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# Genetic variants identified in novel candidate genes for anorexia nervosa and analysis of molecular pathways for diagnostic applications

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**Abstract. – OBJECTIVE: Anorexia nervosa (AN) is a severe psychiatric disorder characterized by an intense fear of gaining weight, a relentless pursuit of thinness, and a distorted body image. Recent research highlights the substantial contribution of genetics to AN's etiology, with genes like *BDNF*, *SLC6A4*, and *DRD2* implicated. However, a comprehensive genetic test for AN diagnosis is lacking. This study aims to elucidate the biological foundations of AN, examining variants in genes associated with syndromic forms, rare variants in AN patients, and candidate genes from GWAS studies, murine models, or established molecular pathways.**

**MATERIALS AND METHODS: The study involved 135 AN patients from Italy, diagnosed based on DSM-V criteria. A specialized Next-Generation Sequencing panel targeting 163 genes was designed. Sequencing was performed on an Illumina MiSeq System, and variants were analyzed using bioinformatics tools. Data on clinical parameters, exercise habits, and AN types were collected.**

**RESULTS: The AN cohort, predominantly female, exhibited diverse clinical characteristics. Our analysis identified gene variants associated with syndromic forms of AN, such as STRA6, NF1, MAT1A, and ABCC6. Variants were also found in known AN-related genes (*CD36, DRD4, GCKR, GHRL, GRIN3B, GPR55, LEPR*) and in other 16 candidate genes (*A2M, AEBP1, ABHD4, ACBD7, CNTNAP, GFRAL, GRIN2D, LIPE, LMNA, NMU, PDE3B, POMC, RYR1, TNXB, TYK2, VPS13B*), highlighting the complexity of AN's genetic landscape. The endocannabinoid and dopamine pathways play crucial roles. Skeletal muscle-related genes and appetite-regulating hormones also revealed potential connections. Adipogenesis-related genes suggest AN's association with subcutaneous adipose tissue deficiency.**

**CONCLUSIONS: This study provides comprehensive insights into the genetic underpinnings of AN, emphasizing the importance of multiple pathways. The identified variants contribute.**

*Key Words:*
Anorexia nervosa, NGS panel, Variants, Candidate genes, Molecular pathways, Metabolic disorders.

## Introduction

Anorexia nervosa (AN) is a complex and potentially life-threatening psychiatric disorder characterized by an intense fear of gaining weight,

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a persistent search for thinness, and a distorted body image. AN predominantly affects young women, with a peak onset during adolescence, although it can occur in individuals of any age and gender[1,2]. Its clinical presentation encompasses a range of symptoms, including significant weight loss, refusal to maintain a minimally average body weight, preoccupation with food, calories, and dieting, as well as various physiological changes such as amenorrhea (in females), lanugo hair growth, and osteoporosis[3]. Alongside these overt manifestations, AN is also associated with a host of psychological symptoms, including anxiety, depression, obsessive-compulsive behaviors, and social withdrawal[4,5].

Previous scholars[5] have consistently demonstrated a higher risk of developing AN among individuals with affected relatives, suggesting a hereditary component. While the exact genetic architecture of AN remains complex and multifactorial, several common genes and genetic pathways have been implicated. Variations in the *BDNF* (brain-derived neurotrophic factor) play a crucial role in regulating appetite and body weight; this gene has been associated with increased susceptibility to AN[6-8]. Additionally, genes involved in serotonin and dopamine signaling pathways, such as the serotonin transporter gene (*SLC6A4*)[9] and the dopamine receptor gene (*DRD2* and *DRD4*)[10], have been linked to the development of AN, as alterations in these systems can affect mood and appetite regulation[11]. Genetic factors are estimated to account for about 80% of the risk of developing AN[9]. Evidence from previous studies[10], along with whole exome sequencing, genome-wide studies, and candidate gene association studies, underscore the fundamental role of genetics in the etiology of AN.

The management of AN necessitates a multidisciplinary approach, yet significant uncertainties persist in the realms of screening, detection, and classification. Genetic counseling has shown promise for individuals with psychiatric disorders like AN and their families[11].

The primary objective of this scientific article is to elucidate the biological foundations and molecular pathways involved in AN through a comprehensive analysis of a gene panel. The designed and validated gene panel facilitates a multi-phased analysis: (1) identification of variants in genes linked to syndromic forms of AN, (2) identification of variants in genes previously associated with rare variants in anorexic patients, and (3) identification of variants in candidate genes for AN based on GWAS studies, murine models, or their known protein functions within established molecular pathways of AN. A deeper understanding of the pathophysiology of AN is needed, and in terms of treatment, continued efforts are required to explore novel nutritional and pharmacological avenues[12].

