Genetics Factors Contributing to Body Weight in Anorexia Nervosa and Bulimia Nervosa

by

Zeynep Yilmaz

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy (PhD)

Institute of Medical Science

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Abstract

Anorexia nervosa (AN) is an eating disorder (ED) with substantial morbidity and the highest mortality among psychiatric disorders. Low body mass index (BMI) is the *sine qua non* of AN, and behaviours associated with reaching it are the primary reason for AN's high morbidity and mortality. Low BMI is also the main criterion diagnostically separating AN from bulimia nervosa (BN). The aim of this dissertation was to determine the role of genes regulating weight and appetite in BMI in AN and BN. Study 1 utilized carefully selected DNA samples to explore the role of markers in the leptin, melanocortin, and neurotrophin system genes with known or putative function in AN, BN, and controls, as well as in lifetime BMIs in EDs. Study 2 investigated dopamine pathway genes and *FTO* in weight regulation in a large sample of AN cases. The results revealed that an *MC4R* variant linked to antipsychotic-induced weight gain was underrepresented in AN, and *AGRP* and *NTRK2* genetic variants were linked to minimum BMI in AN and maximum BMI in BN, respectively. In Study 2, a significant association between *FTO* and BMI at recruitment was observed. To our knowledge, this is the first study to utilize two distinct but complementary genetic approaches in the study of weight in EDs. These genetic findings

may serve as an important first step toward gaining a better understanding of weight regulation in AN and BN, as well as having the potential for developing more effective treatment options and providing a highly specific target for novel medications. Alongside this work, other ED genetic studies utilizing different clinical phenotypes were also carried out during my PhD, and the papers published are inserted as appendices for reasons of thematic unity.

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List of Abbreviations*

α-MSH	Alpha-melanocyte-stimulating hormone
5-HTTLPR	43-bp insertion/deletion polymorphism in the promoter region of *SLC6A4* gene
7R	7-repeat variant of the *DRD4* gene
AAO	Age at onset
ABA	Activity-based anorexia
ADHD	Attention-deficit/hyperactivity disorder
ADRB2	β-2 adrenergic receptor
AGRP	Agouti-related protein
AIWG	Antipsychotic medication-induced weight gain
Ala	Alanine
AN	Anorexia nervosa
AN-ARP	Price Foundation anorexia nervosa affected relative pair study
AN-BP	Anorexia nervosa, binge/purge subtype
AN-R	Anorexia nervosa, restricting subtype
ANT-CW	Price Foundation anorexia nervosa trio/control women study
AIM	Ancestry informative marker
Arg	Arginine
BDNF	Brain derived neurotrophic factor
BED	Binge eating disorder
BMI	Body mass index
BN	Bulimia nervosa
BN-ARP	Price Foundation bulimia nervosa affected relative pair study
bp	Base-pair
CACNA1C	Calcium channel, voltage-dependent, L type, alpha 1C subunit
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine-regulated transcript
CBT	Cognitive behaviour therapy
CCK	Cholecystokinin
CEU	European Caucasian ancestry
CHB	Chinese Han ancestry
CNR1	Cannabinoid receptor 1
CNS	Central nervous system
CNV	Copy number variant
COL4A5	Collagen, type IV, alpha 5
COMT	Catechol-O-methyltransferase
CTL	Control
curBMI	Current body mass index; body mass index at recruitment

CYP2D6	Cytochrome P450 2D6
Cys	Cysteine
DAT	Dopamine transporter
DBH	Dopamine beta hydroxylase
dH_2O	Distilled water
D1	Dopamine 1
D2	Dopamine 2
DISC1	Disrupted in schizophrenia 1
DNA	Deoxyribonucleic acid
DRD1	Dopamine receptor D1
DRD2	Dopamine receptor D2
DRD3	Dopamine receptor D3
DRD4	Dopamine receptor D4
DRD5	Dopamine receptor D5
DSM	Diagnostic and Statistical Manual for Psychiatric Disorders
ED	Eating disorder
EDNOS	Eating disorder not otherwise specified
EMSA	Electromobility shift assay
ESR1	Estrogen receptor 1
ESR2	Estrogen receptor 2
FAAH	Fatty acid amide hydrolase
fMRI	Functional magnetic resonance imaging
FTO	Fat mass and obesity associated
GABA	γ-aminobutyric acid
GCAN	Genetics Consortium for Anorexia Nervosa
GHRL	Ghrelin
GHS-1RA	Growth hormone secretagogue receptor
Gln	Glutamine
GLP2	Glucagon-like peptide 2
GWAS	Genome-wide association study
HRH1	Histamine receptor H1
HTR1A	Serotonin receptor 1A
HTR1B / HTR1Dβ	Serotonin receptor 1B
HTR2A	Serotonin receptor 2A
HTR2C	Serotonin receptor 2C
HWE	Hardy-Weinberg Equilibrium
Ile	Isoleucine
JPT	Japanese ancestry
KCNN3	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3

kb	Kilobase
KO	Knockout
LD	Linkage disequilibrium
L-DOPA	Levodopa (L-3,4-dihydroxyphenylalanine)
LEP	Leptin
LEPR	Leptin receptor
Leu	Leucine
logAAO	Log10-transformed age at onset
logcurBMI	Log10-transformed current body mass index
logmaxBMI	Log10-transformed maximum lifetime body mass index
Lys	Lysine
MAF	Minor allele frequency
MAOA	Monoamine oxidase A
maxBMI	Maximum lifetime body mass index
MC3R	Melanocortin 3 receptor
MC4R	Melanocortin 4 receptor
MDS	Multidimensional Scaling
Met	Methionine
MHC	Major histocompatibility complex
minBMI	Minimum lifetime body mass index
MRI	Magnetic resonance imaging
miRNA	micro ribonucleic acid
mRNA	Messenger ribonucleic acid
NAS	N-acetylserotonin
NIEHS	National Institute of Environmental Health Sciences
NPY	Neuropeptide Y
NT3	Neurotrophin 3
NTRK2 / TrkB	Neurotrophic tyrosine kinase receptor type 2
NTRK3 / TrkC	Neurotrophic tyrosine kinase receptor type 3
OCD	Obsessive compulsive disorder
OPRD1	Opioid receptor D1
OPRM1	Opioid receptor mu 1
PCR	Polymerase chain reaction
PGC	Psychiatric Genomics Consortium
PF	Price Foundation
POMC	Pro-opiomelanocortin
QC	Quality control
RDoC	Research Domain Criteria
RNA	Ribonucleic acid
SCID-I	Structured Clinical Interview for DSM-IV, Axis I Disorders

SIAB-EX	Structured Interview of Anorexia Nervosa and Bulimic Syndromes
SLC6A3	Solute carrier family 6, member 3; dopamine transporter
SLC6A4	Solute carrier family 6, member 4; serotonin transporter
SMR	Standardized mortality ratio
SNP	Single nucleotide polymorphism
TH	Tyrosine hydroxylase
Thr	Threonine
Tyr	Tyrosine
UTR	Untranslated region
VGLL4	Vestigial like 4 (Drosophila)
VNTR	Variable number tandem repeat
WTCCC	Wellcome Trust Case-Control Consortium
WTCCC3	Wellcome Trust Case-Control Consortium, Third Wave
YRI	Yoruba in Ibadan, Nigeria ancestry
ZNF804A	Zinc finger protein 804A
ZNF804B	Zinc finger protein 804B

* Abbreviations that are most relevant for this dissertation and regularly used in the main body of text are in bold.

Note: Names of genes are italicized throughout the text. Human genes are indicated in capital letters, whereas only the first letter is capitalized for animal genes (e.g., *BDNF* in humans versus *Bdnf* in mice).

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CHAPTER 1

INTRODUCTION

1.1. Individual, Collective, and Financial Burden of Anorexia Nervosa and Bulimia Nervosa

Anorexia nervosa (AN) is a serious eating disorder (ED) with substantial morbidity and the highest lifetime mortality among psychiatric disorders (Sullivan, 1995). About 90% of patients afflicted with AN are women, and the prevalence rate is as high as 1% including subsyndromal cases (American Psychiatric Association, 2000). The illness typically begins in adolescence and often runs a chronic course. Biological, psychological, and environmental factors contribute to the development of AN, making it a complex illness with multidimensional etiology.

Since its first description in the 17th century, AN still remains largely a treatment-resistant illness with disturbingly high morbidity and mortality. Medication trials are yet to identify a medication with clear benefit, and rigorously controlled psychotherapy studies are sparse (American Psychiatric Association, 2000). Based on extensive literature reviews and large population cohort investigations, the standardized mortality ratio (SMR) associated with AN is estimated to range between 5.9 and 6.2, meaning that there are up to 520% more cases of death due to AN than in the general population for the same age range, with a weighted annual mortality rate of 5 per 1000 person years (Papadopoulos et al., 2009; Arcelus et al., 2011). Younger age is associated with better outcome, a finding that highlights AN's chronic nature in adult patients (Papadopoulos et al., 2009; Arcelus et al., 2011). Most common reasons for high SMR are AN-related medical complications and suicide (Papadopoulos et al., 2009; Arcelus et

al., 2011). Over two decades, only half of those with AN fully recover from this devastating disorder (Lowe et al., 2001), and due to the misconceptions held by the general public (Darby et al., 2012) and the stigma attached to having an ED, many individuals refrain form seeking much-needed treatment.

In addition to the high mortality and morbidity, AN is also a costly illness to treat, with the cost possibly being as high as US $8,042 purchasing power parities (Stuhldreher et al., 2012). In the previous years, Statistics Canada reported that the province of Ontario spent $8,000,000 for out-of-country treatments for AN, as facilities are limited and have long wait times. However, research dollars spent on EDs in 2012 averaged to be under $7 per affected individual in the United States (National Institutes of Health, 2012), drawing attention to the desperate need for more research funding for this devastating illness.

Bulimia nervosa (BN) has a prevalence rate of 2-3% in a young female population, and similar to AN, 90% of those who suffer from BN are women (American Psychiatric Association, 2000). Effective treatments for BN are available but not widely disseminated to primary care clinicians. Furthermore, due to the secrecy and shame associated with binge eating and purging, many individuals with BN refrain from seeking treatment. Over a decade, while 51% of those with BN will meet criteria for good outcome (Clausen, 2004), 11% will still meet full diagnostic criteria for this disorder, and over an additional 20% will still suffer from subclinical disordered eating (Keel et al., 1999). The SMR for BN is estimated to be 1.9 (Arcelus et al., 2011). In addition to the mortality issue, the financial cost associated with BN is a significant burden to the individual as well as the society: it has been reported that the mean total food cost for binge eating and purging in individuals with BN is close to US $1,600 per annum, which is a third of their annual food cost (Crow et al., 2009). Outpatient treatment costs for BN can run up to US

$2,400 per annum (Koran et al., 1995) and US $5,016 purchasing power parities (Stuhldreher et al., 2012).

In summary, full-syndrome and subsyndromal EDs are relatively common in the general population and are associated with a significant increase in mortality, especially in young women. Considering the high cost of treatment, lost wages and disability claims, AN and BN pose a serious financial burden to the individual, health care system, and the society as a whole. There are no evidence-based treatments for AN and many patients with BN relapse, thus it is of utmost importance to focus research efforts on the etiology and treatment of EDs.

1.2. Diagnostic Criteria for AN and BN[1]

EDs are characterized by the presence of disordered eating behavior as well as characteristic psychological disturbance. The Diagnostic and Statistical Manual of Mental Disorders Fourth Edition (DSM-IV) recognized two EDs: AN and BN (American Psychiatric Association, 2000). Another ED, binge eating disorder (BED), has been officially recognized as an ED diagnosis in the Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (DSM-5; American Psychiatric Association, 2013). As the focus of this dissertation is on the EDs associated with caloric restriction and the pursuit of a low body weight, BED will not be discussed in detail, and the focus will be on AN and BN.

The root of the term anorexia nervosa comes from the Greek word *orexis* (‘appetite’), and the term *anorexia* meaning ‘lack of appetite’ is a misnomer. In his 1689 book entitled “*Treatise of Consumptions,”* English physician Richard Morton described the first documented case in the

[1] This section is largely excerpted with a few updates from the text I wrote originally for a book chapter as a co-author: Kaplan AS, Yilmaz Z. Eating disorders in primary care mental health clinics. In: Ivbijaro G, editor. World Organization of Family Doctors (Wonca) in partnership with the World Health Organization: companion to primary care mental health. Milton Keynes (UK): London; 2012. p. 421-44. Appropriate permissions are included in the Copyright Acknowledgements section of the dissertation.

medical literature of extreme weight loss and wasting without any evidence of known disease as "nervous atrophy, or consumption." The actual term *anorexia nervosa,* however, was coined independently by two physicians. In his 1873 report, Charles Lasegue—a French physician—published his case studies of young women suffering from *l'anorexie hysterique*. English physician Sir William Gull, who was also the attending physician to Queen Victoria, coined the current term *anorexia nervosa* for this illness as a part of his description of this syndrome in an address published in the Lancet around the same time (Brumberg, 2000). Some historians argue that Catherine of Siena and other fasting saints of the Middle Ages were the first high-profile cases of AN; however, it is difficult to validate these terms (Vandereycken and van Deth, 1994). The original DSM published in 1952 featured AN as a diagnostic category (American Psychiatric Association, 1952), and AN has been in the media spotlight since the 1970s.

Although binge eating and purging date back to Roman times, BN as a psychiatric syndrome can be classified as more of a modern illness, first named and described about three decades ago (Russell, 1979). The first inclusion of this disorder as an ED was in the DSM-III in 1980, under the name *bulimia*, which means 'ox hunger' in Greek (American Psychiatric Association, 1980). The name was then changed to *bulimia nervosa* in the DSM-III-R a few years later (American Psychiatric Association, 1987). Finally, binge eating without any compensatory behaviors (as in BED) is now recognized in the DSM-5, published in May 2013, as an official ED diagnosis.

Below are the DSM-IV criteria for AN and BN. Because the studies covered in this dissertation utilized DSM-IV diagnostic criteria, revised criteria of AN and BN as a part of the DSM-5 are listed in the Appendix (Section A1).

1.2.1. DSM-IV Criteria for AN

The pathognomonic feature of AN is low weight. The DSM-IV criteria (American Psychiatric Association, 2000) for AN are:

A. Refusal to maintain body weight at or above a minimally normal weight for age and height (weight loss leading to maintenance of body weight less than 85% of that expected; or failure to make expected weight gain during a period of growth, leading to a body weight less than 85% of that expected).
B. Intense fear of gaining weight or becoming fat even though underweight.
C. Disturbance in the way in which one's body weight is experienced, undue influence of body weight or shape on self evaluation, or denial of the seriousness of the current low body weight.
D. In post-menarchal females, amenorrhea (the absence of three consecutive menstrual cycles)

Subtypes:

1. Restricting Type (AN-R): during the current episode of AN, the person has not regularly engaged in binge-eating or purging behavior.
2. Binge-Eating/Purging Type (AN-BP): during the current episode of AN, the person has regularly engaged in binge-eating or purging behavior.

1.2.2. DSM-IV Criteria for BN

The pathognomonic clinical feature of BN is binge eating with compensation. The DSM-IV criteria for BN are:

A. Recurrent binge eating: An episode of binge eating is characterized by: eating, in a discrete period of time, an amount of food that is definitely larger than most people

would eat during a similar period of time or under similar circumstances. It also consists of a sense of lack of control over eating during the episode (e.g., a feeling that one cannot stop eating or control how much one is eating).

B. Recurrent inappropriate compensatory behavior to prevent weight gain, such as self-induced vomiting, misuse of laxatives, diuretics, enemas or other medications; fasting or excessive exercise.

C. The binge-eating and compensatory behavior in order to prevent weight gain both occur, on average, at least twice a week for 3 months.

D. Self-evaluation is unduly influenced by body weight and shape.

E. The disturbance does not occur exclusively during episodes of AN.

Subtypes:

1. The purging type: during the current episode of BN, a person has regularly engaged in self-induced vomiting or the misuse of laxatives, diuretics or enemas.
2. The non-purging type: the person has used other inappropriate compensatory behaviors, such as fasting or excessive exercise, but has not regularly engaged in the purging methods.

1.3. Clinical Phenotypes Associated with AN and BN[2]

1.3.1. Depression and Anxiety

Many ED patients also present with current or lifetime history of depression. The prevalence rate of major depression is estimated to be up to 86% for AN and 63% for BN

[2] This section is largely excerpted with a few updates from the text I wrote originally for a book chapter as a co-author: Kaplan AS, Yilmaz Z. Eating disorders in primary care mental health clinics. In: Ivbijaro G, editor. World Organization of Family Doctors (Wonca) in partnership with the World Health Organization: companion to primary care mental health. Milton Keynes (UK): London; 2012. p. 421-44. Appropriate permissions are included in the Copyright Acknowledgements section of the dissertation.

(O'Brien and Vincent, 2003). Comorbidity rates obtained from population studies are often lower than the rates observed in clinical settings, with the prevalence of depression estimated to be 39.1% in AN and 50.1% in BN according to a population-based survey (Hudson et al., 2007). There is up to 23-fold increase in suicide rates among ED patients compared to the general population, and these rates are especially high for those who engage in purging behaviours (Foulon et al., 2007). Furthermore, comorbid depression may increase the risk of physiological abnormalities observed in ED patients: individuals with BN and comorbid depression have significantly elevated levels of afternoon cortisol compared to BN patients without comorbid depression (Yilmaz et al., 2012b). This pattern of cortisol nonsupression may be an indicator of ongoing physiological stress response in a subgroup of BN patients.

Anxiety disorders are also common among individuals with EDs. About 55-83% of AN cases and 68-71% of BN cases present with a lifetime history of at least one anxiety disorder (Godart et al., 2000; Kaye et al., 2004a). Population-based studies estimate the prevalence rate of anxiety disorders to be 47.9% in AN and 80.6% in BN (Hudson et al., 2007). Obsessive compulsive disorder (OCD) is one of the most prevalent anxiety disorders among EDs, with as much as a third of ED patients also suffering from OCD (O'Brien and Vincent, 2003). OCD rates are shown to be higher among patients with AN compared to those with BN, and OCD is significantly overrepresented among the siblings of individuals of BN compared to the general population (Bellodi et al., 2001). Social phobia and generalized anxiety disorder are also highly comorbid with EDs: in one study, a large portion of women with EDs, regardless of the specific diagnosis, scored high on social avoidance and distress, the key criterion for social phobia (Penas-Lledo et al., 2010). The main triggers of social phobia in EDs have been proposed to be the fear of negative evaluation and social appearance anxiety (Levinson and Rodebaugh, 2012).

1.3.2. Substance Use/Abuse

ED patients (especially those who engage in binge eating) have elevated rates of substance use—alcohol and street drugs—compared to the general population. Up to 35% of teenagers with EDs have substance abuse problems, and substance use is often associated with poor outcome and serves as an important severity indicator, especially for the binge-purge spectrum of EDs (Castro-Fornieles et al., 2010). Similarly, a large population-based survey found that 27% of individuals with AN and 36.8% of individuals with BN meet criteria for substance abuse (Hudson et al., 2007). One of the most abused substances in EDs is alcohol, with as many as a quarter of ED patients reporting alcohol use-related problems (Baker et al., 2010). It is not uncommon for patients to seek out street drugs that result in weight loss, such as amphetamine-containing substances and other stimulants. Abuse of prescription drugs given for other conditions—especially if they lead to appetite suppression and weight loss—is also common among ED patients. These prescription drugs include but are not limited to methylphenidate (Ritalin) and thyroid preparations for hypothyroidism. A variety of over-the-counter diet pills, diuretics and supplements such as ephedrine are also abused in order to either suppress weight or maintain low weight. While substance abuse often precedes the onset of the ED in BN, the opposite pattern is more common for AN (Baker et al., 2010).

1.3.3. Personality and Temperament

There are significant differences in personality traits between individuals with AN and BN. AN patients are more likely to be compulsive, over-controlled, socially avoidant, conflict-averse, passive and fearful. In contrast, BN patients are often impulsive and thrill-seeking, and the likelihood of engaging in impulsive behaviours such as self-harm, stealing, substance abuse and promiscuity is high (Federici and Kaplan, 2009). A meta-analysis investigating temperament

among psychiatric diagnoses has shown that AN is associated with a significantly higher score on the persistence scale of personality dimensions compared to other psychiatric diagnoses, whereas individuals with BN score highest for novelty seeking (Miettunen and Raevuori, 2012). Women with AN also tend to score higher in harm avoidance compared to nonpsychiatric controls, which also appears to be independent of low body mass index (BMI; Klump et al., 2000), thus likely to precede ED onset. Perfectionism is another trait that is often associated with AN: many individuals with AN, regardless of subtype, report significant childhood perfectionism and rigidity (Halmi et al., 2012), which may be important risk factor for this ED.

1.3.4. Attention-Deficit/Hyperactivity Disorder

Classical symptoms of attention-deficit/hyperactivity disorder (ADHD) are reported by as much as a third of patients who regularly engage in binge eating (Fleming and Levy, 2002). We have recently reported that a history of childhood ADHD may be present in approximately 24% of BN cases (Yilmaz et al., 2011; Yilmaz et al., 2012c; see Appendices A5 and A6), and impulsivity may be an indicator of BN symptom severity (Surman et al., 2006). In a large population-based survey, the prevalence rate of ADHD was approximately 35% in BN (Hudson et al., 2007). From the opposite perspective, females with ADHD are reported to be at 6-fold higher risk for developing an ED compared to non-ADHD controls (Surman et al., 2006; Biederman et al., 2007). Individuals who engage in binge eating may be inattentive to their internal sense of hunger, satiety and amount of food consumed on a daily basis, which is a phenomenon also observed in individuals with ADHD (Fleming and Levy, 2002). Furthermore, elevated impulsivity and a lack of inhibition may also play a role in triggering binges (Schweickert et al., 1997).

1.4. Epidemiology of AN and BN[3]

The lifetime prevalence rate of AN is 0.3-1% among young females (Hoek, 1993; Gotestam and Agras, 1995; Hudson et al., 2007; Treasure et al., 2010), and 90% of the those afflicted are women. A recent nation-wide survey conducted in the United States reported the prevalence rate of AN to be around 0.3% among adolescents aged 13 to18 (Swanson et al., 2011). Onset of the illness often occurs in adolescent years; however, prepubescent onset is not uncommon, and similarly, stressful life events may lead to the development of AN later in life. Research on male patients is scarce, but male patients with AN on average are older, present with higher BMI and are less likely to be suicidal compared to female patients with AN (Gueguen et al., 2012).

Binge eating behaviour is relatively common in the general population (Hudson et al., 2007). With the inclusion of subsyndromal cases, up to 5.9% of women and 1.5% of men in Germany are reported to suffer from disturbed eating (Hilbert et al., 2012), which includes but is not limited to binge eating. Bulimic tendencies often have their onset during adolescence and early adult years, and as many as 13% of North American college students display varying degrees of bulimic symptoms (Halmi et al., 1981; Rush et al., 2009). However, regular binge eating associated with characteristic psychopathology with or without purging is less prevalent and is a syndrome that requires psychiatric attention. BN is estimated to affect 2-3% of young adult women, and similar to the pattern observed in AN, about 90% of patients are female (American Psychiatric Association, 2000).

[3] This section is largely excerpted with a few updates from the text I wrote originally for a book chapter as a co-author: Kaplan AS, Yilmaz Z. Eating disorders in primary care mental health clinics. In: Ivbijaro G, editor. World Organization of Family Doctors (Wonca) in partnership with the World Health Organization: companion to primary care mental health. Milton Keynes (UK): London; 2012. p. 421-44. Appropriate permissions are included in the Copyright Acknowledgements section of the dissertation.

For many years, EDs were thought to occur almost exclusively in young women of European descent with an upper middle-class upbringing (Bruch, 1978). This theory has been seriously challenged in the past few decades, as EDs now cross ethnic, racial and social-class lines (American Psychiatric Association, 2000). Although EDs are still more commonly observed in Western societies, they also occur in non-Western cultures. A brief review of the literature on the prevalence of EDs in non-Western cultures and developing countries is included in the Appendix (Section A2).

1.5. Outcome of AN and BN

AN often follows a chronic course, and as a result of this, ongoing follow-up after completion of a treatment program is the norm rather than the exception (Long et al., 2012). In primary care settings, up to 57% of AN patients and 61% of BN patients are considered to be fully recovered five years after treatment (van Son et al., 2010). However, it is important to consider the possibility that more severe cases of AN and BN are often referred for specialist care, which may explain the relatively high recovery rates reported in primary care cohorts compared to psychiatric settings. In specialist settings, however, less than 50% of AN patients achieve full recovery, roughly a third of the patients improve, and 20% develop a chronic course of the disorder (Steinhausen, 2002). A 12-year outcome study reported more grim statistics, with 27.5% of those with AN having a good outcome, 25.3% having an intermediate outcome, 39.6% having a poor outcome, and close to 8% having been deceased at the end of 12 years (Fichter et al., 2006).

Differences in recovery patterns for AN-R and AN-BP subtypes have been widely reported. AN-R may be associated with a greater degree of recovery compared to AN-BP (Ward et al., 2003), whereas individuals with AN-BP who utilize other purging behaviours in addition

to self-induced vomiting have poorer treatment outcome compared tor restrictors or AN-BP patients for whom self-induced vomiting is the only purging behaviour (Stoving et al., 2012). Upon exiting treatment, although AN-BP patients are more likely to improve on secondary psychometric measures compared to AN-R patients, they are also more likely to be remitted in the future (Long et al., 2012). One possible reason for this counterintuitive pattern is the distress associated with binge eating and purging, which may make the individuals with AN-BP more likely to seek treatment compared to restrictors whose symptoms are more ego-syntonic. Furthermore, due to the complex comorbidities associated with binge eating and purging, weight restoration alone is not likely to be an effective treatment approach for AN-BP (Ward et al., 2003).

Outcome for BN is often better compared to AN: similar to the pattern observed in addictions, the course of BN is defined by cycles of recovery and relapse. Long-term outcome studies have consistently shown that about 55-70% of BN patients fully or partially recover, whereas 30% of the cases either become chronic or crossover to another ED (Garfinkel et al., 1995; Keel et al., 1999; Keski-Rahkonen et al., 2009).

Although treatment is not the focus of this doctoral dissertation, there is merit in a brief review of the state of treatment for both AN and BN, especially considering the chronic nature of the former and recovery/relapse cyclic nature of the latter. Please refer to the Appendix for a review of the most recent psychological and pharmacological approaches (Section A3). In summary, there is dire need for rigorous psychotherapy and medication treatment studies for AN to improve recovery rate, reduce chronicity, and improve patients' quality of life. Although effective treatments exist for BN, development of new methods and protocols could boost

recovery rates, reduce the frequency of relapse cycle, and potentially lead to more cost-effective treatment options.

1.6. The Current State of Psychiatric Genetics

Compared to other medical conditions, psychiatric disorders have their unique set of challenges to overcome due to the complex nature of human behaviour and psychopathology: psychiatric syndrome definitions are based on clinical consensus as opposed to reliable biomarkers, and the presence of significant clinical heterogeneity poses further complications to biological studies of these disorders. As a result of this, psychiatric genetics as a field took longer to bloom than the rest of medical genetics. The majority of the genetic vulnerabilities in psychiatry are non-Mendelian in nature: while Mendelian disorders are typified by a single relatively rare genetic mutation leading to a clear phenotype with high penetrance and dominant versus recessive model, it is more likely that a large number of common variants (each of which has only a small effect) cumulatively increase susceptibility to a possible cluster of syndromes or phenotypes within psychiatric disorders (Sullivan et al., 2012). However, despite these unique challenges, our understanding of the biological etiology of psychiatric disorders has come a long way in the last few decades.

Psychiatric genetics is a large field with thousands of research studies and hundreds of different phenotypes, and a more comprehensive review on the state of psychiatric genetics can be found elsewhere (e.g., Züchner et al., 2007; Burmeister et al., 2008; Sullivan et al., 2012). In this section, I will highlight some of the most significant genetic findings that have changed the way researchers think about the biology of psychiatric disorders, as well as summarize the more commonly used research methods in psychiatric genetics.

Initial groundbreaking genetic findings in psychiatry came from pedigree studies. The discovery of disrupted in schizophrenia 1 (*DISC1*) gene in a Scottish pedigree with a history of psychotic and affective disorders (Millar et al., 2000) and the presence of 22q11 deletion in a subset of schizophrenia patients (Chow et al., 1994; Lindsay et al., 1995) significantly impacted the field of psychiatric genetics. For Alzheimer's Disease, linkage findings on chromosome 19 led to the important discovery of the role of apolipoprotein E E4 variant in decreasing the rate of proteolytic break-down of the peptide beta-amyloid, thus leading to plaque buildup (Pericak-Vance et al., 1991; Corder et al., 1993). Pedigree studies often focus on rare variants, and this method is still used successfully in the study of developmental disorders such as mental retardation and autism (Mir et al., 2009; Mochida et al., 2009; Noor et al., 2010; Rafiq et al., 2010).

Following the initial wave of successful pedigree studies, candidate gene studies have further improved our understanding of psychiatric etiology. By developing *a priori* hypotheses and selecting candidate genes based on biological function (as demonstrated in *in vitro*, *in vivo* or animal studies), candidate gene researchers have significantly contributed to our understanding of the neurobiology and genetics of psychiatric syndromes and psychopathology. One particular example out of many is the 48 base-pair (bp) variable number tandem repeat (VNTR) polymorphism located in exon III of the dopamine receptor D4 (*DRD4*) gene, which was identified as a risk locus for ADHD over a decade ago (LaHoste et al., 1996; Faraone et al., 2001). Since then, research has consistently shown a robust association between the 7-repeat (7R) variant of this polymorphism and ADHD (Gizer et al., 2009), one of the most consistently replicated genetic associations in the field of psychiatric genetics. In addition to its link to childhood ADHD, our extended group has shown that this hypofunctional variant is also

associated with adult ADHD (Muglia et al., 2000), and in the case of BN, with maximum lifetime BMI (Kaplan et al., 2008; Levitan et al., 2010) and a history of childhood ADHD (Yilmaz et al., 2012c). However, alongside findings that stood the test of time, other candidate gene results did not replicate in follow-up studies due to small sample size, clinical heterogeneity, and population stratification (Sullivan et al., 2012). Nevertheless, candidate gene technique has established itself as a valid methodology that builds upon prior basic research in hopes to gain insight into psychiatric etiology informed by biological function.

With the advances in technology and a shift of emphasis from individual research groups to large consortia, genome-wide association studies (GWAS) and high-throughput sequencing projects have become more feasible and affordable to carry out. Recent GWAS collaborations have lead to the identification of new genes and regions of susceptibility for schizophrenia and bipolar disorder (Sklar et al., 2008; International Schizophrenia Consortium, 2008; Walsh et al., 2008; O'Donovan et al., 2008; Stefansson et al., 2009; Green et al., 2012). Calcium channel, voltage-dependent, L type, alpha 1C subunit (*CACNA1C*) gene on chromosome 12 and the major histocompatibility complex (*MHC*) region on chromosome 16 have especially garnered attention, with the former regulating calcium signaling responsible for neuronal excitation and the latter playing an important role in immune response. Despite the small effect size of these genes, carrying an increased number of susceptibility alleles significantly increases risk for psychiatric disorders (International Schizophrenia Consortium et al., 2009), with central nervous system (CNS) genes explaining a larger portion of the total variance compared to the rest of the genome (Lee et al., 2012a). Although initial efforts mostly focused on schizophrenia and bipolar disorder, other GWAS projects have included DNA samples from individuals with other psychiatric diagnoses such as major depression and OCD (McGuffin et al., 2005; Mathews et al., 2012).

While the focus of GWAS is common variants with small effect size, high-throughput sequencing allows researchers to identify *de novo* and/or rare variants with relatively larger effect size (Gershon et al., 2011). Indeed, high-throughput exome sequencing of pedigrees has been fruitful in autism research (Neale et al., 2012). Studies of rare copy number variants (CNVs) have suggested that CNVs may be more frequent in individuals with psychiatric disorders (regardless of the specific diagnosis) compared to the general population (Saus et al., 2010; Van Den Bossche et al., 2012), and variability in phenotype could be due to incomplete penetrance, resulting in some individuals carrying the risk loci not expressing the trait (Van Den Bossche et al., 2012). With the sample sizes continuing to increase as a part of expansion of international consortia, the improved statistical power is likely to lead to more genome-wide discoveries involving both common and rare variants in psychiatric disorders.

1.7. Genetics of AN and BN

In the last few decades, there has been a significant change in our understanding of the etiology of AN and BN. Previously, EDs were thought to be almost entirely due to environmental factors, including the objectification of women and idealization of thinness by the society, as well as dysfunctional family dynamics. More recently, the knowledge of genetics and heritability in psychiatric disorders has greatly increased, and with the development of more sophisticated tools, researchers are now able to make more meaningful connections between complex disorders and genes. It is now understood that EDs have sociocultural, psychological and neurobiological risk factors that contribute to their etiology.

In this section, I will summarize the literature on heritability, linkage and candidate gene studies looking at gene systems that were not studied as a part of this dissertation. More

comprehensive reviews of the genetic findings in EDs are available elsewhere (e.g., Clarke et al., 2012; Trace et al., 2013).

1.7.1. Heritability

EDs are at least moderately heritable. Although both AN and BN tend to run in families, heritability is more significant in the case of AN. The heritability index obtained from identical twin studies is up to 0.71 for AN, meaning that 71% of phenotypic variation can be explained by additive genetic factors (Kipman et al., 1999). In addition, female relatives of AN patients are up to 11 times more likely to develop AN compared to females without a relative with AN (Strober et al., 2000). Heritability of BN is estimated to be around 0.50-0.60 (Bulik et al., 2010), and most of the variance in core BN symptoms (especially vomiting) is due to additive genetic factors (Mazzeo et al., 2010). First-degree relatives of individuals with BN are also more likely to have BN compared to first-degree relatives of controls (reviewed in Thornton et al., 2011). Furthermore, co-twins of individuals who are preoccupied with weight and shape and/or have anorexia-spectrum symptoms are more likely to develop AN, whereas co-twins of individuals with bulimic symptoms are shown to be at a greater risk for developing BN (Bulik et al., 2000). One twin study identified the genetic overlap between and AN and BN to be 0.46 (Bulik et al., 2010), which may explain the high crossover rates between the two EDs: as reviewed in detail in the Methods chapter of this dissertation (Section 3.1.3), AN-to-BN crossover is estimated to be around 36%, with higher baseline and lifetime BMIs being predictors of crossover (Tozzi et al., 2005). On the other hand, a study of AN-R probands with at least five years of illness duration revealed that while the prevalence rate of AN among their first-degree relatives was 1%, no cases of BN were found among them (Grigoroiu-Serbanescu et al., 2003), highlighting the possibility of limited genetic overlap between diagnostically stable AN and BN.

Disordered eating and the dysfunctional eating attitudes that are commonly observed in patients with EDs also have some genetic basis. A large population-based twin study has shown that 43% of variance in individual differences in weight and shape-related concern and 49% of the variance in individual differences in binge eating can be explained by genetic influences (Munn et al., 2010). In addition, similar to the findings on the heritability of weight (see Section 1.8.1), shared environmental factors do not seem to have an effect on disordered eating in adoptive siblings, and the heritability of disordered eating is still high for twins reared apart (Klump et al., 2009), further highlighting the importance of genetic factors.

EDs are complex behaviours with biological, psychological, and sociocultural factors contributing to their development, which make it challenging for genetic researchers to pinpoint the exact genetic cause and contribution to these disorders. However, their complex etiology does not discredit the role that genetic factors may play in an individual's predisposition to developing an ED. Like most other psychiatric illnesses, we can conclude that the heritability of EDs follows a non-Mendelian pattern, with many genes making a small contribution to their development.

1.7.2. Linkage Studies

Initial efforts in the study of AN genetics were funded and carried out by the Price Foundation (PF) Consortium, a privately-funded nonprofit international consortium. Since the first study of my dissertation is obtained using the PF dataset, more information about the nature of the consortium and data collection will be provided in the Methods section.

The first genome-wide linkage analysis in EDs, conducted by PF investigators, detected a strong signal at chromosome 1p34.2, with D1S3721 as a possible susceptibility locus for AN-R (Grice et al., 2002). More recently, this finding has been replicated by a Japanese group as a part

of a genome-wide microsatellite study (Nakabayashi et al., 2009). A later follow-up study on this signal by the PF group identified serotonin 1B (*HTR1B*; also *HTR1Dβ*) and opioid delta 1 receptor (*OPRD1*) loci to be significantly associated with AN (Bergen et al., 2003a), with both findings independently replicated by another research group (Brown et al., 2007). HTR1B is responsible for mediating the effects of the anorectic compound fenfluramine (Grignaschi et al., 1995) and has both presynaptic and postsynaptic functions (Lim et al., 2010). The *HTR1B* rs6296 G/C polymorphism may also predict minimum lifetime BMI in patients with BN (Levitan et al., 2001), with individuals carrying the C allele having a lower minimum BMI compared to G allele carriers, further suggesting a role for *HTR1B* polymorphisms in EDs. Moreover, our group has also demonstrated a possible link between the GG genotype of rs6296 and presence of full-syndrome OCD in BN probands (Levitan et al., 2006a). In the case of OPRD1, the release of endogenous opioids plays at least a partial role in the positive reinforcing effects of food, hence it is important to examine the role of the genes that regulate the opioid system in EDs. Considering the important role endogenous opioids play in high-caloric food consumption in order to regulate negative affect, there is a need for more research to explore the role of opioid gene polymorphisms in AN and BN.

Another linkage study by the PF group uncovered a link between chromosome 2 and obsessionality, chromosome 13 for drive for thinness, and chromosome 1 for the drive for thinness-obsessionality combined trait in AN (Devlin et al., 2002), further highlighting the genetic susceptibility to AN subphenotypes. As for BN, chromosome 10p was identified for a risk factor in over 300 multiplex family samples (Bulik et al., 2003). Since the publication of these studies, additional loci have been identified for ED-related behaviours and phenotypes in

BN, including the involvement of 4q21 for minimum BMI, also using linkage method (Bacanu et al., 2005).

1.7.3. Serotonin System Genes

Because of the important role serotonin plays in eating behaviour, serotonergic genes and their involvement in EDs have been studied extensively. The relationship between food restriction and reduction in L-tryptophan, the precursor to serotonin, is well established (Hu et al., 2003). In addition, there is some evidence suggesting sex differences in serotonin synthesis in the brain (Nishizawa et al., 1997), with serotonin levels being significantly disrupted during periods of caloric restriction in women compared to men, which may explain why food deprivation-related abnormalities are more commonly observed in women. Even after recovery, individuals with a history of AN present with elevated levels of serotonin metabolites in their cerebrospinal fluid (Kaye et al., 1998), possibly pointing to trait-related serotonergic abnormalities.

The serotonin 2A receptor (*HTR2A*) rs6311 polymorphism has been associated with AN and low body weight in patients with AN, BN, as well as in healthy individuals (Ricca et al., 2004; Sorli et al., 2008; Martaskova et al., 2009; Enoch et al., 1998). However, other studies did not report positive associations with this polymorphism in AN (Gorwood et al., 2002; Hinney et al., 1997b). A small-scale meta-analysis has also failed to demonstrate preferential transmission of rs6311 variants to the offspring with AN, but despite clinical and methodological heterogeneity issues, the study confirmed the association of the *HTR2A* gene with AN in a case-control design. In terms of alternate phenotypes, the A allele of rs6311, the same variant that is linked to low weight, was associated with lower dietary fat intake in healthy children and adolescents in a large family-cohort study (Herbeth et al., 2005). Within AN, presence of the A

allele may increase OCD comorbidity rate (Enoch et al., 1998) and lead to a later age of ED onset (Kipman et al., 2002). Finally, although there is preliminary evidence put forward by a single study for the involvement of other *HTR2A* variants in AN diagnosis (Kiezebrink et al., 2010), polymorphisms other than rs6311 have not been widely studied in large samples.

Unlike many of the serotonin receptors and similar to HTR1B, serotonin 1A receptor (HTR1A) has presynaptic and postsynaptic function (Lim et al., 2010). In a small sample of Korean female adolescents, a link between *HTR1A* rs6295 genotype and dysfunctional eating attitudes has been reported (Lim et al., 2010). Otherwise, *HTR1A* polymorphisms have not been investigated in EDs.

Similar to what has been reported with other serotonin receptors, serotonin 2C receptor (*Htr2c*) knockout mice also become obese, thus highlighting this receptor's crucial role in feeding behaviour (Tecott et al., 1995). *HTR2C* genetic variants have been recently linked to antipsychotic medication-induced weigh gain (AIWG; Wallace et al., 2011). Based on the findings of a small trio study, *HTR2C* rs6318 C variant may be more likely to be transmitted to individuals with AN (Hu et al., 2003), and this variant has been also correlated with low weight and increased severity in AN (Hu et al., 2003) as well as low body weight in healthy controls (Westberg et al., 2002). Furthermore, our extended group has provided evidence for the involvement of the rs6318 with weight regulation in an atypical subtype of of major depression called seasonal affective disorder (defined by seasonal overeating, weight gain, and increase in carbohydrate cravings) and BN (Praschak-Rieder et al., 2005).

The serotonin transporter (SLC6A4) plays a key role in the reuptake of extracellular serotonin in the brain. Although a number of individual studies found no evidence for the 43-bp insertion/deletion polymorphism in the promoter region of *SLC6A4* gene (known as *5-HTTLPR*)

in AN (Hinney et al., 1997a; Castellini et al., 2012; Urwin et al., 2003a), two meta-analyses have reported an association for the short variant (S-allele) with AN susceptibility (Calati et al., 2011; Lee and Lin, 2010). In the case of BN, meta-analyses have failed to replicate the previously reported link between *5-HTTLPR* and BN diagnosis (Lee and Lin, 2010; Polsinelli et al., 2012). Interestingly, *5-HTTLPR* S-allele may also increase risk for AN-to-BN crossover (Castellini et al., 2012). The S-allele has been repeatedly associated with impulsivity, novelty seeking, and trauma in BN and BN-spectrum disorders (Steiger et al., 2001; Thaler et al., 2013; Steiger et al., 2011), thus suggesting that although it doesn't necessarily lead to BN susceptibility, the presence of the risk allele may increase the prevalence of certain alternate phenotypes (often linked to impulse dysregulation) within BN. Only two studies have investigated the possible role of *SLC6A4* polymorphisms outside of the *5-HTTLPR* promoter insertion/deletion and found no evidence for their involvement in AN (Kiezebrink et al., 2010) or in ED-related phenotypes such as binge eating and weight/shape concern (Munn-Chernoff et al., 2012).

In summary, there is substantial evidence for the involvement of a number of serotonergic genes in weight regulation. Select serotonin system genes and polymorphisms—due to their known functionality or link with other psychiatric disorders—have been extensively studied in AN and BN; however, conclusiveness of the evidence on their role in EDs is uncertain due to small sample size, as well as heterogeneity in ancestry and study methodology.

1.7.4. Other Gene Systems

Although serotonin and dopamine systems (the latter of which is covered in Section 1.8.5) have been the most studied in the case of EDs, other genes have also been examined in their relation to both susceptibility and association with select phenotypes.

Endocannabinoids are known to play an important role in the regulation of appetite and body weight, and it has recently been shown that the endocannabinoid system is involved in attenuating the weight loss associated with the development of activity-based anorexia (ABA) in rodents (Verty et al., 2011). A small family study reported overtransmission of a three-locus cannabinoid receptor 1 (*CNR1*) haplotype, including the (AAT)n repeat in the 3' flanking region, in AN-R (Siegfried et al., 2004), whereas another study failed to replicate this association (Muller et al., 2008). Since then, a more recent study observed overrepresentation of the *CNR1* rs1049353 A allele in AN and BN probands compared to controls, and this single nucleotide polymorphism (SNP) combined with fatty acid amide hydrolase (*FAAH*) rs324420 polymorphism was overrepresented in AN but not BN (Monteleone et al., 2009). In summary, these data do not yet provide strong evidence for or against the involvement of cannabinoid system genes in EDs, and larger scale studies with better gene coverage and less heterogeneity are essential to further investigate the endocannabinoid involvement in AN and BN.

The role of sex hormones in the development of AN and BN has recently garnered significant research attention. Although an intrauterine masculinization effect has been proposed for female twins with EDs who have a male co-twin (Culbert et al., 2008), other twin studies reported no relationship between sex of the twin pair and disordered eating (Raevuori et al., 2008; Lydecker et al., 2012). A discussion of this complicated line of research is beyond the scope of this dissertation; however, these theories have led to the investigation of the genes regulating sex hormones in ED development. Thus far, the focus has been on the estrogen receptor 1 (*ESR1*) and estrogen receptor 2 (*ESR2*; also known as *ERβ*) genes, yielding variable findings (Versini et al., 2010; Eastwood et al., 2002; Rosenkranz et al., 1998). Considering the inverse correlation between age of menarche and ED susceptibility and that genetic factors

linked to an earlier age of menarche also associated with ED risk (Baker et al., 2012), there is some evidence for at least partial involvement for sex hormones in EDs. Therefore, further research on the genes regulating sex hormones is needed to better understand the role of sex hormones in ED etiology.

