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Molecular Pathways Analysis through Multi-Omics Approaches to Study the Biological Basis of Anorexia Nervosa

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ABSTRACT

Anorexia nervosa is a complex and multifactorial disorder with a high genetic component and severe metabolic consequences. Despite its high heritability, the molecular mechanisms underlying AN remain unclear, and current treatment strategies are often ineffective. Recent advancements in genomics and metabolomics offer promising tools for understanding the genetic and biochemical pathways involved in AN. This study aimed to investigate the genetic and metabolomic basis of AN using next-generation sequencing, polygenic risk scoring, and hair metabolomic profiling, with the ultimate goal of improving diagnosis and treatment strategies.

This study analyzed three distinct case studies involving AN patients. The first case study involved sequencing the DNA of 68 AN patients using a panel of 162 genes to identify rare variants associated with the disorder. The second case study expanded the cohort to 135 AN patients and included the calculation of PRS using a newly designed panel of 163 genes and 730 single nucleotide polymorphisms. The third case study focused on metabolomic analysis of hair samples from 25 AN patients and 25 healthy controls, with an emphasis on amino acid and carnitine profiles. NGS, statistical analysis, and machine learning algorithms were employed to analyze genetic variants and metabolomic profiles.

In the first case study, rare variants in key genes were identified, suggesting a potential monogenic form of AN. The second case study revealed a significant genetic contribution to AN risk, with the PRS explaining 21.3% of the variance in the phenotype. Variants in genes related to dopamine signaling, skeletal muscle function, and appetite regulation were identified, highlighting diverse molecular mechanisms contributing to AN. The third case study

demonstrated significant metabolic alterations in AN patients, particularly deficiencies in branched-chain amino acids and essential amino acids. These findings suggest a biochemical basis for appetite dysregulation observed in AN patients. Given the emerging evidence from genetic studies, genetic testing in AN is becoming a clinical reality, with identified candidate genes such as *NNAT*, *EPHX2*, *LEP*, and *MC4R* offering the potential to tailor treatments, predict disease progression, and assess recurrence risk within families. Moreover, genetic testing is crucial for distinguishing between true AN and syndromic forms that mimic AN but have distinct etiologies, enabling more accurate diagnoses and avoiding mismanagement.

This study provides insights into the genetic and metabolic foundations of AN. The integration of genomic and metabolomic data highlights the potential for personalized medicine approaches in AN diagnosis and treatment. Genetic testing, particularly for rare variants, could play a key role in early diagnosis and risk assessment, while targeted nutritional interventions may address the metabolic imbalances associated with the disorder. This study investigates a large Italian cohort of 228 anorexia nervosa (AN) patients to validate genetic associations with AN. We developed a genetic test targeting syndromic forms that mimic AN phenotypically but have distinct underlying etiologies. Our analysis identified key variants in genes such as *NNAT*, *EPHX2*, and *ESR2*, highlighting their role in AN pathogenesis. Syndromic forms related to genes like *ARG1* and *ALPL* were also explored. This genetic test represents a crucial tool for differential diagnosis, improving clinical outcomes for patients with AN and its syndromic forms. Future research should focus on expanding these findings to larger cohorts and further exploring the interaction between genetic predisposition and environmental factors in AN.

CHAPTER 1 - INTRODUCTION

1.1 ANOREXIA NERVOSA

Anorexia nervosa (AN) is a psychiatric eating disorder and serious mental illness, more common in women than men and often with adolescent onset [1]. According to DSM-V (the Diagnostic and Statistical Manual of Mental Disorders), the diagnostic criteria are restriction of energy intake, abnormally low body weight, intense fear of gaining weight and body-image disturbance [2]. AN causes fatigue, dizziness, and syncope [3], leads to infertility and hair loss, and has the highest mortality rate (5/1000 per year) of all psychiatric disorders [4].

Although AN is being considered predominantly a mental disorder, several indications point to a biochemical basis of this disease. In fact, recent studies identify AN as a complex multifactorial disease with a strong genetic component. Linkage studies show that genetic factors play a major role in the development of AN [5]. Twins show an inheritance of 50-60% [6] and studies on relatives of patients with AN show a higher probability (11-times greater risk [3]) of developing the disease than relatives of unaffected individuals [7].

Patients suffering from AN are characterized by inadequate food intake. Food intake is a conserved behavioral characteristic modulated by genetic polymorphisms (SNPs) [8, 9] and involving at least four biological systems: the serotonin pathway, the dopamine pathway, the endocannabinoid pathway, and appetite-regulating hormones [6].

Serotonin and dopamine are neurotransmitters involved in the regulation of biological processes such as food intake, prevention of depressive states, anxiety control, locomotion, and peripheral functions [10, 11]. Abnormalities in the serotonin pathway can cause persistent

stress and limit neuronal plasticity predisposing to anorexia (**Figure 1a**). While abnormalities in the dopamine pathway can lead to hyperactive exercise and behavioral disturbances, all symptoms associated with AN (**Figure 1b**). Some variants in the *HTR1D* (Serotonin Receptor 1D) [10], *DRD2* (Dopamine Receptor D2) and *DRD4* (Dopamine Receptor D4) genes have recently been identified in patients suffering from eating disorders [11].

The endocannabinoid system plays a significant role in the regulation of appetite. This endogenous cannabinoid neuro- modulator system includes two cannabinoid receptors, CBR1 and CBR2, along with endogenous ligands like anandamide (N-arachidonoylethanolamine), 2-arachidonoylglycerol, as well as the enzymes contributing to their synthesis or degradation, such as monoacylglycerol lipase (MAGL), fatty acid amide hydrolase (FAAH), N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), and diacylglycerol lipase (DAGL) [12]. Genetic variants in the *CNR1* (Endocannabinoid Receptor 1) gene, which encodes the CBR1 (Cannabinoid Receptor Type 1) receptor, might contribute significantly to the susceptibility to AN [13]. The role of the cannabinoid receptors is also supported by studies performed on anandamide (also known as N-arachidonoylethanolamine), a fatty acid neurotransmitter that plays a key role in the in the establishment of synaptic plasticity [14]. Palmitoylethanolamide (PEA) is an endogenous fatty acid amide. The main target of PEA is the peroxisome proliferator-activated receptor α (PPARα). PEA, after the activation of PPARα, brings the signal further through the vagus nerve to the brain. In underweight AN patients, plasma PEA concentration increases after exposure to a non-favorite meal and progressively decrease after eating it, whereas plasma concentrations of PEA progressively decrease in hedonic eating [15].

Genetic factors are estimated to account for about 80% of the risk of developing AN [16]. Various family and twin studies, whole exome analysis and genome-wide and candidate gene association studies suggest a fundamental role of genetics in AN [17]. Management of this disorder requires a multidisciplinary approach. Anorexics expose their bodies to poor nutrition and excessive physical exercise for long periods. This affects their metabolomic profile, making it important to understand which biomarkers and molecular pathways are involved [18]. Metabolomics, one of the omics sciences, is crucial to understand the metabolic impact of diseases. It is concerned with analysis of the metabolome, the set of all metabolites produced by cell regulatory processes [19].

1.2 GENOMICS AND METABOLOMICS OF ANOREXIA NERVOSA

By twin and family studies, it was extensively demonstrated that eating disorders are heritable [17, 20, 21]. Subsequent genome-wide association studies (GWAS) were fundamental to understand the molecular mechanisms involved in eating disorders. In particular, genetic risk score from GWAS results can be very useful in clinical practice [22]. Currently, there are especially GWAS results on anorexia nervosa with multiple genetic loci identification, suggesting that several genetic variants are associated with AN disease risk [23]. Also, the role of rare and structural variants in eating disorders was explored by studies of whole-exome and whole-genome analyses. Instead, gene expression studies had offer insight into the genes and molecular mechanisms that influence phenotypes [17]. Several neuropeptides, neurotransmitters and hormones are involved in eating disorders. The complex brain homeostatic control of feeding involves neural circuits located in the hypothalamus (hunger

signals, initiating feeding behavior) and the brainstem (satiation signals, limiting meal size) [24]. Hypothalamic NPY/AgRP neurons produce Neuropeptide Y and Agouti-related peptide responsible for an orexigenic signal increasing the ACTH, cortisol and prolactin release and involved in appetite regulation. These neuropeptides are associated to high food intake with up-regulation in AN [25]. Instead, orexins are orexigenic neuropeptides involved in endocrine system regulation, with an important function in insulin, glucagon, and leptin secretion in response to glucose [26]. Another neuropeptide involved in eating disorders is Proopiomelanocortin (POMC) is an anorexigenic peptide at the hypothalamic ARC regulated by leptin [27]. Several research connect also the peptidic hormone oxytocin signaling and eating disorders. Specific oxytocin receptor genes polymorphisms have been found [28]. Neurobiological mechanisms underlying eating disorders might involve an overreaction of the immune system, generating, in turn, a dysfunction of neuropeptide signaling. Also, the brain-gut-microbiota axis allows a bidirectional communication between gut microbes and the brain through endocrine, neural, immune, and metabolic pathways [24]. Although determining the cause of eating disorders is complex because there are both genetic and environment factors that can contribute to their development, there is growing scientific interest to identifying causal genes of eating disorders. Genetics research can improve knowledge about the heritability of eating disorders thanks to new molecular technologies such as Next Generation Sequencing [17].

The genetic mechanisms underlying AN have been the most investigated and studies related to this disorder are those that have provided the most results. AN is a multifactorial disorder with a strong genetic component. The familial nature of AN has already been demonstrated for

several decades, with a heritability range of 33-84% [7-29]. Several genetic studies have made it possible to identify many genetic loci involved in molecular pathways that might lead to anorexia [6].

• Serotonergic genes.

The serotonin or 5-hydroxytryptamine (5-HT) system is involved in food intake, mood, and body weight regulation [30] (**Figure 1.2**). It has been hypothesized that 5-HT activity is altered in the acute illness state of AN. Most positron emission tomography studies of AN patients have targeted the 5HT1A and 5HT2A receptors and 5HTT [31]. In particular, important AN targets could be the *5-HT2A* receptor gene and the 5HT-transporter-linked polymorphic region (5-HTTLPR) [32]. An increase in 5-HT reuptake occurs following the administration of estrogens which alter the mRNA and protein levels of some markers of serotonin [33]. Moreover, AN patients may have *HTR1D* gene variations [10]. Recently, it has been identified a positive relationship between the serotonin transporter gene *SLC6A4* methylation levels and resting-state functional connectivity between the dorsolateral prefrontal cortex and the salience network in AN patients [34]. Anyway, it is unlikely that this pathway is the only one involved in the onset of AN in a subject because it is associated with numerous psychiatric disorders and therefore cannot be considered a specific vulnerability factor for AN [28-30].

• Dopaminergic genes.

The dopaminergic system modulates thinking processes, reward, emotional behavior, substance dependence, feeding and motor activity. Dopamine (DA) is a catecholamine that acts primly through of two G protein-coupled DA, D1 (D1R) and D2 (D2R) receptors (**Figure 1.3**). DA has been implicated in the pathophysiology of AN by preclinical and clinical evidence.

A gene that plays an important role in the dopamine system is DAT1 that encodes a transmembrane protein that regulates dopamine reuptake from synapses and possesses variable number of tandem repeats in its 3'-untranslated region. Polymorphisms in the number of repeats influence DAT1 expression (VNTR 10R/9R). The TaqIA restriction endonuclease site in *DRD2* (rs1800497) has been shown to reduce the density of D2 autoreceptors in the striatum. Moreover, the rs6280 variant in *DRD3* increases the affinity for endogenous dopamine. Recently, it has been demonstrated that AN patients carrying the homozygous variant Gly9Gly genotype in the dopamine D3 receptor have worse symptomatology [35].

• Appetite Regulation Genes.

The communication between gut and hypothalamus involves a huge numbers of appetite hormones (**Figure 1.4**). After stimulation, anorexigenic peptides are released while the levels of the orexigenic peptide ghrelin reduce. Ghrelin is an appetite stimulating hormone produced in the stomach and pancreatic cells that is inversely associated with body mass index (BMI) [36]. In response to prolonged starvation the level of ghrelin in the plasma increases [37]. Leptin is a hormone produced by adipocytes and involved in the food intake and regulation of energy balance [38]. In AN patients the level of plasma circulating leptin in cerebrospinal fluid is reduced (hypoleptinemia) [39]. The serum level of leptin is significantly decreased in AN patients but only moderately increased in obese patients [40]. An increased concentration of NPY, which mediates leptin receptors, is associated to body mass deficiency with high concentrations of leptin, suggesting defects in the regulatory axis [41]. The pancreatic polypeptide (PP) peptide tyrosine-tyrosine (PYY) belongs to NPY family and is post-prandially secreted in ileum and colon with an anorexigenic role [42]. Its peripheral administration

decreases appetite along with weight loss through inhibition of the arcuate hypothalamic nucleus expression of NPY/AGRP [43]. Anyway, serum levels of PYY hormone are less diminished in AN as comparated to BN/BED [20]. Cholecystokinin (CCK) is a peptidic hormone of the gastrointestinal system that promotes satiety but has been also associated with anxiety [44]. CCK plasma levels in AN patients and control group are similar both prior to and after a fooding suggesting a hormonal adaptation. However, in older analysis, in AN patients CKK plasma showed a postprandial increase [45-46]. GLP-1 is a brain-gut peptide that exerts a hormone-neurotransmitter action increasing satiety and inhibiting food intake, energetic expenditure, and insulin levels [47]. GLP-1 level decreases in AN patients, while insulin and glucagon levels increase, indicating an alteration in glucose homeostasis [48]. Oxyntomodulin (OXM), which acts through GLP-1 receptor, inhibits food intake, and reduces plasma levels of ghrelin [36].

• Endocannabinoid genes.

Endocannabinoid system controls food intake through both central and peripheral mechanisms. CB1 and CB2, the cannabinoid receptors, are expressed in multiple brain regions that control food intake [49] (**Figure 1.4**). Genetic variants in *CNR1* and *CNR2* genes, influence food intake and body weight and they have been associated to AN [50]. Systemic and local administrations in animals of both exogenous cannabinoids (i.e. THC) and endocannabinoids increase food intake [51]. CB1 receptor antagonists are hypophagic and reduce body weight [52]. Cannabidiol, quite the opposite, can prevent the hyperphagic effect induced by the CB1 receptor agonist [53-54]. Genetic variants in *CNR1*, which encodes the CB1 receptor, are related to the susceptibility to AN. The basis of the non-Mendelian inheritance of AN could be

associated with CNR1 (AAT)n trinucleotide repeats, but functional studies are needed to prove the differential effect [55]. Anandamide, also known as arachidonoylethanolamine, (AEA) plays a key role in feeding behaviour generating pleasure after food consumption [56]. Plasma levels of this lipid mediator are downregulated in AN patients [57]. In fact, anandamide binds to CB1R and inhibits neuronal differentiation [58]. PEA binds the cannabinoid-like G-coupled receptors GPR55 and GPR119. The anorectic action of exogenous PEA is mediated by transcription factor PPARα in the small intestine [59]. After a high-fat feeding in mouse the concentration of PEA decreases [60]. Plasma PEA concentration increases in AN patients after exposure to a non-favorite meal [61].

By linkage and association studies on AN, chromosomes 1, 2, 4, and 13 were identified as possible regions as- sociated to AN. The analyzed genes were associated to neural signaling, either by neurotransmitters or by hormones affecting the satiety regulatory system in subcortical structures of the brain, such as the hypothalamus. However, the small sample size of these type of studies was a limit and meta-analyses gives disaccord evidence [18].

Several GWAS for the identification of genetic variations related to the disorder were conducted on AN. Until 2019 a single genome-wide locus on chromosome 12 (lead SNP: rs4622308) related to AN was identified in a region that regard also diabetes mellitus type 1 and autoimmune disorders. Interestingly, successively the Anorexia Nervosa Genetics Initiative (ANGI), the Genetic Consortium for Anorexia Nervosa (GCAN), and the Wellcome Trust Case Control Consortium-3 (WTC-CC-3) along with UK Biobank have detected eight chromosomal regions, comprising 120 genes, significantly associated with AN. Analyses in silico and research by available large-scale in vitro data have revealed that four of the genes of these

chromosome regions might be more likely to be associated to the AN etiology: *CADM1*, *MGMT*, *FOXP1*, and *PTBP2* [23, 32]. Interestingly, Watson HJ et al. (2017) [23] described three significantly altered loci correlating AN risk with increased BMI. The genes associated to those loci are *CTBP2*, *CCNE1*, *CARF* and *NBEAL1* [62]. In a large screening of 152 candidate genes by GWAS rare variants associated to AN were identified in *EPHX2* that encodes a protein involved in cholesterol metabolism. Moreover, variants in *ESR2*, encoding the estrogen receptor 2, can be associated with AN in female [3]. However, at the base of the limits of these studies there are several factors, such as the winner's curse, small sample size, moderator variables explaining and lack of heterogeneity of the cohorts [17]. Anyway, the results of these GWAS showed that AN is highly polygenic. By whole genome sequencing and linkage analysis to analyze two families with recurrence of eating disorders, were detected a missense variant cosegregating with the affected family members in *ESRRA*, and a potentially damaging variant in *HDAC4* (histone deacetylase 4) that play a significant role in the estrogen system. transcriptional studies revealed that expression of the *HDAC4* deacetylase repressed the transcription of ESRRA-induced target genes, whereas *ESRRA* and *HDAC4* exhibited interaction in both in vivo and in vitro studies. For which variants in *ESRRA* and *HDAC4* cause a decrease in the activity of *ESRRA* and an increase in the likelihood of AN onset [63]. By familial whole-exome analysis, were been identified variants of *NNAT* in two male AN probands: one nonsense variant (p.Trp33*) and one rare variant in the 5′UTR. Moreover, by a large screening were identified 11 *NNAT* variants in AN patient (40% male and 6% female) 2[64]. In an another whole-exome sequencing study were identified genes carrying damaging variants belonged to three pathways: neuropeptide hormone signaling, inflammatory pathway, and

cholinergic neurotransmission [65]. Recently, have been sequenced the whole exome of one family and found three ultra-rare deleterious variants of *DRD4*, *NMS*, and *CCKAR*, linked with the reward pathway, in three affected members. In the other study, the authors identified de novo variants in *CSMD1*, *CREB3*, *PTPRD* and *GAB1* involved in the dopamine pathway and neuron differentiation [66, 67]. Epigenetic mechanism may help initiate and maintain AN. Frieling et al. described higher levels of methylation in the promotors of *DAT1* (dopamine active transporter 1) and *DRD2* (dopamine receptor D2) in AN patients. Other study linked AN weight loss to hypermethylation and reduced expression of POMC [68].

In addition to evaluating rare Mendelian genetic variants in AN, assessing SNPs can also be highly significant. The Polygenic Risk Score (PRS) is an estimation of the common genetic component of an individual towards a specific phenotype. It is calculated by summing the genotypes across the individual's entire genome, weighted based on the estimated effects derived from Genome-Wide Association Study (GWAS) summary statistics. In the context of AN, the PRS enables the calculation of a score reflecting the polygenic risk of developing AN. This score considers the cumulative effect of various common genetic variants, providing a more accurate assessment of individual risk compared to the analysis of single SNPs. Only through the development of algorithms it is possible to calculate the PRS and, therefore, understanding how SNPs accumulate in determining the phenotype of individuals affected by AN. These algorithms facilitate the evaluation of the cumulative effects of multiple genetic variants, aiding in personalized risk assessment and informing prevention and treatment strategies [69].

Metabolomic analysis, a powerful tool that examines the comprehensive profile of small molecule metabolites in biological samples, has emerged as a promising approach to unraveling the underlying metabolic alterations in AN [70, 71]. This approach has been successfully employed in various fields, including clinical diagnostics, drug development, and nutrition research [72]. Metabolomic analysis of biological samples, such as blood, urine, and hair, can offer insights into disease-specific metabolic perturbations and help uncover potential biomarkers for various disorders [73]. The utilization of hair metabolomics has a unique advantage in the study of AN [74]; hair metabolomics captures long-term metabolic changes [75]. I4ndeed, hair strands grow at an average rate of 1 cm per month, preserving metabolites over extended periods [76]. This temporal dimension is particularly relevant for AN, a disorder characterized by chronic and persistent metabolic alterations [77].

Recent advances in mass spectrometry and liquid chromatography have enabled high-throughput and high-resolution metabolomic analysis of hair samples [75]. By profiling the metabolites present in hair, researchers can gain insights into the long-term biochemical processes associated with AN and potentially uncover novel metabolic pathways linked to the disorder [78, 79]. These investigations have identified disturbances in lipid metabolism, amino acid metabolism, and energy production in AN patients [80]. Amino acids, as central components of protein synthesis and energy metabolism, play a pivotal role in maintaining overall health [81]. Their levels are tightly regulated by intricate metabolic pathways, and any disruptions can have profound effects on physiological processes [82].

Recent research [83] has proposed that individuals with AN may also experience alterations in their perception of chemosensory stimuli. These alterations change the response of taste

receptors and regulators of appetite to essential amino acids (EAAs) and branched-chain amino acids (BCAAs) [84]. Amino acids, as central players in this pathway, serve as building blocks for proteins and enzymes, participate in neurotransmitter synthesis, and contribute to energy production through gluconeogenesis and the citric acid cycle [85]. Dysregulation of amino acid metabolism has been implicated in a range of metabolic disorders, including anorexia, diabetes, obesity, and cardiovascular diseases [86].

1.3 CHEMOSENSORY FUNCTION

Chemosensory signals provide us with multiple cues during social interactions [87]. Chemosensory alterations can affect responses to starvation, as individuals with AN exhibit degree of chemosensory dysfunction [83]. Studies [88] on animals in which the availability of suitable food is limited show that EAAs regulate food intake. This response is triggered by the detection of nutrients within a brain region, the anterior piriform cortex (APC) [89-91]. When meals with low essential amino acid content are consumed, their concentrations decrease in plasma and brain [92]. As the concentration of intracellular EAAs decreases, the corresponding transfer RNA (tRNA) becomes deacylated [93]. Subsequently, the enzyme general control nonderepressible kinase 2 (GCN2) phosphorylates eukaryotic initiation factor 2 (eIF2), slowing down protein synthesis. This favors the translation of mRNA, regulating gene expression [94, 95].

Following studies [88] on mice, both APC and GCN2 have been identified as EAA chemosensors. APC exhibits sensory function when EAA levels are depleted [96], with GCN2 playing a pivotal role by detecting meals lacking in EAA, binding to deacylated tRNA, and

phosphorylating eIF2, consequently impairing protein synthesis [97]. Numerous studies [88, 98] highlight that reduced food intake of EAAs involves mechanisms in both the hypothalamus and the APC. Research on mice showed a direct connection between these two regions. The hypothalamus contains appetite regulators, like neuropeptide Y (NPY) [99]. Starvation triggers an increase in ghrelin, the hunger-stimulating hormone, which activates NPY [100]. The **Figure 1.5** shows the pathway involved in chemosensory functions related to the food intake.

Recent research subjected mice to a valine-deficient diet, resulting in significant reductions in food intake and body weight. When a diet rich in valine is reintroduced, normal food intake levels return. This suggests that the taste of a valine-deficient diet might deter mice from consuming an EAA-deficient diet [101]. Similar results were observed in pigs [102]; a valine-deficient diet reduced food intake, and this effect was enhanced when an excessive dose of leucine was administered. This could be attributed to the adverse effects of an imbalanced diet in BCAAs, including valine, leucine, and isoleucine, leading to an anorexic response as a protective measure. This response occurs rapidly, likely to safeguard against neuronal pathologies resulting from the imbalance of these three BCAAs [101].

Additionally, research has explored diets centered around valine, isoleucine, and tryptophan to enhance growth performance while also examining the potential negative impacts on growth associated with high concentrations of leucine and lysine [103]. Tryptophan, a precursor of serotonin, a neurotransmitter known for its role in regulating food intake, plays a key role in these studies [101, 102]. Also, it is well-established that an imbalance in BCAAs can impede the brain's ability to absorb tryptophan. This is due to the shared transporters between large neutral amino acids, such as tryptophan, and BCAAs. Consequently, this imbalance can lead

to reduced serotonin synthesis and a decrease in food intake [104]. This finding could hold significant implications for individuals with eating disorders, including AN [105]. Therefore, there is potential for further investigation into the amino acid pathways involved in these processes.

1.4 METABOLISM AND PATHWAYS OF AMINO ACIDS

Valine-deficient, threonine-deficient, and lysine-deficient diets cause mice to cease eating before reaching satiety [106-108]. Rats can detect EAA-deficient diets within just 20-30 minutes, resulting in meal termination [109]. However, when exposed to an EAA-balanced diet after such an experience, their eating rate increases rapidly [110]. Hence, the biochemical and neurological mechanisms responsible for detecting the presence of EAAs are remarkably swift [88]. The APC has been identified as the brain region responsible for sensing EAAs. An EAA-imbalanced diet can rapidly reduce the levels of limiting amino acids in the APC by up to 56% in just 21 minutes [92]. A deficiency in EAAs leads to an accumulation of deacylated tRNA, resulting in such low levels of aminoacylated tRNA that new protein synthesis cannot be initiated [93].

This rapid mechanism of recognizing EAA-deficient diets involves an essential enzyme in initiating new protein synthesis: the GCN2 enzyme, which phosphorylates eIF2 [111]. To further support the role of uncharged tRNA in detecting EAA-deficient diets, micro-injections of tRNA synthetase inhibitors (L-amino alcohols) were performed in the rat APC, resulting in reduced food intake after 20 minutes, simulating the effects of an EAA-deficient diet [112]. L-amino alcohols inhibit the synthesis of charged tRNA, favoring the synthesis of uncharged tRNA. The pyramidal cells of layer II in the APC serve as the output neurons. These cells are excitatory

glutamatergic neurons regulated by GABAergic proteins [113]. In the absence of new protein synthesis, inhibitory proteins critical for controlling the APC's output circuit are quickly lost from the neural membrane [114]. Consequently, the APC cannot maintain its normal balance between stimulatory and inhibitory neurons within the circuit. This leads to the liberation of excitatory glutamatergic neurons, which transmit signals to various brain areas involved in heightened motor activity and the rejection of EAA-deficient diets, resulting in reduced food intake and impaired growth [98].

Leucine activates the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway. This EAA stimulates ribosomal protein S6 kinase (S6K1) and inhibits eukaryotic translation initiation factor 4E binding protein-1 (4EBP1) [115]. mTOR pathway regulation depends on leucine transport [116], and it triggers protein synthesis and cell growth in the presence of EAA-balanced diets. As mentioned earlier, valine-deficient diets decrease food intake, and this effect is exacerbated when leucine intake is elevated [26]. High levels of leucine cause excessive mTOR signaling, adversely affecting normal growth by reducing food intake [117]. However, since BCAAs (valine, leucine, isoleucine) share the same transporters, valine has been shown to hinder the transport of leucine across the blood-brain barrier, mitigating the excessive stimulation of mTOR [103]. Excessive leucine concentrations stimulate the catabolism of all BCAAs (valine, leucine, isoleucine) and not just leucine [103].

