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Fat depot-specific mRNA expression of novel loci associated with waist–hip ratiox

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Abstract

Objective—We hypothesized that genes within recently identified loci associated with waist–hip ratio (WHR) exhibit fat depot-specific mRNA expression, which correlates with obesity-related traits.

Methods—Adipose tissue (AT) mRNA expression of 6 genes *(TBX15/WARS2, STAB1, PIGC, ZNRF3* and *GRB14)* within these loci showing coincident cis-expression quantitative trait loci was measured in 222 paired samples of human visceral (vis) and subcutaneous (sc) AT. The relationship of mRNA expression levels with obesity-related quantitative traits was assessed by Pearson's correlation analyses. Multivariate linear relationships were assessed by generalized linear regression models.

Results—Whereas only *PIGC, ZNFR3* and *STAB1* mRNA expression in sc AT correlated nominally with WHR ($P < 0.05$, adjusted for age and sex), mRNA expression of all studied genes

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Conflict of Interest
The authors declare no conflict of interest.

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in at least one of the fat depots correlated significantly with vis and/or sc fat area (P ranging from 0.05 to 4.0 x 10^6, adjusted for age and sex). Consistently, the transcript levels of *WARS, PIGC* and *GRB14* were nominally associated with body mass index (BMI) (P ranging from 0.02 to 9.2 x 10^5, adjusted for age and sex). Moreover, independent of sex, obesity and diabetes status, differential expression between vis and sc AT was observed for all tested genes ($P < 0.01$). Finally, the rs10195252 T-allele was nominally associated with increased *GRB14* sc mRNA expression ($P = 0.025$ after adjusting for age, sex and BMI).

Conclusions—Our data including the inter-depot variability of mRNA expression suggests that genes within the WHR-associated loci might be involved in the regulation of fat distribution.

Keywords

fat distribution; gene expression; waist–hip ratio; GWAS

Introduction

Body fat distribution (FD) is one of the main predictors of obesity-associated complications, such as type 2 diabetes, chronic inflammation, coronary heart disease as well as hepatic glucose production.[1–3] There is good evidence that FD is controlled by genetic factors. Individual variation in waist–hip ratio (WHR), a measure of FD, is heritable with estimates ranging from 22-61%.[4–7] However, the mechanisms and exact genetic variants causing adverse or visceral (vis) FD are still poorly understood.

Along with biological candidate genes, genome-wide association studies (GWAS) represent an important source of genes potentially controlling FD. Recently, 14 loci have been identified in a GWAS for WHR independent of BMI.[8] Six out of the 14 loci did not only harbour polymorphisms associated with WHR but also showed coincident cis-expression quantitative trait loci (eQTLs) (expression quantitative trait loci) implicating that the observed associations with WHR are likely to be mediated by gene transcripts *(TBX15, WARS2, PIGC, STAB1, GRB14* and *ZNRF3)* in various tissues/organs.

Although these data indicate a possible role of the six genes in the regulation of FD, more detailed analyses are inevitable to pinpoint causal transcripts underlying the observed association signals. Therefore, the aim of the study was to evaluate whether *TBX15, WARS2, PIGC, STAB1, GRB14* and *ZNRF3* exhibit fat depot-specific mRNA expression and whether their transcript levels correlate with obesity-related traits by analysing paired samples of human vis and subcutaneous (sc) AT from 222 metabolically well-characterized subjects. Further, we investigated the effects of WHR-associated genetic variants within/nearby these genes on AT gene expression.

Materials and Methods

Subjects

A total of 222 Caucasian men (N = 78) and women (N = 144), who underwent open abdominal surgery were included in the study, and paired samples of vis and sc AT were obtained.[9] The subjects had a mean age of 51 ± 15 years, a mean BMI of 41.6 ± 13.7 kg m

