Bipolar disorder:

Genetic analysis of the circadian rhythm associated genes and metabolic syndrome

By

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DECLARATION

I certify that the dissertation hereby submitted for the degree *M.Sc*. in Human Molecular Genetics at the University of the Free State is my independent effort and had not previously been submitted for a degree at another University/Faculty. I furthermore waive copyright of the dissertation in favour of the University of the Free State.

C. Susmak

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Table of contents

List of figures

List of tables

Abbreviations

χ^2	Chi-square
˚C	Degrees Celsius
=	Equals
>	Greater than
≥	Greater than or equal to
<	Less than
µg	Microgram
µl	Microlitre
µM	Micromolar
%	Percentage
3’-UTR	3 prime untranslated region
5’-UTR	5 prime untranslated region
A	Adenine
$A_{260/280}$	Absorbance ratio at 260 nm and 280 nm
APA	American Psychiatric Association
ARNTL	Aryl hydrocarbon receptor nuclear translocator-like
BD	Bipolar disorder
BD-I	Bipolar I disorder
BD-II	Bipolar II disorder
BDNF	Brain-derived neurotrophic factor
bHLH	Basic helix-loop-helix
BMAL1	Brain and muscle Arnt-like protein 1
BMI	Body mass index
bp	Base pair
C	Cytosine
CDS	Coding DNA sequence
CK1d	Casein kinase 1 delta
CK1e	Casein kinase 1 epsilon
CLOCK	Circadian Locomotor Output Cycles Kaput circadian regulator
cm	Centimetre

CNTF	Ciliary neurotrophic factor
CNV	Copy number variant
Cry1	Cryptochrome 1
Cry2	Cryptochrome 2
CVD	Cardiovascular disease
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
df	Degrees of freedom
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic triphosphate
DSM	Diagnostic and Statistical Manual for Mental Disorders
dTTP	Thymidine triphosphate
DZ	Dizygotic
E-box	Enhancer Box
EDTA	Ethylenediaminetetraacetic acid
F	Forward
G	Guanine
G → A	Guanine to adenine
GDNF	Glial cell line-derived neurotrophic factor
GSK-3	Glycogen synthase kinase-3
GSK-3α	Glycogen synthase kinase-3α
GSK-3β	Glycogen synthase kinase-3β
GWAS	Genome-wide association studies
GxE	Gene-environmental
HDL	High-density lipoprotein
Hg	Mercury
HIV	Human Immunodeficiency Virus
HWE	Hardy-Weinberg Equilibrium
ICD-10	10^{th} revision of the International Classification of Diseases
JIS	Joint Interim Statement
kbp	Kilobase pair
kDa	KiloDalton
kg	Kilogram

kg/m^2	Kilogram per square metre
L	Litre
LD	Linkage disequilibrium
LDL	Low-density lipoprotein
m	Metre
M	Molar
MAP	Mitogen-activated protein
Met	Methionine
MetS	Metabolic syndrome
mg	Milligram
$MgCl_2$	Magnesium chloride
mg/µl	Milligrams per microliter
ml	Millilitre
mm	Millimetre
mM	Millimolar
mmHg	Millimetres of mercury
mmol	Millimoles
mmol/L	Millimoles per litre
mRNA	Messenger ribonucleic acid
MZ	Monozygotic
NaCl	Sodium chloride
NCBI	National Center for Biotechnology
NCEP ATP III	The Third Report of The National Cholesterol Education Program (NCEP). Expert Panel on Detection, Evaluation And Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III)
ng	Nanogram
NGF	Nerve growth factor
NGS	Next-generation sequencing
nm	nanometre
NPAS2	Neuronal PAS-domain protein 2
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
OMIM	Online Mendelian Inheritance in Man

PAS	Period-Arnt-single-minded
PCR	Polymerase chain reaction
PER	Period circadian regulator
PER3	Period circadian regulator 3
Pro-BDNF	BDNF precursor
R	Reverse
rpm	Revolutions per minutes
SA	South Africa
SADAG	The South African Depression and Anxiety Group
SANHANES	South African National Health and Nutrition Examination Survey
SASH	South African Stress and Health
SASHG	South African Society for Human Genetics
SCN	Suprachiasmatic nuclei
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
T	Thymine
T→C	Thymine to cytosine
T2D	Type 2 diabetes
T_a	Annealing temperature
TAE	Tris/acetic acid/EDTA
Tris	Trishydroxymethylaminomethane
V	Volts
Val	Valine
V/cm	Volts per centimetre
V/minute	Volts per minute
VNTR	Variable number tandem repeat
vs	Versus
WHO	World Health Organization
WMH	World Mental Health
Wnt	Wingless/Integrated

Abstract

Bipolar disorder (BD) is a serious, lifelong psychiatric disorder characterised by mood dysregulation. It has been rated as the sixth most debilitating disorder in the world. Comorbidity is often observed, with metabolic syndrome (MetS) as the most prevalent medical comorbidity. The pathophysiology observed between BD and MetS is suggested to be the result of common underlying genetic, environmental and lifestyle risk factors. The circadian rhythm and neurotrophins pathway disruptions play a role in BD and MetS aetiology.

The aim of the study was to investigate genetic associations between selected gene variants in these pathways and metabolic risk factors in patients with BD. A case-control design was implemented with 53 BD cases and matched controls. The questionnaire and genotype data were analysed using quantitative and molecular techniques to ensure a thorough comparison of the genetic and environmental components between the two groups investigated.

Selected candidate gene polymorphisms have previously been associated with BD, MetS and the circadian rhythm clock. Genotyping of *CLOCK* (rs1801260), *PER3* VNTR (rs57875989), *GSK-3β* (rs3755557 and rs334558) and *BDNF* (rs6265) was performed with restriction enzyme digestion and bi-directional sequencing. Statistical analysis was done with the Statistical Analysis System Software.

The genotype association with BD and MetS did not deliver statistically significant results. Analysis of multi-locus genotypes, a significant association between *CLOCK* and *GSK-3β* rs334558 (p = 0.012) as well as between *BDNF* and *PER3* (p = 0.022) was observed. When comparing the cases with the controls in terms of smoking cigarettes, exercise habits, following a balanced diet, prevalence of hypercholesterolaemia, cardiovascular conditions and diabetes, no differences were observed. A significant difference was observed in the ‘drinking alcohol on a regular basis’ (p = 0.012), where more controls were regular alcohol consumers as compared to the BD cases.

With regards to the ‘body mass index’ variable, the majority of BD cases were obese (p = 0.005).

This is the first study to investigate the allele and genotype frequencies of the selected polymorphisms from the circadian rhythm and neurotrophin pathways, in a South African BD population. The comorbidity of risk factors contributing to MetS in BD patients may be explained by overlapping genetic, lifestyle and environmental factors. Increasing the sample size and including more genetic variants may assist future researchers.

Keywords: bipolar disorder, metabolic syndrome, psychiatric genetics, genotyping

Chapter 1:
Introduction

1.1. General introduction and background

Bipolar disorder (BD) is a severe mental disorder (Kerner, 2014) whereby the affected individuals experience intense, erratic and repeated mood swings that alternate between mania and depression (Kerner, Lambert and Muthén, 2011; Chen *et al.*, 2013). It creates significant burdens on the patient, their families and society (Miller, Dell'Osso and Ketter, 2014). This chronic mood disorder (Sajatovic, 2005) affects occupational functioning (Miller, Dell'Osso and Ketter, 2014), relationships with individuals (Sajatovic, 2005) and the patient's overall quality of life (Miller, Dell'Osso and Ketter, 2014). Bipolar disorder is one of the leading global causes of disability, especially to individuals aged between 15 and 44 (Sajatovic, 2005). This disorder is associated with extremely high healthcare costs (Hirschfeld and Vornik, 2005) and comorbid conditions (Sajatovic, 2005). It is a common, complex psychiatric disorder with strong genetic risk factors (Kerner, 2015). Family, twin and adoption studies have generated vast amounts of evidence indicating genetic predisposition to BD. However, the association between genotypes and phenotypes is complex, involving the interaction of multiple genes and genetic mechanisms. Bipolar disorder is additionally influenced by non-genetic and stochastic risk factors (Marangoni, Hernandez and Faedda, 2016).

Africa is under-represented in terms of psychiatric genetic research being conducted on the continent (Soteriades *et al*., 2005). However, its broad range of genetic diversity may potentially provide better insight into complex disorders, such as BD (Wright *et al*., 2011). According to the 2018 mid-year population estimates report, South Africa (SA) is estimated at a population size of 57.7 million individuals (Statistics South Africa, 2018). The population can be broken down into the

following fractions: 80.9% are of Black African ethnicity, 7.8% are of White ethnicity, 8.8% are of Coloured ethnicity and 2.5% are of Indian/Asian ethnicity (Statistics South Africa, 2018). South Africa contains a large gene pool that may potentially contribute to a better understanding of BD genetic risk factors. Case-control association studies with genetically homogenous groups and mapping of susceptible loci, or identifying novel genes with genetically diverse groups, would greatly contribute to research on the identification of BD risk alleles (Wright *et al*., 2011). Molecular genetic research and the identification of risk alleles will aid in future BD diagnosis and treatment efforts (Hashimoto, 2010; Kerner, 2014).

Most human physiological processes are controlled through the chronological regulation of circadian rhythms (Bechtold, Gibbs and Loudon, 2010). Sleep-wake cycles, blood pressure, hormone regulation and metabolism, to name a few, are controlled by an endogenous cycle within the body that runs on a 24-hour period (Bechtold, Gibbs and Loudon, 2010). Disruption of these circadian rhythms has been shown to play a significant role in the pathogenesis of BD (Etain *et al*., 2014) and metabolic syndrome (MetS) (Maury, Hong and Bass, 2014). There is a substantial overlap between the shared genetic and environmental risk factors involved in both BD and MetS (Mansur, Brietzke and McIntyre, 2015). Neurotrophins, including the brain-derived neurotrophic factor (BDNF), play a role in mood regulation and are involved in the circadian rhythm system, showing diurnal rhythm (Tirassa, Quartini and Iannitelli, 2015). The functional role of BDNF extends to energy homeostasis as well as being critical for environmental changes in response to metabolic alterations. Impaired BDNF signalling, however, can have a severe impact on the body which can result in metabolic and psychiatric disorders (Marosi and Mattson, 2014). Brain-derived neurotrophic factor demonstrates to be a promising diagnostic marker for BD (Tirassa, Quartini and Iannitelli, 2015).

Comorbidity, specifically MetS comorbidity has become a major concern in BD patients and is found at a higher prevalence than in the general population (Garcia-Portilla *et al*., 2008). In addition to difficulties associated with BD, MetS presents its own negative associations including poor response to BD treatment and poor illness

outcome (McIntyre *et al*., 2007). Affected individuals are additionally at a higher risk of premature mortality (Taylor and MacQueen, 2006).

Considering the above evidence, psychiatric genetic research is greatly needed in SA and can bring new information into the scientific field. This will increase awareness of the problems in SA with regards to psychiatric research. Bipolar disorder alone and together with MetS, may cause severe implications to affected patients. Therefore, further investigation on genes associated with the circadian rhythm and neurotrophin gene family will help identify key risk alleles. In turn, this can help gain more information on the possible role of these genes as clinical diagnostic markers. This will aid in effective treatment and increase the understanding of the complex genetic mechanisms and pathophysiology involved in BD and MetS.

1.2. Research aims and objectives

The aim of this study was to investigate selected MetS risk factors and associations of selected polymorphisms from the circadian rhythm clock and neurotrophin family in patients with BD. Research shows that BD and MetS risk factors are mutually related to each other (Valkanova and Ebmeier, 2013; Vancampfort *et al*., 2014) and share identical biological mechanisms and genetic variants (Amare *et al*., 2017). However, only a limited amount of research has been conducted to investigate this biological and genetic comorbidity. The circadian clock controls brain functions involved in mood regulation and metabolic processes. The disruption of the circadian clock may be the cause of BD and metabolic comorbidity often observed. The involvement of this biological mechanism has not been considered previously in a unified manner (Barandas *et al*., 2015). Therefore, the use of a case-control research study combining the genetic risk alleles in the circadian clock and neurotrophin gene family, together with BD and MetS risk factors, are essential in the investigation of BD and MetS comorbidity. To meet the aims of this study, this dissertation consists of the following objectives:

Part one of this study will determine whether selected lifestyle and associated MetS risk factors are found at a higher prevalence in patients with BD as compared to the general population. Deductions will be made by comparing a BD case group consisting of individuals medically diagnosed with BD to a control group with no personal or familial history of mental illness.

Part two will determine whether the selected polymorphisms play a role in BD aetiology. The allele and genotype frequencies between the BD case and control groups will be compared. Furthermore, multi-locus genotypes will be compiled to determine cumulative risk on BD. Age at BD diagnosis and selected polymorphisms will be tested for allele and genotype associations. Lastly, statistically significant results from part one will be tested for allele and genotype associations of the selected polymorphisms.

1.3. Research layout

To meet the aims of this study, the research project comprised several components as shown in Figure 1.1.

Chapter 1 includes the study's general introduction and background, research aim and objectives as well as the research layout of the thesis.

Chapter 2 consists of a comprehensive literature review of the research topic. An in-depth literature analysis of BD includes its aetiology (genetic and environmental background), clinical phenotype, diagnostic criteria and associated burdens. The associated comorbidities are discussed focusing on MetS and its complications. Furthermore, the biological pathways involved in BD and MetS, with an emphasis on the circadian rhythm, neurotrophins and associated genes are explained.

Chapter 3 consists of the materials and methods used in this study to meet the objectives.

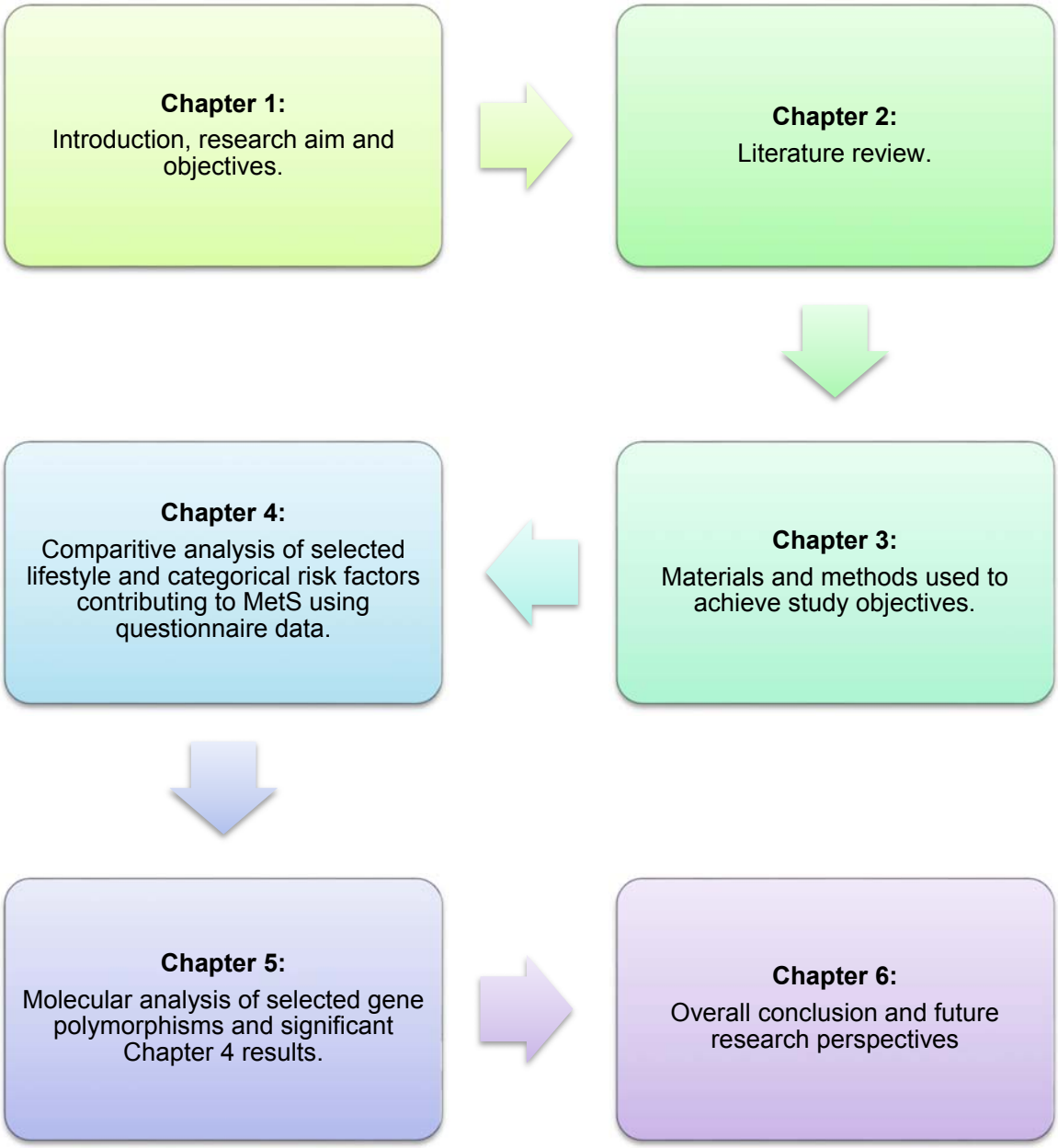


Figure 1.1 Research layout of the study.

Chapter 4 includes the quantitative and statistical analysis of the selected lifestyle and associated MetS risk factors obtained from the questionnaires provided to study participants. From this, a comparison between the BD case and control groups was made to determine if there was a statistical significant difference between the BD case and control groups.

Chapter 5 covers the molecular analysis of the selected polymorphisms. Differences in allele and genotype frequencies between the BD case and control groups was investigated for significant associations. Statistical analyses was also performed on multi-locus genotypes. The age at diagnosis was evaluated to determine whether it can be associated to specific allele and genotype frequencies. Additionally, any significant questionnaire results obtained from Chapter 4 was compared to the allele and genotype frequencies from the selected polymorphisms.

Chapter 6 consists of the overall study conclusion as well as study limitations and future research.

Chapter 7 includes the study references and appendices.

Chapter 2:
Literature review

2.1. The origin of bipolar disorder as a mental illness

Bipolar disorder was originally identified by classical Greek physicians in ancient times (Liddell and Scott, 1889). The categorical approach used in psychiatry was presented by the founders of modern psychiatry, such as Emil Kraepelin (Kraepelin, 1921). Kraepelin distinguished the first category, termed 'manic-depressive psychosis,' known today as the group of affective disorders (Kraepelin, 1921). The term 'bipolar' was originally used by Karl Leonhard (Leonhard, 1957) in 1957 to describe disorders that include both mania and depression. The term 'bipolar disorder' was implemented into the Diagnostic and Statistical Manual for Mental Disorders (DSM) by the American Psychiatric Association (APA) and replaced the previously used phrase, 'manic depression' (APA, 1980).

2.2. Clinical phenotype and diagnostic criteria of bipolar disorder

In modern day psychiatry, BD is referred to as an affective disorder (Bostwick and Pankratz, 2000; Phillips and Kupfer, 2013), where patients present with uncontrolled episodes of mood fluctuations, varying from severe mania to acute depression (Craddock and Jones, 1999; Sklar *et al*., 2008; Chen *et al*., 2013; Grande *et al*., 2016). These cycles often have in-between stages of normal mood known as euthymia (Haenisch *et al*., 2014). In a mixed state, contrasting symptoms can occur simultaneously and in the worst-case scenario, psychotic symptoms can arise (Figure 2.1) (Thomas, 2004; Abreu and Bragança, 2015; Harrison, 2016).

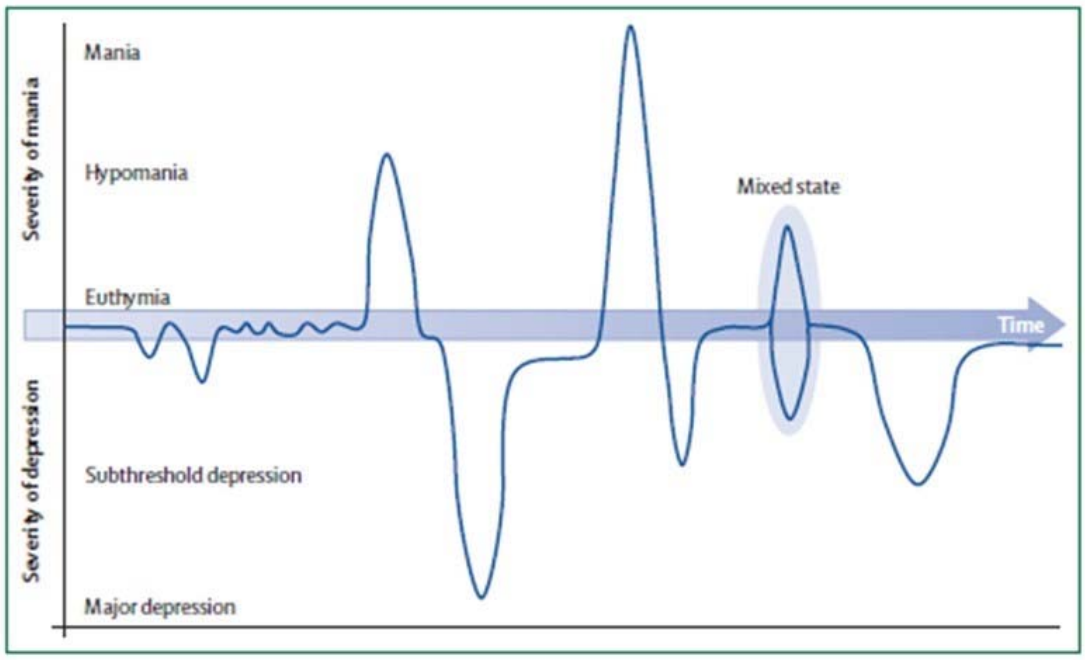


Figure 2.1 A life chart depicting the development and severity of mania and depression in a BD individual over time. The mania and hypomania symptoms are displayed above the baseline (euthymia) and the depressive symptoms are shown below the baseline (Grande *et al*., 2016).

Bipolar disorder is a chronic (Grande *et al*., 2016), lifelong mood disorder (Beyer *et al*., 2005; Wilson *et al*., 2006) with the average age at onset typically occurring in late adolescence or early adulthood (Shih, Belmonte and Zandi, 2004; Hashimoto, 2010; Harrison, 2016), usually before the age of 25 years (Thomas, 2004). Specifically, age at onset refers to the age a patient first presents with the criteria for depression or mania (Benedetti *et al*., 2004a). Onset can occur much later in life, but also as early as the age of 12 years (Shih, Belmonte and Zandi, 2004). The median age for the first major mood episode is at 20 years (Lui *et al*., 2008).

The most widely recognised diagnostic criteria used in psychiatry are based on the 10th revision of the International Classification of Diseases (ICD-10) (World Health Organization [WHO], 1993) and the current fifth edition of the DSM (DSM-V) (APA, 2013) (Grande *et al*., 2016). Diagnosis of the BD phenotype is determined by the patient's self-assessment, their overt behaviour (Frey *et al*., 2013) and clinical symptoms (Craddock and Sklar, 2013), that are frequently characterised by subthreshold symptoms (Bonnín *et al*., 2012). According to the DSM-V panel for diagnosis of BD and related disorders, BD can be classified under the following subtypes: bipolar I disorder (BD-I), bipolar II disorder (BD-II), cyclothymic disorder, other specified bipolar and related disorder, unspecified bipolar and related disorder, substance or drug-induced bipolar and related disorder as well as bipolar and related disorder due to another medical condition (APA, 2013). Bipolar disorder is primarily divided into the two subtypes, BD-I and BD-II (Thomas, 2004; Grande *et al*., 2016). The diagnosis of BD-I requires the patient to present at least one manic episode. Major depressive episodes are usually present but, are not required for diagnosis. The diagnosis of BD-II requires at least one hypomanic episode, thus, a less severe form of mania, and one major depressive episode (APA, 2013). 'Rapid cyclers' are BD patients that experience at least four manic or depressive episodes within a 12-month cycle (Belmaker, 2004). Patients who present with hypomanic or major depressive episodes over a two-year period, but do not fully meet the criteria for hypomania or major depression, are diagnosed as having cyclothymic disorder (APA, 2013).

Bipolar disorder affects both men and women equally (Pini *et al*., 2005) with regards to the age at onset, symptom presentation and total number of mood episodes (Kawa *et al*., 2005). Bipolar I disorder is equally distributed in both men and women, whereas BD-II affects more women (Nivoli *et al*., 2011). Differences are observed in the mood episode type presented at BD onset as well as the comorbidity variations between men and women (Kawa *et al*., 2005). Bipolar disorder affects individuals regardless of their ethnicity, nationality or social status (Alonso *et al*., 2011).

2.2.1. A description of mania and hypomania

Mania is the distinguishing trait of BD and is described as being exceedingly active with a decreased need for sleep (Belmaker, 2004; Hirschfeld, 2007; Phillips and Kupfer, 2013). Patients typically experience an extremely uplifting mood (elation), exceedingly high positive beliefs (Belmaker, 2004), irritable mood (Phillips and Kupfer, 2013), are more talkative, have racing thoughts (Hirschfeld, 2007) and have an increased motor drive that may vary in severity and duration (Grande *et al*., 2016). These symptoms cause disruptions in their judgment and affects the way they conduct themselves (Craddock and Jones, 1999; Belmaker, 2004; Wilson *et al*., 2006), which is typically in complete contrast to the patient's normal behaviour (Belmaker, 2004). Patients may become involved in activities that jeopardise many aspects of their life, including their occupation and personal relationships. Mania may take several weeks to months before reaching peak intensity (Belmaker, 2004). Acute mania is characteristic of BD-I (Thomas, 2004) and viewed as a medical emergency. It can have many destructive effects if not treated promptly (Belmaker, 2004) and often requires hospitalisation (Hirschfeld, 2007).

Hypomania is a mild, less severe form of mania (Thomas, 2004; Phillips and Kupfer, 2013) and symptoms are not as prolonged as those associated with mania (Phillips and Kupfer, 2013). One episode of hypomania does not usually present any social or occupational complications (Thomas, 2004; Hirschfeld, 2007) or the need for

hospitalisation (Hirschfeld, 2007). However, persistent hypomania can cause a reasonable amount of impairment in social and workplace environments (Thomas, 2004). It is usually under-diagnosed due to inadequate communication between patients and medical practitioners (Thomas, 2004). Hypomania of BD-II may only become apparent after the administration of incorrect treatment to a BD patient, such as antidepressants, which may aggravate symptoms and induce mania (Hirschfeld, 2007). It is critical that the correct manic state in the patient is identified before a treatment plan is established (Thomas, 2004).

2.2.2. A description of depression

Many features of unipolar and bipolar depression show extreme similarity (Grande *et al*., 2016) and often result in misdiagnosis (Phillips and Kupfer, 2013). Patients with unipolar depression do not display mania. Bipolar depression is usually the first symptom observed at BD onset. The mood fluctuations observed between unipolar, BD-I and BD-II differ and are displayed in Figure 2.2 (Phillips and Kupfer, 2013). Characteristics that distinguish bipolar depression from unipolar depression include an earlier age at onset, shorter duration of episodes, more frequent episodes and a positive family history (Mitchell, Malone, and Doebbeling, 2008). Bipolar depression is accompanied by reduced amounts of energy, loss of pleasure, having a low mood (Phillips and Kupfer, 2013), hypersomnia, hyperphagia (Mitchell, Malone, and Doebbeling, 2008; Goodwin *et al*., 2008), weight gain and social isolation, to name only a few (Goodwin *et al*., 2008).

2.3. Epidemiology of bipolar disorder

Bipolar disorder has a lifetime prevalence estimated at 1% (Sklar *et al*., 2002; Fajutrao *et al*., 2009). It is ranked as one of the 10 most debilitating disorders (Lopez *et al*., 1998), being placed sixth in the world (Abreu and Bragança, 2015). Bipolar

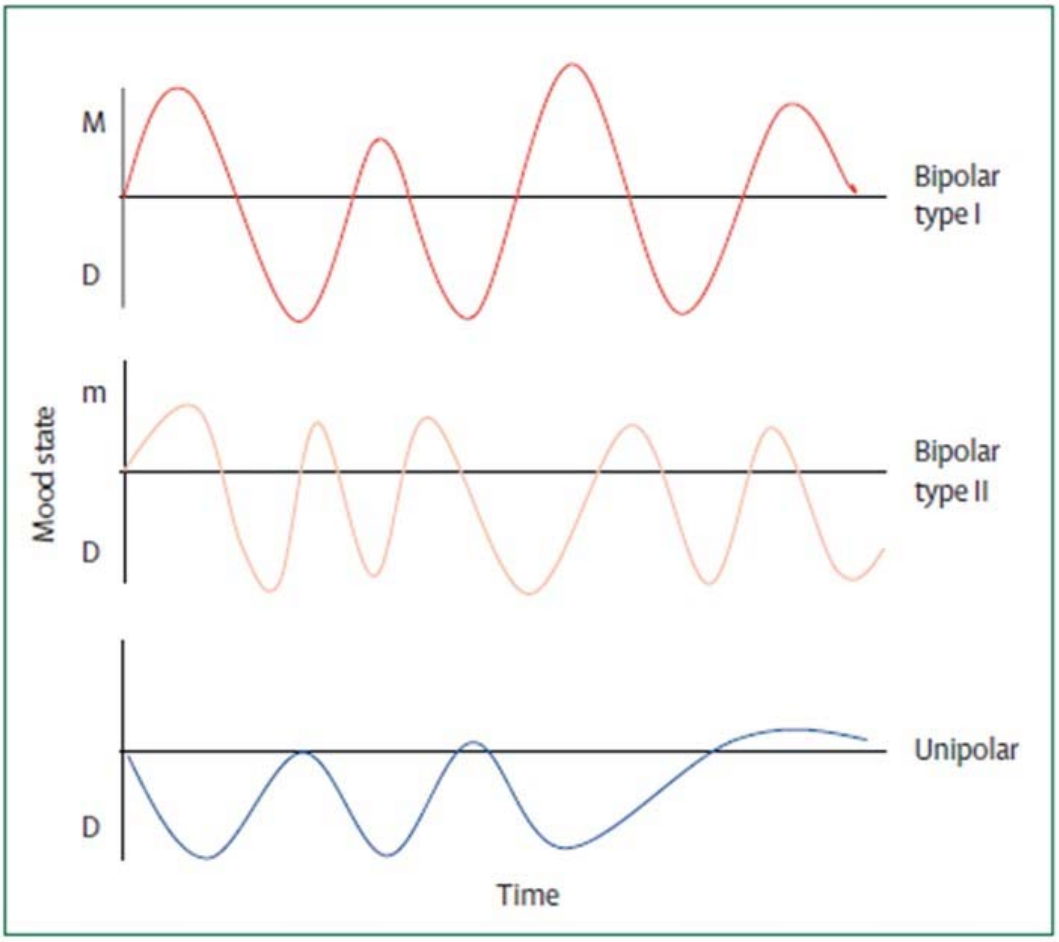


Figure 2.2 The differences in mood variations over time observed in BD-I, BD-II and unipolar depression (M=mania, m=hypomania, D=depression) (Phillips and Kupfer, 2013).

disorder is one of the most clinically severe mental illnesses (Pini *et al*., 2005; McClung, 2013), affecting approximately 1 - 2% of the world population, depending on the criteria used (Harrison, 2016).

2.3.1. Prevalence of bipolar disorder and other psychiatric disorders in South Africa

In SA, over four million individuals are diagnosed with BD (The South African Depression and Anxiety Group [SADAG], 2018). Statistics from the Council of Medical Schemes in SA shows that the incidence of BD in medical aid members, have increased by 228% between 2006 and 2011. In 2015, neuropsychiatric disorders were the third highest contributor of burden in SA (Bateman, 2015). There is a lack of current data on the prevalence of BD in SA. However, due to high misdiagnosis rates, prevalence estimates are likely to be much higher (Fajutrao *et al*., 2009). According to the South African Stress and Health Study (SASH), conducted as part of the WHO World Mental Health (WMH) project (Williams *et al*., 2004), mood disorders have a lifetime prevalence of 9.8% in SA and a total of 1 in 3 South Africans have a lifetime DSM disorder. When compared to other countries, SA has a moderately high 12-month prevalence of mood disorders. A total of 1 in 6 South Africans were diagnosed with a DSM disorder in the preceding 12 months of the study (Herman *et al.*, 2009). When compared to Nigeria, the only other African country that had taken part in the WMH survey, the 12-month prevalence of mood disorders were 1 in 17 (Herman *et al.*, 2009), confirming the strikingly elevated rates of mental disorders in SA (Gureje *et al*., 2006).

When considering SA provinces, the Western Cape had the highest 12-month and lifetime prevalence rates of mental disorders (Herman *et al.*, 2009). It was the first province to be colonised and has one of the highest urbanisation levels. Rural provinces generally have lower mental disorder rates, with the Eastern Cape presenting with the lowest rate in the country (Herman *et al.*, 2009). This might be attributed to the huge deficiency and insufficiency of mental health care services in

the rural parts of SA and the unequal distribution of mental health professionals (Burns, 2011; Vergunst, 2018). Only a total of 0.28 psychiatrists and 0.32 psychologists per 100 000 individuals are available in SA (Lund *et al*., 2010), with the majority located in urban areas (Burns, 2011). Rural areas must rely on general doctors for mental health care. If referred to a psychiatrist, transportation to the nearest city remains challenging (Vergunst, 2018). A total of 34% of individuals in SA live in rural areas (The World Bank, 2018). Many do not have the opportunity to receive the appropriate mental health care and are, therefore, never diagnosed, a scenario that may account for the low statistics in rural areas. In addition, poverty, illiteracy and the stigma associated with mental disorders prevent individuals from rural SA to receive suitable mental health care they deserve (Burns, 2011).

A total of 26% of DSM disorders are considered severe in SA (Herman *et al*., 2009). This is a clear indication that psychiatric disorders are a huge liability in SA (Williams *et al*., 2008). The SASH, conducted between 2002 and 2004, was the first investigation of its nature performed in SA (Williams *et al*., 2004) with regards to the incidence, extremity and medical care of mental illnesses of the population (Williams *et al*., 2008). The SASH remains the primary source of mental health prevalence data for SA (Jacob and Coetzee, 2018). The requirements and challenges of individuals living in rural SA, specifically those living with mental illness, have not been fully investigated (Neille and Penn, 2015; Vergunst, 2018) and remain largely neglected. Only recently, has this gained more interest and attention (Vergunst, 2018).

2.4. Complexity and negative implications associated with bipolar disorder

Bipolar disorder is frequently accompanied by burdensome effects in all aspects of the affected patient's life (Sajatovic, 2005; Chen *et al*., 2013). This includes their health, career, social (Sajatovic, 2005; Chen *et al*., 2013) and personal aspects (Chen *et al*., 2013; Haenisch *et al*., 2014). Cognitive dysfunction plays a crucial factor in disability (Fajutrao *et al*., 2009). These burdens interfere with BD patients'

behaviour and reasoning that can have harsh effects on them and their families (Lin, Huang and Lui, 2013). Overall, their quality of life is significantly reduced (Sajatovic, 2005; Sylvia *et al*., 2017) and they generally have an overall poor health status (Pini *et al*., 2005; Fajutrao *et al*., 2009). Factors such as suicide (Pini *et al*., 2005; Fajutrao *et al*., 2009; Grande *et al*., 2016), high morbidity rates (Harrison, 2016), premature morbidity (Ösby *et al*., 2016), days out of role (Alonso *et al*., 2011), high relapse rates while on treatment (Sajatovic, 2005; Morales-Marín *et al*., 2016), psychiatric and medical comorbidities (Sajatovic, 2005; Fajutrao *et al*., 2009; Song *et al*., 2014), disability, and high misdiagnosis rates, additionally add to the burden of BD itself (Fajutrao *et al*., 2009). Some individuals suffer from BD for many years before being diagnosed and treated, as it often goes unrecognised (Hashimoto, 2010).

2.4.1. Economic burdens

The economic burden of BD is extremely high as it is one of the highest causes of disability around the world (Thomas, 2004). As a result, this inflicts an extensive impact on the healthcare system and the costs involved. Between 2008 and 2012, the number of mental disorder medical aid pay-outs by the largest medical aid scheme in SA, Discovery Health, increased by a staggering 41% (Bateman, 2015). Overall, the annual risk ‘spend’ of all medical schemes for hospital admissions increased from R96.7 million to R464.6 million (Bateman, 2015). Unfortunately, the statistics for the public sector are unavailable, where over 80% of the South African population are treated (Bateman, 2015). It is costing the country more money not to treat mental health patients than to treat them (Bateman, 2015).

According to the WHO’s WMH Surveys, BD has the second highest average days out of role at a total of 17.3 days per year (Alonso *et al*., 2011). Days out of role indicates the number of days an individual is not able to work or perform their usual daily tasks due to their health condition (Andrade *et al*., 2013). In turn, this causes a tremendous economic burden (Fajutrao *et al*., 2009). It places great stress on

society, not only affecting healthcare costs (Bauer *et al*., 2001; Sajatovic, 2005; Miller, Dell'Osso and Ketter, 2014), but also unemployment rates (Sajatovic, 2005; Miller, Dell'Osso and Ketter, 2014) and efficiency in the workplace (Miller, Dell'Osso and Ketter, 2014). Suicide and premature death add to economic stress as it yields reduced productivity (Fajutrao *et al*., 2009). Finding risk alleles to use as diagnostic markers will aid in early BD diagnosis to enable patients to receive appropriate treatment as soon as possible. This will reduce the days out of role and help decrease the economic burden on the country.

