CLINICAL STUDY

Identification and functional characterization of a missense mutation in resistin in two patients with severe obesity and insulin resistance

Sigri Beckers, Fenna de Freitas, Doreen Zegers, An Verrijken¹, Armand V Peeters, Frida Peiffer¹, Stijn L Verhulst², Guy Massa³, Ilse L Mertens¹, Kristine N Desager², Luc F Van Gaal¹ and Wim Van Hul

Department of Medical Genetics, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium, ¹Department of Endocrinology, Diabetology and Metabolism, Antwerp University Hospital, Wilrijkstraat 10, 2650 Antwerp, Belgium, ²Department of Paediatrics, Antwerp University Hospital, Wilrijkstraat 10, 2650 Antwerp, Belgium and ³Department of Paediatrics, Jessa Hospital, Stadsomvaart 11, 3500 Hasselt, Belgium

(Correspondence should be addressed to W Van Hul; Email: wim.vanhul@ua.ac.be)

Abstract

Objective: In this study, we hypothesized that mutations in the resistin encoding gene, *RETN*, may cause a monogenic form of obesity.

Design/methods: We screened the coding region of *RETN* in 81 morbidly obese adults, 263 overweight and obese children/adolescents, and 116 healthy lean subjects. *In vitro* experiments include qPCR, ELISA, and western blot for WT and mutant resistin transfected into 3T3-L1 adipocytes.

Results: Mutation analysis identified five sequence variants in our patient populations: 3'-UTR +87 G/A, 3'-UTR +100 A/G, T73T, IV3-61 C/A, and C78S. In our control population, we only found the 3'-UTR +87 G/A variant. We started functional experiments for the C78S mutation that was found in a 20-year-old obese male (body mass index (BMI)=39.7 kg/m²) and his obese mother (BMI=31.9 kg/m²). *In vitro* testing demonstrated that the mutation does not impair mRNA expression. We identified a 100-fold lower extracellular protein concentration for mutant resistin compared with WT levels using a resistin ELISA on cell culture medium ($P=4.87 \times 10^{-6}$). We also detected a decreased intracellular concentration for the mutant protein (tenfold lower relative levels, $P=0.007$). The plasma resistin levels of the proband and his mother, however, did not differ significantly from lean control individuals.

Conclusions: In conclusion, we identified the first missense mutation in resistin in a morbidly obese proband and his obese mother. Functional testing of the mutant protein suggests that the C78S mutant protein is degraded, possibly resulting in a decreased extracellular concentration, which may predispose to obesity.

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Introduction

Resistin is one of the most controversial adipokines. After its initial discovery in mice, resistin was thought to be the link between obesity and diabetes (1–3). This was suggested because of the reported expression and secretion of resistin by adipocytes and because of the increased concentration of the protein in mice with diet-induced and genetic forms of obesity. Furthermore, it was seen that administration of resistin impaired glucose tolerance and increased insulin resistance (IR) in mice. Neutralization of endogenous resistin by antibodies improved insulin sensitivity in mice with diet-induced obesity (1). An inhibitory effect of resistin on adipose conversion could also be demonstrated *in vitro* (3, 4). In addition, it was also suggested that resistin may be involved in inflammation due to its homology to FIZZ1 (2). Further studies demonstrated

that lowering resistin levels in high fat-fed mice to levels of mice fed a standard chow can completely reverse IR in these animals (5). Resistin knockout mice have reduced blood glucose levels after fasting and are more glucose tolerant when fed a high-fat diet (6). In mice overexpressing resistin, the high levels of the protein cause glucose intolerance, hyperinsulinemia, and hypertriglyceridemia accompanied by whole-body IR (7). Transgenic mice overexpressing dominant inhibitory resistin show increased adiposity and enlarged adipocyte size (4). Together, these results demonstrate that resistin is involved in obesity and IR in rodents.

