ARTICLE

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Mutational landscape of risk variants in comorbid depression and obesity: a next-generation sequencing approach

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Major depression (MD) and obesity are complex genetic disorders that are frequently comorbid. However, the study of both diseases concurrently remains poorly addressed and therefore the underlying genetic mechanisms involved in this comorbidity remain largely unknown. Here we examine the contribution of common and rare variants to this comorbidity through a next-generation sequencing (NGS) approach. Specific genomic regions of interest in MD and obesity were sequenced in a group of 654 individuals from the PISMA-ep epidemiological study. We obtained variants across the entire frequency spectrum and assessed their association with comorbid MD and obesity, both at variant and gene levels. We identified 55 independent common variants and a burden of rare variants in 4 genes (*PARK2*, *FGF21*, *HIST1H3D* and *RSRC1*) associated with the comorbid phenotype. Follow-up analyses revealed significantly enriched gene-sets associated with biological processes and pathways involved in metabolic dysregulation, hormone signaling and cell cycle regulation. Our results suggest that, while risk variants specific to the comorbid phenotype have been identified, the genes functionally impacted by the risk variants share cell biological processes and signaling pathways with MD and obesity phenotypes separately. To the best of our knowledge, this is the first study involving a targeted sequencing approach toward the study of the comorbid MD and obesity. The framework presented here allowed a deep characterization of the genetics of the co-occurring MD and obesity, revealing insights into the mutational and functional profile that underlies this comorbidity and contributing to a better understanding of the relationship between these two disabling disorders.

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INTRODUCTION

Major depression (MD) and obesity are amongst the major causes of disability, morbidity and mortality worldwide [1, 2]. While suffering from any of these conditions independently represent a major burden to personal and public health implications, generating an enormous economic and social cost, the fact of suffering them comorbidly contributes to aggravate the situation [3–7]. Consequently, given the high prevalence of both disorders and their consequences, understanding the nature of their relationship is a challenge in epidemiological and psychosomatic medicine.

The association between MD and obesity has been firmly established [8–15]. In addition, epidemiological evidence, including several longitudinal meta-analyses, strongly supports a bidirectional association between these two common conditions [16–19]. There is evidence that people with MD are more likely to suffer from obesity compared to psychiatrically healthy controls. Likewise, people with obesity are also more likely to develop MD than normal weight people. Despite that, the underlying mechanisms remain unclear. Biological, psychological and behavioral factors have been proposed as plausible explanations [20]. Obesity would lead to MD due to stigma, interpersonal distress, and changes in body image; while MD would lead to obesity as a result of physical inactivity, alcohol abuse, emotional eating and antidepressant treatment [17, 18, 21, 22]. Interestingly, such psychological and behavioral factors are associated with biological dysregulations of depression and obesity [20]. The biological mechanisms described appear to be strongly connected by alterations in the systems involved in homeostatic adjustments and the brain circuitries that integrate mood regulatory responses (e.g., the hypothalamic–pituitary–adrenal (HPA) axis, immuno-inflammatory activation, neuroendocrine regulators of energy metabolism or the microbiome) [17, 20, 23–26].

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The etiology of MD and obesity reveals their complexity and multifactorial nature. Concerning the biological component, different studies have reported that MD and obesity have a similar genetic burden, with additive genetic effects explaining ~35% and ~40% of heritability for MD [27, 28] and body mass index (BMI) [29, 30], respectively. Nowadays, hundreds of single nucleotide polymorphisms (SNPs) associated with MD [31–34] and obesity or BMI [35–37] have been identified thanks to genome-wide association studies (GWASs). Interestingly, the polygenic contribution to BMI heritability was found to be significantly enriched for brain cells as compared to other tissues, suggesting a central role of specific brain regions in the regulation of body mass and energy homeostasis [38]. In addition to the genetic risk factors identified for each disease, a shared genetic susceptibility profile between MD and obesity has also been found [32, 39]. In fact, Bahrami et al. [39] have identified 32 shared loci between both conditions. In this direction, genetic risk for obesity-related traits has been found to be significantly increased in individuals with certain depressive symptoms [40–44]. Likewise, genetic risk for MD has also been found to be significantly increased in individuals with higher BMI values [43]. However, despite the relevance of these findings, the explored variants are mainly SNPs (minor allele frequency (MAF) ≥ 1%). Rare (1% > MAF ≥ 0.1%), ultra-rare genetic variants (MAF < 0.1%) and other structural variations are considerably more poorly explored. Indeed, while common variation constitutes an important part of the heritability of complex diseases, those other genetic variations usually confer a substantially higher risk of disease due to a more deleterious impact on the affected gene functionality [45–48]. Their exploration is therefore necessary to contribute to unraveling the genetic architecture and thus to unravel the molecular mechanisms underlying the MD–obesity relationship.

Next-generation sequencing (NGS) technology captures many types of genetic variation across the entire frequency spectrum including structural variations. Recent whole exome sequencing (WES) studies have provided new insights into the contribution of rare coding variants on depression [49–53] and obesity [54–56]. Furthermore, this approach allowed the identification of causal genes, mainly for monogenic obesity. These genes – including *BDNF*, *MC4R* and *PSCK1* – have also been found to be strongly associated with obesity in GWAS [54]. Nevertheless, while WES has provided clear advantages in comparison to whole genome sequencing (WGS) – including lower cost and easier interpretation of the functional impact of coding variants – noncoding variants are not covered [57]. Targeted sequencing offers an alternative that could not only solve some of the limitations that WGS and WES present, but is also an ideal option to further explore specific genomic regions of relevance in the phenotypes under investigation. Thus, candidate gene sequencing – mainly in Mendelian diseases [58] – or post-GWAS fine mapping studies [59] are among the main applications of this sequencing approach. To the best of our knowledge, no relevant targeted gene sequencing studies for MD have been performed to date. Meanwhile, in the study of obesity genetics, a few studies have applied candidate gene sequencing [60, 61]. Thus, an in-depth genetic screening of candidate regions and genes in MD and obesity has not been carried out so far, neither independently nor combined. This study aimed to further explore the genetic relationship between MD and obesity taking advantage of targeted sequencing strategy. Specifically, we searched for genetic risk variants across the entire frequency spectrum in flanking genomic regions and genes previously associated with MD and obesity, assessed the contribution of those variants to the pathophysiology of comorbid MD and obesity, and provided a general overview of the cellular and molecular pathways mapped by those variants when these complex diseases co-occur. The overall workflow of the study is shown in Fig. 1.

METHODS

Study cohort

Subjects. The study was conducted in a group of 654 individuals from the PISMA-ep cohort, a cross-sectional epidemiological study based on a representative sample of the general adult population living in the entire Andalusia region (Spain) [62] aimed to establish the prevalence of major psychiatric disorders in Andalusia. The methodology and characteristics of the PISMA-ep study have been described in more detail elsewhere [62]. For the present study we selected all depressive cases, an age- and sex-matched group of individuals with obesity, and individuals without any of these disorders.