2.4.2. Comorbidity

Comorbidity is the term used to describe the presence of two disorders co-occurring in one patient (Krishnan, 2005). Over 300 diagnoses are classified in the DSM and many overlap each other. Subsequently, if an individual displays one disorder, that individual is at a high risk of presenting with numerous other disorders (Eisner *et al*., 2017). Individuals with BD are at an increased risk for comorbidity (Czepielewski *et al*., 2012) and display high comorbidity with medical and other psychiatric disorders (Sajatovic, 2005). Approximately, 67 - 99% of BD patients will show comorbidity with other conditions (Krishnan, 2005; Kessler *et al*., 2005) and many will display comorbidity with two or more disorders simultaneously (Bauer and Pfennig, 2005). Relatives of a family member presenting with comorbidity are more inclined to have the comorbid disorder themselves (McElroy *et al*., 2001).

Comorbidity results in numerous anticipated complications in patients diagnosed with BD (Beyer *et al*., 2005), an outcome that negatively influences illness trajectory (Bauer *et al*., 2016). Often, complications and delays in diagnosis and treatment are experienced (Vieta *et al*., 2001; Krishnan, 2005). A large part of misdiagnosis is due to the considerable symptom overlap between disorders (Krishnan, 2005; Nabavi, Mitchell and Nutt, 2015). It is often uncertain whether a disorder is strictly comorbid, as it can be influenced by the medication used (Cassidy *et al*., 1999; McElroy *et al*., 2001). It can however, be a combination of both, as observed for obesity, diabetes

and hypothyroidism (Cassidy *et al*., 1999; McElroy *et al*., 2001). Many comorbid conditions occur at a higher incidence than expected by chance (Krishnan, 2005). Ultimately, the physiological processes that cause comorbidity are unclear (Krishnan, 2005). Researchers consider comorbid disorders to share mutual genetic (Kerner, 2014) and environmental (McElroy *et al*., 2001) risk factors with BD (Kerner, 2014). Awareness of comorbidities and its complications associated with BD are required for optimal medical treatment (Krishnan, 2005) and is essential for future BD research.

2.4.2.1. Substance abuse comorbidity

Substance abuse is extremely common in BD patients, however, the relationship of this comorbidity is unknown (Strakowski *et al*., 2000). Researchers investigating the comorbidity of substance abuse and BD have found that a majority of adult patients were abusing either alcohol, recreational and/or prescription drugs (Strakowski *et al*., 1992; Goldstein and Bukstein, 2010). Substance abuse is found at the highest prevalence in BD patients of all Axis I disorders (Regier *et al*., 1990). Axis I disorders include all clinical disorders excluding personality disorders and mental retardation (APA, 2000). Substance abuse is related to an earlier age at BD onset, impaired treatment response and an overall poor illness outcome (Strakowski *et al*., 2000; Ostacher *et al*., 2006).

2.4.2.2. Psychiatric comorbidity

Psychiatric comorbidity in BD patients is common (Harrison, 2016). Axis I and Axis II disorders (include all personality disorders and mental retardation) (APA, 2000) are frequently comorbid in BD patients and usually precedes the onset of BD (Krishnan, 2005). The lifetime prevalence of psychiatric comorbidity has been estimated to be between 50% and 70% (Vieta *et al*., 2001). Research has shown that BD patients comorbid with an Axis I disorder, have additional negative effects

including earlier age of symptom onset, increasing number and severity of mood episodes, drug abuse (McElroy *et al*., 2001), higher occurrence of depressive episodes, attempted suicides (Vieta *et al*., 2001) and rapid cycling (McElroy *et al*., 2001, Vieta *et al*., 2001).

Anxiety (Krishnan, 2005; Nabavi, Mitchell and Nutt, 2015) and panic disorder frequently coexist with BD (Krishnan, 2005). Pavlova *et al*. (2015) found that anxiety disorders were three-fold more prevalent in BD patients than in the general population. Strakowski and colleagues (1992) also reported obsessive-compulsive disorder and panic disorder to be much higher in BD patients as compared to the general population (Strakowski *et al*., 1992).

2.4.2.3. Medical comorbidity

Beyer and colleagues (2005) found that the highest medical comorbidity with BD was in the endocrine, nutritional and MetS group (diabetes, obesity, thyroid conditions and hypercholesterolaemia), with a prevalence of 13.6% in the study population (Beyer *et al*., 2005). The incidence of MetS in patients with BD has become alarming as compared to the general population (Garcia-Portilla *et al*., 2008; Bly *et al*., 2014; Silarova *et al*., 2015). This co-occurrence increases morbidity and mortality (Cardenas *et al*., 2008) and is associated with a poor response to treatment, a poor illness course and a complex illness presentation. Affected individuals are more inclined to experience depressive episodes and symptoms, while being at a greater risk of suicide (McIntyre *et al*., 2010). Therefore, it is crucial that adequate management of MetS in BD patients be implemented so that BD prognosis and treatment is controlled (Fagiolini *et al*., 2005).

The second highest comorbidity with BD was observed in diseases of the circulatory system (heart disease and hypertension) (Beyer *et al*., 2005). This was observed in 10.7% of the study subjects from Beyer and colleagues (2005). Other comorbid

diseases within this BD study population were disorders of the nervous system and sense organs (migraines, head injuries, stroke and vision loss) as well as infectious and parasitic diseases such as Human Immunodeficiency Virus (HIV) and hepatitis C (Beyer *et al*., 2005).

2.4.3. Metabolic syndrome classification and general overview

The first classification of MetS, as it is known today, was originally described as a syndrome by Eskil Kylin in 1923 as having hypertension, hyperglycaemia and gout (Kylin, 1923). 'Syndrome X' classified by Gerald M. Reaven was used to describe the grouping of factors for coronary artery disease in a single individual (Reaven, 1988). Over the past few decades, many terms and phrases have been used for what we know today as 'metabolic syndrome' (Alberti, Zimmet and Shaw, 2006). Metabolic syndrome is a cluster of risk factors within an individual that contribute to cardiovascular disease (CVD) (Moreira *et al*., 2016) and diabetes (Alberti *et al*., 2009; Vancampfort *et al*., 2013). These factors are linked to an increase in morbidity, mortality (Cardenas *et al*., 2008), premature death (Kilbourne *et al*., 2007) and ischaemic stroke (Boden-Albala *et al*., 2007).

According to the 2009 Joint Interim Statement (JIS) definition (Alberti *et al*., 2009), the requirement of three out of the following five core component risk factors constitute the MetS diagnosis. The requirement for the diagnosis of MetS is shown in Table 2.1 (Alberti *et al*., 2009). Various classifications for MetS have been designed for simplistic use in a clinical context as a way of recognising individuals who are at high risk for CVD (Vancampfort *et al*., 2013; Vancampfort *et al*., 2015; Vancampfort *et al*., 2016). This will allow patients to receive suitable supervision and management for symptoms (Vancampfort *et al*., 2013; Vancampfort *et al*., 2015; Vancampfort *et al*., 2016). Due to multiple definitions of MetS available, direct data comparisons between publications are not possible and leads to confusion (Alberti, Zimmet and Shaw, 2006). It is essential that one standardised international

Table 2.1 Criteria used for the clinical diagnosis of MetS (Alberti *et al*., 2009).

Core component measured	Categorical cut-off points
1) Elevated waist circumference	Elevated waist cut-off points differ between genders and ethnic groups. Different organisations use their own references with regards to waist circumference cut-off points (Alberti *et al*., 2009).
2) Elevated blood pressure (hypertension)	Systolic > 130 millimetre (mm) of mercury (Hg) (mmHg) (or on treatment) and/or diastolic > 85 mmHg (or on treatment).
3) Elevated fasting plasma glucose	≥ 5.6 millimoles per litre (mmol/L) (or on treatment).
4) Reduced high-density lipoprotein (HDL) cholesterol	≤ 1.0 mmol/L for men (or on treatment) and ≤ 1.3 mmol/L for women (or on treatment).
5) Elevated fasting triglycerides	≥ 1.7 mmol/L (or on treatment).

MetS definition or diagnostic tool be created (Eckel, Grundy and Zimmet, 2005; Alberti, Zimmet and Shaw, 2006).

Prevalence rates for MetS are increasing at an alarming rate and constitutes a worldwide public health concern (Alberti, Zimmet and Shaw, 2006; Garcia-Portilla *et al*., 2008; Cardenas *et al*., 2008). An average of 20 - 25% of the world's adult population meets the criteria for having MetS (Eckel, Grundy and Zimmet, 2005). Not only is MetS highly prevalent in the general population, MetS risk factors and CVD have become a major concern in BD patients (Babić *et al*., 2010; Valkanova and Ebmeier, 2013; Vancampfort *et al*., 2014). This is due to the high prevalence rate of MetS as a comorbidity associated with BD (Beyer *et al*., 2005; Fagiolini *et al*., 2005; Czepielewski *et al*., 2012; Vancampfort *et al*., 2015). Fagiolini *et al*. (2005) found that the MetS prevalence rate is 30% among BD patients. Similar results were observed by Vancampfort *et al*. (2013) and Vancampfort *et al*. (2015) with 35 - 40% of BD patients presenting with MetS. When compared to the general population, the number was 1.58 times greater (Vancampfort *et al*., 2015). Individuals with a severe mental illness are three times more prone to MetS as compared to the general population (Carrá *et al*., 2014). McIntyre *et al*. (2010) summarised the MetS prevalence rates in BD patients per country (Figure 2.3). Several differences were observed from the data, most notably that individuals were differently affected by the various MetS definitions. Increased waist circumference was the most consistent reported MetS risk factor in all countries (McIntyre *et al*., 2010).

2.4.3.1. Factors contributing to metabolic syndrome in patients with bipolar disorder

There are many variables that can contribute to MetS (Yoon *et al*., 2004; Taylor and McQueen, 2006). In addition to the core component risk factors required for MetS diagnosis (Table 2.1) lifestyle factors also play a large role. An unbalanced diet (Yoon *et al*., 2004; Brietzke *et al*., 2012; Bly *et al*., 2014), inactive lifestyle, smoking cigarettes (Yoon *et al*., 2004; Brietzke *et al*., 2012) and high alcohol consumption

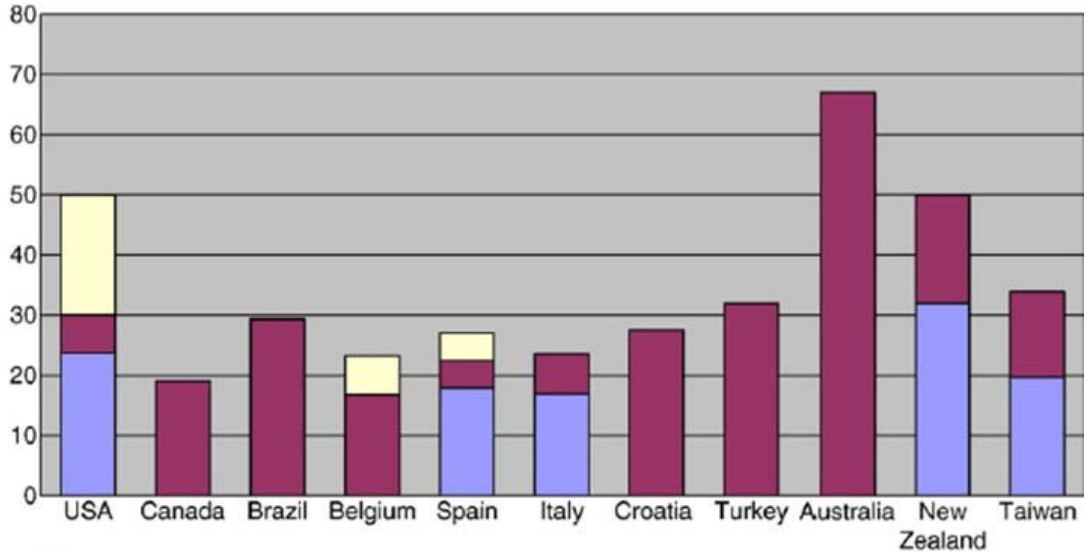


Figure 2.3 A graph representing the comparison of MetS rates in BD patients summarised by country. The light-yellow colour depicts the range, the difference in the lowest and highest MetS rates in the countries where numerous studies were done. The dark purple colour indicates the incidence of MetS in the BD population. The difference between the general population and minimum MetS rates were taken. If this was not available, the general MetS rate for the country was used. The light blue colour depicts the general population. This is only shown for the countries where the statistics were available (McIntyre *et al*., 2010).

(Yoon *et al*., 2004) are some of the harmful factors contributing to MetS and CVD (Vancampfort *et al*., 2015). Patients with severe psychiatric disorders are more inclined to poor, unhealthy lifestyles (Buhagiar, Parsonage and Osborn, 2011; Carrá *et al*., 2014; Vancampfort *et al*., 2015). The mechanism underlying MetS, caused by lifestyle factors is uncertain. However, these patients may have an inclined genetic predisposition (Yoon *et al*., 2004). High alcohol consumption is associated with coronorary artery disease and increased triglycerides, blood pressure as well as blood glucose (Yoon *et al*., 2004). Cigarette smoking and an inactive lifestyle increases the proatherogenic particles in the blood and increases the risk of insulin resistance (Eckel, Grundy and Zimmet, 2005) and abdominal obesity (Chiolero *et al*., 2008). Tobacco may increase blood pressure and reduce HDL cholesterol (Balhara, 2012). A sedentary lifestyle and poor diet increases weight and obesity (Taylor and McQueen, 2006). These factors influence the core component risk factors required for MetS diagnosis.

There are other underlying associated risk factors implicated in MetS including obesity, insulin resistance, aging, a proinflammatory state, hormone imbalance and genetic profile (Alberti, Zimmet and Shaw, 2006). Additionally, the use of prescription antipsychotic (Carrá *et al*., 2014) and mood stabilising (McIntyre *et al*., 2010) medication has been linked to obesity and MetS by accelerating their development (McIntyre *et al*., 2010; Carrá *et al*., 2014). Although, antipsychotics, mood stabilisers and antidepressants can contribute to metabolic complications as a side effect, metabolic disruptions have, however, shown to occur in psychiatric patients independently (Vancampfort *et al*., 2014; Barandas *et al*., 2015). Pathophysiological association between BD and MetS is unknown (Cardenas *et al*., 2008). The complex relationship observed between these two disorders has been suggested to result from common underlying genetic risk factors (Carrá *et al*., 2014; Vancampfort *et al*., 2015). Additionally, evidence proposes that metabolic abnormalities may precede BD onset (Gálvez *et al*., 2015). The term ‘metabolic-mood syndrome’ has been proposed as a possible subtype explaining the association between obesity and mood disorders, where variations observed in mood and metabolism are clinically connected and mutually influence each other (McIntyre *et al*., 2007).

2.4.4. Misdiagnosis and delay in accurate bipolar disorder diagnosis and treatment

The misdiagnosis of BD is common and delays the time for the patient to receive appropriate treatment (Hirschfeld, 2007). This delay can lead to widespread negative consequences (Sajatovic, 2005; Hirschfeld, 2007). When inappropriate treatment is administered, certain medications can aggravate symptoms - such as antidepressants which can accelerate mania and result in rapid cycling. This mistreatment of BD can destabilise and worsen the disorder (Bowden, 2005; Hirschfeld, 2007), prolonging the patient's distress and increasing the risk of suicide (Bowden, 2005).

Kessing (2005) found that 56.2% of BD patients had received diagnosis at their first evaluation, whereas 43.6% received the appropriate BD diagnosis after numerous consultations. On average, it took most patients approximately one year from their first consultation to their first diagnosis, whereas for 25% of the patients, receiving the correct diagnosis took approximately two and a half years (Kessing, 2005). Similar results were observed by Baca-Garcia *et al*. (2006). Their research showed that a mere 30% of patients received BD diagnosis at their first evaluation, whereas 70% received appropriate BD diagnosis during later consultations (Baca-Garcia *et al*., 2006). Patients with BD often consult numerous medical practitioners before the correct diagnosis is made (Thomas, 2004).

Numerous factors can cause the misdiagnoses of BD (Thomas, 2004). This includes the wide range of observable clinical features associated with BD (Thomas, 2004; Fears *et al*., 2014), symptoms that may be unclear at BD onset (Kerner, 2014), medical practitioners not inquiring all relevant information from patients (Thomas, 2004), patients not effectively communicating all symptoms to the medical practitioner (Sajatovic, 2005), the episodic feature of BD not being detected, psychiatric and medical comorbidity (Thomas, 2004), symptom overlap with other psychiatric disorders (Song *et al*., 2014; Kerner, 2014) as well as the age at BD

onset (Thomas, 2004). Additionally, the categories used in the diagnosis of psychiatric disorders display extensive heterogeneity (Phillips and Kupfer, 2013).

The assessment and monitoring of BD is dependant on clinical symtoms of the patient (Goldstein and Young, 2013). Due to the lack of objective clinical diagnostic tests (Barnett and Smoller, 2009; Goldstein and Young, 2013; Phillips and Kupfer, 2013), there is substantial overlap between the categories used for BD diagnosis. Therefore, BD has been classified as a challenging psychiatric disorder to diagnose accurately, which directly affects patient treatment and health (Phillips and Kupfer, 2013). Patients with BD display a subtler course of illness than originally presumed, which is characterised by residual symptoms. Additionally, they experience emotional imbalance, sleep disruptions, circadian rhythm disturbances and cognitive impairment (Leboyer and Kupfer, 2010).

Therefore, the use of accurate, comprehensive assessments and screening tools, together with the awareness of BD, will significantly reduce the misdiagnosis rates (Bowden, 2005). Due to the complexity of BD, a complete analysis including the social, biological and psychological aspects contributing to the pathogenesis of BD needs to be reviewed thoroughly (Grande *et al*., 2016). Although the aetiology and pathophysiology of BD are not well understood, genetic research has provided remarkable evidence of susceptible genetic risk factors that contribute to the pathogenesis of BD (Craddock and Sklar, 2013; Grande *et al*., 2016). This allows psychiatry an opportunity to determine the biological mechanisms involved in BD (Craddock and Sklar, 2013) and will immensely contribute to earlier BD diagnosis and aid in the efficiency of psychiatric treatment (Hashimoto, 2010; Kerner, 2014).

2.5. Multifactorial aspects contributing to the pathogenesis of bipolar disorder

Bipolar disorder is best attributed to the intricate gene-environment (GxE) interaction model (Moffitt, Caspi and Rutter, 2005; Craddock and Sklar, 2013;

Ludwig and Dwivedi, 2016), whereby genetic, environmental and epigenetic factors are connected, contributing to BD aetiology (Martinowich, Schloesser and Manji, 2009; Grande *et al*., 2010; Wright *et al*., 2011; Song *et al*., 2014; Ejmalian *et al*., 2016; Ludwig and Dwivedi, 2016). An exact model for this interaction has not yet been determined (Badner *et al*., 2012; Ludwig and Dwivedi, 2016). Thus, many aspects are still unknown and so, the inheritance patterns of BD remain uncertain (Martinowich, Schloesser and Manji, 2009; Wright *et al*., 2011).

It may be possible that certain genetic effects will only become known under specific environmental circumstances (Barclay *et al*., 2011). Thus, epigenetic changes and GxE interactions are investigated to better understand the genetic vulnerability that may cause BD aetiologly (Sigitova *et al*., 2017). Familial risk factors and gene heritability may aid in assessing the degree of genetic and environmental impacts on BD (Song *et al*., 2014). Factors to consider are individual personality differences and coping mechanisms, which play a crucial role in the correlation between exposure of environmental factors and the trauma it can cause (Williams *et al*., 2004).

2.5.1. Environmental factors in South Africa

There are numerous possible environmental factors that can influence the elevated rate of psychiatric disorders in SA (Stein *et al*., 2008). The emotional distress caused by poor financial conditions (Patel and Kleinman, 2003) and the elevated risk of contracting HIV (Williams *et al*., 2004; Hughes, Hoyo and Puoane, 2006), brutality (Hirschowitz and Orkin, 1997), political violence (Williams *et al*., 2004), sex discrimination (Dunkle *et al*., 2004) as well as the distress and dangers associated with deep surface mining, not only to the miners themselves but, to their families and communities (Donoghue, 2004), can contribute to psychiatric illness in SA.

There are additional individual and familial-specific environmental factors contributing to BD susceptibility (Kerner, 2014). These include chronic stress factors, trauma (Kauer-Sant'Anna *et al*., 2007; Martinowich, Schloesser and Manji, 2009), childhood trauma such as abuse of a physical or sexual nature (Kauer-Sant'Anna *et al*., 2007; Brietzke *et al*., 2012; Aas *et al*., 2014), neglect and the loss of a parent (Brietzke *et al*., 2012). In SA, the environmental risk factors in BD patients are a relatively understudied field (Bortolato *et al*., 2017).

2.5.2. Genetic factors

2.5.2.1. Evidence of heredity through family, twin and adoption studies

Heritability describes the component of an individual's observable characteristics that are contributed by genetic factors (Smoller and Finn, 2003). In a population, heritability is referred to the strength of genetic influences that may vary between studied populations and groups (Smoller and Finn, 2003). Research specifically directed into BD has been widespread (Smoller and Finn, 2003), with heritability estimated to be higher than 80% (Kieseppä *et al*., 2004). This indicates a significant genetic component (Craddock and Jones, 1999; Smoller and Finn, 2003; Martinowich, Schloesser and Manji, 2009) comprising genes that predisposes individuals to BD (Kieseppä *et al*., 2004). The inheritance patterns (Wright *et al*., 2011), the extent of inheritance as well as whether any sex-specific influences are present, remain unknown (Song *et al*., 2014).

Family, twin and adoption studies are the principal methodologies (Shih, Belmonte and Zandi, 2004) used to examine the effects of familial and genetic influences on a specific disorder (Smoller and Finn, 2003; Shih, Belmonte and Zandi, 2004). Bipolar disorder is one of the most investigated disorders using these research approaches (Smoller and Finn, 2003). These studies have contributed to the efforts of identifying susceptible genes that can predispose individuals to BD (Craddock

and Jones, 1999; Smoller and Finn, 2003; Belmaker, 2004) as well as their familial environment (Smoller and Finn, 2003). Information gathered thus far has proven to be useful to medical practitioners, patients and their families regarding inheritance of BD (Smoller and Finn, 2003).

A family-based study performed by Song *et al*. (2014) on a Swedish population found strong familial risks for BD, with higher risks of BD in first-degree relatives of the affected BD probands (Song *et al*., 2014). The estimated heritability was shown to be 58% and the remaining component attributed to non-shared environmental factors. Furthermore, their research shows pleiotropy of BD gene variants with other psychiatric disorders (Song *et al*., 2014). Similar results were observed by Lichtenstein *et al*. (2009), where genetic pleiotropy was observed between BD and schizophrenia. The risk for both schizophrenia and BD were increased in first-degree relatives of probands diagnosed with either disorder. The comorbidity between the two psychiatric disorders were due to additive genetic effects common to both disorders and therefore, share common genetic aetiology. Lichtenstein *et al*. (2009) further showed that adopted children, whose biological parents were diagnoses with a psychiatric disorder, had a higher risk of developing the same psychiatric disorder (Lichtenstein *et al*., 2009).

Adoption studies that analyse mood disorders are extremely rare (Smoller and Finn, 2003; Althoff *et al*., 2005). Such studies may be useful in examining and evaluating the genetic and non-genetic influences on psychiatric disorders by changing environmental factors. Research conducted by Mendlewicz and Rainer (1977) as well as Wender *et al*. (1986) found that biological parents of the adoptees had a higher risk and prevalence of BD than the adoptive parents. Data available on adoption studies is consistent with the genetic inheritance of BD (Taylor, Faraone and Tsuang, 2002; Smoller and Finn, 2003; Althoff *et al*., 2005).

Twin studies are performed to separate genetic factors from environmental influences, while comparing the rate of concordance of a disorder (Shih, Belmonte

and Zandi, 2004). Specifically, this is done by contrasting a specific disorder in monozygotic (MZ) twins, who are genetically identical and dizygotic (DZ) twins, who share 50% of the same genetic material (Smoller and Finn, 2003; Shih, Belmonte and Zandi, 2004). It is also assumed that that both sets of twins share similar environments (known as the equal environments assumption) (Smoller and Finn, 2003). Kieseppä *et al*. (2004) conducted twin studies on BD-I patients and reported a concordance rate of 43% for MZ twins, 6% for DZ twins and a heritability estimate of 93% (Kieseppä *et al*., 2004). Similar results were found by McGuffin *et al*. (2003), who included both BD-I and BD-II in their study. The concordance rate for MZ twins were 40% and 5% for DZ twins, heritability was estimated to be 85% (McGuffin *et al*., 2003). An MZ co-twin has the highest risk of developing a mood disorder if a relative with BD has already been diagnosed, followed by a first-degree relative and then an unrelated individual. The risk for second-degree relatives lies between the risk for the first-degree relative and the general population (Craddock and Jones, 1999).

Concordance rates less than 100% in MZ twins suggest an environmental influence in BD aetiology (Smoller and Finn, 2003; Shih, Belmonte and Zandi, 2004). Twin studies including BD patients show that concordance rates for MZ twins are always higher than for DZ twins (Smoller and Finn, 2003; Kieseppä *et al*., 2004), suggesting a large genetic contribution in BD (Smoller and Finn, 2003; Shih, Belmonte and Zandi, 2004). Such high heritability and familial risk results indicate that molecular genetics has a substantial role in understanding BD aetiology (Grande *et al*., 2010).

2.5.2.2. Molecular research of susceptible genetic risk variants and the complications in detecting genetic anomalies in bipolar disorder

Over the last century, consistent evidence of the high heritability of BD has suggested that genetic studies are key to identifying risk alleles (Neale and Sklar, 2015). With BD presenting as such an intricate, multi-faceted disorder (Martinowich, Schloesser and Manji, 2009; Kerner, 2014; Haenisch *et al*., 2014), detecting

susceptible genetic variants associated with BD have been intangible (Wilson *et al*., 2006). Although molecular genetic research has provided a new frontline into BD aetiology (Belmaker, 2004), much effort has been directed towards the identification of genetic risk alleles (Kerner, 2014). However, no clinical biological diagnostic tests for the diagnosis of BD have yet become available (Hashimoto, 2010).

Linkage studies are one of the earliest genetic research approaches involving the genome to search for disease risk alleles in family members affected with the same disorder (Neale and Sklar, 2015). This has provided the foundation in genetic research for the identification of high-risk disease genes (Craddock and Sklar, 2013; Neale and Sklar, 2015). But then, this is impeded by the presence of limited locus heterogeneity. Linkage studies have not been able to pinpoint to specific risk variants for BD and this shows that there are numerous genetic variants and regions in the genome involved (Neale and Sklar, 2015). A large-scale genome-wide linkage study that included 972 BD pedigrees, evidently showed that no specific locus of large effect is involved in the genetic aetiology of BD for the majority of BD patients (Badner *et al*., 2012). However, there are exceptions where a single gene of large effect was found to be involved in BD aetiology. Rao and colleagues (2017) recently discovered rare, deleterious mutations in gene coding areas in families with BD (Rao *et al*., 2017). Therefore, it is very probable that common and rare genetic variants are involved in the predisposition of BD aetiology (Craddock and Sklar, 2013).

With increasing knowledge being obtained about common genetic variations, with the technology available today, the SNP Consortium (Thorisson and Stein, 2003) and the International HapMap (The International HapMap Consortium, 2005) projects have been able to create a large genome-wide database of common deoxyribonucleic acid (DNA) variants, depicting the linkage disequilibrium (LD) of genetic variants (Neale and Sklar, 2015). Specifically, the HapMap project was able to provide information on allele frequencies from various ethnicities across continents. Such large studies have created a platform for genome-wide association

studies (GWAS), where the identified genetic markers can be tested on a genome-wide level (Neale and Sklar, 2015).

Association studies have been able to identify genetic risk variants at a population-level (Craddock and Sklar, 2013). Such studies can detect specific candidate genes that have a small to moderate effect on disease. Once chromosome gene mapping and high-throughput genotyping became possible, research shifted towards GWAS. This enabled the investigation of numerous genetic variants on all chromosomes simultaneously (Craddock and Sklar, 2013). An unbiased analysis (Neale and Sklar, 2015) of thousands of single nucleotide polymorphisms (SNPs) can be genotyped from thousands of individuals (Craddock and Sklar, 2013). Genome-wide association studies can examine single DNA variants and their association to specific disorders (Neale and Sklar, 2015). Also, it has made the analysis of copy number variants (CNV) possible. Numerous complex genetic traits have been identified with the use of GWAS (Craddock and Sklar, 2013).

Genetic studies have associated genes from the circadian rhythm and neurotrophin family to BD (Table 2.2). With the knowledge gained this far with regards to BD susceptibility genes and circadian rhythm dysfunction, it is possible that genes involved in regulating the circadian rhythm are involved in BD pathophysiology (Milhiet *et al*., 2011). This can potentially be used as an effective biological diagnostic tool. Studies from GWAS, CNV analysis, animal models, transcriptomics/proteomics and functional convergent genomics have provided insightful results in support of circadian clock dysfunction in BD aetiology (summarised by Milhiet *et al*., 2011). The endogenous circadian rhythm clock controls various brain functions relating to behaviour, physiology and metabolism (Barandas *et al*., 2015). Disruptions in the circadian rhythm may have a fundamental role in psychiatric and metabolic comorbidity (Barandas *et al*., 2015). Research has found that neurotrophins, such as the BDNF, are structurally and functionally associated with the circadian rhythm system (Tirassa, Quartini and Iannitelli, 2015) in addition to playing a role in mood disorders (Tirassa, Quartini and Iannitelli, 2015) and MetS (Hristova and Aloe, 2005).

Table 2.2 Association studies conducted on selected circadian rhythm and neurotrophin genes associated with BD.

Candidate gene	Association with BD
Circadian Locomotor Output Cycles Kaput (*CLOCK*) circadian regulator	Schuch *et al*. (2017); Lee *et al*. (2010); Soria *et al*. (2010); Kripke *et al*. (2009); Shi *et al*. (2008).
Period circadian regulator 3 (*PER3)*	Brasil Rocha *et al*. (2017); Karthikeyan *et al*. (2014b); Nievergelt *et al*. (2006); Mansour *et al*. (2006).
Glycogen synthase kinase-3β (*GSK-3β*)	Lin, Huang and Lui, (2013); Luykx *et al*. (2010).
Brain-derived neurotrophic factor (*BDNF*)	Sears *et al*. (2011); Okada *et al*. (2006); Green and Craddock, (2003); Sklar *et al*. (2002).

More recently, next-generation sequencing (NGS) has been developed for whole genome genotyping on thousands of individuals (Craddock and Sklar, 2013; Neale and Sklar, 2015). This will greatly help the psychiatric field in identifying rare risk alleles that have a small or moderate effect on disease susceptibility (Craddock and Sklar, 2013; Neale and Sklar, 2015). This level of sequencing can search for genetic variation only found in specific family lines. The results from GWAS prove that BD is caused by both common and rare genetic variants that vary in their size of effect. This will provide further information into pathogenic alleles and processes underling BD (Neale and Sklar, 2015).

2.5.2.3. Complexity in the genetic mechanisms of inheritance

Identifying the genetic basis involved in psychiatric disorders will provide a foundation for understanding the disease mechanisms involved at a molecular level (Geschwind and Flint, 2015). Research shows that BD does not follow the simple Mendelian mechanism of inheritance (Belmaker, 2004; Craddock and Sklar, 2013) and no highly penetrant Mendelian genes have been discovered (Sklar *et al*., 2008). Instead, a polygenic mechanism of inheritance has been observed (Belmaker, 2004; Sklar *et al*., 2008) as confirmed through GWAS (Sigitova *et al*., 2017). The recurrent risk for BD also massively decreases from MZ co-twin to first-degree relatives and to unrelated individuals. This data is consistent with epistatic gene interactions and more complex genetic mechanisms (Craddock and Jones, 1999). Epistasis is the occurrence of multiple genes interacting to produce a specific phenotype (Craddock and Owen, 1996).

Different genetic profiles, including the presence of preventative genes, relative to disease causing alleles, influence BD susceptibility (Martinowich, Schloesser and Manji, 2009). Potential genetic mechanisms further involved in the complexity of inheritance are presented in Table 2.3. Further research may contribute to the better understanding of the genetic components involved in BD aetiology (Kerner, 2014).

Table 2.3 Genetic mechanisms involved in the complex inheritance of BD.

Genetic mechanism	Description
Incomplete penetrance	Where an individual inherits a predisposing allele and does not manifest the phenotype, while others may manifest the phenotype without inheriting the predisposing allele due to environmental factors (Lander and Schork, 1994; Sklar *et al*., 2002).
Locus, phenotypic and genetic heterogeneity	Where a specific phenotype or trait is caused by multiple genes (Craddock and Owen, 1996; Sklar *et al*., 2002; Sklar *et al*., 2008; Neale and Sklar, 2015).
Epigenetic modifications	Where the environment can have an effect at a molecular level (Tsankova *et al*., 2007). Changes may occur on DNA level without modifying the DNA sequence. Gene expression may be affected and can be inherited by future generations (Barnett and Smoller, 2009).
Genomic imprinting	Also known as the parent-of-origin-effect (McMahon *et al*., 1995) is a phenomenon that causes genetic material, at a chromosomal or allelic level, to be expressed depending on the transmission of the parent, either from the maternal or paternal side (Grigoroiu-Serbanescu *et al*., 1995).
Anticipation	The phenomenon whereby the genetic disorder which is passed on from one generation to the next becomes increasingly more severe in the phenotype of subsequent generations coupled by an earlier age at onset (Lange and McInnis, 2002).
Mitochondrial inheritance	A genetic mechanism whereby the mitochondria are exclusively inherited from the maternal line (Craddock and Owen, 1996), indicating a maternal effect on BD susceptibility (McMahon *et al*., 1995). Mitochondrial inheritance is a genetic mechanism whereby the mitochondria is exclusively inherited from the maternal line (Craddock and Owen, 1996), indicating a maternal effect on BD susceptibility (McMahon *et al*., 1995). However, very recently biparental mitochondrial inheritance was discovered by Luo *et al*. (2018). This research opens novel fields in mitochondrial disease mechanisms and therapeutic action (Luo *et al*., 2018).
Variable expressivity	The occurrence of a variety of clinical phenotypes that can result from the same genes (Kelsoe, 2003).

2.6. The necessity of genetic biomarkers in diagnosing bipolar disorder and their associated benefits

The use of phenomenology-based diagnostic systems for diagnosing BD remains controversial (Grande *et al*., 2016). Psychiatric diagnoses lack any biological validation tests as is found in other fields of medicine (Frey *et al*., 2013). Without clear, objective biomarkers for BD, underdiagnoses or false-positive diagnoses could result (Grande *et al*., 2016). A biological marker, also known as a biomarker, is a naturally occurring measurable indicator that can objectively evaluate normal biological processes, pathogenic processes as well as pharmacological responses to a specific treatment (Biomarker Definitions Working Group, 2001). Biological features such as candidate genes, proteins and other molecules of the body can be used as biomarkers (Pfaffenseller *et al*., 2013).

The field of psychiatry is in great need of biomarkers for the development of drug target interventions (Tamminga *et al*., 2017). More research into the underlying causes of psychiatric illness will aid in breaking the stigma related to mental illness, pinpoint specific BD markers (Scola and Andreazza, 2014; Jacoby *et al*., 2016) and help identify the affected pathways (Jacoby *et al*., 2016). In turn, such a scheme may be able to provide personalised treatment (Scola and Andreazza, 2014; Neale and Sklar, 2015) directed at specific biological targets (Neale and Sklar, 2015). With biomarkers, the diagnosis of BD will be greatly improved (Scola and Andreazza, 2014) and more accurate diagnostic assessments can be done (Pfaffenseller *et al*., 2013). Additionally, the prognosis of BD can be determined as well as the patients' response to treatment (Sigitova *et al*., 2017). Markers can be developed where specific mood episodes can be detected and trait-specific markers developed to recognise BD characteristics (Frey *et al*., 2013). Much research is being focused on neurotrophins and their role in neuroplasticity and have shown to be potential therapeutic targets in BD (Grande *et al*., 2010; Hashimoto, 2010; Goldstein and Young, 2013; Pfaffenseller *et al*., 2013; Fernandes *et al*., 2015).

2.7. The importance of analysing the genetic associations between bipolar disorder and metabolic syndrome risk factors

Literature shows accumulating evidence linking BD with metabolic risk factors (Valkanova and Ebmeier, 2013; Vancampfort *et al*., 2014). There is a two-way association between BD and cardio-metabolic diseases (Kemp *et al*., 2010). Psychotropic medication is related to an increase in risk of metabolic disturbances and physical health complications (Correll *et al*., 2015). Medication used for CVD (which is a risk factor for MetS) has shown to have an effect on mood and increase the risk of mood disorders (Huffman and Stern, 2007). However, metabolic disturbances in psychiatric patients can develop independently of drug effects (Vancampfort *et al*., 2014; Barandas *et al*., 2015).