As homology between mouse and human resistin is low (only 55.8% on protein level), it is not surprising that the role of resistin in humans is less clear. Although resistin is predominantly expressed in adipocytes in mice, its expression in humans is most abundant in

mononuclear cells/macrophages (8, 9). In obesity, it has been shown that macrophages infiltrate the adipose tissue (10, 11) and this could therefore explain the reports of resistin expression in adipose tissue. Furthermore, serum levels of obese humans were found to be higher than those in lean control subjects (12, 13). Other reports, however, did not find any relationship between resistin serum levels, body weight, and obesity (14, 15). Several genetic studies have focused on the possible association between resistin polymorphisms and phenotypes of obesity or IR. We previously reported that we found no association of SNPs in *RETN* with complex obesity in women (16). These results were in line with association studies in other populations (17, 18). Some studies, however, reported that SNPs in resistin were associated with higher body mass index (BMI) or other obesity parameters (19, 20). Other studies have shown that resistin variants may have a role in the development of type 2 diabetes (20, 21), although these associations were not confirmed by others (22, 23). This clearly shows that the role of resistin in humans and its possible involvement in obesity and associated IR remains controversial.

Until now, no mutations in human resistin have been reported. In a strain of diet-resistant rats, an L9V mutation in the signal sequence of resistin was described. This mutation did not affect protein secretion as mutant resistin was detected in the culture medium of transfected 3T3-L1 cells. However, differentiation of 3T3-L1 adipocytes expressing mutant resistin was inhibited *in vitro*, although WT resistin stimulated adipogenesis in these experiments (24).

In this study, we hypothesized that mutations in resistin may be involved in the development of obesity. Discovery of such a mutation would enhance our understanding of resistin's role in humans.

Research design and methods

Study populations

Totally, 263 overweight and obese children and adolescents (98 boys and 165 girls; Table 1) were recruited from the Antwerp University Hospital and Virga Jesse Hospital, Hasselt. The young patient

population has a mixed ethnicity, which may have an effect on resistin expression as previously reported (25, 26). A population of 81 morbidly obese Caucasian adults was recruited from the Antwerp University Hospital outpatient obesity clinic. A control population of 116 lean Caucasian subjects was also included (BMI between 18.5 and 25 kg/m²; Table 1). All participants gave their written informed consent. The study protocol was approved by the local ethics committee.

Anthropometry

Height was measured to the nearest 0.5 cm and body weight was measured with a digital scale to the nearest 0.1 kg. BMI was calculated as weight (kg) over height (m) squared (kg/m²).

Laboratory analyses

A fasting blood sample was taken for analyses. Plasma glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, γ -glutamyltransferase, and uric acid were measured on the Vitros 5.1 FS Chemistry System (Ortho-Clinical Diagnostics, Rochester, NY, USA). Low-density lipoprotein (LDL) cholesterol was calculated with the Friedewald formula (27). High-sensitive C-reactive protein (CRP) was determined on a BN II analyzer (Siemens, Munchen, Germany). Insulin and C-peptide levels were measured on a Modular E170 system (Roche Diagnostics). IR was estimated using the homeostatic model assessment of IR (HOMA-IR) (28). HOMA-IR was calculated as (insulin in μ U/ml $\times$ glucose in mmol/l)/22.5, with 1 as a reference value.

Mutation analysis

We analyzed the coding region of *RETN* (exons 2–4) and part of the 3'-UTR. Exon 2, exon 3, and the respective intron–exon boundaries were screened using denaturing HPLC (DHPLC) or high-resolution melting curve analysis (HRM). DHPLC was performed on a WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Inc., Glasgow, UK). HRM was performed using the Lightcycler LC480 Real-Time PCR System (Roche) with incorporation of LCGreen+ fluorescent dye (Idaho Technology, Inc., Salt Lake City, UT, USA) into the PCR product. Samples with chromatograms or melting curves deviating from WT were sequenced. Due to the presence of several polymorphisms in the amplicon containing exon 4, this exon and the respective intron/exon boundaries were checked using direct sequencing. Sequencing was performed with ABI BigDye Terminator v1.1 Cycle Sequencing kits on an ABI Prism Genetic Analyzer 3130xl (Applied Biosystems, Inc., Foster City, USA). The melanocortin-4 receptor gene (*MC4R*) was screened as described before (29). Primer sequences for mutation analysis of *RETN* are as follows: exon 2 DHPLC forward 5'-gTtcccTcTTTcAgcgcTgc-3'

Table 1 Characteristics of study populations. Mean $\pm$ S.E.M. is given for every parameter. Body mass index (BMI) z-score based on Flemish Growth Charts (30) is shown only for children and adolescents.

