Brief Communication

MOLECULAR METABOLISM

Functional and clinical relevance of novel and known *PCSK1* variants for childhood obesity and glucose metabolism

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ABSTRACT

Objective: Variants in *Proprotein Convertase Subtilisin/Kexin Type 1* (*PCSK1*) may be causative for obesity as suggested by monogenic cases and association studies. Here we assessed the functional relevance in experimental studies and the clinical relevance through detailed metabolic phenotyping of newly identified and known *PCSK1* variants in children.

Results: In 52 obese children selected for elevated proinsulin levels and/or impaired glucose tolerance, we found eight known variants and two novel heterozygous variants (c.1095 + 1G > A and p.S24C) by sequencing the *PCSK1* gene. Patients with the new variants presented with extreme obesity, impaired glucose tolerance, and PCOS. Functionally, c.1095 + 1G > A caused skipping of exon8 translation and a complete loss of enzymatic activity. The protein was retained within the endoplasmic reticulum (ER) causing ER stress. The p.S24C variant had no functional effect on protein size, cell trafficking, or enzymatic activity. The known variants rs6230, rs35753085, and rs725522 in the 5′ end did not affect *PCSK1* promoter activity.

In clinical association studies in 1673 lean and obese children, we confirmed associations of rs6232 and rs6234 with BMI-SDS and of rs725522 with glucose stimulated insulin secretion and Matsuda index. We did not find the new variants in any other subjects.

Conclusions: We identified and functionally characterized two rare novel *PCSK1* variants of which c.1095 + 1G > A caused complete loss of protein function. In addition to confirming rs6232 and rs6234 in *PCSK1* as polygenic risk variants for childhood obesity, we describe an association of rs725522 with insulin metabolism. Our results support the contribution of *PCSK1* variants to obesity predisposition in children.

Keywords PCSK1; PC1/3; Obesity; Children; Prohormone convertase 1/3

1. INTRODUCTION

Variants in the *proprotein convertase subtilisin kexin type 1* (*PCSK1*) gene encoding proprotein convertase 1/3 (PC1/3) contribute to polygenic obesity risk as shown by candidate gene approaches [1] and subsequent genetic association studies [2] as well as genome-wide association studies (GWAS) for BMI and proinsulin [2–4]. Unlike some other genes identified in hypothesis-free, genome-wide association studies, *PCSK1* constitutes a reasonable candidate for functional relevance based on its biological function. The *PCSK1* gene is located on chromosome 5q [5] in a region originally showing linkage with type 2 diabetes [6]. In addition to this, linkage peaks on chromosome 5q have been identified for obesity [7,8].

PCSK1 is mainly expressed in neuroendocrine tissues [9], where it is involved in tissue-specific processing of prohormones and neuropeptide precursors such as proopiomelanocortin, proinsulin, proglucagon, and other known key regulators of energy metabolism [10,11].

Common variants in/near *PCSK1* gene have been associated with obesity risk, body mass index variation, birth weight in association with body mass index, and proinsulin levels in several populations [1,2,4,12,13]. The coding variants rs6232, rs6234, and rs6235 have functional consequences on PCSK1 activity [1,14–16].

Rare mutations in *PCSK1* have also been described in individuals with early onset monogenic obesity. Null mutations in the *PCSK1* gene cause recessive, monogenic, early-onset morbid obesity as a part of a complex syndrome including hypoadrenalism, hypogonadism, intestinal dysfunction, hyperphagia, altered proinsulin to insulin ratio, postprandial hypoglycemia, and diabetes insipidus [11,17–21]. Moreover, heterozygous nonsense mutation p.R80* was linked in one family with

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dominantly inherited obesity and impaired glucose tolerance, and rare heterozygous missense *PCSK1* variants were found in 0.83% of extremely obese individuals [22,23].

These genotype-phenotype links in both common and rare variants are making this gene one of the most relevant players in the etiology of obesity.

In the present study, we aimed to i) identify new variants, ii) investigate their clinical relevance with regard to obesity and glucose metabolism, and iii) assess their functional relevance on secretion, localization, enzymatic activity, and the ability to induce endoplasmic reticulum (ER) stress.

2. MATERIALS AND METHODS

2.1. Study subjects

Genetic analyses were performed in a cohort of 1673 children and adolescents of the *Leipzig school children cohort* [24] and *Leipzig Obesity Childhood cohort* as described previously [25].

The body mass index (BMI) was standardized referring to national reference data [26]. Children with a BMI-standard deviation score (SDS) $\geq$1.88 (=97th percentile) were classified obese.

For the case control setting, 684 obese children (322 boys and 362 girls; mean age 11.53 $\pm$ 3.36 years; mean BMI-SDS 2.41 $\pm$ 0.66) were compared to 989 lean children (467 boys and 522 girls; mean age 11.83 $\pm$ 2.78 years; mean BMI-SDS $-$0.14 $\pm$ 0.78).

Written consent was obtained from both children $>$12 years and parents. The study was approved by the ethics committee of the University of Leipzig (NTC 02208141).

2.2. Genetic analyses

Sanger sequencing was performed using the Big Dye Terminator (Applied Biosystems, Foster City, CA) on an automated DNA capillary sequencer (ABI PRISM 3100 Avant; Applied Biosystem). Sequence information for all oligonucleotide primers used for variant screening is available upon request.

Genotypes of rs6230, rs6232, rs6234, and p.S24C in *PCSK1* were determined using pre- or custom-designed Taqman single-nucleotide polymorphism genotyping assays (ABI) on an ABI Prism 7500 platform. Call rate was $>$95%.

PCR-restriction fragment length polymorphism (RFLP) technique was used for rs6230, rs35753085, rs725522, and c.1095 + 1G > A with the specified restriction enzyme (Supplemental Table 4) and evaluation of fragment length by electrophoresis.

For the p.S24C variant, the DNA sequence conservation (GERP, and phyloP100way provided by UCSC Genome Browser (http://genome.ucsc.edu) [27,28] and pathogenicity was tested using *in silico* analyses (SIFT [29], PROVEAN [30] (http://provean.jcvi.org), MutationAssesor (http://mutationassessor.org) [31], CADD (http://cadd.gs.washington.edu/score) [32], and SNPs&GO (http://snps.biofold.org) [33]. Sequence alignment of PCSK1 proteins was done at www.uniprot.org and by Signal-BLAST at sigpep.services.came.sbg.ac.at/signalblast.html.