The mutual relationship between psychiatric disorders and metabolic disruptions have recently led to the 'metabolic-mood syndrome,' suggesting the presence of shared biological mechanisms (Mansur, Brietzke and McIntyre, 2015) and pathophysiology (Barandas *et al*., 2015), where variations observed in mood and metabolism have a mutual effect on each other (McIntyre *et al*., 2007; Mansur, Brietzke and McIntyre, 2015). Twin studies have shown moderate genetic associations between mood and various cardio-metabolic disorders. The strong association between the two disorders could be explained by mutual underlying biological pathways and pleiotropic genetic factors (Figure 2.4) (Amare *et al*., 2017). Limited data exists to enable better understanding of this biological function and co-occurrence (Barandas *et al*., 2015).

As previously mentioned, certain effects of genes may only become known in specific environmental circumstances (Barclay *et al*., 2011). This not only applies to BD but, can potentially be the connection between mood and cardio-metabolic disorders (Amare *et al*., 2017). Certain environmental factors may regulate the expression of genes found within the cardio-metabolic and neural pathways, leading to GxE interactions. The interactions of genetic and environmental factors such as

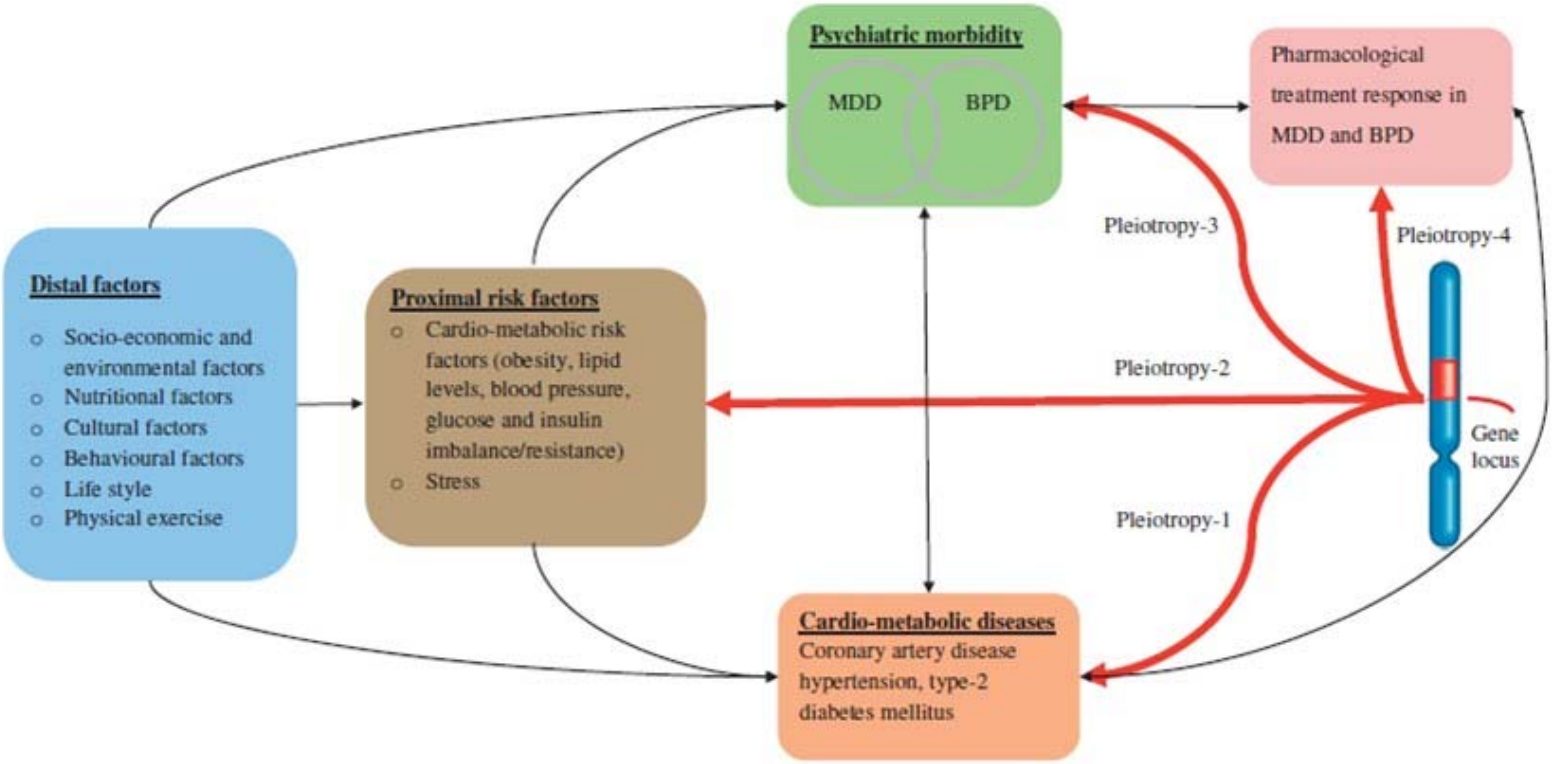


Figure 2.4 A schematic diagram representing possible consequences of pleiotropy. The unidirectional red arrows display the pleiotropic impacts of a single genetic locus on cardio-metabolic disorders, related risk factors, mood disorders and treatment response. The black bi-directional arrows display the two-way epidemiological associations between mood disorders and cardio-metabolic disorders (Amare *et al*., 2017).

stress, physical exercise, diet and lifestyle can have an impact on the development and pathogenesis of both mood and cardio-metabolic disorders (Figure 2.4).

Environmental risk factors can be classified as either proximal or distal. Proximal risk factors can act directly to cause disease, whereas distal risk factors work via intermediate causes and factors to cause disease (Amare *et al*., 2017). Distal factors are the risk factors an individual has some control over. An individual that develops a specific disease may have had the influence of certain environmental risk factors for many years that may, for example, have been shaped by socio-economic influences (WHO, 2002). Furthermore, treatment response can be affected by pleiotropic genes and mutual biological pathways (Amare *et al*., 2017) (Figure 2.4).

2.8. Biological pathways and genes implicated in bipolar disorder and metabolic syndrome

Most BD patients suffer from disrupted circadian rhythms (McClung, 2007). Impaired circadian rhythms may play a pivotal role in psychiatric and metabolic disorders due to the circadian clock regulating areas of the brain that serve in mood regulation, cognitive and metabolic processes (Barandas *et al*., 2015). It is still uncertain as to whether circadian rhythm disturbances are due to BD itself, or due to other underlying conditions. It remains unknown how this influences mood. However, with advancements made in molecular biology, the robust association between BD and circadian rhythms is becoming increasingly understood (McClung, 2007).

2.8.1. Overview and function of the circadian rhythm clock

Chronobiology describes the study of the effects of time and biological rhythms on living processes (Abreu and Bragança, 2015). There are different types of rhythms that control various aspects and processes in the body that run on an approximately 24-hour cycle. This is known as circadian and the term was coined by Franz Halberg (Halberg, 1969). Through his research, Halberg developed the science of chronobiology (Cornelissen *et al*., 2013). Nearly all human physiological processes run on a 24-hour cycle and this includes our body temperature, blood pressure, hormones, metabolism, sleep-wake cycle (Bechtold, Gibbs and Loudon, 2010), immune function (Scheiermann, Kunisaki and Frenette, 2015) and DNA repair (Sancar, Lindsey-Boltz and Kang, 2010). These wide-ranging processes are due to the molecular circadian clock controlling the rhythmic transcription of thousands of genes in the brain and body. This allows transcriptional coordination of wide-ranging body processes (Pantazopoulos *et al*., 2018).

Daily, environmental photic time signals are processed through the retinal input pathways in order for the circadian pacemaker to synchronise to a 24-hour cycle (Archer *et al*., 2003). Without external time signals, the endogenous circadian clock becomes free-running (Archer *et al*., 2003). The circadian clock is able to produce a 24-hour cycle without any external factors but, remains susceptible to environmental factors that can have an influence on the internal clock. The internal clock can predict these changes and will always adjust our physiology accordingly to help us adapt to the changes (Bechtold, Gibbs and Loudon, 2010). The molecular clock consists of core ‘clock’ genes that regulate the circadian rhythms. These genes are expressed throughout the body (Ozburn *et al*., 2016).

2.8.1.1. Components and mechanism of the molecular clock

The core circadian clock is found in the suprachiasmatic nuclei (SCN) located in the hypothalamus (McClung, 2013; Edgar and McClung, 2013) where it functions as the principal molecular clock of the body (Bechtold, Gibbs and Loudon, 2010). Light signals are captured by retinal ganglion cells (Edgar and McClung, 2013) and the signals are received via synaptic transmission by axons of the retinohypothalamic tract. These electrical signals are converted into chemical signals where they are responsible for modifying expression of clock genes in a subset of SCN neurons and promptly establishes a new phase in all SCN neurons. Once completed, signals are further distributed to all other areas of the brain and peripheral organs through neuronal connections, endocrine signals, body temperature rhythms and indirect cues, provoked by oscillating behaviour (Dibner, Schibler and Albrecht, 2010).

The molecular clock is controlled by a series of negative feedback loops (Abreu and Bragança, 2015). Figure 2.5 depicts the molecular clock as well as the transcriptional and translational feedback loops present within the molecular clock (McClung, 2013). The basic helix-loop-helix (bHLH)-period-Arnt-single-minded (PAS)-containing transcription factors, *CLOCK* and the brain and muscle Arnt-like protein 1 (*BMAL1*), also known as Aryl hydrocarbon receptor nuclear translocator-like (*ARNTL*) or *MOP3*, bind together in the nucleus, forming a heterodimer that binds to the Enhancer Box (E-box). The E-box contains sequences in numerous circadian genes including *PER1*, *PER2* and *PER3* as well as the *Cryptochrome* (*CRY1* and *CRY2*) genes. Outside the cell, inside the cytoplasm, the PER and CRY proteins form a dimer and are transferred back into the nucleus inhibiting the transcription of CLOCK and BMAL1 (Abreu and Bragança, 2015; Vadnie and McClung, 2017). This forms a negative feedback cycle occurring every 24 hours (McClung, 2013). The CLOCK and BMAL1 proteins function to control the expression of the nuclear hormones, Rev-erbα and Rorα, either causing transcription or inhibition of the *BMAL1* gene. This process is carried out through the REV-ERB/ROR response element. The *PER* and *CRY* genes can also be regulated by neuronal PAS-domain protein 2 (NPAS2, also known as MOP4), by

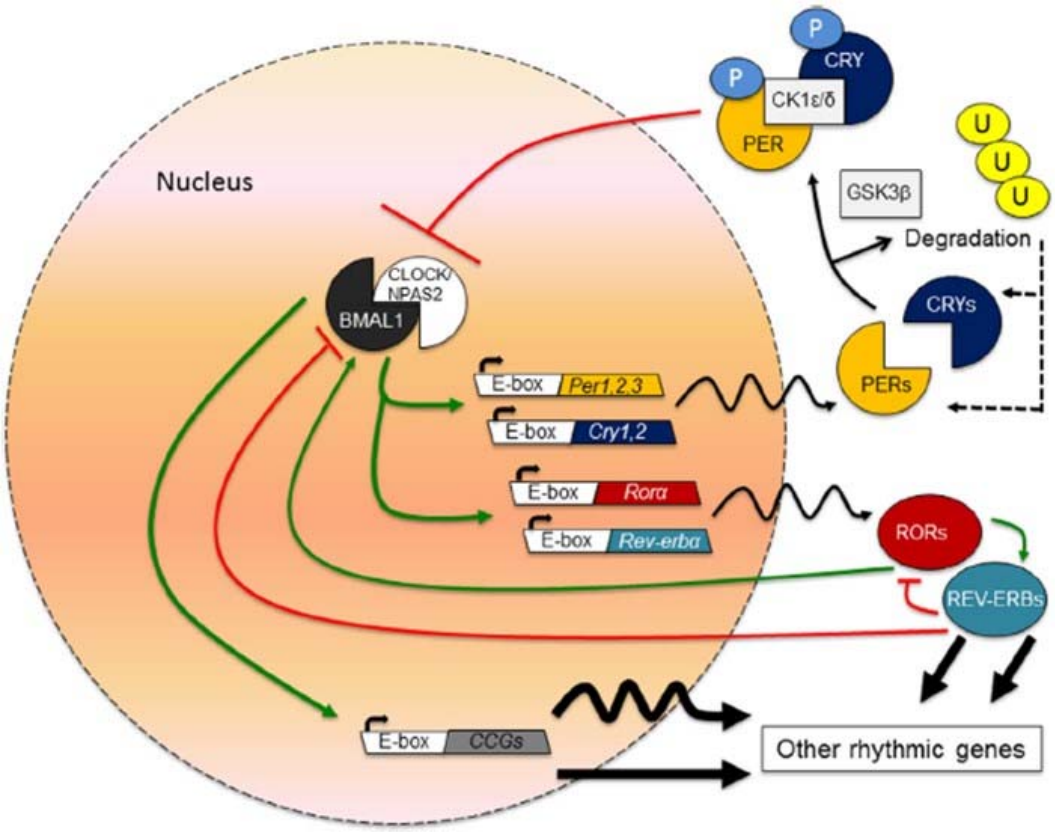


Figure 2.5 A diagram depicting the circadian rhythm clock (McClung, 2013).

forming a dimer with BMAL1 outside the nucleus (McClung, 2013). The PER, CRY and BMAL1 proteins become phosphorylated by Casein kinase 1 delta and epsilon proteins (CK1d and CK1e) to change and restrict their access into the nucleus. Glycogen synthase kinase-3β phosphorylates PER2, but allows it access to the nucleus. The GSK-3β protein phosphorylates Rev-erbα, increasing its stability and additionally and causes proteasomal degradation of the CLOCK and CRY proteins (McClung, 2013).

2.8.1.2. Disruptions of the circadian clock

Circadian rhythm cycles in our bodies are important for the timing and rhythm of essential biological processes (Abreu and Bragança, 2015). It is of utmost importance that these rhythms are maintained for optimal environmental adaption so that information synchronisation can occur within our bodies (Abreu and Bragança, 2015). For the internally produced circadian rhythms to run on a 24-hour cycle, they need external indicators, known as zeitgebers. If the body does not receive the appropriate stimulation to adjust to the environment, the clock will not keep to the 24-hour cycle. Disruption of the circadian rhythm clock has substantial effects on the human body (Bechtold, Gibbs and Loudon, 2010). Acute mood episodes have been demonstrated to be associated with circadian misalignment between BD patients' internal circadian rhythms and their external environment (Moon *et al*., 2016). However, circadian rhythm misalignment can be a characteristic of BD during the euthymic phase (Abreu and Bragança, 2015).

Recently, the effect of circadian disruptions has received increasing attention and is considered a contributing factor in some clinical and pathological diseases (Bechtold, Gibbs and Loudon, 2010). This includes disorders affecting sleep, cancer, MetS, inflammation as well as affective disorders such as BD (Figure 2.6) (Bechtold, Gibbs and Loudon, 2010). Differences in sleep, circadian rhythms and the risk of physical and mental disorders between individuals are to an extent due

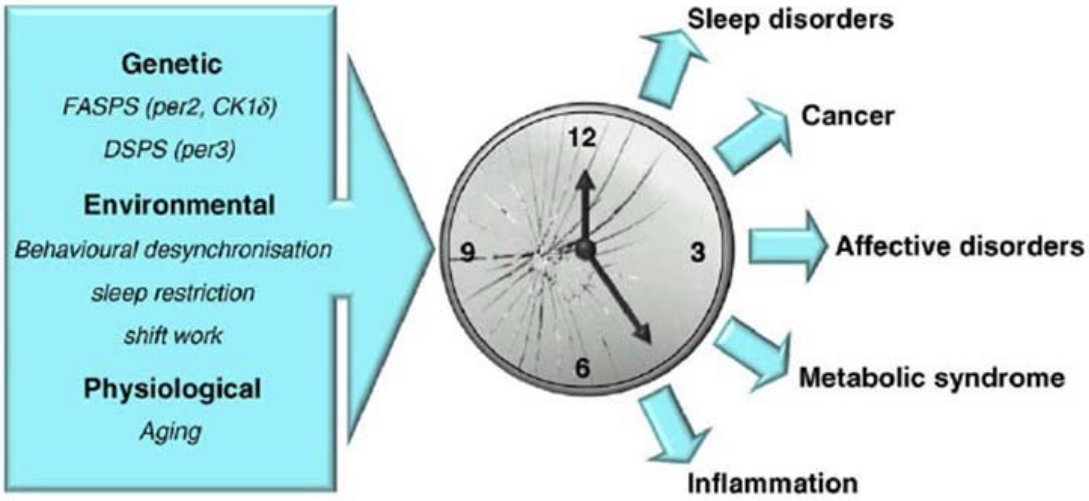


Figure 2.6 The causes and consequences of circadian dysfunction (Bechtold, Gibbs and Loudon, 2010).

to genetic variation (Archer *et al*., 2017). Genes involved have been shown to cause discrepencies in sleep and circadian phenotypes (Dijk and Archer, 2010). Patients with BD often display abnormalities in their circadian rhythms such as sleep (Li, Frye and Shelton, 2012; Ozburn *et al*., 2016), daily activity, body temperature and blood pressure (Ozburn *et al*., 2016). Additional disruptions in other circadian rhythms can include weight variation, CVD and hormone imbalances (Li, Frye and Shelton, 2012).

There are numerous factors that can disturb synchronisation of the circadian clock causing the rhythms to lose their 24-hour cycle, and thus, making the cycle either longer or shorter. Such factors can be pathological, non-pathological, internal or external (Bechtold, Gibbs and Loudon, 2010). External desynchronisation takes place when circadian rhythms in the body are altered by environmental factors (Reinberg and Ashkenazi, 2003). Lifestyle habits such as chronic shift work, sleep deprivation and air travel disrupt the natural internal clock (Bechtold, Gibbs and Loudon, 2010). Jet lag is an example of an external zeitgeber causing desynchronisation. When travelling between time zones, the internal clock is adjusted once the individual has adapted to the new time zone. Other external indicators include food consumption, environmental temperature and the day/night cycle (Abreu and Bragança, 2015) as well as all factors listed in Chapter 2.3.1.

2.8.2. Circadian Locomotor Output Cycles Kaput (*CLOCK*) circadian regulator gene (OMIM 601851)

The human *CLOCK* (Schuch *et al*., 2017; Riestra *et al*., 2017) gene is situated on chromosome 4q12 (Figure 2.7) with a length of 119 kilobase pairs (kbp) that encodes a protein of 846 amino acids. It is regarded as one of the most significant genes of the molecular clock (Schuch *et al*., 2017) and is expressed throughout the body (Ozburn *et al*., 2016). This includes the SCN of the hypothalamus, hippocampus, pyriform cortex and cerebellum (Lee *et al*., 2010).

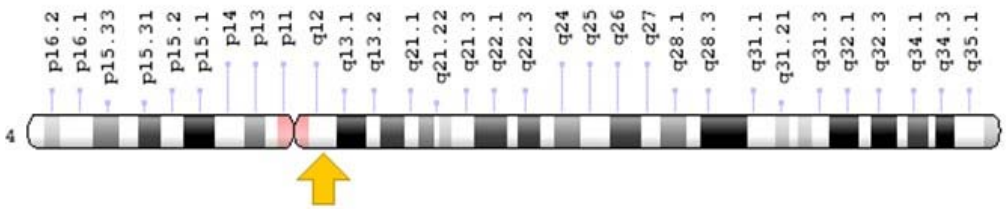


Figure 2.7 The location of the *CLOCK* gene on chromosome 4q12, indicated by the yellow arrow (National Center for Biotechnology [NCBI], 2018).

The protein products of *CLOCK* and *BMAL1* (Schuch *et al*., 2017) heterodimerise to form a positive regulator of the circadian clock (Benedetti *et al*., 2007; Ozburn *et al*., 2016). This positive regulator is a bHLH transcription factor (Turek and Kolker, 2001) that functions to transcribe other genetic components of the circadian clock (Serretti *et al*., 2003; Benedetti *et al*., 2007; Schuch *et al*., 2017). It also regulates chromatin opening and acts an as acetyltransferase (Schuch *et al*., 2017).

Behavioural and molecular studies have proposed CLOCK as a significant factor in the neuronal function, especially the dopaminergic system that has been associated with aetiology of psychiatric disorders (Schuch *et al*., 2017). There is an underlying interplay between *CLOCK* and the environment that has not yet been defined (Serretti *et al*., 2003). Variants of the *CLOCK* gene have been the most strongly associated with BD (McClung, 2007). The most researched variant in *CLOCK* is the 3111 thymine/cytosine (T/C) SNP (Schuch *et al*., 2017).

The 3111 T/C SNP (rs1801260) (Benedetti *et al*., 2007) involves a nucleotide substitution from T to C, at position 3111 of the DNA sequence, resulting in three genotypes (T/T, T/C or C/C) (Benedetti *et al*., 2003). The SNP is positioned in the 3'-untranslated region (3'-UTR) of the *CLOCK* gene (Ozburn *et al*., 2016). This region is particularly important for messenger ribonucleic acid (mRNA) stability, expression and function (Schuch *et al*., 2017). Unstable mRNA affects the translated CLOCK protein levels in the body (Benedetti *et al*., 2003; Monteleone *et al*., 2008; Ozburn *et al*., 2016). This may be the cause of internal dysregulation of the circadian rhythm (Monteleone *et al*., 2008; Ozburn *et al*., 2016). This SNP has also been linked to clinical traits of mood disorders (Lee *et al*., 2010).

The sleep/wake cycle plays a crucial factor in mood disorders and is clinically observed in medical practice (Barbini *et al*., 1996). The C allele of the *CLOCK* 3111 T/C SNP has not been related to sleep disruptions in healthy individuals (Serretti *et al*., 2003). However, it has been associated with sleep disruptions, insomnia (Serretti *et al*., 2003) and decreased need for sleep (Benedetti *et al*., 2003; Serretti

et al., 2003) in patients with BD, thus, triggering manic episodes (Barbini *et al*., 1996). This finding supports a notion that there is a connection between the *CLOCK* gene and mood disorders (Serretti *et al*., 2003). Carriers of the C allele in BD experiencing depression are more active in the evenings, have decreased amounts of sleep and experience a delay in sleep onset (Benedetti *et al*., 2007). Association of the C allele with active evening preferences may cause an extended phase shift in BD patients with the C/C genotype. This has shown to increase the number of mood episodes experienced by BD patients (Wirz-Justice *et al*., 1999). This could have a major effect of the translated protein, having a direct impact on the circadian rhythms. This can cause the sensitivity to the activating effects of zeitgebers to increase (Benedetti *et al*., 2007).

The 3111 T/C SNP is possibly linked to BD antidepressant treatment response (Nievergelt *et al*., 2006). Serretti and colleagues (2005) found that the CC genotype in a cohort of BD patients experiencing a depressive episode had a more severe form of insomnia while on antidepressant medication and specifically had a problem maintaining sleep throughout the whole treatment. The function of the *CLOCK* 3111 T/C SNP on a molecular level is yet to be determined (Serretti *et al*., 2005). The C allele in BD patients has additionally been associated with an increased recurrence of BD episodes (Benedetti *et al*., 2003; Nievergelt *et al*., 2006). It is not certain how *CLOCK* influences the recurrence of mood episodes. It may play a role directly on the recurrence rate or work through additional indirect mechanisms (Benedetti *et al*., 2003).

Research performed on mice indicated that a mutation in their *Clock* gene lengthens their circadian time period and eradicates regular rhythmicity (Vitaterna *et al*., 1994). Roybal and colleagues (2007) have shown that *Clock* mutant mice displayed symptoms very similar to that of mania experienced in humans. Chronic mood stabiliser treatment returned their manic behaviour to their usual wild-type behaviour. This research revealed that *CLOCK* plays an important role in the mood and behavioural regulation in the dopaminergic system (Roybal *et al*., 2007). Mouse behavioural research and human genetic research indicate that *CLOCK* plays an

important role in mood disruptions associated with BD (McClung, 2007) and is a contributing factor to psychological disorders (Schuch *et al*., 2017).

Additionally, *CLOCK* has been associated with the traits of MetS (Garaulet *et al*., 2009) and metabolic dysfunction (Fuller, Lu and Saper, 2008). Components of the MetS in humans that have been associated with the *CLOCK* 3111 SNP include glucose levels, waist circumference, blood pressure (Garaulet *et al*., 2009), body mass index (BMI) (Monteleone *et al*., 2008; Garaulet *et al*., 2009) and obesity (Monteleone *et al*., 2008; Garaulet *et al*., 2010). Overweight and obese indivduals carrying the CC genotype presented with higher BMI values (Monteleone *et al*., 2008) and carriers of the C allele had difficulty in losing weight as compared to non-carriers (Garaulet *et al*., 2010). Therefore, *CLOCK* has an important role in regulating body weight and has also been shown to be responsive to weight loss programmes (Garaulet *et al*., 2010). Animal models with *CLOCK* gene disruptions were inclined to obesity, shifts in eating behaviours as well as glucose and lipid imbalance. This confirms that *CLOCK* plays a significant role in body weight regulation (Turek *et al*., 2005) and glucose homeostasis (Rudic *et al*., 2004).

2.8.3. Period circadian regulator 3 (*PER3*) gene (OMIM 603427)

The *PER* gene family is a core constituent in the circadian clock (Archer *et al*., 2003; Zhu *et al*., 2005; Benedetti *et al*., 2008) and are responsible for their own gene expression through negative auto-feedback regulation (Figure 2.5) (Archer *et al*., 2003). There are numerous phosphorylation sites present on *PER3* that can have an effect on the gene function (Archer *et al*., 2003). In turn, this can have an effect on the post-translational modifications of proteins, their stability and structure (Dijk and Archer, 2010). This ultimately influences the function of the PER3 protein (Archer *et al*., 2003).

The *PER3* gene is located on chromosome 1p36.23 (Figure 2.8) (Nievergelt *et al*., 2006) and comprises of 21 exons that span more than 50 kbp (Ebisawa *et al*., 2001). A 54 base pair (bp) long variable number tandem repeat (VNTR) polymorphism (rs57875989) (Wirth *et al*., 2013; Archer *et al*., 2017) is located in exon 18 (Zhu *et al*., 2005). This VNTR translates into 18 amino acids, repeated either four (*PER3*^{4}) or five (*PER3*^{5}) times, resulting in three genotypes (*PER3*$^{4/4}$, *PER3*$^{4/5}$, *PER3*$^{5/5}$) (Ebisawa *et al*., 2001; Wirth *et al*., 2013; Anderson *et al*., 2017; Archer *et al*., 2017). Within *PER3*^{4}, a 3110 T/C SNP (M1037T, rs2640909) is present (Figure 2.9) (Ebisawa *et al*., 2001).

The *PER3* VNTR has been associated with numerous circadian and pathological disorders including BD (Nievergelt *et al*., 2006; Karthikeyan *et al*., 2014b; Brasil Rocha *et al*., 2017). This length polymorphism has an important interrelationship with sleep homeostasis and circadian rhythmicity (Dijk and Archer, 2010). It is a significant genetic marker for variations observed in sleep and for circadian phase misalignment (Viola *et al*., 2007). Carriers of the *PER3*^{5} repeat show increased sleep pressure and are inclined to present with poor cognitive function when sleep deprived (Karthikeyan *et al*., 2014a).

Karthikeyan and colleagues (2014b) found the *PER3*^{5} allele to occur at a significantly higher frequency in the BD patient group than in the unaffected control group (Karthikeyan *et al*., 2014b. Artioli *et al*. (2007) associated the *PER3*$^{5/5}$ genotype with certain mood traits including novelty seeking, extravagance and integrated conscience in a subset of BD individuals (Artioli *et al*., 2007). The *PER3* VNTR also plays a role in the age at BD onset (Benedetti *et al*., 2008). Specifically, Benedetti *et al*. (2008) found that individuals with the *PER3*$^{5/5}$ genotype are linked to early onset BD, while individuals with the *PER3*$^{4/5}$ genotype have an intermediate onset of BD and individuals with *PER3*$^{4/4}$ genotype had a later BD onset (Figure 2.10) (Benedetti *et al*., 2008).

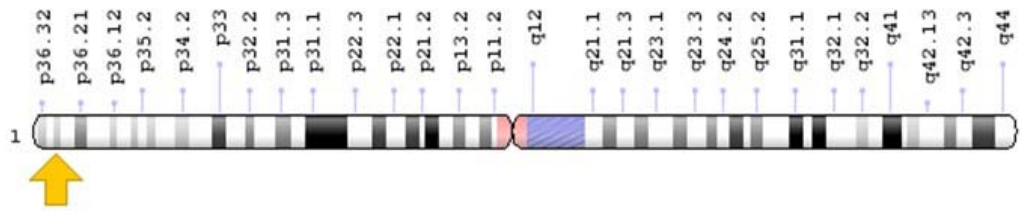


Figure 2.8 The location of the *PER3* gene on chromosome 1p36.23, indicated by the yellow arrow (NCBI, 2018).

Figure 2.9 The illustration of the nucleotide and amino acid sequence of *PER3*[4] and *PER3*[5] of the *PER3* VNTR. The dotted line represents the missing repeat in the sequence observed in individuals with the *PER3*[4] allele (Ebisawa *et al*., 2001). The 3110 T/C (M1037T, rs2640909) polymorphism (Rybakowski *et al*., 2014) is only located on *PER3*[4] and is shown in the boxed areas (Ebisawa *et al*., 2001).

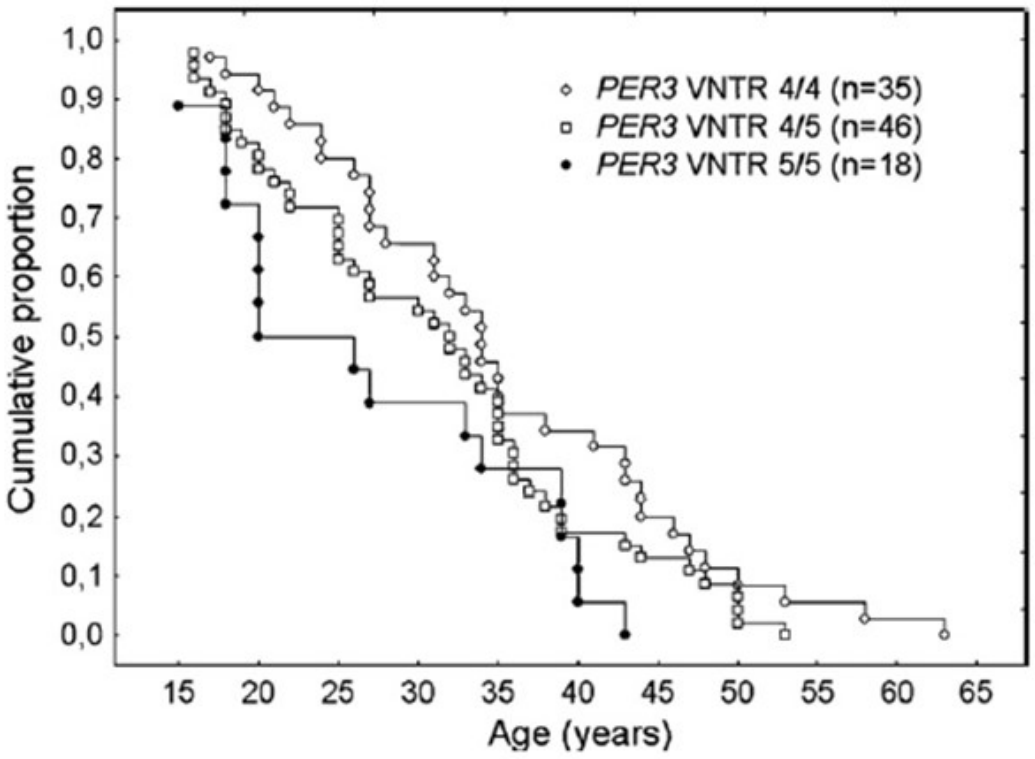


Figure 2.10 The cumulative proportion of the lifetime distributions of BD age at onset in the three genotypic groups (*PER3*$^{4/4}$, *PER3*$^{4/5}$, *PER3*$^{5/5}$) (Benedetti *et al*., 2008).

Due to the role that *PER3* plays in sleep, poor sleep conditions are related to weight gain, obesity (Bienertova-Vasku *et al*., 2014), CVD, diabetes (Li *et al*., 2017) and MetS (Knutson *et al*., 2007). The *PER3*$^{5/5}$ genotype is related to a higher consumption of fat as an energy source and a higher risk of developing poor nutritional habits (Bienertova-Vasku *et al*., 2014). In light of this, type 2 diabetes (T2D) is found more prevalently in individuals with the *PER3*^{5} allele (Karthikeyan *et al*., 2014a). Impaired *PER3* transcription can influence glucose metabolism (Karthikeyan *et al*., 2014a) and diabetes (Ando *et al*., 2007). The factors mentioned can potentially contribute to MetS.

2.8.4. Glycogen synthase kinase-3β (*GSK-3β*) gene (OMIM 605004)

There are numerous mechanisms in GSK-3 that strongly regulate its own function (Jope and Johnson, 2004; Tsai *et al*., 2008). Two homologous isoforms of GSK-3, GSK-3α and GSK-3β are present in the human genome (Woodgett, 1990; Grimes and Jope, 2001; Tsai *et al*., 2008; Yoon and Kim, 2010), whereby GSK-3β is the dominating brain isoform (Yoon and Kim, 2010). This enzyme's activity is controlled through its phosphorylation at serine-9 in GSK-3β and serine-21 in GSK-3α (Grimes and Jope, 2001). If this inhibition activity is impaired, the unusually high GSK-3 activity could result in adverse effects on cell structure, growth, motility and cell survival (Jope and Johnson, 2004). This dysregulation can cause behavioural abnormalities such as affecting BD onset (Lin, Huang and Lui, 2013). Glycogen synthase kinase-3 has been linked to BD and its treatment response (Jope and Roh, 2006).

Glycogen synthase kinase-3β is located on chromosome 3q13.3 (Figure 2.11). This gene is approximately 268 kbp in length and includes 12 exons (Shaw *et al*., 1998; Tsai *et al*., 2008). The GSK-3β enzyme is 47 kiloDalton (kDa) in molecular weight (Benedetti *et al*., 2004b) and serves as a polypeptide with catalytic properties similar to those of GSK-3α (Woodgett, 1990). Glycogen synthase kinase-3β is present in all body tissues and is most abundantly found in the brain (Woodgett, 1990; Tsai *et*

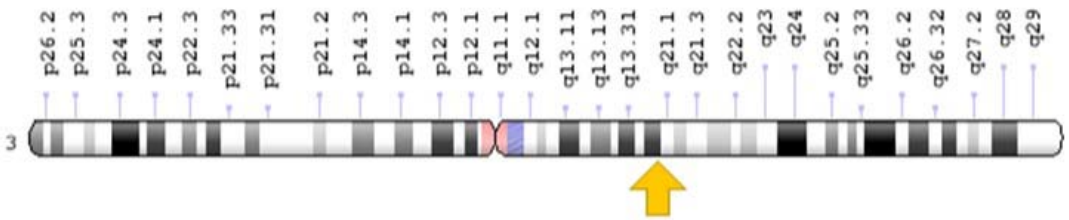


Figure 2.11 The location of the *GSK-3β* gene on chromosome 3q13.3, indicated by the yellow arrow (NCBI, 2018).

al., 2008). The abundance of GSK-3β in the brain signifies its essential role in neuronal signalling systems. Thus, altered GSK-3β influences neuronal dysfunction, consequently causing disease (Grimes and Jope, 2001). Additionally, the specific location on chromosome 3 where *GSK-3β* is located, has been associated with BD (Badenhop *et al*., 2002).

Glycogen synthase kinase-3β is located in the cytoplasm of the cell, where it functions as a serine/threonine protein kinase (Jope and Roh, 2006; Yoon and Kim, 2010; Lin, Huang and Lui, 2013). It was originally named for its role in glycogen synthase inactivation through phosphorylation (Grimes and Jope, 2001; Lin, Huang and Lui, 2013). Once GSK-3β is activated, it regulates substrates by adding a phosphoric acid to either the serine or threonine residue of the substrate (Jope and Roh, 2006). It has a significant role in phosphorylating and regulating numerous transcription factors as well as metabolic signalling structural proteins (Pandey *et al*., 2010; Yoon and Kim, 2010; Shim *et al*., 2012). The Wingless/Integrated (Wnt) signalling pathway, the mitogen-activated protein (MAP) kinase pathway and the phosphatidylinositol-3 kinase pathway are signalling pathways where GSK-3β plays a fundamental role (Mansour, Monk and Nimgaonkar, 2005; Lee and Kim, 2011). These pathways are linked to gene expression, metabolism, neuroplasticity, cell survival and death, neurogenesis and circadian rhythms in neurons (Grimes and Jope, 2001) as well as increased risk of suicidal behaviour in BD patients (Jiménez *et al*., 2013). Glycogen synthase kinase-3β additionally regulates the transcription of circadian rhythm genes in the SCN (Abreu and Bragança, 2015).