	Adults	Children	Adolescents	Controls
<i>n</i>	81	87	176	116
Men/women	14/67	36/51	62/114	14/102
Age (years)	43.6 $\pm$ 1.2	8.9 $\pm$ 0.2	15.9 $\pm$ 0.2	38.8 $\pm$ 0.9
BMI (kg/m ²)	47.8 $\pm$ 0.5	28.2 $\pm$ 0.5	35.0 $\pm$ 0.4	22.6 $\pm$ 0.2
BMI z-score	–	+2.7 $\pm$ 0.1	+2.5 $\pm$ 0.0	–

and reverse 5'-gTgccAgggATcAgTgAggTcT-3', exon 2 HRM forward 5'-ATccAgcTgTgggTcTcTTggT-3' and reverse 5'-TTggggAgcTTgcccAAGTgg-3', exon 2 sequencing forward 5'-gTccAggggcAgATccTAc-3' and reverse 5'-gTggATAAggTggggAcAgT-3', exon 3 DHPLC forward 5'-cTgTcTccTTTccTccTgccc-3' and reverse 5'-ggAcAAcAgTcTcc-TgcAcTcAc-3', exon 3 HRM forward 5'-cTgTcTccTTTccTccTgccc-3' and reverse 5'-ggAcAAcAgTcTccTgcAcTcAc-3', exon 3 sequencing forward 5'-cTccAAAAcAcccAATcAAG-3' and reverse 5'-gAgTgAgggTggAcAcAg-3', exon 4 sequencing forward 5'-cAcTggTgTccA-cccTcAc-3' and reverse 5'-cAGTTggAgAccccATAggA-3'.

Cell culture

3T3-L1 cells were maintained in DMEM supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (basal medium; Invitrogen). Cells were incubated at 37 °C in humidified air containing 5% CO₂. To stimulate differentiation to adipocytes, cells were allowed to grow to confluency. After 2 days' confluency, differentiation was induced by adding 5 µg/ml insulin (Sigma–Aldrich), 0.25 µM dexamethasone (Alexis Biochemicals, Lausen, Switzerland), and 0.5 mM iso-butyl-methyl-xanthine (Sigma–Aldrich) to basal medium. After 2 days, this medium was renewed. On day 4 after inducing differentiation, medium was changed to basal medium supplemented with 5 µg/ml insulin. From day 6, cells were cultured in basal medium until transfection. Medium was renewed every 2 days. Differentiation was visually checked after staining with Oil Red O using a Lipid Staining kit (Zen-Bio, Research Triangle Park, NC, USA).

Vectors

Wild-type resistin cDNA cloned in a pCMV6-XL4 expression vector and an empty pCMV6-XL4 expression vector were obtained from Origene Technologies (Rockville, MD, USA). Mutant resistin carrying a C78S mutation was created using a Quikchange II Site Directed Mutagenesis kit (Stratagene, USA). All cloned products were sequenced as described above. A pEGFP-N1 mammalian expression vector (Clontech) was used as internal control in all transfection experiments.

Transfection

3T3-L1 adipocytes were transfected on day 12 of differentiation. Cells (2×10^6 cells/transfection) were co-transfected with 2 µg WT or mutant resistin in pCMV6-XL4 expression vector and 2 µg pEGFP-N1, as internal control. Transfection was performed using the Nucleofector II device with Cell Line Nucleofector solution L according to the manufacturer's protocol (Amaxa, Germany). After transfection, cells were

cultured in 24-well plates (5×10^5 cells/well) and incubated for 24 h before other assays were performed.

EGFP measurement

EGFP fluorescence was measured with a Wallac Victor² Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA) 24 h after transfection. Fluorescence measurements of 1 s per well were obtained. These measurements were used to determine transfection efficiency (30).

Total protein measurement

The total amount of protein in cell lysates was determined by BCA Protein Assay (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Pre-diluted BSA was used as a standard (Pierce Biotechnology). Absorbance at 562 nm was measured by a Wallac Victor² Multilabel Plate Reader (Perkin Elmer).

