individual. A small grey arrow in the bottom left-hand corner of the shape indicates the proband. A large green arrow indicates the family members for whom WGS was performed

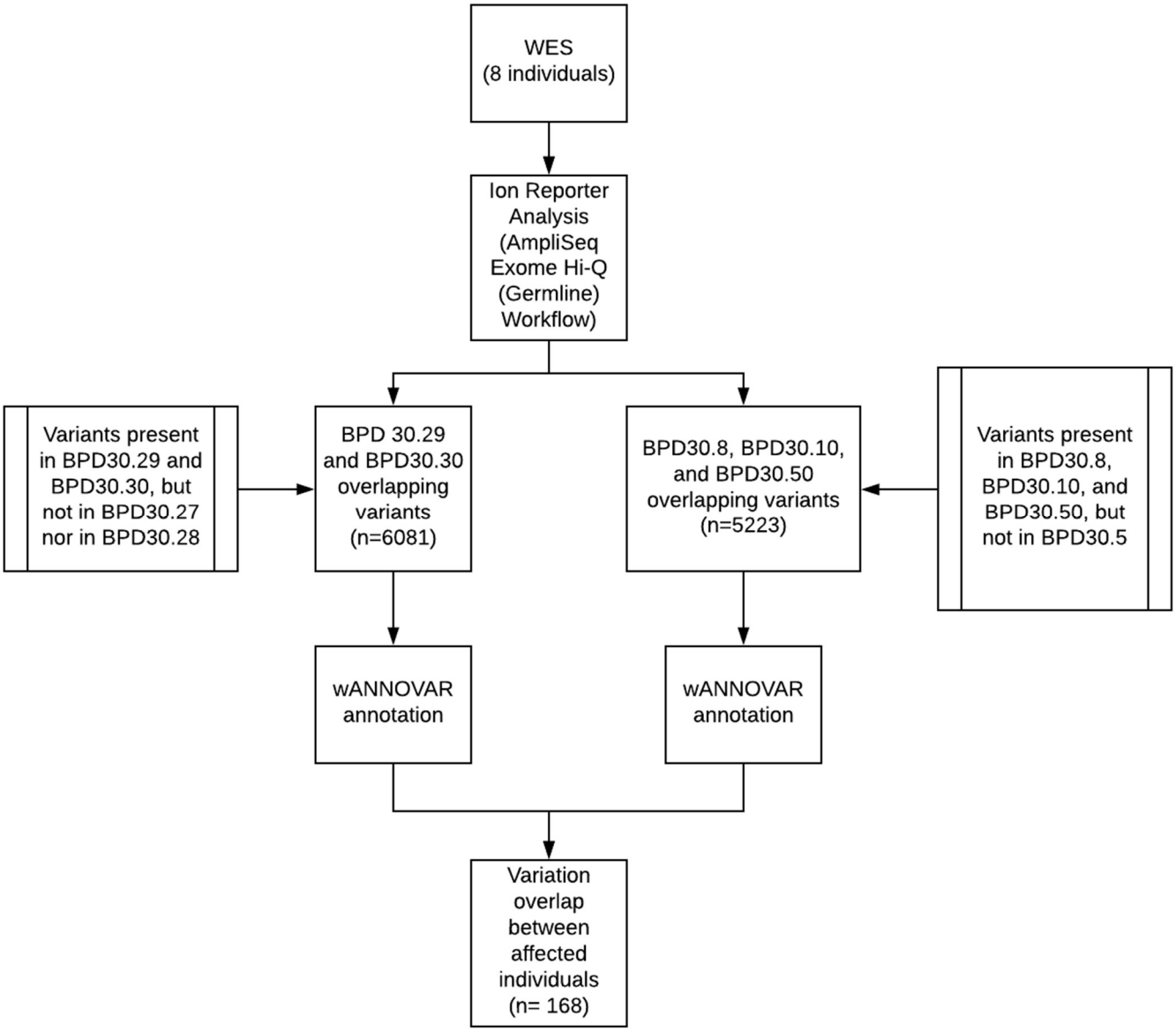


Figure 2.5: Graphical illustration of the workflow used to determine the variation common to all affected individuals used in whole-exome sequencing

CHAPTER 3: RESULTS

3.1 OVERVIEW

This study is an investigation in two parts in order to investigate both common and rare variation that may underlie familial BD. Figure 3.1 is an overview of this study, describing the steps performed for each of the two parts of the study.

Part 1 (common variation): WGS data of four BD-affected family members was compared to Dutch and German controls, and the WGS variation present in affected individuals was used for pathway analysis. The pathways which were identified by three programmes were assessed, and variants were selected from genes in each of these pathways to assess common variation in familial BD, which were then extended to other BD families. In addition, variants were selected from a recent BD GWAS. The selected variants were genotyped and analysed using FBAT.

Part 2 (rare variation): In order to investigate rare variation that may be involved in the aetiology of BD, WES was performed for eight family members, five of whom were affected with BD. The variation detected in only the affected individuals was retained for analysis, which included pathogenicity prediction, MAFs, and pathway analysis.

3.2 PRINCIPAL COMPONENT ANALYSIS

In a previous study, WGS had been performed on four members of Family 30. In the absence of WGS data for an unaffected family member, a PCA was conducted to illustrate whether individuals of Dutch ancestry would serve as appropriate controls for the Afrikaner individuals. Afrikaners are a South African population of mixed ancestry from several populations, as discussed in section 2.2. Figure 3.2 is a PCA of whole-genome genotype data of several world populations and the Afrikaner family 30 members. In Figure 3.2, the European (Iberians from Spain, Finnish, British and Scottish, and Dutch) and Puerto Rican populations are most closely clustered with the Afrikaner Family 30 individuals, in the upper left corner. The Asian populations cluster densely in the upper right corner, while the African and African-American populations tend to cluster towards the lower central point of the figure. The Puerto Rican individuals, as can be expected given the history of that country, are an admixed population with no defined cluster. Based on the data available for this study, this analysis demonstrates that the Dutch represent a suitable proxy as a control for the Afrikaner family.

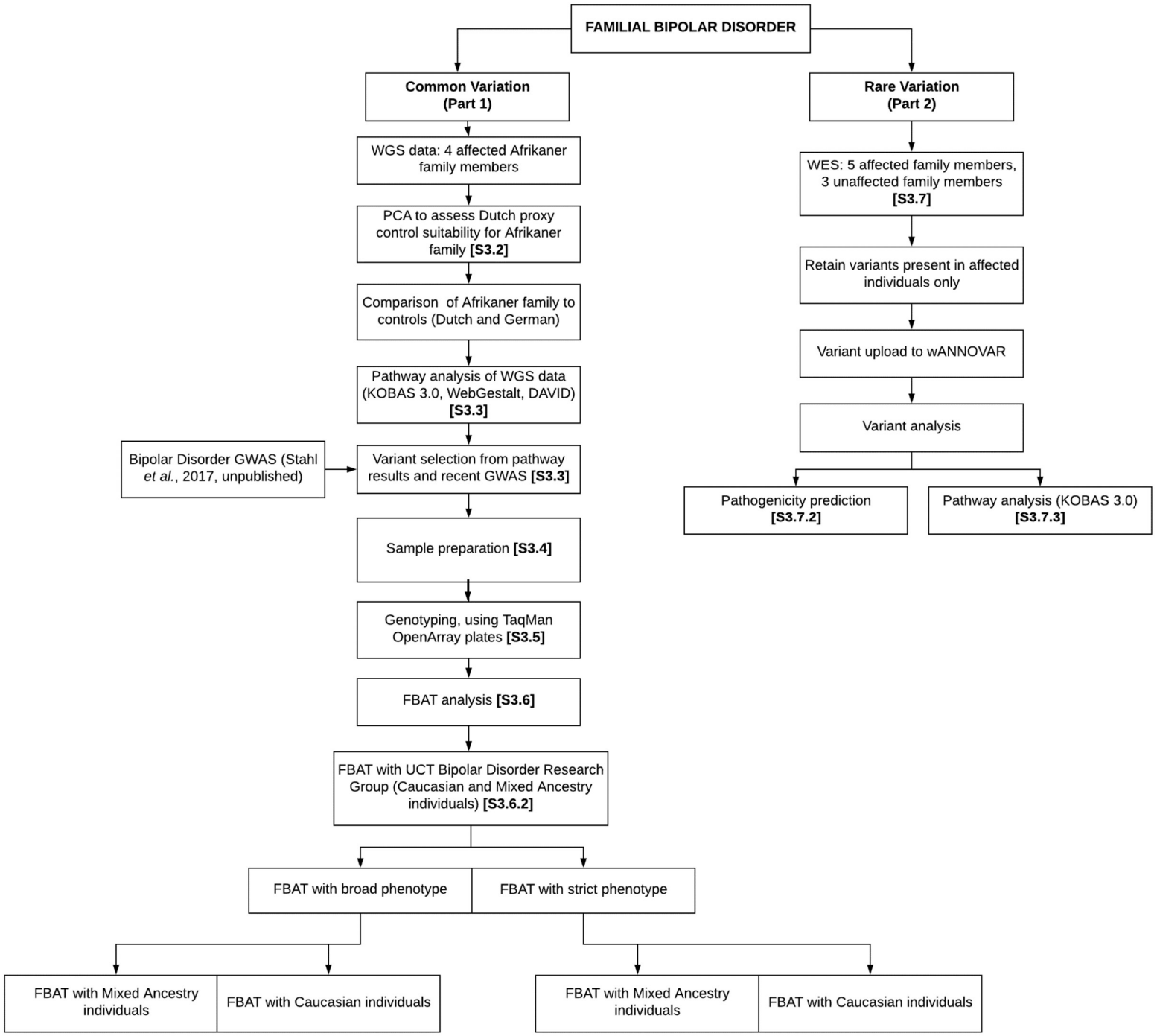


Figure 3.1: Overview of the study design and the presentation of results

The numbers in square brackets indicate the corresponding sections in this chapter describing the results

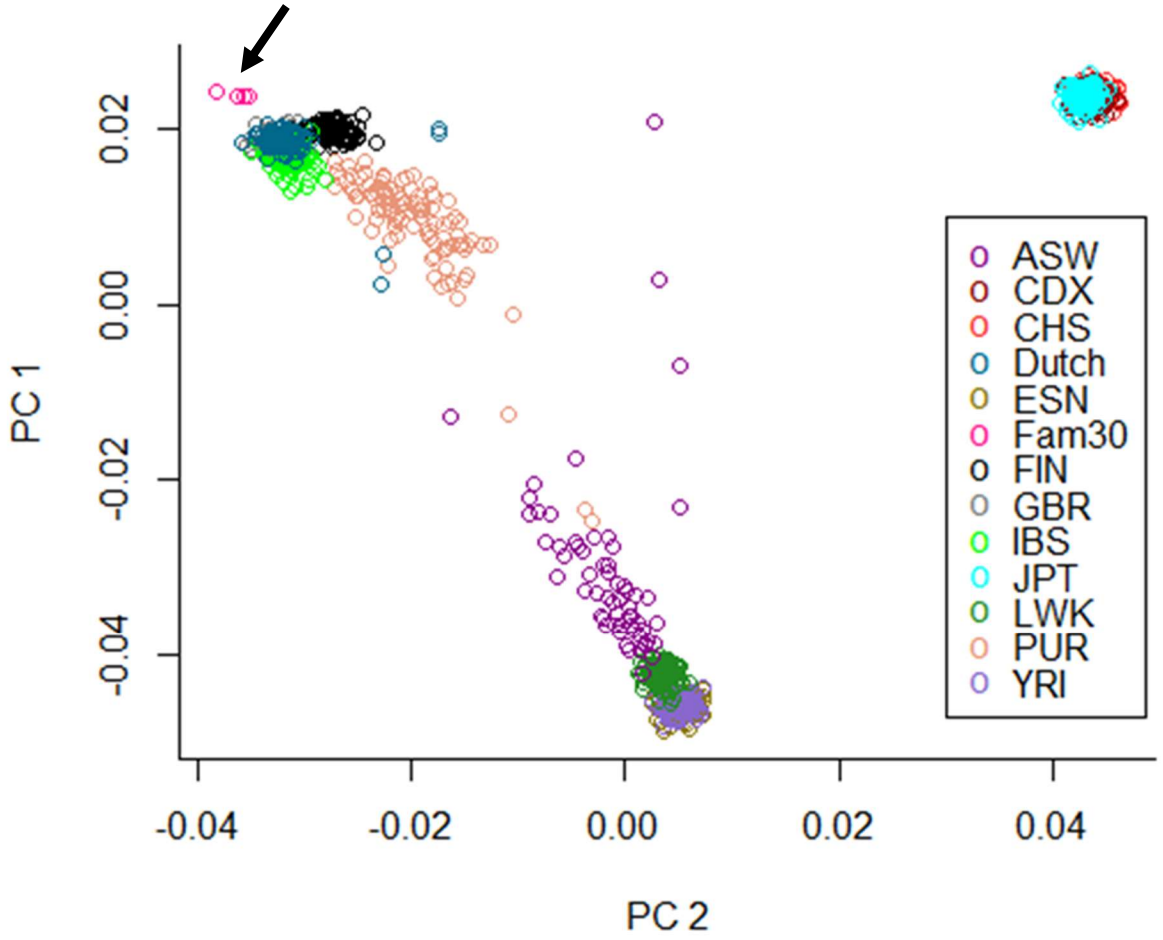


Figure 3.2: Principal component (PC) analysis of 12 populations and the Afrikaner Family 30 individuals

The population groups indicated are African ancestry in South-West USA (ASW), Xishuangbanna in China (CDX), Southern Han Chinese (CHS), Dutch, Esan in Nigeria (ESN), Afrikaner Family 30 (Fam30 – indicated in the plot by a black arrow), Finnish (FIN), English and Scottish (GBR), Iberians in Spain (IBS), Japanese in Tokyo (JPT), Luhya in Kenya (LWK), Puerto Ricans from Puerto Rico (PUR), and Yoruba in Nigeria (YRI)

3.3 PATHWAY ANALYSIS OF WHOLE-GENOME SEQUENCING DATA AND VARIANT SELECTION

Gene-based pathway analysis was performed based on the variants from WGS that were present exclusively in the BD-affected individuals, not the Dutch or German controls. Only intronic and intergenic variation was identified, and these variants were annotated to the closest gene, and the list of genes that emerged was submitted to three pathway analysis platforms, KOBAS 3.0, DAVID, and WebGestalt. KEGG-based pathway analysis indicated that many of the genes constituted part of a regulatory network, or typical cell function, as can be seen in Table 3.1. Four pathways were highly ranked in the ten most over-represented pathways across all three programmes: *pathways in cancer* (hsa05200), *focal adhesion* (hsa04510), *regulation of the actin cytoskeleton* (hsa04810), and the *Wingless-type MMT (Wnt) signalling* pathway (hsa04310). The *pathways in cancer* pathway was excluded from further consideration as it is relatively broad, and encompasses several other pathways, including *Wnt signalling*, *calcium signalling*, and *MAPK signalling* [http://www.genome.jp/kegg-

bin/show_pathway?hsa05200]. After this pathway, the *focal adhesion* and *regulation of the cytoskeleton* were the next two pathways ranked most highly by KOBAS 3.0, DAVID, and WebGestalt. The *focal adhesion pathway* is involved in a broad range of cellular function, including cell growth, differentiation, and proliferation. Focal adhesions refer to connections between the cell and the extracellular matrix. The *focal adhesion pathway* is thus intrinsically linked to the regulation of the actin cytoskeleton, as focal adhesions directly involve links between integrins and the actin cytoskeleton of the cell (Sastry and Burridge, 2000).

Variants from the WGS data which had been annotated to the genes of either the *regulation of the actin cytoskeleton*, or the *focal adhesion* pathways, were investigated. Variants with MAFs between 0.1 and 0.5 were prioritised as putatively involved, as the current hypothesis for psychiatric illness is that both rare and common variants may be involved in disease aetiology (Visscher et al., 2012). Variants from the pathways above were selected, as well as variants from the largest BD GWAS to date (Stahl et al., 2018). The variants selected for genotyping are listed in Table 3.4 below. The variant rs1064395, in the gene *neurocan* (*NCAN*), was used as a proxy for rs111444407 from Stahl et al. (2018), as a catalogue TaqMan® probe was available for the former variant, while it was not guaranteed that a custom probe could be successfully designed for the latter. These variants are only 3 528 bp apart in *NCAN*, and are in high LD in European, African, and Chinese populations as a result of their proximity, according to predictions from LDlink (both D` and $r^2 > 0.9$) [https://analysistools.nci.nih.gov/LDlink/] (Machiela and Chanock, 2015). The variants listed in Table 3.2 were genotyped with the TaqMan® OpenArray®.

Table 3.1: The ten most over-represented KEGG pathways across three programmes

KOBAS 3.0			DAVID			WebGestalt		
Pathway Name	**Pathway ID**	**Corrected *P*-Value**	**Pathway Name**	**Pathway ID**	**Corrected *P*-Value**	**Pathway Name**	**Pathway ID**	**Corrected *P*-Value**
*Pathways in cancer**	hsa05200	0.0011	*Pathways in cancer*	hsa05200	0.0001	Metabolic pathways	hsa01100	0.0000
Axon guidance	hsa04360	0.0880	*Focal adhesion*	hsa04510	0.0005	*Pathways in cancer*	hsa05200	0.0000
PI3K-Akt signalling pathway	hsa04151	0.0067	ErbB signalling pathway	hsa04012	0.0132	*Focal adhesion*	hsa04510	0.0000
cAMP signalling pathway	hsa04024	0.0131	*Regulation of actin cytoskeleton*	hsa04810	0.0335	MAPK signalling pathway	hsa04010	0.0000
Metabolic pathways	hsa01100	0.0159	Renal cell carcinoma	hsa05211	0.0337	*Regulation of actin cytoskeleton*	hsa04810	0.0000
Focal adhesion	hsa04510	0.0172	*Wnt signalling pathway*	hsa04310	0.0673	Neuroactive ligand-receptor interaction	hsa04080	0.0000
Rap1 signalling pathway	hsa04015	0.0193	Glioma	hsa05214	0.2347	Olfactory transduction	hsa04740	0.0000
Regulation of actin cytoskeleton	hsa04810	0.0378	B cell receptor signalling pathway	hsa04662	0.3035	Endocytosis	hsa04144	0.0000
Neuroactive ligand-receptor interaction	hsa04080	0.0383	Vascular smooth muscle contraction	hsa04270	0.3406	*Wnt signalling pathway*	hsa04310	0.0000
Wnt signalling pathway	hsa04310	0.0387	Colorectal cancer	hsa05210	0.3419	Chemokine signalling pathway	hsa04062	0.0000

*Italicised pathway names indicate the pathways which are the in the ten most over-represented KEGG pathways across all three programmes

3.4 SAMPLE INTEGRITY VERIFICATION AND PREPARATION

Samples from the Division's BD project were selected for genotyping based on a family history of BD and other psychiatric disorders, as well as the presence of clinical records, and sufficient DNA. The integrity of the DNA samples used for genotyping on the ThermoFisher OpenArray® was assessed using agarose gel electrophoresis. Figure 3.3 provides an example of the integrity of the DNA samples used for this analysis. A degraded sample, with little intact DNA, is indicated with an arrow in lane M. Each well, A-P, was loaded with DNA from different individuals. Ma denotes the molecular weight marker, used to assess the size of the visible bands in bp. The bands below the wells A-P are sufficiently intact, except for the sample in lane M. Some smearing of sample DNA is evident in wells D, E, F, G, J, K, N, O, and P, but sufficient DNA of usable integrity is present. Well C is empty, indicating that there is little DNA in the sample that was loaded onto the gel. All samples presented in Figure 3.3, aside from those in wells C and M, were sufficiently intact to use for genotyping.

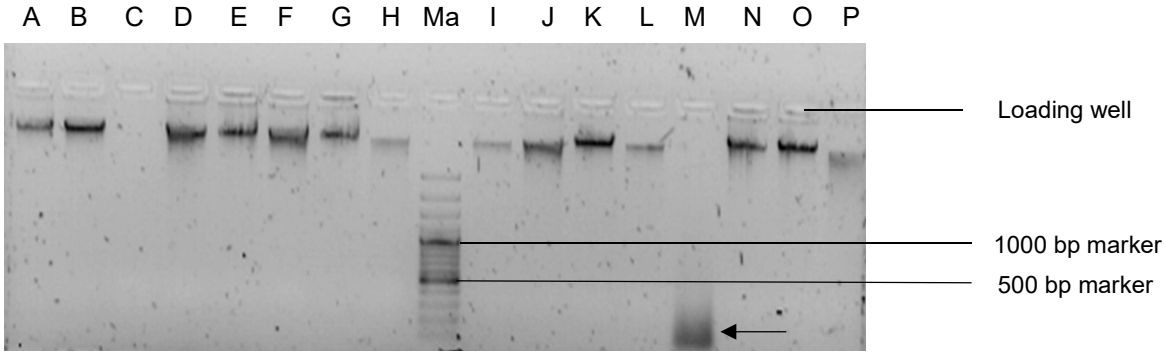


Figure 3.3: An inverted image of a 1% agarose gel used for the assessment of DNA integrity

The arrow pointing to lane M indicates a degraded sample with little DNA integrity. 'Ma' denotes the Gene Ruler™ 100bp DNA Ladder Molecular Weight Marker (Fermentas Life Sciences, ThermoFisher Scientific, Carlsbad, California, USA)

3.5 GENOTYPING WITH TAQMAN® OPENARRAY® REAL-TIME PCR PLATES

All genotyping was performed using the OpenArray® TaqMan® platform, at the CPGR in Cape Town, South Africa. In total, 14 variants were successfully genotyped in approximately 470 individuals, as can be seen in Table 3.2. For several TaqMan® assays, the samples were monomorphic and as such the following variants were excluded from further analysis: rs2177863, rs855201, and rs1662809. A number of variants were not genotyped successfully in a number of individuals as the genomic DNA did not amplify sufficiently for the reaction (NOAMP), or the genotype could not be accurately resolved (UND), or the genotype data fell outside of the accepted range for either homozygous wildtype, heterozygous, or homozygous alternate allele genotypes (INV) and were deemed invalid. The wildtype alleles for rs11355106 (a single bp deletion) and rs6130764 (T) are not the ancestral alleles but are the more prevalent (and thus inferred wild type) alleles.

Table 3.2: Total number of successfully genotyped individuals for the selected variants

Variant	No Amplification	Undetermined	Invalid	Homozygous Reference Allele Genotype (n)	Heterozygous Genotype (n)	Homozygous Alternative Allele Genotype (n)	Total Genotyped Individuals
rs7232062	22	25	0	TT (177)	TG (163)	GG (122)	462
rs9675777	58	3	0	CC (52)	CT (81)	TT (315)	448
rs35195646	19	3	2	AA (313)	Adel (153)	deldel (19)	485
rs11761919	44	42	1	GG (111)	GA (180)	AA (131)	422
rs11355106	15	35	0	deldel (183)	Cdel (276)	CC (0)	459
rs40502	11	16	0	TT (481)	CT (1)	CC (0)	482
rs2177863	23	1	1	CC (490)	CT (0)	TT (0)	490 (monomorphic)
rs819724	19	2	0	AA (306)	AG (170)	GG (12)	488
rs4659702	38	6	1	TT (178)	TC (222)	CC (64)	464
rs35798951	23	5	0	Ins/ins (199)	Ins/A (195)	AA (87)	481
rs855201	31	1	0	AA (482)	AG (0)	GG (0)	482 (monomorphic)
rs10994318	5	85	0	GG (15)	CG (404)	CC (0)	419
rs10744560	24	13	0	CC (252)	CT (173)	TT (47)	472
rs1662809	48	1	1	AA (457)	AC (0)	CC (0)	457 (monomorphic)
rs1064395	20	0	0	GG (264)	GA (189)	AA (36)	489
rs6130764	13	16	0	TT (178)	CT (215)	CC (87)	480
rs133912	19	3	0	TT (486)	GT (1)	GG (0)	487

'Del' indicates a single base pair deletion, while 'ins' denotes a single base pair insertion

3.6 FAMILY-BASED ASSOCIATION TEST

3.6.1 FAMILIAL INFORMATION USED FOR THE FAMILY-BASED ASSOCIATION TEST

The genotypes for the individuals for whom data was available from the OpenArray® genotyping were integrated into a pedigree file with the familial relationships, sex, and affection status of each individual recorded. This file was investigated for any errors using PEDSTATS (Wigginton and Abecasis, 2005). The final pedigree file contained 910 individuals across 105 families. PEDSTATS is able to provide a wide array of summary information regarding the pedigree file, including the family structure, as well as the distribution of sex and disease status. As is evident in Figure 3.4(aii), two generations were collected but in some cases as many as five generations were available and included. The most frequent number of individuals per family was three, but in two families, there was information available for 50 -60 family members. A total of 401 sibling pairs (Figure 3.4bi), 37 half-sibling pairs (Figure 3.4bii), 227 cousin pairs (Figure 3.4b.iii), 477 parent-child pairs (Figure 3.4bv), and 114 grandparent-grandchild pairs were observed in the pedigree file (Figure 3.4biv). Information regarding affection status was available for 621 of the 910 individuals.

