Thesis

Genetic Links of Major Depression and Anorexia Nervosa

submitted by

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Zusammenfassung

Einleitung: Laut vorbekannter Literatur wurden bestimmte Genvarianten, welche mit Anorexia Nervosa (AN) assoziiert sind, auch mit Depressionen in Verbindung gebracht. Im Rahmen dieser Diplomarbeit werden auf Basis der größten Vorstudien vielversprechende Genotypen der Gene 5-HT2A, PPP3CA, OPCML und TSHZ1 ausgewählt und hinsichtlich ihrer assoziierten Depressions-Scores im Zusammenhang mit Anorexia Nervosa untersucht.

Methodik: Von 19 Patient*Innen mit AN und 30 gesunde Kontrollpersonen (HC), wurde als Teil einer Pilotstudie der Hamilton Depression Rating Scale (HAMD) und den Beck Depression Inventory (BDI) Fragebogen herangezogen, um die Proband*Innen auf depressive Symptomatik zu untersuchen. Nach Entnahme einer Blutprobe wurde mittels QIAGEN's QIAsymphony SP Roboter die DNA-Isolierung durchgeführt. Im Life & Brain Center Bonn wurde anschließend mittels Illumina's Infinium Global Screening Array-24 v3.0 Kit eine Genotypisierung durchgeführt. Die hypothesengeleitete Isolierung von den Genotypen rs6311 (5-HT2A), rs10791286 (OPCML), rs17030795 (PPP3CA), und rs56156506 (TSHZ1), erfolgte mittels PLINK1.9.

Ergebnisse: Patient*Innen mit AN haben im Vergleich zur Gruppe gesunder Kontrollpersonen signifikant höhere Werte bei beiden Fragebögen erzielt ($p < .001$). Träger des Genotyps AA auf dem PPP3CA Gen (rs17030795) zeigen zudem signifikant höhere Werte ($p = .046$) bei der Beantwortung der Fragen des HAMD.

Diskussion: Derzeit besteht eine schwache Forschungslage für eine gemeinsame genetische Basis in der Entstehung von AN und Depressionen als Komorbidität. Die Ergebnisse dieser Arbeit liefern somit einen wichtigen Anhaltspunkt für weitere Forschungsfragestellungen mit größeren Proband*Innenzahlen. Je besser die Entstehung von Komorbiditäten bei AN Patient*innen erforscht ist, desto vielfältiger und intensiver kann Prävention und Therapie zur Genesung beitragen.

Abstract

Introduction: According to prior literature, certain genetic variants associated with Anorexia Nervosa (AN) have also been linked to depression. As part of this thesis, promising genotypes of the genes 5-HT2A, PPP3CA, OPCML, and TSHZ1 are selected based on the largest preliminary studies and examined with regard to their associated depression scores in connection with Anorexia Nervosa.

Methods: From 19 patients with anorexia nervosa (AN) and 30 healthy control subjects (HC), the Hamilton Depression Rating Scale (HAMD) and the Beck Depression Inventory (BDI) questionnaires were used as part of a pilot study to examine the participants for depressive symptoms.Following a blood sample collection, DNA isolation was performed using QIAGEN's QIAsymphony SP robot. Genotyping was then conducted at the Life & Brain Center in Bonn using Illumina's Infinium Global Screening Array-24 v3.0 Kit. Hypothesis-driven isolation of the genotypes rs6311 (5-HT2A), rs10791286 (OPCML), rs17030795 (PPP3CA), and rs56156506 (TSHZ1) was carried out using PLINK1.9.

Results: Patients with AN scored significantly higher on both questionnaires compared to the healthy control group ($p < .001$). Carriers of the AA genotype on the PPP3CA gene (rs17030795) also scored significantly higher ($p = .046$) on the HAMD questionnaire.

Conclusion: Current research provides limited evidence for a shared genetic basis in the development of AN and depression as a comorbidity. The results of this study provide an important basis for future research with larger sample sizes. The better the comorbidities of AN patients are understood, the more diverse and effective prevention and treatment strategies can be to support recovery.

Table of Contents

Abbreviations and definitions

5-HT	5-hydroxytryptamine, serotonin
AN	Anorexia Nervosa
ANGI	Anorexia Nervosa Genetics Initiative
ANOVA	Analysis of variance
APA	American Psychiatric Association
ASD	Autism Spectrum Disorder
BDI	Beck Depression Inventory
BED	Binge eating disorder
BMI	Body Mass Index (kg/m^2)
BN	Bulimia Nervosa
CADM1	Cell adhesion molecule 1
CDCV	Common disease common variant hypothesis
CDH10	Cadherin-10
CT	Computer tomography
DNA	Deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
EAT-26	Eating Attitude Test with 26 items
EDE-Q	Eating Disorder Examination Questionnaire
ED	Eating disorder

GWAS	Genome-wide association study
GxE	Gene-environment interaction
HAMD	Hamilton Depression Rating Scale
HC	Healthy controls
HGP	Human genome project
ICD	International Classification of Diseases
IGF1	Insulin-like growth factor 1
ILSE	A study at the psychiatric and psychotherapeutic department at the medical university of Graz: “Vergleich impliziter Lernstrategien bei Patienten mit Anorexia Nervosa und Major Depression”
IPAQ	International Physical Activity Questionnaire
LH	Luteinizing Hormone
MMST	Mini Mental Status Test
MRI	Magnetic resonance imaging
MWT-B	Multiple Choice Vocabulary Test
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PGC-ED	ED Working Group of the psychiatric genomic consortium
PPP3CA	Protein phosphatase 3 catalytic subunit alpha
PRS	Polygenic risk scores
SNP	Single-Nucleotide Polymorphism

SSNRI	Selective Serotonin and Noradrenaline Reuptake Inhibitor
SSRI	Selective Serotonin Reuptake Inhibitor
TCTA	Tricyclic Tertiary Amines
WHO	World Health Organization
WPT	Weather Prediction Task

List of figures

List of tables

1. Introduction

1.1. Anorexia Nervosa

AN is a common eating disorder characterized by a variety of symptoms. Individuals with AN actively strive to lower their body weight or maintain an unhealthily low weight. Due to their distorted body image, patients have a profound fear of gaining weight (Peterson and Fuller, 2019). It predominantly affects young women, with a lifetime prevalence of up to 2.2% (Jagielska and Kacperska, 2017), while men are less frequently affected, with a prevalence of only 0.3% (Watson *et al.*, 2019). The prognosis for AN is generally poor, particularly for hospitalized patients and those who develop the disorder after the age of 19 or before the age of 12. AN has the highest mortality rate among all psychiatric disorders, with an average standardized mortality ratio of 5.9 in the overall AN population (Jagielska and Kacperska, 2017). This may be attributed to the high likelihood of developing psychiatric comorbidities, such as major depression, obsessive-compulsive disorder, or substance abuse, which can occur during recovery or even years after treatment (Moskowitz and Weiselberg, 2017). About 50% of deaths are attributed to the process of slow starvation (Neale and Hudson, 2020).

1.1.1. Diagnostic Criteria

To diagnose mental disorders such as AN, clinicians use different classification systems, especially the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), which was released and updated in its fifth version in 2013 by the American Psychiatric Association (APA) (American Psychiatric Association, 2022). The content of the DSM-5 is based on the latest research studies and is influenced by the International Classification of Diseases (ICD) which is maintained by the World Health Organization (WHO). The updated ICD-11 has been effective since its official announcement in 2019 and differs slightly from the ICD10. The adaptations were made according to the most recent studies

(Harrison *et al.*, 2021). The following table shows some significant diagnostic criteria for AN based on both diagnostic systems.

Table 1: Diagnostic criteria for AN

DSM-5	ICD-11
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.	Significantly low body weight for the individual's height, age, and developmental stage (BMI less than 18.5 kg/m2 in adults and BMI-for-age under 5th percentile in children and adolescents). that is not due to another health condition or to the unavailability of food.
Intense fear of gaining weight or of becoming fat, or persistent behavior that interferes with weight gain, even though at a significantly low weight.	Low body weight is accompanied by a persistent pattern of behaviors to prevent restoration of normal weight, which may include behaviors aimed at reducing energy intake (restricted eating), purging behaviors (e.g. self-induced vomiting, misuse of laxatives), and behaviors aimed at increasing energy expenditure (e.g. excessive exercise), typically associated with a fear of weight gain.
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 bodyweight.	Low body weight or shape is central to the person's self- evaluation or is inaccurately perceived to be normal or excessive.
compared to the DSM- IV the criterion "Amenorrhea" was removed because it can't be applied to men, young women before they reach menarche, and women who take exogenic hormones.	…

own table based on (Zipfel *et al.*, 2015).

Leaving out the criterion of amenorrhea, which is the absence of monthly menstruation, was the main change in the update from ICD10 to ICD11. It can be explained by the argument that female patients before menarche and male patients for example, cannot meet this criterion and therefore should not be excluded.

To sum up: apart from the visual symptoms such as the gaunt, weak appearance, their daily life is determined by worries about food intake, fear of gaining weight and the urge to balance out any calories. Either by excessive exercise, longer periods of no food intake at all, or the abuse of laxatives for example. The patients don't realize that their weight is too low. They always feel "fat" and uncomfortable which leads to actions to constantly lower their weight.

