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**INVESTIGATION OF COMMON AND RARE GENETIC VARIATION IN THE *BAMBI* GENOMIC REGION  
IN LIGHT OF HUMAN OBESITY**

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## Abstract

**Purpose:** To confirm the previously identified link between *BAMBI* and human obesity by means of a genetic and functional analysis.

**Methods:** We performed both a mutation analysis, using high-resolution melting curve analysis, and a genetic association study, including 8 common tagSNPs in the *BAMBI* gene region. Three of the identified genetic variants (R151W, H201R and C229R) were evaluated for their Wnt signaling enhancing capacity in a Wnt luciferase reporter assay.

**Results:** Mutation screening of the *BAMBI* coding region and exon-intron boundaries on our population of 677 obese children and adolescents and 529 lean control subjects resulted in the identification of 18 variants, 10 of which were not previously reported and 12 of which were exclusively found in obese individuals. The difference in variant frequency, not taking into account common polymorphisms, between obese (3.1%) and lean (0.9%) subjects was statistically significant ( $p = 0.004$ ). Our Wnt luciferase assay, using WT and mutant *BAMBI* constructs, showed a significantly reduced activity for all of the investigated variants. Logistic and linear regression analysis on our Caucasian population of 1022 obese individuals and 606 lean controls, did not identify associations with obesity parameters ( $p$ -values  $>0.05$ ).

**Conclusions:** We found several rare genetic variations, which represent the first naturally occurring missense variants of *BAMBI* in obese patients. Three variants (R151W, H201R and C229R) were shown to reduce Wnt signaling enhancing capacity of *BAMBI* and we believe this result should encourage further study of this gene in other obese populations. In addition, we did not find evidence for the involvement of *BAMBI* common variation in human obesity in our population.

Keywords: Bambi, Wnt, Genetics, Human Obesity

## Introduction

Obesity is caused by an excessive amount of body fat that predisposes an individual to numerous comorbidities, ranging from hypertension and cancer to type 2 diabetes [1-3]. It is a chronic disease that has become a major health problem with increasing prevalence worldwide. The World Health Organization (WHO) estimates that overweight affects 30-70% and obesity affects 10-30% of adults in European Union countries [4]. In addition, 20% of children and adolescents are overweight and of these, a third are obese [5]. Obesity and overweight in a population are commonly evaluated by means of the body mass index (BMI). The obese state occurs as a result of an increased ratio of caloric intake vs. energy burning and this leads to both enlargement of existing adipocytes (hypertrophy) and formation of new adipocytes (hyperplasia) from mesenchymal stem cells (MSCs) or preadipocytes, residing in adipose tissue.

The Wnt (wingless-type MMTV integration site family) pathway is an evolutionary conserved transduction cascade that controls differentiation and proliferation of cells in both embryonic and adult mammalian tissues. Activation of this pathway has been shown to result in the inhibition of MSCs differentiation to adipocytes [6]. The pathway is initiated by the binding of Wnt ligands to the 7 transmembrane Frizzled (Fz) receptor and single-pass low density lipoprotein receptor-related protein (LRP) 5 and 6 [7, 8]. Depending on the Fz isoform that is used, and depending on the co-receptors present,  $\beta$ -catenin dependent or independent Wnt pathways are activated. Activation of a  $\beta$ -catenin independent pathway can result in the release of calcium ions from the endoplasmic reticulum, which will then further function as secondary messenger [9, 10], or it involves jun N-terminal kinase (JNK) as an intracellular signaling molecule [11]. However, for this study, we mainly focused on the  $\beta$ -catenin dependent or "canonical" Wnt pathway.  $\beta$ -catenin serves as the central messenger in this pathway, but is degraded when the pathway is inactive. For this purpose, a destruction complex is formed consisting of Axin, casein kinase 1 $\alpha$  (CK1 $\alpha$ ), glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) and Adenomatous polyposis coli (APC) [12, 13]. The destruction complex phosphorylates  $\beta$ -catenin, leading to its subsequent ubiquitinylation and proteasomal degradation. When Wnts bind to Fz and LRP5/6, Axin migrates to LRP5/6 at the plasma membrane leading to the disassembly of the destruction complex [14]. In addition, the cytoplasmic effector protein Dishevelled (DVL) is recruited to the membrane, and its DEP domain is able to interact with Fz [15]. As a consequence,  $\beta$ -catenin accumulates and can migrate to the nucleus where it binds transcription factors of the T-cell factor (TCF)-lymphoid enhancer factor (LEF) family and activates transcription of target genes [13, 16]. Through inhibition of the main adipogenic transcription factors CCAAT/enhancer binding protein  $\alpha$