## Patients and Methods

### Patients and Sample Collection

The genetic association analysis was conducted with a cohort of individuals diagnosed with AN from Italy. Participants meeting the criteria for AN as outlined in the DSM-V (Diagnostic and Statistical Manual of Mental Disorders, 5th edition) from Palazzo Francisci (Todi) were recruited for this study. This research adhered to the principles established in the Declaration of Helsinki and received approval from the Institutional Review Board "Comitato Etico delle Aziende Sanitarie (CEAS) della Regione Umbria" under Protocol Number 29616/12/AV. To ensure data confidentiality, each participant was assigned a unique alphanumeric code. Experienced psychiatrists specializing in eating disorders, following the DSM-V criteria, through comprehensive face-to-face interviews, accurately confirmed the clinical diagnosis. After obtaining informed consent, patients were provided with tailored questionnaires and assessments to describe their clinical profiles. Exclusion criteria encompassed alternative eating disorders and underlying medical conditions leading to weight loss. A blood sample containing EDTA was collected from each participant. Genomic DNA samples from all individuals were isolated from peripheral blood using a commercial kit (SAMAG 120 BLOOD DNA Extraction Kit). The quantity of DNA was measured using a Qubit 4 Fluorometer.

We aim to elucidate the biological foundations of AN, examining variants in genes associated with syndromic forms, rare variants in AN patient, and candidate genes from GWAS studies or established molecular pathways; therefore, there is no output that can be analyzed statistically.

### Gene Selection and Panel Design

A subset comprising 163 genes from a dedicated Next-Generation Sequencing (NGS) panel was analyzed[5]. The collective length of the genomic targets covered by this panel amounted to 3.55 Mb. These analyzed genes were further cate-

gorized into groups: genes linked to syndromic disorders now found in forms of AN, genes previously associated with AN, and "research" candidate genes, chosen based on findings from genome-wide association studies (GWAS), molecular pathways, and animal models. The probe set was precisely designed to capture the coding exons and adjacent 15-base pair flanking regions of each gene within the panel, employing the Twist Custom Panel Design Technology (Twist Bioscience, https://www.twistbioscience.com/products/ngs). The target sequences were mapped to the GRCh38/hg38 genome version for reference.

### Genetic Analysis and Variant Detection

NGS libraries were constructed following the manufacturer's guidelines, utilizing the Twist Library Preparation EF Kit and the Universal Adapter system, incorporating the Standard Hybridization Target Enrichment procedure (Twist Bioscience). Subsequently, the DNA Library samples underwent sequencing on an Illumina MiSeq System (Illumina, San Diego, CA), which produced 150-base pair-long reads, adhering to established laboratory protocols as detailed[13]. Following sequencing, Fastq files were generated, encompassing both forward and reverse reads. Read alignment was executed using the BWA software (version 0.7.17-r1188), while the removal of duplicate reads was carried out through SAMBAMBA (version 0.6.7). For realignment, the GATK (version 4.0.0.0) tool was employed. To identify nucleotide variations, comprehensive databases such as dbSNP (www.ncbi.nlm.nih.gov/SNP/) and the Human Gene Mutation Database professional (HGMD; http://www.biobaseinternational.com/product/hgmd) were checked. Our bioinformatics pipeline also incorporated an assessment for the potential presence of copy number variants (CNVs). Variants meeting the criteria of a minimum depth of coverage of 10X and a quality score (Phred-score) exceeding 18 were considered for inclusion in the analysis. Variant calling was carried out using both Samtools (http://www.htslib.org/) and GATK (https://gatk.broadinstitute.org/hc/en-us) tools, employing their default settings. Furthermore, an *in silico* evaluation of the pathogenicity of nucleotide alterations within exons was conducted. This assessment utilized VarSome (https://varsome.com/), MutationTaster (http://www.mutationtaster.org), Polyphen-2, SIFT, and CADD. To ensure the inclusion of rare variants, we cross-referenced their minor allele frequencies (MAF) with the Genome Aggregation Database (gnomAD; http://gnomad.broadinstitute.org/), specifically selecting variants with a MAF below 1%.

