

Original article

Association between angiotensin-like 6 (*ANGPTL6*) gene polymorphisms and metabolic syndrome-related phenotypes in the French MONICA Study

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Abstract

Aim. – Although the *ANGPTL6* (angiotensin-like 6) gene product is now known to be involved in the regulation of fat mass and insulin sensitivity in mice, its physiological functions in humans have yet to be determined.

Methods. – Subjects from the population-based French MONICA Study ($n = 3402$) were genotyped for single nucleotide polymorphisms (SNPs) in *ANGPTL6*, and associations with anthropometric or biochemical phenotypes were looked for.

Results. – On evaluating the frequency of 17 *ANGPTL6* SNPs in 100 randomly selected subjects on the basis of linkage disequilibrium mapping, four SNPs (rs6511435, rs8112063, rs11671983 and rs15723) were found to cover more than 95% of the known *ANGPTL6* genetic variability. Subjects from the entire MONICA Study were then genotyped for these four SNPs. No significant association was detected for rs11671983 and rs15723. In contrast, the *G* allele of rs8112063 was associated with lower plasma glucose levels ($P = 0.009$). Also, obese subjects carrying the *G* allele of rs6511435 had higher plasma insulin levels than *AA* subjects ($P = 0.0055$). Moreover, the *G* allele of rs6511435 tended to be associated with a 20% higher risk of the metabolic syndrome ($P = 0.034$). However, when false discovery rate testing (40 tests) was applied, these associations were no longer statistically significant.

Conclusion. – These findings constitute the first study in humans of *ANGPTL6* genetic variability. Although there was no evidence that polymorphisms in *ANGPTL6* might be significantly associated with the metabolic syndrome-related phenotypes, a weak association of these polymorphisms with these parameters cannot be excluded. Further association studies are needed to arrive at any definite conclusions.

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Keywords: *ANGPTL6* gene; Polymorphisms; Plasma glucose; Metabolic syndrome; Insulin resistance; Association study

Résumé

Étude d'association entre des polymorphismes du gène *ANGPTL6* et des phénotypes du syndrome métabolique dans l'étude française MONICA.

But. – Bien que le rôle de l'*ANGPTL6* (angiotensin-like 6) dans la régulation de la masse grasse et la sensibilité à l'insuline ait été mis en évidence chez la souris, ses fonctions physiologiques chez l'homme sont encore inconnues.

Méthodes. – Le génotypage des polymorphismes du gène *ANGPTL6* a été réalisé chez les sujets de l'étude MONICA, représentative de la population française âgée de 35 à 65 ans ($n = 3402$), à la recherche d'associations entre ces polymorphismes et les phénotypes anthropométriques et biologiques.

Résultats. – Nous avons évalué la fréquence de 17 polymorphismes du gène *ANGPTL6* chez 100 sujets tirés au sort de l'étude MONICA et, à partir de la carte de déséquilibre de liaison, nous avons montré que quatre polymorphismes (rs6511435, rs8112063, rs11671983 et rs15723) couvraient plus de 95 % de la variabilité génétique connue de *ANGPTL6*. Tous les individus de l'étude MONICA ont alors été génotypés pour ces quatre

Abbreviations: ANGPTL, Angiotensin-like protein; CI, Confidence interval; HOMA-IR, Homoeostasis model assessment–insulin resistance; RFLP, Restriction fragment length polymorphism; SNP, Single nucleotide polymorphism; WHO, World Health Organization.

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polymorphismes. Aucune association n'a été détectée pour les polymorphismes rs11671983 et rs15723. À l'inverse, l'allèle G du polymorphisme rs8112063 était associé à une glycémie plus basse ($P=0,009$). De plus, les individus obèses porteurs de l'allèle G du polymorphisme rs6511435 présentaient une insulïnémie plus élevée que les individus AA ($P=0,0055$). L'allèle G du polymorphisme rs6511435 tendait également à être associé à une augmentation du risque de 20 % de syndrome métabolique ($P=0,034$). Cependant, lorsqu'on applique une correction pour tests multiples (40 tests réalisés), ces associations n'étaient plus significatives.

Conclusion. – En conclusion, ces résultats constituent la première étude de la variabilité génétique du gène *ANGPTL6* chez l'homme. Bien que nous n'ayons pas mis en évidence d'association significative entre les polymorphismes d'*ANGPTL6* et les phénotypes du syndrome métabolique, nous ne pouvons pas exclure pour autant que des polymorphismes de ce gène puissent être faiblement associés à ces phénotypes. D'autres études d'association sont nécessaires pour pouvoir conclure.

