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

Lise Folon, Morgane Baron*, Bénédicte Toussaint, Emmanuel Vaillant, Mathilde Boissel, Victoria Scherrer, Hélène Loiselle, Audrey Leloire, Alaa Badreddine, Beverley Balkau, Guillaume Charpentier, Sylvia Franc, Michel Marre, Soulaimane Aboulouard, Michel Salzet, Mickaël Canouil, Mehdi Derhourhi, Philippe Froguel†, Amélie Bonnefond†*

Lancet Diabetes Endocrinol 2023; 11: 182–90

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*Joint first authors

†Joint last authors

Inserm UMR1283, CNRS UMR8199, European Genomic Institute for Diabetes, Institut Pasteur de Lille, Lille University Hospital, Lille, France (L Folon MSc, M Baron PhD, B Toussaint MSc, E Vaillant MSc, M Boissel MSc, V Scherrer BSc, H Loiselle BSc, A Leloire BSc, A Badreddine MSc, M Canouil PhD, M Derhourhi PhD, Prof P Froguel PhD, A Bonnefond PhD); **Université de Lille, Lille, France** (L Folon, M Baron, B Toussaint, E Vaillant, M Boissel, V Scherrer, H Loiselle, A Leloire, A Badreddine, S Aboulouard MSc, Prof M Salzet PhD, M Canouil, M Derhourhi, Prof P Froguel, A Bonnefond); **Paris-Saclay University, Paris-Sud University, Université de Versailles Saint-Quentin-en-Yvelines, Center for Research in Epidemiology and Population Health, Inserm U1018 Clinical Epidemiology, Villejuif, France** (B Balkau PhD); **Centre d'Étude et de Recherche pour l'Intensification du Traitement du Diabète, Evry, France** (G Charpentier MD, S Franc MD); **Department of Diabetes, Sud-Francilien Hospital, Paris-Sud University, Corbeil-Essonnes, France** (S Franc); **Institut Necker-Enfants Malades, INSERM, Université de Paris, Paris, France** (Prof M Marre PhD); **Clinique Ambroise Paré, Neuilly-sur-Seine, France** (Prof M Marre); **Inserm U1192, Laboratoire Protéomique, Réponse Inflammatoire et Spectrométrie de Masse, Lille, France** (S Aboulouard, Prof M Salzet); **Department of Metabolism, Imperial College London, London, UK** (Prof P Froguel, A Bonnefond)

Summary

Background Rare biallelic pathogenic mutations in *PCSK1* (encoding proprotein convertase subtilisin/kexin type 1 [PC1/3]) cause early-onset obesity associated with various endocrinopathies. Setmelanotide has been approved for carriers of these biallelic mutations in the past 3 years. We aimed to perform a large-scale functional genomic study focusing on rare heterozygous variants of *PCSK1* to decipher their putative impact on obesity risk.

Methods This case-control study included all participants with overweight and obesity (ie, cases) or healthy weight (ie, controls) from the RaDiO study of three community-based and one hospital-based cohort in France recruited between Jan 1, 1995, and Dec 31, 2000. In adults older than 18 years, healthy weight was defined as BMI of less than 25·0 kg/m², overweight as 25·0–29·9 kg/m², and obesity as 30·0 kg/m² or higher. Participants with type 2 diabetes had fasting glucose of 7·0 mmol/L or higher or used treatment for hyperglycaemia (or both) and were negative for islet or insulin autoantibodies. Functional assessment of rare missense variants of *PCSK1* was performed. Pathogenicity clusters of variants were determined with machine learning. The effect of each cluster of *PCSK1* variants on obesity was assessed using the adjusted mixed-effects score test.

Findings All 13 coding exons of *PCSK1* were sequenced in 9320 participants (including 7260 adults and 2060 children and adolescents) recruited from the RaDiO study. We detected 65 rare heterozygous *PCSK1* variants, including four null variants and 61 missense variants that were analysed in vitro and clustered into five groups (A–E), according to enzymatic activity. Compared with the wild-type, 15 missense variants led to complete PC1/3 loss of function (group A; reference) and rare exome variant ensemble learner (REVEL) led to 15 (25%) false positives and four (7%) false negatives. Carrying complete loss-of-function or null *PCSK1* variants was significantly associated with obesity (six [86%] of seven carriers *vs* 1518 [35%] of 4395 non-carriers; OR 9·3 [95% CI 1·5–177·4]; p=0·014) and higher BMI (32·0 kg/m² [SD 9·3] in carriers *vs* 27·3 kg/m² [6·5] in non-carriers; mean effect π 6·94 [SE 1·95]; p=0·00029). Clusters of *PCSK1* variants with partial or neutral effect on PC1/3 activity did not have an effect on obesity or overweight and on BMI.

Interpretation Only carriers of heterozygous, null, or complete loss-of-function *PCSK1* variants cause monogenic obesity and, therefore, might be eligible for setmelanotide. In silico tests were unable to accurately detect these variants, which suggests that in vitro assays are necessary to determine the variant pathogenicity for genetic diagnosis and precision medicine purposes.

Funding Agence Nationale de la Recherche, European Research Council, National Center for Precision Diabetic Medicine, European Regional Development Fund, Hauts-de-France Regional Council, and the European Metropolis of Lille.