2.3. Enzyme activity assay, immunofluorescence, and immunoblot

Activity studies were performed according to the previously published protocol [34]. The enzymatic activity was assayed using a fluorogenic substrate p-Glu-Arg-Thr-Arg-Arg-amino methylcoumarin (Peptides International Inc., Kentucky, USA) and normalized to the amount of enzyme determined by immunoblot analysis using Image Station 440 (Eastman Kodak Co., New Haven).

Chinese hamster ovary (CHO) cells transfected and grown on glass slides were fixed in 4% paraformaldehyde. After washing in PBS, endogenous peroxidase was quenched with NH_4Cl and blocked with blocking reagent (Roche) and 0.2% triton. Cells were stained with anti-FlagM2 primary antibody (1:600; Sigma–Aldrich) followed by incubation with a donkey anti-rabbit Dy488 secondary antibody (1:200/Jackson Immuno, Suffolk, UK).

PCSK1 was co-localized with dsRed-ER (Clontech) labeled ER by laser-scanning microscopy with a plan-neofluar 100$\times$/1.3 oil objective (Carl Zeiss AG, Jena, Germany).

For western blot analysis, cells were lysed (RIPA buffer), samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected by chemiluminescence. All used antibodies are listed in Supplemental Table 5.

2.4. Statistical analyses

Association analyses were performed with Statistica 7.1 software (StatSoft, Tulsa, OK). Differences in the genotypes distribution between cases and controls were tested using an additive model adjusting for sex, age, and pubertal stage. Associations of polymorphisms with quantitative obesity-related metabolic traits were assessed by generalized linear regression models.

Homozygotes for the major allele (MM), heterozygotes (Mm) and homozygotes for the risk allele (mm) were coded to a continuous numeric variable for genotype (as 0, 1 and 2, respectively) in the additive model. A dominant model for the risk allele was defined as contrasting genotyping groups MM versus Mm + mm. Statistical power was calculated using the Quanto software (USC Biostats, Los Angeles, CA). For the rs6230, rs35753085, rs725522, rs6232, and rs6234, we had a statistical power in 12.1%, 13.6%, 10.7%, 56.4%, and 58%, respectively, to detect the observed effects at $\alpha = 0.5$ in our cohort (see ORs in Table 2). To reach the power of 80%, 13005, 10776, 16155, 1727, and 1662 obese individuals with a corresponding number of controls would be required for the studied variants.

Non-normally distributed parameters were logarithmically transformed prior to statistical analysis. Hardy–Weinberg equilibrium was tested using χ^2 test. All *p*-values are reported without Bonferroni corrections for multiple testing, which would require $p < 0.002$ (assuming 6 traits and 5 genetic variants included in the analyses). Therefore, all associations with *p*-values $<$0.05 but $>$0.002 were considered as nominal.

Detailed information about cell culture, transfection, plasmid construction, reporter gene, and apoptosis assay can be find in the Supplemental material.

3. RESULTS

3.1. Identification of variants in PCSK1

To identify novel variants in *PCSK1* with the utmost probability, we selected 52 obese children (BMI SDS 3.0 $\pm$ 0.7) with high proinsulin levels $>$90th centile, impaired glucose tolerance, or high proinsulin:insulin ratio from our *Leipzig Obesity Childhood cohort*. By sequencing all 14 exons (NM 000439) with intron/exon boundaries including 254 bp of the 5′ UTR, we identified eight known and two novel heterozygous variants. Two variants were located in the 5′ UTR, five in the coding region, and three in the intronic region (Table 1, Supplementary Figure 1). The novel variant p.S24C (c.70A $<$ T; in Figures called S24C) is located within exon1 and predicts a missense mutation p.S24C within the signal peptide of PC1/3. The novel c.1095 + 1G > A variant (in Figures called ΔEx8) affects the donor splice site of intron 8.

Table 1 — List of variants identified by sequencing of the *PCSK1* coding region and 5′ UTR in 52 obese children.

dbSNP	SNP position	SNP localization	Amino-acid change	Chromosome 5 position	Carrier/Allele frequency
rs6230	**c.-101T > C**	5′ UTR	–	95,768,847	13/–
rs35753085	**c.-96C > T**	5′ UTR	–	95,768,842	1/–
Novel p.S24C	**c.70A > T**	**Exon 1**	**p.S24C**	**95,768,676**	**1/–**
rs725522	**c.180 + 37C > T**	Intron 1	–	95,768,530	4/0.0188
rs6232	**c.661A > G**	Exon 6	p.N221D	95,751,785	12/0.0398
Novel c.1095 + 1G > A	**c.1095 + 1G > A**	**Intron 8**	**Truncation**	**95,746,477**	**1/–**
rs6233	**c.1650C > T**	Exon 12	p.N550N	95,733,112	24/0.3528
rs271920	**c.1722 + 54A > G**	Intron 12	–	95,732,992	24/0.000009
rs6234	**c.1993C > G**	Exon 14	p.Q665E	95,728,974	34/0.2688
rs6235	**c.2069C > G**	Exon 14	p.S690T	95,728,898	34/0.2653

The novel heterozygous variants are marked in bold. SNP positions refers to the translation initiation site (NM 000439). Chromosome 5 positions are based on GRCh37 (ENSG00000175426). Carrier means the number of patients with the respective mutation in the sequencing cohort (N = 52). Where available, allele frequencies based on ExAC consortium are given.

3.2. Clinical relevance of identified variants

To assess potential associations with obesity and metabolic traits, we genotyped these *PCSK1* variants in 989 lean and 684 obese children (Supplementary Table 1). All genotype distributions were consistent with Hardy–Weinberg equilibrium ($p > 0.05$). The two novel variants were not found in any other obese or lean child in our cohort confirming a survey in large variant databases (1000 genomes Project, NHLBI GO Exome Sequencing Project (ESP), Exome Aggregation Consortium (ExAC); all were accessed through the UCSC Genome Browser January 2016).

In the case control design, the minor alleles for rs6232 ($p = 0.022$) and rs6234 ($p = 0.039$) were significantly more prevalent in the obese group after adjustment for sex, age and pubertal stage (Table 2), conferring a higher risk of obesity for rs6232 (OR = 1.39, $p = 0.022$) and rs6234 (OR = 1.19, $p = 0.039$). Consistently, the minor alleles were nominally modestly associated with higher BMI-SDS (rs6232 $p = 0.033$, rs6234 $p = 0.035$; Figure 1A–B). In subsequent generalized linear regression analyses rs6232 and rs6234 contributed independently to BMI-SDS (Supplementary Table 2). Rs6230, rs35753085, and rs725522 were not associated with obesity.