A deficiency in these amino acids can alter the expression of growth hormone-insulin-like growth factor-1 (GH-IGF-1). GH promotes the secretion of IGF-1, but this process appears to be contingent on the availability of valine, which can, in turn, inhibit the expression of IGF-1. Consequently, as dietary leucine levels increase, food intake decreases [118, 119]. However,

adding valine and isoleucine to the diet can counteract the negative effects of high leucine concentrations [39]. It is crucial, though, to maintain an appropriate balance and not overconsume leucine. Problems related to food intake and growth are associated with an unbalanced intake of valine, leucine, and isoleucine. An EAA-balanced diet not only increases appetite but also enhances growth. Therefore, a direct signaling mechanism operates to maintain a balanced EAA profile in the diet [116]. The **Figure 1.6** represents the molecular pathways related to BCAAs-deficient or imbalanced diets.

1.5 AIM OF THE PROJECT

The objective of this study is to investigate the genetic and metabolomic foundations of anorexia nervosa (AN) to improve the diagnosis and to achieve a personalized therapeutic strategy for the patients. Understanding these molecular mechanisms is essential for developing targeted treatments, early interventions, and precise monitoring of both disease progression and treatment efficacy. AN management requires a multidisciplinary approach, as the disorder involves prolonged periods of poor nutrition and excessive physical activity, which profoundly affect the metabolomic profile. Metabolomics provides insights into the metabolic disturbances caused by the disease, focusing on the comprehensive analysis of metabolites produced by cell regulatory processes. Recent studies have used targeted metabolomic approaches to explore the metabolic alterations in anorexic patients, aiming to identify specific biomarkers and molecular pathways involved in the disorder.

To this end, the main objective of this study is the development of a multi-omic test using both genomic and metabolomic data for improved early diagnosis and personalized treatment of AN.

Genomic analyses, including the identification of relevant genetic variants and the use of PRS, help define individual susceptibility to AN. Simultaneously, metabolomic profiling offers critical information on the biochemical imbalances resulting from the disease, allowing for a deeper understanding of the affected metabolic pathways.

By generating individualized risk profiles and guiding pharmacological therapies, this multi-omic approach seeks to enhance treatment efficacy, reduce relapse rates, and optimize clinical management. Moreover, it will provide a foundation for developing potential nutritional and adjuvant therapies to support the management of AN. This integrated strategy aims to not only improve patient outcomes but also streamline clinical resources by tailoring treatments to the specific needs of each patient.

CHAPTER 2 – MATERIALS AND METHODS

The genetic association study was performed on an Italian AN cohort. Patients were enrolled if the DSM-V (Diagnostic and Statistical Manual of Mental Disorders, 5th edition) criteria for AN were fulfilled and patients willing to participate in this study from *"Centro Disturbi del Comportamento Alimentare Residenza Palazzo Francisci, Todi (PG), USL Umbria 1"* and *"Ambulatorio per Disturbi del Comportamento Alimentare, Umbertide (PG), USL Umbria 1"*. The clinical diagnosis was verified by psychiatrists with long expertise in eating disorders, in according to DSM-V criteria during a face-to-face interview and clinical data were collected from both AN patients to characterize their demographic and clinical characteristics. The data included age, height, weight, BMI, presence of comorbidities, dietary behaviors, biochemical markers. After obtaining the consent, specific questionnaires and tests were performed for every patient to define their clinical characteristics. All patients gave written informed consent to participation at the time of recruitment. Exclusion criteria included other types of eating disorder and medical conditions causing weight loss. Family members who consented to participate in the study underwent a diagnostic interview with a trained research team member. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board *"Comitato Etico delle Aziende Sanitarie (CEAS) della Regione Umbria"* Prot. N. 29616/12/AV and by *"Comitato Universitario Di Bioetica"* Prot. N. 117677 OF 27th of March 2024. For data protection, all participants were assigned a unique alphanumeric code.

Genetic analysis was performed from a blood or saliva sample using collection kits from Isohelix, United Kingdom (GeneFix DNA/RNA Collection). Samples of genomic DNA of all

subjects were extracted from peripheral blood using a commercial kit (SAMAG DNA Extraction Kit, Sacace Biotechnologies, Italy). DNA was quantified on a Qubit 4 Fluorometer. DNA was sequenced by Next Generation Sequencing (NGS), and available family members were analyzed for variant segregation by Sanger sequencing. A bibliographic study in literature, animal models, pathway and genetic databases was carried out for the identification of genes associated with Mendelian syndromes presenting anorexia, genes that confer greater susceptibility to the onset of anorexia nervosa and candidate genes for which animal models are available: PubMed, Scopus, The Human Gene Mutation Database (HGMD), Mouse Genome Informatics (MGI), Kyoto Encyclopedia of Genes and Genomes (KEGG), Online Mendelian Inheritance in Man (OMIM), The NHGRI-EBI Catalog of human genome-wide association studies (GWAS Catalog). NGS libraries were generated according to the manufacturer's protocol using the Twist Library Preparation EF Kit and Universal Adapter system with Standard Hybridization Target Enrichment (Twist Bioscience). The DNA Library samples were sequenced on an Illumina MiSeq System (Illumina, San Diego, CA) using 150 bp-long reads, according to laboratory methods described elsewhere [120]. Fastq (forward–reverse) files were obtained after sequencing. Read alignment was done with BWA (0.7.17-r1188) software. Duplicates were removed with SAMBAMBA (0.6.7) and GATK (4.0.0.0) was used for realignment. We used the international databases dbSNP (www.ncbi.nlm.nih.gov/SNP/) and Human Gene Mutation Database professional (HGMD; http://www.biobaseinternational.com/product/hgmd) for all nucleotide changes [120]. Our bioinformatic pipeline also evaluated the possible presence of copy number variants (CNVs). Variants with a depth of 10X and quality (Phred-score) above 18 were considered for the

analysis. For variant calling both Samtools (http://www.htslib.org/) and GATK (https://gatk.broadinstitute.org/hc/en-us) tools were used with default settings. In silico evaluation of the pathogenicity of nucleotide changes in exons was performed using VarSome (https://varsome.com/), Mutation-Taster (http://www.mutationtaster.org), Polyphen-2, SIFT, and CADD. Minor allele frequencies (MAF) were checked in the Genome Aggregation Database (gnomAD) and selected all variants with a MAF lower than 1%. DNA of available family members was screened by Sanger sequencing to confirm the presence or absence of selected variants identified in the proband.

Sanger sequencing was performed using the Dye Terminator Cycle Sequencing (DTCS) kit (Beckman Coulter, Fullerton, CA, USA) on a CEQ8800 Sequencer (Beckman Coulter, Fullerton, CA, USA, https://www.beckmancoulter.com/wsrportal/techdocs?docname=A16638aa).

The identification of disease-associated variants involves comparing the genomes of individuals affected by a particular disease with those who do not have the disease. This process, known as GWAS, aims to calculate the frequency of variants that are more commonly found in groups of people with the specific disease. To analyze this information, bioinformatics tools like Plink or PRSice are used, along with statistical models that estimate the risk of developing a particular disease. The outcome of this analysis is the production of PRS, which provide a quantitative assessment of an individual's susceptibility to the disease. The statistical model employed in this process is Logistic Regression, which encompasses several steps, such as calculating genotypic frequencies, evaluating the Hardy-Weinberg Equilibrium, and

conducting association studies using Plink and PRSice, along with Multiple Logistic Regressions.

For the metabolomic analysis, healthy volunteers were recruited following the following inclusion criteria: normal or underweight BMI, gender and age matched with the AN patients. Hair samples were collected from participants by cutting a 1 cm diameter hair strand, starting from the scalp base and extending 4–5 cm towards the tip. Each hair sample was cleaned, dried, and cut into 1 cm long pieces. These hair pieces were placed in glass vials and subjected to metabolite extraction.

Metabolites were extracted from the hair samples using a multi-step procedure. First, 30 mg of hair pieces were immersed in 2 mL of methanol in glass vials. The vials were vortexed and incubated at 50°C for 1 hour. After cooling to room temperature, 2 mL of acetonitrile was added, and the vials were vortexed, followed by centrifugation at 13,000 g for 10 minutes. The organic layers were collected, combined, and dried under N_2 gas. The remaining hair residue was further processed by adding water and adjusting the pH to extract both acidic and basic metabolites. The obtained extracts were then reconstituted in H_2O and CH_3OH (70:30).

Metabolomic analysis was performed using liquid chromatography (LC) coupled with mass spectrometry (MS). LC separation was carried out using a Phenomenex Jupiter C18 column (50 x 2.1 mm, 5 μm particle size). Binary gradient elution with mobile phases consisting of water with 0.2% formic acid (mobile phase A) and acetonitrile (mobile phase B) was employed. The LC system was coupled to an HCT Ultra high-capacity ion trap mass spectrometer equipped with an electrospray ionization (ESI) source. The mass spectrometer was operated in both positive and negative ion modes.

The acquired mass spectrometry data were processed and analyzed using the SANIST software suite, a tool specifically designed for metabolomic data analysis. Statistical comparisons between AN patients and healthy controls were conducted using appropriate tests, and p-values were calculated to assess the significance of differences in amino acid concentrations (level of significance $p < 0.05$). Ratios of specific amino acids were calculated to provide insights into their interplay and potential implications for appetite regulation. With the analytical technique used, it was not possible to distinguish Leucine and Isoleucine molecules, so we report them as a sum value.

In the context of our research, we employed multivariate analysis techniques, specifically Principal Component Analysis (PCA) and Receiver Operating Characteristic (ROC) analysis, with a significance threshold set at p-value < 0.05 to evaluate the robustness of our findings. The ratio of patients with AN to healthy controls (CTR) was calculated based on amino acid concentrations in hair, expressed in nanograms per milligram. Additionally, Partial Least Squares Regression (PLSR) was utilized to identify biomarkers most strongly correlated with anorexia nervosa by comparing patient and control data. Furthermore, we conducted ROC analysis on selected metabolites, presenting maximum sensitivity and specificity values, associated cut-off points, and the area under the curve (AUC).

CHAPTER 3 – RESULTS

The cases examined and analyzed in this PhD project were three in total: the first included 68 AN patients and focused solely on genetic analysis, aiming to identify rare variants; the second involved 135 anorexic patients for the analysis of rare variants and SNPs (single nucleotide polymorphisms) for calculating the PRS (polygenic risk score), using genetic data from 98 anonymized controls obtained from the MAGI database. The third case was related to metabolomic analysis, which was independent of the first two, analyzing hair samples from 25 patients and 25 controls.

3.1 FIRST CASE STUDY RESULTS, 68 AN PATIENTS

The panel used to sequence the DNA of 68 anorexic patients (63 females and 5 males) comprised 162 genes [1]. Median age was 21 years for female patients (range 12–57) and 26 years for male patients (range 13–36). The median BMI of AN patients was 14.13 (range 9.26–18.37). In the sample, 49 patients (72%) had a psychiatric comorbidity, 27 (40%) had anxiety disorders, 28 (41%) depressive disorders, 17 (25%) obsessive disorder, seven (10%) self-harm disorder, two (3%) alcohol dependence and one bipolar disorder. All clinical data, related to these 68 patients, are shown in **Table 3.1** [1]. Only the genetic analysis was conducted on these individuals because they did not want to participate in the metabolomics part of the study because of the hair sampling. Sequencing-QC was for all sample above 80% as per diagnostic guidelines. Average depth coverage of all samples was 114.2X with 99.99% of coding regions covered at 10X and 99.98% covered at 25X. For each subject, all 162 genes of the NGS panel were analyzed. None of them carried pathogenic CNVs. After filtering, a total of 307

heterozygous variants were identified, 68 of whom had a minor allele frequency <1%. Sixteen were predicted to be deleterious by 3 bioinformatics algorithms out of 5. The predicted deleterious variants were identified in *CD36*, *CACNA1C*, *DRD4*, *EPHX2*, *ESR1*, *GRIN2A*, *GRIN3B*, *LRP2*, *NPY4R*, *PDE11A*, *PTGS2*, *PTPN22*, *SGPP2* and *SLC25A13.* The genetic variants identified by NGS are shown in **Table 3.2** [1].

3.1.1 Genes already reported to carry variants in AN patients

In *LRP2* we found a rare missense variant: c.6160G > A; p.Asp2054Asn (rs138269726). *LRP2* is involved in the leptinergic system that regulates fatty tissue volume and the sense of hunger and satiety. Primarily expressed in adipose tissues, leptin is an important anorexigenic hormone active in the hypothalamus. *LRP2* (LDL receptor related protein 2), maps to chromosome 2q31.1 and encodes a multi-ligand endocytic receptor that binds leptin, mediating leptin reuptake in renal tubules and promoting leptin transport across the choroid plexus [121]. LRP2 variants have been associated with AN [122].

A rare missense variant, c.1218G>C; p.Glu406Asp (rs142719624), was found in *DRD4* (dopamine receptor D4) in a proband and her affected mother. This gene encodes the D4 subtype of the dopamine receptor, already associated with attention-deficit hyperactivity disorder and AN [123], and highly expressed in the hypothalamus. *DRD4* was previously involved in familial form of AN [124]. Changes in mRNA expression have been reported in D2 receptor genes in AN patients [125]. Missense variants in *DRD4* co-segregating with AN were recently identified [126].

In *SGPP2* (sphingosine-1-phosphate phosphatase 2) we found a rare missense variant: c.631C > T; p.Arg211Cys (rs371268936). This variant was also identified in the affected brother and the proband's unaffected father. The *SGPP2* gene encodes a protein induced by inflammatory responses that has also been associated with eating disorders [127].

We found a rare missense variant in *EPHX2* (epoxide hydrolase 2), c.86 T > A; p.Leu29His (rs369978603), in an affected individual. The gene encodes a soluble, ubiquitously expressed epoxide hydrolase [128]. *EPHX2* transcription is induced by sex hormones and regulated by the hypothalamic–pituitary–gonadal axis [128]. *EPHX2* may regulate inflammation, blood pressure, and lipid and carbohydrate metabolism [127]. Its variants have been associated with anorexia in 2014 [129].

Given the results of Lombardi et al. [130] on the whole *NNAT* gene in AN, we decided to evaluate the exons (included in the NGS panel) and the promoter (not included in the NGS panel) of *NNAT* by Sanger sequencing. Interestingly, out of 57 probands (52 female, 5 male) we found three different variants (rs149148408, rs148287456, rs180832532) in four patients [1]. Two variants had previously been described by Lombardi et al. [130], while the other has never hitherto been associated with AN. The DNA of the affected aunt of Case 19 [1] was available and confirmed segregation of the variant rs149148408 with phenotype.

3.1.2 Variants in candidate genes

A truncating variant, c.524 T > A; p.Leu175* (rs146120263), was found in the *CD36* gene in one patient. *CD36* (leukocyte differentiation antigen CD36) encodes a platelet-specific surface glycoprotein that serves as a thrombospondin receptor and functions as cell adhesion molecule.

It binds long-chain fatty acids and is involved in the regulation of fatty acid transport. Variations in plasma lipids induced by low-fat and fat-free diets are sensed by neurons of the ventromedial hypothalamus [131].

A novel frameshift variant in *GRIN3B*, c.1395_1396insACGT; p.Gly466ThrfsTer18, was found in one patient. *GRIN3B* (glutamate ionotropic receptor NMDA type subunit 3B) encodes a subunit of an N-methyl-d-aspartate (NMDA) receptor. The protein is mainly expressed in motorneurons, where it constitutes an excitatory glycinergic receptor. *GRIN3B* is part of the endocannabinoid system, a fundamental biological pathway that can regulate feeding behavior [132]. We further identified a rare missense variant, c.2929A > C; p.Asn977His (rs776506065), in *GRIN2A* (glutamate ionotropic receptor NMDA type subunit 2A). This variant was inherited from the healthy father, and interestingly, family members reported to have anorexia were on the paternal side. Those cousins were unfortunately not available for genetic testing to confirm segregation of the variant in the family. The gene encodes a member of the glutamate-gated ion channel protein family involved in hyperactivity and eating disorders, including AN [133]. *GRIN2A* is fundamental in determining behavior, and among many other functions, controls addiction formation and mood. Interestingly, anti-NMDA receptor encephalitis has been shown to result in a classical AN-like syndrome in humans [134]. Moreover, the *GRIN2A* gene was recently shown to be involved in the progression of activity-based anorexia through altered transcription levels in treated mice lacking a functional SIRT1 gene in the brain, which normally induces upregulation of *GRIN2A* [135]. Loss-of-function variants in *GRIN2A* have been associated with a severe autosomal dominant neurological disorder. However, our variant is

missense and may have a less drastic effect on protein function and give rise to a reduced developmental delay and to isolated anorexia.

3.1.3 Genes associated with syndromic forms of AN

A variant in *SLC25A13*, c.1367G>A; p.Arg456His (rs764693182), was detected in one patient and her affected mother. *SLC25A13* (solute carrier family 25 member 13) encodes a carrier, stimulated by calcium, localized on the external side of the inner mitochondrial membrane that mediates aspartate/glutamate exchange across that same membrane. Variants in *SLC25A13* have been reported in association with citrin deficiency and severe anorexia mimicking anorexia nervosa [136]. Although citrin deficiency has autosomal recessive inheritance, we cannot exclude that heterozygous *SLC25A13* variants may result in isolated forms of AN. However, we will investigate whether there is a second variant in deep intronic regions or if this specific variant significantly affects *SLC25A13* expression even when in the heterozygous state.

3.2 SECOND CASE STUDY RESULTS, 135 AN PATIENTS

Then, the panel was expanded to include 730 SNPs with the aim to calculate the PRS for the AN patiens. This new panel of 163 genes and 730 SNPs, as well as the metabolomics panel later designed, remains a trade secret until data are published through scientific articles or patents.

The clinical data collected for the 135 anorexic patients are presented in **Table 3.3**. Regarding the gene panel, the DNA of 135 anorexic patients was sequenced. Sequencing-QC was for all sample above 80% as per diagnostic guidelines. Average depth coverage of all samples was

114.2X, with 99.99% of coding regions covered at 10X and 99.98% covered at 25X. The genomic sequencing NGS was performed in all 135 patients recruited in the study. After obtaining the raw data, based on the ACMG guidelines, the results were filtered, and **Table 3.4** reports the variants considered Pathogenic (P), likely pathogenic (LP), and Variable with Uncertain Significance (VUS), 61 patients in total.

3.2.1 Genes associated with syndromic forms of AN

In Patient 17, a noteworthy variant was found in the *STRA6* gene. This gene, responsible for retinol signaling, caught our attention due to its potential role in disrupting vitamin A balance. Notably, disturbances in vitamin A levels, documented in cases of hypervitaminosis A, include symptoms such as AN [137]. In Patient 50, a variant was identified in the *NF1* gene associated with neurofibromatosis. This finding aligns with previous studies suggesting a potential link between neurofibromatosis and the pathogenesis of eating and feeding behavior disorders. The dermatological alterations characteristic of neurofibromatosis may contribute to the distorted body image observed in AN [138]. Patient 60 exhibited a variant in the *MAT1A* gene. This gene's association with hypermethioninemia, a condition that can manifest asymptomatically but may lead to AN and liver diseases, adds a layer of complexity to our study [139]. *In vivo* studies using animal models highlighted that the absence of this enzyme led to a cessation of eating and subsequent weight loss [140]. Further exploration revealed three distinct variants in the *ABCC6* gene in Patients 39, 42, and 45. This gene is renowned for associating with pseudoxanthoma elasticum, a genetic connective tissue disorder. Notably, a compelling case report [141]

describes AN and severe weight loss alongside classic manifestations, emphasizing the importance of recognizing diverse presentations of the disorder.

3.2.2 Genes already reported to carry variants in AN patients

In the genomic analysis of three patients (Cases 2, 39, and 49), the *GCKR* gene (OMIM *600842) exhibited two distinct variants, both resulting in premature stop codons. Notably, this gene encodes the glucokinase regulatory protein, which is essential for the production of leptin, a hormone pivotal for inducing a sense of satiety and prompting the cessation of food intake. The identified variations in the *GCKR* gene suggest a potential mechanism leading to imbalances in leptin levels, thereby elevating the risk for AN [142]. Patient 2 showed a variant in the *GPR55* gene (OMIM *604107), encoding a G Protein-coupled Receptor known for its binding affinity to endocannabinoids. Additionally, this protein forms complexes with PEA, which has been implicated in the context of AN, suggesting a multifaceted interplay in the neurobiological mechanisms associated with the disorder [143]. A noteworthy commonality emerged in the genetic makeup of twelve patients, all harboring the same variant in the *DRD4* gene (OMIM *126452). This gene encodes the D4 subtype of the dopamine receptor, and its association with AN has been previously documented by our group [1]. Patient 8 presented a variant in the *GRIN3B* gene (OMIM *606651), encoding a subunit of the N-methyl-D-aspartate (NMDA) receptor. This gene has already been associated with AN, and the protein is mainly expressed in motor neurons, constituting an excitatory glycinergic receptor and is part of the endo- cannabinoid system, a fundamental biological pathway that regulates feeding behavior [1]. The genetic sequencing of Patient 9 revealed a variant in the *LEPR* gene (OMIM *601007),

known for encoding the Leptin receptor. Leptin, produced by adipocytes, acts both peripherally and centrally by reducing the appetite and creating an overall negative energy balance [6]. Variants in the *CD36* gene (OMIM *173510) were identified in four patients (Cases 22, 33, 51, and 58). This gene, encoding a thrombospondin receptor and involved in fatty acid transport, may contribute to regulating feeding behavior through its influence on plasma lipid levels. Changes in plasma lipids induced by low-fat and fat-free diets are sensed by neurons in the ventromedial hypothalamus [1]. Further, Patient 38 displayed a variant in the *GHRL* gene (OMIM *605353), encoding Ghrelin. Ghrelin signals to the brain when the stomach is empty, regulating hunger. Variations in this gene have been observed in individuals with eating disorders, suggesting its potential relevance in the manifestation of AN [144, 145].

3.2.3. Genetic variants in candidate genes

In the genomic analysis of three patients (Cases 2, 26, and 61), variants were identified in the *RYR1* gene (OMIM *180901), with one patient exhibiting two distinct variants in the same gene. This gene is implicated in skeletal muscle weakness and loss of muscle tone, shedding light on potential connections between muscular abnormalities and AN [146]. Variants in the *VPS13B* gene (OMIM *607817) were identified in two patients (Cases 4 and 46). While this gene is known for its association with Cohen syndrome in patients with compound heterozygous variants, it also plays a role in adipogenesis, suggesting its involvement in AN [147]. Patient 6 presented a variant in the *TYK2* gene (OMIM*176941), crucial for the formation and development of brown adipose tissue and skeletal muscle, underscoring the intricate interplay between genetic factors and tissue development in AN [148]. The *LMNA* gene (OMIM *150330)

exhibited a mis- sense variant in two patients (Cases 7 and 30), both sharing the same genetic alteration (c.1364G>A – p.Arg455His). This gene is already associated with familial partial lipodystrophy characterized by the absence of subcutaneous adipose tissue [149]. A variant in the *A2M* gene was identified in Patient 11. This gene (OMIM *103950) encodes alpha-2 macroglobulin that is involved in zinc homeostasis, and disruptions in zinc homeostasis may lead to cerebral function imbalances [150]. In addition, in a randomized, double-blind study, zinc supplementation improved weight gain in anorexic patients [151]. Patient 14 presented a variant causing a truncated protein in the *NMU* gene (OMIM *605103), known for encoding an anorexigenic hormone. Neuromedin U plays a physiological role in regulating food intake and partially mediates the effects of leptin, whose treatments were also evaluated [152, 153]. A variant in the *AKR1C1* gene (OMIM *600449) was identified in Patient 15, a gene previously associated with lipedema by our group [154]. In Case 16, variants were found in the *CARF* (OMIM *607586) and *NBEAL1* (OMIM *609816) genes. A study [155] with AN revealed intriguing links between polymorphisms (SNPs) in these genes and decreased body weight, particularly highlighting *CARF*'s involvement in the leptin-melanocortin-BDNF pathway. The *GUCY2C* gene (OMIM *601330), with a variant identified in Patient 18, is implicated in appetite regulation as it is expressed in the hypothalamus [156]. Silencing of the intestinal epithelial transmembrane receptor, encoded by *Gucy2c*, led to obesity and metabolic syndrome in a knockout mouse model [157]. Patient 20 exhibited a variant in the *ATXN1* gene (OMIM *601556), associated with spinocerebellar ataxia, a condition linked to dopa- mine pathway alterations and indirectly connected to AN [158]. Patient 23 presented a variant in the *ESR2* gene (OMIM *601663), responsible for encoding the estrogen receptor 2. Considering the gender prevalence

of AN, genetic variations in estrogen receptors may play a role in its manifestation [1]. A variant in the *AGPAT2* gene (OMIM *603100) was observed in Patient 25. Variants in this gene are associated with generalized lipodystrophy, a condition characterized by a lack of subcutaneous adipose tissue and playing a pivotal role in the synthesis of glycerophospholipids and triglycerides [159]. Patient 26 displayed variants in the *ANK2* and *DNAAF1* genes, with *ANK2* (OMIM*106410) linked to anxiety and hyperactivity phenotypes, both recognized features of AN [160]. *DNAAF1* (OMIM *613190) is responsible for ciliary architecture and its inactivating mutations result in primary ciliary dyskinesia [161]. Interestingly, this case also displays a variant in the *RYR1* gene. A splicing variant in the *ACBD7* gene was found in Patient 27, with scholars [162] suggesting its involvement in hypothalamic control of food intake and energy expenditure via the leptin-melanocortin pathway. Variants in the *ABHD4* gene (OMIM*619728) were identified in three patients (Cases 28, 44, and 59). This gene encodes a phospholipase involved in the metabolism of endogenous endocannabinoids, such as anandamide, which plays a role in establishing synaptic plasticity and regulating food intake [163]. In addition, AEA is positively correlated with excessive exercise, a particular phenotype found in anorexic patients [164]. Patient 29 presented a variant in the *GFRAL* gene (OMIM *617837), part of the *GDP15-GFRAL* axis. It has been determined that the GDP15-GFRAL axis is associated with appetite loss, weight loss, and decreased BMI [165, 166]. Patients 29 and 47 exhibited different variants in the *PDE3B* gene (OMIM *602047), playing an important role in mediating leptin signaling in the hypothalamus and in regulating food intake and body weight [167]. Variants in the *TNXB* gene (OMIM *600985) were found in Patients 35 and 55, with studies indicating hypermethylation in this gene sites in anorexic patients [168]. Patient 36 displayed a variant in

the *GRIN2D* gene (OMIM *602717), encoding the GluN2D subunit of the NMDA receptor for glutamate, one of the most important excitatory neurotransmitters in the human brain. NMDA receptors play a key role in synaptic plasticity and neurotransmission and in the regulation of food intake [169]. Patient 40 exhibited a variant in the *CNTNAP2* gene (OMIM *604569), implicated in synaptic plasticity. Studies on mutant mice in *CNTNAP2* revealed differences in food intake and locomotor hyperactivity compared to control mice [170]. Variants in the *POMC* gene (OMIM *176830), encoding proopiomelanocortin, were identified in Patients 43 and 56. Proopiomelanocortin neurons play a role in appetite suppression by releasing alpha-MSH, which is an anorectic melanocortin-4 receptor (MC4R) agonist [171]. Patient 48 presented a variant in the *LIPE* gene (OMIM *151750), associated with familial partial lipodystrophy. Additionally, a variant in the *MC4R* gene (OMIM *155541) was found in the same patient, underscoring the central role of this gene in appetite regulation. Indeed, the peptide released by POMC, alpha-MSH, binds the MC4R receptor, resulting in the stimulation of satiety and an increase in energy expenditure [172]. Patient 52 displayed a variant in the *AEBP1* gene (OMIM *602981), involved in adipose tissue development and, in an *in vivo* animal model study [173], its functional role on adipogenesis was investigated and suggested as a therapeutic target for obesity.