(C/EBP $\alpha$ ) and peroxisome proliferator activated receptor  $\gamma$  (PPAR $\gamma$ ), Wnt signaling is able to maintain preadipocytes in an undifferentiated state, or promote their differentiation to osteoblasts [6, 17-19]. BAMBI is a 260-amino acid single pass transmembrane (Tm) protein (Figure 1) that lacks an intracellular (IC) kinase domain but has an extracellular (EC) domain that is closely related to type I receptors of the transforming growth factor- $\beta$  (TGF $\beta$ ) family. TGF $\beta$ s are multifunctional growth factors which inhibit cell cycle progression in many cell types. TGF $\beta$  receptors type I (T $\beta$ RI) and type II (T $\beta$ RII) are known to function as a heteromeric cell-surface complex when activated by TGF $\beta$ , BMPs or Activin-like ligands. It was shown that human BAMBI, like its *Xenopus* homologue, can inhibit TGF $\beta$  and BMP-mediated transcriptional responses and cell growth arrest, by acting as a decoy receptor [20]. In addition, BAMBI was shown to be an enhancer of Wnt signaling by *in vitro* assays in which ectopic expression of BAMBI increased  $\beta$ -catenin nuclear levels and was able to activate transcription from a LEF-luciferase construct. The C-terminal IC domain of BAMBI can form a complex with both Fz5 and LRP6 and in addition, it can bind DVL. It is presumed that BAMBI enhances the Wnt signal transduction through these interactions, eventually leading to increased expression of target genes such as *Cyclin D1* and *c-myc* [21].

The first link between BAMBI and obesity came with the discovery that fibroblast growth factor 1 (FGF1) promotes adipogenesis of human preadipocytes, and that this is partly mediated by *BAMBI* downregulation. Furthermore, *BAMBI* knockdown was sufficient to recapitulate the effects of FGF1 by promoting commitment of MSCs to the adipocyte lineage [22]. In both *ob/ob* and high fat diet (HFD)- induced obese mice, *Bambi* mRNA expression was decreased [22], further confirming the *in vivo* importance of BAMBI in the light of obesity. In addition, in human non-alcoholic steatohepatitis (NASH) patients, liver BAMBI protein negatively correlated with BMI of the patients [23].

In this study we performed a thorough mutation screening of the human *BAMBI* coding region, in a population of obese children, adolescents and lean controls. In addition, we investigated the association of common polymorphisms in the *BAMBI* gene region with complex obesity in an extensively characterized population of obese and lean individuals.

## Materials and Methods

### *Population for mutation analysis*

Six hundred and seventy-seven overweight and obese children and adolescents (299 boys and 378 girls; adolescent if age  $\geq$  12 yrs; Table 1) consulting the Child Obesity Clinics from the Antwerp University Hospital (Antwerp) and Jessa Hospital (Hasselt), both in Belgium, were recruited for the mutation analysis. The patient population has a mixed ethnicity (about 80% of Caucasian origin). Five hundred and twenty-nine control individuals (186 males and 343 females, BMI 18.5-25 kg/m<sup>2</sup>; Table

1) of mostly Caucasian origin were recruited among the university and hospital personnel and among couples seeking prenatal counselling at the Department of Medical Genetics (due to high maternal age or increased triple test). Couples seeking prenatal genetic counselling because of familial disease history were excluded. All subjects gave their written informed consent before participation and the study protocol was approved by the local ethics committee.

#### *Population for association study*

For the association study, we recruited a population of one thousand and twenty-two individuals (473 men and 549 women, BMI  $\geq$  30 kg/m<sup>2</sup>; Table 2) from patients consulting the outpatient obesity clinic at the Antwerp University Hospital (a tertiary referral facility). Inclusion criteria were obesity (BMI  $\geq$  30 kg/m<sup>2</sup>) and age 21-69 years. Exclusion criteria were pregnancy, diabetes or impaired glucose tolerance. All subjects were Caucasian and at enrolment none were involved in an ongoing weight management program.

Six hundred and six control individuals (223 males and 383 females, BMI 18.5-25 kg/m<sup>2</sup>; Table 2) of Caucasian origin were recruited among the university and hospital personnel and among couples seeking prenatal counselling at the Department of Medical Genetics as described above. This control population includes the lean individuals that were used for the mutation analysis. All subjects gave their written informed consent before participation and the study protocol was approved by the ethics committee of the Antwerp University Hospital.

#### *Anthropometry*

Height was measured to the nearest 0.5 cm; body weight was measured with a digital scale to the nearest 0.2 kg. Body mass index (BMI) was calculated as weight (in kg) over height (in m) squared and BMI cut-off values of 25 and 30 kg/m<sup>2</sup> for overweight and obesity, as determined by the World Health Organisation (WHO), were used for adults. BMI cut-off values for the children and adolescents were determined by using the Flemish Growth Charts [24], where the percentile lines that pass through a BMI of 25 and 30 kg/m<sup>2</sup> at 18 years of age are used as cut-off values for overweight and obesity, respectively. BMI Z-scores were calculated based on the Flemish Growth Charts.

Waist circumference was measured at mid-level between the lower rib margin and the iliac crest and hip circumference was measured at the level of the trochanter major. Using these values, the waist-to-hip ratio (WHR) was calculated. Visceral (VFA), subcutaneous (SFA) and total abdominal (TFA) fat areas were determined with a computerized tomography (CT) scan that was performed at the L4–L5 level, according to the technique described by Van der Kooy and Seidell [25]. Body composition was determined by bio-impedance analysis as described by Lukaski *et al.* [26] and fat mass percentage was calculated, using the formula of Deurenberg *et al.* [27].