Thus far, the most comprehensive candidate gene study of AN, conducted by PF researchers, has investigated the role of 182 genes covered by over 5,000 functional and tag SNPs in 1,085 AN probands and 677 healthy controls. Although none of the SNPs reached statistical significance following correction, the top hits were located in *GLP2* and *KCNN3* genes (the former of which being involved in intestinal function and the latter playin a part in the regulation of potassium channel function), none of which have been further studied in EDs. Despite the comprehensive nature of this study, due to its pure case-control design, no within-AN analyses were performed to evaluate the role of these genes in important subphenotypes such as body weight and psychopathology.

1.7.5. GWAS

Compared to the rest of psychiatric genetics, ED research has been behind in producing GWAS findings and results obtained through more cutting-edge methodologies. The first genome-wide study of highly polymorphic microsatellite regions was conducted in 331 Japanese AN cases, in which the authors reported an association between AN and 1q41 and 11q22 genomic regions (Nakabayashi et al., 2009); further research is needed to confirm and understand the nature of these associations. More recently, a GWAS carried out by the PF group in 1,033 AN cases identified a large and rare CNV on 13q12 present in two individuals; although no SNP reached genome-wide significance, the strongest signal came from *ZNF804B* on 7q21, the paralog of *ZNF804A* which has been previously associated with schizophrenia (Wang et al.,

2011b). A GWAS collaboration on twins has also failed to find a genome-wide significant hit but provided evidence for the involvement of eight loci with various ED-related phenotypes such as drive for thinness, body dissatisfaction, bulimia, weight fluctuations, as well as traits associated with obsessive compulsive personality (Boraska et al., 2012). Another recent GWAS on disordered eating in female twins also did not report any genome-wide significant findings, but a number of genes were implicated in AN- and BN-spectrum disorder phenotypes (*CLEC5A, LOC136242, TSHZ1*, and *SYTL5* genes for the former and *NT5C1B* for the latter; Wade et al., 2013); however, the degree of generalizability of these results obtained from population twin studies to clinical ED syndromes is currently unknown.

Further GWAS efforts are currently underway to update the AN genetic literature, one of which is the Wellcome Trust-funded Genetic Consortium for Anorexia Nervosa (GCAN) initiative. GCAN is included under the Wellcome Trust Case-Control Consortium Third Wave (WTCCC3), and since it is the sample used for the second study in this dissertation looking at the dopamine pathway genes, details of this collaboration will be covered in the Methods section. The manuscript resulting from this case-control GWAS is currently under preparation, and although no SNP reached genome-wide significance, the most significant *p*-value was obtained for a SNP in the *COL4A5* gene, linked to collagen production (Bulik et al., 2012). Further replication studies, larger sample size, and functional experiments are required to gain a better understanding of the role of this gene in AN. Thus far, there have not been any GWAS or high-throughput sequencing studies conducted in BN.

1.8. Genetics of Appetite and Weight Regulation

As highlighted in the previous section, ED researchers have so far focused on a small selection of gene systems and polymorphisms, while not examining other genes known to play a

role in appetite and body weight. Furthermore, studies that have investigated these genes have been hindered by clinical, methodological and ancestral heterogeneity, small sample size, and lack of statistical correction for multiple testing, thus leading to variable findings that have not been consistent (Trace et al., 2013). In the following section, I will summarize the literature on the candidate genes that are studied as a part of this dissertation. In many instances, research on these candidate genes has been very limited in EDs. Therefore, evidence for the possible involvement of some of the genes covered in the upcoming section in EDs came from non-ED fields such as: (1) appetite and weight regulation studies conducted in rodents; (2) obesity literature; and (3) AIWG research.

1.8.1. A Summary on the Heritability of Body Weight

Body weight and BMI, like other anthropometric measures, are multigenic and determined by a combination of genetic and environmental factors. The earliest form of evidence for heritability of weight came from twin studies. One classic study put weight's heritability at 0.78-0.81 in twins over the span of multiple decades, and concordance rates of obesity were twice as much in monozygotic twins as dizygotic twins (Stunkard et al., 1986). Shared environment's effect on weight is shown to be negligible in the case of twins reared together versus twins reared apart, with non-shared environmental factors explaining more of the nonhereditary variance compared to shared environmental factors (Stunkard et al., 1990). In infants who were followed from birth to three years of age, additive genetic factors were the most significant contributors to body weight, with heritability estimates ranging between 0.6 and 0.8 (Demerath et al., 2007). It has also been demonstrated that heritability estimates for BMI increase with age, suggesting that environmental factors may play a bigger role in childhood than in adolescence (Salsberry and Reagan, 2010). Most recently, a large meta-analysis reported the

heritability of BMI to be 0.47-0.90 based on twin studies and 0.24-0.81 based on family studies (Elks et al., 2012). In adults, although not as highly heritable as weight, BMI fluctuation may also have some genetic basis (Bergin et al., 2012). Similarly, weight gain in response to environmental factors may also have genetic etiology; for example, heritability of antipsychotic-induced change in BMI is estimated to be as high as 0.6 for monozygotic twins (Gebhardt et al., 2010).

1.8.2. Leptin System Genes

1.8.2.1. Leptin and Leptin Receptor. The leptinergic system is involved in the regulation of fat tissue volume, as well as the sensations of hunger and satiety. Primarily expressed in adipose tissues, leptin (LEP) is an important anorexigenic hormone that regulates energy intake and expenditure, appetite, metabolism, and eating behaviour through its action on the arcuate nucleus in the hypothalamus. More specifically, LEP signals nutrition status to the hypothalamus by triggering melanocortin production through pro-opiomelanocortin (POMC) neurons. LEP administration activates POMC and cocaine and amphetamine regulated transcript (CART) expressing neurons and inhibits agouti-related protein (AGRP) and neuropeptide Y (NPY) expressing neurons, which in return lead to a decrease in food intake and an increase in energy expenditure. Ghrelin (GHRL) is the natural LEP antagonist, and plasma LEP is inversely correlated with AGRP activity (Moriya et al., 2006). LEP acts through the leptin receptor (LEPR), also a protein that controls fat-tissue mass via the hypothalamus effects on satiety and energy expenditure. Leptin system's involvement in appetite regulation is illustrated in Figure 1.

Since the discovery that disruptions in the *Lepr* gene (also known as *Ob* gene) lead to obesity and hyperphagia in mice, the leptinergic system was one of the earliest targets for the genetic studies of obesity and weight regulation (Grayson and Seeley, 2012). Plasma LEP levels

are correlated with BMI and fat mass, and obese individuals often present with LEP resistance due to alterations in LEP signaling in the arcuate nucleus. In humans, a study of Pakistani consanguineous families with severely obese children reported high frequency of *LEP* mutations (Saeed et al., 2012). Postnatal nutrition may also alter hormonal sensitivity, with undernourishment possibly leading to LEP sensitivity and resistance to weight gain (Stocker et al., 2012). Other *LEP* polymorphisms have also been implicated in weight regulation in South African adolescents (Lombard et al., 2012). A large-scale obesity study has identified a statistically significant link between *LEP* rs17151919 and body weight in African-American participants and a similar nominally significant trend in European subjects (Friedlander et al., 2010). The G variant of *LEP* rs7799039 has been found to be overrepresented in overweight girls in a study of Spanish adolescents, and the A allele of the same polymorphism is linked to lower plasma LEP levels in both sexes (Riestra et al., 2010b). Finally, *LEP* rs7799039 G allele is shown to lead to AIWG in patients with schizophrenia (Opgen-Rhein et al., 2010; Kuo et al., 2011), a finding that is in line with the obesity research.

In the case of the functional *LEPR* rs1137101, located in the extracellular binding domain of the *LEPR* gene and leads to a truncated receptor, research findings have been variable on its possible effect on body weight. A study of postmenopausal women reported that carriers of the *LEPR* rs1137101 A allele may have a higher mean BMI compared to the G homozygotes, and AG heterozygosity has been associated with increased plasma LEP levels (Quinton et al., 2001). However, it has been reported that the opposite allele may have an effect on plasma LEP levels and high BMI in Spanish adolescent girls (Riestra et al., 2010a), Brazilian adults (Hinuy et al., 2010; Angeli et al., 2011) and Japanese men (Pereira et al., 2011), especially in conjunction with a β-2 adrenergic receptor (*ADRB2*) polymorphism. Although the functionality of this

polymorphism has been well documented (Riestra et al., 2010a), its exact role in weight regulation needs to be further studied in general and clinical populations. More recently, two separate meta-analyses of case-control studies failed to find a relationship between *LEP, LEPR* and obesity (Bender et al., 2011; Yu et al., 2012); however, these studies did not look at obesity-related phenotypes such as weight or BMI, suggesting that future studies should focus on continuous measures instead of using discrete weight categories or case-control design.

Patients with acute AN are known to have low plasma LEP levels (Janeckova, 2001; Moriya et al., 2006). Serum LEP levels have also been correlated with BMI in patients with EDs in general (Ferron et al., 1997), and high levels of LEP have been linked to increased body dissatisfaction, binge eating, and bulimic tendencies in adolescents (Lofrano-Prado et al., 2011). Furthermore, LEP deficiency results in anxiety-related behaviours in rodents (Finger et al., 2010). Considering the high comorbidity of anxiety disorders in ED patients, LEP's role in eating behaviour in ED populations may go beyond appetite regulation and may explain the link between eating-related anxiety and weight regulation. At a molecular biology level, research on the role of the *LEPR* gene has yielded variable findings. Although some studies reported no association of *LEPR* with AN (Hinney et al., 1998b; Quinton et al., 2004), there has not been any conclusive evidence on the involvement of *LEPR* in low body weight in AN.

While the *LEPR* gene has been more widely studied in EDs, there has only been one study conducted on the *LEP* gene, showing significant differences in *LEP* expression between two AN-R and two AN-BP probands (Janas-Kozik et al., 2008). Based on the very limited literature on the role of *LEP* in EDs, there is undoubtedly a need for further research involving much larger sample sizes.

1.8.2.2. Histamine Receptor H1. LEP is known to partly exert its effects through the histamine receptor H1 (HRH1) and facilitates the release of histamine via HRH1 in the hypothalamus (Morimoto et al., 2000). HRH1 is expressed postsynaptically and at a high density in hypothalamic regions such as the ventromedial and paraventicular nuclei, which are associated with feeding behaviour (Malmlof et al., 2006). Central histamine signaling is involved in the regulation of food intake and body weight (Yoshimatsu et al., 2002). HRH1 agonists increase and antagonists decrease food intake in rodents (Sakata et al., 1997). The anorexic effect of LEP is attenuated in *Hrh1* deficient mice, and *Hrh1* knockout (KO) mice develop obesity and hyperphagia (Masaki et al., 2004; Jorgensen et al., 2006). LEP administration is known to increase histamine levels, and administration of histidine decarboxylase prior to the injection of LEP prevents LEP-induced reduced food intake (Jorgensen et al., 2006), which highlights the interaction between the histaminergic system and LEP-induced satiety, as well as this pathway's important role in food intake and weight regulation.

Despite its involvement in feeding behaviour, surprisingly the *HRH1* gene and its possible role in obesity have not been well studied, as most of the focus has been on *LEP* and *LEPR* genes. It is promising that *HRH1* genetic polymorphisms have been linked to antipsychotic action of atypical agents and may predict weight gain in patients with non-affective psychosis (Vehof et al., 2011). Another study also reported a statistical trend for the involvement of *HRH1* rs13064530 in clozapine response (Lee et al., 2012b), highlighting the receptor's possible interaction with the dopamine system and its role in weight regulation. To date, there are no published studies investigating *HRH1* or any other histamine system genes in AN or BN. When all the neurobiological findings are considered together, the *HRH1* gene is a prime, but understudied, candidate for genetic association analysis with low body weight in EDs.

1.8.2.3. GHRL. GHRL is the orexigenic peptide ligand of growth hormone secretagogue receptor (GHS-R1A). Often referred to as the 'hunger hormone', it is associated with the regulation of energy balance and food intake. GHRL is secreted by the gut and is expressed in the hypothalamus, as well as in hippocampal and mesolimbic structures. As the natural antagonist of LEP's effect, increased levels of plasma GHRL result in weight gain. A powerful appetite-stimulating peptide, GHRL activates AGRP and NPY neurons in the arcuate nucleus and suppresses POMC neurons through GSH-R1A (Scerif et al., 2011). Healthy human subjects experience a significant increase in appetite and food intake following intravenous GHRL administration (Wren et al., 2001). It has also been shown that GHRL has a secondary role in the rewarding aspects of food and eating, independent of its role in body weight regulation (Perello et al., 2010). Peripheral GHRL administration also increases the consumption of food with high reward properties (e.g., high sugar foods) in rodents, thus it has been proposed that GHRL signaling in the pleasure centres of the brain, such as the ventral tegmental area (VTA), may be crucial for pleasure-driven eating behaviours (Egecioglu et al., 2010).

Despite GHRL's important role as an orexigenic peptide, studies involving *Ghrl* KO mice have yielded variable findings in regards to food intake and weight regulation (Kang et al., 2011), whereas recent animal models utilizing *Ghrl* overexpression documented hyperphagia, glucose intolerance and LEP sensitivity in these transgenic mice (Bewick et al., 2009). In the case of humans, plasma GHRL levels are positively correlated with BMI in obese individuals (Franek et al., 2010). A number of studies have investigated the role of the *GHRL* gene and obesity, and one of the earlier studies reported that the T allele of the *GHRL* rs34911341 mutation and the rs696217 T variant may be linked to obesity in a small group of European females (Ukkola et al., 2001). A more recent Brazilian study, however, failed to replicate a

similar association in men (Dantas et al., 2011), suggesting a possible genotype-sex interaction for *GHRL*'s effect on weight. The rs696217 polymorphism has also been associated with BMI and early-onset obesity in European (Korbonits et al., 2002; Miraglia del Giudice et al., 2004) but not in Chinese children (Zhu et al., 2010).

Plasma GHRL levels are inversely correlated with BMI in ED patients (Cellini et al., 2006; Nakahara et al., 2008). A family trio study has found increased transmission of the *GHRL* rs4684677-rs696217 A-G haplotype in AN, as well as increased transmission of the rs696217 T variant in AN-BP (Dardennes et al., 2007). However, other studies reported conflicting findings, failing to find a relationship between these *GHRL* polymorphisms and AN or BN (Cellini et al., 2006; Monteleone et al., 2006a; Kindler et al., 2011). The *GHRL* rs2075356 polymorphism, which is in high linkage disequilibrium (LD) with rs696217, has been associated with BN purging subtype (Ando et al., 2006), as well as BED and obesity (Monteleone et al., 2007). A nominally significant association between TT genotype of *GHRL* rs2075356 and faster weight recovery has also been reported in a study of 165 Japanese AN-R probands (Ando et al., 2010). In summary, although the role of *GHRL* in weight regulation has not been sufficiently studied, there is reason to believe that it holds potential for future ED research.

1.8.3. Melanocortin System Genes

1.8.3.1. Melanocortin 4 Receptor. Stimulation of brain melanocortin leads to a reduction in food intake and weight. LEP signals nutritional status to the hypothalamus by triggering melanocortin production through POMC neurons. Anorexigenic properties of the melanocortin 4 receptor (MC4R) come from the alpha-melanocyte-stimulating hormone (α-MSH), a naturally occurring endogenous peptide that binds to MC4R. Furthermore, administration of AGRP, the inverse agonist of melanocortin receptors (Ollmann et al., 1997), activates the dopaminergic

neurons in the midbrain, increases dopamine turnover in the prefrontal cortex, and attenuates sucrose-seeking behaviour in rats (Davis et al., 2011). The mechanism behind melanocortinergic role in appetite and weight regulation is summarized in Figure 1.

Mc4r KO mice have been consistently documented to become obese and hyperphagic (Marsh et al., 1999; Atalayer et al., 2010), in addition to developing metabolic abnormalities such as hyperinsulinemia and hyperglycemia (Srisai et al., 2011; Huszar et al., 1997). *Mc4r* KO mice are also insensitive to the administration of d-Fenfluramine—an indirect serotonin agonist—and its anorexigenic effects (Xu et al., 2010). Furthermore, administration of the cyclic heptapeptide melanotan II, a nonselective melanocortin agonist, increases metabolic rate in mice but doesn't lead to any changes in *Mc4r* KO animals (Chen et al., 2000; Mul et al., 2012). On the other hand, rats engaging in higher levels of physical activity show enhanced melanocortin messenger ribonucleic acid (mRNA) expression in the brain compared to rats with lower levels of physical activity (Shukla et al., 2012).

In humans, defects in the *MC4R* gene have been a known cause of autosomal dominant obesity, accounting for 6% of all obesity cases (Farooqi et al., 2003). Various studies in different populations have reported rare *MC4R* genetic mutations in severely obese individuals (Hinney et al., 1999a; Vaisse et al., 2000; Hinney et al., 2006; Alfieri et al., 2010; Scherag et al., 2010; Wang et al., 2010b; Buchbinder et al., 2011; Zegers et al., 2011; Nowacka-Woszuk et al., 2011; van den Berg et al., 2011), although whether the obesity cases with *MC4R* mutations are phenotypically different from those without identified *MC4R* mutations is currently not clear (Melchior et al., 2012). GWAS reports suggest that several markers near *MC4R* (especially rs17782313, rs17700633 and rs571312, all of which are in high LD with one another) are strongly associated with obesity in healthy adults (Loos et al., 2008; Luan et al., 2009), and these

results have been replicated in numerous studies utilizing different methodologies, conducted in different ethnic populations and age groups, and with obesity-related phenotypes including but not limited to BMI (Thorleifsson et al., 2009; Speliotes et al., 2010; Liu et al., 2010; Petry et al., 2010; Elks et al., 2010; Scherag et al., 2010; Wu et al., 2010; Liu et al., 2011; Beckers et al., 2011; Rouskas et al., 2012; Zhao et al., 2011; Paternoster et al., 2011; Hunt et al., 2011; Hong and Oh, 2012; Huang et al., 2011; Dorajoo et al., 2012; Lombard et al., 2012; Okada et al., 2012; Corella et al., 2012; Kvaloy et al., 2013; Warrington et al., 2013). *MC4R* common variants, however, are not believed to influence birth weight (Kilpelainen et al., 2011a). More recently, a GWAS on AIWG reported a peak at the near-*MC4R* region on chromosome 18, corresponding to rs489693 (Malhotra et al., 2012), whereas another study by our group has identified rs8087522 as another possible risk locus for weight gain in schizophrenia patients undergoing antipsychotic medication treatment (Chowdhury et al., 2012). The rare genetic mutations located in the coding region that lead to early-onset severe obesity are believed to result in the misfolding of MC4R in the endoplasmic reticulum (Granell et al., 2010), whereas the function of the near *MC4R* common variants is currently unknown. While the rs17782313 marker has also been linked to satiety as well as enjoyment of food in obese pediatric populations (Valladares et al., 2010; Stutzmann et al., 2009), another study failed to find an association between four *MC4R* common variants and macronutrient consumption (Hasselbalch et al., 2010).

In BN probands, MC4R haploinsufficiency has been associated with maximum lifetime BMI (Hebebrand et al., 2002). However, the role of *MC4R* gene has not yet been studied in AN. *MC4R*'s involvement in binge eating behavior has been more controversial (Branson et al., 2003; Hebebrand et al., 2004), and more research is needed to determine the relationship.

1.8.3.2. Melanocortin 3 Receptor. Melanocortin 3 receptor (MC3R) is associated with increased fat mass despite decreased food intake when deficient in mice, suggesting that *Mc3r* deficiency leads to preferential partition of nutrients into fat mass (Huszar et al., 1997). Similar to MC4R, MC3R is also heavily expressed in the hypothalamic regions of the brain, but unlike *Mc4r* KO mice, *Mc3r* deficiency does not lead to hyperphagia in rodents, but a long-term high-caloric diet results in a decrease in *Mc3r* mRNA expression in the arcuate nucleus (van den Heuvel et al., 2011). Although various lines of research point to the involvement of MC4R in weight regulation, the role of MC3R in energy homeostasis is still debated (Adan et al., 2003), partly due to a lack of definitive studies and the recent research efforts having focused almost exclusively on MC4R. Furthermore, the influence of MC3R on weight regulation may be subtler compared to that of MC4R (Irani et al., 2011). More recently, it has been shown that MC3R may play a role in the response to serotonin agonists with anorexigenic effects, such as WAY-161503 with selective affinity for HTR2B and 2C receptors (Rowland et al., 2010), thus implicating MC3R in the regulation of appetite and food intake. Moreover, overexpression of MC3R may increase sensitivity to the anorexigenic gut hormone cholecystokinin (Atalayer et al., 2010), further separating MC3R from MC4R in terms of mechanism of action.

In line with *MC4R* findings in humans, *MC3R* rare functional mutations are also more commonly observed in obese individuals compared to controls (Mencarelli et al., 2011). In a study of 1,008 obese cases and 313 normal-weight controls, *MC3R* rs3746619 was found to influence weight in obese individuals, but no differences in allele frequency was observed between cases and controls (Zegers et al., 2010). Another study also failed to find case-control differences in *MC3R* common variants but reported an association between two variants (rs3746619 and rs3827103) and glucose oxidation following moderate exercise in obese children

after controlling for fat mass (Obregon et al., 2012). *MC3R* rs6014646 polymorphism may have an effect on weight loss based on a dietary intervention trial involving 760 obese individuals of European descent (Santos et al., 2011). There is some preliminary evidence linking *MC3R* polymorphisms to eating behaviour, specifically emotional eating, in children (Obregon et al., 2010).

Aside from an exploratory study of 158 Dutch AN patients having failed to find an association between four *MC3R* SNPs and AN diagnosis (de Krom et al., 2005b), *MC3R* polymorphisms have not been studied in ED populations despite their potential role in weight regulation.

1.8.3.3. AGRP. AGRP is a neuropeptide that suppresses melanocortin receptor activity, resulting in an increase in appetite and decrease in metabolic rate and energy expenditure (Krashes et al., 2011). AGRP is reported to ameliorate self-starvation and hyperactivity in rats (Kas et al., 2003). As an important orexigenic peptide, AGRP is also the inverse agonist for α-MSH, MC3R, and MC4R (Ollmann et al., 1997) whereas GHRL serves as the upstream regulator of AGRP. Fasting increases AGRP levels and decreases melanocortin activity by signaling via the hypothalamus to increase food intake, and AGRP expressing neurons are inhibited by LEP administration (Kas et al., 2003). Plasma AGRP levels are inversely correlated with BMI (Moriya et al., 2006). Furthermore, administration of AGRP activates the dopaminergic neurons in the midbrain and increases dopamine turnover in the prefrontal cortex (Davis et al., 2011), and injection of AGRP affects sucrose-seeking behaviour in rats (Davis et al., 2011).

Various animal models have been used to study the *Agrp* gene. The agouti mouse (with *Agrp* overexpression) is one of the earliest preclinical models of obesity. An increase in the expression of *Agrp* is observed in the hypothalamus of mice deficient in *Lep* or *Lepr*, suggesting

that LEP mediates the signaling that suppresses AGRP production (Korner et al., 2000). Although *Agrp* mRNA levels increase in starving wild type rodents, the opposite effect is observed in ABA rats (Kas et al., 2003). AGRP injection in ABA rats has been linked to an increase in food intake (Hillebrand et al., 2006), basal temperature (Hillebrand et al., 2006; Kas et al., 2003; Adan et al., 2003), survival rate (Hillebrand et al., 2006), and a decrease in hyperactivity (Hillebrand et al., 2006; Kas et al., 2003).

AGRP rs11575892 CT heterozygosity has been linked a higher BMI in a study of 95 individuals with severe obesity (Kalnina et al., 2009). In ED populations, the majority of *AGRP* research studies have focused on rs5030980, which is located in the coding region of the gene. The A variant of rs5030980 has been associated with low weight in AN in two separate pilot studies with small sample size (Vink et al., 2001; Dardennes et al., 2007). A preliminary trio study reported an overtransmission of the AG genotype in probands with AN-BP (Dardennes et al., 2007). Despite these promising findings, results are far from being generalizable due to the sample size-related issues, and larger genetic analyses are required to further investigate the role of *AGRP* gene polymorphisms in AN. Thus far, *AGRP* polymorphisms have not been explored in BN.

1.8.3.4. POMC. POMC, a precursor polypeptide that is mainly expressed in the arcuate nucleus, is associated with appetite regulation, as well as the secretion of glucocorticoids. LEP signals nutritional status to hypothalamus by triggering melanocortin production through POMC neurons (Cowley et al., 2001), and administration of LEP activates POMC and CART expressing neurons while inhibiting AGRP- and NPY-expressing neurons (Scerif et al., 2011), which in return leads to a decrease in food intake and an increase in energy expenditure (Adan et al., 2003). Taken together, these findings suggest that POMC neurons could be an important link

between leptinergic and melanocortinergic systems and may play a causal role in diet-induced obesity (Gamber et al., 2012).

POMC neurons are inhibited in response to fasting, and reduction of *Pomc* mRNA levels is observed in starved rodents (Kas et al., 2003). POMC overexpression in the VTA decreases the likelihood of diet-induced obesity (Andino et al., 2011), and *Pomc* heterozygous KO mice consume significantly larger amounts of food and have a preference for high-fat foods compared to the wild type animals (Corander and Coll, 2011). Furthermore, POMC-treated mice not only increase their activity levels, but they also lose overall weight and fat mass (Zhang et al., 2011). In ABA rats, POMC expression is upregulated only in the presence of hyperactivity (Hillebrand et al., 2006), a finding that contrasts with the downregulation of other anorexigenic hormones and peptides, suggesting that POMC may have a unique effect of hyperactivity.

Rare missense mutations of the *POMC* gene, located on chromosome 2p23, have been associated with obesity (Hinney et al., 1998a; Adan and Vink, 2001; Mencarelli et al., 2012). A genome-wide multipoint linkage analysis found an association between a chromosome 2p21 microsatellite polymorphism, which is located close to the *POMC* gene, and serum LEP levels and adiposity in Mexican Americans (Comuzzie et al., 1997). *POMC* common variants have also been linked to anthropometric measures such as waist-to-hip ratio, visceral and abdominal fat, as well as preference of macronutrients (Ternouth et al., 2011). Furthermore, rs1042571 has been linked to obesity in 308 European-American individuals (Wang et al., 2012a).

Other than the identification of a *POMC* missense mutation in an AN patient in the 1990s (Hinney et al., 1998a), the role of the *POMC* gene has surprisingly not been studied in AN or BN, especially considering the important role of the POMC peptide in appetite and energy regulation.

1.8.4. Neurotrophic System Genes

1.8.4.1. Brain Derived Neurotrophic Factor. Brain derived neurotrophic factor (BDNF) is a protein that supports the growth, survival, differentiation, and assigned function of neurons. BDNF is expressed mostly in the hippocampus and plays an important role in learning and memory formation (Egan et al., 2003). In terms of eating behaviour, BDNF is involved in appetite suppression by downstream regulation of melanocortin signaling in the hypothalamus (Xu et al., 2003). Long-term BDNF infusion leads to appetite suppression and weight loss via the paraventricular nucleus in the hypothalamus (Toriya et al., 2010), a brain region where MC3R receptors are also heavily expressed. BDNF also plays a critical role in the development and function of serotonergic neurons in the brain (Lyons et al., 1999; Klein et al., 2010), which also links it to appetite regulation. In animal studies, *Bdnf* heterozygous KO rodents show weight gain, increased appetite and adipocyte hypertrophy (Kernie et al., 2000), whereas BDNF injection leads to appetite suppression and weight loss in wild type mice (Pelleymounter et al., 1995; Wang et al., 2010a). ABA mice show a reduction in hippocampus BDNF expressions, which suggests that BDNF signaling may be altered as a function of susceptibility to AN (Gelegen et al., 2008). The mechanism behind the involvement of neurotrophins in appetite and weight regulation is summarized in Figure 1.

Also expressed in the mesolimbic dopamine system, BDNF facilitates motivation, reward-seeking behaviour, drug and palatable food consumption (Cordeira et al., 2010). It has been shown that BDNF plays a critical role in the early development of dopaminergic neurons in the brain: BDNF-depleted mice experience a reduction in dopamine in the nucleus accumbens and dorsal striatum, parts of the mesolimbic pathway in the brain that are responsible for the release of dopamine to the prefrontal cortex (Cordeira et al., 2010). Based on these findings, it

can be concluded that BDNF is likely involved in the positive modulation of the dopaminergic system for hedonic feeding.

BDNF polymorphisms have been the focus of candidate gene studies of weight regulation for some time, especially the rs6265 G/A (Val/Met) substitution at codon 196 (Gunstad et al., 2006; Beckers et al., 2008; Shugart et al., 2009). Similar to the results of the candidate gene literature preceding it, a GWAS has linked the Val variant of rs6265 to obesity (Thorleifsson et al., 2009), and this finding has been replicated in a subsequent study (Wu et al., 2010). More recently, the Genetic Investigation of Anthropometric Traits Consortium GWAS with close to 250,00 individuals further renewed the interest in *BDNF*'s role in weight regulation, as rs10767664 was one of the top hits for obesity (Speliotes et al., 2010). Other studies reported similar associations involving the T allele of *BDNF* rs10767664 and obesity (Hong and Oh, 2012; Zhao et al., 2011; Dorajoo et al., 2012; Guo et al., 2013). The rs2030323, which is in complete LD with rs10767664, is a predictor of BMI in East Asian populations (Okada et al., 2012). A near-*BDNF* polymorphism (rs925946) has been reportedly involved in waist-to-hip ratio in European men (Kvaloy et al., 2013), as well as early infancy weight gain alongside other GWAS SNPs linked to obesity (Elks et al., 2010). However, rs6265 does not appear to be associated with birth weight (Kilpelainen et al., 2011a; Kvaloy et al., 2013) or weight at the earlier stages of development (Kvaloy et al., 2013). The reported connection between Met66 and restricted energy intake in school-age children (Arija et al., 2010) further emphasizes the need to study rs6265 in ED susceptibility.

The *BDNF* gene has been implicated in AN and BN by various studies, but the nature of the findings has not been consistent. Among all SNPs, rs6265 has undoubtedly been the most investigated in EDs. The first finding on the possible involvement of the A allele (Met66) with

AN and low BMI in AN came from a small study with 64 Spanish AN probands (Ribases et al., 2003), which was replicated by follow-up studies of varying sample sizes in European populations (Ribases et al., 2004; Ribases et al., 2005b; Dmitrzak-Weglarz et al., 2007; Gelegen et al., 2008). The *BDNF* Met66 allele has also been associated with BN diagnosis (Ribases et al., 2004). While a cross-disorder meta-analysis on rs6265 has yielded positive findings (Gratacos et al., 2007), a more recent larger meta-analysis of case-control studies has failed to replicated the association between *BDNF* rs6265 and AN (Brandys et al., 2011), suggesting that while the role of this polymorphism may be limited in ED diagnosis, its effect may show itself during the study of ED-related phenotypes such as BMI. Indeed, our group has reported a significant association between rs6265 Met allele and maximum lifetime BMI in BN (Kaplan et al., 2008), whereas another study linked the Met allele to increased binge eating frequency in BN probands (Monteleone et al., 2006b). A population study in Estonian adolescents showed that Met carriers who engage in extreme weight control behaviours are more likely to report bulimic tendencies and increased binge eating (Akkermann et al., 2011), which would explain the previously reported association of Met allele with maximum BMI as well as binge eating. More interestingly, rs6265 is in moderately high LD ($r^2 = 0.768$) with rs10767664, one of the top obesity SNPs, which further emphasizes the potential of its involvement in weight regulation in ED.

The *BDNF* rs56164415 (also know as -270C/T) polymorphism and its role in EDs has been more controversial (Ribases et al., 2003; de Krom et al., 2005a; Dmitrzak-Weglarz et al., 2007), possibly resulting from the low minor allele frequency (MAF) and issues with statistical power due to small sample size. It has been reported that the C allele may be more common in BN probands and may predict later age of onset for weight loss (Ribases et al., 2004). In terms of

family studies, preferential transmission on the -270C/Met66 haplotype (rs56164415 and rs6265) has been reported in families of AN probands (Ribases et al., 2005b), but other studies failed to replicate this association (Dardennes et al., 2007; Mercader et al., 2007).

1.8.4.2. Neurotrophic Tyrosine Kinase Receptor Types 2 and 3. Neurotrophic tyrosine kinase receptor type 2 (NTRK2; also known as TrkB) is the main receptor for the BDNF peptide and plays an important part in the differentiation and survival of neurons (Klein et al., 1991). Via its expression in the hypothalamus, NTRK2 is also involved in appetite and weight regulation. Peripheral and central administrations of NTRK2 agonists lead to appetite and weight suppression in animals and reduce obesity in *Bdnf* KO mice (Xu et al., 2003). In mice with diet-induced obesity with impaired fear conditioning, expression of full-length NTRK2 is significantly reduced in the amygdala (Yamada-Goto et al., 2012), suggesting a role for NTRK2 in emotional regulation and fear response. NTRK2 is also expressed in the mesolimbic dopamine system and is localized to VTA dopamine cells (Cordeira et al., 2010), which implies involvement in reward mechanisms and possibly hedonic eating. In mice, deletion of NTRK2 receptors from dopamine 1 (D1) neurons leads to obesity due to increased feed efficiency, and loss of NTRK2 signaling in D1 neurons causes hyperphagia in mice on a high-fat diet, suggesting two separate diet-dependent mechanisms of weight regulation (Mason et al., 2013).

The *NTRK2* gene is mapped on 9q22.1; composed of 21 exons, it has five mRNA isoforms that code for five separate proteins (Ribases et al., 2005a). A recent meta-analysis of over 100,000 individuals has linked the presence of the *NTRK2* rs1211166 A allele with an increase in BMI (Guo et al., 2013). The first study of *NTRK2* in EDs reported the C homozygosity of rs1187325 to be more common in AN-BP and rs1078947 to be linked to maximum lifetime BMI in 83 AN probands (Ribases et al., 2005a). *NTRK2* rs1439050 may be

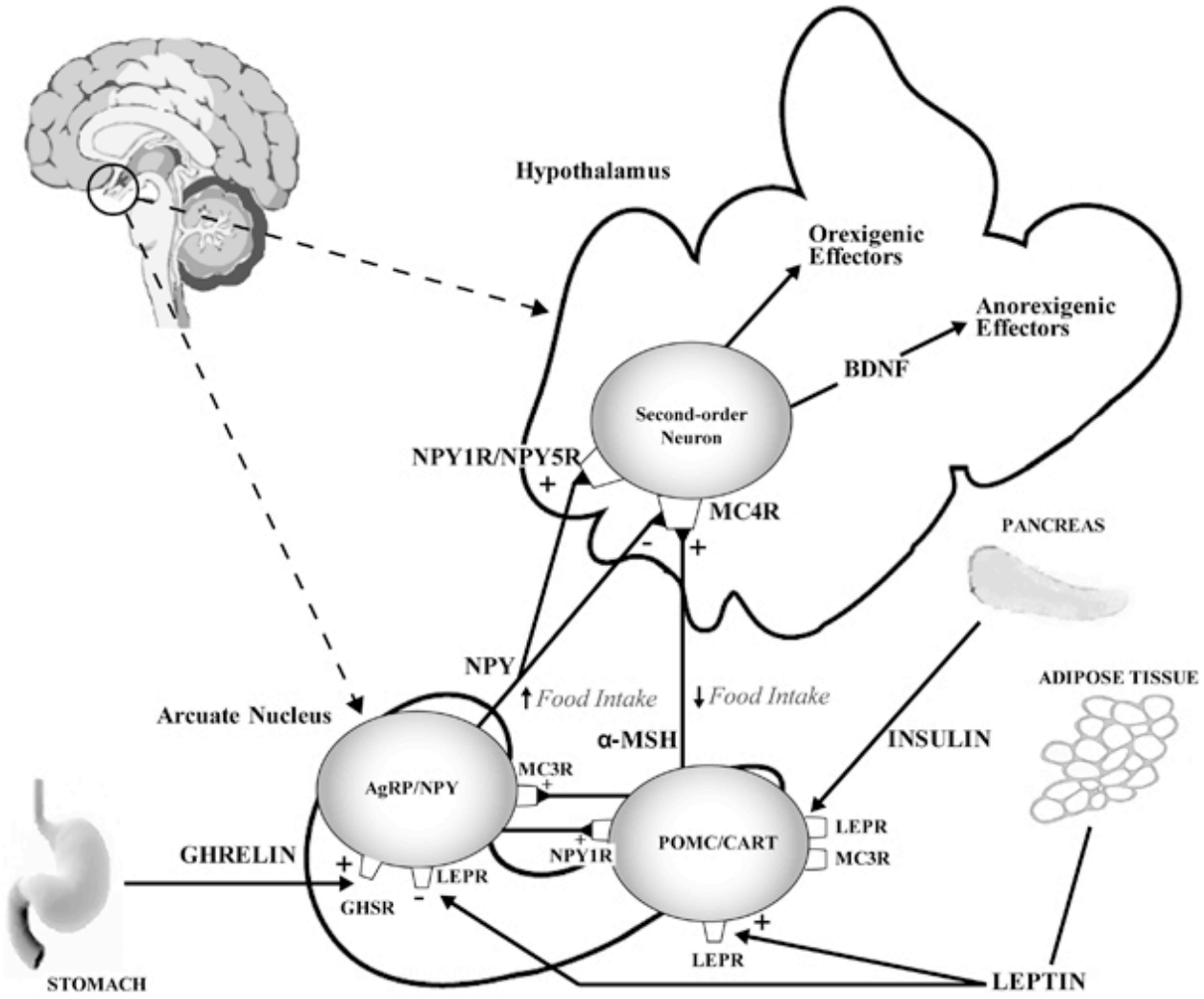
associated with ED diagnosis and early age of onset (Gratacos et al., 2010). Despite the positive findings from these initial studies, the role of *NTKR2* genes in weight regulation in AN patients has not been sufficiently examined.

Neurotrophic tyrosine kinase receptor type 3 (NTRK3; also known as TrkC) is the major binding site and the physiologic receptor for neurotrophin 3 (NT3), another important neurotrophic peptide alongside BDNF. NT3 affects the development of neurons expressing the BDNF gene (Kolbeck et al., 1999). Mice with diet-induced obesity suffer from an imbalance in the interaction between the BDNF and NT3 systems in the cerebral cortex, hippocampus and amygdala related to cognition and fear (Yamada-Goto et al., 2012). In a group of 146 women with varying degrees of adiposity, NT3 levels were correlated with the individuals' lipid profiles (Bullo et al., 2007). Since a large proportion of the research attempts have focused on BDNF and yielded promising findings, other neurotrophic receptors have not been thoroughly studied in obesity or EDs. One study reported an overrepresentation of the C allele of *NTRK3* rs7180942, located in intron 8, in AN patients and overtransmission of the CT genotype to the offspring with an ED (Mercader et al., 2008), but these promising results are in need of replication to understand the role of NTRK3 in EDs.

1.8.5. Dopamine System Genes

Dopamine is an important monoamine neurotransmitter that belongs in the catecholamine family. Produced in several different areas of the brain, including the substantia nigra and VTA, it is involved in a large variety of brain functions, including feeding behaviour and reward systems. Levodopamine (L-DOPA) is the precursor of dopamine, and dopamine is a precursor of noradrenaline and adrenaline. Administration of AGRP activates the dopaminergic neurons in the midbrain and increases dopamine turnover in the prefrontal cortex (Davis et al., 2011). Early

Figure 1. Schematic representation of the leptin, melanocortin, and neurotrophin systems and their involvement in appetite and weight regulation[4]



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development of dopaminergic neurons in the brain is at least partially guided by BDNF, as BDNF-depletion leads to reduction in dopamine in the nucleus accumbens and dorsal striatum in mice (Cordeira et al., 2010).

Dopamine acts through five receptors. Dopamine receptor D1 (DRD1), the most abundant dopamine receptor in the CNS, regulates neuronal growth and development. D1 and D1-like receptors play an excitatory role by activating adenylyl cyclase and increasing the intracellular concentration of the second messenger cyclic adenosine monophosphate (cAMP). DRD1 is believed to play a key role in working memory formation. It has also been demonstrated that MC4R may play a role in the signaling of D1 receptor neurons in procedural memory learning (Cui et al, 2012) and in stress-induced synaptic adaptations in nucleus accumbens (Lim et al, 2012), thus suggesting an interaction between the dopamine (specifically DRD1) and the melanocortin systems, which may have implications for appetite and weight regulation. Dopamine receptor D2 (DRD2) directly inhibits the formation of cAMP by inhibiting adenylyl cyclase. DRD2's influence on weight and appetite regulation is well documented and is reviewed in more detail below. Dopamine receptor D3 (DRD3) is a D2-like receptor, heavily expressed in the Islands of Calleja and nucleus accumbens. D3 may potentially play a role in the modulation of CART, an anorexigenic peptide, thus affect feeding behaviour and weight regulation (Beaudry et al., 2004). DRD4, which also possesses D2-like properties, plays a key role in executive functions through its expression in the frontal regions of the brain. DRD4 has been shown to influence weight regulation in numerous studies, also summarized below. Dopamine receptor D5 (DRD5) is expressed in limbic system neurons. Due to the structural similarities between D1 and D5, there are only a handful of ligands that are able to distinguish

between these two receptors; however, D5 has a much higher affinity to dopamine compared to D1. Similar to D1, DRD5 receptors also play an excitatory role and activate adenylyl cyclase.

Dopamine and the mesolimbic pathway regulate incentive motivation (Gilpin and Koob, 2008), and incentive motivation is an important aspect in maintaining addictive behaviours, including compulsive overeating and binge eating. Hedonistic behaviours and substances with abuse potential result in an increase in intrasynaptic dopamine levels in the nucleus accumbens (Goodman, 2008). Highly palatable food, like substances with high abuse potential, has very strong rewarding properties; ingestion of palatable foods activates the dopaminergic system and the nucleus accumbens in the brain (Davis et al., 2008). Repeated stimulation of this pathway is associated with the development of addictions, including binge eating or 'food addiction' (Mathes et al., 2009). Animal studies have consistently demonstrated that chronic sugar intake leads to increased dopamine receptor binding in the mesolimbic pathway, leading to a significant decrease in dopamine release, which in return further increases sugar-seeking behaviour (Avena et al., 2008). These findings closely mimic the phenomenon observed in ED patients who binge eat, where the desire to cope with negative affect and anxiety increases the likelihood of relapse, creating a downward spiral (Mathes et al., 2009). Overweight individuals with compulsive overeating are shown to be hyper-responsive to reward, in a fashion similar to what has been reported in individuals with addictions (Filbey et al., 2012). Functional magnetic resonance imaging (fMRI) studies show that recovered bulimics may have altered response in the anterior ventral striatum and display difficulties in differentiating positive and negative feedback in a monetary reward task, possibly due to disturbances of systems modulating reward contributing to ED risk (Wagner et al., 2010). Moreover, abnormalities in reward learning have been reported in recovered bulimic following a catecholamine challenge (Grob et al., 2012), providing evidence

for the possibility that the disturbances observed in the dopamine system in BN are state-independent.

On the opposite end of the spectrum, there are also several lines of evidence suggesting that dopamine dysfunction may predispose to AN by disturbing reward association with food intake. Food restriction has been shown to alter dopaminergic transmission, resulting in an increased interest in novel stimuli to boost dopamine levels (Carr, 2007). This finding suggests that the alternating pattern between restriction and binge eating may magnify the abnormalities in dopaminergic transmission, especially in AN-BP patients. ABA mice administered olanzapine are more likely to survive compared to those administered fluoxetine (Klenotich et al., 2012), which implicates dopaminergic abnormalities in the presence of hyperactivity, one of the key symptoms of AN (American Psychiatric Association, 2000).

ED patients demonstrate reduced cognitive flexibility and impaired executive functioning, two domains in which dopamine plays a pivotal role, compared to healthy controls in neuropsychological assessments (Tchanturia et al., 2012b). In addition, individuals with acute and recovered AN are more likely to report social anhedonia compared to healthy controls (Tchanturia et al., 2012a), further pointing to a disturbance in the dopaminergic pathways. Imaging studies also lend support to the possibility that individuals with AN experience disruption in the reward mechanisms leading to hyperactivity and disturbed hedonic response to food even after recovery (Frank et al., 2005). Dopaminergic abnormalities and the resulting excessive self-restraint may not be limited to the realm of food, as AN patients often show less temporal discounting and can delay reward for longer periods of time compared to the general population (Steinglass et al., 2012). Individuals recovered from AN also show different patterns of [11C]raclopride binding in ventral striatum (associated with euphoria) compared to controls

following an amphetamine challenge, which may explain the food-related anxiety experienced by individuals with AN while at the acute stage and following recovery (Bailer et al., 2012). Dopaminergic system is also central to the regulation of motor activity, and individuals with AN also display a disturbance in motor activity in that they often engage in compulsive exercising, even in the face of ongoing weight loss and starvation (Davis et al., 1997).