Depending on the course and symptoms of AN and the trigger factors there are different subtypes of the disease, according to the underlying clinical classification system (DSM 5, ICD 10, ICD 11) that experts are using:

1. Anorexia Nervosa, restricting type (F50.00)
   Patients with AN share an unhealthy low body weight in common. However there are subtypes of AN that describe their actions to lose more weight and maintain it at a low level. Patients suffering from the restrictive type of AN limit all their food intake to a minimum and therefore look extremely gaunt (Miyata *et al.*, 2021).

2. Anorexia Nervosa, binge-/ purging type (F50.01)
   Patients with AN of the binge-/ purging type also severely limit their food intake. However, they also show episodes of binge eating, which are defined as the uncontrolled eating of large amounts of food at a time. These episodes are followed by self induced vomiting or the abuse of laxatives to compensate for what they've eaten (Miyata *et al.*, 2021).

3. Atypical Anorexia nervosa (F50.1)
   The symptoms of patients with atypical AN (ANN) are basically the same as those of patients with common AN. The difference lies in the severity of their manifestation and frequency. The patients often present with body weights within the normal range or even above, whereas other

psychopathologies may present more strongly (Walsh, Hagan and Lockwood, 2023). These may include fasting, vomiting, laxative abuse, and a strong fear of gaining weight, just as with typical AN. Since body shape and body weight do not send any alarming signals to friends, family, doctors or the affected person, the correct diagnosis for AAN is made with a delay (Vo and Golden, 2022).

The lack of nutrition, however, is just as harmful and can lead to abnormalities in electrolytes, heart failure, gastrointestinal and digestive issues, and low bone mineral density, to name a few (Vo and Golden, 2022).

4. Anorexia Athletica

   For some disciplines in sports, such as professional climbing, gymnastics, or ballet, it can be beneficial to keep body weight extremely low for those who want to compete at a professional level. To achieve that, these athletes choose methods like overexercising or restricting calories. Similar to regular AN the long term consequences can be fatal, leading to metabolic and endocrine disruptions (Sudi *et al.*, 2004).

1.1.2. Epidemiology

To improve the treatment and outcome of AN, it is crucial to understand who is affected and what contributes to the development of the disorder, considering multiple factors (Slof-Op ’t Landt *et al.*, 2005).

For both men and women, the highest incidence of AN occurs between the ages of 15 and 19. As shown in the table below, the incidence rates have increased since 2000. However, further research is needed to determine whether this increase can be attributed to improved detection or an actual rise in the number of affected individuals (Micali *et al.*, 2013).

Table 2: Crude and age-standardized incidence rates for eating disorders in 2000 and 2009 per 100 000 population.

	2000			2009		
	N	Crude incidence (95% CI)	Age-standardized incidence (95% CI)	N	Crude incidence (95% CI)	Age-standardized incidence (95% CI)
Overall	789	33.0	32.3	897	36.8	37.2
Females	732	53.2	51.8	816	62.7	62.6
Males	57	5.6	5.6	81	7.1	7.1

own table based on (Micali *et al.*, 2013).

More recent studies show that the incidence rates have been growing over the past decades for patients under 15 years of age, whereas the overall rates have remained nearly stable. A meta-analysis from 2021 shows that, depending on the country and the population, incidence rates vary from 120 to 580 per 100.000 people (van Eeden, van Hoeken and Hoek, 2021).

A Norwegian study reported overall rates from 33.2 to 33.5 between the years 2010 and 2016 using a broad definition of AN (Reas and Rø, 2018).

A study reporting on incidence rates in the UK and Ireland mentioned a rate of 25.7 per 100.000 for the female population and 2.3 per 100.000 for males (Petkova *et al.*, 2019).

All these rates have in common that they are significantly higher for women than for men. When provided by health care facilities, these rates tend to be lower than those provided by population-based studies. This might be due to the high estimated numbers of undiagnosed affected people (van Eeden, van Hoeken and Hoek, 2021).

Only about 46.9% of the patients achieve full recovery, indicating the absence of the defined clinical symptoms. For 20.08%, the symptoms persist and lead to chronic AN (Steinhausen, 2002). There is very little literature available on long term recovery rates after 20 or 30 years of treatment.

One follow-up study with 228 participants, published in 2017, reported that after 9

years, 31.4% of the AN patients were defined as recovered, and after 22 years, the percentage doubled (Eddy *et al.*, 2017).
Another follow-up study with 51 participants reported recovery rates of 64% after 30 years (Dobrescu *et al.*, 2020).
Poor recovery rates are confirmed by many other studies, with rates varying from 33% after two years, as summarized in a meta-analysis by Gowers et al. from 2007 (Gowers *et al.*, 2007).

1.1.3. Etiology

Family and twin studies suggest that eating disorders, including AN, frequently exhibit familial patterns. For instance, the likelihood of developing AN increases by up to 11.4 times when there is an affected family member. These findings suggest that genetic and environmental factors within family settings contribute to the development of AN (Slof-Op ’t Landt *et al.*, 2005).

In addition to the higher occurrence of eating disorders within families, genetic factors have also been investigated through genome-wide association studies (GWAS). These studies allow researchers to associate single nucleotide polymorphisms (SNPs) within the genome with specific disorders. This has led to the discovery of nine SNPs and their associated genes with genome-wide significance ($p < 5x10^{-8}$), which will be further explained and discussed in the following chapters (Duncan *et al.*, 2017; Watson *et al.*, 2019)

A study review published in 2022 highlights the interplay between environmental factors and genetics, emphasizing their mutual influence. It evaluates GWAS, family and twin studies, cross- disorder analysis and polygenic risk scores (PRS) to conclude that a genetic predisposition to AN, combined with a stressful life event such as childhood trauma, increases the risk of actually developing the disorder (Baker, Schaumberg and Munn-Chernoff, 2017).

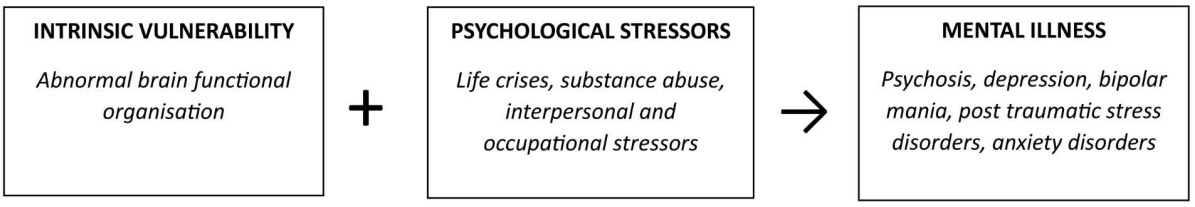
Given the fact that so many young people develop the disorder during adolescence, there are studies looking more closely at those environmental factors. An ideal body weight and shape can be internalized at a very young age.

Children whose parents are on a strict diet or apply restrictive rules on meal intake are at a higher risk of developing EDs (Gonçalves *et al.*, 2013). Parents, other family members, and friends serve as role models, and negative comments on weight and eating behavior of their children are disastrous (Krug *et al.*, 2009).
Social media acts as another major factor. Being thin is associated with being beautiful, healthy, and popular, which makes the body shapes that are shown in TV-shows and magazines desirable (Gonçalves *et al.*, 2013).

1.1.3.4. The vulnerability-stress model

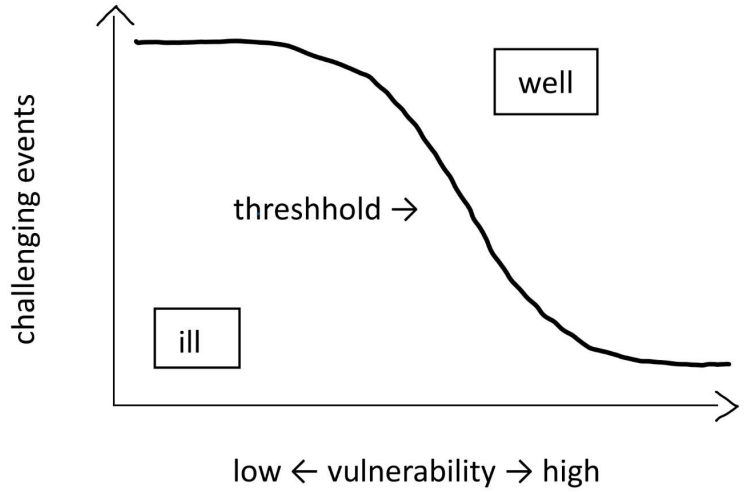
Another model that can be applied to observe the correlation between multiple factors is the vulnerability-stress model by Zubin and Spring (1977).
In theory, each individual possesses a vulnerability to develop mental disorders, which is influenced by both congenital and acquired factors. The former includes the individual's genes and neurophysiology, while the latter consists of life events during early childhood or adolescence that can either protect the individual from or contribute to the development of mental disorders. These acquired factors may encompass physical diseases, trauma, or unstable family relationships. The higher an individual's vulnerability, the greater the likelihood of developing a mental disorder if specific events disrupt this stability. These events can be categorized as endogenous or exogenous. Endogenous events can arise, for example, from hormonal changes, a lack of nutrition, or infections, while exogenous events may involve significant life changes such as the loss of a close family member or termination of employment (Zubin and Spring, 1977). The interaction of these factors is illustrated in Figures 1 and 2.

