

Jan Windholz, Peter Kovacs, Marina Schlicke, Christin Franke, Anubha Mahajan, Andrew P. Morris, Johannes R. Lemke, Jürgen Klammt, Wieland Kiess, Torsten Schöneberg, Roland Pfäffle and Antje Körner*

Copy number variations in “classical” obesity candidate genes are not frequently associated with severe early-onset obesity in children

DOI 10.1515/jpem-2016-0435

Received November 22, 2016; accepted March 6, 2017

Abstract

Background: Obesity is genetically heterogeneous and highly heritable, although polymorphisms explain the phenotype in only a small proportion of obese children. We investigated the presence of copy number variations (CNVs) in “classical” genes known to be associated with (monogenic) early-onset obesity in children.

Methods: In 194 obese Caucasian children selected for early-onset and severe obesity from our obesity cohort we screened for deletions and/or duplications by multiplex ligation-dependent probe amplification reaction (MLPA). As we found one MLPA probe to interfere with a polymorphism in *SIMI* we investigated its association with obesity and other phenotypic traits in our extended cohort of 2305 children.

Results: In the selected subset of most severely obese children, we did not find CNV with MLPA in *POMC*, *LEP*, *LEPR*, *MC4R*, *MC3R* or *MC2R* genes. However, one *SIMI* probe located at exon 9 gave signals suggestive for *SIMI* insufficiency in 52 patients. Polymerase chain reaction (PCR) analysis identified this as a false positive result due to interference with single nucleotide polymorphism (SNP) rs3734354/rs3734355. We, therefore, investigated for associations of this polymorphism with obesity and metabolic traits in our extended cohort. We found rs3734354/rs3734355 to be associated with body mass index-standard deviation score (BMI-SDS) ($p=0.003$), but not with parameters of insulin metabolism, blood pressure or food intake.

Conclusions: In our modest sample of severely obese children, we were unable to find CNVs in well-established monogenic obesity genes. Nevertheless, we found an association of rs3734354 in *SIMI* with obesity of early-onset type in children, although not with obesity-related traits.

Keywords: children; copy number variations; genetic variants; obesity; *SIMI*.

*Corresponding author: Dr. Antje Körner, Pediatric Research Center, University Hospital for Children and Adolescents, University of Leipzig, Liebigstraße 20a, 04103 Leipzig, Germany, E-mail: Antje.Koerner@medizin.uni-leipzig.de; Leipzig University Medical Center, IFB AdiposityDiseases, University of Leipzig, Leipzig, Germany; and Institute of Biochemistry, Molecular Biochemistry, Medical Faculty, University of Leipzig, Leipzig, Germany

Jan Windholz, Marina Schlicke, Jürgen Klammt, Wieland Kiess and Roland Pfäffle: Pediatric Research Center, University Hospital for Children and Adolescents, Medical Faculty, University of Leipzig, Leipzig, Germany

Peter Kovacs: Leipzig University Medical Center, IFB AdiposityDiseases, University of Leipzig, Leipzig, Germany

Christin Franke and Torsten Schöneberg: Institute of Biochemistry, Molecular Biochemistry, Medical Faculty, University of Leipzig, Leipzig, Germany

Anubha Mahajan: Wellcome Trust Centre for Human Genetics, Nuffield Department of Medicine, University of Oxford, Oxford, UK

Andrew P. Morris: Wellcome Trust Centre for Human Genetics, Nuffield Department of Medicine, University of Oxford, Oxford, UK; and Department of Biostatistics, University of Liverpool, Liverpool, UK

Johannes R. Lemke: Institute of Human Genetics, Medical Faculty, University of Leipzig, Leipzig, Germany

Introduction

Obesity is etiologically heterogeneous. The assumed heritability for obesity-related phenotypes is estimated to be above 60% [1], although it varies to a large extent [2]. The effects of single nucleotide polymorphisms (SNP) have been investigated extensively under the assumption that common diseases are attributable to common variants. However, common loci identified by genome-wide association studies (GWAS) were able to explain around 3% of body mass index (BMI) variance only [3]. This gap moved the focus towards copy number variations (CNVs) that are estimated to account for about 18% of heritable variance in gene expression [4]. Recent findings started to elucidate the role of CNVs in non syndromic diseases and metabolic

disorders, as was shown for an association of a common deletion near the neuronal growth regulator 1 (*NEGR1*) with BMI [5], large CNVs (>1 Mb) were found to be overrepresented in obese cases vs. control subjects [6], and CNVs to be associated with BMI and gene expression of *AMY1* as well as serum amylase enzyme levels [7]. Also, associations with syndromic forms of obesity were recently reported for CNVs on chromosome 16 in adults [8], and are well described for specific entities such as Charcot-Marie-Tooth [9], Angelman [10] and Prader-Willi syndrome [11], attention deficit hyperactivity disorder [12] or autism [13].

We hypothesized that CNVs around obesity candidate genes might explain the gap between expected and measured heritability for BMI and contribute to BMI variation, potentially causing severe early-onset obesity. In this regard, particularly genes with rare variants causing monogenetic early-onset obesity, such as melanocortin receptors (*MC4R*, *MC3R*, *MC2R*) [14, 15], leptin receptor (*LEPR*) [16], single minded homolog 1 (*SIM1*) [17], proopiomelanocortin (*POMC*) [18] would be interesting. As only children are less affected by comorbidities, medication and environmental factors, they are an attractive population to identify contributing genetic determinants in such a complex polygenic disease like obesity. We assumed that children with a high BMI-standard deviation score (SDS) have a higher risk for gene dosage variation and we consequently selected patients with the utmost BMI-SDS of our cohort. In this subset, we screened for CNVs in *POMC*, *LEP*, *LEPR*, *MC4R*, *MC3R*, *MC2R* and *SIM1* gene regions using multiplex ligation-dependent probe amplification (MLPA) technique, which has been proven to be efficient and robust [19] and was previously successfully applied for screening of variations in children with idiopathic mental retardation [20], autism [21] and obsessive-compulsive disorder [22].

