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Contribution of heterozygous *PCSK1* variants to obesity and implications for precision medicine: a case-control study

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Abstract

Background. Recessive deficiency of proprotein convertase 1 (PC1/3; encoded by *PCSK1*) leads to early-onset obesity associated with severe endocrinopathy. Patients recessively deficient for *PCSK1* are eligible for MC4R agonist setmelanotide. Few heterozygous *PCSK1* mutations were associated with obesity risk, but their clinical impact and interest for precision medicine remain elusive. Here, we performed a large-scale functional genomic study focusing on rare, heterozygous variants of *PCSK1* to decipher the putative impact of these variants on obesity risk.

Methods. *PCSK1* was analysed in a case-control study for obesity, including 9,320 participants from three community- and one hospital-based cohort studies (France), who were recruited between Jan 1, 1995, and Dec 31, 2000. Functional assessment of rare missense variants was performed. Pathogenicity clusters of variants were determined through machine learning. Effect of each cluster on obesity was assessed through adjusted mixed-effects score test.

Findings. We identified 65 rare, heterozygous *PCSK1* variants including four null variants and 61 missense variants that were analysed *in vitro*. Missense variants were clustered into five groups (A-E) based on their enzymatic activity. Group_A variants had complete loss of function. Taking as reference this group, REVEL led to false positive (25%) and false negative (7%) results. Along with null variants, Group_A variants increased obesity risk (odds ratio of 9.3 [95% CI 1.5–177]) and body mass index (effect of 6.9 [SE 1.9]). In contrast, groups of variants with none or partial loss of function did not contribute to adiposity.

Interpretation. Our data suggest that only the carriers of heterozygous, null or complete loss-of-function *PCSK1* variants develop dominant obesity and therefore may be eligible for setmelanotide. *In silico* prediction did not enable an accurate sensibility and specificity for the detection of these variants. Therefore, *in vitro* tests are required to determine the variant pathogenicity for genetic diagnosis and precision medicine purposes.

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Research in context

Evidence before this study

We searched, PubMed, from Jan 1, 1990, to Aug 15, 2022, for “PCSK1”, “heterozygous”, “mutation”, “obesity” and “human”. This search revealed 10 studies. Of these, three reported biallelic *PCSK1* mutations (*i.e.* compound heterozygous) in patients with obesity; one was focused on a mouse model (knockout for *Pax6*); three sequenced *PCSK1* (among other genes linked with monogenic obesity) in patients with obesity ($n=1014$, $n=463$, $n=485$) without highlighting associations between pathogenic, heterozygous *PCSK1* mutations (with no evidence of functional effect) and obesity risk or bariatric surgery outcomes; one found a nonsense, heterozygous *PCSK1* mutation co-segregating with obesity in a three-generation family; one found a significant association between the burden of rare, null, heterozygous mutations in *PCSK1*, *MC4R* and *LEPR* and increased body mass index; and one sequenced 845 people with obesity and found seven rare, heterozygous *PCSK1* variants that had a deleterious effect on enzyme activity according to *in vitro* analyses and that contributed to obesity in the general population.

Added value of this study

The present study was motivated by the fact that a Phase 3 clinical trial is evaluating the *MC4R* agonist setmelanotide in patients with obesity who carry rare, heterozygous *PCSK1* variants. This is based on the assumption that rare, heterozygous mutations in *PCSK1* cause a dominant form of obesity. However, their true contribution to obesity remains elusive. The added value of the present study is the demonstration that *in vitro* functional analyses are necessary to assess the pathogenic effect of *PCSK1* mutations on obesity.

Implications of all the available evidence

Only the patients with obesity who carry complete loss-of-function, heterozygous *PCSK1* variants are likely to be considered for future treatment by setmelanotide. This study advocates for performing systematic *in vitro* studies before considering precision medicine.

Introduction

Overweight, obesity and their health comorbidities constitute a major growing medical burden. According to the World Health Organization, they are recognized as the 5th leading cause of death in the world. Obesity is a complex multifactorial disease with a strong genetic basis¹. Although common obesity is defined as a polygenic disease, monogenic obesity is caused by the presence of a single rare mutation with strong effect on increased weight. Monogenic obesity affects less than 5% of the population with obesity¹. Genes linked with non-syndromic monogenic obesity are mostly involved in the leptin-melanocortin pathway, which is critical for controlling appetite in the central nervous system. These genes include *LEP* (encoding leptin), *LEPR* (encoding leptin receptor), *POMC* (encoding pro-opiomelanocortin), *PCSK1* (encoding prohormone convertase subtilisin/kexin type 1 [PC1/3]), and *MC4R* (encoding melanocortin receptor type 4) whose deficiency is the most prevalent in Europe¹. Importantly, the identification and investigation of these genes have enabled the development of a new drug, the MC4R agonist setmelanotide, for patients with monogenic obesity. This drug that is administered by subcutaneous injections was shown to efficiently lead to weight loss in patients with obesity who are deficient for *POMC*, *LEPR* or *PCSK1*, in whom traditional interventions have usually poor impact on weight²⁻⁴. The U.S. Food and Drug Administration and the European Medicines Agency have recently approved the use of setmelanotide in these deficiencies.