### *Mutation analysis*

Blood samples were obtained from all subjects and DNA extraction was performed by standard procedures [28].

The coding region of *BAMBI* (783bp, GenBank accession number NC\_000010.10) consists of three exons. All exons and exon-intron boundaries were screened for mutations using high-resolution melting curve analysis (HRM). HRM was performed using the Lightcycler 480 Real-Time PCR System (Roche, Penzberg, Germany) with incorporation of LCGreen+ fluorescent dye (Idaho Technology Inc., Salt Lake City, UT, USA) into the PCR product. Samples with melting curves deviating from the wild-type (WT) were sequenced. Sequencing was performed with ABI BigDye Terminator v1.1 Cycle Sequencing kits on an ABI Prism Genetic Analyzer 3130xl (Applied Biosystems Inc, Foster City, CA, USA). Primer sequences for HRM and sequencing are available on request.

For analysis of identified variants that result in an amino acid change we consulted following *in silico* prediction programmes: SIFT [29], Polyphen-2 [30], MutPred [31], SNPs&GO [32] and the ConSurf database server [33].

### *Expression constructs and in vitro mutagenesis*

Human *BAMBI* cDNA tagged with myc tag at the C terminus and subcloned into a pCMV5 expression vector was kindly provided by Ye-Guang Chen's Lab (School of Life Sciences, Tsinghua University, Beijing). Three variants that were found in our population, and that resulted in a non-synonymous amino acid change (R151W, H201R, C229R) were introduced in the full-length wild type construct using the QuickChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). Primer sequences were designed according to the recommendations for the QuickChange Site-directed Mutagenesis kit. The insert sequence was verified for the presence of the mutation and absence of PCR errors by DNA sequencing.

A mouse *wnt1-V5* expression construct was provided by Dr. Bart Williams (Van Andel Research Institute, Grand Rapids, MI), a mouse *mesdc-2* expression construct was provided by Dr. Bernadette Holdener (State University of New York, Stony Brook, NY), and Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD) kindly provided the Topflash Wnt reporter construct (pGL3-OT). The Renilla Luciferase construct pRL-TK was purchased from Promega Corporation.

### *Western blot*

Western blot was performed in triplicate on membrane proteins present on HEK293T cells, cotransfected with a myc-tagged *BAMBI* WT or mutant construct and a pEGFP-N1 vector, isolated by use of the Pierce Cell Surface Protein Isolation Kit (Thermo scientific, Waltham, MA) according to the

manufacturer's guidelines. Detection of BAMBI in membrane samples was performed by western blotting. Protein concentrations were measured with Qubit protein assay (Life technologies, Carlsbad, California, USA). Samples were mixed with NuPAGE® LDS sample buffer and sample reducing agent (Invitrogen, Carlsbad, CA, USA) and heated to 70 °C for 10 min prior to gel electrophoresis. Samples were then loaded on a 4–12% NuPAGE® Novex Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA, USA) and separated under denaturing conditions. Proteins were transferred by semi-dry transfer to nitrocellulose membranes. Non-specific binding sites were blocked by incubation in TBS containing 5% milk powder.

The membranes were then incubated overnight with the primary mouse anti-c-myc monoclonal antibody (1:5000) (Sigma-Aldrich, Saint Louis, MO, USA) at 4°C. Membranes were subsequently incubated with the secondary antibody anti-mouse IgG horse-radish peroxidase (HRP) conjugate (1:5000) (Bio-Rad, Hercules, CA, USA) for 2 h at room temperature. Membranes were incubated with ECL Western Blotting Substrate (Pierce Biotechnology, Rockford, IL, USA) and visualized with the use of Image Quant LAS400 Mini system (GE). Obtained images were analyzed with ImagequantTL software (GE). Novex® Sharp Pre-stained Protein Standard was loaded to estimate protein size. Human BAMBI travels at a molecular weight around 28–29 kDa [34]. To correct for transfection efficiency, samples were also assayed for GFP signal using the primary antibody rabbit anti-GFP (1:2500)(Sigma-Aldrich) and secondary antibody goat anti-rabbit igG HRP (1:10000) (Bio-Rad)).

#### *Luciferase reporter assay*

The human embryonic kidney (HEK) cell line 293T was grown in DMEM (Invitrogen) supplemented with FBS (10%v/v, Invitrogen). Twenty-four hours prior to transfection, cells were plated at  $3 \times 10^4$  cells/well in 96-well plates. Cells were transfected using Fugene 6 (Roche Applied Science) according to the manufacturer's instructions. In HEK293T cells, Wnt1-V5 (1ng), mesdc-2 (2ng), pRL-TK (2.5ng) and pGL3-OT (50ng) constructs were cotransfected with WT or different mutant *BAMBI* constructs (32ng). When needed, empty pcDNA3.1 vector was added to ensure an equal total DNA amount for all transfection experiments. As a negative control, cell were transfected with mesdc-2, pRL-TK and pGL3-OT but no Wnt1 was added. Each transfection was carried out in triplicate and repeated independently in at least three separate experiments. Forty-eight hours after transfection, cells were lysed and firefly and renilla luciferase activity was measured on a Glomax Multi+ Luminometer (Turner Designs, Sunnyvale, CA) using the dual luciferase reporter assay system (Promega Corporation) according to manufacturer's instructions.