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$^{-2}$, a mean WHR of 0.94 ± 0.11 and a mean height of 1.69 ± 0.09 m. All subjects had a stable weight with no fluctuation > 2% of the body weight for at least 3 m before surgery. Patients with severe conditions including generalized inflammation or end-stage malignant diseases were excluded from the study. Samples of vis and sc AT were immediately frozen in liquid nitrogen after explantation.[9] Among the 222 subjects, 42 were lean (mean age 63 ±12 years, mean BMI 22.0 ± 0.4 kg m $^{-2}$), 21 were overweight (mean age 71 ± 10 years, mean BMI 27.1 ± 0.3 kg m $^{-2}$) and 159 were obese (mean age 45 ± 13 years, mean BMI 48.6 ± 0.7 kg m $^{-2}$). An oral glucose tolerance test was performed after an overnight fast with 75 g of standardized glucose solution (Glucodex Solution 75 g; Merieux, Montreal, Quebec, Canada). Fasting plasma insulin was measured with an enzyme immunometric assay for the IMMULITE automated analyzer (Diagnostic Products Corporation, Los Angeles, CA, USA). Insulin sensitivity was assessed with euglycemic-hyperinsulinemic clamps.[10] In addition to above mentioned clinical parameters, abdominal vis and sc fat area were calculated using computed tomography scans at the level of L4-L5 and percentage body fat was measured by dual-energy x-ray absorptiometry.

The ethics committee at the Medical Faculty of the University of Leipzig specifically approved this study and all subjects gave written informed consent before taking part in the study.

Analysis of human mRNA expression

Total RNA was isolated from paired sc and vis AT samples using TRIzol (Life Technologies, Grand Island, NY, USA), and 1 μ g RNA was reverse transcribed with standard reagents (Life Technologies). Human gene expression was measured by quantitative real-time RT–PCR by using TaqMan methodology and fluorescence was detected on an ABI PRISM 7500 sequence detector (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions (Applied Biosystems; assay Hs00537087_m1 with ACCESSION AK096396 for *TBX15;* Hs00210571_m1 with ACCESSION AJ242739 for *WARS2;* Hs00267516_s1 with ACCESSION AK308201 for *PIGC;* Hs01109068_m1 with ACCESSION AB052956 for *STAB1;* Hs00610307_m1 with ACCESSION AK301961 for *GRB14;* Hs00393094_m1 with ACCESSION AB051436 and AB032959 for *ZNRF3).* Human mRNA expression was calculated relative to the mRNA expression of *HPRT* and *18S rRNA,* determined by a premixed assay on demand (PE Biosystems, Darmstadt, Germany). The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

Genotyping of single-nucleotide polymorphisms

Genotyping of the single-nucleotide polymorphisms (SNPs) (rs984222 G > C, rs6784615 T > C, rs1011731 A > G, rs4823006 A > G and rs10195252 T > C was done using the TaqMan SNP Genotyping assays according to the manufacturer's protocol (Applied Biosystems, Inc., Foster City, CA, USA). To limit genotyping errors, according to recommendation by Pompanon *et al.,* [11] a random ~5% selection of the sample was re-genotyped in all SNPs; all genotypes matched initial designated genotypes.

Statistical analyses

Prior to statistical analysis, non-normally distributed parameters were logarithmically transformed to approximate a normal distribution. Differences in mRNA expression between vis and sc AT were assessed using the paired Student's t-test. Multivariate linear relationships were assessed by generalized linear regression models. P-values were adjusted for age, sex and BMI (if appropriate) and the genetic analyses were done under the additive model of inheritance. Furthermore, SNP × sex interaction term has been included to test possible interaction effects of sex in linear regression models in the whole sample.

P-values ≤ 0.05 were considered to provide evidence for nominal association and are presented without correction for multiple hypothesis testing. Only two-sided *P*-values are provided. Pearson correlation coefficients (r) are provided for relationships of mRNA expression levels with obesity-related quantitative traits.

All statistical analyses were performed using SPSS version 20 (SPSS, Inc.; Chicago, IL, USA).