Very rare biallelic pathogenic mutations in *PCSK1* cause early-onset obesity associated with various endocrinopathies⁵. PC1/3 is a major endoprotease involved in the processing of various prohormones and neuropeptides that regulate appetite and energy homeostasis. In the leptin-melanocortin pathway, PC1/3 cleaves POMC into adrenocorticotrophic hormone (ACTH) that is the precursor of α -melanocyte-stimulating hormone (α -MSH), *i.e.* the agonist of MC4R. PC1/3 is highly expressed in neural and endocrine tissues and it includes a prodomain, a catalytic domain, a P domain and a carboxy-terminal domain. PC1/3 is synthesized as an inactive precursor (preproPC1/3) that is converted into a proPC1/3 zymogen (94 kDa) by auto cleavage in the endoplasmic reticulum (ER). The prodomain is then cleaved in the ER and in the Golgi where PC1/3 is converted into a partially active form (87 kDa). Autocatalytic cleavages lead to the mature activation of PC1/3 (74/66 kDa) in the acidic environment of the secretory granules⁶. In addition to the role of *PCSK1* in recessively inherited forms of obesity, linkage analyses and genome-wide association studies showed that frequent single nucleotide polymorphisms in *PCSK1* were associated with an increased risk of common obesity^{7,8}. In contrast, the putative impact of rare, heterozygous mutations of *PCSK1* on obesity has not been clearly defined in large-scale studies, so far. Two previous studies identified rare, heterozygous, null variants (p.R80* and c.1095+1G>A) in *PCSK1* with a deleterious functional effect on PC1/3, which apparently caused monogenic obesity^{9,10}. In addition, based on the sequencing of 845 participants with morbid obesity, seven rare heterozygous *PCSK1* variants had a deleterious effect on PC1/3 activity according to *in vitro* analyses and contributed to obesity in the general population¹¹. However, several studies that investigated the functional effect of *PCSK1* variants (including reference¹¹) were possibly biased as the authors used a *PCSK1* plasmid which included a non-synonymous mutation (encoding p.S357G) that lacked normal subcellular regulation and produced anomalous substrate processing when compared to wild-type (WT) *PCSK1*¹².

The question of the impact of rare, heterozygous *PCSK1* mutation on obesity is crucial for genetic diagnosis and treatment purposes, as a company is advancing a Phase 3 clinical trial (ClinicalTrials.gov Identifier: NCT05093634) evaluating setmelanotide in patients with obesity who carry rare, heterozygous *PCSK1* variants, without functional *in vitro* assessment of identified variants.

Here, we aimed to perform a large-scale functional genomic study focusing on rare, heterozygous variants of *PCSK1* to decipher the putative impact of these variants on obesity risk in humans.

Methods

Study participants. The present analysis is primarily a case-control study for obesity, including 9,320 participants from the RaDiO (*Rare variants involved in Diabetes and Obesity*) study that included three community- and one hospital-based cohort studies (France), who were recruited between Jan 1, 1995, and Dec 31, 2000 (**Appendix, p5**)^{13,14}. The study protocols were approved by local ethics committees. All participants older than 18 years signed an informed consent form. Oral assent from children/adolescents was obtained and parents (or legal guardians) signed and informed consent form. Study enrollment was voluntary. Therefore, biases of this study are similar to those of any biobank with voluntary enrollment.

In participants older than 18 years, normal weight was defined as $BMI < 25 \text{ kg/m}^2$, overweight as $25 \leq BMI < 30 \text{ kg/m}^2$ and obesity as $BMI \geq 30 \text{ kg/m}^2$. In children and adolescents younger than 18 years, normal weight was defined as BMI-for-age < 85th percentile and obesity as BMI-for-age $\geq 95^{\text{th}}$ percentile. Sex was determined based on self-reporting. Participants with type 2 diabetes (T2D) had fasting glucose $\geq 7.0 \text{ mmol/L}$ and/or used treatment of hyperglycaemia, and were negative for islet or insulin autoantibodies. Control individuals had fasting glucose $< 5.6 \text{ mmol/L}$ and did not use treatment for hyperglycaemia. Importantly, in the case-

control study for T2D, only controls known to have fasting glucose <5.6 mmol/L after age 40 were included.

No sample size calculation was performed before analysis. We included all study participants recruited and accurately sequenced in the RaDiO study.

Procedures. *PCSK1* (NM_000439.5) was sequenced by next generation sequencing, as previously described^{13,14}. All rare coding *PCSK1* variants (with a minor allele frequency <1%) were covered with >40 reads and had a QUAL score >150. Furthermore, no variants had >5% missing genotype across the participants and no participants had >5% of missing genotypes across *PCSK1*. 61 rare nonsense or missense variants of *PCSK1* were created by mutagenesis and inserted into plasmids from the WT *PCSK1* plasmid (Origene) using the Quik Change II XL Site Directed Mutagenesis Kit (Agilent). Sequence of each plasmid was checked by Sanger sequencing. Two plasmids including the *PCSK1* variant encoding p.K293R or p.T366M were purchased from Twist Bioscience.

All functional analyses were done in human embryonic kidney 293 (HEK293) cells. The culture of these cells is described in the **Appendix (p2)**. For the enzymatic assays, HEK293 cells were transfected using FuGENE HD (Promega) with 150 ng/mL of plasmid encoding the β -galactosidase gene and 1,000 ng/mL of *PCSK1* plasmid (WT or with variant). 48 hours after transfection, the β -galactosidase activity in cells was measured as previously described¹³, and the enzymatic activity of secreted PC1/3 in supernatants was measured by adding fluorescent substrate of PC1/3. The detailed protocol for enzymatic assays is described in the **Appendix (p2)**. The experiments were performed in technical triplicate and each experiment was repeated four times. We used three different controls in each experiment: 1/ one positive control that was a plasmid including WT *PCSK1*; 2/ one negative control that was a plasmid including p.R80* PC1/3 mutant; 3/ one negative control where WT or mutated *PCSK1* was not transfected ('non-transfected' condition). Statistical analyses for enzymatic assays were