This section will briefly summarize the relevant literature on the dopamine receptor genes and related genes in the extended dopamine system genes. The vast majority of research efforts in EDs and weight gain studies have thus far focused on DRD2 and DRD4, as well as solute carrier family 6, member 3 (SLC6A3; also known as dopamine transporter or DAT) and catechol-O-methyltransferase (COMT), which will be reviewed in significant detail below. Other dopamine system genes, however, have not been widely studied with regards to their potential involvement in weight regulation or EDs but still merit consideration due to their functional significance.

1.8.5.1. DRD2. DRD2 plays a key role in the dopaminergic control of motor activity (Baik et al., 1995). D2 inhibits adenylate cyclase activity. Expressions of D2 in the caudate putamen and BDNF in the hippocampus are significantly reduced in ABA mice in response to scheduled feeding and increased activity level (Gelegen et al., 2008). *Drd2* KO mice display LEP sensitivity (Kim et al., 2010), an occurrence that highlights DRD2's role in energy homeostasis. Similar to the mechanisms proposed for addiction disorders, striatal D2 expression is significantly reduced in obese rodents, and *Drd2* deficient rats develop compulsive eating (Johnson and Kenny, 2010). In line with the animal research, obese individuals have lower striatal D2 receptor availability compared to normal-weight controls (Wang et al., 2001), and there is an inverse correlation between D2 receptor availability and BMI (Wang et al., 2001).

One field in which DRD2 has been thoroughly studied is schizophrenia. Both typical and atypical antipsychotic drugs are high-affinity DRD2 antagonists, and the fact that antipsychotic medications were quickly identified as D2 receptor antagonists led to the dopamine hypothesis of schizophrenia (Carlsson and Lindqvist, 1963; Seeman et al., 1976; Abi-Dargham et al., 2000). It has also been demonstrated *in vitro* that D2 occupancy closely correlates with the antipsychotic potencies of the different agents (Seeman et al., 1976). A two-step meta-analysis published by our group confirmed that D2 occupancy is positively correlated with treatment response to antipsychotics in schizophrenia (Yilmaz et al., 2012d). DRD2 also plays a role in the weight gain side effect of atypical antipsychotics: D2 activation partly redirects high fat diet-induced metabolic anomalies in obesity-prone mice (de Leeuw van Weenen et al., 2011), and administration of atypical agents has no effect on weight or food intake *Drd2* KO mice (Yoon et al., 2010). Numerous pharmacogenetic studies have linked DRD2 polymorphisms to AIWG (Lencz et al., 2010; Hong et al., 2010; Lett et al., 2012; Muller et al., 2012a). Researchers have also identified nominally significant associations with DRD2 polymorphisms and obesity-related phenotypes such as waist circumference (Kvaloy et al., 2013; Chen et al., 2012).

In AN, the insertion of the C nucleotide in the *DRD2* rs1799732 (-141 C in/del) polymorphism is preferentially transmitted to AN probands from parents (Bergen et al., 2005), and the haplotype including rs1799732 C insertion and rs6277 C allele is overrepresented in AN compared to controls (Bergen et al., 2005). *DRD2* rs1800497 (Taq1A) T allele, especially in conjunction with the 10-repeat variant of the *SLC6A3* rs28363170, has been linked to a higher BMI in probands with bulimic-spectrum disorders (Thaler et al., 2012). In addition, *DRD2*'s involvement in binge eating has been well documented (Davis et al., 2006; Davis et al., 2008;

Davis et al., 2009b; Davis et al., 2012). To date, however, there have not been any studies systematically screening *DRD2* polymorphisms and low body weight in AN.

1.8.5.2. DRD4. DRD4 is one of the main dopamine receptors, encoded by the *DRD4* gene on chromosome 11p15.5. Considered to be a D2-like receptor, DRD4 inhibits adenylate cyclase upon activation and reduces the intracellular concentration of the second messenger cyclic AMP (Asghari et al., 1995). However, while the DRD2 is believed to regulate reward and motor centers via mesolimbic and nigrostriatal pathways, DRD4 may influence satiety via the through its action on the mesohypothalamus (Huang et al., 2005).

In EDs, our extended group has shown the 7R variant of the exon III VNTR to be associated with maximum lifetime BMI (Kaplan et al., 2008; Levitan et al., 2010) and a history of childhood ADHD in BN (Yilmaz et al., 2012c; Appendix A6), as well as maximum BMI in seasonal affective disorder (Levitan et al., 2006a; Levitan et al., 2004). In the case of AN, a study of 109 probands did not report a significant association between *DRD4* exon III VNTR and BMI (Hinney et al., 1999b), but these results are difficult to generalize because of the modest sample size. Another study reported an association between the C allele of the *DRD4* rs1800955 SNP and AN in 202 trios (Bachner-Melman et al., 2007). Otherwise, there has not been sufficient research conducted on *DRD4* polymorphisms other than the 48-bp exon III VNTR. In light of the promising findings on the involvement of *DRD4* variants in weight regulation, ED research needs to expand the study of *DRD4* to include other SNPs in AN and BN.

1.8.5.3. COMT. The *COMT* gene encodes the COMT enzyme responsible for degrading catecholamines, including dopamine and norepinephrine, particularly in frontal areas of the brain (Allen Brain Atlas, 2010; Matsumoto et al., 2003). In healthy individuals, COMT function is associated with executive functions such as mental flexibility (Mitaki et al., 2013). Also in the

frontal regions of the brain, it is believed that serotonin contributes to dopamine regulation: decrease in serotonin levels results in an increase in dopamine levels, and vice versa (Kapur and Remington, 1996; Sasaki-Adams and Kelley, 2001). BN has been often associated with low levels of serotonin, which may persist even after recovery (Kaye et al., 1998), and it has been suggested that the binge eating behaviour may be an attempt to increase tryptophan production to normalize serotonin levels in frontal regions of the brain (Kaye et al., 2001). Many BN patients also suffer from mood and anxiety disorders, further suggesting a possible imbalance of the dopamine-serotonin ratio. Considering the pivotal role COMT plays in dopamine breakdown, *COMT* gene is a prime candidate to study in EDs.

In psychiatric genetics, one of the most studied variants of the *COMT* gene has been the G/A single nucleotide polymorphism at codon 158, exon 3 (rs4680; also known as Val158Met). Functional studies have identified the Val158Met polymorphism as a marker of trimodal function (Chen et al., 2004), leading to high, intermediate, and low enzyme activities. More specifically, Val158 has been associated with increased enzyme activity, which may result in faster breakdown of the catecholamines, potentially leading to lower levels of dopamine in the synaptic cleft. Furthermore, it has been shown that variations in the *COMT* haplotype consisting of rs6269, rs4633, rs4818 and rs4680 are linked to mRNA folding as well as COMT enzyme activity (Nackley et al., 2006). In BN, although a preliminary study of 42 probands reported that the Val allele of *COMT* rs4680 was overrepresented among BN probands compared to healthy controls (Mikolajczyk et al., 2006), our group failed to replicate this association but instead found the medium activity haplotype to be preferentially transmitted to the offspring with BN (Yilmaz et al., 2011; see Appendix A5). Furthermore, we reported an association between the presence of the Val allele and a history of childhood ADHD in BN probands (Yilmaz et al.,

2011). This publication is included in the Appendix of this dissertation (Section A5). In line with our ADHD findings, the Met allele has been significantly associated with decreased impulsivity, and a statistical trend was reported with lower binge eating frequency in BN (Groleau et al., 2012). In the case of AN, a meta-analysis of eight case-control studies failed to find a link between rs4680 and AN diagnosis (Brandys et al., 2012); however, this lack of association does not rule out a role for *COMT* rs4680 or other *COMT* variants in weight regulation within AN.

1.8.5.4. SLC6A3. SLC6A3 terminates the action of dopamine by its high affinity sodium-dependent reuptake into presynaptic terminals. Functional studies have shown that *SLC6A3* polymorphisms influence frontostriatal inhibition networks and may play a role in disorders of impulse dysregulation (Cummins et al., 2012).

SLC6A3 gene has been another candidate for the association studies of EDs, particularly for binge eating. A study of over 20,000 American adolescents revealed that carriers of the 10-repeat variant of the rs28363170, a VNTR polymorphism located in the 3' end of the gene, were more likely to consume foods with high-sugar content when in a depressed mood compared to the carriers of the 9-repeat (Agurs-Collins and Fuemmeler, 2011). In addition, the 10-repeat allele, especially in conjunction with DRD2 Taq1A T allele, predicts a higher BMI in probands with bulimic-spectrum disorders (Thaler et al., 2012). Although this has not yet been replicated in patients with a full BN diagnosis, this preliminary finding suggests a possible association between the *SLC6A3* VNTR and binge-eating behaviour. The role of SLC6A3 has not been well studied in AN.

1.8.5.5. MAOA. MAOA catalyzes the oxidation of monoamines, including noradrenaline, adrenaline, serotonin, and dopamine. Food deprivation decreases *Maoa* mRNA levels in mice (Jahng et al., 1998), thus suggesting an involvement in the appetite regulation. The *MAOA* gene,

especially the 30-bp VNTR located in the promoter region, has been implicated in several psychiatric disorders, such as OCD (Taylor, 2012), panic disorder (Reif et al., 2012), major depression (Fan et al., 2010), and bipolar disorder (Preisig et al., 2000; Muller et al., 2007; Fan et al., 2010). *MAOA* genetic polymorphisms have also been linked to obesity (Need et al., 2006; Fuemmeler et al., 2008), food reinforcement and BMI in healthy controls (Carr et al., 2013), and high BMI in depressed men (Fuemmeler et al., 2009).

The number of studies investigating the role of *MAOA* in EDs has been limited. Preferential transmission of the *MAOA* VNTR long allele in AN-R was reported in two separate family studies (Urwin et al., 2003b; Urwin and Nunn, 2005). There are no published studies of *MAOA* polymorphisms in BN, and more studies are required to investigate *MAOA* loci other than the VNTR in AN and ED-related phenotypes such as BMI.

1.8.6 Fat Mass and Obesity Associated (*FTO*) Gene

The *FTO* gene on chromosome 16, which covers over 400,000 bp and consists of nine exons, has been shown to be strongly associated with BMI, obesity, and weight gain in numerous studies including different ancestral populations (Frayling et al., 2007; Luan et al., 2009; Thorleifsson et al., 2009; Ruiz et al., 2010; Wangensteen et al., 2010; Hassanein et al., 2010; Zhang et al., 2010a; Adeyemo et al., 2010; Ng et al., 2010; Kaakinen et al., 2010; Scherag et al., 2010; Bressler et al., 2010; Holzapfel et al., 2010; Speliotes et al., 2010; Gonzalez et al., 2012; Paternoster et al., 2011; Rouskas et al., 2012; Huang et al., 2011; Okuda et al., 2011; Dorajoo et al., 2012; Prakash et al., 2011; Wang et al., 2012b; Hong et al., 2012; Lombard et al., 2012; Okada et al., 2012) and three meta-analyses (Peng et al., 2011; Yang et al., 2012; Guo et al., 2013). The vast majority of associations are reported with SNPs located in intron 1, and the most significant SNP linked to obesity varies in different populations. The involvement of *FTO* SNPs

located in intron 1 in weight regulation has also been shown in infants, children, and adolescents (Frayling et al., 2007; Bollepalli et al., 2010; Elks et al., 2010; Fang et al., 2010; Xi et al., 2010; Zavattari et al., 2011; Liu et al., 2011; Zhao et al., 2011; Wang et al., 2011a; Hallman et al., 2012; Warrington et al., 2013). In addition to the direct relationship between *FTO* and body weight, a number of studies reported significant gene-environment interactions involving diet and physical activity (Lee et al., 2010a; Sonestedt et al., 2011; Kilpelainen et al., 2011b; Corella et al., 2012), whereas others failed to find a SNP-lifestyle association (Holzapfel et al., 2010). More recently, *FTO* polymorphisms have been implicated in AIWG (Tiwari et al., 2011; Reynolds et al., 2012). *FTO* haploblock linked to obesity is also shown to influence body weight composition in a study of 4,523 middle-aged or older female twins (Livshits et al., 2012).

Currently, there is no evidence that *FTO* risk variants influence macronutrient or total energy intake (Hasselbalch et al., 2010), and population studies point to changes in adiposity as the possible mechanism of FTO's action. Interestingly, although *FTO* has little influence on birth weight (Lopez-Bermejo et al., 2008; Kilpelainen et al., 2011a) and the most drastic BMI differences are observed in adolescence for different genotypes (Kvaloy et al., 2013), it appears that the impact of carrying *FTO* risk alleles is maintained well into adulthood, most likely due to the weight-related effects of the early developmental years (Kaakinen et al., 2010). Although some sex differences have been reported (Hasselbalch et al., 2010; Kvaloy et al., 2013; Warrington et al., 2013), data have not been consistent, and studies with better statistical power are needed to understand the nature of these potential sex differences.

Although the genetic findings on the role of *FTO* in weight regulation are robust and well replicated, the expression and function of the intron 1 SNPs are not clear. FTO is involved in cerebrocortical insulin response, memory, synapse plasticity, and adipose tissue metabolism

(Jacobsson et al., 2012). *Fto* deficiency in mice leads to postnatal growth retardation (Yeo and Heisler, 2012), lean body mass (Fischer et al., 2009; Yeo and Heisler, 2012), and resistance to diet-induced obesity (Fischer et al., 2009). On the other hand, overexpression of *Fto* intron 1 SNPs leads to increased body mass (Berulava and Horsthemke, 2010; Church et al., 2010), possibly by altering the binding site of transcription factors (Berulava and Horsthemke, 2010) and leading to increased food intake (Church et al., 2010). FTO is also linked to nucleic acid demethylation, and feeding behaviour plays an important part in the regulation of *Fto* mRNA levels (Gerken et al., 2007). Although expressed abundantly across several tissues, FTO is most highly expressed in the brain (McTaggart et al., 2011), particularly the hypothalamus (Gerken et al., 2007), further pointing to a crucial function in appetite and weight regulation. However, the data from a more recent study suggest that FTO affects body weight not through changes in food intake, but through a decrease in energy expenditure (Fischer et al., 2009). Moreover, preliminary evidence suggests that *FTO* risk alleles may alter the perception of satiety (Papathanasopoulos et al., 2010), increase eating behaviour in the absence of hunger in subjects experiencing acute stress (Rutters et al., 2010), and may result in more eating episodes per day independent of body weight (McCaffery et al., 2012).

Two studies have investigated the possible involvement of *FTO* in EDs. While the authors of the first study (performed using the PF DNA samples) failed to find an association between the seven *FTO* SNPs and psychological or behavioural ED phenotypes in AN (Jonassaint et al., 2011), the other group reported a nominally significant association of the A allele of *FTO* rs9939609— obesity risk allele— in intron 1 with both AN and BN (Muller et al., 2012b), but it is important to note that there was no correction for multiple testing and these results need to be replicated. The role of the *FTO* obesity loci in AN weight regulation is to be

investigated by our collaborators using the GCAN dataset in the near future, with the hopes that the large sample size will provide a more conclusive answer. Furthermore, considering that *FTO* spans over a 400-kilobase (kb) region, it is too early to rule out the role of other *FTO* variants in AN or weight regulation in EDs. In light of the promising literature on obesity, *FTO* in general is a candidate gene with great potential for studies on BMI-related differences in AN and BN.

CHAPTER 2

OBJECTIVES AND HYPOTHESES

2.1. Objectives

AN and BN are serious psychiatric disorders with complex non-Mendelian genetic etiology. Thus far, researchers have focused on a small selection of gene systems and polymorphisms, in the meanwhile neglecting other genes known to play a role in appetite and weight regulation. In addition, the vast majority of the ED genetic studies have been hindered by clinical, methodological and ancestral heterogeneity, small sample size, and lack of statistical correction for multiple testing. One of the biggest shortcomings has been the issue of diagnostic heterogeneity: as it will be discussed in the Methods chapter of this dissertation, many BN probands have a history of AN, and the converse is less common but does occur. This high rate of diagnostic crossover threatens the validity of genetic findings in any study that compares AN and BN probands. Furthermore, BMI histories of AN patients with a history of BN are significantly different from those with AN with no history of BN (also discussed in detail in Section 3.1.3), thus further complicating genetic studies of weight regulation in EDs. This factor may have also contributed to many of the genes and polymorphisms having yielded at best variable findings in EDs, highlighting the need to conduct trials with larger sample sizes and reduced clinical heterogeneity in terms of ED diagnosis. Finally, on many occasions, ED genetic studies have not reflected the advances in the obesity and AIWG literature, which may provide useful and novel insights to our understanding of weight regulation in AN and BN.

This doctoral dissertation aims to utilize two distinct but complementary candidate gene approaches in the hopes of dissecting the genetic etiology of weight regulation in AN and BN: the first approach is targeted in terms of both the phenotype and the selection of loci, whereas the

second approach consists of a more systematic screening and thorough coverage of the select candidate genes in a larger sample. More specifically, utilizing novel methodology that reduces phenotypic group heterogeneity, the goal of Study 1 is to investigate on average two polymorphisms with known or putative function in the leptin, melanocortin, and neurotrophin system genes in AN probands with no history of BN, selected as a part of Study in in the PF cohort. The case-control component of this study utilizes BN probands with no history of AN as a comparative ED group and women with no psychiatric history as controls. The second component of this study explores the role of the select candidate genes on minimum lifetime BMI, maximum lifetime BMI, and BMI at the time of recruitment in AN and BN groups separately. Study 2, on the other hand, is a secondary analysis of GCAN AN GWAS data and investigates the role of dopamine system genes and *FTO* in a large dataset of AN probands. More specifically, the SNPs of a select number of candidate genes covered by the GWAS genotyping will be extracted to study their possible role on minimum lifetime BMI, maximum lifetime BMI, and BMI at recruitment in AN.

2.2. Hypotheses

The main hypothesis for Study 1 is that specific functional variants of the candidate genes in the leptin, melanocortin, and neurotrophin systems, associated with appetite and weight suppression, are more likely to be present in AN patients compared to BN patients with no history of AN or nonpsychiatric controls. We also hypothesize that these leptin, melanocortin, and neurotrophin system genetic variants will be linked to weight regulation (as measured by lowest lifetime BMI, highest lifetime BMI, and BMI at recruitment) within AN and BN groups, respectively. The main hypothesis for Study 2 is that candidate genes and loci implicated in obesity and weight regulation studies will be associated with the lifetime BMI measures (also as

measured by lowest lifetime BMI, highest lifetime BMI, and BMI at recruitment) in AN probands. Considering that the set of candidate genes that we chose for this study has either resulted in variable findings in AN patients or not been sufficiently studied, setting detailed *a priori* hypotheses as to which alleles will be associated with low body weight in AN is a challenge.

We have three sets of general hypotheses for Study 1: leptinergic, melanocortinergic, and neurotropic. We also have one leptinergic, three melanocortinergic, and four neurotrophic specific hypotheses based on the ED and obesity literature reviewed above for Study 1:

1) *HRH1* polymorphisms associated with AIWG will have a protective effect on AN;
2) *MC4R* rs17782313 T allele will be linked to AN diagnosis and lower minimum lifetime BMI in AN and BN;
3) *MC4R* alleles linked to AIWG will be underrepresented in AN probands compared to BN and control groups, and the alleles with protective effect for weight gain will be associated with lower BMI in AN;
4) The G (Thr) allele of *AGRP* rs5030980 will be more common in AN compared to BN and controls, and this allele will predict lower BMI in AN;
5) The Met66 variant of *BDNF* rs6265 will be associated with low BMI in AN;
6) *NTRK2* rs1187325 C homozygosity will be overrepresented in AN probands compared to BN and controls;
7) *NTRK2* rs1078947 will predict low BMI in AN; and
8) CT heterozygotes of the *NTRK3* rs7180942 will be underrepresented in EDs compared to controls, and C allele will be more common in AN.

For Study 2, our general hypotheses concern dopaminergic pathway and *FTO*, predicting their involvement in weight regulation in AN probands. Furthermore, we have four dopamine pathway-specific hypotheses, also based on the ED and obesity literature reviewed above:

1) *DRD2* genetic polymorphisms will be linked to lifetime BMI in AN.
2) *DRD2* SNPs implicated in the previous PF study (e.g., rs1799732, rs6277) will be linked to BMI in AN;
3) *COMT* Met158 allele (rs4680) will be more common in AN probands with lower current and lifetime BMI measures; and
4) *MC4R* markers previously linked to AIWG will be linked to low lifetime BMI measures in AN.

CHAPTER 3

METHODS

3.1. Study 1: PF Candidate Gene Analysis

3.1.1. Proband inclusion/exclusion criteria

3.1.1.1. PF cohort. The main sample used for the selection of suitable probands was derived from the PF Genetic Consortium. The recruitment process for this consortium included careful phenotyping of all participants, and these procedures and sample characteristics have been previously outlined in detail (Kaye et al., 2000; Bergen et al., 2003b; Kaye et al., 2004b).

PF genetic collaboration initially started as a cooperative arrangement between the PF in Geneva, Switzerland, the University of Pittsburgh, and a number of high-profile academic sites in North America and Europe. DNA extraction, as well as all initial genetic work and statistical analyses took place at the University of Pittsburgh, USA. Currently, the DNA samples are stored in SeraCare Life Sciences—a biorepository located in Maryland, USA—and the data analysis component has been taken over by the individual investigators and their groups who have participated in the data collection. The initial founding sites under the PF umbrella were: (1) the University of Pittsburgh, USA; (2) University of Toronto, Canada; (3) Cornell University, USA; (4) University of California at Los Angeles, USA; (5) King's College London, UK; and (6) University of Munich, Germany with established clinical and research eating disorder programs. Since then, other institutions have also been included in the consortium, the list of which consists of but is not limited to: (1) University of Pisa, Italy; (2) University of North Dakota, USA; (3) University of North Carolina at Chapel Hill, USA; (4) University of Minnesota, USA; (5) Harvard University, USA; (6) University of Pennsylvania, USA; and (7) University of California

at San Diego, USA. Today, PF International Genetic Consortium houses one of the largest and best characterized ED DNA samples in the world, and this fruitful collaboration has lead to numerous genetic and clinical publications that have made significant contributions to our understanding of AN and BN (Grice et al., 2002; Devlin et al., 2002; Bergen et al., 2003b; Bergen et al., 2003a; Bulik et al., 2003; Bulik et al., 2004; Bacanu et al., 2005; Tozzi et al., 2005; Jacobs et al., 2009; Baker et al., 2010; Root et al., 2010; Pinheiro et al., 2010; Bloss et al., 2011; Jonassaint et al., 2011; Wang et al., 2011b; Dellava et al., 2012; Halmi et al., 2012; Hoffman et al., 2012).

PF DNA samples were collected for three separate studies: AN affected relative pair (AN-ARP), BN affected relative pair (BN-ARP), and AN trio/control women (ANT-CW). Core inclusion criteria for the AN-ARP study included: (1) lifetime DSM-IV diagnosis of AN, where the amenorrhea criterion was not strictly enforced; (2) 13-65 years of age; (3) three years minimum duration for AN; and (4) no binge eating for at least once a week for 3 or more consecutive months, i.e., exclusion of AN-BP and BN. Exclusion criteria included: (1) organic brain syndrome; (2) IQ < 70; (3) history of psychosis or bipolar disorder; (4) obesity; and (5) any medical illness that may affect appetite, food intake, or weight. Affected relatives were not subjected to criteria as stringent as those for the cases, and the ED diagnoses of AN-R, AN-BP, BN, and EDNOS were all acceptable.

The BN-ARP study included individuals with current or past BN, purging subtype (as per DSM-IV criteria), where regular purging was defined as binge eating and self-induced vomiting for at least twice a week for at least six months prior to study enrollment. Prior AN history was not an exclusion criterion. Individuals with a history of obesity were only excluded if their lifetime maximum BMI was > 35 kg/m^2. Bipolar Disorders I and II were under the exclusion

criteria if the BN symptoms occurred exclusively during manic or hypomanic episodes. Otherwise, the inclusion and exclusion criteria regarding age and medical history were as per described for AN-ARP. Also similar to the AN-ARP study, the definition of EDs was more broadly defined for affected relatives in the BN-ARP study. The assessment process for the PF studies was very thorough in order to properly characterize all cases. Assessments administered by trained interviewers included the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I), Structured Interview of Anorexia Nervosa and Bulimic Syndromes (SIAB-EX), the Yale-Brown Obsessive Compulsive Scale, and the Yale-Brown-Cornell Eating Disorder Scale. The extensive self-report assessment package included well-validated and utilized scales such as the Eating Disorder Inventory-2, State-Trait Anxiety Inventory, Multidimensional Perfectionism Scale, Temperament and Character Inventory, Revised NEO Personality Inventory, and Barrett Impulsivity Scale.

For the present study, only a select number of probands recruited for the PF studies were included in the analysis. The detailed inclusion criteria can be found in Table 1. More specifically, this study consisted of a subgroup of participants who either had AN with no history of BN or BN with no history of AN. The rationale behind this selection was to keep the samples of the two ED phenotypes distinct, especially considering the high crossover rates between AN and BN (as discussed in Sections 3.1.2 and 3.1.3). In the end, 787 (100%) of the AN and 147 (55.1%) of the BN DNA samples included in the present study were obtained from the PF studies. All individuals included in the analysis were unrelated, and in the case of affected relatives, the participant selection focused on maximizing the BN group due to a smaller number of BN DNA samples being available for the purpose of this study.

3.1.1.2. Toronto Bulimia Nervosa Genetics Study cohort. Additional BN samples were

Table 1. Detailed inclusion criteria for the AN, BN, and nonpsychiatric control groups

AN	BN	Nonpsychiatric controls
1. Women with a DSM-IV-TR/SIAB-EX diagnosis of either current or lifetime AN (amenorrhea not strictly enforced)	1. Women with a DSM-IV-TR/SIAB-EX diagnosis of either current or lifetime BN, purging subtype	1. Women 18-65 years of age
2. European Caucasian ancestry	2. European ancestry	2. No current or past psychiatric disorder, as assessed by a check-list prior to phlebotomy;
3. 13-65 years of age	3. 13-65 years of age	3. European ancestry
4. Minimum lifetime BMI < 18.5 kg/m^2	4. Minimum lifetime BMI > 18.5 kg/m^2	4. Current BMI between 19 kg/m^2 and 28 kg/m^2
5. Maximum lifetime BMI < 35 kg/m^2	5. Maximum lifetime BMI < 35 kg/m^2	
6. Onset of AN before age 25	6. No DSM-IV-TR diagnosis of current or lifetime AN	
7. Fulfillment of AN diagnostic criteria for at least three years at the time of recruitment	7. No DSM-IV-TR diagnosis of current or lifetime psychotic episode (i.e., schizophrenia, schizophreniform disorder, or schizoaffective disorder)	
8. No DSM-IV-TR diagnosis of current or lifetime BN	8. In the case of a DSM-IV-TR diagnosis of current or lifetime bipolar disorder, binge eating and purging episodes not limited to manic or hypomanic phases	
9. No DSM-IV-TR diagnosis of current or lifetime psychotic episode (i.e., schizophrenia, schizophreniform disorder, or schizoaffective disorder)	9. No current or past medical or metabolic condition that may affect appetite, weight, or eating	
10. No current or past medical or metabolic condition that may affect appetite, weight, or eating		

selected from the Toronto Bulimia Nervosa Genetics Study, stored at the Centre for Addiction and Mental Health (CAMH) Neurogenetics Laboratory in Toronto, Canada. The Toronto Bulimia Nervosa Genetic Study includes DNAs from 242 women with current or past BN (purging subtype), who were recruited through advertisements posted at various clinical and community settings in Toronto, Canada. Eligibility criteria for BN probands in the principal study included: (1) between 18 and 65 years of age; (2) DSM-IV diagnosis for current or past BN, purging subtype; and (3) European descent, with no more than one grandparent identified as non-European. Exclusion criteria included: (1) a maximum lifetime BMI $\geq$ 35 kg/m^2; (2) history of a psychotic episode; (3) history of bipolar disorder if binge eating and purging occurs only during manic/ hypomanic phases; (4) diabetes preceding the onset of ED; (5) thyroid or endocrine disorders; and (6) medical conditions that could affect appetite, weight, or eating behaviour. Subgroups of this sample have been described in previous publications by our group elsewhere (Levitan et al., 2001; Levitan et al., 2006a; Kaplan et al., 2008; Levitan et al., 2010; Yilmaz et al., 2011; Yilmaz et al., 2012c).

All BN probands in the original study completed the SCID-I and the Eating Disorder Examination (Fairburn and Cooper, 1993). Participants were weighed during the in-person assessment, whereas height information as well as minimum and maximum weights were obtained via self-report. BMIs were calculated for each BN patient based on the following formula: *weight in kilograms / height in metres squared*. All participants received oral and written summaries of the purposes, procedures, and potential risks of the study and gave informed written consent. The study protocol was approved by the Research Ethics Boards within the University Health Network and CAMH in Toronto, Canada.

Out of the original 242 BN cases, we selected a subgroup of 120 probands with no

history of AN to include in the present study. The details of the selection process are summarized in Table 1.

3.1.1.3. Control cohort. Finally, 322 female controls with no psychiatric history were obtained from the Toronto Centre for Applied Genomics in Toronto, Canada. All controls were of European ancestry. All individuals whose DNA samples were included in the biorepository completed an extensive list self-report questionnaires on health history, which included but was not limited to the following categories: (1) pregnancy; (2) smoking; (3) bronchial emphysema; (4) asthma; (5) diabetes; (6) high blood pressure; (7) heart disease; (8) cancer; (9) rheumatoid and osteoarthritis; (10) epilepsy; (11) migraine; and (12) mental disorders. The reason for the thorough health history screening was to ensure the individuals recruited could serve as controls for a diverse selection of medical research studies.

Considering that the control sample was not specifically screened for EDs, we only included individuals with a current BMI between 19 kg/m^2 and 28 kg/m^2 to avoid any extreme weight phenotypes (Table 1).

All aspects of this present research study were reviewed and approved by the CAMH Research Ethics Board, and informed consent for providing genetic materials and inclusion of these materials in future collaborative studies was obtained from all individuals whose DNA samples were included in our analysis according to the Declaration of Helsinki.

3.1.2. Low body weight as the primary phenotype

Low weight or BMI is the *sine qua non* of AN and the primary target of initial treatment (American Psychiatric Association, 2006). Low weight and behaviours associated with reaching and maintaining it are also the primary reason for the high morbidity and mortality in this illness. Low BMI is also an important indicator of AN severity and often predicts poor treatment

outcome (American Psychiatric Association, 2006). While low weight may serve as a positive reinforcement for the maintenance of the anorexic state on a cognitive level (Brockmeyer et al., 2012), the main diagnostic criterion that separates AN from BN is low body weight according to the DSM-IV-TR (American Psychiatric Association, 2000).

On an etiological level, there is some evidence for premorbid low BMI distinguishing teenage girls who go on to develop AN in young adulthood from those who develop BN (Tyrka et al., 2002). Furthermore, less than 5% of AN patients report a history of obesity compared to a third of BN patients (Villarejo et al., 2012). A recent study reported that as much as 64% of those with a BN diagnosis had a BMI of 25 kg/m^2 or above, which is the cutoff of overweight, in a community sample (Masheb and White, 2012). In addition, lower body fat percentage, an anthropomorphic measure linked to BMI, is a significant risk factor for relapse in AN (Bodell and Mayer, 2011). With all these lines of evidence combined, low BMI is a very suitable primary phenotype for the genetic studies of AN in our quest to better understand the biological etiology of this disorder.

It has been proposed that the study of *endophenotypes* holds great potential in understanding the genetic etiology of complex behaviours and disorders associated with them (Bulik et al., 2007; Gottesman and Gould, 2003). Over the last few years, there has been a shift from looking for the disease gene (i.e., case-control studies) toward the investigation of endophenotypes in psychiatric candidate gene studies. Endophenotypes are measurable traits that (1) are heritable; (2) co-segregate with a psychiatric illness in the general population; (3) are state-independent; and (4) are found in family members at a higher rate than in the general population (Gershon and Goldin, 1986). Researchers are now working toward identifying suitable and reliable endophenotypes for various psychiatric disorders. For example, there is

strong evidence suggesting that prepulse inhibitions may be a viable endophenotype in schizophrenia (Gottesman and Gould, 2003). Thinking of psychiatric diagnoses as a continuum and focusing on the underlying traits (e.g., investigating the genetic determinants of impulsivity as opposed to AN or BN as a diagnostic category) will likely be a more fruitful approach that solves the current issue of non-replication in psychiatric candidate gene research. Currently there are no agreed-upon endophenotypes identified in AN or BN. Although both low BMI and intentional weight loss in AN are at least partially heritable (Bulik et al., 2010) and the prevalence of familial obesity is reported to be lower among AN probands compared to the rates observed in the general population (Villarejo et al., 2012), there is insufficient evidence for low weight to be state-independent. Based on these findings, low weight associated with AN cannot be fully classified as an endophenotype, but it is a very strong candidate for a valid subphenotype or alternate phenotype that may help researchers reduce clinical heterogeneity and identify a true and replicable signal in AN.

3.1.3 Issue of high diagnostic crossover rates in the genetic studies

An important factor that complicates genetic study design is the high crossover rate between diagnoses and subtypes within a diagnostic category. For instance, 50% of individuals with AN-R develop AN-BP in a few years' span (Eddy et al., 2002). AN to BN crossover rate may be as high as 36%, with higher baseline and lifetime BMIs being predictors of crossover (Tozzi et al., 2005). Crossover from BN to AN may be less common, with the estimates ranging from 4% to 27% (Tozzi et al., 2005; Fichter and Quadflieg, 1997). Diagnostic instability makes it difficult to tease apart genetic contributions to AN versus BN, and maintaining these groups diagnostically distinct is an important step toward reducing phenotypic heterogeneity in genetic studies. However, studies also report a sharp decline in crossover rates after five years (Tozzi et

al., 2005; van Son et al., 2010), with most cases of crossovers taking place during the first three to five years of illness (Eddy et al., 2002). Thus, a minimum of three-year course of illness could serve as an important genetic study inclusion criterion, especially for AN, to reduce the probability of crossover after study participation.

The issue of heterogeneity caused by high ED crossover rates is especially problematic when studying ED-associated phenotypes such as BMI. There is evidence suggesting that weight histories of AN patients differ significantly if they have a history of BN versus no history of BN. For example, individuals with AN-R have been found to have a significantly lower maximum BMI compared to AN probands with a history of BN. Similarly, BN probands with a history of AN tend to report significantly lower current, maximum and minimum BMIs compared to those without a history of AN (Kaye et al., 2004b). Another confounder that may affect the upper end of BMI between groups is the higher prevalence of premorbid obesity in BN compared to AN (33.2% vs. 4.6%, respectively; Villarejo et al., 2012). Furthermore, higher maximum lifetime BMI is a predictor of diagnostic crossover from AN to BN (Monteleone et al., 2011). In summary, it is important to consider the high crossover rates between AN and BN when designing genetic studies that compare these two diagnostic groups, especially when BMI measures are studied as the principal associated phenotype.

3.1.4. Inclusion of BN probands in the case-control design

Our study was specifically designed to optimize the use of BN probands as a comparative ED group. Thus far, the vast majority—if not all—of the candidate gene studied in the ED literature have included AN probands with a history of BN and BN probands with a history of AN in their analyses. This method helps with increasing sample size in both groups, especially considering that the diagnostic crossover rates between ED are high. However, when comparing

two groups in a genetic study, it is important to diagnostically and phenotypically separate them as much as possible to maximize between-group and minimize within-group differences. Via our thorough selection process of probands for both AN and BN groups (discussed in detail in Section 3.1.1), we aimed to ensure that AN and BN groups were diagnostically distinct from each other. In summary, by including AN probands with no BN history in our AN group and BN probands with no AN history in our BN group, we hope to reduce phenotypic heterogeneity between ED groups while maximizing genetic similarities within each cohort.

3.1.5. Use of nonpsychiatric controls

Women with no psychiatric history were used as the control group in this study. The use of healthy controls is the hallmark of case-control study design; in order to establish the presence of any genetic differences in the affected group, researchers have to provide evidence that a similar significant pattern is not present in unaffected individuals who are otherwise comparable to the affected individuals.

Inclusion of healthy controls is an efficient and inexpensive method that allows researchers to attribute observed differences to the medical condition being studied in the cases. The case-control design is especially informative in the study of rare diseases or for preliminary analyses for conditions about which little is known. Furthermore, longitudinal design is unlikely to be informative for classical genetic studies as to who will develop AN or not, since the genetic risk factors are presumed to be present from birth on, and unless researchers are interested in gene-environment interaction or phenotypic risk factors, long-term follow-up of individuals is not likely to help us pinpoint the genetic risk (i.e., DNA sequence-derived risk) for ED diagnosis.

Case-control design is still very much utilized in psychiatric studies, including candidate gene analyses (LaHoste et al., 1996; Muglia et al., 2000; Ribases et al., 2004; Yilmaz et al.,

2012c), as well as the recent GWAS efforts (Sklar et al., 2008; O'Donovan et al., 2008; International Schizophrenia Consortium et al., 2009; Green et al., 2012; Bulik et al., 2012).

3.1.6. Candidate gene approach

As highlighted in the Introduction, the candidate gene approach has been thus far very fruitful in psychiatric genetics and helped researchers uncover very important associations in the etiology of psychiatric disorders. Designing studies based on *a priori* hypotheses informed by biological function, candidate gene studies aim to build upon prior knowledge provided by basic science. The present study included a total of 11 candidate genes from the leptin, melanocortin, and neurotrophin gene systems, which are listed in Table 2, selected for their involvement in appetite, weight regulation, and energy homeostasis.

It is important to acknowledge that not all candidate gene findings have been successfully replicated, with the most important reason behind this being small sample size. In addition, phenotypic heterogeneity and population stratification (i.e., genetic heterogeneity due to ancestral background) have also posed issues for replication in follow-up studies and in some instances led to variable findings. Instead of completely putting aside the candidate gene approach and shifting solely to GWAS and high-throughput sequencing, it is important for researchers to have adequate sample size for the analyses they plan to perform, control for population stratification, and characterize the study sample thoroughly to reduce phenotypic heterogeneity.

Although some would argue against the utilization of the candidate gene approach in the age of GWAS, it is unfair to deny that the candidate gene technique has established itself as a valid methodology and has greatly contributed to our current understanding of psychiatric

Table 2. List of candidate genes included in the PF study

Gene	Abbreviation	Location	Gene size (kb)
Leptin receptor	*LEPR*	1p31.3	217.2
Leptin	*LEP*	7p31.3	16.4
Ghrelin	*GHRL*	3p25.3	72
Histamine receptor H1	*HRH1*	3p25.3	191.7
Melanocortin 3 receptor	*MC3R*	20q13.2	1.1
Melanocortin 4 receptor	*MC4R*	18q21.32	1.4
Agouti-related protein	*AGRP*	16q22.1	1.2
Pro-opiomelanocortin	*POMC*	2p23.3	7.8
Neurotrophic tyrosine kinase receptor type 2	*NTRK2*	9q21.33	355.3
Neurotrophic tyrosine kinase receptor type 3	*NTRK3*	15q25.3	379.7
Brain-derived neurotrophic factor	*BDNF*	11p14.1	67.2

illness. Candidate gene and GWAS methods are not diametrically opposite as some would argue, but they are rather complementary: while GWAS relies on the power of very large sample sizes and the study of the whole genome, the hypothesis-free approach disregards decades of functional preclinical research which can really inform and guide study design. Furthermore, a blind scan of the genome also requires harsh multiple testing correction to reduce Type I error rates, whereas a targeted candidate gene approach based on the literature and known function helps reduce the number of tests and does not require stringent multiple testing correction. While GWAS utilizes thousands of individuals with more broadly defined phenotypes, candidate gene studies can complement our understanding of the biological etiology of psychiatric disorders by focusing on a smaller number of carefully characterized cases, thus leading to less heterogeneity and potentially better able to zero in on a distinct signal that separates the cases from controls.

3.1.7. Selection of functional SNPs

For the present candidate gene study, we decided to pursue a very targeted approach that focuses on SNPs with known or putative function, as assessed by *in silico* analysis. This approach has a number of advantages over the tag SNP approach; first, the study of functional variants help us make more biologically meaningful discoveries as to the effects of any genetic differences associated with the phenotype being studied. In addition, focusing on a small number of carefully selected loci reduces multiple testing and requires less stringent statistical correction. Below is a brief summary of the rationale for the inclusion of the selected loci that falls under each gene system studied, which is also outlined in Table 3. On average, two markers per gene were selected. Priority was given to SNPs that have been studied in EDs and/or weight regulation literature (obesity and AIWG). Primarily two *in silico* tools were utilized: the National Institute of Environmental Health Sciences (NIEHS; http://snpinfo.niehs.nih.gov) and BrainArray

(http://brainarray.mbni.med. umich.edu). Both websites utilize HapMap sequences and the University of California Santa Cruz Genome Bioinformatics website (http://genome.ucsc.edu) for conservation, regulatory potential, and transcription factor-binding sites. Further details on the *in silico* algorithms used by NIEHS are outlined elsewhere (Xu and Taylor, 2009).

Among the leptin system genes, we selected a total of seven SNPs to study. *LEPR* rs1137100, a missense mutation that results in Lys/Arg amino acid substitution, and rs1137101, also a missense mutation that results in Gln/Arg substitution, have been shown to affect plasma soluble LEPR levels in health individuals in a GWAS in 1,504 women of European descent (Sun et al., 2010). *LEP* rs7799039 is located in the promoter region of the gene and also significantly alters mRNA expression and plasma LEP levels. An electromobility shift assay (EMSA) study revealed that nuclear extracts derived from both U937 cells and human adipocytes form a protein-DNA complex with the rs7799039 polymorphic site and bind with higher affinity to the A allele (Hoffstedt et al., 2002). The function of *GHRL* rs696217 has not been fully determined. However, according to *in silico* analysis, this locus is predicted to be a putative transcription factor-binding site, thus may regulate the transcription of genetic information from DNA to mRNA. In addition, it is a nonsynonymous amino acid change (Leu/Met) and may lead to changes in the protein. Similarly, *GHRL* rs4684677, an A to T transversion that leads to a nonconservative amino acid change from Gln to Leu at codon 90, is highly conserved among species. Although its exact function is unknown, it is exonic and may affect protein coding. *In silico* analysis also predicts rs4684677 to be a putative splicing site. Since these two *GHRL* polymorphisms have been previously studied in EDs, led to variable findings due to sample size issues, and are predicted to have functional importance, they were included in the present study. *HRH1* polymorphisms were selected solely by *in silico* predictions of function, since they have

not been studied in EDs or weight regulation. The rs12490160 SNP is located upstream of the *HRH1* gene, thus it is likely to be a transcription factor-binding site and play a regulatory role. *HRH1* rs3732941, on the other hand, is located in the 3' untranslated region (UTR) and may be a microRNA (miRNA) binding site.