Results

mRNA expression according to sex, obesity and diabetes status As sexual dimorphism has been reported for WHR and waist circumference in the initial GWAS,[8] we analysed the expression data not only in the total cohort but also in men and women separately. Except for *STAB1* with higher mRNA expression in sc AT in men when compared with women (P<0.05), there were no gender differences in gene expression in either tissue (Figure 1). Changes in expression activity of a gene/pathway in AT may be linked (either as a cause or a consequence) to a certain FD pattern or metabolically relevant phenotypes. Therefore, we compared the mRNA expression levels between lean (BMI < 25 kg m $^{-2}$), overweight (BMI 25–30 kg m $^{-2}$) and obese (BMI > 30kg m $^{-2}$) subjects. *WARS2, PIGC, STAB1* and *GRB14* showed significantly different transcript levels between obese and lean subjects (Figure 2). Surprisingly, only *PIGC, ZNFR3* and *STAB1* mRNA expression in sc AT correlated significantly with WHR (P < 0.05 after adjusting for age and sex; Table 1). As WHR might be masked in severely obese conditions, we additionally included sc and vis fat area measurements as a more precise measure of FD. As summarized in Table 1, mRNA expression of all studied genes in at least one of the fat depots correlated with vis and/or sc fat area *(P* ranging from 0.05 to 4.0 × 10^6 after adjusting for age and sex; Supplementary Figure). Correlations of *GRB14* (vis), *WARS* (vis) and *PIGC* (sc) AT mRNA expression with both vis and sc fat area were in accordance with correlations of their gene expression with BMI (Table 1).

As glucose homeostasis status may eventually have an impact on expression of the transcripts, we tested this hypothesis as well. Compared with subjects with normal glucose tolerance, *WARS2* and *PIGC* mRNA levels in vis AT were significantly lower in subjects with T2D *(P* <0.05; Figure 3). However, this appeared to be driven by obesity as no significant association was found after adjusting the analyses for BMI (all *P*> 0.05).

Fat depot specific mRNA expression

The novel FD candidate genes *TBX15, WARS2, PIGC, STAB1, GRB14* and *ZNRF3* were differentially expressed between vis and sc AT. *WARS2, PIGC, STAB1, GRB14* and *ZNRF3* were predominantly expressed in vis fat, whereas *TBX15* transcript levels were significantly higher in sc AT ($P < 0.05$;Figure 1). These differences seemed to be independent of sex, obesity or diabetes status, as they were observed in stratified analyses as well (Figures 1–3).

mRNA expression according to genotypes at WHR-associated loci

eQTL data can implicate regional transcripts that mediate trait associations[8] and may help to sort out the expected direction. To elucidate the possible link between the initially described WHR-associated genetic variants[8] and gene expression, we investigated the effects of rs984222 G > C (TBX15/WARS2), rs6784615 T > C (*STAB1*), rs1011731 A > G (PIGC), rs4823006 A > G (*ZNRF3*) and rs10195252 T > C (*GRB14*) within/nearby these genes on AT gene expression. All SNPs were in Hardy–Weinberg Equilibrium (all $P > 0.05$) and had following minor allele frequencies: rs984222— 38.8%, rs6784615—6.2%, rs1011731—40.7%, rs4823006—43% and rs10195252—41.1%.

We found no sex-SNP interaction effects in the linear regression analyses in the whole sample and thus, subsequent analyses were performed without further sex stratifications. Except for rs10195252 in *GRB14,* none of the SNPs showed association with the respective gene transcript. The rs10195252 T-allele was nominally associated with increased *GRB14* sc mRNA expression ($P < 0.025$ after adjusting for age, sex and BMI; Figure 4).

Discussion

In the present study, we measured the AT mRNA expression of 6 genes *(TBX15/WARS2, STAB1, PIGC, ZNRF3* and *GRB14)* within the recently reported WHR loci[8] in paired samples of human vis and sc AT. We observed differential expression between vis and sc AT for all tested genes. Furthermore, the mRNA expression of the studied genes correlated with measures of FD and obesity such as BMI, WHR and vis and sc fat area.

In general, mechanisms underlying differential gene expression between sc and vis AT are poorly understood. Nevertheless, many of these genes, including *RBP4, LEP, PPARG, SERPINA12, AR* and *CB1R* are not only differentially expressed in various fat depots, but their mRNA expression is also associated with traits related to obesity, such as insulin resistance or adipokine levels.[3,12–14] Moreover, it has been shown that for some physiologically plausible candidate genes involved in the regulation of FD such as *BMPRs,* associations of genetic polymorphisms with anthropometric and metabolic measures might be mediated by changes in mRNA expression.[15,16] Inter-depot specific differences have also been reported for genes involved in cytokine secretion or lipolysis but also for developmental genes involved in Wnt signaling.[17] Also developmental genes such as *TBX15, HOXA5* or *GPC4* seem to play a relevant role in obesity and body FD.[18] These genes did not only show strong inter-depot differences in mRNA expression in both mice and humans, but they also exhibited changes in expression that closely correlated with BMI and/or WHR.[18] As postulated by Gesta *et* al.,[18] these differences in gene expression are