Clinical assessments and measures. The Mini-International Neuropsychiatric Interview (MINI) was used to ascertain the diagnosis of MD following DSM-IV criteria [63]. Interviews were conducted by fully trained psychologists and took place either in the participant's local primary healthcare center or in their homes. For each participant, self-reported height and weight were obtained to calculate their body mass index (BMI) using the formula: weight in kilograms divided by height in meters squared (kg/m^2). Participants were grouped into four categories, following WHO criteria [64]: underweight (BMI < 18.5 kg/m^2), normal weight (BMI 18.5–24.99 kg/m^2), overweight (BMI 25.0–29.99 kg/m^2) and obesity (BMI ≥ 30 kg/m^2). In addition, a biological sample was obtained from each participant with an Oragene® saliva DNA (OG-500; DNA Genotek Inc., Kanata, ON, Canada) collection kit. The Oragene® Saliva Collection Kit protocol was used for DNA extraction. The original DNA samples were prepared to be stored at −80 °C in matrix plate format. DNA quantification was measured using the Infinite® M200 PRO Multimode Microplate Reader (Tecan, Research Triangle Park, NC, USA).

Sequencing

Targeted genomic regions sequencing. Specific genomic regions of interest in MD and obesity, respectively, based on traditional candidate genes for these disorders as well as associated loci reported in GWAS meta-analysis – available at the time of the study design – of MD [32] and obesity [35] were included in the design of the targeted DNA sequencing panel (see details in Fig. 2). Capture libraries were prepared by hybridization according to the SeqCap EZ HyperCap v2.3 protocol (Roche) and libraries were sequenced at 75 base pairs (bp) paired-end read length with Illumina NextSeq 500 system. All coding exons of 357 genes and 979 noncoding regions, distributed in 414 genes, (~1.5 Mb) were sequenced for the 654 included individuals (for all covered regions, see Supplementary Table 1).

Data processing. Primary bioinformatics analysis of the obtained reads was carried out in accordance with GATK best practice [65] (https://software.broadinstitute.org/gatk/best-practices/). The quality of the reads generated by the sequencer in FASTQ format was analyzed and those of low quality were discarded. The remaining sequences were aligned to the reference genome GRCh37(hg19), eliminating low-quality alignments. Subsequently, variants (SNVs and small INDELs) were identified. Resulting variants were annotated using Cellbase [66]: information was added regarding its genotype, population frequency, the sequence ontology (SO) of the variant, the gene and transcript(s) it might affect, its HGVS nomenclature, the degree of conservation (GERP) [67], its pathogenicity level (SIFT [68], Polyphen [69], CADD [70]) and its clinical significance according to the OMIM [71] (https://www.omim.org/) and ClinVar [72] (https://www.ncbi.nlm.nih.gov/clinvar/) databases.

Variant analysis

Classification of variants. Autosomal variants found in individuals were classified according to their MAF in the European population of the 1000 Genomes Project Phase 3 [73] (1 KG-EUR, https://www.internationalgenome.org) as common (those variants with an MAF ≥ 0.05); rare (0 < MAF < 0.05); and undetected (undescribed in the aforementioned database). Additionally, rare autosomal variants at 1 KG-EUR or undetected, but common in the control groups of our cohort (MAF≥ 0.05) were also considered common variants. A different approach was used to treat common and rare variants in the subsequent association analyses.

Disease association studies. Comorbid MD and obesity status was the main binary outcome analyzed in this study. Thus, cases consisted of individuals in the study cohort diagnosed with MD who also had obesity

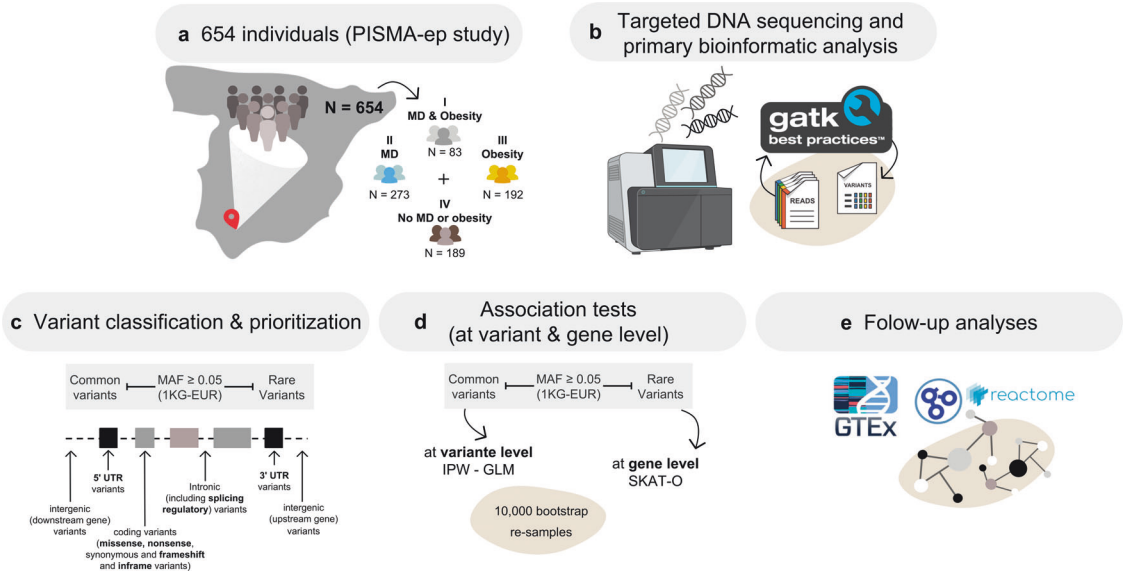

Fig. 1 Study flowchart. a Biological sample (saliva) was obtained from 654 participants. This group included (I) individuals with comorbid MD and obesity (N = 83), from among (II) MD individuals (N = 273) and (III) obese individuals (N = 192), and (IV) individuals without MD or obesity (N = 189). **b** Specific genomic regions of interest in MD and obesity were sequenced. **c** The identified variants were classified according to their MAF in the European population of the 1000 Genomes Project Phase 3 (1 KG-EUR) as common (MAF ≥ 0.05) and rare (0 ≤ MAF < 0.05). Internal common variants in the control group of our cohort (MAF ≥ 0.05) were also included as common variants. A different prioritization criterion was used to treat common and rare variants. **d** All autosomal common variants annotated with the sequence ontology (SO) terms detailed in (**c**) were included in the variant level analysis. Weighted logistic regression models were performed to evaluate the association of these variants with the comorbid phenotype. For gene level analysis, all rare variants with SO annotations highlighted in bold in (**c**) were collapsed into genes and the association of these genes with the phenotype of interest was evaluated by performing a sequence kernel association test-optimal (SKAT-O). Ten thousand bootstrap samples were carried out on both variant and gene level analyses. **e** Finally, (i) public expression Quantitative Trait Loci (eQTL) datasets were checked to find out genes whose expression was altered by the risk variants (eGenes) using eQTL datasets across brain regions from GTEx (Release V8 data) and (ii) a gene-set enrichment analysis (GSEA) was performed to determine in which biological processes and cell signaling pathways mapped the variants and genes previously identified and thus contribute to a better understanding of the mechanisms underlying the relationship between depression and obesity.

(BMI ≥ 30) and controls consisted of individuals with no co-occurring MD and obesity. Sex and age were included as covariates in the analyses. In addition, MD and obesity phenotypes separately were also considered as binary outcomes in the study. BMI and MD status were included as covariates when MD and obesity were considered outcomes, respectively. An additive inheritance model was assumed for the variants to be assessed.