A total of eight loci were selected from the melanocortin system candidate genes. Although the function of *MC4R* rs17782313 is not known, it has been repeatedly linked to obesity in the literature (as reviewed in Section 1.8.3.1), and it merits a thorough investigation in EDs. Similarly, rs489693 has been associated with AIWG by our extended group (Malhotra et al., 2012), and although its function is currently unknown, its study in EDs and weight regulation could be informative. *MC4R* rs8087522 is located in the promoter region of the gene, and in an EMSA study conducted by our group, it has been shown that the presence of the A allele may create a transcription factor-binding site (Chowdhury et al., 2012). Two *MC3R* markers were selected, one of which (rs6127698) is located in the promoter region and is predicted to be a transcription factor-binding site. The second locus, rs3827103, is a missense mutation that results in a Val/Ile amino acid substitution and has been investigated in one preliminary ED study (de Krom et al., 2005b). This exonic polymorphism is also highly conserved, and an *in vitro* study using transfected HEK-293 cells linked rs3827103 to diminished functionality and expression of the MC3R receptor (Feng et al., 2005). In addition to being studied in EDs, *AGRP* rs5030980 comes before the C-terminal Cys rich part of the protein with receptor binding activity (Vink et al., 2001). A missense mutation (Ala/Tyr substitution), this polymorphism is also located two amino acids from the proteolytic site that generates a higher affinity isoform (Ollmann et al., 1997). *AGRP* rs13338499 is yet to be studied in EDs or weight regulation, however it merits inclusion in the present study since it is located upstream of the gene and a putative transcription-

Table 3. SNPs included in the PF candidate gene study and the rationale behind their inclusion

Gene	dbSNP #	SNP type	Function	Studied in EDs?	Limitations of previous studies
LEPR	rs1137100	Missense (Lys/Arg)	Affects plasma soluble leptin receptor levels	Quinton et al. (2004)	Small sample size
LEPR	rs1137101	Missense (Gln/Arg)	Affects plasma soluble leptin receptor levels	Quinton et al. (2004)	Small sample size
LEP	rs7799039	In the promoter region	Affects mRNA expression and plasma leptin levels	Janas-Kozik et al. (2008)	Small sample size
GHRL	rs696217	Missense (Leu/Met)	Putative transcription factor-binding site; might be of regulatory nature; nonsynonymous amino acid change	Ando et al. (2006); Dardennes et al. (2007); Cellini et al. (2006); Monteleone et al. (2006a); Monteleone et al. (2007); Kindler et al. (2011)	Conflicting findings
GHRL	rs4684677	Missense (Gly/Leu)	Highly conserved among species; exonic; putative splicing site	Cellini et al. (2006), Dardennes et al. (2007); Kindler et al. (2011)	Conflicting findings
HRH1	rs12490160	Upstream of the gene	Putative transcription factor-binding site	No	Not studied in EDs
HRH1	rs3732941	In the 3' UTR	Putative miRNA binding site	No	Not studied in EDs
AGRP	rs5030980	Missense (Ala/Tyr)	Exonic; located before the C-terminal Cys rich part of the protein with receptor binding activity; also located two amino acids from the proteolytic site that generates higher affinity isoform	Vink et al. (2001); Dardennes et al. (2007)	Small sample size

Gene	dbSNP #	SNP type	Function	Studied in EDs?	Limitations of previous studies
AGRP	rs13338499	Upstream of the gene	Putative transcription factor-binding site; located upstream of AGRP; possible regulatory role	No	Not studied in EDs
NTRK2	rs1078947	Intronic	Based on past significant ED findings; functionality unknown	Ribases et al. (2005a)	Small sample size; needs replication
NTRK2	rs1187325	In the 5' UTR	May affect the length and stability of the mRNA isoforms	Ribases et al. (2005a)	Small sample size
MC3R	rs6127698	In the promoter region	Putative transcription factor-binding site;	No	Not studied in EDs
MC3R	rs3827103	Missense (Val/Ile)	Exonic; highly conserved; *in vitro* diminished functionality and expression of the receptor	de Krom et al. (2005b)	Small sample size
MC4R	rs17782313	Intergenic	Significant findings in the obesity literature; functionality unknown	No; studied in obesity (see Section 1.8.3.1)	Not studied in EDs
MC4R	rs489693	Intergenic	Significant findings in the AIWG literature; function unknown	No; studied in AIWG (see Section 1.8.3.1)	Not studied in EDs
MC4R	rs8087522	In the promoter region	Putative transcription factor binding site; located in the promoter region	No; studied in AIWG (see Section 1.8.3.1)	Not studied in EDs

Gene	dbSNP #	SNP type	Function	Studied in EDs?	Limitations of previous studies
NTRK3	rs7180942	Intronic	Heterozygosity may reduce expression levels	Mercader et al. (2008)	Needs replication
NTRK3	rs1128994	Synonymous	Putative splicing site	No	Not studied in EDs
BDNF	rs6265	Missense (Val/Met)	Affects the secretion and dendritic trafficking of BDNF; alters hippocampal volume; linked to impaired cognitive skills	Ribases et al. (2003); Ribases et al. (2004); Ribases et al. (2005b); de Krom et al. (2005); Monteleone et al. (2006b); Dmitrzak-Weglarz et al. (2007); Gratacos et al. (2007); Gelegen et al. (2008); Kaplan et al. (2008); Brandys et al. (2011)	Conflicting findings
BDNF	rs56164415	In the 5' UTR	Affects mRNA folding; possible splicing site	Ribases et al. (2003); Ribases et al. (2004); Ribases et al. (2005b); de Krom et al. (2005a); Dmitrzak-Weglarz et al. (2007); Dardennes et al. (2007); Mercader et al. (2008)	Small sample sizes, conflicting findings
POMC	rs1042571	In the 3' UTR	Putative transcription factor-binding site; potential miRNA binding site	Ternouth et al., 2011; Wang et al., 2012a;	Not studied in EDs

factor binding site. Finally, we chose to include one *POMC* marker (rs1042571), which is located in the 3' UTR and predicted to be a transcription factor- and miRNA-binding site.

From the neurotrophin system genes, six loci were selected for the present study. The functionality of *BDNF* rs6265 (Val66Met) has been extensively studied. For example, Met allele has been shown to disrupt dendritic trafficking of the *BDNF* mRNA (Chiaruttini et al., 2009). A magnetic resonance imaging (MRI) study reported that the Val66Met polymorphism was associated with local variation in gray matter volume in newborns (Knickmeyer et al., 2013). In 19 first-episode schizophrenia patients and 25 healthy individuals, Val/Val genotype was linked to a larger hippocampal volume (Szeszko et al., 2005), and a separate study on healthy individuals also confirmed that those carrying the Met variant of rs6265 may have reduced hippocampal volume compared to Val/Val carriers (Pezawas et al., 2004). Similarly, presence of the Met allele may lead to reduced short-term plasticity and making more errors in short-term motor learning tasks (McHughen et al., 2010). In addition, researchers have reported that the Met allele leads to poorer emotional decision making in healthy subjects, as assessed via Iowa Gambling Task (Kang et al., 2010b). *In silico*, Val66Met is predicted to be a transcription factor-binding site and play a regulatory role. The second *BDNF* SNP, rs56164415, is located in the 5' UTR and is thought to be involved in mRNA folding, and based on *in silico* predictions, it may also be a putative splicing site. As for the *NTRK2* loci, rs1187325 is located in the 5' UTR and speculated to affect the length and stability of the mRNA isoforms (Ribases et al., 2005a). On the other hand, the function of rs1078947 is unknown, and it was selected due to having been linked to maximum BMI in AN in a preliminary study (Ribases et al., 2005a). Finally, one of the two *NTRK3* loci of interest (rs1128994) is a synonymous substitution that may be located at a

splicing site, whereas rs7180942–located in intron 8—has been shown to reduce expression levels in the case of heterozygosity in lymphoblastoid cell lines (Mercader et al., 2008).

3.1.8. Laboratory methods

Genomic DNA for the PF samples were extracted at the University of Pittsburgh, and the extraction of genomic DNA was performed at the Hospital for Sick Children in the case of the nonpsychiatric controls. For the BN samples collected as a part of the Toronto Bulimia Nervosa Genetics Study, genomic DNA was extracted using the high salt method (Lahiri and Nurnberger, 1991) by a laboratory technician in the Neurogenetics Laboratory, CAMH. Stock DNAs for the 787 AN and 147 BN DNA samples collected as a part of the PF studies were received from SeraCare biorepository in the USA, and dilutions for all AN and BN samples were prepared in-house (20 ηg of DNA per 1 µl, in TE buffer). Control stock DNA was at a higher concentration, thus the samples were diluted in order to obtain a concentration of 20 ηg/µl.

The variants present at biallelic SNP loci were determined by polymerase chain reaction (PCR), and all genotyping was performed by me as a part of my PhD at the CAMH Neurogenetics Laboratory. PCR is a commonly used and reliable technique that amplifies the number of copies of a specific region of DNA, in order to produce enough DNA to be adequately tested. PCR consists of three steps: denaturation, annealing, and elongation. The denaturation step consists of the DNA being heated to a temperature as high as 98°C (depending on the protocol). This causes the paired strands to separate, making the single strands accessible to primers. During the annealing step, large excess of primers relative to the amount of DNA being amplified are added, and the reaction is cooled to allow double-strands to form again. During the elongation step, DNA polymerase (such as Taq polymerase) is added, which detects the opposing strand's sequence and extends the primer's sequence by binding the nucleotides

together in the order in which they pair across from one another (A:T and C:G). With each annealing/elongation cycle, more primers are added, and the cycle is repeated. This results in amplification of the DNA, i.e., a very large number of copies of the DNA and the genomic region of interest to be produced. More information on the basic principles behind PCR can be found elsewhere (Rychlik et al., 1990).

For the following five loci, Life Technologies Applied Biosystems Inc. (ABI) TaqMan Allelic Discrimination custom and pre-designed assays were used: rs56164415, rs13338499, rs1078947, rs1187325, and rs4684677. Assay and primer sequence information is provided in Tables 4 and 5. TaqMan assays use a 5' exonuclease PCR-based fluorescent multiplexed end-point reaction to classify unknown samples as homozygotes or heterozygotes at specific loci of interest. The total volume of all PCR reactions was 10 µl, which consisted of 1µl of DNA, 5µl of 2X TaqMan Buffer, 0.25µl of 40X TaqMan Assay and 3.75µl of distilled water (dH_2O). The PCR cycling conditions included initial denature for 10 minutes at 95°C followed by 50 cycles of the following: 92°C for 15 seconds and 60°C for 1 minute. Although majority of the loci required 50 cycles, the exact number varied between SNPs, and the information on the number of PCR cycles for each SNP can be found in Tables 4 and 5. Results of the reaction are visualized on the ABI ViiA7 Sequence Detection System in the presence of variation of alleles by comparing to No Template Controls (NTCs).

The primary genotyping of the remaining 16 loci were performed using the ABI OpenArray Genotyping System, and all ambiguous genotypes were repeated using ABI TaqMan assays (Tables 4 and 5). Similar to the TaqMan single SNP assays, OpenArray uses PCR reagents to provide qualitative detection of targets using post-PCR analysis. This platform also makes it possible for between 16 and 256 TaqMan assays to be genotyped in 12 to 144 samples

Table 4. Details on the primer sequence and allele call on the positive strand for the SNPs genotyped using the ABI on demand assays

SNP	Primer Sequence [VIC/FAM]	ABI Assay	Allele call (+)	# of PCR cycles
rs1137100	TTATGTGCAGACAACATTGAAG GAA**[A/G]**GACATTTGTTTCAACA GTAAATTCT	C____518168_20	1=G, 2=A	50-60
rs1137101	ATCACATCTGGTGGAGTAATTTT CC**[A/G]**GTCACCTCTAATGTCAG TTCAGCCC	C___8722581_10	1=A, 2=G	40
rs7799039	TTGTTTTGTTTTGCGACAGGGTT GC**[A/G]**CTGATCCTCCCGCCTCA GTCTCCCT	C___1328079_10	1=A 2=G	50-60
rs696217	GCAGAGGTACCGACCCGGACTT CCA**[G/T]**TTCATCCTCTGCCCCTT CTGCTTGA	C___3151003_20	1=T, 2=G	60
rs4684677	CAGGGCCTGGCTGTGCTGCTGG TAC**[A/T]**GAACCCCTGACAGCTT GATTCCAAC	C__25607748_10	1=T, 2=A	50-60
rs12490160	GACCTGGTTCCCTGCAGCAACA CCT**[C/T]**GGGAGGTTAAAAAATA CAGATGACT	C___1417464_10	1=T, 2=C	60
rs3732941	TGGTATACAGAGGGCACTCCTA TGC**[A/G]**TTTTTAAAACATGCTG AGCACATAC	C___1288856_10	1=G, 2=A	60
rs5030980	GCAGTTACCTCTGCCAAGGCCT GAG**[C/T]**CTCCTGCAACAGATCC TCTTCTGCC	C__29708280_10	1=T, 2=C	50
rs1078947	GTTATTCCTCAGTTATTAGCACA AA**[C/T]**GTTATTTCCTTAGGAAC TAGGCTGT	C____581246_1_	1=T, 2=C	50-60
rs6127698	CCCCTGTCTTGCCATGAAAAGA GCT**[G/T]**TAACTGTAGCAGCCGG TGGCAGGTT	C___9485714_10	1=T, 2=G	60
rs3827103	TGAGCAGGTCTTCATCAAGCCC GAG**[A/G]**TTTTCCTGTCTCTGGG CATCGTCAG	C___9485713_10	1=G, 2=A	50
rs17782313	GTTTAAAGCAGGAGAGATTGTA TCC**[C/T]**GATGGAAATGACAAGA AAAGCTTCA	C__32667060_10	1=T, 2=C	50
rs8087522	TAAGAACCCAGCCAGTAGTGGT TCA**[A/G]**TTAAAATACCTGAAAA ACAGAGAGG	C__29004626_10	1=G, 2=A	50-60

SNP	Primer Sequence [VIC/FAM]	ABI Assay	Allele call (+)	# of PCR cycles
rs489693	TCTTAATTCTGTTGTCATTAGTTCC**[A/C]**GTTTGTTAAATGTTTACAGCGTGGC	C___3058718_10	1=C, 2=A	50-60
rs7180942	GAACCAGCAGATATTCTCTAACTAC**[C/T]**TGTGGGGAATCGGATACTTTCTGTG	C__31854596_10	1=T, 2=C	50
rs1128994	AGAGAGGAAGCTGGGAGCCATCAGC**[A/G]**TTGATGCAGTAGAGGTTCTGGCTGT	C___9487317_20	1=G, 2=A	50-60
rs6265	TCCTCATCCAACAGCTCTTCTATCA**[C/T]**GTGTTCGAAAGTGTCAGCCAATGAT	C__11592758_10	1=T, 2=C	50-60
rs1042571	GCTGGGAGGCGGCAGCAGGGCAGGG**[A/G]**AGAGCAAGGGGCTTTGGGGTCGACC	C___8722914_10	1=G, 2=A	40

Table 5. Details on the primer sequence, probe and allele call on the positive strand for the SNPs genotyped using the ABI by design assays

SNP	Forward Primer Sequence	Reverse Primer Sequence	FAM probe	VIC Probe	ABI assay	Allele call (+)	# of PCR cycles
rs13338499	GGTGACTGAGCGAGACTCTCT	GATTACAGGCGTGAGCCACT	TTTCACAGCCGGATGC	TCACGGCCGGATGC	AHRSGBX	1=A, 2=G	50-60
rs1187325	CAAGCACCGAGGAGTTAAGAGA	CACCGGCCGCTTTCC	AAGGCCTCCCCGCACG	AAGGCCTCCCGGCACG	AHS1EH5	1=C, 2=G	50-60
rs56164415	CCAGCGCTTGCCTACCT	GGAGCCAGAATCGGAACCA	CTCACGGGTCCCC	CCTCACGAGTCCCC	AHUACOD	1=C, 2=T	50

loaded with the assays for the loci of interest, and the total volume of reaction mix was 3 µl, which consisted of 1.5 µl of DNA and 1.5 µl of 2X TaqMan OpenArray Master Mix. After the reaction mix was loaded onto the arrays, the PCR cycling conditions included initial denature for 10 minutes at 93°C followed by 50 cycles of the following: 95°C for 45 seconds, and 94°C for 13 seconds, and 53°C for 2 minutes and 14 seconds. Genotypes then were visualized using the ABI OpenArray SNP Genotyping Analysis Software.

For each SNP, roughly 5% of the DNA samples were re-genotyped for quality control (QC) purposes. All ambiguous genotypes were retyped, and if they remained ambiguous, they were excluded from the analysis. Genotyping of the DNA was performed by me as a part of my PhD at the Neurogenetics Laboratory at CAMH, blind to psychiatric diagnosis.

3.1.9. Statistical analysis

Chi-squared, t-test and analysis of variance (ANOVA) on baseline characteristics between AN, BN, and control groups were performed using SPSS Statistics v17 (SPSS Inc., Chicago, USA, 2008). QC steps prior to data analysis consisted of checking for deviations from Hardy-Weinberg Equilibrium (HWE; cutoff $p < 0.01$), removal of SNPs with low MAF (< 0.03) and low genotyping rate (< 90%), and exclusion of individuals with low genotyping rate (< 90%). For the case-control component of the analysis, genotype and ED diagnosis were treated as categorical variables. The chi-squared test was performed for the case-control comparisons using PLINK. (Purcell et al., 2007). Power calculations were completed using Quanto v.1.2.4 (available through http://hydra.usc.edu/gxe/), and for the case-control component, we have over 90% power to detect an odds ratio as low as 1.5 (MAF 0.10, log additive model).

For the quantitative phenotypic analysis, we investigated the role of the genetic polymorphisms on three BMI measures: current BMI (curBMI), maximum lifetime BMI

(maxBMI) and minimum lifetime BMI (minBMI). In the case of the nonpsychiatric controls, minBMI and maxBMI information was not available, thus the analysis was limited to curBMI. Quantitative data were analyzed separately in each diagnostic group using linear and logistic regression in PLINK. Age, age at onset (AAO), AN subtype (for AN only) and site (PF versus Toronto; for BN only) were entered as covariates. For the quantitative phenotypic analyses, we have over 80% power to detect a mean change of 0.6 kg/m^2 in BMI for the AN group (MAF 0.10, log additive model).

Due to multiple testing, appropriate statistical correction was necessary to reduce the rate of Type I error and reporting false positives. Nyholt (gene-based) and Bonferroni (experiment-wise) corrections were used for this purpose. Nyholt correction is a simple statistical method that calculates the number independent loci for the SNPs on the same gene using LD information (Nyholt, 2004). Once the number of independent loci was determined per gene, Bonferroni correction was applied to correct for multiple testing experiment-wise by dividing the uncorrected *p*-value of 0.05 by the number of independent loci. In our study, the number of independent loci was determined to be 18.75, which set the adjusted *p* at 0.0027. All statistical analyses were two-tailed, and *p*-values under 0.01 were considered as statistical trends.

3.2. Study 2: Secondary Analysis of Dopamine System Genes in the GCAN Dataset

3.2.1. Consortium description

GCAN is a Wellcome Trust-funded initiative under WTCCC3 that aims to uncover the genetic basis of AN. Led by the Sanger Institute in the UK and the University of North Carolina at Chapel Hill, USA, the GCAN initiative was established in 2007 and has been formed by researchers from the following 13 countries: (1) Canada; (2) Czech Republic; (3) Finland; (4) France; (5) Germany; (6) Greece; (7) Italy (two sites); (8) the Netherlands (two sites); (9)

Poland; (10) Spain; (11) Sweden; (12) the UK (two sites); and (13) the USA (three sites). In addition to the case DNAs, Greece and Italy also provided the consortium with a small number of ethnicity-matched control DNA samples. The replication phase has included additional DNA samples from Austria, Japan, and Estonia, as well as the PF DNA samples. The majority of control DNA samples (currently not available for secondary analysis) were collected as a part of the WTCCC initiatives. For the purpose of secondary analysis, thus far access has been granted only to the samples included in the discovery phase, which included 3,111 AN cases and 133 controls. The manuscript resulting from this case-control GWAS is currently in preparation, and although no SNP reached genome-wide significance, the most significant *p*-value was obtained for a SNP in the *COL4A5* gene, linked to collagen production (Bulik et al., 2012). Although GCAN initially started with a focus on AN, more recently BN samples have also been included as a part of future GWAS and high-throughput sequencing efforts.

In contrast with the careful phenotypic characterization of the PF Genetic Consortium DNA samples, the GCAN phenotype was more broadly defined. Diagnostic determination was established using semi-structured or structured interview or population assessment strategy based on DSM diagnostic criteria. AN probands were all female and met the DSM-IV criteria for lifetime AN or lifetime EDNOS AN-subtype (i.e., exhibiting the core feature of AN but not meeting either the duration or < 85% of ideal body weight criteria). Amenorrhea criterion was not strictly enforced. A DSM-IV diagnosis of BN was also permitted. Exclusion criteria included medical or psychiatric conditions that might have confounded the diagnosis of AN (e.g., psychotic disorders, mental retardation, or a medical or neurological condition causing weight loss). Furthermore, the collection of assessment and self-report materials were allowed to vary between sites, as long as the cases included in the study met the core AN or EDNOS-AN criteria

according to the DSM guidelines. Aside from the exclusion of control samples and some AN cases during the QC process, the present study included in this PhD dissertation did not apply additional inclusion/exclusion criteria and utilized all available AN cases. Each study obtained ethical approval from their local ethics committee, and informed consent for providing genetic materials and inclusion of these materials in future collaborative studies was obtained from all individuals whose DNA samples were included in the consortium according to the Declaration of Helsinki.

3.2.2 List of candidate genes

Due to the important role dopamine system plays in appetite and weight regulation (as outlined in the Introduction), the GCAN candidate gene analysis focused on dopaminergic genes, as well as other candidate genes known to interact with dopamine system genes (i.e., *MC4R* and *BDNF*). Because of its significant association with body weight and obesity (see Section 1.8.6), we also chose to include *FTO* in our analysis. The candidate genes included in this study are listed in Table 5.

Also compared to the highly targeted approach of focusing on a select number of functional loci, our efforts focused on gene-based analysis for the GCAN study. We utilized all SNPs genotyped for the genes of interest for a more systematic screening and thorough coverage to inquire their possible role on minBMI, maxBMI, and curBMI in AN. Gene extraction details and the list of SNPs captured following post-QC for each candidate gene are outlined in Tables 6 and 7, respectively. Also in contrast with the PF study in which all genotyping was carried out in-house, all GCAN DNA samples were genotyped using Illumina 660W-Quad arrays (Illumina, Inc., San Diego, CA, USA) at the Wellcome Trust Sanger Institute in London, UK.

3.2.3. Statistical analyses

GWAS QC and candidate gene secondary analysis were performed using PLINK and R v2.15.1 (available through http://www.r-project.org). Power calculations using Quanto indicate that we have over 80% power to detect an R^2 of 0.004 for MAF of 0.05 under the additive model. SPSS Statistics v17 (SPSS Inc., Chicago, USA, 2008) was used for descriptive statistics and preparation of baseline data summary.

As a part of this dissertation, QC was conducted on raw GWAS genotype data obtained from the Sanger Institute as a part of the WTCCC3 GCAN study. QC was performed according to the established guidelines outlines elsewhere (Anderson et al., 2010). The steps of the genome-wide QC included removal of SNPs with no genotype, sex check to ensure only female probands were included, exclusion of control samples, removal of SNPs with MAF < 0.05 and genotype frequency < 95%, exclusion of probands with < 99% successful genotyping rate, exclusion of individuals with excess heterozygosity (cutoff of ± 2 *SD* from the mean), exclusion of related individuals (identity by descent; PI_HAT > 0.1), multidimensional scaling (MDS) run to assess population substructure, and removal of SNPs that deviated from HWE (genome-wide $p < 10^{-6}$). Candidate gene extraction was performed following the QC. Cutoffs were chosen according to guidelines published elsewhere (Anderson et al., 2010) and with the guidance given by the Sanger Institute.

During the QC process, pruned GWAS data were also merged with the HapMap data (also pruned) to assess the ancestral background of the AN cases. Population stratification is a possible confounding factor that arises due to the presence of a systematic difference in allele frequencies between different ancestral and geographical populations, mostly attributable to non-random mating between groups and genetic drift of allele frequencies in each group. If it is not properly controlled for, population stratification can pose a great challenge to the validity of

Table 6. List of dopamine pathway candidate genes included in the GCAN secondary analysis

Gene	Role in the Dopamine Pathway
DRD1	Dopamine receptor
DRD2	Dopamine receptor
DRD3	Dopamine receptor
DRD4	Dopamine receptor
DRD5	Dopamine receptor
TH	Catalyzes the rate-limiting step in the biosynthesis of the conversion of L-DOPA, precursor of dopamine.
DBH	Converts dopamine to noradrenaline
SLC6A3	Dopamine transporter; terminates the action of dopamine by its high affinity sodium-dependent reuptake into presynaptic terminals
COMT	Involved in metabolism of catecholamines, including dopamine
MAOA	Catalyzes the oxidation of monoamines, including dopamine
MC4R	Co-expressed with DRD1 in the ventral striatum (an important reward centre); upregulation of MC4R expression occurs via DRD1 and DRD2-dependent mechanisms
BDNF	Trophic factor for mesencephalic dopaminergic neurons and their survival; dopaminergic signaling plays a role in the upregulation of BDNF

Table 7. GCAN candidate gene extraction details

Gene	Chromosome	Bp (downstream)	Bp (upstream)	Size (kb)[a]	# of SNPs covered (post-QC)[c]
DRD1	5	174795281	174813769	18.5	0
DRD2	11	112775528	112881091	105.6	29
DRD3	3	115325247	115390589	65.3	17
DRD4	11	617305	635703	18.4	2
DRD5	4	9382701	9399730	17.0	0
SLC6A3	5	1440909	1508543	67.6	24
COMT	22	18299309	18341528	42.2	23
DBH	9	135481306	135519287	38.0	24
TH	11	2136736	2159611	22.9	5
MC4R	18	55989544	56200981	211.4[b]	33
MAOA	X	43390353	43496011	105.7	5
BDNF	11	27628018	27709872	81.9	6
FTO	16	52285376	52710882	425.5	87

[a] Rounded off to the first decimal place; includes additional 5 kb downstream and 10 kb upstream

[b] Includes additional 5 kb downstream and 200 kb upstream

[c] SNP count after the removal of Speliotes et al. (2010) loci or the proxies thereof, which will be analyzed in their relation to BMI by our collaborators under the GCAN umbrella.

genetic findings. In order to assess the population structure of the GCAN cases, HapMap3-r2 dataset was used and the data files for the HapMap populations were downloaded in PLINK format from the project's website (ftp://ftp. ncbi.nlm.nih.gov/hapmap/ genotypes/200901_ phaseIII/plink_format/). Following the download, GCAN dataset was merged with the three principal HapMap populations: European Caucasian (CEU), combined Chinese Han and Japanese (CHB +JPT), and Yoruba in Ibadan, Nigeria (YRI).

The focus of the analysis was to investigate the possible link between dopamine system genes with the quantitative variables of minBMI, maxBMI and curBMI, for which we used linear regression analysis in PLINK. Principal components C1 through C10 (for population stratification, as determined by MDS), AAO, AN subtype, and history of BN were entered as covariates in the model. Bonferroni correction was applied to *p*-values for multiple comparisons for a total of 255 comparisons (which corresponds to the number of SNPs studied), and all tests were two-tailed with adjusted-Bonferroni $p = 0.05$.

CHAPTER 4

RESULTS

4.1. Study 1: PF Candidate Gene Analysis

4.1.1. Sample Description

As described in the Methods section, after applying the carefully selected inclusion criteria, 787 AN probands, 267 BN probands, and 322 nonpsychiatric female controls were suitable to be included in our study. Following QC, 42 individuals with AN, 22 individuals with BN, and one control were removed due to low genotyping rate, bringing the final study sample to 745 AN cases, 245 BN cases, and 321 nonpsychiatric controls. Of the AN probands, 369 met the criteria for AN-R (49.5%), whereas 376 were classified as AN-BP (50.5%). All AN probands and 128 BN probands (52.2%) came from the PF Consortium, and 117 BN probands (47.8%) came from the Toronto Bulimia Nervosa Genetics Study. Individuals removed due to low genotyping rate did not differ from those who passed QC, and none of the descriptive statistics were significantly altered following QC (results not shown).

In terms of the characteristics of the sample (Table 8), all variables with the exception of AAO for BN (AAO-BN) were normally distributed. Individuals in the control group were significantly older than AN and BN probands ($M_{control}$ = 49.4 ± 8.8, M_{AN} = 26.1 ± 8.5, M_{BN} = 27.2 ± 8.3, $p < 0.0001$). AN probands had a significantly lower curBMI at the time of study recruitment compared to the other two groups (M_{AN} = 18.05 ± 2.71, M_{BN} = 23.28 ± 3.01, $M_{control}$ = 23.60 ± 2.16, $p < 0.0001$). There were also significant differences between AN and BN groups in terms of minBMI (M_{AN} = 13.82 ± 1.95, M_{BN} = 20.09 ± 1.54, $p < 0.0001$) and maxBMI (M_{AN} = 21.07 ± 2.42, M_{BN} = 25.82 ± 3.06, $p < 0.0001$).

Table 8. Characteristics of AN, BN, and control participants

	AN (n=745)	**BN (n=267)**	**Control (n=321)**	***F***	***p***
Age (years)[a,b]	26.1 ± 8.5	27.2 ± 8.3	49.4 ± 8.8	867.909	< 0.0001
CurBMI (kg/m^2)[c]	18.05 ± 2.71	23.28 ± 3.01	23.60 ± 2.16	671.019	< 0.0001
MinBMI (kg/m^2)[c]	13.82 ± 1.95	20.09 ± 1.54	-	-51.198[d]	< 0.0001
MaxBMI (kg/m^2)[c]	21.07 ± 2.42	25.82 ± 3.06	-	-22.003[d]	< 0.0001

[a] Age data missing for one AN and one BN proband

[b] Control > AN = BN

[c] BMI data missing for four BN probands

[d] Because minBMI and maxBMI information was not available for controls, independent *t*-test was run to compare AN and BN groups, and the statistic reported here is the *t*-value.

We also observed a number of differences between AN-R and AN-BP subtypes (Table 9). Probands in the AN-R group were younger at the time of recruitment ($M_{AN\text{-}R} = 25.0 \pm 8.5$, $M_{AN\text{-}BP} = 27.2 \pm 8.4$, $p < 0.0001$), weighed less at the time of recruitment ($M_{AN\text{-}R} = 17.85 \pm 2.79$, $M_{AN\text{-}BP} = 18.25 \pm 2.62$, $p = 0.042$), and reported lower minBMI and maxBMI ($M_{AN\text{-}R} = 13.66 \pm 1.89$, $M_{AN\text{-}BP} = 13.98 \pm 1.99$, $p = 0.025$; $M_{AN\text{-}R} = 20.86 \pm 2.42$, $M_{AN\text{-}BP} = 21.29 \pm 2.40$, $p = 0.013$; respectively). AN AAO did not differ between subtypes ($M_{AN\text{-}R} = 16.2 \pm 3.2$, $M_{AN\text{-}BP} = 16.2 \pm 2.9$, $p = 0.758$). When BN probands were stratified by site (Table 10), the results showed that those selected from the PF sample were older at the time of recruitment ($M_{PF} = 29.0 \pm 9.6$, $M_{Toronto} = 25.2 \pm 6.7$, $p < 0.0001$) and had an earlier AAO compared to the probands selected from the Toronto Bulimia Nervosa Genetics Study sample ($M_{Price} = 17.0 \pm 3.7$, $M_{Toronto} = 18.0 \pm 4.2$, $p = 0.015$).

4.1.2. Genetic QC summary

Following QC, *NTRK3* rs1128994 was removed due to excess heterozygosity, most likely caused by a design issue involving the probes of the ABI assay. No additional markers were excluded due to low MAF or genotyping rate, thus all analyses were carried out with 20 markers. Successful genotype rate was 93%, with the majority of the SNPs reaching 98-100% genotype rate. None of the SNPs deviated from HWE in any of the three groups, and the pairwise LD (r^2) information for the SNPs in the same gene for the entire sample can be found in Table 11.

4.1.3. Case-control results

Results of the case-control comparisons are summarized in Tables 12, 13, and 14. Our analysis revealed a statistical trend for the A allele of *MC4R* rs489693 to be underrepresented in 745 AN probands compared to 321 nonpsychiatric controls (28.3% vs. 47.8%, $p = 0.0044$, $OR = 0.75$ [0.62-0.91]; Table 12 and Figure 2). Interestingly, we observed a nominally significant

Table 9. Characteristics of AN probands, stratified by subtype

	AN-R (n=369)	**AN-BP (n=376)**	***t***	***p***
Age (years)[a]	25.0 ± 8.5	27.2 ± 8.4	-3.499	< 0.0001
CurBMI (kg/m^2)	17.85 ± 2.79	18.25 ± 2.62	-2.041	0.042
MinBMI (kg/m^2)	13.66 ± 1.89	13.98 ± 1.99	-2.040	0.025
MaxBMI (kg/m^2)	20.86 ± 2.42	21.29 ± 2.40	-2.497	0.013
AAO (years)[b]	16.2 ± 3.2	16.2 ± 2.9	-0.309	0.758

[a] Age data missing for one AN-R proband

[b] AAO data missing for 27 AN-R and 51 AN-BP probands

Table 10. Characteristics of BN probands, stratified by site

	PF (n=128)	**Toronto (n=117)**	***t***	***p***
Age (years)[a]	29.0 ± 9.6	25.2 ± 6.7	3.608	< 0.0001
CurBMI (kg/m^2)[b]	23.31 ± 2.88	23.25 ± 3.16	0.157	0.876
MinBMI (kg/m^2)[b]	19.99 ± 1.29	20.20 ± 1.78	-1.042	0.289
MaxBMI (kg/m^2)[b]	25.75 ± 2.84	25.91 ± 3.29	-0.402	0.688
AAO (years)[c]	17.0 ± 3.7	18.0 ± 4.2	5286.500[d]	0.015

[a] Age data missing for one PF probands

[b] BMI data missing for four Toronto probands

[c] AAO data missing for one Toronto and 15 PF probands

[d] Nonparametric Mann-Whitney test was performed for AAO since its distribution deviated from normal distribution, and the statistic reported here is Mann-Whitney's *U*.

Table 11. Pairwise r^2 for SNPs located in the same gene

Gene	SNP 1	SNP 2	r^2
AGRP	rs13338499	rs5030980	0.54
BDNF	rs6265	rs56164415	0.12
GHRL	rs696217	rs4684677	0.07
HRH1	rs12490160	rs3732941	0.03
LEPR	rs1137100	rs1137101	0.62
NTRK2	rs1187325	rs1078947	0.04
MC4R	rs17782313	rs489693	0.77
MC4R	rs17782313	rs8087522	0.25
MC4R	rs489693	rs8087522	0.28
MC3R	rs3827103	rs6127698	0.26

relationship between *MC4R* rs489693 and AN diagnosis when AA and AC genotypes were grouped together: while 57.9% of controls carried at least one copy of the A allele, this rate was only at 47.9% for AN probands ($p = 0.0027$; Figure 3).

AN and BN cases did not differ in terms of genotype or allele frequencies of the SNPs included in this study (Table 13). Similarly, we failed to provide any evidence for group differences between BN and controls (Table 14).

4.1.4. Genetics of body weight in AN

For the within-AN analysis of BMI, we entered age, AAO-AN and AN subtype as covariates. As summarized in Tables 8-10, we observed significant baseline differences in terms of age and all three BMI measures between AN-R and AN-BP probands. It is well documented that AN-R probands often weigh less than AN-BP probands (summarized in Methods 2.1.2 and 2.1.3). Although AAO was comparable between subtypes, we chose to control for it since AN onset and weight suppression at an earlier age may act as a confounder in the analysis.

Our findings pertaining to curBMI, minBMI, and maxBMI in AN are described in Tables 15, 16, and 17. In summary, none of the 20 SNPs studied were associated with either curBMI or maxBMI in AN probands. However, we found that *AGRP* rs13338499 was significantly correlated with minBMI under the additive model. More specifically, each copy of the A allele carried resulted in a lower BMI ($M_{GG} = 14.89$ kg/m^2, $M_{AG} = 14.32$ kg/m^2, $M_{AA} = 13.70$ kg/m^2, $p = 0.0013$; Table 16 and Figure 4).

4.1.5. Genetics of body weight in BN

In BN phenotypic analysis, age, AAO and site were entered as covariates as a result of the findings of the descriptive analysis.

Table 12. Case-control allelic analysis for AN vs. controls

Locus	Minor allele	MAF (AN)	MAF (CTL)	χ^2	*p*
rs1137100	G	0.238	0.252	0.4843	0.4865
rs1137101	G	0.427	0.450	0.9593	0.3274
rs7799039	A	0.463	0.427	2.3860	0.1224
rs696217	T	0.077	0.076	0.0046	0.9457
rs4684677	A	0.063	0.056	0.3060	0.5801
rs12490160	G	0.097	0.098	0.0024	0.9610
rs3732941	G	0.126	0.114	0.6154	0.4327
rs5030980	T	0.038	0.042	0.2229	0.6368
rs13338499	G	0.099	0.099	0.0096	0.9220
rs1078947	T	0.162	0.137	2.6040	0.1066
rs1187325	C	0.442	0.472	1.7540	0.1853
rs6127698	T	0.479	0.467	0.2524	0.6154
rs3827103	G	0.069	0.079	0.1559	0.6930
rs17782313	C	0.240	0.231	0.2351	0.6278
rs489693	**A**	**0.283**	**0.478**	**8.1130**	**0.0044**
rs8087522	A	0.307	0.329	1.0130	0.3141
rs7180942	C	0.488	0.475	0.2954	0.5868
rs6265	T	0.198	0.218	1.0120	0.3145
rs56164415	T	0.059	0.063	0.1314	0.7170
rs1042571	A	0.197	0.178	0.9773	0.3229

Note 1: Bonferroni-corrected *p*-value set at 0.0027 for statistical significance and 0.01 for statistical trend. Results in bold represent statistical significance or trend.

Table 13. Case-control allelic analysis for AN vs. BN

Locus	Minor allele	MAF (AN)	MAF (BN)	χ^2	*p*
rs1137100	G	0.238	0.264	1.3540	0.2446
rs1137101	G	0.427	0.436	0.1208	0.7282
rs7799039	A	0.463	0.441	0.7369	0.3907
rs696217	T	0.077	0.088	0.5615	0.4537
rs4684677	A	0.063	0.056	0.3087	0.5785
rs12490160	G	0.097	0.102	0.0876	0.7673
rs3732941	G	0.126	0.132	0.1127	0.7371
rs5030980	T	0.038	0.041	0.0944	0.7586
rs13338499	G	0.100	0.091	0.2885	0.5912
rs1078947	T	0.165	0.146	0.9211	0.3372
rs1187325	C	0.448	0.450	0.0083	0.9274
rs6127698	T	0.479	0.439	2.4330	0.1188
rs3827103	G	0.068	0.090	2.4580	0.1169
rs17782313	C	0.240	0.236	0.0430	0.8357
rs489693	A	0.283	0.331	4.1000	0.0429
rs8087522	A	0.307	0.280	1.2800	0.2579
rs7180942	C	0.488	0.429	5.2050	0.0225
rs6265	T	0.198	0.169	1.9330	0.1644
rs56164415	T	0.059	0.051	0.3981	0.5281
rs1042571	A	0.197	0.201	0.0465	0.8293

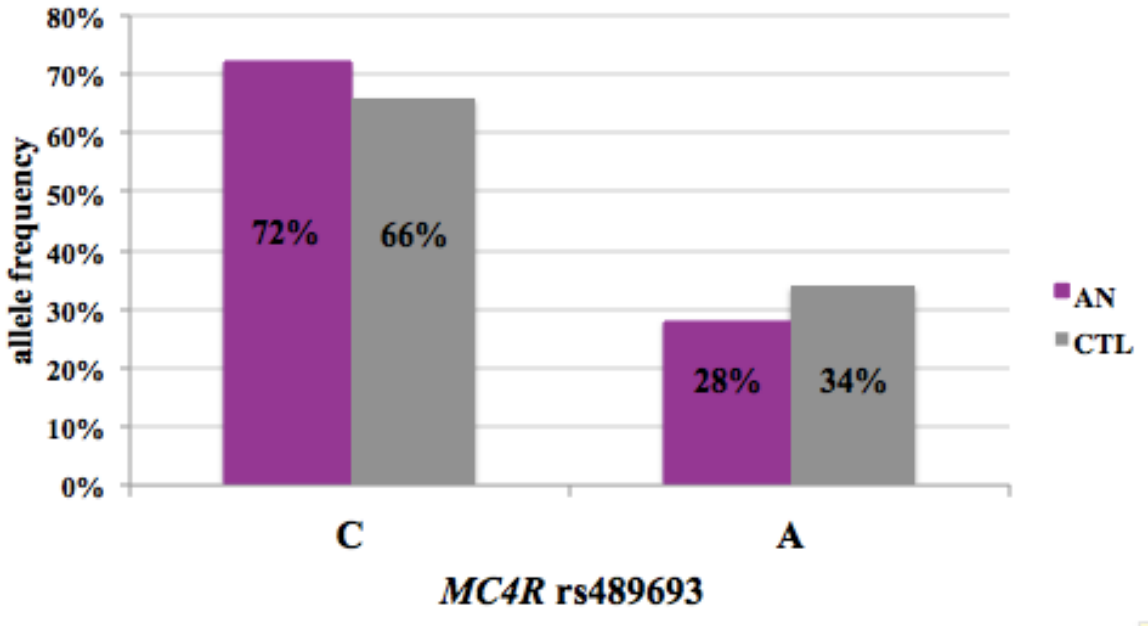
Note 1: Bonferroni-corrected *p*-value set at 0.0027 for statistical significance and 0.01 for statistical trend.

Table 14. Case-control allelic analysis for BN vs. controls

Locus	Minor allele	MAF (BN)	MAF (CTL)	χ^2	*p*
rs1137100	G	0.264	0.252	0.2091	0.6475
rs1137101	G	0.436	0.450	0.2178	0.6407
rs7799039	A	0.441	0.427	0.2227	0.6370
rs696217	T	0.088	0.076	0.4836	0.4856
rs4684677	A	0.056	0.056	0.0025	0.9599
rs12490160	G	0.102	0.098	0.0473	0.8279
rs3732941	G	0.132	0.114	0.8380	0.3600
rs5030980	T	0.041	0.042	0.0107	0.9175
rs13338499	G	0.092	0.099	0.1588	0.6903
rs1078947	T	0.146	0.137	0.1867	0.6657
rs1187325	C	0.450	0.479	0.9039	0.3417
rs6127698	T	0.439	0.467	0.9252	0.3361
rs3827103	G	0.090	0.073	1.0340	0.3092
rs17782313	C	0.236	0.231	0.0408	0.8399
rs489693	A	0.331	0.344	0.2304	0.6312
rs8087522	A	0.280	0.329	3.1430	0.0763
rs7180942	C	0.429	0.475	2.4240	0.1195
rs6265	T	0.169	0.179	0.1827	0.6691
rs56164415	T	0.051	0.063	0.6957	0.4042
rs1042571	A	0.201	0.178	0.9445	0.3311

Note 1: Bonferroni-corrected *p*-value set at 0.0027 for statistical significance and 0.01 for statistical trend.