performed using R version 4.1.3. Measures were normalized by dividing PC1/3 activity measures by background measures. Then each set of experiments was normalized using non-transfected and WT PC1/3 activity measures. The non-transfected factor was computed as the geometric mean of the non-transfected PC1/3 activity measures. The WT factor was computed as the geometric mean of the WT PC1/3 activity measures at 300 minutes minus the non-transfected condition. Finally, the normalized PC1/3 activity measures were computed as PC1/3 activity measures minus the non-transfected factor divided by the WT factor. The minimal value of the normalized PC1/3 activity measures was set to zero, if needed, by adding the absolute minimum to all normalized measures. The normalized PC1/3 activity trajectories were clustered using the R package kml with 10 resampling iterations and a number of cluster from two to eight. The optimal number of clusters was found to be five (Groups_A, _B, _C, _D and _E) based on five criteria, namely: AIC, BIC, Calinski-Harabatz, Ray-Turi, and Davues-Bouldin. The protein expression of *PCSK1* variants in cell lysates of HEK293 cells was assessed by Western blotting. The detailed protocol for Western blotting assays is described in the **Appendix (p3)**. Three independent experiments for each mutation were performed. Protein expression of two *PCSK1* variants in HEK293 cell supernatants was assessed by liquid chromatography-mass spectrometry (LC-MS). The detailed protocol for LC-MS assays is described in the **Appendix (p4)**. Protein identification was performed by comparing all MS and MS/MS data with the *Homo Sapiens* proteome database (Uniprot, June 2021 version), using MaxQuant software (version 1.6.10.43). For the protein and peptide identification parameters, we used a false discovery rate of 1%, and a minimum of two peptides per protein including one unique. The statistical analysis was done using Perseus software (version 1.6.10.43). They were three replicates per group. The results were normalized by Z-score.

Statistical analysis for genetic association studies. The rare variants were divided into four clusters (I=Group_A+Null variants; II=Group_B; III=Group_C; and IV=Group_D + Group_E) based on enzymatic assays. Each cluster was analyzed using the mixed-effects score test (MiST) method¹⁷. MiST provides a score statistic $S(\pi)$ for the mean effect (π) of the cluster, and a score statistic $S(\tau)$ for the heterogeneous effect (τ) of the cluster. Let the equation of the model be : $Y = \alpha X + \pi GZ$, where Y is the trait of interest, X is the matrix of covariates (*i.e.* age, sex, BMI, ancestry for assessing T2D and cholesterol levels, and age, sex, ancestry for assessing adiposity), G is the matrix of *PCSK1* variants and Z is a vector of ones repeated n times, with n the number of rare *PCSK1* variants, leading to: $\pi GZ = \pi \sum_{i=1}^n G_i$. The ancestry of participants was assessed using the first five genotypic principal components (from PC1 to PC5), as previously described¹⁴. As none of the association studies had significant heterogeneity, we only showed the P -values associated with the score $S(\pi)$ representing the mean effect of the cluster. These statistical analyses were performed using the R software (version 4.0.2).

Role of funding source

Funding sources were not involved in study design, data collection, data analysis, data interpretation, writing of the manuscript or decision to submit the manuscript for publication.

Results

All 13 coding exons of *PCSK1* (NM_000439.5) were accurately sequenced in 9,320 participants including 7,260 adults and 2,060 children from the RaDiO study (**Appendix, p5**). Among adults, 21% had obesity, 39% had overweight and 40% had normal weight; among children, 50% had obesity and 50% had normal weight (**Appendix, p5**). We detected 65 rare, heterozygous, coding variants in *PCSK1* including four null variants (*i.e.* nonsense in which a point mutation results in a premature stop codon, frameshift or canonical ± 1 or 2 splice sites; p.R80*, p.S340Pfs*94, p.Q363* and c.1095+1G>A) and 61 missense variants (*i.e.* point mutations in which a single nucleotide change results in a codon that encodes a different amino acid; **Table 1**).

Each nonsense or missense *PCSK1* variant, or WT *PCSK1* was expressed in HEK293 cells and the enzymatic activity was measured using a fluorescent substrate of PC1/3. When compared to WT condition, most variants significantly decreased the enzymatic activity of PC1/3 to varying degrees (**Appendix, pp7-11**). We performed a cluster analysis *via* machine learning that determined five clusters of variants according to their pathogenicity on PC1/3 activity (*i.e.* Group_A to Group_E) (**Figure 1**). When compared to WT condition, 17 variants led to complete PC1/3 loss of function (Group_A), 11 variants led to partial enzymatic activity (Group_B), 16 variants showed a slightly different activity (Group_C) and 19 variants did not affect the PC1/3 enzymatic activity (Group_D and Group_E) (**Figure 1**). Group_A *PCSK1* variants were located in the prodomain, catalytic domain and P domain of PC1/3 (**Figure 2**). Western blots in cell lysates showed that most Group_A *PCSK1* variants had no or poor expression of the PC1/3 active form whereas the precursor forms of PC1/3 were well expressed (**Figure 3**, and **Appendix pp12-13**). In addition, two Group_A variants encoding p.Q363* and p.G390S did not show both precursor and active forms of PC1/3 but a single band at about 50 kDa (**Figure 3**, and **Appendix pp12-13**). LC-MS analyses confirmed that

PC1/3 protein was not present in supernatants of HEK293 cells transfected with p.Q363* or p.G390S mutant, suggesting an incorrect cleavage of PC1/3 precursor forms (**Appendix p14**).