Figure 1: The vulnerability- stress model



own figure based on (Goh and Agius, 2010), (p. 198)

Figure 2: Relation between vulnerability and challenging events



own figure based on (Zubin and Spring, 1977, p.110)

If this model is applied to AN, it might explain the simultaneous occurrence of other psychiatric disorders, such as major depression, anxiety disorders, or obsessive compulsive disorders: If an individual is suffering from one of these disorders already, it adds to the challenging events he or she has to deal with.

1.1.4. Physiological regulation of food intake

To understand the complex development of eating disorders and the potential role of genetic predispositions, it is essential to understand the physiological regulation of food intake. It is a complex interaction of feedback systems in which the brain, gut, muscles, and fat tissue work together closely to keep body weight within a healthy range.

The process of food intake regulation involves one pathway for acute regulation and one for the long term regulation to keep our bodyweight stable.
Ghrelin, for example, is a peptide that stimulates food intake in the hypothalamic region. It is secreted by the stomach during fasting periods.
If food intake has reached a certain level, our intestines get stretched, which is

registered by mechanoreceptors. In addition, several peptides are secreted to inhibit further food intake. Neuropeptide Y (PYY) acts via the vagus nerve in the arcuate nucleus (ARC) by inhibiting Neuropeptide Y (NPY) and orexigenic agouti-related peptide (AgRP) neurons, which would otherwise stimulate hunger. The other populations of neurons in the ARC are cocaine- and amphetamine-transcript (CART) and proopiomelanocortin (POMC). They send inhibiting signals to other brain regions when stimulated.
Glucagon-like-peptide 1 (GLP- 1) stimulates the secretion of insulin in the pancreas and inhibits hunger in the ARC as well.
Blood levels of cholecystokinin (CCK) rise and fall quickly after meal consumption. It not only stimulates the contraction of the gallbladder, the secretion of the pancreas, and the contraction of our stomach, it also inhibits food intake via our lateral hypothalamus (Druce and Bloom, 2006).
The hypothalamus plays a central role as it manages hunger in its lateral region and saturation in the ventromedial nucleus and paraventricular nucleus (Behrends, 2021).

For the long-term regulation of our weight, fat tissue is very important. It secretes adiponectin and resistin and, most importantly, the hormone leptin in white adipose tissue cells. A high fat mass in an individual correlates with high measurable levels of leptin. Leptin inhibits the orexigenic AgRP/ NPY neurons and stimulates the inhibitory neurons. Together with insulin, which is secreted by the ß-cells of the pancreas, it activates a catabolic metabolism (Druce and Bloom, 2006).

These are only some of the most important stimuli that regulate how much we eat, and at what point we feel hungry or satiated. To ensure that an individual eats enough food to provide the amount of energy that is burned, food intake activates reward systems that motivate the individual to eat even more. In evolutionary terms it made sense to eat as much as possible whenever food was available to gain weight for longer periods of starvation. Today, food is available almost at all times. Regardless of this, these mechanisms still work, which is why food intake also has a high emotional connection. Dopamine, for example, is one of the most frequently studied neurotransmitters that get released as soon as appetizing food

is presented. It encourages further food intake until satisfaction (Volkow, Wang and Baler, 2011; Wallace and Fordahl, 2022).

1.1.4. Pathology of restrictive eating behavior

So far, there is little understanding of what contributes to the development of an eating disorder. It is also hard to find answers to the question of whether changes in neurobiology and neuropeptide pathways are the cause or consequence of AN. There are imaging studies where the investigators use computerized tomography (CT) and magnetic resonance imaging (MRI). Cerebral atrophy and increased volume of the ventricles were common findings, but most of the studies are limited to small sample sizes. Functional MRI investigations found high activation of the amygdala when showing AN patients pictures of food, which normally is an indicator of feelings of anxiety (Fox, 2008).

Looking at the hormone ghrelin, which is the only known hormone that increases the feeling of hunger and promotes food intake, there are recent studies showing increased levels in AN patients (Dostálová and Haluzík, 2009). Whereas the blood levels would fall after a meal in healthy control subjects, levels in AN patients are harder to predict and very inconsistent. It is possible that they remain high for hours, even after food intake (Nedvídková *et al.*, 2003; Otto *et al.*, 2005).

Another factor that should be considered is our gut microbiome. Each individual harbors thousands of distinct bacterial species in their digestive system. This microbiome can be influenced by factors such as the mode of birth, early-life and adolescent diet, medication usage, physical activity, and illnesses. These bacteria play an important role in fermenting indigestible fiber and facilitating the absorption of nutrients from the food we consume (Cresci and Bawden, 2015). Additional research is required to gather insights into the alterations of our microbiome in individuals with chronic AN and its changes during the recovery phase. Many studies have limited sample sizes or show inconsistent findings. However, it is known that there is a noticeable change in the diversity of species within our gut. This downshift can potentially be reversed as part of the recovery process (Ruusunen *et al.*, 2019). The alpha diversity still seems to be lower than in healthy

control groups, as was shown in one study by (Kleiman *et al.*, 2015). That means that the frequency of different microbiota species in one place - our gut system in this case - at the time of the investigation, is limited.

1.1.5. Therapy

The gradual starvation and significant weight loss can lead to cardiovascular instability, dehydration, confusion, and delirium in acute settings (Neale and Hudson, 2020). The long-term consequences can affect nearly every organ system and include reduced bone density, fertility problems due to amenorrhea, decreased renal function, and neurocognitive deficits (Peterson and Fuller, 2019). Therefore, a diagnosed eating disorder should lead to the immediate initiation of therapy. This is particularly important because in a study from Morris & Twaddle (2007), it is mentioned that the process of seeking treatment can take up to six years from the actual onset of symptoms. The sooner therapy is initiated, the better the prognosis for the patient (Morris and Twaddle, 2007).

In the mid-20th century, pharmaceutical treatment dominated the field (Steinhausen, 2002). Studies such as the one by Neale and Hudson (2020) show that this has nowadays shifted towards an interdisciplinary treatment approach involving nutritionists, psychiatrists, and the involvement of the family to create a supportive environment for the patient's recovery. Furthermore, they state that it is essential that the patient is cooperative and willing to undergo treatment. Otherwise, there is a high likelihood of therapy failure or a relapse into old patterns and weight loss (Neale and Hudson, 2020).

The main focus is to achieve a healthy weight and address any nutritional imbalances and organ dysfunctions that may have occurred, depending on the severity and duration of the eating disorder. To ensure sustainable therapy, it is important to provide education on healthy nutrition and eating behaviors, as well as to address the treatment of any co-occurring psychiatric disorders (Peterson and Fuller, 2019).

The selection of the most suitable therapy for each individual must be assessed in every case. It depends on the severity of the symptoms, current age and age at onset, comorbidities, and the primary triggering factors that led to the ED.
Psychotherapy options can include individual therapy at regular intervals, which can include cognitive-behavioral therapy or interpersonal psychotherapy, focusing on the patient's relationships, for example. Other options are group therapy in addition to individual therapy or family therapy, which can involve separate sessions with the family and patient or joint sessions together. Weight gain might take more time but seems to be more sustainable when the whole family is involved (Morris and Twaddle, 2007). This is particularly preferred for adolescents and very young patients, as a positive parent-child relationship is also an indicator of a better long- term prognosis (Steinhausen, 2002; Morris and Twaddle, 2007).

The effects of inpatient treatment are still discussed controversially. For instance, being in the presence of other patients who struggle with their food intake could potentially create the challenge of concentrating on weight gain under unfavorable circumstances. The expenses associated with this form of treatment are higher compared to other therapy options, and there are notable rates of recurrence (Morris and Twaddle, 2007).
Hospitalization should be considered when the patient's safety and life are at risk, such as in cases of severe dehydration, or electrolyte imbalances, heart failure, or other organ malfunctions. Refeeding the patient via feeding tubes should be considered when other treatment options have proven unsuccessful (Peterson and Fuller, 2019).

Medications are still utilized to facilitate the appropriate treatment of psychiatric comorbidities, but the evidence regarding their benefits for weight gain in individuals with eating disorders is limited. Antipsychotic medications, such as olanzapine, for instance, are known to have weight gain as a potential side effect. However, in anorexic patients, they are more commonly prescribed to alleviate obsessive exercising and fearful thoughts related to food (Herpertz, Herpertz-Dahlmann, *et al.*, 2011).

Antidepressants, including selective serotonin reuptake inhibitors (SSRIs) or tricyclic antidepressants, may be prescribed to address depressive and anxiety

symptoms in individuals with AN. However, studies do not show a significant benefit in terms of recovery for children. Therefore, in such cases, antidepressants should only be considered if symptoms persist even after achieving a normal BMI (Herpertz, Herpertz-Dahlmann, *et al.*, 2011).

Other medications, such as drugs with appetitive effects, vitamin supplements, or anxiolytic medication, can be used to support recovery alongside psychotherapy (Herpertz, Herpertz-Dahlmann, *et al.*, 2011).

1.2. Major Depression

MD is one of the most common psychiatric disorders in the world, affecting one in five people on average (Filatova, Shadrina and Slominsky, 2021). Symptoms, according to the ICD-10 criteria, may include lethargy, loss of appetite and weight, feelings of worthlessness, and lack of happiness, and can potentially lead to suicidal thoughts or suicide (Schramm *et al.*, 2020). Since the release of the ICD-11, there have been changes in the diagnosis of MD concerning changes to core and accompanying features of the disorder (Lundin, Möller and Forsell, 2023).