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Mots clés : Gène de l'*ANGPTL6* ; Polymorphismes ; Glycémie ; Syndrome métabolique ; Insulinorésistance ; Études d'associations

1. Introduction

Angiopoietin-like proteins [ANGPTLs] or 'angiopoietin-related proteins' [ARPs] are orphan ligands with a degree of similarity to angiopoietins: they contain a coiled-coil domain and a fibrinogen-like domain, and have angiogenic effects [1–10]. The ANGPTL family has seven members (ANGPTL 1–7), which have been found in both humans and mice [11] (except for ANGPTL5, identified only in humans [12]). The ANGPTLs are secreted proteins that serve as endocrine signals in the peripheral tissues. Its most interesting member (in terms of glucose, lipid homeostasis and insulin sensitivity) is ANGPTL6 (also called 'angiopoietin-related growth factor' [AGF]). ANGPTL6 was first identified in 2003, and has been characterized as an angiogenic factor involved in epidermal proliferation and wound healing [9,13]. Recently, it was shown that ANGPTL6 is involved in the development of obesity and its related insulin resistance in mouse models [14]. Indeed, Oike et al. have shown that mice surviving deletion of the *Angptl6* gene develop marked obesity, characterized by an increase in fat depots and adipocyte size, hypercholesterolaemia, elevated plasma non-esterified fatty acid (NEFA) levels, hyperinsulinaemia and glucose intolerance. The increased fat mass was due to a decrease in energy expenditure rather than an increase in food intake. In addition, transgenic mice in which *Angptl6* was overexpressed displayed a significant reduction in body weight (resulting from increased energy expenditure) and improved insulin sensitivity [14]. These mice were also protected against high-fat diet-induced obesity and non-adipose-tissue steatosis.

Little is known of the physiology of *ANGPTL6*. The mechanisms by which it stimulates energy expenditure and insulin sensitivity, and the nature of the tissues targeted by the protein have yet to be established. In mice, *Angptl6* is expressed predominantly in the liver, but also in haematopoietic cells [13]. In humans, the *ANGPTL6* gene is located on chromosome 19p13.2, but nothing is known of its expression and function. For this reason, we aimed to establish whether *ANGPTL6* genetic variability could be associated with anthropometric or biochemical phenotypes in humans using a sample population ($n=3402$) from the French population-based MONICA Study.

2. Materials and methods

2.1. Subjects

Participants were recruited from the WHO–MONICA population survey carried out from 1995 to 1997 in different regions of France: the Lille Urban Community in the north (Lille, $n=1195$); the Bas-Rhin county in the east (Strasbourg, $n=1131$); and the Haute-Garonne county in the south (Toulouse, $n=1182$), as described elsewhere [15]. The study protocol was approved by the independent ethics committee of each respective region. After signing an informed consent form, participants were administered a standard questionnaire, while physical measurements were taken by a specially trained nurse. Physical activity was defined as at least 15 min/day of walking and/or the daily lifting or carrying of heavy objects at work and/or doing sports or physical exercise for more than 2 h/week. Current cigarette smokers were defined as those who smoked at least one cigarette/day. Total alcohol intake (in mL) was calculated as the total sum per week of wine, beer, cider and spirits. Anthropometric measurements were taken with the subjects wearing light clothing and no shoes. Body mass index (BMI) was calculated according to the Quetelet equation, and the metabolic syndrome was assessed using the WHO criteria [16].

2.2. Laboratory methods

A 20 mL blood sample was drawn on disodium EDTA after the subject had fasted for at least 10 h. Lipid and lipoprotein levels were all measured at the Purpan Hospital Biochemical Laboratory (Toulouse), as described elsewhere [15]. The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as the product of fasting plasma insulin (in $\mu\text{U/mL}$) and fasting plasma glucose (in mmol/L), divided by 22.5 [17].