In terms of polygenic risk score (PRS), using our developed algorithm, we could achieve a preliminary result only, and the cumulative effect of several polymorphisms was calculated, which were found to be statistically significant in the case-control study. SNPs panel sequencing was performed on 135 anorexic patients and 98 controls (selected from our database). We then built a machine learning model to create a predictive model of PRS. The

statistical model used was a logistic regression. We evaluated the Hardy-Weinberg Equilibrium, and we examined the association between a set of SNPs and the disease using logistic regression (**Figure 3.1a**) with Plink and PRSice. The SNPs were weighted (**Table 3.5**), and we calculated the final PRS score for each patient. The mean PRS score was -0.15 for the patients and -0.32 for the controls (**Figure 3.1b**). The PRS had an R-squared value of 0.213 and a p-value of 2.1e-57 (**Table 3.6**).

3.3 THIRD CASE STUDY RESULTS, 25 AN PATIENTS AND 25 HEALTHY CONTROLS

The study was designed to investigate the metabolic alterations in AN through hair metabolomic analysis. A total of 25 female AN patients and 25 age-matched healthy female controls were recruited with the clinical data reported in **Table 3.7**. The metabolomic analysis of hair samples from AN patients and healthy controls revealed significant alterations in amino acid concentrations that exhibited a key role in the metabolic disturbances associated with AN. To achieve this, we employed targeted metabolomics using a panel that included amino acids, carnitines, lipids and endocannabinoids allowing us to specifically identify changes in metabolic profiles and highlight their contribution to the observed metabolic alterations in AN patients. For the evaluation of the study results, only the data related to amino acids and carnitines were evaluated, as they were considered the most significant.

3.3.1 Amino Acid Concentrations

Comparative analysis of amino acid concentrations in hair samples showed distinct differences between AN patients and healthy controls (**Table 3.8**). Notably, propionyl-carnitine and

carnitine concentrations were significantly lower in anorexic patients compared to controls ($p < 0.001$), indicating potential disruptions in lipid metabolism. Propionyl-carnitine and carnitine are involved in fatty acid metabolism, and their reduced levels may suggest altered lipid utilization in anorexic individuals. With the results obtained, it was possible to define the true metabolomic profiles of anorexic patients and of controls. Their comparison enables to build the profiles of an affected person and a healthy one, as shown in **Figure 3.2**.

Leucine, isoleucine, and valine are BCAAs crucial for muscle maintenance and protein synthesis [174]. AN patients exhibited significantly lower concentrations of leucine ($p < 0.001$), isoleucine ($p < 0.001$), and valine ($p < 0.001$) compared to controls, suggesting compromised protein metabolism and muscle preservation in AN. This observation aligns with the known catabolic state associated with anorexia [175].

Alanine, tyrosine, and phenylalanine concentrations were found to be higher in AN patients than in controls. Alanine, a key player in carbohydrate metabolism [176] may reflect increased protein degradation or altered metabolic pathways linked to protein catabolism in AN. Tyrosine and phenylalanine are precursors of neurotransmitters [177], and their elevated levels might contribute to the neurochemical imbalances often observed in AN. **Figure 3.3** and **Figure 3.4** illustrate the alterations in amino acid concentrations in anorexic patients compared to healthy controls. **Figure 3.3** shows the different metabolomic profiles between patients and controls, while principal component analysis (**Figure 3.4**) shows that patients and controls clustered in two distinct groups. Significant differences were observed for propionyl-carnitine, carnitine, leucine/isoleucine, valine, and other amino acids. These alterations point to potential metabolic disruptions associated with AN.

3.3.2 Essential Amino Acid Deficiency and Tryptophan Ratio

An important finding is the significant deficiency of EAAs in AN patients. The total concentration of EAAs was notably lower in AN patients (9.81 ng/mg) compared to healthy controls (18.72 ng/mg), resulting in a ratio of 1.91 (Healthy controls in ratio to AN patients). EAAs play a pivotal role in regulating appetite and food intake, and their deficiency in anorexic patients may contribute to appetite dysregulation and compromised nutritional status [178].

The ratio of tryptophan to valine, leucine, and isoleucine was elevated in AN patients (0.27) compared to controls (0.19), resulting in a ratio of 0.70 (CTR/AN). This finding suggests that anorexic individuals preferentially transport tryptophan over these BCAAs due to their shared transporters [104].

The observed alterations in amino acid concentrations provide insights into the metabolic disruptions in AN. The deficiencies in BCAAs and EAAs point to the importance of these amino acids in maintaining muscle mass and regulating appetite. These findings have potential clinical implications for AN treatment. Restoring BCAAs and EAAs levels through targeted supplementation may support muscle preservation and address appetite dysregulation [179].

3.3.3 Machine Learning Algorithms

The metabolites that emerged as the most significant throughout these iterations are of particular interest, as they possess a remarkable capacity to differentiate between patients with AN and healthy controls. In this regard, we performed a ROC analysis on the selected metabolites, as outlined in **Table 3.9**. This analysis facilitated the determination of optimal cut-off values that classify a sample as either a patient or a control. The ROC curve provides a comprehensive evaluation of the trade-offs between sensitivity and specificity across various thresholds. Then, we utilized the PLSR to assess the potential

of classification models in the context of Anorexia Nervosa (AN) [181]. As demonstrated in **Table 3.9**, the features analyzed correspond to various metabolites, which are pivotal for identifying key biomarkers associated with AN. To enhance the reliability of our findings, we conducted 100 iterations of analysis [182], where each iteration involved randomly partitioning the dataset into a training set (75% of the data) and a testing set (25% of the data). The results were consistently encouraging, with the model achieving a high correlation of 98%.

For instance, both Propionyl-Carnitine and Carnitine exhibited maximum sensitivity and specificity values of 1.0 at cut-off points of 0.5825 and 0.0255, respectively, indicating their exceptional utility as potential biomarkers. Leucine/Isoleucine and Valine also demonstrated high sensitivity (0.88) and specificity (1.0), with area under the curve (AUC) values of 0.94, thereby reinforcing their significance in the classification process.

Table 3.10 further elucidates the importance of these metabolites in the context of AN. The importance scores reveal that Propionyl-Carnitine ranked the highest, with a score of 100, followed by Carnitine (93.04), Leucine/Isoleucine (82.01), and Valine (71.64). These values indicate the degree of correlation each metabolite has with the classification outcome, substantiating their potential role as biomarkers.

In summary, our analysis not only highlights key metabolites with strong associations to AN but also underscores their diagnostic potential through rigorous machine learning methodologies and ROC analysis.

CHAPTER 4 – DISCUSSION

About the first casuistry, female AN patients had at least one affected family member, whereas male patients with AN are so rare that our population only included sporadic cases. The extreme rarity of male AN makes a genetic origin of the disease seem extremely likely, as already shown in other studies [130]. Familial segregation studies were used to confirm the possible involvement of identified variants in the etiology of AN.

We concentrated on the familial form of AN for a number of reasons. Although AN is highly heritable and forms family clusters, the genetic causes are difficult to ascertain. To date, GWAS studies have encountered problems in identifying associated genetic loci, probably due to the high genetic and etiological heterogeneity of AN. Choosing familial AN was fundamental, because it enabled us to perform segregation analysis and to select only those variants found in affected individuals. To our knowledge, few studies have conducted NGS in families with recurrence of AN [130, 124, 183], and only one had a similarly numerous cohort [130].

The major finding of our study is the confirmation that *NNAT* promoter variants are consistently found in patients with AN. The first study to report this association was published in 2020, when Lombardi et al. reported that *NNAT* variants were associated with AN in 40% of male and 6% of female AN patients [130]. In our cohort, we found three variants in *NNAT* in four probands (6%), all located in the promoter region. Two variants were previously reported by Lombardi et al. [130], while one variant has never hitherto been associated with AN. The *NNAT* gene encodes a proteolipid protein that functions as a regulatory subunit of ion channels. It is involved in the maintenance of segment identity in the hindbrain and pituitary development, and maturation or maintenance of the overall structure of the nervous system [184]. Furthermore,

we may have identified a patient affected by a syndromic AN form with a likely pathogenic variant in *PDE11A*, a gene responsible for autosomal dominant Cushing syndrome, that includes anorexia among its features [185, 186]. Genetic testing is essential to characterize the molecular pathways involved in AN and to allow a differential diagnosis with other syndromes that might be confused with AN. Our study also suggests that monogenic forms of AN are probably more common than previously thought and their identification may allow a tailored treatment. It is now clear that implementation of a comprehensive genetic test for the diagnosis of AN is necessary. This was also clearly stated in an editorial of The British Journal of Psychiatry, which stressed the need to elucidate the genetic determinants of various psychiatric disorders, including AN, to identify their specific causes in each patient and to estimate the recurrence risk of AN in other family members [187].

Our study also showed that, among various pathways involved in AN, the endocannabinoid one is the most involved. We identified many variants in genes involved in this pathway, five of which have a predicted high impact. Since there are currently no drug treatments for AN, new and tailored treatment options with good efficacy and safety profiles are required. Molecules that modulate specific pathways might be useful for targeted treatment of AN, and this could be the case for patients with variants in genes involved in the endocannabinoid metabolic pathway. Interestingly, in a controlled trial using a cannabinoid receptor agonist (tetrahydrocannabinol), AN patients were found to have high plasma endocannabinoid levels, suggesting dysregulation of the endocannabinoid system [188]. Indeed, PEA has been proposed as targeted treatment for AN patients [189].

Regarding the second casuistry, the primary aim of our study was to investigate the biological underpinnings of AN using a gene panel designed to identify variants associated not only with anorexia itself but also with syndromic conditions that mimic AN but represent distinct pathologies, as we found in the first casuistry. As well as our first case study, Our gene analysis aimed at: identifying variants in genes associated with syndromic forms of AN, pinpointing variants in genes previously linked to rare variants in anorexic patients, and identifying variants in candidate genes for AN based on GWAS studies, murine models, or their known protein functions in molecular pathways relevant to AN [190].

4.1 SYNDROMIC FORMS OF AN

Our study revealed two potential syndromic forms of AN, i.e. hypervitaminosis A and hypermethioninemia. Hypervitaminosis A, characterized by acute Vitamin A toxicity, includes AN among its symptoms. Similarly, hypermethioninemia, validated through *in vivo* studies, includes AN as one of its symptoms. These findings offer potential therapeutic avenues by targeting specific molecular pathways to compensate for metabolic imbalances associated with these syndromes [137, 139].

4.2 ENDOCANNABINOIDS PATHWAY

The endocannabinoid pathway is known for its substantial role in appetite regulation. Notably, cannabinoids induce rewarding effects, including the pleasure sensation experienced after consuming tasty food. Our study pinpointed *GPR55* and *ABHD4* as genes of particular interest within the endocannabinoid pathway. GPR55's binding affinity for endocannabinoids,

coupled with its connection to PEA, is implicated in the intricate regulation of anorexic behaviors. On the other hand, ABHD4, a key player in endocannabinoid metabolism, deserves attention due to its association with AEA. The positive correlation between anandamide and excessive physical activity observed in anorexic patients accentuates the potential relevance of *ABHD4* variants in causing AN. Using an integrated approach and combining genomics and metabolomics data could be a crucial future avenue to gain insight into the molecular mechanisms of endocannabinoids underlying AN [6, 143, 164].

4.3 DOPAMINE PATHWAY

Our study highlighted the presence of the same frameshift variant in *DRD4* in 12 AN patients. *DRD4* encodes the D4 subtype dopamine receptor, a neuromodulating catecholamine crucial in regulating emotional behavior, natural motivation, reward, and cognitive functions. In addition to the *DRD4* findings, our study brought attention to a variant in the *ATXN1* gene, which is associated with the dopamine pathway. This reinforces the significance of the dopamine signaling cascade in the context of AN. *ATXN1*, known for its involvement in Ataxin-1-related disorders, adds a layer of complexity to our understanding of how the dopa- mine pathway may contribute to the manifestation of anorexic behaviors. The presence of consistent variants in these genes suggests a potential link between disruptions in the dopamine pathway and the development of AN [6, 158].

4.4 SKELETAL MUSCLE AND APPETITE LOSS

Two notable variants in *RYR1* and *TYK2* genes, both associated with the skeletal muscle,

emerged in our study. *RYR1*, known for its role in skeletal muscle function, is implicated in conditions characterized by skeletal muscle weakness and muscle hypotonia. Our study suggests a potential link between *RYR1* variants and the release of myokines during muscle loss. Myokines, signaling molecules released by muscles, have been recognized for their role in influencing appetite regulation [191]. The interplay between *RYR1* variants and myokine release could explain how skeletal muscle alterations affect appetite modulation in the context of AN [145]. *TYK2*, involved in the formation and structuring of the musculoskeletal system, further underscores the connection between skeletal muscle alterations and appetite regulation [148].

4.5 APPETITE-REGULATING HORMONES

The gastrointestinal tract houses endocrine cells releasing an array of hormones in response to nutrient intake, intricately regulating post- prandial satiety through the gut-hypothalamus axis. Identified variants in *LEPR* and *GCKR* genes highlight their roles in the intricate dynamics of appetite regulation. Leptin, produced by adipocytes, exerts profound control over food intake and energy expenditure. Acting both peripherally and centrally, it fosters reduced appetite and an overall negative energy balance. Variants in *LEPR* underscore its relevance in AN, bridging adipose tissue signaling with appetite regulation [6, 142]. We also identified a frameshift variant in the *GHRL* gene, encoding ghrelin. Ghrelin, produced in the stomach and pancreatic cells, exerts a wide range of effects on feeding behavior, reproduction, and growth, resulting in a positive energy balance. The finding of variants in this gene in individuals with eating disorders suggests its possible relevance in the onset of AN [144]. Another variant of considerable interest has been identified in the *NMU* gene, known to encode an anorectic

hormone Neuromedin U that plays a crucial physiological role in regulating food intake and partially con- tributes to the effects of leptin [152, 153]. The melanocortin pathway revealed intriguing variations in the *POMC* and *MC4R* genes. This pathway holds significant interest due to the pivotal role played by proopiomelanocortin. Proopiomelanocortinergic neurons contribute to appetite suppression by releasing alpha-melanocyte-stimulating hormone (α-MSH), an agonist for the anorexigenic melanocortin-4 receptor (MC4R) [171]. Identifying a variant in the *MC4R* gene underscores its role in appetite regulation. The binding of α-MSH to the MC4R receptor stimulates satiety and enhances energy expenditure [172] A noteworthy finding in a candidate gene, *PDE3B*, was observed in two patients. This gene plays a key role in mediating leptin signaling in the hypothalamus, thereby significantly affecting hypothalamic functions related to energy homeostasis, regulation of food intake, and body weight [167].

4.6 ADIPOGENESIS AND ADIPOGENIC CONTROL PATHWAY

The adipogenesis pathway is pivotal in understanding the challenges faced by anorexic patients, marked by the deficiency of subcutaneous adipose tissue. In this context, our study highlighted two genes, *LMNA* and *VPS13B*, emerging as significant players with variants detected in four cases each (two variants in *LMNA* and two in *VPS13B*). *LMNA*'s association with familial partial lipodystrophy, characterized by the absence of subcutaneous adipose tissue, underscores its potential implication in the pathogenesis of AN [149]. Simultaneously, the involvement of *VPS13B* in adipogenesis suggests a multifaceted genetic contribution to AN [147]. Furthermore, genetic variants in the *AGPAT2* and *LIPE* genes, both linked to lipodystrophies, emphasize the intricate genetic landscape associated with AN [159, 192]. The *AEBP1* gene,

crucial for adipose tissue development, was scrutinized for its functional role in adipogenesis, presenting itself as a potential therapeutic target for obesity based on insights gleaned from an *in vivo* animal model study. Identifying these genetic markers provides valuable clues to the complex interplay between genetics and AN, offering potential avenues for targeted therapeutic interventions [173].

The PRS case/control study results have provided valuable insights into the genetic variability associated with the phenotype under study. Specifically, the analysis revealed that the PRS explains approximately 21.3% of the genetic variance in the phenotype according to our casuistry. In other words, the SNPs we selected for the PRS calculation are responsible for about the 21.3% of the variability observed between cases and controls. This is a substantial contribution, indicating that the genetic markers included in our model play a significant role in determining susceptibility to the condition.

Furthermore, the statistical significance of these findings is extremely strong, with a p-value of 2.1×10^{-57}. This very low p-value means that the observed association between the PRS and the phenotype is highly unlikely to have occurred by chance. The strength of this evidence suggests that the selected genetic variants are not only relevant but also provide a robust and meaningful prediction of the risk for the condition in question. However, it is important to note that our study has limitations related to our sample, and the results obtained are preliminary, requiring further investigation for a more comprehensive understanding.

The third case study was carried out to analyze metabolic alterations in AN through the analysis of amino acid concentrations in hair samples. The results provide valuable insights into the intricate biochemical changes associated with this complex and debilitating disorder. However,

it is essential to consider the role of the chemosensory pathway in these metabolic changes. This pathway plays a pivotal role in shaping eating behaviors and is influenced by various amino acids. The observed alterations in amino acid concentrations underscore the extent of metabolic disturbances in AN and other metabolic disorders [193]. The reduced levels of propionyl-carnitine and carnitine are of particular interest, as they reflect potential disruptions in lipid metabolism [194]. These two molecules play a crucial role in fatty acid transportation into mitochondria for energy production [195]. The scarcity of propionyl-carnitine and carnitine in AN patients may suggest altered lipid utilization pathways, aligning with the energy conservation strategies often observed in individuals with AN [196]. The decreased concentrations of leucine, isoleucine, and valine in AN patients underscore the alterations in chemosensory function and, therefore, in reduced food intake and excessive physical activity, the two main phenotypes of the AN patients[197]. BCAAs are vital for maintaining muscle mass, and their deficiency in AN may contribute to muscle wasting and catabolism [198]. The reduction of BCAAs is a noteworthy finding, as it provides a biochemical basis for the skeletal muscle loss commonly seen in AN patients [199].

The elevated levels of alanine, tyrosine, and phenylalanine in AN patients indicate potential disturbances in amino acid metabolism [200]. Alanine's role in carbohydrate metabolism makes its increase in AN a plausible reflection of altered metabolic pathways associated with protein catabolism [176]. Similarly, the elevation of tyrosine and phenylalanine aligns with their role as precursors for neurotransmitter synthesis [177], suggesting that neurochemical imbalances might contribute to the neuropsychiatric features of AN. The marked deficiency of essential amino acids (EAAs) in AN patients carries significant implications [201]. EAAs are not only

building blocks for proteins but also key regulators of appetite and food intake [202]. The notable decrease in total EAAs in AN patients points to a potential mechanism contributing to appetite dysregulation [203], and the overall malnourished state observed in AN. The increased ratio of tryptophan to valine, leucine, and isoleucine indicates preferential transport of tryptophan in AN patients. As these amino acids share the same transporters, this alteration might lead to an imbalance in the central nervous system's neurotransmitter production [204]. The preferential transport of tryptophan over the BCAAs could contribute to decreased appetite and the perpetuation of the catabolic state [205].

While the findings of this study offer valuable insights into the metabolic alterations associated with AN, it is crucial to acknowledge the role of the chemosensory pathway in shaping the appetite and eating behaviors of individuals with this disorder. The deficiencies in BCAAs and EAAs underscore the potential benefits of targeted nutritional interventions that aim to restore these amino acid levels [206] Such interventions could have a dual effect of preserving muscle mass and modulating appetite [179].

Given the emerging evidence from genetic studies, the role of genetic testing in AN could be an important tool to be used in clinics. As more candidate genes such as *NNAT*, *EPHX2*, *LEP*, and *MC4R* are identified in AN patients, genetic testing can be employed to diagnose and to tailor treatments, predict disease progression, and assess the risk of recurrence within families. Moreover, it is crucial for differentiating between true AN and syndromic forms that present similar clinical features but have different etiologies, enabling more accurate diagnoses and avoiding mismanagement of the disorder.

The inclusion of genetic testing as a standard clinical tool for AN has the potential to

revolutionize the management of the disorder, providing a personalized medicine approach that could improve outcomes by targeting the underlying genetic mechanisms. This will be particularly valuable in cases where rare genetic variants play a significant role, as evidenced by the growing body of research. Genetic testing could become an essential step in the early diagnosis of AN, especially in individuals with a family history of the disorder, enabling targeted interventions and prevention strategies. As sequencing technologies continue to advance, the ability to identify rare variants associated with AN will improve, making genetic testing more accessible and informative in everyday clinical practice.

While the findings of this study offer valuable insights into the metabolic alterations associated with AN, it is crucial to acknowledge the limitations inherent the preliminary nature of these results. Further research on an independent casuistry is needed to deepen our understanding of these complex metabolic pathways and their implications. Additionally, the deficiencies in BCAAs and EAAs underscore the potential benefits of targeted nutritional interventions aimed at restoring these amino acid levels, which could help preserve muscle mass and modulate appetite and physical and locomotor activity.

CHAPTER 5 - INDUSTRIAL APPLICATION, GENETIC TESTING IN AN

In this project, we present results from one of the largest Italian cohorts, comprising 228 patients with AN, to investigate and validate Mendelian genes associated with both isolated and syndromic forms of the disease. To our knowledge, MAGI is one of the first laboratories in Italy to develop genetic testing for the Mendelian forms of AN. The development of the test considered the canonical criteria for analyzing a gene in diagnostics: identified in at least two independent families where the disease cosegregates with the identified variant, studies conducted to demonstrate the role of this gene in the manifestation of the disease through models, and demonstration of the effect of the variant on the loss of protein function.

Our study, confirming the involvement of genes already identified as causative of AN in other studies of different populations, has demonstrated the utility of this genetic test in the Italian population, identifying both isolated and syndromic forms (listed in **Table 5.1**).

Regarding the genes on isolated AN, *NNAT*, with a role for the ion channel regulation and brain development exhibited rare variants in families with male patients affected by AN, and dysfunction of this gene may influence feeding behaviors and weight regulation. The variants were identified in two independent families with male AN patients, and also variants reported in our study [1, 130], suggesting a role in males affected by AN.

ESRRA is also strongly implicated in isolated AN, particularly among women [3, 63]. This gene plays a key role in the estrogen signaling pathway, which regulates mood, appetite, and energy balance. Genome and exome sequencing in two families with recurrent eating disorders identified variants in the *ESRRA* gene [63]. Additionally, *ESRRA* interacts with *HDAC4*, a gene that regulates energy metabolism, further supporting the hypothesis that dysregulation of the

estrogen pathway contributes to AN pathogenesis. Variants in *HDAC4* were also found in sequencing studies of these families [63, 183].

Another gene involved in the neurobiological mechanisms of isolated AN is *NTS*, a neuropeptide that regulates appetite and body weight. Rare variants in NTS and its receptor *NTSR1* were found in a cohort of 93 AN patients. Animal models have demonstrated the involvement of NTS in appetite and weight regulation [207, 208].

Moreover, the DRD4 gene is important because of its role in mood and behavioral regulation, and dysregulations could contribute to distorted eating behaviors. Variants in *DRD4* were identified through whole-exome sequencing in one family with AN, and in 14 patients reported in this project [1, 124].

In syndromic AN, anorexia occurs alongside other conditions. Variants in the *ARG1* gene, associated with argininemia, cause ammonia buildup in the blood, leading to nervous system damage and AN [209]. Another example is *ALPL*, the gene responsible for infantile hypophosphatasia, a disorder characterized by skeletal abnormalities due to defective bone mineralization. Severe forms of this syndrome can cause also AN [210]. The mitochondrial gene *MTTL1*, associated with cyclic vomiting syndrome, can present with AN with recurrent nausea and vomiting [211]. *SLC4A1* variants, responsible for distal renal tubular acidosis with hemolytic anemia, impair the body's ability to remove acids, causing AN with growth failure [212]. Variants in *SLC25A13*, associated with citrin deficiency, can present with severe AN and with significant weight loss. Genetic testing for *SLC25A13* variants is essential for diagnosing the syndromic AN and implementing proper dietary interventions. Studies, including our own, have identified *SLC25A13* variants [1, 136].

The genetic test for AN, developed as a primary outcome of this project, represents a significant advancement in both diagnosis and management of AN. The ability to distinguish the different forms through genetic testing enables more accurate diagnoses, improving patient outcomes. Our validation of variants associated with both isolated and syndromic AN was performed in a large Italian cohort. In our cohort, considering the genes listed in **Table 5.1**, the genetic test for AN yielded a positivity rate of about 8%. This validation provides a strong basis for integrating genetic testing into diagnostic practice, offering substantial support to healthcare professionals managing patients with AN. The United Kingdom Eating Disorders Genetics Initiative has already begun incorporating genetic testing for AN into clinical diagnostics.

CHAPTER 6 – CONCLUSION AND FUTURE PERSPECTIVES

In this PhD project, we explored the biological basis of AN, a multifactorial disorder with complex biological underpinnings. Our study, divided into three distinct case studies, sought to identify key genetic variants and metabolomic disruptions associated with AN, advancing the current understanding of this devastating condition. Through a combination of NGS, PRS, and metabolomic profiling, we have uncovered valuable insights that hold potential for improving diagnosis, treatment, and prevention strategies for AN.

The first case study highlighted the necessity of comprehensive genetic testing to identify pathogenic variants early on and to detect syndromic forms of AN and enabling personalized treatment approaches. The discovery of genetic variants in syndromic forms of AN, which can mimic its clinical presentation, also underscores the importance of differential diagnosis. This helps distinguish AN from other disorders with overlapping symptoms, allowing for more accurate therapeutic interventions.

The second case study expanded the analysis to a larger cohort using PRS, which demonstrated that genetic factors play a significant role in AN development. The PRS explained a substantial portion of the variability in the phenotype, providing robust statistical support for the role of genetics in AN. Also, we identify rare variants in genes involved in dopamine signaling, skeletal muscle function, and appetite regulation offer new avenues for future research into the molecular pathways contributing to AN. These findings open the possibility of developing pharmacological treatments that target specific pathways in genetically predisposed individuals.

The third case study, which employed metabolomic profiling of hair samples, revealed metabolic alterations that shed light on the biochemical impact of AN. Deficiencies and imbalances in BCAAs and EAAs point to reduced food intake and excessive physical activity, common phenotypes of AN. These findings support the potential for nutritional interventions aimed at restoring metabolic balance, with the possibility of improving health of AN patients.

The implications of these results are interesting. First, they highlight the growing importance of omics sciences—such as genomics and metabolomics—in studying the molecular foundations of complex disorders like AN. Genomic data provide insights into the heritable factors that predispose individuals to AN, while metabolomics offers a detailed understanding of the downstream metabolic consequences of the disease. Together, these approaches can help to elucidate the intricate network of genetic, biochemical, and environmental factors that contribute to AN, paving the way for the development of more effective, personalized therapeutic strategies.