Resistin mRNA expression

RNA was isolated from transfected 3T3-L1 adipocytes 24 h after transfection. RNA isolation and cDNA synthesis was performed using a TaqMan Gene Expression Cells-to-Ct Kit (Applied Biosystems, Inc.). Expression analysis was performed with TaqMan Gene Expression assays (Applied Biosystems, Inc.) according to manufacturer's protocol. The *RETN* transcription levels were normalized by three stably expressed reference genes (*GAPDH*, *UBC*, and *ACTB*). The following assays were used: *RETN*, Hs00982492_g1; *GAPDH*, Mm99999915_g1; *UBC*, Mm01201237_m1; *ACTB*, Mm01205647_g1. Real-time PCR was performed on a Lightcycler LC480 Real-Time PCR System. We compared 14 WT samples with 14 mutant samples. Every run contained a no-template control and all samples were analyzed in duplex. Raw data were imported into the relative quantification software qBase (31) for further analysis. A Mann–Whitney *U* non-parametrical test was used to check the statistical significance ($P < 0.05$) of the obtained results (SPSS, Inc., Version 15.0, Chicago, IL, USA).

RNA from patients carrying the *RETN* mutation was isolated from blood collected in PAXgene Blood RNA Tubes using the PAXgene Blood RNA kit according to the manufacturer's protocol (PreAnalytiX, Hombrechtikon, Switzerland). cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR with random hexamers (Invitrogen).

PCR on cDNA was performed with primers specific for human resistin mRNA (sequences available on request) using standard conditions. Resulting PCR products were sequenced according to the protocol described above to determine whether the mutant mRNA is expressed.

Resistin protein expression

Detection of WT and mutant resistin protein in cell lysates and conditioned media from transfected 3T3-L1 cells was carried out by western blotting. Samples were separated by electrophoresis on 4–20% polyacrylamide-SDS gels under reducing conditions. The proteins were transferred to PVDF membranes by semi-dry transfer and blocked overnight with 5% (w/v) skimmed milk. Membranes were blotted with polyclonal rabbit anti-resistin antibody (1:5000 dilution; Abcam, Cambridge, UK), followed by goat anti-rabbit IgG HRP conjugate (1:10 000; Bio-Rad). After incubation with ECL western blotting substrate (Pierce Biotechnology) according to the manufacturer's conditions, bands were visualized using a Fuji Medical Film Processor FPM-100A (Fuji Photo, Tokyo, Japan).

Resistin ELISA

Resistin concentrations in conditioned media of transfected 3T3-L1 adipocytes and cell lysates were determined by ELISA (Biovendor Medical Laboratory, Brno, Czech Republic) following instructions from the manufacturer. Absorbance was read at 450 nm in a Wallac Victor² Multilabel Plate Reader (Perkin Elmer). The amount of measured resistin was corrected for transfection efficiency based on the EGFP measurements and total amount of protein by BCA protein assay. The relative levels of mutant protein was compared with WT levels (set at 100%) by a one-sample *t*-test (SPSS, Inc., Version 15.0). Plasma resistin levels in blood samples were also measured by ELISA. Each sample was measured in duplicate in three different experiments. Mean values were compared using a one-way ANOVA with LSD *post hoc* tests (SPSS, Inc., Version 15.0).

Resistin tissue expression

We performed a PCR on cDNA from multiple human tissues (Clontech) to examine resistin expression differences. cDNA from five tissues (lung, skeletal muscle, small intestine, and prostate) was compared with adipose tissue (PrimerDesign, Southampton, UK) as a positive control. We also included cDNA from the proband and his parents (originating from RNA extracted from blood). All samples were amplified in a 25 µl reaction volume with GoTaq polymerase according to the manufacturer's protocol (Promega). Primers used for resistin were 5'-cTggggcTgTTggTgTcT-3' and 5'-cTggcAgTgAcATgTggTcT-3'. GAPDH was amplified as internal control using following primers: 5'-TgccAAATATgATgAcAT-cAAGAA-3' and 5'-ggAgTgggTgTcgcTgTTg-3'. The PCR was paused after 22, 26, 30, and 34 cycles to remove 5 µl of product from the wells. To quantify the expression, 5 µl of PCR product was loaded onto a 1% agarose gel stained with ethidium bromide.

Results

Mutation analysis

We recruited two populations of obese individuals: one cohort of 81 morbidly obese adults (mean BMI = 47.8 ± 0.5 kg/m², 21–69 years) and a cohort of 263 overweight/obese children and adolescents (mean BMI = 32.8 ± 0.4 kg/m², 0–20 years). Characteristics of the patient populations are shown in Table 1.

Several known polymorphisms were identified in all study populations (rs3219177, rs12981326, rs1042265, rs3745368, rs3745369, and rs3833230; data not shown).