The mood stabilisers lithium and valproic acid, are effective treatments used in the acute and maintenance phases of BD (Lee and Kim, 2011). Lithium directly inhibits GSK-3β (Klein and Melton, 1996), whereas valproic acid indirectly suppresses GSK-3β through phosphorylation of serine-9 (Kim *et al*., 2005). This suggests GSK-3β plays a significant role in the therapeutic action of BD (Lee and Kim, 2011). Pandey *et al*. (2010) found that GSK-3β levels significantly increased in BD patients after the administration of mood stabiliser treatment, increasing GSK-3β levels similar to that observed in control subjects (Pandey *et al*., 2010). Thus, GSK-3β

levels in BD patients are corrected after mood stabilising treatment (Pandey *et al*., 2010). A more recent study has shown that GSK-3β activity differs between the various BD mood states as well as between euthymic BD patients and healthy individuals (Jacoby *et al*., 2016). An increased amount of GSK-3β was observed in BD patients in depressive and mixed mood states as compared to euthymic BD patients. However, a reduced amount of GSK-3β was seen in the euthymic BD patients as compared to healthy individuals. There were no dissimilarities detected between BD patients in the manic state as compared to BD patients in the euthymic state (Jacoby *et al*., 2016). Therefore, *GSK-3β* is an important candidate gene for the action of lithium treatment response as well as for BD aetiology (Nishiguchi *et al*., 2006; Iwahashi *et al*., 2014).

Russ and colleagues (2001) identified two common SNPs located at the –50C/T (rs334558) and –1727 A/T (rs3755557) positions of *GSK-3β*. These SNPs are found on the transcriptional start site and upstream of the coding sequence (Russ, Lovestone and Powell, 2001; Lee and Kim, 2011). They are of great interest due to their location in the effective promotor region (Figure 2.12) (Iwahashi *et al*., 2014). The T/T genotype of the –50C/T SNP has been linked to an earlier age at BD onset (Benedetti *et al*., 2004a; Benedetti *et al*., 2004b) whereas C/C homozygotes have been linked to a later age at BD onset. Bipolar disorder patients with the C/C genotype present with a less severe form of the disorder and may have a protective role in BD (Benedetti *et al*., 2004b). Association between the –50C/T SNP and lithium has been recognised, where the C allele has been associated with a better treatment response to lithium (Rybakowski *et al*., 2013). Jiménez *et al*. (2014) showed that the C allele is associated with higher attentional-cognitive impulsivity in BD patients, who had problems focusing and maintaining attention (Jiménez *et al*., 2014). The T allele has better transcriptional strength and is better influenced by lithium, inhibiting the activity of GSK-3β (Iwahashi *et al*., 2014). Lee and Kim, (2011) associated the A allele of the –1727A/T SNP to an earlier age at BD onset as compared to carriers of the T allele. Furthermore, the A allele was associated with psychotic mania in BD patients (Lee and Kim, 2011). The A allele enhances the binding affinities of transcription factors and in turn increases *GSK-3β* expression (Li *et al*., 2011).

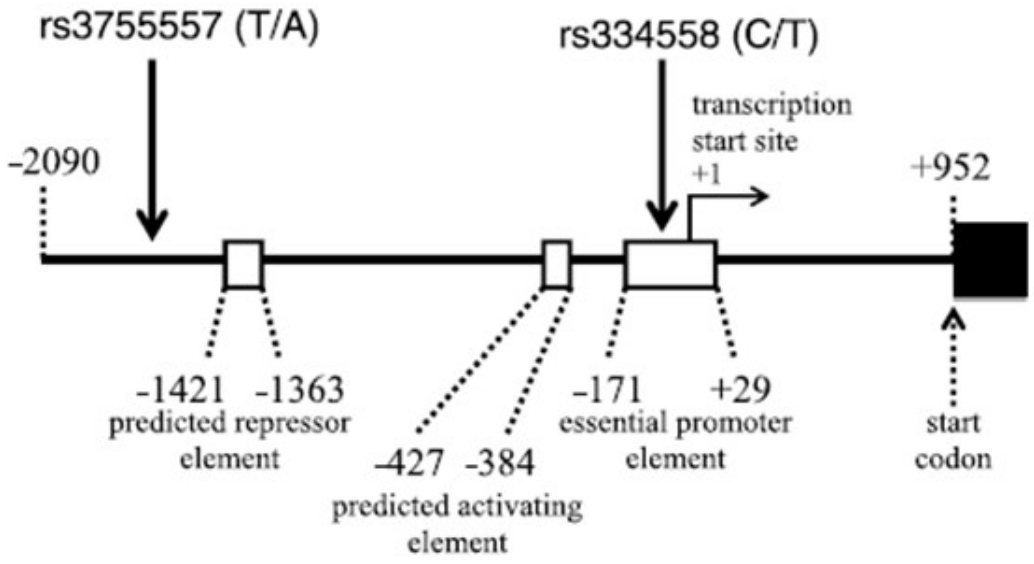


Figure 2.12 The *GSK-3β* gene located on chromosome 3q13.3 depicting rs3755557 SNP located at –1727 and the rs334558 SNP located at –50 relative to the transcriptional start site, upstream of the coding sequence on the *GSK-3β* promotor (Russ, Lovestone and Powell, 2001; Lee and Kim, 2011).

2.8.5. Neurotrophins

Neurotrophins are important in brain maintenance and regulation (Huang and Reichardt, 2001). They are mainly regulated during the development stage and are expressed throughout the brain in adulthood (Scola and Andreazza, 2015). The neurotrophic factors include BDNF, nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), glial cell line-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF). They are growth factors have many functions in brain cells (promote neuroplasticity, neurogenesis, survival, differentiation and brain cell maintenance) (Sigitova *et al*., 2017). As a neurotrophin, dysregulation of the BDNF protein may contribute to pathophysiology of mood disorders (Mai, Jope and Li, 2002; Ejmalian *et al*., 2016). The BDNF protein is one of the most studied proteins in BD research (Fernandes *et al*., 2015).

2.8.5.1. Brain-derived neurotrophic factor (*BDNF*) gene (OMIM 113505)

Brain-derived neurotrophic factor is the most widely distributed neurotrophin in the central nervous system (Frey *et al*., 2013; Pfaffenseller *et al*., 2013). It is mainly produced in the brain and spinal cord (Sears *et al*., 2011) and has a significant role in neurogenesis (Grande *et al*., 2010; Rakofsky, Ressler and Dunlop, 2012), neural development pathways (Grande *et al*., 2010), neuronal survival, structure, function (Kauer-Sant'Anna *et al*., 2009), maturation, differentiation and migration (Rakofsky, Ressler and Dunlop, 2012). The BDNF orchestrates mechanisms of neuronal plasticity and survival together with other biological factors (Grande *et al*., 2010). Brain-derived neurotrophic factor has many roles in brain development, physiology and pathology (Pruunsild *et al*., 2007). Additionally, BDNF is a dominant regulator of the circadian clock (Cain *et al*., 2017), demonstrating a daily rhythm that is affected by light (Tirassa, Quartini and Iannitelli, 2015), which depicts morningness and eveningness dimensions (Cain *et al*., 2017).

Animal and human studies show *BDNF* as a candidate gene in the aetiology of psychiatric disorders (Sears *et al*., 2011). Research shows that BDNF protein levels fluctuate during mood episodes (Kauer-Sant'Anna *et al*., 2009; Rakofsky, Ressler and Dunlop, 2012). It has also been found that a negative correlation exists between BDNF levels and the number of mood episodes. The BDNF levels decrease significantly from early to late stage BD (Kauer-Sant'Anna *et al*., 2009). Recent studies show that BDNF levels decrease during manic and depressive episodes (Pfaffenseller *et al*., 2013; Cattaneo *et al*., 2016) and return to normal levels in the euthymic state (Cattaneo *et al*., 2016). Mood stabilisers such as lithium (Muneer, 2017), play a role by increasing BDNF levels (Fernandes *et al*., 2011; Muneer, 2017). Glycogen synthase kinase-3 inhibitors and silencing of specific protein kinases, enhance transcription of *BDNF* and is a downstream target of BDNF (Muneer, 2017). Elevated GSK-3 activity is consequence of reduced BDNF signalling, a main cause of development and advancement of mood disorders. Therefore, BDNF may be a promising potential biomarker to use in mood disorders (Rakofsky, Ressler and Dunlop, 2012; Cattaneo *et al*., 2016) and its importance has promoted further research of BDNF into the pathophysiology of BD (Pfaffenseller *et al*., 2013).

The *BDNF* gene is situated on chromosome 11p14.1 (Figure 2.13) (Sears *et al*., 2011; González-Castro *et al*., 2014) and is approximately 70 kb in length (Pruunsild *et al*., 2007). The structure of *BDNF* is extremely complex, consisting of 11 exons (I–IX, Vh and VIIIh) located on the 5' end, and contains nine promoters. These exons combine and form many various transcripts. Exon I, II (with transcripts IIa, IIb and IIc), III, IV, V (with transcripts Va, Vb, Vc and V–VIII–VIIIh), VI (with transcripts VIb, VIb–IXabd and VIb–IXbd), VII (with transcripts VIIa and VIIb) and IX (with transcripts IXabd and IXabcd) are the promotors used in brain and tissue areas. Exon IX contains the coding DNA sequence (CDS) as well as eight upstream exons encoding promoters that control regional and cell-type-specific expression (Figure 2.14) (Cattaneo *et al*., 2016).

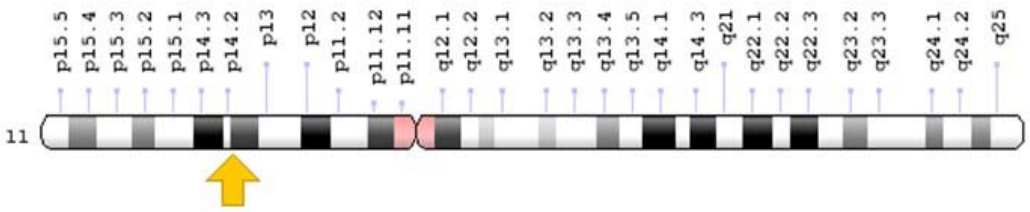


Figure 2.13 The location of the *BDNF* gene on chromosome 11p14.1, indicated by the yellow arrow (NCBI, 2018).

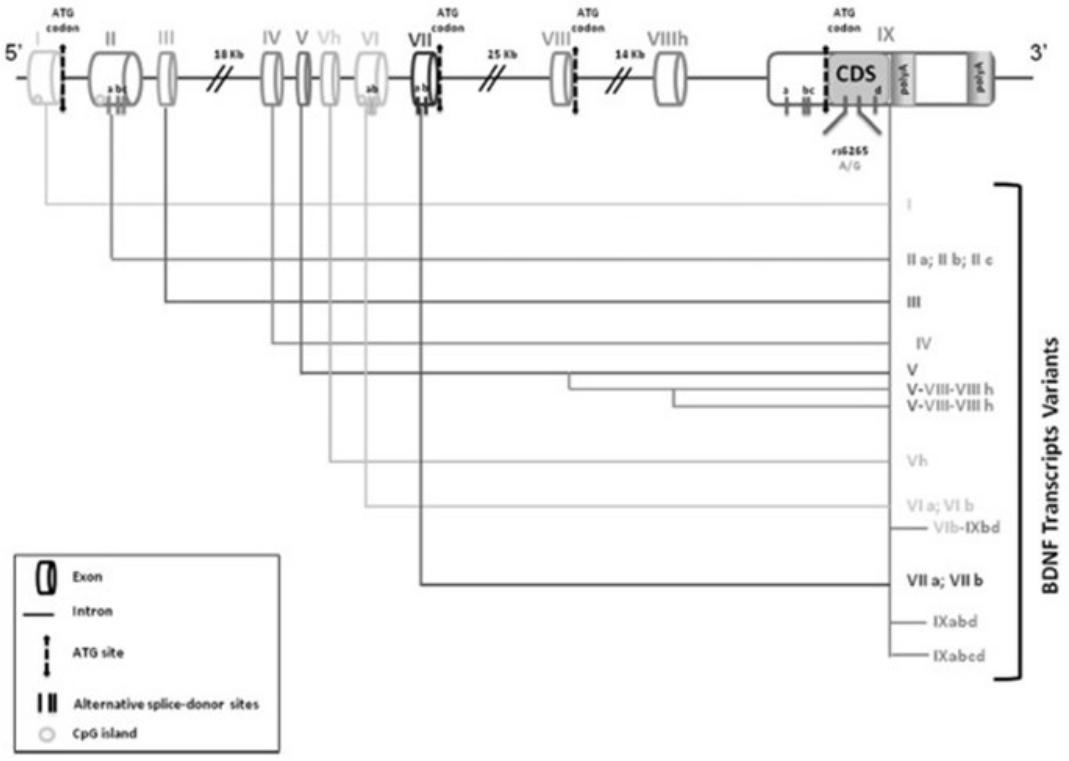


Figure 2.14 The *BDNF* gene structure depicting the 11 exon, the CDS in exon IX as well as the alternative splice-donor sites and CpG islands (Cattaneo *et al*., 2016).

The *BDNF* rs6265 SNP consists of a guanine to adenine (G→A) substitution on nucleotide number 196 of exon 2, which represents codon number 66 (Grande *et al*., 2010; Ejmalian *et al*., 2016). A missense change (Daily and Park, 2017) resulting in a valine (Val) (encoded by the G allele) to methionine (Met) (encoded by the A allele) amino acid substitution (Grande *et al*., 2010; Ejmalian *et al*., 2016; Zeni *et al*., 2016) constitutes a change in the 5' pro-region of *BDNF* (Sheikh *et al*., 2010). This SNP appears to affect mRNA and protein movement (Pruunsild *et al*., 2007; Morales-Marín *et al*., 2016). The Met amino acid influences the intracellular processing of BDNF and has an effect on its secretion, leading to changes in the hippocampal function and memory performance (Egan *et al*., 2003). The Met allele has also been shown to correlate with a decrease in BDNF activity (González-Castro *et al*., 2014) and decrease in hippocampal volume (Ribeiro *et al*., 2007). In patients with BD, the Val allele is over-transmitted (Neves-Pereira *et al*., 2002; Sklar *et al*., 2002; Geller *et al*., 2004; Lohoff *et al*., 2005). The BDNF transcript has been shown to be reduced in the hippocampus in BD patients (Ejmalian *et al*., 2016). The functional nature of the rs6265 SNP makes it a plausible risk factor for psychiatric disorders such as BD (Sears *et al*., 2011).

The *BDNF* gene is also related to BMI, obesity, eating disorders and MetS (Morales-Marín *et al*., 2016). The GG (Val/Val) genotype of the rs6265 SNP is significantly associated with the risk of obesity in BD patients. Carriers of the AA (Met/Met) genotype or Met allele have a lower risk of obesity. Additionally, the G allele was found to significantly increase the risk of being overweight or obese (Morales-Marín *et al*., 2016). In healthy individuals similar results were observed, whereby individuals with the GG or GA genotype had a higher BMI than individuals with an AA genotype (Gunstad *et al*., 2006; Timpano *et al*., 2011). The *BDNF* gene is strongly associated with T2D (Wang *et al*., 2012). Daily and Park (2017) found the GG genotype to be associated with higher risk for T2D than carriers of the AA genotype. The GA genotype and was found to reduce the risk of insulin resistance and T2D (Daily and Park, 2017). It has been found that BDNF levels are greatly reduced in patients with MetS and atherosclerotic CVD (Chaldakov *et al*., 2004). Therefore, BDNF may play a large role in the aetiology of human coronary atherosclerosis and MetS.

2.9. Summary

After reviewing the literature, it is clear that BD is a very serious, complex mood disorder that inflicts substantial burdens on the affected individual, as well as their families and society as a whole. Bipolar disorder is a highly heritable, multifactorial psychiatric illness. Diagnosis of BD currently relies exclusively on phenotypes presented by patients as assessed with the use of phenomenology-based diagnostic systems, which can easily cause misdiagnosis. The discovery of biomarkers for BD diagnosis will be essential by allowing early detection and treatment to target BD risk alleles and biological pathways involved. Identifying risk alleles will aid in diagnosis and allow for personalised treatment targeting the specific risk alleles. With this approach, misdiagnosis can be avoided, and appropriate treatment administered effectively without delay. South Africa will have approximately 1 in 3 individuals suffering from a mental disorder in their lifetime.

Due to various genetic mechanisms involved in BD inheritance, the search for risk alleles has been a daunting task. The inheritance of BD is polygenic, with numerous rare and common risk alleles involved with small to moderate effect. Gene-gene and GxE interactions, epistasis and pleiotropy further complicate the search for risk alleles. The circadian rhythm is responsible for driving the 24-hour rhythm in humans. It is controlled internally by the clock genes and externally by zeitgebers. Disruptions in the circadian clock can cause misalignment and contribute to the pathology of BD and MetS. Comorbidity, especially with MetS, is frequently observed in patients with BD. A mutual relationship exists between BD and MetS and disruptions of the circadian rhythm and neurotrophins may have an important role in this comorbidity. Metabolic syndrome in BD patients has become a worldwide concern with up to 40% of these patients presenting with MetS, a scenario that increases the risk of morbidity and mortality.

In SA, more research is needed to investigate the genetic association of BD and MetS. This will highlight the genetic diversity present in the country and greatly

contribute to the scientific world. The aim of this research project is to inform more individuals of the role that genetics play in BD, through the sampling process and scientific discussions and aims to highlight the importance of research needed in SA for all future scientists.

Chapter 3:
Materials and methods

3.1. Introduction

This study employed a quantitative research paradigm which is generally used to determine relationships, cause and effect between variables in controlled environments. Quantitative genetics estimates the cumulative effect of genetic influence irrespective of the number of genes involved or the size or complexity of their effects (Haworth and Plomin, 2010). It is useful in indicating both genetic and environmental factors that may contribute to the variances observed between different populations (Plomin, Owen and McGuffin, 1994). Quantitative genetic research has been useful in depicting the importance of genetic factors in many complex disorders and has created a foundation for the use of molecular genetic research (Plomin, Owen and McGuffin, 1994).

3.2. Research design

In the robust search for candidate genes that influence the aetiology of psychiatric disorders, the design of this study focused on the most basic aspect of genetics, thus, genetic variants and their association to BD aetiology. Therefore, multiple BD susceptibility genes of small effect were selected and investigated (Collins *et al*., 1998; Craddock and Sklar, 2013; Kerner, 2014). Evidence of comorbidity between BD and MetS, as well as the risk factors influencing MetS, have shown the large extent of their mutual, bidirectional association with underlying shared genetic influences. Though research suggests that environmental factors are important and undeniably play a role in the comorbidity of risk factors influencing MetS onset, the

influence of lifestyle-associated environmental factors could not be ignored in this study.

This study employed a quantitative research paradigm. Therefore, in order to control for influential variables discussed as contributing to BD aetiology (Chapter 2), a case-control design was implemented. The BD case group comprised individuals affected with BD. The control group, with no personal or family history of BD or mental illness, was matched to the BD case group based on gender, ethnicity and age within a 5-year interval. Matching controls to BD cases was done to eliminate confounding factors like population stratification, which plays a major role in genetic association studies. Also, matching in case-control studies would lead to a balance between the two groups, which can reduce the variance in parameters of interest. The Hardy–Weinberg equilibrium (HWE) is calculated to determine whether the genotype frequencies of each polymorphism are in equilibrium for case and control groups. This is especially important in population-based genetic association studies as it serves as a quality control step. Deviations from HWE in the control group may signal important problems including genotyping errors, selection bias and population stratification. Violations in HWE may cause significant bias in studies (Namipashaki, Razaghi-Moghadam and Ansari-Pour, 2015). Furthermore, this is a candidate polymorphic association study that intends to associate genetic variants in genes that play a role in specific disorders with the phenotypic characteristics of individuals.

In order to carry out this research project, each study participant was asked to complete a questionnaire and provide a 1 - 2 millilitre (ml) saliva sample that was collected in a 15 ml sterile tube. Questionnaires and DNA samples were analysed using quantitative and molecular techniques to ensure a thorough comparison of the genetic and environmental components between the two groups.

3.3. Study population

A cohort of 103 individuals above the age of 18 voluntarily participated in this research study. The BD case group consisted of 53 diagnosed BD-I or BD-II men and women of White, Black African and Coloured ethnicity (classified as recommended by the South African Society for Human Genetics [SASHG, 2018] and the South African population census [Statistics South Africa, 2011]) who were previously diagnosed by psychiatrists, and are currently on treatment for their symptoms. The majority of study participants were recruited from support groups located in Gauteng and Free State provinces. Outpatients from private psychiatric hospitals located in the North West, Northern Cape, Eastern Cape and Mpumalanga provinces were also recruited. This method of sampling, thus, using support groups allowed for random sampling by including a wide range of individuals with different ethnicities across different provinces in SA. It is important to note that no BD diagnoses were made by the researchers in the study. Individuals from the control group were identified at random from the general population.

3.4. Ethical consideration

The study was approved by the Ethics Committee of the Health Sciences Faculty of the University of the Free State in Bloemfontein (ECUFS 77/2014) on 3 June 2014 (Appendix A). Information letters were distributed explaining the aim and protocol of this research study (Appendix B [English] and Appendix C [Afrikaans]). All participants did so voluntarily and were assigned a unique code to maintain their anonymity and confidentiality. Every study participant was asked to provide written informed consent (Appendix B [English] and Appendix C [Afrikaans]). No hospital inpatients were included, only outpatients were recruited for this study.

3.5. The comparative analysis of selected lifestyle and associated risk factors contributing to metabolic syndrome between the BD case and control groups

3.5.1. Data collection and questionnaire information

A basic questionnaire consisting of short questions of a quantitative nature was provided to each study participant. The primary purpose of the questionnaire was to collect factual data and was not used for diagnostic purposes. The questionnaire was designed in English (Appendix D) and translated to Afrikaans (Appendix E) and constructed to be concise. The questionnaire used for the collection of data included the following three sections:

a) Section A: Included general questions pertaining to background and demographical information. The quantitative variables included age, gender, ethnicity, geographical location and home language. This section was completed by both the BD case and control groups and was used as a comparison tool to ensure the control group matched the BD case group.

b) Section B: Included questions relating to lifestyle and associated risk factors contributing to MetS and was completed by both the BD case group and control group. The MetS risk factor information gathered included:

- Weight in kilograms (kg) and height in centimetres (cm) which was used to calculate the BMI (kg/m^2) and categorized as underweight (<18.5), normal weight (18.5 - 24.9), overweight (25.0 - 29.9) or obese (> 30) (WHO, 1995). These values relied on the information directly from the questionnaire given by the study participant.
- Waist measurement in cm. Waist circumference cut-off points used were: ≥ 102 cm for men and ≥ 88 cm for women. The Third Report of The National Cholesterol Education Program (NCEP), Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult

Treatment Panel III) (NCEP ATP III) recommended waist circumference threshold for abdominal obesity was used. Due to the various ethnicity populations present in SA, ethnic and country specific waist circumference cut-off points could not be used. Additionally, it was not always possible to measure the waist circumference and therefore the waist circumference was determined by the study participants' clothing sizes (Han *et al*., 2005).

- Lifestyle factors including:
  (1) Exercising less than 2 times a week;
  (2) Smoking cigarettes (and the number of cigarettes smoked per day was specified by the study participant);
  (3) Drinking alcohol on a regular basis (alcoholic drinks consumed per week was specified by the study participants). Moderate drinking is considered as one drink per day for women and two drinks a day for men (Jacobs and Steyn, 2013; The Heart and Stroke Foundation South Africa, 2017). One drink is equal to 340 ml beer, 120 ml wine, 60 ml sherry or 25 ml spirits (The Heart and Stroke Foundation South Africa, 2017).
  (4) Whether a balanced diet is followed (consisting of the correct proportion of all the nutrients including carbohydrates, fats, proteins, minerals and vitamins along with sufficient amounts of water and roughage material [Sylvia *et al*., 2013]).
  (5) Medical comorbid conditions contributing to MetS including: cardiovascular conditions, diabetes, hypertension and hyper-cholesterolaemia, as well as the age at diagnosis of the comorbid disorder and treatment received.

Due to limited resources and inability to draw blood for laboratory analysis, blood pressure, fasting plasma glucose, fasting triglycerides and HDL cholesterol levels were not tested. Therefore, the study relied on treatment prescribed to study participants by medical professionals to confirm diagnosis of the above MetS risk factors. Consequently, the ability to determine MetS in study participants could not be done. Instead, the number of core component risk factors (elevated waist circumference, study participants on medication for both hypertension and hypercholesterolaemia) were determined for each individual (with CVD, with

diabetes, without CVD or diabetes) and a comparison made between the BD case and control groups.

(c) Section C: Included questions relating to BD. The information gathered here included: BD diagnosis type, age at diagnosis, current medication prescribed for treatment, BD family history, comorbidity (with psychiatric disorders and substance abuse) and symptoms relating to BD prior to diagnosis and treatment. No information regarding the reaction or side effects of the medication was collected. The list of symptoms provided in the questionnaire was gathered from journal articles as the most frequently experienced symptoms experienced prior to BD diagnosis. Only the BD case group was required to answer this section. No diagnosis of BD was made in this study.

3.5.2. Statistical analysis of the questionnaire data

All statistical analyses of the data were performed with assistance from the Department of Mathematical Statistics and Actuarial Science at the University of the Free State. For this purpose, the SAS™ software package (v14.2, SAS Institute Inc, 2016) was used. Descriptive statistics, thus, mean, standard deviation (SD), minimum, median and maximum values were determined for the quantitative variables: age and age at diagnosis (for the BD case group only) using the FREQ Procedure. Furthermore, frequencies and proportions (counts and percentages) of the categorical variables for sections A, B and C were also performed.

The two sample groups (BD cases versus [vs] controls) were compared with respect to the prevalence of MetS risk factors using the chi-square (χ^2) test. In BD cases where information on the MetS risk factor was binary (i.e. yes/no; all variables except BMI), the Pearson χ^2 statistic and associated *p*-value were calculated. For BMI, which was reported as an ordinal variable (i.e. underweight, normal, overweight and obese), the Cochran-Mantel-Haenszel row mean score test and associated *p*-value were calculated. All statistical analyses were conducted at a

95% confidence interval. Results are significant when $p < 0.05$. Incomplete and unanswered questions were omitted from statistical analysis.

3.6. Molecular analysis of selected polymorphisms in *CLOCK*, *PER3*, *GSK-3β* and *BDNF* genes in relation to bipolar disorder and metabolic syndrome

3.6.1. Gene and polymorphism selection

Candidate genes and their corresponding variants of interest were chosen based on their association to BD and its treatment (see Chapter 2). Additionally, genes and their respective variants were chosen because they have been associated with risk factors influencing MetS as well. A total of four genes and five variants within the selected genes were identified (Table 3.1). Two types of genetic markers were used in this study to test for their association with BD: SNPs and a VNTR.

3.6.2. DNA extraction

Genomic DNA was extracted from saliva samples using the salting out protocol adapted from Quinque *et al*. (2006). Each study participant deposited 1 - 2 ml saliva into a sterile 15 ml collection tube to which 1 ml lysis buffer (50 mM [millimolar] trishydroxymethylaminomethane [Tris] [pH 8.0], 50 mM ethylenediaminetetraacetic acid [EDTA], 50 mM sucrose, 100 mM sodium chloride [NaCl] and 10% sodium dodecyl sulphate [SDS]) was added. Thereafter, 20 microlitres (µl) (20 milligram [mg]/ml) proteinase K (Roche, Basel, Switzerland) and 150µl of 10% SDS were added whereafter the sample was incubated overnight at 53 degrees Celsius (˚C) in a water bath. An amount of 400 µl of 5 molar (M) NaCl was added to the sample and incubated on ice for 10 minutes. Thereafter, the sample was centrifuged at 45,000 rpm (revolutions per minute) for 20 minutes, instead of 13,000 rpm for 10

Table 3.1 Candidate genes and polymorphisms selected for this study.

Gene	Chromosomal location	Variant type	Variant identification
CLOCK	4q12	SNP	rs1801260
PER3	1p36.23	VNTR	rs57875989
GSK-3β	3q13.3	SNP	rs3755557 (–1727)
GSK-3β	3q13.3	SNP	rs334558 (–50)
BDNF	11p14.1	SNP	rs6265

minutes as used by Quinque *et al*. (2006). Once completed, 1 ml supernatant was transferred into a 1.7 ml sterile tube whereby 700 µl of 96% ethanol was added and not 800 µl of isopropanol as used by Quinque *et al*. (2006). The sample was placed onto an orbital shaker at 99 rpm for 10 minutes and then centrifuged at 13,000 rpm for 15 minutes. The supernatant was discarded, and the pellet washed with 500 µl 70% ethanol and again placed onto the orbital shaker at 99 rpm for 30 minutes. This step was repeated, however, was not included in the method by Quinque *et al*. (2006). The supernatant was discarded, and the sample left open overnight for the pellet to air dry. Once air dried, 100 µl nuclease-free water was added to the pellet to allow it to dissolve by a short heating step of 50˚C for 60 minutes. During DNA extraction, a minimum of 3 aliquots were made for each sample to yield as much DNA as possible to be used for backup samples.

Quantification of the extracted DNA was performed using the NanoDrop™ Lite spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The quantity (nanogram [ng]/µl) and purity ($A_{260/280}$) of the DNA was measured. The average of 3 readings were taken for every sample. Upon quantification, samples were diluted with nuclease-free water to a concentration of 50 ng/µl. The isolated DNA was stored at –20°C.

3.6.3. Conventional polymerase chain reaction (PCR)

Conventional PCR was performed using the primer sequences identified from previously published research articles as shown in Table 3.2. The primers for rs6265 (*BDNF*) and rs334558 (*GSK-3β*) were designed using Primer3 (Rozen and Skaletsky, 2000), OligoAnalyzer 3.0 (https://www.idtdna.com/analyzer/Applications/OligoAnalyzer/) and Primer Blast (http://www.ncbi.nlm.nih.gov/). All primers were synthesised by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, RSA). The reaction conditions for all primer sets, except for s3755557 (*GSK-3β*) and rs334558 (*GSK-3β*), were previously optimised by the human genetics research team at the

Table 3.2 The primer sequences used for the genotyping of SNPs in *CLOCK*, *PER3*, *GSK-3β* and *BDNF*.

Gene and polymorphism	Forward (F) and reverse (R) primer sets	References
CLOCK **rs1801260**	F: 5'- TCCAGCAGTTTCATGAGATGC -3' R: 5'- GAGGTCATTTCATAGCTGAGC -3'	Katzenberg *et al*. (1998); Serretti *et al*. (2003); Benedetti *et al*. (2003); Bailer *et al*. (2005); Suzuki *et al*. (2017).
PER3 **VNTR**	F: 5'- CAAAATTTTATGACACTACCAGAATGGCTGAC -3' R: 5'- AACCTTGTACTTCCACATCAGTGCCTGG -3'	Ebisawa *et al*. (2001); Archer *et al*. (2003); Barbosa *et al*. (2010); Barclay *et al*. (2011).
GSK-3β **rs3755557 (–1727)**	F: 5'- CCAGCGTCCATTGTTCTACC -3' R: 5'- GCTTTGAAACACTGATGAAGGAC -3'	Custom designed.
***GSK*-3β** **rs334558 (–50)**	F: 5'- AGCCCAGAGCCCTGTCAG -3' R: 5'- GACGTCCGTGATTGGCTC -3'	Russ, Lovestone and Powell, (2001); Lee *et al*. (2011).
BDNF **rs6265**	F: 5'- TCTCCCTACAGTTCCACCAG -3' R: 5'- CTACTGAGCATCACCCTGGA -3'	Custom designed.

Department of Genetics, University of the Free State. A PCR gradient protocol was applied to optimise rs3755557 (*GSK-3β*) and rs334558 (*GSK-3β*). A gradient protocol was added to the reference annealing temperatures (T_a) provided by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, RSA) with each primer set. The T_a were optimised with a range between 57°C and 65°C for rs3755557 (reference temperature: 60°C), and 62°C and 67°C for rs334558 (reference temperature: 64.2°C).

Amplification was performed using the reaction conditions in Table 3.3 using the T100™ Bio-Rad Thermal Cycler (Bio-Rad Laboratories [pty] Ltd, Hercules, USA). For the *CLOCK*, *PER3* and *GSK-3β* variants, every 20 µl PCR consisted of 200 ng DNA, 0.2 µM (micromolar) of the forward and reverse primer, 10 µl DreamTaq™ Master Mix (containing 2x DreamTaq buffer, 4 mM magnesium chloride [$MgCl_2$] and 0.4 mM of each dNTP [dATP, dCTP, dTTP and dGTP]) (Thermo Fisher Scientific, Waltham, USA) and 4 µl nuclease-free water. For the *BDNF* variant, every 20 µl reaction mixture consisted of 100 ng of DNA, 0.5 µM of the forward and reverse primer, 10 µl DreamTaq™ Master Mix and 6 µl nuclease-free water.

3.6.4. Gel electrophoresis

The amplified products were visualised on a 2% weight/volume (w/v) agarose gel to confirm successful amplification. The gels were prepared using Seakem® LE Agarose (Lonza Group Ltd, Basel, Switzerland) and 1x TAE buffer (40 mM Tris-acetate and 1 mM EDTA). The amplified products were mixed with GelRed® (Biotium Inc, Fremont, USA) and bromophenol blue to enable the tracking and visualisation of the PCR products. A 50 bp O'GeneRuler DNA ladder (Fermentas, Thermo Fisher Scientific, Waltham, USA) was also loaded onto the gel along with amplified products to determine fragment sizes. All gels were subjected to electrical current at 100 volts per cm (V/cm) for 40 minutes.

Table 3.3 The PCR protocols used for rs1801260 (*CLOCK*), *PER3* VNTR, rs3755557 (*GSK-3β*) rs334558 (*GSK-3β*) and rs6265 (*BDNF*).

Gene and variant amplified	PCR steps	Temperature	Duration
CLOCK **rs1801260**	Step 1: Initial denaturation	95°C	5 minutes
	Step 2: Denaturation	95°C	30 seconds
	Step 3: Annealing	61°C	30 seconds
	Step 4: Extension	72°C	1 minute (steps 2-4 x 35 cycles)
	Step 5: Final extension	72°C	5 minutes
PER3 **VNTR**	Step 1: Initial denaturation	94°C	3 minutes
	Step 2: Denaturation	94°C	45 seconds
	Step 3: Annealing	61°C	45 seconds
	Step 4: Extension	72°C	1 minute (steps 2-4 x 38 cycles)
	Step 5: Final extension	72°C	10 minutes
GSK-3β **rs3755557 (–1727)**	Step 1: Initial denaturation	95°C	3 minutes
	Step 2: Denaturation	95°C	30 seconds
	Step 3: Annealing	57.6°C	1 minute
	Step 4: Extension	72°C	1 minute (steps 2-4 x 35 cycles)
	Step 5: Final extension	72°C	5 minutes
GSK**-3β** **rs334558 (–50)**	Step 1: Initial denaturation	95°C	3 minutes
	Step 2: Denaturation	95°C	30 seconds
	Step 3: Annealing	63°C	1 minute
	Step 4: Extension	72°C	1 minute (steps 2-4 x 35 cycles)
	Step 5: Final extension	72°C	5 minutes
BDNF **rs6265**	Step 1: Initial denaturation	95°C	3 minutes
	Step 2: Denaturation	95°C	30 seconds
	Step 3: Annealing	62.5°C	1 minute
	Step 4: Extension	72°C	1 minute (steps 2-4 x 35 cycles)
	Step 5: Final extension	72°C	5 minutes

3.6.5. Genotyping

3.6.5.1. Restriction enzyme digestion and visualisation

Restriction enzyme digestion of amplified PCR products (*CLOCK*, *PER3*, *GSK-3β* and *BDNF*) were done according to the manufacturer's protocol (New England Biolabs® Inc, Ipswich, USA). Each 32 µl reaction contained 10 µl PCR reaction mixture, 18 µl nuclease-free water, 2 µl CutSmart® Buffer and 1 µl (10 units/µg DNA) of the particular restriction enzyme. The amplification lengths of the PCR products, restriction enzymes used for digestion of the target loci, their respective restriction sites, expected fragment sizes after digestion as well as incubation temperatures and duration used are depicted in Table 3.4 and Table 3.5. Due to amplified fragments in *PER3* consisting of a VNTR with fragments of somewhat similar length (5R = 635 bp; 4R = 581 bp), digestion was done to improve visualisation of the alleles, specifically in individuals who were homozygous for the alleles. To this end, an SNP, M1037T (rs2640909), located in *PER3*^{4} of the VNTR was, therefore, targeted for digestion.

Restriction products for *CLOCK*, *PER3* and *GSK-3β* (rs334558) were separated and visualised on a 2% (w/v) TAE agarose gel (100 V/minute for 40 minutes), restriction fragments for *BDNF* were visualised on a 3% (w/v) TAE agarose gel (100 V/minute for 40 minutes) prepared as above. The fragment banding patterns present on the gels were analysed and genotypes for each study participant determined. For agarose gel visualisations indicating smears, atypical or faint banding patterns that were unable to be analysed, the appropriate troubleshooting steps were performed, and the process repeated.

Table 3.4 The amplification lengths of the PCR products, restriction enzymes used for digestion, their respective restriction sites, amplification lengths, expected fragment sizes after digestion, incubation temperatures and duration used for rs1801260 (*CLOCK*), *PER3* VNTR, rs3755557 (*GSK-3β*), rs334558 (*GSK-3β*) and rs6265 (*BDNF*).