According to the ICD 10, major depression ranks among the affective disorders and is subdivided into gradations depending on severity and manifestation of symptoms. It ranges from mild depressive episodes (F32.0) with only a few symptoms affecting the patient to severe depressive episodes without psychotic symptoms (F32.2) or with psychotic symptoms (F32.3). These symptoms may include hallucinations or the inability to move, eat, or speak (Sjöberg *et al.*, 2017).
Atypical depression (F32.8) is another subtype and presents with symptoms that can be contrary to the typical depressive symptoms including hypersomnia or overeating (Juruena *et al.*, 2018).

1.2.1. Diagnostic Criteria

As for AN, there are diagnostic criteria established by the APA in the DSM-5 standard (Salk, Hyde and Abramson, 2017). It was published in 2013 and includes several changes compared to the DSM-IV.

The symptoms must persist for at least most of the days for a minimum of two years, except for children or adolescents, for whom the duration is just one year. The absence of these symptoms should not exceed two months. At least two or more of the following symptoms may occur simultaneously: poor appetite or overeating, insomnia or hypersomnia, low energy or fatigue, low self-esteem, feelings of hopelessness, and poor concentration. There are no indications of other psychotic disorders that can account for these manifestations, and the patients are significantly affected by them in their daily lives (Filatova, Shadrina and Slominsky, 2021).

1.2.2. Epidemiology

All in all, the global 12-month prevalence of major depressive disorder (MDD) is slightly higher for women, at 5.8%, compared to only 3.8% in men. There are multiple theories to explain this gap, including sociological theories such as income differentials or acts of violence against women. A social structural theory takes into account gender inequality and other factors, such as ethnicity or the underdiagnosis of men (Salk, Hyde and Abramson, 2017; Swetlitz, 2021).

Patients diagnosed with AN, who are predominantly female as well, exhibit symptoms of MD in approximately 51.4%-56% of cases, both during the acute phase of their illness and after successful treatment for their eating disorder (Jagielska and Kacperska, 2017).

1.2.3. Therapy

Alongside the treatment of psychological issues, such as the distorted body image and the treatment of underweight, therapy needs to be adjusted if any comorbidities, like major depression, are diagnosed (Herpertz, Hagenah, *et al.*, 2011).
Just as for AN, the guidelines suggest a treatment for major depression that suits each patient individually. Depending on the social environment of the patient, the severity of symptoms, age, or whether it is a chronic or acute setting, it can involve psychotherapy or psychoeducational options with or without additional use of antidepressants. First-line antidepressants are usually selective serotonin reuptake inhibitors (SSRIs), as they have lower risks of side effects such as tricyclic tertiary amines (TCTA), for example (Gautam *et al.*, 2017).

To sum up, in addition to therapy options of AN above, even though there is poor evidence in the use of medication during recovery from AN, it can be a good option to treat comorbidities like MD.

1.3. How Genes influence the development of Anorexia Nervosa

There are both mental and physical disorders, such as cancer or, as in our case, AN, where researchers and medical doctors are still investigating all the pathways as to why one person might develop the disease while another may not. In 1990, the Human Genome Project was founded with the goal of sequencing the entire human genome to gain a deeper understanding of it. Researchers from around the world worked together to advance the necessary technology, gather, and analyze the data (Green, Watson and Collins, 2015).
Since then, the number of studies focusing on the interaction between genes and environmental factors in the onset of EDs in affected people has been growing.

As previously mentioned, the vulnerability-stress model is one way to explain how various factors contribute to the development of AN (Zubin and Spring, 1977). The best way to get an idea about the important role of genes in this setting is through family-, and more precisely, twin studies. Concordance rates for monozygotic twins are at 44%, whilst dizygotic twins have lower rates, with 12.5% on average.

This is particularly important when the question arises of whether genes or the family setting itself is to be blamed for the clusters of EDs (Kipman *et al.*, 1999).
One study used a next-generation-sequencing panel (NGS) to identify genetic variants that are associated with AN. All female patients (N= 63) had at least one family member who was also affected by an ED (Ceccarini *et al.*, 2022).
The chances of developing AN are 11.4 times higher for relatives of affected AN patients and even higher for relatives of affected bulimia nervosa (BN) patients, with a hazard ratio from 12.1 (Strober *et al.*, 2000).
Other study reviews suggest a heritability range from 33% to 84% for AN (Strober *et al.*, 2000) and a range from 22% to 62% in EDs overall. The cause for the high variance in these ranges might be due to gene-environment interactions (GxE). These interactions can lead to changes in the transcription and function of certain genes but up to this point the evidence for DNA methylation processes in AN patients is limited (Iranzo-Tatay *et al.*, 2022).

1.3.1 Genome wide association studies

The human genome varies by only about 0.01% from one person to another, which is roughly equivalent to around 3.2 million base pairs. In certain locations, these base pairs vary by just a single nucleotide, being substituted for a different one. These variations are referred to as single nucleotide polymorphisms (SNPs), which are basically “established point mutations”. Usually, they occur as alleles, which means that for one SNP there are two different options: G or C, and A or T (Kwok and Chen, 2003). Techniques like polymerase chain reaction (PCR) aid in amplifying even minute quantities of DNA and targeting specific regions to identify SNPs (Lorenz, 2012).
Since the early twenty-first century, thanks to the 1000 Genomes Project (1000 Genomes Project Consortium *et al.*, 2015), and the HapMap project (Morton, 2008) among others, genomic data banks have been expanding and studies called Genome wide association studies (GWAS) have emerged. Based on the collected genomic data, these studies aim to examine genetic variants such as SNPs that are likely to be associated with particular common diseases. To understand how one SNP can be associated with certain diseases, it is necessary to explain the term “genome-wide significance”: To statistically prove the influence

of a genomic variant, it has to meet a certain threshold, which is usually the *p-value* (Xu *et al.*, 2014). If the null hypothesis assumes no connection between an SNP and an illness, it will be stated as true if the results are above the *p-value* threshold of < 5x10^-8. If the results are below this threshold, the null hypothesis is overruled, and the alternative hypothesis stating that there is a genome-wide association between the SNP and disease will be accepted as true (Uffelmann *et al.*, 2021).

This is where the Common Disease Common Variant hypothesis (CDCV) becomes important. It implies that some SNPs are commonly detected within a population in around 5-10% of individuals. Many of these are located in non-coding regions of the DNA. However, in conjunction with specific other SNPs and environmental factors, they are linked to common psychiatric and physical disorders, with some SNPs even contributing to more than one condition. Some examples are presented below. The precise mechanisms through which they affect the development of these illnesses are not yet fully understood (Hemminki, Försti and Bermejo, 2008).

To sum up, GWAS help to identify regions on our genome that are likely to be co-responsible for the development of diseases. Increased knowledge among researchers and doctors about disease triggering factors can pave the way for new therapeutic options or even the prevention of illnesses. Polygenic risk scores (PRS), for example, combine all the relevant SNP variants to identify patients with high genetic risk for a disease, such as MD, when combined with environmental triggering factors (Lewis and Vassos, 2020).

1.4. Relevant Genes linked to Anorexia Nervosa and Major Depression

The first GWAS study involving AN patients was conducted by Nakabayashi *et al.* in 2009. They identified and mapped 9 loci on the genome that are associated with AN, along with two associated genes. Among these, SPATA17 encodes a protein highly expressed in the testis, while CNTN5 encodes a cell-adhesion protein that plays an important role in neural development and function. Nevertheless, the robustness of these findings is debatable, considering the relatively small sample size of only 320 AN cases and 341 HCs and particularly due to the study's limited scope, which focused on Japan rather than being internationally diverse (Nakabayashi *et al.*, 2009).

In the following years, 2011 and 2014, two further studies with larger sample sizes were presented by Wang *et al.* (2011) and Boraska *et al.* (2014). Their sample sizes were larger with 1.033 AN cases and 3.733 HCs (Wang *et al.*, 2011) and 2.907 AN cases and 14.860 HCs (Boroska *et al.*, 2014) and from a larger variety of countries. Even though the association with certain genes, such as CDH9 and AKAP6 (Wang *et al.*, 2011), or PPP3CA and SPATA13 (Boraska *et al.*, 2014), was strong, it was not significant. Both studies emphasized the need for even more expansive sample sizes and additional research to draw significant conclusions.

Duncan *et al.* (2017) were the first researchers to identify the first genome-wide significant locus on chromosome 12: SNP rs4622308. The six genes ERBB3, PA2G4, RPL41, IKZF4, RPSL41 and ZC3H10 overlap in this region (Duncan *et al.*, 2017).

Only two years later, the largest GWAS on an AN sample to date was conducted by Watson *et al.* (2019). They included data from the UK Biobank, the Anorexia Nervosa Genetics Initiative (ANGI), and the Eating Disorder Working Group of the Psychiatric Genomic Consortium (PGC-ED). The ANGI and the PGC-ED were designed to collect more AN samples to provide additional data in a short period of time (Thornton *et al.*, 2018). These samples sum up to 16.992 in the AN group and 55.525 HCs from 17 countries. They detected eight genome-wide significant

risk loci in total. Four of them are single-gene loci with the most significant association: CADM1, MGMT, FOXP1 and PTBP2 (Watson *et al.*, 2019).

Table 3 provides an overview of all the genome-wide significant SNPs mentioned above with their p-values and odds ratios (OR).

Table 3: An overview of the Genome wide significant SNPs for Anorexia Nervosa with their location and associated genes.