First, all identified common variants (MAF ≥ 0.05 in 1 KG-EUR and in our control groups) were included. Weighted logistic regression models (generalized linear model, GLM) were performed using Stats R package (v 4.1.2) to assess the association of each of these variants with each of the three phenotypes under study (i comorbid MD and obesity, ii MD and iii obesity). Weights were constructed by inverse probability weighting (IPW) with the WeightIt R package (v 0.13.1). To assess the viability of a causal analysis based on IPW, the proportion of covariates that could be effectively balanced was checked using the standardized mean differences test as implemented in the Cobalt R package (v 4.4.0). A 0.05 threshold was considered as suggested in Stuart et al. [74]. In order to obtain a reliable estimate of the covariate-adjusted odds ratio, bias-corrected and accelerated bootstrap interval (BCa CI) method with 10,000 resamples was performed to measure the association between variants and outcomes using the Boot R package (v 1.3.28). 95% confidence intervals (CI) were obtained for the covariate-adjusted odds ratios (OR). Therefore, associations in which 1 was not included in this interval were considered significant. In order to identify independent associations, the patterns of linkage disequilibrium (LD) between those variants were explored using European ancestry TOPMed WGS data [75] through the TOP-LD tool [76]. For all pairs of variants within 500 Kb a maximum R^2 threshold of 0.2 was considered to determine independence between them. To validate these findings, the independence of these pairs of variants in a window of 500 Kb was also assessed in our sequencing data by using chi-square statistic implemented in vcftools [77].

The sequence kernel association test-optimal (SKAT-O) [78] as provided in the SKAT R package (v 2.2.4) was conducted using a small-sample (<2000) adjustment and a maximum cutoff of internal cohort MAF of 0.05 to determine the difference in the aggregate burden of prioritized rare variants between cases and controls at gene level. Rare and undetected variants in 1 KG-EUR (0 ≤ MAF < 0.05) with functional relevance – (i) missense variants; (ii) Loss of function (LoF) variants: nonsense, frameshift and inframe (insertions and deletions) variants and splicing regulatory variants; and (iii) variants in UTR regions – were prioritized. In addition, genes carrying variants annotated as pathogenic or probably pathogenic in Clinvar were included. SKAT-O was applied to aggregate genetic information across genes with at least two prioritized rare variants to test for associations with the main outcome of the study (comorbid MD and obesity) and with the phenotypes of MD and obesity separately. Similarly as in the variant level exploration, sex and age were used as covariates in the analyses. Also, BMI and MD status were included as covariates when MD and obesity were considered outcomes, respectively. 10,000 bootstrap replicates were carried out to obtain a reliable estimate of the statistic. Genes with a resampled p value < 0.05 were considered significant.

Follow-up of significantly associated variants and genes. To explore the functional impact of the common associated variants, all the significant signals were considered. First, in order to gain insights into the effects of noncoding variants, public expression Quantitative Trait Loci (eQTL) datasets were checked to find out other genes whose expression was altered by these variants (eGenes) using eQTL datasets across 13 brain regions from the GTEx portal (Release V8 data, www.gtexportal.org) [79]. Additionally, to provide functional meaning to our detected associations, a gene-set enrichment analysis (GSEA) was performed. Thus, for each explored phenotype, a list of input genes was built by including genes mapped by those risk common variants (carrier genes), genes resulting from SKAT-O and eGenes. GSEA was performed using the gprofiler2 R package (v 0.2.1). Gene-sets were obtained using data from Gene Ontology (GO) Consortium (http://www.geneontology.org/) [80, 81] and Reactome (https://reactome.org) [82] with the aim of having a representation of descriptive terms of biological processes involving a group of genes at cellular level (defined in GO:BP) and canonical cell signaling pathways (defined in Reactome) [82]. GO terms Inferred from Electronic Annotation

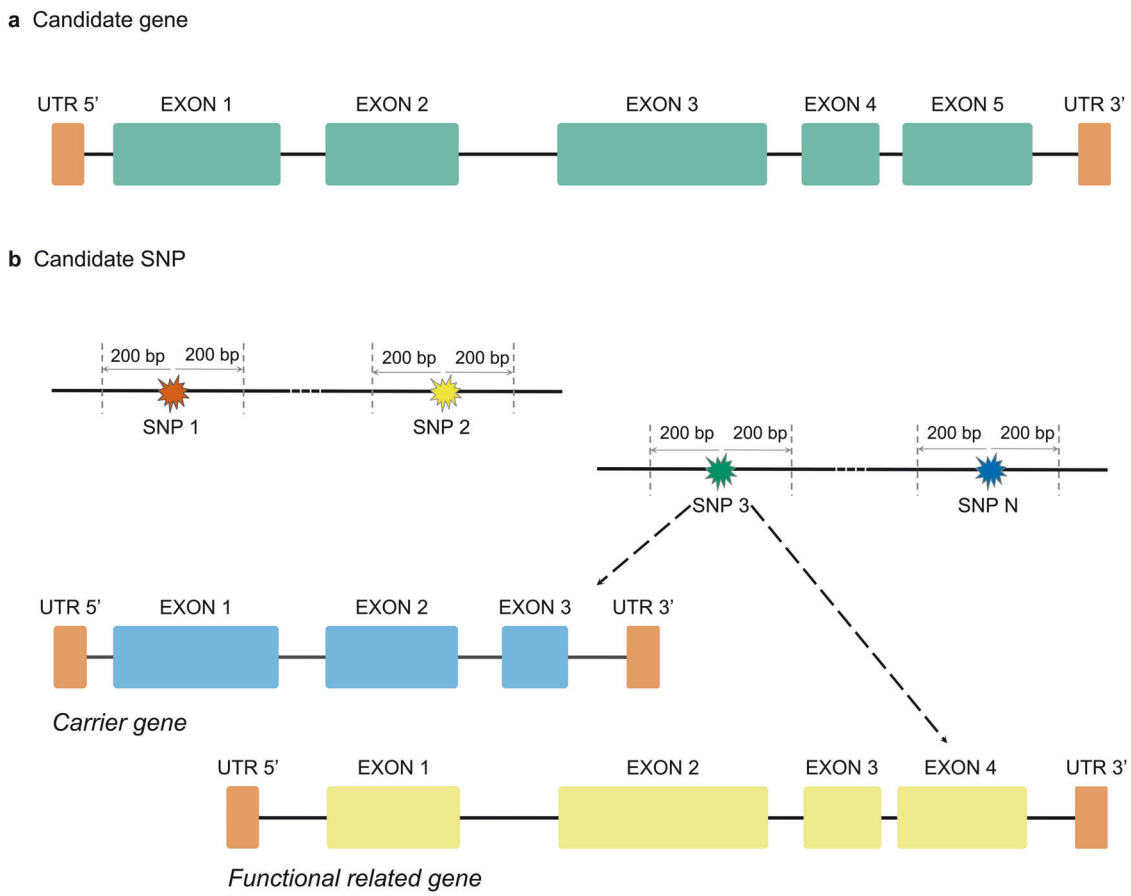

Fig. 2 Overview of the targeted sequencing panel design strategy from candidate genes and SNPs for MD and obesity. a For all genes included in the panel, coding sequences and UTR regions were captured, except for *FTO* and *MC4R* genes, for which the complete sequence was captured, given their high interest according to candidate gene studies in MD and obesity. **b** Capture of the 200 bp flanking to candidate SNPs located in intronic regions and capture of the exonic and UTR regions of functionally related genes (eQTLs, DEPICT, GRAIL, miRNA binding site, etc.) to these polymorphisms and of carrier genes.