The affection status of the remaining 288 individuals was unknown and recorded as such in the pedigree file. Of these 621 individuals, 194 were affected with BD-I (156 individuals), BD-II (35 individuals), or BD-NOS (three individuals), with a larger proportion of females (n = 105) to males (n = 89). The remainder were either unaffected, or affected with a psychiatric disorder other than BD, including SCZ, schizoaffective disorder, MDD, OCD, specific phobias, psychotic disorder not-otherwise-specified, and alcohol abuse and/or dependence. The individuals classified as controls in the strict BD research group were also predominantly female (n = 264). For the second phase of FBAT analysis, individuals affected with these other psychiatric disorders were classified as affected in a broad spectrum psychiatric phenotype (n = 398), and the majority of the affected individuals were female and of Mixed Ancestry (total females n = 229; MA females n = 137; females n = 92). This is demonstrated in Table 3.3. A Pearson's Chi-square test indicated that while there are more females than males in this sample group, it is not statistically different ($p = 0.0848$).

Table 3.3: Demographic information of the affected and unaffected individuals of the research group

	Males [%]	Females [%]	Total [%]
Cases (Strict BD)	89 [46] (45 MA + 44 C)	105 [54] (67 MA + 38 C)	194 (112 MA + 82 C) [58 MA + 42 C]
Controls	163 [38] (85 MA + 78 C)	264 [62] (147 MA + 117 C)	427 (232 MA + 125 C) [54 MA + 46 C]
Cases (Broad Psychiatric Phenotype)	169 [42] (94 MA + 75 C)	229 [58] (137 MA + 92 C)	398 (231 MA + 167 C) [58 MA + 42 C]
Controls	83 [37] (36 MA + 47 C)	140 [63] (77 MA + 63 C)	223 (113 MA + 110 C) [51 MA + 49 C]

MA denotes Mixed Ancestry individuals, while C indicates individuals of Caucasian ancestry

3.6.2 PERFORMING THE FAMILY-BASED ASSOCIATION TEST

Briefly, FBAT was performed for the following groups under the following conditions:

i) Strict BD phenotype

- Dominant, recessive, and additive genetic models
- Offset 0, and offset 0.01
- Combined ethnicities (Mixed Ancestry and Caucasian individuals), and split ethnicities

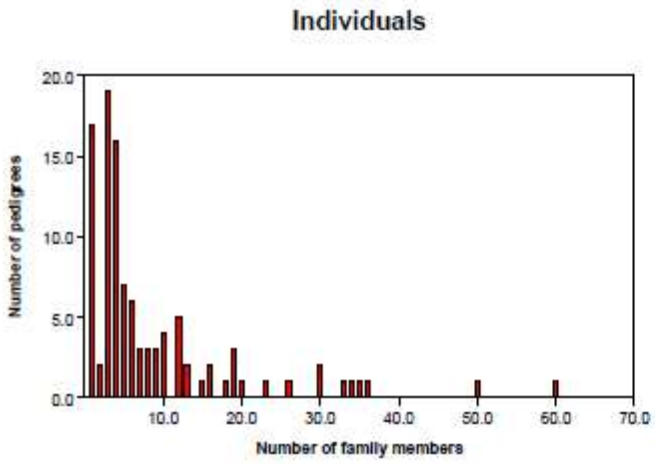
ii) Broad spectrum psychiatric phenotype

- Dominant, recessive, and additive genetic models
- Offset 0, and offset 0.3
- Combined ethnicities (Mixed Ancestry and Caucasian individuals), and split ethnicities

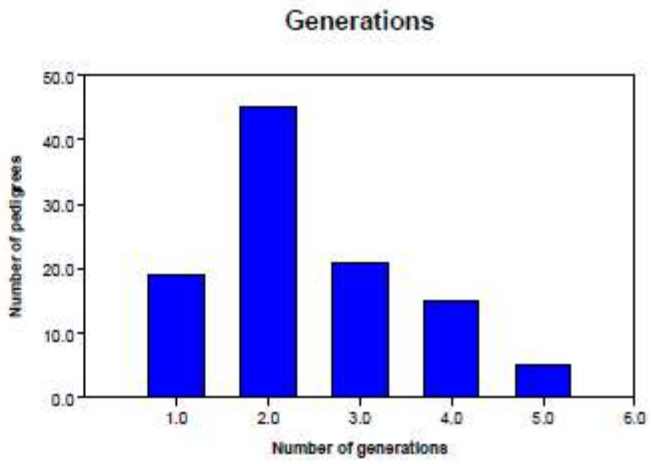
FBAT immediately excluded variants which displayed non-Mendelian inheritance patterns within families. FBAT was initially performed considering only individuals with BD-I, BD-II, or BD-NOS (strict phenotype) as affected. The affection status of the other individuals was considered either unaffected, or as unknown. Both ancestry groups (Caucasian and Mixed Ancestry) were analysed together. In this test, when using a recessive or dominant genetic model, a marginally significant association was observed for the variant rs10994318, in *ankyrin 3* (*ANK3*), and BD (p = 0.0414) with

an offset of 0, as can be seen in Table 3.4. The marginal significance of the rs10994318 association was maintained under recessive and dominant models when the offset μ was increased to 0.01, to reflect the 1% global prevalence of BD (Merikangas et al., 2011, 2007). However, this association was not observed under an additive genetic model. When the research group was split by ethnicity (Mixed Ancestry, n = 508; Caucasian, n = 401) and a strict BD phenotype, the only variant to reach significance was rs11355106 in *actin 2* (*ACTN2*) (p = 0.0397) in the Mixed Ancestry group. The variant rs4659702 in *ACTN2*, was significantly associated with the strict BD phenotype when analysing the combined ethnicities (p = 0.0339; μ = 0.01), under a recessive and dominant inheritance model, and this association was maintained when assessing the Caucasian strict BD phenotype under a recessive or dominant model (p = 0.0449; μ = 0.01), and when assessing a broad spectrum psychiatric phenotype in the individuals of Mixed Ancestry under an additive inheritance model (p = 0.0224; μ = 0.3), as can be seen in Table 3.5. This variant approached significant association with a broad psychiatric phenotype in the Mixed Ancestry group (p = 0.0595; μ = 0.3) under a dominant or recessive model, as shown in Table 3.6. All results can be found in appendix G (FBAT Results). No variants remained significant after correction for multiple testing (Bonferroni corrected p-value threshold = 0.0036). Any significant values referred to hereafter imply significance without consideration of the Bonferroni correction threshold. The intention underlying this study is to focus on biological significance, rather than a zealous over-interpretation of statistical significance, as determining biological importance through statistical thresholds is not always appropriate nor is it necessary (Altman and Krzywinski, 2015; Wasserstein and Lazar, 2016).

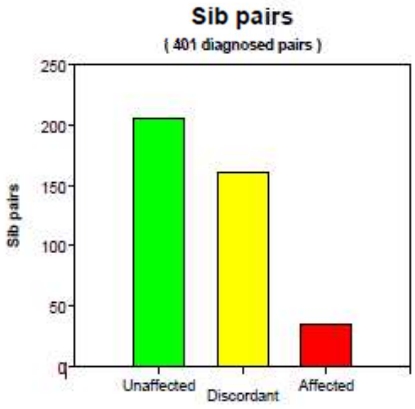
3.4.a.i



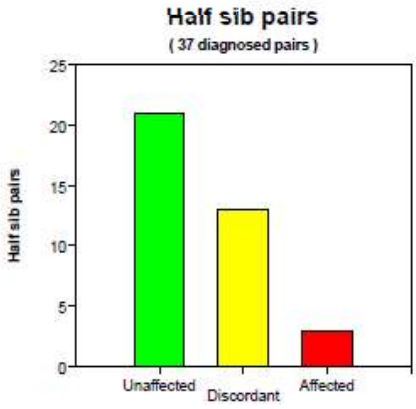
3.4.a.ii



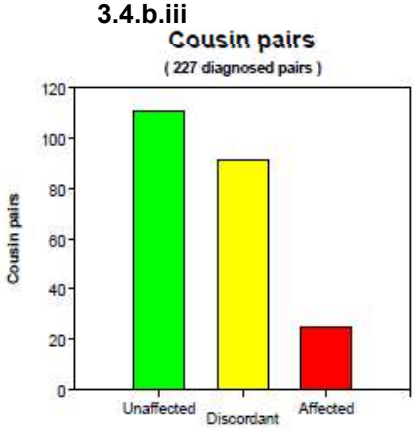
3.4.b.i



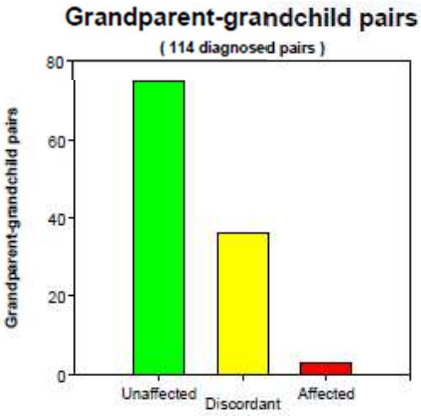
3.4.b.ii



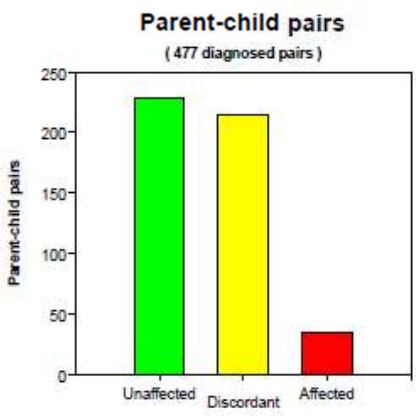
3.4.b.iii



3.4.b.iv



3.4.b.v



3.4.b.vi

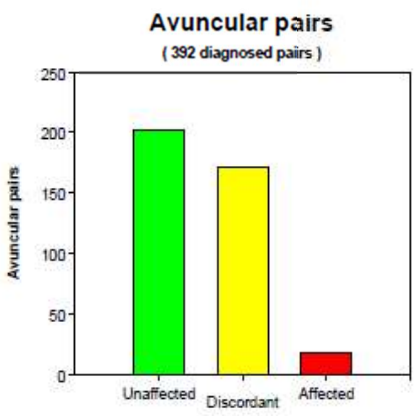


Figure 3.4: A summary of family structure observed in the pedigree file

Table 3.4: Variants significantly associated with bipolar disorder under dominant and recessive inheritance models in a research group of Caucasian (C) and Mixed Ancestry (MA) individuals (before correction for multiple testing)

Ethnicity	Phenotype	Model	Offset (μ)	Marker	Gene	Allele	Allele Frequency	Number of Informative Families	*P*-Value
MA+C	Strict	Dominant	0.01	rs4659702	*ACTN2*	T	0.614	14	0.0339
MA+C	Strict	Recessive	0.01	rs4659702	*ACTN2*	C	0.386	14	0.0339
MA+C	Strict	Dominant	0.01	rs10994318	*ANK3*	C	0.484	23	0.0443
MA+C	Strict	Recessive	0.01	rs10994318	*ANK3*	G	0.516	23	0.0443
MA+C	Strict	Dominant	0.00	rs10994318	*ANK3*	C	0.484	23	0.0414
MA+C	Strict	Recessive	0.00	rs10994318	*ANK3*	G	0.516	23	0.0414

Table 3.5: Variants significantly associated with bipolar disorder or a broad psychiatric phenotype under dominant and recessive inheritance models in a research group of either Mixed Ancestry (MA) or Caucasian (C) individuals using the Family-based Association Test (before correction for multiple testing)

Ethnicity	Phenotype	Model	Offset (μ)	Marker	Gene	Allele	Allele Frequency	Number of Informative Families	*P*-Value
MA	Broad	Dominant	0.3	rs11355106	*ACTN2*	C	0.379	22	0.0083
MA	Broad	Recessive	0.3	rs11355106	*ACTN2*	del	0.621	22	0.0083
MA	Broad	Additive	0.3	rs4659702	*ACTN2*	T	0.713	25	0.0224
MA	Broad	Additive	0.3	rs4659702	*ACTN2*	C	0.287	25	0.0224
MA	Strict	Dominant	0.01	rs11355106	*ACTN2*	C	0.379	24	0.0397
MA	Strict	Recessive	0.01	rs11355106	*ACTN2*	del	0.621	24	0.0397
C	Strict	Recessive	0.01	rs4659702	*ACTN2*	C	0.508	10	0.0449
C	Strict	Dominant	0.01	rs4659702	*ACTN2*	T	0.492	10	0.0449

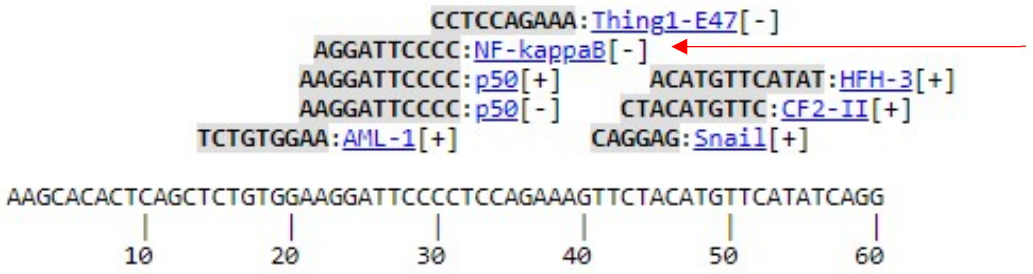
Table 3.6: Variants approaching significance using the Family-based Association Test analysis under broad or strict psychiatric phenotypes, in both Caucasian (C) and Mixed Ancestry (MA) populations

Ethnicity	Phenotype	Model	Offset (μ)	Marker	Gene	Allele	Allele Frequency	Number of Informative Families	*P*-Value
MA	Broad	Dominant	0.3	rs6130764	*WFDC12*	C	0.38	24	0.0504
MA	Broad	Recessive	0.3	rs6130764	*WFDC12*	T	0.62	24	0.0504
MA	Strict	Dominant	0.01	rs11761919	*RELN*	A	0.387	19	0.0506
MA	Strict	Recessive	0.01	rs11761919	*RELN*	G	0.613	19	0.0506
MA	Broad	Dominant	0.3	rs4659702	*ACTN2*	C	0.287	21	0.0595
MA	Broad	Recessive	0.3	rs4659702	*ACTN2*	T	0.713	21	0.0595
MA+C	Strict	Dominant	0.01	rs10994318	*ANK3*	G	0.516	22	0.0624
MA+C	Strict	Recessive	0.01	rs10994318	*ANK3*	C	0.484	22	0.0624
MA	Strict	Dominant	0.01	rs10994318	*ANK3*	C	0.477	14	0.0663
MA	Strict	Recessive	0.01	rs10994318	*ANK3*	G	0.523	14	0.0663
MA	Strict	Dominant	0.01	rs7232062	*AC010754.1*	T	0.723	10	0.0674
MA	Strict	Recessive	0.01	rs7232062	*AC010754.1*	G	0.277	10	0.0674

3.6.3 *IN SILICO* FUNCTIONALITY PREDICTION

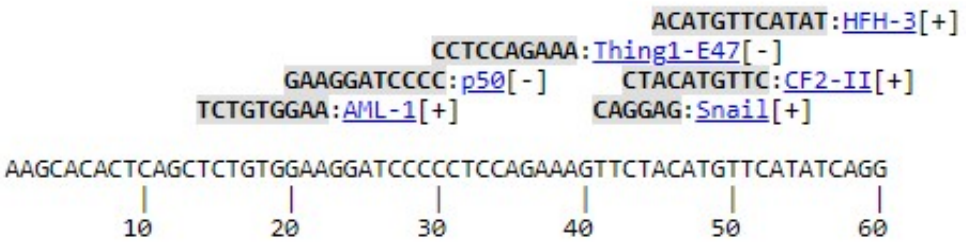
Intronic or intergenic variants that were associated with either strict BD or a broad psychiatric phenotype were further assessed for functional prediction using ConSite (Sandelin et al., 2004). This programme facilitates prediction of transcription factor binding sites, and changes to the motifs thereof in the presence of variation. The variants rs4659702, rs10994318 are both intronic variants. As is visible in Figure 3.5b, the alternative C allele of rs4659702 in *ACTN2* disrupts a binding site for nuclear factor-κβ (NF-κβ) which is visible in Figure 3.5a. The *ANK3* variant rs10994318 caused no such disturbance to any binding motif. The variant rs11355106 is an exonic deletion that results in a frameshift, and was not assessed using ConSite.

3.5.a



Transcription factor binding sites for NF-κB and p50 present on the negative strand in sequence with reference allele

3.5.b



Transcription factor binding sites for NF-κB and p50 absent on the negative strand in sequence with variant allele

Figure 3.5: ConSite prediction of transcription factor binding sites along the nucleotide sequence of DNA incorporating the variant rs4659702 in *ACTN2*

Symbols in square brackets indicate the positive [+] or negative [-] strands. A red arrow indicates the location of the transcription factor binding sites in the reference sequence

3.6.4 HARDY-WEINBERG EQUILIBRIUM TESTING OF VARIATION

The PEDSTATS programme (Wigginton and Abecasis, 2005) was used to perform HWE calculations using the Chi-square statistical test for each variant. Several variants did not meet HWE, and these are summarised in Table 3.7 below. The variants that did not meet HWE are mostly those variants that are associated with BD or a broad psychiatric phenotype according to FBAT analyses. The variants rs7232062, rs11355106, and rs10994318 did not meet HWE in either the Mixed Ancestry or Caucasian

population groups. Of these variants, rs10994318 was identified as a significant variant in the FBAT of the combined pedigree, including both Caucasian and Mixed Ancestry families (Table 3.4), and approached significance in the strict BD FBAT conducted on solely the Mixed Ancestry families (Table 3.5). The variant rs11355106 did not meet HWE in either population and was associated with both strict BD and a broad psychiatric phenotype in the Mixed Ancestry population. The variant rs9675777 did not meet HWE in the Mixed Ancestry population, but this variant was not significant nor approaching significance in any FBAT analyses. The variants rs40502 and rs133912 were monomorphic in both the Mixed Ancestry and Caucasian populations, and no HWE test could be performed for these variants.

3.7 WHOLE-EXOME SEQUENCING OF FAMILY MEMBERS

3.7.1 FAMILY 30 MEMBERS USED FOR WHOLE-EXOME SEQUENCING

As a means of investigating potential rare, damaging variants that may be involved in the aetiology of BD in this particular Afrikaner family, two sets of individuals were chosen from Family 30 for WES. Of these individuals, four had been used for the WGS used to undertake pathway analysis (BPD 30.10, BPD 30.29, BPD 30.50, BPD 30.30). Figure 2.4 in Chapter 2 supra illustrates the pedigree structure of a subset of Family 30 from which members were selected for WGS and WES. For the WES analysis, two types of family structures within the greater pedigree were chosen. Firstly, a family quartet, comprising of a BD-affected father (BPD 30.29), a BD-affected son (BPD 30.30), an unaffected daughter (BPD 30.28), and a mother (BPD 30.27), who has had one major depressive episode. The second family substructure comprised of three siblings and their paternal cousin. The father of the quartet family is a maternal cousin to the three siblings but is not related to the siblings' paternal cousin. This structure facilitated observation of familial transmission between generations of affected individuals, as provided by the quartet, as well as similarities within affected individuals of the same generation, as provided by the siblings. An unaffected cousin to the siblings was used as a control to aid in the identification of variants which may be specific to the Afrikaner population of South Africa, as this population is known to possess founder mutations for several disorders, as addressed in Section 2.2.

3.7.2 QUALITY OF WHOLE-EXOME SEQUENCING

The mean depth of WES achieved for each individual was approximately 107.7 X. There were over 60% usable reads for each sample. The Ion Sphere™ Particle loading rating, an indication of how comprehensively the wells of the chip were filled, was above 90% for each sample.

Table 3.7: Variants not in Hardy-Weinberg Equilibrium (HWE) in strict bipolar disorder in Mixed Ancestry (MA) and Caucasian (C) population groups

Variant	HWE *P*-Value (Strict BD, Mixed Ancestry)	HWE *P*-Value (Strict BD, Caucasian)
rs7232062	**0.0271**	**0.0050**
rs9675777	**1.8×10^{-11}**	-
rs11355106	**6.1×10^{-28}**	**0.0001**
rs10994318	**1.6×10^{-55}**	**2.3×10^{-47}**
rs40502	Monomorphic in MA	Monomorphic in C
rs133912	Monomorphic in MA	Monomorphic in C

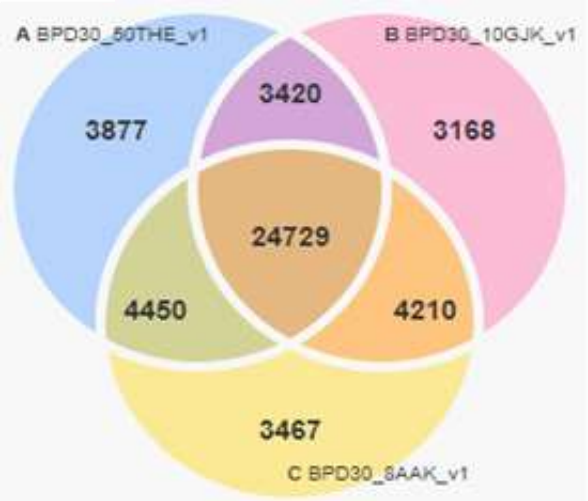
3.7.3 EXOMIC VARIATION SHARED BY AFFECTED INDIVIDUALS

The variation shared by the family members affected with BD was identified by determining the variation shared by the affected father (BPD 30.29) and son (BPD 30.30) of the quartet, that was not present in either the unaffected mother (BPD 30.27) or daughter (BPD 30.28) (n = 6 081). This was followed by the determination of the variation shared by the siblings (BPD 30.8, BPD 30.10, and BPD 30.50) that was not present in their unaffected paternal cousin (BPD 30.5) (n = 5 285). The Ion Torrent™ Ion Reporter™ generated Venn diagrams as depicted in Figure 3.6 to demonstrate variation overlap between three individuals. The total variation (n = 24 729) shared by the three affected siblings (BPD 30.8, BPD 30.10, and BPD 30.50) is illustrated in Figure 3.6.a, the variation shared by the affected father and son (BPD 30.29 and BPD 30.30 respectively), which is not present in the unaffected sister, (BPD 30.28) is shown in Figure 3.6.b (n = 4 440), while the variation shared by the affected father and son (BPD 30.29 and BPD 30.30) but which is not present in the son's mother (BPD 30.27) is illustrated in the Venn diagram in Figure 3.6.c (n = 7 448). All other comparisons were performed using single-line bash script commands. In total, these five affected family members share 168 variants which are not present in the unaffected family members, 151 of which are exonic, and 17 of which are not in the exome (but rather in the regions surrounding coding sequences), following rsID annotation using wANNOVAR (Wang et al., 2010; Yang & Wang, 2015). These 168 variants occur in 130 genes.