The necessity of further investigation into these molecular pathways is evident. Expanding the genomic analysis to larger, more diverse cohorts and integrating multi-omic data will be crucial to refining our understanding of AN's etiology. Moreover, the inclusion of gene-environment interactions in future studies could offer a more comprehensive view of how genetic predispositions are modulated by external factors. Metabolomic studies should also be extended to include longitudinal data to monitor how metabolic profiles evolve during disease progression and treatment.

Genetic testing will play an increasingly crucial role in the future of AN research and clinical practice. As we continue to identify specific genetic markers linked to AN, genetic testing could

become a standard tool in early diagnosis, risk assessment, and treatment planning. Personalized therapeutic approaches, informed by a patient's unique genetic and metabolic profile, could revolutionize the management of AN, reducing relapse rates and improving patient outcomes. Additionally, understanding an individual's genetic risk may enable preventative measures for those at high risk, potentially altering the course of the disease before it fully manifests. The incorporation of genetic testing as a standard clinical and diagnostic tool for AN has the potential to revolutionize its management, enabling personalized medicine approaches that target specific genetic mechanisms, particularly in cases where rare variants play a crucial role in disease development.

One of the most significant outcomes of this PhD project is the development of a genetic test for AN. This test allows for the identification of syndromic forms that mimic AN but have distinct genetic causes. By enabling accurate differentiation between primary AN and these syndromic forms, the test offers a powerful diagnostic tool that improves patient care and personalized treatment strategies. This innovation stands as a major achievement in advancing the clinical management of AN. In conclusion, this work has demonstrated the value of integrating genomics and metabolomics in the study of AN. The insights gained provide a foundation for the future development of precision medicine approaches, aimed at diagnosing, treating, and preventing this complex disorder. The findings not only advance our understanding of the molecular mechanisms driving AN but also set the stage for future research that will further unravel the complexities of its genetic and metabolic architecture. Through continued exploration and innovation, the hope is that we can develop more effective, tailored interventions to improve the lives of individuals affected by AN.

TABLES

Table 3.1. Clinical data of 68 AN patient (first casuistry).

CHARACTERISTICS	VALUES
Gender: female; male	N=63; 5
Mean age ± SD (years)	21 ± 8
Mean height ± SD (cm)	161 ± 0.06
Lowest mean weight ± SD (kg)	36.69 ± 6.65
Highest mean weight ± SD (kg)	57.95 ± 10.41
Mean BMI lowest ± SD (kg/m^2)	14.13 ± 2.12
Mean BMI highest ± SD (kg/m^2)	21.64 ± 5.09
Mean duration of amenorrhea ± SD (months)	34.9 ± 47.9
Vomiting (number of patients)	34
Water restriction (number of patients)	32
Use of diet pills (number of patients)	7
Abuse of purgatives (number of patients)	17
Abuse of diuretics (number of patients)	5
Psychiatric comorbidities	Count
Anxiety disorders (number of patients)	27
Depressive disorders (number of patients)	28
Alcohol dependence (number of patients)	2
Self-harm (number of patients)	7
Obsessive disorder (number of patients)	17
Bipolar disorder (number of patients)	1
Biochemical blood parameters	Mean
Mean glycemia ± SD (mg/dl)	79 ± 25
Mean azotemia ± SD (mg/dl)	30 ± 11
Mean cholesterol concentration ± SD (mg/dl)	176 ± 59
Mean triglycerides concentration ± SD (mg/dl)	108 ± 136

Mean creatinine concentration ± SD (mg/dl)	0.7 ± 0.1
Mean uric acid concentration ± SD (mg/dl)	3.7 ± 1.3
Mean sodium concentration ± SD (mmol/L)	141 ± 3
Mean potassium concentration ± SD (mmol/L)	4.3 ± 0.4
Mean magnesium concentration ± SD (mg/dl)	2.4 ± 1.4
Mean calcium concentration ± SD (mg/dl)	8.8 ± 2.4
Mean vitamin D3 concentration ± SD (ng/dl)	25.6 ± 14.8
Mean TSHconcentration ± SD (*μIU/ml*)	2.6 ± 4.2
Mean GOT concetration ± SD (U/L)	26.2 ± 12.3
Mean GPT concetration ± SD (U/L)	28.9 ± 22

Table 3.2. Genetic variants identified by NGS in the first casuistry of 68 AN patients. All variants identified are indicated by cDNA base sequence and by protein sequence according to the HGVS (Human Genome Variation Society) nomenclature guidelines. Minor allele frequency percentage as reported in GnomAD. DC = disease causing; LP = likely pathogenic; MAF = minor allele frequency; P = pathogenic; US = variant with uncertain significance.

PATIENT	SEX	GENE	NUCLEOTIDE VARIANT	AMINO ACID VARIANT	SNP ID	MAF (%)	VARIANT TASTER PREDICTION	VARSOME EVALUATION	ACMG EVALUATION
Case 1	F	*PDE11A*	NM_001077197.1: c.169C>T	NP_001070665.1: p.Arg57*	rs76308115	0.3	DC	VUS	VUS
Case 2	F	*PTPN22*	NM_015967.7: c.2291dup	NP_057051.3: p.Asn764Lysfs*16	rs751082679	0.006	/	VUS	VUS
Case 3	F	*GRIN2A*	NM_000833.5: c.2929A>C	NP_000824.1: p.Asn977His	rs776506065	0.002	DC	VUS	VUS
Case 4	F	*CD36*	NM_000072.3: c.524T>A	NP_000063.2: p.Leu175*	rs146120263	0.007	DC	P	VUS

Case 5	F	*PTGS2*	NM_000963.4: c.625G>A	NP_000954.1: p.Gly209Arg	rs751815873	0.0004	DC	VUS	VUS
Case 6	F	*CACNA1C*	NM_000719.7: c.2573G>A	NP_000710.5: p.Arg858His	rs786205753	/	DC	LP	VUS
Case 7	F	*CACNA1C*	NM_000719.7: c.1519C>T	NP_000710.5: p.Arg507Cys	rs1416110017	0.005	DC	VUS	VUS
Case 8	F	*DRD4*	NM_000797.4: c.1218G>C	NP_000788.2: p.Glu406Asp	rs142719624	0.001	DC	VUS	VUS
Case 9	F	*SGPP2*	NM_001320833.2: c.631C>T	NP_001307762.1: p.Arg211Cys	rs371268936	0.008	DC	VUS	VUS
Case 10	F	*LRP2*	NM_004525.3: c.6160G>A	NP_004516.2: p.Asp2054Asn	rs138269726	0.1	DC	VUS	VUS
Case 11	F	*SLC25A13*	NM_001160210.1: c.1367G>A	NP_001153682.1: p.Arg456His	rs764693182	0.004	DC	LP	VUS
Case 12	F	*EPHX2*	NM_001256482.2: c.86T>A	NP_001243411.1: p.Leu29His	rs369978603	0.004	DC	VUS	VUS
Case 13	F	*ESR1*	NM_000125.3: c.805C>T	NP_000116.2: p.Arg269Cys	rs142712646	0.09	DC	LP	VUS
Case 14	F	*ESR1*	NM_000125.3: c.1301G>A	NP_000116.2: p.Arg434Gln	rs1356474609	0.004	DC	LP	VUS
Case 15	F	*GRIN3B*	NM_138690.3:	NP_619635.1:	/	/	/	LP	VUS

			c.1395_1396insACGT	p.Gly466Thrfs*18					
Case 16	F	*NPY4R*	NM_005972.6: c.105_108dup	NP_005963.4: p.Ser37Glyfs*16	/	/	/	VUS	VUS
Case 17	F	*PDE11A*	NM_001077196.2: c.328del	NP_001070664.1: p.Cys110Valfs*14	rs573163079	0.1	DC	LP	VUS

Table 3.3. Clinical data of 135 AN patients (134 females, 1 male), second casuistry.

CHARACTERISTICS	**VALUES**
Gender	
Male	1
Female	134
Demographic Data	
Mean age ± SD (years)	22.29 ± 9.03
Mean height ± SD (m)	1.61 ± 0.06
Lowest mean weight ± SD (kg)	37.59 ± 5.73
Highest mean weight ± SD (kg)	56.90 ± 10.55
Mean BMI lowest ± SD (kg/m2)	14.46 ± 1.97
Mean BMI highest ± SD (kg/m2)	21.59 ± 4.29
Excessive physical exercise	
No	30
Yes	89
Not Reported	16
AN Type	
Restrictive	97
Purging	29

Atypical	3
Orthorexia	1
Unknown	5

Table 3.4. Genetic variants identified in 61 patients out of the total 135 patients analyzed by NGS. All variants identified are indicated by cDNA base sequence and by protein sequence according to the HGVS (Human Genome Variation Society) nomenclature guidelines. DC = disease causing; LP = likely pathogenic; P = pathogenic; VUS = variant with uncertain significance.

PATIENT	SEX	GENE	NUCLEOTIDE VARIANT	AMINO ACID VARIANT	RS ID	ACMG VERDICT
Case 1	F	*NAT1*	c.641C>A	p.Ser214Ter	-	LP
Case 2	F	*GCKR*	c.1618C>T	p.Arg540Ter	rs146053779	LP
		GPR55	c.53del	p.(Leu18ArgfsTer2)	-	VUS
		RYR1	c.1654C>T	p.Arg552Trp	rs193922770	P
Case 3	F	*DRD4*	c.849_850insGGCCTCCCCCCGG	p.Ser284GlyfsTer170	rs1290906588	VUS
Case 4	F	*VPS13B*	c.9406-1G>T	-	rs386834119	P
Case 5	F	*MAPK12*	c.536_539del	p.Asp179ValfsTer3	rs746412981	LP

Case 6	F	*PALB 2*	c.104T>C	p.Leu35Pro	rs14104 7069	P
		TYK2	c.2047+1G>T	-	rs15683 33687	P
Case 7	F	*DRD4*	c.849_850insGGCCTCC CCCCGG	p.Ser284GlyfsTer 170	rs12909 06588	VUS
		LMNA	c.1364G>A	p.Arg455His	rs26760 7597	LP
		MAPK 12	c.101dup	p.Ser35LeufsTer1 8	rs53216 3968	LP
Case 8	F	*GRIN 3B*	c.1396_1397insCGTG	p.Gly466AlafsTer1 8		LP
Case 9	F	*DRD4*	c.849_850insGGCCTCC CCCCGG	p.Ser284GlyfsTer 170	rs12909 06588	VUS
		LEPR	c.3495_3496del	p.Ter1166IlefsTer 14	rs75657 1131	LP
Case 10	F	*DRD4*	c.849_850insGGCCTCC CCCCGG	p.Ser284GlyfsTer 170	rs12909 06588	VUS
Case 11	F	*A2M*	c.2126-5_2126-1del	-	-	LP
Case 12	F	*CNBD 1*	c.406G>T	p.Glu136Ter	-	LP
Case 13	F	*DRD4*	c.849_850insGGCCTCC CCCCGG	p.Ser284GlyfsTer 170	rs12909 06588	VUS
Case 14	F	*NMU*	c.186T>A	p.Cys62Ter	-	LP

Case 15	F	*AKR1C1*	c.969T>G	p.Tyr323Ter	rs201500205	LP
Case 16	F	*CARF*	c.2146dup	p.Thr716AsnfsTer5	rs201520695	LP
		NBEAL1	c.3463G>T	p.Glu1155Ter	rs200689887	P
Case 17	F	*STRA6*	c.866-2A>G	-	rs749139729	LP
Case 18	F	*GUCY2C*	c.2662del	p.Arg888GlyfsTer4	rs764325331	LP
Case 19	F	*DRD4*	c.849_850insGGCCTCCCCCCGG	p.Ser284GlyfsTer170	rs1290906588	VUS
Case 20	F	*ATXN1*	c.672_673insTAG	p.Gln224_Gln225insTer	-	LP
Case 21	F	*DRD4*	c.849_850insGGCCTCCCCCCGG	p.Ser284GlyfsTer170	rs1290906588	VUS
Case 22	F	*CD36*	c.1255-1G>A	-	rs375042355	LP
		DRD4	c.849_850insGGCCTCCCCCCGG	p.Ser284GlyfsTer170	rs1290906588	VUS
Case 23	F	*ESR2*	c.335C>A	p.Ser112Ter	rs141516067	LP
Case 24	F	*DRD4*	c.849_850insGGCCTCCCCCCGG	p.Ser284GlyfsTer170	rs1290906588	VUS
Case 25	F	*AGPAT2*	c.37_38insAGC	p.Leu13Ter	-	LP

Case 26	F	*ANK2*	c.11716C>T	p.Arg3906Trp	rs121912706	LP
		DNAAF1	c.1698+1G>A	-	rs139519641	LP
		RYR1	c.4711A>G	p.Ile1571Val	rs146429605	LP
Case 27	F	*ACBD7*	c.194-2A>G	-	rs149110813	LP
Case 28	F	*ABHD4*	c.130del	p.Leu44TrpfsTer16	-	LP
Case 29	F	*GFRAL*	c.1015dup	p.Ile339AsnfsTer36	rs527905870	LP
		PDE3B	c.527_542dup	p.Ser183GlyfsTer162	-	LP
Case 30	F	*AKR1E2*	c.763C>T	p.Arg255Ter	rs763570731	LP
		LMNA	c.1634G>A	p.Arg455His	rs142191737	LP
Case 31	F	*AMT*	c.959G>A	p.Arg320His	rs121964985	P
Case 32	F	*CEP290*	c.1593C>A	p.Tyr531Ter	rs763559949	P
Case 33	F	*CD36*	c.787_808del	p.Val263IlefsTer16	rs754365623	P
		GSDMB	c.622C>T	p.Arg208Ter	rs139970728	LP

Case 34	F	*DRD4*	c.849_850insGGCCTCCCCCCGG	p.Ser284GlyfsTer170	rs12909 06588	VUS
Case 35	F	*MC1R*	c.88C>T	p.Gln30Ter	rs75657 9024	LP
		TNXB	c.12463+2T>C	-	rs54571 9209	LP
Case 36	F	*GRIN 2D*	c.3684_3685insGA	p.Pro1229AspfsTer290	-	LP
Case 37	F	*DRD4*	c.849_850insGGCCTCCCCCCGG	p.Ser284GlyfsTer170	rs12909 06588	VUS
Case 38	F	*GHRL*	c.102_103del	p.Arg34SerfsTer37	rs77152 7525	LP
Case 39	F	*ABCC 6*	c.1553G>A	p.Arg518Gln	rs72653 772	LP
		DRD4	c.849_850insGGCCTCCCCCCGG	p.Ser284GlyfsTer170	rs12909 06588	VUS
		GCKR	c.1618C>T	p.Arg540Ter	rs14605 3779	LP
Case 40	F	*CNTN AP2*	c.3979_3994dup	p.?	-	LP
Case 41	F	*KIT*	c.1247T>G	p.Leu416Arg	-	LP
Case 42	F	*ABCC 6*	c.1799G>A	p.Arg600His	rs76143 3545	LP
Case 43	F	*POMC*	c.706C>G	p.Arg236Gly	rs28932 472	LP

Case 44	F	*ABHD 4*	c.832C>T	p.Arg278Ter	rs37165 1543	LP
Case 45	F	*ABCC 6*	c.801_803del	p.Asn267del	rs11855 30488	LP
		ZMPS TE24	c.1204-5_1210del	-	rs31226 2689	LP
Case 46	F	*VPS1 3B*	c.9406-1G>T	-	rs38683 4119	P
Case 47	F	*MCTP 1*	c.2987dup	p.Asn996LysfsTer 11	rs11915 26672	LP
		PDE3 B	c.463_482del	p.Ala156ProfsTer 177	-	LP
Case 48	F	*LIPE*	c.235del	p.Gln79LysfsTer4 1	rs77070 7778	LP
		MC4R	c.291T>G	p.Asn97Lys	-	LP
Case 49	F	*GCKR*	c.679C>T	p.Arg227Ter	rs14984 7328	LP
Case 50	F	*A2ML 1*	c.2072del	p.Pro691GlnfsTer 2	rs76966 9380	LP
		NF1	c.1260+1G>C	-	-	P
Case 51	F	*CD36*	c.949dup	p.Ile317AsnfsTer3 6	rs70961 716	P
Case 52	F	*AEBP 1*	c.1485+1G>A	-	-	LP

Case 53	F	*NAT1*	c.546dup	p.Asp183ArgfsTer 13	rs77020 9499	LP
Case 54	F	*SCPE P1*	c.619+2T>C	-	-	LP
Case 55	F	*TNXB*	c.12463+2T>C	-	rs54571 9209	LP
Case 56	F	*ALOX 12*	c.1288_1303del	p.Arg430SerfsTer 2	-	LP
		POM C	c.706C>G	p.Arg236Gly	rs28932 472	LP
Case 57	F	*APOA 1*	c.284T>A	p.Phe95Tyr	rs13840 7155	LP
Case 58	F	*CD36*	c.1105_1125+29dup	-	rs78000 2632	LP
Case 59	F	*ABHD 4*	c.679del	p.Arg227AlafsTer 33	rs56568 0365	LP
Case 60	F	*MAT1 A*	c.540dup	p.Lys181Ter	-	LP
Case 61	F	*MAPK 12*	c.101dup	p.Ser35LeufsTer1 8	rs53216 3968	LP
		RYR1	c.4711A>G	p.Ile1571Val	rs14642 9605	LP
		RYR1	c.11798A>G	p.Tyr3933Cys	rs14713 6339	LP

Table 3.5 SNPs identified as a result of PRS analysis performed with an internally validated algorithm by MAGI and reporting: P-Value, Odd-Ratio and PRS-Score for each.

DBSNP	GENE	P-VALUE	ODD-RATIO	PRS-SCORE
rs36083386	*ESR1*	1,27E-04	0,15	-1,91
rs4988235	*MCM6*	1,14E-11	0,21	-1,55
rs536706	*OPRD1*	0	0,28	-1,28
rs12444979	*LOC105371116*	0	0,28	-1,27
rs56149994	*INSR*	0	0,3	-1,2
rs11174202	*TAFA2*	0	0,31	-1,18
rs2346061	*CNDP1*	6,71E-05	0,38	-0,96
rs266728	*RFC4*	0	0,39	-0,95
rs12773846	*LHPP*	0	0,39	-0,93
rs1194197	*CD36 (Intron)*	0	0,41	-0,9
rs1518395	*VRK2*	0	0,44	-0,83

Table 3.6. Summary of PRS-score results for AN patients and controls.

GROUP	MEAN PRS SCORE	PRS R-SQUARED	P-VALUE
Anorexic Patients	-0.15	0.213	2.1e-57
Controls	-0.32		

Table 3.7. Clinical data of 25 all-female AN patients and 25 all-female healthy controls third casuistry. *Mean ± SD

CHARACTERISTICS	VALUES (AN)	VALUES (CTR)
Mean Age (Years)*	20 ± 5	24 ± 2
MEAN HEIGHT (cm)*	162.78 ± 1.52	163.37 ± 3.24
LOWEST MEAN WEIGHT (kg)*	43.96 ± 5.6	59.54 ± 5.63
HIGHEST MEAN WEIGHT (kg)*	59.14 ± 9.43	71.36 ± 8.78
MEAN BMI LOWEST (kg/m²)*	16.14 ± 1.97	19.06 ± 1.42
MEAN BMI HIGHEST (kg/m²)*	21.30 ± 4.21	26.24 ± 5.76
Fasting (Number of Patients)	16	-
Excessive Physical Exercise (Number of Patients)	20	-

Table 3.8 Amino Acid Concentrations in AN patients and healthy controls. The ratio is between healthy controls, where we found the amino acid in nanograms on milligrams of hair, and anorexia nervosa patients.

MOLECULE	HEALTHY CONTROLS (N=25) [ng/mg]	ANOREXIC PATIENTS (N=25) [ng/mg]	RATIO CONTROLS/ANOREXIC	P-Value
Propionyl-Carnitine	145.784	0.00788	185	1.76E-21

Carnitine	0.08468	0.00396	21	6.12E-14
Leucine/Isoleucine	347.448	133.596	2,6	6.08E-08
Valine	904.788	392.512	2,3	4.38E-07
Threonine	355.312	22.454	1,58	1.38E-05
Aspartate	622.304	431.704	1,44	0.008059868
Methionine	0.05872	0.0408	1,49	0.004126336
Glycine	473.776	418.512	1,13	0.269643362
Alanine	135.496	2.492	0,5	0.005098476
Histidine	0.8236	161.072	0,51	1.29E-06
Tyrosine	144.988	286.876	0,5	2.02E-08
Glutamate	119.932	331.216	0,36	0.008621941
Phenylalanine	0.26048	0.85112	0,3	2.27E-07

Table 3.9 ROC analysis of selected metabolites. The maximum sensitivity and specificity values and their correlated cut-off are shown. Then, we were able to calculate the area under the curve (AUC).

MOLECULES	CUT-OFF	SENSIBILITY	SPECIFICITY	AUC
Propionyl-Carnitine	0.5825	1	1	1
Carnitine	0.0255	1	1	1
Leucine/Isoleucine	23.875	0.88	1	0.94
Valine	73.105	0.88	1	0.94

Table 3.10 Partial Least Squares Regression to identify the biomarkers more correlated to anorexia nervosa, comparing patients and controls data.

MOLECULES	IMPORTANCE_MEAN
Propionyl-Carnitine	100
Carnitine	93.04
Leucine/Isoleucine	82.01
Valine	71.64

Table 5.1 Overview of Genetic Studies and Diagnostic Genetic Testing in AN

GENE	DATA PUBLISHED	DIAGNOSTIC PERSPECTIVE	PATHWAY INVOLVED
NNAT	Identified in two independent families with male individuals affected by AN, also reported in this project.	Helps in identifying male-specific cases of AN; involved in brain development and energy metabolism.	Ion channel regulation, brain development, and energy metabolism.
ESRRA	Exome sequencing, WGS, and linkage analysis in two families with recurrent eating disorders.	Identifies estrogen-related metabolic processes, crucial for female-specific AN cases.	Estrogen signaling, energy balance, interaction with *HDAC4*.
HDAC4	Exome sequencing, WGS, and linkage analysis in two families with recurrent eating disorders.	Provides insights into gene regulation and metabolic dysfunction in AN.	Gene expression regulation, energy metabolism, interaction with *ESRRA*.
NTS	Whole-exome sequencing in 93 individuals with AN and an animal model.	Identifies neuropeptide signaling dysregulation contributing to appetite and weight control.	Neuropeptide regulation, appetite, and body weight regulation.
DRD4	Whole exome study in one family with AN, also reported in this project.	Detects dopamine-related behavioral and mood dysregulation in AN.	Dopamine signaling, mood and behavior regulation.
ARG1	12 novel ARG1 mutations, totaling 66 mutations in 112 patients.	Identifies metabolic conditions like argininemia that mimic AN symptoms.	Urea cycle dysfunction, ammonia accumulation, nervous system damage.
ALPL	Reported by Weiss et al. (1988), consistent with autosomal recessive inheritance.	Distinguishes bone-related metabolic disorders that resemble AN.	Bone metabolism, skeletal abnormalities, autosomal recessive inheritance.
MTTL1	Reported in a family with 4 members spanning 3 generations with cyclic vomiting syndrome.	Identifies mitochondrial dysfunction mimicking AN through cyclic vomiting syndrome.	Mitochondrial function, cyclic vomiting, and energy dysregulation.
SLC4A1	Reported in two Thai families with recessive dRTA due to compound heterozygous mutations.	Differentiates renal tubular acidosis from AN through metabolic	Renal tubular function, acid-base balance, growth regulation.

		dysfunction identification.	
SLC25A13	Two studies, along with our own, identified variants in families with citrin deficiency.	Identifies citrin deficiency presenting as AN, aiding in dietary intervention and management.	Citrin metabolism, energy production, and weight loss regulation.

FIGURES

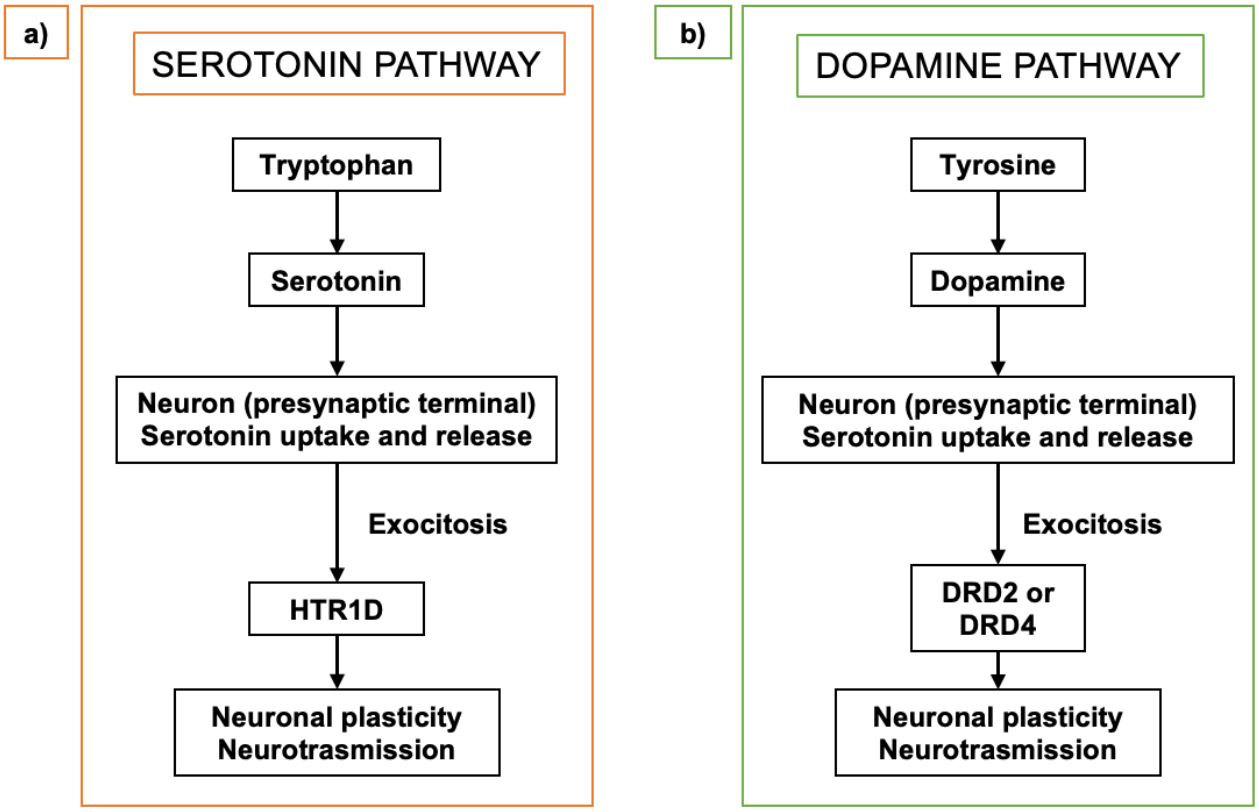

Figure 1.1 a) Serotonin pathway: serotonin is synthesized from tryptophan and by binding the HTR1D receptor has a key role in neuronal plasticity and neurotransmission; **b)** Dopamine pathway: Dopamine is synthesized from tyrosine and by binding DRD2 and DRD4 receptors has an important role in neuronal plasticity and neurotransmission.