Subjects and methods

Cohort design

We selected 194 Caucasian children and adolescents (86 male, 108 female) with a BMI of ≥ 2 SDS (mean BMI was 2.89 ± 0.41 SDS) recruited from our obesity clinic for the initial CNV screen. For a further description see Table 1.

To validate our findings on the *SIM1* polymorphism rs3734354, we extended our cohort with exome chip data of a further 1074 obese children as well as 1231 lean controls, altogether 2305 children (Table 2).

A comprehensive metabolic work-up including lipid profiles, an oral glucose tolerance test and blood pressure measurements were collected. All children were free of severe diseases and of medications. We excluded participants that had one of the following characteristics: chromosome disorders (Klinefelter's syndrome, trisomia),

Table 1: Demographic characteristics of patients analyzed for copy number variations.

Variable	Mean \pm SD [min–max]
n (Girls/boys)	194 (108/86)
Age, years	11.0 \pm 3.8 [3–18]
BMI, kg/m ²	31.0 \pm 5.3 [21–47]
BMI-SDS	2.89 \pm 0.41 [2.2–4.8]
WHR	0.89 \pm 0.1 [0.73–1.49]
PH	2.69 \pm 1.8 [1–6]

Range is given in squared brackets. BMI, body mass index; WHR, waist-to-hip ratio; PH, Tanner pubertal state.

Table 2: Demographic characteristics of patients analyzed for *SIM1* variant rs3734354.

Variable	Mean \pm SD [min–max]
n (Girls/boys)	2305 (1182/1123)
Age, years	11.3 \pm 3.36 [0.2–17.99]
BMI, kg/m ²	22.4 \pm 6.55 [11.4–56]
BMI-SDS	1 \pm 1.48 [–4.4 to 4.9]
Height SDS	0.34 \pm 1.2 [–6 to 4.8]
WHR	0.85 \pm 0.08 [0.58–1.49]
PH	2.53 \pm 1.7 [1–6]

Range is given in squared brackets. BMI, body mass index; WHR, waist-to-hip ratio; PH, Tanner pubertal state.

other genetic disorders (Prader-Willi syndrome, Bardet-Biedl syndrome), administration of valproic acid or metformin. Height was measured to the nearest of 0.1 cm and weight to the nearest of 0.1 kg using a digital balance. BMI data were standardized to age and sex of the children applying German reference data [23], and is given as BMI-SDS or z-score. To classify overweight (BMI between 1.23 and 1.88 SDS) and obesity (BMI ≥ 1.88 SDS) we applied consensus guidelines of the German Working Group for Pediatric Obesity [24]. Blood samples were obtained in the fasted state at 08:00 am and were immediately centrifuged, aliquoted and stored at -80 °C. Blood lipids, glucose and insulin were measured by a certified laboratory applying standard clinical biochemistry methods.

Written informed consent was obtained from all guardians and children older than 12 years. Both studies were approved by the Ethical Committee of the University of Leipzig.

MLPA

We used a kit nr. P220 by MCR-Holland (Amsterdam, The Netherlands) covering our target genes, probes are listed in Supplemental Table 1. A total of 100 ng of genomic DNA was used for the reaction which was carried out with MLPA-SALSA kit by MCR-Holland according to the manufacturer's instructions. To measure the probe amplitude we injected polymerase chain reaction (PCR) products into capillary electrophoresis on an ABI PRISM 7500 sequence detector (Applied Biosystems Inc., Foster City, CA, USA) and extracted the

data using GeneMapper (Applied Biosystems). For data normalization, based on a healthy, non obese control, the relative peak height (RPH) method recommended by MRC-Holland was used [25, 26]. Peak heights below 0.75 were considered as deletions and above 1.30 as duplications. Positive samples were repeated twice. Repeatedly found deletions were confirmed twice with MLPA and with PCR followed by sequencing to verify the alteration and to make sure that the MLPA probe properly bound to its binding site.

Genotyping *SIM1*

To verify the expected deletion in *SIM1* exon 9 we used DNA, for which MLPA indicated a homozygous loss of both alleles and performed PCR with four overlapping primers flanking the exon 9 MLPA probe binding site (see Supplemental Figure 1). Genomic DNA was extracted from peripheral leukocytes using a Qiagen DNA extraction kit (Qiagen, Hilden, Germany). The region in *SIM1* was amplified by PCR under the following conditions: denaturation at 96 °C for 3 min, then 25 cycles of denaturation at 96 °C for 30 s, annealing at 56 °C for 15 s and elongation at 60 °C for 4 min, in the end followed by elongation at 72 °C for 10 min. We inspected the PCR products for the presence of single bands of the expected size on a 1% agarose gel and extracted them using the QIAquick Gel Extraction Kit (Qiagen).