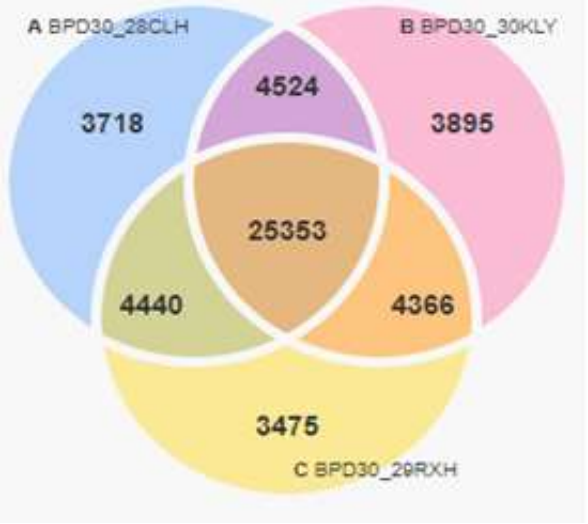
The non-synonymous exomic variation was annotated using wANNOVAR, which provided several prediction tool outputs. The variation was filtered by functional annotation based on SIFT and PolyPhen-2 scores. Of the 151 exomic variants in the WES data, this method identified seven non-synonymous variants as damaging (D), and these variants are listed in Table 3.8. However, as can be seen in Table 3.8, the prediction was not consistent across several prediction tools. One stop-gain mutation was shared by the affected family members, rs250426 in *steroid receptor RNA activator 1*

(*SRA1*). This stop-gain mutation has a MAF above 0.2 in all populations listed in the wANNOVAR output.

3.6.a



3.6.b



3.6.c

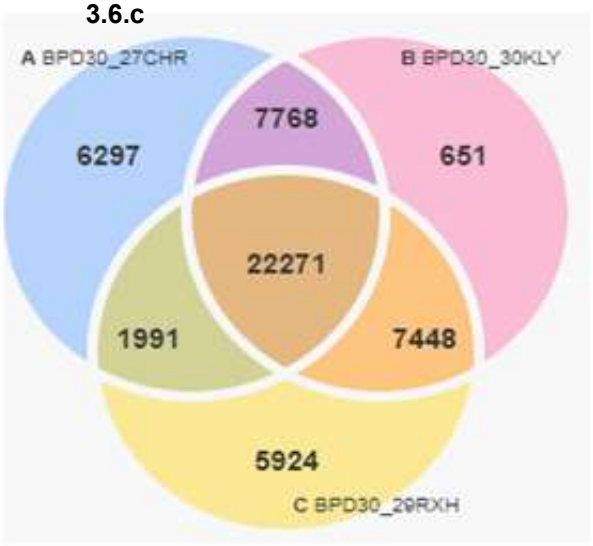


Figure 3.6: Variation shared by family members both affected and unaffected with bipolar disorder

Table 3.9 indicates the relatively rare variation (MAF < 0.1) identified by WES in these affected Afrikaner family members. The 1000 Genomes Project phase 3 data (The 1000 Genomes Project Consortium, 2015) (data accessed via wANNOVAR) and the Exome Aggregation Consortium version 0.3.1 (ExAC; data accessed via wANNOVAR) (Lek et al., 2016).

Table 3.8: Non-synonymous variation identified by whole-exome sequencing and shared by five Afrikaner family members affected with bipolar disorder

Chromosome	rsID	Gene	SIFT Prediction	PolyPhen-2 Prediction	MutationTaster Prediction	FATHMM Prediction	MetaSVM Prediction
1	rs34228541	*HHAT*	D	D	P*	T**	T
2	rs1035834	*FBXO36*	D	D	P	T	T
2	rs71428454	*AQP12A*	D	D	P	T	T
5	rs1129770	*CMYA5*	D	D	P	T	T
5	rs9324624	*ARHGEF37*	D	D	P	T	T
7	rs2285744	*THSD7A*	D	D	P	T	T
7	rs11556924	*ZC3HC1*	D	D	P	T	T

*A MutationTaster prediction of P is defined as "polymorphism_automatic," and is probably not damaging. **A FATHMM prediction score of T is defined as "Tolerated," and is not predicted to be damaging. A D score from SIFT and PolyPhen-2 indicate "Deleterious" or "Probably Damaging" variation respectively [http://annovar.openbioinformatics.org/en/latest/user-guide/filter/#-mutationtaster-annotation]

MAFs for global, as well as for European (EUR), African (AFR), and Finnish (FIN) populations are provided in Table 3.9 to provide an indication of the degree of rarity of these variants. Of these rare variants, rs142375896, rs16856462, rs147753755, rs41275305, and rs35884480 are non-synonymous, while the other rare variation does not result in an amino acid change. As shown in Table 3.9, the rs142375896 in *solute carrier family 26 member 9* (*SLC26A9*) is predicted as damaging by four well-established prediction tools: PolyPhen-2, MutationTaster, FATHMM, and MetaSVM. This variant is rare in both African and European populations, according to both the 1000 Genomes Project information (MAF EUR: 0.006) and the ExAC database (MAF AFR: 0.0012; MAF FIN: 0.0012). The variant rs17854844 in *RB binding protein 5* (*RBBP5*) is also rare in both African and European populations, but no prediction information was provided by wANNOVAR for this synonymous SNP. Table 3.10 indicates the ten genes from WES which have been listed on BDgene in relation to BD, and the WES-identified variants that these genes possess.

These genes are: *cytotoxic T-lymphocyte associated protein 4* (*CTLA4*), *calpain 10* (*CAPN10*), *hepatocyte growth factor activator* (*HGFAC*), *ankyrin 2* (*ANK2*), *exocyst complex component 3* (*EXOC3*), *WW and C2 domain containing 1* (*WWC1*), *solute carrier family 17 member 3* (*SLC17A3*), *major histocompatibility complex class IG* (*HLA-G*), *thrombospondin type 1 domain containing 7A* (*THSD7A*), and *protease serine 55* (*PRSS55*).

3.7.4 PATHWAY ANALYSIS OF EXOMIC VARIATION SHARED BY AFFECTED INDIVIDUALS

In order to determine whether pathway analysis of WGS and WES data would implicate the same biological pathways in BD, KEGG-based pathway analysis was completed using KOBAS 3.0. The input list contained 131 unique genes. In this analysis, the most significant pathway in which these genes acted was the *circadian entrainment* pathway (hsa04713; $p = 4.45 \times 10^{-18}$). This was followed by the *dopaminergic synapse* pathway (hsa04728; $p = 1.18 \times 10^{-17}$), *retrograde endocannabinoid signalling* pathway (hsa04723; $p = 1.79 \times 10^{-16}$), *pathways in cancer* pathway (hsa05200; $p = 1.06 \times 10^{-15}$), and the *amphetamine addiction* pathway (hsa05031; $p = 6.70 \times 10^{-15}$).

Table 3.9: Rare variation identified by whole-exome sequencing and shared by five Afrikaner family members affected with bipolar disorder

Chromosome	rsID	Gene	1000G MAF (EUR)	1000G MAF (AFR)	ExAC MAF	ExAC MAF (AFR)	ExAC MAF (FIN)	SIFT Prediction*	PolyPhen2 Prediction**	MutationTaster Prediction***	FATHMM Prediction#	MetaSVM Prediction##
1	rs142375896	*SLC26A9*	0.006	.	0.0031	0.0012	0.0012	T	D	D	D	D
1	rs17854844	*RBBP5*	0.005	.	0.0024	0.0007	0.0008	(none)	(none)	(none)	(none)	(none)
1	rs16856470	*SLC26A9*	0.079	.	0.0635	0.0767	0.0479	(none)	(none)	(none)	(none)	(none)
1	rs9438438	*SLC26A9*	0.089	.	0.0764	0.163	0.064	(none)	(none)	(none)	(none)	(none)
1	rs33943971	*SLC26A9*	0.081	.	0.0713	0.1663	0.0464	(none)	(none)	(none)	(none)	(none)
1	rs34803033	*SLC26A9*	0.075	.	0.0712	0.1748	0.0476	(none)	(none)	(none)	(none)	(none)
1	rs16856462	*SLC26A9*	0.081	0.0008	0.0742	0.1742	0.0482	T	B	P	D	T
2	rs35945835	*KIF1A*	0.053	0.0015	0.0381	0.0076	0.0159	(none)	(none)	(none)	(none)	(none)
5	rs147753755	*FAM71B*	0.002	0.025	0.0032	0.0006	0	D	B	N	T	T
5	rs72647759	*ADAMTS16*	0.007	0.0038	0.0038	0.0006	0.0103	(none)	(none)	(none)	(none)	(none)
5	rs368799133	*PWWP2A*	0.008	0.0038	0.008	0.0018	0.0012	(none)	(none)	(none)	(none)	(none)
5	rs56278697	*SOX30*	0.006	0.07	0.0062	0.0004	0.0014	(none)	(none)	(none)	(none)	(none)
5	rs61748187	*FBXL7*	0.032	0.12	0.0248	0.0067	0.0198	(none)	(none)	(none)	(none)	(none)
5	rs41275305	*TTC1*	0.0089	0.15	0.0103	0.025	0.0014	T	B	N	T	T
6	rs35884480	*SLC25A27*	0.058	0.17	0.0486	0.0078	0.0624	T	B	D	T	T
6	rs3752417	*HIST1H3C*	0.084	0.18	0.0725	0.1089	0.0452	(none)	(none)	(none)	(none)	(none)
7	rs1432	*THSD7A*	0.025	0.18	0.0153	0.0038	0.0087	(none)	(none)	(none)	(none)	(none)

In this table, MAF indicates the minor allele frequency; 1000G indicates the 1000 Genomes Project; ExAC indicates the Exome Aggregation Consortium; EUR the European population supergroup; AFR the African population supergroup, and FIN the Finnish population. A "." in any MAF column indicates that no information was available for the variant. Of the prediction tools listed, SIFT is the Sorting Intolerant From Tolerant programme; PolyPhen-2 is Polymorphism Phenotyping version 2; FATHMM refers to Functional Analysis through Hidden Markov Models; and MetaSVM refers to Meta-analytic Framework Based on Support Vector Machine software. *SIFT predictions of T indicate Tolerated

Table 3.10: Whole-exome sequencing derived genes and the variants therein, identified in members of Family 30, and which have previously been listed on BDgene [http://bdgene.psych.ac.cn/index.do]

Chromosome	Gene	Variant	Variant Type
2	*CTLA4*	rs231775	non-synonymous
2	*CAPN10*	rs3792269	synonymous
2	*CAPN10*	rs7607759	non-synonymous
4	*HGFAC*	rs28468427	synonymous
4	*ANK2*	rs3736575	synonymous
4	*ANK2*	rs3733615	synonymous
4	*ANK2*	rs2293324	synonymous
5	*EXOC3*	rs2672722	synonymous
5	*WWC1*	rs12054944	synonymous
6	*SLC17A3*	rs1165165	non-synonymous
6	*HLA-G*	rs1130356	synonymous
7	*THSD7A*	rs1432	synonymous
7	*THSD7A*	rs2285744	non-synonymous
8	*PRSS55*	rs4406360	non-synonymous

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 DISCUSSION

BD is a complex psychiatric condition with an hypothesised polygenic aetiology. Given this context, NGS has been applied in two parts in order to investigate the contributing factors across the genome. The first part of this study involved analysis of WGS data of four affected family members of Afrikaner origin, and pathway analysis of the variation present in these individuals compared to the controls, which were individuals of Dutch and German descent. Variants from the genes in the most over-represented pathways, *regulation of the actin cytoskeleton*, and *focal adhesion*, were selected for genotyping in South African BD families which comprised of Caucasian and Mixed Ancestry individuals. Several variants reached significance in the combined group or when stratified by ethnicity. While these variants did not reach significance after correction for multiple testing (for which the Bonferroni threshold was 0.0036), for the purposes of distinguishing variants with *p*-values below 0.05 from those with *p*-values above 0.05, they are called significant in this discussion.

The second part of this study aimed to identify whether WES would prove successful in identifying rare, damaging variation in a family of Afrikaner origin. WES indicated that the affected individuals from Family 30 share 168 variants. Several of these variants lie in genes that have previously been investigated in BD and variation with a potential damaging effect was identified, but the pathogenicity of these variants could not be clearly determined.

4.1.1 FINDINGS FROM THE FAMILY-BASED ASSOCIATION TEST

In a combined ancestry group including both Caucasian and Mixed Ancestry individuals, variation in several genes from the *regulation of the actin cytoskeleton* and *focal adhesion* pathways were significantly associated with BD. The variant rs4659702 in *ACTN2* was significantly associated with BD under dominant and recessive inheritance models (p = 0.0339; μ = 0.01). When stratifying by ethnicity, this variant remained significant in the Caucasian strict BD cohort under recessive and dominant genetic models (p = 0.0449; μ = 0.01). When the affection status was broadened to include other psychiatric disorders, such as alcohol use disorder, SCZ, schizoaffective disorder, and MDD, this *ACTN2* variant (rs4659702) remained significant in the Mixed Ancestry cohort under an additive genetic model.

ACTN2 encodes the actinin-α2 protein which is involved in bundling of actin filaments and is expressed primarily in cardiac muscle, skeletal muscle, and the brain (Mills et al., 2001; Tiso et al., 1999). The Human Protein Atlas [https://www.proteinatlas.org/] (Thul et al., 2017) lists the more precise location of *ACTN2* expression in the neuropil of the cerebral cortex, as well as in the neuronal cells of the caudate nuclei [https://www.proteinatlas.org/ENSG00000077522-ACTN2/tissue]. Magnetic resonance

imaging of BD individuals has demonstrated that the shape of the left caudate nucleus is significantly different to the shape of the caudate nucleus in unaffected individuals (Ong et al., 2012). Damage to the caudate nucleus in humans has been shown to result in behavioural abnormalities, including an inability to act in a decisive manner, and a loss of inhibition (Bhatia and Marsden, 1994). In several case studies, caudate infarction has been shown to result in psychiatric symptomatology (Cheng & Liu, 2015; Varoglu, 2010).

The role of the intronic *ACTN2* variant rs4659702 is not entirely known, but the alternate C allele results in the loss of a transcription factor binding site for NF-κB on the reverse strand, and p50 on the positive strand. However, the exact role in the regulation of *ACTN2* by these transcription factors is not clear. Further investigation of the effect of this variant on *ACTN2* expression, and thereafter the role of actinin-α2 in the caudate nucleus is necessary. Presently, there is no information in the literature regarding the association of rs4659702 with BD, NGS has previously identified two *ACTN2* mutations involved in familial cardiomyopathies (Bagnall et al., 2014; Girolami et al., 2014). Lithium, a commonly prescribed drug for BD (Oruch et al., 2014), is known to exert cardiotoxic effects, notably in long-term treatment (Anantha Narayanan et al., 2015; Serinken et al., 2009; Waring, 2007). Given that lithium may exert cardiotoxic effects in some cases, and that *ACTN2* variants have been observed in cardiomyopathy, the effects of rs4659702 may be associated with cardiotoxic lithium response in BD. *ACTN2* encodes an F-actin crosslinking protein. The precise mechanistic action of lithium remains relatively unclear, but the compound is known to have an effect on phosphoinositides via a potential inhibition of inositol monophosphatase (Saiardi and Mudge, 2018; Soares et al., 2000). The actin cytoskeleton is regulated by phosphoinositides, and lithium has been shown to affect the organisation of F-actin in neurons (Calabrese and Halpain, 2014; Saarikangas et al., 2010). The lithium-modulated reorganisation of actin may be implicated in the long-term cardiotoxicity of the drug.

The second *ACTN2* variant, rs11355106, is a frameshift variant; a single bp deletion in exon ten of *ACTN2*, which disturbs the reading frame of the protein product, actinin-α2. This variant has not previously been described in relation to BD, nor to any other disorder. This variant was identified by WGS in the four affected Afrikaner family members and was not present in the control Dutch or German genomes. All individuals were heterozygous for the deletion, which is fairly prevalent in several global populations, especially amongst Europeans [http://www.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=1:236736100-236737100;v=rs11355106;vdb=variation;vf=6630876]. The 1000 Genomes MAF data on ENSEMBL lists the lowest MAF for the rs11355106 deletion in a European group as 0.69 amongst the Utah residents of Northern and Western European ancestry. The Iberians from Spain have a reported MAF of 0.71. It seems unlikely then that this variant is disease-causing, but it is possible that it is one of the common variants of small effect size thought to be involved in BD, such as those involved in other complex traits (Yang et al., 2010). It is interesting to note that the *ACTN2* variant rs4659702 was

significantly associated with the strict BD diagnosis in both the Mixed Ancestry families and the Caucasian families, while the second *ACTN2* variant, rs11355106, was only significant amongst Mixed Ancestry families with a broad-spectrum phenotype. This underscores the heterogeneity of psychiatric disorders, both genetically and across populations.

A third variant that was significant for several of the FBAT conditions is rs10994318 in *ANK3*. This variant was selected from the GWAS by Stahl et al. (2018). This PGC study represents the largest GWAS of BD to date, comprising of 50 352 cases and 31 358 controls of European ancestry. The gene *ANK3* is a well-established BD candidate, and the association scores using this large GWAS dataset were significant (combined $p = 6.8 \times 10^{-9}$). The protein product, ankyrin G, is expressed in many tissues, including the neuropil of the cerebral cortex and granular cells of the cerebellum [https://www.proteinatlas.org/ENSG00000151150-ANK3/tissue] (Thul et al., 2017). Ankyrin G is involved in the organisation of the vertebrate cell membrane and assists in linking components of the cell membrane to the actin cytoskeleton of the cell. The expression of *ANK3* in the peripheral blood of BD patients has been shown to be significantly elevated when compared to either SCZ cases and healthy controls ($p = 2.8610 \times 10^{-4}$) (Wirgenes et al., 2014).

Mouse models of ankyrin G deficiency have demonstrated that a knockout of this gene in the juvenile stages of development results in disorganisation and disintegration of the myelin sheath, responsible for axon insulation, and can result in a slow process of destabilisation at the nodes of Ranvier along axons, leading to disturbed signal transduction (Saifetiarova et al., 2017). A second study investigated the effects of ankyrin G loss in the adult mouse forebrain and observed that mice with this loss developed symptoms reminiscent of human mania, including hyperactivity, a state of activity during normal sleeping hours, lower levels of anxiety, and an increased desire for exploration (Zhu et al., 2017). These manic-like symptoms were relieved following the administration of valproate or lithium.

A study of adult heterozygous mouse knockouts of exon 37 in *Ank3* demonstrated that anxious behaviour increased with reduction of *Ank3* expression, and decreased brain volume in four regions, including the cingulate cortex, primary motor cortex, hippocampal fimbria, and the retrosplenial cortex (van der Werf et al., 2017). A volume reduction of the fimbria of the hippocampus has been observed in human BD-II (Elvsåshagen et al., 2013). The variant rs10994318 is intronic, with no predictable effects on the protein or on any transcription factor binding sites in the *ANK3* gene. Many studies of *ANK3* variation and BD have indicated that other intronic *ANK3* variants are associated with the disorder. One such variant in relatively close proximity to rs10994318, rs10994336, has been associated with BD (Ferreira et al., 2008; Roby, 2017; Schulze et al., 2009). These *ANK3* variants, rs10994318 and rs10994336, are 53 956bp apart [https://www.ncbi.nlm.nih.gov/gene/288], and calculations performed in LDlink indicate that these variants are in LD ($r^2 > 0.7$) in both European and African populations [https://analysistools.nci.nih.gov/LDlink/?tab=home] (Machiela and Chanock, 2015).

Studies of Nordic (Tesli et al., 2011), German (Schulze et al., 2009), and other European populations (Ferreira et al., 2008) have observed significant associations between rs10994336 and BD. Other *ANK3* variants which have also been associated with BD include rs9804190 (Baum et al., 2008; Tesli et al., 2011), rs10761482 (Athanasiu et al., 2010), and rs1938526 (Smith et al., 2009). All of the aforementioned *ANK3* variants are intronic, with no clear functional role in BD pathology. However, a brain imaging study of rs9804190 has demonstrated a significant difference in uncinate fasciculus fractional anisotropy between BD-affected alternate allele carriers and controls (both homozygous reference allele carriers, and alternate allele carriers), as well as BD homozygous reference allele carriers (Lippard et al., 2017).

The uncinate fasciculus is a tract of white matter that connects the orbitofrontal cortex with the anterior temporal lobes of the brain, with a role in memory processing, decision making, and emotional regulation (Olson et al., 2015; Von Der Heide et al., 2013). This white matter brain tract is one of the last to reach maturity, which occurs during the third decade of life, and differences in maturation profiles may explain behavioural differences between individuals (Olson et al., 2015). The authors of the brain study suggest that affected individuals who carry the alternate allele at the rs9804190 locus display compromised integrity of this white matter tract, but that carriers of the alternate allele without BD are not at risk (Lippard et al., 2017). The variant in the present study, *ANK3* rs10994318, was significantly associated with BD in the combined research group of both Mixed Ancestry and Caucasian families but did not attain significance when stratified by ethnicity. However, this SNP did approach significance in the Mixed Ancestry cohort of individuals with strict BD ($p = 0.0663$; $\mu = 0.01$). This variant was not associated with the broad psychiatric phenotype of this research cohort, which included BD, SCZ, MD, schizoaffective disorders, substance abuse, alcohol abuse, and anxiety disorders. It seems that in this cohort the variant was involved in BD alone, but the other *ANK3* SNP, rs10994336, has previously been associated with SCZ in a Chinese study (Guo et al., 2016). While the *ANK3* variant rs10994318 may have an effect specific to BD pathogenesis, the effects is small and cannot be detected once the research group is stratified due to the reduction in cases and controls (Ikeda et al., 2018).

Both of these variants, *ACTN2* rs11355106 and *ACTN2* rs4659702, were selected for genotyping based on their presence in WGS data from affected Afrikaner individuals. A large GWAS of European American and African American individuals (1 001 European American cases, 1 033 European American controls, 345 African American cases, 670 African American controls) has previously shown distinct genetic associations with BD within each ethnic group, with rs5907577 (intergenic on X chromosome), rs10193871 in *NCK associated protein 5* (*NCKAP5*) reaching significance amongst European Americans, and rs2111504 in *dpy-19 like C-mannosyltransferase 3* (*DPY19L3*), and rs2769605 in *neurotrophic receptor tyrosine kinase 2* (*NTRK2*) reaching significance amongst African Americans (Smith et al., 2009). However, the FBAT in the present study was conducted in a cohort predominantly composed of Mixed Ancestry females. The association of BD with *ANK3* rs10994318,

observed in the combined cohort of strict BD under dominant or recessive inheritance models (p = 0.0443; μ = 0.01), was driven by the Mixed Ancestry individuals despite being selected from a GWAS of mostly European individuals (Stahl et al., 2018). However, this distinction between ethnic groups could be due to the larger number of Mixed Ancestry individuals in the FBAT analysis (112 Mixed Ancestry cases, 82 Caucasian cases, 232 Mixed Ancestry controls, and 125 Caucasian controls), or because the Mixed Ancestry individuals have inherited this variant from their European ancestors.

The alternative hypothesis of the FBAT (H_A) assumes that association occurs in the presence of linkage (Laird et al., 2000). Hence it may be worthwhile to consider the regions to which these associated variants map along the genome, and whether these regions have been reported previously. The genes *ACTN2* and *ANK3* are found in cytogenic regions 1q43 and 10q21.2, respectively. Previous research has shown linkage between these regions with both BD and SCZ. The regions 1q32-42 have been implicated in a linkage study of familial SCZ in Finland (Ekelund et al., 2001), and BD has been linked to chromosomal regions 1q32 (Detera-Wadleigh et al., 1999), and 1q42 (Macgregor et al., 2004). Moreover, the region 1q44 has been linked to suicidal behaviour in a study of BD, psychosis, and suicidality (Cheng et al., 2006). Previous studies have observed linkage at 10q21-24 with BD (Liu et al., 2003), and a meta-analysis of BD, schizoaffective disorder and SCZ observed nominal linkage at 10q11.21-10q22.1 in BD-I and schizoaffective cases (Segurado et al., 2003). Previous whole-genome linkage analysis of BD-I in the Afrikaner Family 30 has indicated that region 10p14-10p15.1 is suggestively linked with BD in this family (Dalvie, 2015). The region 1q43 has previously been linked to cardiac malfunction (Wellcome Trust Case Control Consortium, 2007), and MDD (Zubenko et al., 2003), but not BD specifically.