Chro	Genes	SNP	*p-value*	OR	Reference
12	IKZF4, ERBB3, ZC3H10, RPL41, RPS26, PA2G4	rs4622308	4.252 x 10-9	1.2	Duncan et. al., 2017
11	CADM1	rs6589488	6.31 x 10-11	1.14	Watson et al., 2019
10	MGMT	rs2008387	1.73 x 10-8	1.08	Watson et al., 2019
5	CDH10	rs370838138	3.17 x 10-8	1.08	Watson et al., 2019
3	NSUN3	rs13100344	4.21 x 10-8	1.08	Watson et al., 2019
3	FOXP1	rs9874207	2.05 x 10-8	1.08	Watson et al., 2019
3	NCKIPSD	rs9821797	6.99 x 10-15	1.17	Watson et al., 2019
2	ERLEC1, ASB3	rs2287348	5.62 x 10-9	1.11	Watson et al., 2019
1	PTBP2	rs10747478	3.13 x 10-8	1.08	Watson et al., 2019

It is incredible to see how far genetic research on AN has come since the first GWAS on the disease was performed in 2009 (Nakabayashi *et al.*, 2009). To identify even more genes, the constant collection of genomic data from patients

to increase sample sizes is essential, as well as examining discrepancies among countries and continents (Wang, Cordell and Van Steen, 2019).

However, there are diseases such as EDs, whose heritability can only partly be explained by the most recent genetic findings. This is particularly true for their most frequent comorbidities, which can also be genetically influenced. The term “missing heritability” refers to the notion that these disorders do indeed have a genetic component, aspects of which may have only been partially identified. The existence of smaller studies with reduced sample sizes and focused deep phenotyping is crucial to uncover the missing, more complex links (Manolio *et al.*, 2009).

This leads to the focus of this diploma thesis, which investigates candidate genes using a hypothesis-driven approach based on their function in MD pathways. The genes discussed below have been mentioned and investigated in previous studies before (Kennedy *et al.*, 2006; Boraska *et al.*, 2014; Brunoni *et al.*, 2020). Literature suggests links between these genes and AN, as well as connections to MD. This makes them particularly interesting to examine further, as MD is such a common comorbidity in AN patients. Investigating these genes further is valuable because they are not among the most frequently studied SNPs for AN to date. Each new finding contributes significantly to understanding the etiology of AN and MD and aids in developing effective prevention and treatment strategies.

1.4.1. 5-HT2A

There are four studies from the late 20th century that confirm a significant association between AN and the 5-HT2A gene (Nishiguchi *et al.*, 2001), (Sorbi *et al.*, 1998), (Enoch *et al.*, 1998), (Collier *et al.*, 1997).
However, one other study did not confirm this association (Ziegler *et al.*, 1999). Meta-analyses, such as the one by (Plana *et al.*, 2019), describe irregularities that might be due to varying sample sizes or population samples.

5-HT2A (5- hydroxytryptamine 2A) is a gene located on chromosome 13q14-q21 that encodes the subtype 2A of the serotonin receptors. These receptors are

postsynaptic G-protein-coupled receptors, crucial for the serotonergic system, which regulates intracellular long-term responses. They are distributed throughout the central nervous system and are likely associated with psychiatric disorders (Brunoni *et al.*, 2020). The SNP rs6311 in particular is located in the promoter region of the gene and regulates the 5- HT2A expression (Yan *et al.*, 2021).

Studies show very inconsistent results regarding the connection between 5-HT2A and AN. The assumption of a genetic foundation for the receptor's functioning, regardless of the illness itself, is based on the following knowledge: First, serotonin plays an important role in mood regulation, impulse control, and food intake. Second, medication that interacts with the serotonin pathways is prescribed for patients with AN only when it helps to address comorbidities such as MD. Third, in a study by Kaye *et al*. (2005) using PET scans, irregular functioning of the 5-HT2A pathways persisted in patients even after recovery (Kaye *et al.*, 2005).

That is what makes this gene an interesting choice for this study. Since medication is already known to benefit affected AN patients with depressive symptoms, the results of this study contribute to the existing data and potentially confirm an association with both disorders.

1.4.2. OPCML

So far, the evidence for a linkage of OPCML to AN remains unclear. The study by Huckins *et al.* (2018) emphasizes important low-frequency variants in the development of physical and psychiatric diseases. These variants are found in <5% of the population and are typically less likely to be detected than common variants. However, risk alleles appear more frequently in these exact regions (Kido *et al.*, 2018). Even though the study from Huckins *et al.* (2018) did not confirm genome-wide significance for OPCML, they found an association for the SNP rs10791286 within all their cohorts, which is located on that exact gene (Huckins *et al.*, 2018).

The OPCML gene is localized on chromosome 11q25 and encodes the opioid-binding protein/cell adhesion molecule-like, which is involved in both short- and

long-term effects of opioids and acts by linking G-proteins to the receptor. Often located near serotonin and other neurotransmitters, it is distributed throughout our nervous system (Schol-Gelok *et al.*, 2010).

Apart from studies suggesting OPCML as a tumor suppressor (Shao *et al.*, 2021), its involvement in the development of MD is discussed in some studies. Kennedy *et. al* (Kennedy *et al.*, 2006), showed a significant decrease in mu-receptor bindings in women diagnosed with MD compared to an HC group.

Given the previously investigated association of the SNP and its role as a potential rare variant influencing AN, it is crucial to gather more data on its role in the development of the disorder. The additional effect identified by Kennedy *et al.* in their study with MD patients prompted the inclusion of this gene in the present study to further explore its potential influence on both AN and MD.

1.4.3. TSHZ1

This gene is localized on chromosome 18q22. It encodes a protein called t-shirt zinc finger protein, which is a transcription factor involved in processes such as neuronal development and skeletal growth. It is also downregulated in patients with type 2 diabetes and is part of the gene family that regulates the endocrine cell development in the endocrine pancreas (Raum *et al.*, 2015).

One study from Wade *et al*. (2013) couldn't find any genes with genome- wide significance, but TSHZ1 was one gene, alongside CLEC5A and three different kinds of taste receptor genes, that showed at least a strong association to AN. It is linked to weight change and glucose levels (Wade *et al.*, 2013).

It is known that glucose levels in AN patients tend to be too low due to calorie restriction, but there are mechanisms that seem to protect the affected person from life-endangering hypoglycemia (Germain *et al.*, 2023). This makes it particularly interesting to have a closer look at the genetic variants of this gene in AN patients, since evidence is weak so far.

A study investigating the connection in patients with MD has not yet been conducted, making it particularly interesting to closely examine the results of the questionnaires regarding depressive symptoms and a possible connection with this gene.

1.4.4. PPP3CA

Even though the evidence is not genome-wide significant, one study suggests a strong association with AN and connects the gene to other neuropsychiatric diseases like Alzheimer's (Boraska *et al.*, 2014). This is particularly interesting since AN often co-occurs with other psychiatric disorders, which is why this gene was selected for investigation in this study.

Protein phosphatase 3 catalytic subunit alpha (PPP3CA) is a gene that is suggestively involved in the pathways of the gut-brain axis, as investigated by a study examining the genetic connections between schizophrenia, MD, Crohn's disease, and ulcerating colitis. The gene encodes a protein from the calcineurin family. Expressed in the gastrointestinal tract, it can be involved in inflammatory processes and is found in the brain, where it supports neuronal signal transduction (Uellendahl-Werth *et al.*, 2022).

As mentioned in the chapter "Pathology of restrictive eating behavior", the pathways that regulate hunger and satiation are disrupted in AN patients. This makes it particularly interesting to gather more data on a gene involved in the gut-brain axis.

1.5. Hypothesis

So far, it is known that family members of patients with AN have a significantly increased risk of developing an eating disorder themselves. Studies with monozygotic twins in particular, confirm the hypothesis that genes are involved in this process, as the concordance rates are even higher than those for dizygotic twins, which limits the influence of the family background (Kipman *et al.,* 1999).

Psychological as well as physical comorbidities are a common occurrence for affected patients. These include, among others, obsessive-compulsive disorder, schizophrenia, substance abuse, and MD for example.

The pathways for many of these mentioned disorders are not fully understood yet. In addition to environmental factors such as the previously mentioned family background, friends, or the influence of social media, genetics seem to have a huge impact. Ever since GWAS studies were first conducted on patients with EDs and other diseases, it has been known that not just one, but multiple genes might influence AN and its comorbidities (Watson *et al.*, 2019). This leads to the assumption that the genetic variations, also known as SNPs, might be the same for AN and its most frequent comorbidities.

There are currently 9 SNPs that have reached genome-wide significance for AN. They are presented in table 3 above with their associated genes, as well as some less common variants with a suggested strong association to AN that require further investigation (Watson *et al.*, 2019).

In this thesis, the goal is to determine whether less common genetic variations within the genes PPP3CA, 5-HT2A, TSHZ1, and OPCML occur in a significantly different way in the group of AN patients compared to the group of HC. Since the rates of depressive disorders in patients with EDs are striking (Casper, 1998; Jaite *et al.*, 2013; Jagielska and Kacperska, 2017) and might be linked to the same genes as well (Thornton *et al.*, 2016; Harder *et al.*, 2022). The author of this study also aims to demonstrate that their scores on questionnaires vary significantly when assessing those symptoms, compared to the HCs.