Mutation analysis of the coding region of the *RETN* gene revealed no coding mutations in the adult cohort. One 34-year-old female (BMI = 43.6 kg/m²) was found to carry an intronic variation, IV3-61 C/A. Screening of resistin in the population of children and adolescents identified two heterozygous variations in the 3'-UTR, one heterozygous synonymous coding variation, and one heterozygous missense mutation; one male adolescent (20 years old) with a BMI of 50.6 kg/m² carried a 3'-UTR +100 A/G variation; and two patients harbored a 3'-UTR +87 G/A variant: one 19-year-old boy with a BMI of 30.2 kg/m² and one 8-year-old boy with a BMI of 28.7 kg/m², which equals obesity at this young age (based on the Flemish Growth Charts (32)). We also found a T73T variation in a 13-year-old boy (BMI = 33.37). Furthermore, we identified a heterozygous C78S mutation in a 20-year-old Caucasian male with severe obesity (BMI = 39.7 kg/m²). Clinical characteristics of all mutation and variation carriers are shown in Table 2. Screening of our control population identified the 3'-UTR +87 G/A variation in one lean female (BMI = 19.6 kg/m²). None of the other variations were identified in control subjects.

Clinical characteristics of C78S carriers

The C78S mutation is the first missense mutation reported in resistin. This mutation was found in a severely obese, insulin-resistant male with a BMI of 39.7 at 20 years of age (weight = 128.6 kg, height = 1.80 m). The proband reported that he had longstanding obesity since childhood, although he was born with a normal birth weight. He has been enrolled in a weight management program and was considered to be a good weight loss respondent. With a classical life style intervention program in addition to treatment with orlistat, he was able to lose 18 kg over 4 months. However, he suffered from progressive weight regain after withdrawal of dietary measures. At the age of 28, the proband weighed 141.6 kg (BMI = 43.7 kg/m²). Major metabolic features of the proband and his mother are shown in Table 3.

The C78S mutation was found to be inherited from his obese, insulin-resistant mother (BMI = 31.9 kg/m²).

Table 2 Clinical characteristics of variation/mutation carriers. Body mass index (BMI) z-scores are based on Flemish Growth Chart data (30).

Variation/mutation	Age (years)	Sex	Weight (kg)	Height (m)	BMI (kg/m ²)	BMI z-score	Weight class
IV3-61 C/A	34	F	143.0	1.81	43.6	–	Obesity
T73T	13	M	102.2	1.75	33.4	+2.4	Obesity
C78S	20	M	128.6	1.80	39.7	+3.2	Obesity
	49	F	85.8	1.64	31.9	–	Obesity
3'-UTR +87 G/A	8	M	69.0	1.55	28.7	+2.8	Obesity
	19	M	95.6	1.78	30.2	+2.2	Obesity
	22	F	1.68	55.4	19.6	–	Normal weight
3'-UTR +100 A/G	20	M	186.7	1.92	50.6	+3.7	Obesity

F, female; M, male.

She was reported to suffer from hypercholesterolemia and high blood pressure by her family doctor. Furthermore, she has an impaired fasting glucose with an elevated HbA1c suggestive for disturbed glucose tolerance (Table 3). His father was also morbidly obese (BMI=42.5 kg/m²) but did not carry a mutation in resistin. He also had increased HOMA-IR but showed normal fasting glucose levels (Table 3). Screening of *MC4R* did not identify any additional mutations.

mRNA expression of mutant and WT resistin

To confirm that the C78S mutation in resistin does not influence resistin mRNA expression, we isolated RNA from 3T3-L1 cell cultures and performed RT-PCR. We were able to demonstrate that the C78S mutation does not impair absolute mRNA expression of resistin. Sequencing of cDNA confirmed that the isolated mRNA harbors the C78S mutation (Fig. 1A–C). Quantitative analysis showed no significant difference in expression levels between WT and mutant resistin ($P > 0.05$).

Furthermore, we also studied mRNA levels of resistin in blood from the proband and his parents. We found

that both mutation carriers showed expression of resistin. Sequencing of cDNA revealed that both WT and mutant mRNA were present (Fig. 1D).

Analysis of mutant and WT resistin protein

Differentiated 3T3-L1 adipocytes were transfected with WT or mutant human resistin. To confirm proper differentiation to adipocytes, we stained 3T3-L1 cells with Oil Red O for every experiment. Differentiation was always present (data not shown).