4.1.2 FINDINGS FROM WHOLE-EXOME SEQUENCING

WES of several members of an Afrikaner family was performed in order to identify rare, damaging variation that could be further investigated for a putative role in BD. The five affected individuals shared a total of 168 variants, 151 of which are in the exome, while a further 17 are in the genomic regions surrounding the exons, which are detected by WES. When using SIFT and PolyPhen-2 HDIV to predict the effect of non-synonymous variation, seven variants were identified as damaging, in *HHAT*, *FBXO36*, *AQP12A*, *CMYA5*, *ARHGEF37*, *THSD7A*, and *ZC3HC1*. However, these variants were not predicted to be damaging by MutationTaster, FATHMM, nor MetaSVM. FATHMM has been identified as having a higher discriminative power than PolyPhen-2, SIFT, and MutationTaster (Dong et al., 2015), but previously the PolyPhen-2 HVAR algorithm, trained to differentiate between variants involved in Mendelian disorders (Adzhubei et al., 2013) has been identified as more sensitive relative to either PolyPhen-2 HDIV, or SIFT (Frousios et al. 2013). Due to the complexity of BD, and the small effect sizes of associated variants, the PolyPhen-2 HDIV algorithm was used for predictions in an attempt to retain the majority of any potentially informative variation. Sensitivity gives an indication of the true positive rate of the prediction tool, not the specificity of the tool (Vihinen, 2012).

When considering the importance of rare variation, as was the intention for this component of the study, 17 variants with a MAF below 0.1 in the 1000 Genomes EUR population, ExAC global, and ExAC Finnish populations were identified. Five of these rare variants are non-synonymous, exonic variants. Of these variants, rs142375896 in *SLC26A9*, was predicted as damaging by PolyPhen-2, MutationTaster, FATHMM, and MetaSVM. This variant appears to be a rare, damaging variant, non-synonymous candidate for BD in this Afrikaner family. The gene *SLC26A9* is a member of a conserved family of sulphate/anion transporters, expressed in the gastrointestinal tract and salivary glands [https://www.proteinatlas.org/ENSG00000174502-SLC26A9/tissue].

Of the rare variants presented in Table 3.9, six are present in *SLC26A9*. This suggested that LD may be a factor in the apparent burden of variation in this gene. Calculations performed in LDlink (Machiela and Chanock, 2015), in both European and in African populations, indicated that many of these *SLC26A9* variants are in LD. The variants rs16856470, and rs16856462 are in LD in European populations, with r^2 and D` scores above 0.9. In African populations, these variants are also in weak LD according to the r^2 score ($r^2 = 0.306$). The variant of interest, rs142375896, is not in LD with any other detected *SLC26A9* variation, including the neighbouring rs34803033, which is 252bp away. Of the variants in *SLC26A9*, the lack of LD with the rare variant rs142375896 by surrounding *SLC26A9* variation indicates that this variant is worth investigating further in other family members, and perhaps within the Afrikaner population, without necessarily investigating the other *SLC26A9* variants detected in this study.

The rare variation present in the affected family members must be carefully considered in the context of the history of the Afrikaner population. For example, the variants rs34803033 and rs16856462, both in *SLC26A9*, are both rare in European populations. The latter is also predicted to be damaging by FATHMM. However, both of these variants have MAFs above 0.15 in the ExAC African population subgroup (as provided by the wANNOVAR output), and are in LD. Without considering the possible African influence on the Afrikaner genome, one would conclude rs16856462 to be worthy of further investigation. However, given the relatively high frequency with which this variant is observed in African population groups, it should perhaps not be prioritised over other, more obviously rare, damaging variants, such as *SLC26A9* rs142375896, which has low MAFs (< 0.01) in all populations according to the wANNOVAR output. WES also identified a single stop-gain variant in *steroid receptor RNA activator 1* (*SRA1*), rs250426, in affected individuals, annotated as such by wANNOVAR. However, this variant has a relatively high MAF in several world populations (0.29-0.84 in African, European, American, and East Asian populations according to ExAC and the 1000 Genomes Project), and is listed by ENSEMBL as a synonymous variant [http://www.ensembl.org/Homo_sapiens/Variation/Explore?r=5:140556675-140557675;v=rs250426;vdb=variation;vf=138331; accessed 8 March 2018]. Two of the BD-I individuals of this family were homozygous for the variant.

In order to determine whether any of the variant-containing genes identified by WES had been previously investigated in BD, the gene list of the 168 variants shared by affected individuals from WES was compared to the list of genes on BDgene (Chang et al., 2013) which have previously been investigated in the context of BD. Of the 168 variants shared by all affected individuals, ten genes to which variants mapped from WES have been identified or investigated in previous studies of BD (not necessarily associated), listed in Table 3.10, including: *CTLA4* (Jun et al., 2004; Liu et al., 2011), *CAPN10* (Moskvina et al., 2009), *HGFAC* (Georgi et al., 2014), *ANK2* (Winham et al., 2014), *EXOC3* (Noor et al., 2014), *WWC1* (Cruceanu et al., 2013), *SLC17A3* (Johnson et al., 2009), (*HLA-G*) (Debnath et al., 2013), *THSD7A* (Moskvina et al., 2009), and *PRSS55* (Xu et al., 2014). Of the aforementioned genes, a single variant identified by WES in this study has been previously investigated in a study of BD. This variant, rs231775 in *CTLA4*, was investigated in a Han Chinese cohort, but was not associated with BD (Liu et al., 2011).

It is interesting that of the 130 variant-carrying genes identified by WES amongst affected family members, such a relatively small number of genes have previously been investigated, however, this serves to underscore the complexity of BD. The variants and genes investigated in BD studies are diverse, and many do not have clear functional roles linking them to this complex disorder. As such, methods such as pathway analysis have been developed as the ability to generate larger datasets has grown.

4.1.3 FINDINGS FROM PATHWAY ANALYSIS OF NEXT-GENERATION SEQUENCING DATA

The pathway analysis used in this study can be referred to as knowledge base-driven pathway analysis, specifically an over-representation analysis approach, as it depends on public repositories of knowledge of pathways and the components thereof, rather than a method that relies on molecular kinetics (Khatri et al., 2012). Pathway analysis was performed using both WES and WGS data for individuals from the same Afrikaner family, Family 30. In both cases, the variation present exclusively in affected individuals was annotated to a corresponding gene, and the resultant list of genes was used as the list for over-representation analysis. This approach provides an indication of which biological pathways harbour the "burden of disease" in this family. Pathway analysis of WGS data was performed with three tools: KOBAS 3.0, DAVID, and WebGestalt. The four pathways which occurred in the top ten most over-represented pathways in each programme were: *pathways in cancer* (hsa05200), *focal adhesion* (hsa04510), *regulation of the actin cytoskeleton* (hsa04810), and the *Wnt signalling pathway* (hsa04310). The first of these, *pathways in cancer*, was also implicated in pathway analysis of WES data. This pathway is involved in a variety of functions, which include cell growth and proliferation, damage to DNA, metastasis, and other typically "cancerous" features [http://www.kegg.jp/kegg-bin/show_pathway?map05200] (Hanahan and Weinberg, 2000), all of which are related to the regular function of the cell. The other pathways identified in the WGS data, *focal adhesion*, *regulation of the*

actin cytoskeleton, and the *Wnt signalling pathway* also implicate core developmental and regulatory pathways in BD aetiology. A key component of the *Wnt signalling pathway* is *glycogen synthase kinase-3β* (*GSK3β*), which is a well-known BD candidate gene (Benedetti et al., 2003; Bureau, et al., 2017; Munkholm et al., 2018; Nievergelt et al., 2006), however only three intronic *GSK3β* variants, and one variant 50 849bp from *GSK3β* were identified by WGS. Cellular features related to actin control have been previously implicated by Bousman et al. (2010), who identified *actin cytoskeleton signalling* in a majority Caucasian sample group from San Francisco. The most over-represented KEGG pathways identified in the WES data indicated that *circadian entrainment* (hsa04713), the *dopaminergic synapse pathway* (hsa04728), *retrograde endocannabinoid signalling* (hsa04723), *pathways in cancer* (hsa05200), and the *amphetamine addiction pathway* (hsa05031) were the pathways that held the majority of the genetic variation. There is a large body of research investigating the role of circadian rhythm abnormalities and circadian variants in BD, which incorporates circadian gene variant association with BD (Benedetti et al., 2003; Gonzalez et al., 2015; Mansour et al., 2006; Nievergelt et al., 2006; Soria et al., 2010), cognitive function (Thomas et al., 2017), lithium response (Geoffroy et al., 2016), age of onset (Benedetti et al., 2008), sleep disturbances in BD (Bradley et al., 2017; Geoffroy et al., 2015; Pagani et al., 2016), seasonal patterning of disease episodes (Geoffroy, et al., 2015), and abnormal biological rhythmicity (Duarte Faria et al., 2015; Gonzalez et al.,s 2014).

A postulated model of omnigenic complex disease indicated that it is likely that peripheral genes (and the variants therein), which are disease genes with small but non-zero effect size, are more likely to dominate the phenotypic variance of a disease (Boyle et al., 2017). This may indicate that pathway analysis performed using Family 30 is more likely to find pathways that modulate how BD presents in this family than the "core" causative genes underlying BD, given that so few family members were assessed. If this is the case, given that affective moods are more likely associated with genetic components than other personality or behavioural characteristics (Sperry et al., 2017; Vandeleur et al., 2013), it may be pertinent to further classify the nature of the BD individual's moods with a greater granularity, when manic, depressive, or euthymic, to create endophenotypes for further investigation.

4.2 PRÉCIS AND IMPLICATIONS OF MAIN FINDINGS

The findings described in this study indicate that the underlying genetic model of BD is complex, possibly consisting of both common and rare variation as has been previously described (Gratten et al., 2014). The associated variants, rs4659702 and rs10994318, located in the genes *ACTN2* and *ANK3* respectively, have demonstrated that populations of differing ancestry, in this case Mixed Ancestry and Caucasian individuals, may share particular risk variants for BD. The former of these variants was selected from research involving Caucasian individuals. This study serves as a successful replication of the association of rs10994318, selected from a GWAS, within a smaller research group. The associated variant rs11355106 in *ACTN2* was associated with both strict BD and a broad-spectrum psychiatric phenotype within a Mixed Ancestry cohort, despite being selected from WGS of Afrikaner individuals.

This may indicate that the variant may not be associated in Caucasian individuals at large but may affect a broad range of psychiatric phenotypes in the Afrikaner population. The ancestral inheritance of this variant in both the Caucasian and Mixed Ancestry groups could suggest its European origin. WES provided a list of rare variants which can be investigated further, but also provokes the conversation that not all variants that are rare in the population of consideration (in this case, Caucasians), are rare in other populations (such as African populations). In a group such as the Afrikaners, who possess genetic input from a variety of non-Caucasian sources, this must be considered before such variants are investigated further.

Three component arguments can be discussed given the findings outlined above. Firstly, the contribution of rare and common variation to BD and other psychiatric disorders has been extensively investigated, and more recent research has indicated that there is no single type of variation involved in the genetic architecture of BD. The present study seems to confirm this, as associations have been identified between BD and common variation using FBAT, and rare variation was identified using WES. Much of the intronic variation identified in BD research, as has been mentioned earlier in this discussion, is relatively common. However, other studies have also identified *de novo* and rare variation in BD (Cruceanu et al., 2017; Cruceanu et al., 2013; Georgieva et al., 2014; Kataoka et al., 2016; Kerner et al., 2013; Lescai et al., 2017; O'Brien et al., 2018; Rao et al., 2017; Sharp et al., 2017). While it is difficult to comment on the precise extent to which each type of variation contributes to BD in this study, given the limited sample size, it is likely that common variation may contribute less strongly, while rare variation will have a larger effect on disease manifestation, as has been described by others (Boyle et al., 2017; Craddock and Sklar, 2013; Schork et al., 2009).

The second element to be addressed is whether coding or regulatory variation is more likely to be involved in BD. Once again, this is a challenging issue to discuss, and genetic studies are often biased towards the investigation of coding, non-synonymous variation, especially with the rise of WGS and WES methods, and access to the resultant large datasets. These methods facilitate investigation of a variety of variation, but the focus remains on variation with more obviously damaging consequences, as it remains difficult to fully interpret variation that does not meet these criteria (Ward and Kellis, 2012). The investigation of ostensibly pathogenic exonic variation remains a "low-hanging fruit", and a residual effect from the historical investigation of Mendelian disorders. The lack of damaging exonic variation from the WGS of Afrikaner individuals (Dalvie, 2015), and the lack of obvious, extremely damaging mutations identified by this study seem to indicate that regulatory variation may have a larger effect than previously supposed. Previous analysis of non-coding variation identified by GWAS of complex diseases has indicated an enrichment of these variants in regulatory sites, such as DNase hypersensitive sites (Maurano et al., 2012). A study of BD GWAS susceptibility variants similarly identified an enrichment of *cis*-regulatory variation that affects both mRNA and methylation loci (Gamazon et al., 2013). Pathway analysis has indicated a variety of regulatory mechanisms involved in

BD, which either indicates that BD is a disease of dysregulation or implies that the omnigenic model proposed by Boyle et al. (2017) is at play (which suggests that pathway analysis will only be able to give an indication of the peripherally involved genes, not the causative ones). However, methods including pathway analysis should be used to further investigate variants that are not coding nor obviously pathogenic.

Lastly, the variant rs11355106 in *ACTN2*, associated with both strict BD and the broad psychiatric phenotype, raises the issue of variation in psychiatric disorders that is disorder specific, or which affects multiple phenotypes. There is substantial evidence to suggest that BD and other psychiatric disorders may share common variation and disease pathways, as well as family evidence that indicates that family members of BD probands are at increased risk of BD as well as other psychiatric disorders, compounded by the fact that BD individuals often have comorbid psychiatric afflictions. To address the lattermost point first, BD often occurs alongside anxiety disorders, substance use disorders, ADHD, behaviour disorders, and eating disorders (Kim et al., 2016; Krishnan, 2005; McElroy et al., 2001; Merikangas et al., 2011; Sachs et al., 2000; Simon et al., 2004).

The next point, that families that contain BD individuals are at increased risk of several psychiatric disorders, is indicated by findings from several population-based studies. A study of families of Taiwanese SCZ probands indicated that family members had a relative risk of 3.23 (95% CI: 3.12-3.35) for BD, as well as relative risks greater than 1.3 for MDD and ADHD (Cheng et al., 2017). A similar study in Swedish families indicated that families of probands with either SCZ or BD were at greater risk of developing either disorder, and that the comorbidity between these disorders was 63% (Lichtenstein et al., 2009). A meta-analysis of familial coaggregation of SCZ and BD confirmed this observation (Van Snellenberg and de Candia, 2009). Finally, there is substantial genetic evidence indicating that many psychiatric disorders share genetic variation. These included overlaps between BD and borderline personality disorder (Witt et al., 2017), between BD, SCZ, and ASD (Carroll & Owen, 2009; Rzhetsky et al., 2007), between BD and SCZ (Forstner et al., 2017; Hennah et al., 2009; Moskvina et al., 2009), and between five psychiatric disorders, BD, SCZ, ASD, MDD, and ADHD (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013).

This can be illustrated using a mouse model of *Cacna1c* deletion, *CACNA1C* being a common risk gene for several disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Green et al., 2010). One study found that by deleting *Cacna1c* from glutamatergic forebrain neurons during embryonic development, mice with the deletion developed symptoms which could be broadly related to psychiatric endophenotypes, including anxiety, cognitive differences, and a maladaptive response to stress (Dedic et al., 2017).

4.3 LIMITATIONS AND FUTURE DIRECTIONS

There exist several limitations of this study which can be broadly classified as: i) what was not investigated, ii) inherent limits of the study design based on the small sample sizes, and iii) technical error. In the first category of limitations (i), neither mitochondrial genome data from WGS nor copy number variation were investigated, and several studies have indicated that mitochondrial (Andreazza et al., 2010; Chang et al., 2014) and copy number variants (Green et al., 2016; Noor et al., 2014) may be involved in the aetiology of several psychiatric disorders, including BD and SCZ (Gonçalves et al., 2018; Rollins et al., 2009). This does not detract from the findings of this study but may have limited the possible scope of the findings. A second limitation, of the second class of limitations (ii), is that WES and WGS data analysis only considered familial variation shared by multiple affected family members. The intention of this study was to pursue the familial factors that influence BD, but nonetheless, *de novo* variation may have an influence on the phenotypes and severity of BD (Kataoka et al., 2016). Thirdly, considering the nature of BD complexity, the genotyping component of this study has a relatively small number (n = ~470) compared to larger GWAS that have identified BD variation (Psychiatric GWAS Consortium Bipolar Disorder Working Group, 2011; Stahl et al., 2018), and which have included over 1 000 cases. The WES (n = 8) and WGS (n = 4) components also have relatively few samples relative to other NGS studies of BD (Lescai et al., 2017; Rao et al., 2017), which have over ten cases and 200 controls. Fourthly, relating to technical limitations (iii), the several Mendelian errors in the genotyping data which were identified by FBAT indicate that either the sample family pedigrees were incorrect (which, given the age of the project, may be a factor worth considering) or the genotyping method chosen was not the most reliable method at this scale. Fifthly, while the study research group was stratified by ethnicity for analysis in FBAT, it was not stratified by sex. This was not done as there was a greater number of women in the sample. However, a study of the *BDNF* variant rs6265 in a cohort of individuals with MDD indicated that this variant may have a greater role in the aetiology of the disorder in men (Verhagen et al., 2010). Thus, despite the equal prevalence of BD between the sexes, certain variation may be considered sex-specific.

In order to extend the work of this study of BD in South Africa, the variants rs4659702, rs11355106, rs10994318, and rs10994336 should be examined in a larger South African research group of Caucasian, Mixed Ancestry, and African individuals. This could be completed using SNP-focused genotyping, or by NGS methods in order to observe potentially linked variants. The roles of these variants in BD pathology should be functionally assessed by tissue culture, in order to determine whether they affect cell structure and cytoskeletal integrity, as suggested by the genes in which these variants are located, and to determine whether they are involved in psychiatric drug metabolism or response, for example, to lithium. To complement the functional assays, potential brain imaging studies to observe the effects of the variants on brain structure and volume could be established. The potential effect of NF-κB regulation of *ACTN2* should also be determined, as one such transcription factor

binding site is disrupted by rs4659702. The role of the mitochondrial genome should be assessed in future studies involving WGS of BD in order to investigate what could be a neglected source of BD-specific modifying variation, as should the contribution of genome copy-number variants to BD, which have recently been identified as potential but weak sources of BD association in a study of British individuals (Green et al., 2016; Noor et al., 2014)

4.4 CONCLUSION

This pilot study has indicated that variants selected from WGS data from a particular family can potentially be extended to a larger population, as demonstrated by the significant associations of rs4659702 and rs11355106 within BD or a broad-spectrum phenotype within the Mixed Ancestry population of South Africa. Both of these variants were selected for genotyping based on pathway analysis of WGS data from an Afrikaner family. The variant rs10994318 was also selected based on data from European-ancestry individuals and was associated with BD in the Mixed Ancestry subgroup of this study, indicating that the Mixed Ancestry population of South Africa may be susceptible to BD at these loci based on variation inherited from European ancestors.

The significance of the association between rs11355106 and both strict BD and a broader phenotype, as well as the BD-specific association of rs10994318 indicates that some variants may be linked to broader psychiatric symptomatology, while others are disorder-specific. This study also intimated that different segments of the genome may have distinct effects on BD aetiology. Pathway analysis of intronic and intergenic WGS data and WES data did not implicate the same pathways, thus demonstrating that different biological mechanisms may be involved in BD depending on whether it is involved in either a non-coding or exomic manner. It is apparent that intronic and intergenic variation within the genome is intrinsically linked to BD aetiology and cannot be ignored in future investigations. The functional effects of such variation should be examined in order to make progress towards understanding the biological process of BD pathogenesis.

This work has contextualised the variation apparent in BD-affected individuals in South Africa by indicating potentially useful biological pathways and variants from these pathways, such as rs4659702, rs11355106, rs10994318, which may be used to further examine the role of core cellular regulation mechanisms in the pathogenesis of BD. This work also indicates that international findings from larger studies may apply to South African individuals, and provides a small step forward in the process of establishing critically necessary knowledge about BD and how it may be diagnosed and treated.

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APPENDIX A: EXAMPLE 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)_____

Fax:

Contact Address: _____ Town: _____ Tel: _____

Fax:

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

Fax:

Hospital or Address: _____ Town: _____ 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: _____

<u>For Laboratory use only:</u>

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

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

CONSENT FOR DNA ANALYSIS AND STORAGE

1. **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:_____**

   _____

2. **I understand that the genetic material for analysis is to be obtained from: blood cells/skin sample/other (specify) (DELETE WHERE NOT APPLICABLE) :**

3. **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.

4. **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:

   _____

   _

5. **I authorise / do not authorise my doctor(s) (DELETE WHERE NOT APPLICABLE) to provide relevant clinical details to the Division of Human Genetics, UCT.**

6. **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.

7. **I understand that I may withdraw my consent for any aspect of the above at any time without this affecting my future medical care.**

8. **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 B: DNA EXTRACTION PROTOCOL

A) Blood: Salting-Out Method

The extraction procedure from whole blood involved the addition of red blood cell (RBC) lysis buffer to lyse erythrocytes, phosphate buffered saline (PBS) to wash the pellet, cell lysis buffer to lyse the white blood cells (WBCs), SDS to denature the protein components of the sample, and proteinase K to degrade the protein components (Section C, Appendix A).

1. Centrifuge the whole blood sample at 2 500rpm for 10 minutes
2. Draw 500µl of white blood cell layer and transfer it into a 2ml Eppendorf tube
3. Add 900µl of RBC lysis buffer to the tube and vortex the sample
4. Incubate at 37^{o}C for an hour
5. Centrifuge the sample at 2 500rpm for 10 minutes
6. Discard the supernatant
7. Aliquot 2ml of PBS to the pellet and vortex the sample
8. Centrifuge the sample at 2 500rpm for 10 minutes
9. Discard the supernatant
10. Add 60µl of cell lysis buffer, 20µl of 20% SDS and 2µl of proteinase K (20mg/ml) to the pellet
11. Incubate at 37^{o}C overnight, and vortex in between until the pellet dissolves
12. Add 400µl of 6M NaCl to the sample
13. Vortex well and observe the solution becoming cloudy
14. If the solution does not become cloudy, store the sample at 4^{o}C for 5 minutes
15. Centrifuge at 2 500rpm for 15 minutes
16. Transfer the supernatant into a clean 2ml Eppendorf tube, and if the DNA is floating, then make sure to transfer it to the new tube
17. Add 100µl of absolute ethanol (100%) to the supernatant in the new tube
18. Invert the tube multiple times until the DNA is visible and aggregated
19. Centrifuge the sample at 10 000rpm for 2 minutes
20. Discard the supernatant and observe the pellet's position in the tube

21. Add 1ml of 70% ethanol to the tube and vortex well

22. Centrifuge the sample at 10 000 rpm for 2 minutes

23. Repeat step 20

24. Allow the pellet to air-dry at room temperature for no longer than 2 hours

25. Re-suspend the pellet in 200µl 1X TE buffer and leave at room temperature for at least two days to allow the pellet to dissolve

B) Saliva: Oragene® Saliva Kit Method

1. Invert the Oragene® saliva tube to mix and incubate it at 50^{o}C for an hour

2. Transfer the saliva to a 15ml Greiner tube

3. Add 1/25 of the volume of prepIT, the Oragene® DNA purifier fluid (supplied with kit), to the sample and vortex thoroughly

4. Incubate the sample at -20^{o}C for 10 minutes and centrifuge it at 2 400rpm for 10 minutes

5. Transfer the supernatant into a clean 15ml tube, add twice the volume of absolute ethanol (100%) to the sample, and invert the tube to mix

6. Centrifuge the tube for 10 minutes at 2 400rpm and decant the supernatant

7. Re-suspend the pellet in ice-cold 70% ethanol and invert to mix

8. Centrifuge the sample at 2 400rpm for 10 minutes and decant the supernatant

9. Allow the pellet to air dry for at least 2 hours

10. Reconstitute the pellet in 200µl 1X TE buffer for at least 2 days

C) Composition of Reagents

DNA Extraction: Salting Out Method Reagents

- Red blood cell lysis buffer (RBC lysis buffer):

16.56 g Ammonium chloride (NH_4Cl) (Sigma-Aldrich, Johannesburg, GT, RSA)

1.58 g Ammonium bicarbonate powder (NH_4CO_3) (Sigma-Aldrich)

0.4 ml EDTA (0.5M, pH 7.4) (B&M Scientific, Cape Town, WC, RSA)

Make up to 2 L with ddH2O

- Cell lysis buffer:

25 ml Tris-HCl (1 M, pH 7.5)

16.7 ml NaCl (3 M) (Sigma-Aldrich)

1 ml EDTA (0.5 M) (B&M Scientific)

Make up to 500 ml with dH2O

- Phosphate buffered saline:

10 pellets (Sigma-Aldrich) dissolved in 1 l H2O

- Proteinase K (20mg/ml):

0.20g Proteinase K (Inqaba Biotec, Pretoria, GT, RSA)

5ml CaOAc (2mM, pH 8) (Sigma-Aldrich)

5ml SABAX (Adcock Ingram)

Filter sterilise (0.2µ)

Store at -20ºC

- 10% SDS:

10g SDS (Sigma-Aldrich)

100ml dH2O

- 70% Ethanol:

700ml ethanol (100%) (Kimix, Cape Town, WC, RSA)

300ml dH_2O

- EDTA (0.5M, pH 8)

37.224g EDTA (B&M Scientific)

>2g NaOH pellets (B&M Scientific)

- Tris-HCl:

12.1g Tris (Separations Scientific, Johannesburg, GT, RSA)

60ml dH_2O

Adjusted pH to 7.5 with HCl

- Tris-Cl (1M, pH 8):

10.6g Tris/Trizma base (Melford Biolaboratories, Ipswich, UK)

17.7g Tris-HCl

- TE buffer:

400µl EDTA (0.5 M) (B&M Scientific)

2ml Tris-Cl (pH 8)

APPENDIX C: WHOLE-GENOME SEQUENCING PROTOCOL

This protocol was taken directly from the thesis, "Genetic Analysis of Bipolar Disorder and Alcohol Use Disorder" by Dalvie, 2015.