(IEA) were excluded. Enrichment *p* values were computed using Fisher's exact test and corrected for multiple testing with false discovery rate (FDR) [83]. Results for the comorbid phenotype were compared with those obtained considering MD and obesity phenotypes separately.

RESULTS

Study cohort

The main case-control study included 83 individuals with comorbid MD and obesity, and 571 individuals with no co-occurring MD and obesity (named as controls). Sex distribution between cases and controls was significantly different ($\chi^2 = 6.56$, p value < 0.05). Furthermore, a significant difference in the age means between these two groups was found ($t = -86.81$, p value < 0.05) (see Table 1). The sex, age and BMI distributions considering MD and obesity phenotypes separately are detailed in Supplementary Tables 2, 3 and Supplementary Fig. 1.

Sequencing

The average percentage of positions with coverage depth ≥30× for these 654 individuals was 92.1% (± 5.2) (Supplementary Fig. 2). Overall, 38,528 autosomal variants were identified, out of which 3165 were common and 35,393 were rare or undetected (based on the 1 KG-EUR database using a threshold of ≥5% for common variants). Table 2 details the number of variants found according to their classification, based on their frequencies and their consequence annotation.

Variants analysis

Common variants association study. Common autosomal variants found in individuals were selected according to their MAF in 1 KG-EUR and in the control group of our cohort. Thus, the number of variants included in the comorbid MD and obesity study was 3757. From these, in a first step, 151 significant variants (95% CI covariate-adjusted OR) were obtained in the explored comorbid phenotype (Supplementary Table 4). In addition, considering the MD and obesity phenotypes independently, a total of 166 and 168 significant variants (95% CI covariate-adjusted OR) were identified, respectively (Supplementary Tables 5, 6). Comparing the results of these three contrasts, among the 151 variants associated with the comorbid phenotype, 101 were exclusive to that phenotype. Among the remaining 50 variants, 49 overlapped with the variants identified in the MD or obesity groups separately, and only one variant (rs74991234) overlapped in all three evaluated phenotypes (Fig. 3). Considering the direction of association of the overlapping variants with the comorbid phenotype, all of them were found to be opposite, except for the one that overlapped in the three groups. This could be explained by the fact that MD and obesity association studies were corrected for the effect that BMI or MD status might have on the assessed phenotype, respectively. Therefore, 102 common variants were finally considered as risk variants for comorbid MD and obesity. These correspond to the exclusive variants of the comorbid phenotype and the overlapping variant in the three outcomes. Among the 102 risk variants, 55 independent signals

Table 1. Sex and age distribution between cases with comorbid MD and obesity and controls.

		Cases 83 (12.7%)	Controls 571 (87.3%)	Test (χ^2/t-test; df)	*p*
Sex N (%)	Female	66 (79.5)	369 (64.6)	6.56; 1	0.010
	Male	17 (20.5)	202 (35.4)		
Age mean (SD)		55.05 (14.32)	50.96 (15.18)	−86.81; 653.63	$<2.2 \times 10^{-16}$

χ^2 chi-square test, *df* degree of freedom, *p* *p* value, *SD* standard deviation.

Table 2. Classification of the genetic variants identified according to their MAF in 1 KG-EUR and their consequence annotations.

	Intergenic upstream	5′ UTR	Coding				Intronic	3′ UTR	Intergenic downstream	Total
			Missense	Nonsense	Synonymous	Ins/Del				
Common	18	52	240	0	341	9	2371	85	19	3165
Rare	32	114	736	0	597	14	3122	125	17	4757
Undetected	103	651	4509	10	11109	168	13,334	710	42	30,636
Total	153	817	5485	10	12,047	191	18,827	920	78	38,528

Ins/Del insertion/deletion.

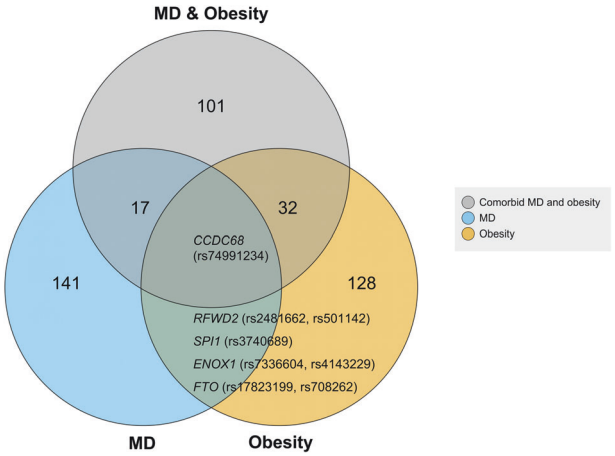

Fig. 3 Venn diagram of the variants significantly associated with each of the three phenotypes evaluated. A total of 151, 166 and 168 significant variants (95% CI covariate-adjusted OR) were identified for (i) comorbid MD and obesity, (ii) MD and (iii) obesity, respectively. Only the variant rs74991234 in *CCDC68* gene was significant in the three phenotypes. Other 17 and 32 variants overlapped between the comorbid phenotype and the independently explored MD and obesity phenotypes, respectively. An overlap of 8 significant variants (rs74991234, rs2481662, rs501142, rs3740689, rs7336604, rs4143229, rs17823199 and rs708262) was found among MD and obesity.

were identified after checking their independence with TOP-LD and vcftools (Table 3). The haplotypes determined, as well as the independent variants, are detailed in Supplementary Table 7. It should be noted that the *FTO* gene, whose sequence was completely captured, concentrates 25 variants (8 independent signals), being the gene in which the highest number of independent signals have been mapped. Additionally, it is noteworthy that up to 3 independent variants were mapped in the *LRP1B* gene.

Rare variants association study. After prioritizing rare variants according to their potential functional relevance, the role of the remaining 8009 rare variants was assessed by a variant burden analysis using SKAT-O at the gene level. These variants were grouped in 274 carrier genes, which were included in the study. The number of rare variants found in each gene and their classification according to their consequence annotation are shown in Supplementary Table 8 and Supplementary Fig. 3.

After performing a gene-based association test, 4 genes (*PARK2*, *FGF21*, *HIST1H3D* and *RSRC1*) were found to be significantly associated with comorbid MD and obesity (Table 4). Figure 4 shows the distribution of rare variants in these genes according to their consequence annotation. Considering the genes obtained for the independently evaluated MD and obesity phenotypes (Supplementary Tables 9, 10), one gene (*MTIF3*) was overlapped between MD and the comorbid phenotypes (Fig. 5). Therefore, taking into account that the gene level analysis for the MD phenotype was also adjusted with BMI, this gene was excluded from those significantly associated with comorbid MD and obesity and it was not considered for subsequent analyses.