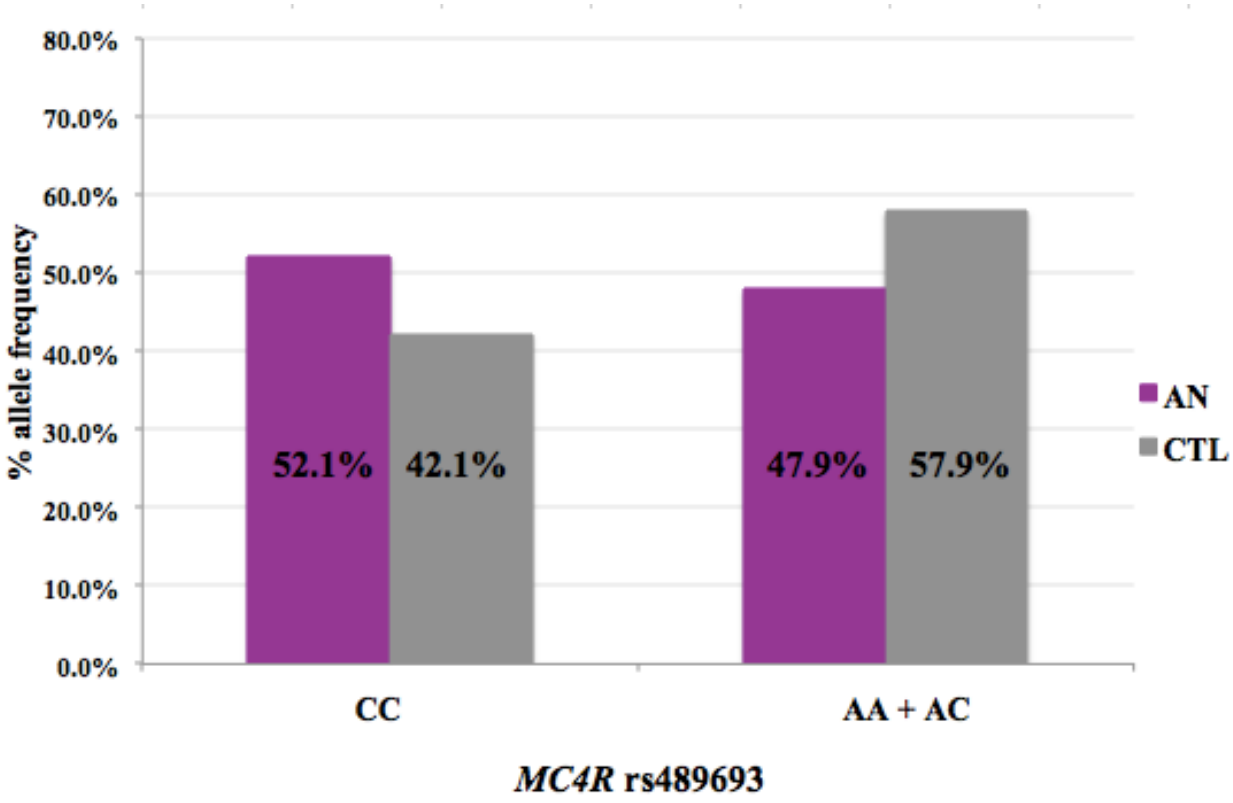
Figure 2. Association of *MC4R* rs489693 in AN vs. controls under the allelic model



Note 1: CTL = controls

Note 2: $p = 0.0044$ (trend), $OR = 0.75$ ($CI_{95} = 0.62\text{-}0.91$)

Figure 3. Association of *MC4R* rs489693 in AN vs. controls, with AA and AC genotypes grouped together



Note 1: CTL = controls

Note 2: $p = 0.0027$ (nominally significant)

Table 15. Analysis of the 20 SNPs in relation to their role on curBMI in AN

SNP	Genotype	*M* ± *SD*	*β*	*t*	*p*
	G/G	17.96 ± 2.63			
rs1137100	G/A	18.13 ± 2.62	-0.0713	-0.396	0.6923
	A/A	18.00 ± 2.78			
	G/G	17.76 ± 2.40			
rs1137101	G/A	18.15 ± 2.77	-0.1818	-1.189	0.2348
	A/A	18.05 ± 2.80			
	A/A	17.59 ± 2.84			
rs1042571	A/G	18.19 ± 2.72	0.04902	0.2479	0.8043
	G/G	17.94 ± 2.74			
	A/A	18.73 ± 2.12			
rs4684677[a]	A/T	17.99 ± 2.80	-0.0088	-0.0295	0.9765
	T/T	18.04 ± 2.72			
	T/T	17.74 ± 1.88			
rs696217	T/G	18.48 ± 2.70	0.4889	1.7530	0.08005
	G/G	17.99 ± 2.72			
	G/G	18.42 ± 2.62			
rs12490160	G/T	17.86 ± 2.82	-0.0810	-0.3209	0.7484
	T/T	18.09 ± 2.70			
	G/G	18.31 ± 1.62			
rs3732941	G/A	17.71 ± 2.86	-0.4505	-1.993	0.04668
	A/A	18.15 ± 2.67			
	A/A	18.23 ± 3.02			
rs7799039	A/G	18.01 ± 2.67	0.1010	0.6763	0.4991
	G/G	17.98 ± 2.54			
	C/C	18.07 ± 2.81			
rs1187325	C/G	18.01 ± 2.73	0.05081	0.3366	0.7365
	G/G	18.07 ± 2.67			
	T/T	17.19 ± 2.65			
rs1078947	T/C	18.02 ± 2.72	-0.2818	-1.3930	0.1640
	C/C	18.12 ± 2.72			
	T/T	17.95 ± 2.01			
rs6265	T/C	18.07 ± 2.72	0.04513	0.2319	0.8167
	C/C	18.04 ± 2.75			
	T/T	19.69 ± 1.78			
rs56164415[a]	T/C	17.42 ± 2.68	-0.4943	-1.6080	0.1083
	C/C	18.10 ± 2.70			
	C/C	17.96 ± 2.63			
rs7180942	C/T	18.13 ± 2.62	-0.0713	-0.396	0.6923
	T/T	18.00 ± 2.78			

SNP	Genotype	*M* ± *SD*	*β*	*t*	*p*
	T/T	18.38 ± 2.70			
rs5030980[a]	T/G	18.05 ± 2.71	0.3549	2.3830	0.0174
	G/G	17.74 ± 2.72			
	G/G	14.79 ± 0.00			
rs13338499	G/A	18.26 ± 2.76	0.1102	0.2809	0.7789
	A/A	18.05 ± 2.70			
	C/C	19.17 ± 2.51			
rs17782313	C/T	18.12 ± 2.55	0.1153	0.4611	0.6449
	T/T	18.03 ± 2.73			
	A/A	18.08 ± 2.79			
rs489693	A/C	18.27 ±2.75	0.2586	1.5350	0.1252
	C/C	17.92 ± 2.68			
	A/A	18.19 ± 2.83			
rs8087522	A/G	18.14 ± 2.73	0.1839	1.1150	0.2651
	G/G	17.96 ± 2.68			
	T/T	17.37 ± 2.86			
rs6127698	T/G	18.11 ± 2.67	-0.1958	-1.2080	0.2276
	G/G	18.13 ± 2.71			
	G/G	17.80 ± 2.63			
rs3827103	G/A	18.16 ± 2.69	-0.1568	-1.0430	0.2974
	A/A	18.04 ± 2.82			

Note 1: Age, AAO, and AN subtype entered as covariates

Note 2: Bonferroni-corrected *p*-value set at 0.0027 for statistical significance and 0.01 for statistical trend

[a] MAF < 0.07

Table 16. Analysis of the 20 SNPs in relation to their role on minBMI in AN

SNP	Genotype	*M* ± *SD*	*β*	*t*	*p*
rs1137100	G/G	14.29 + 2.02	-0.0105	-0.0837	0.9333
	G/A	13.72 + 1.99			
	A/A	13.85 + 1.91			
rs1137101	G/G	14.05 + 1.92	-0.0520	-0.4905	0.6239
	G/A	13.62 + 1.96			
	A/A	13.97 + 1.93			
rs1042571	A/A	13.89 + 2.09	0.0739	0.5455	0.5856
	A/G	13.86 + 1.84			
	G/G	13.75 + 1.99			
rs4684677[a]	A/A	14.62 + 1.11	0.1125	0.5441	0.5865
	A/T	13.85 + 1.84			
	T/T	13.79 + 1.97			
rs696217	T/T	13.87 + 2.35	0.3407	1.7610	0.0786
	T/G	14.07 + 2.07			
	G/G	13.78 + 1.93			
rs12490160	G/G	13.63 + 1.61	0.0511	0.2916	0.7707
	G/T	13.84 + 1.81			
	T/T	13.82 + 1.99			
rs3732941	G/G	14.12 + 2.06	0.08594	0.5472	0.5845
	G/A	13.92 + 1.87			
	A/A	13.79 + 1.97			
rs7799039	A/A	13.59 + 2.06	-0.1644	-1.5890	0.1125
	A/G	13.88 + 1.84			
	G/G	13.88 + 2.05			
rs1187325	C/C	13.71 + 1.93	-0.0553	-0.5300	0.5963
	C/G	13.83 + 1.97			
	G/G	13.81 + 1.94			
rs1078947	T/T	13.92 + 1.86	-0.1050	-0.7491	0.4541
	T/C	13.81 + 2.01			
	C/C	13.84 + 1.93			
rs6265	T/T	14.49 + 1.37	0.1277	0.9475	0.3437
	T/C	13.74 + 2.02			
	C/C	13.82 + 1.94			
rs56164415[a]	T/T	14.29 + 2.80	-0.1962	-0.9157	0.3602
	T/C	13.54 + 2.05			
	C/C	13.85 + 1.94			
rs7180942	C/C	14.29 + 2.02	-0.0105	-0.0837	0.9333
	C/T	13.72 + 1.99			
	T/T	13.85 + 1.91			

SNP	Genotype	*M* ± *SD*	*β*	*t*	*p*
rs5030980[a]	T/T	10.76 + 0.00	0.4402	1.6160	0.1066
	T/G	14.23 + 1.86			
	G/G	13.79 + 1.96			
rs13338499	**G/G**	**14.89 + 2.33**	**0.5609**	**3.2380**	**0.0013**
	G/A	**14.32 + 1.86**			
	A/A	**13.70 + 1.95**			
rs17782313	C/C	13.88 + 2.15	0.1435	1.2270	0.2203
	C/T	13.89 + 1.79			
	T/T	13.77 + 2.02			
rs489693	A/A	13.87 + 1.99	0.0654	0.5712	0.5680
	A/C	13.85 + 1.82			
	C/C	13.79 + 2.04			
rs8087522	A/A	13.91 + 1.97	0.2083	1.8570	0.0638
	A/G	13.88 + 1.85			
	G/G	13.75 + 2.04			
rs6127698	T/T	13.76 + 1.99	0.0523	0.5018	0.6160
	T/G	13.91 + 1.89			
	G/G	13.70 + 2.02			
rs3827103	G/G	11.69 + 1.27	-0.2987	-1.4530	0.1466
	G/A	13.87 + 1.88			
	A/A	13.83 + 1.96			

Note 1: Age, AAO, and AN subtype entered as covariates

Note 2: Bonferroni-corrected *p*-value set at 0.0027 for statistical significance and 0.01 for statistical trend. Results in bold represent statistical significance or trend.

[a] MAF < 0.07

Table 17. Analysis of the 20 SNPs in relation to their role on maxBMI in AN

SNP	Genotype	*M* ± *SD*	*β*	*t*	*p*
	G/G	20.96 ± 2.69			
rs1137100	G/A	21.04 ± 2.41	-0.2048	-1.3320	0.1834
	A/A	21.10 ± 2.41			
	G/G	20.70 ± 2.40			
rs1137101	G/A	21.17 ± 2.45	-0.2649	-2.0290	0.0428
	A/A	21.13 ± 2.39			
	A/A	21.36 ± 2.30			
rs1042571	A/G	21.07 ± 2.48	0.0372	0.2201	0.8258
	G/G	21.05 ± 2.45			
	A/A	21.59 ± 1.74			
rs4684677[a]	A/T	20.95 ± 2.24	0.0424	0.1660	0.8682
	T/T	21.07 ± 2.44			
	T/T	21.67 ± 2.43			
rs696217	T/G	21.46 ± 2.74	0.4076	1.7100	0.0878
	G/G	21.00 ± 2.36			
	G/G	20.75 ± 2.13			
rs12490160	G/T	20.96 ± 2.48	-0.0104	-0.0486	0.9613
	T/T	21.11 ± 2.41			
	G/G	21.11 ± 1.75			
rs3732941	G/A	20.94 ± 2.66	-0.1563	-0.8092	0.4187
	A/A	21.11 ± 2.36			
	A/A	21.33 ± 2.58			
rs7799039	A/G	20.96 ± 2.42	0.1156	0.9069	0.3648
	G/G	21.06 ± 2.29			
	C/C	21.21 ± 2.66			
rs1187325	C/G	21.11 ± 2.43	0.2018	1.5730	0.1161
	G/G	20.85 ± 2.23			
	T/T	20.37 ± 2.73			
rs1078947	T/C	21.14 ± 2.42	-0.2220	-1.2850	0.1993
	C/C	21.08 ± 2.41			
	T/T	21.05 ± 1.95			
rs6265	T/C	20.96 ± 2.41	-0.0882	-0.5313	0.5954
	C/C	21.12 ± 2.45			
	T/T	20.77 ± 1.47			
rs56164415[a]	T/C	21.06 ± 2.28	0.0348	0.1320	0.8950
	C/C	21.07 ± 2.44			
	C/C	21.12 ± 2.43			
rs7180942	C/T	21.16 ± 2.45	0.1315	1.0310	0.3030
	T/T	20.86 ± 2.38			

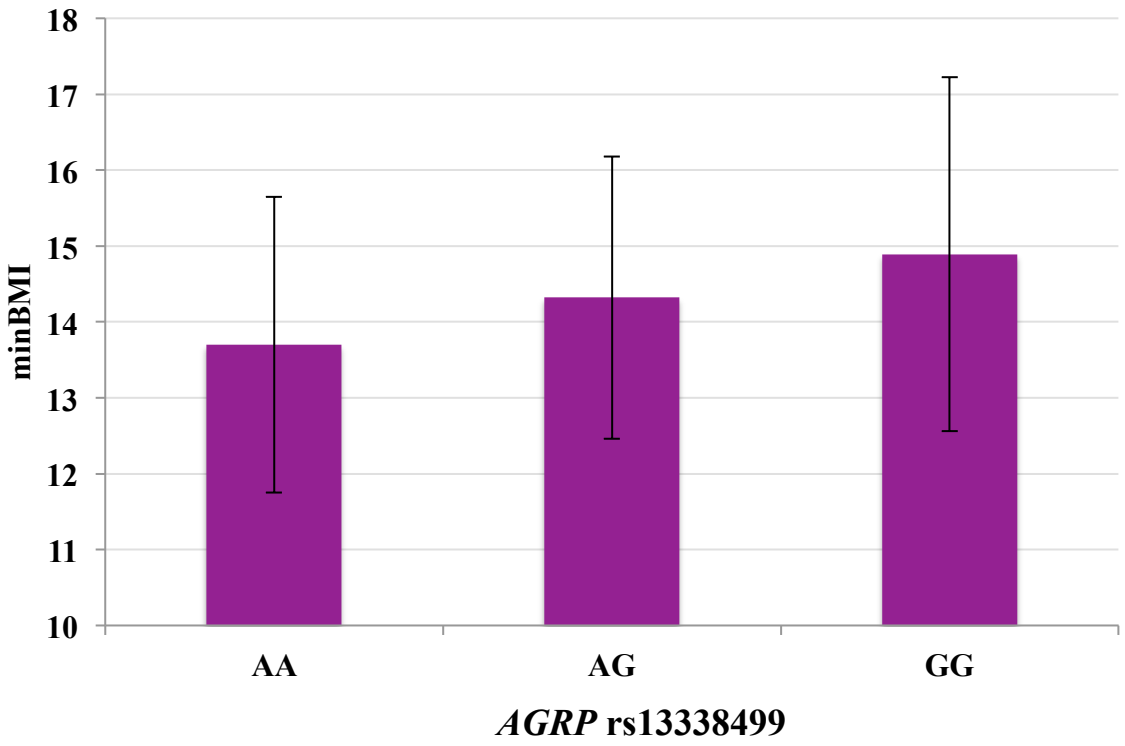
SNP	Genotype	M ± SD	*β*	*t*	*p*
	T/T	23.94 ± 0.00			
rs5030980[a]	T/G	21.28 ± 2.37	0.1513	0.4502	0.6527
	G/G	21.06 ± 2.43			
	G/G	21.66 ± 2.22			
rs13338499	G/A	21.20 ± 2.21	0.02925	0.1362	0.8917
	A/A	21.03 ± 2.46			
	C/C	21.43 ± 2.69			
rs17782313	C/T	21.15 ± 2.46	0.2544	1.7670	0.0776
	T/T	20.99 ± 2.36			
	A/A	21.39 ± 2.82			
rs489693	A/C	21.12 ± 2.41	0.2397	1.7020	0.0892
	C/C	20.98 ± 2.36			
	A/A	21.04 ± 2.26			
rs8087522	A/G	20.94 ± 2.46	-0.1334	-0.9629	0.3360
	G/G	21.18 ± 2.42			
	T/T	20.98 ± 2.46			
rs6127698	T/G	20.96 ± 2.38	-0.1317	-1.025	0.3055
	G/G	21.34 ± 2.46			
	G/G	20.58 ± 2.66			
rs3827103	G/A	21.63 ± 2.59	0.2598	1.025	0.3058
	A/A	20.99 ± 2.39			

Note 1: Age, AAO, and AN subtype entered as covariates

Note 2: Bonferroni-corrected *p*-value set at 0.0027 for statistical significance and 0.01 for statistical trend.

[a] MAF < 0.07

Figure 4. Distribution of the mean minBMI in AN based on *AGRP* rs13338499 genotype



Note 1: $p = 0.0013$ (significant); age, AAO and AN subtype entered as covariates

Note 2: Mean minBMI in kg/m^2 per genotype: AA = 13.70 ± 1.95, AG = 14.32 ± 1.86, GG = 14.89 ± 2.33

Tables 18-20 summarize the results of our analysis in BN probands. In summary, we did not find a significant association or statistical trend between the markers of interest and curBMI or minBMI. On the other hand, we observed a link between *NTRK3* rs1042571 and maxBMI in the BN group (Table 20). Indeed, each copy of the T allele was correlated with a mean increase over 1 kg/m^2 in BMI (M_{TT} = 27.68, M_{TC} = 26.63, M_{CC} = 25.47, p = 0.001; Figure 5).

4.1.6. Genetics of body weight in controls

Although the focus of the present study was not weight regulation in healthy controls, we investigated the relationship between BMI and the chosen candidate genes to see if similar trends emerged in the control group as well. Since the controls were significantly older than the AN or BN cases and age is often associated with an increase in body weight, we entered age as a covariate in the analysis pertaining to curBMI in control women. As summarized in Table 21, none of the markers were linked to curBMI in women without a psychiatric history in our study. Since we did not have lifetime BMI information on the controls, we were not able to look at the possible role of the selected loci on minBMI or maxBMI in this group.

4.2. Study 2: Secondary analysis of dopamine system genes in GCAN dataset

3.2.1. QC summary

Prior to QC, 594,398 SNPs were covered by the GWAS genotyping, and 3,244 DNA samples (3,111 AN cases and 133 controls) were included in the dataset. As a part of the individual-level QC, 11 probands were removed due to no sex information or being male, 42 probands were removed for low genotype rate, 25 probands were removed for high heterozygosity, 24 probands were removed for high relatedness.

The plot resulting from the merge of GCAN dataset with the three major HapMap3

Table 18. Analysis of the 20 SNPs in relation to their role on curBMI in BN

SNP	Genotype	*M* ± *SD*	*β*	*t*	*p*
rs1137100	G/G	21.43 ± 8.23	-0.2937	-0.5629	0.5740
	G/A	22.82 ± 5.55			
	A/A	22.9 ± 4.15			
rs1137101	G/G	22.73 ± 5.96	0.0613	0.1245	0.9011
	G/A	22.85 ± 4.91			
	A/A	22.43 ± 4.91			
rs1042571	A/A	24.49 ± 3.76	1.0780	2.0200	0.04465
	A/G	23.72 ± 3.28			
	G/G	22.36 ± 5.34			
rs4684677[a]	A/A	23.30 ± 1.55	0.6163	0.6568	0.5120
	A/T	23.38 ± 2.41			
	T/T	22.67 ± 5.31			
rs696217	T/T	23.04 ± 3.37	0.0614	0.0833	0.9337
	T/G	23.09 ± 3.17			
	G/G	22.69 ± 5.36			
rs12490160	G/G	19.85 ± 2.36	-0.6449	-0.8641	0.3885
	G/T	22.61 ± 5.81			
	T/T	22.82 ± 4.91			
rs3732941	G/G	25.21 ± 3.25	0.3437	0.5370	0.5918
	G/A	22.84 ± 2.70			
	A/A	22.64 ± 5.62			
rs7799039	A/A	23.4 ± 3.56	0.7473	1.6430	0.1018
	A/G	22.76 ± 6.03			
	G/G	22.34 ± 4.24			
rs1187325	C/C	22.74 ± 3.48	-0.4877	-1.0740	0.2841
	C/G	22.18 ± 6.68			
	G/G	23.40 ± 2.94			
rs1078947	T/T	25.70 ± 3.86	1.1450	1.7750	0.0773
	T/C	23.44 ± 5.22			
	C/C	22.36 ± 5.07			
rs6265	T/T	22.09 ± 3.55	0.1781	0.2741	0.7843
	T/C	23.03 ± 2.71			
	C/C	22.64 ± 5.89			
rs56164415[a]	T/T	20.20 ± 0.00	0.1008	0.1041	0.9172
	T/C	23.19 ± 2.60			
	C/C	22.72 ± 5.27			
rs7180942	C/C	23.04 ± 2.89	-0.3070	-0.6672	0.5053
	C/T	22.07 ± 6.48			
	T/T	23.62 ± 3.15			

SNP	Genotype	*M* ± *SD*	*β*	*t*	*p*
rs5030980[a]	T/T	-	-0.1884	-0.1622	0.8713
	T/G	22.76 ± 8.33			
	G/G	22.76 ± 4.70			
rs13338499	G/G	22.11 ± 1.53	0.4401	0.5185	0.6047
	G/A	23.30 ± 6.53			
	A/A	22.64 ± 4.99			
rs17782313	C/C	20.87 ± 8.52	-0.3190	-0.5934	0.5535
	C/T	22.65 ± 4.49			
	T/T	23.01 ± 4.95			
rs489693	A/A	21.01 ± 6.55	-0.5167	-1.0510	0.2942
	A/C	22.81 ± 4.06			
	C/C	23.11 ± 5.57			
rs8087522	A/A	23.64 ± 2.64	0.0071	0.0143	0.9886
	A/G	22.22 ± 6.10			
	G/G	23.05 ± 4.36			
rs6127698	T/T	22.72 ± 5.60	0.4987	1.0760	0.2830
	T/G	23.11 ± 4.34			
	G/G	22.23 ± 5.77			
rs3827103	G/G	23.91 ± 4.49	0.3853	0.4945	0.6214
	G/A	22.60 ± 5.97			
	A/A	22.78 ± 4.91			

Note 1: Age, AAO, and site entered as covariates

Note 2: Bonferroni-corrected *p*-value set at 0.0027 for statistical significance and 0.01 for statistical trend.

[a] MAF < 0.07

Table 19. Analysis of the 20 SNPs in relation to their role on minBMI in BN

SNP	Genotype	*M* ± *SD*	*β*	*t*	*p*
	G/G	20.39 ± 1.58			
rs1137100	G/A	20.21 ± 1.48	0.1935	1.1060	0.2701
	A/A	19.97 ± 1.59			
	G/G	20.37 ± 1.45			
rs1137101	G/A	20.03 ± 1.58	0.1736	1.0760	0.2830
	A/A	19.96 ± 1.42			
	A/A	19.91 ± 1.16			
rs1042571	A/G	20.18 ± 1.37	0.0833	0.4281	0.6690
	G/G	20.11 ± 1.64			
	A/A	19.09 ± 0.38			
rs4684677[a]	A/T	20.20 ± 1.47	-0.0032	-0.0103	0.9918
	T/T	20.08 ± 1.56			
	T/T	20.11 ± 1.59			
rs696217	T/G	20.02 ± 1.50	-0.0635	-0.2605	0.7947
	G/G	20.10 ± 1.56			
	G/G	17.99 ± 2.54			
rs12490160	G/T	19.83 ± 1.35	-0.4594	-1.8570	0.0647
	T/T	20.17 ± 1.57			
	G/G	20.19 ± 1.54			
rs3732941	G/A	20.19 ± 1.44	0.1076	0.5053	0.6139
	A/A	20.06 ± 1.59			
	A/A	20.18 ± 1.69			
rs7799039	A/G	20.26 ± 1.65	0.2662	1.7610	0.0797
	G/G	19.77 ± 1.20			
	C/C	19.95 ± 1.87			
rs1187325	C/G	20.17 ± 1.49	-0.1059	-0.6949	0.4879
	G/G	20.15 ± 1.48			
	T/T	20.74 ± 2.16			
rs1078947	T/C	20.47 ± 1.78	0.5443	2.5560	0.0113
	C/C	19.97 ± 1.44			
	T/T	19.86 ± 1.04			
rs6265	T/C	19.85 ± 1.37	-0.3435	-1.6030	0.1104
	C/C	20.21 ± 1.62			
	T/T	20.20 ± 0.00			
rs56164415[a]	T/C	20.19 ± 1.46	0.0533	0.1637	0.8701
	C/C	20.08 ± 1.56			
	C/C	20.08 ± 1.41			
rs7180942	C/T	19.87 ± 1.38	-0.1905	-1.251	0.2122
	T/T	20.42 ± 1.78			

SNP	Genotype	*M* ± *SD*	*β*	*t*	*p*
	T/T	-			
rs5030980[a]	T/G	20.34 ± 1.40	0.2117	0.5252	0.6000
	G/G	20.07 ± 1.56			
	G/G	19.65 ± 0.66			
rs13338499	G/A	20.32 ± 1.51	0.1364	0.4896	0.6250
	A/A	20.06 ± 1.57			
	C/C	20.10 ± 1.46			
rs17782313	C/T	19.85 ± 1.44	-0.2310	-1.2990	0.1953
	T/T	20.24 ± 1.60			
	A/A	19.59 ± 1.12			
rs489693	A/C	20.01 ± 1.49	-0.3507	-2.1600	0.0318
	C/C	20.29 ± 1.66			
	A/A	20.19 ± 1.44			
rs8087522	A/G	20.01 ± 1.44	-0.0393	-0.2382	0.8120
	G/G	20.14 ± 1.65			
	T/T	20.12 ± 1.59			
rs6127698	T/G	20.17 ± 1.63	0.0871	0.5602	0.5759
	G/G	19.95 ± 1.39			
	G/G	20.13 ± 1.70			
rs3827103	G/A	20.02 ± 1.47	-0.0353	-0.1354	0.8925
	A/A	20.10 ± 1.56			

Note 1: Age, AAO, and site entered as covariates

Note 2: Bonferroni-corrected *p*-value set at 0.0027 for statistical significance and 0.01 for statistical trend.

[a] MAF < 0.07

Table 20. Analysis of the 20 SNPs in relation to their role on maxBMI in BN

SNP	Genotype	*M* ± *SD*	*β*	*t*	*p*
	G/G	25.45 ± 2.29			
rs1137100	G/A	26.18 ± 3.05	0.1668	0.4898	0.6248
	A/A	25.62 ± 3.14			
	G/G	25.58 ± 2.90			
rs1137101	G/A	25.80 ± 3.05	-0.0602	-0.1906	0.8491
	A/A	25.85 ± 3.10			
	A/A	26.92 ± 3.50			
rs1042571	A/G	26.39 ± 3.26	0.9228	2.4310	0.0159
	G/G	25.51 ± 2.94			
	A/A	24.62 ± 0.33			
rs4684677[a]	A/T	26.07 ± 2.54	0.2143	0.3567	0.7216
	T/T	25.78 ± 3.12			
	T/T	28.66 ± 2.37			
rs696217	T/G	25.46 ± 3.18	0.0904	0.1908	0.8488
	G/G	25.83 ± 3.03			
	G/G	22.85 ± 1.77			
rs12490160	G/T	25.56 ± 3.39	-0.5864	-1.2130	0.2264
	T/T	25.91 ± 2.98			
	G/G	27.29 ± 3.34			
rs3732941	G/A	25.72 ± 2.89	0.1697	0.4103	0.6820
	A/A	25.80 ± 3.11			
	A/A	25.73 ± 3.19			
rs7799039	A/G	25.95 ± 3.10	0.2258	0.7633	0.4461
	G/G	25.69 ± 2.93			
	C/C	25.29 ± 3.12			
rs1187325	C/G	25.83 ± 3.03	-0.3860	-1.3250	0.1866
	G/G	26.06 ± 3.04			
	T/T	**27.68 ± 2.88**			
rs1078947	**T/C**	**26.63 ± 3.37**	**1.2940**	**3.1660**	**0.0018**
	C/C	**25.47 ± 2.90**			
	T/T	23.99 ± 2.54			
rs6265	T/C	25.56 ± 2.97	-0.4694	-1.1240	0.2622
	C/C	25.98 ± 3.10			
	T/T	24.27 ± 0.00			
rs56164415[a]	T/C	25.42 ± 2.89	-0.6231	-0.9878	0.3244
	C/C	25.87 ± 3.08			
	C/C	25.51 ± 3.25			
rs7180942	C/T	25.45 ± 2.29	-0.1425	-0.4807	0.6312
	T/T	26.18 ± 3.05			

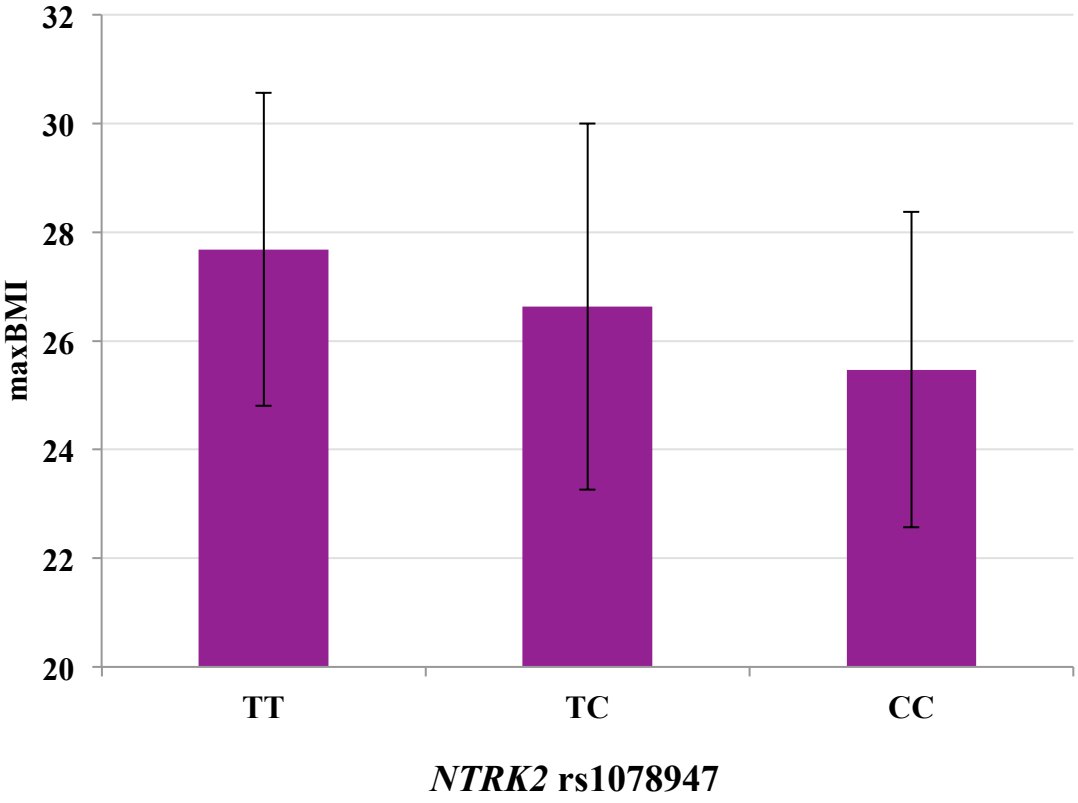
SNP	Genotype	*M* ± *SD*	*β*	*t*	*p*
	T/T	-			
rs5030980[a]	T/G	26.36 ± 3.53	0.2113	0.2699	0.7875
	G/G	25.78 ± 3.02			
	G/G	25.18 ± 0.59			
rs13338499	G/A	26.35 ± 3.17	0.3122	0.5814	0.5617
	A/A	25.76 ± 3.08			
	C/C	25.32 ± 2.73			
rs17782313	C/T	25.78 ± 3.26	-0.3659	-1.0570	0.2916
	T/T	25.90 ± 2.99			
	A/A	24.57 ± 2.42			
rs489693	A/C	25.85 ± 3.04	-0.7598	-2.4170	0.0165
	C/C	26.09 ± 3.16			
	A/A	26.14 ± 3.35			
rs8087522	A/G	25.87 ± 3.03	0.2007	0.6266	0.5316
	G/G	25.74 ± 3.06			
	T/T	26.19 ± 3.52			
rs6127698	T/G	25.78 ± 2.97	0.3548	1.1770	0.2403
	G/G	25.65 ± 2.91			
	G/G	27.93 ± 3.46			
rs3827103	G/A	26.08 ± 3.48	0.2860	0.5656	0.5723
	A/A	25.75 ± 2.97			

Note 1: Age, AAO, and site entered as covariates

Note 2: Bonferroni-corrected *p*-value set at 0.0027 for statistical significance and 0.01 for statistical trend. Results in bold represent statistical significance or trend.

[a] MAF < 0.07

Figure 5. Distribution of the mean maxBMI in BN based on *NTRK3* rs1078947 genotype



Note 1: $p = 0.0018$ (significant); age, AAO and site entered as covariates

Note 2: Mean maxBMI in kg/m^2 per genotype: TT = 27.68 ± 2.88, TC = 26.63 ± 3.37, CC = 25.47 ± 2.90

Table 21. Analysis of the 20 SNPs in relation to their role on curBMI in controls

SNP	Genotype	M ± SD	*β*	*t*	*p*
rs1137100	G/G	23.49 ± 2.32	-0.0431	-0.2256	0.8217
	G/A	23.57 ± 2.14			
	A/A	23.63 ± 2.16			
rs1137101	G/G	23.73 ± 2.10	-0.0083	-0.0517	0.9588
	G/A	23.47 ± 2.05			
	A/A	23.68 ± 2.34			
rs1042571	A/A	23.65 ± 2.28	0.2756	1.2370	0.2169
	A/G	23.85 ± 2.19			
	G/G	23.50 ± 2.13			
rs4684677[a]	A/A	23.39 ± 0.00	-0.1267	-0.3445	0.7307
	A/T	23.48 ± 1.97			
	T/T	23.62 ± 2.19			
rs696217	T/T	20.20 ± 0.00	0.0815	0.2512	0.8018
	T/G	23.83 ± 2.42			
	G/G	23.57 ± 2.11			
rs12490160	G/G	21.77 ± 1.41	-0.2800	-0.9678	0.3339
	G/T	23.46 ± 2.05			
	T/T	23.65 ± 2.18			
rs3732941	G/G	22.09 ± 1.35	-0.3873	-1.5230	0.1289
	G/A	23.51 ± 2.20			
	A/A	23.66 ± 2.16			
rs7799039	A/A	23.41 ± 2.03	0.1659	1.0000	0.3180
	A/G	23.90 ± 2.21			
	G/G	23.31 ± 2.13			
rs1187325	C/C	24.24 ± 2.13	0.3374	1.996	0.0468
	C/G	23.39 ± 2.19			
	G/G	23.53 ± 1.97			
rs1078947	T/T	23.53 ± 2.71	0.0292	0.1217	0.9032
	T/C	23.59 ± 2.02			
	C/C	23.60 ± 2.19			
rs6265	T/T	22.58 ± 2.49	-0.2457	-1.1030	0.2711
	T/C	23.49 ± 2.20			
	C/C	23.69 ± 2.12			
rs56164415[a]	T/T	23.31 ± 2.85	0.3414	0.9989	0.3186
	T/C	24.01 ± 1.99			
	C/C	23.57 ± 2.17			
rs7180942	C/C	23.46 ± 2.17	-0.0972	-0.5867	0.5578
	C/T	23.61 ± 2.13			
	T/T	23.70 ± 2.216			

SNP	Genotype	M ± SD	*β*	*t*	*p*
rs5030980[a]	T/T	-	0.4439	1.0330	0.3026
	T/G	24.01 ± 2.01			
	G/G	23.56 ± 2.17			
rs13338499	G/G	21.42 ± 1.00	0.1310	0.4598	0.6460
	G/A	24.00 ± 1.82			
	A/A	23.55 ± 2.22			
rs17782313	C/C	23.78 ± 2.37	0.5038	2.4890	0.0133
	C/T	24.02 ± 1.86			
	T/T	23.32 ± 2.28			
rs489693	A/A	23.88 ± 2.27	0.2583	1.4250	0.1550
	A/C	23.72 ± 2.03			
	C/C	23.39 ± 2.26			
rs8087522	A/A	23.38 ± 2.39	-0.0204	-0.1137	0.9095
	A/G	23.71 ± 2.23			
	G/G	23.54 ± 2.04			
rs6127698	T/T	23.87 ± 2.27	0.1236	0.7479	0.4551
	T/G	23.48 ± 2.24			
	G/G	23.58 ± 1.93			
rs3827103	G/G	25.78 ± 0.00	0.3263	0.9904	0.3227
	G/A	23.80 ± 2.12			
	A/A	23.56 ± 2.17			

Note 1: Age entered as a covariate

Note 2: Bonferroni-corrected *p*-value set at 0.0027 for statistical significance and 0.01 for statistical trend.

[a] MAF < 0.07

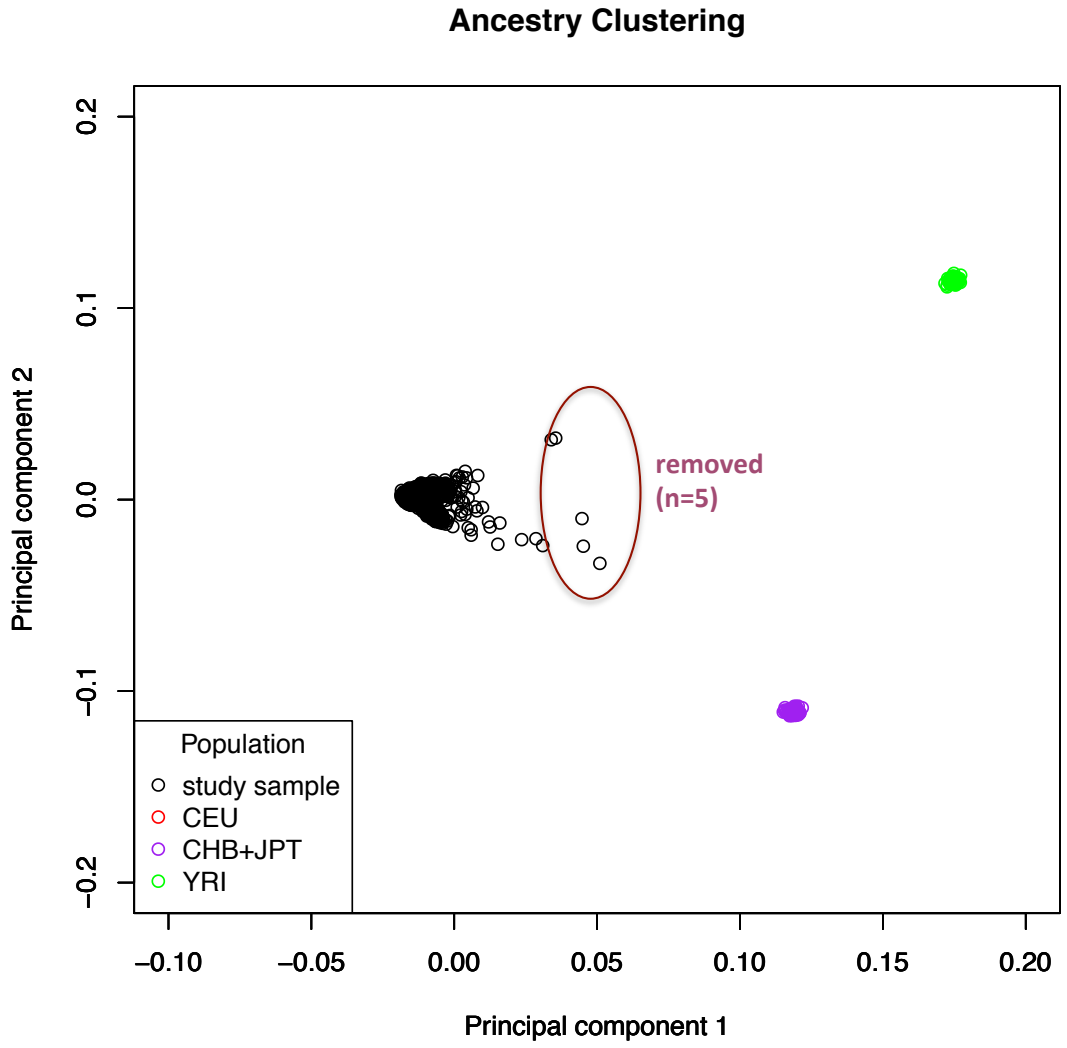
populations is included as Figure 6, in which it can be seen that GCAN population merged remarkably with the CEU-ancestry HapMap population. Based on the clustering of the data, 5 individuals were removed from the GCAN data set for potential population stratification. We also ran MDS to look at principal components C1 through C10, clusters derived from ancestry informative markers (AIMs) in the genome, in the GCAN sample alone. Figure 7 features the plot of C1 and C2 components.

As a part of the SNP-level QC, 18 SNPs were removed for no genotype, 5,408 SNPs were removed for low genotype rate (GENO > 0.05), 66,304 SNPs were removed for low minor allele frequency (MAF < 0.05), and 5,119 SNPs were removed for being out of Hardy-Weinberg Equilibrium (HWE < 10^{-6}). After the QC, the following 13 candidate genes were extracted from the genome-wide data (+10 kb upstream and 5 kb downstream, with the exception of MC4R which included 200 kb downstream): *DRD1, DRD2, DRD3, DRD4, DRD5, SLC6A3, DBH, TH, COMT, MAOA, BDNF, MC4R,* and *FTO* (see Table 7 in the Methods chapter). Following the extraction of these genes, genotype information was available for 264 post-QC SNPs. A total of nine SNPs that have been associated with obesity (or their proxies as defined by $r^2 \geq 0.8$) according to an influential GWAS paper (Speliotes et al., 2010) were then removed from analysis (two in *BDNF*, four in *FTO*, and three in near-*MC4R* region), since the secondary analysis involving these markers will be carried out by other GCAN collaborators (see Table 8 in the Methods chapter). *DRD1* and *DRD5* genes were not covered by the GWAS chip post-QC and therefore excluded. The final analysis consisted of 11 genes and 255 SNPs.

4.2.2. Sample description

Following QC, the final GWAS sample was 3,004 AN probands, and clinical phenotype

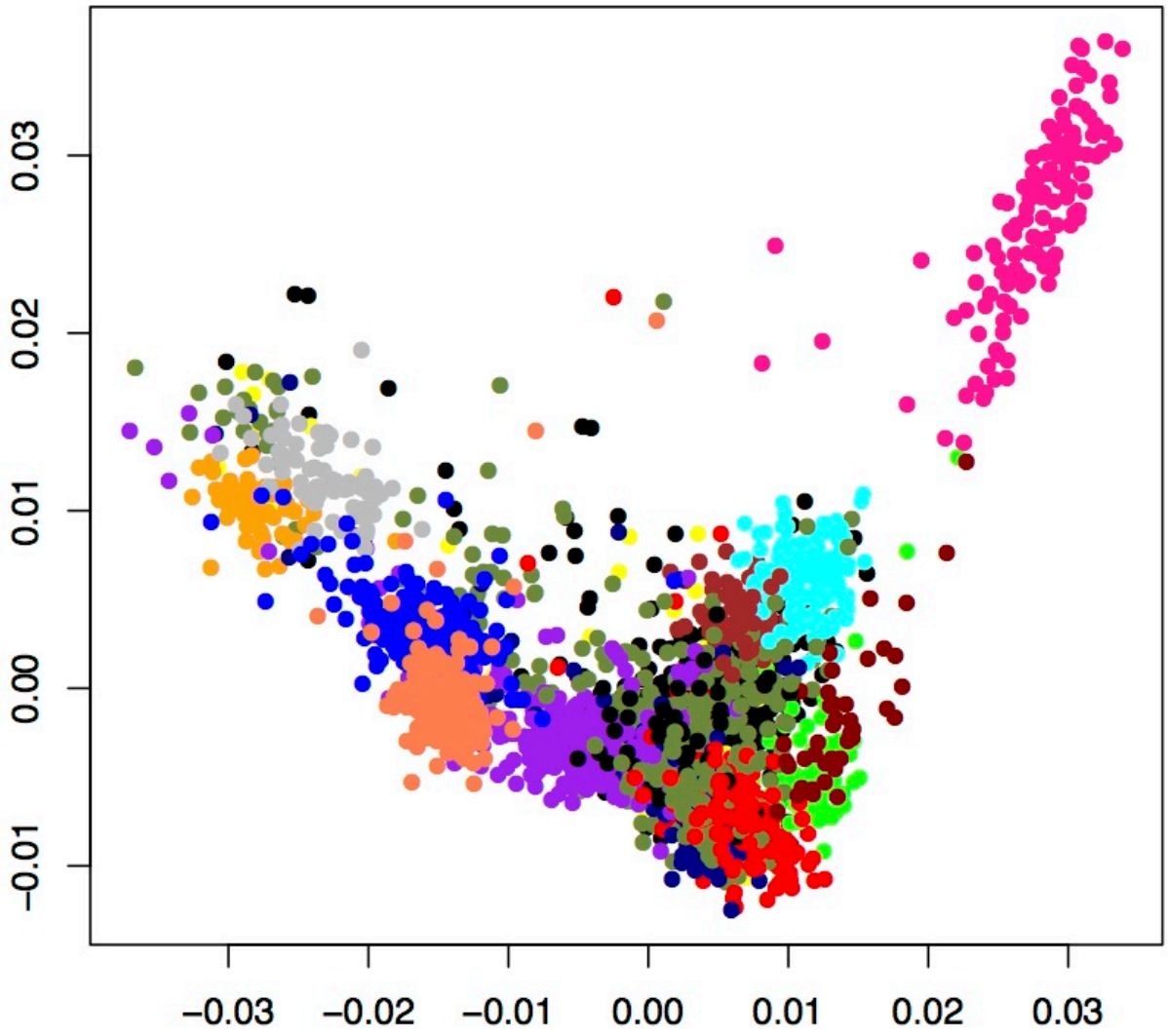
Figure 6. MDS plot of components C1 and C2 of the GCAN dataset, merged with HapMap3 populations



Note 1: CEU = HapMap European Caucasian ancestry population; CHB+JPT = HapMap Chinese Han and Japanese ancestry populations; YRI = HapMap Yoruba in Ibadan, Nigeria ancestry (African American) population

Note 2: CEU population is not clearly visible, as it was plotted underneath the study sample data points, indicating that the probands included in the GCAN analysis were all of European descent.

Figure 7. MDS plot of principal components C1 and C2 for GCAN sites



Note 1: The x-axis represents principal component C1, and the y-axis represents principal component C2.