WGS consists of three steps: library creation, clustering and sequencing, each of which are described below.

1) Library Creation

DNA shearing

- Random DNA shearing on Covaris instrument
- Switch on machine 1hr before use. Add distilled water to chamber (between 10 and 15 left-hand side measure)
- Require temperature to be between 3-4°C
- Setting at P1
- De-gas
- Shearing: duration 40 seconds- should give fragments of 300bp
- Clean-up of fragmented DNA
- Use Qiagen kit

End repair (ER)

- This step gets rid of 3' and 5' overhangs
- Consists of: ER control, ER mix, resuspension buffer
- Use 96-well plate
- Add 10µl ER control added to each sample
- Add all DNA sample (~50µl fragmented DNA)
- Add 40µl ER mix
- Place seal on plate
- Place plate in thermal cycler at 30°C for 30 minutes

Clean-up with AmPure XP beads

- Shake beads
- Add 160µl beads to sample
- Mix well

- Incubate at room temp for 15 mins
- Incubate at room temp for 15 mins on magnetic plate (pulls beads to the bottom of well)
- Discard supernatant without disturbing pellet (brown clump of beads)
- Add 200µl 80% ethanol and incubate for 30 seconds at room temperature
- Discard supernatant and add 200µl 80% ethanol
- Incubate at room temp for 15 minutes to dry out pellet (may see a crack in the pellet)
- Add 17.5µl resuspension buffer, mix with pellet (mix well), make sure pellet is evenly dispersed and no pellet remains on the walls of the well
- Incubate at room temperature for 2 minutes
- Incubate at room temperature for 5 minutes on magnetic plate (or until liquid is clear)
- Transfer 15µl supernatant to clean well
- Adenylate 3' ends
- Add 2.5µl A-tailing control
- Add 12.5µl A-tailing mix
- Incubate at 37°C on thermal cycler for 30 minutes

Ligate Adapters

- Add 2.5µl ligase control to each sample
- Add 2.5µl ligase mix
- Add 2.5µl adapter index (different adapter index for each sample)
- Mix thoroughly
- Incubate at 30°C for 10 minutes

Clean-up with AmPure XP beads

- Shake beads
- Add 45µl beads to sample
- Follow protocol as above
- Transfer 20µl of supernatant to clean well

Purify products on gel (Invitrogen e-gel)

- Load 20µl DNA product into each well (x2 for each sample)

- Load 10µl 50bp MWM
- Load water into empty wells
- Add 20µl water into second lane of wells after 400bp fragment reaches second lane
- Collect (pipette from second lane wells) before 500bp fragment reaches second lane

Clean-up with Qiagen kit

PCR of products

- Add 5µl PCR primer cocktail to wells in PCR plate
- Add 25µl PCR master mix
- Add all of the cleaned up product
- Pop air bubbles and seal plate

Clean-up PCR products with AmPure XP beads

- Add 50µl AmPure beads
- Step 12- take out entire volume. Do not remove any beads

Check concentration of product: Nanodrop (concentration) and Agilent Analyser (size of fragments and molar conc.)

Agilent analyser

- Use DNA 1000 chip
- add 9µl of gel dye mix (from 4°C fridge) in the well marked ―G‖
- Place chip on priming station
- Push down syringe until chip is locked into position (―pressurising‖ spreads gel into capillaries)
- Add 9µl gel-dye mix into other G-wells
- load 5µl marker (DNA 1000) into every other well not marked ―G‖
- load 1µl ladder into ladder well
- load 1µl sample into other wells (not G-wells)
- load 1µl water into empty wells
- ensure that there are no air bubbles
- vortex for 1 minute

- Computer setting- DNA 1000 series assay

2) Clustering

- Thaw reagent plate and hybridisation buffer at room temp

DNA dilution

- Need to have a DNA conc. of 10nm
- Therefore, dilute with elution buffer (EB) and Tween
- End concentration of DNA= 10nM

Denaturation

- Starting [DNA]= 10nM
- Add 17µl EB
- Add 2µl DNA template
- Add 1µl 2N NaOH (always add last)
- Vortex and centrifuge
- Incubate at room temperature for 5 minutes
- Place on ice
- Final [DNA]= 1pmol/µl

Serial Dilution

- Add 4µl denatured sample to clean tube
- Add 496µl hybridisation buffer
- Vortex and centrifuge
- Final [DNA]= 8 pmol
- Add 125µl diluted sample to clean tube
- Add 208µl hybridisation buffer
- Vortex and centrifuge
- Final [DNA]= 3pmol
- Add 148.5µl diluted sample to 8 strip tubes
- Add 1.5µl 8pmol phiX control (20µl 20pmol phiX and 30µl hybridisation buffer)
- Put on strip cap, vortex and centrifuge

- At cluster station (cBot Illumina)
- Wash reservoir (blot water/wash buffer)
- Load reagents (remove red strip)
- Scan barcode
- Load flowcell (wash with water beforehand)- rainbow side-up
- Put on manifold
- Load template (without strip cap, NB note direction)
- Do pre-check
- Start clustering (runs for ~4 hours)

3) Sequencing (Illumina HiSeq 2000)

- Thaw sequencing reagents at 4°C overnight (except dNTPs and polymerase)
- Wash instrument
- Place water bottles in panel (A/B)
- Change PE reagents to water (optional)
- Wash ~1hr

Sequencing

- Place cleavage mix in water at room temperature
- Add reagent bottles from TruSeq kit to corresponding colours of black rack
- Split incorporation mix into two bottles (other half for paired end reaction)
- Add both tubes of long read FFN mix to incorporation mix
- Add 1.1ml of polymerase to incorporation mix
- Shake
- Place cleavage mix in rack
- Put on caps with holes
- Add rack to sequencer
- Prime X2 (only for version 3)
- Once primed, remove old gaskets and flowcell
- Wipe flowcell station with alcohol swab

- Put in flowcell (wash with water, then alcohol swab, then dry with wipe) of interest and new gaskets
- Engage vacuum
- Wait for first base read (approx. 30 minutes- 1hr)

Paired-end (PE) cluster generation (“turn-around chemistry”)

- Use PE cluster kit
- Thaw reagents
- Prepare 0.1N NaOH (HP3)
- Add 2.85ml of PW1 to 15ml conical tube
- Add 150µl HP3
- Use PE module rack (black, thin rack)
- Add reagents to module- uncap
- Scan lot no. of PE kit
- Add module to PE rack
- Pull lever down

APPENDIX D: WHOLE-GENOME SEQUENCING BIOINFORMATICS PROTOCOL

This protocol was taken directly from the thesis, "Genetic Analysis of Bipolar Disorder and Alcohol Use Disorder" by Dalvie, 2015.

A) Bioinformatic Analysis of WGS Data

In order to derive useful information from the WGS data, the appropriate bioinformatic analysis was performed by the Computational Biology (CBIO) Group (UCT). Computations were performed using facilities provided by UCT's ICTS High Performance Computing team [http://hpc.uct.ac.za]. Outlined below is the workflow and programs used in the analysis:

B) Quality Control

The raw sequence reads were obtained from the sequencer in fastq format. The files were divided in terms of lane number or run number (each sample comprised 5 runs), direction (forward and reverse), and read number (each lane and direction file consisted of 26 or 27 reads). Therefore, each individual sample consisted of a minimum of 260 raw sequence files. The program FastQC [http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/] was used to ensure that the quality of the raw sequencing data was sufficient for downstream analysis. This program allows for the visual inspection of the read quality and statistics. Included in the output are the following:

Basic statistics: contains details about the file, the Illumina system used, the total and filtered number of sequences, the sequence length and percentage GC content

- Per base sequence quality: quality score for each of the bases
- Per sequence quality scores: a plot of the quality scores for each of the sequences
- Per base sequence content: base content across all sequences
- Per base GC content: percentage GC content across all bases
- Sequence GC content: plot of percentage GC content across all sequences
- Per base N content: percentage N content across all bases
- Sequence length distribution: distribution of sequence length across all sequences
- Sequence duplication levels: percentage duplicate compared to unique sequences
- Over-represented sequences: presence of over-represented sequences
- K-mer content: relative enrichment of K-mers across read length

C) Alignment to Reference Genome

The Burrows-Wheeler Aligner (BWA) (bwa- 0.5.9) [Li and Durbin, 2009] tool was used to align the short sequence reads to the reference human genome sequence (hg19 assembly). The BWA-backtrack algorithm was implemented as this algorithm is able to align relatively short sequence reads to a long reference sequence. Alignments were run on each read for each sample and subsequently combined, creating a sequence alignment map (SAM) file. 33

D) Post-Alignment Processing and Variant Calling

SAMtools [Li et al., 2009], Picard [http://broadinstitute.github.io/picard/] and GATK [McKenna et al., 2010] were used for post-alignment processing and for variant calling. Post-processing includes the sorting and indexing of the binary alignment map (BAM) file.

Firstly, the combined aligned reads were sorted using SAMtools (samtools-0.1.17), which created a binary SAM file called a BAM file. Statistics for the sorted BAM file were obtained, duplicates were removed, and the BAM file was indexed. Picard (picard-tools-1.51) is a java–based program with command line functionality allowing for the manipulation of SAM and BAM files. This program was used to obtain various metrics regarding the aligned sequence, such as an alignment summary and quality score distribution. Qualimap [Garcia-Alcalde et al., 2012] was used to determine the sequence coverage, percentage GC content, percentage of the reads which were successfully mapped and the read length. Indel realignment was performed using GATK (gatk v1.2). Insertion/deletion (indel) realignment consists of two steps, firstly the identification of target intervals, and secondly, the actual realignment process using the *IndelRealigner* utility in GATK. To ensure that all mate-pair information was correct across reads, the *FixMateInformation* utility was used in Picard (picard-tools-1.51).

At this point of the data analysis, the quality scores of each of the bases sequenced were recalibrated from the aligned BAM file using GATK. This process is performed since the quality scores assigned by various sequencers are often inaccurate and biased. Thus the base quality score recalibration (BQSR) process assigns quality scores based on the actual probability that the base mismatches the reference genome. Also, this process is able to improve the variation in quality with machine cycle and sequence context. This is achieved by examining the covariation of the base based on the following features [http://www.broadinstitute.org/gatk/guide/article?id=44]:

i) Reported quality score

ii) Position within the read

iii) Preceding and current nucleotide

In order to call variants from the aligned sequence, the *UnifiedGenotyper* in GATK was used. Once the variants were called, variant quality score recalibration (VQSR) was performed. This process allows for the assignment of well calibrated probability scores to each of the variants (known and novel) in a variant call list [http://www.broadinstitute.org/gatk/guide/article?id=39]. It is achieved by the development of a continuous, covarying estimate of the relationship between SNP call annotations and the probability that a variant is truly a variant as opposed to a sequencing or data analysis error. This is a two-step process whereby the following two options are used: *VariantRecalibrator* and *ApplyRecalibration*. The former generates a recalibration file based on a Gaussian mixture model which assesses the annotation values over a high quality subset of the input call set and then evaluates all input variants. This model is based on data from ―known sites‖ or ―true sites training resource‖ such as HapMap 3.3 [Gibbs et al., 2003], dbSNP 132 and the Omni 2.5 SNP human microarray. The HapMap resource is a SNP call set with a very high degree of confidence assigned to each of the variants. GATK will select the variants in this resource as representative of true sites and will use these variants to train the recalibration model [http://www.broadinstitute.org/gatk/guide/article?id=1259]. The result of this is a VCF file which is a text file containing a list of the variants called. Thereafter, the variant list was filtered to only include variants with a high quality and high coverage.

APPENDIX E: TBE BUFFER PREPARATION

10X TBE buffer:

0.89 M (216g) Tris (B&M Scientific, South Africa)

0.89 M (110g) Boric acid (ICN, Biomedicals Inc, USA)

0.04 M (148g) EDTA (BDH Electron® Laboratory Supplies, UK)

Make up to 2L with distilled H_2O

1X TBE buffer, used to make gels:

10X stock solution diluted to 1X

APPENDIX F: CPGR OPENARRAY® GENOTYPING PROTOCOL

This protocol was taken directly from the CPGR Report provided after the completion of genotyping.

A) DNA quality control

The quality of the submitted DNA samples was assessed using the NanoDrop 8000 Spectrophotometer. For good quality DNA samples, the A260/280 and A260/230 ratios should be between 1.7 and 1.9 and the concentrations should be at least 50ng/µl. Very few samples had concentrations lower than 50ng/µl and the majority of the samples had ratios within the expected range. Please see 1409PCR_DNA Quality control.xlsx file for quality control analysis of the samples.

Well C4 of Plate 2 (corresponding to sample 109.1 EVA) was found empty.

B) Genotyping

The 17 customized SNP assays were run with all submitted samples on the OpenArray plates. The samples la-belled BLANK1, BLANK2, BLANK3 and BLANK4 were used as NTCs, and the allele calling was done automatically by the TaqMan Genotyper Software. Tabular data and Allelic Discrimination plots were generated for report-ing. The plots are attached as jpeg files and the results of the genotypes are also attached (1049PCR_20171207_121633_Results.txt)

Downstream analysis of the experiments can be performed with the TaqMan Genotyper Software which is freely available for download from Thermo Fisher Scientific website. Six files with the .eds extension are pro-vided for analysis using the software.

C) Experimental procedures

- DNA quality control

DNA was quantified and the quality of the samples was determined on the NanoDrop 8000 spectrophotometer.

- Genotyping

The concentrations of DNA samples were normalized to 50ng/µl and 2µl of each sample was mixed with 2.0 µl of TaqMan OpenArray Genotyping Master Mix in a 384-well plate. Samples that had initial concentrations lower than 50ng/µl were used without pre-dilution. Samples were subsequently loaded onto the OpenArray plate using the QuantStudio 12 K Flex OpenArray AccuFill System. The loaded plates were then sealed and filled with an immersion fluid. PCR amplification was performed on the QuantStudio 12K Flex Real-Time qPCR instrument according to the manufacturer's recommendations. The genotype profiles for the 17 SNPs in each OpenArray plate were determined with the TaqMan Genotyper software version 1.3 using autocalling as the call method for all samples. The quality value of the data point's genotype was determined by a threshold above 0.95 and real-time allelic discrimination plots were generated for each assay. Homozygous alleles amplify on the x or y axis, with heterozygous alleles amplifying along the x=y slope. The SNP genotype profiles of all samples were exported into an Excel file for reporting.

APPENDIX G: FAMILY-BASED ASSOCIATION TEST RESULTS

Ethnicity	Phenotype	Model	Offset (μ)	Marker	Allele	Allele Frequency	Number of Informative Families	S-E(S)	Var(S)	Z	*P*-Value
MA	Broad	Recessive	0	**rs11355106**	del	0.621	20	-7.3	6.47	-2.87	**0.004105**
MA	Broad	Dominant	0	**rs11355106**	C	0.379	20	7.3	6.47	2.87	**0.004105**
MA	Broad	Recessive	0.02	**rs11355106**	del	0.621	22	-7.165	6.256	-2.865	**0.004174**
MA	Broad	Dominant	0.02	**rs11355106**	C	0.379	22	7.165	6.256	2.865	**0.004174**
MA+C	Broad	Dominant	0	**rs10994318**	G	0.516	16	6	4.5	2.828	**0.004678**
MA+C	Broad	Recessive	0	**rs10994318**	C	0.484	16	-6	4.5	-2.828	**0.004678**
MA+C	Broad	Dominant	0.01	**rs10994318**	G	0.516	22	5.9	4.411	2.809	**0.004965**
MA+C	Broad	Recessive	0.01	**rs10994318**	C	0.484	22	-5.9	4.411	-2.809	**0.004965**
MA+C	Broad	Dominant	0.02	**rs10994318**	G	0.516	22	5.8	4.323	2.79	**0.005278**
MA+C	Broad	Recessive	0.02	**rs10994318**	C	0.484	22	-5.8	4.323	-2.79	**0.005278**
MA	Broad	Dominant	0.3	**rs11355106**	C	0.379	22	5.275	3.997	2.639	**0.008326**
MA	Broad	Recessive	0.3	**rs11355106**	del	0.621	22	-5.275	3.997	-2.639	**0.008326**
MA+C	Broad	Dominant	0	**rs11355106**	del	0.696	12	4.5	3.375	2.449	**0.014306**
MA+C	Broad	Recessive	0	**rs11355106**	C	0.304	12	-4.5	3.375	-2.449	**0.014306**
MA	Broad	Recessive	0	**rs11355106**	C	0.379	12	-4.5	3.375	-2.449	**0.014306**
MA	Broad	Dominant	0	**rs11355106**	del	0.621	12	4.5	3.375	2.449	**0.014306**
MA+C	Broad	Dominant	0.01	**rs11355106**	del	0.696	15	4.425	3.308	2.433	**0.014978**
MA+C	Broad	Recessive	0.01	**rs11355106**	C	0.304	15	-4.425	3.308	-2.433	**0.014978**
MA	Broad	Recessive	0.02	**rs11355106**	C	0.379	14	-4.355	3.242	-2.419	**0.015579**
MA	Broad	Dominant	0.02	**rs11355106**	del	0.621	14	4.355	3.242	2.419	**0.015579**
MA+C	Broad	Dominant	0.02	**rs11355106**	del	0.696	15	4.35	3.242	2.416	**0.015699**
MA+C	Broad	Recessive	0.02	**rs11355106**	C	0.304	15	-4.35	3.242	-2.416	**0.015699**
MA	Broad	Recessive	0	**rs10994318**	C	0.477	10	-4	3	-2.309	**0.020921**
MA	Broad	Dominant	0	**rs10994318**	G	0.523	10	4	3	2.309	**0.020921**
MA	Broad	Additive	0.3	**rs4659702**	T	0.713	25	5.521	5.842	2.284	**0.022349**
MA	Broad	Additive	0.3	**rs4659702**	C	0.287	25	-5.521	5.842	-2.284	**0.022349**

MA	Broad	Recessive	0.02	**rs10994318**	C	0.477	13	-3.865	2.882	-2.277	**0.022806**
MA	Broad	Dominant	0.02	**rs10994318**	G	0.523	13	3.865	2.882	2.277	**0.022806**
MA+C	Strict	Dominant	0.01	**rs4659702**	T	0.614	14	2.883	1.847	2.122	**0.033855**
MA+C	Strict	Recessive	0.01	**rs4659702**	C	0.386	14	-2.883	1.847	-2.122	**0.033855**
MA	Strict	Recessive	0	**rs11355106**	del	0.621	17	-4.233	4.157	-2.076	**0.037857**
MA	Strict	Dominant	0	**rs11355106**	C	0.379	17	4.233	4.157	2.076	**0.037857**
MA	Strict	Dominant	0.01	**rs11355106**	C	0.379	24	4.166	4.102	2.057	**0.039696**
MA	Strict	Recessive	0.01	**rs11355106**	del	0.621	24	-4.166	4.102	-2.057	**0.039696**
MA+C	Broad	Dominant	0	**rs11355106**	C	0.304	28	6.3	9.47	2.047	**0.040635**
MA+C	Broad	Recessive	0	**rs11355106**	del	0.696	28	-6.3	9.47	-2.047	**0.040635**
MA+C	Broad	Dominant	0.01	**rs11355106**	C	0.304	31	6.245	9.312	2.046	**0.040709**
MA+C	Broad	Recessive	0.01	**rs11355106**	del	0.696	31	-6.245	9.312	-2.046	**0.040709**
MA+C	Broad	Dominant	0.02	**rs11355106**	C	0.304	31	6.19	9.157	2.046	**0.040796**
MA+C	Broad	Recessive	0.02	**rs11355106**	del	0.696	31	-6.19	9.157	-2.046	**0.040796**
MA+C	Strict	Dominant	0	**rs10994318**	C	0.484	10	3.083	2.285	2.04	**0.041363**
MA+C	Strict	Recessive	0	**rs10994318**	G	0.516	10	-3.083	2.285	-2.04	**0.041363**
MA+C	Strict	Dominant	0.01	**rs10994318**	C	0.484	23	3.013	2.244	2.011	**0.044276**
MA+C	Strict	Recessive	0.01	**rs10994318**	G	0.516	23	-3.013	2.244	-2.011	**0.044276**
C	Strict	Recessive	0.02	**rs4659702**	C	0.508	10	-1.992	0.981	-2.011	**0.044365**
C	Strict	Dominant	0.02	**rs4659702**	T	0.492	10	1.992	0.981	2.011	**0.044365**
C	Strict	Recessive	0.01	**rs4659702**	C	0.508	10	-1.996	0.99	-2.006	**0.044901**
C	Strict	Dominant	0.01	**rs4659702**	T	0.492	10	1.996	0.99	2.006	**0.044901**
MA	Broad	Recessive	0.02	**rs6130764**	T	0.62	24	-5.497	7.581	-1.997	**0.045876**
MA	Broad	Dominant	0.02	**rs6130764**	C	0.38	24	5.497	7.581	1.997	**0.045876**
MA	Broad	Recessive	0	**rs6130764**	T	0.62	22	-5.577	7.825	-1.994	**0.046165**
MA	Broad	Dominant	0	**rs6130764**	C	0.38	22	5.577	7.825	1.994	**0.046165**
MA	Strict	Recessive	0	**rs11761919**	G	0.613	13	-3.586	3.347	-1.96	**0.049967**
MA	Strict	Dominant	0	**rs11761919**	A	0.387	13	3.586	3.347	1.96	**0.049967**
MA	Broad	Dominant	0.3	rs6130764	C	0.38	24	4.374	4.999	1.956	0.050428
MA	Broad	Recessive	0.3	rs6130764	T	0.62	24	-4.374	4.999	-1.956	0.050428
MA	Strict	Dominant	0.01	rs11761919	A	0.387	19	3.553	3.304	1.955	0.050639
MA	Strict	Recessive	0.01	rs11761919	G	0.613	19	-3.553	3.304	-1.955	0.050639
MA+C	Broad	Dominant	0	rs10994318	C	0.484	18	4.333	4.944	1.949	0.051321
MA+C	Broad	Recessive	0	rs10994318	G	0.516	18	-4.333	4.944	-1.949	0.051321
MA+C	Broad	Dominant	0.01	rs10994318	C	0.484	24	4.263	4.855	1.935	0.053009