We investigated by western blotting whether the mutant protein is expressed in differentiated 3T3-L1 adipocytes. We found that WT resistin was present in cell culture medium of transfected cells (Fig. 2) but was undetectable in cell lysates (data not shown). For cells transfected with mutant resistin, we could not detect the protein in medium nor in cell lysates (Fig. 2). In cells transfected with both WT and mutant resistin, we were only able to identify a faint band at about 12.5 kDa in medium (Fig. 2). These results suggest that the mutation might cause impaired secretion of the protein. Furthermore, we found no evidence for intracellular accumulation of mutant protein. Some aspecific banding remains at 10 and 15 kDa, which we suspect to represent mouse resistin or 'resistin-like molecule' (RELM) proteins as these are highly related to resistin. These additional bands also show that equal loading was present in all lanes.

We performed an ELISA to confirm these initial results. The concentration of WT or mutant human resistin was determined in the cell culture medium and cell lysates by ELISA 24 h after transfection. We used a human resistin-specific ELISA, which shows no cross-reactivity with mouse protein; therefore, we are confident that our results only represent human resistin levels. After correction for transfection efficiency and total amount of protein, we found that the relative amount of mutant resistin secreted in the cell culture medium was significantly reduced compared with secretion of the WT protein. The relative levels of mutant resistin in medium were 100-fold lower than the WT levels ($P = 4.87 \times 10^{-6}$; Fig. 3A). When we co-transfected cells with equimolar amounts of WT and mutant resistin, we found that the relative level of

Table 3 Biochemical characterization of the mutation carriers.

Parameter	Proband	Mother	Father
Age (years)	20	49	52
Glucose (mmol/l)	4.22	6.33	5.06
Insulin (μU/ml)	25	27.2	18.1
C-peptide (nmol/l)	0.53	1.76	1.43
HOMA-IR	4.7	7.6	4.1
HbA1c (%)	ND	6.3	ND
γ-GT (U/l)	23	38	ND
Cholesterol (mg/dl)	210	248	236
HDL cholesterol (mg/dl)	45	56	46
LDL cholesterol (mg/dl)	138	161	148
Triglycerides (mg/dl)	136	155	209
Uric acid (mg/dl)	7.1	5.9	ND
CRP (mg/dl)	ND	1.24	0.39

CRP, C-reactive protein; γ-GT, γ-glutamyl-transpeptidase; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; ND, not determined. HOMA-IR is calculated as $(\text{insulin } (\mu\text{U/ml}) \times \text{glucose (mmol/l)}) / 22.5$ with 1 as a reference value for normal insulin sensitivity.

Figure 1 Detection of resistin mRNA expression. (A–C) Detection of resistin mRNA expression in transfected 3T3-L1 cells: mRNA was isolated from 3T3-L1 adipocytes transfected with empty pCMV6-XL4 expression vector, pCMV6-XL4 with WT resistin, pCMV6-XL4 with mutant resistin, or a combination of both. Cells transfected without DNA or without application of the electroporation were used as negative controls. RT-PCR was performed and resulting products were sequenced. Electropherograms are shown for cells transfected with WT RETN (A), mutant RETN (B), or a combination of both (C). (D) Detection of resistin mRNA expression in plasma from the proband and his parents: RNA was isolated from blood collected in PAXgene Blood RNA Tubes using the PAXgene Blood RNA kit. RT-PCR was performed and the resulting PCR products were sequenced. Electropherograms showing the resistin sequence is shown from the proband, his mother who also carries the mutation and his WT father.

secreted resistin was about half of the level seen in cells transfected with WT protein ($P=2.048 \times 10^{-4}$; Fig. 3A).

When comparing WT and mutant levels in cell lysates (corrected for transfection efficiency and total amount of protein), it became clear that the amount of intracellularly stored mutant resistin was also lower than the amount of intracellular WT resistin (tenfold lower relative levels, $P=0.007$; Fig. 3B). In cells expressing WT and mutant resistin together, a reduction in intracellular resistin levels was observed, but this difference was not statistically significant ($P=0.451$; Fig. 3B). Together, these results suggest that the mutant resistin is degraded, most likely due to misfolding.