## Results

The study recruited 135 AN patients (134 females and 1 male) with an average age of 22 years. Participants had a mean height of 161 cm (± 6 cm) and a range of weights from 37.59 kg (± 5.73 kg) to 56.90 kg (± 10.55 kg), indicating significant weight variability. The average BMI at the lowest weight was approximately 14.46 kg/m² (± 1.97), while at the highest weight, it was about 21.59 kg/m² (± 4.29). Notably, 89 participants engaged in excessive physical exercise, while 30 did not, and 16 had unknown exercise habits. Regarding AN type, the study predominantly comprised participants with the restrictive type (n=97), followed by purging (n=29), with smaller numbers exhibiting atypical or orthorexia AN type. Additionally, 5 participants had an unknown AN type. All clinical data is shown in Table I. 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. Genetic variants identified in the AN population are reported in Table II.

**Table I.** Clinical data of recruited patients in the study.

| Characteristics | Values |
|---|---|
| **Gender** | |
| Male | 1 |
| Female | 134 |
| 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/m$^2$) | 14.46 ± 1.97 |
| Mean BMI highest ± SD (kg/m$^2$) | 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 II.** Genetic variants identified in our population by NGS.

| 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_850insGGCCT CCCCCCGG | 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 | *PALB2* | c.104T>C | p.Leu35Pro | rs141047069 | P |
| | | *TYK2* | c.2047+1G>T | - | rs1568333687 | P |
| Case 7 | F | *DRD4* | c.849_850insGGCCT CCCCCCGG | p.Ser284GlyfsTer170 | rs1290906588 | VUS |
| | | *LMNA* | c.1364G>A | p.Arg455His | rs267607597 | LP |
| | | *MAPK12* | c.101dup | p.Ser35LeufsTer18 | rs532163968 | LP |
| Case 8 | F | *GRIN3B* | c.1396_1397insCGTG | p.Gly466AlafsTer18 | | LP |
| Case 9 | F | *DRD4* | c.849_850insGGCCT CCCCCCGG | p.Ser284GlyfsTer170 | rs1290906588 | VUS |
| | | *LEPR* | c.3495_3496del | p.Ter1166IlefsTer14 | rs756571131 | LP |
| Case 10 | F | *DRD4* | c.849_850insGGCCT CCCCCCGG | p.Ser284GlyfsTer170 | rs1290906588 | VUS |
| Case 11 | F | *A2M* | c.2126-5_2126-1del | - | - | LP |
| Case 12 | F | *CNBD1* | c.406G>T | p.Glu136Ter | - | LP |
| Case 13 | F | *DRD4* | c.849_850insGGCCT CCCCCCGG | p.Ser284GlyfsTer170 | rs1290906588 | 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_850insGGCCT CCCCCCGG | p.Ser284GlyfsTer170 | rs1290906588 | VUS |
| Case 20 | F | *ATXN1* | c.672_673insTAG | p.Gln224_Gln225insTer | - | LP |
| Case 21 | F | *DRD4* | c.849_850insGGCCT CCCCCCGG | p.Ser284GlyfsTer170 | rs1290906588 | VUS |
| Case 22 | F | *CD36* | c.1255-1G>A | - | rs375042355 | LP |
| | | *DRD4* | c.849_850insGGCCT CCCCCCGG | p.Ser284GlyfsTer170 | rs1290906588 | VUS |
| Case 23 | F | *ESR2* | c.335C>A | p.Ser112Ter | rs141516067 | LP |
| Case 24 | F | *DRD4* | c.849_850insGGCCT CCCCCCGG | 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_850insGGCCT CCCCCCGG | p.Ser284GlyfsTer170 | rs1290906588 | VUS |
| Case 35 | F | *MC1R* | c.88C>T | p.Gln30Ter | rs756579024 | LP |
| | | *TNXB* | c.12463+2T>C | - | rs545719209 | LP |