MA+C	Broad	Recessive	0.01	rs10994318	G	0.516	24	-4.263	4.855	-1.935	0.053009
MA+C	Broad	Dominant	0.02	rs10994318	C	0.484	24	4.193	4.767	1.921	0.054794
MA+C	Broad	Recessive	0.02	rs10994318	G	0.516	24	-4.193	4.767	-1.921	0.054794
MA	Broad	Dominant	0.3	rs4659702	C	0.287	21	-4.096	4.725	-1.884	0.059498
MA	Broad	Recessive	0.3	rs4659702	T	0.713	21	4.096	4.725	1.884	0.059498
MA	Broad	Additive	0.02	rs4659702	T	0.713	25	5.381	8.296	1.868	0.061705
MA	Broad	Additive	0.02	rs4659702	C	0.287	25	-5.381	8.296	-1.868	0.061705
MA+C	Strict	Dominant	0.01	rs10994318	G	0.516	22	2.65	2.022	1.864	0.062376
MA+C	Strict	Recessive	0.01	rs10994318	C	0.484	22	-2.65	2.022	-1.864	0.062376
MA	Broad	Additive	0	rs4659702	T	0.713	23	5.371	8.53	1.839	0.065899
MA	Broad	Additive	0	rs4659702	C	0.287	23	-5.371	8.53	-1.839	0.065899
MA	Strict	Dominant	0.01	rs10994318	C	0.477	14	2.516	1.876	1.837	0.066272
MA	Strict	Recessive	0.01	rs10994318	G	0.523	14	-2.516	1.876	-1.837	0.066272
MA	Strict	Dominant	0.01	rs7232062	T	0.723	10	2.545	1.935	1.829	0.067352
MA	Strict	Recessive	0.01	rs7232062	G	0.277	10	-2.545	1.935	-1.829	0.067352
MA	Broad	Recessive	0	rs10994318	G	0.523	12	-3.333	3.444	-1.796	0.072486
MA	Broad	Dominant	0	rs10994318	C	0.477	12	3.333	3.444	1.796	0.072486
MA	Broad	Recessive	0.02	rs10994318	G	0.523	15	-3.198	3.326	-1.754	0.079498
MA	Broad	Dominant	0.02	rs10994318	C	0.477	15	3.198	3.326	1.754	0.079498
MA	Strict	Additive	0.01	rs6130764	T	0.62	31	-5.435	9.972	-1.721	0.085203
MA	Strict	Additive	0.01	rs6130764	C	0.38	31	5.435	9.972	1.721	0.085203
MA	Broad	Dominant	0.3	rs11355106	del	0.621	14	2.325	1.839	1.714	0.086473
MA	Broad	Recessive	0.3	rs11355106	C	0.379	14	-2.325	1.839	-1.714	0.086473
MA	Strict	Additive	0	rs6130764	T	0.62	27	-5.44	10.092	-1.713	0.086795
MA	Strict	Additive	0	rs6130764	C	0.38	27	5.44	10.092	1.713	0.086795
MA	Strict	Dominant	0.01	rs10994318	G	0.523	13	2.183	1.654	1.697	0.089718
MA	Strict	Recessive	0.01	rs10994318	C	0.477	13	-2.182	1.654	-1.697	0.089718
MA+C	Strict	Dominant	0	rs11355106	C	0.304	20	3.733	4.907	1.685	0.091911
MA+C	Strict	Recessive	0	rs11355106	del	0.696	20	-3.733	4.907	-1.685	0.091911
MA	Strict	Recessive	0	rs6130764	T	0.62	21	-4.138	6.031	-1.685	0.09199
MA	Strict	Dominant	0	rs6130764	C	0.38	21	4.138	6.031	1.685	0.09199
MA	Strict	Dominant	0.01	rs6130764	C	0.38	24	4.098	5.951	1.68	0.092994
MA	Strict	Recessive	0.01	rs6130764	T	0.62	24	-4.098	5.951	-1.68	0.092994
C	Strict	Recessive	0.01	rs6130764	T	0.57	10	2.064	1.524	1.672	0.09446
C	Strict	Dominant	0.01	rs6130764	C	0.43	10	-2.064	1.524	-1.672	0.09446

MA+C	Strict	Dominant	0.01	rs11355106	C	0.304	33	3.678	4.847	1.671	0.094773
MA+C	Strict	Recessive	0.01	rs11355106	del	0.696	33	-3.678	4.847	-1.671	0.094773
C	Strict	Recessive	0.02	rs6130764	T	0.57	10	2.045	1.513	1.663	0.096377
C	Strict	Dominant	0.02	rs6130764	C	0.43	10	-2.045	1.513	-1.663	0.096377
MA	Broad	Additive	0.3	rs35798951	(blank)	0.746	22	3.867	5.429	1.659	0.097029
MA	Broad	Additive	0.3	rs35798951	A (ins)	0.254	22	-3.867	5.429	-1.659	0.097029
MA	Strict	Additive	0.01	rs35195646	A	0.811	17	-3.476	4.743	-1.596	0.11047
MA	Strict	Additive	0.01	rs35195646	del	0.189	17	3.476	4.743	1.596	0.11047
MA	Strict	Dominant	0.01	rs11355106	del	0.621	14	1.928	1.471	1.589	0.111952
MA	Strict	Recessive	0.01	rs11355106	C	0.379	14	-1.927	1.471	-1.589	0.111952
MA+C	Strict	Dominant	0.01	rs11355106	del	0.696	15	1.925	1.471	1.587	0.11242
MA+C	Strict	Recessive	0.01	rs11355106	C	0.304	15	-1.925	1.471	-1.587	0.11242
MA	Strict	Additive	0	rs35195646	A	0.811	13	-3.476	4.797	-1.587	0.11248
MA	Strict	Additive	0	rs35195646	del	0.189	13	3.476	4.797	1.587	0.11248
C	Strict	Additive	0.01	rs6130764	T	0.57	15	2.642	2.915	1.547	0.12181
C	Strict	Additive	0.01	rs6130764	C	0.43	15	-2.642	2.915	-1.547	0.12181
MA	Broad	Additive	0.3	rs6130764	T	0.62	28	-4.338	7.874	-1.546	0.122123
MA	Broad	Additive	0.3	rs6130764	C	0.38	28	4.338	7.874	1.546	0.122123
C	Strict	Additive	0.02	rs6130764	T	0.57	15	2.617	2.887	1.54	0.123559
C	Strict	Additive	0.02	rs6130764	C	0.43	15	-2.617	2.887	-1.54	0.123559
MA	Broad	Dominant	0.3	rs10994318	G	0.523	13	1.975	1.656	1.535	0.124803
MA	Broad	Recessive	0.3	rs10994318	C	0.477	13	-1.975	1.656	-1.535	0.124803
MA	Strict	Additive	0.01	rs7232062	T	0.723	24	4.035	7.592	1.464	0.143078
MA	Strict	Additive	0.01	rs7232062	G	0.277	24	-4.035	7.592	-1.464	0.143078
MA+C	Broad	Dominant	0.02	rs4659702	T	0.614	13	2.683	3.375	1.461	0.144113
MA+C	Broad	Recessive	0.02	rs4659702	C	0.386	13	-2.683	3.375	-1.461	0.144113
MA+C	Broad	Dominant	0.01	rs4659702	T	0.614	13	2.7	3.43	1.458	0.144893
MA+C	Broad	Recessive	0.01	rs4659702	C	0.386	13	-2.7	3.43	-1.458	0.144893
MA+C	Broad	Dominant	0	rs4659702	T	0.614	11	2.717	3.486	1.455	0.145683
MA+C	Broad	Recessive	0	rs4659702	C	0.386	11	-2.717	3.486	-1.455	0.145683
MA	Strict	Additive	0	rs7232062	T	0.723	20	4	7.656	1.446	0.148273
MA	Strict	Additive	0	rs7232062	G	0.277	20	-4	7.656	-1.446	0.148273
MA	Broad	Dominant	0.3	rs35798951	A (ins)	0.254	21	-2.931	4.328	-1.409	0.158945
MA	Broad	Recessive	0.3	rs35798951	(blank)	0.746	21	2.931	4.328	1.409	0.158945
MA+C	Strict	Additive	0.01	rs35195646	A	0.798	29	-3.491	6.16	-1.407	0.159545

MA+C	Strict	Additive	0.01	rs35195646	del	0.202	29	3.491	6.16	1.407	0.159545
MA+C	Strict	Additive	0	rs35195646	A	0.798	18	-3.476	6.226	-1.393	0.163561
MA+C	Strict	Additive	0	rs35195646	del	0.202	18	3.476	6.226	1.393	0.163561
MA+C	Broad	Additive	0.02	rs35798951	(blank)	0.599	33	4.737	11.72	1.384	0.166478
MA+C	Broad	Additive	0.02	rs35798951	A (ins)	0.401	33	-4.737	11.72	-1.384	0.166478
MA	Broad	Recessive	0.02	rs4659702	T	0.713	21	3.606	6.795	1.383	0.166515
MA	Broad	Dominant	0.02	rs4659702	C	0.287	21	-3.606	6.795	-1.383	0.166515
MA+C	Broad	Additive	0.01	rs35798951	(blank)	0.599	33	4.702	11.887	1.364	0.172667
MA+C	Broad	Additive	0.01	rs35798951	A (ins)	0.401	33	-4.702	11.887	-1.364	0.172667
MA	Broad	Recessive	0	rs4659702	T	0.713	19	3.571	6.995	1.35	0.176911
MA	Broad	Dominant	0	rs4659702	C	0.287	19	-3.571	6.995	-1.35	0.176911
MA+C	Broad	Additive	0	rs35798951	(blank)	0.599	29	4.667	12.057	1.344	0.178960
MA+C	Broad	Additive	0	rs35798951	A (ins)	0.401	29	-4.667	12.057	-1.344	0.178960
MA	Broad	Additive	0.02	rs6130764	T	0.62	28	-4.478	11.689	-1.31	0.190273
MA	Broad	Additive	0.02	rs6130764	C	0.38	28	4.478	11.689	1.31	0.190273
MA+C	Strict	Dominant	0	rs35798951	A (ins)	0.401	20	2.972	5.273	1.294	0.195534
MA+C	Strict	Recessive	0	rs35798951	(blank)	0.599	20	-2.972	5.273	-1.294	0.195534
MA	Broad	Additive	0	rs6130764	T	0.62	26	-4.488	12.056	-1.293	0.196156
MA	Broad	Additive	0	rs6130764	C	0.38	26	4.488	12.056	1.293	0.196156
MA+C	Strict	Dominant	0.01	rs35798951	A (ins)	0.401	30	2.947	5.225	1.289	0.197281
MA+C	Strict	Recessive	0.01	rs35798951	(blank)	0.599	30	-2.947	5.225	-1.289	0.197281
C	Broad	Recessive	0.3	rs35798951	A (ins)	0.58	10	-1.73	1.816	-1.284	0.199065
C	Broad	Dominant	0.3	rs35798951	(blank)	0.42	10	1.73	1.816	1.284	0.199065
MA+C	Strict	Additive	0.01	rs1064395	G	0.748	37	-3.8	8.842	-1.278	0.201322
MA+C	Strict	Additive	0.01	rs1064395	A	0.252	37	3.8	8.842	1.278	0.201322
MA+C	Broad	Additive	0.02	rs4659702	T	0.614	36	4.411	11.988	1.274	0.202624
MA+C	Broad	Additive	0.02	rs4659702	C	0.386	36	-4.411	11.988	-1.274	0.202624
MA	Broad	Additive	0.3	rs7232062	T	0.723	24	3.55	7.814	1.27	0.204108
MA	Broad	Additive	0.3	rs7232062	G	0.277	24	-3.55	7.814	-1.27	0.204108
MA	Strict	Additive	0	rs11761919	G	0.613	18	-3	5.609	-1.267	0.205267
MA	Strict	Additive	0	rs11761919	A	0.387	18	3	5.609	1.267	0.205267
MA	Strict	Additive	0.01	rs11761919	G	0.613	24	-2.97	5.538	-1.262	0.206918
MA	Strict	Additive	0.01	rs11761919	A	0.387	24	2.97	5.538	1.262	0.206918
MA+C	Broad	Dominant	0.02	rs35798951	(blank)	0.599	15	2.39	3.588	1.262	0.207034
MA+C	Broad	Recessive	0.02	rs35798951	A (ins)	0.401	15	-2.39	3.588	-1.262	0.207034

MA+C	Broad	Additive	0.01	rs4659702	T	0.614	36	4.391	12.174	1.259	0.208178
MA+C	Broad	Additive	0.01	rs4659702	C	0.386	36	-4.391	12.174	-1.259	0.208178
MA+C	Strict	Additive	0	rs1064395	G	0.748	27	-3.76	8.95	-1.257	0.208884
MA+C	Strict	Additive	0	rs1064395	A	0.252	27	3.76	8.95	1.257	0.208884
MA+C	Broad	Dominant	0.01	rs35798951	(blank)	0.599	15	2.38	3.639	1.248	0.21216
MA+C	Broad	Recessive	0.01	rs35798951	A (ins)	0.401	15	-2.38	3.639	-1.248	0.21216
MA+C	Broad	Additive	0	rs4659702	T	0.614	31	4.371	12.364	1.243	0.213784
MA+C	Broad	Additive	0	rs4659702	C	0.386	31	-4.371	12.364	-1.243	0.213784
MA+C	Broad	Dominant	0	rs35798951	(blank)	0.599	12	2.37	3.691	1.234	0.21733
MA+C	Broad	Recessive	0	rs35798951	A (ins)	0.401	12	-2.37	3.691	-1.234	0.21733
MA+C	Strict	Additive	0.01	rs7232062	T	0.55	37	4.06	11.577	1.193	0.23277
MA+C	Strict	Additive	0.01	rs7232062	G	0.45	37	-4.06	11.577	-1.193	0.23277
C	Strict	Additive	0.02	rs4659702	T	0.492	15	1.863	2.454	1.189	0.234298
C	Strict	Additive	0.02	rs4659702	C	0.508	15	-1.863	2.454	-1.189	0.234298
C	Strict	Additive	0.01	rs4659702	T	0.492	15	1.848	2.463	1.178	0.238881
C	Strict	Additive	0.01	rs4659702	C	0.508	15	-1.848	2.463	-1.178	0.238881
MA+C	Strict	Additive	0	rs7232062	T	0.55	28	4	11.674	1.171	0.241713
MA+C	Strict	Additive	0	rs7232062	G	0.45	28	-4	11.674	-1.171	0.241713
MA	Broad	Recessive	0	rs9675777	T	0.679	10	2.169	3.463	1.165	0.243831
MA	Broad	Dominant	0	rs9675777	C	0.321	10	-2.169	3.463	-1.165	0.243831
MA	Broad	Recessive	0.02	rs9675777	T	0.679	11	2.141	3.376	1.165	0.243885
MA	Broad	Dominant	0.02	rs9675777	C	0.321	11	-2.141	3.376	-1.165	0.243885
MA	Strict	Recessive	0	rs35798951	(blank)	0.746	17	-2.472	4.523	-1.162	0.245041
MA	Strict	Dominant	0	rs35798951	A (ins)	0.254	17	2.472	4.523	1.162	0.245041
MA+C	Strict	Additive	0.01	rs4659702	T	0.614	40	3.194	7.634	1.156	0.247702
MA+C	Strict	Additive	0.01	rs4659702	C	0.386	40	-3.194	7.634	-1.156	0.247702
MA	Strict	Dominant	0.01	rs35798951	A (ins)	0.254	23	2.443	4.48	1.154	0.24838
MA	Strict	Recessive	0.01	rs35798951	(blank)	0.746	23	-2.443	4.48	-1.154	0.24838
C	Broad	Recessive	0	rs819724	A	0.826	10	-2.25	3.812	-1.152	0.249185
C	Broad	Dominant	0	rs819724	G	0.174	10	2.25	3.813	1.152	0.249185
MA+C	Strict	Additive	0	rs4659702	T	0.614	26	3.174	7.697	1.144	0.252623
MA+C	Strict	Additive	0	rs4659702	C	0.386	26	-3.174	7.697	-1.144	0.252623
C	Broad	Additive	0.3	rs35798951	(blank)	0.42	11	1.85	2.657	1.135	0.256401
C	Broad	Additive	0.3	rs35798951	A (ins)	0.58	11	-1.85	2.657	-1.135	0.256401
MA+C	Strict	Dominant	0	rs11761919	A	0.541	17	2.336	4.285	1.129	0.259024

MA+C	Strict	Recessive	0	rs11761919	G	0.459	17	-2.336	4.285	-1.129	0.259024
MA	Broad	Dominant	0.3	rs9675777	C	0.321	11	-1.751	2.424	-1.125	0.260638
MA	Broad	Recessive	0.3	rs9675777	T	0.679	11	1.751	2.424	1.125	0.260638
MA+C	Strict	Dominant	0.01	rs11761919	A	0.541	25	2.298	4.238	1.116	0.264371
MA+C	Strict	Recessive	0.01	rs11761919	G	0.459	25	-2.298	4.238	-1.116	0.264371
C	Broad	Additive	0	rs6130764	T	0.57	11	2.833	6.509	1.111	0.266744
C	Broad	Additive	0	rs6130764	C	0.43	11	-2.833	6.509	-1.111	0.266744
MA	Broad	Additive	0.3	rs11355106	del	0.621	22	-2.95	7.062	-1.11	0.266975
MA	Broad	Additive	0.3	rs11355106	C	0.379	22	2.95	7.062	1.11	0.266975
C	Broad	Additive	0.01	rs6130764	T	0.57	13	2.808	6.408	1.109	0.267271
C	Broad	Additive	0.01	rs6130764	C	0.43	13	-2.808	6.408	-1.109	0.267271
MA+C	Strict	Dominant	0.01	rs1064395	A	0.252	30	2.387	4.687	1.103	0.270193
MA+C	Strict	Recessive	0.01	rs1064395	G	0.748	30	-2.387	4.687	-1.103	0.270193
C	Broad	Recessive	0.3	rs819724	A	0.826	12	-1.65	2.252	-1.099	0.271598
C	Broad	Dominant	0.3	rs819724	G	0.174	12	1.65	2.252	1.099	0.271598
MA+C	Strict	Dominant	0	rs1064395	A	0.252	20	2.382	4.731	1.095	0.27346
MA+C	Strict	Recessive	0	rs1064395	G	0.748	20	-2.382	4.731	-1.095	0.27346
MA+C	Strict	Additive	0	rs11761919	G	0.459	24	-3	8.109	-1.053	0.292115
MA+C	Strict	Additive	0	rs11761919	A	0.541	24	3	8.109	1.053	0.292115
MA+C	Strict	Additive	0.01	rs11761919	G	0.459	34	-2.955	8.028	-1.043	0.296982
MA+C	Strict	Additive	0.01	rs11761919	A	0.541	34	2.955	8.028	1.043	0.296982
MA+C	Strict	Dominant	0	rs9675777	C	0.196	10	-1.834	3.132	-1.036	0.300021
MA+C	Strict	Recessive	0	rs9675777	T	0.804	10	1.834	3.132	1.036	0.300021
MA+C	Strict	Dominant	0.01	rs7232062	T	0.55	19	1.99	3.702	1.034	0.300988
MA+C	Strict	Recessive	0.01	rs7232062	G	0.45	19	-1.99	3.702	-1.034	0.300988
MA+C	Strict	Dominant	0.01	rs9675777	C	0.196	13	-1.82	3.097	-1.034	0.301006
MA+C	Strict	Recessive	0.01	rs9675777	T	0.804	13	1.82	3.097	1.034	0.301006
C	Broad	Additive	0.3	rs6130764	T	0.57	13	2.083	4.064	1.033	0.301418
C	Broad	Additive	0.3	rs6130764	C	0.43	13	-2.083	4.064	-1.033	0.301418
MA+C	Strict	Dominant	0	rs7232062	T	0.55	14	1.956	3.729	1.013	0.310997
MA+C	Strict	Recessive	0	rs7232062	G	0.45	14	-1.956	3.729	-1.013	0.310997
C	Broad	Additive	0.01	rs35798951	(blank)	0.42	11	1.995	3.976	1.001	0.317063
C	Broad	Additive	0.01	rs35798951	A (ins)	0.58	11	-1.995	3.976	-1.001	0.317063
MA	Broad	Additive	0.02	rs35798951	(blank)	0.746	22	2.747	7.803	0.983	0.325479
MA	Broad	Additive	0.02	rs35798951	A (ins)	0.254	22	-2.747	7.803	-0.983	0.325479

MA+C	Broad	Dominant	0.02	rs6130764	C	0.404	32	3.044	10.126	0.957	0.3387
MA+C	Broad	Recessive	0.02	rs6130764	T	0.596	32	-3.044	10.126	-0.957	0.3387
MA+C	Broad	Dominant	0.01	rs6130764	C	0.404	32	3.066	10.285	0.956	0.339147
MA+C	Broad	Recessive	0.01	rs6130764	T	0.596	32	-3.066	10.285	-0.956	0.339147
MA+C	Broad	Dominant	0	rs6130764	C	0.404	30	3.087	10.448	0.955	0.339614
MA+C	Broad	Recessive	0	rs6130764	T	0.596	30	-3.087	10.448	-0.955	0.339614
MA	Strict	Dominant	0.01	rs1064395	G	0.681	14	-1.655	3.07	-0.945	0.344883
MA	Strict	Recessive	0.01	rs1064395	A	0.319	14	1.655	3.07	0.945	0.344883
MA	Broad	Additive	0	rs35798951	(blank)	0.746	20	2.667	8.021	0.942	0.3464
MA	Broad	Additive	0	rs35798951	A (ins)	0.254	20	-2.667	8.021	-0.942	0.3464
MA	Strict	Dominant	0.01	rs35195646	del	0.189	16	1.642	3.112	0.931	0.351961
MA	Strict	Recessive	0.01	rs35195646	A	0.811	16	-1.642	3.112	-0.931	0.351961
MA	Strict	Recessive	0	rs35195646	A	0.811	12	-1.643	3.152	-0.925	0.354782
MA	Strict	Dominant	0	rs35195646	del	0.189	12	1.643	3.152	0.925	0.354782
MA	Strict	Recessive	0	rs1064395	A	0.319	13	1.628	3.115	0.922	0.356442
MA	Strict	Dominant	0	rs1064395	G	0.681	13	-1.628	3.115	-0.922	0.356442
MA	Broad	Recessive	0	rs11761919	G	0.613	20	-2.404	6.905	-0.915	0.36031
MA	Broad	Dominant	0	rs11761919	A	0.387	20	2.404	6.905	0.915	0.36031
MA	Broad	Recessive	0.02	rs11761919	G	0.613	20	-2.336	6.685	-0.904	0.366213
MA	Broad	Dominant	0.02	rs11761919	A	0.387	20	2.336	6.685	0.904	0.366213
MA	Broad	Dominant	0.3	rs10994318	C	0.477	15	1.308	2.1	0.903	0.366621
MA	Broad	Recessive	0.3	rs10994318	G	0.523	15	-1.308	2.1	-0.903	0.366621
MA+C	Broad	Dominant	0.02	rs35798951	A (ins)	0.401	26	-2.347	7.165	-0.877	0.380647
MA+C	Broad	Recessive	0.02	rs35798951	(blank)	0.599	26	2.347	7.165	0.877	0.380647
MA	Strict	Additive	0.01	rs11355106	del	0.621	24	-2.238	6.553	-0.874	0.381897
MA	Strict	Additive	0.01	rs11355106	C	0.379	24	2.238	6.553	0.874	0.381897
MA+C	Broad	Dominant	0	rs10744560	T	0.286	25	-2.75	10.015	-0.869	0.384867
MA+C	Broad	Recessive	0	rs10744560	C	0.714	25	2.75	10.015	0.869	0.384867
MA+C	Broad	Dominant	0	rs9675777	C	0.196	14	-1.919	4.901	-0.867	0.386041
MA+C	Broad	Recessive	0	rs9675777	T	0.804	14	1.919	4.901	0.867	0.386041
MA+C	Broad	Dominant	0.01	rs9675777	C	0.196	15	-1.905	4.837	-0.866	0.386376
MA+C	Broad	Recessive	0.01	rs9675777	T	0.804	15	1.905	4.837	0.866	0.386376
MA	Strict	Additive	0	rs11355106	del	0.621	17	-2.233	6.657	-0.866	0.386701
MA	Strict	Additive	0	rs11355106	C	0.379	17	2.233	6.657	0.866	0.386701
MA+C	Broad	Dominant	0.02	rs9675777	C	0.196	15	-1.891	4.774	-0.866	0.386738