2.3. Genotyping

Single nucleotide polymorphism (SNP) typing was performed using the restriction fragment length polymorphism (RFLP) method (see Supplementary Data Table). The genotyping success rate was greater than 96% for all SNPs. All SNPs

respected the Hardy–Weinberg equilibrium ($P > 0.05$), except for rs6511435 ($P = 0.002$). However, the RFLP method can lead to genotyping errors due to incomplete digestion so, to avoid this, the restriction enzyme was always added in excess. The genotypes in excess for the rs6511435 SNP corresponded to both the GG homozygote (uncut) and AA homozygote (cut) genotypes, ruling out the hypothesis that this was due to uncut digestion products. Moreover, on checking 30% of the genotypes of rs6511435 ($n = 934$) with another primer pair and another restriction enzyme that cuts the other (G) allele (see [Supplementary Data Table](#)), only six discordant genotypes (0.6%) were found, ruling out a problem with the genotyping technique. Indeed, no reason for deviation from the Hardy–Weinberg equilibrium can be identified in some cases [18]. Paired linkage disequilibrium estimates were calculated using GOLD software [19].

2.4. Statistical analyses

Statistical analyses were performed using SAS statistical software, version 8 (SAS Institute Inc., Cary, NC). Inter-group comparisons of quantitative variables were tested using a general linear model (the GLM procedure in SAS). Data for triglyceride, HOMA, glucose and insulin levels were log-transformed to obtain a normal distribution. For anthropometric variables, the confounding variables were age, gender, centre, smoking status, alcohol consumption and physical activity. For biochemical variables, the confounding variables were age, gender, centre, BMI, smoking status, alcohol consumption and physical activity. Multivariate logistic-regression analyses were used to calculate the odds ratios (OR) for the metabolic syndrome adjusted for age, gender, centre, smoking status, alcohol consumption and physical activity. Reported P values are nominal.

3. Results

ANGPTL6 SNPs were extracted from the Children's Hospital Informatics Program SNP (snpper.chip.org) and the NCBI dbSNP databases, out of which 17 SNPs were selected, located from 1000 bp upstream of the first exon to 1000 bp downstream of the last exon: rs6511435; rs7260319; rs8109578; rs8101501; rs10417676; rs9749206; rs10410922; rs8112063; rs11671983; rs8107814; rs2336689; rs6511432; rs6511431; rs10408727; rs10408244; rs1044611; and rs15723. In the HapMap database (release 23), only the rs8109578, rs8112063, rs10408244 and rs15723 SNPs were reported, and no tag SNPs were described.

To build the linkage disequilibrium map, 100 randomly selected individuals from the MONICA Study were genotyped for the 17 selected SNPs. However, rs7260319, rs8101501, rs10417676, rs9749206, rs2336689, rs6511431, rs10408727 and rs1044611 were not detected in this sample, and the minor allele frequencies of the rs8109578, rs6511432 and rs10408244 SNPs were too low (0.05, 0.04 and 0.01, respectively) to yield sufficient statistical power in the analyses and so were not pursued. The minor allele frequencies of the rs6511435, rs10410922, rs8112063, rs11671983, rs8107814 and rs15723 SNPs were 0.25, 0.37, 0.37, 0.13, 0.10 and 0.10, respectively. The linkage disequilibrium pattern across the SNPs was assessed using both D' and r^2 (Fig. 1). Although the rs10410922, rs8112063, rs11671983, rs8107814 and rs15723 SNPs were in linkage disequilibrium ($|D'| = 1$), complete allelic association ($r^2 = 1$) was only observed for rs10410922 and rs8112063, and for rs8107814 and rs15723. Rs6511435 was independent of the other SNPs (maximum $|D'| = 0.3$ and $r^2 = 0.03$).

Subjects from the MONICA population study ($n = 3402$) were then genotyped for the four SNPs — rs6511435, rs8112063 (tagging rs10410922), rs11671983 and rs15723 (tagging rs8107814) — that covered more than 95% of the known genetic