Follow-up of significantly associated variants and genes. Using eQTLs datasets across 13 brain regions from the GTEx portal [78], 35 of the total of 102 variants significantly associated with comorbid MD and obesity (Table 3 and Supplementary Table 7) were found to be eQTLs in at least one of those brain regions for 36 eGenes (Supplementary Table 11). After performing GSEA considering these eGenes, along the carrier genes of the common risk variants and genes resulting from SKAT-O, 210 statistically significant gene-sets (FDR < 0.05) were obtained from GO:BP (178 significant group of genes) and from Reactome (32 significant pathways) (Supplementary Table 12). Regarding MD and obesity phenotypes independently, 40 and 15 variants were found to be brain eQTL for 36 and 16 genes, respectively (Supplementary Tables 13, 14). Likewise, after applying GSEA in these phenotypes, 396 and 132 gene-sets were determined to be significantly enriched, respectively (Supplementary Tables 15, 16). Comparing the results obtained from GSEA for the three evaluated phenotypes, 32 significant gene-sets were obtained exclusively for the comorbid phenotype (20 reported from GO and 12 reported from Reactome) (Fig. 6). Figure 7 shows a summary of these significant gene-sets for the comorbid phenotype and the mapped genes derived from the previous analyses. Most GO terms defined biological processes related to carbohydrate metabolism or peptide metabolism (GO:0005975, GO:0045912, GO:0034248,

Table 3. Fifty-five independent variants identified in the analysis of 83 cases with comorbid MD and obesity and 571 controls.

chrN:pos:REF:ALT	rs ID	Gene	SO	MAF[a]	OR[b] estimate	95% CI OR[b]	Linked[c] variants (gene)
chr1:47224170:C:G	rs34087210	*TAL1*	Intron	0.603	1.720	[1.13–2.30]	rs145295250 (*TAL1*)
chr1:48591023:T:C	rs7543402	*AGBL4*	Intron	0.349	1.446	[1.01–1.88]	
chr1:65609903:A:G	rs74733149	*LEPR*	Intron	0.085	0.498	[0.09–0.91]	
chr1:74250840:T:C	rs548378187	*FPGT–TNNI3K, TNNI3K*	Intron	0.054	0.553	[0.16–0.94]	
chr1:181771607:A:G	rs199929	*CACNA1E*	Intron	0.260	0.705	[0.42–0.99]	
chr2:86591745:A:G	rs12623509	*CHMP3, RNF103–CHMP3*	Intron	0.488	0.729	[0.49–0.96]	rs966477 (*CHMP3, RNF103–CHMP3*)
chr2:140510004:G:A	rs35821928	*LRP1B*	Synonymous	0.072	0.369	[0.07–0.67]	
chr2:140702395:C:T	rs16844555	*LRP1B*	Intron	0.138	1.761	[1.00–2.52]	
chr2:142130807:G:C	rs1375610	*LRP1B*	UTR	0.431	1.527	[1.04–2.01]	
chr2:211420378:T:C	rs2289086	*ERBB4*	Intron	0.334	1.426	[1.01–1.84]	
chr2:218568874:G:A	rs526897	*RQCD1*	UTR	0.620	1.561	[1.05–2.07]	rs549026, rs10932782 (*USP37*); rs485765, rs7574429 (*PLCD4*); rs3770213, rs3770214 (*ZNF142*); rs1863704 (*STK36*)
chr3:44254911:T:C	rs4682960	*TOPAZ1*	Intron	0.522	1.522	[1.05–1.99]	
chr3:53186113:G:T	rs45513792	*PRKCD*	Intron	0.042	2.295	[1.05–3.54]	rs41275531, rs41315886 (*PRKCD*)
chr3:116444807:C:-	–	*LSAMP*	Intron	NA	1.745	[1.01–2.48]	
chr4:3074877:CAGCAG:-	rs71180116	*HTT*	Deletion	0.327	1.739	[1.16–2.32]	
chr4:76144324:AA:-	rs33913434	*NUP54*	Intron	0.224	10.360	[1.24–19.48]	
chr4:144652803:A:-	rs3215015	*HHIP*	Splice	0.368	0.713	[0.44–0.99]	
chr5:154404193:C:T	rs6580076	*GALNT10*	Synonymous	0.157	1.820	[1.14–2.50]	
chr5:168062045:-:T	rs11411759	*TENM2*	Intron	0.385	3.17E + 06	[1.18–6.33E + 06]	
chr6:50843402:A:T	rs2857513	*TFAP2B*	UTR	0.114	0.605	[0.22–0.99]	
chr6:147508937:C:T	rs66812182	*SAMD5*	Synonymous	0.272	0.697	[0.44–0.95]	
chr7:83134219:AT:-	rs61658777	*PCLO*	Intron	NA	2.618	[1.06–4.18]	
chr7:83156404:A:-	rs71074628	*PCLO*	Intron	NA	2.102	[1.03–3.17]	
chr7:93441559:AA:-	rs3068357	*CALCR*	Intron	0.121	4.216	[1.37–7.06]	
chr9:36999027:G:T	rs4880050	*PAX5*	Intron	0.928	8.552	[1.38–15.72]	
chr9:109174508:C:T	rs10979727	*EPB41L4B*	UTR	0.425	0.745	[0.49–1.00]	
chr11:31681804:C:T	rs10767903	*ELP4*	Synonymous	0.753	1.06E − 04	[5.93E − 08–2.12E −04]	
chr12:122276383:-:CACACACACACACA	rs112247423	*CLIP1*	Intron	0.160	2.564	[1.12–4.00]	
chr13:33063777:-:A	rs34137141	*KL*	Intron	NA	1.953	[1.17–2.74]	rs564481 (*KL*)
chr13:43298316:-:A	rs35698699	*ENOX1*	Intron	0.478	1.659	[1.05–2.27]	
chr13:98466336:C:T	rs9584854	*STK24*	Intron	0.267	0.700	[0.42–0.98]	
chr14:24812637:C:T	rs712491	*STXBP6*	UTR	0.304	0.654	[0.39–0.91]	
chr14:24819006:A:C	rs71405872	*STXBP6*	Intron	0.072	2.128	[1.23–3.03]	
chr14:29632819:T:C	rs1209180	*PRKD1*	Intron	0.581	1.739	[1.08–2.40]	rs3783299, rs1191601 (*PRKD1*)