Introduction

Overweight (BMI 25·0–29·9 kg/m²), obesity (BMI ≥30·0 kg/m²), and related health comorbidities (including cardiovascular diseases, diabetes, musculoskeletal disorders, and some cancers) constitute a major growing medical burden and are recognised as the fifth leading cause of death worldwide, according to WHO. Obesity is a complex multifactorial disease with a strong genetic basis.[1] 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 and it affects less than 5% of people with obesity.[1] Genes linked with non-syndromic monogenic obesity are mostly involved in the leptin-melanocortin pathway, which is crucial for controlling appetite in the CNS. These genes include *LEP* (encoding leptin), *LEPR* (encoding the leptin receptor), *POMC* (encoding pro-opiomelanocortin), *PCSK1* (encoding proprotein convertase subtilisin/kexin type 1 [PC1/3]; neuroendocrine convertase 1), and *MC4R* (encoding melanocortin receptor 4) with deficiencies most prevalent in Europe.[1] Importantly, identification of these genes has enabled the development of setmelanotide, an MC4R agonist, for people with monogenic obesity. This novel drug, administered by subcutaneous injection, was shown to efficiently lead to weight loss in people with obesity who

Research in context

Evidence before this study

We searched, PubMed from Jan 1, 1990, to Aug 15, 2022, using the search terms "PCSK1", "heterozygous", "mutation", "obesity", and "human". This search revealed ten studies. Of these, three studies reported biallelic *PCSK1* mutations (ie, compound heterozygous) in people with obesity; one was focused on a mouse model (knockout of *Pax6*); three sequenced *PCSK1* among other genes linked with monogenic obesity (n=1014, n=463, and 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 showed a significant association between rare, null, heterozygous mutations in *PCSK1*, *MC4R*, and *LEPR* and increased BMI; 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 contributed to obesity in the general population. Currently a phase 3 clinical trial (NCT05093634) evaluating the melanocortin 4 receptor (MC4R) agonist setmelanotide in people with obesity who carry rare heterozygous *PCSK1* variants is underway. This trial assumes that *PCSK1* mutations cause a dominant form of obesity; however, the true contribution of these mutations remains elusive.

Added value of this study

Based on in vitro functional analyses in a large-scale case-control study, our results suggest that only complete loss-of-function heterozygous *PCSK1* variants cause monogenic obesity. Therefore, in vitro functional analyses are necessary to assess the pathogenic effect of *PCSK1* mutations in people with obesity.

Implications of all the available evidence

Only people with obesity who carry complete loss-of-function heterozygous *PCSK1* variants are likely to be considered for future treatment with setmelanotide. This study advocates for performing systematic in vitro analyses before considering precision medicine. Research evaluating setmelanotide in people with overweight or obesity should use the same strategy as our study to assess the functional effect of these variants and their impact on monogenic obesity.

Correspondence to:
Dr Amélie Bonnefond,
Inserm UMR1283,
CNRS UMR8199, European Genomic Institute for Diabetes,
Université de Lille,
Institut Pasteur de Lille,
Lille University Hospital,
59045 Lille Cedex, France
amelie.bonnefond@cnrs.fr

For more on **WHO statistics** see https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight

are deficient for *POMC*, *LEPR*, or *PCSK1*, in whom traditional interventions usually have a poor effect on weight.[2–4] This finding led to the US Food and Drug Administration and European Medicines Agency approving the use of setmelanotide in the past 3 years for people with obesity.

Rare biallelic pathogenic mutations in *PCSK1* cause early-onset obesity associated with various endocrinopathies.[5] PC1/3 is a major endoprotease involved in processing various prohormones and neuropeptides that regulate appetite and energy homoeostasis. In the leptin-melanocortin pathway, PC1/3 cleaves POMC into adrenocorticotropic hormone (ACTH), the precursor of α-melanocyte-stimulating hormone—ie, the agonist of MC4R. PC1/3 is highly expressed in neural and endocrine tissues and includes a prodomain, catalytic domain, P domain, and a carboxy-terminal domain. PC1/3 is synthesised as an inactive precursor known as preproPC1/3, which is converted into a proPC1/3 zymogen (94 kDa) by autocleavage in the endoplasmic reticulum. The prodomain is then cleaved in the endoplasmic reticulum and Golgi, where PC1/3 is converted into a partly active form (87 kDa). Autocatalytic cleavages lead to the mature activation of PC1/3 (74/66 kDa) in the acidic environment of secretory granules.[6] 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 obesity in the general population.[7,8] However, the putative impact of rare heterozygous mutations in *PCSK1* on obesity has not been clearly defined in large-scale studies. Two previous studies identified rare, heterozygous, null variants (Arg80Ter and 1095+1G→A) in *PCSK1* with a deleterious functional effect on PC1/3, which was suggested as the cause of monogenic obesity.[9,10] Additionally, based on sequencing of 845 participants with severe obesity (BMI 40·0 kg/m² or higher) and in vitro analysis, a study by Creemers and colleagues[11] found that seven rare heterozygous *PCSK1* variants had a deleterious effect on PC1/3 activity and contributed to obesity in the general population. However, this study, alongside several others that investigated the functional effect of *PCSK1* variants, used a *PCSK1* plasmid which included a non-synonymous mutation (encoding Ser357Gly) that did not have normal subcellular regulation and produced anomalous substrate processing when compared with wild-type *PCSK1*^{WT}, which might have led to biases in the interpretation of variant effect on PC1/3 activity.[12]

Understanding the impact of a rare heterozygous *PCSK1* mutation on obesity is crucial for genetic diagnosis and treatment purposes. An ongoing phase 3 clinical trial (NCT05093634) is evaluating setmelanotide in people with obesity who carry rare heterozygous *PCSK1* variants but does not include 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 their putative impact on obesity risk in humans.