Gene and polymorphism	Amplification length of PCR product in bp	Restriction enzyme and buffer	Restriction enzyme recognition site	Incubation temperature and duration	Allelic variation	Expected digested fragment sizes in bp
***CLOCK* rs1801260**	221	Bsp1286I 10 x Buffer SduI™ (New England Biolabs® Inc, Ipswich, USA)	5'- GDGCH↓C- 3' 3' -C↑HCGDG- 5'	37°C for 30 minutes	T: Ancestral C: Variant	**T/T**: 221 **C/C**: 125 + 96 **C/T**: 221 + 125 + 96
***PER3* M1037T (rs2640909)**	4-repeat: 581 5-repeat: 635	NcoI 10 x Buffer Tango™ (New England Biolabs® Inc, Ipswich, USA)	5'- C↓CATGG -3' 3'- GGTAC↑C -5'	37°C for 30 minutes	4-Repeat 5-Repeat	See Table 3.5 below.
***GSK-3β* rs3755557 (–1727)**	250	MseI 10 x Buffer R™ (New England Biolabs® Inc, Ipswich, USA)	5'- T↓TAA -3' 3'- AAT↑T -5' (recognised twice in the amplified region)	37°C for 30 minutes	A: Ancestral T: Variant	**A/A**: 163 + 87 **T/T**: 163 + 59 + 28 **A/T**: 163 + 87 + 59 + 28
***GSK-3β* rs334558 (–50)**	344	AluI 10 x Buffer Tango™ (New England Biolabs® Inc, Ipswich, USA)	5'- AG↓CT -3' 3'- TC↑GA -5'	37°C for 30 minutes	G: Ancestral A: Variant	**G/G**: 344 **A/A**: 220 + 124 **G/A**: 344 + 220 + 124
***BDNF* rs6265**	324	NlaIII 10 x Buffer G™ (New England Biolabs® Inc, Ipswich, USA)	5'- CATG↓ -3' 3'- ↑GTAC -5' (recognised three times in the amplified region)	37°C for 30 minutes	G: Ancestral A: Variant	**G/G**: 170 + 81 + 58 + 15 **A/A**: 94 + 81 + 76 + 58 + 15 **G/A**: 170 + 94 + 81 + 76 + 58 + 15

Table 3.5 The genotypes and associated fragment sizes of *PER3* observed after NcoI digestion of the M1037T (rs2640909) SNP for the *PER3*$^{4/4}$, *PER3*$^{4/5}$ and *PER3*$^{5/5}$ genotypes[a].

Genotypes	C/C (rs2640909)	C/T (rs2640909)	T/T (rs2640909)
***PER3*$^{4/4}$**	581bp	581bp + 428bp + 154bp	428bp + 154bp
***PER3*$^{4/5}$**	635bp + 581bp	T allele in 4R and C allele in 5R: 635bp + 428bp + 154bp C allele in 4R and T allele in 5R: 581bp + 428bp + 208bp	428bp + 208bp + 154bp
***PER3*$^{5/5}$**	635bp	635bp + 428bp + 208bp	428bp + 208bp

[a]Product sizes were determined from the reference sequence PER3 ENSG00000049246 [accessed April 2013].

3.6.5.2. Sequencing

Bi-directional DNA sequencing was performed on 20% of all study samples for *CLOCK*, *PER3*, *GSK-3β* (rs334558 and rs3755557) and *BDNF*. This was done to confirm the genotyping results observed on the agarose gels. Sequencing was performed by the use of the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, Foster City, USA). Every 20 µl sequencing reaction was set up using the following protocol: 15.7 µl nuclease-free water, 1 µl BigDye® premix, 1 µl of 5x sequencing buffer, 3.2 mM primer (either forward or reverse) and 1 µl PCR product adding to a volume of 20 µl per reaction. The sequencing programme consisted of denaturation at 96°C for 10 seconds, annealing for 5 seconds at the annealing temperature of the specific gene's primers minus 2°C and lastly extension for 4 minutes at 60°C. These steps were repeated for 30 cycles.

Cycle sequencing products were purified using the ZR DNA Sequencing Clean-up Kit™ (Zymo Research, Irvine, USA). The suggested manufacturer's protocol was used, adding 240 µl Sequencing Binding Buffer to the 20 µl sequencing PCR reaction whereafter it was transferred to a Zymo-Spin™ IB Column in a collection tube and centrifuged at 13,000 rpm for 30 seconds. Thereafter, 300 µl Sequencing Wash Buffer was added to the Column and centrifuged at 13,000 rpm for 30 seconds. An amount of 10 µl Hi-Di™ Formamide (Applied Biosystems, Foster City, USA) was added to the column matrix and transferred into a 1.5 ml microcentrifuge tube and centrifuged at 13,000 rpm for 15 seconds to elute the DNA. Ultra-pure DNA was then loaded onto a 96 well microtiter plate for analysis on the ABI3500 Genetic Analyser (Applied Biosystems, Foster City, USA).

Electropherograms were analysed using the sequence analysis software (Chromas version 2.6.4; Technelysium, Au) to confirm the presence or absence of an SNP or VNTR. Sequences were aligned with the reference sequence for each gene using Ensembl genome browser 90 (CLOCK ENSG0000013485, GSK-3B NC_000003, PER3 ENSG00000049246, BDNF ENSG00000176697) (Ensembl, 2018) (Zerbino

et al., 2018). The sequences were aligned to the reference sequences using Showalign from The European Molecular Biology Open Software Suite (Emboss) (http://emboss.bioinformatics.nl/cgi-bin/emboss/show align) (Rice, Longden and Bleasby, 2000).

3.6.6. Statistical analysis of molecular data

All statistical analysis of the data was performed with the assistance of the Department of Mathematical Statistics and Actuarial Science at the University of the Free State. The SAS^{TM} procedure FREQ software program (SAS Institute Inc, 2016) was used to perform the analysis. The primary objective of the analysis was to assess differences between the BD case and control group with regards to allele and genotype frequencies. The Cochran-Mantel-Haenszel χ^2 test was used to calculate and compare the allelic and genotype frequencies between the BD case and the control group. The *p*-value for each polymorphism was reported and *p*-values less than 0.05 are considered to be significant. Unknown genotypes were omitted from the statistical analysis. The HWE for each polymorphism was tested using a χ^2 test. A population is considered in equilibrium if the χ^2 is less than 3.841. The allele and genotype study results were further compared to those reported by the 1000 Genomes Project. This project aims to provide descriptive data on common human genetic variation. It provides a benchmark for other human genetic association studies to compare allele and genotype frequences (The 1000 Genomes Project Consortium, 2015). This is especially useful in SA, where allele and genotypic data for the selected polymorphisms used in the study is very limited. Therefore, the 1000 Genomes Project, as well as research studies focusing on BD with the selected polymorphisms of this study, were used as a comparison tool.

Correlations between genotypes were calculated with the Cochran-Mantel-Haenszel correlation test (1 df). The odd ratios were calculated by pooling the two most frequent genotypes for a gene with three genotypes, so that the two categories

created are the rarest genotype vs the other two genotypes. Correlations between the BD age at diagnosis and selected gene polymorphisms of the BD base group were calculated. The descriptive statistics for age at diagnosis were tabulated. A nonparametric test for the association between genotype and age at diagnosis for all polymorphisms were also calculated. The χ^2 statistic and associated *p*-values were reported. The data was further stratified by grouping the age at BD diagnosis into the following three groups: < 20 years, 21 - 29 years and > 30 years (Coryell *et al*., 2013) and the associated *p*-values calculated.

Additional analyses were done on the significant questionnaire results from Chapter 4. In the first part of the analysis, the data from the two groups were pooled together and in the second part, the data was stratified between the two groups. The Cochran-Mantel-Haenszel correlation test was done and associated *p*-values reported (1 df).

Chapter 4:
Questionnaire results from the comparative analysis between the BD case and control groups, for selected lifestyle and associated risk factors contributing to metabolic syndrome

4.1. Introduction

Bipolar disorder is associated with numerous psychiatric and medical comorbid disorders (Sajatovic, 2005), as discussed in Chapter 2. These comorbid disorders can have many adverse effects on BD patients' course of illness (Bauer *et al*., 2016). Metabolic syndrome is defined by grouping risk factors that contribute to CVD and diabetes (Alberti, Zimmet and Shaw, 2006). Cardiovascular disease is of particular concern in sub-Saharan Africa, as the mortality rate due to CVD has increased by 81% from 1990 to 2013 (Mensah *et al*., 2015). In SA, 2.2% of the population suffer from CVD (Shisana *et al*., 2014) with 18% of deaths in the country due to CVD (Statistics South Africa, 2016). Diabetes is found in 5% of the population (Shisana *et al*., 2014) and accounts for 5.5% of deaths (Statistics South Africa, 2016).

Researchers have pointed out that BD and comorbid disorders, such as MetS, share mutually joined genetic (Kerner, 2014) and environmental (McElroy *et al*., 2001) risk factors. The importance of "nature (genetics)" vs "nurture (non-genetic factors)" has gained momentum over the past few decades and escalated in the 1960s and 1970s (Plomin, Owen and McGuffin, 1994). Quantitative genetics, also known as the genetics of complex traits, studies varied genetic influences on the trait of interest, whereby non-genetic factors may also contribute (Hill, 2010). Quantitative genetic

studies would seek to determine that environmental influences accounts for as much variance as genetic factors. Additionally, every individual develops their own experiences towards environmental factors (Plomin, Owen and McGuffin, 1994). This approach allows for a better understanding of phenotypic variation as observed within families and different population types (Hill, 2010).

Questionnaires are often used in human behavioural studies as a means of collecting data for research quantification, especially when face-to-face interviews are not possible (Munn, Drever and Scottish Council for Research in Education, 1990). Advantages of questionnaires are that they are extremely time efficient, usually have a high return rate, every participant receives the same standardised set of questions and retain the anonymity of every participant (Munn, Drever and Scottish Council for Research in Education, 1990). However, disadvantages of using questionnaires includes language barriers, different interpretations of questions as well as the validity of answers. Study participants may be untruthful and answer the questions in a socialy desirable manner (McDonald, 2008). Questionnaires allow researchers to gather information from the study participants and can be used in large groups of individuals, ranging from hundreds to thousands (Riemann, Angleitner and Strelau, 1997). Therefore, this research project was comprised of both questionnaire and molecular genetics data to investigate the association between BD and MetS.

4.2. Questionnaire results

4.2.1. Section A: Biographical information

Questionnaire data from 53 BD case group and 36 control group individuals were received and used for statistical analysis. Of the 53 BD participants, females were over-represented in this study at 69.81% (n = 37) compared to males at 30.19% (n = 16). The BD case group consisted of White (English and Afrikaans) individuals (n

= 40; 75.47%), Black African individuals (Sotho, Tswana, Xhosa and Zulu) (n = 10; 18.87%) and Coloured individuals (n = 3; 5.66%). Individuals from the BD case group originated from six provinces across SA, resulting in a variety of individuals from different locations (Figure 4.1). The control group was collected at random from the general population in the Free State, Gauteng and Mpumalanga provinces. The results from the control group were identical to the BD case group, as they matched in age, gender and ethnicity (data not shown). Individuals from the BD case group were divided into six age category groups (Figure 4.2) to assist with the control group sampling process. The ages ranged from 19 - 69 years with a mean age and SD of 41.74 ± 11.96 years. The majority (n = 21; 39.62%) of patients in the BD case group were in the 45 - 54 age category. Of the 53 BD cases, a total of 37 questionnaires were completed fully.

4.2.2. Section B: Information on selected lifestyle and associated risk factors contributing to metabolic syndrome

In this section, a comparison of selected lifestyle and associated risk factors between the BD case and control groups were made. When comparing the selected lifestyle risk factors contributing to MetS using the Cochran-Mantel-Haenszel correlation test (Table 4.1), the BD case group had a higher prevalence of cigarette smokers (33.33% vs 22.22%; p = 0.280), a higher number of individuals who exercised less than twice a week (51.15% vs 45.71%; p = 0.634), and more individuals who were not following a balanced diet (39.53% vs 30.56%; p = 0.409). Overall, both groups had a high prevalence of individuals who were following a balanced diet. However, there were no statistically significant differences observed between the two groups. Analysing the ‘drink alcohol on a regular basis’ variable, a statistically significant difference was found between the two groups. More individuals from the control group drank alcohol on a regular basis (36.11% vs 11.9%; p = 0.012).

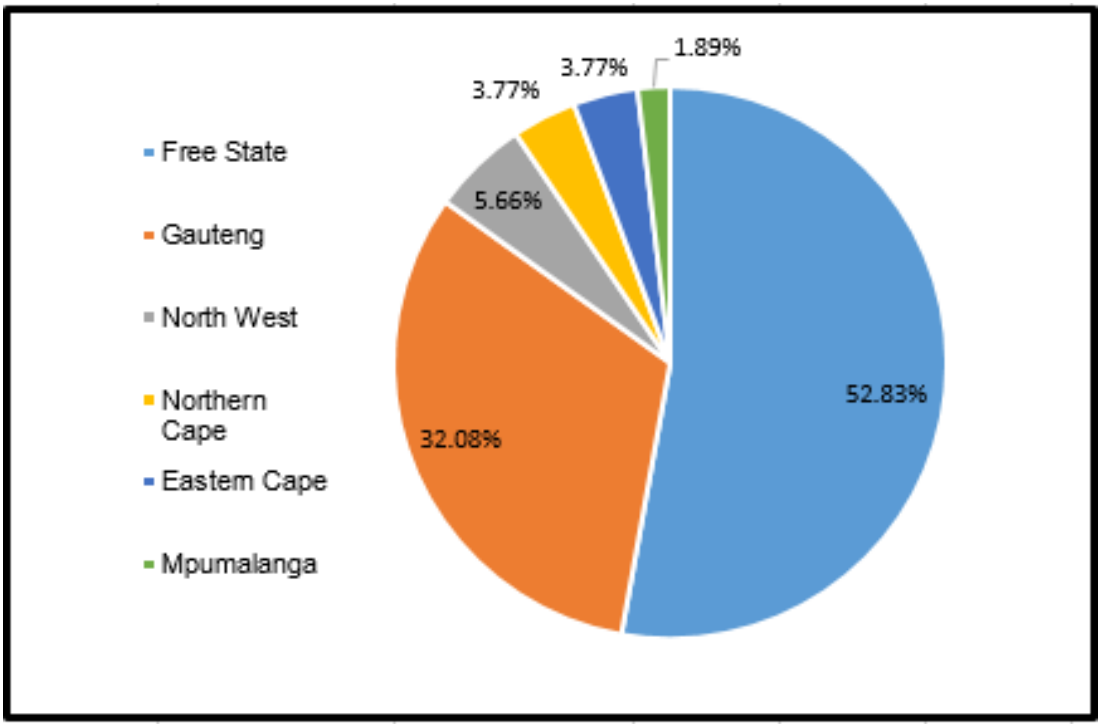


Figure 4.1 A pie chart depicting the percentages of BD cases collected from each province within SA.

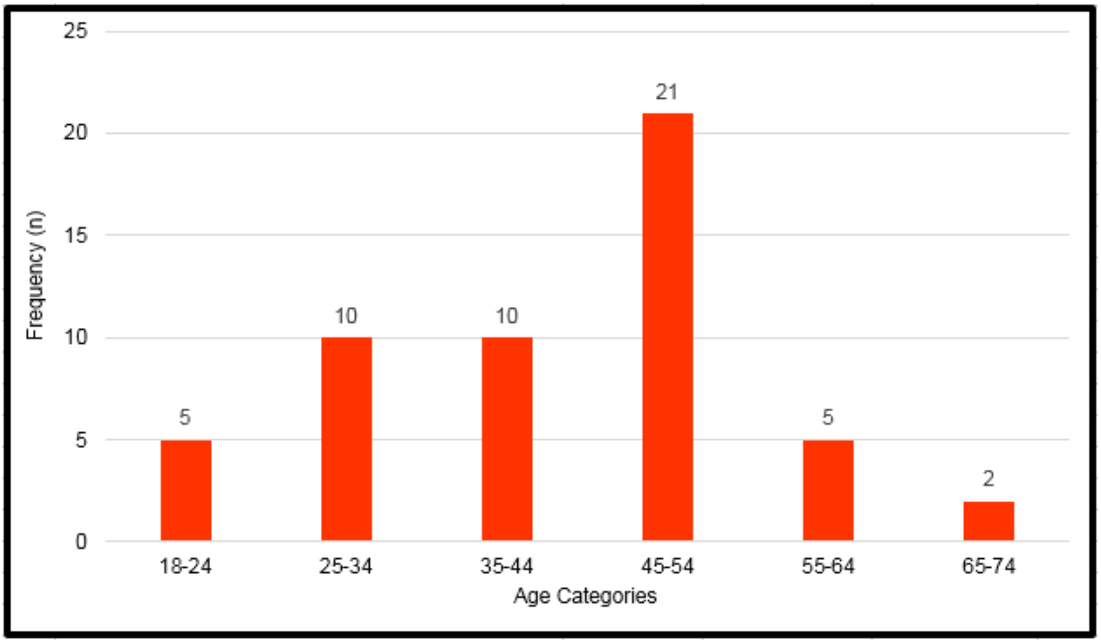


Figure 4.2 A bar graph displaying the number of the BD cases within each age category.

Table 4.1 A comparison of selected lifestyle factors contributing to MetS between the BD cases and controls using the Cochran-Mantel-Haenszel correlation test. The value highlighted in red is statistically significant ($p < 0.05$).

Variable	BD case group [n (%)]	Control group [n (%)]	*p*-value
Smoke cigarettes			0.280
Yes	14 (33.33)	8 (22.22)	
No	28 (66.67)	28 (77.78)	
Total [n (%)]	42 (100)	36 (100)	
Exercise less than twice a week			0.634
Yes	22 (51.16)	16 (45.71)	
No	21 (48.84)	19 (54.29)	
Total [n (%)]	43 (100)	35 (100)	
Follow a balanced diet			0.409
Yes	26 (60.47)	25 (69.44)	
No	17 (39.53)	11 (30.56)	
Total [n (%)]	43 (100)	36 (100)	
Drink alcohol on a regular basis			**0.012**
Yes	5 (11.90)	13 (36.11)	
No	37 (88.10)	23 (63.89)	
Total [n (%)]	42 (100)	36 (100)	

The core component risk factors evaluated in this study are shown in Table 4.2. The cut-off points for 'elevated waist circumference' differ between men and women. For this study, the results for men and women were grouped together due to the comparison being made between the BD case and control groups, and not between genders. The prevalence of the elevated waist circumference (≥ 102 cm for men and ≥ 88 cm for women) in both groups was observed in more than half of the BD cases (55.56% vs 41.67%; p = 0.242). Hypertension was found at an equal prevalence between the two groups (25%; p = 1), whereas the hypercholesterolaemia variable was elevated in the BD case group (25% vs 16.67%; p = 0.377). However, the Cochran-Mantel-Haenszel correlation test indicated that BD cases did not differ from the controls.

Assessing the overall body fat in relation to BMI values (Table 4.3), more than half of the individuals in both groups (70.27% and 58.33%, BD case and control group respectively) were either overweight (BMI 25.0 - 29.9) or obese (BMI > 30). A statistically significant difference was observed in the 'obese' category as it was found that the majority of BD cases were obese (43.24% vs 8.33%; p = 0.005). Due to the categorical risk factors contributing to MetS (which increases the risk of diabetes and CVD), the number of study participants already presenting with CVD and/or diabetes was determined. Overall, more individuals within the BD case group presented with cardiovascular conditions (7.5% vs 2.75%; p = 0.361) and diabetes (12.2% vs 2.78%; p = 0.127). Comparisons of the BD cases with the controls, revealed that none of the differences were statistically significant (Table 4.4).

Further analysis was performed by dermining the number of core componental risk factors present in each individual that may contribute to MetS. A comparison was made between the BD case and control groups. This was done by analysing elevated waist circumference, hypertension and hypercholesterolaemia in study participants with CVD, with diabetes and participants without CVD or diabetes. Individuals with diabetes and/or CVD are more likely to present with the core component risk factors (Albert, Zimmet and Shaw, 2006). There were 11 categories and were divided as follows: zero, one, two or three core component risk factors in

Table 4.2 A comparison of categorical risk factors contributing to MetS between the BD cases and controls using the Cochran-Mantel-Haenszel correlation test.

Variable	BD case group [n (%)]	Control group [n (%)]	*p*-value
Elevated waist circumference			0.242
Yes	20 (55.56)	15 (41.67)	
No	16 (44.44)	21 (58.33)	
Total [n (%)]	36 (100)	36 (100)	
Hypertension			1
Yes	10 (25)	9 (25)	
No	30 (75)	27 (75)	
Total [n (%)]	40 (100)	36 (100)	
Hypercholesterolaemia			0.377
Yes	10 (25)	6 (16.67)	
No	30 (75)	30 (83.33)	
Total [n (%)]	40 (100)	36 (100)	

Table 4.3 A comparison of the BMI scores in the underweight, normal, overweight and obese categories, between the BD case and control groups.

BMI group	Underweight (< 18.5)	Normal (18.5 - 24.9)	Overweight (25.0 - 29.9)	Obese (> 30)	Total
BD case group [n (%)]	1 (2.7)	10 (27.03)	10 (27.03)	16 (43.24)	37 (100)
Control group [n (%)]	0	15 (41.67)	18 (50)	3 (8.33)	36 (100)
Total [n (%)]	1 (2.7)	25 (68.7)	28 (77.03)	19 (51.57)	73 (100)

Table 4.4 A comparison of the presence of CVDs and diabetes between the BD case and control groups.

Medical conditions	BD case group [n (%)]	Control group [n (%)]	*p*-value
CVD			0.361
Yes	3 (7.5)	1 (2.75)	
No	37 (92.5)	35 (97.22)	
Total [n (%)]	40 (100)	36 (100)	
Diabetes			0.127
Yes	5 (12.2)	1 (2.78)	
No	36 (87.8)	35 (97.22)	
Total [n (%)]	41 (100)	36 (100)	

participants without CVD and diabetes, diabetic with zero, one, two or three core component risk factors, zero, one, two or three core component risk factors in participants with CVD (Table 4.5). The Fisher's exact test was performed on each category. The results show that more BD cases presented with diabetes or CVD and displayed more core component risk factors. The majority of BD cases had 1 risk factor (40.54% vs 30.56%; p = 0.466) whereas most controls had zero risk factors (41.67% vs 24.32%; p = 0.140).

Furthermore, it was determined whether the BD cases presenting with at least one diagnosed MetS risk factor (hypertension, hypercholesterolaemia, CVD, or diabetes), were diagnosed before or after BD diagnosis. It was found that 42.11% of BD cases were diagnosed before their BD diagnosis, 47.37% were diagnosed after their BD diagnosis and 10.53% were diagnosed in the same year of their BD diagnosis. Familial history analysis revealed that 72.97% of the individuals from the BD case group stated that they had at least one family member presenting with a MetS risk factor, CVD or diabetes, compared to 63.89% of the individuals from the control group (p = 0.404).

4.2.3. Section C: Information relating to bipolar disorder

Of the 53 individuals from the BD case group, 43.40% (n = 23) were diagnosed with BD-I, 41.51% (n = 22) were diagnosed with BD-II and 1.88% (n = 1) were diagnosed as being a rapid cycler. A total of 13.21% (n = 7) were unsure of their BD diagnostic classification and were therefore not classified herein. All individuals diagnosed with BD were clustered together in one group for statistical purposes, as this study focused on the BD case group as a whole regardless of BD diagnosis type. Both BD-I (43.40%) and BD-II (41.51%) were equally distributed and represented within this study.

Table 4.5 A comparison of the number of categorical risk factors present (elevated waist circumference, hypertension and hypercholesterolaemia) contributing to MetS (in study participants without diabetes or CVD, with diabetes and with CVD), between the BD case and control groups.

Number of variable risk factors	0 risk factors	1 risk factor	2 risk factors	3 risk factors	Diabetic + 0 risk factors	Diabetic + 1 risk factor	Diabetic + 2 risk factors	Diabetic + 3 risk factors	CVD + 0 risk factors	CVD + 1 risk factor	CVD + 2 risk factors	Total
BD case group [n (%)]	9 (24.32)	15 (40.54)	6 (16.22)	0	0	1 (2.7)	2 (5.41)	1 (2.7)	0	1 (2.7)	2 (5.41)	37 (100)
Control group [n (%)]	15 (41.67)	11 (30.56)	6 (16.67)	2 (5.56)	0	1 (2.78)	0	0	1 (2.78)	0	0	36 (100)
Total [n (%)]	24 (32.88)	26 (35.62)	12 (16.44)	2 (2.74)	0	2 (2.74)	2 (2.74)	1 (1.37)	1 (1.37)	1 (1.37)	2 (2.74)	73 (100)
***p*-value (*p*)**	0.140	0.466	1.0000	0.240	-	1.0000	0.493	1.0000	0.493	0.493	0.493	-

Evaluating the age at BD diagnosis, the mean age and SD for the BD case group were 32.09 ± 11.75 years old, with the youngest age of diagnosis at 11 years old and the oldest at 58 years old (Figure 4.3). The average duration of illness was 10.8 years with the minimum duration being 1 year of illness and the maximum being 36 years. A total of 41 BD cases answered the section on symptoms experienced before their BD diagnosis. Symptoms most often experienced were 'impaired judgement and impulsiveness' (n = 38, 92.68%), whereas the least experienced symptoms were 'delusions and hallucinations' (n = 12, 29.27%).

Family history analysis of 37 BD cases that answered this section, shows that a total of 24.32% (n = 9) have at least one family member diagnosed with BD and 8.11% (n = 3) of BD cases had a family history of depression. Of the 42 that answered the comorbid questions, a total of 12 (28.57%) individuals from the BD case group presented comorbidity with other psychiatric disorders and/or substance addiction (Figure 4.4). Of these 12 individuals, 41.67% (n = 5) had more than one comorbid disorder simultaneously. Out of the 42 BD cases, the majority of individuals from the BD case group (n = 30, 71.43%) did not present with psychiatric or substance addiction comorbidity.

4.3. Discussion

4.3.1. Lifestyle factors contributing to metabolic syndrome

The main lifestyle factor with a statistically significant difference (p = 0.012) observed between the two study groups was 'drinking alcohol on a regular basis.' Only a few individuals in the BD case group consumed alcohol on a regular basis. Individuals in the BD case and control groups who indicated they were regular alcohol consumers, did so moderately. Thus, they did not exceed one drink per day (for women) or two drinks per day (for men) (Jacobs and Steyn, 2013). These results are contradictory to those found in the literature. For instance, research shows that

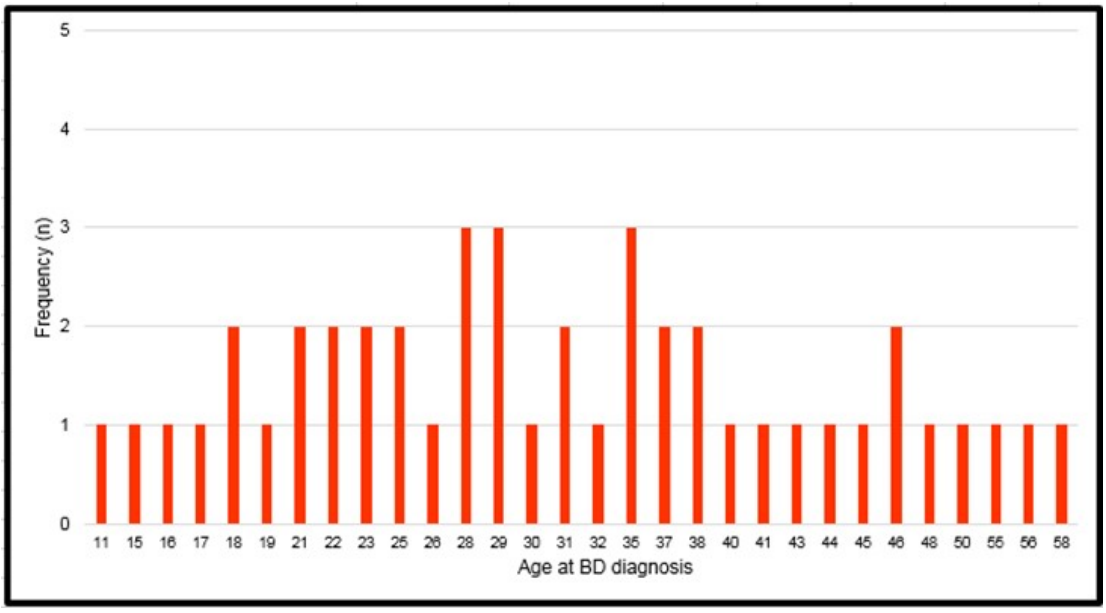


Figure 4.3 A bar graph representing the frequency of BD cases within each age at BD diagnosis.

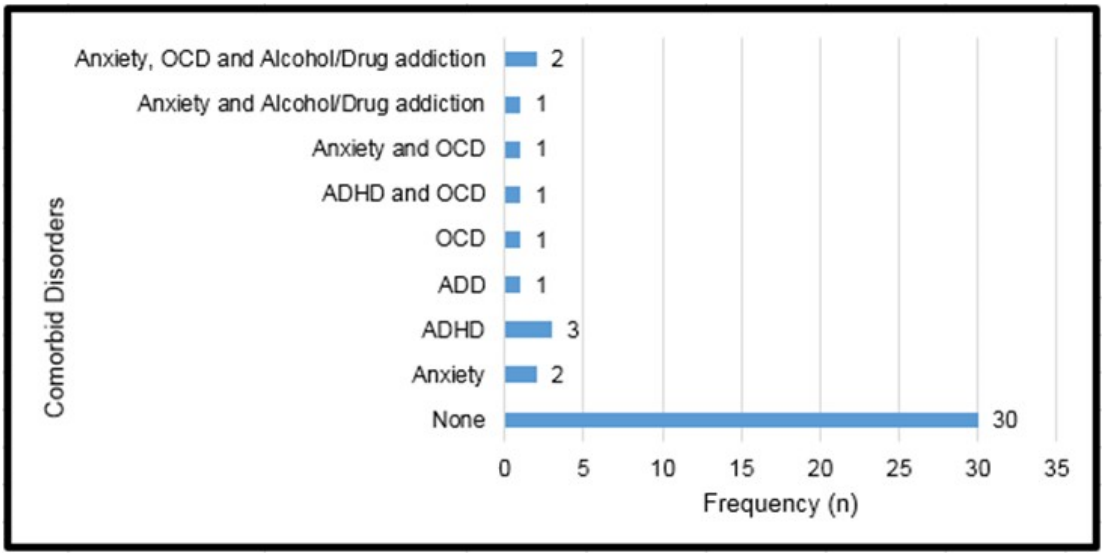


Figure 4.4 A clustered bar graph representing the frequency of comorbid psychiatric disorders and substance addictions present within the BD case group.

alcohol dependence frequently co-occurs in individuals with BD (Brown *et al*., 2014; Carmiol *et al*., 2014) with more than 50% of individuals with BD also being diagnosed with an alcohol use disorder in their lifetime (Carmiol *et al*., 2014). Alcohol dependence has been associated with BD severity (Frye *et al*., 2003), reduced BD treatment response and recovery (Strakowski *et al*., 2000) and it has also been shown to drastically worsen the symptoms and other complications associated with BD (Brown *et al*., 2014).

There are numerous reasons for the contradictory findings of this study with regards to alcohol consumption. First, females were over-represented in this study (69.81% vs 30.19% males) and research has shown that men have a higher tendency to abuse alcohol than women (Frye *et al*., 2003). Second, age is an important factor, as younger adults consume more alcohol (Nolen-Hoeksema, 2004) but, in this study, the majority of study participants were older and aged between 45 - 54 years. Another important factor to consider is that the study particpants may not have been truthful when answering the questionnaire, due to alcohol not being recommended when on BD medication (Weich and Pienaar, 2009). Alcohol may interact with psychiatric medication in a pharmacokinetic or pharmacodynamic manner (Cheng *et al*., 2018). This will subsequently influence drug metabolism and can cause adverse reactions. Alternatively, psychiatric drugs can also affect alcohol metabolism and increase alcohol-related side effects (Cheng *et al*., 2018). Therefore, it is likely that study BD cases do not consume alcohol due to the medication and psychotherapy treatment (Weich and Pienaar, 2009).

When comparing BD cases to controls, there were more BD cases who smoked cigarettes (33.33% vs 22.22%). Within the BD case group, most individuals were non-smokers (66.67%). In addition to increasing the risk of CVD, cigarette smoking is associated with impaired insulin action and abdominal obesity (Chiolero *et al*., 2008). The low rates of non-smokers in the BD case group could potentially be due to the fact that many individuals have quit the ‘bad habit’ for the sake of their health with the help of psychotherapy. Many may have been untruthful in this section. According to the South African National Health and Nutrition Examination Survey

(SANHANES) a total of 20.8% of South Africans reported a history of ever having smoked tobacco (Shisana *et al*., 2014).

Additionally, the majority of the BD cases stated that they eat a balanced diet (60.47% vs 69.44%). However, 51.16% of BD cases reported exercising less than twice a week (vs 45.71% of controls). Similar results were found by Bly *et al*. (2014), whereby the BD cohort consumed more fibre, fewer carbohydrates and calories compared to the control group. Literature has shown that these factors are frequently observed in patients with BD (Kilbourne *et al*., 2007; Fagiolini *et al*., 2008; Brietzke *et al*., 2011; Buhagiar *et al*., 2011; Bly *et al*., 2014; Carrá *et al*., 2014; Vancampfort *et al*., 2015) and have a significant impact on MetS (Fagiolini *et al*., 2008). Within this study, the highest frequency of BD patients were found to have suboptimal exercise habits. According to the SANHANES, 26% of adults consume a high fat diet and 25.6% consume a diet with low amounts of fruit and vegetables. In SA, 27.9% of men and 45.2% of women do not exercise (Shisana *et al*., 2013). Similar findings were observed by Kilbourne *et al*. (2007). Depression plays a major role in contributing to an inactive lifestyle of BD patients. Depressive episodes are frequently associated with lack of social interaction and an inactive lifestyle (Baldessarini, 2002; McElroy *et al*., 2004). This causes a serious concern as it directly influences weight gain, further increasing the risk of CVD and potentially other medical comorbid disorders (Kilbourne *et al*., 2007).

Overall, most individuals from the BD case group displayed a healthy lifestyle. These results are comparable to those by Bly *et al*. (2014), where the BD cohort had a low incidence of lifestyle risk factors contributing to MetS. Poor dietary habits (Bly *et al*., 2014; Padmavati, 2016) and lifestyle factors contributing to MetS have not yet been fully investigated in patients with psychiatric disorders and further research is required on MetS mechanisms (Bly *et al*., 2014). Findings by Bly and colleagues (2014) suggested that lifestyle factors may not be the primary cause of MetS, but rather complex underlying, ambiguous mechanisms in patients with severe mental disorders may be at play (Bly *et al*., 2014). However, the pathophysiology of this interaction remains unclear (Fagiolini *et al*., 2008).

4.3.2. Core component and associated risk factors contributing to metabolic syndrome

Analysing the individual component MetS risk factors, the primary finding in the BD case group was the prevalence of obesity observed when compared to the control group. A total of 43.24% of the BD patients were classified as obese and 55.56% had an elevated waist circumference. These results are consistent with those by McElroy *et al*. (2016), where 44.4% of the BD patients were obese and 48.0% presented with abdominal obesity. Various studies found similar results (Fagiolini *et al*., 2005; Garcia-Portilla *et al*., 2008; Cardenas *et al*., 2008; Moreira *et al*., 2017). Obesity is frequently associated with BD (Fiediorowicz *et al*., 2008; Weiner, Warren and Fiedorowicz, 2011; McElroy *et al*., 2016) and abdominal obesity is the most strongly associated MetS component in BD (Silarova *et al*., 2015). Compared to the general population, these rates are extremely high (Fagiolini *et al*., 2005). Although there are various definitions used to define MetS, increased waist circumference has been the most constant abnormality reported in research (McIntyre *et al*., 2010; Grover *et al*., 2014). Obesity and abdominal obesity is associated with an overall poor improvement in health and may predict the course of BD more strongly than MetS. It is uncertain why obesity is related to a poor BD outcome but, may possibly be a marker for BD severity (McElroy *et al*., 2016). According to the SANHANES, about two out of three women (65.1%) and almost one in three men (31.2%) are overweight or obese. A total of 40.1% of women are obese compared to 11.6% of men. One in ten males (9.9%) have a waist circumference ≥ 102 cm, while one in two (50.5%) females have a waist circumference ≥ 88 cm (Shisana *et al*., 2014). The high number of individuals in the BD case group that do not exercise regularly, in conjunction with an unbalanced diet, can lead to obesity and subsequent medical comorbidities (Young and Grunze, 2013; McElroy *et al*., 2016). With this in mind, obesity can increase the number of cyclic depressive episodes in BD patients (Young and Grunze, 2013).