Furthermore, we assessed the association of *PCSK1* variants with detailed traits related to glucose (including fasting blood glucose (BG), BG 120 min post oGTT, area under the curve BG), and insulin metabolism (including fasting plasma insulin, proinsulin, C-peptide, peak insulin, area under the curve insulin, Matsuda ISI, HOMA, QUICKI, Belfiore, and Stumvoll-index). Rs725522 was nominally significantly associated with Matsuda ISI ($p = 0.016$), AUC Ins-to-AUC BG ratio ($p = 0.015$), and peak insulin ($p = 0.004$) but not with proinsulin levels (Figure 1C–F). Furthermore, we found significant higher blood glucose 30min after glucose load and a significantly elevated first phase insulin response during oral glucose tolerance test (oGTT) in the group of the risk allele carriers (Figure 1G). In subsequent generalized linear regression analyses, rs725522 contributed independently from BMI-SDS and age to insulin secretion (Supplementary Table 3). The other variants were not associated with insulin traits (data not shown).

Table 2 — Association of *PCSK1* genetic variants with obesity.

SNP	Controls BMI SDS <1.28 (N = 989)	Cases BMI SDS ≥1.28 (N = 681)	*p* Value (adjusted for sex, age and PH)	OR (95% CI)
rs6230				0.93
TT	69.0%	70.3%	0.349	(0.77; 1.12)
TC	27.3%	27.0%		
CC	3.7%	2.7%		
MAF	17.4%	16.3%		
rs35753085				0.71
CC	98.2%	98.7%	0.289	(0.30; 1.67)
CT	1.8%	1.3%		
TT	0.0%	0.0%		
MAF	0.9%	0.6%		
rs725522				1.16
AA	95.1%	94.0%	0.530	(0.76; 1.79)
AG	4.7%	6.0%		
GG	0.2%	0.0%		
MAF	2.6%	3.0%		
rs6232				1.39
AA	90.9%	87.4%	**0.022**	(1.00; 1.93)
AG	8.8%	12.2%		
GG	0.3%	0.4%		
MAF	4.7%	6.5%		
rs6234				1.19
CC	57.9%	52.5%	**0.039**	(1.01; 1.42)
CG	35.7%	39.6%		
GG	6.4%	7.9%		
MAF	24.3%	27.8%		

p Value and odds ratio (OR) with 95% confidence intervals (CI) were calculated under logistic regression analyses in additive mode of inheritance, adjusted for sex, age, and PH. MAF – minor allele frequency; PH – pubertal stages on basis of pubic hair development.

3.3. Functional characterization of PCSK1 variants

We first focused on the functional characterization of the two novel variants performing expression-, secretion-, and cellular localization studies and an enzyme activity assay. We also included the rs725522 variant since no functional investigations have been performed for this variant. However, we did not re-investigate rs6232 and rs6234 since similar investigations have already been published [1]. Second, we performed reporter gene assays to investigate all variants (rs35753085, rs6230, rs725522, p.S24C) that may affect *PCSK1* promotor activity. Third, we investigated if variants can induce endoplasmic reticulum (ER) stress. For this, we preselected variants that were already described as variants with disturbed function (p.N221D [1], p.S307L [11], and p.R80Q [16] as well as our novel variants. Rs6234 and rs6235 were not investigated since former studies [1,16] did not suggest an altered function of these variants.

c.1095 + 1G > A variant. The occurrence of this variant in only one patient with morbid obesity, and the molecular localization with base-pair exchange of the first nucleotide of the intron 8 donor splice site suggested a potential functional relevance affecting splicing (Figure 2A).