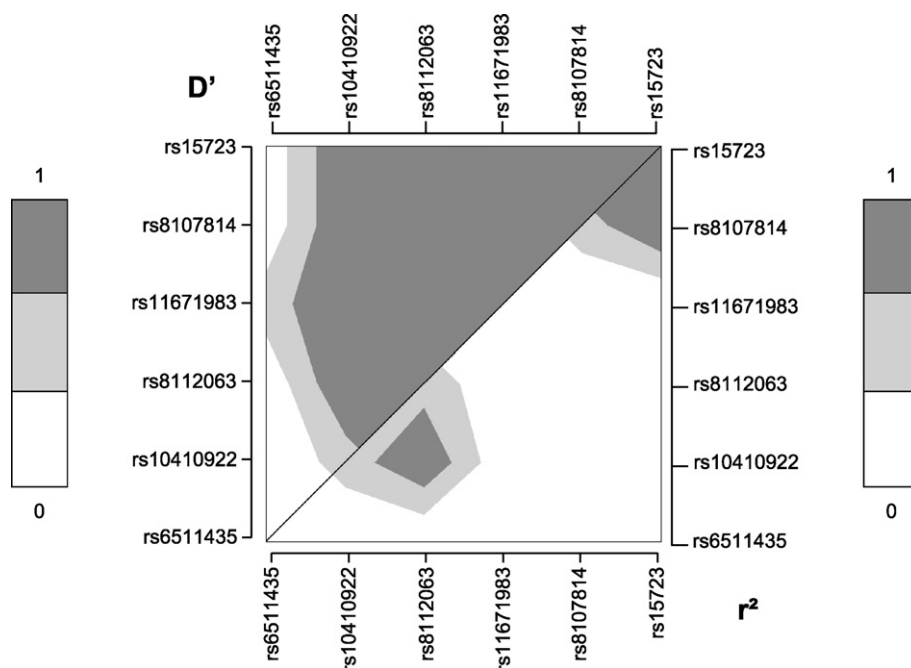


Fig. 1. Linkage disequilibrium across six *ANGPTL6* polymorphisms. The D' is displayed in the upper left corner and the r^2 is displayed in the lower right corner.

Table 1

Association between rs15723 and anthropometric and biochemical parameters.

rs15723 (<i>n</i>)	TT (2751)	TC (528)	CC (35)	P CC + TC vs TT
BMI (kg/m ²)	26.3 ± 4.6	26.5 ± 4.7	27.8 ± 5.9	0.444
Waist/hip ratio	0.89 ± 0.09	0.89 ± 0.09	0.89 ± 0.09	0.683
Cholesterol (mmol/L)	5.89 ± 1.06	5.94 ± 1.01	5.70 ± 0.88	0.870
HDL cholesterol (mmol/L)	1.47 ± 0.44	1.49 ± 0.44	1.51 ± 0.49	0.391
LDL cholesterol (mmol/L)	3.85 ± 1.00	3.90 ± 0.94	3.65 ± 0.84	0.965
Triglycerides (mmol/L)	1.30 ± 0.97	1.25 ± 0.92	1.47 ± 1.21	0.176
Glucose (mmol/L)	5.56 ± 1.43	5.46 ± 0.90	6.10 ± 2.00	0.090
Insulin (uU/mL)	11.24 ± 9.33	11.54 ± 7.84	10.91 ± 4.21	0.552
HOMA-IR	2.90 ± 3.19	2.86 ± 2.14	3.14 ± 2.21	0.974

Data are expressed as means ± S.D.

Table 2

Association between rs11671983 and anthropometric and biochemical parameters.

rs11671983 (<i>n</i>)	CC (2488)	CG (782)	GG (51)	P GG + CG vs CC
BMI (kg/m ²)	26.4 ± 4.7	26.1 ± 4.5	21A ± 4.4	0.698
Waist/hip ratio	0.88 ± 0.09	0.89 ± 0.10	0.88 ± 0.10	0.108
Cholesterol (mmol/L)	5.90 ± 1.06	5.89 ± 1.03	5.82 ± 0.97	0.695
HDL cholesterol (mmol/L)	1.49 ± 0.45	1.47 ± 0.43	1.46 ± 0.47	0.328
LDL cholesterol (mmol/L)	3.86 ± 0.99	3.86 ± 0.98	3.75 ± 0.94	0.654
Triglycerides (mmol/L)	1.28 ± 0.95	1.30 ± 0.96	1.55 ± 1.56	0.215
Glucose (mmol/L)	5.53 ± 1.37	5.61 ± 1.36	5.60 ± 1.42	0.064
Insulin (uU/mL)	11.14 ± 9.00	11.81 ± 9.48	10.16 ± 4.68	0.062
HOMA-IR	2.84 ± 2.89	3.10 ± 3.52	2.63 ± 1.74	0.023

Data are expressed as means ± S.D.

variability of *ANGPTL6*. The minor allele frequencies of the *ANGPTL6* rs6511435, rs8112063, rs11671983 and rs15723 SNPs in the MONICA sample were 0.28, 0.43, 0.13 and 0.09, respectively.

Next, the association of these four *ANGPTL6* SNPs was assessed against anthropometric and biochemical phenotypes — namely, mean BMI, waist-to-hip ratio, plasma cholesterol (total, HDL and LDL), triglycerides, glucose and insulin levels, and insulin sensitivity (estimated by the HOMA-IR). No significant interaction between the SNPs and either gender or physical activity could be detected with these parameters.