Patients with BD have a five times higher risk of developing CVD (Gálvez *et al*., 2015). In this study, there were no statistically significant differences between the

BD case and control groups observed for CVD, diabetes, hypercholesterolaemia and hypertension. Hypertension was observed to be equally prevalent in the BD case and control groups. However, analysis of the individuals within the BD case group showed that most individuals did not present with any MetS risk factors. On a national level in SA, the SANHASNES found that 16.5% have hypertension and 4.3% have hypercholesterolaemia (Shisana *et al*., 2014). Various studies found that the prevalence of MetS increases substantially with age (Garcia-Portia *et al*., 2008; Vancampfort *et al*., 2013; Grover *et al*., 2014; Silarova *et al*., 2015). Additionally, MetS in BD patients seem to have seasonal variation, with the highest MetS rates being evident during winter (Altinbas *et al*., 2013). Remarkably, depression in these BD-MetS patients was also considerably frequent during winter than in BD patients without MetS (Altinbas *et al*., 2013). However, the season of data collection may in fact influence the study results. It may be possible that more individuals within this group do present with MetS, but due to limited study resources and not being able to perform tests on actual blood pressure, fasting glucose, triglycerides and cholesterol levels, it was not possible to collect this information and had to rely on the medication that was being prescribed.

We also found that 42.11% of BD case group individuals were diagnosed with a metabolic abnormality before being diagnosed with BD. In contrast, a total of 47.37% of the BD patients were diagnosed with a metabolic abnormality after their BD diagnosis and 10.53% were diagnosed in the same year of their BD diagnosis. This may suggest that metabolic abnormalities are not just influenced by the medication administered but, by various other factors such as genetics and the environment. The familial aggregation of MetS components in both the BD case (72.97%) and control (63.89%) groups were extremely high. These results are consistent with a genetic cause with heritability estimates for each MetS component exceeding 50% (Ziki and Mani, 2016). Pleiotropy can potentially explain the comorbidity of mental and body disorders and how conditions such as glucose intolerance, dyslipidaemia and hypertension are found at a higher prevalence in patients with BD, even before these patients are treated with medications causing the iatrogenic effect and amplifying metabolic symptoms (Nasrallah, 2013).

4.3.3. Information relating to bipolar disorder

Consistent evidence has shown that BD aggregates in families (Taylor *et al*., 2002; Smoller and Finn, 2003; Song *et al*., 2014), with the highest risk of BD in first-degree relatives (Smoller and Finn, 2003; Song *et al*., 2014). Our findings seem to correlate with that concept. A total of 24.3% of the BD case group has at least one family member diagnosed with BD and, of this percentage, 18.21% has a first-degree relative diagnosed with BD and the remaining percentage has at least one second-degree relative diagnosed with BD. Bipolar disorder frequently co-exists with numerous other psychiatric disorders (Shih *et al*., 2004). Not only was BD found to aggregate in some families within this study, but also depression. A total of 8.12% of individuals within the BD case group has at least one first-degree family member with depression. Evidence in psychiatry shows that more than one psychiatric disorder can aggregate in one family (Nasrallah, 2013). Due to the high heritability of BD, this comorbidity is likely due to pleiotropy, where one gene can have an effect on numerous clinical phenotypes (Nasrallah, 2013; Song *et al*., 2014).

The comorbidity of additional psychiatric disorders with BD is often observed in individuals (McElroy *et al*., 2001; Song *et al*., 2014; Harrison, 2016). Results from this study shows that 28.57% of the BD patients presented comorbidity with other psychiatric disorders and many of the individuals presented with more than one comorbid disorder simultaneously. Anxiety was the most frequent comorbid psychiatric disorder observed in the BD case group. Research has found anxiety disorders to be a frequent comorbid disorder in patients with BD (McElroy *et al*., 2001; Sasson *et al*., 2003; Krishnan, 2005; Nabavi, Mitchell and Nutt, 2015). Not only does this comorbidity cause diagnostic challenges, but therapeutic challenges, where the medication administered for anxiety may induce mania if not monitored carefully (Sasson *et al*., 2003). Research has also shown that anxiety may potentially be a risk factor for the onset of BD itself (Krishnan, 2005).

4.4. Conclusion

The aim of this chapter was to determine whether the selected lifestyle and associated risk factors contributing to MetS, are found more predominantly in the BD case group. The study results confirm the high comorbidity of MetS risk factors frequently associated with BD, whereby a higher prevalence of BD cases smoked cigarettes, were physically inactive and did not follow a balanced diet. This demonstrates that BD patients are at risk to an unhealthy lifestyle. However, the majority of BD cases in this study are living a healthy lifestyle. This may be due to treatment and psychotherapy received for BD, where certain behaviours (consuming alcohol) are discouraged whilst on treatment, as confimed by the high amount of BD cases that do not consume alcohol in this study.

The core component and associated MetS risk factors were elevated in the BD case group, with the exception of hypertension being found at an equal prevalence in both the BD cases and controls. Obesity was significantly higher in the BD case group, a variable consistently observed in patients with BD. Comorbidity is often observed in BD patients, usually presenting with more than one comorbid disorder, as observed in this study. The study further highlights that BD patients have a higher risk of MetS abnormalities that contribute to CVD and diabetes. This was observed in the higher prevalence of the BD cases already presenting with CVD and diabetes. The lifestyle factors assessed in this study, may play a pivitol role in MetS abnormalities. However, the results from the study may have occurred by chance. This may be due to the high prevalence rates of obesity, elevated waist circumference, hypertension, hypercholesterolaemia, diabetes and CVD in SA as observed by the SANHANES (Shisana *et al*., 2014). This emphasises the need for further nationwide research on MetS risk factors, specifically in the BD population. Therefore, significant results from this chapter (drinking alcohol on a regular basis and BMI variables) were compared to the genotypes from the selected polymorphisms in Chapter 5 to establish whether there are significant genetic associations.

The familial aggregation of associated MetS risk factors was high in the BD cases, thus, showing a genetic association between MetS and BD. This may confirm the hypothesis surrounding ‘metabolic-mood syndrome’ and the complexity of underlying genetic mechanisms. Furthermore, BD and MetS additionally share environmental risk factors that may explain the differences observed in phenotypic variation. Therefore, gene-gene and GxE interactions needs further investigation.

The clinical association between BD and metabolic abnormalities has become a field of growing interest (Gálvez *et al*., 2015), but remains poorly understood (Vancampfort *et al*., 2015). Future studies will be needed to further investigate the genetic and environmental influences that may have an impact on MetS and BD. Physicians should take extra care in patients with BD, as metabolic abnormalities are extremely prevalent in BD patients and can have dire consequences if not attended to efficiently.

Chapter 5:

The molecular analysis of selected polymorphisms in *CLOCK*, *PER3*, *GSK-3β* and *BDNF* on the influence of bipolar disorder

5.1. Introduction

Bipolar disorder is a polygenic disorder that involves numerous genes with small effects (Craddock and Sklar, 2013) as well as environmental factors that may largely differ among affected individuals (Barnett and Smoller, 2009). Due to its complexity, it has been challenging to define a basic genetic model of BD or to identify specific risk alleles (Kerner, 2015). Case-control studies aid in focusing on specific alleles of interest and may help identify genetic aetiological risk factors. The candidate gene approach has been used to select variants of interest to further aid in detecting important associations. Analysis of SNPs has come to the forefront in gene-mapping studies to associate numerous variants in the genome simultaneously (Mansour *et al*., 2009).

The pursuit for genetic variations that contribute to disease is crucial towards understanding genetic disorders (Sheikh *et al*., 2010). Polymorphisms in circadian rhythm genes influence pathogenesis and symptomatology of BD, as well as treatment response (Dallaspezia and Benedetti, 2009). The *BDNF* gene is the most acknowledged for its role in BD aetiology (Ejmalian *et al*., 2016), with the Val66Met SNP being an important potential high-risk allele and biomarker (Sklar *et al*., 2002). Therefore, genes chosen were associated with the circadian rhythm clock and neurotrophin family with the hope to possibly be used as biomarkers in future. Much research has been focused on the *BDNF* as a potential biomarker for BD.

Additionally, these selected variants were chosen because of very limited information on them in SA populations.

5.2. Results and discussion

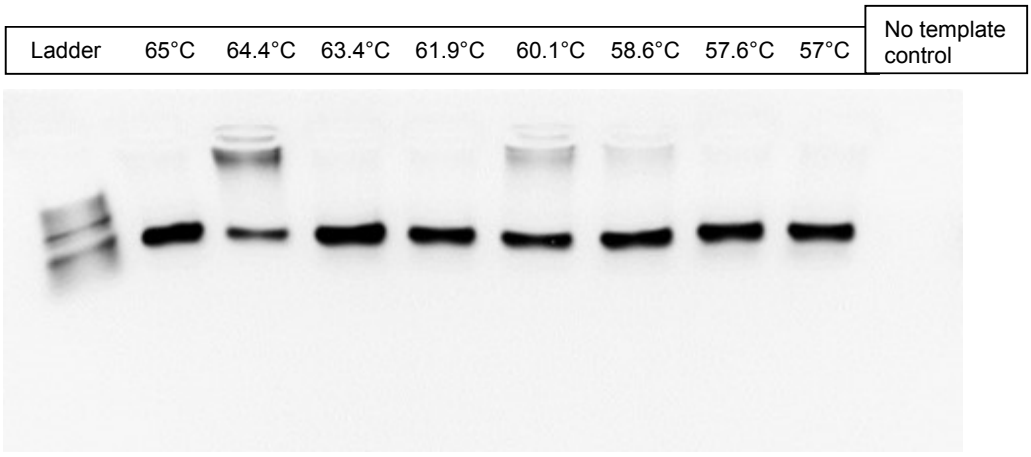
5.2.1. Results from the primer optimisations for rs3755557 (*GSK-3β*) and rs334558 (*GSK-3β*)

Following the PCR temperature gradient analysis in Table 3.3 (Chapter 3), amplified products were visualised on a 2% agarose gel (w/v) to verify successful amplification of the targeted fragments. The T_a showing the optimum fragment amplification was selected as the one to use in further PCR protocols (Table 3.2, [Chapter 3]). No primer dimers were visible on agarose gels. A no template control showed no contamination of the PCR reagents. A 50 bp molecular weight marker (O'RangeRuler DNA ladder, Fermentas, Thermo Fisher Scientific, Waltham, USA) was also included on each gel. For rs3755557 (*GSK-3β*), the T_a = 57.6°C was selected and PCR amplification using this temperature yielded PCR products of 250 bp (Figure 5.1a). For rs334558 (*GSK-3β*), the T_a = 63°C was used to generate PCR products of 344 bp (Figure 5.1b).

5.2.2. Allelic and genotype frequencies of selected polymorphisms between the BD case group and control group

A total of 50 individuals from the BD case group and 50 individuals from the control group provided DNA samples for further analysis. The sampling process had taken an extensive 30-month duration. Table 5.1 displays the number of samples successfully amplified by PCR for each selected polymorphism. Adjustments to the temperatures of the in-house PCR protocols were made and the concentrations of the DNA were increased if needed. Samples that did not amplify were excluded

a)



b)

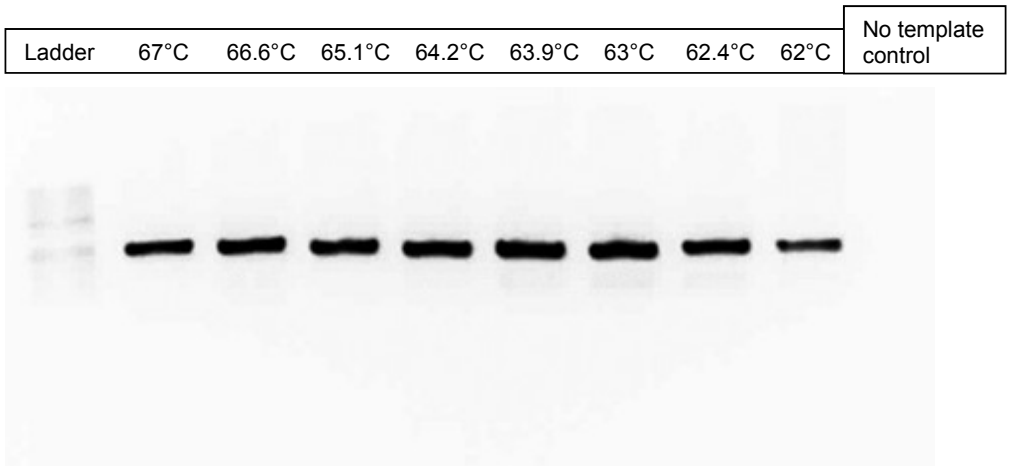


Figure 5.1

a) Optimisation of the T_a for the PCR amplification of the 250 bp product of rs3755557 (*GSK-3β*). A temperature gradient ranging from 57°C to 65°C was used. The selected T_a = 57.6°C was used for further PCR protocols.

b) Optimisation of the T_a for the PCR amplification of the 344 bp product of rs334558 (*GSK-3β*). A temperature gradient ranging from 62°C to 67°C was used. The selected T_a = 63°C was used for further PCR protocols.

Table 5.1 The number of samples successfully amplified for *CLOCK* rs1801260, *PER3* VNTR, *GSK-3β* rs3755557, *GSK-3β* rs334558 and *BDNF* rs6265.

Gene and polymorphism	*CLOCK* rs1801260	*PER3* VNTR	*GSK-3β* rs3755557	*GSK-3β* rs334558	*BDNF rs*6265	Total
BD case group (n)	49	47	48	47	49	240
Control group (n)	50	48	47	42	49	236
Total (n)	99	95	95	89	98	476

from further study procedures. Amplicons for *CLOCK*, *PER3*, *GSK-3β* (rs334558) and *BDNF* were successfully digested with their respective restriction enzymes and protocols, which resulted in the DNA fragments of different lengths (Table 3.4 [Chapter 3]).

Agarose gels that presented with smears, atypical or faint banding patterns, sample aliquotes were switched out with one of the other aliquotes to control for DNA degradation. If the problem persisted, the DNA quantity was decreased when smears were present in the gel and the DNA quantity was increased when agorose gels presented with faint banding. Some gels were subjected to electrical current for more than 40 minutes for better fragment separation. This, however, caused the gels to heat up more than usual, causing atypical banding patterns, when DNA fragments from the samples did not correspond to the ladder. In samples where the fragment sizes was not certain, sequencing was perfomed to confirm the fragment sizes. However, the digestion of the *GSK-3β* rs3755557 SNP presented with several limitations and all samples were, therefore, sequenced instead. In order to determine whether BD was associated with any of the selected variants, the allele and genotype frequencies were compared between the BD case and control groups.

5.2.2.1. Circadian Locomotor Output Cycles Kaput (*CLOCK*) gene

The region of *CLOCK* containing the rs1801260 SNP was successfully amplified and digested with Bsp1286I. The *CLOCK* rs1801260 SNP for this sample population was in HWE according to the χ^2 statistic ($\chi^2 = 0.0042$; df = 1) as $\chi^2 < 3.841$. The allelic and genotype frequencies for the BD case and control groups are depicted in Table 5.2. The length of undigested PCR amplicons is 221 bp. Amplicons with the T allele were not digested and remained 221 bp in length. In the presence of a C allele, the amplicon will be digested into two fragments with lengths of 125 bp and 96 bp. Heterozygotes will, therefore, have three fragments present:

Table 5.2 The allele and genotype frequencies for *CLOCK* rs1801260 in the BD case and control groups.

***CLOCK* rs1801260**							
	Genotype frequency				**Allele frequency**		
	TT	**TC**	**CC**	**Total**	**T**	**C**	**Total**
BD case group [n (%)]	28 (57.14)	17 (34.69)	4 (8.16)	49	73 (74.49)	25 (25.51)	98
Control group [n (%)]	31 (62)	18 (36)	1 (2)	50	80 (80)	20 (20)	100
Total [n (%)]	59 (59.60)	35 (35.35)	5 (5.05)	99	153 (77.27)	45 (22.73)	198
	$\chi^2 = 2.0985$; df = 2; $p = 0.350$				$\chi^2 = 0.8526$; df = 1; $p = 0.356$		

221 bp, 125 bp and 96 bp. The gel electrophoresis and sequencing electropherograms for the resulting three genotypes can be seen in Figure 5.2. Allele frequencies of the rs1801260 SNP (BD case and control groups combined) were 77.27% for the T allele and 22.73% for the C allele. These results mirrored those reported by the 1000 Genomes Project (The 1000 Genomes Project Consortium, 2015), where T = 77% and C = 23% for the global population. The derived C allele was found at a higher prevalence in the BD case group, whereas the ancestral T allele was found to be more common in the control group. However, the allele frequencies between the two groups were not significant ($\chi^2 = 0.8526$; df = 1; $p = 0.356$).

Comparing the genotype frequencies between the two groups, more individuals in the BD case group were homozygous for the CC genotype (8.16%) vs 2% in the control group. A higher prevalence for the homozygous TT genotype was found within the control group (62%) vs 57.14% in the BD case group. These results are comparable to those reported by the 1000 Genomes Project (The 1000 Genomes Project Consortium, 2015), whereby global results showed TT = 60.8%; TC = 32.4% and CC = 6.7%. In this study, no significant differences were observed between the genotype frequencies for *CLOCK* rs1801260 ($\chi^2 = 2.0985$; df = 2; $p = 0.350$) between the two groups. Therefore, no association between *CLOCK* rs1801260 SNP and BD was observed. Positive results have associated the C allele with high BD recurrence rates (Benedetti *et al*., 2003; Serretti *et al*., 2003), sleep disruptions with high evening activity (Benedetti *et al*., 2007) causing a phase-shift that increases the number of mood episodes (Wirz-Justice *et al*., 1999). However, other studies did not find any association of *CLOCK* rs1801260 with BD (Bailer *et al*., 2005; Nievergelt *et al.,* 2006; Kishi *et al*., 2009). Differences in allele frequencies of the *CLOCK* rs1801260 between ethnic groups may be that the polymorphism has different effects in each ethnic group. A study by Barbosa *et al*. (2010) found that Asians living in Brazil had a higher frequency of the T allele compared to the Caucasians (0.84 vs 0.71) in Brazil. This scenario may possibly be due to environmental differences such as latitude, which may act as a selection force on the specific allele on different populations (Lee *et al*., 2010).

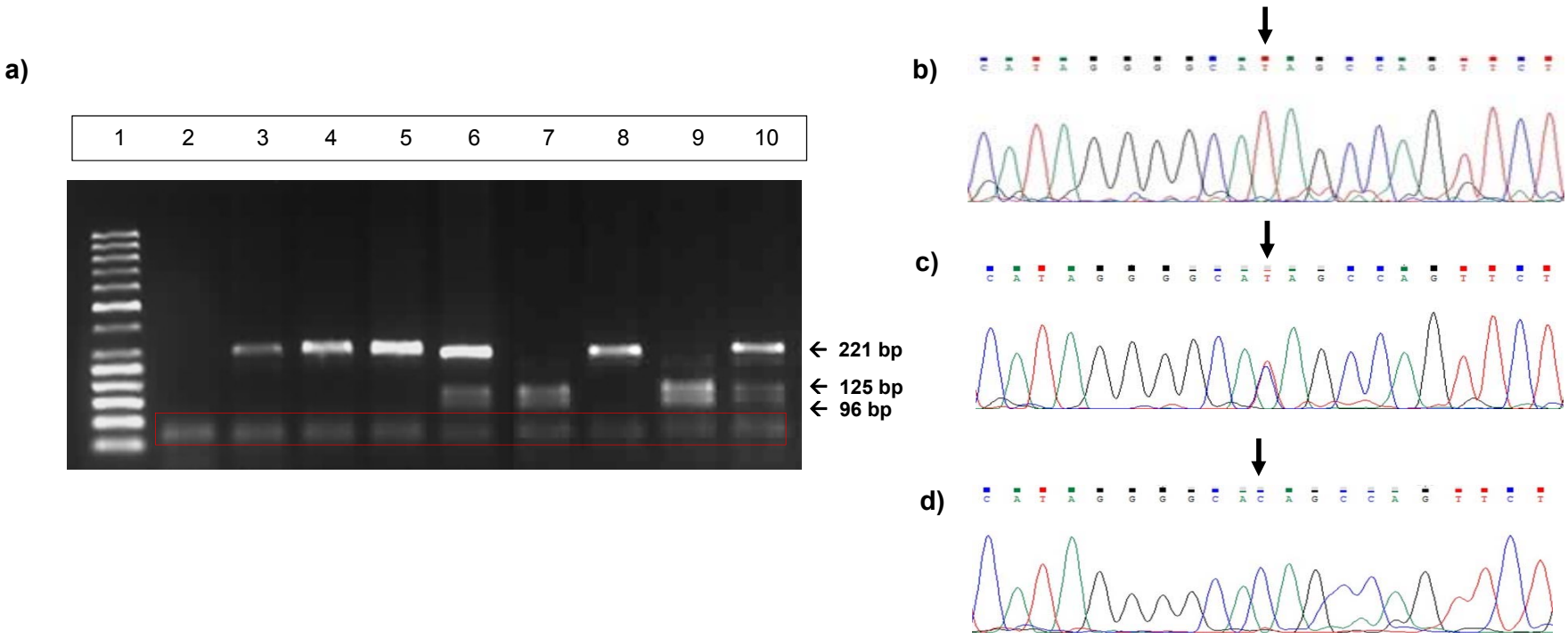


Figure 5.2 a) The visualisation of a 2% agarose gel depicting the digested *CLOCK* rs1801260 SNP. Lane 1 contains the 50 bp O'GeneRuler DNA ladder (Fermentas, Thermo Fisher Scientific, Waltham, USA), lane 2 contains the no template control, lane 3 contains the undigested DNA fragment (221 bp), lanes 4, 5 and 8 each consists of one digested DNA fragment (221 bp) which indicate the individuals are homozygous TT, lanes 6 and 10 each consists of three digested DNA fragments (221 bp, 125 bp and 96 bp) which indicate the individuals are heterozygous CT and lanes 7 and 9 each consists of two digested DNA fragments (125 bp and 96 bp) which indicate the individuals are homozygous CC. Primer dimers are visible at the bottom of the figure as indicated in the red block. Sequencing electropherograms of a **b)** homozygous TT individual **c)** heterozygous CT individual and **d)** homozygous CC individual in the *CLOCK* gene for the rs1801260 SNP. The black arrow indicates the SNP position.

5.2.2.2. Period circadian regulator 3 (*PER3*) gene

The *PER3* VNTR was successfully amplified and digested with the NcoI restriction enzyme. The sample population for *PER3* VNTR was in HWE according to the χ^2 statistic ($\chi^2 = 0.7357$; df = 1) as $\chi^2 < 3.841$. NcoI recognises the T allele at the M1037T (rs2640909) polymorphism (Ebisawa *et al*., 2001) that occurs in the *PER3*^{4} of the *PER3* VNTR. The allelic and genotype frequencies obtained from the current study population are depicted in Table 5.3. The length of the undigested *PER3*^{4} DNA fragment is 581 bp, whereas the undigested *PER3*^{5} DNA fragment is 635 bp in length. Figure 5.3 depicts a virtual gel representing the expected fragment sizes after digestion with NcoI and the resulting genotypes are shown Figure 5.4. Multiple sequence alignment of the *PER3*^{4} and *PER3*^{5} repeats was performed and compared to the reference sequence from Ensembl (Figure 5.5) (Ensembl, 2018). Evaluating the allele frequencies, *PER3*^{5} (BD case group [35.11%] and control group [33.33%]) and *PER3*^{4} (BD case group [64.89%] and control group [66.67%]) was found at similar prevlances in both groups. (Table 5.3). There were no statistically significant differences observed in the allele frequencies between the two groups in this study ($\chi^2 = 0.0603$; df = 1; $p = 0.806$). Similar allele frequencies for *PER3*^{4} and *PER3*^{5} were found in the general population of SA (Shawa and Roden, 2016).

Analysing genotype frequencies, more individuals from the BD case group (17.02%) relative to the control group (10.42%) were homozygous for the *PER3*$^{5/5}$ genotype. Similarly, more BD cases were also heterozygous for *PER3*$^{4/4}$ (46.81%) compared to controls (43.75%). However, more controls were shown to be heterozygoes *PER3*$^{4/5}$ (45.83%) vs 36.17% in BD cases. This may show that the *PER3*^{4} is found more prevalently and does not seem to be dominant over *PER3*^{5}. A study by Karthikeyan *et al*. (2014b) found the *PER3*$^{5/5}$ genotype to be significantly more prevalent in the BD-I cohort and compared to the control cohort ($p = 0.02$). The *PER3*^{5} allele was also found to be higher in the BD cohort ($p < 0.03$). Additionally, *PER3*$^{5/5}$ was found to be associated with certain traits of BD (Artiolo *et al*., 2007) as

Table 5.3 The allele and genotype frequencies for the *PER3* VNTR in the BD case and control groups.

***PER3* VNTR**							
	Genotype frequency				**Allele frequency**		
	4R4R	**4R5R**	**5R5R**	**Total**	**4R**	**5R**	**Total**
BD case group [n (%)]	22 (46.81)	17 (36.17)	8 (17.02)	47	61 (64.89)	33 (35.11)	94
Control group [n (%)]	21 (43.75)	22 (45.83)	5 (10.42)	48	64 (66.67)	32 (33.33)	96
Total [n (%)]	43 (45.26)	39 (42.05)	13 (13.68)	95	125 (65.79)	65 (34.21)	190
	χ^2 =1.3541; df = 2; p = 0.508				χ^2 = 0.0603; df = 1; p = 0.806		

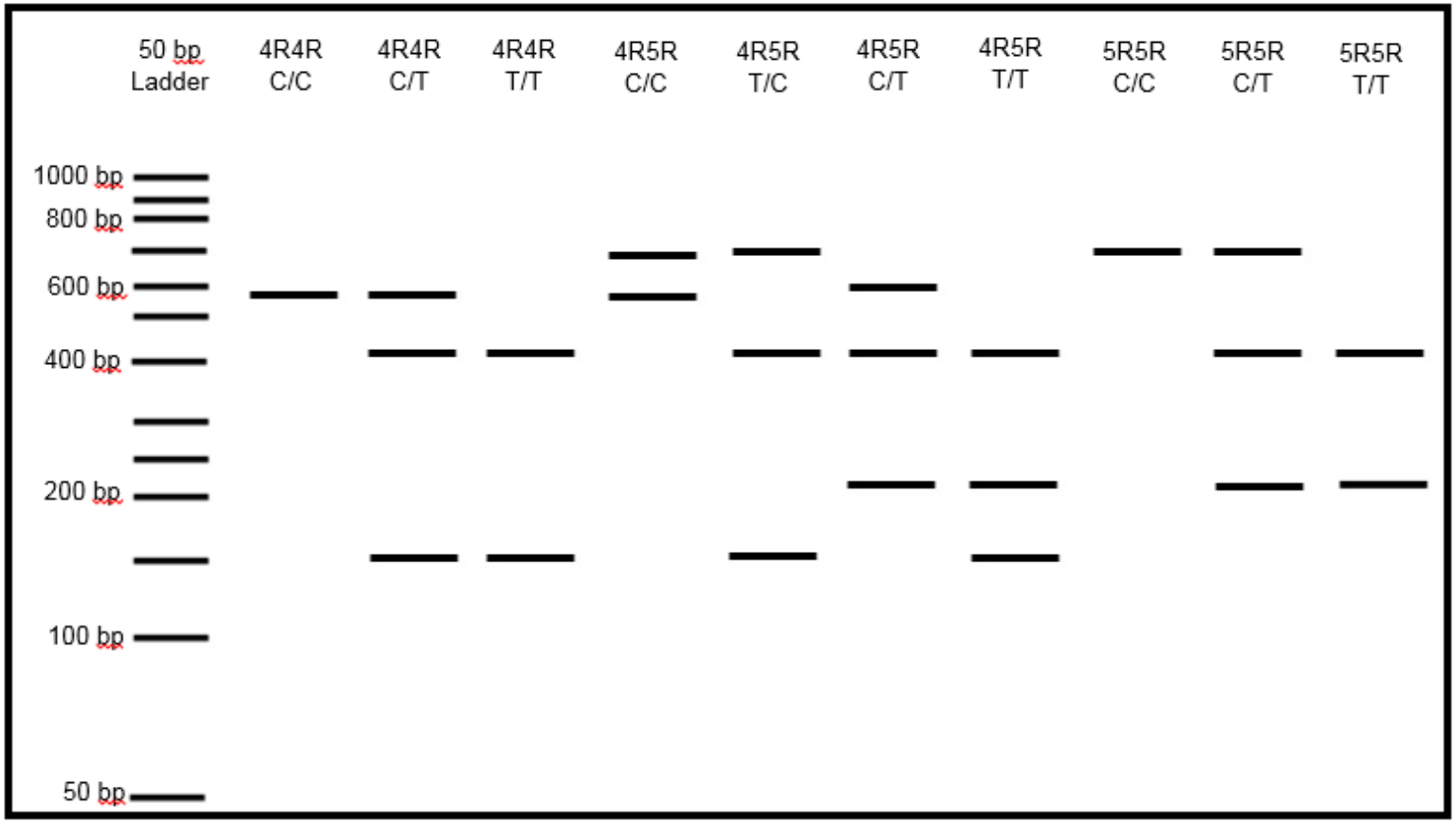


Figure 5.3 A virtual gel representing the expected fragment sizes for the *PER3* VNTR genotypes after digestion.

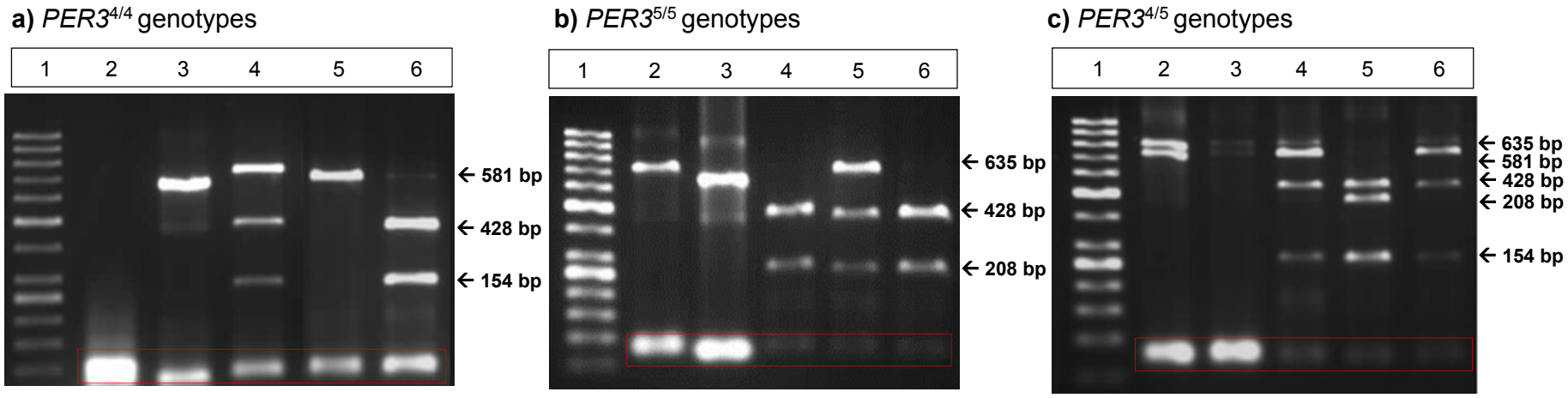


Figure 5.4 The visualisation of 2% agarose gels depicting the digested *PER3* VNTR. Lane 1 contains the 50 bp O'GeneRuler DNA ladder (Fermentas, Thermo Fisher Scientific, Waltham, USA), **a)** *PER3*$^{4/4}$ genotypes: lane 2 contains the no template control, lane 3 contains the undigested DNA fragment (581 bp), lane 4 contains three digested (heterozygous CT M1037T) fragments (581 bp, 428 bp and 154 bp), lane 5 consists of one digested (homozygous CC M1037T) fragment (581 bp), lane 6 contains two digested (homozygous TT M1037T) fragments (428 bp and 154 bp). **b)** *PER3*$^{5/5}$ genotypes: lane 2 contains the undigested DNA fragment (635 bp), lane 3 contains one digested (homozygous CC M1037T) fragment (635 bp), lanes 4 and 6 contain two digested (homozygous TT M1037T) fragments (428 bp and 208 bp), lane 5 contains three digested (heterozygous CT M1037T) fragments (635 bp, 428 bp and 208 bp). **c)** *PER3*$^{4/5}$ genotypes: lane 2 contains the undigested DNA fragments (635 bp and 581 bp), lane 3 consists of two digested (homozygous CC M1037T) fragments (635 bp and 581 bp), lanes 4 and 6 contain three digested (heterozygous CT M1037T) fragments (635 bp, 428 bp and 154 bp), lane 5 consists of three digested (homozygous TT M1037T) fragments (428 bp, 208 bp and 154 bp). Primer dimers are visible at the bottom of the figures as indicated in the red blocks.

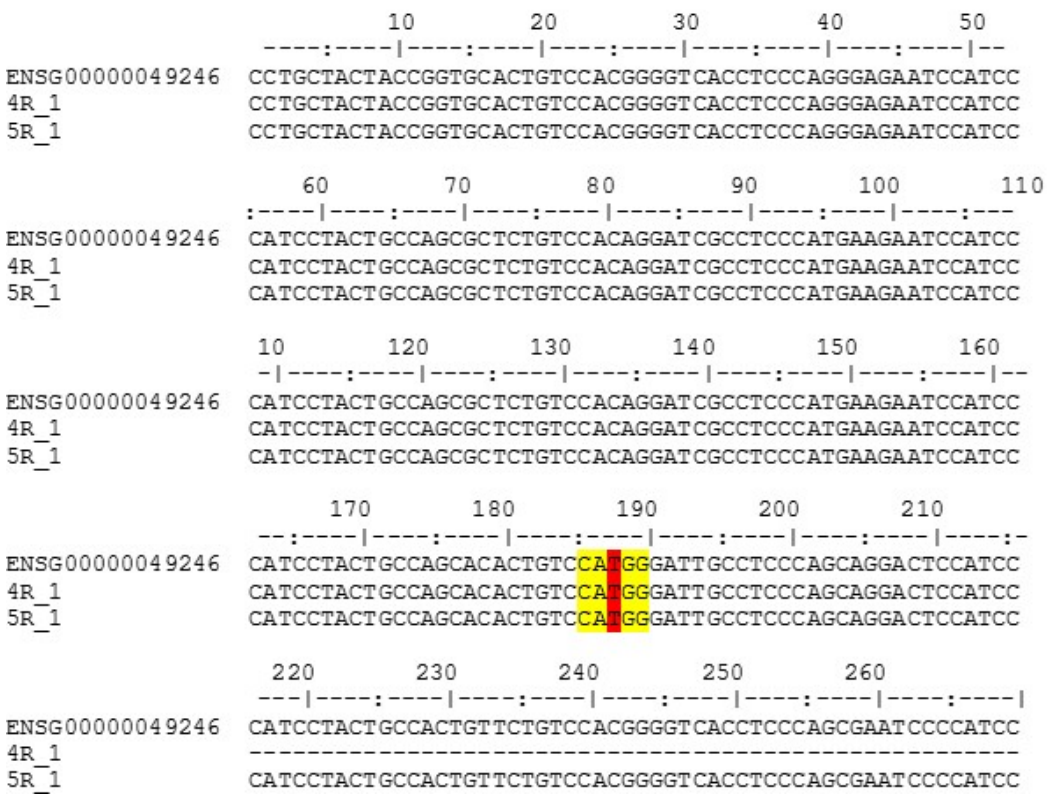


Figure 5.5 An output file alignment presenting the multisequence alignment of the *PER3*[4] and *PER3*[5] repeats. The M1037T (rs2640909) SNP is highlighted in red. The restriction enzyme site is highlighted in yellow.

well as an early onset of BD (Benedetti *et al*., 2008). Other studies found no associations of *PER3* VNTR with BD (Kripke *et al*., 2009; Casiraghi *et al*., 2010). Global populations of the *PER3* VNTR display large variations in the distributions of allele frequencies for *PER3* and can be contributed to genetic drift and human migration (Nadkarni *et al*., 2005).

5.2.2.3. Glycogen synthase kinase-3β (*GSK-3β*) gene

5.2.2.3.1. *GSK-3β* rs3755557

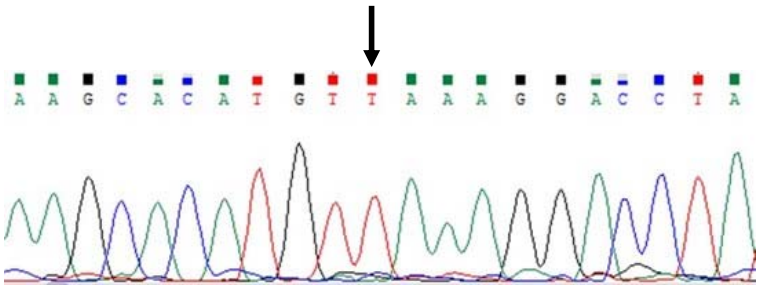
Samples were successfully sequenced with the use of the ABI3500 Genetic Analyser (Applied Biosystems®, Foster City, USA). The *GSK-3β* rs3755557 SNP for this sample population was in HWE according to the χ^2 statistic ($\chi^2 = 1.4669$; df = 1) as $\chi^2 < 3.841$. The allele and genotype frequencies of the SNP in the current study population are shown in Table 5.4. Sequencing electropherograms of each genotype can be seen in Figure 5.6. The allele and genotype frequencies were similarly distributed between the BD case and control group. The allele frequencies from the current study population (T = 88.95% vs A = 11.05) were similar to the frequencies from the 1000 genomes (T = 84.8%, A = 15.2%) (The 1000 Genomes Project Consortium, 2015). There were no statistically significant differences observed in the allele frequencies between the BD case and control groups in this study ($\chi^2 = 0.0902$; df = 1; $p = 0.764$).