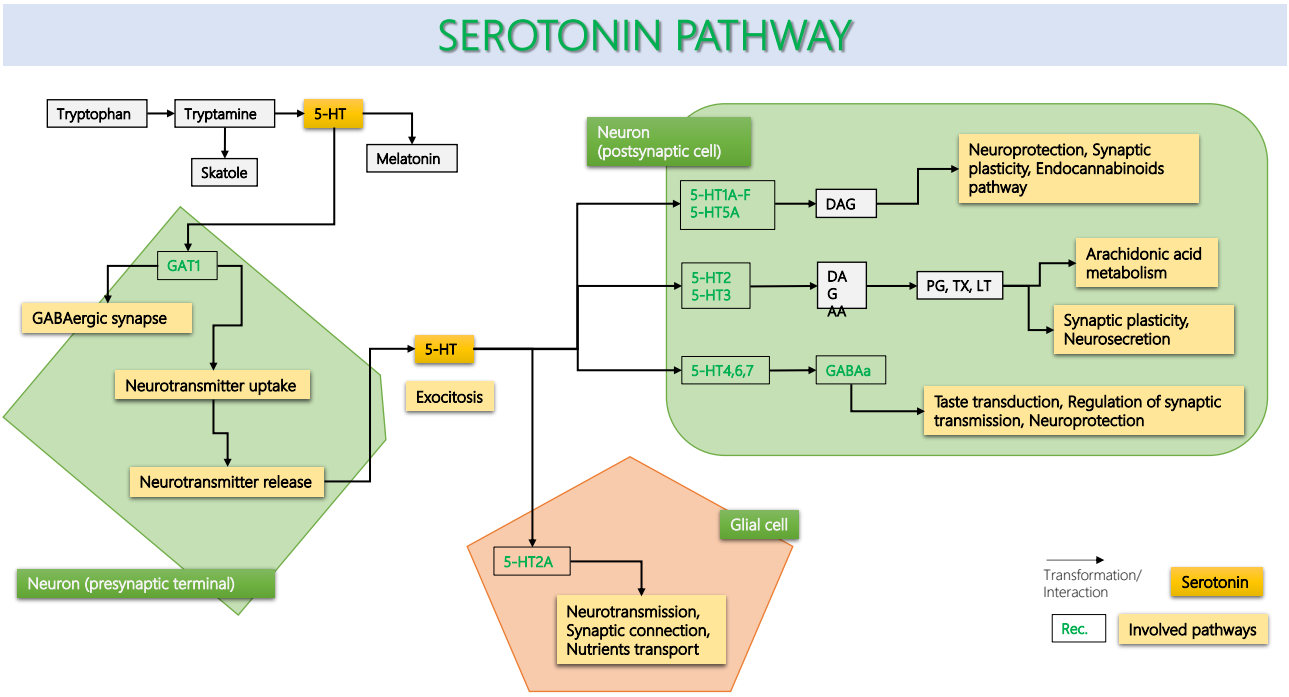

Figure 1.2. Serotonin pathway including key genes and metabolites involved in its biosynthesis, transport, and degradation.

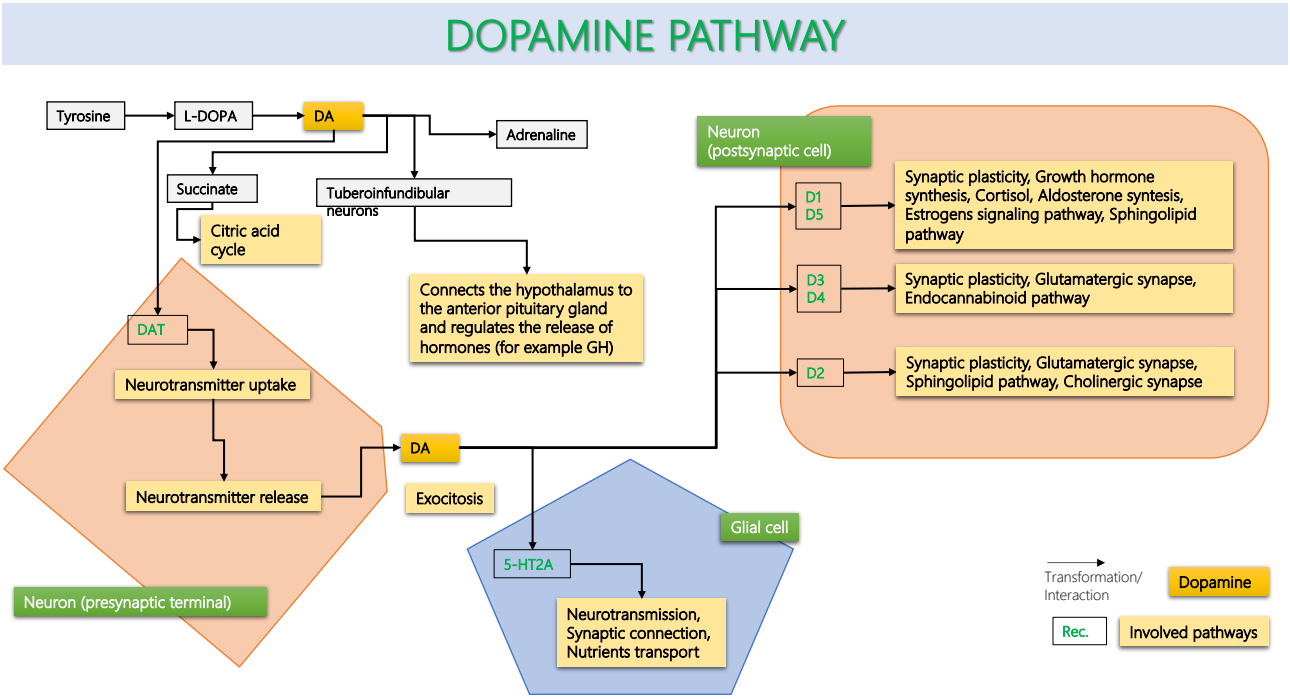

Figure 1.3. Dopamine pathway including key genes and metabolites involved in its biosynthesis, transport, and degradation.

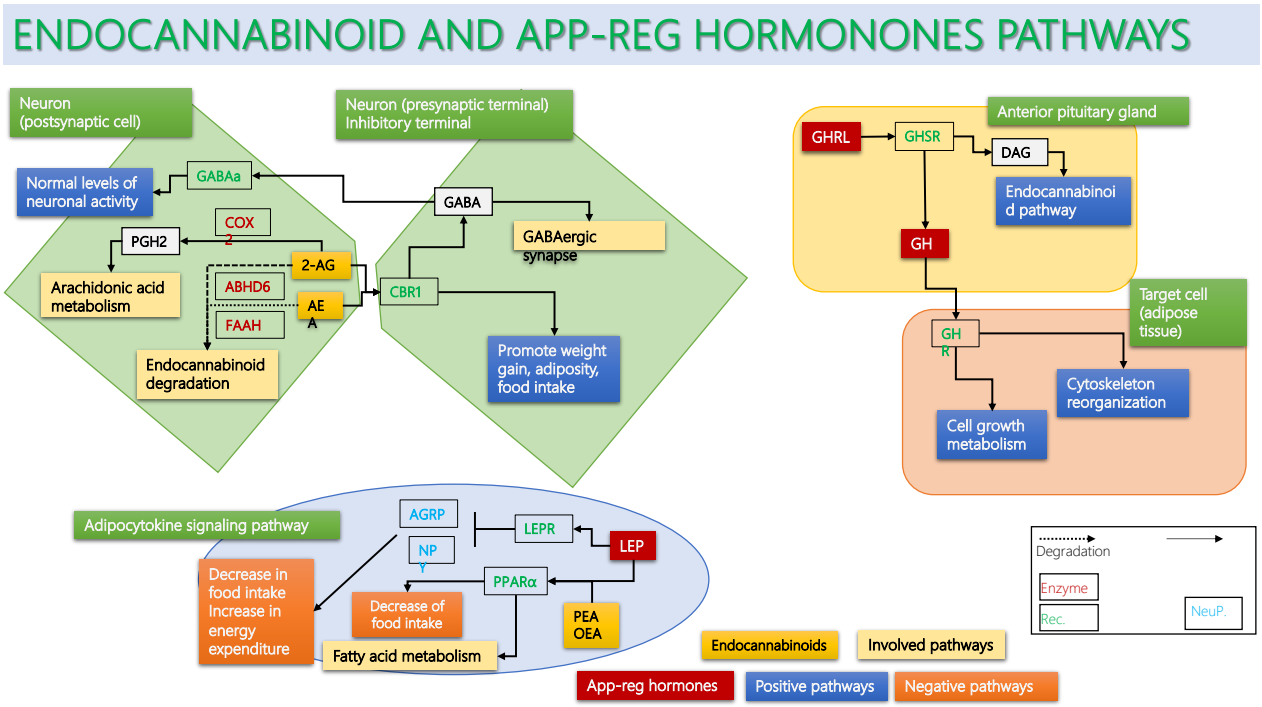

Figure 1.4. Endocannabinoid and Appetite-Regulating Hormones pathway

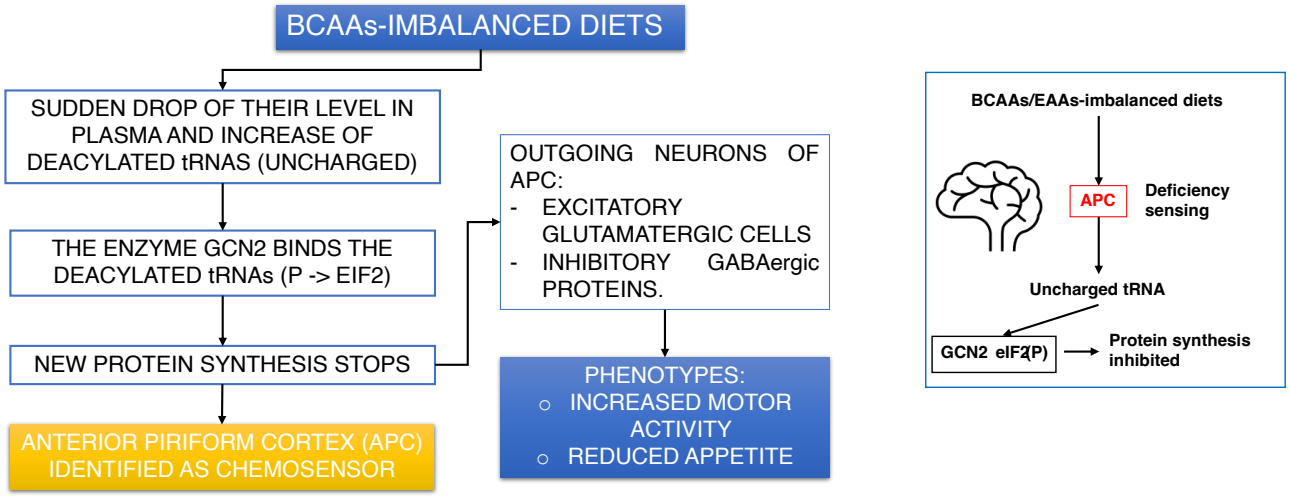

Figure 1.5. Representation of metabolic pathways of the chemosensory function related to the food intake.

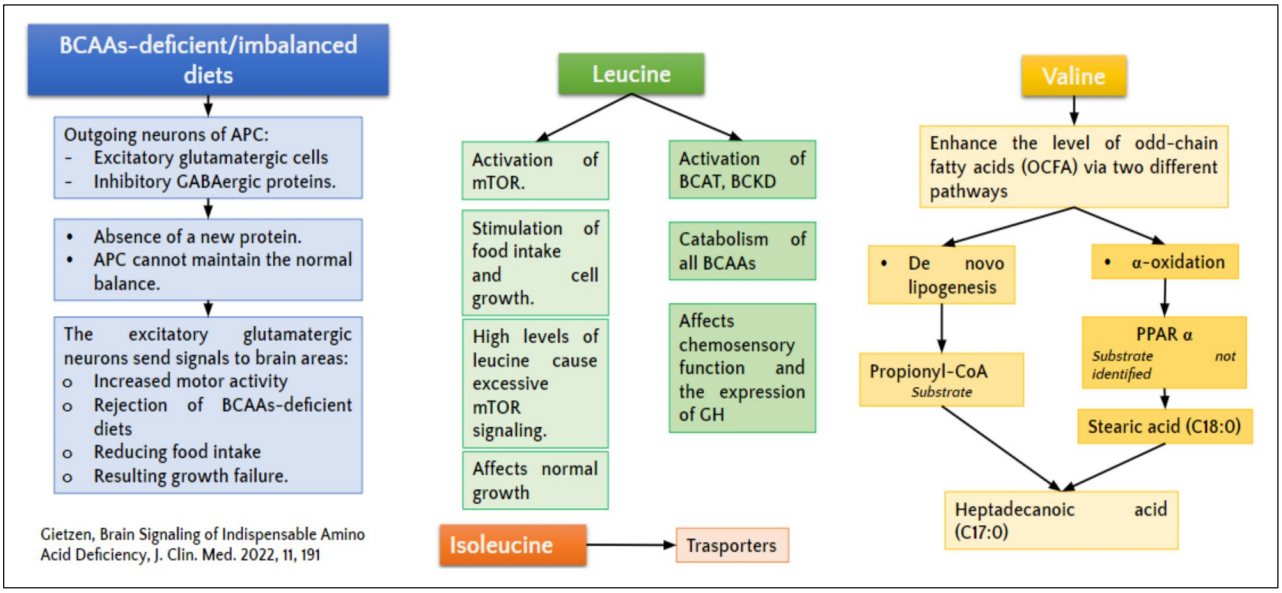

Figure 1.6. Representation of metabolic pathways of BCAAs: leucine, valine, and isoleucine.

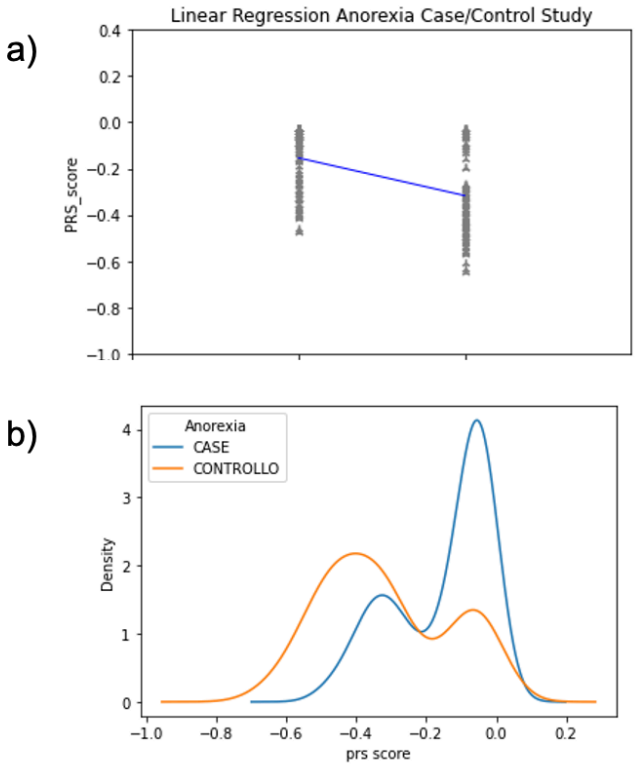

Figure 3.1. a) Linear Regression Model. **b)** PRS-score curve of cases and controls.

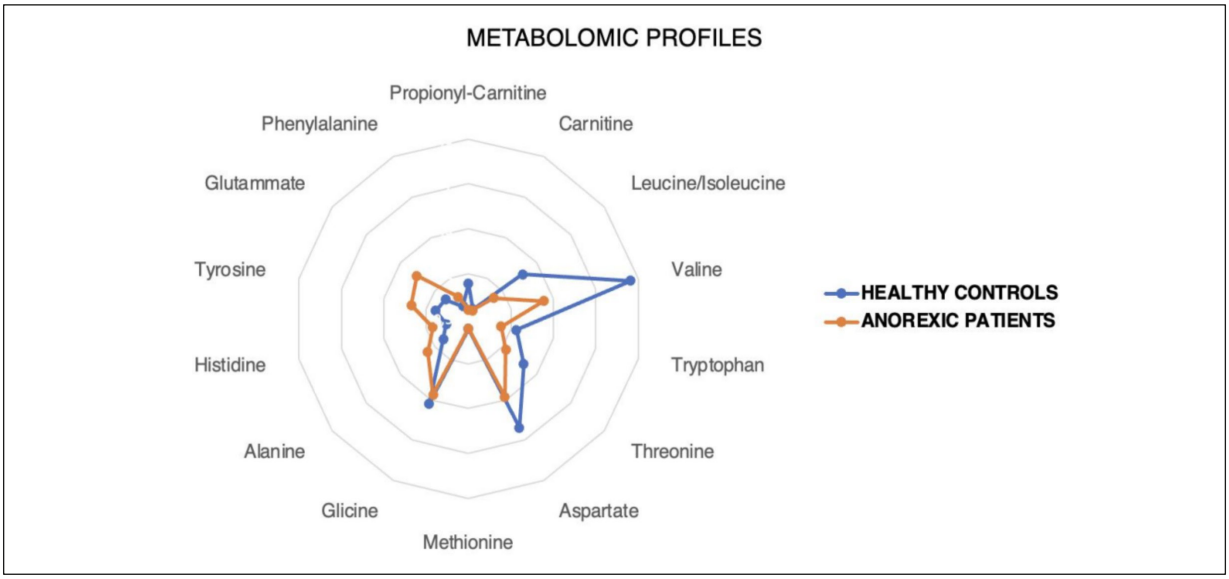

Figure 3.2. Metabolomic profiles of AN patients and controls.

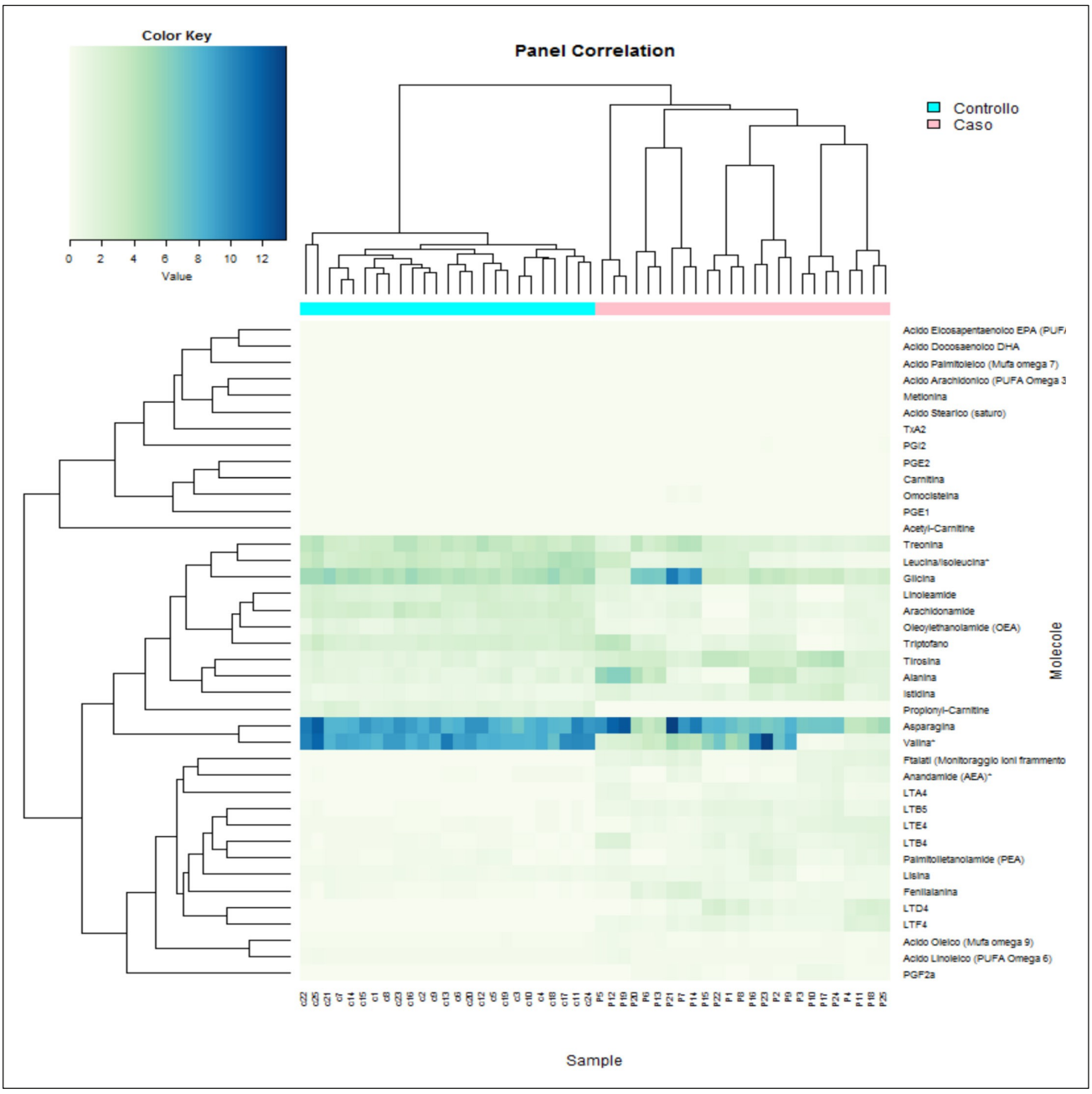

Figure 3.3 Heat map to compare metabolomic profiles of AN patients and controls. Patients are shown in red, and controls are shown in light blue.

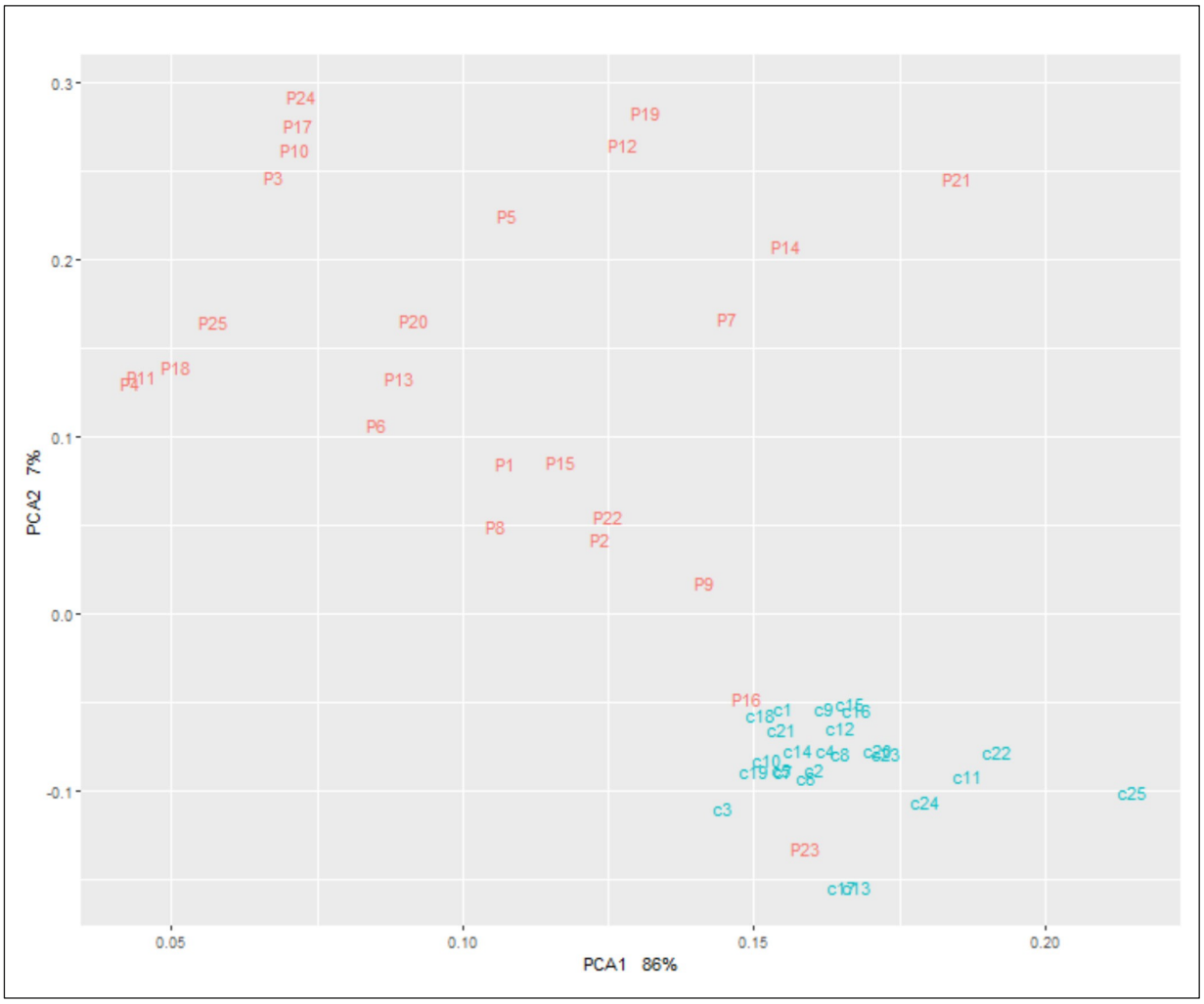

Figure 3.4. Principal component analysis (PCA). Metabolic Alterations in Amino Acid Concentrations. Patients are shown in red, and controls are shown in light blue.

REFERENCES

[1] Ceccarini MR, Precone V, Manara E, Paolacci S, Maltese PE, Benfatti V, Dhuli K, Donato K, Guerri G, Marceddu G, Chiurazzi P, Dalla Ragione L, Beccari T, Bertelli M. A next generation sequencing gene panel for use in the diagnosis of anorexia nervosa. Eat Weight Disord. 2022 Jun;27(5):1869-1880. doi: 10.1007/s40519-021-01331-0. Epub 2021 Nov 25. PMID: 34822136.

[2] Baker JH, Schaumberg K, Munn-Chernoff MA. Genetics of Anorexia Nervosa. Curr Psychiatry Rep. 2017 Sep 22;19(11):84. doi: 10.1007/s11920-017-0842-2. PMID: 28940168; PMCID: PMC6139670.

[3] Zipfel S, Giel KE, Bulik CM, Hay P, Schmidt U. Anorexia nervosa: aetiology, assessment, and treatment. Lancet Psychiatry. 2015 Dec;2(12):1099-111. doi: 10.1016/S2215-0366(15)00356-9. Epub 2015 Oct 27. PMID: 26514083.

[4] Smink FR, van Hoeken D, Hoek HW. Epidemiology of eating disorders: incidence, prevalence and mortality rates. Curr Psychiatry Rep. 2012 Aug;14(4):406-14. doi: 10.1007/s11920-012-0282-y. PMID: 22644309; PMCID: PMC3409365.