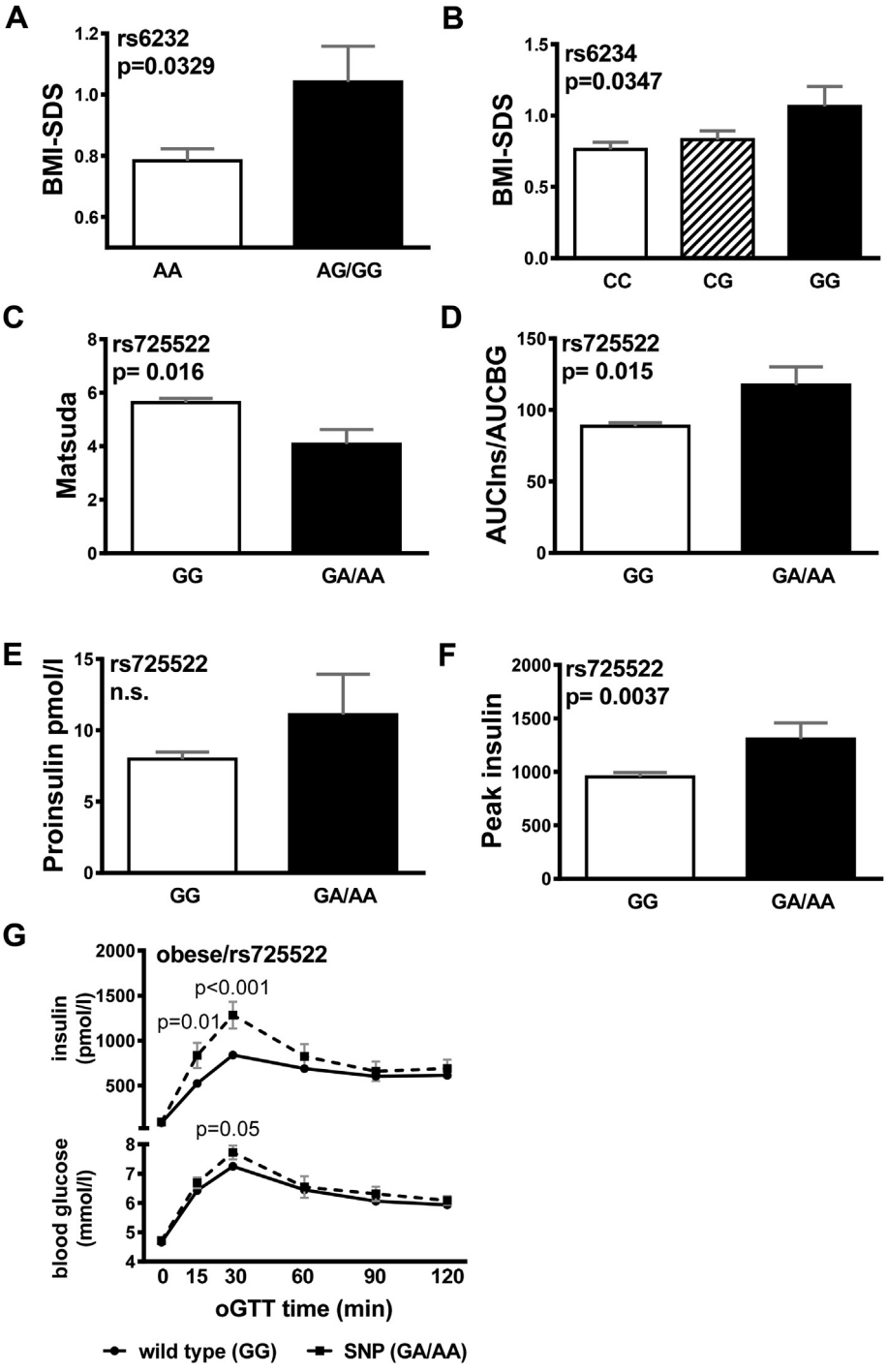

Figure 1: Association with obesity and metabolic factors. Carriers for rs6232 (A) and rs6234 (B) had higher BMI-SDS compared to wt. Carriers for rs725522 had higher (C) AUCIns/AUCBG and lower (D) Matsuda Insulin sensitivity indices (ISI) as compared to wt carriers. There were no significant differences in (E) proinsulin and (F) peak insulin levels. (G) Course of blood glucose and (H) insulin levels during oGTT in carriers of rs725522 wt (N = 473) and minor allele (N = 26). Data are given as mean ± SEM. AUC, area under the curve; wt, wild-type.

To verify the alternative splice effect, we constructed mini-genes (gwt, c.1095 + 1G > A (gΔEx8)) containing the complete introns 7 and 8 of *PCSK1*. These mini-gene constructs were compared with cDNA plasmids expressing wild-type (WT) cDNA (wtcDNA) and a c.1095 + 1G > A (ΔEx8) cDNA plasmid (c.1095 + 1G > A cDNA (ΔEx8)) (Figure 2A). The c.1095 + 1G > A (ΔEx8) coding plasmids generated mRNA with a molecular weight reduced by 213 bp compared to the WT constructs (Figure 2B; 2.5 kb versus 2.3 kb) predicted to result in a 71-amino-acids in-frame deletion (exon 8-skipping) within the catalytic domain of PC1/3. Cells transfected with the gwt-construct expressed both *PCSK1* mRNA species (2.5 + 2.3 kb) and an additional third fragment of about 2.4 kb. We confirmed the identity of the WT and c.1095 + 1G > A (ΔEx8) fragments, and identified the third amplicon as a hybrid species generated during PCR reaction.