A higher frequency of individuals within this study were homozygous for the TT genotype (77.89%) as compared to the 72.5% reported by the 1000 Genomes Project (The 1000 Genomes Project Consortium, 2015). Further, similar frequencies of the heterozygous genotypes were observed in the two studies (22.11% herein vs 24.6%, 1000 Genomes Project). It is interesting to note that the AA genotype was not observed in any study participant and was also the lowest frequency (2.8%) observed in the 1000 Genomes Project, which was only observed in the South Asian

Table 5.4 The allele and genotype frequencies for the *GSK-3β* rs3755557 SNP in the BD case and control groups.

***GSK-3β* rs3755557**							
	Genotype frequency				**Allele frequency**		
	TT	**TA**	**AA**	**Total**	**T**	**A**	**Total**
BD case group [n (%)]	38 (79.17)	10 (20.83)	0	48	86 (89.58)	10 (10.42)	96
Control group [n (%)]	36 (76.60)	11 (23.40)	0	47	83 (88.30)	11 (11.70)	94
Total [n (%)]	74 (77.89)	21 (22.11)	0	95	169 (88.95)	21 (11.05)	190
	$\chi^2 = 0.0912$; df = 1; $p = 0.763$				$\chi^2 = 0.0902$; df = 1; $p = 0.764$		

a)



b)

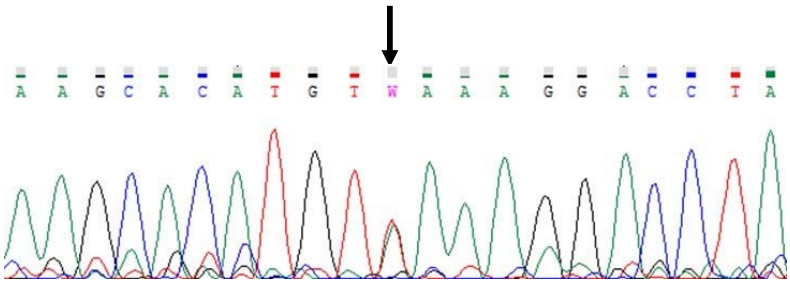


Figure 5.6 Sequencing electropherograms of a **a)** homozygous TT individual and **b)** heterozygous TA individual in the *GSK-3β* gene for the rs3755557 SNP. The black arrow indicates the SNP position.

population. No individuals of the Asian population were included in this study. This SNP was included in this study as it has not yet been investigated in South African BD patients. The TT (BD case group [79.17%] and control group [76.60%]) and TA (BD case group [20.84%] and control group [23.40%]) genotypes were found at similar frequencies in the two groups. No statistically significant differences were observed between the genotype frequencies for *GSK-3β* rs3755557 ($\chi^2 = 0.0912$; df = 1; $p = 0.763$). Therefore, no association between *GSK-3β* rs3755557 and BD was observed. However, the A allele has been positively associated with an earlier age at BD onset as well as psychotic mania episodes (Lee and Kim, 2011).

5.2.2.3.2. *GSK-3β* rs334558

Samples for the rs334558 SNP (*GSK-3β*) were successfully digested with the restriction enzyme AluI and subsequently visualised through 2% agarose gels (Figure 5.6). The *GSK-3β* rs334558 SNP for this sample population was in HWE according to the χ^2 statistic ($\chi^2 = 1.9528$; df = 1) as $\chi^2 < 3.841$. Allele and genotype frequencies are shown in Table 5.5. Sequencing electropherograms of each resulting genotype is depicted in Figure 5.7. The undigested PCR amplicons for *GSK-3β* is 344 bp, and thus, the size of undigested amplicons homozygous for the GG genotype. The restriction enzyme AluI recognises an A allele and, therefore, homozygous AA genotypes will result in two fragments (220 bp and 124 bp), whereas the GA genotype will result in three fragments (344 bp, 220 bp and 124 bp). In this study, the G allele was found at a prevalence of 57.45% and the A allele at 42.55%. Similar results were reported by the 1000 Genomes Project, where G = 59.9% and A = 40.1% (The 1000 Genomes Project Consortium, 2015). There were no statistically significant differences observed in the allele frequencies between the BD case and control groups in this study ($\chi^2 = 0.0019$; df = 1; $p = 0.965$).

The genotype frequencies between the BD case and control groups in this study were similar. The 1000 Genomes Project (The 1000 Genomes Project Consortium, 2015), reported the following genotypes: AA = 21.60%, GA = 37% and GG = 41.40%

Table 5.5 The allele and genotype frequencies for the *GSK-3β* rs334558 SNP in the BD case and control groups.

***GSK-3β* rs334558**							
	Genotype frequency				**Allele frequency**		
	GG	**GA**	**AA**	**Total**	**G**	**A**	**Total**
BD case group [n (%)]	14 (29.79)	26 (55.32)	7 (14.89)	47	54 (57.45)	40 (42.55)	94
Control group [n (%)]	12 (28.57)	24 (57.14)	6 (14.29)	42	48 (57.14)	36 (42.86)	84
Total [n (%)]	26 (29.21)	50 (56.18)	13 (14.61)	89	102 (57.30)	76 (42.70)	178
	$\chi^2 = 0.0300$; df = 2; $p = 0.985$				$\chi^2 = 0.0019$; df = 1; $p = 0.965$		

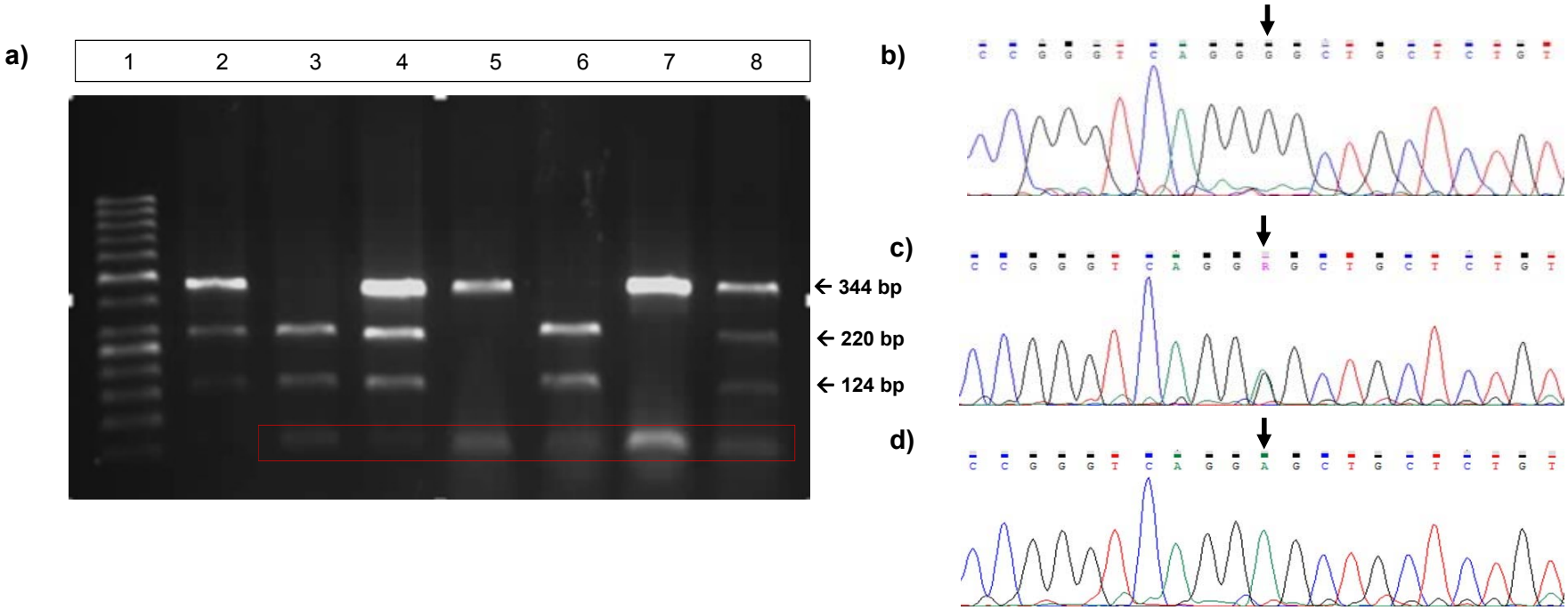


Figure 5.7 a) The visualisation of a 2% agarose gel depicting the digested *GSK-3β* rs334558 SNP. Lane 1 contains the 50 bp O'GeneRuler DNA ladder (Fermentas, Thermo Fisher Scientific, Waltham, USA), lanes 2, 4 and 8 each consists of three digested DNA fragments (344 bp, 220 bp and 124 bp) which indicate the individuals are heterozygous GA, lanes 3 and 6 each contain two digested DNA fragments (220 bp and 124 bp) which indicate the individuals are homozygous AA, lane 5 consists of one digested DNA fragment (344 bp) which indicate the individual is homozygous GG and lane 7 contains the undigested DNA fragment (344 bp). Primer dimers are visible at the bottom of the figure as indicated in the red block. Sequencing electropherograms of a **b)** homozygous GG individual **c)** heterozygous GA individual and **d)** homozygous AA individual in the *GSK-3β* gene for the rs334558 SNP. The black arrow indicates the SNP position.

which was slightly different to the genotype frequencies of this study (AA = 14.61%, GA = 56.18% and GG = 29.21%). Remarkably, most individuals in both groups were heterozygous GA (BD case group = 55.32%; control group = 57.14%). The AA genotype was found to be the least prevalent (BD case group = 14.89%; control group = 14.29%) with the GG genotype found at an intermittend prevalence (BD case group = 29.79%; control group = 28.57%). No significant differences were observed between the genotype frequencies for *GSK-3β* rs334558 ($\chi^2 = 0.0300$; df = 2; $p = 0.985$) and thus no association between *GSK-3β* rs334558 and BD was observed. An association between the TT genotype with poor lithium response has been reported (Lin *et al*., 2013) as well as early BD onset (Benedetti *et al*., 2004a). A study by Lee *et al*. (2006) found no association between *GSK-3β* rs334558 and BD.

5.2.2.4. Brain-derived neurotrophic factor (*BDNF*) gene

A total of 98 samples were successfully digested with NlaIII and visualised on 3% agarose gels. The denser 3% agarose gel allows the smaller DNA fragments to be better separated, therefore allowing for better visualisation of similar sized fragments. According to the χ^2 statistic, this sample group for the *BDNF* (rs6265) was in HWE ($\chi^2 = 0.3151$; df = 1) as $\chi^2 < 3.841$. The allele and genotype frequencies are shown in Table 5.6. The undigested PCR amplicon was 324 bp in length. The restriction enzyme NlaIII recognises the DNA fragment three times in the amplified region. The amplicons homozygous for the GG genotype resulted in four fragments (170 bp, 81 bp, 58 bp, and 15 bp), whereas amplicons with the homozygous AA genotype resulted in five fragments (94 bp, 81 bp, 76 bp, 58 bp, and 15 bp). Heterozygous GA genotypes, on the other hand, will yield six fragments (170 bp, 94 bp, 81 bp, 76 bp, 58 bp, and 15 bp). The 58 bp and 15 bp fragments were not always clearly visible on the gels due to their small fragment sizes (Figure 5.8).

The G allele was found at a frequency of 89.58% and the A allele at 10.42% in the study population. The G allele frequency was slightly higher in this study as

Table 5.6 The allele and genotype frequencies for *BDNF* rs6265 in the BD case and control groups.

***BDNF* rs6265**							
	Genotype frequency				**Allele frequency**		
	GG	**GA**	**AA**	**Total**	**G**	**A**	**Total**
BD case group [n (%)]	32 (65.31)	16 (32.65)	1 (2.04)	49	80 (89.58)	18 (10.42)	98
Control group [n (%)]	35 (71.43)	13 (26.53)	1 (2.04)	49	83 (88.30)	15 (11.70)	98
Total [n (%)]	67 (68.37)	29 (29.59)	2 (2.04)	98	163 (83.16)	33 (16.84)	196
	χ^2 =0.4453; df = 2; p = 0.800				χ^2 = 0.3441; df = 1; p = 0.556		

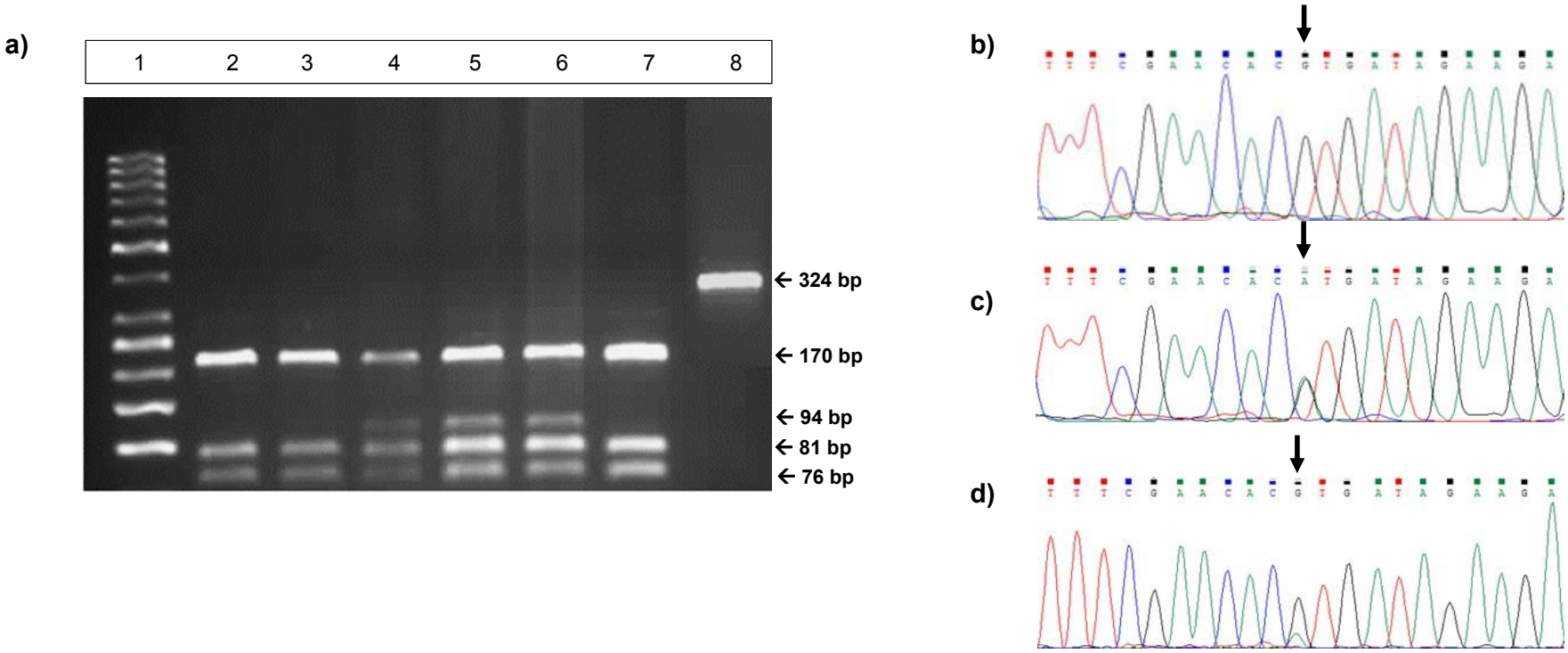


Figure 5.8 a) The visualisation of a 3% agarose gel depicting the digested *BDNF* rs6265 SNP. Lane 1 contains the 50 bp O'GeneRuler DNA ladder (Fermentas, Thermo Fisher Scientific, Waltham, USA), lanes 2, 3 and 7 each consist of three digested DNA fragments (94 bp, 81 bp, 76 bp, 58 bp, and 15 bp) which indicate the individuals are homozygous AA, lanes 4, 5 and 6 each consist of four digested DNA fragments (170 bp, 94 bp, 81 bp, 76 bp, 58 bp, and 15 bp) which indicate the individuals are heterozygous GA and lane 8 contains the undigested DNA fragment (324 bp). Sequencing electropherograms of a **b)** homozygous GG individual **c)** heterozygous GA individual and **d)** homozygous AA individual in the *BDNF* gene for the rs6265 SNP. The black arrow indicates the SNP position.

compared to the 1000 Genomes Project (79.9%), whereas the A allele occurred at a prevalence of 20.1% (The 1000 Genomes Project Consortium, 2015). There were no statistically significant differences observed in the allele frequencies between the BD case and control groups ($\chi^2 = 0.3441$; df = 1; $p = 0.556$).

Regarding genotype frequencies, a higher prevalence of individuals in the BD case group were heterozygous for the GA genotype (32.65%) relative to the control (26.53%), whereas more individuals in control group were homozygous for GG (71.43%) vs 65.31% in the BD case group. When pooled together, the total genotype results of this study (GG = 68.37%, GA = 29.59% and AA = 2.04%) were similar to the 1000 Genomes Project (GG = 66.7%, GA = 26.4% and AA = 6.9%) (The 1000 Genomes Project Consortium, 2015). No statistically significant differences were observed between the genotype frequencies for SNP rs6265 in *BDNF* ($\chi^2 = 0.4453$; df = 2; $p = 0.800$) and, therefore, no association between *BDNF* rs6265 and BD was observed. Associations of *BDNF* rs6265 with BD have been reported where the G allele is over-transmitted in BD patients (Neves-Pereira *et al*., 2002; Sklar *et al*., 2002; Geller *et al*., 2004; Lohoff *et al*., 2005). Other studies, however, found no association between *BDNF* rs6265 and BD (González-Castro *et al*., 2015; Ejmalian *et al*., 2016).

There may be various reasons for the inconsistent findings and discrepancies between studies involving the same genetic variant. The clinical criteria for diagnosing patients may be vary between the studies, genetic heterogeneity might play a role (González-Castro *et al*., 2015), whereby the frequency of alleles of the rs6265 SNP is ethnic dependant (Vincze *et al*., 2008). It may be possible that results are dependent on another modifying gene allowing the rs6265 SNP to have an effect at the clinical level. This may lead to the development of BD. Environmental factors and GxE interactions may need to be evaluated (Vincze *et al*., 2008)

5.2.3. Multi-locus genotype associations

The Cochran-Mantel-Haenszel test was used to test for multi-locus genotypes to determine cumulative risk on BD. The BD case and control groups were first separated (adjusted for group). Furthermore, the test was performed with the two groups merged together (not adjusted for group). These results are depicted in Table 5.7 with the associated *p*-values. Significant associations are marked in red.

Significant associations were obtained for the *CLOCK* rs1801260 and *GSK-3β* rs334558 SNPs for both the adjusted ($p = 0.012$) (Table 5.8) and not adjusted ($p = 0.012$) (Table 5.9) for group. Both correlations show that the ancestral G allele from *GSK-3β* rs334558 and the ancestral T allele from *CLOCK* rs1801260 are associated with each other. Most individuals that carry the GA and GG genotype from *GSK-3β* rs334558 will also be carriers of the TT genotype of *CLOCK* rs1801260. Similarly, the association between the *BDNF* rs6265 and *PER3* VNTR polymorphisms adjusted for group ($p = 0.022$) (Table 5.10) and not adjusted for group ($p = 0.021$) (Table 5.11) were statistically significant. The $PER3^{4}$ allele is significantly associated with the ancestral G allele of *BDNF* rs6265. More individuals who carry the $PER3^{4/4}$ genotype also carry the GG genotype of *BDNF* rs6265.

It is possible that these results occurred by chance, as these alleles do appear to be dominant within the study population (for both BD case and control groups as shown in Table 5.5 and Table 5.6). The significant results found in both the 'adjusted' and 'not adjusted for' groups may suggest the existence of epistasis (gene-gene interactions), and thus, complicating the search for disease-causing alleles. This interaction may not be specific to BD but, may influence the functioning of other processes in the body too. Many of these circadian rhythm proteins are part of much larger complexes or form heterodimers with each other and have overlapping functions. As such, gene-gene interactions are highly likely (Nievergeld *et al*., 2006).

Table 5.7 The association between gene polymorphisms adjusted and not adjusted for group. The values highlighted in red are statistically significant ($p < 0.05$).

Gene and polymorphism		Adjusted for group	Not adjusted for group
***CLOCK* rs1801260**	***BDNF rs6265***	$p = 0.469$; df = 1	$p = 0.515$; df = 1
***CLOCK* rs1801260**	***GSK-3β* rs334558**	$p =$ **0.012**; df = 1	$p =$ **0.012**; df = 1
***CLOCK* rs1801260**	***PER3* VNTR**	$p = 0.347$; df = 1	$p = 0.353$; df = 1
***CLOCK* rs1801260**	***GSK-3β* rs3755557**	$p = 0.107$; df = 1	$p = 0.103$; df = 1
BDNF rs6265	***GSK-3β* rs334558**	$p = 0.061$; df = 1	$p = 0.060$; df = 1
BDNF rs6265	***PER3* VNTR**	$p =$ **0.022**; df = 1	$p =$ **0.021**; df = 1
BDNF rs6265	***GSK-3β* rs3755557**	$p = 0.895$; df = 1	$p = 0.913$; df = 1
***GSK-3β* rs334558**	***PER3* VNTR**	$p = 0.878$; df = 1	$p = 0.875$; df = 1
***GSK-3β* rs334558**	***GSK-3β* rs3755557**	p = 0.265; df = 1	$p = 0.263$; df = 1
***PER3* VNTR**	***GSK-3β* rs3755557**	$p = 0.407$; df = 1	$p = 0.412$; df = 1

Table 5.8 The association between the *CLOCK* rs1801260 and *GSK-3β* rs334558 SNPs adjusted for group.

BD case group					**Control group**				
	***GSK-3β* rs334558**					***GSK-3β* rs334558**			
***CLOCK* rs1801260**	**AA**	**GA**	**GG**	**Total**	***CLOCK* rs1801260**	**AA**	**GA**	**GG**	**Total**
CC	1	2	1	4	**CC**	0	1	0	1
CT	5	10	2	17	**CT**	4	8	4	16
TT	1	14	11	26	**TT**	2	15	8	25
Total	7	26	14	47	**Total**	6	24	12	42
***p* = 0.012; df = 1**									

Table 5.9 The association between the *CLOCK* rs1801260 and *GSK-3β* rs334558 SNPs not adjusted for group.

	***GSK-3β* rs334558**			
***CLOCK* rs1801260**	**AA**	**GA**	**GG**	**Total**
CC	1	3	1	5
CT	9	18	6	33
TT	3	29	19	51
Total	13	50	26	89
***p* = 0.012; df = 1**				

Table 5.10 The association between the *BDNF* rs6265 and *PER3* VNTR polymorphisms adjusted for group.

BD case group					**Control group**				
	***PER3* VNTR**					***PER3* VNTR**			
***BDNF* rs6265**	**4R4R**	**4R5R**	**5R5R**	**Total**	***BDNF* rs6265**	**4R4R**	**4R5R**	**5R5R**	**Total**
AA	0	0	1	1	**AA**	0	1	0	1
GA	5	9	2	16	**GA**	4	6	3	13
GG	17	8	5	30	**GG**	16	15	2	33
Total	22	17	8	47	**Total**	20	22	5	47
***p* = 0.022; df = 1**									

Table 5.11 The association between the *BDNF* rs6265 and *PER3* VNTR polymorphisms not adjusted for group.

	***PER3* VNTR**			
***BDNF* rs6265**	**4R4R**	**4R5R**	**5R5R**	**Total**
AA	0	1	1	2
GA	9	15	5	29
GG	33	23	7	63
Total	42	39	13	94
***p* = 0.021; df = 1**				

5.2.4. Associations between the age at diagnosis and selected gene polymorphisms in patients with bipolar disorder

Although no information on the actual age at BD onset was collected, the age at diagnosis data was available. Literature states that the age at BD onset to diagnosis takes approximately one year for the majority of patients (Kessing, 2005) and, therefore, age at diagnosis was used for statistical analysis. Descriptive statistics for the comparison of genotypes of each selected polymorphism relative to age at diagnosis is summarised in Table 5.12. Results from Chapter 4 showed the mean age at diagnosis and SD for the BD case group was 32.09 ± 11.75 years old, with the youngest age of diagnosis at 11 years old and the oldest at 58 years old. These results are comparable to Benedetti *et al*. (2008) where the average age at onset was 31.78 ± 11.18 years.

Regarding the *CLOCK* rs1801260 SNP, the mean age at diagnosis between the three genotypes were similar (CC = 31.75 ± 11.27; CT = 31.94 ± 14.42; TT = 31.91 ± 10.83). The non-parametric test for association between genotypes and age at diagnosis yielded no significant results (p = 1; df = 2) and no association between age at diagnosis and *CLOCK* rs1801260 was found. Similarly, Lee *et al*. (2010) did not find any significant correlation (p = 0.126) between *CLOCK* rs1801260 and the age at onset.

Benedetti and colleagues were able to find association between the *PER3* VNTR and age at onset (p = 0.023). (Benedetti *et al*., 2008). Specifically, they found an early age at onset in $PER3^{5/5}$ homozygotes (26.61 ± 10.49), an intermediate age at onset in $PER3^{4/5}$ heterozygotes (31.17 ± 10.56) and later age at BD onset in the $PER3^{4/4}$ homozygotes (35.23 ± 11.43). We also found similar results in the current research study. Particularly, individuals with the $PER3^{5/5}$ genotype had an early age at diagnosis (28.00 ± 12.68), individuals with the $PER3^{4/5}$ genotype had an intermediate age at diagnosis (31.38 ± 11.24) and individuals with the $PER3^{4/4}$ had a later age at diagnosis (34.12 ± 12.54). However, no statistical significance was

Table 5.12 The descriptive statistics for age at diagnosis for the selected polymorphisms.

***CLOCK* rs1801260**				***PER3* VNTR**				***GSK-3β* rs3755557**			***GSK-3β* rs334558**				***BDNF* rs6265**			
	CC	**CT**	**TT**		**4R4R**	**4R5R**	**5R5R**		**TA**	**TT**		**AA**	**GA**	**GG**		**AA**	**GA**	**GG**
N	4	16	22	**N**	17	16	7	**N**	8	33	**N**	7	22	11	**N**	1	15	26
Mean	31.75	31.94	31.91	**Mean**	34.12	31.38	28.00	**Mean**	27.13	32.33	**Mean**	37.43	29.64	31.73	**Mean**	50.00	32.73	31.15
Std	11.27	14.42	10.83	**Std**	12.54	11.24	12.68	**Std**	9.79	11.89	**Std**	13.16	10.46	14.59	**Std**	-	11.83	11.74
Min	18.00	11.00	16.00	**Min**	18.00	11.00	15.00	**Min**	11.00	15.00	**Min**	23.00	15.00	11.00	**Min**	50.00	18.00	11.00
Median	32.50	27.00	30.50	**Median**	30.00	31.00	22.00	**Median**	28.50	31.00	**Median**	38.00	29.00	35.00	**Median**	50.00	29.00	30.00
Max	44.00	58.00	56.00	**Max**	58.00	56.00	50.00	**Max**	45.00	58.00	**Max**	58.00	55.00	56.00	**Max**	50.00	56.00	58.00
***p* = 1; df = 2**				***p* = 0.507; df = 2**				***p* = 0.254; df = 1**			***p* = 0.337; df = 2**				***p* = 0.288; df = 2**			

observed (p = 0.507; df = 2) and no association between age at diagnosis and *PER3* VNTR was found.

Comparing the *GSK-3β* rs3755557 SNP, results from Lee and Kim, (2011) had found a significant association (p = 0.034) with the TT genotype and an older age at BD onset (AA = 27.4 ± 9.1; AT = 30.1 ± 11.8; T/T = 42.3 ± 19.9) and individuals with the A allele had an earlier age at onset. For this study, it was found that the age at diagnosis was higher for individuals with the TT genotype (32.33 ± 11.89) and a slightly younger age at onset was observed for individuals with the TA genotype (27.13 ± 9.79). However, there was no statistical significance observed (p = 0.254) and no association between age at diagnosis and *GSK-3β* rs3755557 was found. Analysing the *GSK-3β* rs334558 SNP, an older age at onset was observed for individuals that are AA homozygous (37.43 ± 13.16). A younger age at onset was observed for individuals with the G allele (GA = 29.64 ± 10.46; GG = 31.73 ± 14.59). Research by Benedetti *et al*. (2004a, 2004b) linked the A allele to a younger age at onset in BD-I patients (p = 0.005) (Benedetti *et al*., 2004a). This study did not find any statistical significance (p = 0.337), similar to the results from Lee and Kim, (2011), who found no association between *GSK-3β* rs334558 and age at onset.

Analysing the *BDNF* rs6265 SNP, this study did not find any significant associations between with the age at BD diagnosis (p = 0.288). The mean age at diagnosis between the GA genotype (32.73 ± 11.85) and GG genotype (31.15 ± 11.74) was similar. Only one individual presented with the AA genotype with the age at BD onset at 50 years and, therefore, could not take this result into consideration. The results from this study are comparable to those by Kunugi *et al*. (2004) and Neves-Pereira *et al*. (2002) who also did not find any association *BDNF* rs6265 to age at onset.

The data in the current study was further stratified into three ‘age at diagnosis’ categories (< 20, 21 - 29, >30). Coryell *et al*. (2013) previously used the same approach, but with age at onset. The results were compared to the genotypes of the

selected polymorphisms and presented in Table 5.13. However, the results did not produce any of significance. Age at onset in BD patients can be used to determine homogeneous BD subtypes and, in turn, susceptibility genes (Bellivier *et al*., 2003). The genetic aetiology of early-onset BD may differ to the late-onset subtype. However, the importance of environmental factors, including developmental influences on the effect of BD onset, have not always been substantially analysed. Linkage studies show that early and late-onset BD are due to numerous common polymorphisms of small effect and rare variants with large effects. Numerous types of methodologies have been used and may explain the lack of replication of these types of studies. In future, the difference between early-onset and late-onset will need to be clearly defined and larger sample sizes will be needed, with the use of GWAS and related large-scale techniques (Kennedy *et al*., 2015).

The age at diagnosis was assessed and not age at onset, to which the results were compared to. This could also have affected results as research has shown that the number of years between average age at onset and age at diagnosis could be a large difference. Morken *et al*. (2009) has shown that the younger the patient, the longer it took to receiving pharmacological treatment. The duration from onset to first pharmacological treatment and the duration from onset to first admission to hospital were inversely correlated with age at onset. Early-onset and late-onset BD will need to be clearly defined and remain constant in genetic research, as the definition of early and late onset BD differs vastly between studies. This complicates the comparison process and may affect the results obtained. Study participants in one study may be referred to as early-onset and in another referred to as late-onset when researching the same polymorphisms (Kennedy *et al*., 2015). Early-onset BD is associated with a more severe phenotype, more psychotic symptoms, greater overall comorbidity and a delay from first episode occurrence to treatment (Suominen *et al*., 2007).

Table 5.13 The comparison between age at diagnoses (stratified into three groups) and genotypes from selected polymorphisms.

CLOCK rs1801260					*PER3* VNTR					*GSK-3β* rs3755557			
	CC	CT	TT	Total		4R4R	4R5R	5R5R	Total		TA	TT	Total
<20 years	1	3	3	7	**<20 years**	2	2	2	6	**<20 years**	1	6	7
21-29 years	1	6	7	14	**21-29 years**	6	5	3	14	**21-29 years**	4	10	14
>30years	2	7	12	21	**>30years**	9	9	2	20	**>30years**	3	17	20
Total	4	16	22	42	**Total**	17	16	7	40	**Total**	8	33	41
***p* = 0.808; df = 2**					***p* = 0.584; df = 2**					***p* = 0.581; df = 2**			

GSK-3β rs334558					*BDNF* rs6265				
	AA	GA	GG	Total		AA	GA	GG	Total
<20 years	0	4	3	7	**<20 years**	0	1	5	6
21-29 years	3	9	2	14	**21-29 years**	0	7	8	15
>30years	4	9	6	19	**>30years**	1	7	13	21
Total	7	22	11	40	**Total**	1	15	26	42
***p* = 0.274; df = 2**					***p* = 0.500; df = 2**				

5.2.5. Associations between statistically significant questionnaire results and genotypes from selected polymorphisms

Statistically significant results from Chapter 4 were used to further seek for possible genotype associations through the selected polymorphisms. The BMI and alcohol intake variables were merged with the genotype data and analysed using the Cochran-Mantel-Haenszel test. The statistical results for the BMI comparison with the genotypes of selected polymorphisms are shown in Table 5.14. The results were further stratified by group (Table 5.15).

Genotypes from the *CLOCK* rs1801260 SNP were also compared to the BMI groups. There were no correlations found between the BMI value and genotype (p = 0.130). The majority of individuals with a normal BMI did present with the TT genotype. No correlation with the overweight or obese variables could be made as they were equally distributed between the CT and TT genotypes. Monteleone *et al*., (2008) found a significant association between BMI and genotype, with the CC genotype associating with higher BMI values (p = 0.001) as compared to individuals with the TT genotype. It was further found that the C allele may predispose obese individuals to reach a higher BMI value. Garaulet *et al*. (2010) also found that individuals with the C allele may be at a higher risk for obesity and more resistant to weight loss (p = 0.008). However, this study found no association between BMI and *CLOCK* rs1801260.

After comparing genotypes of *PER3* VNTR to various BMI groups, no association was found between the polymorphism and BMI (p = 0.965). Genotypes were dispersed between various BMI categories. Compared to existing literature, some studies also failed to detect association between genotype and BMI (Bienertova-Vasku *et al*., 2014). Then again, a strong correlation was found between the *PER3*$^{5/5}$ genotype and a higher daily intake of fats and the individuals were also more likely to have poor nutritional eating habits (p = 0.006) (Bienertova-Vasku *et al*., 2014). However, this study found no association between BMI and *PER3* VNTR.

Table 5.14 The comparison between BMI and genotypes from selected polymorphisms not adjusted for group.

***CLOCK* rs1801260**					***PER3* VNTR**					***GSK-3β* rs3755557**				
	BMI					**BMI**					**BMI**			
	Normal	**Overweight**	**Obese**	**Total**		**Normal**	**Overweight**	**Obese**	**Total**		**Normal**	**Overweight**	**Obese**	**Total**
CC	1	3	1	5	**4R4R**	12	11	8	31	**TA**	6	3	6	15
CT	7	12	10	29	**4R5R**	7	15	7	29	**TT**	18	22	13	53
TT	17	13	8	38	**5R5R**	5	2	3	10					
Total	25	28	19	72	**Total**	24	28	18	70	**Total**	24	25	19	68
***p* = 0.130; df = 1**					***p* = 0.965; df = 1**					***p* = 0.686; df = 1**				

***GSK-3β* rs334558**					***BDNF* rs6265**				
	BMI					**BMI**			
	Normal	**Overweight**	**Obese**	**Total**		**Normal**	**Overweight**	**Obese**	**Total**
AA	5	3	3	11	**AA**	1	1	0	2
GA	16	11	14	41	**GA**	8	8	9	25
GG	1	10	1	4	**GG**	16	18	10	44
Total	22	24	18	64	**Total**	25	27	19	71
***p* = 0.588; df = 1**					***p* = 0.689; df = 1**				

Table 5.15 The comparison between BMI and genotypes from selected polymorphisms adjusted for group.

BD case Group					Control Group				
***CLOCK* rs1801260**					***CLOCK* rs1801260**				
	BMI					**BMI**			
	Normal	**Overweight**	**Obese**	**Total**		**Normal**	**Overweight**	**Obese**	**Total**
CC	1	2	1	4	**CC**	0	1	0	1
CT	3	3	8	14	**CT**	4	9	2	15
TT	6	5	7	18	**TT**	11	8	1	20
Total	10	10	16	36	**Total**	15	18	3	36
***p* = 0.555; df = 2**					***p* = 0.205; df = 2**				
***PER3* VNTR**					***PER3* VNTR**				
	BMI					**BMI**			
	Normal	**Overweight**	**Obese**	**Total**		**Normal**	**Overweight**	**Obese**	**Total**
4R4R	6	3	7	16	**4R4R**	6	8	1	15
4R5R	1	6	6	13	**4R5R**	6	9	1	16
5R5R	3	1	2	6	**5R5R**	2	1	1	4
Total	10	10	15	35	**Total**	14	18	3	35
***p* = 0.366; df = 2**					***p* = 0.973; df = 2**				
***GSK-3β* rs3755557**					***GSK-3β* rs3755557**				
	BMI					**BMI**			
	Normal	**Overweight**	**Obese**	**Total**		**Normal**	**Overweight**	**Obese**	**Total**
TA	2	1	5	8	**TA**	4	2	1	7
TT	8	8	11	27	**TT**	10	14	2	26
Total	10	9	16	35	**Total**	14	16	3	33
***p* = 0.444; df = 1**					***p* = 0.660; df = 1**				
***GSK-3β* rs334558**					***GSK-3β* rs334558**				
	BMI					**BMI**			
	Normal	**Overweight**	**Obese**	**Total**		**Normal**	**Overweight**	**Obese**	**Total**
AA	2	2	3	7	**AA**	3	1	0	4
GA	8	1	11	20	**GA**	8	10	3	21
GG	0	6	1	7	**GG**	1	4	0	5
Total	10	9	15	34	**Total**	12	15	3	30
***p* = 1; df = 2**					***p* = 0.330; df = 2**				
***BDNF* rs6265**					***BDNF* rs6265**				
	BMI					**BMI**			
	Normal	**Overweight**	**Obese**	**Total**		**Normal**	**Overweight**	**Obese**	**Total**
AA	0	1	0	1	**AA**	1	0	0	1
GA	3	3	8	14	**GA**	5	5	1	11
GG	7	6	8	21	**GG**	9	12	2	23
Total	10	10	16	36	**Total**	15	17	3	35
***p* = 0.558; df = 2**					***p* = 0.562; df = 2**				

The association between BMI and genotypes for the *GSK-3β* rs3755557 and rs334558 analysed in this study sample were also not significant. Thus, no associations could be detected between the SNPs in *GSK-3β* and BMI (rs3755557 $p = 0.686$; rs334558 $p = 0.588$) as this study was the first to perform this comparison. On the contrary, the *BDNF* rs6265 polymorphism has previously been associated with BMI. Shugart *et al*. (2009) were able to find significant correlation between the AA genotype and lower BMI values, as compared to the GA or GG genotypes in women. Likewise, Timpano *et al*. (2011) found that carriers of the GG genotype were more likely to be categorized as obese. However, data from our study population did not yield any significant association between *BDNF* rs6265 and BMI ($p = 0.689$) and no association between BMI and *BDNF* rs6265. Similar, Perkovic *et al*. (2013), also found no association.