Table 3. continued

chrN:pos:REF:ALT	rs ID	Gene	SO	MAF[a]	OR[b] estimate	95% CI OR[b]	Linked[c] variants (gene)
chr14:64125250:C:T	rs9323446	*SYNE2,ESR2*	Intron	0.110	1.803	[1.01–2.59]	
chr14:64224927:TT:-	rs35455974	*SYNE2,ESR2*	Intron	0.038	1.74E − 08	[4.42E − 09–3.04E − 08]	
chr16:19831660:A:-	rs60105902	*IQCK*	Intron	NA	3.67E + 06	[2.00–7.33E + 06]	
chr16:28495551:A:G	rs149271	*APOBR*	Synonymous	0.348	1.563	[1.09–2.03]	rs180743, rs180744, rs151174 (*APOBR*), rs762634, rs1059491, rs4149406 (*SULT1A2*)
chr16:53705018:T:-	rs35814419	*FTO*	Intron	NA	0.498	[0.06–0.94]	
chr16:53773852:A:G	rs17817288	*FTO*	Intron	0.528	1.531	[1.05–2.01]	rs28429148, rs8047395, rs199952722, rs8055197, rs1861866, rs10852521, rs9922047, rs8057044, rs11075987 (*FTO*)
chr16:53817392:G:A	rs9972717	*FTO*	Intron	0.165	1.795	[1.09–2.50]	
chr16:53822032:T:C	rs78965501	*FTO*	Intron	0.035	2.578	[1.30–3.86]	
chr16:53869542:T:-	rs35983302	*FTO*	Intron	0.450	1.948	[1.12–2.77]	
chr16:53880000:T:-	rs373705985	*FTO*	Intron	NA	2.143	[1.18–3.10]	
chr16:53928220:A:G	rs11646505	*FTO*	Intron	0.509	0.696	[0.46–0.93]	
chr16:54093771:C:T	rs12932373	*FTO*	Intron	0.151	1.867	[1.09–2.65]	rs79014830, rs967515, rs1015279, rs17236232, rs10492872, rs17833492, rs62034137, rs62637747 (*FTO*)
chr17:2061466:-:G	rs145926620	*SMG6*	UTR	0.526	0.549	[0.20–0.90]	
chr17:5356444:C:T	rs61635291	*RABEP1*	Intron	0.082	2.269	[1.13–3.41]	rs34594998, rs7213832 (*RABEP1*)
chr17:80923747:C:T	rs7217786	*RPTOR*	Missense	0.326	1.526	[1.00–2.00]	rs2271603 (*RPTOR*)
chr18:23544944:C:-	rs879174633	*NPC1*	Intron	NA	2.002	[1.00–3.00]	
chr18:54936852:T:C	rs74991234	*CCDC68*	Missense	0.055	0.355	[0.06–0.65]	
chr18:79863692:-:GGC	rs71338073	*KCNG2*	Insertion	0.417	1.581	[1.03–2.13]	
chr19:18343004:-:T	rs35175980	*PGPEP1*	Intron	NA	1.982	[1.09–2.87]	
chr19:30444797:C:A	rs61744158	*ZNF536*	Missense	0.052	2.012	[1.04–2.99]	
chr22:41154950:C:T	rs4822011	*EP300*	Intron	0.635	1.638	[1.10–2.17]	rs4822002, rs20552, rs2076577 (*EP300*); rs139437, rs139438, rs139450, rs139451 (*L3MBTL2*)

chr chromosome, *pos* position GRCh38(hg38), *REF* reference allele, *ALT* alternative allele, *SO* sequence ontology, *MAF* minor allele frequency, *OR* odds ratio, *CI* confidence interval.
[a]MAF in 1 KG EUR (1000 genome European population).
[b]OR estimate adjusted for the covariates sex and age for the independent variants significantly associated with the comorbid phenotype of MD and obesity. The estimate OR corresponds to the mean of the lower and upper 95% CI of the 10,000 bootstrap resamples. Positive or negative association is indicated by an estimate above or under 1, respectively.
[c]Linked variants among the 102 significant signals according to TOP-LD and chi-square statistic (vcftools). Variant with the largest effect was considered the index variant (see Supplementary Table 7).

Table 4. Genes associated with comorbid MD and obesity through rare variation.

Gene[a]	Variants (N)	Missense	LoF[b]	UTR	SKAT-O resamp[c] *p*
PARK2	23	14	6	3	0.006
MTIF3	3	3	0	0	0.011
FGF21	5	4	0	1	0.021
HIST1H3D	3	0	1	2	0.024
RSRC1	7	5	2	0	0.045

LoF loss of function, *resamp p* resampled *p* value.
[a]*MTIF3* gene was excluded because it was also significant in the MD phenotype analysis, which was adjusted with BMI.
[b]LoF variants include nonsense, frameshift, inframe and splicing regulatory variants.
[c]10,000 bootstrap replicates were carried out to obtain a reliable estimate of the statistic. Genes with a resampled *p* value < 0.05 were considered significant. Sex and age were used as covariates.

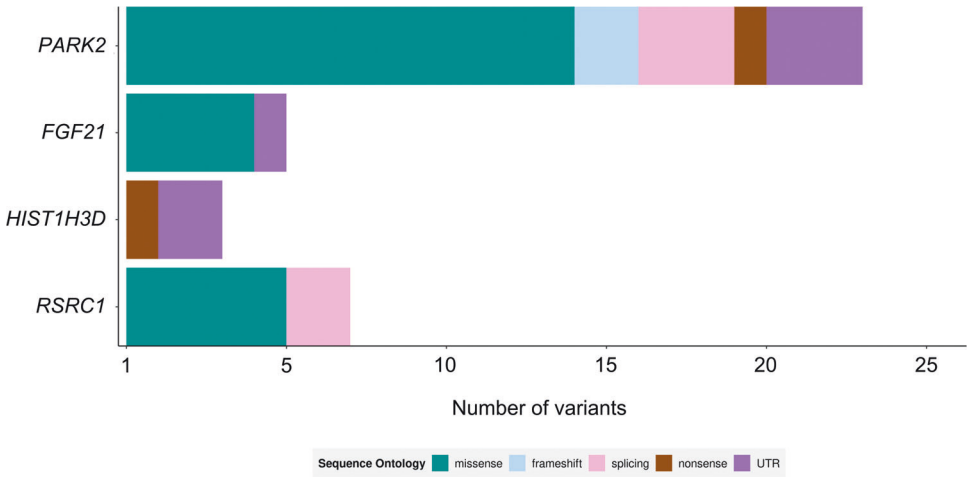

Fig. 4 Distribution of the identified rare variants among the significantly associated genes with comorbid MD and obesity. Barplot showing the number of rare variants identified in each gene and their classification based on their SO for the four genes (*PARK2*, *FGF21*, *HIST1H3D*, *RSRC1*) obtained in the burden analysis.

GO: 0006109, GO:0044281), intracellular transport (GO:0051169, GO:0006913, GO:0032386) and steroid hormone signaling (GO:0071383, GO:0030518, GO:0048545, GO:0043401) (see Fig. 7a). Concerning the significant Reactome pathways, we found some related to lipid and lipoprotein metabolism (HSA-9024446, HSA-9029569, HSA-174824, HSA-8964043) and others linked to chromatin organization and modification (HSA-4551638, HSA-3247509, HSA-4839726) (see Fig. 7b). We also found groups of cell cycle-related genes among both GO (GO:0007049) and Reactome (HSA-6791312) terms (Fig. 7).

DISCUSSION

Here we present a framework that addresses an unexplored approach in the study of the genetics of comorbid MD and obesity: the contribution of rare variants in this comorbidity. Through targeted sequencing of specific genomic regions of high interest in both MD and obesity phenotypes, we have identified common and rare variants for this comorbid phenotype, providing initial insight for the molecular mechanisms underlying the co-occurrence of both diseases, and contributing to complement the biological knowledge available on the field. Over the last few years, several studies have contributed to elucidating the shared genetic profile between these disorders [39–44], being the meta-GWAS by Barahmi et al. [39] the only study to date that has identified shared loci between both depression and obesity. Despite these studies have pointed toward significant findings that explore common variants shared between these diseases, to the best of our knowledge, the study of the contribution of rare variants in the comorbid phenotype has not been addressed yet. Although WES-based studies exploring the burden of rare variants in MD [49–53] and in obesity [54–56] have been conducted in recent years, these phenotypes were considered separately. There are no NGS approaches that identify variants, across the entire frequency spectrum, associated with these two complex diseases to date.