	Minor allele count in gnomAD	Minor allele count in RaDiO	References*	Functional group†	REVEL (score)‡
34G→C; Ala12Pro	0	1	..	D	Neutral (0·28)
173T→C; Leu58Ser	0	1	..	B	Neutral (0·15)
209T→G; Phe70Cys	0	1	..	A	Neutral (0·48)
238C→T; Arg80Ter	2	1	Philippe et al (2015)[9]; Qian et al (2021)[17]	A and null	NA
239G→A; Arg80Gln	585	1	..	B	Neutral (0·21)
243G→C; Arg81Ser	1	1	..	A	Neutral (0·24)
254A→G; His85Arg	0	1	..	C	Neutral (0·10)
281A→G; Asp94Gly	1	2	..	D	Neutral (0·063)
284G→A; Arg95His	3	1	..	A	Neutral (0·23)
334G→A; Ala112Thr	0	2	..	D	Neutral (0·015)
375G→A; Met125Ile	51	13	Creemers et al (2012)[11]	B	Neutral (0·33)
395T→C; Leu132Ser	0	1	..	A	Deleterious (0·92)
470C→T; Thr157Met	5	1	..	B	Deleterious (0·83)
524C→T; Thr175Met	41	2	Creemers et al (2012)[11]	A	Deleterious (0·83)
541T→C; Tyr181His	29	4	Creemers et al (2012)[11]	B	Deleterious (0·94)
559T→C; Tyr187His	4	2	..	D	Deleterious (0·83)
577G→T; Asp193Tyr	0	1	..	A	Deleterious (0·91)
608C→T; Thr203Ile	0	1	..	C	Deleterious (0·64)
646A→G; Ile216Val	1	1	..	C	Neutral (0·36)
650C→T; Ala217Val	15	3	..	C	Deleterious (0·87)
844C→T; Arg282Trp	7	1	..	D	Deleterious (0·65)
869A→G; Tyr290Cys	16	1	..	C	Deleterious (0·75)
878A→G; Lys293Arg	2	1	..	B	Neutral (0·23)
910G→A; Val304Ile	35	3	..	A	Deleterious (0·63)
934C→T; Arg312Cys	1	1	..	B	Deleterious (0·76)
970G→T; Asp324Tyr	0	1	..	A	Deleterious (0·72)
974G→A; Ser325Asn	0	1	Creemers et al (2012)[11]	C	Deleterious (0·73)
985A→G; Ile329Val	1	1	..	C	Deleterious (0·55)
987C→G; Ile329Met	0	2	..	C	Deleterious (0·78)
1018del; Ser340ProfsTer94	0	1	..	Null	NA
1019C→A; Ser340Tyr	0	1	..	B	Deleterious (0·60)
1061C→G; Ser354Cys	2	1	..	D	Deleterious (0·62)
1087C→T; Gln363Ter	2	1	..	A and null	NA
1095+1G→A; ?§	0	1	Löffler et al (2016)[10]; Martín et al (2013)[18]	Null	NA
1097C→T; Thr366Met	9	1	..	B	Neutral (0·37)
1123A→G; Thr375Ala	0	1	..	A	Deleterious (0·87)
1130C→T; Thr377Met	4	1	..	E	Deleterious (0·60)
1168G→A; Gly390Ser	2	2	..	A	Deleterious (0·96)
1193C→G; Ala398Gly	0	1	..	C	Deleterious (0·55)
1198C→T; Pro400Ser	0	1	..	A	Deleterious (0·70)
1283A→C; Lys428Thr	0	1	..	D	Neutral (0·33)
1346T→C; Leu449Pro	0	1	..	A	Deleterious (0·96)
1381G→A; Val461Met	7	1	..	D	Neutral (0·32)
1405G→A; Val469Ile	6	2	..	E	Neutral (0·069)
1406T→C; Val469Ala	1	1	..	C	Neutral (0·19)
1441G→A; Ala481Thr	0	1	..	C	Neutral (0·054)
1460T→C; Ile487Thr	6	1	..	A	Neutral (0·31)

(Table 1 continues on next page)

Methods

Study design and participants

This case-control study included participants from the RaDiO study of three community-based and one hospital-based cohort in France (appendix p 5).[13,14] Cases include adults or children with obesity or overweight, whereas controls include adults or children with healthy weight. In adults older than 18 years, healthy weight was defined as BMI of less than 25·0 kg/m², overweight as BMI 25·0–29·9 kg/m² or higher, and obesity as BMI 30·0 kg/m² or higher. In children and adolescents younger than 18 years, healthy weight was defined as BMI-for-age of less than the 85th percentile and obesity as BMI-for-age in the 95th percentile or higher. Sex was determined by self-reporting. Participants with type 2 diabetes had fasting glucose of 7·0 mmol/L or higher or used treatment for hyperglycaemia (or both) and were negative for islet or insulin autoantibodies. Participants in the control group had fasting glucose of less than 5·6 mmol/L at age older than 30 years and did not use treatment for hyperglycaemia. All participants and parents (or legal guardians) provided written informed consent. Children and adolescents provided oral assent. The study protocols were approved by local ethics committees.

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 more than 40 reads and had a quality score higher than 150. Furthermore, no variants had more than 5% of missing genotypes across participants and none of the participants had more than 5% of missing genotypes across *PCSK1*. 61 rare nonsense or missense variants of *PCSK1* were created by mutagenesis and inserted into plasmids from the *PCSK1*^{WT} plasmid (Origene, Rockville, MD, USA) using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA). The sequence of each plasmid was checked by Sanger sequencing. Two plasmids, including the *PCSK1* variant encoding Lys293Arg or Thr366Met, were purchased from Twist Bioscience (South San Francisco, CA, USA).

All functional analyses were done in human embryonic kidney 293 (HEK293) cells, which were cultured according to standard conditions (appendix p 2). For the enzymatic assays, HEK293 cells were transfected using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA) with 150 ng/mL of plasmid encoding the β-galactosidase gene and 1000 ng/mL of *PCSK1* plasmid (*PCSK1*^{WT} or with each variant *PCSK1*^{mut}). 48 h after transfection, the β-galactosidase activity in cells was measured as previously described,[13] and the enzymatic activity of secreted PC1/3 in supernatants was measured by adding a fluorescent substrate of PC1/3 (appendix p 2). The experiments were performed in technical triplicate and each experiment was

repeated four times. We used three different controls in each experiment: one positive control plasmid including *PCSK1*^{WT}; one negative control plasmid encoding Arg80Ter PC1/3^{mut}; and one negative control, in which *PCSK1*^{WT} or *PCSK1*^{mut} was not transfected (defined as a non-transfected cell).