#### *Genotyping*

Eighteen SNPs were identified in the *BAMBI* gene region, including 10 kb upstream and downstream of the gene by using information from the international HapMap project [35] (HapMap release 28 Phase II+III, August10, ncbi36 assembly, included region: Chr22:37372604..37407900). Data from the CEPH population was selected, containing SNPs identified in Utah residents with ancestry from Northern and Western Europe (CEU). Only SNPs with a minor allele frequency (MAF)  $\geq 0.05$  were included in the Tagger analysis, using aggressive tagging of 2- and 3-marker haplotypes and with  $r^2$  and LOD thresholds at 0.8 and 3.0, respectively (Haploview version 4.2) [36]. Based on linkage disequilibrium (LD) data between the eighteen SNPs in the *BAMBI* region, eight tagSNPs (rs624032, rs3758365, rs17753457, rs663378, rs17832466, rs655766, rs9663912 and rs12570151, figure 3) were randomly selected by the Haploview software. Genotyping of these tagSNPs provides information on most of the known common variation in the 25kb *BAMBI* region in the CEU population.

TaqMan Pre-Designed Genotyping Assays (Applied Biosystems Inc., Foster City, CA, USA) were used for genotyping, according to the manufacturer's protocol, on a Lightcycler 480 Real-Time PCR System (Roche, Penzberg, Germany). Blank samples were included as negative controls and a minimum of 28 samples were placed in duplicate on each run. Duplicate samples were 100% concordant and thus correct genotyping could be confirmed. The minimal genotyping success rate was 95,3% and samples with one or more missing genotypes were excluded.

#### *Statistical analysis*

**Mutation analysis.** We used Chi<sup>2</sup> analysis (Fisher's exact test) for comparison of variant frequencies between case and control groups [37].

**Luciferase reporter assay.** Data are expressed as mean values  $\pm$  SD and comparison between two measurements for a single experiment was performed using a Student's t-test. Per experiment, values were then recalibrated relative to the luciferase activity value from the WT *BAMBI* transfection, which was set at 100%, and a Student's t-test was performed on these values to investigate the overall significant differences between measurements in the different experiments. Values of  $p < 0.05$  were considered significant.

**Association study.** Hardy-Weinberg equilibrium (HWE) was calculated for all tagSNPs by use of the LINKUTIL package [38] with the significance level set at 0.01.

Odds ratios (OR) were calculated using univariate logistic regression with correction for age and sex. Linear regression was used to quantify the effect of a SNP on BMI, waist circumference, height, WHR, fat mass, fat mass percentage and total, visceral and subcutaneous fat with age and sex as covariates. All analyses were performed under an additive mode of inheritance and the significance level was set at  $p = 0.05$ . All statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, USA).

Power calculations were performed using Quanto and in our total population (cases + controls) we have 80 % power to detect a risk of 1.55-1.24 with a SNP with MAF of 5-50 %. In the obese part of our population (males + females), used for linear regression analysis, we have >80% power to detect an effect of 1-1.5% for the selected parameters [39].

## Results

### *Identification of BAMBI variants and pedigree analysis*

By performing a thorough mutation screening in 677 obese children and adolescents, and 529 lean control individuals, we were able to identify a total of eighteen sequence variants in the *BAMBI* coding region and exon-intron boundaries (Table 3). Ten of these variants were, to our knowledge, not previously reported [40, 41]. Previously known variants were found with similar frequencies as reported in the dbSNP database. Twelve out of eighteen variants were found exclusively in obese individuals, including five variants resulting in a non-synonymous amino acid change (P58R, T77K, R151W, H201R, C229R, Table 3, Figure 1). On the non-synonymous *BAMBI* variants we performed an *in silico* analysis, using several online prediction programs, to estimate their effect on protein function (Table 4) [29-33]. Both amino acid positions 151 and 229 are highly conserved across species and we thus categorized R151W and C229R as interesting variants to investigate further. When including information on known *BAMBI* protein domains, we saw that H201R, V208I and C229R are located in the IC protein domain that was predicted to be responsible for enhancing the Wnt pathway activity. However, V208I was found with a similar frequency in both cases (1.6%) and controls (2.1%) and was therefore not selected as a candidate for further investigation. Based on these arguments, we decided to select c.451C>T (R151W), c.602A>G (H201R) and c.685T>C (C229R) for functional analysis.

R151W was found in a 10-year-old girl with a BMI of 25.5 kg/m<sup>2</sup> (height: 1.40 m; weight: 50 kg; Z-score: +1.97), but no information on the parents was available. We found the H201R variant in a 10-year-old girl with a BMI of 30,1 kg/m<sup>2</sup> (height: 1.47 m; weight: 65 kg; Z-score: +2.45), and a fat excess of 15.1%. The girl has an overweight father (BMI of 27.4 kg/m<sup>2</sup>) but a normal weight mother and two lean brothers. We were not able to obtain DNA samples from the family members. The C229R variant was found in a 20-year-old woman with a BMI of 40.8 kg/m<sup>2</sup> (height: 1.71 m; weight: 118.6 kg). This patient has an overweight mother (BMI of 27.7 kg/m<sup>2</sup>) and an obese father (BMI of 30.1 kg/m<sup>2</sup>) and brother (BMI of 31.8 kg/m<sup>2</sup>). The C229R variant was inherited from mother to daughter, both father and brother were non-carriers of this variant.