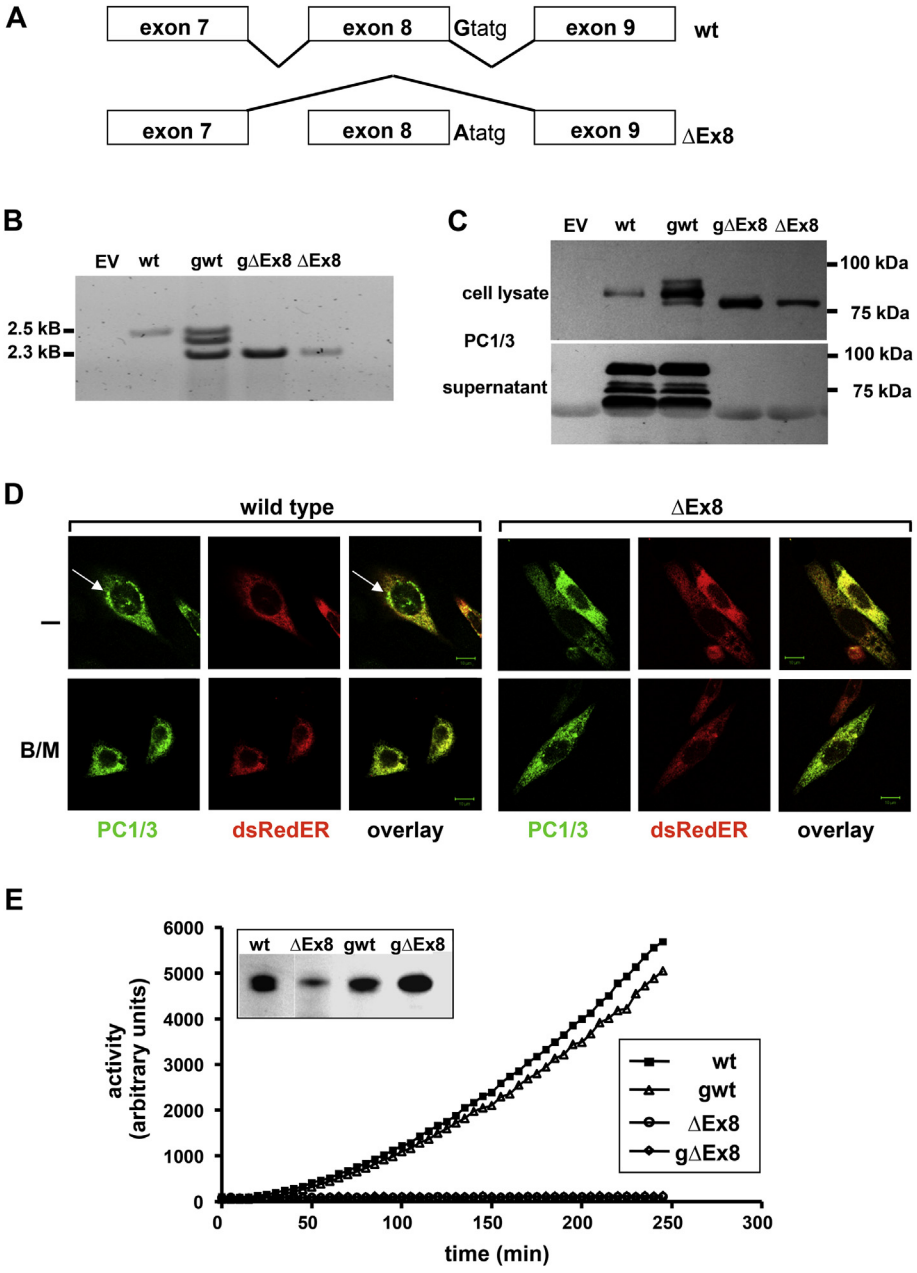

Figure 2: Functional characterization of the c.1095 + 1G > A mutation. The mutation c.1095 + 1G > A was named as ΔEx8. (A) Schematic representation of exon 8 skipping. Hek293 cells were transiently transfected with expression vectors encoding the Flag-tagged PC1/3 variants (wtcDNA; gwt; c.1095 + 1G > A (ΔEx8) cDNA; gc.1095 + 1G > A (ΔEx8)). (B) PCR products exons 1 to exon 14 showing different sized amplicons for gwt and c.1095 + 1G > A (ΔEx8) variants. EV, empty vector. (C) Immunoblot with antibodies to the Flag epitope showing protein in the cell lysates but not in the supernatants of c.1095 + 1G > A (ΔEx8) variants. (D) Confocal microscopy of CHO cells transfected with Flag-tagged PC1/3 variants wtcDNA (wild-type) and c.1095 + 1G > A cDNA (ΔEx8) as indicated and with dsRedER plasmids shows assembly of PC1/3 in the ER in wild-type and perinuclear localization (which was inhibited by Brefeldin/Monensin (B/M)) treatment. The c.1095 + 1G > A (ΔEx8) variant was detectable in the ER without assembly in perinuclear vesicles. (E) Enzymatic activity was completely abolished in Hek293 cells expressing the ΔEx8 variants compared to wildtype protein.

In concordance with mRNA expression, PC1/3 protein immunoblotting of cell lysates and supernatants revealed a 7 kDa smaller protein expressed from mutant cDNA and mini-gene constructs (Figure 2C). In contrast to the WT proteins, the mutant proteins were not secreted into the supernatant (Figure 2C). This cellular retention may be due to impaired cellular trafficking, which we subsequently investigated by immunofluorescence in transiently transfected CHO cells (Figure 2D). WT PC1/3 was readily detectable in the ER and in perinuclear vesicles; detection was lost after incubation with Golgi inhibitors BrefeldinA/Monensin (B/M) [35] as positive control, leading to accumulation of PC1/3 within the ER. The mutant protein was exclusively detected within the ER but not in perinuclear vesicles. Similar results were obtained in human embryonic kidney (Hek293) cells (data not shown). Finally, exon 8 deletion (c.1095 + 1G > A (ΔEx8)) led to a complete loss of enzymatic activity of PC1/3 compared to WT protein (Figure 2E).

Thus, the c.1095 + 1G > A (ΔEx8) variant leads to a truncated protein with a complete loss of function and which is retained within the ER.

p.S24C variant. We similarly characterized potential functional consequences of the p.S24C variant on PC1/3 maturation, secretion, localization, and enzymatic function. The detection in whole cell lysates, release into supernatant, and the subcellular localization in the ER and perinuclear vesicles was identical to the WT protein (Figure 3A–C). The enzymatic activity of the p.S24C variant was not impaired in enzyme assays, providing evidence that enzymatic function is pertained when transfected into neuro2A (N2A) neuroblastoma (Figure 3D) or Hek293 cells (data not shown).

In *in silico* analyses, p.S24C was found tolerated/benign (MutationAssessor — Low impact, Provean — neutral, score −1.52, SIFT — tolerated, score 0.056, SNPs&GO: neutral polymorphism CADD: 13.37, PredictSNP — neutral with 89% accuracy) and not particularly conserved (phyloP100way: −0.115669, and GERP 0.168). According to the Uniprot, the first 27 amino acids are forming a signal peptide. Hence, p.S24C does not alter PC1/3 protein maturation and function.

rs725522 variant. To assess whether the nucleotide exchange in intron 1 may affect mRNA splicing, we again constructed mini-genes (gintron1wt and gintron1-37), containing the complete cDNA plus the first 218bp and the last 502bp of intron 1. PCR analysis revealed a product of predicted size for both the WT and variant (Supplementary Figure 4A) and hence no effect on *PCSK1* mRNA splicing or PC1/3 maturation and secretion.

Promoter variants. We designed reporter constructs including −779bp to +109bp relative to the transcription start site (Supplementary Figure 5) to assess whether variants in 5′UTR or intron