The protein expression of each *PCSK1*^{mut} in cell lysates of HEK293 cells was assessed by Western blotting (appendix p 3). Three independent experiments for each mutation were performed. Protein expression of Gln363Ter PC1/3^{mut} and Gly390Ser PC1/3^{mut} in HEK293 cell supernatants was assessed by liquid chromatography-mass spectrometry (LC-MS; appendix p 3).

We performed prediction analysis to separate complete loss-of-function variants following in vitro analyses from other variants using rare exome variant ensemble learner (REVEL), an ensemble method for predicting the pathogenicity of rare missense variants.[15] The main outcome was the effect of *PCSK1* variants on obesity, according to their functional impact on PC1/3 activity.

Statistical analysis

No sample size calculation was performed. All participants recruited and sequenced in the RaDiO study were included. Statistical analyses for enzymatic assays were performed using R (version 4.1.3). PC1/3 activity measures were divided by β-galactosidase activity measures. Each set of experiments was then normalised using non-transfected and PC1/3^{WT} activity measures. The non-transfected factor was computed as the geometric mean of non-transfected PC1/3 activity measures. The wild-type factor was computed as the geometric mean of the PC1/3^{WT} activity measures at 300 min minus the non-transfected measures. The normalised PC1/3^{mut} activity measures were computed as PC1/3^{mut} activity measures minus the non-transfected factor divided by the wild-type factor. The minimum value of normalised PC1/3^{mut} activity measures was set to zero, when needed, by adding the absolute minimum to all normalised measures. The resulting PC1/3 activity trajectories were clustered by machine learning using R package k-means for longitudinal data (kml) with ten resampling iterations and a number of clusters from two to eight. The optimal number of clusters was five (groups A, B, C, D, and E) based on five criteria (Akaike information criterion, Bayesian information criterion, Calinkski-Harabatz, Ray-Turi, and Davies-Bouldin).

Protein identification was performed by comparing all MS and tandem mass spectrometry-data with the Homo Sapiens proteome database (Uniprot; June, 2021), using MaxQuant software (version 1.6.10.43). For the protein and peptide identification, we used a false discovery rate of 1% and a minimum of two peptides per protein, including one unique peptide. The statistical analysis was done using MaxQuant Perseus software, with three replicates per group. The results were normalised by Z score.

	Minor allele count in gnomAD	Minor allele count in RaDiO	References*	Functional group†	REVEL (score)‡
(Continued from previous page)					
1472C→T; Thr491Ile	0	1	..	D	Neutral (0·41)
1582G→A; Ala528Thr	0	2	..	B	Neutral (0·24)
1585G→A; Ala529Thr	0	1	..	C	Neutral (0·13)
1621C→T; Arg541Trp	11	1	..	C	Neutral (0·45)
1622G→A; Arg541Gln	1	1	..	C	Neutral (0·35)
1669C→T; His557Tyr	0	1	..	A	Deleterious (0·84)
1745G→C; Gly582Ala	0	1	..	A	Deleterious (0·88)
1813C→T; Arg605Cys	0	1	..	B	Neutral (0·40)
1823C→T; Thr608Met	7	1	..	D	Neutral (0·16)
1855G→A; Gly619Arg	0	3	..	D	Neutral (0·27)
1918A→G; Thr640Ala	368	32	..	D	Neutral (0·026)
1961G→A; Arg654Gln	8	1	..	D	Neutral (0·069)
1978G→A; Glu660Lys	2	1	..	D	Neutral (0·046)
2076G→C; Lys692Asn	126	1	..	D	Neutral (0·16)
2129C→G; Pro710Arg	1	1	..	D	Neutral (0·17)
2180T→A; Val727Asp	0	1	..	D	Neutral (0·12)
2219G→A; Arg740Gln	7	1	..	C	Neutral (0·26)
2236G→A; Val746Met	10	3	..	C	Neutral (0·065)

gnomAD=genome aggregation database (version 2.1.1). NA=not applicable. REVEL=rare exome variant ensemble learner. *References in which the *PCSK1* mutations were reported as pathogenic, according to Human Gene Mutation Database Professional (version 2022.3). †According to our in vitro assays. ‡Pathogenicity threshold for REVEL is 50%. §No protein annotation.

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

For analysis of genetic association studies, the rare variants were divided into four pathogenicity clusters (group A and null variants; group B; group C; and groups D and E) on the basis of enzymatic assays. Each cluster was analysed using the mixed-effects score test method,[16] which provides a score statistic for the mean effect (π) and heterogeneous effect (τ) of the cluster. The equation of this model is,

See Online for appendix

$$Y = aX + \pi GZ$$

where Y is the trait of interest, X is the matrix of covariates (ie, age, sex, BMI, and ancestry for assessing type 2 diabetes and lipid concentrations and age, sex, and ancestry for assessing adiposity), G is the matrix of *PCSK1* variants, and Z is a vector of *PCSK1* variants ones repeated *n* times in all participants with *n* as the number of rare *PCSK1* variants found in participants, leading to:

$$\pi GZ = \pi \sum_{i=1}^{n} G_i$$

For the **MaxQuant software** see https://www.maxquant.org/

The ancestry of participants was assessed using the first five genotypic principal components (PC1–PC5), as previously described.[14] PC1–PC5 were computed using a principal component analysis on 15 020 common variants available in RaDiO and in the 1000 Genomes