We analyzed the effect of rare *PCSK1* variants on metabolic homeostasis. Using the MiST method adjusted for sex, age and ancestry, the burden of all rare variants was significantly associated with a higher risk of overweight or obesity ($P=0.018$ with an odds ratio [OR] of 1.7, 95% confidence interval [CI]: 1.1-2.8; **Table 2**) and with a higher BMI (28 ± 5.6 kg/m² in carriers *versus* 27 ± 6.5 kg/m² in non-carriers; $P=0.039$ with an effect [π] of 1.6 ± 0.79 ; **Table 2**). However, the burden of all rare variants was not significantly associated with T2D risk or cholesterol levels (**Table 2**).

We then performed the same analyses according to the pathogenicity of variants based on *in vitro* assays (**Figure 1**). The burden of complete loss-of-function or null *PCSK1* variants (*i.e.* Cluster_I merging Group_A variants and the two rare null variants c.1018del and c.1095+1G>A) had a significant effect on a higher BMI (32 ± 9.3 kg/m² in carriers *versus* 27 ± 6.5 kg/m² in non-carriers; $P=0.00030$ with π of 6.9 ± 1.9 ; **Table 2**). In the same line, this burden of complete loss-of-function or null *PCSK1* variants was significantly higher among individuals with obesity only (six out of seven [86%] individuals with obesity among carriers *versus* 35% of individuals with obesity among non-carriers; $P=0.014$ with OR of 9.3, 95% CI: 1.5-177; **Table 2**) or among individuals with overweight or obesity (16 out of 17 [94%] among carriers *versus* 60% among non-carriers; $P=0.013$ with OR of 8.5, 95% CI: 1.7-154; **Table 2**). We found the same trend of association between obesity and the burden of complete loss-of-function or null *PCSK1* variants in children although the statistical power was more limited unfortunately (five out of six [83%] children with obesity among carriers *versus* 50% of children with obesity among non-carriers; $P=0.18$ with OR of 4.7, 95% CI: 0.57-103; **Appendix p6**). In contrast, the different burdens of *PCSK1* variants with partial or neutral effect on PC1/3 activity did not have any significant effect on the risk of obesity or

overweight, and on BMI or other metabolic traits (**Table 2**). Our present results were in line with a recent paper that showed that heterozygous protein-truncating variants in *PCSK1* were strongly associated with increased risk of obesity, based on 640,000 exome data¹⁸. In this study, the genetic effect seen on adiposity was higher in the carriers of null, heterozygous *PCSK1* variants when compared to the carriers of null, heterozygous *MC4R* variants¹⁸.

We performed prediction analysis in order to discriminate complete loss-of-function variants (Group_A) from other variants using REVEL that is an ensemble method for predicting the pathogenicity of rare missense variants (**Table 1**)¹⁹. A total of four complete loss-of-function mutations (encoding p.F70C, p.R81S, p.R95H, p.I487T) were not predicted as pathogenic by REVEL. In contrast, 15 variants (encoding p.T157M, p.Y181H, p.Y187H, p.T203I, p.A217V, p.R282W, p.Y290C, p.R312C, p.S325N, p.I329V, p.I329M, p.S340Y, p.S354C, p.T377M, p.A398G) that were part of Group_B, Group_C, Group_D or even Group_E were detected as deleterious by REVEL (**Table 1**). The distribution of the carriers of a missense *PCSK1* variant according to their sex, their obesity status, the enzymatic activity of missense variants (Group A versus Group B), and the REVEL prediction of missense variants (neutral versus deleterious) shows an enrichment of carriers with obesity in Group A, regardless of REVEL prediction (**Appendix, p15**). These results highlighted the requirement of *in vitro* analyzes to pinpoint complete loss-of-function missense variants in *PCSK1*.

Discussion

Our results unambiguously showed that only the heterozygous, null or functionally proven complete loss-of-function *PCSK1* variants have a dominant effect on obesity risk, whereas missense variants with neutral or partial deleterious effect on PC1/3 activity do not contribute to obesity risk or to increased BMI in a large population. In our study, this dominant, complete deficiency of PC1/3 affects 0.4% of participants with obesity.

We also found that the PC1/3 protein expression of complete loss-of-function *PCSK1* variants was impaired in most cases. Indeed, among the 17 complete loss-of-function variants, three mutations (encoding p.R80*, p.V304I, p.P400S) did not show any form of PC1/3, three mutations (encoding p.F70C, p.T175M, p.L449P) had only the zymogen form (94 kDa), six mutations (encoding p.L132S, p.D193Y, p.D324Y, p.I487T, p.H557T, p.G582A) had both precursor forms (94 kDa and 87 kDa) but poorly the active form of PC1/3 (74/66 kDa), and two mutations (encoding p.Q363* and p.G390S) showed an incorrectly cleaved protein (**Figure 3**). Only three Group_A mutations had all forms of PC1/3 expressed in the same way as the WT condition (p.R81S, p.R95H, p.T375A), suggesting that PC1/3 encoded by Group_A mutations was normally secreted but that its enzymatic activity was impaired. These results are in line with a classification of *PCSK1* mutations which was previously proposed²⁰: 1/ PC1/3 proteins that are retained within the ER, 2/ PC1/3 proteins that are able of trafficking to the secretory granules but that are altered in the secretory pathway, and 3/ PC1/3 proteins that are correctly folded and secreted but that have impaired enzymatic activity. Furthermore, the authors proposed that there was a gradation of the severity of obesity from the third category (carriers with a more severe obesity) to the first category (carriers with a less severe obesity)²⁰. We did not replicate these findings; indeed, we did not observe any increase in the severity of phenotypes according to the classification of the mutant protein in these three mechanistic categories. Some of the carriers of the mutations that did not show any form of

PC1/3 presented with overweight or even normal weight (**Figure 3**). More generally, we observed that the carriers of a heterozygous, null or complete loss-of-function variant did not have extreme obesity despite the dominant effect of these mutations. Although a previous study suggested that some carriers of a heterozygous mutation with predicted loss of function in *LEP*, *LEPR*, *POMC* and *PCSK1* could present with dramatic phenotypes similar to the carriers of a biallelic mutation²¹, the present results are in line with other studies where the obesity of the carriers of a heterozygous *PCSK1* variant was less severe and less penetrant than in the carriers of a biallelic *PCSK1* variant^{9,22,23}.