*Continued*

**Table II *(Continued)*.** Genetic variants identified in our population by NGS.

| Patient | Sex | Gene | Nucleotide variant | Amino acid variant | rs ID | ACMG verdict |
|---|---|---|---|---|---|---|
| Case 36 | F | *GRIN2D* | c.3684_3685insGA | p.Pro1229AspfsTer290 | - | LP |
| Case 37 | F | *DRD4* | c.849_850insGGCCT CCCCCCGG | p.Ser284GlyfsTer170 | rs1290906588 | VUS |
| Case 38 | F | *GHRL* | c.102_103del | p.Arg34SerfsTer37 | rs771527525 | LP |
| Case 39 | F | *ABCC6* | c.1553G>A | p.Arg518Gln | rs72653772 | 2 |
| | | *DRD4* | c.849_850insGGCCT CCCCCCGG | p.Ser284GlyfsTer170 | rs1290906588 | VUS |
| | | *GCKR* | c.1618C>T | p.Arg540Ter | rs146053779 | LP |
| Case 40 | F | *CNTNAP2* | c.3979_3994dup | p.? | - | LP |
| Case 41 | F | *KIT* | c.1247T>G | p.Leu416Arg | - | LP |
| Case 42 | F | *ABCC6* | c.1799G>A | p.Arg600His | rs761433545 | LP |
| Case 43 | F | *POMC* | c.706C>G | p.Arg236Gly | rs28932472 | LP |
| Case 44 | F | *ABHD4* | c.832C>T | p.Arg278Ter | rs371651543 | LP |
| Case 45 | F | *ABCC6* | c.801_803del | p.Asn267del | rs1185530488 | LP |
| | | *ZMPSTE24* | c.1204-5_1210del | - | rs312262689 | LP |
| Case 46 | F | *VPS13B* | c.9406-1G>T | - | rs386834119 | P |
| Case 47 | F | *MCTP1* | c.2987dup | p.Asn996LysfsTer11 | rs1191526672 | LP |
| | | *PDE3B* | c.463_482del | p.Ala156ProfsTer177 | - | LP |
| Case 48 | F | *LIPE* | c.235del | p.Gln79LysfsTer41 | rs770707778 | LP |
| | | MC4R | c.291T>G | p.Asn97Lys | - | LP |
| Case 49 | F | *GCKR* | c.679C>T | p.Arg227Ter | rs149847328 | LP |
| Case 50 | F | *A2ML1* | c.2072del | p.Pro691GlnfsTer2 | rs769669380 | LP |
| | | *NF1* | c.1260+1G>C | - | - | P |
| Case 51 | F | *CD36* | c.949dup | p.Ile317AsnfsTer36 | rs70961716 | P |
| Case 52 | F | *AEBP1* | c.1485+1G>A | - | - | LP |
| Case 53 | F | *NAT1* | c.546dup | p.Asp183ArgfsTer13 | rs770209499 | LP |
| Case 54 | F | *SCPEP1* | c.619+2T>C | - | - | LP |
| Case 55 | F | *TNXB* | c.12463+2T>C | - | rs545719209 | LP |
| Case 56 | F | *ALOX12* | c.1288_1303del | p.Arg430SerfsTer2 | - | LP |
| | | *POMC* | c.706C>G | p.Arg236Gly | rs28932472 | LP |
| Case 57 | F | *APOA1* | c.284T>A | p.Phe95Tyr | rs138407155 | LP |
| Case 58 | F | *CD36* | c.1105_1125+29dup | - | rs780002632 | LP |
| Case 59 | F | *ABHD4* | c.679del | p.Arg227AlafsTer33 | rs565680365 | LP |
| Case 60 | F | *MAT1A* | c.540dup | p.Lys181Ter | - | LP |
| Case 61 | F | *MAPK12* | c.101dup | p.Ser35LeufsTer18 | rs532163968 | LP |
| | | *RYR1* | c.4711A>G | p.Ile1571Val | rs146429605 | LP |
| | | *RYR1* | c.11798A>G | p.Tyr3933Cys | rs147136339 | LP |

P: Pathogenic, LP: Likely Pathogenic, VUS: Variant with Uncertain Significance.

### *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[14]. 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[15].

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[16]. *In vivo* studies using animal models highlighted that the absence of this enzyme led to a cessation of eating and subsequent weight loss[17]. 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[18] describes AN and severe weight loss alongside classic manifestations, emphasizing the importance of recognizing diverse presentations of the disorder.

### 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[19]. 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 Palmitoylethanolamide (PEA), which has been implicated in the context of AN, suggesting a multifaceted interplay in the neurobiological mechanisms associated with the disorder[20]. 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[5]. 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 endocannabinoid system, a fundamental biological pathway that regulates feeding behavior[5]. 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[8]. 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[5]. 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[21,22].