Primary Hypothesis: The occurrence of the following less common genetic variants, known as SNPs, differs between patients with AN and those in the HC group. These include SNP rs6311 (5- HT2A), rs10791286 (OPCML), rs17030795 (PPP3CA), and rs56156506 (TSHZ1).

Secondary Hypothesis: The total scores of the Hamilton Depression Rating Scale (HAMD) and Beck Depression Inventory (BDI) questionnaires differ between the group of AN patients and the HC group.

2. Methods

The DNA of the patients was collected in the so-called ILSD study (Implement Learning Strategies in Patients with AN and MD) at the department of Psychiatry and Psychotherapeutic Medicine at the Medical University of Graz, Austria. The recruitment of patients with AN and HC began in 2015.

The study was approved by the local ethics committee of the Medical University of Graz in accordance with the Declaration of Helsinki (ICH Guidelines for Good Clinical Practice). That means that all participants must sign a written consent after receiving detailed information about the study and the process involved in becoming part of it. The ethics reference number is 27-481 ex 14/15.

2.1. Participants

Since 2015, patients have been recruited incrementally over the years from the ward of the Department of Psychiatry and Psychotherapeutic Medicine at the Medical University of Graz. Additionally, leaflets were distributed to other psychotherapeutic institutions and private doctors to expand the search for participants.

The diagnosis for AN was initially made by clinicians prior to the patients' inpatient admission. During recruitment, participants were asked to complete the questions of the Eating Attitude Test (EAT-26) (Garner *et al.*, 1982) and the Eating Disorder Examination Questionnaire (EDE-Q) (Fairburn and Beglin, 1994), to confirm their current healthstate.

Healthy participants were recruited through Probando (a tool for finding study participants) and flyers. The HC group included students (medicine, psychology, molecular biology), friends and family members of the research team, scientific and non-scientific employees from several wards and departments of the Medical University of Graz (Psychiatric and Psychotherapeutic Medicine, Internal Medicine, Human Genetics, Surgery), as well as study participants found by Probando.

Participation was voluntary, and participants could withdraw from the study any time. To ensure that participants were fully informed about the study's procedure, objectives, inclusion and exclusion criteria, and any potential risks, each participant received verbal information through an interview prior to the start of the tests.
Each participant was then anonymized by assigning a code, which was used for all testing, including the genetic analysis. Their data is collected on a protected USB flash drive.

To ensure that both patients and healthy controls meet all the criteria, each participant was confirmed through a brief semi-structured interview in advance. The weight from AN patients was directly taken from their hospital chart, while the weight of HCs was self-reported by asking each individual to state their current weight.

For this thesis, data from 79 participants were examined. Of these, 28 were AN patients, and 51 were in the HC group. In some cases, relevant test results were not collected or were only partially completed, leading to the exclusion of these datasets. This resulted in a total sample of 19 AN patients with 2 males and 17 females, and 30 individuals in the HC group with 2 males and 28 females. That sums up to a total of 49 participants included in the statistical analysis. Further details about the sample are provided below the inclusion and exclusion criteria.

2.1.1. Inclusion and exclusion criteria

Inclusion criteria for participants diagnosed with AN:

- The participant meets the diagnostic criteria for AN in the ICD-10 (F.50.0).
- The current BMI is lower than 17.5 kg/m^2.
- The participant scores a minimum of 26 points at the Mini- Mental- Status-Test to ensure the absence of any other neurological conditions, such as learning impairments, mental retardation, or dementia.
- At the time of the admission to the study, the participant is at least 18 years and older

Inclusion criteria for the Healthy control participants:

- The participant has no history of psychiatric disorders.
- The current BMI is higher than 17,5 kg/m^2
- The participant scores a minimum of 26 points at the Mini- Mental- Status-Test to ensure the absence of any other neurological conditions, like learning impairments, mental retardation, or dementia.
- At the time of the admission to the study, the participant is at least 18 years and older.

Exclusion criteria for both groups:

- Genetic diseases that could run in a family, such as Huntington's Chorea, Alzheimer's disease, or chronic immunological disorders, are excluded
- Additionally, for participants in the HC group: Axis-1 diagnoses in first-degree relatives should be excluded.

2.2. Measurements

Prior to the admission appointment, participants receive a letter at their homes containing the EDE-Q, the BDI, the International Physical Activity Questionnaire (IPAQ), and the EAT-26. These questionnaires are to be completed by participants beforehand and brought with them to the appointment. After obtaining consent and collecting a blood sample from each participant, four additional tests are conducted with the interviewer. Two of these tests, described below, are simple assessments to rule out any cognitive deficits that could potentially compromise the accuracy of the results obtained from other tests:

When taking the Mini-Mental Status Test (MMST), participants are required to respond to questions that assess various cognitive aspects such as memory, attention, and orientation. Only individuals who achieve a minimum total score of 26 out of 30 will be eligible for inclusion in the study (Norris, Clark and Shipley, 2016).

The second test is the updated version of the multiple-choice vocabulary test (MWT-B) by Lehrl *et al.* in 2005. High scores on the MWT-B are positively correlated with IQ, providing a simple way to estimate one's approximate IQ score. Participants receive a form containing 37 lines of word options that progressively increase in difficulty throughout the test. Within each line, only one word is grammatically correct and not a neologism. The participant's task is to identify and mark this correct word without any given time limitation. A point is awarded for each word correctly identified and marked (Lehrl, Triebig and Fischer, 1995).

The remaining tests, whether conducted at home or under the guidance of the interviewer, help assess the current mental health status and its connection to food intake for each individual. As a result, they are important for determining whether a participant should be categorized as an AN patient or a member of the HC group. The tests relevant to the thesis will be described more precisely below:

2.2.1. Hamilton Depression Rating Scale (HAMD)

For this test, it is essential that the interviewer observes the subject's body language, facial expressions, and voice. The interviewer asks 21 questions about general mood, stress symptoms, insomnia, weight loss, and other characteristics linked to depression. Based on the responses and the patient's reactions, the interviewer assigns scores of either 0 to 2 points, or 0 to 4 points for each question. If the total score is below 8, the client is considered to have healthy mood regulation with normal variations. Scores between 9 and 16 indicate minor depression symptoms, while scores from 17 to 24 suggest moderate depression, and scores exceeding 25 are indicative of major depressive disorder.

The close observation of the participant provides an advantage over the Beck Depression Inventory (BDI), presented below, which relies solely on a self-report questionnaire (Hamilton, 1960).

2.2.2. Beck Depression Inventory (BDI)

As mentioned above, the BDI is a self-report questionnaire designed to assess the severity of depressive symptoms in a patient. The questionnaire consists of 21 questions, each offering four answer options. Depending on the severity of the symptoms, these options are assigned scores ranging from 0 points, when the symptom is not experienced, to 3 points, when the symptom is most intense.

A total score of up to 9 points indicates a healthy mental state without any signs of depression. Scores ranging from 10 to 18 points are associated with mild depression, scores between 19 and 29 points suggest moderate depression, and any score exceeding 30 points indicates severe major depression (Beck *et al.*, 1961).

2.3 Genotyping

After signing the consent form by the participants and the investigator, venous blood samples were collected from each participant.
Ethylenediaminetetraacetic (EDTA) tubes were used for this purpose, and the samples were promptly frozen at -20°C within the psychiatry ward of the clinic. In October 2021, once a sufficient number of samples had been collected, they were transferred to the Institute of Human Genetics at the Medical University of Graz in Austria.
At this location, the DNA extraction was performed by the QIAGEN's QIAsymphony SP, a fully automated system capable of processing up to 96 samples simultaneously (Parham *et al.*, 2012).

From the Medical University of Graz in Austria, the samples were sent on dry ice to the Life & Brain Center in Bonn, Germany. There, the required procedures were carried out using the Illumina's Infinium Global Screening Array-24 v3.0 Kit.
The Illumina Infinium Global Screening Array-24 v3.0 Kit is a microarray system, designed for the analysis of biological sample material, such as the DNA samples. The process operates as follows:

Within each DNA sample, sequences at a molecular level vary from one individual to another. These variations are referred to as single nucleotide polymorphisms which were explained in the chapters above.

Through an assay, these variations can be identified using probe molecules. These molecules are situated on a solid support system and, in our case, consist of nucleotides that are precisely complementary to the target sequences in our DNA sample. For instance, if our target molecule is the nucleotide pair GG, it will bind to the complementary probing nucleotides CC.
To detect these bonds, specific laser techniques or fluorescent labeling methods are used (Bednár, 2000).

To ensure that there are no Mendelian incompatibilities or familial correlations for quantitative traits and to apply the Hardy-Weinberg equilibrium, standard QC was

conducted beforehand. Hypothesis-driven genotypes of the target genes were extracted from the PED files with PLINK 1.9.

The genotypes relevant to this diploma thesis were the SNPs rs6311 (in gene 5-HT2A), rs10791286 (in gene OPCML), rs56156506 (in gene TSHZ1) and rs17030795 (in gene PPP3CA).

2.4. Statistical analysis

For the statistical analysis, version 28.0 version of the Statistical Package for the Social Sciences (SPSS) was used, with the sample consisting of 49 participants in total across all investigations.

First, all potential outliers were excluded and the data were verified for adherence to a normal distribution. To ensure that no demographic parameters, such as age, sex, or years of education, correlated with the results of the HAMD and BDI, a Spearman correlation was performed subsequently. This step is essential to identify any potential covariates that might affect the test scores and therefore influence the results of the following analyses.