Innovative medicine should be given to right patients to maximize their efficiency and to minimize side effects. That is the principle of precision medicine. So far, setmelanotide has been given to patients with obesity who carry biallelic pathogenic mutations in *LEP*, *LEPR*, *POMC* and *PCSK1* that are not very numerous. As a company is currently trialing setmelanotide for patients with heterozygous *PCSK1* variants, it is crucial to know whether these mutations are truly functional, especially because they represent a much larger population than the carriers of homozygous variants²¹. Unfortunately, the disability of *in silico* tests to accurately detect complete loss-of-function *PCSK1* variants suggested that *in vitro* assays are necessary to determine the variant pathogenicity before proposing a treatment targeting the leptin-melanocortin pathway. Of note, the current trial is also assessing the effect of setmelanotide on obesity in patients with heterozygous variants in *POMC* or *LEPR*, while their true contribution to dominant obesity is unclear. Therefore, future research directions could be focused on deciphering the functional effect of heterozygous variants in *POMC* or *LEPR* and their contribution to obesity.

Our study was limited by the fact that it was not possible to have access to levels of insulin and ACTH (which are both cleaved by PC1/3) in the carriers of null or complete loss-of-function *PCSK1* variants. However, we did not find any association of these variants with

T2D or lipids, which suggests a minimal impact on these metabolic pathways. Furthermore, the *in vitro* functional studies were done in HEK293 cells, where PC1/3 is not expressed in physiological conditions. These cells strongly differ from neuronal cells which embody the main actors of the obesity phenotype. However, this limitation was overcome by the use of a large genetic association case-control study following our standard *in vitro* assays. Importantly, our study does not imply that the patients with severe, early-onset obesity carrying a neutral, heterozygous *PCSK1* mutation should not be further investigated as another mutation might have a deleterious effect on the leptin-melanocortin pathway, and therefore these patients could be eligible for setmelanotide treatment.

Figure Legends

Figure 1. Cluster analysis according to enzymatic activity of 63 rare missense or nonsense variants of *PCSK1*

The figure shows the PC1/3 activity trajectories clustering of *PCSK1* variants by kml ($k = 5$). The Y-axis represents PC1/3 catalytic activity normalised by β -galactosidase activity. Enzymatic activity was measured for 300 minutes. Five groups according to enzymatic activity of variants were determined by machine learning. Pink variants are included in Group_A; orange variants are included in Group_B; yellow variants are included in Group_C; green variants are included in Group_D and blue variants are included in Group_E. Non-transfected and WT conditions are highlighted in black. Data are the mean of four independent experiments performed in technical triplicate. *NT*, non-transfected; *WT*, wild-type.

Figure 2. Localization of rare coding missense or nonsense variants of *PCSK1*

The different domains of PC1/3 protein are represented. Pink variants are amino acids changes linked to complete loss of enzymatic activity (Group_A) according to our *in vitro* assays. Black variants are amino acids changes linked to variants with partial or neutral effect on PC1/3 activity (Group_B, Group_C, Group_D and Group_E) according to our *in vitro* assays. *S*, peptide signal; *C-term*, C-terminal domain

Figure 3. Protein expression of complete loss-of-function *PCSK1* variants (Group_A) on cell lysates by Western blotting

This table recapitulates the protein expression of Group_A *PCSK1* variants by Western blotting. Sizes of precursor forms of PC1/3: 94 kDa and 87kDa; size of active form of PC1/3: 74/66 kDa. Three independent experiments were performed for each mutation (images of Western blotting available in **Appendix, pp12-13**). N_{nwt} , number of normal-weight

participants carrying the mutation in the RaDiO study; N_{ob} , number of participants with obesity carrying the mutation in the RaDiO study; N_{owt} ; number of overweight participants carrying the mutation in the RaDiO study.

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Declaration of Interests

Authors declare that they have no competing interests.

Contributors statement

LF, MBar, PF and ABon conceived and designed the research question; LF, MBar, VS, HL and AL contributed to functional analyses of each variant; BT and EV contributed to next-generation sequencing; MBoi, MC and ABon contributed to statistical analyses; ABad and MD contributed to computer analyses; MS and SA contributed to proteomic analyses; BB, GC, SF, MM and PF contributed to cohort data; LF and ABon wrote the first draft of the manuscript; LF, MBar and ABon have directly accessed and verified the underlying data. All

authors provided input on the interpretation of results. All authors revised the manuscript for important intellectual content and read and approved the final manuscript.

Data sharing

All relevant data have been included in the manuscript and supplementary tables. Given the sensitivity and risk of re-identification, all clinical data linked with NGS data for this study is available only upon request from the corresponding author.