#### *Effects of R151W, H201R and C229R on the cellular localisation of BAMBI*

First we analysed the cellular localisation of WT and mutant (R151W, H201R, C229R) BAMBI proteins by performing a western blot analysis on the membrane fraction of cells transfected with a myc-tagged *BAMBI* construct. As expected, wild-type BAMBI could be detected in the membrane fraction. In addition, our results showed membrane localisation for all three mutant BAMBI forms (data not shown).

#### *Effects of R151W, H201R and C229R on the signaling ability of BAMBI*

By performing a Wnt luciferase assay, including the WT or mutant BAMBI protein, we wanted to investigate the effect of each of these variants on the ability of BAMBI to activate the Wnt pathway *in vitro*. As expected, we observed a significant increase in Wnt signaling when the wild-type *BAMBI* construct was added, compared to signaling activated by WNT1 alone ( $p=3.8 \cdot 10^{-4}$ , Figure 2). In addition, we observed a significant decrease in Wnt activity when cells were transfected with *BAMBI* R151W ( $p=3.4 \cdot 10^{-4}$ ), *BAMBI* H201R ( $p=0.003$ ) or *BAMBI* C229R ( $p=0.035$ ) (Figure 2).

#### *Association study on BAMBI common polymorphisms*

In addition to a mutation screening, we performed an association analysis including nearly all the common variation ( $MAF > 0.05$ ) in the *BAMBI* gene and its 10kb surrounding region (Figure 3). Eight TagSNPs, selected from the HapMap CEU population, were genotyped on an extensively characterized population of 1022 obese cases and 606 lean control individuals. In our Caucasian population, minor allele frequencies for rs624032, rs3758365, rs17753457, rs663378, rs17832466, rs655766, rs9663912 and rs12570151 were 35.0%, 12.1%, 3.8%, 16.9%, 31.3%, 27.0%, 15.4% and 8.5% respectively, which is similar to the frequencies found in the HapMap CEU population. HWE was present for all SNPs analyzed ( $p > 0.01$ ; data not shown). We calculated odds ratios by means of logistic regression analysis but no significant p-values were found ( $p > 0.05$ ; data not shown). In our obese population, we performed linear regression analysis, but no association was found with either BMI, weight, height, waist, hip, waist-to-hip ratio, fat free mass, fat mass, fat mass percentage, total abdominal, visceral or subcutaneous fat ( $p > 0.05$ ; Table 5).

## **Discussion**

After BAMBI was identified as a decoy receptor for TGF $\beta$ -signaling, because of its T $\beta$ RI-like extracellular domain, it was shown that BAMBI also enhances Wnt signaling [20, 21]. Activation of Wnt signaling is known to enhance osteoblastogenesis of MSCs and inhibit adipogenesis [6].

Consequently, the pathway has been linked to the development of human obesity [42]. Furthermore, a direct link between BAMBI activity and adipogenesis was found, both *in vitro* in human pre-adipocytes, and *in vivo* in the *ob/ob* and high fat diet (HFD)- induced obese mice, where BAMBI mRNA expression was decreased [22]. These results prompted us to investigate the existence of *BAMBI* mutations and/or polymorphisms that could lead to the development of monogenic or complex obesity, respectively, in our population of Belgian obese individuals. This study is, to our knowledge, the first evaluation of *BAMBI* as a candidate gene for human obesity.

By performing a mutation analysis on the *BAMBI* coding region and intron-exon boundaries in our population of 677 obese children and adolescents and 529 lean control individuals, we were able to identify 18 genetic variations (Table 3). Two variants (c.364+13C>T, c.622G>A) were found with similar frequencies in the obese and lean population and were previously reported as polymorphisms in SNP repositories [35, 40, 43]. Not taking into account these polymorphisms, we were able to identify variants in the *BAMBI* coding region in 21 obese individuals vs. 5 lean controls. This difference in variant frequency between obese (3.1%) and lean (0.9%) subjects was statistically significant ( $p = 0.004$ ). Out of 18 identified variants, 5 were located inside an intron and another 7 were located inside an exon, but resulted in a synonymous amino acid change. For these variants it is challenging to evaluate the effect on protein function and therefore, at this time, we chose not to investigate them further. However, 5 additional variants resulted in a non-synonymous amino acid change, and all of these variants were found exclusively in obese individuals (Table 3). We performed *in silico* analysis for the non-synonymous variants, using several online prediction programs, to estimate their effect on protein function [29-33]. It is clear that both R151W and C229R are most likely to influence normal protein functioning (Table 4). Because the IC domain of BAMBI was predicted to interact with both Fz and LRP6, and because these interactions presumably elicit the Wnt signalling stimulating action, we were especially interested in variants that were found in the C-terminal region. Therefore, based on evolutionary conservation and/or position in the IC domain of BAMBI, we postulated that R151W, H201R and C229R might affect the stimulatory action of BAMBI in the Wnt pathway, and as such influence normal adipogenesis in the obese patients in whom these variants were found. Co-segregation analysis of *BAMBI* C229R with obesity was uninformative because both parents of the proband are either obese or overweight, and the family members' dietary habits are unknown.