Sequencing was performed on overlapping fragments (primer see Supplemental Table 2). The detected *SIM1* variant was verified by reamplification and resequencing. Sequencing of the purified PCR products was performed using the ABI Prism sequence detector in combination with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

The rs3734354 genotype data for the extended cohort were derived from the HumanExome BeadChip v1.0 (Illumina, Inc., San Diego, CA, USA) using standard protocols suggested by the manufacturer.

Statistical analyses

Statistical analyses were performed using the SPSS software package (version 17.0) (SPSS, Inc., Chicago, IL, USA). We used logistic regression to compare allelic frequencies between obese and lean healthy controls. To test for associations of genetic variants with binary obesity/overweight and metabolic or anthropometric characteristics we applied generalized linear regression models under the additive mode of inheritance. All qualitative and quantitative analyses were adjusted for age, sex and pubertal stage. Pubertal stage was clinically assessed by Tanner stage ranging from PH1 (=prepubertal) to PH5 (=adolescent, puberty completed). Hence, we regarded the PH as an ordinal variable. A p-value <0.05 was considered to provide nominal evidence for association.

Results

Screen for CNVs in severe obesity subset

We did not find CNVs in *POMC*, *LEP*, *LEPR*, *MC4R*, *MC3R*, *MC2R* or *SIM1* in 194 obese children.

Initially, MLPA results indicated a homozygous *SIM1* exon 9 deletion in three cases and a heterozygous deletion of the same exon in 49 cases (Figure 1). We then tried to validate this finding by PCR, which was, however, not indicative for deletions nor insertions or duplications large enough to be detected by agarose gel electrophoresis (Figure 2). Specifically, PCR products from samples for which MLPA indicated loss of one or both alleles were of equal size as the ones with the wild-type control (Figure 2). By Sanger sequencing of these fragments we found rs3734355 (NM_005068.2:c.1112C>T, predicting amino acid substitution p.Ala371Val) to be located at the binding site of the MLPA *SIM1* Exon 09A probe (Figure 3). This SNP was in complete linkage disequilibrium with rs3734354, which predicts a Pro-352Thr change. The rs3734354 AA genotype corresponded in all three cases with the absence of a MLPA-signal, falsely indicating the absence of *SIM1* copies and the AC-variant in 49 cases corresponded with a blunted MLPA-signal, falsely indicating hemizygosity, whereas the CC-variant retained full MLPA-signal indicating the presence of two copies.

Hence, we did not find CNV in any of the obesity candidate genes investigated, but found a frequent SNP in *SIM1* in our subset.

Association of *SIM1* genetic variant with childhood obesity

To investigate the role of the polymorphisms we used the exome chip data of all 194 cases and additional 880 obese and overweight children and 1231 lean children. We found rs3734354/rs3734355 to be associated with binary obesity/overweight (see Table 3) and concordantly after applying linear regression under the additive model, to be associated with BMI-SDS (p=0.003, adjusted to age, sex and pubertal state), and nominally with height SDS (p=0.04). We did not find an association with further parameters of obesity such as waist-to-hip ratio, pubertal state, leptin levels, the SDS corrected blood pressure or parameters of insulin homeostasis.

Furthermore we did not observe any association with quantity and quality of food intake of the patients, e.g. calories intake, intake of proteins, fats, carbohydrates and fiber (see Table 4). Other 13 variants of *SIM1* on the exome chip were either not polymorphic in the probands tested in our study or were not analyzed due to low frequency.

Discussion

In this study, we aimed to investigate the role of CNVs in childhood obesity. We concentrated on “classical”