Note 2: The colour codes for the participating countries is as follows: Finland = dark pink, Germany = black, USA = dark green, Canada = yellow, Netherlands = red, Norway = green, Czech Republic = brown, Poland = cyan, Italy South = orange, Italy North = blue, UK = dark blue, France = purple, Spain = coral, Sweden = dark red, Greece = gray.

data (maxBMI, minBMI, curBMI, AAO, history of BN and AN subtype) were available for 2,396 individuals. Baseline characteristics of the cases are outlined in Table 22. Among the sample, 813 probands had AN-R (50.7%), 789 had AN-BP (49.3%), and 211 had a history of BN (8.9%). The distribution of three of the phenotypic variables (curBMI, maxBMI and AAO) deviated from a normal distribution, thus they were log10-transformed to ensure normality. No phenotypic data were available for Greece and Italy North, and the Polish site did not provide information on BMI measures or AAO.

The final breakdown of the DNA samples from each site included in phenotypic analysis is as follows: 66 from Canada (2.8%), 50 from Czech Republic (2.1%), 102 from Finland (4.3%), 249 from France (10.4%), 446 from Germany (18.6%), 59 from Italy (2.5%), 281 from the Netherlands (11.7%), 86 from Norway (3.6%), 175 from Poland (7.3%), 166 from Spain (6.9%), 38 from Sweden (1.6%), 220 from the UK (9.2%), and 457 from the USA (19.1%).

4.2.3. LogcurBMI analysis

Figure 8 features the list of the most significant 25 SNPs (i.e., Top 25 hits) in association with logcurBMI. We detected a significant association between *FTO* rs1075440 and logcurBMI in 1,992 AN probands, with logAAO, AN subtype, history of BN and ancestry principal components C1 through C10 entered as covariates in the model. This finding stayed significant following Bonferroni correction. Furthermore, all of the three most significant SNPs and 11 of the 25 top SNPs were located in the *FTO* gene. Another SNP that reached significance prior to Bonferroni correction, rs1861868, is also in the *FTO* gene and is in moderate LD with rs1075440 ($r^2 = 0.45$). Visualization of the SNPs associated with logcurBMI using LocusZoom (Pruim et al., 2010) revealed that a majority of the *FTO* SNPs and rs1075440 are separated by a

Table 22. Baseline characteristics of the GCAN AN cases included in the final analysis

Phenotype variable	**N**	***M* ± *SD***
curBMI (kg/m^2)	1,992	17.97 ± 3.27
minBMI (kg/m^2)	1,797	14.80 ± 2.15
maxBMI (kg/m^2)	1,611	22.24 ± 3.38
AAO (years)	1,457	17.2 ± 4.5
logcurBMI	1,992	1.25 ± 0.08
logmaxBMI	1,611	1.34 ± 0.06
logAAO	1,457	1.22 ± 0.10

Figure 8. PLINK output of the Top 25 SNPs for logcurBMI in AN

CHR	SNP	UNADJ	GC	BONF	HOLM	SIDAK_SS	SIDAK_SD	FDR_BH	FDR_BY
16	**rs1075440**	**2.367e-005**	**7.474e-005**	**0.006035**	**0.006035**	**0.006017**	**0.006017**	**0.006035**	**0.03694**
16	rs10852521	0.002706	0.004963	0.6901	0.6874	0.4989	0.4976	0.2903	1
16	**rs1861868**	**0.003416**	**0.006102**	**0.871**	**0.8642**	**0.5821**	**0.5792**	**0.2903**	**1**
18	rs12958350	0.00488	0.008375	1	1	0.7128	0.7085	0.3111	1
16	rs8044769	0.01086	0.01704	1	1	0.9382	0.9354	0.4974	1
11	rs7125415	0.01494	0.02264	1	1	0.9785	0.9768	0.4974	1
9	rs2097628	0.01526	0.02307	1	1	0.9802	0.9783	0.4974	1
16	rs6499653	0.01561	0.02353	1	1	0.9819	0.9798	0.4974	1
16	rs2111112	0.01852	0.02741	1	1	0.9915	0.9901	0.518	1
18	rs9960448	0.02352	0.03391	1	1	0.9977	0.9971	0.518	1
18	rs11152221	0.02531	0.03619	1	1	0.9986	0.9981	0.518	1
16	rs9937234	0.02605	0.03713	1	1	0.9988	0.9984	0.518	1
11	rs10891556	0.02641	0.03759	1	1	0.9989	0.9985	0.518	1
16	rs9302652	0.03624	0.04983	1	1	0.9999	0.9999	0.6238	1
16	rs7190492	0.0367	0.05039	1	1	0.9999	0.9999	0.6238	1
11	rs4436578	0.04668	0.06247	1	1	1	1	0.7261	1
5	rs40184	0.04841	0.06453	1	1	1	1	0.7261	1
18	rs9675886	0.05221	0.06904	1	1	1	1	0.7397	1
18	rs1943222	0.06168	0.08012	1	1	1	1	0.792	1
16	rs12596210	0.06212	0.08062	1	1	1	1	0.792	1
9	rs1611119	0.07098	0.09084	1	1	1	1	0.7947	1
18	rs7230734	0.07422	0.09453	1	1	1	1	0.7947	1
5	rs2617596	0.07817	0.09902	1	1	1	1	0.7947	1
16	rs1362570	0.08116	0.1024	1	1	1	1	0.7947	1
5	rs2617605	0.0863	0.1082	1	1	1	1	0.7947	1

Note 1: SNP in red font is significantly associated with logcurBMI following Bonferroni correction. SNP in blue font is in moderate LD with the top SNP ($r^2 = 0.45$).

Note 2: The following covariates were entered into the model: principal components C1-C10, logAAO, history of BN, and AN subtype.

recombination hot spot, which explains the lack of LD between these loci (Figure 9).

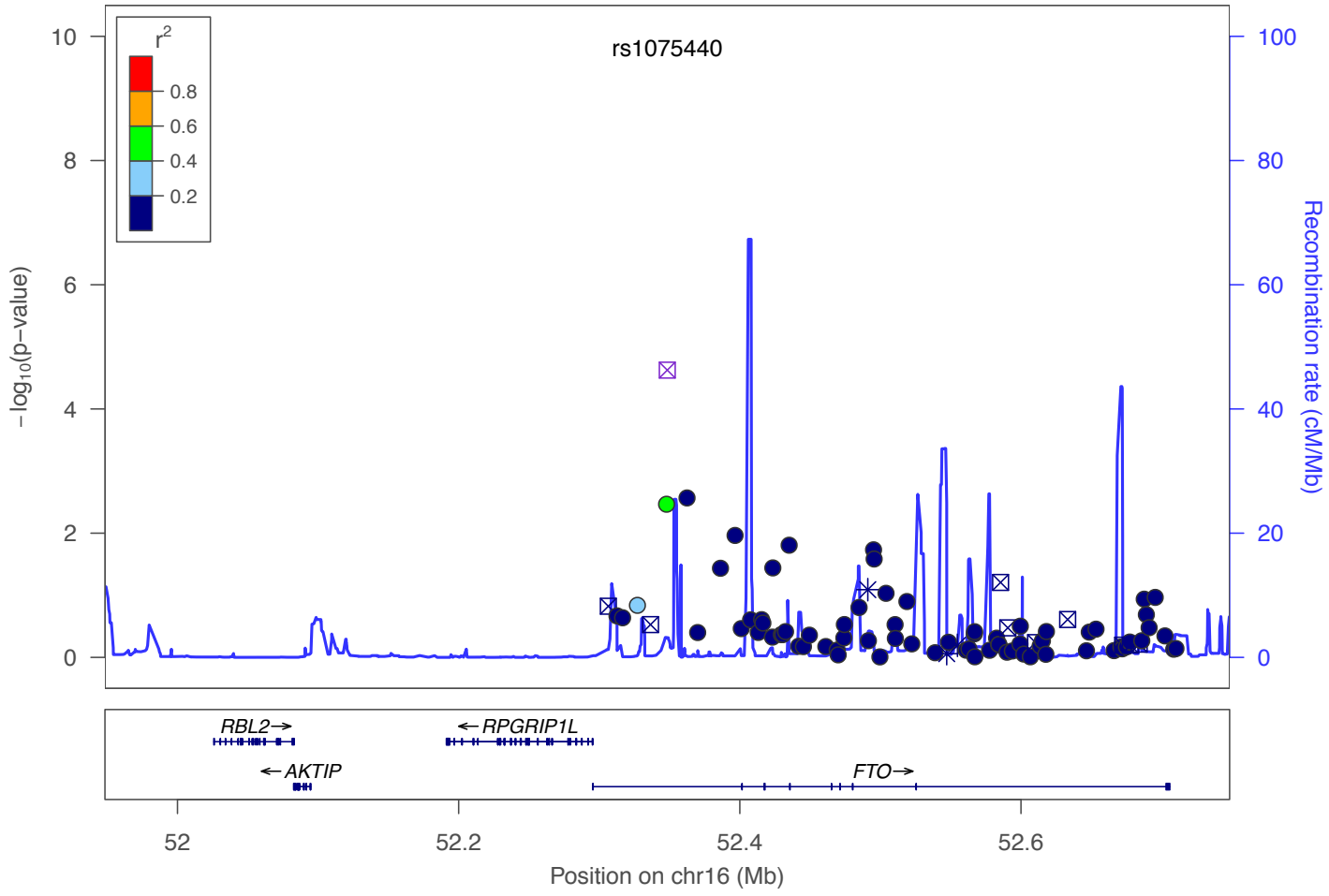
4.2.4. MinBMI analysis

The list of the 25 most significant SNPs associated with minBMI in 1,797 AN cases is included in Figure 10. Similar to logcurBMI, we controlled for logAAO, AN subtype, history of BN and principal components C1 through C10. The most significant SNP was *MAOA* rs2072743, but the association was not statistically significant after Bonferroni correction. In fact, four of the Top 10 SNPs were located in the *MAOA* gene, and the most significant 25 SNPs also featured loci in *FTO*; however, none of the *p*-values reached Bonferroni-corrected threshold.

4.2.5. LogmaxBMI analysis

The list of the 25 most significant SNPs associated with logmaxBMI in 1,611 probands can be found in Figure 11. LogAAO, AN subtype, history of BN and principal components C1 through C10 were entered as covariates. *DRD2* rs4648318 had the most significant uncorrected *p*-value in association with logmaxBMI. Furthermore, three of *DRD2* SNPs were among the Top 10 SNPs; however, none of them reached significance following correction. In addition, five of the Top 10 SNPs were located in the *FTO* gene, but similar to *DRD2*, none of the associations involving these markers were significant post-Bonferroni.

Figure 9. LocusZoom plot for the *FTO* rs1075440 and logcurBMI



Note 1: *FTO* rs1075440 is indicated by the purple square, which is located n a region highly conserved in placental mammals.

Note 2: SNP highlighted with the green dot (rs1861868) is in moderate LD with the rs1075440 ($r^2 = 0.45$), whereas the blue do represent another marker that is not among the Top 25 hits and in weak LD with rs1075440. The spike on the right of rs1075440 indicates recombination hotspot, thus SNPs separated by this zone (i.e., rs1075440 and the SNPs that fall on the right side of the hotspot) are not in LD.

Figure 10. PLINK output of the Top 25 SNPs for minBMI in AN

CHR	SNP	UNADJ	GC	BONF	HOLM	SIDAK_SS	SIDAK_SD	FDR_BH	FDR_BY
23	rs2072743	0.004594	0.007489	1	1	0.6909	0.6909	0.7202	1
9	rs2097628	0.005664	0.009042	1	1	0.7651	0.7638	0.7202	1
16	rs10852521	0.0149	0.0216	1	1	0.9783	0.9776	0.7202	1
16	rs8044769	0.0166	0.02381	1	1	0.986	0.9853	0.7202	1
23	rs2235186	0.01673	0.02398	1	1	0.9865	0.9855	0.7202	1
23	rs909525	0.02008	0.02827	1	1	0.9943	0.9937	0.7202	1
22	rs1544325	0.0205	0.02881	1	1	0.9949	0.9943	0.7202	1
3	rs1486009	0.02259	0.03144	1	1	0.9971	0.9965	0.7202	1
3	rs11706283	0.03409	0.04558	1	1	0.9999	0.9998	0.7991	1
23	rs1137070	0.03506	0.04675	1	1	0.9999	0.9998	0.7991	1
22	rs5746847	0.03667	0.04868	1	1	0.9999	0.9999	0.7991	1
22	rs5993883	0.04535	0.05899	1	1	1	1	0.7991	1
9	rs2519148	0.04896	0.06322	1	1	1	1	0.7991	1
16	rs16952686	0.05151	0.0662	1	1	1	1	0.7991	1
3	rs7633291	0.05715	0.07271	1	1	1	1	0.7991	1
3	rs10934256	0.05715	0.07271	1	1	1	1	0.7991	1
16	rs16953047	0.05885	0.07466	1	1	1	1	0.7991	1
18	rs12958350	0.06015	0.07616	1	1	1	1	0.7991	1
3	rs167771	0.06176	0.078	1	1	1	1	0.7991	1
9	rs129885	0.06267	0.07904	1	1	1	1	0.7991	1
23	rs1465108	0.07163	0.0892	1	1	1	1	0.8059	1
18	rs9956274	0.07443	0.09236	1	1	1	1	0.8059	1
9	rs129886	0.07595	0.09407	1	1	1	1	0.8059	1
22	rs174675	0.08047	0.09912	1	1	1	1	0.8059	1
16	rs2689264	0.0806	0.09927	1	1	1	1	0.8059	1

Note: The following covariates were entered into the model: principal components C1-C10, logAAO, history of BN, and AN subtype.

Figure 11. PLINK output of the Top 25 SNPs for logmaxBMI in AN

CHR	SNP	UNADJ	GC	BONF	HOLM	SIDAK_SS	SIDAK_SD	FDR_BH	FDR_BY
11	rs4648318	0.004112	0.01473	1	1	0.6503	0.6503	0.674	1
16	rs1861868	0.008285	0.02484	1	1	0.8801	0.8791	0.674	1
11	rs7125415	0.01007	0.02875	1	1	0.9243	0.9228	0.674	1
22	rs165656	0.01394	0.03667	1	1	0.9721	0.9709	0.674	1
16	rs1075440	0.01821	0.0448	1	1	0.9908	0.9901	0.674	1
11	rs4436578	0.02312	0.05359	1	1	0.9974	0.9971	0.674	1
16	rs4784346	0.02369	0.05458	1	1	0.9978	0.9974	0.674	1
16	rs16945088	0.02473	0.05637	1	1	0.9983	0.998	0.674	1
22	rs4680	0.02537	0.05747	1	1	0.9986	0.9982	0.674	1
16	rs9924877	0.0289	0.06338	1	1	0.9994	0.9993	0.674	1
22	rs165722	0.0315	0.06763	1	1	0.9997	0.9996	0.674	1
16	rs7200972	0.04128	0.08293	1	1	1	1	0.674	1
16	rs7194243	0.04131	0.08298	1	1	1	1	0.674	1
5	rs2617605	0.04196	0.08396	1	1	1	1	0.674	1
11	rs4620755	0.04362	0.08645	1	1	1	1	0.674	1
22	rs5993891	0.04551	0.08927	1	1	1	1	0.674	1
11	rs2242592	0.04915	0.09463	1	1	1	1	0.674	1
3	rs2630351	0.05434	0.1021	1	1	1	1	0.674	1
18	rs8094523	0.05484	0.1028	1	1	1	1	0.674	1
11	rs2587550	0.05488	0.1029	1	1	1	1	0.674	1
16	rs1345390	0.05792	0.1072	1	1	1	1	0.674	1
11	rs4581480	0.05815	0.1075	1	1	1	1	0.674	1
18	rs9956274	0.06183	0.1126	1	1	1	1	0.6855	1
3	rs2630349	0.06564	0.1178	1	1	1	1	0.6974	1
16	rs2111118	0.07726	0.1333	1	1	1	1	0.7656	1

Note: The following covariates were entered into the model: principal components C1-C10, logAAO, history of BN, and AN subtype.

CHAPTER 5

DISCUSSION

5.1. Summary of PF candidate gene findings (Study 1)

The research included in this dissertation involving the PF DNA samples (Study 1) was designed to: (1) genotype on average two polymorphisms with known or putative function in the leptin, melanocortin, and neurotrophin system genes in AN probands with no history of BN, and vice versa. The high crossover rates between ED diagnosis, especially in the case of AN to BN has been a significant souce of bias in genetic studies, which has been acknowledged by genetic researchers as well (Muller et al., 2012b). The case-control component BN probands with no history of AN as the comparative ED group and women with no psychiatric history as controls. The second part of the study explored the role of the select candidate genes on minimum lifetime BMI, maximum lifetime BMI, and BMI at the time of recruitment in AN and BN groups separately.

In line with the literature, we observed significant phenotypic differences between the two AN subtypes. As expected, AN-R probands reported lower current and lifetime BMIs; however, there was no difference in AAO between AN-R and AN-BP probands. When we looked at site differences for BN probands, we discovered that the Toronto BN cases were on average four years younger at recruitment compared to the PF cases. This difference can be attributed to recruitment methods utilized by the two studies: while the PF consortium sites had a budget for large-scale advertising efforts (such as newspaper and radio ads) in addition to traditional hospital and treatment program-driven recruitment, the limited budget of the Toronto BN study led to a different approach that consisted of posting flyers at hospitals and local university campuses in addition to contacting individuals on the waitlist for treatment programs.

This difference in outreach may explain why the Toronto probands were younger in terms of age at recruitment. Furthermore, PF BN participants on average were one year younger when they developed BN, which may also be an artifact of university campus based-recruitment: it is possible that Toronto study included younger women with less severe BN compared to the PF study having access to the more severe BN cases through the wide outreach and better utilization of hospital resources across multiple participating countries. However, these systematic differences between recruitment sites as well as AN subtypes prompted us to control for AN subtype in all AN analyses and age-related variables (AAO and age) in all BN analyses to ensure that observed genetic differences were not due to these potential confounders.

5.1.1. Summary of the case-control analysis

As a part of the case-control analysis, there was a statistical trend for the *MC4R* rs489693 A allele to be less common in AN cases compared to nonpsychiatric controls. When AA and AC genotypes were combined under the dominant model for the A allele, this association reached nominal significance after Bonferroni correction. This finding is particularly interesting, considering that the rs489693 A allele has been implicated in AIWG based on GWAS results and three independent replication samples (Malhotra et al., 2012). The underrepresentation of the variant linked to weight gain in the AIWG literature suggests that the A allele may have a protective effect against severe and sustained weight suppression, as observed as a part of AN diagnosis. The fact that we did not observe a link between this marker and any of the BMI measures could possibly mean that *MC4R* is not responsible for within-AN BMI variations and that its involvement is in the overall susceptibility to maintained low body weight. Although rs489693 is not currently known to be functional, it may either be in LD with a functional polymorphism or indirectly responsible for alterations in MC4R function or expression, which

requires further studies to be conducted on its functionality. This finding at least partially supports our general melanocortinergic hypothesis and the specific hypothesis (hypothesis #3 for Study 1) concerning the involvement of *MC4R* genetic variants in AN compared to controls.

Despite the methodological strengths of this dissertation in maximizing phenotypic differences between AN and BN groups, we did not observe any differences between the ED diagnoses and SNPs selected from the candidate genes of interest. One possible reason for this is that the BN sample size may not have been sufficient for the required statistical power (745 AN cases versus 245 BN cases) to detect differences. The most significant *p*-value was for *NTRK3* rs7180942 ($p = 0.0225$), with the C allele slightly overrepresented in AN compared to BN, similar to what has been reported by a European study (Mercader et al., 2008). It would be interesting to see if this difference would become statistically significant if the BN sample size were increased. Alternately, the results could be due to the fact that we may not have selected the right markers or the right genes that are responsible for diagnostic differences. Furthermore, difference between the diagnostic categories may not be a function of being able to suppress one's body weight, and further research should focus on different gene systems based on different *a priori* phenotypic hypotheses. Finally, we also failed to observe any differences in allele frequencies of the SNPs included in the study between BN and control groups, thus suggesting that leptin-melanocortin-neurotrophin system genes may not confer risk for BN. It is also important to acknowledge the sample size limitations (245 BN and 321 control samples), which may have resulted in an underpowered analysis for markers with low MAF. Finally, similar to the AN vs. BN analysis, the results could have been entirely due to the selection of the wrong candidate SNPs and genes in the gene systems studied in this project. However,

considering that the BN cases did not differ from the nonpsychiatric female controls in terms of their BMI histories, the lack of association was not entirely surprising.

In summary, the results provided partial support for our general melanocortinergic hypothesis in AN compared to controls. However, none of the leptin (*LEP, LEPR, GHRL,* and *HRH1*) and neutrophin system genes (*BDNF, NTRK2,* and *NTRK3*) were linked to AN or BN, thus our general and specific leptinergic and neutrophic hypotheses (hypothesis #1 involving *HRH1* for the former and hypotheses #6 and #8 for the latter) for case-control analysis were not supported in this study sample. Similarly, our specific hypothesis concerning *AGRP* rs5030980 (hypothesis #4) was also not supported in our case-control sample, a reason for which could be lack of power for the low MAF. *AGRP* rs13338499 and the *POMC* gene were also not linked to AN or BN in the PF candidate gene analysis.

5.1.2. Summary of the within-group BMI analyses

In the AN group, *AGRP* rs13338499 was significantly linked to minBMI following correction for multiple testing. To our knowledge, this is the first time this marker has been studied in weight regulation, and it is not in high (or moderate) LD with any other *AGRP* loci previously investigated in EDs. According to *in silico* analysis, rs13338499 is a putative transcription factor-binding site, and since it is located upstream of the *AGRP* gene, it is likely to play a regulatory role as well. Considering the key orexigenic role AGRP plays through the hypothalamus, this finding further emphasizes the importance of the melanocortin system in weight regulation. Plasma AGRP levels are known to be inversely correlated with BMI (Moriya et al., 2006), and *Agrp* overexpression is one of the earliest animal models of obesity. Based on these findings, it can be speculated that the A allele is linked to a loss of function, thus leading to haploinsufficiency via reduction of AGRP expression and resulting in a lower weight in a

subgroup of AN cases. Functional experiments are needed to uncover whether rs13338499 plays a regulatory role as per *in silico* predictions. For curBMI and maxBMI, we did not observe any trends or significant associations in AN probands. This finding provides support for our melanocortinergic general hypothesis for low body weight in AN.

In the case of BN, *NTRK2* rs1078947 T allele predicted a higher maxBMI, and the *p*-value stayed significant after correction. Interestingly, the T allele was also linked to a higher mean minBMI in BN, which did not quite pass the statistical trend cutoff set at $p < 0.01$ ($p = 0.0113$). It would be interesting to see if this observation would reach statistical significance in a larger BN sample. Our finding appears to contradict what has previously been reported with this SNP, as it was found that the C allele was linked to a higher maxBMI in AN probands (Ribases et al., 2005a). A few possible explanations exist for this discrepancy. First, since rs1078947 did not yield any significant association with any of the three BMI measures in our 745 AN probands, the results of the first study might have been affected by the small sample size (N = 83). Second, it is also possible that the AN group in the previous study may have included individuals with a history of BN, which may have also led to the difference in the reported findings due to phenotypic heterogeneity. Nonetheless, these results need to be replicated to understand the true role *NTRK2* rs1078947 may play in weight regulation in EDs. This marker is not predicted to have function *in silico*, and functional research is needed to understand the nature of this association. Similar to AN, there was no significant association with the 20 SNPs studied with curBMI or minBMI in BN. In summary, our general neurotrophic hypothesis is partially supported by this finding in BN, but we reject the specific hypothesis on *NTRK2* rs1078947 in low BMI in AN (hypothesis #7 for Study 1).

Although this study was not designed to explore the genetics of BMI in controls, we repeated the genetic analysis with curBMI in our nonpsychiatric female control group to determine whether any significant associations observed in EDs are indeed unique to ED status. Although none of the SNPs was linked to BMI in controls, we observed that those with at least one copy of the C allele of *MC4R* rs17782313 on average reported a higher curBMI. This finding, although short of being statistically significant, is very much in line with the vast literature on the role of near-*MC4R* common variants in body weight (reviewed in detail in Introduction chapter, Section 1.8.3.1). It is also interesting that this obesity-linked marker did not yield any positive findings in the ED groups, and this finding provides further evidence that *MC4R* rs17782313 may influence body weight in the general population but is independent from ED diagnosis. Again, it is important to consider that the control sample size was smaller compared to the AN sample size, which could explain the nonsignificant *p*-value. Nonetheless, this observation of the C allele being linked to a higher BMI fits well with what has been well reported and well replicated in the literature.

Contrary to our specific hypothesis # 5 for Study 1, we failed to find a link between *BDNF* rs6265 polymorphism and ED status or weight regulation in EDs in the PF sample. Considering the repeated reports on the association of the hypofunctional Met allele with ED status and low body weight, this nonsignificance was somewhat surprising. However, this finding is at least in partial agreement with our previous research, where we reported that the Val66Met polymorphism by itself was not associated with BMI in BN but predicted a higher maxBMI in the presence of the *DRD4* 7R variant (Kaplan et al., 2008). We were not able to retest the entire premise of the *DRD4-BDNF* gene-gene interaction from our previous study, since our arrangement with PF did not include permission for the analysis of dopamine system

genes. Thus far, there have been two meta-analyses conducted on the role of rs6265 on EDs. Although the first study linked the Met allele to ED diagnosis (Gratacos et al., 2007), the second meta-analysis did not find an association with AN (Brandys et al., 2011), and our findings appear to agree with the latter report. Possible explanations for the multiple positive associations of rs6265 with EDs and BMI published in the literature include small sample size, phenotypic heterogeneity (especially in the form of ED history), and population stratification.

We were also unable to conclusively study four of the 21 SNPs included in the study: three markers had a low MAF (*AGRP* rs5030980, *BDNF* rs56164415, and *GHRL* rs4684677), thus making our analysis underpowered, and *NTRK3* rs1128994 was excluded from our analysis due to excess heterozygosity, most likely due to technical issues with the probes of the ABI assay. Since these four markers were specifically included in the analysis due to either being studied in EDs before and yielding mixed results that need replication or having known or putative function, we believe there is merit in including these loci in future studies with a much larger sample size for more meaningful conclusions.

Finally, since a GWAS has been performed by our PF collaborators on the larger AN sample consisting of 1,033 cases (Wang et al., 2011b), we compared our results with the GWAS *p*-values for the SNPs included in our study. Out of the 21 SNPs, only the following six overlapped with the GWAS: rs1137100, rs1137101, rs696217, rs3732941, rs6265, rs6127698, and rs3827103. None of these six SNPs was in the Top 100 hits in the GWAS case-control analysis, and the highest *p*-value was 0.1247 for *MC3R* rs3827103 in the female-only analysis. The three SNPs with which we report significant findings were not covered by the GWAS chip. Despite this PF GWAS effort, we firmly believe that our targeted candidate gene project with refined ED phenotypes is relevant and novel, especially considering that the GWAS included all

unrelated AN cases regardless of their BN history and did not investigate any quantitative traits such as BMI.

In summary, the second part of the Study 1 investigating the role of the selected candidate genes in weight regulation in AN and BN provided some support for our general melanocortinergic hypothesis for AN and the general neurotrophic hypothesis for BN. Our results did not support the general leptinergic hypothesis in weight regulation in EDs, as none of the *LEP, LEPR, GHRL* or *HRH1* markers were associated with BMI in AN or BN. Furthermore, our hypothesis involving the *MC4R* obesity locus rs17782313 (specific hypothesis #2) was also not supported in our sample. Other *MC4R* loci and the *POMC* marker were also not associated with BMI in EDs in our sample. Similar to what was observed in the case-control analysis, the *AGRP*-related specific hypothesis was not supported, with low MAF being a possible issue. Finally, we failed to find supporting evidence for all of the specific neurotrophic hypotheses regarding BMI in and BN in our sample. We also failed to find a association of *BDNF, NTRK2* rs1187325 and *NTRK3* genes with any of the three BMI measures in EDs.

5.2. Summary of GCAN findings (Study 2)

The Study 2 included in this PhD dissertation investigated the role of dopamine pathway genes and *FTO* in body weight in a large sample of AN cases. Compared to the targeted approach of Study 1, the aim of this secondary analysis was a more thorough exploration of entire genes and a focus on maximizing sample size. Since the samples came from several different countries, population stratification is an issue that may have influenced the results. In order to account for the effects of population stratification, we only included European probands that clustered well with the HapMap3 CEU populations and controlled for principal components C1 through C10 by entering them as covariates in all analyses. From a phenotypic perspective,

history of BN and AN subtype are factors known to influence BMI (as reviewed in Methods chapter, sections 3.1.2 and 3.1.3). In addition, there is increasing evidence that different genetic etiology may be present in cases with early AAO as opposed to later AAO for psychiatry disorders (Goncalves et al., 2012; De Luca et al., 2012; Lett et al., 2013). In summary, a total of 13 covariates were entered into the model: C1-10, logAAO, AN subtype, and history of BN.

FTO rs1075440 G allele was significantly associated with a lower logcurBMI in AN probands, which stayed statistically significant after correcting for multiple testing. According to the functional annotation displayed by LocusZoom, this polymorphism is located in a region highly conserved in placental mammals. Although *in silico* analysis did not predict a function for this locus, both BrainArray and NIEHS databases confirmed its high conservation across mammals. Despite not being statistically significant, the G allele was correlated with a lower logmaxBMI in AN as well, ranking as the eighth top SNP for this measure. Thus far, this locus has not come up in the weight regulation literature, as rs1075440 has not been linked to BMI in any research studies, which makes this association novel. Furthermore, rs1075440 is separated from the majority of the other *FTO* loci by a recombination hot spot, and as a result of this, it is not in LD with most of the *FTO* SNPs, including those that have been linked to obesity in the literature. That being said, the fact that another *FTO* SNP—rs1861868—which is in moderate LD with rs1075440 ($r^2 = 0.452$) ranked #3 for the logcurBMI phenotype and had a significant unadjusted association suggests against the possibility that our finding with rs1075440 is a statistical anomaly. This analysis did not include *FTO* SNPs linked to obesity, since secondary analysis involving these loci will be carried out by other GCAN collaborators. In summary, our *FTO*-related general and specific hypotheses (hypothesis #1 for Study 2) were supported by our results.

In the case of minBMI, none of the SNPs crossed the Bonferroni-corrected significance threshold. However, *MAOA* rs2072743 came up as the top SNP for this phenotype in AN. This marker is not predicted to have a functional role by *in silico* programs; however, it has been reported that the *MAOA* mRNA levels may be significantly elevated in depressed individuals of Chinese ancestry carrying the A allele of rs2072743 (Zhang et al., 2010b). In addition, two other *MAOA* SNPs were among the Top 10 most significant loci for minBMI and were in relatively high LD with the top SNP (r^2 = 0.703 and 0.751, respectively). This pattern implies a possible small role for the *MAOA* gene in minimum lifetime BMI in AN, and although not significant, it would be interesting to see if these results would pass the Bonferroni-corrected significance threshold in a larger sample size.

As for logmaxBMI, we did not find any statistically significant associations with the dopamine system SNPs. However, it is noteworthy that the top locus (rs4648318) was located in the *DRD2* gene, as well as two other SNPs in the Top 10 and a total of seven SNPs in the Top 25 most significant uncorrected *p*-values. This locus has been linked to depressive symptoms in Finnish men as a part of a two-marker haplotype (Nyman et al., 2012), but it has not been implicated in weight regulation thus far. Furthermore, the two addition *DRD2* loci in the Top 10 were (rs7125415 and rs4436578) in moderate LD with rs4648318 (r^2 of 0.318 and 0.355, respectively); in addition, three more *DRD2* loci were among the Top 25 hits and were in moderate LD with the top SNP *(*r^2 range of 0.405 and 0.458). Interestingly, the two additional Top 10 *DRD2* polymorphisms for logmaxBMI were among the 10 most significant *p*-values for logcurBMI as well. Furthermore, rs4436578 has previously been associated with AIWG in Chinese patients with schizophrenia (Hong et al., 2010), which further supports the connection of this polymorphism to weight regulation. *In silico* analysis does not predict a functional role for

any of the three most significant *DRD2* markers, but it is possible that they may be in high LD with another marker with function. *DRD2* rs6277, previously linked to AN (Bergen et al., 2005), was not among the top hits, and the top *DRD2* SNP was only in weak LD with it ($r^2 = 0.272$). *DRD2* rs17799732, also associated with BMI by our PF collaborators (Bergen et al., 2005), could not be covered by the GWAS chip since it is an insertion/deletion polymorphism, so we were unable to evaluate its role in BMI in AN as a part of this secondary analysis. Based on these results, it can be speculated that *DRD2* may at least weakly influence maximum lifetime BMI in AN, and it would be interesting to further explore this possible link in a larger cohort. Thus, although our *DRD2*-specific hypotheses were not supported in this sample, the role of the *DRD2* markers in weight regulation in AN merits further consideration in future studies.

It is surprising that the only statistically significant association was obtained for logcurBMI as opposed to the lifetime BMI measures that capture the weight extremes. There are two possible explanations for this phenomenon. First, data for curBMI was available for more cases compared to the lifetime BMIs, thus it might have been more adequately powered, or that the lifetime BMI variables did not have adequate power for detecting a significant association. Second, depending on site and data collection methods, it is more likely that curBMI was obtained objectively (especially in clinics and treatment programs) compared to lifetime BMIs, which are more likely to be self-reported. This was the case for the samples our site provided to the GCAN; while curBMI was obtained in an objective manner, information on minBMI and maxBMI were collected through the completion of Eating Disorder Inventory self-report questionnaire. Based on this scenario, we can speculate that the curBMI is likely to be a more accurate measure for the GCAN sample, whereas assuming that in many instances lifetime BMIs were obtained through self-report, they may be affected by recall bias. Furthermore, it is

important to note that the majority of the studies using BMI as a subphenotype have relied on current BMI measures, thus our results would be in line with the phenotype reported in the weight regulation literature.

Despite not being statistically significant, a few additional observations merit discussion. First, one *COMT* marker, rs165656, was ranked as the fourth most significant SNP prior to correction for logmaxBMI in AN. Although not known to be functional, this locus is in complete LD with rs4680 (Val158Met), which is known to influence COMT enzyme activity. Our work on the *COMT* gene's role in BN is summarized in detail in the Appendix (Section A5). Moreover, rs4680 was one of the Top 10 markers for logmaxBMI as well. Although it was hypothesized that the Met variant would be linked to lower minBMI, the results indicate that the Val variant was associated with a lower logmaxBMI in AN. It is important to note that this finding was not statistically significant (thus not supporting our specific hypothesis #3 for Study 2 regarding *COMT*), but the possible role of the *COMT* gene may merit further investigation in weight regulation in AN. Second, *MC4R* rs489693, which we found to be underrepresented in AN compared to controls in Study 1, was not covered by the genotyping in GCAN, so we were not fully able to evaluate our specific hypothesis #4 on the role of AIWG SNPs in the *MC4R* gene and BMI in AN. It is interesting to note that five of the Top 25 loci for logcurBMI phenotype were *MC4R* markers, none of which was in LD with rs489693 or rs8087522. This finding was not entirely surprising; we also did not detect an association involving this variant with any of the three BMI measures in the PF genetic analysis. The five loci that came up in the logcurBMI analysis, however, were in weak LD with the obesity variant rs17782313 (not included in the analysis), and none of them is predicted to be functional. *MC4R* markers were not especially represented among the logmaxBMI and minBMI top hits.

In summary, our GCAN results support our general hypothesis involving the *FTO* gene and BMI in AN. *DRD2, DRD3, DRD4, DBH, TH, SCL6A3, MAOA, COMT, MC4R* and *BDNF* genes were not linked to weight regulation in AN following Bonferroni correction, thus we did not find any direct evidence supporting the general and specific dopaminergic hypotheses in our sample. Similarly, our specific hypothesis #4 regarding *MC4R* AIWG loci was not supported.

5.3. Strengths

The studies included in this dissertation are innovative and benefit from a number of significant strengths. For the PF candidate gene study and the GCAN secondary analysis, we had a large number of AN probands (N = 745 and 1,992, respectively), both of which are among the largest candidate gene sample sizes in EDs. Considering that the majority of genetic studies in EDs have suffered from a small sample size, our studies were adequately powered for the proposed analysis and allowed us to more confidently reach conclusions based on our findings.

One of the major strengths of the PF genetic analysis is the careful selection of probands in order to reduce phenotypic heterogeneity. Alongside the issue of sample size, the high crossover rates between ED diagnoses and subtypes (which can be up to 40% in some instances) is a major problem for genetic studies wishing to compare AN and BN cases. This issue is likely to be one of the reasons for the nonreplication of candidate gene results and the conflicting findings reported in the literature. By excluding AN probands with a history of BN and BN probands with a history of AN, we were able to maximize between-group differences—thus increase the potential to magnify genetic differences as well—while minimizing phenotypic heterogeneity within each ED group.

Another strength of this dissertation is the combination of methodologically distinct but complementary approaches to genetic analysis. The inclusion of SNPs with known or putative

function in the PF analysis is a unique approach that has not been often utilized in ED genetic studies. After developing our *a priori* hypotheses regarding the possible etiology of body weight in EDs based on the rich literature on animal and human obesity studies, we included genes in the leptin, melanocortin, and neurotrophin systems that have either not been sufficiently studied or led to mixed findings due to methodological issues. Based on functionality experiments reported in the literature as well as with the assistance of *in silico* prediction websites, we selected on average two markers with known or putative function per gene, which is an approach that is more likely to provide researchers with a biologically meaningful insight for the etiology of EDs. Moreover, recent evidence suggests that there may be a consistent pattern of enrichment among functional SNPs in the human genome (Schork et al., 2013), further highlighting the need to prioritize markers based on known or putative function. In the case of the GCAN analysis, our focus was shifted toward maximizing sample size and obtaining adequate coverage of each candidate gene included as a part of the dopamine pathway plus *FTO*. The GWAS already having been performed and genotype data being accessible to GCAN collaborators are two factors that have made this gene-based approach feasible and practical for this study by reducing genotyping costs that we would have experienced otherwise for thorough gene coverage. Moreover, it has been argued that the gene-based approach may be the best way to proceed in the age of GWAS, especially since the gene is still deemed as the basic building block of biology (Neale and Sham, 2004). We believe that while the PF and GCAN approaches are different, they provide us with a better opportunity at getting a more complete glimpse of the big picture by complementing thoroughness with a systematic focus on the biological function and clinical phenotype. Indeed, detailed phenotyping is often not an option for GWAS aimed to reach the high sample sizes, but a large *N* helps us detect signals that a smaller sample size cannot. On the

other hand, a targeted study with a well-defined but smaller sample may help with focusing on a genetic signal while significantly reducing background noise.

From a statistical perspective, both PF and GCAN studies utilized appropriately stringent statistical corrections for multiple testing. In fact, another major reason for replication issues in psychiatric genetics (especially in EDs) has to do with the lack of correction; considering that each test introduces a 5% rate of Type I error (two-sided), it is crucial for researchers to account for this accumulating false positive error rate. In the case of the GCAN, Bonferroni correction was applied to determine the significance threshold by dividing the alpha of 0.05 by 255, the number of SNPs included in the analysis. In the case of the PF analysis, however, we used a hybrid of Nyholt and Bonferroni corrections: Nyholt correction provided us with the number of independent loci per gene based on the LD information between the SNPs, and then Bonferroni correction was utilized based on the results of the Nyholt test. This statistically rigorous but not overly conservative approach helps ensure that any significant finding is not an artifact of multiple testing.

5.4. Limitations

Despite the significant strengths of this dissertation, a number of limitations also merit careful consideration. For instance, in the case of the PF study, nonpsychiatric female controls were significantly older than the AN and BN cases. Although we acknowledge that it is best to use age-matched controls recruited specifically for the study being conducted, it can be argued that the older age of the controls does not pose a risk to our findings for two reasons: first, age of an individual does not have an effect on their DNA sequence in classical molecular genetic studies (unlike gene expression or epigenetic studies); second, we could argue that since, in the majority of cases, EDs are developed during teen years or early adulthood (American Psychiatric

Association, 2000), inclusion of older controls compared to age-matched controls reduces the likelihood of these nonpsychiatric women going on to developing an ED after study inclusion. Because of these two reasons, we did not enter age as a covariate for case-control analyses involving the women with no psychiatric history. Another possible shortcoming involving the controls is the lack of ED-specific screening. However, considering the low prevalence rate of EDs in the general population and that the AN and BN groups are enriched for any genetic factors that may increase risk for EDs, this is a conservative bias that is not likely to affect the outcome of the genetic analysis.

In terms of the BN probands in the PF study, we observed significant site differences: PF BN cases were older and had an earlier AAO compared to Toronto BN cases. Although these differences were limited to age-related data and not observed for BMI measures, we included AAO and age at recruitment as covariates in BN analyses to control for site effects. However, it is possible that the results may still have been influenced by these systematic site-related phenotypic differences in BN cases.

In the PF study, although the sample size for AN was large for a candidate gene analysis, BN and control sample sizes were average and comparable to other ED genetic studies, which may have reduced the statistical power of within-group BMI analyses for BN and control groups. Furthermore, the sample sizes for the three groups were not well balanced (745 AN vs. 245 BN vs. 321 controls), and we readily acknowledge that this could have also resulted in a lack of power to detect differences in allele frequencies between groups. Although 745 AN cases for PF and 1,992 AN cases for GCAN individually make these studies one of the largest in ED genetics literature, it is entirely possible that despite our power calculations, we were unable to capture the role of some of the SNPs with lower MAF or smaller effect size. It would be interesting to

repeat these analyses in a larger sample size to see if our current findings would replicate or more SNPs become statistically significant.

Although we believe that one of the strengths of the PF analysis is the utilization of functional variants, the genotyping of a small number of SNPs and not using any tag SNPs for better gene coverage could also be posed as a criticism. For the reasons explained in the Objectives section of this dissertation, we decided to focus our efforts in a very targeted way on SNPs with known or putative function in order to maximize the limited operating budget and the chance to obtain results that are more meaningful compared to an approach that utilizes tag SNPs not predicted to have a functional role. Through a careful literature review and *in silico* analysis we prioritized a small number of markers that are more likely to yield more clinically and functionally relevant results for weight regulation in EDs. However, it is entirely possible that the risk loci for EDs located in these candidate genes of interest were outside of those we selected for genetic analysis, and that a tag SNP approach may provide more complete information and complementary findings to our more targeted study.

For the GCAN secondary analysis, SNP coverage for each gene on the GWAS chip varied significantly. For example, *DRD1* and *DRD5* genes had to be excluded due to not being directly genotyped, and only two loci were covered for *DRD4*. As a result of this, our findings may have been affected by the inconsistent gene coverage as a part of the GWAS genotyping. Although it would have been ideal to work with the imputed data as opposed to raw genotype data, WTCCC3 is not planning to make the imputed genotypes available at least until the GWAS case-control study is published (manuscript currently under preparation). In the future, it would be ideal to repeat the secondary analysis using the imputed dataset. We also acknowledge that GWAS genotype data only cover polymorphisms of biallelic nature; as a result of this, we cannot

comment on the possible role of VNTRs, insertion/deletion polymorphisms, and CNVs located in the dopamine pathway genes on weight regulation in AN.

Another limitation of the two genetic analyses performed as a part of this doctoral dissertation is the possible effect of population stratification on the genetic results. This is especially of concern due to both studies consisting of DNA samples collected as a part of international consortia. Although all participants included in the PF analysis were of European ancestry, we did not have access to genome-wide data to analyze AIMs to determine population substructure. In the case of the GCAN analysis, although we took precautions by controlling for principal components C1 through C10, it is still possible that the findings were influenced by differences in allelic frequencies between European subpopulations.

It is also important to acknowledge that the effect sizes associated with the statistically significant observations for BMI in the PF study were relatively small. Indeed, the mean differences between genotypes for *AGRP* rs13338499 in the case of minBMI in AN and for *NTRK2* rs1078947 in the case of maxBMI in BN were between 0.5 and 1 kg/m^2, so their clinical significance could be deemed to be limited. There are two possible reasons for these observations. First, since one of our aims for Study 1 was to reduce phenotypic variance within each diagnostic group, this methodological approach also led to a reduction in variance in the three BMI measures among probands, and as a result of this, genetic effects that are statistically significant are no longer likely to result in large differences in BMI among genotypes. Second, with the recent discoveries in genetic studies, it is becoming more and more evident that the etiology of psychiatric disorders can be attributed to the presence of a large number of common risk variants, each of which having a small effect (International Schizophrenia Consortium et al., 2009; Sullivan et al., 2012). This means that unless a marker has high penetrance and major

deleterious effects, it is highly unlikely that risk variants will lead to drastic changes in mean BMI.

Although our goal was to systematically examine the variants in the dopamine system genes and *FTO* as a part of the GCAN secondary analysis, we excluded nine SNPs that resulted in significant associations with obesity (or the proxies of these SNPs, as defined by $r^2 = 0.8$) as per the Speliotes et al.'s study (Speliotes et al., 2010) from our analysis, since the investigation on the role of these loci on BMI will be carried out by our collaborators. This is a limitation that did not allow us to explicitly test the obesity loci in relation to weight regulation in AN; however, it is important to note that the number of excluded SNPs was very small compared to the SNPs included in our analysis. Furthermore, based on the results of the PF genetic analysis, we could cautiously speculate that these loci are not likely to play a major role in AN weight regulation in the GCAN sample.