Our results report 55 independent common variants associated with comorbid MD and obesity. *FTO* and *LRP1B* were the genes with the highest number of independent signals. Interestingly, both genes have been found to be relevant in the two explored phenotypes. Several studies have shown that depression increases the effect of the *FTO* gene on BMI [84–86]. *LRP1B* has been reported in GWAS including large populations as a factor in obesity susceptibility [35, 87–89]. In addition, this gene is highly expressed in the adult human brain [90, 91] and it has been reported in various studies of neurological disorders [92–96], which suggests that *LRP1B* is a gene of interest for psychiatric disorders. Furthermore, considering *CCDC68* gene, in which the only overlapping variant between the comorbid phenotype and the independently explored MD and obesity phenotypes mapped, some interesting findings emerged from previous studies. Several signals at *CCDC68* have been previously reported to be associated with depression [31–34, 97–100] and with obesity-related diseases, such as diabetes [101] or coronary artery disease [102]. Finally, comparing our results with the results by Bahrami et al. [39], no overlap at variant level was found. However, taking into account the genes in which the identified signals were mapped, 4 overlapping genes were found (*IQCK*, *ZNF536*, *AGBL4*, *STK24*).

Nevertheless, the target phenotype in Bahrami et al. [39] was not comorbid MD and obesity, since GWAS of both diseases independently were meta-analyzed.

At the gene level, we found a burden of rare variants in *PARK2*, *FGF21*, *HIST1H3D* and *RSRC1* genes for the comorbid phenotype. These results are consistent with previous studies identifying a significant role of these genes in both MD and obesity – or other related physical conditions. First, *PARK2* gene has been associated with mitochondrial dysfunction [103, 104], present in patients with MD [103]. Song et al. [105] found that Parkinson's disease (PD) patients with mutations in *PARK2* had more depressive symptoms and fewer motor symptoms. In addition, this gene is involved in the development of metabolic syndrome, which includes obesity – among others [104]. Second, *FGF21* gene is a crucial hormonal regulator of metabolic function and has been found to act directly on the nervous system suppressing sweet and alcohol preference, increasing thermogenesis and improving insulin sensitivity [106–108]. Also, it affects biological signaling cascades involved in depression, such as corticotropin-releasing hormone (CRH), leptin and sympathetic nervous system pathways [106]. Third, *HIST1H3D* gene has been reported to be downregulated in women with postpartum depression [109]. Meanwhile, another study identified an association between this gene and adiposity by assessing differential methylation between colon cancer patients with and without obesity [110]. Finally, risk loci in the *RSRC1* gene have been identified in GWAS studies of depression [34] and BMI [111]. Despite the evidence reported for the relationship of these genes with the diseases of interest, the studies addressed the evaluation of depression and obesity independently – with the exception of previous evidence found for the *FGF21* gene, in which these diseases were considered together. Therefore, these results constitute the first evidence of the involvement of *PARK2*, *HIST1H3D* and *RSRC1* genes in the pathophysiology of comorbid depression and obesity. Further studies in this direction are needed to validate these results and to continue providing evidence at the gene level.

We further explored the functional impact of the variants and genes found for comorbid MD and obesity. Approximately 50% of the identified eQTLs were intronic variants distributed in 9 genes (*RNF103*, *EP300*, *L3MBTL2*, *PLCD4*, *PRKCD*, *SULT1A2*, *TAL1*, *TOPAZ1* and *USP37*). These eQTLs would account for the impact on the expression levels of nearly 80% of the eGenes for the comorbid phenotype. This highlights the relevance of noncoding variants in this phenotype and suggests that more extensive follow-up of these variants is necessary. Regarding GSEA results, a substantial number of the biological processes defined by the enriched gene-

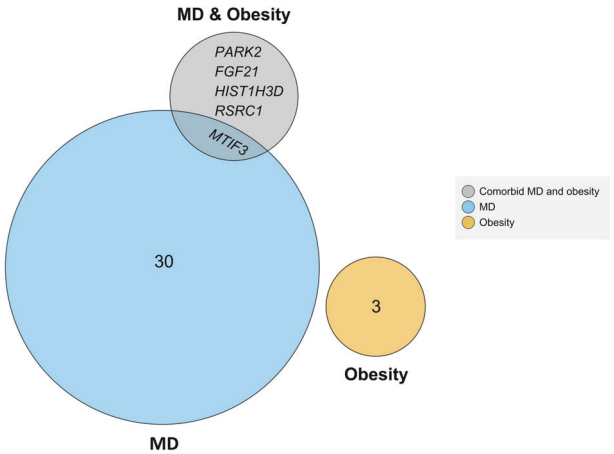

Fig. 5 Venn diagram of the genes significantly associated with each of three phenotypes evaluated through rare variation. A significant burden of rare variants (SKAT-O resampled *p* value < 0.05) in the comorbid MD and obesity phenotype were identified in *PARK2*, *FGF21*, *HIST1H3D*, *RSRC1* and *MTIF3* genes. *MTIF3* gene was also among the 31 significant genes in the MD phenotype. No overlap was identified between the three genes with a significant burden in the obesity phenotype and the ones obtained in the comorbid phenotype. Neither with those obtained when considering the MD phenotype.