Before conducting any analysis of variance (ANOVA) in this study, a Levene's test was performed to assess variance homogeneity. Normal distribution was tested using the Kolmogorov-Smirnov test. The non-parametric data were analyzed for group differences using the U-test.
An ANOVA was then conducted to compare the results of BDI and HAMD between the AN patient group and the HC group.

To test whether depression scores differed between genotype carriers, it was necessary to group certain genotypes partially when one of the genetic variants occurred in fewer than 10 cases. For instance, the genotype AA of the OPCML gene appeared in only 8 patients, which is why it was combined with the next most frequent genotype, GG. This approach ensured a representative analysis. For the statistical analysis, we conducted four separate ANOVAs and Levene’s tests, one for each gene mentioned earlier. In each analysis, the independent variable was

either the two main genotypes or the grouped genotypes with the third genotype. This approach allowed us to identify significant differences that may exist between these genotypes in the scores from HAMD and BDI.

3. Results

The final sample of HC participants comprised 30 participants. One of them was male, and the others were female. Their ages ranged from 20 to 61 (MD = 25.5; IQR= 23-36) and none of them received psychotropic drugs while taking part in the study.

The group of AN patients includes 19 individuals aged 18 to 52 (MD = 24.0; IQR= 20-34). Medication was prescribed for 8 patients, which included mainly Selective Serotonin and Noradrenaline Reuptake inhibitors (SSNRIs), mirtazapine, trazodone and nicotine.

The ages at which the AN patients were diagnosed with their disorder range from 12 to 40 years (M= 19.7; SD= 6.6).

Table 4 visualizes the clinical and demographic parameters that define the two groups from the study. The following parameters significantly differentiate the AN patients from the HC group ($p < .001$): BMI (kg/m^2), educational years, and the test results from HAMD and BDI.

Table 4: Overview of the parameters in the HC and AN group.

	AN n = 19 M (SD)	HC n = 30 M (SD)	*p- value*
Age (years)	27.63 (10.82)	29.63 (9.54)	.500
Educational (years)	12.89 (2.18)	17.45 (3.77)	< 0.001**
BMI (kg/m^2)	14.92 (4.75)	21.76 (2.28)	< 0.001**

** p-value significant <0.05

3.1 Results of the Questionnaires

3.1.1. BDI

The u-test in the group of AN patients showed significantly higher rates than the HC group ($p < .001$). The scores range from 1 to 44 (Md = 25; IQR = 16-35). The scores in the HC group, on the other hand, range from 0 to 17 (Md = 2.00; IQR: 0-4,25) and are not normally distributed. Only two HC participants presented with a score above 10 points. To sum up, the scores for symptoms of depression in the AN group are significantly higher ($p < .001$) than those in the HC group.

3.1.2. HAMD

As presented in Table 5 just like with the BDI, the scores of the AN patients are significantly higher ($p < .001$) according to the u- test than those in the HC group. The scores in the AN group range from 4 to 28 points (Md = 18; IQR: 11-21), whereas the lowest score is 0 points and the highest 8 points in the HC group (Md = 1.5; IQR: 0-4,25).

The boxplot diagrams below Table 5 give an overview of the distribution of the scores in both groups.

Table 5: Overview of the results from HAMD and BDI

	AN n=19 Md; IQR	HC n=30 Md; IQR	*p-value*
BDI (0-63)	25; 19	2; 4	< 0.001**
HAMD (0-66)	18; 10	1,5; 4	< 0.001**

** p-value significant < 0.05

Figure 3: Boxplot diagram of HAMD scores in both groups.

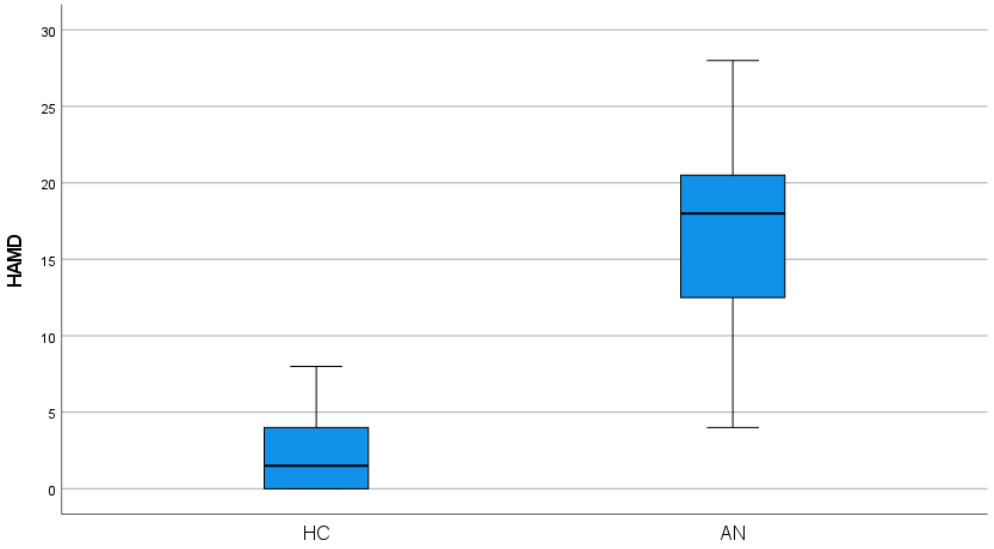
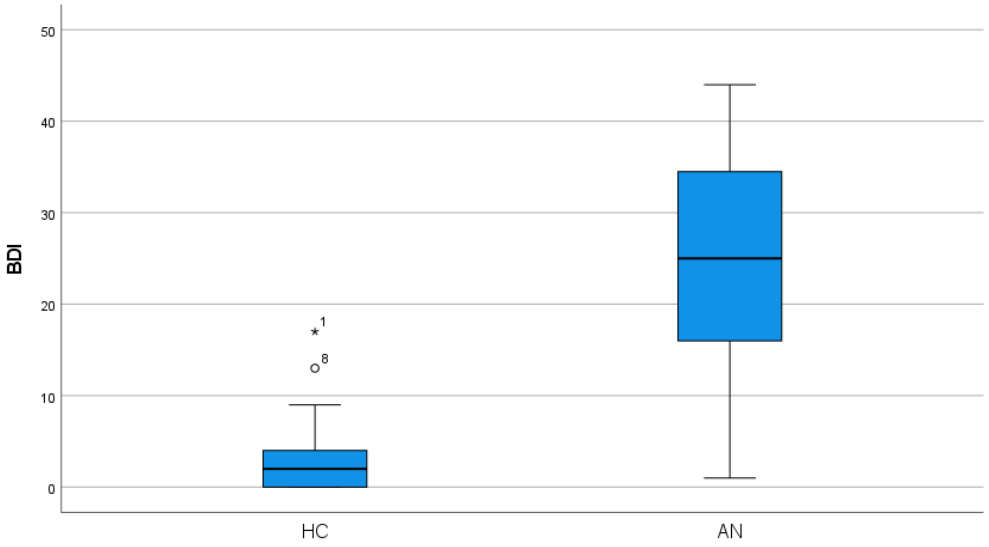


Figure 4: Boxplot diagram of BDI scores in both groups.



3.2. Genotypes

Table 6 and 7 below provide a brief overview of the results from the ANOVA. Each genotype variant was tested twice: once in the AN group (Table 5) and once in the HC group (Table 6), to determine whether the results from HAMD and BDI differed significantly depending on which genotype was present in the participants.
No significant differences were found.

Table 6: Results of the ANOVA comparing genotype variations for each SNP in the AN group with the test results from HAMD and BDI.

Gene	SNP	Test	AN n = 19			
			F	df	np^2	*p*
5HT2A	rs6311	HAMD	1.111	1	0.061	0.307
		BDI	1.127	1	0.062	0.303
OPCML	rs10791286	HAMD	1.084	1	0.059	0.312
		BDI	1.131	1	0.062	0.303
PPP3CA	rs17030795	HAMD	0.351	1	0.020	0.561
		BDI	0,469	1	0.268	0.503
TSHZ1	rs56156506	HAMD	0.622	1	0.035	0.441
		BDI	1.227	1	0,067	0.283

Table 7: Results of the ANOVA comparing genotype variations for each SNP in the HC group with the test results from HAMD and BDII.

Gene	SNP	Test	HC n = 30			
			F	df	np^2	*p*
5HT2A	rs6311	HAMD	1.993	1	0.066	0.169
		BDI	1.028	1	0.035	0.319
OPCML	rs10791286	HAMD	0.436	1	0.015	0.515
		BDI	0.126	1	0.004	0.725
PPP3CA	rs17030795	HAMD	0.081	1	0.002	0.779
		BDI	0.000	1	0.00001	0.986
TSHZ1	rs56156506	HAMD	2.442	1	0.080	0.129
		BDI	0.103	1	0.003	0.750

However, it is worth taking a closer look at the PPP3CA gene. When the groups are not split into AN and HC, there is a significant difference between the genotypes and test results for the HAMD (p = 0.046), as shown in Table 8 presented below. However, these results do not apply to the BDI for PPP3CA, nor for any of the other genes, since p- values are always above 0.05.
Using a cross table, it becomes evident that the genotype AA occurs in 94.4% of the AN patients and only 61.3% of the HCs.