References

- 1 El-Sayed Moustafa JS, Froguel P. From obesity genetics to the future of personalized obesity therapy. *Nat Rev Endocrinol* 2013; **9**: 402–13.
- 2 Kühnen P, Clément K, Wiegand S, *et al.* Proopiomelanocortin Deficiency Treated with a Melanocortin-4 Receptor Agonist. *N Engl J Med* 2016; **375**: 240–6.
- 3 Clément K, Biebermann H, Farooqi IS, *et al.* MC4R agonism promotes durable weight loss in patients with leptin receptor deficiency. *Nat Med* 2018; **24**: 551–5.
- 4 Wabitsch M, Farooqi S, Flück CE, *et al.* Natural History of Obesity Due to POMC, PCSK1, and LEPR Deficiency and the Impact of Setmelanotide. *J Endocr Soc* 2022; **6**: bvac057.
- 5 O’Rahilly S, Gray H, Humphreys PJ, *et al.* Impaired Processing of Prohormones Associated with Abnormalities of Glucose Homeostasis and Adrenal Function. *N Engl J Med* 1995; **333**: 1386–91.
- 6 Stijnen P, Ramos-Molina B, O’Rahilly S, Creemers JWM. PCSK1 Mutations and Human Endocrinopathies: From Obesity to Gastrointestinal Disorders. *Endocr Rev* 2016; **37**: 347–71.
- 7 Benzinou M, Creemers JWM, Choquet H, *et al.* Common nonsynonymous variants in PCSK1 confer risk of obesity. *Nat Genet* 2008; **40**: 943–5.
- 8 Wen W, Cho Y-S, Zheng W, *et al.* Meta-analysis identifies common variants associated with body mass index in east Asians. *Nat Genet* 2012; **44**: 307–11.
- 9 Philippe J, Stijnen P, Meyre D, *et al.* A nonsense loss-of-function mutation in PCSK1 contributes to dominantly inherited human obesity. *Int J Obes* 2015; **39**: 295–302.
- 10 Löffler D, Behrendt S, Creemers JWM, *et al.* Functional and clinical relevance of novel and known PCSK1 variants for childhood obesity and glucose metabolism. *Mol Metab* 2017; **6**: 295–305.
- 11 Creemers JWM, Choquet H, Stijnen P, *et al.* Heterozygous Mutations Causing Partial Prohormone Convertase 1 Deficiency Contribute to Human Obesity. *Diabetes* 2012; **61**: 383–90.
- 12 Blanco EH, Peinado JR, Martín MG, Lindberg I. Biochemical and cell biological properties of the human prohormone convertase 1/3 Ser357Gly mutation: a PC1/3 hypermorph. *Endocrinology* 2014; **155**: 3434–47.
- 13 Baron M, Maillet J, Huyvaert M, *et al.* Loss-of-function mutations in MRAP2 are pathogenic in hyperphagic obesity with hyperglycemia and hypertension. *Nat Med* 2019; **25**: 1733–8.
- 14 Bonnefond A, Boissel M, Bolze A, *et al.* Pathogenic variants in actionable MODY genes are associated with type 2 diabetes. *Nat Metab* 2020; **2**: 1126–34.
- 15 Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods* 2009; **6**: 359–62.

- 16 Bache N, Geyer PE, Bekker-Jensen DB, *et al.* A Novel LC System Embeds Analytes in Pre-formed Gradients for Rapid, Ultra-robust Proteomics *. *Molecular & Cellular Proteomics* 2018; **17**: 2284–96.
- 17 Sun J, Zheng Y, Hsu L. A unified mixed-effects model for rare-variant association in sequencing studies. *Genet Epidemiol* 2013; **37**: 334–44.
- 18 Akbari P, Gilani A, Sosina O, *et al.* Sequencing of 640,000 exomes identifies GPR75 variants associated with protection from obesity. *Science* 2021; **373**. DOI:10.1126/science.abf8683.
- 19 Ioannidis NM, Rothstein JH, Pejaver V, *et al.* REVEL: An Ensemble Method for Predicting the Pathogenicity of Rare Missense Variants. *Am J Hum Genet* 2016; **99**: 877–85.
- 20 Ramos-Molina B, Martin MG, Lindberg I. PCSK1 Variants and Human Obesity. In: *Progress in Molecular Biology and Translational Science*. Elsevier, 2016: 47–74.
- 21 Courbage S, Poitou C, Le Beyec-Le Bihan J, *et al.* Implication of Heterozygous Variants in Genes of the Leptin–Melanocortin Pathway in Severe Obesity. *The Journal of Clinical Endocrinology & Metabolism* 2021; **106**: 2991–3006.
- 22 Farooqi IS, Keogh JM, Yeo GSH, Lank EJ, Cheetham T, O’Rahilly S. Clinical Spectrum of Obesity and Mutations in the Melanocortin 4 Receptor Gene. *N Engl J Med* 2003; **348**: 1085–95.
- 23 Huvenne H, Le Beyec J, Pépin D, *et al.* Seven Novel Deleterious LEPR Mutations Found in Early-Onset Obesity: a Δ Exon6–8 Shared by Subjects From Reunion Island, France, Suggests a Founder Effect. *The Journal of Clinical Endocrinology & Metabolism* 2015; **100**: E757–66.
- 24 Qian Y, Wu B, Liu R, *et al.* Case Report: Complete Maternal Uniparental Isodisomy of Chromosome 5 (iUPD(5)mat) With PCSK1 Nonsense Variant in an Infant With Recurrent Diarrhea. *Front Genet* 2021; **12**: 668326.
- 25 Martín MG, Lindberg I, Solorzano-Vargas RS, *et al.* Congenital proprotein convertase 1/3 deficiency causes malabsorptive diarrhea and other endocrinopathies in a pediatric cohort. *Gastroenterology* 2013; **145**: 138–48.