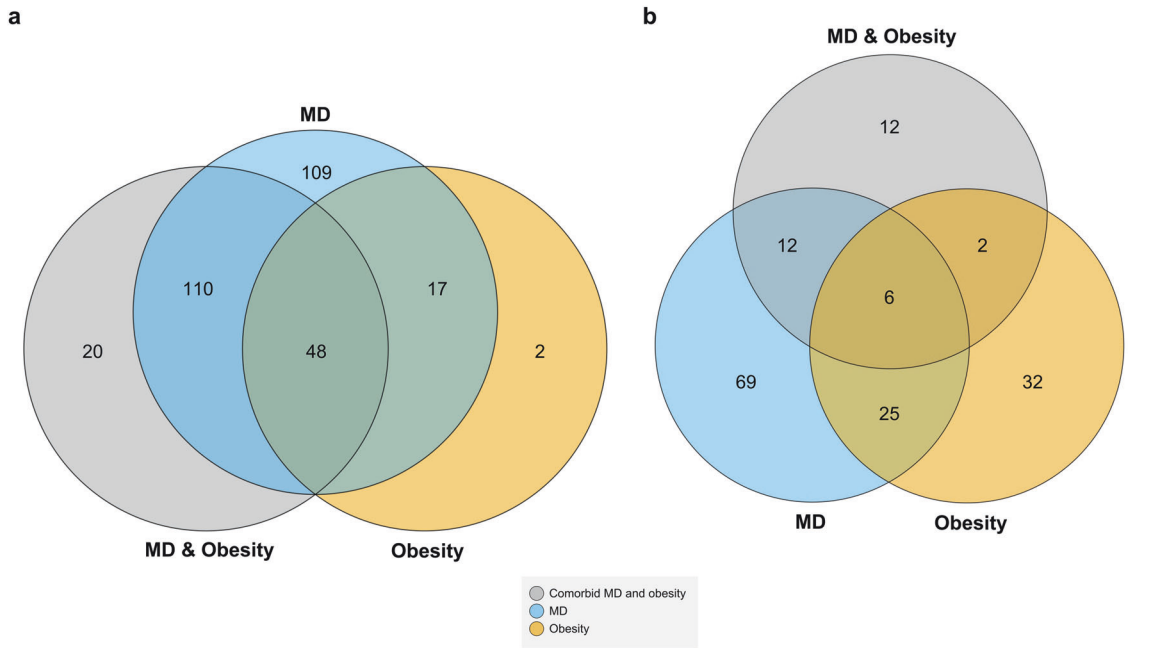

Fig. 6 Venn diagram of the gene-sets obtained in the GSEA for each of three phenotypes evaluated. a GO:BP results. A total of 178 significant gene-sets were obtained for the comorbid phenotype, while 284 and 67 significant terms were obtained for the MD and obesity phenotypes, respectively. Among these, 20 exclusive gene-sets were identified for the comorbid phenotype. **b** Reactome results. A total of 32 significant signaling pathways were found in the comorbid phenotype, while 112 and 66 pathways were obtained in MD and obesity phenotypes, respectively. Among these, 12 gene-sets were exclusive to the comorbid phenotype.

Fig. 7 Heatmap with GO and Reactome GSEA summary results. Exclusive significant gene-sets (FDR < 0.05) for the comorbid MD and obesity phenotype were plotted. The color of the dots shows the phenotype for which each gene was considered as an input gene in the GSEA of the corresponding phenotype ("All": input gene in the three phenotypes, "Comorbid MD & Obesity": input gene in comorbid MD and obesity, "MD": input gene in MD, "Obesity": input gene in obesity and "MD + Obesity": input gene in MD and obesity, separately). The shape inside the dot denotes the source from which each gene was included as an input gene in the GSEA of the phenotype that the color indicates ("carrier": considered because of being a risk variant carrier, "SKAT-O": considered because of being significant in the rare variant at gene level, "eGene": considered because of being determined as an eGene). For the "All" phenotype, the comorbid phenotype gene source was assumed. **a** Dot plot shows significantly enriched GO:BP terms (FDR < 0.05) identified for comorbid MD and obesity phenotype. **b** Dot plot shows significantly enriched Reactome pathways (FDR < 0.05) identified for comorbid MD and obesity phenotype.

sets have been reported to be crucial in metabolic dysregulation and hormone signaling mechanisms linking MD and obesity, which supports the general approach presented here. For instance, lipid dysregulation is an important component in the relationship between MD and obesity [112, 113]. The metabolic dysregulations observed in atypical depression mainly affect lipid/fat metabolism [112]. The role of steroid hormone signaling in the link between the diseases of interest should also be highlighted. These hormones include glucocorticoids (cortisol, cortisone and corticosterone), which are involved in the regulation of

metabolism, immune response and anti-inflammatory function [20]. Finally, we obtained cell cycle-related groups of genes in both GO:BP and Reactome GSEA. Some studies have suggested the role of cell cycle epigenetic regulation in neurodevelopmental and emotional disorders [114, 115]. It is worth highlighting that genes mapped in enriched gene-sets for the comorbid phenotype come from the input gene lists not only of the comorbid phenotype, but also of both MD and obesity phenotypes. This suggests that, despite the specific mutational profile of comorbidity – revealed in our results at the variant and gene level, the genes functionally impacted by the risk variants share biological cell signaling pathways.

The genetic associations reported here are the result of comparing three study groups. In this direction, the associations identified, both at variant and gene level, are validated by taking into account the MD and obesity groups separately. Associations with MD phenotype were corrected for the effect of BMI, and those with obesity were corrected for the effect of MD. Thus, only exclusive associations with the comorbid phenotype were considered, as well as overlapping associations in the three phenotypes and, in the analysis at the variant level, those with a similar direction. Nevertheless, this study presents certain potential limitations. First, the moderate sample size for genetic studies and the lack of an external validation cohort. Nonetheless, to overcome such constraints, we used bootstrapping to find a better estimate of the variability of the association measures, while minimizing the risk of spurious associations by controlling for possible confounding factors. Second, the use of self-reported height and weight measures to calculate BMI values could be another limitation. Nonetheless, in a study of an interaction effect between *FTO* gene, BMI and depressive disorder conducted by Rivera et al. [85] both self-reported and non-self-reported measures were used and no differences due to this factor were found, which prevented us from having reported mistaken results. Thirdly, SKAT-O is powerful, but computes only set-wise association *p* values and does not provide single-variant effect estimates, nor does it provide association direction. However, studying the role of rare variants by grouping them at the gene level is an approach that addresses the lack of statistical power in the study of these variants due to their low frequency. Finally, the contribution of single variants to a phenotype is difficult to estimate and, as such, clinical applicability remains complex and requires further in-depth functional follow-up, which remains speculative so far. Future efforts including WGS data to investigate the role of all common and rare coding and noncoding genetic variation would allow the identification of new loci and genes with functional impact on these phenotypes, as well as, the discovery of other structural variations, a very difficult task from WES and targeted sequencing. However, the larger targets may require a bigger sample size. Also, investigating more accurately the functional impact of these associations, as well as the specific functional profile of the comorbid phenotype, would be highly interesting.

In conclusion, to the best of our knowledge this is the first study using a targeted sequencing approach to study common and rare genetic variants in the comorbid MD and obesity phenotype, providing insights into the genetic mechanisms involved in the etiology and development of this comorbidity. Interestingly, although risk variants specific to the comorbid phenotype have been identified, the genes functionally impacted by the risk variants share biological cell signaling pathways with MD and obesity phenotypes separately. These findings will be essential in future approaches, where a more sophisticated modular perspective should be considered to contribute to a better understanding of the relationship between these two conditions, as well as, of the shared pathophysiological mechanisms. From a broader view, these findings could facilitate the identification of causal variants and, consequently, an easier interpretation of the functional impact of previously associated genes and loci with MD and obesity.

DATA AVAILABILITY

The datasets analyzed during the current study are available in the European Genome-Phenome Archive repository (https://ega-archive.org/datasets/EGAD50000000476).

CODE AVAILABILITY

Code for processing sequencing data is extensively explained in the "Methods" section of this manuscript and could be made available to editor and reviewers upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization: MR and LJMG. Methodology: MR, LJMG, JD, ELI, RC, CL and AMPG. Formal analysis: AMPG. Analysis supervision and results interpretation: RC, CL and ELI. Resources: MR, JAC and BG. Data curation: JPF and DLL. Writing – original draft: AMPG. Writing – review & editing: all authors. Visualization: AMPG. Supervision: MR, LJMG and JD. Funding acquisition: MR, EM, JAC and BG.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in compliance with the Helsinki Declaration. The Research Ethics Committee of the University of Granada (UGR) approved the study and the written informed consents. Accordingly, the aforementioned Ethics Committee authorized the collection of biological samples and the development of genetic analyses on them at the meeting held on 24 April 2019.

ADDITIONAL INFORMATION

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