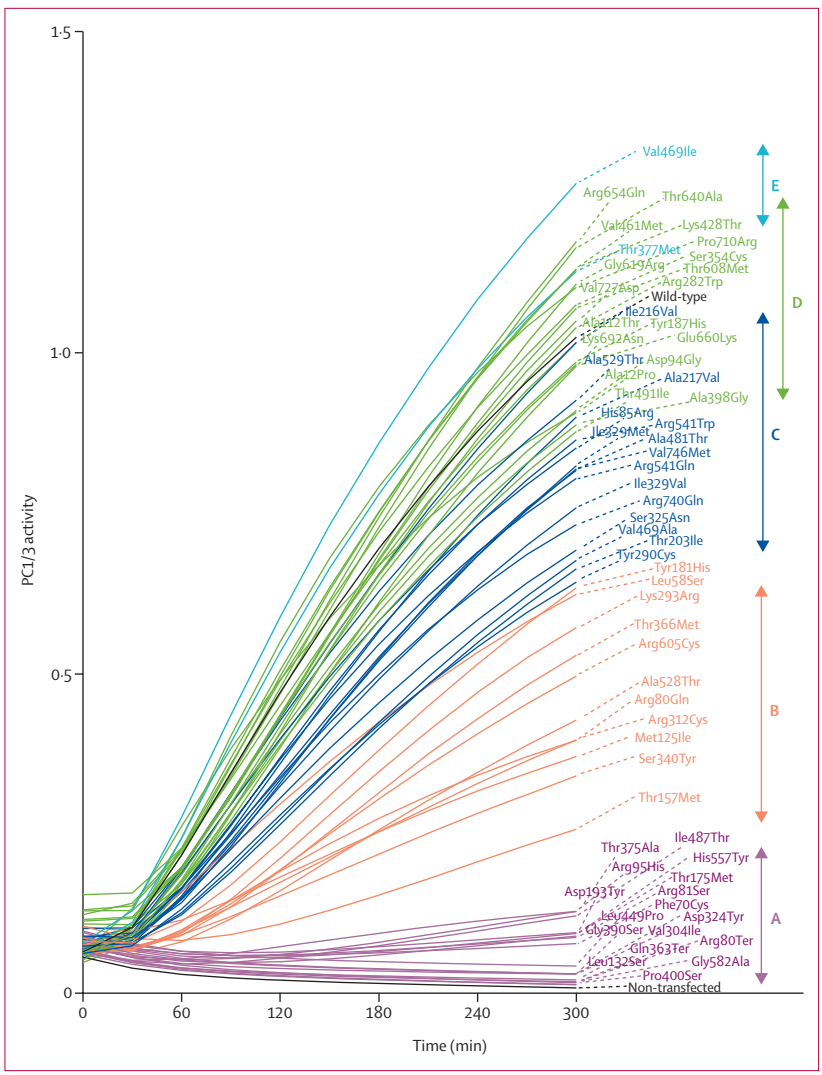


Figure 1: **Cluster analysis according to enzymatic activity of 63 rare missense or nonsense variants of *PCSK1***
PC1/3 activity trajectories clustering of *PCSK1* variants by k-means for longitudinal data (k=5). Data are the mean of four independent experiments performed in technical triplicate. PC1/3 activity represents the enzymatic fluorescence of PC1/3 normalised by the fluorescence of β-galactosidase.

Project. Because none of the association studies between *PCSK1* variants and obesity, overweight, BMI, type 2 diabetes, or lipids had a significant p value for heterogeneous effect (τ), we only showed the p values associated with the score representing the mean effect of the cluster. These statistical analyses were performed using the R software (version 4.0.2).

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

All 13 coding exons of *PCSK1* were sequenced in 9320 participants (including 7260 adults and 2060 children and adolescents) recruited from the RaDiO study between Jan 1, 1995, and Dec 31, 2000 (appendix p 5). 1524 (21%) of 7260 adults had obesity, 4382 (60%) had overweight and obesity, and 2878 (40%) had a healthy weight. 1032 (50%) children and adolescents had obesity and 1028 (50%) had a healthy weight (appendix p 5). We detected 65 rare, heterozygous, coding variants in *PCSK1* including four null variants (ie, a nonsense variant in which a point mutation results in a premature stop codon [Arg80Ter and Gln363Ter], frameshift [Ser340ProfsTer94], or canonical splice sites [1095+1G→A]) and 61 missense variants (ie, point mutations in which a single nucleotide change results in a codon that encodes a different amino acid; table 1).

Each nonsense or missense $PCSK1^{mut}$ or $PCSK1^{WT}$ was expressed in HEK293 cells and the enzymatic activity was measured using a fluorescent substrate of PC1/3. Compared with wild-type, most variants substantially decreased the enzymatic activity of PC1/3 (appendix pp 7–11). From the cluster analysis, we determined five clusters of variants according to their pathogenicity on PC1/3 activity (ie, groups A–E; figure 1). Compared with wild-type, 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 lower activity (group C), and 19 variants did not affect the enzymatic activity of PC1/3 (groups D and E). 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; appendix pp 12–13). Among 17 complete PC1/3 loss-of-function variants (group A), three mutations (encoding Arg81Ser, Arg95His, and Thr375Ala) had all forms of PC1/3 expressed in the same way as the wild-type, three mutations (encoding Arg80Ter, Val304Ile, and Pro400Ser) did not show any form of PC1/3, three mutations (encoding Phe70Cys, Thr175Met, and Leu449Pro) had the zymogen form (94 kDa), six mutations (encoding Leu132Ser, Asp193Tyr, Asp324Tyr, Ile487Thr, His557Thr, and Gly582Ala) had both precursor forms (94 kDa and 87 kDa) but the active form of PC1/3 was less visible (74/66 kDa). Additionally, two group A variants encoding Gln363Ter and Gly390Ser did not show precursor or active forms of PC1/3, but a single band at around 50 kDa. LC-MS analyses showed that PC1/3 protein was not present in supernatants of HEK293 cells transfected with Gln363Ter or Gly390Ser, suggesting an incorrect cleavage of PC1/3 precursor forms (appendix p 14). Notably, among carriers of heterozygous complete loss-of-function variants (group A) from the RaDiO study, 11 individuals had obesity and two had a healthy weight (figure 3).