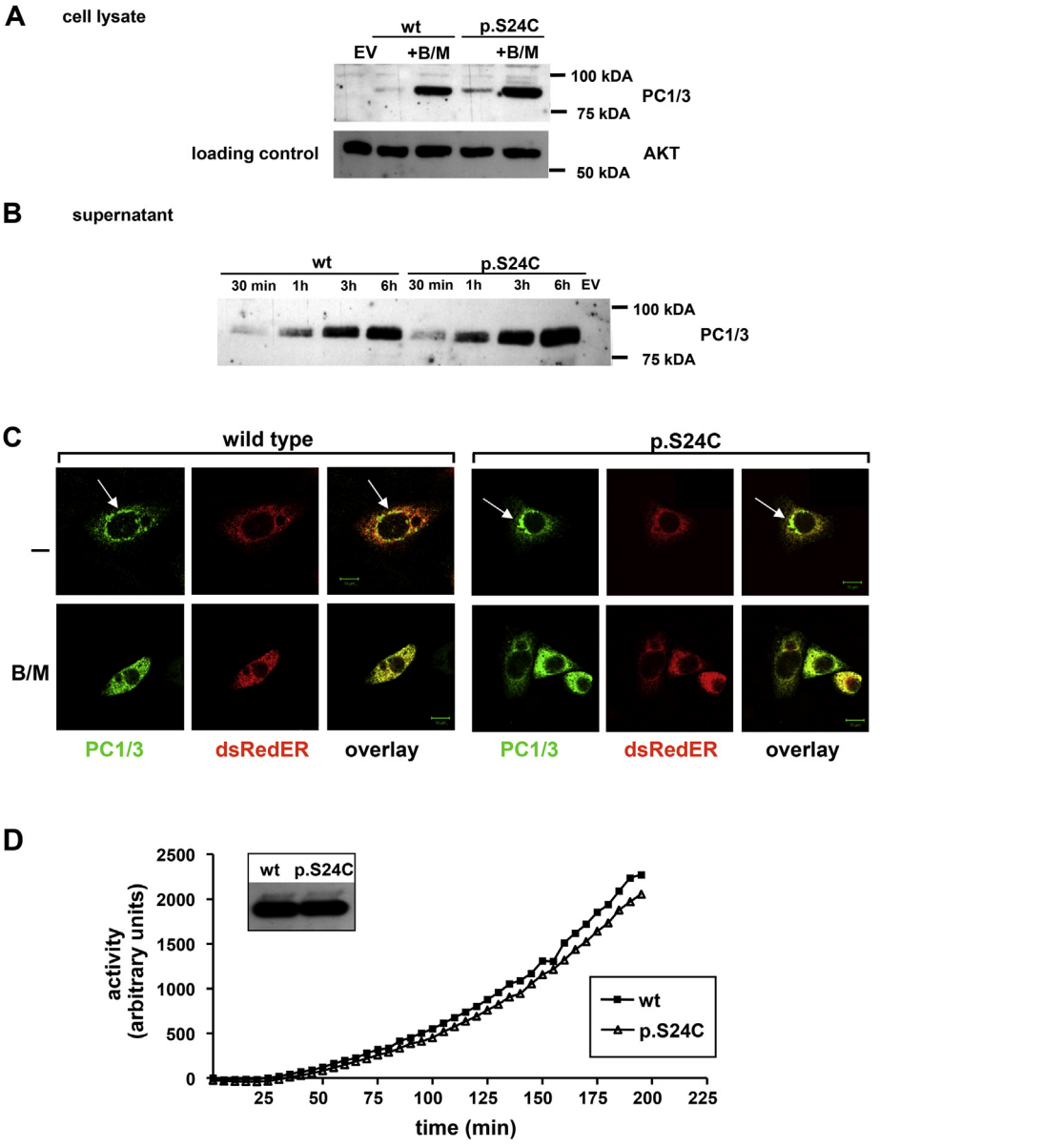

Figure 3: Functional characterization of the p.S24C mutation. Immunoblot (A + B) with antibodies to the Flag epitope showing protein in the cell lysates and in the supernatants of wild-type (WT) and mutated variant. After Brefeldin/Monensin (B/M) treatment, proteins were retained in the cell. EV, empty vector. (C) Confocal microscopy of CHO cells transfected with Flag-tagged PC1/3 variants wtcDNA (wild-type) and p.S24CcDNA (p.S24C) as indicated and with dsRedER plasmids shows equally subcellular assembly of PC1/3 wt and variants in the ER and perinuclear localization (which was inhibited by Brefeldin/Monensin (B/M) treatment). (D) Enzymatic activity of PC1/3 was retained in p.S24C variant similar to the wild-type.

1 affect promoter activity in Hek293 (non-neuroendocrine origin) and mouse insulinoma cells (βTC3; neuroendocrine origin). We included the p.S24C variant, for which we did not expect regulatory effects as an unregulated control. Compared to the WT construct we did not observe regulatory effects for any of the studied variants (rs35753085, rs6230, and p.S24C) (Supplementary Figure 5B).

As the presence of introns could elevate mRNA levels [36] by increasing the synthesis of mRNA, we analyzed the effects of rs725522 located in intron 1 on *PCSK1* expression.

In a second promoter construct containing the exon 1 + 197bp of intron 1 (Supplementary Figure 5A), the presence of WT intronic sequence (fullex1, Supplementary Figure 5A) doubled luciferase activity in Hek293 and βTC3 cells compared to the WT construct (Supplementary Figure 5C). The insertion of the SNP rs725522, however, did not affect luciferase activity.

Overall, the analyzed variants did not affect promoter activity of *PCSK1* in our cell systems.

Effects of *PCSK1* variants on ER-stress. The retention of misfolded proteins in the ER can lead to ER stress and unfolded protein response (UPR) activation [37,38] and eventually apoptosis [39,40]. We, therefore, evaluated whether non-synonymous variants c.1095 + 1G > A and p.S24C and common variants p.R80Q (rs1799904), p.S307L and p.N221D (rs6232) in *PCSK1* may lead to ER stress and the three branches of UPR activation (Inositol-Requiring Enzyme 1 (IRE1), Activating Transcription Factor 6 (ATF6), and PRKR-Like Endoplasmic Reticulum Kinase (PERK) [37].

The mutated p.S24C, p.R80Q, and p.N221D were readily detectable in cell lysates and supernatants, whereas release of c.1095 + 1G > A and p.S307L was blocked (Figure 4A).

Brefeldin was employed as positive control for inducing ER stress [41]. To assess the activation of the IRE1 pathway, we analyzed the processing of X-Box Binding Protein (XBP) 1 mRNA in an alternatively spliced variant [42] as described [43]. Expression of c.1095 + 1G > A and p.S307L resulted in an alternative XBP1 splicing pattern comparable to that of the Brefeldin control (Figure 4B), whereas the other variants did not affect XBP1 mRNA splicing. To evaluate activation of the ATF6 pathway we analyzed BIP (HSPA5) and CHOP (DDIT3) mRNA expression [42]. We did not detect an activation of the ATF6 pathway in any investigated variant. Thirdly, we examined the phosphorylation of the PERK target eIF2α but, again, did not find significant changes for the different variants (Figure 4C).

Finally, since persistently activated UPR can cause apoptosis, we analyzed the apoptosis and did not find an increased apoptosis rate after transfection with any of the variants, whereas camptothecin/etoposide as positive controls exerted the expected increase.

In summary, c.1095 + 1G > A and p.S307L cause an activation of the IRE1 pathway in Hek293 cells, indicating ER stress, whereas ATF6 and PERK pathway as well as apoptosis were not affected by *PCSK1* variants.

3.4. Clinical phenotype of patients carrying novel variants

The patient carrying the c.1095 + 1G > A variant was a 15.4 year old girl with severe obesity (BMI-SDS 2.95 to 3.25 in sequential visits), acanthosis nigricans, and signs of hirsutism, hypertension, and polycystic ovary (PCO) syndrome. Metabolically, she had impaired glucose tolerance with hyperinsulinemia upon glucose provocation (Supplementary Figure 2A,C) and elevated proinsulin levels >4× upper reference limit (between 43.2 and 85.5 pmol/L). Both parents of the patient were obese (BMIs mother: 35.5 kg/m^2, father: 34.7 kg/m^2) but were, unfortunately, not available for further analysis.

The girl carrying the p.S24C variant had a similar phenotype with complete metabolic syndrome including severe obesity (BMI-SDS 3.45 to 3.64), acanthosis, hypertension, and PCO syndrome. She also had impaired fasting glucose and impaired glucose tolerance with delayed although elevated insulin response in oral glucose tolerance test (oGTT; Supplementary Figure 2B,D) and elevated basal proinsulin levels (42.8—71.2 pmol/L).

To assess whether those phenotypes differ significantly from "common obesity," we matched controls, applying the criteria same sex (female), completed pubertal development, and similar age (between 14.4 and 18 years), which was fulfilled by 73 subjects from our cohort. Since the patient with p.S24C was also heterozygous for rs725522 and the patient with c.1095 + 1G > A did not carry any other variants, we also tried to match the genotype of the controls which left us with 4 controls for the p.S24C patient and 28 for the c.1095 + 1G > A patient. The phenotype of the carriers of novel variants appeared to be more severe, particularly with respect to insulin secretion and insulin resistance, compared to matched obese controls (Supplementary Figure 3).