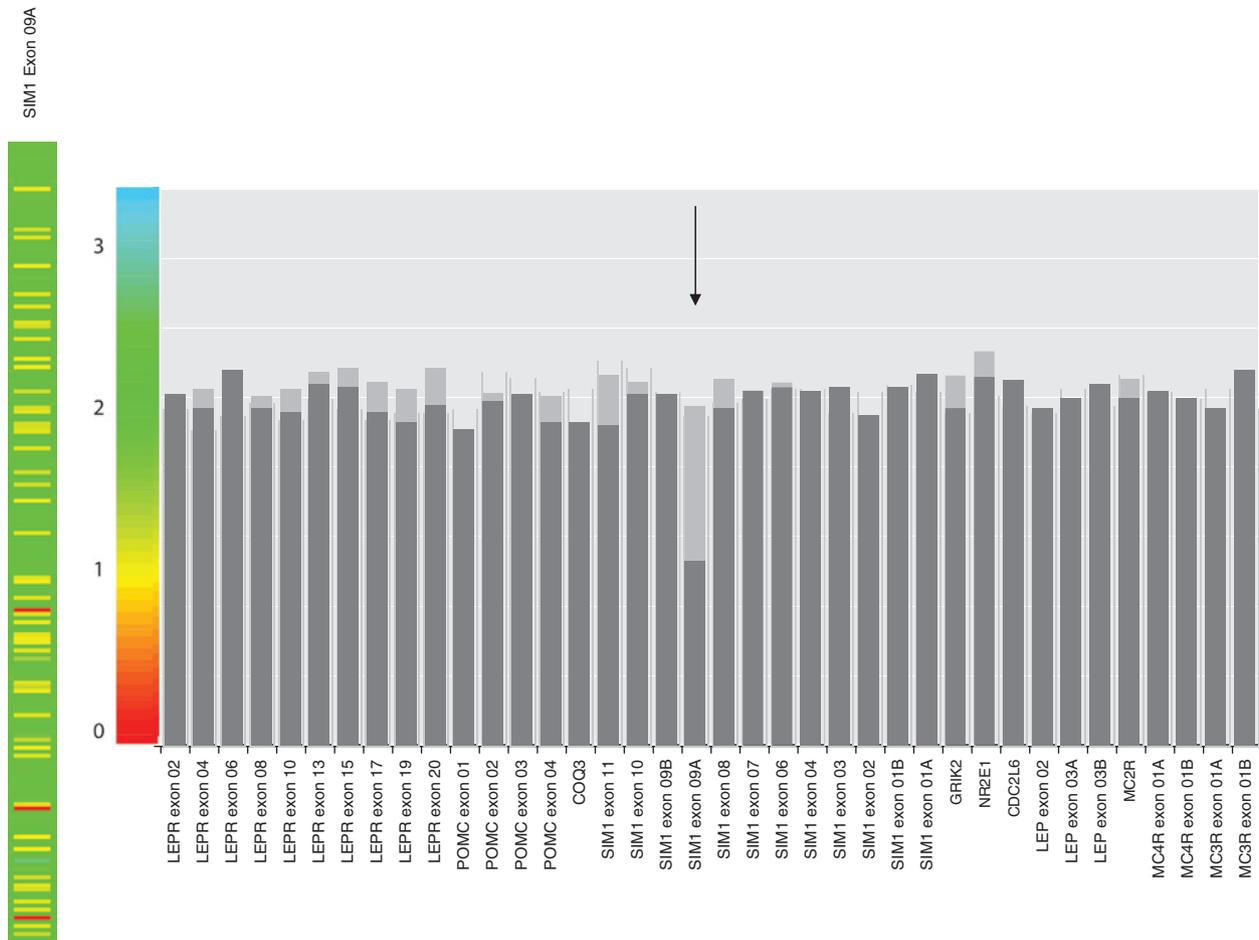


Figure 1: Overview of MLPA-results.

Left panel: copy number signals of the *SIM1* probe (“*SIM1* exon 09A”) for all patients coded in colors. A green stripe represents one patient with two gene copies; yellow a heterozygous deletion; red a homozygous deletion. $n=194$. Right panel: overlay of two MLPA result charts. Y-axis: number of gene copies. X-axis: regions of the binding probe. The arrow shows a sample with one missing *SIM1* copy, for the light gray control the copy is present.

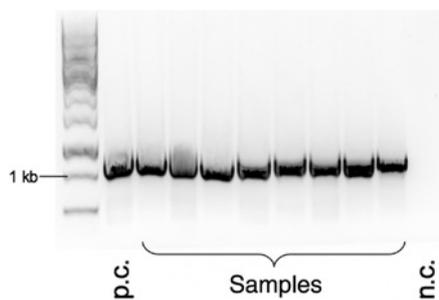


Figure 2: Agarose gel electrophoresis of the PCR amplifications of *SIM1*, covering the binding site of the exon 9 MLPA probe. The first band is a reference ladder with the 1 kb position being marked. Second band is a reference positive control containing two copies of *SIM1* exon 9, bands 3–10 are amplicon one homozygous and heterozygous samples showing same size as the positive control, hence lack of partial deletion, band 11 is a non-template control. For further information on primer location see Supplement Figure 1.

monogenic obesity genes, in which lack of one DNA strand may not have been detectable in conventional sequencing. The MLPA probes we used are set around genes that interact with the regulation of the leptin-melanocortin system: leptin, being mainly synthesized in adipocytes, acts at the hypothalamus and interacts with neuropeptide Y/agouti related peptide (*AGRP*) and *POMC* neurons [27]. Both influence the energy balance. *POMC* neurons secrete the α -melanocyte stimulating hormone (α -MSH) which binds the melanocortin receptors (*MC3R*, *MC4R*) in the paraventricular nucleus and ventromedial hypothalamus [28]. Deficiency in *MC4R* resulted in hypertrophy of adipose tissue, dramatically increased body weight and hyperinsulinemia in mice [29]. Although *MC2R* is not considered a monogenic obesity gene, we included it in our study as previous findings have shown its important role in the hypothalamic-pituitary-adrenal axis [30]. *SIM1*, instead, is

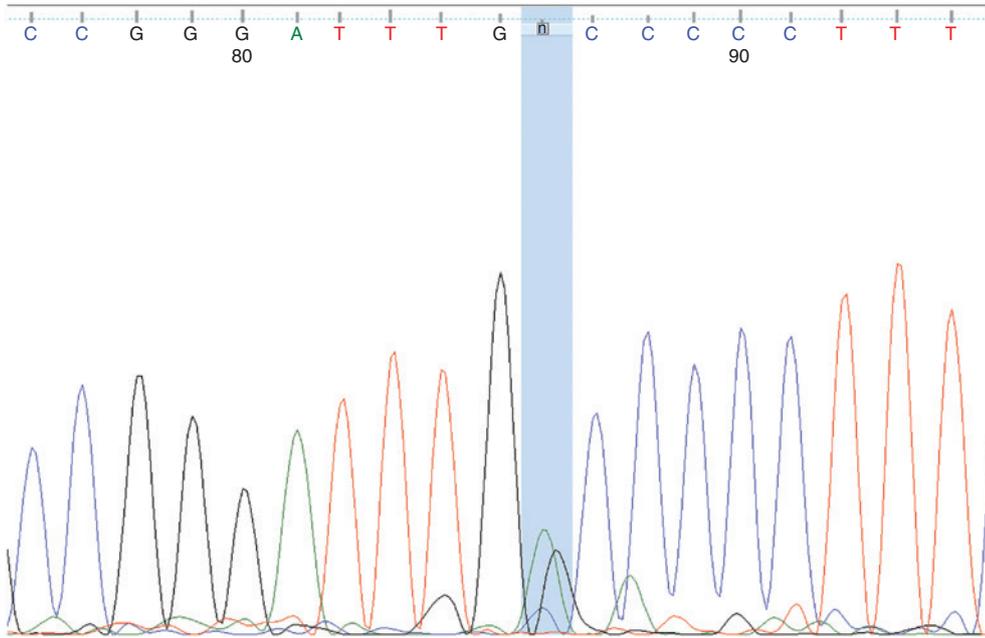


Figure 3: Heterozygous variant of rs3734354.