Using statistical analyses, we assessed the effect of rare *PCSK1* variants on metabolic homoeostasis. After adjustment for sex, age, and ancestry, all rare variants

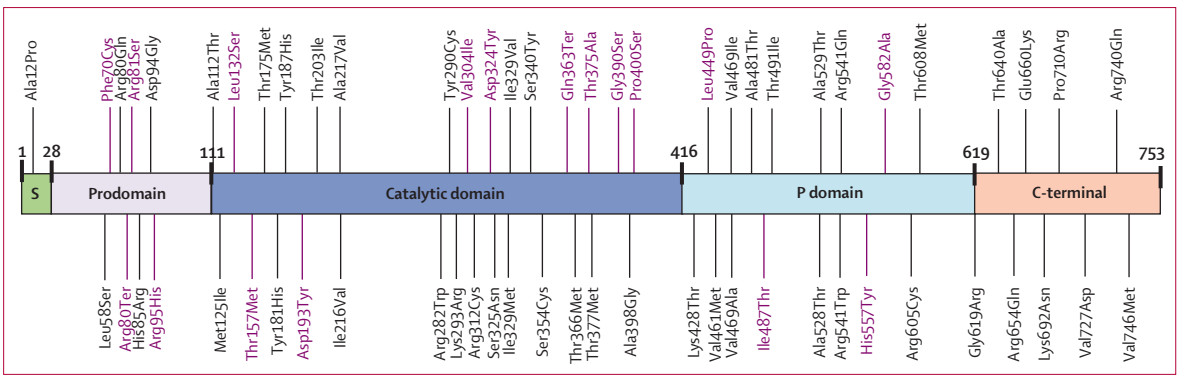


Figure 2: **Localisation of rare coding missense or nonsense variants of *PCSK1***
Different domains of the PC1/3 protein are shown. Purple variants are amino acid changes linked to complete loss of enzymatic activity (group A), according to our in vitro assays. Black variants are amino acid changes linked to variants with partial or neutral effect on PC1/3 activity (groups B, C, D, and E), according to our in vitro assays. S=peptide signal.

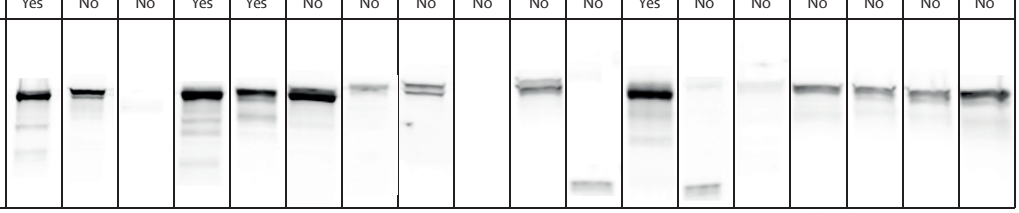
Complete loss-of-function *PCSK1* variants																			
Variants	**Wild-type**	**Phe70 Cys**	**Arg80 Ter**	**Arg81 Ser**	**Arg95 His**	**Leu132 Ser**	**Thr175 Met**	**Asp193 Tyr**	**Val304 Ile**	**Asp324 Tyr**	**Gln363 Ter**	**Thr375 Ala**	**Gly390 Ser**	**Pro400 Ser**	**Leu449 Pro**	**Ile487 Thr**	**His557 Tyr**	**Gly582 Ala**
$n_{obesity}$	..	0	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	0
$n_{overweight}$	..	1	0	0	0	0	2	0	1	0	1	0	1	0	0	0	1	1
$n_{healthy}$	..	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	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	Yes	No	No	No	No	No	No	Yes	No	No	No	No	No	No
Example kDa 100 70 55																		

Figure 3: **Protein expression of complete loss-of-function *PCSK1* variants (group A) on cell lysates 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 (appendix pp 12–13). $n_{healthy}$ shows the number of healthy-weight participants, $n_{obesity}$ shows the number of participants with obesity, and $n_{overweight}$ shows the number of participants with BMI 25·0–29·9 kg/m², all of whom are carrying the mutation in the RaDiO study.

were significantly associated with a higher risk of overweight and obesity (odds ratio [OR] 1·7 [95% CI 1·1–2·8]; p=0·018) and a higher BMI (27·9 kg/m² [SD 5·6] in carriers *vs* 27·3 kg/m² [6·5] in non-carriers; mean effect π 1·65 [SE 0·79]; p=0·039; table 2). However, carrying all rare variants was not significantly associated with type 2 diabetes risk, obesity risk or concentrations of triglycerides or high-density lipoprotein cholesterol.

We then performed the same analyses according to the pathogenicity of variants based on in vitro assays (figure 1). The risk of carrying complete loss-of-function or null *PCSK1* variants (ie, including group A and the two rare null variants [Ser340ProfsTer94 and 1095+1G→A]) was significantly associated with higher BMI (32·0 kg/m² [SD 9·3] in carriers *vs* 27·3 kg/m² [6·5] in non-carriers; mean effect π 6·94 [SE 1·95]; p=0·00029; table 2). Similarly, the risk of carrying complete loss-of-function or null *PCSK1* variants was significantly higher among individuals with obesity (six [86%] of seven carriers *vs* 1518 (35%) of 4395 non-carriers; OR 9·3 [95% CI 1·5–177·4]; p=0·014) or those with overweight and obesity (16 [94%] of 17 *vs* 4366 (60%) of 7243; OR 8·5 [1·7–154·0]; p=0·013; table 2). We found a similar but not statistically significant association between obesity and the risk of carrying complete loss-of-function or null *PCSK1* variants in children and adolescents (five [83%] of six carriers with obesity *vs* 1039 (50%) of 2080 non-carriers with obesity; OR 4·7 [0·6–103·0]; p=0·18; appendix p 6). Whereas carrying different clusters of *PCSK1* variants with partial or neutral effect (ie, group B, group C, and groups D and E) 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).