4. DISCUSSION

In this study, we characterized two novel and 8 known variants that had been identified by sequencing of the *PCSK1* gene in a subset of obese patients, which we regarded as prone for *PCSK1* mutations. In functional studies, we confirmed that the c.1095 + 1G > A (ΔEx8) variant caused a complete loss of enzymatic activity of the protein and cellular retention leading to ER stress. The other novel variant p.S24C did not have an effect on protein size, secretion, or enzymatic activity. Neither this nor the known variants rs6230, rs35753085, and rs725522 located in the 5end of the gene affected *PCSK1* promoter activity. Moreover, we performed detailed clinical studies for association with glucose or insulin metabolism, in which we confirmed association of rs6232 and rs6234 with obesity and describe a nominal association for rs725522 with insulin metabolism.

4.1. Novel rare *PCSK1* variants

There are several reports of patients with monogenic obesity due to homozygous or compound heterozygous *PCSK1* mutations [11,17—21,34] and on the heterozygous mutation carriers [22,23]. Some of the previously reported heterozygous *PCSK1* mutations have not co-segregated with the obesity phenotype in all of the patients [22,23]. Functionally, Our newly identified c.1095 + 1G > A variant leads to abnormal splicing and subsequent skipping of exon 8 in the *PCSK1* mRNA. The resulting truncated protein lacks 71 amino acids in the catalytic domain and, not surprisingly, has a complete loss of enzymatic function *in vitro*. In addition, it shows abnormal cellular localization. This retention of the mutated protein in the ER may be caused by binding to chaperones [44], as previously reported for other PC1/3 mutants [34], and lead to the induction of IRE1, an early ER stress marker. The activation of ER stress by *PCSK1* variants, recently also shown by others [14], may present an additional impairment of the cells expressing PC1/3. In conclusion, our *in vitro* data provide strong evidence for a complete loss of function of the c.1095 + 1G > A mutation. Previously described rare heterozygous *PCSK1* mutations were linked to obesity [22] or obesity and impaired glucose tolerance [23] but were not reported to present any other phenotypic features described in homozygous mutation carriers [11]. Nevertheless, our proband has some additional clinical features including PCO syndrome, acanthosis nigricans, and hypertension. Moreover, her proinsulin levels were 4-fold higher than in similarly

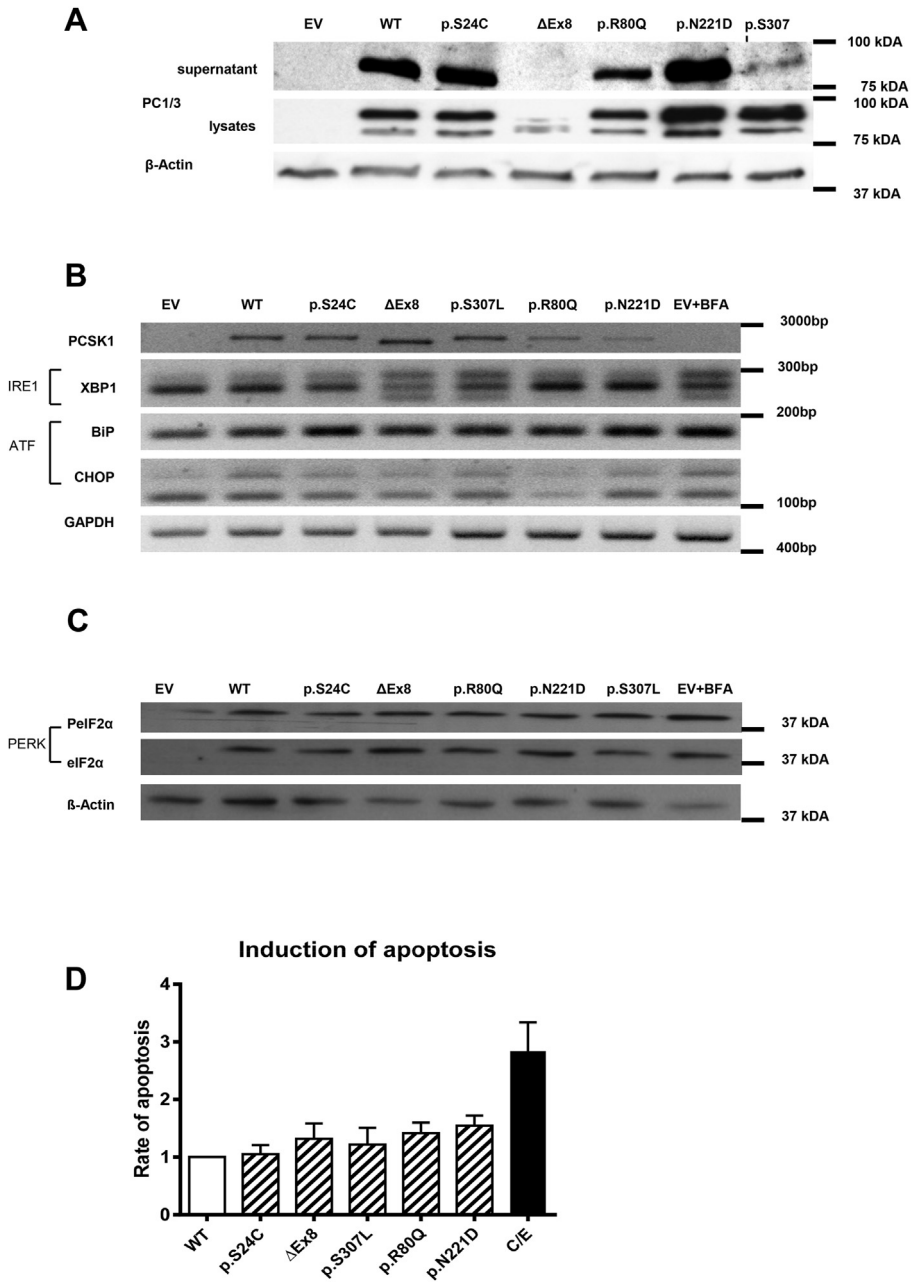

Figure 4: Endoplasmic reticulum stress. (A) Influence of *PCSK1* variants on expression/maturation and release of PC1/3 wild-type (WT) and different variants as indicated by immunoblot analysis (EV = empty vector). (B) UPR activation was assessed via PCR for the IRE1 (XBP1) and ATF (BiP and CHOP) pathway (BFA = BrefeldinA [1 μg/ml]). (C) For analysis of PERK pathway activation, phosphorylated (PeIF2α) and unphosphorylated eIF2α was analyzed by western blotting. (D) Apoptosis was assessed by FACS identification of Annexin-V/Propidium Iodide stained cells. Results were normalized to the apoptosis rate of the wild-type transfected cells (C/E = camptothecin [2 μM]/etoposide [85 μM]).