Table 1. Rare *PCSK1* variants detected in the RaDiO study

Mutation in <i>PCSK1</i> (NM_000439.5)	MAC in gnomAD	MAC in RaDiO	References [†]	Functional group [‡]	REVEL (score)*
c.34G>C, p.A12P	0	1	-	Group D	Neutral (0-28)
c.173T>C, p.L58S	0	1	-	Group B	Neutral (0-15)
c.209T>G, p.F70C	0	1	-	Group A	Neutral (0-48)
c.238C>T, p.R80*	2	1	^{9,24}	Group A and Null	NA
c.239G>A, p.R80Q	585	1	-	Group B	Neutral (0-21)
c.243G>C, p.R81S	1	1	-	Group A	Neutral (0-24)
c.254A>G, p.H85R	0	1	-	Group C	Neutral (0-10)
c.281A>G, p.D94G	1	2	-	Group D	Neutral (0-063)
c.284G>A, p.R95H	3	1	-	Group A	Neutral (0-23)
c.334G>A, p.A112T	0	2	-	Group D	Neutral (0-015)
c.375G>A, p.M125I	51	13	¹¹	Group B	Neutral (0-33)
c.395T>C, p.L132S	0	1	-	Group A	Deleterious (0-92)
c.470C>T, p.T157M	5	1	-	Group B	Deleterious (0-83)
c.524C>T, p.T175M	41	2	¹¹	Group A	Deleterious (0-83)
c.541T>C, p.Y181H	29	4	¹¹	Group B	Deleterious (0-94)
c.559T>C, p.Y187H	4	2	-	Group D	Deleterious (0-83)
c.577G>T, p.D193Y	0	1	-	Group A	Deleterious (0-91)
c.608C>T, p.T203I	0	1	-	Group C	Deleterious (0-64)
c.646A>G, p.I216V	1	1	-	Group C	Neutral (0-36)
c.650C>T, p.A217V	15	3	-	Group C	Deleterious (0-87)
c.844C>T, p.R282W	7	1	-	Group D	Deleterious (0-65)
c.869A>G, p.Y290C	16	1	-	Group C	Deleterious (0-75)
c.878A>G, p.K293R	2	1	-	Group B	Neutral (0-23)
c.910G>A, p.V304I	35	3	-	Group A	Deleterious (0-63)
c.934C>T, p.R312C	1	1	-	Group B	Deleterious (0-76)
c.970G>T, p.D324Y	0	1	-	Group A	Deleterious (0-72)
c.974G>A, p.S325N	0	1	¹¹	Group C	Deleterious (0-73)
c.985A>G, p.I329V	1	1	-	Group C	Deleterious (0-55)
c.987C>G, p.I329M	0	2	-	Group C	Deleterious (0-78)
c.1018del, p.S340Pfs*94	0	1	-	Null	NA
c.1019C>A, p.S340Y	0	1	-	Group B	Deleterious (0-60)
c.1061C>G, p.S354C	2	1	-	Group D	Deleterious (0-62)
c.1087C>T, p.Q363*	2	1	-	Group A and Null	NA
c.1095+1G>A, p.?	0	1	^{10,25}	Null	NA
c.1097C>T, p.T366M	9	1	-	Group B	Neutral (0-37)
c.1123A>G, p.T375A	0	1	-	Group A	Deleterious (0-87)
c.1130C>T, p.T377M	4	1	-	Group E	Deleterious (0-60)
c.1168G>A, p.G390S	2	2	-	Group A	Deleterious (0-96)
c.1193C>G, p.A398G	0	1	-	Group C	Deleterious (0-55)
c.1198C>T, p.P400S	0	1	-	Group A	Deleterious (0-70)
c.1283A>C, p.K428T	0	1	-	Group D	Neutral (0-33)
c.1346T>C, p.L449P	0	1	-	Group A	Deleterious (0-96)
c.1381G>A, p.V461M	7	1	-	Group D	Neutral (0-32)
c.1405G>A, p.V469I	6	2	-	Group E	Neutral (0-069)
c.1406T>C, p.V469A	1	1	-	Group C	Neutral (0-19)
c.1441G>A, p.A481T	0	1	-	Group C	Neutral (0-054)
c.1460T>C, p.I487T	6	1	-	Group A	Neutral (0-31)

c.1472C>T, p.T491I	0	1	-	Group D	Neutral (0.41)
c.1582G>A, p.A528T	0	2	-	Group B	Neutral (0.24)
c.1585G>A, p.A529T	0	1	-	Group C	Neutral (0.13)
c.1621C>T, p.R541W	11	1	-	Group C	Neutral (0.45)
c.1622G>A, p.R541Q	1	1	-	Group C	Neutral (0.35)
c.1669C>T, p.H557Y	0	1	-	Group A	Deleterious (0.84)
c.1745G>C, p.G582A	0	1	-	Group A	Deleterious (0.88)
c.1813C>T, p.R605C	0	1	-	Group B	Neutral (0.40)
c.1823C>T, p.T608M	7	1	-	Group D	Neutral (0.16)
c.1855G>A, p.G619R	0	3	-	Group D	Neutral (0.27)
c.1918A>G, p.T640A	368	32	-	Group D	Neutral (0.026)
c.1961G>A, p.R654Q	8	1	-	Group D	Neutral (0.069)
c.1978G>A, p.E660K	2	1	-	Group D	Neutral (0.046)
c.2076G>C, p.K692N	126	1	-	Group D	Neutral (0.16)
c.2129C>G, p.P710R	1	1	-	Group D	Neutral (0.17)
c.2180T>A, p.V727D	0	1	-	Group D	Neutral (0.12)
c.2219G>A, p.R740Q	7	1	-	Group C	Neutral (0.26)
c.2236G>A, p.V746M	10	3	-	Group C	Neutral (0.065)

†References where the *PCSK1* mutations were reported pathogenic according to HGMD Professional (version 2022.3);

‡According to our *in vitro* assays;

*Pathogenicity threshold for REVEL: 50%.