We then assessed the relevance of in silico analyses to separate complete loss-of-function *PCSK1* variants from

	Trait in carriers	Trait in non-carriers	Odds ratio (95% CI)	Mean effect π (SE)	p value
BMI, kg/m²					
Group A and null	32·0 (9·3)	27·3 (6·5)	..	6·94 (1·95)	0·00029
Group B	26·2 (3·6)	27·3 (6·5)	..	−0·99 (1·69)	0·56
Group C	27·4 (4·5)	27·3 (6·5)	..	0·87 (2·28)	0·70
Groups D and E	27·3 (4·0)	27·3 (6·5)	..	1·18 (1·14)	0·30
All	27·9 (5·6)	27·3 (6·5)	..	1·65 (0·79)	0·039
Obesity					
Group A and null	6/7 (86%)	1518/4395 (35%)	9·3 (1·5–177·4)	..	0·014
Group B	4/11 (36%)	1520/4391 (35%)	1·0 (0·3–3·4)	..	0·97
Group C	3/7 (43%)	1521/4395 (35%)	1·4 (0·3–6·3)	..	0·68
Groups D and E	10/24 (42%)	1514/4378 (35%)	1·2 (0·5–2·7)	..	0·66
All	23/49 (47%)	1501/4353 (34%)	1·5 (0·9–2·7)	..	0·16
Overweight and obesity					
Group A and null	16/17 (94%)	4366/7243 (60%)	8·5 (1·7–154·0)	..	0·013
Group B	15/22 (68%)	4367/7238 (60%)	1·4 (0·6–3·6)	..	0·50
Group C	8/12 (67%)	4374/7248 (60%)	1·1 (0·3–4·3)	..	0·85
Groups D and E	34/48 (71%)	4348/7212 (60%)	1·6 (0·9–3·1)	..	0·16
All	72/98 (73%)	4310/7162 (60%)	1·7 (1·1–2·8)	..	0·018
Type 2 diabetes					
Group A and null	11/17 (65%)	2167/5485 (40%)	1·2 (0·4–3·8)	..	0·79
Group B	9/19 (47%)	2169/5483 (40%)	1·7 (0·6–4·3)	..	0·29
Group C	5/9 (56%)	2173/5493 (40%)	1·3 (0·3–5·9)	..	0·71
Groups D and E	16/41 (39%)	2162/5461 (40%)	0·8 (0·4–1·7)	..	0·61
All	41/85 (48%)	2137/5417 (39%)	1·1 (0·7–1·8)	..	0·67
High-density lipoprotein, mmol/L					
Group A and null	1·4 (0·6)	1·5 (0·4)	..	0·07 (0·12)	0·54
Group B	1·5 (0·3)	1·5 (0·4)	..	−0·05 (0·10)	0·65
Group C	1·4 (0·4)	1·5 (0·4)	..	−0·02 (0·14)	0·87
Groups D and E	1·5 (0·5)	1·5 (0·4)	..	0·04 (0·07)	0·58
All	1·4 (0·4)	1·5 (0·4)	..	0·02 (0·05)	0·71
Triglycerides, mmol/L					
Group A and null	1·5 (1·1–1·7)	1·1 (0·8–1·6)	..	−0·02 (0·13)	0·91
Group B	1·1 (0·8–1·7)	1·1 (0·8–1·6)	..	0·10 (0·11)	0·39
Group C	1·0 (0·9–1·4)	1·1 (0·8–1·6)	..	−0·08 (0·16)	0·61
Groups D and E	1·1 (0·9–1·5)	1·1 (0·8–1·6)	..	0·00 (0·08)	0·95
All	1·2 (0·9–1·6)	1·1 (0·8–1·6)	..	0·01 (0·05)	0·89

Data are n/N (%), mean (SD), or median (IQR), unless stated otherwise. Triglyceride data were log-transformed before analysis.

Table 2: **Effect of rare *PCSK1* variant clusters on obesity and metabolic traits in adults, according to variant pathogenicity on PC1/3 activity**

other variants. A total of four (7%) of 61 complete loss-of-function mutations (encoding Phe70Cys, Arg81Ser, Arg95His, and Ile487Thr) were not predicted as deleterious by REVEL and are shown as neutral (false negatives; table 1). Whereas 15 variants (25%; encoding Thr157Met, Tyr181His, Tyr187His, Thr203Ile, Ala217Val, Arg282Trp, Tyr290Cys, Arg312Cys, Ser325Asn, Ile329Val, Ile329Met, Ser340Tyr, Ser354Cys, Thr377Met, and Ala398Gly) that were part of groups B–E were detected as deleterious by REVEL (false positives). The distribution of carriers of a missense *PCSK1* variant according to sex, obesity status, enzymatic activity of missense variants (group A vs group B), and the REVEL prediction of missense variants (neutral vs deleterious) shows an enrichment of carriers with obesity in group A, regardless of the REVEL prediction (appendix p 15). These results highlighted the requirement of in vitro analyses to pinpoint complete loss-of-function missense variants in *PCSK1*.