Sequence of the rs3734354 region showing two dyes at the same position indicating a heterozygous polymorphism in one of our patients with a MLPA signal suggestive for loss of one *SIM1* exon 9 copy.

Table 3: Cross tables of rs3734354 genotype and binary overweight/obesity with according odds ratios (95% KI) and p-values.

	CC		AC		AA		Total		OR [95% C.I.]	p-Value
	Count	%	Count	%	Count	%	Count	%		
Lean	877	52.5	317	54.6	35	70.0	1229	53.4	0.93 [0.88 ± 0.99]	0.014
Overweight and obese	794	47.5	264	45.4	15	30.0	1073	46.6		
Total	1671	100.0	581	100.0	50	100.0	2302	100.0		
Lean	877	59.4	317	60.4	35	71.4	1229	59.9	0.95 [0.90 ± 1.01]	0.095
Obese	600	40.6	208	39.6	14	28.6	822	40.1		
Total	1477	100.0	525	100.0	49	100.0	2051	100.0		

Lean is defined as BMI below 1.23 SDS, overweight as BMI between 1.23 and 1.88 SDS, obese as BMI above 1.88 SDS; OR is odds ratio with 95% confidence interval given in square brackets; p and OR are given after logistic regression for binary obesity ± overweight in the additive mode of inheritance, adjusted to age, sex and pubertal stage.

a transcriptional factor expressed in the central nervous system that plays a role in the development and function of the hypothalamus [31]. Its influence on obesity has previously been demonstrated in knock-out mice: while *SIM1* homozygous null mice die shortly after birth, heterozygous mice survive and develop early-onset obesity and hyperphagia [32]. In humans, Varela et al. were able to find a deletion in *SIM1* in one patient who presented not only with learning disability and hypotonia but also with hyperphagia and obesity [33]. Altogether we hypothesized that deficiencies or duplications in those genes may influence the BMI through direct or indirect interactions with the leptin-melanocortin system [34]. As described above common SNPs identified by GWAS could so far explain only a small

percentage of inherited obesity. Recently focus shifted towards CNVs, revealing rare variants that influence the leptin-melanocortin system [35]. We expected that CNVs in our selected genes will have an effect on BMI and other parameters of obesity that will appear more evidently and early in severely obese children.

To investigate the presence of CNVs, we performed a copy number quantification in our cohort of extremely obese children with BMI > 2.2 SDS and up to 4.2 SDS. After verifying our data, all our patients had two copies of the analyzed sections of genes. Previous studies with similar approaches show ambiguous results: Glessner et al. identified several CNVs in obese children and controls by deriving common CNVs from SNP data [36].

Table 4: Metabolic characteristics stratified for the gene variants of rs3734354 (\pm SEM) and according effect sizes and p-values.

rs3734354	n	CC (n=1673)	AC (n=582)	AA (n=50)	β	p-Value
Parameters of obesity						
BMI-SDS	2302	1.01 \pm 0.04	1 \pm 0.06	0.37 \pm 0.22	-0.06	0.003
Sex	2305	0.49 \pm 0.01	0.48 \pm 0.02	0.44 \pm 0.07	-0.02	0.35
Age	2305	11.33 \pm 0.08	11.33 \pm 0.14	11.39 \pm 0.53	0.007	0.59
Height SDS	2300	0.34 \pm 0.03	0.37 \pm 0.05	-0.05 \pm 0.17	-0.04	0.04
Waist-to-hip ratio	2024	0.85 \pm 0.002	0.85 \pm 0.004	0.85 \pm 0.02	0.01	0.58
Pubertal state	2280	2.53 \pm 0.04	2.53 \pm 0.07	2.48 \pm 0.25	-0.008	0.53
Birth weight, g	1054	3387 \pm 24	3455 \pm 39	3289 \pm 174	-0.001	0.97
Leptin	730	22 \pm 0.7	24 \pm 1.5	21 \pm 4.1	-0.003	0.93
Insulin homeostasis						
FBG, mmol/L	778	4.66 \pm 0.02	4.59 \pm 0.04	4.56 \pm 0.1	-0.03	0.36
120 min BG, mmol/L	916	5.96 \pm 0.04	6.05 \pm 0.09	5.44 \pm 0.23	-0.05	0.16
FPI, pmol/L	765	133 \pm 7	137 \pm 14	111 \pm 23	-0.01	0.71
120 min Insulin, pmol/L	898	636 \pm 30	765 \pm 71	482 \pm 106	-0.008	0.8
HOMA	903	2.87 \pm 0.1	2.9 \pm 0.15	2.49 \pm 0.47	-0.02	0.57
HbA _{1c}	821	5.37 \pm 0.01	5.42 \pm 0.02	5.46 \pm 0.07	0.04	0.23
RR						
Systolic RR SDS, mmHg	2180	0.95 \pm 0.03	0.93 \pm 0.05	0.67 \pm 0.19	-0.03	0.14
Diastolic RR SDS, mmHg	2180	0.6 \pm 0.02	0.64 \pm 0.03	0.53 \pm 0.13	-0.004	0.84
Food intake						
Energy, kcal	153	2065 \pm 51	2056 \pm 81	1659 \pm 754	-0.05	0.51
Protein, g	153	71 \pm 1.9	70 \pm 3.2	50 \pm 25.1	-0.08	0.28
Fat, g	153	81 \pm 2.5	80 \pm 4	59 \pm 17.6	-0.07	0.37
Carbohydrates, g	153	254 \pm 7	254 \pm 11	225 \pm 118	-0.02	0.83
Fiber, g	153	16.78 \pm 0.5	17.64 \pm 1.23	15.49 \pm 4.86	0.02	0.84