We first confirmed the presence of WT and mutant (R151W, H201R or C229R) BAMBI protein forms on the membrane of HEK293T cells, and subsequently investigated their ability to activate the canonical WNT pathway in this cell type.

Firstly, a significant increase in Wnt pathway activity was observed when we transfected the cells with both *Wnt1* and WT *BAMBI* constructs, in comparison to transfection with *Wnt1* alone, which is a

replication of a previous study [21]. Although the observed increase that was seen when adding WT BAMBI was not as explicit as described by Lin *et al.*, a similar result may be obtained by further optimization of the assay. Secondly, we observed a significant decrease in luciferase transcription from the TCF response element containing TOPFLASH vector when transfecting cells with a construct containing the R151W, H201R or C229R mutant forms of *BAMBI*, in comparison with transfection with the WT *BAMBI*. Relative to the Wnt pathway activity in HEK293T cells transfected with *Wnt1* and wild-type *BAMBI*, which is set at 100%, transfection with *BAMBI* R151W, *BAMBI* H201R or *BAMBI* C229R resulted in a decrease in Wnt signaling of 16% ( $p= 3.4 \cdot 10^{-4}$ ), 12% ( $p= 0.003$ ) and 9% ( $p=0.035$ ) respectively (Figure 2). Wnt pathway activation in cells transfected with a mutant *BAMBI* is still higher than cells transfected with *Wnt1* only, without overexpression of a BAMBI protein (relative decrease in activity of 28%,  $p= 3.8 \cdot 10^{-4}$ ). However, this may be due to the activity of endogenous BAMBI and a limitation of our study is that we did not knock down wild-type BAMBI presence in HEK293T cells which was shown in a previous study to impair Wnt signalling [21]. In addition, we did not investigate the effects of the mutant BAMBI proteins on endogenous TGF $\beta$  signalling.

It is thus clear that without cosegregation analysis in the pedigree and additional functional research, including investigation of the effect of the mutant BAMBI proteins on differentiation of hMSCs, we cannot firmly establish that the identified variants are causative for the obese phenotype of the patients.

Furthermore, we also performed an association analysis on nearly all of the genetic variation in the *BAMBI* gene region including BMI and several adiposity parameters. However, both logistic and linear regression analysis, with several obesity parameters, did not result in statistically significant p-values (Table 5). At this time, it thus appears that common polymorphisms in the *BAMBI* gene region are not influencing complex obesity in our population, although this lack of association might be due to a lack of power. Because effect sizes of polymorphisms associated with complex diseases are typically small, large populations are needed to detect them and increasing our population size may thus reveal hidden association signals.

In conclusion, we performed a mutation analysis on *BAMBI* and identified 18 variants in our population of obese children, adolescents and lean controls. When excluding previously reported variants that were found in both our obese and lean population, the total variant frequency was significantly higher in obese vs. controls and in addition, all 5 variants resulting in a non-synonymous amino acid change were found in the obese. These genetic variations represent the first naturally occurring missense variants of *BAMBI* in obese patients. R151W, H201R and C229R were selected for further functional analysis and all three variants significantly reduced Wnt signaling enhancing capacity of BAMBI in a Wnt luciferase reporter assay, even though their cellular localisation appears unchanged. Although we were not able to perform pedigree analysis on the variants, the finding of

several non-functioning *BAMBI* alleles in human families affected by obesity should encourage further study of this gene in other obese populations. In addition, we excluded the involvement of common *BAMBI* polymorphisms with human complex obesity in our population.

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## Figure Legends

**Figure 1:** BAMBI protein structure and the position of identified non-synonymous variants.

Graphical representation of the human BAMBI protein. Non-synonymous amino acid changes found in our population are indicated in white boxes. The variant depicted in a grey box was found both in obese and lean individuals. Asterisks were placed with variants that were previously found and have been described in the dbSNP database. Numbers indicate amino acid position. SP, signal peptide; EC, extracellular domain; Tm, transmembrane domain; IC, intracellular domain.

**Figure 2:** Wnt luciferase assay results. Results from the Wnt luciferase assays are shown, whereby HEK293T cells were transfected with either Wnt1, or Wnt1 in addition to a *BAMBI* WT or mutated construct. The data represent the mean  $\pm$  SD, relative to the luciferase activity from the experiment in which WT *BAMBI* was used, which was set at 100 (\*\*,  $p < 0.005$ ; \*,  $p < 0.05$ ).

**Figure 3:** *BAMBI* gene structure and position of genotyped tagSNPs. The human *BAMBI* gene structure is depicted, including the 10kb up- and downstream region. The position of the tagSNPs that were genotyped in this study is shown. Numbers indicate position on chromosome 10.