When adjusting for groups, there were no statistically significant associations between BMI and the selected polymorphisms of the study. The circadian rhythm genes as well as *BDNF* have been associated with obesity (Monteleone *et al*., 2008; Shugart *et al*., 2009). To increase that chance of detecting associations, ethnic and gender stratification will be essential in genetic research, especially with BMI. If research is conducted in separate population groups, additional risk alleles may be uncovered (Shugart *et al*., 2009). As seen with Shugart *et al*. (2009), it may be possible that certain polymorphisms are only associated with one of the gender types. It is also important that all environmental influences that can have an impact on the phenotype be included in the study (Timpano *et al*., 2011), so as to test for any GxE interactions as well as any epistatic and environmental interactions.

Results on the statistical analysis of associations between alcohol intake and selected gene polymorphisms are displayed in Table 5.16. Overall, we did not find any significant associations between *CLOCK* rs1801260 ($p = 0.417$), *PER3* VNTR ($p = 0.714$), *GSK-3β* rs3755557 ($p = 0.953$), *GSK-3β* rs334558 ($p = 0.312$) and *BDNF* rs6265 ($p = 0.475$) variants and alcohol consumption. Also, adjusting the group data did not yield any significant associations (Table 5.17).

Table 5.16 The comparison between alcohol intake and genotypes from selected polymorphisms not adjusted for group.

***CLOCK* rs1801260**				***PER3* VNTR**				***GSK-3β* rs3755557**			
	Drink alcohol regularly				**Drink alcohol regularly**				**Drink alcohol regularly**		
	Yes	**No**	**Total**		**Yes**	**No**	**Total**		**Yes**	**No**	**Total**
CC	1	4	5	**4R4R**	7	26	33	**TA**	3	12	15
CT	5	25	30	**4R5R**	6	26	32	**TT**	12	46	58
TT	11	31	42	**5R5R**	3	7	10				
Total	17	60	77	**Total**	16	59	75	**Total**	15	58	73
***p* = 0.417; df = 1**				***p* = 0.714; df = 1**				***p* = 0.953; df = 1**			

***GSK-3β* rs334558**				***BDNF* rs6265**			
	Drink alcohol regularly				**Drink alcohol regularly**		
	Yes	**No**	**Total**		**Yes**	**No**	**Total**
AA	1	10	11	**AA**	0	2	2
GA	10	33	43	**GA**	5	20	25
GG	4	11	5	**GG**	12	38	50
Total	15	54	69	**Total**	17	60	77
***p* = 0.312; df = 1**				***p* = 0.475; df = 1**			

Table 5.17 The comparison between alcohol intake and genotypes from selected polymorphisms adjusted for group.

BD case group				**Control group**			
***CLOCK* rs1801260**				***CLOCK* rs1801260**			
	Drink alcohol regularly				**Drink alcohol regularly**		
	Yes	**No**	**Total**		**Yes**	**No**	**Total**
CC	1	3	4	**CC**	0	1	1
CT	0	15	15	**CT**	5	10	15
TT	3	19	22	**TT**	8	12	20
Total	4	37	41	**Total**	13	23	36
***p* = 0.226; df = 2**				***p* = 0.696; df = 2**			
***PER3* VNTR**				***PER3* VNTR**			
	Drink alcohol regularly				**Drink alcohol regularly**		
	Yes	**No**	**Total**		**Yes**	**No**	**Total**
4R4R	2	16	18	**4R4R**	5	10	15
4R5R	1	15	16	**4R5R**	5	11	16
5R5R	1	5	6	**5R5R**	2	2	4
Total	4	36	40	**Total**	12	23	35
***p* = 0.757; df = 1**				***p* = 0.781; df = 2**			
***GSK-3β* rs3755557**				***GSK-3β* rs3755557**			
	Drink alcohol regularly				**Drink alcohol regularly**		
	Yes	**No**	**Total**		**Yes**	**No**	**Total**
TA	0	8	8	**TA**	3	4	7
TT	3	29	32	**TT**	9	17	26
Total	3	37	40	**Total**	12	21	33
***p* = 0.374; df = 1**				***p* = 0.692; df = 1**			
***GSK-3β* rs334558**				***GSK-3β* rs334558**			
	Drink alcohol regularly				**Drink alcohol regularly**		
	Yes	**No**	**Total**		**Yes**	**No**	**Total**
AA	0	7	7	**AA**	1	3	4
GA	3	19	22	**GA**	7	14	21
GG	1	9	10	**GG**	3	2	5
Total	4	35	39	**Total**	11	19	30
***p* = 0.0593; df = 2**				***p* = 0.483; df = 2**			
***BDNF* rs6265**				***BDNF* rs6265**			
	Drink alcohol regularly				**Drink alcohol regularly**		
	Yes	**No**	**Total**		**Yes**	**No**	**Total**
AA	0	1	1	**AA**	0	1	1
GA	2	12	14	**GA**	3	8	11
GG	3	24	27	**GG**	9	14	23
Total	5	37	42	**Total**	12	23	35
***p* = 0.895; df = 2**				***p* = 0.615; df = 1**			

Yoshihara *et al*. (2011) also found no statistical significance between the *GSK-3β* rs3755557 and rs334558 SNPs and alcoholism (Yoshihara *et al*., 2011). However, BD is highly comorbid with alcohol use disorder and is observed in more than 50% of BD patients in their lifetime. Shared genetic risk factors may explain the high association of alcohol use disorders in BD patients (Carmiol *et al*., 2014). For better detection of associations, genders should be stratified, as research shows that, on average, women drink much less alcohol than men on average (Nolen-Hoeksema, 2004). A larger study population size with a case-control group should be included in future research. Stressful life events and circumstances play a major role in mental health and in individuals vulnerable to addiction. Adverse environmental factors, together with clock genes, may be key in GxE interactions that lead to alcohol addiction (Perreau-Lenz and Spanagel, 2015).

5.3. Conclusion

The present chapter primarily aimed to investigate whether the selected polymorphisms in the circadian rhythm and neurotrophin pathways played a role in BD aetiology. The selected genes and associated polymorphisms in this study (*CLOCK* rs1801260, *PER3* VNTR, *GSK-3β* rs334558 and rs3755557, *BDNF* rs6265) have previously been associated with BD, however, the literature has presented with inconsistent results. It was, therefore, important to investigate these polymorphisms and their association in a BD South African population. A case-control study was executed on the DNA samples of 50 BD cases and 50 controls.

Bipolar disorder is a genetically complex disorder influenced by multiple genes and pathways. The BD phenotype is further complicated by genetic mechanisms (Chapter 2), thus, increasing the difficulty in the search for BD risk alleles. This may explain why no association between BD and the selected polymorphisms were found. This is typically observed in the field of psychiatric genetics. Additionally, non-genetic factors may interact with alleles which may explain the variations observed in the BD phenotype.

Multi-locus genotypes were analysed to evaluate the cumulative risk of BD. Significant associations were obtained for the *CLOCK* rs1801260 and *GSK-3β* rs334558 SNPs as well as for *BDNF* rs6265 and *PER3* VNTR polymorphisms. These results may confirm possible overlapping roles of these polymorphisms and propose a gene-gene interaction. Further research is required to confirm these results. Strong evidence exists for the association of age at onset and certain selected polymorphisms in the study. However, in the study, the use of age at BD diagnosis found no association to the selected polymorphisms. Larger study populations are needed to investigate the age at onset. Using the age at diagnosis may not be ideal to use in research, as onset may have occurred many years before the patients were diagnosed. Furthermore, clear definitions need to be established to define early BD and late BD onset.

Obesity and high alcohol consumption in BD patients are common. These variables provided statistically significant results in Chapter 4. Therefore, the 'BMI' and 'drinking alcohol on a regular basis' variables were further investigated for allele and genotype associations of the selected polymorphisms in this study. However, no statistically significant associations with the selected polymorphisms were found. It has been shown that gender stratification may provide better statistical power, as certain genes may interact differently between men and women. Again, environmental factors such as stress, need to be incorporated to establish any GxE interactions.

Bipolar disorder is a highly heritable, polygenic disorder. The link between the observed phenotype and genotype is complex and requires further investigations. South Africa has very limited data available for allele and genotypic association studies specifically for the circadian rhythm and neurotrophin pathways. The country consists of many ethnic groups and a large gene pool that should be utilised, as it may provide additional information into the genetic mechanisms underlying BD aetiology. Non-genetic factors provide just as much importance and should be included in psychiatric molecular research. Further research into the genes of other neurological pathways may provide insightful information. Identifying BD risk alleles

will not only increase the understanding of the underlying genetic mechanism of BD, but will aid as biomarkers, allowing fast diagnosis and assist with effective BD treatment.

Chapter 6:
Conclusion

6.1. Summary

Bipolar disorder is a multi-factorial mental disorder characterised by episodic mood swings, ranging from mania to depression. It negatively affects the patient's overall quality of life and daily functioning. Bipolar disorder is frequently associated with the comorbidity of other psychiatric disorders and medical conditions, which often causes a more severe course of illness and poor treatment response. Metabolic syndrome is one of the most frequently recognised comorbidity in BD patients. The pathophysiology and complexity of BD are attributed to genetic, non-genetic and stochastic risk factors. Molecular research has indicated that BD and MetS share mutual genetic factors. Research shows that the circadian rhythm and neurotrophin genes both play a role in BD and MetS aetiology and are important for BD drug targeting. Very limited research exists in SA with regards to the circadian rhythm and neurotrophin pahways and were, therefore, further investigated.

This was the first study in SA to investigate the genetic associations of selected circadian rhythm (*CLOCK* rs1801260; *PER3* VNTR; *GSK-3β* rs3755557 and rs334558) and the neurotrophin gene (*BDNF* rs6265) variants in a South African BD population. Additionally, the high comorbidity risk factors and environmental factors influencing MetS were also investigated. The study group consisted of a total of 53 BD patients (BD case group) and 50 individuals who matched in age (within a 5-year interval), gender and ethnicity, with no history of mental illness (control group). A quantitative approach was used to evaluate both genetic and environmental influences and a case-control design was implemented to regulate for influential variables contributing to the aetiology of BD.

In this study, the questionnaire variables that presented with statistical significance was ‘drinking alcohol on a regular basis’ and ‘BMI.’ When associated with the selected polymorphisms, no statistical significance was found. Furthermore, multi-locus genotypes were compiled to test for cumulative genetic risk to BD. The *CLOCK* rs1801260 and *GSK-3β* rs334558 presented with statistical significance as well as the *BDNF* rs6265 and *PER3* VNTR polymorphisms. These results highlight the complexity and polygenicity of BD, possible epistasis and GxE interactions involved in both BD and MetS. Furthermore, this indicates the involvement of many genes of small effects and low penetrance from different neurological pathways. This increases the difficulty of detecting risk alleles for BD.

The genetics of complex disorders, such as BD, is further complicated by their multi-factorial nature. They may additionally present with an indirect phenotype or only pertain to certain characteristics of the disorder. The genetic effects may only become known within a certain environment that may differ from one individual to the next. The phenotype may additionally be regulated by the environment that may influence the epigenetics of the disorder, changing their expression. The risk factors contributing to MetS in this study population can be explained by overlapping genetic mechanisms and pleiotropy, where one gene can influence numerous traits and further explain comorbidity observed between BD and MetS. This may define how neurological disorders and disorders of the body are related.

South Africa presents with an abundant gene pool that should be utilised in genetic research, which may increase the knowledge of BD, its physiological processes and risk alleles. Furthermore, the need for BD diagnostic tools are critical to decrease the misdiagnoses rates and increase effective treatment in the future.

This study was strengthened using a case-control group with the inclusion of both BD-I and BD-II individuals of both genders, different ages and various ethnicities found within SA. This study highlights the importance of genetic research in BD, the neuropathological pathways involved and provides insight into its complexity and

multi-factorial nature. Much needed research is required in SA for patients suffering with BD and MetS comorbidity.

6.2. Limitations and future research

There are several limitations noted in this study and it is important to be aware of them. Self-report questionnaires present with some disadvantages (Munn, Drever and Scottish Council for Research in Education, 1990). Firstly, questionnaires can have a low response rate and may not always be fully completed (Cook, Heath and Thompson, 2000). Sensitive study participants may intently skip questions and avoid disclosing certain information about themselves. Questionnaires were distributed to 53 BD case group and 50 control group individuals. Only a total of 37 questionnaires from the BD case group and 36 questionnaires from the control group were fully completed. Secondly, responses to the questionnaires may entail the need for further explanations to some extent. The participants' judgment to the questions may not fully indicate their true behaviour (Riemann, Angleitner and Strelau, 1997). Thirdly, study participants may not be completely honest when answering the questions and in turn manipulate or adapt their answers, affecting the results of the study.

Due to limited contact with the participants, ethical considerations and resources, it was not possible to measure the waist measurement, height and weight of every study participant. Furthermore, each study participant evaluated their own diet quality. The answers provided by the participants were done so by their own discretion and may not have reflected the true results. It was also not possible to measure blood pressure, fasting plasma glucose, fasting triglycerides and LDL and HDL cholesterol levels. Having this information available would have provided greater statistical value to this research project and would have aided in determining possible MetS risk factors in the study participants. Cardiovascular conditions and diabetes diagnoses relied on the memory of the patients and the medications they take for their conditions. However, due to the chronicity of the medication taken,

patients could recall the medications they take regularly. It is clear that a universal definition for MetS be established that will allow for better diagnoses and comparison between research studies. In addition, waist circumference measurements do not differentiate between subcutaneous and abdominal fat (Tchernof and Després, 2013), and BMI does not take an individual's gender, age, bone structure, muscle mass or fat distribution into consideration (Rothman, 2008). Therefore, these two measurements are not the most optimal measurements for body fat (McElroy *et al*., 2016). It would be wise to include the effects of pharmacotherapy in determining their effect of MetS in patients with BD (Fagiolini *et al*., 2005). A study by Saloojee, Burns and Motala, (2017) was the first study to demonstrate the elevated risk of MetS in black South African women taking antipsychotic medication. Future research should include and utilise as many resources as possible, together with more sensitive and validated methods, to provide the best statistical value, and to quantify environmental factors.

A larger sample size including inpatients and outpatients of all ages, from various ethnicities around SA would greatly improve future research. It may be beneficial to investigate the effects of genetic variants within each population as alleles may have different effects on different ethnicities. Additionally, analysing the gender differences could provide additional information on BD and MetS. The duration of illness could provide more important information as to whether it may have a statistical significance on the effect of MetS risk factors and MetS itself. Another very important variable to include would be to determine whether the MetS risk factors are found more prevalently in individuals with certain mood episodes. It has been shown that MetS risk factors are found more prevalently in BD patients in the depressive episode (Moreira *et al*., 2017). It will also be important to determine the age the current lifestyle factors have been ongoing as to determine if an association exists with BD onset. Factors such as socioeconomic status, full history of treatment medication exposure and a complete background of all variables will be important for future research.

From a molecular genetics perspective, future BD studies should use larger case-control cohorts and identify variants involved in specific BD endophenotypes and clinical indicators, such as age at onset. These sub-groups may simplify the search for risk alleles. All possible genetic mechanisms (Chapter 2.5.2.3) that can influence the BD should be acknowledged and incorporated. Conflicting study results can be avoided by acknowledging and not underestimating the heterogeneity of BD (Leboyer and Kupfer, 2010). Interacting neurological pathways should be considered, as epistasis and pleiotropy will have an influence, especially in the neurotrophin and circadian rhythm pathways. This is due to the cyclic nature of BD and how these pathways may influence them. One should be mindful of incorporating the environmental influences and their impact on the genes' expression and the BD phenotype. With the large gene pool present in SA, future researchers should consider using the South African population. This will lead to a better understanding of BD aetiology and explain the comorbidity of MetS in these patients. This will contribute to the understanding of underling mechanisms involved and in turn, hope to produce valuable biomarkers for all BD patients.

Chapter 7:
References and appendices

7.1. General references

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7.3. Appendices

Appendix A

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA
UFS
UV

[redacted]

Research Division
Internal Post Box G40
☎(051) 4052812
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E-mail address: StraussHS@ufs.ac.za

Ms H Strauss/hv

2014-06-06

REC Reference nr 230408-011
IRB nr 00006240

MS C SUSMAK
C/O MS S SCHNEIDER
DEPARTMENT OF GENETICS
FACULTY OF HEALTH SCIENCES
UFS

Dear Ms Susmak

ECUFS NR 77/2014
MS C SUSMAK **DEPARTMENT OF GENETICS**
PROJECT TITLE: BIPOLAR DISORDER: INFLUENCE OF CIRCADIAN RHYTHM GENES AND METABOLIC SYNDROMES.

1. You are hereby kindly informed that at the meeting on 3 June 2014 the Ethics Committee approved the above study after all conditions have been met.

2. Committee guidance documents: Declaration of Helsinki, ICH, GCP and MRC Guidelines on Bio Medical Research. Clinical Trial Guidelines 2000 Department of Health RSA; Ethics in Health Research: Principles Structure and Processes Department of Health RSA 2004; Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa, Second Edition (2006); the Constitution of the Ethics Committee of the Faculty of Health Sciences and the Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines.

3. Any amendment, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.

4. The Committee must be informed of any serious adverse event and/or termination of the study.

5. All relevant documents e.g. signed permission letters from the authorities, institutions, changes to the protocol, questionnaires etc. have to be submitted to the Ethics Committee before the study may be conducted (if applicable).

6. A progress report should be submitted within one year of approval of long term studies and a final report at completion of both short term and long term studies.

7. Kindly refer to the ETOVS/ECUFS reference number in correspondence to the Ethics Committee secretariat.

Appendix B

Participation in a Genetic Research Study

Dear Participant

You are being invited to take part in a Master's degree research study carried out by the Genetics department at the University of the Free State. This study will investigate the influence of genes on the development of **bipolar disorder** and **metabolic syndromes**.

Before deciding whether or not to part take in the study, it is advised that you carefully read through the information stated below. You are more than welcome to discuss the given information with friends, family or your doctor. You are free to ask any questions regarding the study before making your finally decision.

If you agree to participate, you will be given a signed copy of this document as well as the participant information sheet, which is a written summary of the research.

Participant Information

The purpose of this study is to determine the role genes play in the development of bipolar disorder and metabolic syndrome. In order to achieve this, genetic material (DNA) will be collected to be analysed in a laboratory, whereafter it will be stored for future research. The information gathered by researchers from the DNA might lead to better understanding the genetic causes of these disorders which in turn could lead to better treatment options.

You will be asked to complete a short questionnaire. This will not require more than 15 minutes of your time. If you wish to take part in this study, your informed consent is needed. Participation in this study is voluntary. You are more than welcome to withdraw from this study at any time without penalization. Withdrawing from the study will not affect your future medical care nor will your medical care be compromised in anyway.

Any individual willing to give consent will be asked for a saliva sample. From the saliva sample, DNA will be extracted. The DNA will purely be used for research purposes. There are no risks involved in the collection of the saliva sample as it requires you to donate saliva into a sterile collection tube. There are no costs involved in participating. Similarly, you will not receive any money or other compensation if you participate in this study.

The findings of this study will not benefit you directly or have direct bearing on your management. However, the findings may benefit others in the future, such as your family members and future generations, which might develop these disorders, in terms of prevention or treatment.

Your identity, personal and genetic information will be kept confidential. The following arrangements have been made to ensure privacy and confidentiality of your personal, medical and genetic information. Your genetic material and information will be used in an unidentifiable form by assigning you an unique research number. We ensure that all of your information will be safely stored and access will be limited to authorized researchers. Because your material or information is to be made unidentifiable, it will not be possible to provide you with personal research results. The results will however be published in group format. We assure confidentiality and anonymity.

Your participation is highly appreciated.

If you have any questions with respect to this study please contact:

Miss Chantay Susmak (0715763828) or Mrs Sue-Rica Schneider (051 401 3730) at the Department of Genetics, University of the Free State.

You may also contact the Secretariat of the Ethics Committee of the Faculty of Health Sciences, University of the Free State at telephone number (051) 4052812 if you have questions about your rights as a research subject.

Consent to participate

I, _____ (name and surname), hereby AGREE to participate in the research described above. I fully understand the purpose of this study and I confirm that my participation in my study is completely voluntary. I furthermore understand that my DNA will be required for the research project. I understand that my participation or choosing to withdraw from the study will not hold any negative consequences for myself, or cost anything. I further understand that the data gathered in this study may be published in a scientific journal, but if this should happen, I will remain completely anonymous.

_____ _____

Signature Date

_____ _____

Witness Signature Date

Consent for the handling of genetic material

I, _____ (Name and Surname), hereby understand that providing my genetic material in the form of a saliva sample will hold no negative effects for me. I further understand that my genetic material will be handled only by the Department of Genetics researchers and that it will only be used for research purposes. I understand that this genetic material will need to be stored for the duration of the study. By signing this I recognise that I will be handing my genetic information over to the researchers and I trust that it will be handled with the utmost care and secrecy.

_____ _____

Signature Date

Appendic C

Deelname aan ‘n Genetiese Navorsing Studie

Geagte Deelnemer

U word uitgenooi om deel te neem aan ‘n Meestersgraad navorsingstudie wat uitgevoer word deur die Departement van Genetika aan die Universiteit van die Vrystaat. Hierdie studie sal die invloed van gene op die ontwikkeling van **bipolêre versteuring** en **metaboliese sindroom** ondersoek.

Voordat u besluit om deel te neem aan hierdie studie, raadpleeg ons u om aandagtig deur die onderstaande inligting lees. U is meer as welkom om die gegewe inligting met vriende, familie of ‘n dokter te bespreek. U is ook welkom om enige vrae oor die studie te vra voordat u ‘n finale besluit neem.

Indien u saamstem om deel te neem, sal u 'n getekende afskrif van hierdie dokument gegee word sowel as die deelnemer inligtingsblad, wat 'n skriftelike opsomming van die navorsing het.

Deelnemer Inligting

Die doel van hierdie studie is om die rol wat gene in die ontwikkeling van bipolêre versteuring en metaboliese sindroom speel te bepaal. Om dit te bereik, sal genetiese materiaal (DNA) versamel word om in 'n laboratorium geanaliseer te word, waarna dit vir toekomstige navorsing sal gestoor word. Navorsing oor die DNA sal ons n beter begrip van die genetiese oorsake van die versteurings gee en dit kan ook tot beter behandeling opsies lei.

U sal gevra word om 'n kort vraelys te voltooi. Dit sal nie meer as 15 minute van u tyd neem nie. As u sou besluit om aan hierdie studie deel te neem, is u ingeligte toestemming nodig. Deelname aan hierdie studie is vrywillig. Indien u, u gedagtes sou verander oor die studie, is u meer as welkom om op enige tyd te onttrek van die studie, sonder on gepenaliseerd te word. 'n Besluit om nie deel te neem nie sal nie u mediese sorg of toekomstige mediese sorg in enige wyse beïnvloed nie.

Enige individu wat bereid is om toestemming te gee om aan die studie deel te neem, sal gevra word vir 'n speeksel monster. Van die speeksel monster sal DNA onttrek word. Die DNA sal slegs gebruik word vir navorsingsdoeleindes vir hierdie studie. Daar is geen risiko's verbonde aan die versameling van die speeksel monster nie omdat dit slegs vereis dat die deelnemer speeksel in 'n plastiese buisie skenk. Daar is ook geen kostes aan verbonde vir die deelnemer nie. Net so ook, sal u nie enige geld, inligting of enige ander vergoeding vir hierdie studie ontvang nie.

Die bevindinge van hierdie studie sal nie u direk voordeel nie of 'n direkte invloed op u bestuur hê nie. Dit kan, alhoewel, ander in die toekoms voordeel, soos u familie en toekomstige geslagte, wat hierdie versteurings kan ontwikkel, in terme van voorkoming of behandeling.

U identiteit, persoonlike en genetiese inligting sal vertroulik hanteer word. Die volgende reëlings is getref om u privaatheid en vertroulikheid van u persoonlike, mediese en genetiese inligting te verseker. U genetiese materiaal en inligting sal in 'n anonieme vorm gebruik word deur om vir u 'n

unieke navorsing nommer te gee. Ons verseker dat al u inligting veilig bewaar sal word en toegang sal beperk word tot gemagtigde navorsers. Om materiaal en inligting vertroulik te hou, sal persoonlike navorsingsresultate nie moontlik wees nie. Die resultate sal vertroulik gehou word en die data-insameling en DNA-ontleding sal gerapporteer word as groep inligting of gepubliseer word in 'n wetenskaplike joernaal.

Jou deelname word opreg waardeer.

As u enige vrae het met betrekking tot hierdie studie kontak:

Mej Chantay Susmak (0715763828) of Mev Sue-Rica Schneider (051 401 3730) by die Departement van Genetika, Universiteit van die Vrystaat.

U kan ook die Sekretariaat van die Etiekkomitee van die Gesondheidswetenskappe Fakulteit kontak, Universiteit van die Vrystaat by telefoonnommer (051) 4052812 as u vrae het oor u se regte as 'n onderwerp.

Toestemming om deel te neem

Ek, _____ (naam en van), gee hiermee my toestemming om deel te neem aan die navorsing wat hierbo beskryf is. Ek verstaan ten volle die doel van hierdie studie en ek bevestig ook dat my deelname aan hierdie studie ten volle vrywillig is. Ek verstaan verder ook dat my DNA benodig sal word vir die navorsingsprojek. Ek verstaan dat my deelname of die keuse om te onttrek van die studie, nie enige negatiewe gevolge sal inhou vir myself, of enige geld kos nie. Ek verstaan verder dat die data wat ingesamel word in hierdie studie gepubliseer kan word in 'n wetenskaplike joernaal, maar indien dit sou gebeur, sal ek heeltemal anoniem bly.

_____ _____

Handtekening Datum

_____ _____

Getuie handtekening Datum

Toestemming vir die hantering van genetiese materiaal

Ek, _____ (naam en van), verstaan dat die verskaffing van my genetiese materiaal in die vorm van 'n speeksel monster, geen negatiewe gevolge vir my sal hê nie. Ek verstaan verder dat my genetiese materiaal net deur die Departement van Genetika se navorsers gebruik sal word, slegs vir die doel van die studie. Ek verstaan ook dat die genetiese materiaal vir die durasie van die studie bewaar moet word. Om hier onder te teken erken ek dat

ek my genetiese informasie vir die navorsers oorhandig en ek vertrou dat dit met die grootste sorg en geheimhouding hanteer sal word.

_____ _____

Handtekening Datum

Appendix D

Background Information

Please provide the following information by checking (✓) the appropriate response or filling in the blank.

Date of birth:	_____
Gender:	☐ Male ☐ Female
Ethnic origin (check ✓ one only):	☐ Caucasian ☐ African ☐ Coloured ☐ Indian ☐ Asian ☐ Other, please specify: _____
Is the ethnic origin of your parents the same as yours?	☐ Yes ☐ No If No, please specify the ethnic origin of your Father_____ Mother_____
Is the ethnic origin of your grandparents the same as yours?	☐ Yes ☐ No If No, please specify the ethnic origin of your Grandfather (Father side)_____ Grandmother(Father side)_____ Grandfather(Mother side)_____ Grandmother(Mother side)_____
Home language:	☐ Afrikaans ☐ English ☐ isiZulu ☐ IsiNdebele ☐ IsiXhosa ☐ Sepedi ☐ Setswana ☐ Sesotho ☐ Xitsonga ☐ SiSwati ☐ Tshivenda ☐ Other, please specify: _____
Have you been diagnosed with Bipolar Disorder 1 or 2?	☐ 1 ☐ 2
What age have you been diagnosed with Bipolar Disorder?	_____
What medication(s) are you taking for your condition?	_____

Please complete the following questionnaire by checking (✓) either yes or no or answering where appropriate.

Health and Medical History related to Metabolic Syndrome

1.	Do you exercise less than 2 times a week?	Yes	No
2.	Do you smoke cigarettes on a regular basis? If yes, how many do you smoke per day? _____	Yes	No
3.	Do you drink alcohol on a regular basis? How many alcoholic drinks do you consume per week? _____	Yes	No
4.	Do you follow a balanced diet? (consists of the correct proportion of all the nutrients like carbohydrates, fats, proteins, minerals and vitamins along with sufficient of water and roughage material).	Yes	No
5.	What is your weight in kilograms? _____		
6.	What is your height in meters? _____		
7.	Do you have a waist measurement of more than 102 cm (40 inches) or more if male, or 89 cm (35 inches) or more if female?	Yes	No
8.	Do you suffer from cardiovascular (heart) condition or disease? If yes, please specify _____ At which age were you diagnosed? _____ What medication are you taking for your condition? _____	Yes	No
9.	Do you suffer from diabetes (1 or 2)? If yes, please specify _____ At which age were you diagnosed? _____ What medication are you taking for your condition? _____	Yes	No
10.	Do you have high blood pressure? At which age were you diagnosed? _____ What medication are you taking for your condition? _____	Yes	No
11.	Do you suffer from high cholesterol levels? At which age were you diagnosed? _____ What medication are you taking for your condition? _____	Yes	No
12.	Do you suffer from any other diagnosed medical condition not mentioned? If yes, please specify _____ At which age were you diagnosed? _____ What medication are you taking for your condition? _____	Yes	No
13.	Are you obese?	Yes	No
14.	Do you have any family members who suffer from any of the above mentioned conditions? If yes, please specify condition and family member(s)_____ _____ If no, are there any other conditions which a family member suffers from. Please specify family member and condition _____	Yes	No

Bipolar disorder

The following questionnaire evaluates the symptoms experienced by you before your bipolar was being treated.

Please check (✓) the appropriate phrases which best suit you. You can check more than one.

1.	Feeling abnormally “high” and optimistic	
2.	Feeling extremely irritable	
3.	Unrealistic, grandiose beliefs about one’s abilities or powers	
4.	Sleeping very little, but feeling extremely energetic	
5.	Talking so rapidly that others can’t keep up	
6.	Racing thoughts, jumping quickly from one idea to the next	
7.	Highly distractible, unable to concentrate	
8.	Impaired judgment and impulsiveness	
9.	Acting recklessly without thinking about the consequences	
10.	Delusions and hallucinations	
11.	Feeling hopeless, sad, or empty	
12.	Irritability	
13.	Inability to experience pleasure	
14.	Fatigue or loss of energy	
15.	Physical and mental sluggishness	
16.	Appetite or weight changes	
17.	Sleep problems	
18.	Concentration and memory problems	
19.	Feelings of worthlessness or guilt	
20.	Thoughts of death or suicide	

Thank you for your participation, it is highly appreciated.

Official use only:

Laboratory ID number:_____

Appendix E

Agtergrondinligting

Verskaf asseblief die volgende informasie deur om 'n regmerk (✓) by die gepaste antwoord te gee en om die vermiste informasie in te vul.

Geboortedatum:	_____
Geslag:	☐ Manlik ☐ Vroulik
Etniese oorsprong (merk ✓ net een):	☐ Wit ☐ Swart ☐ Kleurling ☐ Indiër ☐ Asiër ☐ Ander, spesifiseer asseblief: _____
Is die etniese oorsprong van jou ouers die selfde as joune?	☐ Ja ☐ Nee Indien nee, spesifiseer asseblief die etniese oorsprong van jou Vader_____ Moeder_____
Is die etniese oorsprong van jou grootouers die selfde as joune?	☐ Ja ☐ Nee Indien nee, spesifiseer asseblief die etniese oorsprong van jou Oupa (Vader se kant)_____ Ouma(Vader se kant)_____ Oupa(Moeder se kant)_____ Ouma(Moeder se kant)_____
Huis taal:	☐ Afrikaans ☐ Engels ☐ isiZulu ☐ IsiNdebele ☐ IsiXhosa ☐ Sepedi ☐ Setswana ☐ Sesotho ☐ Xitsonga ☐ SiSwati ☐ Tshivenda ☐ Ander, spesifiseer asseblief: _____
Is jy met bipolêre versteuring 1 of 2 gediagnoseer?	☐ 1 ☐ 2
Watter ouderdom was jy met bipolêre versteuring gediagnoseer?	_____
Watse medikasie neem jy vir jou toestand?	_____

Verskaf asseblief die volgende informasie deur om 'n regmerk (✓) by die gepaste antwoord te gee en om die vermiste informasie in te vul.

Gesondheid en mediese geskiedenis verwant aan metaboliese sindroom

1.	Oefen u minder as 2 keer per week?	Ja	Nee
2.	Rook u sigarette op 'n gereelde basis? Indien ja, hoeveel rook u per dag?_____	Ja	Nee
3.	Drink jy alkohol op 'n gereelde basis? Hoeveel alkoholiese drankies drink u per week?_____	Ja	Nee
4.	Volg u 'n gebalanseerde dieet? (bestaan van die korrekte verhouding van al die voedingstowwe soos koolhidrate, vette, proteïene, minerale en vitamiene saam met voldoende water en vesel materiaal).	Ja	Nee
5.	Wat is u gewig in kilogram? _____		
6.	Wat is u hoogte in meter? _____		
7.	Het jy 'n middellyf meting van meer as 102 cm (40 duim) of meer as u manlik is, of 89 cm (35 duim) of meer as u vroulik is?	Ja	Nee
8.	Ly u aan enige kardiovaskulêre (hart) kondisies of siektes? Indien ja, spesifiseer asseblief_____ Op watter ouderdom was jy gediagnoseer?_____ Watse medikasie neem jy vir jou toestand? _____	Ja	Nee
9.	Ly u aan diabetes (1 of 2)? Indien ja, spesifiseer asseblief _____ Op watter ouderdom was u gediagnoseer?_____ Watse medikasie neem u vir u toestand? _____	Ja	Nee
10.	Het u hoë bloeddruk? Op watter ouderdom was u gediagnoseer?_____ Watse medikasie neem u vir u toestand? _____	Ja	Nee
11.	Ly u aan hoë cholesterol vlakke? Op watter ouderdom was u gediagnoseer?_____ Watse medikasie neem u vir u toestand? _____	Ja	Nee
12.	Ly jy aan enige ander gediagnoseer mediese toestand nie genoem nie? Indien ja, spesifiseer asseblief_____ Op watter ouderdom was u gediagnoseer?_____ Watse medikasie neem u vir u toestand? _____	Ja	Nee
13.	Is u vetsugtig?	Ja	Nee
14.	Het u enige familie-lede wat aan enige van die bogenoemde kondisies ly? Indien ja, spesifiseer asseblief kondisie en familie lid_____ _____ Indien nie, is daar enide ander kondisies wat in u se familie loop? Asseblief spesifiseer kondisie en familie lid _____ _____	Ja	Nee

Bipolêre Versteuring

Die volgende vraelys evalueer die simptome wat u ondervind het voordat u behandelling ontvang het. Merk asseblief (✓) die antwoorde wat die beste pas. Jy kan meer as een merk.

1.	Voel abnormaal "hoog" en optimisties	
2.	Voel baie prikkelbare	
3.	Onrealisties, grootse oortuigings oor 'n mens se vermoëns of magte	
4.	Slaap baie min, maar voel vol energie	
5.	Praat so vinnig dat ander nie kan byhou nie	
6.	Wedrenne gedagtes, spring vinnig van die een gedagte na die volgende	
7.	Hoogs afleibaar, moeilikheid om te konsentreer	
8.	Verswakte oordeel en impulsiwiteit	
9.	Roekeloos gedrag sonder om oor die gevolge te dink	
10.	Delusies en hallusinasies	
11.	Voel hopeloos, hartseer, of leeg	
12.	Prikkelbaarheid	
13.	Moeilikheid om plesier te ervaar	
14.	Moegheid en verlies van energie	
15.	Fisiese en geestelike traagheid	
16.	Eetlus of gewig veranderinge	
17.	Slaap probleme	
18.	Konsentrasie en geheue probleme	
19.	Gevoelens van waardeloosheid of skuld	
20.	Gedagtes van dood of selfmoord	

Dankie vir jou deelname, dit word opreg waardeer.

Vir Amptelike gebruik:

Laboratorium ID-nommer:_____