All data is adjusted to age, sex and pubertal state. β is non-standardized per-allele beta (SE) in the additive model and refers to the major allele. FBG, fasting blood glucose; BG, blood glucose; FPI, fasting plasma insulin; HOMA, homeostatic model assessment; RR, blood pressure.

Zhang et al. investigated the interactions of common CNVs and dietary behavior in children using the AccuCopy[®] assay (Genesky BioTech, Shanghai, China), similar to our multiplex reaction, but focused on other gene loci [37]. They found three loci to be significantly associated with obesity (10q11.22, 4q25 and 11q11). Interestingly in this study, a meat diet increased the odds ratio (OR) of obesity for one deletion, while for another deletion and duplication this was done by salty food – showing complex multiplicative interactions.

We are aware of the limitations posed by the method we used: as MLPA probes cover only a part of the gene, short regions which might contain CNV remain unexamined, as well as SNPs causing a gain or loss of function are usually not detectable with MLPA. Also the use of predesigned probes in commercial kits limits the selection of genes. Genes that are both associated with obesity and interact with the hypothalamic regulation of energy intake like *PCKS1* [38] (that performs the proteolytic cleavage of *POMC*) [39] and *MRAP2* (that enhances cAMP signaling through *MC4R*) [40] are interesting candidates for further CNV studies. Furthermore, we acknowledge the number of examined patients is rather low and larger

cohorts ($n > 1000$) would be desirable to increase the odds of detecting CNVs associated with severe obesity.

In addition to this, SNPs like rs3734354/5 can influence the binding characteristics of the MLPA-probe to deliver a false result. As we found rs3734354 altered in >25% of our extremely obese children, we wanted to further evaluate its effects in a larger cohort of obese children compared to lean control children. In our extended cohort of 2305 children we verified an association of rs3734354 with BMI-SDS ($p = 0.003$; $B = -0.06$). Carriers of the rare “AA” variant were not only less prone to obesity but also had a lower height (height SDS: $B = -0.04$, $p = 0.04$). Yet no associations with other parameters of obesity or metabolic deregulation were found, neither did different variants of the SNP influence blood pressure nor show interaction with a specific diet. It is, however, noteworthy that according to our data the minor allele of rs3734354 is protective from obesity in our cohort of children. In previous studies the effects of rs3734354/5 were either inconclusive or contrary: a study on 3.479 adults found rs3734354 associated with higher BMI [41] while Ghossaini et al. performed an analysis on 3.248 obese and lean adults and children (mean age 11 and 41,

respectively) detecting a nominal association ($p=0.01$) with parameters of obesity [42]. A study in Pima Indians found rs3734354/5 not to be associated with BMI [43]. Other rare as well as common variants in *SIM1* have been discovered as important risk factors for obesity [44, 45] underlining the significance of *SIM1* in energy homeostasis. It has been discussed that the incoherent association with BMI described above is age dependent and may not become apparent “until well into middle age” [41]. Our data suggest that variations of rs3734354 interact with obesity of early-onset type in children and have measurable effects at an early age already. The pathomechanism of these interactions is mostly unknown. It remains unclear why adult carriers of the rs3734354 minor allele develop a higher BMI, whereas in children it seems to be protective from obesity. In their previously mentioned study Swarbrick et al. could show that rs3734354/5 can impair *SIM1*'s ability to activate target gene transcription [41]. In mice *SIM1* itself was found to be expressed in the paraventricular and supraoptic nuclei, the amygdala and lateral hypothalamus [46], areas broadly associated with regulation of energy balance. It is likely that *SIM1* regulates the expression of specific peptides like corticotropin-releasing hormone (CRH), oxytocin or somatostatin in the paraventricular nuclei and thus modulate feeding and fasting. This process as well as the transcription activation of target genes needs better understanding.

We found rs3734354 to interfere with one *SIM1* MLPA probe on exon 9 causing a non-ligation pretending a deletion. MRC-Holland updated specifications of the used kit number 220 and later removed the concerned probe. As our study was performed with children, it has to be taken into account that a variant-effect can manifest in later stages of life and is possibly modified by gene-environment interactions – therefore not being detectable or being only detectable with advanced stages of disease, which are less prevalent in children. Also our study was carried out in a cohort enriched in obesity cases compared to the general pediatric population. Studying a secondary outcome in a population ascertained for a primary outcome can introduce a bias in the estimation of the effect.