## Tables

**Table 1:** Characteristics of overweight and obese children and adolescents and of the control population

	Children	Adolescents	Control samples
<b>N</b>	325	352	529
<b>Age (years)</b>	8 ± 2	15 ± 2	35 ± 7
<b>Male/female</b>	155/170	144/208	186/343
<b>Weight (kg)</b>	53.5 ± 16.2	96.9 ± 19.8	65.3 ± 8.8
<b>Height (m)</b>	1.4 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
<b>BMI (kg/m<sup>2</sup>)</b>	27.4 ± 4.1	34.7 ± 5.3	22.1 ± 1.7
<b>BMI Z-score</b>	2.6 ± 0.6	2.5 ± 0.4	-

Mean value ± standard deviation is given. BMI Z-score for children and adolescents was based on data from the Flemish Growth Charts [24].

**Table 2:** Characteristics of obese subjects and of the control samples used in the association study

Parameter	Obese cases	Controls
<b>N</b>	1022	606
<b>Age (years)</b>	41 ± 12	35 ± 7
<b>Male/female</b>	473/549	223/383
<b>Weight (kg)</b>	111.3 ± 21.7	65.6 ± 9.0
<b>Height (m)</b>	1.7 ± 0.1	1.7 ± 0.1
<b>BMI (kg/m<sup>2</sup>)</b>	38.1 ± 6.2	22.0 ± 1.6

Mean value ± standard deviation is given for the obese and control population. Only individuals with a minimum genotype call rate of 87.5% (7 of 8 tagSNPs genotyped) were included.

**Table 3:** *BAMBI* variations found in our cohort of obese children and adolescents and/or in control samples

Nucleotide change	Amino acid change	Type of variation	Frequency in cases	Frequency in control samples	rs-number
c.76+28G>A	-	Intronic	1/677	1/529	-
c.76+71C>T	-	Intronic	-	1/529	-
c.77-4T>C	-	Intronic	5/677	2/529	-
c.105C>T	A35A	Synonymous	-	1/529	-
c.173C>G	P58R	Non-synonymous	1/677	-	rs143207916
c.228G>A	T67T	Synonymous	1/677	-	-
c.230C>A	T77K	Non-synonymous	1/677	-	rs138814781
c.315T>C	N105N	Synonymous	2/677	-	rs146990085
c.363A>C	S121S	Synonymous	1/677	-	rs61729233
c.364+13C>T	-	Intronic	9.45%	9.66%	rs56281747
c.365-6C>G	-	Intronic	1/677	-	-
c.376A>C	R126R	Synonymous	3/677	-	rs34535452
c.441G>A	E147E	Synonymous	1/677	-	-
c.451C>T	R151W	Non-synonymous	1/677	-	-
c.602A>G	H201R	Non-synonymous	1/677	-	-
c.622G>A	V208I	Non-synonymous	11/677	11/529	rs72809669
c.685T>C	C229R	Non-synonymous	1/677	-	-
c.717C>T	N239N	Synonymous	1/677	-	rs142589357

Nucleotide change describes nucleotide position of the sequence variant, whereby the first A of the translation start site was counted as number 1. All variants were found in heterozygous state except for c.364+13C>T, for which the MAF is given as a percentage. Rs-numbers of variants are given if they were described previously in the dbSNP database (build GRCh37.p10).

**Table 4:** Non-synonymous variants in Bambi and their *in silico* predicted effect on protein function

Amino acid change	SIFT	Polyphen2	MutPred	SNP&Go	Consurf
P58R	Tolerated	Probably damaging	$g = 0.622$ ; Gain of helix ( $p=0.012$ ), Loss of loop ( $p=0.013$ )	Neutral (RI=1)	3
T77K	Tolerated	Benign	-	Neutral (RI=4)	1
R151W	Damaging	Probably damaging	-	Disease (RI=2)	9
H201R	Tolerated	Benign	-	Neutral (RI=7)	6
V208I	Tolerated	Benign	-	Neutral (RI=8)	4
C229R	Damaging	Probably damaging	$g = 0.80$ ; Gain of disorder ( $p = 0.023$ )	Disease (RI=6)	8

All non-synonymous amino acid substitutions that were identified in the *BAMBI* coding region in our population are listed here. All variants were exclusively found in obese individuals, except for V208I, which was found with a similar frequency in obese and lean individuals. The effect of the genetic variants on protein function or an estimate of conservation as predicted by SIFT [29], Polyphen-2 [30], MutPred [31], SNPs&GO [32] and the Consurf database server [33] is given. Mutpred output contains both a probability score ( $g$ ) and a  $p$ -value. Only the output with  $g > 0.5$  and  $p < 0.05$  is given, which are the cut-off values for a actionable hypothesis. A score with a  $g > 0.75$  and  $p < 0.05$  is considered a (very) confident hypothesis. The SNP&Go RI-score reports the Reliability Index of the prediction, scoring from 0 (unreliable) to 10 (reliable). The Consurf conservation score is defined as follows: 1-3: variable conservation; 4-6: average conservation and 7-9: high conservation.