Despite the utilization of rigorous correction for multiple testing is one of the strengths of these dissertation, it is also important to acknowledge the possibility of overcorrection. Especially with the GCAN sample, where the loci located in the same gene are likely to be in varying degrees of LD, it is possible that assuming independence for each of the 255 markers might have resulted in Type II errors, where a true significant finding could have been missed because of overly-conservative statistical correction. However, use of Bonferroni is the gold standard for GWAS and GWAS-derived data, so our practice is in line with the literature. Furthermore, even with the hybrid use of Nyholt and Bonferroni, it is highly unlikely that the *p*-values for the top SNPs which were nonsignificant in our analysis would become statistically significant. Considering the variability in genetic findings in ED research due to lack of proper

correction for multiple testing, we would argue that stringent correction would be a less risky and more appropriate approach when trying to balance Type I and Type II errors.

Finally, we acknowledge the possibility that the genetic underpinnings of BMI may not be the key factors leading to the development of AN versus BN or weight regulation within EDs. One possibility is that other psychopathology and traits linked to EDs, such as obsessionality in AN and impulsivity in BN, could play a more important role in weight regulation that genes known to influence appetite and weight. It has been shown that the prevalence of OCD is significantly elevated among the first-degree relatives of ED probands compared to controls (Lilenfeld et al., 1998), and a recent pilot study has reported shared genetic etiology between AN and OCD (Mas et al., 2013), which could have important implications for how we view and classify these disorders if replicated in a larger sample. This possible etiological link points to perfectionistic and obsessive traits being key factors in the suppression of body weight and its maintenance over a prolonged period of time. In the case of BN, impulsivity could be among the reasons for the inability to suppress body weight despite the drive for thinness. Indeed, as reported in the Introduction, childhood ADHD rates are considerably high among BN cases compared to the general population (Fleming and Levy, 2002; Yilmaz et al., 2011; Yilmaz et al., 2012c; see Appendix A5 and A6); furthermore, there could be clinical, neurobiological, and genetic associations between alcohol/substance use disorders and BN, all of which are believed to be driven by impulse dysregulation (as reviewed in Yilmaz et al., 2012a). In summary, it is plausible that low body weight in AN is not the genetic opposite of high BMI in obesity; weight regulation in EDs could be regulated by psychological traits as opposed to weight-related genes, and study of genetic systems linked to obsessionality and impulsivity could possibly be responsible for ED susceptibility.

5.5. 'The Big Picture' and possible implications of the findings

In this final section of Chapter 5, a brief review of the current state of research and emerging trends in psychiatric genetics will be provided. There will also an overview of the possible clinical implications of the results reported in this dissertation.

5.5.1. Challenges associated with reaching large sample sizes

With the promising advances in the field of psychiatric genetics, it is becoming evident that sample sizes much larger than previously anticipated are necessary for the discovery of genome-wide significant loci with small effect sizes. As with other psychiatric disorders, it is highly unlikely that researchers will find any large genetic effects for the overall AN diagnosis (Sullivan et al., 2012). Recent GWAS findings in AN have been negative (Wang et al., 2012b), and it has been proposed that the minimum number of cases needed to reach the low end of sufficient power for detecting common variants with small effect sizes is 10,000 (Sullivan et al., 2012). With this new goal of reaching samples sizes in the tens of thousands, the sample sizes for the studies included in this dissertation are indeed very modest. In order to maximize sample size in the study of psychiatric disorders, the most likely outcome is for researchers to focus their genetic research efforts into forming international consortia and working together in a collaborative manner to further our understanding of the genetic etiology of psychiatric disease.

Although consortia studies exist for EDs (two of which are PF and GCAN), these collaborations have directed their efforts almost solely on the genetics of AN, and thus far no large-scale genetic analyses have been conducted for BN. Furthermore, although the prevalence rate of AN is comparable to that of schizophrenia and that BN is significantly more common, there are likely to be additional obstacles in reaching the high sample sizes for EDs. First, genetic research in EDs is still at least a decade behind many other psychiatric illnesses. Until recently,

some of the main causes for EDs were believed to be the influence of the Western culture, dysfunctional family environment, and other psychological vulnerabilities. Although there is no doubt that these factors often serve as important triggers that may lead to the development of EDs, we now know that the picture is far from complete without the study of genetic etiology. After all, research has consistently shown the high heritability of EDs through additive genetic risk, and it is very difficult for environment alone to trigger full-syndromal disordered eating without the presence of this genetic susceptibility.

The second factor that may limit ED research to immediately reach the desired sample sizes is the stigma attached to having an ED (Darby et al., 2012), resulting in individuals feeling the need to hide their AN or BN out of shame. It is not uncommon for lay people to blame patients for their ED. Furthermore, studies have shown that there are misunderstandings and lack of knowledge about EDs even among professionals working in the medical field (Kaplan and Garfinkel, 1999; Gowers and Shore, 1999; Thompson-Brenner et al., 2012). As a result of this, it is no surprise that many individuals with EDs are not willing to seek treatment or participate in research studies. The good news is that there are various outreach and educational efforts being carried out worldwide to inform people about ED facts and dispel myths, and these programs have significantly increased the general population's understanding of EDs and their etiology. With the continuation of these efforts, hopefully the stigma against AN and BN will be reduced and the individuals with EDs will be more likely to see treatment and participate in research efforts without embarrassment or fear of being blamed for their illness.

5.5.2. Phenotype-driven candidate gene research as a complementary approach to GWAS

As much as we should strive for the collection of tens of thousands of ED cases, it is important for the field to also consider other approaches that may augment the GWAS and large-

scale genetic searches. An alternative but complementary way to maximize genetic research efforts is to focus on intermediate phenotypes (i.e., low body weight). Indeed, with the increasing demand for mega sample sizes for case-control GWAS, many researchers are emphasizing the importance of an intermediate phenotype-driven approach that does not ignore the core symptoms of the disease in favour of reaching very large sample sizes (Meyer-Lindenberg, 2010; Niculescu and Le-Niculescu, 2010; Almasy, 2012). The main rationale for not giving up on phenotype is that risk genes should have greater penetrance with these intermediate phenotypes, since they are more objective, and more proximal to the gene, in comparison to overall diagnosis (such as AN and BN). This complementary approach, similar to the one taken in this dissertation, may not require sample sizes in the thousands, but the careful sample characterization of the ED samples and related phenotypes, especially due to high crossover rates, would be of utmost importance.

Furthermore, ED-related phenotypes and potential endophenotypes are going to become even more relevant with the recent release of the DSM-5. In fact, the revised diagnostic classification system has been under fire for being arbitrarily decided upon by clinicians for purely clinical purposes, and many scientists have made the comment that genes and biology do not follow DSM. Moreover, National Institute of Mental Health has publicly stated that it will not be supporting DSM-5 in biological research context and is in the works of developing its own classification system called Research Domain Criteria (RDoC). According the Dr. Thomas Insel, this classification effort is designed to guide biological and genetic research, and RDoC will be derived from the research on brain mapping, neurocircuitry, and genetics in a dimensional way (details can be found at http://www.nimh.nih.gov/about/director/index.shtml#p145045). Although it is beyond the scope of this dissertation to discuss the merits

of one diagnostic tool over the other, it is important to highlight that as opposed to a dichotomous case-control approach, the benefits of using continuous and multi-dimensional phenotype-driven methodology is back on the genetic research agenda, and this approach is likely to stay as a complementary framework for the foreseeable future. Finally, the characterization of RDoC based on the literature also suggests that the candidate gene methodology, with its valuable *a priori* hypothesis concept and utilization of previous preclinical and clinical work, is here to stay to supplement GWAS efforts. The lack of replication of candidate genes studies is an issue that needs to be addressed (Hart et al., 2013; Sullivan et al., 2012), and the main reasons behind various false positives being produced are small sample size, lack of correction for multiple testing, populations stratification, inconsistencies with assessment and experimental methods, and phenotypic heterogeneity (Li and Meyre, 2013). That being said, evidence provided by candidate gene studies (especially those that have yielded large effect sizes) cannot be entirely discarded (Siontis et al., 2010; Hart et al., 2013), and if the researchers focus on either correcting or controlling for the methodological issues listed above, the critics of the candidate gene studies will likely see the benefit in augmenting other genetic approaches with this hypothesis-driven methodology.

5.5.3. Possible clinical implications of the current findings

To our knowledge, this is the first study to investigate the role of leptin, melanocortin, neurotrophin, and dopamine system genes in body weight in EDs. Considering the major role these systems play in appetite and weight regulation, our significant results pertaining to some of these genes may have a number of implications on different points of the clinical spectrum.

We have demonstrated that the *MC4R* rs489693 may confer risk to AN, whereas *AGRP NTRK2*, and *FTO* loci may play a role in weight regulation in EDs. If our findings are replicated

in studies with larger samples, one of the first lines of translating the genetic results to clinical care would be through early detection. This could be done through using these genetic markers as possible screening tools for susceptibility to AN or weight dysregulation. Considering that the effect sizes associated with these markers were small in our study, genotyping individuals just for these variants would not produce reliable results. However, if these results were combined with other risk variants determined to affect either ED status or weight regulation (of course following thorough replication), then it is plausible to include all these risk markers on a chip and test at-risk individuals for all loci to calculate a composite risk score by adding up the number of risk alleles present. This additive burden assessment method, using loci identified in GWAS, has been successfully implemented to separate schizophrenia cases from healthy controls (International Schizophrenia Consortium et al., 2009). Moreover, that Psychiatric Genomics Consortium (PGC) is in the process of developing a 'PsychChip,' an Illumina custom array that will cover 20,000 SNPs and CNV probes thus far linked to psychiatric illness in PCG projects (details available at https://pgc.unc.edu/SciPlan.php#scope). In line with these exciting new developments, a similar method for ED susceptibility screening can be developed using the four markers identified in this study in combination with other previously identified ED risk loci in the future (contingent upon rigorous replication and validation).

The significant associations reported in this dissertation can also guide the development of new biomarkers to study in AN and BN. Two loci in the melanocortin gene system (specifically in the *MC4R* and *AGRP* genes) were implicated in AN and weight regulation in AN in our PF study. In addition to melanocortin system's well documented interactions with LEP, BDNF, and dopamine, melanocortin signaling may also play a role in the regulation of circulating cholesterol: it has been reported that in rodents, the inhibition of melanocortin system

in the CNS leads to a drastic increase in HDL cholesterol levels in a manner independent of food intake or body weight (Perez-Tilve et al., 2010). Furthermore, *FTO* gene is known to be involved in the breakdown of lipids (Loos and Bouchard, 2008) and has been implicated in HDL levels in a GWAS report (Asselbergs et al., 2012). Interestingly, one of the Top 10 SNPs identified in the PF AN GWAS was in the *VGLL4* gene, which is located within the bp boundaries of the quantitative locus trait regions previously linked to BMI (Wu et al., 2002; Gorlova et al., 2003; Norris et al., 2005; Kraja et al., 2005) and HDL cholesterol (Pollin et al., 2004; Yang et al., 2005). Considering that patients with AN often present with elevated cholesterol levels (Ohwada et al., 2006; Matzkin et al., 2006; Rigaud et al., 2009; Jauregui-Garrido et al., 2012), this clinical abnormality could be speculated to be a sign of a disruption in the melanocortin systems and FTO expression. Furthermore, based on these results and how they fit with the existing literature on hypercholesterolemia in AN, it could be further speculated that cholesterol abnormalities could be a biomarker associated with AN.

Finally, if replicated, our results may have the potential for developing more effective pharmacological treatment approaches and providing a highly specific target for novel medications. AGRP is the natural inverse agonist of MC4R, and considering that the genes that encode for both of these peptides yielded significant result in AN, future research focus on the possible use of AGRP and SHU9119 (an MC4R competitive antagonist) in the treatment of AN. In rodents, administration of AGRP ameliorates the behaviours associated with ABA, whereas SHU9119 does not result in increase in food intake or decrease in wheel running (Hillebrand et al., 2006). Despite these promising findings, we are not aware of any clinical trials of MC4R antagonists or inverse agonists in the treatment of AN symptoms. In addition, the Introduction chapter (Sections 1.8.3.1 and 1.8.5) reviewed the important role melanocortins play in the

regulation of dopamine signaling. Moreover, *MC4R* polymorphisms, including rs489693, have been associated with weight gain in schizophrenia patients treated with atypical antipsychotic medications (Chowdhury et al., 2012; Malhotra et al., 2012). These results suggest a possible indirect role for the melanocortin system in the mechanism of action of antipsychotic medications. In the recent years, there has been an interest in the study of antipsychotic medications such as olanzapine (Zyprexa) in AN in an effort to identify evidence-based pharmacological treatments. Because symptoms of depression, anxiety, hyperactivity and obsessionality are also in part mediated by dopamine and successfully treated with olanzapine, it has been suggested that the core symptoms of AN, which include these symptoms as well as near-delusional body image distortion, may also be effectively treated with this medication. Olanzapine has been linked to a reduction in OCD symptoms in AN (Bissada et al., 2008), as well as an increase in BMI and a trend toward a reduction in anxiety scores by our group (Attia et al., 2011). Following up on these promising results, a larger five-site trial is currently being carried out by our extended group to investigate the possible use of olanzapine in the treatment of AN, and in line with our genetic findings involving the melanocortin system genes, atypical antipsychotic medications may prove to be a viable pharmacological treatment in AN.

We also detected a link between *NTRK2* rs1078947 and a higher maximum lifetime BMI in BN probands. High body weight is an important phenotype in BN, especially considering the high prevalence of personal or familial history of overweight or obesity in this population (Villarejo et al., 2012). This genetic finding involving *NTRK2* is in accordance with the role neurotrophins play in weight regulation and their interaction with leptin and melanocortin systems (reviewed in Introduction, Section 1.8.4). Since BN probands with BMI > 35 were not included in our analysis to avoid extreme obesity-related phenotypes, future studies should look

into the effect of this *NTRK2* polymorphism in BN cases who have a personal history of class II obesity as well. Meanwhile, our findings suggest a possible role for NTRK2 receptor agonists for a subgroup of individuals with BN who are overweight or obese. BDNF is the natural NTRK2 agonist, and to our knowledge, there are no clinical studies investigating the administration of BDNF in BN or obesity. Interestingly, N-acetylserotonin (NAS), the chemical intermediate in the endogenous production of melatonin from serotonin, has been shown to mediate the anti-depressive effects of selective serotonin reuptake inhibitors (SSRIs) through NTRK2 agonism (Jang et al., 2010). Considering that fluoxetine (Prozac) is one of the psychotropic medications approved for the treatment of BN and lead to reduction in core BN symptoms such as binge eating and purging (American Psychiatric Association, 2006), our findings are very much in line with the clinical evidence on the use of SSRIs in the treatment of BN, and if replicated, these results may provide with an alternate, non-serotonergic mechanism of action of SSRIs in BN through neurotrophin agonism. Furthermore, our results—if replicated in a larger sample—also provide rationale for the search of novel BDNF and NTRK2 agonist in the treatment of BN, especially for a subset of patients who are prone to becoming overweight or obese.

CHAPTER 6

FUTURE DIRECTIONS AND CONCLUDING REMARKS

6.1. Future Directions

Our sample size can be considered large in the context of other ED candidate gene studies; however, it is possible that power issues may have either prevented us from detecting association or created false positives. The first and foremost future step would be the replication of the results reported in this dissertation in a separate replication sample to confirm the validity of our findings. It would also be valuable to increase the current sample size for both the PF and GCAN studies to reassess the *p*-values for the top-ranking SNPs and whether increase in statistical power would lead to more SNPs making it past the Bonferroni-corrected significance threshold.

Although we reported significant findings with the some of the genes and SNPs included in our PF analysis, the targeted focus on a small number of functional variants per gene may have resulted in omission of other markers and genes that may be associated with ED diagnosis or BMI in EDs. If the PF study was to be repeated on a larger budget, the ideal way to proceed would be getting a better coverage of the genes by choosing a larger number of SNPs with known or putative function across each gene. When these SNPs are in high LD with those not covered (thus serving as functional tag SNPs), this design would allow us to get a more complete picture of the candidate gene of interest without sacrificing function. In the case of GCAN, future analyses could also prioritize markers based on functional annotation, a process described elsewhere (Hindorff et al., 2009; Lee and Shatkay, 2009). Furthermore, GCAN dataset will be added to the PGC pipeline in the upcoming months, and once the case-control results are

accepted for publication, it is expected that the WTCCC3 will grant access to the imputed dataset. As opposed to being limited to 517,549 SNPs following QC, this method would allow us to impute the missing SNPs and have access to over 1,000,000 markers. Imputation would also solve the problem of gene coverage we faced in this study; for example, *DRD1* and *DRD5* genes were excluded from analysis due to not being on the GWAS chip. Repeating the genetic analysis on the imputed dataset would provide us with an opportunity explore the possible role of all loci in the dopamine system genes and *FTO* on body weight in AN.

Another improvement to be implemented in both PF and GCAN studies is more rigorous control of population stratification. Although all PF probands were of European descent, our arrangement with PF did not allow for the testing of AIMs to determine population substructures within our samples. As a result of this, our findings may be influenced by population stratification, especially considering that the DNA samples were collected as a part of a large international consortium. It is also important to note that the 321 control samples have passed QC for populations stratification after being genotyped for 64 AIMs on the ABI OpenArray as part of another study by our extended group. An important next step would be to genotype a minimum of 64 AIMs in AN and BN groups to identify any individuals who may be different in terms of ancestry and properly control for ancestral heterogeneity. In the GCAN secondary analysis, we controlled for principal components C1 through C10, as determined by MDS, and we also merged the pruned GCAN data with HapMap3 main populations and excluded individuals that deviated from the CEU cluster. However, even with these precautions, we cannot rule out the potential confounding effects of population stratification. Future directions would consist of analyzing the BMI data separately for the samples provided by each country after

MDS and then performing a meta-analysis. This method would treat each subpopulation as a separate cohort and minimize the effects of population stratification.

Thus far, our efforts have focused on the study of common variants. We implemented a MAF of 5% for the GCAN analysis and 3% for the PF analysis. The rationale behind the lower MAF for the PF study was to include *AGRP* rs5030980, a marker with known function and a MAF around 0.03, in the genetic analysis; otherwise, the remaining SNPs had a MAF > 0.05. Thus far, ED genetic researchers have concentrated their efforts on common variants, since these are easier to detect and do not require very large sample sizes to study. With the recent advancements in high-throughput sequencing over the last few years, it is now much easier to detect rare variants (MAF < 0.01) and significantly cheaper to sequence a larger number of cases than ever before. While common variants are easier to detect and may confer risk for a larger population of individuals, they also have very small effect sizes by themselves. Rare variants, on the other hand, often have higher penetrance but are only present in a smaller number of cases. Currently, the most commonly used high-throughput sequencing methods are whole gene sequencing and whole exome sequencing. Whole gene sequencing allows researchers to focus their efforts on capturing a select number of candidate genes, whereas whole exome sequencing consists of sequencing all exons in the human genome. In psychiatric genetics, the latter method has been more commonly utilized thus far. Indeed, whole exome sequencing has vastly improved our understanding of the role of rare and *de novo* variants in psychiatric illnesses such as autism, bipolar disorder and schizophrenia (Neale et al., 2012; Timms et al., 2013; Liu et al., 2013; Shi et al., 2013). Rare variants hypothesis of psychiatric disorders (e.g., sequencing) needs to be studied alongside the common variants hypothesis (e.g, GWAS and candidate gene association), which may give researchers a better chance to explain the missing heritability (Manolio et al.,

2009; Schork et al., 2009; Gershon et al., 2011; Visscher et al., 2012). A whole exome sequencing project has already been proposed and funded as a part of the GCAN initiative, and our PF collaborators have conducted a whole-gene high-throughput sequencing experiment of candidate genes, results of which are in preparation for publication. In the future, whole exome sequencing of FP samples could yield important new information on the effects of rare variants in AN. Moreover, future study design should also include whole exome sequencing in BN.

Although we limited our efforts to a small number of candidate genes carefully selected for their role in appetite and weight regulation, there are other candidate genes that merit consideration in relation to ED diagnosis and BMI. Although numerous associations have been reported with serotonin system genes in EDs (Hinney et al., 1997b; Collier et al., 1997; Enoch et al., 1998; Gorwood et al., 2002; Bergen et al., 2003a; Kipman et al., 2002; Levitan et al., 2006a), there is still need for replication of these findings. Studies on the serotonergic genes have been conducted and are still being carried out by our collaborators using the PF dataset. As for the GCAN, since it is not reasonable for individual investigators to claim a large number of genes for secondary analysis purposes, we decided to focus our efforts on a well-formulized *a priori* hypothesis involving dopamine pathway genes. An opportunity to explore the serotonergic genes and their role in BMI in AN would be a welcome next step. Some of the other promising genes that can benefit from further research or require replication in EDs include *OPRD1* and *OPRM1* (Colantuoni et al., 2001; Bergen et al., 2003a; Zheng et al., 2010; Reece, 2011; Chamberlain et al., 2012; Giuliano et al., 2012; Haghighi et al., 2013; Blasio et al., 2013), *CART* (Kristensen et al., 1998; Rigoli et al., 2010), *NPY* (Xu et al., 1998; Beck, 2006; Zheng et al., 2010; Minor et al., 2011; Hunt et al., 2011; Mercer et al., 2011), *CCK* (de Krom et al., 2006; Hannon-Engel, 2012), endocannabinoid genes (Siegfried et al., 2004; Muller et al., 2008; Monteleone et al., 2009;

Lewis and Brett, 2010; Ishiguro et al., 2011; Schroeder et al., 2012; Scherma et al., 2013), genes that encode liver enzymes such as *CYP2D6* (Penas-Lledo et al., 2012), and GABAergic genes (Bloss et al., 2011; Vong et al., 2011; Aoki et al., 2012).

It is also possible that the genetic answers pertaining to the etiology of EDs and low body weight are to be found outside of the DNA sequence. In recent years, the study of epigenetics has risen in popularity. In contrast to the classical genetic studies, epigenetic research focuses on the functionally relevant modifications (such as DNA methylation and histone modification) in the genome that do not involve the nucleotide sequence, which may affect gene expression. Epigenetic studies also allow researchers to explore the effects of environmental factors on gene expression and etiology of disease. For example, the latest research findings on body weight point toward an active biological control mechanism (i.e., set-point) for body weight regulation—which may be overwritten by environmental factors such as diet—and stability of minimal body weight even after long-term weight cycling (Muller et al., 2010). It has been proposed that in the case of obesity, environmental factors such as abundance of high-fat and high-carbohydrate foods may increase the expression of adiposity-related in genes in vulnerable individuals (Rokholm et al., 2011); in a similar fashion, it is also possible that an environment that promotes thinness can lead to the overexpression of genes that suppress appetite and/or weight in individuals who are already genetically vulnerable to weight suppression. In accordance with these points, although there may not necessarily be any differences in nucleotide sequence between AN cases and controls, it is possible that gene expression or methylation patterns may significantly vary in EDs. A number of epigenetic studies have been conducted in psychiatric disorders (Iwamoto et al., 2011; Aberg et al., 2012), weight regulation (Stevens et al., 2011; Cordero et al., 2011), and EDs in recent years (Frieling et al., 2007; Frieling et al., 2010;

Ehrlich et al., 2010; Ehrlich et al., 2012; Pjetri et al., 2013; Steiger et al., 2013), yielding promising findings. In summary, study of methylation patterns in the candidate genes (especially their promoter regions) included in the this dissertation could provide genetic answers that go beyond DNA sequence, and considering that *FTO* is involved in nucleic acid demethylation (Gerken et al., 2007), exploring the methylation of intron 1 could especially yield valuable information regarding its function and involvement in weight regulation.

Finally, we believe there could be merit in stepping beyond the DSM diagnostic criteria of full-syndromal EDs to explore the important phenotypes in a cross-disorder manner. As highlighted in the Discussion chapter (section 5.5.2), many biological researchers argue that the DSM categorical diagnoses are too clinically oriented and do not provide biological and genetic studies with accurate dimensions to investigate. With additional efforts going into the development of an alternative classification system that is geared toward biological and genetic research, it is important to revisit the diagnostic boundaries of EDs and other psychiatric disorders, as well as considering the potential overlaps. Indeed, the most recent publication by the PGC highlights the importance of shared genetic risk loci in the etiology five separate psychiatric disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium et al., 2013). With the addition of the GCAN dataset to the PGC pipeline, future directions include the study of important ED-related phenotypes (e.g., lifetime BMIs, anxiety, anhedonia, obsessionality, impulsivity, etc.) in a cross-disorder manner in order to maximize sample size and explore the possibility of common genetic etiology for these important phenotypes across psychiatric diagnoses.

6.2. Concluding Remarks

Through the studies conducted as a part of this doctoral dissertation, we conclude that:

1) Due to the high crossover rates between subtypes and diagnostic categories in ED, inclusion of AN cases with a history of BN and BN cases with a history of BN is a serious study design issue in genetic studies designed to compare AN and BN cases. Furthermore, BMI histories often differ between AN and BN probands, so studies interested in investigating the genetics of body weight need to be especially cautious about the confounding effects of diagnostic crossover. Through a process of careful proband selection and exclusion of BN history in AN group and AN history in BN group, we were able to successfully maximize between-group differences and minimize within-group phenotypic differences.

2) We observed a trend toward the *MC4R* rs489693 A allele, which has been previously associated with AIWG, to be underrepresented in AN cases compared to the nonpsychiatric controls. This association became nominally significant post-Bonferroni correction under the dominant model for the A allele. Based on these findings, it can be speculated that the A variant of *MC4R* rs489693 may have a protective effect against sustained severe weight suppression, as in the case of AN. Future studies should focus on determining the functionality of the polymorphism to better explain the role it may play on weight regulation in psychiatric disorders.

3) *AGRP* rs13338499 was significantly linked to minBMI in individuals with AN. Although we did not come across any studies involving this particular variant, these findings are promising because of the predicted function of rs1338499 as a transcription factor-binding site and its potential regulatory role. Further studies are needed to replicate this novel finding and study the role of the *AGRP* gene on BMI in AN.

4) *NTRK2* rs1078947 T allele was significantly associated with a higher maxBMI in BN probands. Although it did not reach statistical significance, a similar effect was observed for minBMI in BN as well. Despite its function not being known, this marker was included in our analysis in order to replicate the findings of a previous ED study, with which our results disagree, thus there is need for another replication study with a larger sample size to explore the possible role of this *NTRK2* marker on weight regulation in BN.

5) As a part of the GCAN secondary analysis, we discovered a novel association between *FTO* rs10754470 in intron 1 and logcurBMI in 1,992 AN cases. This finding stayed statistically significant following Bonferroni correction and after controlling for principal components C1-C10 (for population stratification), AN subtype, history of BN, and logAAO. This is the first mention of this *FTO* SNP in the genetic literature, and rs1075440 is separated from most of the other *FTO* markers by a recombination hot spot, which explains the complete lack of LD with the known *FTO* markers. The function of this *FTO* variant is unknown, but if replicated, this novel finding could expand on the role of *FTO* markers in weight regulation, and considering that the *FTO* gene is over 400kb in size, it highlights the need to look beyond the known obesity loci of this gene.

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APPENDICES

A1. Changes to the EDs Diagnostic Criteria in the DSM-5[5]

A1.1 AN Criteria in the DSM-5

Below is a summary of the new AN diagnostic criteria in the DSM-5 (American Psychiatric Association, 2013):

A. Restriction of energy intake relative to requirements leading to a significantly low body weight in the context of age, sex, developmental trajectory, and physical health. Significantly low weight is defined as a weight that is less than minimally normal, or, for children and adolescents, less than that minimally expected.

B. Intense fear of gaining weight or becoming fat, or persistent behavior that interferes with weight gain, even though at a significantly low weight.

C. Disturbance in the way in which one's body weight or shape is experienced, undue influence of body weight or shape on self-evaluation, or persistent lack of recognition of the seriousness of the current low body weight.

Subtypes:

1. Restricting Type: during the last three months, the person has not engaged in recurrent episodes of binge eating or purging behavior (i.e., self-induced vomiting or the misuse of laxatives, diuretics, or enemas)

[5] This section is largely excerpted with a few updates from the text I wrote originally for a book chapter as a co-author: Kaplan AS, Yilmaz Z. Eating disorders in primary care mental health clinics. In: Ivbijaro G, editor. World Organization of Family Doctors (Wonca) in partnership with the World Health Organization: companion to primary care mental health. Milton Keynes (UK): London; 2012. p. 421-44. Appropriate permissions are included in the Copyright Acknowledgements section of the dissertation.

2. Binge-Eating/Purging Type: during the last three months, the person has engaged in recurrent episodes of binge eating or purging behavior (i.e., self-induced vomiting or the misuse of laxatives, diuretics, or enemas)

For the Criterion A, the word *refusal* has been removed as it may be confusing and difficult to assess and replaced with a more detailed description of the syndrome (Bravender et al., 2007; Becker et al., 2009). Criterion B has been changed to reflect the fact that some patients with anorexia nervosa deny fear of weight gain; the new criterion includes the behavioral correlates as well (Becker et al., 2009; Bravender et al., 2007). The elimination of the amenorrhea criterion has been a result of several reasons. Amenorrhea cannot be applied to men, pre-menarchal patients, as well as female patients taking oral contraceptives. There are many descriptions of patients with AN still menstruating at a very low weight. In addition, research has shown that patients with and without amenorrhea do not differ in terms of various clinical measures, suggesting that amenorrhea may be more useful as a severity indicator as opposed to a diagnostic criterion (Attia and Roberto, 2009). Finally, subtype classification has been limited only to the last three months due to the high rate of crossover from restriction to binge/purge and vice versa (Peat et al., 2009).

A1.2 BN Criteria in the DSM-5

The new diagnostic criteria for BN in DSM-5 are summarized below:

A. Recurrent episodes of binge eating. An episode of binge eating is characterized by eating an amount of food that is definitely larger than most people would eat during a similar period of time and under similar circumstances in a discrete period of time (for example, within any 2-hour period). It also consists of a sense of lack of control over

eating during the episode (for example, a feeling that one cannot stop eating or control what or how much one is eating).

B. Recurrent inappropriate compensatory behavior in order to prevent weight gain, such as self-induced vomiting; misuse of laxatives, diuretics, or other medications, fasting; or excessive exercise.

C. The binge eating and inappropriate compensatory behaviors both occur, on average, at least once a week for 3 months.

D. Self-evaluation is unduly influenced by body shape and weight.

E. The disturbance does not occur exclusively during episodes of anorexia nervosa.

Recent research has shown that clinical characteristics of individuals reporting a lower frequency of binge eating and purging (i.e., once a week) are similar to those who binge/purge twice or more per week (Wilson and Sysko, 2009). As a result of this, binge eating/purging frequency threshold in Criterion C has been decreased from twice a week to once a week.

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A2. Prevalence Rates in Non-Western Cultures and Developing Countries[6]

EDs were originally thought to occur almost exclusively in the Western cultures, but now their prevalence in the non-Western societies is well documented. According to recent epidemiological reports, lifetime prevalence rates for EDs are 1.4% among teenagers in Brazil (Moya et al., 2006), 1% among female university students in Turkey (Uzun et al., 2006), and 1.25% among children and adolescents in India (Mammen et al., 2007). In Asia, Hong Kong, Japan and Taiwan's ED prevalence rates are shown to be comparable to those in Western societies (Stark-Wroblewski et al., 2005; Lee et al., 2010b), and a similar pattern is observed in the Middle Eastern nations such as Iran (Nobakht and Dezhkam, 2000).

There may also be cultural variations in the specific ED diagnosis prevalence rates. For example, although AN rates are comparable to those observed in the Western cultures, BN is shown to be relatively rare in Turkey (Uzun et al., 2006). In addition, rates for treatment seeking and mental health service utilization may be lower in developing countries and also among ethnic minorities living in developed countries. For example, African Americans and Hispanic Americans, although having ED rates comparable the national average, are reported to be much less likely to seek treatment (Marques et al., 2011). Furthermore, it is also possible that milder ED cases may be either not brought to clinical attention at all or treated by primary care physicians as opposed to specialists, which may affect the calculation of the prevalence statistics (Chandra et al., 2012). Fortunately, which individuals may go on to develop an ED among visible minorities can be predicted by the presence of certain risk factors. For example, childhood impulsivity may be a predictor of future bulimic symptoms in African-American girls

[6] This section is largely excerpted with a few updates from the text I wrote originally for a book chapter as a co-author: Kaplan AS, Yilmaz Z. Eating disorders in primary care mental health clinics. In: Ivbijaro G, editor. World Organization of Family Doctors (Wonca) in partnership with the World Health Organization: companion to primary care mental health. Milton Keynes (UK): London; 2012. p. 421-44. Appropriate permissions are included in the Copyright Acknowledgements section of the dissertation.

(Bodell et al., 2012). Longitudinal studies on temperament, as well as social, cultural and other psychological risk factors in specific populations may also help with identifying those at high risk for EDs.

It is important to note that the presentation of ED symptoms may be culture-bound, as there may be variations even in core symptoms depending on culture. Although fear of fatness, body image distortion and weight and shape obsessions have been believed to be the *sine qua non* of EDs for many years, these symptoms may not be present or may be verbalized differently in individuals from non-Western cultures (Pike and Borovoy, 2004). In fact, patients with EDs in non-Western societies often report more somatic complaints such as bloating, abdominal discomfort, and distaste for food, as opposed to the cognitive symptoms such as fear of weight gain and body dissatisfaction (Rieger et al., 2001; Cummins and Lehman, 2007). Furthermore, in Hong Kong, Mainland China, Singapore, Japan, Malaysia, and India, absence of fat phobia among AN cases has been well documented (Becker, 2007). Similarly, Filipino and Omani populations have significantly lower rates of fat phobia, and ED patients present with higher rates of somatic complaints compared to European and North American cases (Viernes et al., 2007). In Ghana, individuals with AN often deny the fear of becoming fat and view their symptoms positively based on religious attitudes in support of restraint in general (Bennett et al., 2004). Among Chinese adolescents, facial appearance and acne—instead of the fat phobia—have been identified as possible triggers of AN (Jackson and Chen, 2007). However, it is also important to note that studies have also reported considerable ED rates with weight concerns and fat phobia in Hong Kong and Mainland China, which could be attributed to the increased influence and internalization of Western beauty ideals (Jackson and Chen, 2007). In summary,

clinicians should consider the cultural background of the patient and assess ED symptoms in a manner that accounts for possible cultural differences in symptom presentation.

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A3. A Summary on the Treatment of AN and BN

A3.1. Most Recent Psychological Approaches in the Treatment of AN and BN

As previously mentioned, there are currently no evidence-based treatments available for adult AN. Treatment programs focus primarily on weight restoration, nutritional status, and addressing the psychological factors that help with maintaining AN. Family therapy is the only evidence-based approach for adolescents with AN (American Psychiatric Association, 2006), shown to be especially effective for those under the age of 19 and have an illness duration of less than three years (Ellison et al., 2012). Even in treatment programs that utilize other methods of treatment, adding family therapy to the program may improve treatment effectiveness in AN patients under the age of 21 (Godart et al., 2012). Unlike AN, there are a number of first-line psychological treatments available for BN (American Psychiatric Association, 2006).

Cognitive behavioural therapy (CBT) utilizes both behavioural and cognitive approaches, and it aims to solve problems concerning dysfunctional emotions, behaviours and cognitions through a goal-oriented, manualized way (Magill and Ray, 2009). Currently, CBT is not recommended as a first-line treatment for AN (American Psychiatric Association, 2006), but some studies suggest that a subgroup of AN patients with lower levels of anxiety at the beginning of treatment may be more likely to see gain weight as a result of CBT (Lockwood et al., 2012). One of the main reasons for CBT often not working for AN is the cognitive deficits experienced by the patients due to starvation and malnutrition. Furthermore, CBT consists of a fair amount of homework geared toward identifying dysfunctional thoughts and beliefs, and many AN patients lack motivation to carry out tasks that require them to be actively engaged in the treatment process on an ongoing basis (Bamford and Mountford, 2012). In the case of BN, CBT is recommended as one of the first-line treatments (Treasure et al., 2010) and has been

systematically shown to be effective in the reduction of binge eating/purging and dealing with self-esteem and body image disturbance (Ramoz et al., 2007). Similarly, online adaptations of CBT also lead to sustained improvements in BN (Ruwaard et al., 2012).

Another psychotherapy that has emerged in the last few years with promise to treat disorders with underlying impulsivity is the dialectic behaviour therapy (DBT). DBT focuses on emotional regulation strategies and is most commonly used in patients with borderline personality disorder and high risk of suicide. Since behaviours such as binge eating and purging are often used as a means of regulating negative affect, variations of DBT have been successfully used in patients with BN (Safer et al., 2001). Group-based adaptation of DBT may also help with binge eating and cognitive aspects of BN (Klein et al., 2012). Interpersonal psychotherapy has been shown to be as effective as CBT in BN in the long term, but there is currently no evidence supporting its use in the treatment of AN (American Psychiatric Association, 2006; Murphy et al., 2012). Finally, although there has been research interest in motivational interviewing in AN in the recent years, a recent review of the literature found motivational interviewing to be only moderately helpful in individuals engaged in binge eating and not effective for combatting restrictive behaviours associated with AN-R (Knowles et al., 2013).

A3.2 Most Recent Pharmacological Approaches in the Treatment of AN and BN

Because of the crucial role serotonin plays in food intake and addictions, many studies have looked at the effectiveness of antidepressants, especially the SSRIs, in the treatment of eating disorders. SSRIs are routinely prescribed to patients with AN, especially for their depressive symptoms and anxiety issues. However, a multi-site, randomized, placebo-controlled study of AN has failed to find a statistical difference between fluoxetine and placebo for relapse prevention (Walsh et al., 2006). On the other hand, fluoxetine is the only FDA-approved

medication for the treatment of BN (American Psychiatric Association, 2006). A meta-analysis reported that antidepressants in general reduce binge frequency by 50%, corresponding to a moderate clinical improvement (Bacaltchuk and Hay, 2003). Fluoxetine at higher doses (60 mg/day compared to 20-40 mg/day in the treatment of depression) has been shown to be effective in reducing binge eating and purging (Shapiro et al., 2007), as well as decreasing anxiety and depressive symptoms in BN (Ramoz et al., 2007). Although currently not FDA-approved for the treatment of ED, sertraline has also been found to be effective in reducing binge eating and purging symptoms in BN in a number of clinical trials (Leombruni et al., 2006; Milano et al., 2004; Sloan et al., 2004).

Olanzapine, an atypical antipsychotic medication used in the treatment of psychotic symptoms and bipolar disorder, may hold promise in the treatment of AN. AN patients in a day hospital treatment setting gained more weight and reported significant improvement in their OCD symptoms when prescribed olanzapine compared to the placebo group (Bissada et al., 2008). Another randomized, placebo-controlled pilot study of olanzapine has found that AN patients in the medication group had significantly higher BMI compared to those assigned to placebo, and there was a nonsignificant trend toward a reduction in anxiety scores in the olanzapine group compared to the placebo group (Attia et al., 2011). Low dose olanzapine treatment adjunct to the regular treatment protocol has also been shown to be beneficial in adolescents with AN (Leggero et al., 2010). Following up on these promising results, a larger five-site trial is currently being carried out by our extended group to further investigate the possibly use of olanzapine in the treatment of AN. A small pilot study of quetiapine treatment found no favourable effect for the medication on AN outcome compared to placebo (Powers et al., 2012).

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A4. Sociocultural and Environmental Factors of AN and BN[7]

EDs are most prevalent in Western culture where the female beauty ideal is currently tied to being thin (Eddy et al., 2007), and it is porposed that dieting and thinness-related cultural messages play a considerable role in the development of weight and shape preoccupation and dissatisfaction in females. These cultural preferences are communicated through a complex and multi-dimensional set of channels, such as the media, fashion, popular culture, economic structures and values, and expectations (Jung and Forbes, 2007).

It is important to note that not every woman in Western nations suffers from an ED. A two-year follow-up study of close to 3,000 women revealed that only a small proportion of dieters went onto develop an ED (Fairburn et al., 2005), and women who often engage in social comparison may be at a higher risk for developing AN (Ahren et al., 2012). Genetic susceptibility is a key susceptibility factor for EDs. Similarly, just because a person has genetic vulnerability to disordered eating does not mean that he or she will develop ED if the environmental or psychological risk factors are not present. This interaction is the foundation of the biopsychosocial model of disease: *the genes load the gun, and the environment pulls the trigger*.

Despite the strong link between genes and EDs, environmental factors also play an important role in the development of EDs. It has been shown that children who grow up in a family that is overly preoccupied with weight and shape are at an increased risk for disordered eating (Francis and Birch, 2005; Neumark-Sztainer et al., 2010). In one study, 17% of mothers whose children had an ED reported a personal history of EDs (Watkins et al., 2012). In addition,

[7] This section is largely excerpted with a few updates from the text I wrote originally for a book chapter as a co-author: Kaplan AS, Yilmaz Z. Eating disorders in primary care mental health clinics. In: Ivbijaro G, editor. World Organization of Family Doctors (Wonca) in partnership with the World Health Organization: companion to primary care mental health. Milton Keynes (UK): London; 2012. p. 421-44. Appropriate permissions are included in the Copyright Acknowledgements section of the dissertation.

professions that focus unduly on weight and shape can also act as triggers for body image problems and EDs. Research has shown that as much as 83% of ballet dancers may meet lifetime diagnostic criteria for an ED (Ringham et al., 2006), and female dancers with perfectionist tendencies and who work in an environment that emphasizes the benefits of thinness or how to stay thin are at a higher risk for developing an ED (Penniment and Egan, 2012). Also not surprisingly, fashion models are much more likely to report full or partial ED symptoms compared to the general population (Preti et al., 2008).

More recently, prenatal hormone exposure as a possible susceptibility factor for EDs has garnered attention among ED researchers. Indeed, females with a male twin are reported to exhibit increased levels of disordered eating, a finding independent of socialization (Culbert et al., 2008). This observation suggests a possible link between masculinization in utero through androgen exposure and increased risk for EDs. Similarly, males with a female twin are as likely to develop AN as females with a female twin (Procopio and Marriott, 2007), a possible role for intrauterine female hormone exposure and increased ED risk in men. There is need for more research to develop a better understanding of hormonal exposure during fetal development in twins and how this may elevate ED risk.

Another possible trigger for EDs is early traumatic events; it has been reported that up to 35% of individuals with an ED report a history of trauma, especially in the form of sexual abuse (Federici and Kaplan, 2009; Mitchell et al., 2012). A US-wide survey has revealed that as much as 16% of women with AN and as much as 47% of women with BN report a lifetime history of full- or partial-syndrome post-traumatic stress disorder (Mitchell et al., 2012). Women with AN-BP are more likely to have elevated childhood trauma scores compared to those with AN-R, who often do not differ from the general population in terms of childhood trauma history (Jaite et al.,

2012). It can be speculated that traumatic events encountered in early life act as a vulnerability factor for EDs, resulting in a dissociative experience that blunts negative emotions. In fact, women who have trauma history are much more likely to rely on binge eating and purging to cope with negative affect (Stewart et al., 2006), and parental abuse has been reported as a predictor of binge eating in patients with EDs (Sachs-Ericsson et al., 2012).

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A5. *COMT* Val158Met Variant and Functional Haplotypes Associated with Childhood ADHD History in Women with Bulimia Nervosa[8]

A5.1 Abstract

Up to one third of patients with bulimia nervosa (BN) report a history of ADHD symptoms, and both disorders may also be associated with dopaminergic abnormalities. *COMT* gene, coding for an enzyme responsible for the degradation of dopamine, may play a part in the etiology of ADHD and BN. This study aimed to (1) examine if certain variants of the *COMT* genetic markers (rs6269, rs4633, rs4818 and rs4680) are more common in BN versus controls; (2) assess transmission of *COMT* alleles in BN families; and (3) explore the role of *COMT* genotypes and haplotypes in bulimic women with childhood ADHD history. 72 BN probands and unaffected relatives were genotyped for *COMT* rs4680 (Val158Met) and three adjacent markers. The remaining 165 probands were matched with nonpsychiatric controls. We also investigated if *COMT* variants and haplotypes were associated with childhood ADHD history in a subgroup of 86 BN probands who completed the Wender Utah Rating Scale (WURS). Our results showed that cases and controls did not differ in *COMT* allele and haplotype frequencies. In contrast, specific alleles of all four *COMT* markers and the medium- activity haplotype were preferentially transmitted to the offspring with BN. *COMT* Val158 allele was overrepresented and the medium-activity haplotype was underrepresented in BN with childhood ADHD history ($p = 0.010$). These findings suggest a possible role for *COMT* variants and related haplotypes in BN and its subphenotypes. If replicated, these preliminary findings may have implications for the prevention and treatment of BN that emerges in the context of childhood ADHD.