### 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[23]. Variants in the *VPSI3B* 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[24]. 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[25]. The *LMNA* gene (OMIM *150330) exhibited a missense 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[26]. 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[27]. In addition, in a randomized, double-blind study, zinc supplementation improved weight gain in anorexic patients[28]. 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[29,30]. A variant in the *AKR1C1* gene (OMIM *600449) was identified in Patient 15, a gene previously associated with lipedema by our group[31]. In Case 16, variants were found in the *CARF* (OMIM *607586) and *NBEAL1* (OMIM *609816) genes. A study[32] 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[33]. Silencing of the intestinal epithelial transmembrane receptor, encoded by *Gucy2c*, led to obesity and metabolic syndrome in a knockout mouse model[34]. Patient 20 exhibited a variant in the *ATXN1* gene (OMIM *601556), associated with spinocerebellar ataxia, a condition linked to dopamine pathway alterations and indirectly connected to AN[35]. Patient 23 presented a variant in the *ESR2* gene (OMIM *601663), responsible for encoding the estrogen receptor 2. We have already reported two variants in the *ESR1* gene, and, considering the gender prevalence of AN, genetic variations in estrogen receptors may play a role in its manifestation[5]. 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[36]. 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[37]. *DNAAF1* (OMIM *613190) is responsible for ciliary architecture and its inactivating mutations result in primary ciliary dyskinesia[38]. 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[39] 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[40]. In addition, anandamide is positively correlated with excessive exercise, a particular phenotype found in anorexic patients[41]. 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[42,43]. 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[44]. 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[45]. 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[46]. 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[47]. 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[48]. 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[49]. Patient 52 displayed a variant in the *AEBP1* gene (OMIM *602981), involved in adipose tissue development and, in an *in vivo* animal model study[50], its functional role on adipogenesis was investigated and suggested as a therapeutic target for obesity.

## Discussion

The primary aim of our study was to investigate the biological underpinnings of AN using a gene panel[5] designed to identify variants associated not only with anorexia itself but also with syndromic conditions that mimic AN but represent distinct pathologies. Our gene analysis aimed at: (1) identifying variants in genes associated with syndromic forms of AN, (2) pinpointing variants in genes previously linked to rare variants in anorexic patients, and (3) identifying variants in candidate genes for AN based on GWAS studies, murine models, or their known protein functions in molecular pathways relevant to AN.

### Syndromic Forms

Our study revealed two potential syndromic forms of AN, i.e. hypervitaminosis A and hypermethioninemia. Hypervitaminosis A, character-

ized 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[14,16].

### 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 Palmitoylethanolamide (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 anandamide. 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[8,20,41].

### Dopamine Pathway

Our study highlighted the presence of the same frameshift variant in *DRD4* in 12 anorexic 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 dopamine 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[8,35].

### 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[51]. The interplay between *RYR1* variants and myokine release could explain how skeletal muscle alterations affect appetite modulation in the context of AN[22]. *TYK2*, involved in the formation and structuring of the musculoskeletal system, further underscores the connection between skeletal muscle alterations and appetite regulation[25].

### Appetite-Regulating Hormones

The gastrointestinal tract houses endocrine cells releasing an array of hormones in response to nutrient intake, intricately regulating postprandial 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[8,19]. 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[21]. 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 contributes to the effects of leptin[29,30]. 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)[48]. 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[49]. 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[44].

### 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[26]. Simultaneously, the involvement of *VPS13B* in adipogenesis suggests a multifaceted genetic contribution to AN[24]. Furthermore, variations in the *AGPAT2* and *LIPE* genes, both linked to lipodystrophies, emphasize the intricate genetic landscape associated with AN[36,49]. 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[50].

## Conclusions

In this study, we conducted an in-depth genetic exploration within the context of AN, by employing a designed and validated genetic panel[5]. Expanding upon our previously published case studies, we investigated various categories of genes, focusing on those associated with syndromic forms of the disorder as well as candidate genes, aiming to enhance our understanding of the genetic determinants of this complex clinical condition. The detailed analysis of genetic variants revealed new insights into molecular pathways that may be pivotal in the pathogenesis of AN. We specifically focused on primary phenotypes, such as appetite loss, severe weight loss, and excessive physical exercise, aiming to identify the underlying genetic connections to these clinical manifestations. Special emphasis was also placed on identifying syndromic variants, as understanding conditions that may mimic AN but have fundamentally distinct etiologies is crucial for determining effective and personalized treatments.

Illuminating the genetic mechanisms underlying this pathology provides a solid foundation for the development of more targeted and personalized therapeutic strategies, thereby improving the approach to clinical management of AN and opening new avenues for future research in the field of eating disorders.

### Conflict of Interest

The authors declare that they have no conflict of interests.

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### Ethics Approval

This research adhered to the principles established in the Declaration of Helsinki and received approval from the Institutional Review Board "Comitato Etico delle Aziende Sanitarie (CEAS) della Regione Umbria" under Protocol Number 29616/12/AV.

### Authors' Contribution

KD and MCM, article writing and variant analysis; AM, support in analysis and article review; GM, data analysis; MB, idea development and article review; TB and MRC, data recruitment and analysis, article review; SC, VG, LS, VB, LDR, PC, CM, KD, GM and GB, article revision and research consultations.

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### Availability of Data and Materials

All data are contained within the text.

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