gnomAD, genome aggregation database browser (v2.1.1); **MAC**, minor allele count; **NA**, not available

Table 2. Protein expression of complete loss-of-function *PCSK1* variants (Group_A) on cell lysates by Western blotting

	Complete loss-of-function <i>PCSK1</i> variants																	
Variants	WT	F70C	R80*	R81S	R95H	L132S	T175M	D193Y	V304I	D324Y	Q363*	T375A	G390S	P400S	L449P	I487T	H557Y	G582A
$N_{ob} / N_{owt} / N_{nwt}$	-	0/1/0	1/0/0	1/0/0	1/0/0	1/0/0	0/2/0	0/0/1	1/1/1	1/0/0	0/1/0	1/0/0	1/1/0	1/0/0	1/0/0	1/0/0	0/1/0	0/1/0
Precursor protein	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	No	No	Yes	Yes	Yes	Yes
Active protein	Yes	No	No	Yes	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No
Examples																		

This table recapitulates the protein expression of Group_A *PCSK1* variants by Western blotting. Sizes of precursor forms of PC1/3: 94 kDa and 87kDa; size of active form of PC1/3: 74/66 kDa. Three independent experiments were performed for each mutation (images of Western blotting available in **Appendix, pp9-10**). N_{nwt} , number of normal-weight participants carrying the mutation in the RaDiO study; N_{ob} , number of participants with obesity carrying the mutation in the RaDiO study; N_{owt} ; number of overweight participants carrying the mutation in the RaDiO study.

Table 3. Effect of different clusters of rare *PCSK1* variants on obesity and other metabolic traits in adults, according to variant pathogenicity on PC1/3 activity

Trait	<i>n</i> _{total}	Cluster	%, mean \pm SD or median [IQR] of the trait in carriers	%, mean \pm SD or median [IQR] of the trait in non-carriers	OR [CI]	$\pi \pm$ SE	<i>P</i>
BMI (kg/m²)	7,260	I	32 \pm 9.3	27 \pm 6.5	-	6.9 \pm 1.9	0.00030
		II	26 \pm 3.6	27 \pm 6.5	-	-0.99 \pm 1.7	0.56
		III	27 \pm 4.5	27 \pm 6.5	-	0.87 \pm 2.3	0.70
		IV	27 \pm 4.0	27 \pm 6.5	-	1.2 \pm 1.1	0.30
		All	28 \pm 5.6	27 \pm 6.5	-	1.6 \pm 0.79	0.039
Obesity	4,402	I	86%	35%	9.3 [1.5–177]	-	0.014
		II	36%	35%	1.0 [0.27–3.4]	-	0.97
		III	43%	35%	1.4 [0.27–6.3]	-	0.68
		IV	42%	35%	1.2 [0.51–2.7]	-	0.66
		All	47%	35%	1.5 [0.85–2.7]	-	0.16
OWT or obesity	7,260	I	94%	60%	8.5 [1.7–154]	-	0.013
		II	68%	60%	1.4 [0.57–3.6]	-	0.50
		III	67%	60%	1.1 [0.34–4.3]	-	0.85
		IV	71%	60%	1.6 [0.85–3.1]	-	0.16
		All	73%	60%	1.7 [1.1–2.8]	-	0.018
T2D	5,496	I	65%	40%	1.2 [0.39–3.8]	-	0.79
		II	47%	40%	1.7 [0.64–4.3]	-	0.29
		III	56%	40%	1.3 [0.29–5.9]	-	0.71
		IV	39%	40%	0.83 [0.39–1.7]	-	0.61
		All	48%	40%	1.11 [0.68–1.8]	-	0.67
HDL (mmol/L)	6,864	I	1.4 \pm 0.56	1.5 \pm 0.44	-	0.074 \pm 0.12	0.54
		II	1.5 \pm 0.34	1.5 \pm 0.44	-	-0.048 \pm 0.10	0.65
		III	1.4 \pm 0.41	1.5 \pm 0.44	-	-0.024 \pm 0.14	0.87
		IV	1.5 \pm 0.45	1.5 \pm 0.44	-	0.039 \pm 0.070	0.58
		All	1.4 \pm 0.44	1.5 \pm 0.44	-	0.018 \pm 0.049	0.71
TG (mmol/L)	6,912	I	1.5 [1.1–1.7]	1.1 [0.77–1.6]	-	-0.015 \pm 0.13	0.91
		II	1.1 [0.75–1.7]	1.1 [0.77–1.6]	-	0.097 \pm 0.11	0.39
		III	1.0 [0.86–1.4]	1.1 [0.77–1.6]	-	-0.080 \pm 0.16	0.61
		IV	1.1 [0.93–1.5]	1.1 [0.77–1.6]	-	-0.0047 \pm 0.076	0.95
		All	1.2 [0.86–1.6]	1.1 [0.77–1.6]	-	0.0073 \pm 0.053	0.89

The associations between the different burdens of rare *PCSK1* variants and adiposity were analyzed using the MiST method adjusted for age, sex and ancestry. The associations between the different burden of rare *PCSK1* variants and T2D risk or cholesterol levels were analyzed using the MiST method adjusted for age, sex, BMI and ancestry. Cluster I includes Group_A and Null variants; Cluster II includes Group_B variants; Cluster III includes Group_C variants and Cluster IV includes Group_D and Group_E variants. TG data were log-transformed before analysis. *BMI*, body mass index; *CI*, confidence interval; *HDL*, high-density lipoprotein; *IQR*,

interquartile range; π , mean effect; **OR**, odds ratio; **OWT**, overweight; **SD**, standard deviation; **SE**, standard error; **T2D**, type 2 diabetes; **TG**, triglycerides.