In summary, we were unable to find CNVs in well-established obesity genes *POMC*, *LEP*, *LEPR*, *MC4R*, *MC3R* and *SIM1* in our modest sample of severely obese children. Nevertheless, we found an association of rs3734354 with obesity of early-onset type in children, although not with obesity-related traits.

Acknowledgments: We thank all the children and their parents who participated in this study. We also gratefully acknowledge the help of the study nurses and physicians

for patient care, and the technical assistants for performing these investigations.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: This work was supported by grants from the German Research Foundation for the Clinical Research Center “Obesity Mechanisms” CRC1052/1 C05, by the Federal Ministry of Education and Research (BMBF), Germany, FKZ: 01EO1001, by the European Community's Seventh Framework Programme (FP7/2007-2013) project Beta-JUDO under grant agreement n° 279153, and the LIFE Child (Leipzig Research Center for Civilization Diseases, Universität Leipzig) funded by the European Union, by the European Regional Development Fund (ERFD) by means of the Free State of Saxony within the framework of the excellence initiative.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

- Hinney A, Vogel CI, Hebebrand J. From monogenic to polygenic obesity: recent advances. *Eur Child Adolesc Psychiatry* 2010;19:297–310.
- Yang W, Kelly T, He J. Genetic epidemiology of obesity. *Epidemiol Rev* 2007;29:49–61.
- Peterson RE, Maes HH, Lin P, Kramer JR, Hesselbrock VM, et al. On the association of common and rare genetic variation influencing body mass index: a combined SNP and CNV analysis. *BMC Genomics* 2014;15:368.
- Stranger BE, Forrest MS, Dunning M, Ingle CE, Beazley C, et al. Relative impact of nucleotide and copy number variation on gene expression phenotypes. *Science* 2007;315:848–53.
- Willer CJ, Speliotes EK, Loos RJ, Li S, Lindgren CM, et al. Six new loci associated with body mass index highlight a neuronal influence on body weight regulation. *Nat Genet* 2009;41:25–34.
- Wang K, Li W-D, Glessner JT, Grant SF, Hakonarson H, et al. Large copy-number variations are enriched in cases with moderate to extreme obesity. *Diabetes* 2010;59:2690–94.
- Falchi M, El-Sayed Moustafa JS, Takousis P, Pesce F, Bonnefond A, et al. Low copy number of the salivary amylase gene predisposes to obesity. *Nat Genet* 2014;46:492–7.
- Bochukova EG, Huang N, Keogh J, Henning E, Purmann C, et al. Large, rare chromosomal deletions associated with severe early-onset obesity. *Nature* 2010;463:666–70.
- Høyer H, Braathen GJ, Eek AK, Nordang GB, Skjelbred CF, et al. Copy number variations in a population-based study of Charcot-Marie-Tooth disease. *Biomed Res Int* 2015;2015:960404.