probably cell autonomous and independent of tissue microenvironment as indicated by their intrinsic nature and by persisting during *in vitro* culture and differentiation. One of the previously reported developmental genes, *TBX15,* was differentially expressed between vis and sc tissue also in the present study with significantly higher mRNA levels in sc AT. This direction is consistent with data obtained in mice studies, and data of an Australian child cohort,[19] but opposite to human studies by Gesta *et al.* [18] One possible explanation might be the substantially smaller sample size of the previous study (53 vs 222) as well as the fact that only lean subjects have been investigated previously, whereas a wide range of BMI was included in the present work. Although the exact role in the regulation of either obesity or FD remains elusive, it is noteworthy that *TBX15* encodes a transcription factor, which was shown to be involved in developmental processes as in dorsoventral patterning[20] and skeletal development.[21] Moreover, differential expression of *TBX15* between fat depots is linked with differences in adipocyte differentiation, triglyceride accumulation and mitochondrial function.[22]

As for any GWAS in general, one of the greatest challenges is to understand the biological consequences of the identified loci associated with the respective trait. Therefore, systematic strategies to elucidate how WHR-associated variants exert their effects will be inevitable. No doubt that fine-mapping efforts are crucial to understand the complete architecture of genetic variation in the associated regions and to narrow down the number of functionally interesting variants. However, for non-Mendelian traits, the majority of the variants identified in GWAS maps within non-coding regions and thus, it is likely that WHR-associated alleles exert their effects by affecting gene transcription. Therefore, identifying the variant associated with gene transcription in specific target tissues, often referred to as eQTLs appears to be one of the major steps to determine potential causality of the revealed associations. Since eQTLs might explain a greater proportion of phenotypic variance than usually observed for risk alleles and clinical traits, eQTL studies do not require as large sample sizes as mostly needed for clinical association studies.[23] Still, even though the sample size in our study may be appropriate in the context of expression studies, we are aware that we may have been lacking adequate statistical power to detect significant associations between the SNPs and mRNA expression in AT. Indeed, only one of the SNPs (rs10195252) showed nominal association with the corresponding gene transcript *(GRB14* mRNA levels in sc AT). However, by applying correction for multiple testing (for example, Bonferroni corrections would require a $P < 0.01$ considering five SNPs analysed and taken into account the strong correlations between clinical traits), none of the SNPs would be associated with the respective gene expression. Nevertheless, the present work provides correlations between mRNA expression and metabolic traits related to obesity and FD, which seem to be statistically robust and resisting corrections for multiple testing. In this context, our study provides novel data on mRNA expression of several WHR-associated genes in AT, suggesting their role in the regulation of FD and so strongly supports the previous GWAS for WHR.[8] It is of note, however, that further mechanisms have to be taken into account when elucidating the causative mechanisms behind genetic associations. For instance, epigenetic regulation of gene expression including DNA-methylation, histone modifications and altered function of non-coding RNAs or miRNAs have to be considered as

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well. Ultimately, with sufficient evidence for a causal variant or a susceptibility gene, cell and tissue models as well as *in vivo* models of disease development may be employed.