[5] Devlin B, Jones BL, Bacanu SA, Roeder K. Mixture models for linkage analysis of affected sibling pairs and covariates. Genet Epidemiol. 2002 Jan;22(1):52-65. doi: 10.1002/gepi.1043. PMID: 11754473.

[6] Paolacci S, Kiani AK, Manara E, Beccari T, Ceccarini MR, Stuppia L, Chiurazzi P, Dalla Ragione L, Bertelli M. Genetic contributions to the etiology of anorexia nervosa: New perspectives in molecular diagnosis and treatment. Mol Genet Genomic Med. 2020 Jul;8(7):e1244. doi: 10.1002/mgg3.1244. Epub 2020 May 5. PMID: 32368866; PMCID: PMC7336737.

[7] Strober M, Freeman R, Lampert C, Diamond J, Kaye W. Controlled family study of anorexia nervosa and bulimia nervosa: evidence of shared liability and transmission of partial syndromes. Am J Psychiatry. 2000 Mar;157(3):393-401. doi: 10.1176/appi.ajp.157.3.393. PMID: 10698815.

[8] Precone V, Beccari T, Stuppia L, Baglivo M, Paolacci S, Manara E, Miggiano GAD, Falsini B, Trifirò A, Zanlari A, Herbst KL, Unfer V, Bertelli M; Geneob Project. Taste, olfactory and texture related genes and food choices: implications on health status. Eur Rev Med Pharmacol Sci. 2019 Feb;23(3):1305-1321. doi: 10.26355/eurrev_201902_17026. PMID: 30779105.

[9] Precone V, Paolacci S, Beccari T, Dalla Ragione L, Stuppia L, Baglivo M, Guerri G, Manara E, Tonini G, Herbst KL, Unfer V, Bertelli M. Pheromone receptors and their putative ligands: possible role in humans. Eur Rev Med Pharmacol Sci. 2020 Feb;24(4):2140-2150. doi: 10.26355/eurrev_202002_20394. PMID: 32141584.

[10] Brown KM, Bujac SR, Mann ET, Campbell DA, Stubbins MJ, Blundell JE. Further evidence of association of OPRD1 & HTR1D polymorphisms with susceptibility to anorexia nervosa. Biol Psychiatry. 2007 Feb 1;61(3):367-73. doi: 10.1016/j.biopsych.2006.04.007. Epub 2006 Jun 27. PMID: 16806108.

[11] Ceccarini MR, Fittipaldi S, Ciccacci C, Granese E, Centofanti F, Dalla Ragione L, Bertelli M, Beccari T, Botta A. Association Between DRD2 and DRD4 Polymorphisms and Eating Disorders in an Italian Population. Front Nutr. 2022 Mar 14;9:838177. doi: 10.3389/fnut.2022.838177. PMID: 35369087; PMCID: PMC8964431.

[12] Doris JM, Millar SA, Idris I, O'Sullivan SE. Genetic polymorphisms of the endocannabinoid system in obesity and diabetes. Diabetes Obes Metab. 2019 Feb;21(2):382-387. doi: 10.1111/dom.13504. Epub 2018 Sep 16. PMID: 30129173.

[13] Siegfried Z, Kanyas K, Latzer Y, Karni O, Bloch M, Lerer B, Berry EM. Association study of cannabinoid receptor gene (CNR1) alleles and anorexia nervosa: differences between restricting and binging/purging subtypes. Am J Med Genet B Neuropsychiatr Genet. 2004 Feb 15;125B(1):126-30. doi: 10.1002/ajmg.b.20089. PMID: 14755457.

[14] Goodfellow CE, Glass M. Anandamide receptor signal transduction. Vitam Horm. 2009;81:79-110. doi: 10.1016/S0083-6729(09)81004-2. PMID: 19647109.

[15] Monteleone AM, Di Marzo V, Aveta T, Piscitelli F, Dalle Grave R, Scognamiglio P, El Ghoch M, Calugi S, Monteleone P, Maj M. Deranged endocannabinoid responses to hedonic eating in underweight and recently weight-restored patients with anorexia nervosa. Am J Clin Nutr. 2015 Feb;101(2):262-9. doi: 10.3945/ajcn.114.096164. Epub 2014 Dec 10. PMID: 25646322.

[16] Moskowitz L, Weiselberg E. Anorexia Nervosa/Atypical Anorexia Nervosa. Curr Probl Pediatr Adolesc Health Care. 2017 Apr;47(4):70-84. doi: 10.1016/j.cppeds.2017.02.003. PMID: 28532965.

[17] Watson HJ, Palmos AB, Hunjan A, Baker JH, Yilmaz Z, Davies HL. Genetics of eating disorders in the genome-wide era. Psychol Med. 2021 Oct;51(13):2287-2297. doi: 10.1017/S0033291720005474. Epub 2021 Feb 15. PMID: 33583449; PMCID: PMC8790815.

[18] Miyata N, Hata T, Takakura S, Yoshihara K, Morita C, Mikami K, Nomoto K, Miyazaki K, Tsuji H, Sudo N. Metabolomics profile of Japanese female patients with restricting-type anorexia nervosa. Physiol Behav. 2021 Jan 1;228:113204. doi: 10.1016/j.physbeh.2020.113204. Epub 2020 Oct 11. PMID: 33053407.

[19] Tomášová P, Procházková P, Roubalová R, Dvořák J, Tlaskalová-Hogenová H, Čermáková M, Pelantová H, Šedivá B, Vecka M, Papežová H, Kuzma M. NMR- and MS-Based Untargeted Metabolomic Study of Stool and Serum Samples from Patients with Anorexia Nervosa. J Proteome Res. 2022 Mar 4;21(3):778-787. doi: 10.1021/acs.jproteome.1c00537. Epub 2021 Oct 4. PMID: 34606283.

[20] Himmerich H, Bentley J, Kan C, Treasure J. Genetic risk factors for eating disorders: an update and insights into pathophysiology. Ther Adv Psychopharmacol. 2019 Feb 12;9:2045125318814734. doi: 10.1177/2045125318814734. PMID: 30800283; PMCID: PMC6378634.

[21] Yao S, Larsson H, Norring C, Birgegård A, Lichtenstein P, D'Onofrio BM, Almqvist C, Thornton LM, Bulik CM, Kuja-Halkola R. Genetic and environmental contributions to diagnostic fluctuation in anorexia nervosa and bulimia nervosa. Psychol Med. 2021 Jan;51(1):62-69. doi: 10.1017/S0033291719002976. Epub 2019 Oct 29. PMID: 31658910; PMCID: PMC7856409.

[22] Wray NR, Lin T, Austin J, McGrath JJ, Hickie IB, Murray GK, Visscher PM. From Basic Science to Clinical Application of Polygenic Risk Scores: A Primer. JAMA Psychiatry. 2021 Jan 1;78(1):101-109. doi: 10.1001/jamapsychiatry.2020.3049. PMID: 32997097.

[23]. Watson HJ, Yilmaz Z, Thornton LM, Hübel C, Coleman JRI, Gaspar HA, Bryois J, Hinney A, Leppä VM, Mattheisen M, Medland SE, Ripke S, Yao S, Giusti-Rodríguez P; Anorexia Nervosa Genetics Initiative; Hanscombe KB, Purves KL; Eating Disorders Working Group of the Psychiatric Genomics Consortium; Adan RAH, Alfredsson L, Ando T, Andreassen OA, Baker JH, Berrettini WH, Boehm I, Boni C, Perica VB, Buehren K, Burghardt R, Cassina M, Cichon S, Clementi M, Cone RD, Courtet P, Crow S, Crowley JJ, Danner UN, Davis OSP, de Zwaan M, Dedoussis G, Degortes D, DeSocio JE, Dick DM, Dikeos D, Dina C, Dmitrzak-Weglarz M, Docampo E, Duncan LE, Egberts K, Ehrlich S, Escaramís G, Esko T, Estivill X, Farmer A, Favaro A, Fernández-Aranda F, Fichter MM, Fischer K, Föcker M, Foretova L, Forstner AJ, Forzan M, Franklin CS, Gallinger S, Giegling I, Giuranna J, Gonidakis F, Gorwood P, Mayora MG, Guillaume S, Guo Y, Hakonarson H, Hatzikotoulas K, Hauser J, Hebebrand J, Helder SG, Herms S, Herpertz-Dahlmann B, Herzog W, Huckins LM, Hudson JI, Imgart H, Inoko

H, Janout V, Jiménez-Murcia S, Julià A, Kalsi G, Kaminská D, Kaprio J, Karhunen L, Karwautz A, Kas MJH, Kennedy JL, Keski-Rahkonen A, Kiezebrink K, Kim YR, Klareskog L, Klump KL, Knudsen GPS, La Via MC, Le Hellard S, Levitan RD, Li D, Lilenfeld L, Lin BD, Lissowska J, Luykx J, Magistretti PJ, Maj M, Mannik K, Marsal S, Marshall CR, Mattingsdal M, McDevitt S, McGuffin P, Metspalu A, Meulenbelt I, Micali N, Mitchell K, Monteleone AM, Monteleone P, Munn-Chernoff MA, Nacmias B, Navratilova M, Ntalla I, O'Toole JK, Ophoff RA, Padyukov L, Palotie A, Pantel J, Papezova H, Pinto D, Rabionet R, Raevuori A, Ramoz N, Reichborn-Kjennerud T, Ricca V, Ripatti S, Ritschel F, Roberts M, Rotondo A, Rujescu D, Rybakowski F, Santonastaso P, Scherag A, Scherer SW, Schmidt U, Schork NJ, Schosser A, Seitz J, Slachtova L, Slagboom PE, Slof-Op 't Landt MCT, Slopien A, Sorbi S, Świątkowska B, Szatkiewicz JP, Tachmazidou I, Tenconi E, Tortorella A, Tozzi F, Treasure J, Tsitsika A, Tyszkiewicz-Nwafor M, Tziouvas K, van Elburg AA, van Furth EF, Wagner G, Walton E, Widen E, Zeggini E, Zerwas S, Zipfel S, Bergen AW, Boden JM, Brandt H, Crawford S, Halmi KA, Horwood LJ, Johnson C, Kaplan AS, Kaye WH, Mitchell JE, Olsen CM, Pearson JF, Pedersen NL, Strober M, Werge T, Whiteman DC, Woodside DB, Stuber GD, Gordon S, Grove J, Henders AK, Juréus A, Kirk KM, Larsen JT, Parker R, Petersen L, Jordan J, Kennedy M, Montgomery GW, Wade TD, Birgegård A, Lichtenstein P, Norring C, Landén M, Martin NG, Mortensen PB, Sullivan PF, Breen G, Bulik CM. Genome-wide association study identifies eight risk loci and implicates metabo-psychiatric origins for anorexia nervosa. Nat Genet. 2019 Aug;51(8):1207-1214. doi: 10.1038/s41588-019-0439-2. Epub 2019 Jul 15. PMID: 31308545; PMCID: PMC6779477.

[24] Cuesto G, Everaerts C, León LG, Acebes A. Molecular bases of anorexia nervosa, bulimia nervosa and binge eating disorder: shedding light on the darkness. J Neurogenet. 2017 Dec;31(4):266-287. doi: 10.1080/01677063.2017.1353092. Epub 2017 Aug 1. PMID: 28762842.

[25] Galusca B, Prévost G, Germain N, Dubuc I, Ling Y, Anouar Y, Estour B, Chartrel N. Neuropeptide Y and α-MSH circadian levels in two populations with low body weight: anorexia nervosa and constitutional thinness. PLoS One. 2015 Mar 23;10(3):e0122040. doi: 10.1371/journal.pone.0122040. PMID: 25798605; PMCID: PMC4370702.

[26] Park JH, Shim HM, Na AY, Bae JH, Im SS, Song DK. Orexin A regulates plasma insulin and leptin levels in a time-dependent manner following a glucose load in mice. Diabetologia. 2015 Jul;58(7):1542-50. doi: 10.1007/s00125-015-3573-0. Epub 2015 Mar 28. PMID: 25813215.

[27] Zhou Y, Litvin Y, Piras AP, Pfaff DW, Kreek MJ. Persistent increase in hypothalamic arginine vasopressin gene expression during protracted withdrawal from chronic escalating-dose cocaine in rodents. Neuropsychopharmacology. 2011 Sep;36(10):2062-75. doi: 10.1038/npp.2011.97. Epub 2011 Jun 15. PMID: 21677651; PMCID: PMC3158323.

[28] Burmester V, Nicholls D, Buckle A, Stanojevic B, Crous-Bou M. Review of eating disorders and oxytocin receptor polymorphisms. J Eat Disord. 2021 Jul 13;9(1):85. doi: 10.1186/s40337-021-00438-0. PMID: 34256847; PMCID: PMC8278600.

[29] Munn-Chernoff MA, Baker JH. A Primer on the Genetics of Comorbid Eating Disorders and Substance Use Disorders. Eur Eat Disord Rev. 2016 Mar;24(2):91-100. doi: 10.1002/erv.2424. Epub 2015 Dec 14. PMID: 26663753; PMCID: PMC4738088.

[30] Yokokura M, Terada T, Bunai T, Nakaizumi K, Kato Y, Yoshikawa E, Futatsubashi M, Suzuki K, Yamasue H, Ouchi Y. Alterations in serotonin transporter and body image-related cognition in anorexia nervosa. Neuroimage Clin. 2019;23:101928. doi: 10.1016/j.nicl.2019.101928. Epub 2019 Jul 3. PMID: 31491815; PMCID: PMC6627582.

[31] Boehm I, Walton E, Alexander N, Batury VL, Seidel M, Geisler D, King JA, Weidner K, Roessner V, Ehrlich S. Peripheral serotonin transporter DNA methylation is linked to increased salience network connectivity in females with anorexia nervosa. J Psychiatry Neurosci. 2020 May 1;45(3):206-213. doi: 10.1503/jpn.190016. PMID: 31823595; PMCID: PMC7828979.

[32] Trace SE, Baker JH, Peñas-Lledó E, Bulik CM. The genetics of eating disorders. Annu Rev Clin Psychol. 2013;9:589-620. doi: 10.1146/annurev-clinpsy-050212-185546. PMID: 23537489.

[33] Compan V. Serotonin 4 receptors: A cornerstone in anorexia nervosa? Autism Open Access 2017, 7:2. doi: 10.4172/2165-7890.1000207

[34] Boraska V, Franklin CS, Floyd JA, Thornton LM, Huckins LM, Southam L, Rayner NW, Tachmazidou I, Klump KL, Treasure J, Lewis CM, Schmidt U, Tozzi F, Kiezebrink K, Hebebrand J, Gorwood P, Adan RA, Kas MJ, Favaro A, Santonastaso P, Fernández-Aranda F, Gratacos M, Rybakowski F, Dmitrzak-Weglarz M, Kaprio J, Keski-Rahkonen A, Raevuori A, Van Furth EF, Slof-Op 't Landt MC, Hudson JI, Reichborn-Kjennerud T, Knudsen GP, Monteleone P, Kaplan AS, Karwautz A, Hakonarson H, Berrettini WH, Guo Y, Li D, Schork NJ, Komaki G, Ando T, Inoko H, Esko T, Fischer K, Männik K, Metspalu A, Baker JH, Cone RD, Dackor J, DeSocio JE, Hilliard CE,

O'Toole JK, Pantel J, Szatkiewicz JP, Taico C, Zerwas S, Trace SE, Davis OS, Helder S, Bühren K, Burghardt R, de Zwaan M, Egberts K, Ehrlich S, Herpertz-Dahlmann B, Herzog W, Imgart H, Scherag A, Scherag S, Zipfel S, Boni C, Ramoz N, Versini A, Brandys MK, Danner UN, de Kovel C, Hendriks J, Koeleman BP, Ophoff RA, Strengman E, van Elburg AA, Bruson A, Clementi M, Degortes D, Forzan M, Tenconi E, Docampo E, Escaramís G, Jiménez-Murcia S, Lissowska J, Rajewski A, Szeszenia-Dabrowska N, Slopien A, Hauser J, Karhunen L, Meulenbelt I, Slagboom PE, Tortorella A, Maj M, Dedoussis G, Dikeos D, Gonidakis F, Tziouvas K, Tsitsika A, Papezova H, Slachtova L, Martaskova D, Kennedy JL, Levitan RD, Yilmaz Z, Huemer J, Koubek D, Merl E, Wagner G, Lichtenstein P, Breen G, Cohen-Woods S, Farmer A, McGuffin P, Cichon S, Giegling I, Herms S, Rujescu D, Schreiber S, Wichmann HE, Dina C, Sladek R, Gambaro G, Soranzo N, Julia A, Marsal S, Rabionet R, Gaborieau V, Dick DM, Palotie A, Ripatti S, Widén E, Andreassen OA, Espeseth T, Lundervold A, Reinvang I, Steen VM, Le Hellard S, Mattingsdal M, Ntalla I, Bencko V, Foretova L, Janout V, Navratilova M, Gallinger S, Pinto D, Scherer SW, Aschauer H, Carlberg L, Schosser A, Alfredsson L, Ding B, Klareskog L, Padyukov L, Courtet P, Guillaume S, Jaussent I, Finan C, Kalsi G, Roberts M, Logan DW, Peltonen L, Ritchie GR, Barrett JC; Wellcome Trust Case Control Consortium 3; Estivill X, Hinney A, Sullivan PF, Collier DA, Zeggini E, Bulik CM. A genome-wide association study of anorexia nervosa. Mol Psychiatry. 2014 Oct;19(10):1085-94. doi: 10.1038/mp.2013.187. Epub 2014 Feb 11. PMID: 24514567; PMCID: PMC4325090.

[35] Duncan L, Yilmaz Z, Gaspar H, Walters R, Goldstein J, Anttila V, Bulik-Sullivan B, Ripke S; Eating Disorders Working Group of the Psychiatric Genomics Consortium; Thornton L, Hinney A, Daly M, Sullivan PF, Zeggini E, Breen G, Bulik CM. Significant Locus and Metabolic Genetic Correlations Revealed in Genome-Wide Association Study of Anorexia Nervosa. Am J Psychiatry. 2017 Sep 1;174(9):850-858. doi: 10.1176/appi.ajp.2017.16121402. Epub 2017 May 12. PMID: 28494655; PMCID: PMC5581217.

[36] Perry B, Wang Y. Appetite regulation and weight control: the role of gut hormones. Nutr Diabetes. 2012 Jan 16;2(1):e26. doi: 10.1038/nutd.2011.21. PMID: 23154682; PMCID: PMC3302146.

[37] Blauwhoff-Buskermolen S, Langius JA, Heijboer AC, Becker A, de van der Schueren MA, Verheul HM. Plasma Ghrelin Levels Are Associated with Anorexia but Not Cachexia in Patients with NSCLC. Front Physiol. 2017 Mar 1;8:119. doi: 10.3389/fphys.2017.00119. PMID: 28298897; PMCID: PMC5331052.

[38] Steiner AA, Romanovsky AA. Leptin: at the crossroads of energy balance and systemic inflammation. Prog Lipid Res. 2007 Mar;46(2):89-107. doi: 10.1016/j.plipres.2006.11.001. Epub 2006 Dec 21. PMID: 17275915; PMCID: PMC1976277.

[39] Föcker M, Timmesfeld N, Scherag S, Bühren K, Langkamp M, Dempfle A, Sheridan EM, de Zwaan M, Fleischhaker C, Herzog W, Egberts K, Zipfel S, Herpertz-Dahlmann B, Hebebrand J. Screening for anorexia nervosa via measurement of serum leptin levels. J Neural Transm (Vienna). 2011 Apr;118(4):571-8. doi: 10.1007/s00702-010-0551-z. Epub 2011 Jan 22. PMID: 21258826.

[40] Monteleone P, Matias I, Martiadis V, De Petrocellis L, Maj M, Di Marzo V. Blood levels of the endocannabinoid anandamide are increased in anorexia nervosa and in binge-eating disorder, but not in bulimia nervosa. Neuropsychopharmacology. 2005 Jun;30(6):1216-21. doi: 10.1038/sj.npp.1300695. PMID: 15841111.

[41] Monteleone P, Serritella C, Martiadis V, Maj M. Decreased levels of serum brain-derived neurotrophic factor in both depressed and euthymic patients with unipolar depression and in euthymic patients with bipolar I and II disorders. Bipolar Disord. 2008 Feb;10(1):95-100. doi: 10.1111/j.1399-5618.2008.00459.x. PMID: 18199246.

[42] Jean A, Conductier G, Manrique C, Bouras C, Berta P, Hen R, Charnay Y, Bockaert J, Compan V. Anorexia induced by activation of serotonin 5-HT4 receptors is mediated by increases in CART in the nucleus accumbens. Proc Natl Acad Sci U S A. 2007 Oct 9;104(41):16335-40. doi: 10.1073/pnas.0701471104. Epub 2007 Oct 3. PMID: 17913892; PMCID: PMC2042207.

[43] Miller CA, Sweatt JD. Covalent modification of DNA regulates memory formation. Neuron. 2007 Mar 15;53(6):857-69. doi: 10.1016/j.neuron.2007.02.022. Erratum in: Neuron. 2008 Sep 25;59(6):1051. PMID: 17359920.

[44] Ehrlich S, Franke L, Schott R, Salbach-Andrae H, Pfeiffer E, Lehmkuhl U, Uebelhack R. Platelet monoamine oxidase activity in underweight and weight-recovered females with anorexia nervosa. Pharmacopsychiatry. 2008 Nov;41(6):226-31. doi: 10.1055/s-2008-1078749. Epub 2008 Dec 9. PMID: 19067259.

[45] Rask-Andersen M, Olszewski PK, Levine AS, Schiöth HB. Molecular mechanisms underlying anorexia nervosa: focus on human gene association studies and systems controlling food intake. Brain Res Rev. 2010 Mar;62(2):147-64. doi: 10.1016/j.brainresrev.2009.10.007. Epub 2009 Nov 18. PMID: 19931559.

[46] Beckers S, Zegers D, de Freitas F, Mertens IL, Van Gaal LF, Van Hul W. Association study of MC4R with complex obesity and replication of the rs17782313 association signal. Mol Genet Metab. 2011 May;103(1):71-5. doi: 10.1016/j.ymgme.2011.01.007. Epub 2011 Jan 22. PMID: 21303735.

[47] Misra M, Klibanski A. Endocrine consequences of anorexia nervosa. Lancet Diabetes Endocrinol. 2014 Jul;2(7):581-92. doi: 10.1016/S2213-8587(13)70180-3. Epub 2014 Apr 2. PMID: 24731664; PMCID: PMC4133106.

[48] Dalton B, Bartholdy S, Robinson L, Solmi M, Ibrahim MAA, Breen G, Schmidt U, Himmerich H. A meta-analysis of cytokine concentrations in eating disorders. J Psychiatr Res. 2018 Aug;103:252-264. doi: 10.1016/j.jpsychires.2018.06.002. Epub 2018 Jun 7. PMID: 29906710.

[49] Monteleone AM, Pellegrino F, Croatto G, Carfagno M, Hilbert A, Treasure J, Wade T, Bulik CM, Zipfel S, Hay P, Schmidt U, Castellini G, Favaro A, Fernandez-Aranda F, Il Shin J, Voderholzer U, Ricca V, Moretti D, Busatta D, Abbate-Daga G, Ciullini F, Cascino G, Monaco F, Correll CU, Solmi M. Treatment of eating disorders: A systematic meta-review of meta-analyses and network meta-analyses. Neurosci Biobehav Rev. 2022 Nov;142:104857. doi: 10.1016/j.neubiorev.2022.104857. Epub 2022 Sep 6. PMID: 36084848; PMCID: PMC9813802.

[50] Giannunzio V, Degortes D, Tenconi E, Collantoni E, Solmi M, Santonastaso P, Favaro A. Decision-making impairment in anorexia nervosa: New insights into the role of age and decision-making style. Eur Eat Disord Rev. 2018 Jul;26(4):302-314. doi: 10.1002/erv.2595. Epub 2018 Apr 17. PMID: 29665149.

[51] O'Brien KM, Vincent NK. Psychiatric comorbidity in anorexia and bulimia nervosa: nature, prevalence, and causal relationships. Clin Psychol Rev. 2003 Feb;23(1):57-74. doi: 10.1016/s0272-7358(02)00201-5. PMID: 12559994.

[52] Mattar L, Thiébaud MR, Huas C, Cebula C, Godart N. Depression, anxiety and obsessive-compulsive symptoms in relation to nutritional status and outcome in severe anorexia nervosa. Psychiatry Res. 2012 Dec 30;200(2-3):513-7. doi: 10.1016/j.psychres.2012.04.032. Epub 2012 Jun 15. PMID: 22703719.

[53] Arcelus J, Mitchell AJ, Wales J, Nielsen S. Mortality rates in patients with anorexia nervosa and other eating disorders. A meta-analysis of 36 studies. Arch Gen Psychiatry. 2011 Jul;68(7):724-31. doi: 10.1001/archgenpsychiatry.2011.74. PMID: 21727255.

[54] Kaye WH, Bulik CM, Thornton L, Barbarich N, Masters K. Comorbidity of anxiety disorders with anorexia and bulimia nervosa. Am J Psychiatry. 2004 Dec;161(12):2215-21. doi: 10.1176/appi.ajp.161.12.2215. PMID: 15569892.

[55] Santonicola A, Gagliardi M, Guarino MPL, Siniscalchi M, Ciacci C, Iovino P. Eating Disorders and Gastrointestinal Diseases. Nutrients. 2019 Dec 12;11(12):3038. doi: 10.3390/nu11123038. PMID: 31842421; PMCID: PMC6950592.

[56] Allen KL, Byrne SM, Hii H, van Eekelen A, Mattes E, Foster JK. Neurocognitive functioning in adolescents with eating disorders: a population-based study. Cogn Neuropsychiatry. 2013;18(5):355-75. doi: 10.1080/13546805.2012.698592. Epub 2012 Jul 17. PMID: 22803827.

[57] Hemmingsen SD, Lichtenstein MB, Sjögren M, Gudex C, Larsen PV, Støving RK. Cognitive performance in hospitalized patients with severe or extreme anorexia nervosa. Eat Weight Disord. 2023 Oct 21;28(1):86. doi: 10.1007/s40519-023-01585-w. PMID: 37864583; PMCID: PMC10590307.

[58] Gordon CM, Ackerman KE, Berga SL, Kaplan JR, Mastorakos G, Misra M, Murad MH, Santoro NF, Warren MP. Functional Hypothalamic Amenorrhea: An Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab. 2017 May 1;102(5):1413-1439. doi: 10.1210/jc.2017-00131. PMID: 28368518.

[59] Badaeva AV, Danilov AB, Clayton P, Moskalev AA, Karasev AV, Tarasevich AF, Vorobyeva YD, Novikov VN. Perspectives on Neuronutrition in Prevention and Treatment of Neurological Disorders. Nutrients. 2023 May 28;15(11):2505. doi: 10.3390/nu15112505. PMID: 37299468; PMCID: PMC10255487.

[60] Frank GK, Shott ME, Riederer J, Pryor TL. Altered structural and effective connectivity in anorexia and bulimia nervosa in circuits that regulate energy and reward homeostasis. Transl Psychiatry. 2016 Nov 1;6(11):e932. doi: 10.1038/tp.2016.199. PMID: 27801897; PMCID: PMC5314116.