Table 8: Overall results of the ANOVA when groups are not split into AN and HC

Gene	**SNP**	**Test**	**AN + HC n = 49**			
			F	df	np^2	p
PPP3CA	rs17030795	HAMD	4.202	1	0.082	0.046
		BDI	1.028	1	0.035	0.319
OPCML	rs10791286	HAMD	0.001	1	2,51 E-5	0.973
		BDI	0.042	1	0.0009	0.893
5- HT2A	rs6311	HAMD	1.194	1	0.025	0.280
		BDI	1.219	1	0.025	0.275
TSHZ1	rs56156506	HAMD	0.858	1	0,018	0.359
		BDI	0.085	1	0,002	0.772

Table 9: Frequency of the AA and GA alleles in AN patients and HCs

Diagnosis		PPP3CA		
		AA	GA	in total
HC	number of patients	19	12	31
	percentage	61.3%	38,7%	100%
AN	number of patients	17	1	18
	percentage	94.4%	5.6%	100%

4. Discussion

The aim of this thesis was to confirm whether there is a significant difference in the results of the HAMD and BDI between the HC and AN groups and various genotypes of four selected SNPs. The chapter above, where these SNPs are presented, shows that they have been investigated previously to identify a connection to the development of AN and various psychiatric comorbidities through genetics (Kaye *et al.*, 2005; Kennedy *et al.*, 2006). However, there are few studies available that address an explicit connection between genetics, AN, and MD as a comorbidity, so the existing evidence is limited. This is why this thesis provides additional data and results to the rare pre-existing findings, such as those from GWAS (Duncan *et al.*, 2017; Watson *et al.*, 2019).
The genes relevant to this thesis are all described in the chapter “Relevant Genes linked to Anorexia Nervosa" above. Associating the genotypes with our test results, which serve as indicators of depressive symptoms, may demonstrate that there are genetic variants influencing not only the development of AN but also depressive symptoms.

Furthermore, the scores of HAMD and BDI were compared between the AN and HC groups to determine whether there is an overall significant difference in the results.

Little is known about whether depressive and anxious symptoms result from malnutrition, an overall fear of food intake and weight gain, or if they have a common genetic basis. The literature generally indicates that a balanced diet is beneficial to maintaining healthy mood regulation (Boulkrane *et al.*, 2020; Gómez-Donoso *et al.*, 2020).

In the sample of AN patients, the scores of both HAMD and BDI are significantly higher than in the HC group. No difference was found when comparing patients taking medication to those not on medication.

According to a study by (Pleplé *et al.*, 2021), research focusing on mood regulation in AN patients often relies solely on BMI measurements to assess nutritional status. However, BMI can be influenced by factors such as sex, muscle mass, and body fat percentage. This study was the first to use bioelectrical impedance alongside BMI to evaluate whether BDI scores changed after treatment for AN and an improvement in nutritional status. However, this hypothesis could not be confirmed (Pleplé *et al.*, 2021).

This again highlights the need to explore other possible reasons for the high co-occurrence of depressive symptoms in AN patients.

Duncan et. al (2017) identified one SNP significantly correlated with AN, while Watson et. al (2019) identified 8 SNPs. An overview can be found in Table 3 in the chapter about relevant genes linked to AN. According to their investigations, it is impossible to pinpoint a single gene as causative. AN is a multifactorial and polygenic disease, suggesting that not all relevant genes have yet been identified (Harder *et al.*, 2022). Given this, genotypes of 4 SNPs located on genes less commonly studied for AN were investigated in this study to explore new approaches and connections between depression and AN.
Among the four SNPs, the SNP rs17030795 (PPP3CA) genotypes show significantly different results on the HAMD. The genotype AA occurs in 94,4% of AN cases and in only 61,3% of the HC group. Participants with genotype AA show significantly higher scores (M = 9.47, SD = p = 0.046) on the HAMD compared to those with genotype GA (M = 4.08). This deviation is not observed when the

groups are analyzed separately, which might be due to the relatively small sample size of this study. Nevertheless, the higher frequency of genotype AA in the AN group compared to the HC group indicates a trend that warrants further investigation.

A different approach to explaining the higher scores of the genotype AA on the HAMD test is the following idea: Regardless of whether one is officially diagnosed with MD or an ED, a person might be at higher risk of developing psychiatric disorders in this category compared to others with different genotypes. The vulnerability-stress model, as discussed in the chapter above, offers an explanation how individual environmental factors influence this development for each person.
The gene PPP3CA was investigated in two further studies and is suspected of being a risk factor for MD (Verma and Shakya, 2021; Uellendahl-Werth *et al.*, 2022).

Interestingly, performing an ANOVA on the genotypes of rs17030795 with the scores of the BDI reveals no significant connection. The difference between these two questionnaires lies in their evaluation and scoring system. BDI relies on self-assessment, which is known to be unreliable in AN patients, particularly regarding their own body image (Hick and Katzman, 1999). This may suggest that this issue could extend to other characteristics of affected AN patients as well.

So far, there is only limited data available about SNP rs17030795 in particular. As described in the study from Boraska *et al.* (2014), it is located on the PPP3CA gene at position hg18 102267099. The gene PPP3CA in turn, encodes the serine/ threonine phosphatase calcineurin and is located on chromosome 4. It is involved in the pathways of calcineurin signal transduction as a phosphatase catalytic subunit (Boraska *et al.*, 2014). Calcium and calmodulin are essential for its functioning, so PPP3CA transmits signals via calcium (Li and Cao, 2022).

A GWAS study by Boraska *et al.* in 2014 found a suggestive association of PPP3CA with AN in a European sample of 2,907 AN patients and 14,860 HC candidates. Since the sample in this thesis consists of considerably fewer

participants and still yields similar results, this gene could serve as a reference point for further research with larger sample sizes.

The authors Li and Cao (2022) describe developmental and epileptic encephalopathy 91 as a single-gene disease caused by mutations on PPP3CA in their study. Another study refers to the calcineurin pathways as a key role in the development of other neurodegenerative diseases, given that misfolded proteins that accumulate in cells disrupt the physiological pathways of calcium (Shah *et al.*, 2017).

AN and MD, both known as psychiatric disorders, have previously been investigated for degenerative or inflammatory processes within certain brain structures as well (Frank, Shott and DeGuzman, 2019), (Beurel, Toups and Nemeroff, 2020). The results for AN patients are very inconsistent, ranging from increased to decreased volumes of certain brain structures, such as white or gray matter, or even no changes at all (Frank, Shott and DeGuzman, 2019). Investigating whether PPP3CA could contribute to these variations would be another interesting indication for larger study samples on this subject.

4.1. Limitations

As indicated by other GWAS studies, the need for large sample sizes to obtain significant outcomes is fundamental to the investigation of genetic phenomena. Recruiting AN patients is challenging, which makes it difficult to find enough participants. Many patients withdraw their agreement to participate in the study when they realize how difficult it is to confront the struggles of their disease again. Additionally, to complete the tests, especially the WPT, participants need good focus and concentration. Due to a lack of nutrition and self-confidence, some patients prematurely ended the tests, which contributed to the small sample size in this study.

Most of the recruited patients are inpatients from the LKH in Graz in Austria, which presents two issues: First, the sample size is limited to a very small population. Ideally, studies should collect data from many different hospitals and countries to

include people of various backgrounds. That makes a study more powerful, as the results are more relevant and valid across different regions. Second, recruiting mostly inpatients differs from other studies that include more outpatients as well. Inpatients and outpatients may differ in their nutritional status, weight, and BMI, potentially leading to different results in the questionnaires. Therefore, it is not straightforward to compare our results to existing data.

Another point to mention is that, to find available pre- existing data, a decision had to be made regarding which genes, and more specifically which SNPs, the thesis would focus on. Unfortunately not all the SNPs we could have investigated were available on the GSA Array.
Also, the number of studies investigating genetic overlap in AN and MD is limited. Even if the results suggest a possible link between these two psychiatric disorders, further research with larger, more heterogeneous sample sizes and additional SNP options is necessary. This will help obtain significant results and enhance our understanding of the connection between MD and AN regarding genetic inheritance and the role of less common variants.

4.2. Conclusions

In this thesis, a significant connection between the genotype AA of the SNP rs17030795 and elevated scores on the HAMD was identified. It was particularly noticeable that participants affected by AN showed significantly higher results in both HAMD and BDI overall. As previous studies have shown, this strengthens the evidence for the idea that both conditions could share common genetic causes.

The knowledge that affected patients tend to have higher depression scores has important clinical implications. The more researchers and clinicians learn about the factors influencing the development of AN and its comorbidities, the more effective new therapeutic approaches can be introduced for affected individuals. Additionally, prevention efforts for those at a high risk for an ED can be improved. The fact that not all of the variables contributing to the development of the disease have been revealed yet, and that these factors are highly individual, makes this even more essential. Given the close linkage between AN and symptoms of major

depression, as well as the possible common genetic basis discovered in this thesis, therapists should be particularly attentive to signs of depression in the early stages of therapy. Any improvement in quality of life for the patient might aid in the recovery process from AN.

By focusing on genes with limited pre-existing data, this study provides an opportunity to expand knowledge about genes associated with EDs and MD. Although high depression rates in AN patients are well-established, the evidence for the underlying cause of this phenomenon remains weak.
Replicating the investigations of this thesis with much larger and more diverse sample sizes could confirm the results and suggest new directions for scientific research.

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