Analysis of rs15723 (a T>C substitution in the 3' untranslated region) showed no associations with the considered phenotypes (Table 1). Table 2 shows the associations for rs11671983 (a C>G change in intron 1) with the studied parameters. HOMA-IR values tended to be higher in *G* allele bearers

($P=0.023$) but, as this was due to the impact of the heterozygote subjects only, this association was considered spurious.

As for rs6511435 (an A>G substitution in the promoter region), subjects carrying the *G* allele had higher plasma fasting insulin concentrations ($P=0.022$) and were more insulin resistant ($P=0.034$) than AA subjects (Table 3). We detected an interaction ($P=0.02$) between rs6511435, plasma insulin levels and obesity, defined as a BMI less than 30 ($n=2766$) or greater or equal to 30 kg/m² ($n=597$). Indeed, mean insulin levels were similar ($P=0.23$) between non-obese rs6511435 *G* allele carriers and AA subjects whereas obese subjects carrying the *G* allele had higher insulin levels than obese AA subjects (18.00 ± 10.57 vs 16.17 ± 8.67 μU/mL, respectively; $P=0.0055$). Given that insulin resistance is a major risk factor for the occurrence of the metabolic syndrome, logistic-regression analyses were performed to evaluate the risk of the metabolic syndrome. In the

Table 3

Association between rs6511435 and anthropometric and biochemical parameters.

rs6511435 (<i>n</i>)	AA (1735)	AG (1278)	GG (303)	P GG + AG vs AA
BMI (kg/m ²)	26.3 ± 4.6	26.3 ± 4.7	26.4 ± 4.6	0.782
Waist/hip ratio	0.89 ± 0.09	0.88 ± 0.10	0.88 ± 0.09	0.289
Cholesterol (mmol/L)	5.91 ± 1.04	5.89 ± 1.09	5.85 ± 0.97	0.689
HDL cholesterol (mmol/L)	1.49 ± 0.46	1.48 ± 0.43	1.45 ± 0.39	0.144
LDL cholesterol (mmol/L)	3.85 ± 0.96	3.88 ± 1.04	3.85 ± 0.92	0.419
Triglycerides (mmol/L)	1.31 ± 1.01	1.26 ± 0.92	1.27 ± 0.84	0.743
Glucose (mmol/L)	5.56 ± 1.49	5.54 ± 1.23	5.52 ± 1.18	0.721
Insulin (uU/mL)	11.02 ± 9.20	11.55 ± 9.35	11.53 ± 6.55	0.022
HOMA-IR	2.82 ± 2.98	2.97 ± 3.20	2.97 ± 2.61	0.034

Data are expressed as means ± S.D.

Table 4

Association between rs8112063 and anthropometric and biochemical parameters.

rs8112063 (n)	AA (1068)	AG (1631)	GG (623)	P GG vs AA + AG
BMI (kg/m ²)	26.4 ± 4.8	26.3 ± 4.6	26.4 ± 4.4	0.689
Waist/hip ratio	0.89 ± 0.10	0.89 ± 0.09	0.89 ± 0.09	0.848
Cholesterol (mmol/L)	5.90 ± 1.06	5.89 ± 1.03	5.82 ± 0.97	0.058
HDL cholesterol (mmol/L)	1.47 ± 0.43	1.48 ± 0.44	1.50 ± 0.48	0.396
LDL cholesterol (mmol/L)	3.85 ± 1.02	3.88 ± 0.99	3.83 ± 0.94	0.190
Triglycerides (mmol/L)	1.30 ± 0.97	1.31 ± 1.01	1.23 ± 0.80	0.096
Glucose (mmol/L)	5.57 ± 1.38	5.57 ± 1.44	5.45 ± 1.13	0.009
Insulin (uU/mL)	11.56 ± 9.66	11.22 ± 9.41	10.99 ± 6.80	0.414
HOMA-IR	3.01 ± 3.39	2.89 ± 3.11	2.74 ± 2.02	0.089

Data are expressed as means ± S.D.

MONICA Study, 19.0% ($n=645$) of the subjects presented with the metabolic syndrome. Those carrying the *G* allele of rs6511435 tended to have a higher risk of the metabolic syndrome (adjusted OR = 1.21 [1.01–1.45]; $P=0.034$) compared with AA subjects.