In conclusion, besides the correlations of expression levels with BMI or WHR, the most striking feature of the expression of the six studied genes is the inter-depot variability as well as correlations with the vis and sc fat area. Even though the nature of our study does not allow clarifying causative chains underlying the observed genotype–phenotype and phenotype–phenotype correlations, it clearly supports the role of recently reported WHR-associated genes in the regulation of FD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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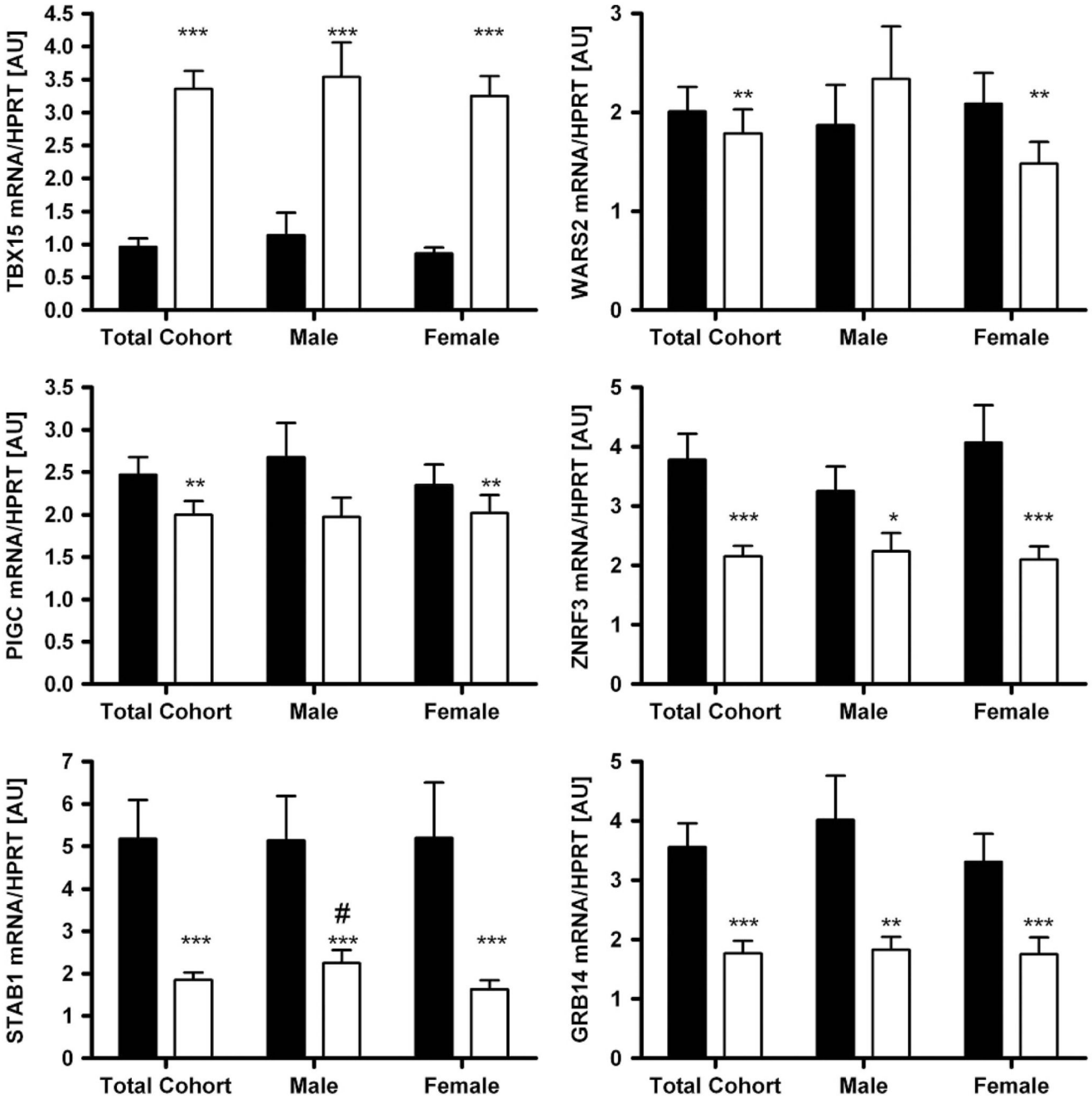

Figure 1.

Expression of *TBX15, WARS2, PIGC, ZNRF3, STAB1* and *GRB14* in 222 paired human samples of visceral (vis) and subcutaneous (sc) adipose tissue (AT) in the total cohort and grouped by gender (78 males, 144 females). Mean±s.e.m. Black bars represent vis and white bars sc AT. *sc vs vis AT depot; #male vs female in the same AT depot. $*^{/\#}P < 0.05$; $**^{/\#\#}P < 0.01$; $***^{/\#\#\#}P < 0.001$.