[61] Fonville L, Giampietro V, Williams SC, Simmons A, Tchanturia K. Alterations in brain structure in adults with anorexia nervosa and the impact of illness duration. Psychol Med. 2014 Jul;44(9):1965-75. doi: 10.1017/S0033291713002389. Epub 2013 Sep 27. Erratum in: Psychol Med. 2014 Jul;44(9):1976. PMID: 24074139.

[62] Pandit R, Mercer JG, Overduin J, la Fleur SE, Adan RA. Dietary factors affect food reward and motivation to eat. Obes Facts. 2012;5(2):221-42. doi: 10.1159/000338073. Epub 2012 Apr 20. PMID: 22647304.

[63] O'Hara CB, Keyes A, Renwick B, Leyton M, Campbell IC, Schmidt U. The Effects of Acute Dopamine Precursor Depletion on the Reinforcing Value of Exercise in Anorexia Nervosa. PLoS One. 2016 Jan 25;11(1):e0145894. doi: 10.1371/journal.pone.0145894. PMID: 26808920; PMCID: PMC4726788.

[64] Holsen LM, Lawson EA, Blum J, Ko E, Makris N, Fazeli PK, Klibanski A, Goldstein JM. Food motivation circuitry hypoactivation related to hedonic and nonhedonic aspects of hunger and satiety in women with active anorexia nervosa and weight-restored women with anorexia nervosa. J Psychiatry Neurosci. 2012 Sep;37(5):322-32. doi: 10.1503/jpn.110156. PMID: 22498079; PMCID: PMC3447131.

[65] Monteleone AM, Castellini G, Volpe U, Ricca V, Lelli L, Monteleone P, Maj M. Neuroendocrinology and brain imaging of reward in eating disorders: A possible key to the treatment of anorexia nervosa and bulimia nervosa. Prog Neuropsychopharmacol Biol Psychiatry. 2018 Jan 3;80(Pt B):132-142. doi: 10.1016/j.pnpbp.2017.02.020. Epub 2017 Mar 1. PMID: 28259721.

[66] Gaudio S, Carducci F, Piervincenzi C, Olivo G, Schiöth HB. Altered thalamo–cortical and occipital–parietal–temporal–frontal white matter connections in patients with anorexia and bulimia nervosa: a systematic review of diffusion tensor imaging studies. J Psychiatry Neurosci. 2019 Sep 1;44(5):324-339. doi: 10.1503/jpn.180121. PMID: 30994310; PMCID: PMC6710091.

[67] Monteleone AM, Treasure J, Kan C, Cardi V. Reactivity to interpersonal stress in patients with eating disorders: A systematic review and meta-analysis of studies using an experimental paradigm. Neurosci Biobehav Rev. 2018 Apr;87:133-150. doi: 10.1016/j.neubiorev.2018.02.002. Epub 2018 Feb 8. PMID: 29428393.

[68] Mayer LE, Schebendach J, Bodell LP, Shingleton RM, Walsh BT. Eating behavior in anorexia nervosa: before and after treatment. Int J Eat Disord. 2012 Mar;45(2):290-3. doi: 10.1002/eat.20924. Epub 2011 Apr 14. PMID: 21495053; PMCID: PMC4469276.

[69] Chatwin H, Holde K, Yilmaz Z, Larsen JT, Albiñana C, Vilhjálmsson BJ, Mortensen PB, Thornton LM, Bulik CM, Petersen LV. Risk factors for anorexia nervosa: A population-based investigation of sex differences in polygenic risk and early life exposures. Int J Eat Disord. 2023 Sep;56(9):1703-1716. doi: 10.1002/eat.23997. Epub 2023 May 25. PMID: 37232007; PMCID: PMC10524536.

[70] Shih PB. Metabolomics Biomarkers for Precision Psychiatry. Adv Exp Med Biol. 2019;1161:101-113. doi:

10.1007/978-3-030-21735-8_10. PMID: 31562625; PMCID: PMC7141790.

[71] Qiu S, Cai Y, Yao H, Lin C, Xie Y, Tang S, Zhang A. Small molecule metabolites: discovery of biomarkers and therapeutic targets. Signal Transduct Target Ther. 2023 Mar 20;8(1):132. doi: 10.1038/s41392-023-01399-3. PMID: 36941259; PMCID: PMC10026263.

[72] Villas-Bôas SG, Rasmussen S, Lane GA. Metabolomics or metabolite profiles? Trends Biotechnol. 2005 Aug;23(8):385-6. doi: 10.1016/j.tibtech.2005.05.009. PMID: 15939497.

[73] Mussap M, Loddo C, Fanni C, Fanos V. Metabolomics in pharmacology - a delve into the novel field of pharmacometabolomics. Expert Rev Clin Pharmacol. 2020 Feb;13(2):115-134. doi: 10.1080/17512433.2020.1713750. Epub 2020 Jan 20. PMID: 31958027.

[74] Jang WJ, Choi JY, Park B, Seo JH, Seo YH, Lee S, Jeong CH, Lee S. Hair Metabolomics in Animal Studies and Clinical Settings. Molecules. 2019 Jun 12;24(12):2195. doi: 10.3390/molecules24122195. PMID: 31212725; PMCID: PMC6630908.

[75] Chen Y, Guo J, Xing S, Yu H, Huan T. Global-Scale Metabolomic Profiling of Human Hair for Simultaneous Monitoring of Endogenous Metabolome, Short- and Long-Term Exposome. Front Chem. 2021 May 12;9:674265. doi: 10.3389/fchem.2021.674265. PMID: 34055742; PMCID: PMC8149753.

[76] Eisenbeiss L, Steuer AE, Binz TM, Baumgartner MR, Kraemer T. (Un)targeted hair metabolomics: first considerations and systematic evaluation on the impact of sample preparation. Anal Bioanal Chem. 2019 Jul;411(17):3963-3977. doi: 10.1007/s00216-019-01873-4. Epub 2019 May 23. PMID: 31123781.

[77] Cobo-Golpe, Markus R. Baumgartner, Tina M. Binz, Thomas Kraemer, Andrea E. Steuer, Detection of hair metabolome changes in cocaine users using untargeted metabolomics, Toxicologie Analytique et Clinique, 2022, https://doi.org/10.1016/j.toxac.2022.06.030.

[78] Rosenberg AM, Rausser S, Ren J, Mosharov EV, Sturm G, Ogden RT, Patel P, Kumar Soni R, Lacefield C, Tobin DJ, Paus R, Picard M. Quantitative mapping of human hair greying and reversal in relation to life stress. Elife. 2021 Jun 22;10:e67437. doi: 10.7554/eLife.67437. PMID: 34155974; PMCID: PMC8219384.

[79] Föcker M, Cecil A, Prehn C, Adamski J, Albrecht M, Adams F, Hinney A, Libuda L, Bühlmeier J, Hebebrand

J, Peters T, Antel J. Evaluation of Metabolic Profiles of Patients with Anorexia Nervosa at Inpatient Admission, Short- and Long-Term Weight Regain-Descriptive and Pattern Analysis. Metabolites. 2020 Dec 24;11(1):7. doi: 10.3390/metabo11010007. PMID: 33374417; PMCID: PMC7823299.

[80] Mayo-Martínez L, Rupérez FJ, Martos-Moreno GÁ, Graell M, Barbas C, Argente J, García A. Unveiling Metabolic Phenotype Alterations in Anorexia Nervosa through Metabolomics. Nutrients. 2021 Nov 26;13(12):4249. doi: 10.3390/nu13124249. PMID: 34959800; PMCID: PMC8706417.

[81] Lopez MJ, Mohiuddin SS. Biochemistry, Essential Amino Acids. 2024 Apr 30. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan–. PMID: 32496725.

[82] Church DD, Hirsch KR, Park S, Kim IY, Gwin JA, Pasiakos SM, Wolfe RR, Ferrando AA. Essential Amino Acids and Protein Synthesis: Insights into Maximizing the Muscle and Whole-Body Response to Feeding. Nutrients. 2020 Dec 2;12(12):3717. doi: 10.3390/nu12123717. PMID: 33276485; PMCID: PMC7760188.

[83] Kinnaird E, Stewart C, Tchanturia K. Taste sensitivity in anorexia nervosa: A systematic review. Int J Eat Disord. 2018 Aug;51(8):771-784. doi: 10.1002/eat.22886. Epub 2018 Jul 8. PMID: 29984498; PMCID: PMC6282513.

[84] Anthony TG, Gietzen DW. Detection of amino acid deprivation in the central nervous system. Curr Opin Clin Nutr Metab Care. 2013 Jan;16(1):96-101. doi: 10.1097/MCO.0b013e32835b618b. PMID: 23222708.

[85] Gutiérrez-Preciado, A., Romero, H. & Peimbert, M. (2010) An Evolutionary Perspective on Amino Acids. Nature Education 3(9):29

[86] Zhu H, Bai M, Xie X, Wang J, Weng C, Dai H, Chen J, Han F, Lin W. Impaired Amino Acid Metabolism and Its Correlation with Diabetic Kidney Disease Progression in Type 2 Diabetes Mellitus. Nutrients. 2022 Aug 15;14(16):3345. doi: 10.3390/nu14163345. PMID: 36014850; PMCID: PMC9415588.

[87] Stevenson RJ. An initial evaluation of the functions of human olfaction. Chem Senses. 2010 Jan;35(1):3-20. doi: 10.1093/chemse/bjp083. Epub 2009 Nov 25. PMID: 19942579.

[88] Gietzen DW, Hao S, Anthony TG. Mechanisms of food intake repression in indispensable amino acid deficiency. Annu Rev Nutr. 2007;27:63-78. doi: 10.1146/annurev.nutr.27.061406.093726. PMID: 17328672.

[89] Maurin AC, Jousse C, Averous J, Parry L, Bruhat A, Cherasse Y, Zeng H, Zhang Y, Harding HP, Ron D, Fafournoux P. The GCN2 kinase biases feeding behavior to maintain amino acid homeostasis in omnivores. Cell Metab. 2005 Apr;1(4):273-7. doi: 10.1016/j.cmet.2005.03.004. PMID: 16054071.

[90] Gloaguen M, Le Floc'h N, Corrent E, Primot Y, van Milgen J. Providing a diet deficient in valine but with excess leucine results in a rapid decrease in feed intake and modifies the postprandial plasma amino acid and α-keto acid concentrations in pigs. J Anim Sci. 2012 Sep;90(9):3135-42. doi: 10.2527/jas.2011-4956. Epub 2012 May 14. PMID: 22585822.

[91] Hawkins RA, O'Kane RL, Simpson IA, Viña JR. Structure of the blood-brain barrier and its role in the transport of amino acids. J Nutr. 2006 Jan;136(1 Suppl):218S-26S. doi: 10.1093/jn/136.1.218S. PMID: 16365086.

[92] Koehnle TJ, Russell MC, Morin AS, Erecius LF, Gietzen DW. Diets deficient in indispensable amino acids rapidly decrease the concentration of the limiting amino acid in the anterior piriform cortex of rats. J Nutr. 2004 Sep;134(9):2365-71. doi: 10.1093/jn/134.9.2365. PMID: 15333730.

[93] Huynh LN, Thangavel M, Chen T, Cottrell R, Mitchell JM, Praetorius-Ibba M. Linking tRNA localization with activation of nutritional stress responses. Cell Cycle. 2010 Aug 1;9(15):3112-8. doi: 10.4161/cc.9.15.12525. PMID: 20714220.

[94] Baird TD, Wek RC. Eukaryotic initiation factor 2 phosphorylation and translational control in metabolism. Adv Nutr. 2012 May 1;3(3):307-21. doi: 10.3945/an.112.002113. PMID: 22585904; PMCID: PMC3649462.

[95] Kilberg MS, Balasubramanian M, Fu L, Shan J. The transcription factor network associated with the amino acid response in mammalian cells. Adv Nutr. 2012 May 1;3(3):295-306. doi: 10.3945/an.112.001891. PMID: 22585903; PMCID: PMC3649461.

[96] Hansen HS, Vana V. Non-endocannabinoid N-acylethanolamines and 2-monoacylglycerols in the intestine. Br J Pharmacol. 2019 May;176(10):1443-1454. doi: 10.1111/bph.14175. Epub 2018 Apr 2. PMID: 29473944; PMCID: PMC6487557.

[97] Hao S, Sharp JW, Ross-Inta CM, McDaniel BJ, Anthony TG, Wek RC, Cavener DR, McGrath BC, Rudell JB, Koehnle TJ, Gietzen DW. Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex.

Science. 2005 Mar 18;307(5716):1776-8. doi: 10.1126/science.1104882. PMID: 15774759.

[98] Gietzen DW, Aja SM. The brain's response to an essential amino acid-deficient diet and the circuitous route to a better meal. Mol Neurobiol. 2012 Oct;46(2):332-48. doi: 10.1007/s12035-012-8283-8. Epub 2012 Jun 7. PMID: 22674217; PMCID: PMC3469761.

[99] Aponte Y, Atasoy D, Sternson SM. AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. Nat Neurosci. 2011 Mar;14(3):351-5. doi: 10.1038/nn.2739. Epub 2010 Jan 5. PMID: 21209617; PMCID: PMC3049940.

[100] Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes. 2001 Aug;50(8):1714-9. doi: 10.2337/diabetes.50.8.1714. PMID: 11473029.

[101] Goto S, Nagao K, Bannai M, Takahashi M, Nakahara K, Kangawa K, Murakami N. Anorexia in rats caused by a valine-deficient diet is not ameliorated by systemic ghrelin treatment. Neuroscience. 2010 Mar 10;166(1):333-40. doi: 10.1016/j.neuroscience.2009.12.013. Epub 2009 Dec 17. PMID: 20006681.

[102] Siebert D, Khan DR, Torrallardona D. The Optimal Valine to Lysine Ratio for Performance Parameters in Weaned Piglets. Animals (Basel). 2021 Apr 27;11(5):1255. doi: 10.3390/ani11051255. PMID: 33925439; PMCID: PMC8144975.

[103] Kerkaert HR, Cemin HS, Woodworth JC, DeR- ouchey JM, Dritz SS, Tokach MD, Goodband RD, Haydon KD, Hastad CW, Post ZB. Improving per- formance of finishing pigs with added valine, iso- leucine, and tryptophan: validating a meta-analy- sis model. J Anim Sci 2021; 99: skab006.

[104] Fernstrom JD. Large neutral amino acids: dietary effects on brain neurochemistry and function. Amino Acids. 2013 Sep;45(3):419-30. doi: 10.1007/s00726-012-1330-y. Epub 2012 Jun 8. PMID: 22677921.

[105] Hu, J., Le, Q., Zhang, M., Kuang, S., Gu, W., Sun, Y., Jean Jacques, K., Zhang, Y., Li, Y., Sun, J., Yang, Y., Wang, Y., Xu, S. and Yan, X. (2021), Effects of amino acids on olfactory-related receptors regulating appetite in silver pomfret. Aquaculture Research, 52: 2528-2539. https://doi.org/10.1111/are.15102

[106] D.W. Gietzen, P.M.B. Leung, T.W. Castonguay, W.J. Hartman, Q.R. Rogers, Time Course of Food Intake

and Plasma and Brain Amino Acid Concentrations in Rats Fed Amino Acid-Imbalanced or -Deficient Diets, 1986, https://doi.org/10.1016/B978-0-12-397855-4.50030-X.

[107] Mori M, Kawada T, Ono T, Torii K. Taste preference and protein nutrition and L-amino acid homeostasis in male Sprague-Dawley rats. Physiol Behav. 1991 May;49(5):987-95. doi: 10.1016/0031-9384(91)90212-7. PMID: 1653438.

[108] Koehnle TJ, Russell MC, Gietzen DW. Rats rapidly reject diets deficient in essential amino acids. J Nutr. 2003 Jul;133(7):2331-5. doi: 10.1093/jn/133.7.2331. PMID: 12840202.

[109] Feurté S, Nicolaidis S, Berridge KC. Conditioned taste aversion in rats for a threonine-deficient diet: demonstration by the taste reactivity test. Physiol Behav. 2000 Jan;68(3):423-9. doi: 10.1016/s0031-9384(99)00202-4. PMID: 10716554.

[110] Rogers QR, Leung PM. The influence of amino acids on the neuroregulation of food intake. Fed Proc. 1973 Jun;32(6):1709-19. PMID: 4710879.

[111] Pezeshki A, Chelikani PK. Low Protein Diets and Energy Balance: Mechanisms of Action on Energy Intake and Expenditure. Front Nutr. 2021 May 13;8:655833. doi: 10.3389/fnut.2021.655833. PMID: 34055853; PMCID: PMC8155302.

[112] Gietzen DW, Hao S, Anthony TG. “Amino acid-sensing mechanisms: biochemistry and behavior”. Handbook of Neurochemistry and Molecular Neurobiology. Springer US, 2007; 249-269.

[113] Ekstrand JJ, Domroese ME, Johnson DM, Feig SL, Knodel SM, Behan M, Haberly LB. A new subdivision of anterior piriform cortex and associated deep nucleus with novel features of interest for olfaction and epilepsy. J Comp Neurol. 2001 Jun 4;434(3):289-307. doi: 10.1002/cne.1178. PMID: 11331530.

[114] Sharp JW, Ross-Inta CM, Baccelli I, Payne JA, Rudell JB, Gietzen DW. Effects of essential amino acid deficiency: down-regulation of KCC2 and the GABAA receptor; disinhibition in the anterior piriform cortex. J Neurochem. 2013 Nov;127(4):520-30. doi: 10.1111/jnc.12403. Epub 2013 Sep 12. PMID: 24024616; PMCID: PMC3858386.

[115] Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. Cell. 2017 Apr 6;169(2):361-

371. doi: 10.1016/j.cell.2017.03.035. Erratum for: Cell. 2017 Mar 9;168(6):960-976. doi: 10.1016/j.cell.2017.02.004. PMID: 28388417.

[116] Gietzen DW. Brain Signaling of Indispensable Amino Acid Deficiency. J Clin Med. 2021 Dec 30;11(1):191. doi: 10.3390/jcm11010191. PMID: 35011932; PMCID: PMC8745678.

[117] Cemin HS, Tokach MD, Dritz SS, Woodworth JC, DeRouchey JM, Goodband RD. Meta-regression analysis to predict the influence of branched-chain and large neutral amino acids on growth performance of pigs1. J Anim Sci. 2019 May 30;97(6):2505-2514. doi: 10.1093/jas/skz118. PMID: 30959521; PMCID: PMC6541811.

[118] Wiltafsky MK, Pfaffl MW, Roth FX. The effects of branched-chain amino acid interactions on growth performance, blood metabolites, enzyme kinetics and transcriptomics in weaned pigs. Br J Nutr. 2010 Apr;103(7):964-76. doi: 10.1017/S0007114509992212. Epub 2010 Mar 3. PMID: 20196890.

[119] Kwon WB, Touchette KJ, Simongiovanni A, Syriopoulos K, Wessels A, Stein HH. Excess dietary leucine in diets for growing pigs reduces growth performance, biological value of protein, protein retention, and serotonin synthesis1. J Anim Sci. 2019 Oct 3;97(10):4282-4292. doi: 10.1093/jas/skz259. PMID: 31410464; PMCID: PMC6776264.

[120] Foldi CJ, Liknaitzky P, Williams M, Oldfield BJ. Rethinking Therapeutic Strategies for Anorexia Nervosa: Insights From Psychedelic Medicine and Animal Models. Front Neurosci. 2020 Feb 4;14:43. doi: 10.3389/fnins.2020.00043. PMID: 32116500; PMCID: PMC7015070.

[121] Gil SY, Youn BS, Byun K, Huang H, Namkoong C, Jang PG, Lee JY, Jo YH, Kang GM, Kim HK, Shin MS, Pietrzik CU, Lee B, Kim YB, Kim MS. Clusterin and LRP2 are critical components of the hypothalamic feeding regulatory pathway. Nat Commun. 2013;4:1862. doi: 10.1038/ncomms2896. Erratum in: Nat Commun. 2013;4:2912. PMID: 23673647.

[122] Clarke TK, Crist RC, Doyle GA, Weiss AR, Brandt H, Crawford S, Crow S, Fichter MM, Halmi KA, Johnson C, Kaplan AS, La Via M, Mitchell JE, Strober M, Rotondo A, Treasure J, Woodside DB, Keel P, Klump KL, Lilenfeld L, Plotnicov K, Magistretti PJ, Bergen AW, Kaye WH, Schork NJ, Berrettini WH. Characterization of genetic variation in the VGLL4 gene in anorexia nervosa. Psychiatr Genet. 2014 Aug;24(4):183-4. doi: 10.1097/YPG.0000000000000040. PMID: 24983835; PMCID: PMC4104366.

[123] Gervasini G, Gordillo I, García-Herráiz A, Flores I, Jiménez M, Monge M, Carrillo JA. Influence of dopamine polymorphisms on the risk for anorexia nervosa and associated psychopathological features. J Clin Psychopharmacol. 2013 Aug;33(4):551-5. doi: 10.1097/JCP.0b013e3182970469. PMID: 23775054.

[124] Bienvenu T, Lebrun N, Clarke J, Duriez P, Gorwood P, Ramoz N. Exome sequencing in a familial form of anorexia nervosa supports multigenic etiology. J Neural Transm (Vienna). 2019 Nov;126(11):1505-1511. doi: 10.1007/s00702-019-02056-2. Epub 2019 Aug 6. PMID: 31388831.

[125] Frieling H, Römer KD, Scholz S, Mittelbach F, Wilhelm J, De Zwaan M, Jacoby GE, Kornhuber J, Hillemacher T, Bleich S. Epigenetic dysregulation of dopaminergic genes in eating disorders. Int J Eat Disord. 2010 Nov 1;43(7):577-83. doi: 10.1002/eat.20745. PMID: 19728374.

[126] Jatana N, Thukral L, Latha N. Structural signatures of DRD4 mutants revealed using molecular dynamics simulations: Implications for drug targeting. J Mol Model. 2016 Jan;22(1):14. doi: 10.1007/s00894-015-2868-x. Epub 2015 Dec 17. PMID: 26680992.

[127] Wade TD, Gordon S, Medland S, Bulik CM, Heath AC, Montgomery GW, Martin NG. Genetic variants associated with disordered eating. Int J Eat Disord. 2013 Sep;46(6):594-608. doi: 10.1002/eat.22133. Epub 2013 Apr 9. PMID: 23568457; PMCID: PMC3775874.

[128] Newman JW, Morisseau C, Hammock BD. Epoxide hydrolases: their roles and interactions with lipid metabolism. Prog Lipid Res. 2005 Jan;44(1):1-51. doi: 10.1016/j.plipres.2004.10.001. Epub 2005 Jan 25. PMID: 15748653.

[129] -Van Zeeland AA, Bloss CS, Tewhey R, Bansal V, Torkamani A, Libiger O, Duvvuri V, Wineinger N, Galvez L, Darst BF, Smith EN, Carson A, Pham P, Phillips T, Villarasa N, Tisch R, Zhang G, Levy S, Murray S, Chen W, Srinivasan S, Berenson G, Brandt H, Crawford S, Crow S, Fichter MM, Halmi KA, Johnson C, Kaplan AS, La Via M, Mitchell JE, Strober M, Rotondo A, Treasure J, Woodside DB, Bulik CM, Keel P, Klump KL, Lilenfeld L, Plotnicov K, Topol EJ, Shih PB, Magistretti P, Bergen AW, Berrettini W, Kaye W, Schork NJ. Evidence for the role of EPHX2 gene variants in anorexia nervosa. Mol Psychiatry. 2014 Jun;19(6):724-32. doi: 10.1038/mp.2013.91. Epub 2013 Sep 3. PMID: 23999524; PMCID: PMC3852189.

[130] Lombardi L, Blanchet C, Poirier K, Lebrun N, Ramoz N, Rose Moro M, Gorwood P, Bienvenu T. Anorexia nervosa is associated with Neuronatin variants. Psychiatr Genet. 2019 Aug;29(4):103-110. doi: 10.1097/YPG.0000000000000224. PMID: 30933048.

[131] Le Foll C, Dunn-Meynell A, Musatov S, Magnan C, Levin BE. FAT/CD36: a major regulator of neuronal fatty acid sensing and energy homeostasis in rats and mice. Diabetes. 2013 Aug;62(8):2709-16. doi: 10.2337/db12-1689. Epub 2013 Apr 4. PMID: 23557700; PMCID: PMC3717873.

[132] Scherma M, Fattore L, Castelli MP, Fratta W, Fadda P. The role of the endocannabinoid system in eating disorders: neurochemical and behavioural preclinical evidence. Curr Pharm Des. 2014;20(13):2089-99. doi: 10.2174/13816128113199990429. Epub 2013 Jul 9. PMID: 23829365.

[133] Hopf FW. Do specific NMDA receptor subunits act as gateways for addictive behaviors? Genes Brain Behav. 2017 Jan;16(1):118-138. doi: 10.1111/gbb.12348. Epub 2016 Nov 18. PMID: 27706932; PMCID: PMC5351810.

[134] Mechelhoff D, van Noort BM, Weschke B, Bachmann CJ, Wagner C, Pfeiffer E, Winter S. Anti-NMDA receptor encephalitis presenting as atypical anorexia nervosa: an adolescent case report. Eur Child Adolesc Psychiatry. 2015 Nov;24(11):1321-4. doi: 10.1007/s00787-015-0682-8. Epub 2015 Feb 8. PMID: 25663428.

[135] Robinette TM, Nicholatos JW, Francisco AB, Brooks KE, Diao RY, Sorbi S, Ricca V, Nacmias B, Brieño-Enríquez MA, Libert S. SIRT1 accelerates the progression of activity-based anorexia. Nat Commun. 2020 Jun 4;11(1):2814. doi: 10.1038/s41467-020-16348-9. PMID: 32499508; PMCID: PMC7272424.

[136] Takeuchi S, Yazaki M, Yamada S, Fukuyama T, Inui A, Iwasaki Y, Ikeda S. An Adolescent Case of Citrin Deficiency With Severe Anorexia Mimicking Anorexia Nervosa. Pediatrics. 2015 Aug;136(2):e530-4. doi: 10.1542/peds.2014-4172. Epub 2015 Jul 20. PMID: 26195537.

[137] SCCS (Scientific Committee on Consumer Safe- ty), Opinion on Vitamin A (Retinol, Retinyl Acetate, Retinyl Palmitate), SCCS/1576/16, 20 April 2016, final version of 6 October 2016, CORRIGENDUM on 23 December 2016.

[138] Fitzpatrick JE, McDermott M, May D, Hofeldt FD. Eruptive neurofibromatosis associated with anorexia nervosa. Arch Dermatol. 1983 Dec;119(12):1019-21. PMID: 6418080.

[139] Schweinberger BM, Wyse AT. Mechanistic basis of hypermethioninemia. Amino Acids. 2016

Nov;48(11):2479-2489. doi: 10.1007/s00726-016-2302-4. Epub 2016 Jul 27. PMID: 27465642.