MA+C	Broad	Recessive	0.02	rs9675777	T	0.804	15	1.891	4.774	0.866	0.386738
MA+C	Broad	Dominant	0.01	rs35798951	A (ins)	0.401	26	-2.322	7.271	-0.861	0.389254
MA+C	Broad	Recessive	0.01	rs35798951	(blank)	0.599	26	2.322	7.271	0.861	0.389254
MA+C	Strict	Dominant	0.01	rs7232062	G	0.45	25	-2.07	5.781	-0.861	0.38931
MA+C	Strict	Recessive	0.01	rs7232062	T	0.55	25	2.07	5.781	0.861	0.38931
MA	Strict	Dominant	0.01	rs9675777	C	0.321	10	-1.32	2.357	-0.86	0.389837
MA	Strict	Recessive	0.01	rs9675777	T	0.679	10	1.32	2.357	0.86	0.389837
MA+C	Broad	Dominant	0.01	rs10744560	T	0.286	27	-2.684	9.849	-0.855	0.392383
MA+C	Broad	Recessive	0.01	rs10744560	C	0.714	27	2.684	9.849	0.855	0.392383
MA+C	Strict	Dominant	0	rs7232062	G	0.45	21	-2.044	5.84	-0.846	0.397773
MA+C	Strict	Recessive	0	rs7232062	T	0.55	21	2.044	5.84	0.846	0.397773
MA+C	Broad	Dominant	0	rs35798951	A (ins)	0.401	22	-2.296	7.379	-0.845	0.397877
MA+C	Broad	Recessive	0	rs35798951	(blank)	0.599	22	2.296	7.379	0.845	0.397877
MA+C	Broad	Dominant	0.02	rs10744560	T	0.286	27	-2.618	9.684	-0.841	0.400138
MA+C	Broad	Recessive	0.02	rs10744560	C	0.714	27	2.618	9.684	0.841	0.400138
MA	Broad	Recessive	0.02	rs35798951	(blank)	0.746	21	2.114	6.381	0.837	0.40268
MA	Broad	Dominant	0.02	rs35798951	A (ins)	0.254	21	-2.114	6.381	-0.837	0.40268
MA	Strict	Additive	0.01	rs1064395	G	0.681	29	-2.29	7.606	-0.83	0.406454
MA	Strict	Additive	0.01	rs1064395	A	0.319	29	2.29	7.606	0.83	0.406454
MA	Broad	Additive	0.02	rs11355106	del	0.621	22	-2.81	11.659	-0.823	0.410538
MA	Broad	Additive	0.02	rs11355106	C	0.379	22	2.81	11.659	0.823	0.410538
MA	Strict	Additive	0	rs1064395	G	0.681	23	-2.26	7.7	-0.814	0.4155
MA	Strict	Additive	0	rs1064395	A	0.319	23	2.26	7.7	0.814	0.4155
MA	Broad	Additive	0	rs11355106	del	0.621	20	-2.8	12.095	-0.805	0.420755
MA	Broad	Additive	0	rs11355106	C	0.379	20	2.8	12.095	0.805	0.420755
MA	Broad	Recessive	0	rs35798951	(blank)	0.746	19	2.056	6.568	0.802	0.422529
MA	Broad	Dominant	0	rs35798951	A (ins)	0.254	19	-2.056	6.568	-0.802	0.422529
C	Broad	Additive	0	rs10744560	C	0.666	10	1.5	3.536	0.798	0.425031
C	Broad	Additive	0	rs10744560	T	0.334	10	-1.5	3.536	-0.798	0.425031
C	Broad	Additive	0.01	rs10744560	C	0.666	11	1.485	3.485	0.795	0.426362
C	Broad	Additive	0.01	rs10744560	T	0.334	11	-1.485	3.485	-0.795	0.426362
MA	Broad	Additive	0.02	rs7232062	T	0.723	24	2.57	10.498	0.793	0.427673
MA	Broad	Additive	0.02	rs7232062	G	0.277	24	-2.57	10.498	-0.793	0.427673
MA+C	Strict	Dominant	0.01	rs35798951	(blank)	0.599	18	1.149	2.11	0.791	0.429071
MA+C	Strict	Recessive	0.01	rs35798951	A (ins)	0.401	18	-1.149	2.11	-0.791	0.429071

MA	Strict	Dominant	0.01	rs6130764	T	0.62	13	-1.338	2.862	-0.791	0.429158
MA	Strict	Recessive	0.01	rs6130764	C	0.38	13	1.338	2.862	0.791	0.429158
MA+C	Strict	Dominant	0.01	rs1064395	G	0.748	15	-1.412	3.254	-0.783	0.433596
MA+C	Strict	Recessive	0.01	rs1064395	A	0.252	15	1.412	3.254	0.783	0.433596
MA+C	Strict	Additive	0.01	rs6130764	T	0.596	46	-2.794	12.887	-0.778	0.43642
MA+C	Strict	Additive	0.01	rs6130764	C	0.404	46	2.794	12.887	0.778	0.43642
MA+C	Strict	Dominant	0.01	rs35195646	del	0.202	27	1.65	4.529	0.775	0.438277
MA+C	Strict	Recessive	0.01	rs35195646	A	0.798	27	-1.65	4.529	-0.775	0.438277
MA+C	Strict	Additive	0	rs6130764	T	0.596	35	-2.774	13.037	-0.768	0.442348
MA+C	Strict	Additive	0	rs6130764	C	0.404	35	2.774	13.037	0.768	0.442348
MA+C	Strict	Dominant	0	rs35195646	del	0.202	17	1.643	4.581	0.768	0.442719
MA+C	Strict	Recessive	0	rs35195646	A	0.798	17	-1.643	4.581	-0.768	0.442719
MA	Strict	Recessive	0	rs6130764	C	0.38	12	1.303	2.889	0.766	0.443493
MA	Strict	Dominant	0	rs6130764	T	0.62	12	-1.303	2.889	-0.766	0.443493
MA	Broad	Additive	0	rs7232062	T	0.723	24	2.5	10.743	0.763	0.445618
MA	Broad	Additive	0	rs7232062	G	0.277	24	-2.5	10.743	-0.763	0.445618
MA	Strict	Additive	0	rs35798951	(blank)	0.746	19	-1.833	5.82	-0.76	0.447284
MA	Strict	Additive	0	rs35798951	A (ins)	0.254	19	1.833	5.82	0.76	0.447284
MA+C	Strict	Dominant	0	rs1064395	G	0.748	14	-1.378	3.303	-0.758	0.448425
MA+C	Strict	Recessive	0	rs1064395	A	0.252	14	1.378	3.303	0.758	0.448425
MA+C	Broad	Dominant	0.02	rs7232062	G	0.45	26	-2.143	8.05	-0.755	0.450082
MA+C	Broad	Recessive	0.02	rs7232062	T	0.55	26	2.143	8.05	0.755	0.450082
MA+C	Strict	Dominant	0	rs6130764	C	0.404	27	2.055	7.566	0.747	0.455073
MA+C	Strict	Recessive	0	rs6130764	T	0.596	27	-2.055	7.566	-0.747	0.455073
MA	Strict	Additive	0.01	rs35798951	(blank)	0.746	25	-1.793	5.769	-0.747	0.455288
MA	Strict	Additive	0.01	rs35798951	A (ins)	0.254	25	1.793	5.769	0.747	0.455288
MA+C	Strict	Dominant	0.01	rs6130764	C	0.404	34	2.034	7.475	0.744	0.456996
MA+C	Strict	Recessive	0.01	rs6130764	T	0.596	34	-2.034	7.475	-0.744	0.456996
MA+C	Broad	Dominant	0.01	rs7232062	G	0.45	26	-2.117	8.152	-0.741	0.458513
MA+C	Broad	Recessive	0.01	rs7232062	T	0.55	26	2.117	8.152	0.741	0.458513
MA	Broad	Dominant	0.3	rs35195646	del	0.189	16	-1.319	3.18	-0.739	0.459628
MA	Broad	Recessive	0.3	rs35195646	A	0.811	16	1.319	3.18	0.739	0.459628
MA	Broad	Dominant	0.3	rs7232062	G	0.277	19	-1.563	4.472	-0.739	0.459758
MA	Broad	Recessive	0.3	rs7232062	T	0.723	19	1.563	4.472	0.739	0.459758
MA+C	Broad	Dominant	0.02	rs35195646	del	0.202	26	-2.03	7.651	-0.734	0.462953

MA+C	Broad	Recessive	0.02	rs35195646	A	0.798	26	2.03	7.651	0.734	0.462953
MA+C	Broad	Dominant	0.01	rs35195646	del	0.202	26	-2.037	7.778	-0.73	0.465159
MA+C	Broad	Recessive	0.01	rs35195646	A	0.798	26	2.037	7.778	0.73	0.465159
MA+C	Broad	Dominant	0	rs7232062	G	0.45	26	-2.09	8.255	-0.727	0.466944
MA+C	Broad	Recessive	0	rs7232062	T	0.55	26	2.09	8.255	0.727	0.466944
MA+C	Broad	Dominant	0	rs35195646	del	0.202	22	-2.044	7.907	-0.727	0.467369
MA+C	Broad	Recessive	0	rs35195646	A	0.798	22	2.044	7.907	0.727	0.467369
MA+C	Broad	Additive	0	rs10744560	C	0.714	32	2.667	13.951	0.714	0.475257
MA+C	Broad	Additive	0	rs10744560	T	0.286	32	-2.667	13.951	-0.714	0.475257
MA+C	Broad	Dominant	0	rs819724	G	0.202	27	2.25	10.035	0.71	0.477539
MA+C	Broad	Recessive	0	rs819724	A	0.798	27	-2.25	10.035	-0.71	0.477539
MA+C	Broad	Dominant	0.01	rs819724	G	0.202	31	2.223	9.866	0.708	0.479056
MA+C	Broad	Recessive	0.01	rs819724	A	0.798	31	-2.223	9.866	-0.708	0.479056
MA+C	Broad	Dominant	0.02	rs819724	G	0.202	31	2.197	9.7	0.705	0.480626
MA+C	Broad	Recessive	0.02	rs819724	A	0.798	31	-2.197	9.7	-0.705	0.480626
MA	Strict	Dominant	0.01	rs7232062	G	0.277	18	-1.49	4.505	-0.702	0.482664
MA	Strict	Recessive	0.01	rs7232062	T	0.723	18	1.49	4.505	0.702	0.482664
MA+C	Broad	Additive	0.01	rs10744560	C	0.714	34	2.592	13.724	0.7	0.484184
MA+C	Broad	Additive	0.01	rs10744560	T	0.286	34	-2.592	13.724	-0.7	0.484184
C	Broad	Additive	0.3	rs10744560	C	0.666	11	1.05	2.333	0.688	0.491762
C	Broad	Additive	0.3	rs10744560	T	0.334	11	-1.05	2.333	-0.688	0.491762
MA	Strict	Recessive	0	rs7232062	T	0.723	16	1.466	4.555	0.687	0.492086
MA	Strict	Dominant	0	rs7232062	G	0.277	16	-1.466	4.555	-0.687	0.492086
MA+C	Broad	Additive	0.02	rs10744560	C	0.714	34	2.517	13.5	0.685	0.493372
MA+C	Broad	Additive	0.02	rs10744560	T	0.286	34	-2.517	13.5	-0.685	0.493372
MA	Broad	Dominant	0.3	rs11761919	A	0.387	20	1.39	4.226	0.676	0.49905
MA	Broad	Recessive	0.3	rs11761919	G	0.613	20	-1.39	4.226	-0.676	0.49905
MA+C	Strict	Additive	0.01	rs11355106	del	0.696	33	-1.753	7.298	-0.649	0.516323
MA+C	Strict	Additive	0.01	rs11355106	C	0.304	33	1.753	7.298	0.649	0.516323
MA	Strict	Additive	0.01	rs819724	A	0.779	21	1.412	4.84	0.642	0.521081
MA	Strict	Additive	0.01	rs819724	G	0.221	21	-1.412	4.84	-0.642	0.521081
MA	Strict	Additive	0	rs819724	A	0.779	15	1.417	4.902	0.64	0.522258
MA	Strict	Additive	0	rs819724	G	0.221	15	-1.417	4.902	-0.64	0.522258
MA+C	Strict	Additive	0	rs11355106	del	0.696	20	-1.733	7.407	-0.637	0.524191
MA+C	Strict	Additive	0	rs11355106	C	0.304	20	1.733	7.407	0.637	0.524191

MA+C	Strict	Additive	0	rs35798951	(blank)	0.599	26	-1.833	8.32	-0.636	0.525037
MA+C	Strict	Additive	0	rs35798951	A (ins)	0.401	26	1.833	8.32	0.636	0.525037
MA+C	Broad	Dominant	0.02	rs1064395	A	0.252	29	1.745	7.546	0.635	0.52527
MA+C	Broad	Recessive	0.02	rs1064395	G	0.748	29	-1.745	7.546	-0.635	0.52527
MA+C	Broad	Dominant	0.01	rs1064395	A	0.252	29	1.74	7.656	0.629	0.529463
MA+C	Broad	Recessive	0.01	rs1064395	G	0.748	29	-1.74	7.656	-0.629	0.529463
MA+C	Strict	Additive	0.01	rs35798951	(blank)	0.599	39	-1.798	8.26	-0.626	0.53149
MA+C	Strict	Additive	0.01	rs35798951	A (ins)	0.401	39	1.798	8.26	0.626	0.53149
MA+C	Broad	Dominant	0	rs1064395	A	0.252	26	1.735	7.767	0.622	0.533641
MA+C	Broad	Recessive	0	rs1064395	G	0.748	26	-1.735	7.767	-0.622	0.533641
MA	Broad	Additive	0.3	rs1064395	G	0.681	29	-1.729	7.758	-0.621	0.534866
MA	Broad	Additive	0.3	rs1064395	A	0.319	29	1.729	7.758	0.621	0.534866
MA+C	Broad	Dominant	0.02	rs4659702	C	0.386	27	-1.728	7.959	-0.613	0.540184
MA+C	Broad	Recessive	0.02	rs4659702	T	0.614	27	1.728	7.959	0.613	0.540184
MA+C	Broad	Dominant	0	rs6130764	T	0.596	17	1.432	5.572	0.607	0.544145
MA+C	Broad	Recessive	0	rs6130764	C	0.404	17	-1.432	5.572	-0.607	0.544145
MA	Broad	Recessive	0.02	rs6130764	C	0.38	10	-1.019	2.893	-0.599	0.54908
MA	Broad	Dominant	0.02	rs6130764	T	0.62	10	1.019	2.893	0.599	0.54908
MA+C	Broad	Dominant	0.01	rs4659702	C	0.386	27	-1.691	8.077	-0.595	0.551739
MA+C	Broad	Recessive	0.01	rs4659702	T	0.614	27	1.691	8.077	0.595	0.551739
MA+C	Broad	Dominant	0.01	rs6130764	T	0.596	20	1.391	5.491	0.594	0.552837
MA+C	Broad	Recessive	0.01	rs6130764	C	0.404	20	-1.391	5.491	-0.594	0.552837
MA	Broad	Recessive	0.02	rs35195646	A	0.811	16	1.295	4.776	0.593	0.553356
MA	Broad	Dominant	0.02	rs35195646	del	0.189	16	-1.295	4.776	-0.593	0.553356
MA	Strict	Additive	0.01	rs4659702	T	0.713	25	1.345	5.171	0.592	0.554064
MA	Strict	Additive	0.01	rs4659702	C	0.287	25	-1.345	5.171	-0.592	0.554064
MA	Strict	Additive	0	rs4659702	T	0.713	19	1.34	5.225	0.586	0.55757
MA	Strict	Additive	0	rs4659702	C	0.287	19	-1.34	5.225	-0.586	0.55757
MA	Broad	Recessive	0	rs35195646	A	0.811	14	1.294	4.934	0.582	0.560301
MA	Broad	Dominant	0	rs35195646	del	0.189	14	-1.294	4.934	-0.582	0.560301
MA+C	Broad	Dominant	0.02	rs6130764	T	0.596	20	1.35	5.411	0.58	0.561756
MA+C	Broad	Recessive	0.02	rs6130764	C	0.404	20	-1.35	5.411	-0.58	0.561756
MA+C	Broad	Dominant	0	rs4659702	C	0.386	23	-1.655	8.197	-0.578	0.563274
MA+C	Broad	Recessive	0	rs4659702	T	0.614	23	1.655	8.197	0.578	0.563274
C	Strict	Recessive	0.01	rs6130764	C	0.43	10	-0.577	1.024	-0.571	0.568246

C	Strict	Dominant	0.01	rs6130764	T	0.57	10	0.577	1.024	0.571	0.568246
C	Strict	Recessive	0.02	rs6130764	C	0.43	10	-0.571	1.013	-0.568	0.570287
C	Strict	Dominant	0.02	rs6130764	T	0.57	10	0.571	1.013	0.568	0.570287
MA+C	Broad	Dominant	0.02	rs11761919	G	0.459	17	1.25	5.025	0.557	0.577256
MA+C	Broad	Recessive	0.02	rs11761919	A	0.541	17	-1.25	5.025	-0.557	0.577256
MA+C	Broad	Dominant	0.01	rs11761919	G	0.459	17	1.243	5.091	0.551	0.58159
MA+C	Broad	Recessive	0.01	rs11761919	A	0.541	17	-1.243	5.091	-0.551	0.58159
C	Broad	Additive	0.01	rs35195646	A	0.787	11	0.985	3.23	0.548	0.583671
C	Broad	Additive	0.01	rs35195646	del	0.213	11	-0.985	3.23	-0.548	0.583671
MA+C	Broad	Additive	0	rs35195646	A	0.798	23	1.738	10.144	0.546	0.585261
MA+C	Broad	Additive	0	rs35195646	del	0.202	23	-1.738	10.144	-0.546	0.585261
MA+C	Broad	Additive	0.01	rs35195646	A	0.798	28	1.723	9.98	0.545	0.585455
MA+C	Broad	Additive	0.01	rs35195646	del	0.202	28	-1.723	9.98	-0.545	0.585455
MA+C	Broad	Additive	0.02	rs35195646	A	0.798	28	1.708	9.819	0.545	0.585679
MA+C	Broad	Additive	0.02	rs35195646	del	0.202	28	-1.708	9.819	-0.545	0.585679
MA+C	Broad	Dominant	0	rs11761919	G	0.459	15	1.237	5.157	0.545	0.585907
MA+C	Broad	Recessive	0	rs11761919	A	0.541	15	-1.237	5.157	-0.545	0.585907
MA+C	Strict	Additive	0.01	rs819724	A	0.799	36	1.422	6.825	0.544	0.586325
MA+C	Strict	Additive	0.01	rs819724	G	0.201	36	-1.422	6.825	-0.544	0.586325
MA	Broad	Dominant	0.3	rs1064395	G	0.681	14	-0.914	2.872	-0.539	0.58959
MA	Broad	Recessive	0.3	rs1064395	A	0.319	14	0.914	2.872	0.539	0.58959
MA+C	Strict	Additive	0	rs819724	A	0.799	22	1.417	6.902	0.539	0.589717
MA+C	Strict	Additive	0	rs819724	G	0.201	22	-1.417	6.902	-0.539	0.589717
C	Broad	Additive	0.01	rs4659702	T	0.492	11	-0.985	3.762	-0.508	0.611576
C	Broad	Additive	0.01	rs4659702	C	0.508	11	0.985	3.762	0.508	0.611576
C	Broad	Additive	0.01	rs7232062	T	0.339	11	-0.975	3.971	-0.489	0.624649
C	Broad	Additive	0.01	rs7232062	G	0.661	11	0.975	3.971	0.489	0.624649
MA+C	Broad	Additive	0.02	rs11355106	del	0.696	31	-1.84	14.561	-0.482	0.629662
MA+C	Broad	Additive	0.02	rs11355106	C	0.304	31	1.84	14.561	0.482	0.629662
MA+C	Broad	Additive	0.01	rs11355106	del	0.696	31	-1.82	14.826	-0.473	0.636443
MA+C	Broad	Additive	0.01	rs11355106	C	0.304	31	1.82	14.826	0.473	0.636443
MA+C	Broad	Additive	0	rs10994318	G	0.516	18	1.667	12.444	0.472	0.636602
MA+C	Broad	Additive	0	rs10994318	C	0.484	18	-1.667	12.444	-0.472	0.636602
MA+C	Broad	Additive	0.01	rs10994318	G	0.516	24	1.637	12.206	0.468	0.639461
MA+C	Broad	Additive	0.01	rs10994318	C	0.484	24	-1.637	12.206	-0.468	0.639461

MA+C	Broad	Additive	0.02	rs10994318	G	0.516	24	1.607	11.972	0.464	0.642405
MA+C	Broad	Additive	0.02	rs10994318	C	0.484	24	-1.607	11.972	-0.464	0.642405
MA+C	Broad	Additive	0	rs11355106	del	0.696	28	-1.8	15.095	-0.463	0.643153
MA+C	Broad	Additive	0	rs11355106	C	0.304	28	1.8	15.095	0.463	0.643153
MA	Strict	Dominant	0.01	rs10744560	T	0.244	18	0.854	3.557	0.453	0.650733
MA	Strict	Recessive	0.01	rs10744560	C	0.756	18	-0.854	3.557	-0.453	0.650733
C	Broad	Additive	0	rs819724	A	0.826	11	-1	5	-0.447	0.654721
C	Broad	Additive	0	rs819724	G	0.174	11	1	5	0.447	0.654721
C	Strict	Recessive	0.01	rs35798951	A (ins)	0.58	12	-0.499	1.245	-0.447	0.654734
C	Strict	Dominant	0.01	rs35798951	(blank)	0.42	12	0.499	1.245	0.447	0.654734
C	Strict	Recessive	0.02	rs35798951	A (ins)	0.58	12	-0.498	1.241	-0.447	0.654834
C	Strict	Dominant	0.02	rs35798951	(blank)	0.42	12	0.498	1.241	0.447	0.654834
C	Broad	Additive	0.01	rs819724	A	0.826	13	-0.99	4.916	-0.447	0.655217
C	Broad	Additive	0.01	rs819724	G	0.174	13	0.99	4.916	0.447	0.655217
MA+C	Broad	Additive	0	rs9675777	C	0.196	16	1.25	7.898	0.445	0.656482
MA+C	Broad	Additive	0	rs9675777	T	0.804	16	-1.25	7.898	-0.445	0.656482
MA+C	Broad	Additive	0.01	rs9675777	C	0.196	17	1.24	7.793	0.444	0.656904
MA+C	Broad	Additive	0.01	rs9675777	T	0.804	17	-1.24	7.793	-0.444	0.656904
MA+C	Broad	Additive	0.02	rs9675777	C	0.196	17	1.23	7.689	0.444	0.657344
MA+C	Broad	Additive	0.02	rs9675777	T	0.804	17	-1.23	7.689	-0.444	0.657344
MA+C	Broad	Additive	0.02	rs7232062	T	0.55	35	1.62	14.405	0.427	0.669501
MA+C	Broad	Additive	0.02	rs7232062	G	0.45	35	-1.62	14.405	-0.427	0.669501
MA	Strict	Recessive	0	rs10744560	C	0.756	13	-0.806	3.612	-0.424	0.671654
MA	Strict	Dominant	0	rs10744560	T	0.244	13	0.806	3.612	0.424	0.671654
C	Broad	Additive	0.3	rs11761919	G	0.27	11	0.7	2.875	0.413	0.679726
C	Broad	Additive	0.3	rs11761919	A	0.73	11	-0.7	2.875	-0.413	0.679726
MA+C	Broad	Dominant	0	rs11761919	A	0.541	24	1.154	7.843	0.412	0.680332
MA+C	Broad	Recessive	0	rs11761919	G	0.459	24	-1.154	7.843	-0.412	0.680332
MA+C	Broad	Additive	0.01	rs7232062	T	0.55	35	1.56	14.591	0.408	0.682982
MA+C	Broad	Additive	0.01	rs7232062	G	0.45	35	-1.56	14.591	-0.408	0.682982
MA	Broad	Dominant	0.3	rs1064395	A	0.319	22	0.814	4.009	0.407	0.684189
MA	Broad	Recessive	0.3	rs1064395	G	0.681	22	-0.814	4.009	-0.407	0.684189
C	Broad	Additive	0.3	rs819724	A	0.826	13	-0.7	2.99	-0.405	0.685609
C	Broad	Additive	0.3	rs819724	G	0.174	13	0.7	2.99	0.405	0.685609
MA+C	Broad	Dominant	0.01	rs11761919	A	0.541	26	1.115	7.728	0.401	0.68835