Analysis of plasma resistin in patients and controls

Plasma resistin levels were measured by ELISA in the proband, his parents, and three lean control individuals (Table 4). We found that no difference in resistin levels was present between the mutation carriers, the lean controls, and the obese father carrying WT resistin ($P=0.596$). *Post hoc* comparisons also showed no difference in plasma levels between the lean controls and the mutation carriers ($P=0.598$). Furthermore, the resistin plasma levels in the WT obese father did not differ from resistin concentrations in the mutation carriers ($P=0.590$) or the lean controls ($P=0.329$).

Resistin tissue expression

Because of the discrepancy between resistin levels *in vitro* (3T3-L1 adipocytes) and plasma, we decided to compare resistin expression in multiple human tissues

using a semi-quantitative PCR. We found that resistin expression is highest in lung, followed by adipose tissue as expected (Fig. 4).

Discussion

Resistin is considered as one of the most controversial adipokines to date. In mice, it is now accepted that this protein is expressed in adipocytes and has a role in linking obesity and type 2 diabetes (33). However, in

Figure 2 Detection of resistin protein in conditioned medium by western blot. Differentiated 3T3-L1 adipocytes were co-transfected with a WT or mutant resistin containing expression vector or a combination of both and a pEGFP-N1 expression vector to correct for transfection efficiency. Conditioned media and lysates were collected 24 h after transfection. Resistin was detected by western blot by incubation with polyclonal rabbit anti-resistin antibody followed by goat anti-rabbit IgG HRP conjugate. A clear band representing resistin is visible in cells transfected with WT and a faint band is also seen in cells transfected with both WT and mutant resistin (indicated with an arrow). Aspecific bands probably representing mouse RETN or RELM family members are visible at 10 and 15 kDa. Lane 1, cells transfected with empty pCMV6-XL4 expression vector. Lane 2, cells transfected with WT resistin. Lane 3, cells transfected with mutant resistin. Lane 4, cells transfected with both WT and mutant resistin.

Figure 3 Relative amount of WT or mutant resistin present in cell culture medium and cell lysates of transfected cells. Differentiated 3T3-L1 adipocytes were co-transfected with a WT or mutant resistin containing expression vector or a combination of both and a pEGFP-N1 expression vector to correct for transfection efficiency. Conditioned media and lysates were collected 24 h after transfection and the amount of resistin was measured by ELISA. The amount of secreted resistin was corrected for transfection efficiency by EGFP measurements and for total amount of protein by BCA measurement on cell lysates. The amount of resistin relative to WT is given. Differences between constructs were calculated by *t*-tests. (A) Relative amount of resistin present in conditioned media. * $P=2.048 \times 10^{-4}$; ** $P=4.87 \times 10^{-6}$. (B) Relative amount of resistin present in cell lysates. * $P=0.007$.

humans, much more debate exists about its expression and possible function. First of all, it is not clear whether resistin can be considered to be an adipokine as most reports now agree that resistin is expressed in mononuclear cells and not adipocytes (8, 9). Although, in obesity, adipose tissue is infiltrated by macrophages and may as such secrete resistin (10, 11). Reports linking the protein to obesity and type 2 diabetes have also been conflicting (20–23). Furthermore, recently, it was suggested that resistin may have an important function in inflammation (34–36).

In this study, we aimed to help clarify the role of resistin in obesity by undertaking a mutation analysis of the gene. We identified two variants in the 3'-UTR: a synonymous coding variant and one intronic variation. One of these (the 3'-UTR +87 G/A variant) was found both in obese and in lean individuals, indicating that this variant is not causative for the obesity. Despite the fact that we were unable to find the remaining ones in our control cohort, we do not expect them to have a functional effect, although this cannot be formally excluded at this point.

More interestingly, we identified the first missense mutation in resistin. The C78S mutation was found in a 20-year-old male with severe obesity and IR.

Re-investigation of the proband at age 28 showed that he was still morbidly obese (BMI = 43.7 kg/m²). We found that the mutation was inherited from his mother who is also obese and insulin resistant. However, this is not very indicative since his father is obese and insulin resistant as well. Although, this does not exclude that the mutation may cause the obesity seen in the proband, as obesity has a very complex etiology. Furthermore, we found that the phenotype of the mutation carriers is worse than that of the WT father, as they show higher HOMA-IR values and the mother also suffers from impaired fasting glucose.

We believe it to be possible that the morbid obesity of the proband could be due to the resistin mutation in combination with other genetic and/or environmental factors. Screening of the MC4R gene, responsible for most monogenic obesity to date, showed no additional mutations in the proband. Further screening of other functional candidate genes could be performed to potentially identify additional genetic components of the proband's obesity phenotype.