As regards rs8112063 (an A>G substitution in intron 1), GG subjects had lower fasting plasma glucose levels compared with A allele bearers ($P=0.009$; Table 4), although the risk of the metabolic syndrome in GG subjects was not significantly modified (OR = 0.82 [0.65–1.04]; $P=0.106$).

Multiple testing (40 tests) was performed in this study and, when applying a correction such as the Benjamini and Hochberg false discovery rate (FDR) [20], the best corrected P value was 0.22, suggesting that none of the associations described above was statistically significant.

4. Discussion

In the present study, we explored the genetic variability of the *ANGPTL6* gene. We assessed the frequency of 17 SNPs and found that four common SNPs covered 95% of the known genetic variability in the gene. We studied the associations between these four SNPs and several anthropometric and biochemical phenotypes (including risk of the metabolic syndrome) in the French MONICA Study sample ($n=3402$). Statistically, no significant associations were detected between any of the four SNPs and the studied phenotypes. However, we observed that the *G* allele of rs8112063 was associated with lower plasma glucose levels. In addition, obese subjects carrying the *G* allele of rs6511435 had higher plasma insulin levels than the AA subjects. The *G* allele of rs6511435 also tended to be associated with a 20% higher risk of the metabolic syndrome. We looked at the publicly available genome-wide association datasets for quantitative glycaemic (glucose, insulin, HOMA-IR) traits (Diabetes Genetics Initiative, <http://www.broad.mit.edu/diabetes/>) and at the risk of type 2 diabetes (DIAGRAM, <http://www.well.ox.ac.uk/DIAGRAM/>) [21] to see whether or not *ANGPTL6* SNPs had been previously associated with glycaemic traits and/or type 2 diabetes. Unfortunately, no *ANGPTL6* SNP had been explored in any of those studies.

Nevertheless, our findings could be consistent with a physiological role of Angptl6 in terms of insulin sensitivity and

glucose metabolism as reported by Oike et al. in transgenic mice [14]. Also, it has recently been shown that Angptl6 inhibits glucose production in rat hepatocytes by preventing expression of glucose-6-phosphatase, a key enzyme in gluconeogenesis [22]. Furthermore, Hato et al. reported that diet-induced obese mice receiving Angptl6 showed increased adenosine monophosphate-activated protein kinase activity (which improves insulin signaling in skeletal muscle) and that, in C2C12 myoblasts, the addition of insulin after pretreatment with Angptl6 increased IRS-1 (insulin receptor substrate-1) phosphorylation, PI3-K (phosphatidylinositol 3-kinase) activity and glucose uptake, thereby resulting in enhanced insulin signaling [11].

Although Oike et al. showed that Angptl6 is involved in fat metabolism and may counteract obesity in mice [14], we detected no significant association between *ANGPTL6* polymorphisms and body-weight-related variables in the French MONICA sample. This was not because of a lack of power as, even when considering the most rare allele (frequency 0.09), we would have detected a 0.7 kg/m² difference in mean BMI in our study. However, an increased fat mass (as seen in obesity) might accentuate the role of *ANGPTL6* on insulin metabolism.

The *ANGPTL6* SNPs we studied are non-coding polymorphisms. Indeed, rs6511435 is located in the gene promoter region, 1066 bp upstream of the transcription start site. This *ANGPTL6* SNP was totally independent of the other SNPs tested. Rs8112063 and rs11671983 are located in the first intron of *ANGPTL6*, which stretches out over nearly 6 kbp. As rs8112063 also tagged rs10410922, the associations observed for rs8112063 could be functionally explained by rs10410922, also located in the first intron, 775 bp away. As the introns (especially the first one) are known to be regulatory regions that modulate gene expression, these SNPs may have an influence on *ANGPTL6* expression levels. In addition, rs15723 is located in the 3' untranslated region of the gene. Functional analyses of these SNPs could shed light on the regulation of *ANGPTL6* gene expression, of which nothing is currently known.

In conclusion, these results constitute the first study findings in humans of *ANGPTL6* genetic variability. We found no evidence that polymorphisms of *ANGPTL6* had any significant influence on the metabolic syndrome-related phenotypes, but a minor role of these polymorphisms on these parameters can-

not be excluded either. Further association studies are needed to allow any definite conclusions to be made.

5. Conflict of interests

No potential conflicts of interest relevant to this article were reported.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.diabet.2008.12.005.

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