Discussion

Our results suggest that heterozygous, null, or proven complete loss-of-function *PCSK1* variants cause monogenic obesity, whereas missense variants with neutral or partial deleterious effect on PC1/3 activity do not contribute to obesity risk or increased BMI in a large population. Our results are in line with a 2021 study,[19] which showed that heterozygous protein-truncating variants in *PCSK1* were strongly associated with increased risk of obesity on the basis of 640 000 exome sequencing data. The genetic effect seen on adiposity was higher among carriers of null heterozygous *PCSK1* variants than among carriers of null heterozygous *MC4R* variants.[19]

We also found that the PC1/3 protein expression of complete loss-of-function *PCSK1* variants was impaired in most cases. Among 17, three mutations did not show any form of PC1/3, three had the zymogen form, six had both precursor forms, and two showed an incorrectly cleaved protein. Three group A mutations had all forms of PC1/3 expressed in the same way as the wild-type, suggesting that PC1/3 encoded by these mutations was secreted as usual, but enzymatic activity was impaired. These results are in line with the classification of *PCSK1* mutations proposed previously.[20] PC1/3 proteins can be classified as those retained within the endoplasmic reticulum (category 1); those able to travel to secretory granules, but which become altered in the secretory pathway (category 2); and those correctly folded and secreted, but with impaired enzymatic activity (category 3). Furthermore, Ramos-Molina and colleagues[20] proposed a gradation of obesity severity in the carriers of mutations from category 1 (ie, carriers with more severe obesity) to the carriers of mutations from category 3 (ie, carriers with less severe obesity). We did not replicate these findings and 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 carriers of mutations that did not show any form of PC1/3 had a BMI lower than 30·0 kg/m². More generally, we observed that carriers of a heterozygous, null, or complete loss-of-function variant did not have extreme obesity (average BMI of 32·0 kg/m²) despite the dominant effect of these mutations. However, a previous study[21] suggested that some carriers of a heterozygous mutation with predicted loss-of-function in *LEP*, *LEPR*, *POMC*, and *PCSK1* could present with phenotypes similar to the carriers of a biallelic mutation. Our

findings are in line with other studies,[9,22,23] in which the obesity in carriers of a heterozygous *PCSK1* variant was less severe and was harboured by a lower proportion of individuals with obesity among all carriers than among carriers of a biallelic *PCSK1* variant.

Innovative medicine should be given to the appropriate patients to maximise efficiency and minimise side-effects, which is the principle of precision medicine. So far, setmelanotide has been given to people with obesity who carry rare, biallelic, pathogenic mutations in *LEP*, *LEPR*, *POMC*, and *PCSK1*. An ongoing trial (NCT05093634) is testing setmelanotide for patients with heterozygous *PCSK1* variants. Thus, establishing whether these mutations are truly functional is crucial, especially because carriers of heterozygous variants represent a larger population than carriers of homozygous variants. Unfortunately, in silico tests were unable to accurately detect complete loss-of-function *PCSK1* variants, which suggests that in vitro assays are necessary to determine the variant pathogenicity before proposing a treatment targeting the leptin-melanocortin pathway. Notably, the ongoing trial (NCT05093634) is also assessing the effect of setmelanotide on obesity in patients with heterozygous variants in *POMC* or *LEPR*, although their true contribution to monogenic obesity is unclear. Therefore, future research directions should focus on determining the functional effect of heterozygous variants in *POMC* or *LEPR* and their contribution to obesity.

Our study was limited by the fact that access to concentrations of insulin and ACTH (which are both cleaved by PC1/3) was not possible for carriers of null or complete loss-of-function *PCSK1* variants. However, we did not find any association of these variants with type 2 diabetes or lipids, which suggests a minimal effect on these metabolic pathways. Notably, biases of this study are similar to those of any biobank with voluntary enrolment. Furthermore, the in vitro functional analyses were done in HEK293 cells, which do not express PC1/3 in physiological conditions. These cells largely differ from neuronal cells that have a strong impact on the obesity phenotype. However, this limitation was overcome by designing a large genetic association case-control study using our standard in vitro assays. Importantly, this study does not imply that patients with severe early-onset obesity carrying a neutral heterozygous *PCSK1* mutation should not be further investigated because another mutation might have a deleterious effect on the leptin-melanocortin pathway, and therefore these patients could be eligible for setmelanotide treatment.

Contributors

LF, MoB, PF, and AmB conceptualised and designed the study. LF, MoB, VS, HL, and AL contributed to functional analyses of each variant. BT and EV contributed to next-generation sequencing. MaB, MC, and AmB contributed to statistical analyses. AlB and MD contributed to computer analyses. MS and SA contributed to proteomic analyses. BB, GC, SF, MM, and PF contributed to collection of cohort data. LF and AmB wrote the first draft of the manuscript. LF, MoB, and AmB have accessed and verified all the data in the study. All authors provided input on the interpretation of results, revised and approved the final manuscript, had full access to all the data in the study, and accept responsibility to submit for publication.

Declaration of interests

We declare no competing interests.

Data sharing

Given the sensitivity and risk of reidentification, all clinical data linked with next-generation sequencing data for this study are only available upon reasonable request to the corresponding author.

Acknowledgments

This study was funded by the French National Research Agency (Agence Nationale de la Recherche [ANR]-10-LABX-46 [European Genomics Institute for Diabetes] and ANR-10-EQPX-07-01 [Lille Integrated Genomics Advanced Network for personalized medicine]), European Research Council (GEPIDIAB 294785 and OpiO 101043671), and the National Center for Precision Diabetic Medicine (PreciDIAB), which is jointly supported by the French National Agency for Research (ANR-18-IBHU-0001), European Regional Development Fund, Hauts-de-France Regional Council, and the European Metropolis of Lille. We are grateful to all individuals included in the cohort studies. We thank Frédéric Allegaert and Timothée Beke for technical assistance and the France Génomique consortium (ANR-10-INBS-009).

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