**Table 5:** Results of the linear regression analysis for the total obese population

	<b>Rs62403</b>	<b>Rs375836</b>	<b>Rs1775345</b>	<b>Rs66337</b>	<b>Rs1783246</b>	<b>Rs65576</b>	<b>Rs966391</b>	<b>Rs1257015</b>
	<b>2</b>	<b>5</b>	<b>7</b>	<b>8</b>	<b>6</b>	<b>6</b>	<b>2</b>	<b>1</b>
<b>BMI</b> (kg/m <sup>2</sup> )	0.964	0.942	0.383	0.289	0.996	0.453	0.778	0.427
<b>Weight</b> (kg)	0.704	0.615	0.401	0.235	0.718	0.471	0.752	0.438
<b>Height</b> (m)	0.446	0.372	0.887	0.945	0.841	0.595	0.499	0.782
<b>Waist</b> (cm)	0.397	0.767	0.134	0.688	0.923	0.444	0.370	0.719
<b>Hip</b> (cm)	0.859	0.569	0.565	0.716	0.447	0.965	0.515	0.234
<b>WHR</b>	0.660	0.559	0.207	0.583	0.572	0.384	0.263	0.658
<b>ffm</b> (kg)	0.073	0.878	0.932	0.316	0.533	0.434	0.154	0.620
<b>Fm (kg)</b>	0.702	0.565	0.335	0.151	0.754	0.273	0.941	0.355
<b>Fm%</b>	0.438	0.326	0.334	0.563	0.481	0.260	0.407	0.158
<b>TAT</b> (cm <sup>2</sup> )	0.800	0.708	0.499	0.640	0.816	0.707	0.787	0.326
<b>VAT</b> (cm <sup>2</sup> )	0.223	0.525	0.152	0.851	0.490	0.482	0.553	0.567
<b>SAT</b> (cm <sup>2</sup> )	0.488	0.267	0.889	0.828	0.658	0.838	0.854	0.190

P-values are given and were calculated by linear regression with age and sex as a covariates (SPSS 20.0). Hip, hip circumference; WHR, waist-to-hip ratio; ffm, fat free mass; fm, fat mass; fm%, fat mass percentage; TAT, total adipose tissue; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue

Figures

Figure 1

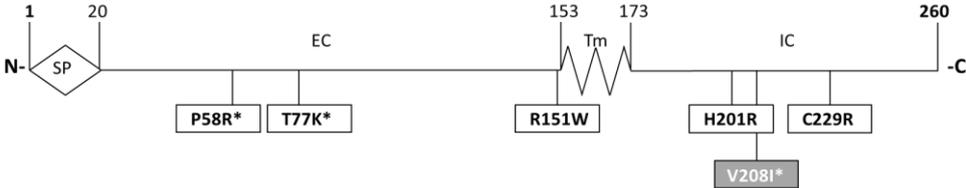


Figure 2

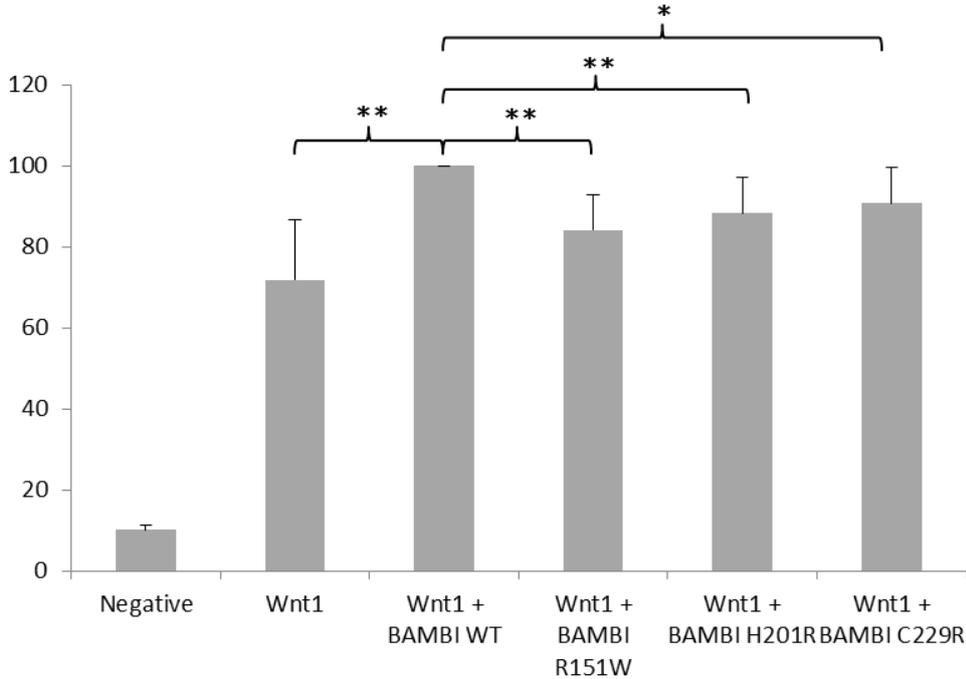


Figure 3