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A5.2 Introduction

Bulimia nervosa (BN) is characterized by episodes of binge eating followed by the use of one or more compensatory behaviors and has a lifetime prevalence rate of 2–3% (American Psychiatric Association, 2000). The heritability of BN has been estimated to be around 0.5–0.6 based on twin studies (Bulik et al., 1998; Bulik et al., 2010), and like other psychiatric disorders, BN is a complex disease with non-Mendelian multigenic etiology. ADHD, characterized by inattention, hyperactivity and impulsivity, is usually first diagnosed in children, although recent studies have found that ADHD has an adult prevalence rate of up to 4% (Cortese et al., 2007; Surman et al., 2006), classifying it as a life-span disorder (Davis et al., 2006; Robeva et al., 2004).

There are a number of prior findings suggesting a mechanistic link between ADHD and BN. First, up to a third of BN patients report classical symptoms of ADHD (Fleming and Levy, 2002). In addition, girls with ADHD may be up to six times more likely to develop BN than non-ADHD controls (Biederman et al., 2007; Surman et al., 2006). Second, ADHD, like BN, is defined by problems in self-regulation, which may affect areas such as memory, attention, arousal, organizational skills, and dietary regulation (Davis et al., 2006). One might expect impulsivity and poor organization skills to be associated with co-morbid ADHD and BN in many cases. Finally, dopaminergic genes have been implicated in the etiology of BN (Frieling et al., 2010; Kaplan et al., 2008; Kaplan et al., 2009; Levitan et al., 2010), ADHD (Gizer et al., 2009; LaHoste et al., 1996), as well as binge eating with comorbid ADHD symptoms by our group (Davis et al., 2007; Davis et al., 2008; Davis et al., 2009a; Davis et al., 2009b).

The catechol-O-methyltransferase (*COMT*) gene encodes the COMT enzyme responsible for degrading catecholamines, including dopamine and norepinephrine, particularly in frontal

areas of the brain (Allen Brain Atlas, 2010; Matsumoto et al., 2003). To date, one of the most studied variants of the *COMT* gene has been the G/A single nucleotide polymorphism resulting in valine–methionine substitution at codon 158 (Val158Met; rs4680). Functional studies have identified the Val158Met polymorphism as a marker of trimodal function (Chen et al., 2004), leading to high, intermediate, and low enzyme activities. More specifically, the valine variant has been associated with increased enzyme activity, which may result in faster breakdown of the catecholamines, potentially leading to lower levels of dopamine in the synaptic cleft. *COMT* polymorphisms are of interest for the researchers focusing on ADHD, as the current understanding of ADHD etiology emphasizes the possibility of reduced dopaminergic activity in the frontal regions of the brain. Thus far, research on the role of *COMT* rs4680 variant has led to variable findings in ADHD (Gizer et al., 2009). In the case of BN, the only published case–control study reported that the Val158 allele of *COMT* rs4680 was overrepresented among BN probands compared to healthy controls (Mikolajczyk et al., 2006). This study also investigated the role of *COMT* haplotypes consisting of rs4633 and rs4680, and the authors reported an association between the low activity haplotype consisting of these two markers and elevated scores on EDI subscales. However, these results need to be replicated in order to be conclusive, as there were only 42 BN probands included in that study. Finally, there are currently no published reports investigating the role of *COMT* genetic variants in BN with a history of ADHD or on the transmission of the *COMT* alleles to the affected offspring with BN in a family-based study design.

Although the Val158Met variant is associated with the thermostability of the COMT protein, its haplotypes may also be predictors of COMT function in a different way. It has been shown that variations in the *COMT* haplotype consisting of rs6269, rs4633, rs4818 and rs4680

are linked to mRNA folding as well as COMT enzyme activity (Nackley et al., 2006). More specifically, the A-C-C-G haplotype has been associated with high COMT protein level and enzyme activity, whereas the G-C-G-G haplotype has been associated with low COMT protein level and enzyme activity (Nackley et al., 2006). It has been reported that *COMT* haplotype variants may be associated with ADHD severity (Halleland et al., 2009). To our knowledge, however, the role of these *COMT* functional haplotypes has not yet been studied in eating disorders.

The main aim of this pilot study is to investigate the role of catecholamines through the *COMT* functional genetic variants in BN and childhood ADHD history. More specifically: (1) to look at the distribution of the *COMT* alleles in the family members of BN probands, as well as to assess a possible preferential transmission of the *COMT* variants and haplotypes to the affected offspring; (2) to compare the distribution of *COMT* genotypes and haplotypes in a separate group of BN probands compared to nonpsychiatric controls; and (3) to investigate the possible association of *COMT* genotype and haplotypes in a subgroup of BN probands with a history of childhood ADHD compared to those without a history of ADHD.

A5.3. Methods

A5.3.1. Participants. A total of 243 women with current or past BN (purging subtype) were recruited through advertisements posted at various clinical and community settings in Toronto, Canada. Eligibility criteria for BN probands included (a) between 18 and 65 years of age; (b) DSM-IV diagnosis for current or past BN, purging subtype; and (c) European Caucasian descent, with no more than one grandparent identified as non-Caucasian. Exclusion criteria included (a) a maximum lifetime BMI ≥35 kg/m2; (b) history of a psychotic episode; (c) history of bipolar disorder if binge eating and purging occurs only during manic/ hypomanic phases; (d)

diabetes preceding the onset of eating disorder; (e) thyroid or endocrine disorders; and (f) medical conditions that could affect appetite, weight, or eating behavior. Subgroups of this sample have been described in previous publications by our group elsewhere (Kaplan et al., 2008; Levitan et al., 2001; Levitan et al., 2006a; Levitan et al., 2010).

75 BN probands and their 148 unaffected first-degree relatives participated in the family component of the study (65 triads, 8 parent- sibling pairs and 2 sibling-pairs). The remaining 166 BN cases were paired with ethnicity-matched female controls, who had been screened for lack of major psychiatric disorder.

All participants received oral and written summaries of the purposes, procedures, and potential risks of the study and gave informed written consent. The study protocol was approved by the Research Ethics Boards within the University Health Network and Centre for Addiction and Mental Health in Toronto, Canada.

A5.3.2. Clinical assessment. All BN probands completed the Structural Clinical Interview for DSM-IV for Axis I Disorders (SCID-I; First et al., 1995) and the Eating Disorder Examination (EDE-12) (Fairburn and Cooper, 1993). Participating siblings were phone-screened for eating disorders, and those with a history of disordered eating were excluded from the study. We obtained current, minimum and maximum weight as well as height information via self-report, and BMIs were calculated for each BN patient based on the formula: *weight in kilograms / height in meters squared*.

History of childhood ADHD in BN probands was assessed using the Wender Utah Rating Scale (WURS) (Ward et al., 1993). A 25-item subscale of the WURS, which focuses on core symptoms of ADHD, correctly identifies 86% of probands with a history of ADHD and 99% of controls using a cut-off score of N45 (Ward et al., 1993), and has been validated for use in

research settings. The WURS was added to the current study protocol later in the study recruitment and was completed by 86 consecutive BN probands.

A5.3.3 Laboratory methods. Blood samples for genetic analysis from BN probands were collected on the day of the clinical interview. Blood lymphocyte DNA was extracted using the high-salt method (Lahiri and Nurnberger, 1991). The details on the *COMT* polymorphisms genotyped as part of this study and their locations are shown elsewhere (Nackley et al., 2006). In all, we genotyped four *COMT* polymorphisms: rs6269 (S-COMT promoter), rs4633 (His62His), rs4818 (Leu136Leu), and rs4680 (Val158Met). Polymerase chain reactions of 10 μl volume using 20 ng genomic DNA were performed using Assays-on-Demand by Applied Biosystems Inc. (ABI; Carlsbad, CA) under the following conditions: 95°C for 10 min, followed by 60 cycles of 92°C 15 s, 60°C 1 min. Determination of alleles was performed using the ABI 7500 Sequence Detection System with the Allelic Discrimination software. Genotyping of the DNA was performed at the Neurogenetics Laboratory at the Centre for Addiction and Mental Health in Toronto, Canada, with lab staff blind to psychiatric diagnosis. All ambiguous genotypes were retyped and if they remained ambiguous, they were excluded from the analysis.

A5.3.4. Statistical methods. Quanto version 1.2.3 was used to perform power calculations (assuming minor allele frequency of 0.2, log-additive genetic model) for the various components of our study. For the 75 families included in the study, we have over 80% power to detect an odds ratio (OR) of 2.1. For the case–control component, our sample has over 80% power to detect an OR of 1.68. Finally, for our within-BN analysis for the association of *COMT* variants and haplotypes with a history of childhood ADHD, we have over 80% power to detect an OR of 3.2.

In order to assess transmission of *COMT* alleles to the affected offspring, FBAT version

2.0.2 was used to perform a bi-allelic transmission disequilibrium test. The case–control allele frequency comparisons were analyzed by using a chi-square test through Haploview version 4.0. A chi-square test using the SPSS version 15.0 was performed to compare *COMT* genotype counts for all four markers in BN probands with and without a history of childhood ADHD. Finally, UNPHASED version 3.0.13 was used to determine *COMT* haplotype frequencies in case–control, family–control as well as within-BN analyses. Nyholt correction (Nyholt, 2004) was used for multiple testing, determining the effective number of independent marker loci to be 2. All statistical analyses were two-tailed with a significance threshold α of 0.025, as set by the Nyholt correction.

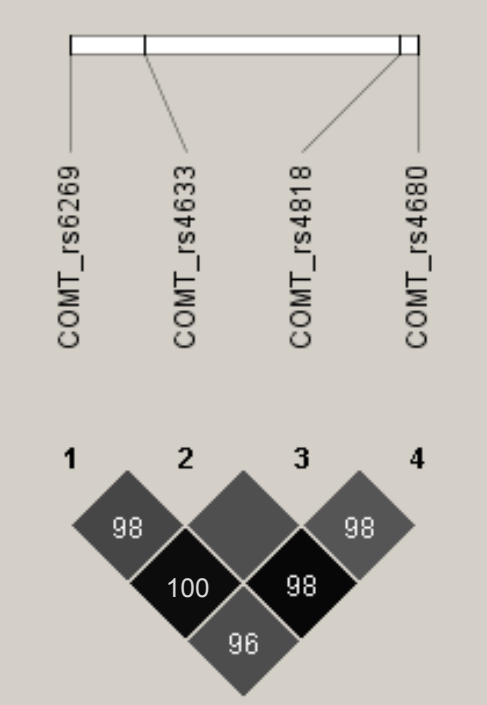
A5.3 Results

The percentage of successful genotyping was at least 98.2% for the case–controls, at least 98.7% for familial transmission analysis (N = 72 following 2 Mendelian errors), and 100% for within-BN childhood ADHD analysis (N = 86). All four *COMT* markers were in Hardy–Weinberg Equilibrium (HWE) for the family study; however, *COMT* rs6269 deviated from HWE in the case–control study (p = 0.048). The four markers were tightly linked, and because their haplotypes have been shown to be functional, we conducted four-marker haplotype analyses (Figure 12).

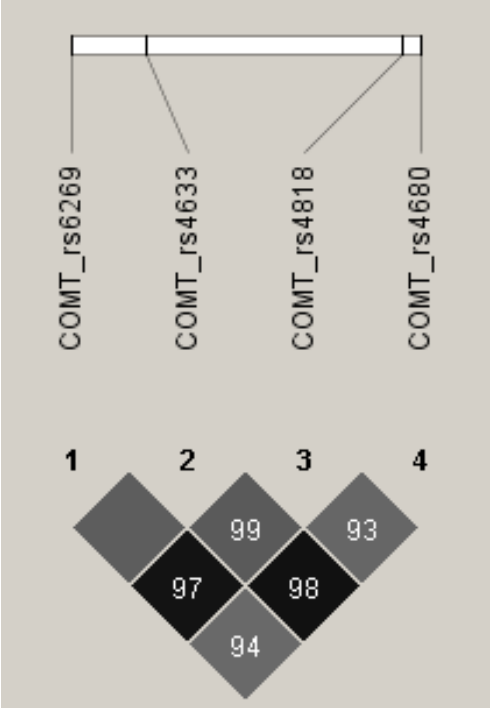
Among the BN probands (*N*=240), the mean age at the time of the study was 26.0 ± 7.0, and the mean BMI at the time of study was 22.2 ± 3.4. The self-reported highest and lowest BMIs were 25.1 ± 3.4 and 18.3 ± 2.7, respectively. Among the 86 BN probands who completed the WURS, 20 (23.3%) scored above the clinical cutoff score of 45 for childhood ADHD, a prevalence rate much higher than previously reported for adults in the general population (Cortese et al., 2007; Surman et al., 2006). The mean age for the 86 BN probands who completed

Figure 12. Linkage disequilibrium plots for the four *COMT* markers in family and case-control samples

Family



Case-control



the WURS was 24.7 ± 6.7, the mean BMI at the time of the study was 22.6 ± 3.2, and the highest and lowest BMI means were 25.0 ± 3.4 and 18.8 ± 2.7, respectively. Probands who completed the WURS did not differ significantly from probands who did not complete WURS in either age or these three BMI measures.

For family analysis, we observed preferential transmission for all four markers (Table 23). More specifically, the A allele of rs6269, the T allele of rs4633, the C allele of rs4818, and the Met allele of rs4680 were significantly overtransmitted to the affected offspring with BN (p = 0.009, 0.006, 0.005, and 0.012, respectively). As for the haplotypes, FBAT revealed that the medium-activity haplotype was significantly overtransmitted to the BN probands (p = 0.011; Table 24). The high-activity haplotype was undertransmitted in BN families, but it did not pass the Nyholt-adjusted threshold (p = 0.026). In post-hoc analyses, paternal versus maternal transmission was examined, and there was no difference, thus providing no evidence for imprinting. There were no differences between bulimic women and nonpsychiatric controls in terms of genotype, allele, and haplotype frequencies for any of the four *COMT* markers (Tables 25 and 26).

Regarding the effect of *COMT* markers on history of childhood ADHD, the Val158 allele was overrepresented among the childhood ADHD group (p = 0.010, OR = 2.56 [95% confidence interval: 1.24– 5.32]; Table 25). In addition, we observed that the C allele of rs4633 was more common in the childhood ADHD group, but it did not pass the Nyhold-adjusted threshold (p = 0.027). The Val158 homozygosity was overrepresented among those with a history of childhood ADHD but the p-value for this observation also failed to pass the adjusted threshold (p = 0.033).

Table 23. Transmission of *COMT* alleles to the affected offspring with BN in 72 families

COMT markers	Allele	Allele frequency	# of informative families	z	*p*
rs6269	A	0.62	54	2.61	*0.009*[1]
rs4633	T	0.55	49	2.75	*0.006*[1]
rs4818	C	0.63	50	2.84	*0.005*[1]
rs4680 (Val158Met)	A (met)	0.56	49	2.5	*0.012*[1]

[1] *p*-value passed the Nyholt-adjusted threshold of 0.025

Table 24. Transmission of *COMT* functional haplotypes to the affected offspring with BN in 72 families

rs6269-rs4633-rs4818-rs480	Haplotype defined by *COMT* activity (Nackley et al., 2006)	Allele frequency	# of informative families	*z*	*p*
2211 (GCGG)	High	0.39	46	-2.23	*0.026*
1122 (ATCA)	Medium	0.51	46	2.55	*0.011*[1]
1221 (ACCG)	Low	0.08	15	-1.80	0.071

[1] *p*-value passed the Nyholt-adjusted threshold of 0.025

Table 25. Genotype frequencies of *COMT* markers for (a) 165 BN cases and ethnicity-matched female nonpsychiatric controls; as well as (b) 20 BN probands with comorbid childhood ADHD and 66 BN probands without a history of childhood ADHD

		Case-control			Within-BN		
COMT markers	Genotypes	BN (yes/no)	*p (genotype)*	*p (allele)*	Childhood ADHD (yes/no)	*p (genotype)*	*p (allele)*
rs6269[1]	1/1 (A/A)	60/60			7/34		
	1/2 (A/G)	90/80	0.186	0.418	13/29	0.204	0.464
	2/2 (G/G)	13/23			0/3		
rs4633	1/1 (T/T)	39/42			4/26		
	1/2 (T/C)	92/86	0.802	1.000	9/29	0.097	*0.027*
	2/2 (C/C)	34/37			7/10		
rs4818[2]	1/1 (G/G)	20/24			3/7		
	1/2 (G/C)	83/79	0.794	0.748	9/25	0.648	0.347
	2/2/ (C/C)	61/61			8/34		
rs4680 (Val158 Met)[1]	1/1 (G/G; val/val)	35/32			8/10		
	1/2 (G/A; val/met)	91/86	0.590	0.388	9/32	*0.033*	*0.010*[3]
	2/2 (A/A; met/met)	37/45			3/24		

[1] $N = 163$ for case-control pairs

[2] $N = 164$ for case-control pairs

[3] *p*-value passed the Nyholt-adjusted threshold of 0.025

Table 26. *COMT* functional haplotype frequencies for (a) 163 BN cases and ethnicity-matched female nonpsychiatric controls; as well as (b) 20 BN probands with comorbid childhood ADHD and 66 BN probands without a history of childhood ADHD

		Case-Control		Within-BN	
rs6269-rs4633-rs4818-rs480	Haplotype defined by *COMT* activity (Nackley et al., 2006)	BN (yes/no)	*p*	Childhood ADHD (yes/no)	*p*
2211 (GCGG)	High	118/119	0.900	11/35	0.979
1122 (ATCA)	Medium	167/169	0.876	15/79	*0.010*[1]
1221 (ACCG)	Low	34/29	0.546	8/10	*0.032*

[1] *p*-value passed the Nyholt-adjusted threshold of 0.025

In terms of the haplotypes, the medium- activity haplotype was significantly underrepresented (p = 0.010, OR = 0.39 [95% confidence interval: 0.19–0.80]; Table 26). The low- activity haplotype appeared to be overrepresented among the BN probands with a history of childhood ADHD, but this finding was not statistically significant after the Nyholt adjustment (p = 0.032).

A5.5. Discussion

This study is the first to assess transmission pattern of *COMT* alleles and haplotypes in BN probands and their first-degree relatives, as well as to explore the possible role of *COMT* functional genotypes and haplotypes, as defined by Nackley et al. (2006) in BN.

In the family study component, we observed preferential transmission for certain variants of all four *COMT* markers. More specifically, we found that the met variant of rs4680, the allele associated with low COMT activity, was significantly overtransmitted to the offspring with BN. We also observed that the medium-activity haplotype was associated with increased risk for BN. In the past, the Met158 allele has been associated with mood as well as anxiety disorders (Hosak, 2007; Kang et al., 2010a), both of which are characterized by low levels of serotonin. In the frontal regions of the brain, serotonin is theorized to contribute to regulating dopamine: when serotonin levels decrease, dopamine levels rise, and vice versa (Kapur and Remington, 1996; Sasaki-Adams and Kelley, 2001). If COMT enzyme activity is low due to the presence of Met158 variant, it may lead to an increase in dopaminergic activity, hence leading to dopamine-serotonin imbalance. BN has been often associated with low levels of serotonin, which may persist even after recovery (Kaye et al., 1998), and it has been suggested that the binge eating behaviour may be an attempt to increase tryptophan production to normalize serotonin levels in frontal brain areas (Kaye et al., 2001). Many BN probands suffer from mood and anxiety disorders, suggesting a possible imbalance of the dopamine-serotonin ratio. Thus, the association

we detected in our family sample could have been due to an underlying association between *COMT* and mood or anxiety disorder.

We did not observe a significant difference between the BN cases and the nonpsychiatric controls in terms of *COMT* allele, genotype, and haplotype frequencies in our sample, hence failing to replicate previous findings that identified the Val158 allele as the risk variant for BN (Mikolajczyk et al., 2006). It is possible that our samples had significant heterogeneity due to population stratification. Although we matched the cases and controls for ethnicity in terms of approximate geographical origin of grandparents, we cannot rule out the possibility that allele frequencies may have differed between groups due to the effects of population stratification. Our significant results in the family sample may be due to the fact that family-based analyses are virtually free of population stratification effects.

In the case of BN with a history of childhood ADHD, we found that the Val158 allele of rs4680 of the *COMT* gene was overrepresented in BN probands with a history of childhood ADHD. This suggests that while the Met158 allele may be associated with BN in general, the presence of the Val158 allele, associated with high COMT enzyme activity, may serve as a risk factor for a subgroup of BN probands with ADHD symptoms. As high COMT activity is associated with faster breakdown and thus lower levels of dopamine (Chen et al., 2004), this pattern would be consistent with the classic models of ADHD that are based on low activity of frontal dopamine neurons (Biederman et al., 2007).

In terms of haplotypes, we observed that the medium-activity haplotype was underrepresented in the childhood ADHD group. This finding points to a second possible mechanism contributing to co- morbid ADHD in BN. More specifically, deviation from an optimal level of COMT enzyme activity may result in behavioral problems such as increased

inattention and impulsivity in a subgroup of BN probands. Alternately, the protective finding of the medium-activity haplotype could also be due to heterosis: heterozygosity may be associated with a higher relative fitness than homozygosity. Furthermore, these findings could be due to the different functionality of Val158Met and *COMT* haplotypes: while the Val158Met influences the thermostability of the protein, the four-marker haplotype affects mRNA stability. Further functional studies are required to clarify the pleiotropic effects of Val158Met on COMT function.

A number of limitations of the current study merit consideration. First of all, the small sample size in the family and ADHD components of the study may have affected our findings, and some of our nominally significant findings did not pass the Nyholt correction. ADHD data were limited to a relatively small subgroup of BN probands participating in the family study, preventing us from investigating the transmission of *COMT* alleles and haplotypes to the affected offspring with a history of ADHD versus without a history of ADHD. In addition, SCID-II was not administered during the clinical interview to the BN probands, so we do not have information on adult ADHD diagnosis on BN probands with a history of childhood ADHD. Finally, our results from the ADHD portion of the study suggest more than one pathway linking COMT activity with ADHD-BN co-morbidity. As a result of this, the ADHD subphenotype may require further refinement in order to be more informative at a genetic level.

If replicated, our preliminary results may have a number of clinical implications. First, close to a quarter of our BN sample met the criteria for childhood ADHD as assessed by WURS. Considering that high impulsivity in BN probands is associated with poorer treatment outcome (Surman et al., 2006), our findings highlight the importance of assessing probands for ADHD in clinical settings. In addition, BN probands with a history of ADHD may be different from those

without a history of ADHD in terms of dopaminergic function. As a result of this, pharmacological treatments with dopamine agonists such as methylphenidate may be helpful for a subgroup of BN probands who report a history of ADHD.

A5.6. Conclusion

In conclusion, we observed a preferential transmission for the A allele of rs6269, the T allele of rs4633, the C allele of rs4818, and the Met allele of rs4680 to the affected offspring with BN. In addition, the medium-activity *COMT* haplotype was significantly overtransmitted to the BN probands. BN cases and healthy controls did not differ in terms of *COMT* genotype and haplotype frequencies. Finally, the Val158 variant of *COMT* rs4680 was significantly overrepresented whereas the medium-activity haplotype was significantly underrepresented among BN probands with a history of childhood ADHD. If replicated, these findings may have important implications for prevention and treatment of BN that emerges in the context of childhood ADHD.

A5.7. References

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A6. Possible Association of the *DRD4* Gene with a History Of Attention-Deficit/Hyperactivity Disorder in Women with Bulimia Nervosa[9]

A6.1 Abstract

Objective: Up to one-third of patients with bulimia nervosa (BN) report a his- tory of attention-deficit/hyperactivity disorder (ADHD) symptoms, and the dopaminergic genes, especially the dopamine receptor D4 (*DRD4*), have been associated independently with both binge eating and ADHD. **Method:** The purpose of this study was to (1) compare the frequency of *DRD4* exon III VNTR variants in 157 BN probands and equal number of healthy controls; (2) assess transmission of *DRD4* alleles in 68 families of patients with BN; and (3) explore the possible role of *DRD4* gene in childhood ADHD history in a sub- group of 81 women with BN. **Results:** Our results showed that the BN probands and controls did not differ in *DRD4* allele frequency. We also did not find evidence for preferential transmission of any *DRD4* variant to the affected offspring with BN. However, the presence of either the 2-repeat or 7-repeat allele was associated with a history of childhood ADHD in BN (p = .038, odds ratio = 3.2). **Discussion:** Our findings suggest that although *DRD4* may not be associated with the diagnosis of BN, its variants are associated with a history of childhood ADHD in BN probands. This may have relevance for the understanding, prevention, and treatment of BN that evolves in the context of childhood ADHD.

A6.2. Introduction

Bulimia nervosa (BN) is an eating disorder with a lifetime prevalence rate of 2-3% and is characterized by episodes of binge eating followed by the use of one or more compensatory

[9] Journal article reprinted with permission from John Wiley & Sons, Inc.: International Journal of Eating Disorders (Yilmaz Z, Kaplan AS, Levitan RD, Zai CC, Kennedy JL. 2012. Possible association of the DRD4 gene with a history of childhood attention-deficit/hyperactivity disorder in women with bulimia nervosa. Int J Eat Disord 45:622-625. doi: 10.1002/eat.20986), copyright 2012. http://onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291098-108X.

behaviors (American Psychiatric Association, 2000). Like many other psychiatric disorders, BN often co-occurs with other psychiatric diagnoses and has environmental, psychological and genetic factors contributing to its etiology.

ADHD, characterized by inattention, hyperactivity and impulsivity, is usually first diagnosed in children, although recent studies have found that ADHD has an adult prevalence rate of up to 4% (Surman et al., 2006; Cortese et al., 2007). However, the symptoms appear to change over time and in adulthood patients present with more inattention-related problems and less hyperactivity(Robeva et al., 2004). With the recent wave of studies done on ADHD in adults, researchers now consider ADHD as a life-span disorder (Robeva et al., 2004; Davis et al., 2006).

There are a number of reasons to suspect a possible etiologic link between ADHD and BN. First, a large percentage of patients diagnosed with BN are also diagnosed with comorbid ADHD (Surman et al., 2006). As much as a third of patients with BN report classical symptoms of ADHD (Fleming and Levy, 2002). In addition, females with ADHD appear to be at 6-fold higher risk for developing BN than non-ADHD controls (Surman et al., 2006; Biederman et al., 2007). Second, impulsivity and poor organization skills are often associated with BN, which are also hallmarks of ADHD. ADHD, just like BN, is defined by problems in self-regulation, which may affect areas such as memory, attention, arousal, organizational skills, and dietary regulation (Davis et al., 2006). It has been reported that patients with BN may be inattentive to their internal sense of hunger, satiety, and amount of food consumed on a daily basis, which is a phenomenon also observed in patients with ADHD (Fleming and Levy, 2002). Impulsivity and lack of inhibition may play a large role in triggering binges (Schweickert et al., 1997). It has also been suggested that domains with ambiguous or contradictory rules may prove to be more difficult to

master for individuals with ADHD (Fleming and Levy, 2002). Eating and food consumption is governed by implicit and often contradictory rules in our society: Fast food advertisements are followed by health shows emphasizing the dangers of obesity on television. These mixed messages might hinder one's ability to make good choices regarding what, when, and how much to eat, especially in the presence of attention- and impulse control-related problems. In addition, impulsivity may be an important factor in determining the severity of BN (Surman et al., 2006).

The dopamine receptor D4 (*DRD4*) gene and its exon III VNTR 7-repeat allele (7R) have been strongly implicated as a risk factor for ADHD (LaHoste et al., 1996; Faraone et al., 2001). Research has consistently shown a robust association between the 7R and ADHD (Gizer et al., 2009), and it is one of the most consistent and replicated genetic associations in behavioral genetics. Over recent years, our group has shown that the 7R allele is associated with a diagnosis of adult ADHD (Muglia et al., 2000), and with maximum lifetime body mass index (BMI) in both BN (Kaplan et al., 2008; Levitan et al., 2010) and seasonal affective disorder, a mood disorder characterized by the presence of overeating(Levitan et al., 2006a; Levitan et al., 2004). However, to our knowledge, no study has investigated whether the 7R is overrepresented in patients in BN compared to either family controls or unrelated nonpsychiatric controls. There is also no published research to date looking at the transmission of the *DRD4* 7R allele to the affected offspring in BN families. In addition, despite the consistent replication of an association between the 7R and ADHD, no study has investigated if a similar association is present in BN patients with comorbid ADHD.

Finally, there is evidence suggesting that 7R may not be the only *DRD4* variant with hypofunctionality. The 7R version of the receptor has been shown to be hypofunctional due to a reduced pharmacological affinity to dopamine: compared to the ancestral 4-repeat allele (4R),

the 7R forms a longer intracellular loop, which in turn results in decreased affinity for dopamine(Asghari et al., 1995). However, more recent research has shown that the 7R may not be the only *DRD4* variant resulting in lower dopaminergic signal transmission. The 2-repeat allele (2R) of the *DRD4* gene may also be hypofunctional for a different reason. Immunohistochemistry findings suggest that 2R may lead to folding inefficiencies of the D4 protein, possibly leading to the misfolded protein being transported back to cytosol for degrading(Van Craenenbroeck et al., 2005). Due to the 2R variant's lower potency for dopamine-mediated cyclic AMP coupling, it has been speculated that the 2R and its chaperone may move more slowly through the endoplasmic reticulum compared to 4R, thus causing less 2R to arrive at the cell surface(Asghari et al., 1995; Van Craenenbroeck et al., 2005). Functional studies have suggested that the 2R allele is intermediate between 4R and 7R in terms of cAMP reduction(Asghari et al., 1995; Reist et al., 2007). As a result of these findings, it is important to investigate whether the hypofunctional 2R variants are also associated with ADHD symptoms in patients with BN.

The goals of this pilot study were threefold: (1) to compare the distribution of *DRD4* hypofunctional alleles in BN patients compared to nonpsychiatric controls; (2) to look at the distribution of the 2R and 7R alleles in the family members of BN patients, as well as to assess a possible preferential transmission of the hypofunctional *DRD4* variants to the affected offspring; and (3) to investigate the possible association of the 2R and 7R alleles of the *DRD4* gene in BN patients with a history of childhood ADHD compared to those without a history of ADHD.

A6.3. Experimental Procedures

A6.3.1. Participants. A total of 243 women with current or past BN purging subtype were recruited through advertisements posted at various clinical and community settings in Toronto,

Canada. Eligibility criteria for the BN group included (a) between 18 and 65 years of age; (b) DSM-IV diagnosis for current or past BN, purging subtype; and (c) European Caucasian descent, with no more than one grandparent identified as non-Caucasian. Exclusion criteria included (a) a maximum lifetime BMI $\geq$ 35 kg/m^2; (b) history of a psychotic episode; (c) history of bipolar disorder if binge eating and purging occurs only during manic/hypomanic phases; (d) diabetes preceding the onset of eating disorder; (e) thyroid or endocrine disorders; and (f) medical conditions that could affect appetite, weight, or eating behavior. Subgroups of this sample have been described in previous publications by our group elsewhere (Levitan et al., 2006a; Levitan et al., 2010; Kaplan et al., 2008).

Out of the 243 patients who participated in the study, we were able to obtain the DNA samples of 148 unaffected first-degree relatives of 75 BN probands for the family component of the study (N=225; 65 triads, 8 parent-sibling triads, 2 sibling-pairs). The 166 BN cases without available first-degree relatives were paired with ethnicity-matched female controls, who had been screened for lack of major psychiatric disorder.

All participants received oral and written summary of the purposes, procedures, and potential risks of the study and gave informed written consent. The study protocol was approved by the Research Ethics Boards within the University Health Network and Centre for Addiction and Mental Health in Toronto, Canada.

A6.3.2. Clinical Assessment. Participants in the BN group met in person with the study coordinator and completed the Structural Clinical Interview for DSM-IV for Axis I Disorders (SCID-I) (First et al., 1995) and the Eating Disorder Examination (EDE-12) (Fairburn and Cooper, 1993). If participating, siblings of BN patients were screened on the telephone for the presence of an eating disorder, and those with a history of disordered eating were excluded from

the study. Current, minimum and maximum weight as well as height information was obtained through self-report, and BMIs were calculated for each BN patient based on the formula: *weight in kilograms / corresponding height in metres squared*.

Childhood ADHD symptoms in BN probands were assessed by using the Wender-Utah Rating Scale (WURS) (Ward et al., 1993). WURS is a 25-item self-report questionnaire that retrospectively assesses the presence of childhood ADHD symptoms in adults. A cut-off score of 46 and above has been reported to correctly identify 86% of patients with ADHD and 99% of controls,[22] demonstrating the validity of its use in research settings. WURS was added to the study protocol later in the study recruitment and was completed by 86 consecutive BN probands.

A6.3.3. Laboratory Methods. Blood lymphocyte DNA was extracted using the high-salt method(Lahiri and Nurnberger, 1991). *DRD4* exon III VNTR was amplified from 20ng of genomic DNA using the following primers: [forward (5'-AGG ACC CTC ATG GCC TTG-3') and reverse (5'-GCG ACT ACG TGG TCT ACT CG-3')] (Lichter et al., 1993). The reaction mix also consisted of 7-deaza-dGTP and DMSO, and the reaction was run in thermocyclers under the following PCR conditions: 95^{o}C 5min, 40 cycles of 95^{o}C 20sec, 64^{o}C 20sec, and 72^{o}C 1min, followed by 10min extension step at 72^{o}C. The PCR products were then visualized via gel electrophoresis using 3.5% agarose prepared with ethidium bromide. Genotyping of the DNA was performed at the Neurogenetics Laboratory at the Centre for Addiction and Mental Health in Toronto, Canada, blind to psychiatric diagnosis. All ambiguous genotypes were retyped and if they remained ambiguous, they were excluded from the analysis.

A6.3.4. Statistical Methods. Quanto version 1.2.3 was used to perform power calculations for the various components of our study. For the 65 triads included in this pilot study, we have over 80% power to detect an odds ratio (OR) of 2.2. For the case-control component, our sample

has over 80% power to detect an OR of 1.7. Finally, for our preliminary within-BN analysis for the role of *DRD4* variants in predicting history of childhood ADHD, we have over 80% power to detect an OR of 3.1.

FBAT version 2.0.2 was used to perform a bi-allelic modified transmission disequilibrium test (2R or 7R present/absent) to assess transmission of *DRD4* alleles to the affected offspring with BN, whereas Haploview version 4.0 was used to compare case-control allele frequencies. Finally, a chi-square test using the SPSS version 15.0 was performed to compare *DRD4* hypofunctional genotype counts (2R or 7R present/absent) in BN patients with and without a history of childhood ADHD. All statistical analyses were two-tailed with a significance threshold a of 0.05.

A6.4. Results

The percentage of successful genotyping was 94.5% for the case-controls (N=157), 95.8% for familial transmission analysis (N=68 following 4 Mendelian errors), and 94.2% for within-BN ADHD history analysis (N=81). *DRD4* was in Hardy-Weinberg Equilibrium for BN probands, family members and nonpsychiatric controls, as assessed by Haploview.

Among the BN probands (final N=234), the mean age at the time of the study was 26.0 $\pm$ 7.1, and the mean BMI at the time of study was 22.10 $\pm$ 3.22. Self-reported highest and lowest BMIs were 25.09 $\pm$ 3.30 and 18.27 $\pm$ 2.68 respectively. Mean age for the 81 BN probands who completed the WURS was 25.1 $\pm$ 7.0, the mean BMI at the time of the study was 22.3 $\pm$ 3.1, whereas the highest and lowest BMI means were 25.00 $\pm$ 3.45 and 18.78 $\pm$ 2.63, respectively. Probands who completed the WURS did not differ significantly from those who did not complete it in terms of age and the three BMI measures.

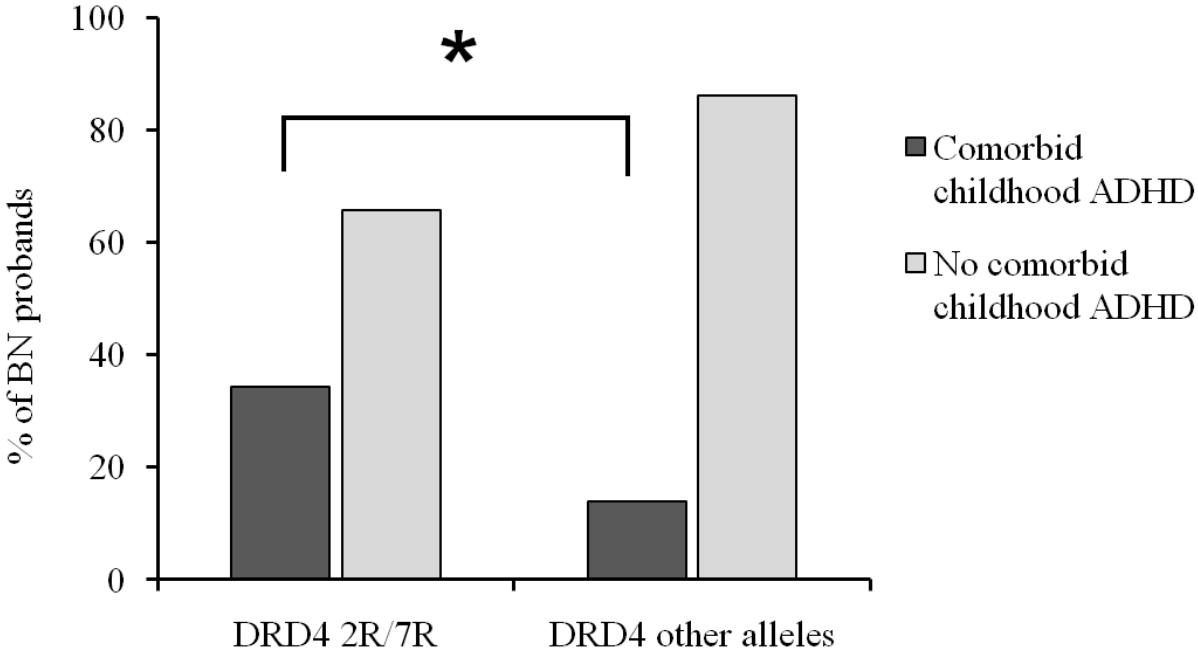
There were no differences between bulimic women and nonpsychiatric controls in terms of *DRD4* allele frequencies: 11.8% of BN probands and 11.8% of controls carried 2R ($p = 1.00$), while the allele frequency for 7R was 16.2% in BN versus 20.0% in controls ($p = 0.21$). Therefore, we failed to find any evidence that either 2R or 7R is associated with the overall diagnosis of BN in our sample.

FBAT revealed that neither 2R nor 7R was preferentially transmitted to the affected offspring from parents (for 2R: informative family count = 16, $Z = 0.00$, $p = 1.00$; for 7R: informative family count = 35, $Z = -0.78$, $p = 0.43$). These preliminary results suggest that the transmission of 2R or 7R to probands with BN does not deviate from the rates seen in the general population.

Among the BN probands who completed the WURS, 19 (23.5%) scored above the clinical cutoff score of 45 for childhood ADHD. In terms of other psychiatric co-diagnoses, 76.3% of the patients for whom we had childhood ADHD information had a lifetime mood disorder, 57.5% had a lifetime anxiety disorder, 33.7% had a lifetime substance use disorder, and 32.9% had a lifetime diagnosis of anorexia nervosa. There was no association of *DRD4* genotype with lifetime history of mood disorders, anxiety disorders, substance use/abuse, or anorexia nervosa.

Regarding the effect of *DRD4* genotype on a history of childhood ADHD, 34.2% of BN probands with WURS scores above established cut-offs carried at least one copy of either the 2R or 7R allele. In contrast, only 14% of BN probands who did not meet WURS cut-off criteria carried one or both of these alleles ($p = 0.038$, OR = 3.2; Figure 13). We were unable to look at 2R and 7R separately due to the small sample size.

Figure 13. *DRD4* exon III VNTR genotype count (2R/7R present or absent) among BN probands with and without history of childhood ADHD.



A6.5. Discussion

To our knowledge, this is the first report to (1) compare the frequency of *DRD4* exon III VNTR variants in BN patients and healthy controls; (2) to assess transmission pattern for *DRD4* alleles in families with an affected offspring; and (3) to explore the possible role of both the 2R and 7R alleles of the *DRD4* gene in the expression of childhood ADHD symptoms in BN probands.

We failed to observe preferential transmission for the hypofunctional 2R and 7R alleles of the *DRD4* gene or any difference between the BN patients and nonpsychiatric controls in terms of *DRD4* allele frequency. These results suggest that either our sample size lacked sufficient power to detect any differences between groups, or that the *DRD4* exon III VNTR is not a risk locus for BN in general. Similarly, past research has shown that *DRD4* gene is unlikely to be related to anorexia nervosa, or to low and high extremes of BMI in nonpsychiatric controls (Hinney et al., 1999b), suggesting that the *DRD4* gene is unlikely to be associated with eating disorder diagnoses as a whole or to extremes of BMI in the general population. However, patients with BN are quite heterogeneous in terms of clinical presentation and psychiatric comorbidities; as the field of psychiatric genetics is moving away from studying 'disease genes' to the study of the endophenotypes and their genetic determinants, it is important to focus on subphenotypes in eating disorders in order to make the study sample more homogeneous.

In support of this subphenotype approach to BN, we found that the 2R and 7R alleles of the DRD4 gene were overrepresented in BN patients with a history of childhood ADHD. This suggests that while the *DRD4* gene may not be associated with BN in general, the presence of *DRD4* hypofunctional variants may serve as a risk factor for a subgroup of BN patients with ADHD symptoms. Although history of childhood ADHD is not an endophenotype by definition,

it appears to serve as a distinct subphenotype within BN. Bulimic patients with ADHD symptoms may form a more homogeneous group that is more impulsive and inattentive compared to BN patients without ADHD, and *DRD4*'s association with these traits has been well documented (Gizer et al., 2009). Finally, our results also suggest that the 2R allele may be involved in childhood ADHD history in BN probands. This finding underlines the need for further functional studies of the 2R, as it was shown to result in reduced folding efficiency in a number of preliminary studies in the past.

In the recent years, there has been a significant amount of attention given to the evolutionary role of the *DRD4* variants, especially in the case of 7R. In terms of its origin, 7R appears to differ from the other *DRD4* variants by greater than six combinations, hence being a younger allele in terms of human evolution as a result of positive selection(Ding et al., 2002). Other studies have shown that the 7R variant may be as young as 50,000 years in human evolution, further highlighting the possibility of its positive selection(Wang et al., 2004). Indeed, many researchers have theorized that those carrying the 7R allele of the *DRD4* may have had an evolutionary advantage due to their willingness to try new food sources or relocate when food became scarce(Levitan et al., 2006b). Similarly, binge eating behavior, which has been associated with 7R as well, may be beneficial for survival when food scarcity is an ongoing threat(Levitan et al., 2006b). The 2R allele is associated with impulsivity, novelty-seeking as well as the presence of ADHD in populations where the 7R variant is rare (Reist et al., 2007), further suggesting that the 2R and 7R may be related in terms of biological function, their association with certain personality traits, as well as positive selection in human evolution.

This pilot study has a number of limitations that merit consideration. First, the small sample size in the family and ADHD components of the study may have played a role in the

nonsignificance of findings. We had childhood ADHD data on a relatively small subgroup of BN probands participating in the family study, which did not allow us to investigate the transmission of the *DRD4* 2R and 7R variants to the affected offspring with a history of ADHD versus without a history of ADHD. In addition, we do not have information on adult ADHD diagnosis on BN probands with a history of childhood ADHD, as SCID-II was not administered as a part of the clinical interviews. However, despite these limitations, this pilot project is the first to examine: (1) the transmission of *DRD4* exon III VNTR alleles to the affected proband with BN; (2) the frequency of *DRD4* variants in BN probands versus nonpsychiatric controls; and (3) the role of the *DRD4* exon III VNTR in BN with a history of childhood ADHD. We hope that the preliminary findings of this study will assist researchers with hypothesis generation for future studies with larger sample size in order to further our understanding of the role of *DRD4* exon III VNTR in BN, particularly in a subgroup of BN patients who report a history of childhood ADHD.

If replicated, our preliminary results may have clinical implications. BN patients with history of ADHD may be different from those without history of ADHD in terms of dopaminergic function, as the presence of comorbid ADHD may be a sign of underlying dopaminergic abnormalities. As a result of this, pharmacological treatments with dopamine agonists such as methylphenidate may be helpful for a subgroup of BN patients who report a history of ADHD. To our knowledge, D4-specific compounds have not been thoroughly tested in either BN or ADHD but may hold potential for treatment.

In conclusion, our findings suggest that while hypofunctional DRD4 alleles are not associated with the overall diagnosis of BN, they are associated with co-morbid childhood

ADHD in female BN probands. If replicated, these results may have relevance for the understanding, prevention and treatment of BN that evolves in the context of childhood ADHD.

A6.6. References

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