MA+C	Broad	Recessive	0.01	rs11761919	G	0.459	26	-1.115	7.728	-0.401	0.68835
MA+C	Broad	Additive	0.02	rs6130764	T	0.596	41	-1.695	17.999	-0.399	0.689545
MA+C	Broad	Additive	0.02	rs6130764	C	0.404	41	1.695	17.999	0.399	0.689545
MA+C	Broad	Dominant	0	rs1064395	G	0.748	15	0.906	5.159	0.399	0.689925
MA+C	Broad	Recessive	0	rs1064395	A	0.252	15	-0.906	5.159	-0.399	0.689925
MA	Strict	Additive	0	rs9675777	C	0.321	10	0.833	4.391	0.398	0.690851
MA	Strict	Additive	0	rs9675777	T	0.679	10	-0.833	4.391	-0.398	0.690851
MA	Strict	Additive	0.01	rs9675777	C	0.321	13	0.823	4.346	0.395	0.692881
MA	Strict	Additive	0.01	rs9675777	T	0.679	13	-0.823	4.346	-0.395	0.692881
MA	Broad	Additive	0	rs9675777	C	0.321	12	1	6.461	0.393	0.694011
MA	Broad	Additive	0	rs9675777	T	0.679	12	-1	6.461	-0.393	0.694011
MA+C	Broad	Additive	0.01	rs6130764	T	0.596	41	-1.675	18.28	-0.392	0.695269
MA+C	Broad	Additive	0.01	rs6130764	C	0.404	41	1.675	18.28	0.392	0.695269
MA+C	Strict	Dominant	0.01	rs11761919	G	0.459	16	-0.657	2.82	-0.392	0.695415
MA+C	Strict	Recessive	0.01	rs11761919	A	0.541	16	0.657	2.82	0.392	0.695415
MA	Broad	Additive	0.02	rs9675777	C	0.321	13	0.98	6.291	0.391	0.696002
MA	Broad	Additive	0.02	rs9675777	T	0.679	13	-0.98	6.291	-0.391	0.696002
MA+C	Broad	Additive	0	rs7232062	T	0.55	33	1.5	14.779	0.39	0.696405
MA+C	Broad	Additive	0	rs7232062	G	0.45	33	-1.5	14.779	-0.39	0.696405
MA+C	Broad	Dominant	0.02	rs11761919	A	0.541	26	1.076	7.615	0.39	0.696538
MA+C	Broad	Recessive	0.02	rs11761919	G	0.459	26	-1.076	7.615	-0.39	0.696538
C	Broad	Additive	0.3	rs35195646	A	0.787	11	0.55	2.022	0.387	0.698949
C	Broad	Additive	0.3	rs35195646	del	0.213	11	-0.55	2.023	-0.387	0.698949
MA+C	Broad	Dominant	0.01	rs1064395	G	0.748	16	0.871	5.078	0.387	0.699011
MA+C	Broad	Recessive	0.01	rs1064395	A	0.252	16	-0.871	5.078	-0.387	0.699011
C	Broad	Recessive	0.3	rs35195646	A	0.787	10	0.525	1.847	0.386	0.699264
C	Broad	Dominant	0.3	rs35195646	del	0.213	10	-0.525	1.847	-0.386	0.699264
MA+C	Strict	Dominant	0.01	rs6130764	T	0.596	23	-0.76	3.886	-0.386	0.699748
MA+C	Strict	Recessive	0.01	rs6130764	C	0.404	23	0.76	3.886	0.386	0.699748
MA+C	Broad	Additive	0	rs6130764	T	0.596	37	-1.655	18.565	-0.384	0.700939
MA+C	Broad	Additive	0	rs6130764	C	0.404	37	1.655	18.565	0.384	0.700939
MA+C	Broad	Dominant	0.02	rs1064395	G	0.748	16	0.836	4.999	0.374	0.708307
MA+C	Broad	Recessive	0.02	rs1064395	A	0.252	16	-0.836	4.999	-0.374	0.708307
C	Broad	Additive	0.3	rs4659702	T	0.492	11	-0.55	2.186	-0.372	0.709886
C	Broad	Additive	0.3	rs4659702	C	0.508	11	0.55	2.186	0.372	0.709886

C	Strict	Recessive	0.01	rs819724	A	0.826	14	-0.48	1.735	-0.364	0.715588
C	Strict	Dominant	0.01	rs819724	G	0.174	14	0.48	1.735	0.364	0.715588
MA	Broad	Additive	0	rs10744560	C	0.756	22	1.167	10.415	0.362	0.717721
MA	Broad	Additive	0	rs10744560	T	0.244	22	-1.167	10.415	-0.362	0.717721
MA	Broad	Recessive	0.02	rs7232062	T	0.723	19	0.897	6.282	0.358	0.72048
MA	Broad	Dominant	0.02	rs7232062	G	0.277	19	-0.897	6.282	-0.358	0.72048
MA	Broad	Additive	0.3	rs819724	A	0.779	22	0.767	4.613	0.357	0.721118
MA	Broad	Additive	0.3	rs819724	G	0.221	22	-0.767	4.613	-0.357	0.721118
MA	Broad	Additive	0.3	rs35195646	A	0.811	17	0.738	4.399	0.352	0.724914
MA	Broad	Additive	0.3	rs35195646	del	0.189	17	-0.738	4.399	-0.352	0.724914
C	Strict	Recessive	0.02	rs819724	A	0.826	14	-0.46	1.722	-0.351	0.725924
C	Strict	Dominant	0.02	rs819724	G	0.174	14	0.46	1.722	0.351	0.725924
MA	Broad	Additive	0.02	rs819724	A	0.779	22	0.907	7.236	0.337	0.736072
MA	Broad	Additive	0.02	rs819724	G	0.221	22	-0.907	7.236	-0.337	0.736072
MA	Broad	Dominant	0.3	rs10744560	T	0.244	19	0.7	4.321	0.337	0.736297
MA	Broad	Recessive	0.3	rs10744560	C	0.756	19	-0.7	4.321	-0.337	0.736297
MA	Broad	Additive	0	rs819724	A	0.779	20	0.917	7.48	0.335	0.737492
MA	Broad	Additive	0	rs819724	G	0.221	20	-0.917	7.48	-0.335	0.737492
MA	Broad	Additive	0.3	rs9675777	C	0.321	13	0.7	4.377	0.335	0.737926
MA	Broad	Additive	0.3	rs9675777	T	0.679	13	-0.7	4.377	-0.335	0.737926
MA	Broad	Recessive	0	rs7232062	T	0.723	19	0.849	6.445	0.335	0.737998
MA	Broad	Dominant	0	rs7232062	G	0.277	19	-0.849	6.445	-0.335	0.737998
MA	Broad	Additive	0.02	rs10744560	C	0.756	23	1.047	10.064	0.33	0.741453
MA	Broad	Additive	0.02	rs10744560	T	0.244	23	-1.047	10.064	-0.33	0.741453
MA	Strict	Dominant	0.01	rs1064395	A	0.319	22	0.635	3.758	0.327	0.743407
MA	Strict	Recessive	0.01	rs1064395	G	0.681	22	-0.635	3.758	-0.327	0.743407
MA	Strict	Recessive	0	rs1064395	G	0.681	16	-0.632	3.793	-0.324	0.745595
MA	Strict	Dominant	0	rs1064395	A	0.319	16	0.632	3.793	0.324	0.745595
MA	Strict	Additive	0.01	rs10744560	C	0.756	21	-0.727	5.138	-0.321	0.748516
MA	Strict	Additive	0.01	rs10744560	T	0.244	21	0.727	5.138	0.321	0.748516
MA	Broad	Recessive	0.02	rs1064395	G	0.681	22	-0.74	5.495	-0.316	0.752233
MA	Broad	Dominant	0.02	rs1064395	A	0.319	22	0.74	5.495	0.316	0.752233
MA	Broad	Recessive	0	rs1064395	G	0.681	20	-0.735	5.642	-0.309	0.75707
MA	Broad	Dominant	0	rs1064395	A	0.319	20	0.735	5.642	0.309	0.75707
MA	Broad	Additive	0.3	rs10994318	G	0.523	15	0.667	4.859	0.302	0.762329

MA	Broad	Additive	0.3	rs10994318	C	0.477	15	-0.667	4.859	-0.302	0.762329
MA	Strict	Additive	0	rs10744560	C	0.756	15	-0.667	5.214	-0.292	0.770323
MA	Strict	Additive	0	rs10744560	T	0.244	15	0.667	5.214	0.292	0.770323
MA	Broad	Additive	0.02	rs35195646	A	0.811	17	0.738	6.643	0.286	0.774589
MA	Broad	Additive	0.02	rs35195646	del	0.189	17	-0.738	6.643	-0.286	0.774589
MA	Broad	Additive	0	rs35195646	A	0.811	15	0.738	6.858	0.282	0.778067
MA	Broad	Additive	0	rs35195646	del	0.189	15	-0.738	6.858	-0.282	0.778067
MA	Broad	Recessive	0	rs10744560	C	0.756	18	0.75	7.23	0.279	0.780294
MA	Broad	Dominant	0	rs10744560	T	0.244	18	-0.75	7.23	-0.279	0.780294
MA	Broad	Additive	0.3	rs11761919	G	0.613	25	0.733	7.06	0.276	0.782551
MA	Broad	Additive	0.3	rs11761919	A	0.387	25	-0.733	7.06	-0.276	0.782551
MA+C	Strict	Additive	0.01	rs10744560	C	0.714	32	-0.742	7.539	-0.27	0.787074
MA+C	Strict	Additive	0.01	rs10744560	T	0.286	32	0.742	7.539	0.27	0.787074
MA+C	Strict	Dominant	0.01	rs10744560	C	0.714	10	-0.37	1.901	-0.269	0.788259
MA+C	Strict	Recessive	0.01	rs10744560	T	0.286	10	0.37	1.901	0.269	0.788259
MA+C	Broad	Dominant	0	rs7232062	T	0.55	13	-0.59	4.838	-0.268	0.788486
MA+C	Broad	Recessive	0	rs7232062	G	0.45	13	0.59	4.838	0.268	0.788486
MA	Broad	Additive	0.02	rs1064395	G	0.681	29	-0.889	11.236	-0.265	0.790943
MA	Broad	Additive	0.02	rs1064395	A	0.319	29	0.889	11.236	0.265	0.790943
MA	Broad	Additive	0.3	rs10744560	C	0.756	23	-0.633	6.136	-0.256	0.798198
MA	Broad	Additive	0.3	rs10744560	T	0.244	23	0.633	6.136	0.256	0.798198
MA+C	Broad	Dominant	0.01	rs7232062	T	0.55	15	-0.557	4.769	-0.255	0.798845
MA+C	Broad	Recessive	0.01	rs7232062	G	0.45	15	0.557	4.769	0.255	0.798845
MA	Broad	Recessive	0.02	rs10744560	C	0.756	19	0.653	6.989	0.247	0.804805
MA	Broad	Dominant	0.02	rs10744560	T	0.244	19	-0.653	6.989	-0.247	0.804805
MA	Broad	Additive	0	rs1064395	G	0.681	27	-0.829	11.576	-0.244	0.807594
MA	Broad	Additive	0	rs1064395	A	0.319	27	0.829	11.576	0.244	0.807594
MA+C	Broad	Additive	0.02	rs1064395	G	0.748	37	-0.909	14.137	-0.242	0.809056
MA+C	Broad	Additive	0.02	rs1064395	A	0.252	37	0.909	14.137	0.242	0.809056
MA+C	Broad	Dominant	0.02	rs7232062	T	0.55	15	-0.523	4.7	-0.241	0.809403
MA+C	Broad	Recessive	0.02	rs7232062	G	0.45	15	0.523	4.7	0.241	0.809403
MA+C	Strict	Additive	0	rs10744560	C	0.714	23	-0.667	7.643	-0.241	0.809442
MA+C	Strict	Additive	0	rs10744560	T	0.286	23	0.667	7.643	0.241	0.809442
MA	Broad	Additive	0.02	rs10994318	G	0.523	15	0.667	8.13	0.234	0.815131
MA	Broad	Additive	0.02	rs10994318	C	0.477	15	-0.667	8.13	-0.234	0.815131

MA	Broad	Additive	0	rs10994318	G	0.523	12	0.667	8.444	0.229	0.818546
MA	Broad	Additive	0	rs10994318	C	0.477	12	-0.667	8.444	-0.229	0.818546
MA+C	Broad	Additive	0.01	rs1064395	G	0.748	37	-0.869	14.355	-0.229	0.818675
MA+C	Broad	Additive	0.01	rs1064395	A	0.252	37	0.869	14.355	0.229	0.818675
MA	Strict	Dominant	0.01	rs4659702	C	0.287	22	-0.458	4.192	-0.224	0.823007
MA	Strict	Recessive	0.01	rs4659702	T	0.713	22	0.458	4.192	0.224	0.823007
MA+C	Broad	Additive	0	rs1064395	G	0.748	34	-0.829	14.576	-0.217	0.828187
MA+C	Broad	Additive	0	rs1064395	A	0.252	34	0.829	14.576	0.217	0.828187
MA	Strict	Recessive	0	rs4659702	T	0.713	16	0.44	4.235	0.214	0.830507
MA	Strict	Dominant	0	rs4659702	C	0.287	16	-0.44	4.235	-0.214	0.830507
MA+C	Strict	Dominant	0.01	rs10744560	T	0.286	26	0.371	5.214	0.163	0.870795
MA+C	Strict	Recessive	0.01	rs10744560	C	0.714	26	-0.371	5.214	-0.163	0.870795
C	Broad	Additive	0.3	rs7232062	T	0.339	11	-0.25	2.45	-0.16	0.87311
C	Broad	Additive	0.3	rs7232062	G	0.661	11	0.25	2.45	0.16	0.87311
MA	Strict	Dominant	0.01	rs819724	G	0.221	18	-0.284	3.251	-0.158	0.874652
MA	Strict	Recessive	0.01	rs819724	A	0.779	18	0.284	3.251	0.158	0.874652
MA	Strict	Additive	0.01	rs10994318	G	0.523	14	-0.333	4.634	-0.155	0.876937
MA	Strict	Additive	0.01	rs10994318	C	0.477	14	0.333	4.634	0.155	0.876937
MA+C	Strict	Additive	0.01	rs10994318	G	0.516	23	-0.363	5.614	-0.153	0.878129
MA+C	Strict	Additive	0.01	rs10994318	C	0.484	23	0.363	5.614	0.153	0.878129
MA	Strict	Recessive	0	rs819724	A	0.779	12	0.278	3.299	0.153	0.878453
MA	Strict	Dominant	0	rs819724	G	0.221	12	-0.278	3.299	-0.153	0.878453
C	Broad	Additive	0.01	rs11761919	G	0.27	11	0.265	3.205	0.148	0.882322
C	Broad	Additive	0.01	rs11761919	A	0.73	11	-0.265	3.205	-0.148	0.882322
MA+C	Strict	Additive	0	rs9675777	C	0.196	12	0.333	5.141	0.147	0.883118
MA+C	Strict	Additive	0	rs9675777	T	0.804	12	-0.333	5.141	-0.147	0.883118
MA+C	Strict	Additive	0.01	rs9675777	C	0.196	16	0.323	5.086	0.143	0.885996
MA+C	Strict	Additive	0.01	rs9675777	T	0.804	16	-0.323	5.086	-0.143	0.885996
MA+C	Strict	Additive	0	rs10994318	G	0.516	10	-0.333	5.722	-0.139	0.889176
MA+C	Strict	Additive	0	rs10994318	C	0.484	10	0.333	5.722	0.139	0.889176
MA+C	Strict	Dominant	0.01	rs4659702	C	0.386	31	-0.31	5.165	-0.137	0.891332
MA+C	Strict	Recessive	0.01	rs4659702	T	0.614	31	0.31	5.165	0.137	0.891332
C	Broad	Recessive	0.3	rs6130764	C	0.43	10	-0.164	1.679	-0.126	0.899418
C	Broad	Dominant	0.3	rs6130764	T	0.57	10	0.164	1.679	0.126	0.899418
MA	Broad	Dominant	0.3	rs819724	G	0.221	19	-0.2	3.819	-0.102	0.918482

MA	Broad	Recessive	0.3	rs819724	A	0.779	19	0.2	3.819	0.102	0.918482
MA+C	Strict	Dominant	0.01	rs819724	G	0.201	32	0.196	4.987	0.088	0.930217
MA+C	Strict	Recessive	0.01	rs819724	A	0.799	32	-0.196	4.987	-0.088	0.930217
MA	Broad	Recessive	0.02	rs1064395	A	0.319	14	0.149	4.389	0.071	0.943487
MA	Broad	Dominant	0.02	rs1064395	G	0.681	14	-0.149	4.389	-0.071	0.943487
MA+C	Broad	Dominant	0.02	rs10744560	C	0.714	11	-0.102	3.162	-0.057	0.954404
MA+C	Broad	Recessive	0.02	rs10744560	T	0.286	11	0.102	3.162	0.057	0.954404
MA+C	Broad	Dominant	0.01	rs10744560	C	0.714	11	-0.092	3.208	-0.052	0.958812
MA+C	Broad	Recessive	0.01	rs10744560	T	0.286	11	0.093	3.208	0.052	0.958812
MA	Broad	Additive	0	rs11761919	G	0.613	25	-0.167	11.264	-0.05	0.960394
MA	Broad	Additive	0	rs11761919	A	0.387	25	0.167	11.264	0.05	0.960394
MA+C	Broad	Dominant	0	rs10744560	C	0.714	10	-0.083	3.255	-0.046	0.963159
MA+C	Broad	Recessive	0	rs10744560	T	0.286	10	0.083	3.255	0.046	0.963159
MA+C	Broad	Additive	0.02	rs11761919	G	0.459	36	0.173	14.103	0.046	0.963186
MA+C	Broad	Additive	0.02	rs11761919	A	0.541	36	-0.173	14.103	-0.046	0.963186
MA	Broad	Recessive	0	rs1064395	A	0.319	13	0.094	4.534	0.044	0.964856
MA	Broad	Dominant	0	rs1064395	G	0.681	13	-0.094	4.534	-0.044	0.964856
MA+C	Broad	Additive	0.01	rs11761919	G	0.459	36	0.128	14.294	0.034	0.972921
MA+C	Broad	Additive	0.01	rs11761919	A	0.541	36	-0.128	14.294	-0.034	0.972921
MA	Broad	Additive	0.02	rs11761919	G	0.613	25	-0.107	10.916	-0.032	0.974244
MA	Broad	Additive	0.02	rs11761919	A	0.387	25	0.107	10.916	0.032	0.974244
C	Strict	Additive	0.02	rs35195646	A	0.787	12	-0.03	1.406	-0.025	0.979816
C	Strict	Additive	0.02	rs35195646	del	0.213	12	0.03	1.406	0.025	0.979816
C	Strict	Additive	0.02	rs7232062	T	0.339	13	0.05	3.952	0.025	0.979935
C	Strict	Additive	0.02	rs7232062	G	0.661	13	-0.05	3.952	-0.025	0.979935
MA	Broad	Dominant	0.3	rs6130764	T	0.62	10	0.036	2.045	0.025	0.980052
MA	Broad	Recessive	0.3	rs6130764	C	0.38	10	-0.036	2.045	-0.025	0.980052
MA+C	Broad	Additive	0	rs819724	A	0.798	31	-0.083	12.48	-0.024	0.981180
MA+C	Broad	Additive	0	rs819724	G	0.202	31	0.083	12.48	0.024	0.981180
MA+C	Broad	Additive	0.01	rs819724	A	0.798	35	-0.078	12.272	-0.022	0.98216
MA+C	Broad	Additive	0.01	rs819724	G	0.202	35	0.078	12.272	0.022	0.98216
MA+C	Broad	Additive	0	rs11761919	G	0.459	33	0.083	14.488	0.022	0.982533
MA+C	Broad	Additive	0	rs11761919	A	0.541	33	-0.083	14.488	-0.022	0.982533
MA+C	Broad	Additive	0.02	rs819724	A	0.798	35	-0.073	12.068	-0.021	0.983158
MA+C	Broad	Additive	0.02	rs819724	G	0.202	35	0.073	12.068	0.021	0.983158

C	Strict	Additive	0.02	rs10744560	C	0.666	11	-0.03	2.376	-0.019	0.984471
C	Strict	Additive	0.02	rs10744560	T	0.334	11	0.03	2.376	0.019	0.984471
C	Strict	Additive	0.02	rs11761919	G	0.27	10	0.03	2.481	0.019	0.984804
C	Strict	Additive	0.02	rs11761919	A	0.73	10	-0.03	2.481	-0.019	0.984804
C	Strict	Additive	0.02	rs819724	A	0.826	15	0.02	1.972	0.014	0.988638
C	Strict	Additive	0.02	rs819724	G	0.174	15	-0.02	1.972	-0.014	0.988638
C	Strict	Recessive	0.02	rs35195646	A	0.787	11	-0.015	1.406	-0.013	0.989906
C	Strict	Dominant	0.02	rs35195646	del	0.213	11	0.015	1.406	0.013	0.989906
C	Strict	Additive	0.01	rs35195646	A	0.787	12	-0.015	1.417	-0.013	0.989946
C	Strict	Additive	0.01	rs35195646	del	0.213	12	0.015	1.417	0.013	0.989946
C	Strict	Additive	0.01	rs7232062	T	0.339	13	0.025	3.985	0.013	0.990008
C	Strict	Additive	0.01	rs7232062	G	0.661	13	-0.025	3.985	-0.013	0.990008
C	Strict	Additive	0.01	rs10744560	C	0.666	11	-0.015	2.402	-0.01	0.992278
C	Strict	Additive	0.01	rs10744560	T	0.334	11	0.015	2.402	0.01	0.992278
C	Strict	Additive	0.01	rs11761919	G	0.27	10	0.015	2.49	0.01	0.992416
C	Strict	Additive	0.01	rs11761919	A	0.73	10	-0.015	2.49	-0.01	0.992416
C	Strict	Additive	0.01	rs819724	A	0.826	15	0.01	1.986	0.007	0.994338
C	Strict	Additive	0.01	rs819724	G	0.174	15	-0.01	1.986	-0.007	0.994338
C	Strict	Additive	0.02	rs35798951	(blank)	0.42	14	-0.01	2.482	-0.006	0.994935
C	Strict	Additive	0.02	rs35798951	A (ins)	0.58	14	0.01	2.482	0.006	0.994935
C	Strict	Recessive	0.01	rs35195646	A	0.787	11	-0.008	1.417	-0.006	0.994973
C	Strict	Dominant	0.01	rs35195646	del	0.213	11	0.008	1.417	0.006	0.994973
MA	Broad	Recessive	0.02	rs819724	A	0.779	19	0.013	6.018	0.005	0.995664
MA	Broad	Dominant	0.02	rs819724	G	0.221	19	-0.013	6.018	-0.005	0.995664
C	Strict	Additive	0.01	rs35798951	(blank)	0.42	14	-0.005	2.49	-0.003	0.997472
C	Strict	Additive	0.01	rs35798951	A (ins)	0.58	14	0.005	2.49	0.003	0.997472
MA	Broad	Recessive	0	rs819724	A	0.779	17	0	6.223	0	1
MA	Broad	Dominant	0	rs819724	G	0.221	17	0	6.223	0	1