A C78S mutation can be expected to have a major effect on the protein. A cysteine residue can have important functions through its involvement in the formation of disulfide bridges. C78 of resistin is located in the highly conserved cysteine-rich C-terminus of resistin. All members of the resistin-like family of proteins (resistin, RELM- α , RELM- β , and RELM- γ) share a common C-terminus domain with a high number of cysteines of which the spacing is invariant (¹C-X₁₁-²C-X₈-³C-X-⁴C-X₃-⁵C-X₁₀-⁶C-X-⁷C-X-⁸C-X₉-⁹C-¹⁰C) (1, 2). C78 is the 5th cysteine of this motif and is most probably involved in the formation of a disulfide bridge (37). Loss of a disulfide bridge is predicted to have large consequences on the tertiary structure of the protein. By use of the POLYPHEN prediction program (38), we also found that this mutation is probably damaging to the protein structure. Furthermore, this residue is highly conserved between species and homologous genes (Fig. 5).

Functional characterization of the C78S mutation showed that the mutant protein was not secreted *in vitro*, although mRNA expression was not different from WT. These data suggest that the mutant protein is degraded before it enters the secretory pathway. On the

Table 4 Comparison of plasma resistin levels between lean control individuals, mutation carriers, and obese father carrying WT resistin. Mean $\pm$ S.E.M. is given for age, BMI, and resistin concentration, except for the obese father for age and BMI.

	Lean controls	Mutation carriers	WT obese father	P value
<i>n</i>	3	2	1	—
Age (years)	25 $\pm$ 1	38.5 $\pm$ 5	52	5.6×10^{-5}
BMI (kg/m ²)	22.1 $\pm$ 0.9	37.8 $\pm$ 2.6	42.5	1.16×10^{-6}
Resistin (ng/ml)	4.26 $\pm$ 0.06	4.10 $\pm$ 0.37	3.89 $\pm$ 0.04	0.596

P values are calculated by one-way ANOVA.

increase in body weight and adiposity with enlarged adipocyte size and higher expression of adipocyte markers. *In vitro* data showed that conditioned media containing the dominant inhibitory resistin prevented the inhibitory effect of normal resistin on adipocyte differentiation of 3T3-L1 cells (4). Another strategy could be to repeat the our experiments in a human pre-adipocyte cell line as our current experiments were all performed in 3T3-L1 cells which are of mice origin.

Further evidence strengthening our belief that a mutation in resistin may play a role in the pathogenesis of obesity comes from the leptin–resistin double knock-out mouse (*ob/ob*-RKO). Deletion of resistin from mice lacking leptin (*ob/ob* mice) dramatically worsens their obese phenotype. The increase in body weight was not caused by a further increase in food intake, but by lowering energy expenditure. The increase in body weight was paralleled by a significant increase in percent body fat in *ob/ob*-RKO mice compared with *ob/ob* mice (42, 43).

Together these studies support our hypothesis that resistin plays a role in controlling the amount of adipose tissue. A mutation in the resistin gene could, therefore, ablate this function and may cause obesity as observed in our proband. However, as there has been large debate about the homology of human resistin to rodent resistin and its function, we cannot conclude at the moment that the C78S mutation is the definitive cause of obesity. However, the finding of a mutation in an obese proband and his obese mother proves that a possible function of the protein in obesity may not be dismissed. Molecular characterization of the mutant protein indicates that the mutation has an effect and most likely leads to degradation of the protein. Further functional studies on WT and mutant resistin will be necessary to further elucidate the role of this effect in the pathogenesis of obesity.

As we only identified one mutation in a single proband and his mother, it is difficult to firmly conclude that resistin mutations might lead to obesity. Therefore, identification of other mutation carriers would be necessary to confirm the causality of resistin mutations in the development of obesity and to further study the phenotype associated with these mutations.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S Beckers and W Van Hul researched data, contributed to discussion, and wrote the manuscript. D Zegers, F de Freitas, A Verrijken, F Peiffer, S L Verhulst, G Massa, I L Mertens, and K N Desager researched data and reviewed manuscript. A V Peeters contributed to discussion and reviewed manuscript. L F Van Gaal researched data, contributed to discussion, and reviewed/edited manuscript.

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