obese controls; this is potentially caused by some degree of dysfunction of PC1/3 [34], which plays a major role in processing proinsulin in PC1/3-null mice [45]. The overall phenotype of these PC1/3-null mice with growth retardation but not obesity, however was not comparable with our patient [45]. Similarly, another mouse model with complete deletion of *Pcsk1* was embryonically lethal and the heterozygous mice also had retarded growth [46]. Interestingly, however, a mouse model with a single nucleotide variation (N222D) showed a similar phenotype [47] as the initially reported first patient with hyperphagic severe early onset obesity with PC1/2 deficiency including hyperproinsulinemia [17].

On the other hand, it has been shown that human carriers with heterozygous mutations for PC1/3 do not exhibit significantly impaired proinsulin processing [34]. Moreover, because of high total insulin levels and apparent signs of insulin resistance, major defects in the insulin secretion seem rather unlikely in our patient. The phenotype of the c.1095 + 1G > A carrier including obesity, hypertension, impaired glucose tolerance, and PCO syndrome was found in less than <5% of obese females aged 16–18 years in our Leipzig childhood obesity cohort. Nevertheless, the proband carrying p.S24C variant presented a similar the phenotype, although this variant had no effect on the protein function in functional and in *in silico* analyses. The first 27 amino acids

of the protein are forming a signal peptide, and hence the mutation may affect cell trafficking. As we showed that the intracellular localization of the p.S24C protein is normal even in homozygous experimentally mutated protein (release into supernatants, ER and Golgi localization, and, finally, enzymatic function), the mutation does apparently not affect protein function, particularly in the heterozygous state.

One major limitation of our study is that DNA or a more detailed metabolic phenotype of the parents was not available to verify the co-segregation of the novel variant with obesity in the family. As both parents were obese, the prevalence and the phenotype of the obesity in the affected family may be influenced by an obesogenic environment, as was the case in the p.R80* family [23]. We have not found any other patient with the c.1095 + 1G > A variant in our sample nor is there any case report in the literature that would corroborate our findings. Hence, we cannot speculate whether the phenotype of our patient is affected by the heterozygous variant, which would have deleterious effects, however, in the homozygous state.

4.2. Known common *PCSK1* variants and their contribution to childhood onset polygenic obesity

PCSK1 variants contribute also to the polygenic type of obesity [2,3]. Children present earlier stages of the disease and are less biased by co-morbidities. Hence, studies in children may allow a better discrimination between primary and secondary associations. In fact, recent reports indicated that the common variants in PCSK1 have a stronger effect on the obesity phenotype in children than in adults [2].

We confirmed the reported association of the common variants rs6232 and rs6234 with obesity and BMI-SDS in our cohort [1] but not the associations of these variants with proinsulin conversion and glucose homeostasis [48]. Our findings that both variants are only mild predictors for BMI-SDS or weight suggest that they are not major contributors to common obesity, which is in line with previous findings [49,50]. Interestingly, we found that the *PCSK1* rs725522 minor allele was significantly associated with parameters of insulin resistance (Matsuda ISI, peak insulin, AUCIns/AUCBG). These results suggest declined insulin sensitivity in the carriers. Albeit we did not find a significant association with proinsulin, the insulin levels were significantly higher after 15 and 30min of oGTT in the obese carrier group compared to non-carriers. This may reflect a difference in kinetics of insulin secretion in carriers and non-carriers at least under the conditions of a glucose load. Associations of obesity risk variants in *PCSK1* with insulin sensitivity have so far only been described for the *PCSK1* variants rs6232 [48] and rs3811951 [51].

Additionally, we investigated the potential functional relevance of the promoter variants. rs6230 and rs35753085 did not have significant effects on *PCSK1* promoter activity in our cell systems. This is in line with results that cAMP-responsive elements [52] and thyroid hormone response element binding sites [53] were not affected by the investigated SNPs. The insertion of an intronic sequence *per se* into the reporter constructs increased the promoter activity. This enhancing effect of intronic sequences on transcription efficiencies is well documented in plants [36] and mammals [54]. Enhancement is perhaps mediated by the predicted binding sites for SP1, AP2α, or TCF-4E. The putative AP2α binding site is lost after insertion of rs725522, and recent publications indicated suppressive effects by AP2α on gene expression [55]. This may explain the slightly but not significantly increased promoter activity by rs725522 in Hek293 cells. We did not see evidence for effects of rs725522 on *PCSK1* mRNA splicing and protein maturation.

There are several limitations of our study on common variants. First, a subset of variants was genotyped using RFLP experiments. This method may be prone to be subjective in the evaluation of PCR products and thereby prone to methodological bias. However, we objected to using pre-designed TaqMan assays due to poor discrimination quality. In addition, we confirmed suspicious samples by Sanger sequencing. Another limitation is that our sample size is small (<60% statistical power) regarding all of the analyzed common variants. We also report nominal *p*-values and Bonferroni correction would likely weaken some associations of common variants (including in rs725522). Nevertheless, the associations between rs6232 and rs6234 and obesity confirm recent findings.

Overall, our and previous publications coherently report an association of variants in *PCSK1* with early onset obesity along a continuum on the severity of phenotype. The continuum ranges from extreme hyperphagic obesity with additional neuroendocrine defects in compound heterozygous individuals [17,20] to dominant monogenic form of obesity in patients with heterozygous loss-of-function mutations [23] or oligogenic forms of obesity lacking severe additional phenotypes in patients with partial *PCSK1* deficiency [22] to common variants reliably associated with obesity and elevated proinsulin [2], although not to the extent as described in monogenic forms of *PCSK1* deficiency.

Our findings of the novel heterozygous loss of function *PCSK1* variant c.1095 + 1G > A in a proband with severe early onset obesity, PCO syndrome, hypertension, and increased proinsulin levels, and associations of *PCSK1* SNPs rs6232 and rs6234 with BMI-SDS and rs725522 with insulin resistance in a large pediatric cohort, support the role of *PCSK1* variants contributing to obesity in children.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.12.002.

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