10. Mertz LG, Christensen R, Vogel I, Hertz JM, Nielsen KB, et al. Angelman syndrome in Denmark. birth incidence, genetic findings, and age at diagnosis. *Am J Med Genet A* 2013;161A: 2197–203.
11. Butler MG, Fischer W, Kibiryeve N, Bittel DC. Array comparative genomic hybridization (aCGH) analysis in Prader-Willi syndrome. *Am J Med Genet A* 2008;146A:854–60.
12. Lionel AC, Crosbie J, Barbosa N, Goodale T, Thiruvahindrapuram B, et al. Rare copy number variation discovery and cross-disorder comparisons identify risk genes for ADHD. *Sci Transl Med* 2011;3:95ra75.
13. Sanders SJ, Ercan-Sencicek AG, Hus V, Luo R, Murtha MT, et al. Multiple recurrent de novo CNVs, including duplications of the 7q11.23 Williams syndrome region, are strongly associated with autism. *Neuron* 2011;70:863–85.
14. Farooqi IS, Keogh JM, Yeo GS, Lank EJ, Cheetham T, et al. Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. *N Engl J Med* 2003;348:1085–95.
15. Melchior C, Schulz A, Windholz J, Kiess W, Schöneberg T, et al. Clinical and functional relevance of melanocortin-4 receptor variants in obese German children. *Horm Res Paediatr* 2012;78:237–46.
16. Farooqi IS, Wangenstein T, Collins S, Kimber W, Matarese G, et al. Clinical and molecular genetic spectrum of congenital deficiency of the leptin receptor. *N Engl J Med* 2007;356:237–47.
17. Hung CC, Luan J, Sims M, Keogh JM, Hall C, et al. Studies of the SIM1 gene in relation to human obesity and obesity-related traits. *Int J Obes (Lond)* 2007;31:429–34.
18. Farooqi IS, Drop S, Clements A, Keogh JM, Biernacka J, et al. Heterozygosity for a POMC-null mutation and increased obesity risk in humans. *Diabetes* 2006;55:2549–53.
19. Boggula VR, Shukla A, Danda S, Hariharan SV, Nampoothiri S, et al. Clinical utility of multiplex ligation-dependent probe amplification technique in identification of aetiology of unexplained mental retardation: a study in 203 Indian patients. *Indian J Med Res* 2014;139:66–75.
20. Mandal K, Boggula VR, Borkar M, Agarwal S, Phadke SR. Use of multiplex ligation-dependent probe amplification (MLPA) in screening of subtelomeric regions in children with idiopathic mental retardation. *Indian J Pediatr* 2009;76:1027–31.
21. Depienne C, Moreno-De-Luca D, Heron D, Bouteiller D, Gennetier A, et al. Screening for genomic rearrangements and methylation abnormalities of the 15q11-q13 region in autism spectrum disorders. *Biol Psychiatry* 2009;66:349–59.
22. Delorme R, Moreno-De-Luca D, Gennetier A, Maier W, Chaste P, et al. Search for copy number variants in chromosomes 15q11-q13 and 22q11.2 in obsessive compulsive disorder. *BMC Med Genet* 2010;11:100.
23. Kromeyer-Hauschild K, Wabitsch M, Kunze D, Geller F, Geiß H, et al. Perzentile für den Body-mass-Index für das Kindes- und Jugendalter unter Heranziehung verschiedener deutscher Stichproben. *Monatsschr Kinderh* 2001;149:818, 807.
24. Consensus guidelines 2005. 2005. <http://www.a-g-a.de>.
25. Schouten JP, McElgunn CJ, Waaijer R, Zwiijnenburg D, Diepvens F, et al. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res* 2002;30:e57.
26. Slater H, Bruno D, Ren H, La P, Burgess T, et al. Improved testing for CMT1A and HNPP using multiplex ligation-dependent probe amplification (MLPA) with rapid DNA preparations: comparison with the interphase FISH method. *Hum Mutat* 2004;24:164–71.
27. Elias CF, Aschkenasi C, Lee C, Kelly J, Ahima RS, et al. Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. *Neuron* 1999;23:775–86.
28. Beales PL. Obesity in single gene disorders. *Prog Mol Biol Transl Sci* 2010;94:125–57.
29. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, et al. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 1997;88:131–41.
30. Metherell LA, Chapple JP, Cooray S, David A, Becker C, et al. Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2. *Nat Genet* 2005;37:166–70.
31. Michaud JL, Rosenquist T, May NR, Fan CM. Development of neuroendocrine lineages requires the bHLH-PAS transcription factor SIM1. *Genes Dev* 1998;12:3264–75.
32. Michaud JL, Boucher F, Melnyk A, Gauthier F, Goshu E, et al. Sim1 haploinsufficiency causes hyperphagia, obesity and reduction of the paraventricular nucleus of the hypothalamus. *Hum Mol Genet* 2001;10:1465–73.
33. Varela MC, Simões-Sato AY, Kim CA, Bertola DR, De Castro CI, et al. A new case of interstitial 6q16.2 deletion in a patient with Prader-Willi-like phenotype and investigation of SIM1 gene deletion in 87 patients with syndromic obesity. *Eur J Med Genet* 2006;49:298–305.
34. Willer CJ, Speliotes EK, Loos RJ, Li S, Lindgren CM, et al. Six new loci associated with body mass index highlight a neuronal influence on body weight regulation. *Nat Genet* 2009;41:25–34.
35. Walley AJ, Asher JE, Froguel P. The genetic contribution to non-syndromic human obesity. *Nat Rev Genet* 2009;10:431–42.
36. Glessner JT, Bradfield JP, Wang K, Takahashi N, Zhang H, et al. A genome-wide study reveals copy number variants exclusive to childhood obesity cases. *Am J Hum Genet* 2010;87:661–6.
37. Zhang D, Li Z, Wang H, Yang M, Liang L, et al. Interactions between obesity-related copy number variants and dietary behaviors in childhood obesity. *Nutrients* 2015;7:3054–66.
38. Löffler D, Behrendt S, Creemers JW, Klammt J, Aust G, et al. Functional and clinical relevance of novel and known PCSK1 variants for childhood obesity and glucose metabolism. *Mol Metab* 2016;6:295–305.
39. D'Angelo CS, Koifmann CP. Copy number variants in obesity-related syndromes: review and perspectives on novel molecular approaches. *J Obes* 2012;2012:845480.
40. Asai M, Ramachandrapa S, Joachim M, Shen Y, Zhang R, et al. Loss of function of the melanocortin 2 receptor accessory protein 2 is associated with mammalian obesity. *Science* 2013;341:275–8.
41. Swarbrick MM, Evans DS, Valle MI, Favre H, Wu S-H, et al. Replication and extension of association between common genetic variants in SIM1 and human adiposity. *Obesity (Silver Spring)* 2011;19:2394–403.
42. Ghossaini M, Stutzmann F, Couturier C, Vatin V, Durand E, et al. Analysis of the SIM1 contribution to polygenic obesity in the french population. *Obesity (Silver Spring)* 2010;18:1670–5.
43. Traurig M, Mack J, Hanson RL, Ghossaini M, Meyre D, et al. Common variation in SIM1 is reproducibly associated with BMI in Pima Indians. *Diabetes* 2009;58:1682–9.
44. Bonnefond A, Raimondo A, Stutzmann F, Ghossaini M, Ramachandrapa S, et al. Loss-of-function mutations in SIM1

contribute to obesity and Prader-Willi-like features. *J Clin Invest* 2013;123:3037–41.

45. Ramachandrappa S, Raimondo A, Cali AM, Keogh JM, Henning E, et al. Rare variants in single-minded 1 (SIM1) are associated with severe obesity. *J Clin Invest* 2013;123:3042–50.
46. Holder JL, Zhang L, Kublaoui BM, DiLeone RJ, Oz OK, et al. Sim1 gene dosage modulates the homeostatic feeding response to

increased dietary fat in mice. *Am J Physiol Endocrinol Metab* 2004;287:E105–13.

Supplemental Material: The online version of this article (DOI: 10.1515/jpem-2016-0435) offers supplementary material, available to authorized users.