[140] Sáenz de Urturi D, Buqué X, Porteiro B, Folgueira C, Mora A, Delgado TC, Prieto-Fernández E, Olaizola P, Gómez-Santos B, Apodaka-Biguri M, González-Romero F, Nieva-Zuluaga A, Ruiz de Gauna M, Goikoetxea-Usandizaga N, García-Rodríguez JL, Gutierrez de Juan V, Aurrekoetxea I, Montalvo-Romeral V, Novoa EM, Martín-Guerrero I, Varela-Rey M, Bhanot S, Lee R, Banales JM, Syn WK, Sabio G, Martínez-Chantar ML, Nogueiras R, Aspichueta P. Methionine adenosyltransferase 1a antisense oligonucleotides activate the liver-brown adipose tissue axis preventing obesity and associated hepatosteatosis. Nat Commun. 2022 Mar 1;13(1):1096. doi: 10.1038/s41467-022-28749-z. PMID: 35232994; PMCID: PMC8888704.

[141] Omarjee L, Nitschke Y, Verschuere S, Bourrat E, Vignon MD, Navasiolava N, Leftheriotis G, Kauffenstein G, Rutsch F, Vanakker OM, Martin L. Severe early-onset manifestations of pseudoxanthoma elasticum resulting from the cumulative effects of several deleterious mutations in ENPP1, ABCC6 and HBB: transient improvement in ectopic calcification with sodium thiosulfate. Br J Dermatol. 2020 Aug;183(2):367-372. doi: 10.1111/bjd.18632. Epub 2019 Dec 22. PMID: 31646622.

[142] Peters T, Antel J, Naaresh R, Laabs BH, Föcker M, Albers N, Bühlmeier J, Hinney A, Libuda L, Hebebrand J. Suggestive Evidence for Causal Effect of Leptin Levels on Risk for Anorexia Nervosa: Results of a Mendelian Randomization Study. Front Genet. 2021 Sep 14;12:733606. doi: 10.3389/fgene.2021.733606. PMID: 34594363; PMCID: PMC8476861.

[143] Donato K, Ceccarini MR, Dhuli K, Bonetti G, Medori MC, Marceddu G, Precone V, Xhufi S, Bushati M, Bozo D, Beccari T, Bertelli M. Gene variants in eating disorders. Focus on anorexia nervosa, bulimia nervosa, and binge-eating disorder. J Prev Med Hyg. 2022 Oct 17;63(2 Suppl 3):E297-E305. doi: 10.15167/2421-4248/jpmh2022.63.2S3.2772. PMID: 36479493; PMCID: PMC9710388.

[144] Schalla MA, Stengel A. The Role of Ghrelin in Anorexia Nervosa. Int J Mol Sci. 2018 Jul 20;19(7):2117. doi: 10.3390/ijms19072117. PMID: 30037011; PMCID: PMC6073411.

[145] Méquinion M, Langlet F, Zgheib S, Dickson S, Dehouck B, Chauveau C, Viltart O. Ghrelin: central and peripheral implications in anorexia nervosa. Front Endocrinol (Lausanne). 2013 Feb 26;4:15. doi: 10.3389/fendo.2013.00015. PMID: 23549309; PMCID: PMC3581855.

[146] Witherspoon JW, Meilleur KG. Review of RyR1 pathway and associated pathomechanisms. Acta Neuropathol Commun. 2016 Nov 17;4(1):121. doi: 10.1186/s40478-016-0392-6. PMID: 27855725; PMCID: PMC5114830.

[147] Limoge F, Faivre L, Gautier T, Petit JM, Gautier E, Masson D, Jego G, El Chehadeh-Djebbar S, Marle N, Carmignac V, Deckert V, Brindisi MC, Edery P, Ghoumid J, Blair E, Lagrost L, Thauvin-Robinet C, Duplomb L. Insulin response dysregulation explains abnormal fat storage and increased risk of diabetes mellitus type 2 in Cohen Syndrome. Hum Mol Genet. 2015 Dec 1;24(23):6603-13. doi: 10.1093/hmg/ddv366. Epub 2015 Sep 10. PMID: 26358774.

[148] Raje V, Derecka M, Cantwell M, Meier J, Szczepanek K, Sisler JD, Strobl B, Gamero A, Harris TE, Larner AC. Kinase Inactive Tyrosine Kinase (Tyk2) Supports Differentiation of Brown Fat Cells. Endocrinology. 2017 Jan 1;158(1):148-157. doi: 10.1210/en.2015-2048. PMID: 27802075; PMCID: PMC5412977.

[149] Monteiro L, Foss-Freitas MC, Navarro A, Pereira F, Coeli F, Carneseca E, Júnior RM, Foss M. Evaluation of Dietary Intake, Leisure-Time Physical Activity, and Metabolic Profile in Women with Mutation in the LMNA Gene. J Am Coll Nutr. 2017 May-Jun;36(4):248-252. doi: 10.1080/07315724.2016.1262299. Epub 2017 Apr 26. PMID: 28443701.

[150] Mocchegiani E, Malavolta M. Zinc dyshomeostasis, ageing and neurodegeneration: implications of A2M and inflammatory gene polymorphisms. J Alzheimers Dis. 2007 Aug;12(1):101-9. doi: 10.3233/jad-2007-12110. PMID: 17851198.

[151] Su JC, Birmingham CL. Zinc supplementation in the treatment of anorexia nervosa. Eat Weight Disord. 2002 Mar;7(1):20-2. doi: 10.1007/BF03354425. PMID: 11930982.

[152] Jethwa PH, Small CJ, Smith KL, Seth A, Darch SJ, Abbott CR, Murphy KG, Todd JF, Ghatei MA, Bloom SR. Neuromedin U has a physiological role in the regulation of food intake and partially mediates the effects of leptin. Am J Physiol Endocrinol Metab. 2005 Aug;289(2):E301-5. doi: 10.1152/ajpendo.00404.2004. Epub 2005 Mar 22. PMID: 16014357.

[153] Botticelli L, Micioni Di Bonaventura E, Del Bello F, Giorgioni G, Piergentili A, Quaglia W, Bonifazi A, Cifani C, Micioni Di Bonaventura MV. The neuromedin U system: Pharmacological implications for the treatment of

obesity and binge eating behavior. Pharmacol Res. 2023 Sep;195:106875. doi: 10.1016/j.phrs.2023.106875. Epub 2023 Jul 29. PMID: 37517560.

[154] Michelini S, Chiurazzi P, Marino V, Dell'Orco D, Manara E, Baglivo M, Fiorentino A, Maltese PE, Pinelli M, Herbst KL, Dautaj A, Bertelli M. Aldo-Keto Reductase 1C1 (*AKR1C1*) as the First Mutated Gene in a Family with Nonsyndromic Primary Lipedema. Int J Mol Sci. 2020 Aug 29;21(17):6264. doi: 10.3390/ijms21176264. PMID: 32872468; PMCID: PMC7503355.

[155] Hinney A, Kesselmeier M, Jall S, Volckmar AL, Föcker M, Antel J; GCAN; WTCCC3; Heid IM, Winkler TW; GIANT; Grant SF; EGG; Guo Y, Bergen AW, Kaye W, Berrettini W, Hakonarson H; Price Foundation Collaborative Group; Children's Hospital of Philadelphia/Price Foundation; Herpertz-Dahlmann B, de Zwaan M, Herzog W, Ehrlich S, Zipfel S, Egberts KM, Adan R, Brandys M, van Elburg A, Boraska Perica V, Franklin CS, Tschöp MH, Zeggini E, Bulik CM, Collier D, Scherag A, Müller TD, Hebebrand J. Evidence for three genetic loci involved in both anorexia nervosa risk and variation of body mass index. Mol Psychiatry. 2017 Feb;22(2):192-201. doi: 10.1038/mp.2016.71. Epub 2016 May 17. Erratum in: Mol Psychiatry. 2017 Feb;22(2):321-322. doi: 10.1038/mp.2016.126. PMID: 27184124; PMCID: PMC5114162.

[156] Nobis S, Goichon A, Achamrah N, Guérin C, Azhar S, Chan P, Morin A, Bôle-Feysot C, do Rego JC, Vaudry D, Déchelotte P, Belmonte L, Coëffier M. Alterations of proteome, mitochondrial dynamic and autophagy in the hypothalamus during activity-based anorexia. Sci Rep. 2018 May 8;8(1):7233. doi: 10.1038/s41598-018-25548-9. PMID: 29740148; PMCID: PMC5940678.

[157] Valentino MA, Lin JE, Snook AE, Li P, Kim GW, Marszalowicz G, Magee MS, Hyslop T, Schulz S, Waldman SA. A uroguanylin-GUCY2C endocrine axis regulates feeding in mice. J Clin Invest. 2011 Sep;121(9):3578-88. doi: 10.1172/JCI57925. Epub 2011 Aug 25. PMID: 21865642; PMCID: PMC3223926.

[158] Goold R, Hubank M, Hunt A, Holton J, Menon RP, Revesz T, Pandolfo M, Matilla-Dueñas A. Down-regulation of the dopamine receptor D2 in mice lacking ataxin 1. Hum Mol Genet. 2007 Sep 1;16(17):2122-34. doi: 10.1093/hmg/ddm162. Epub 2007 Jun 28. PMID: 17599952.

[159] Santos JL, Cortés VA. Eating behaviour in contrasting adiposity phenotypes: Monogenic obesity and congenital generalized lipodystrophy. Obes Rev. 2021 Jan;22(1):e13114. doi: 10.1111/obr.13114. Epub 2020

Aug 2. PMID: 33030294.

[160] Oh H, Lee S, Oh Y, Kim S, Kim YS, Yang Y, Choi W, Yoo YE, Cho H, Lee S, Yang E, Koh W, Won W, Kim R, Lee CJ, Kim H, Kang H, Kim JY, Ku T, Paik SB, Kim E. Kv7/KCNQ potassium channels in cortical hyperexcitability and juvenile seizure-related death in Ank2-mutant mice. Nat Commun. 2023 Jun 15;14(1):3547. doi: 10.1038/s41467-023-39203-z. PMID: 37321992; PMCID: PMC10272139.

[161] Paz-Filho G, Boguszewski MC, Mastronardi CA, Patel HR, Johar AS, Chuah A, Huttley GA, Boguszewski CL, Wong ML, Arcos-Burgos M, Licinio J. Whole exome sequencing of extreme morbid obesity patients: translational implications for obesity and related disorders. Genes (Basel). 2014 Aug 25;5(3):709-25. doi: 10.3390/genes5030709. PMID: 25158045; PMCID: PMC4198926.

[162] Lanfray D, Caron A, Roy MC, Laplante M, Morin F, Leprince J, Tonon MC, Richard D. Involvement of the Acyl-CoA binding domain containing 7 in the control of food intake and energy expenditure in mice. Elife. 2016 Feb 15;5:e11742. doi: 10.7554/eLife.11742. PMID: 26880548; PMCID: PMC4821795.

[163] N. Zhu, A. P. A. Janssen, M. van der Stelt, Isr. J. Chem. 2023, 63, e202200115.

[164] Tam FI, Steding J, Steinhäuser JL, Ritschel F, Gao W, Weidner K, Roessner V, Kirschbaum C, Ehrlich S. Hair endocannabinoid concentrations in individuals with acute and weight-recovered anorexia nervosa. Prog Neuropsychopharmacol Biol Psychiatry. 2021 Apr 20;107:110243. doi: 10.1016/j.pnpbp.2021.110243. Epub 2021 Jan 11. PMID: 33444649.

[165] Wang D, Jabile MJT, Lu J, Townsend LK, Valvano CM, Gautam J, Batchuluun B, Tsakiridis EE, Lally JSV, Steinberg GR. Fatty Acids Increase GDF15 and Reduce Food Intake Through a GFRAL Signaling Axis. Diabetes. 2024 Jan 1;73(1):51-56. doi: 10.2337/db23-0495. PMID: 37847913; PMCID: PMC10784653.

[166] Sabatini PV, Frikke-Schmidt H, Arthurs J, Gordian D, Patel A, Rupp AC, Adams JM, Wang J, Beck Jørgensen S, Olson DP, Palmiter RD, Myers MG Jr, Seeley RJ. GFRAL-expressing neurons suppress food intake via aversive pathways. Proc Natl Acad Sci U S A. 2021 Feb 23;118(8):e2021357118. doi: 10.1073/pnas.2021357118. PMID: 33593916; PMCID: PMC7923658.

[167] Degerman E, Ahmad F, Chung YW, Guirguis E, Omar B, Stenson L, Manganiello V. From PDE3B to the

regulation of energy homeostasis. Curr Opin Pharmacol. 2011 Dec;11(6):676-82. doi: 10.1016/j.coph.2011.09.015. Epub 2011 Oct 14. PMID: 22001403; PMCID: PMC3225700.

[168] Kesselmeier M, Pütter C, Volckmar AL, Baurecht H, Grallert H, Illig T, Ismail K, Ollikainen M, Silén Y, Keski-Rahkonen A, Bulik CM, Collier DA, Zeggini E, Hebebrand J, Scherag A, Hinney A; GCAN and WTCCC3. High-throughput DNA methylation analysis in anorexia nervosa confirms TNXB hypermethylation. World J Biol Psychiatry. 2018 Apr;19(3):187-199. doi: 10.1080/15622975.2016.1190033. Epub 2016 Jul 1. PMID: 27367046.

[169] Sasaki T, Matsui S, Kitamura T. Control of Appetite and Food Preference by NMDA Receptor and Its Co-Agonist d-Serine. Int J Mol Sci. 2016 Jul 7;17(7):1081. doi: 10.3390/ijms17071081. PMID: 27399680; PMCID: PMC4964457.

[170] Scott R, Sánchez-Aguilera A, van Elst K, Lim L, Dehorter N, Bae SE, Bartolini G, Peles E, Kas MJH, Bruining H, Marín O. Loss of Cntnap2 Causes Axonal Excitability Deficits, Developmental Delay in Cortical Myelination, and Abnormal Stereotyped Motor Behavior. Cereb Cortex. 2019 Feb 1;29(2):586-597. doi: 10.1093/cercor/bhx341. PMID: 29300891.

[171] Sohn JW. Network of hypothalamic neurons that control appetite. BMB Rep. 2015 Apr;48(4):229-33. doi: 10.5483/bmbrep.2015.48.4.272. PMID: 25560696; PMCID: PMC4436859.

[172] Chapman KL, Kinsella GK, Cox A, Donnelly D, Findlay JB. Interactions of the melanocortin-4 receptor with the peptide agonist NDP-MSH. J Mol Biol. 2010 Aug 20;401(3):433-50. doi: 10.1016/j.jmb.2010.06.028. Epub 2010 Jun 19. PMID: 20600126; PMCID: PMC3101337.

[173] Ro HS, Zhang L, Majdalawieh A, Kim SW, Wu X, Lyons PJ, Webber C, Ma H, Reidy SP, Boudreau A, Miller JR, Mitchell P, McLeod RS. Adipocyte enhancer-binding protein 1 modulates adiposity and energy homeostasis. Obesity (Silver Spring). 2007 Feb;15(2):288-302. doi: 10.1038/oby.2007.569. PMID: 17299101.

[174] Wolfe RR. Branched-chain amino acids and muscle protein synthesis in humans: myth or reality? J Int Soc Sports Nutr. 2017 Aug 22;14:30. doi: 10.1186/s12970-017-0184-9. PMID: 28852372; PMCID: PMC5568273.

[175] Bishop CA, Machate T, Henning T, Henkel J, Püschel G, Weber D, Grune T, Klaus S, Weitkunat K. Detrimental effects of branched-chain amino acids in glucose tolerance can be attributed to valine induced glucotoxicity in skeletal muscle. Nutr Diabetes. 2022 Apr 13;12(1):20. doi: 10.1038/s41387-022-00200-8. PMID: 35418570; PMCID: PMC9008040.

[176] Sookoian S, Pirola CJ. Alanine and aspartate aminotransferase and glutamine-cycling pathway: their roles in pathogenesis of metabolic syndrome. World J Gastroenterol. 2012 Aug 7;18(29):3775-81. doi: 10.3748/wjg.v18.i29.3775. PMID: 22876026; PMCID: PMC3413046.

[177] Fernstrom JD, Fernstrom MH. Tyrosine, phenylalanine, and catecholamine synthesis and function in the brain. J Nutr. 2007 Jun;137(6 Suppl 1):1539S-1547S; discussion 1548S. doi: 10.1093/jn/137.6.1539S. PMID: 17513421.

[178] Rigamonti AE, Tamini S, Cicolini S, De Col A, Caroli D, Mai S, Rondinelli E, Saezza A, Cella SG, Sartorio A. Evaluation of an Amino Acid Mix on the Secretion of Gastrointestinal Peptides, Glucometabolic Homeostasis, and Appetite in Obese Adolescents Administered with a Fixed-Dose or ad Libitum Meal. J Clin Med. 2020 Sep 22;9(9):3054. doi: 10.3390/jcm9093054. PMID: 32971830; PMCID: PMC7564111.

[179] VanDusseldorp TA, Escobar KA, Johnson KE, Stratton MT, Moriarty T, Cole N, McCormick JJ, Kerksick CM, Vaughan RA, Dokladny K, Kravitz L, Mermier CM. Effect of Branched-Chain Amino Acid Supplementation on Recovery Following Acute Eccentric Exercise. Nutrients. 2018 Oct 1;10(10):1389. doi: 10.3390/nu10101389. PMID: 30275356; PMCID: PMC6212987.

[181] Boulesteix AL, Strimmer K. Partial least squares: a versatile tool for the analysis of high-dimensional genomic data. Brief Bioinform. 2007 Jan;8(1):32-44. doi: 10.1093/bib/bbl016. Epub 2006 May 26. PMID: 16772269.

[182] Cassel C, Hackl P, Westlund AH. Robustness of partial least-squares method for estimating latent variable quality structures. Journal of Applied Statistics, 1999. 26(4), 435–446. https://doi.org/10.1080/02664769922322

[183] Cui H, Moore J, Ashimi SS, Mason BL, Drawbridge JN, Han S, Hing B, Matthews A, McAdams CJ, Darbro BW, Pieper AA, Waller DA, Xing C, Lutter M. Eating disorder predisposition is associated with ESRRA and HDAC4 mutations. J Clin Invest. 2013 Nov;123(11):4706-13. doi: 10.1172/JCI71400. PMID: 24216484; PMCID: PMC3809805.

[184] GeneCards. Available online: https://www.genecards.org/cgi-bin/ carddisp.pl?gene=NNAT&keywords=nnat. Accessed 1 Apr 2021

[185] Sawicka N, Gryczyńska M, Sowiński J, Tamborska-Zedlewska M, Ruchała M. Two diagnoses become one? Rare case report of anorexia nervosa and Cushing's syndrome. Neuropsychiatr Dis Treat. 2013;9:431-5. doi: 10.2147/NDT.S40398. Epub 2013 Mar 31. PMID: 23579693; PMCID: PMC3621711.

[186] Hatakeyama M, Nakagami T, Yasui-Furukori N. Adrenal Cushing's syndrome may resemble eating disorders. Gen Hosp Psychiatry. 2014 Nov-Dec;36(6):760.e9-10. doi: 10.1016/j.genhosppsych.2014.06.006. Epub 2014 Jun 28. PMID: 25085718.

[187] Curtis D, Adlington K, Bhui KS. Pursuing parity: genetic tests for psychiatric conditions in the UK National Health Service. Br J Psychiatry. 2019 May;214(5):248-250. doi: 10.1192/bjp.2019.48. Epub 2019 Mar 22. PMID: 30900968.

[188] Andries A, Støving RK Cannabinoid-1 receptor agonists: A therapeutic option in severe, chronic anorexia nervosa? Neuropsychiatry 2011.1:467–476. https://doi.org/10.2217/npy.11.50

[189] Costa B, Comelli F, Bettoni I, Colleoni M, Giagnoni G. The endogenous fatty acid amide, palmitoylethanolamide, has anti-allodynic and anti-hyperalgesic effects in a murine model of neuropathic pain: involvement of CB(1), TRPV1 and PPARgamma receptors and neurotrophic factors. Pain. 2008 Oct 31;139(3):541-550. doi: 10.1016/j.pain.2008.06.003. Epub 2008 Jul 3. PMID: 18602217.

[190] Donato K, Medori MC, Macchia A, Cecchin S, Ceccarini MR, Beccari T, Gatta V, Stuppia L, Benfatti V, Dalla Ragione L, Micheletti PCC, Dhuli K, Madeo G, Bonetti G, Marceddu G, Bertelli M. Genetic variants identified in novel candidate genes for anorexia nervosa and analysis of molecular pathways for diagnostic applications. Eur Rev Med Pharmacol Sci. 2023 Dec;27(6 Suppl):77-88. doi: 10.26355/eurrev_202312_34692. Erratum in: Eur Rev Med Pharmacol Sci. 2024 Mar;28(6):2627. doi: 10.26355/eurrev_202403_35779. PMID: 38112957.

[191] Grannell A, Kokkinos A, le Roux CW. Myokines in Appetite Control and Energy Balance. Muscles. 2022; 1(1):26-47. https://doi.org/10.3390/muscles1010003

[192] Chapman KL, Kinsella GK, Cox A, Donnelly D, Findlay JB. Interactions of the melanocortin-4 receptor with the peptide agonist NDP-MSH. J Mol Biol. 2010 Aug 20;401(3):433-50. doi: 10.1016/j.jmb.2010.06.028. Epub 2010 Jun 19. PMID: 20600126; PMCID: PMC3101337.

[193] Chen C, Hou G, Zeng C, Ren Y, Chen X, Peng C. Metabolomic profiling reveals amino acid and carnitine alterations as metabolic signatures in psoriasis. Theranostics. 2021 Jan 1;11(2):754-767. doi: 10.7150/thno.51154. PMID: 33391503; PMCID: PMC7738860.

[194] Bene J, Hadzsiev K, Melegh B. Role of carnitine and its derivatives in the development and management of type 2 diabetes. Nutr Diabetes. 2018 Mar 7;8(1):8. doi: 10.1038/s41387-018-0017-1. PMID: 29549241; PMCID: PMC5856836.

[195] Virmani MA, Cirulli M. The Role of l-Carnitine in Mitochondria, Prevention of Metabolic Inflexibility and Disease Initiation. Int J Mol Sci. 2022 Feb 28;23(5):2717. doi: 10.3390/ijms23052717. PMID: 35269860; PMCID: PMC8910660.

[196] Wu T, Guo A, Shu Q, Qi Y, Kong Y, Sun Z, Sun S, Fu Z. L-Carnitine intake prevents irregular feeding-induced obesity and lipid metabolism disorder. Gene. 2015 Jan 10;554(2):148-54. doi: 10.1016/j.gene.2014.10.040. Epub 2014 Oct 25. PMID: 25445284.

[197] Adibi SA. Metabolism of branched-chain amino acids in altered nutrition. Metabolism. 1976 Nov;25(11):1287-302. doi: 10.1016/s0026-0495(76)80012-1. PMID: 790082.

[198] Mann G, Mora S, Madu G, Adegoke OAJ. Branched-chain Amino Acids: Catabolism in Skeletal Muscle and Implications for Muscle and Whole-body Metabolism. Front Physiol. 2021 Jul 20;12:702826. doi: 10.3389/fphys.2021.702826. PMID: 34354601; PMCID: PMC8329528.

[199] Shimomura Y, Murakami T, Nakai N, Nagasaki M, Harris RA. Exercise promotes BCAA catabolism: effects of BCAA supplementation on skeletal muscle during exercise. J Nutr. 2004 Jun;134(6 Suppl):1583S-1587S. doi: 10.1093/jn/134.6.1583S. PMID: 15173434.

[200] Miflin B, Lea P. Amino acid metabolism. Annu Rev Plant Physiol 1977; 28: 299-329.

[201] Xiao F, Guo F. Impacts of essential amino acids on energy balance. Mol Metab. 2022 Mar;57:101393. doi: 10.1016/j.molmet.2021.101393. Epub 2021 Nov 14. PMID: 34785395; PMCID: PMC8829800.

[202] Wang W, Xu Y, Chi S, Yang P, Mai K, Song F. Dietary lysine regulates body growth performance via the nutrient-sensing signaling pathways in largemouth bass (Micropterus salmoides). Front Mar Sci 2020; 7: 595682.

[203] Moris JM, Heinold C, Blades A, Koh Y. Nutrient-Based Appetite Regulation. J Obes Metab Syndr. 2022 Jun 30;31(2):161-168. doi: 10.7570/jomes22031. Epub 2022 Jun 20. PMID: 35718856; PMCID: PMC9284573.

[204] Höglund E, Øverli Ø, Winberg S. Tryptophan Metabolic Pathways and Brain Serotonergic Activity: A Comparative Review. Front Endocrinol (Lausanne). 2019 Apr 8;10:158. doi: 10.3389/fendo.2019.00158. PMID: 31024440; PMCID: PMC6463810.

[205] Harper AE, Miller RH, Block KP. Branched-chain amino acid metabolism. Annu Rev Nutr. 1984;4:409-54. doi: 10.1146/annurev.nu.04.070184.002205. PMID: 6380539.

[206] Nie C, He T, Zhang W, Zhang G, Ma X. Branched Chain Amino Acids: Beyond Nutrition Metabolism. Int J Mol Sci. 2018 Mar 23;19(4):954. doi: 10.3390/ijms19040954. PMID: 29570613; PMCID: PMC5979320.

[207] Lutter M, Bahl E, Hannah C, Hofammann D, Acevedo S, Cui H, McAdams CJ, Michaelson JJ. Novel and ultra-rare damaging variants in neuropeptide signaling are associated with disordered eating behaviors. PLoS One. 2017 Aug 28;12(8):e0181556. doi: 10.1371/journal.pone.0181556. PMID: 28846695; PMCID: PMC5573281.

[208] Roman CW, Derkach VA, Palmiter RD. Genetically and functionally defined NTS to PBN brain circuits mediating anorexia. Nat Commun. 2016 Jun 15;7:11905. doi: 10.1038/ncomms11905. PMID: 27301688; PMCID: PMC4912612.

[209] Diez-Fernandez C, Rüfenacht V, Gemperle C, Fingerhut R, Häberle J. Mutations and common variants in the human arginase 1 (ARG1) gene: Impact on patients, diagnostics, and protein structure considerations. Hum Mutat. 2018 Aug;39(8):1029-1050. doi: 10.1002/humu.23545. Epub 2018 Jun 21. PMID: 29726057.

[210] Weiss MJ, Cole DE, Ray K, Whyte MP, Lafferty MA, Mulivor RA, Harris H. A missense mutation in the human liver/bone/kidney alkaline phosphatase gene causing a lethal form of hypophosphatasia. Proc Natl Acad Sci U S A. 1988 Oct;85(20):7666-9. doi: 10.1073/pnas.85.20.7666. PMID: 3174660; PMCID: PMC282253.

[211] Haan J, Kors EE, Ferrari MD. Familial cyclic vomiting syndrome. Cephalalgia. 2002 Sep;22(7):552-4. doi: 10.1046/j.1468-2982.2002.00420.x. PMID: 12230597.

[212] Sánchez-López JY, Camacho-Torres AL, Ibarra B, Tintos JA, Perea FJ. Analysis of the SLC4A1 gene in three Mexican patients with hereditary spherocytosis: Report of a novel mutation. Genet Mol Biol. 2010 Jan;33(1):9-11. doi: 10.1590/S1415-47572009005000109. Epub 2010 Mar 1. PMID: 21637597; PMCID: PMC3036091.