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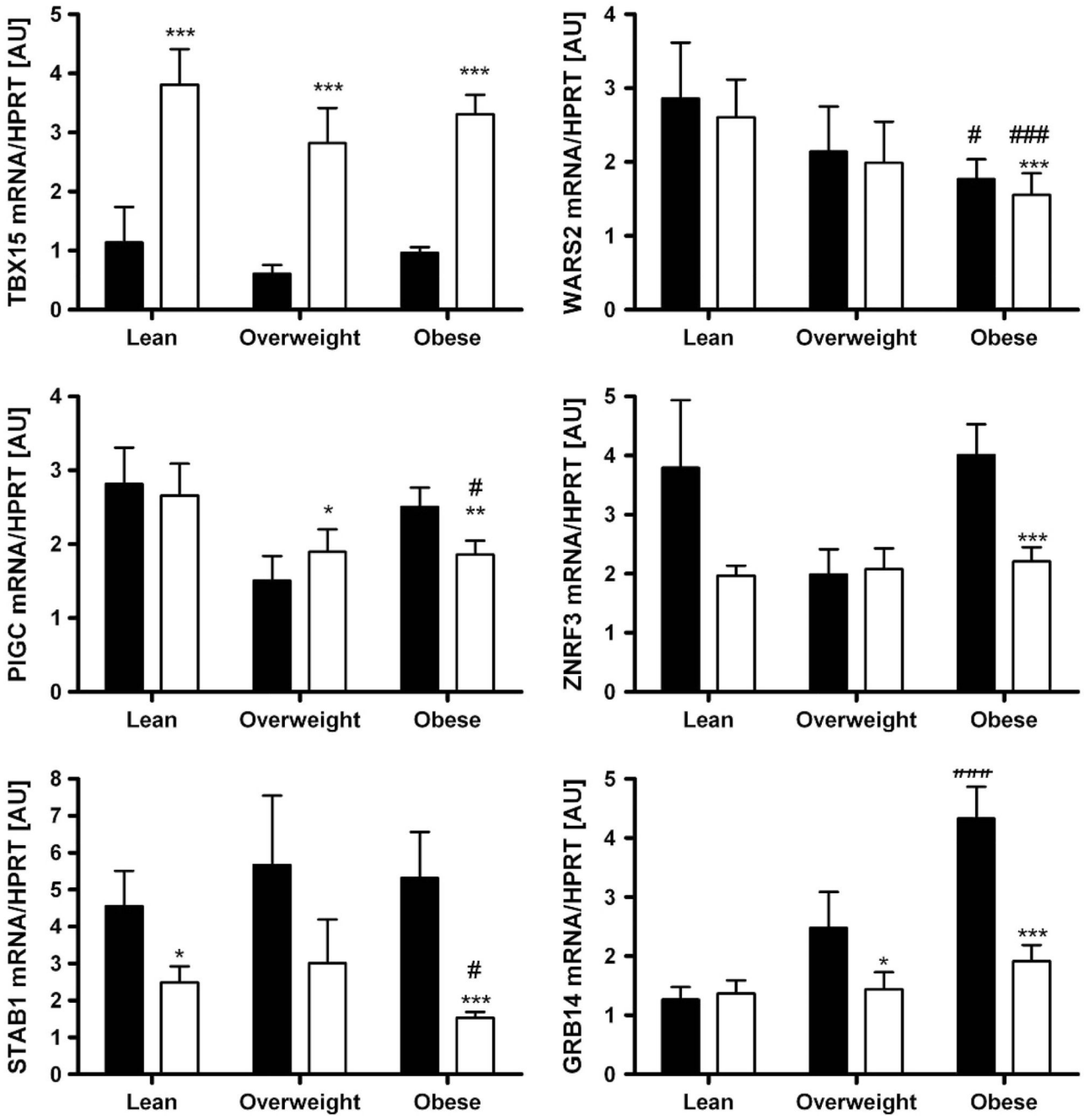

Figure 2.

Expression of *TBX15, WARS2, PIGC, ZNRF3, STAB1* and *GRB14* in paired human samples of visceral (vis) and subcutaneous (sc) adipose tissue (AT) grouped by obesity status (lean *N* = 42, overweight N = 21, obese N = 159). Mean ±s.e.m. Black bars represent vis and white bars sc AT. *sc vs vis AT depot; $^{\#}$vs lean depot. $^{*/\#}P<0.05$; $^{**/\#\#}P < 0.01$; $^{***/\#\#\#}P < 0.001$.

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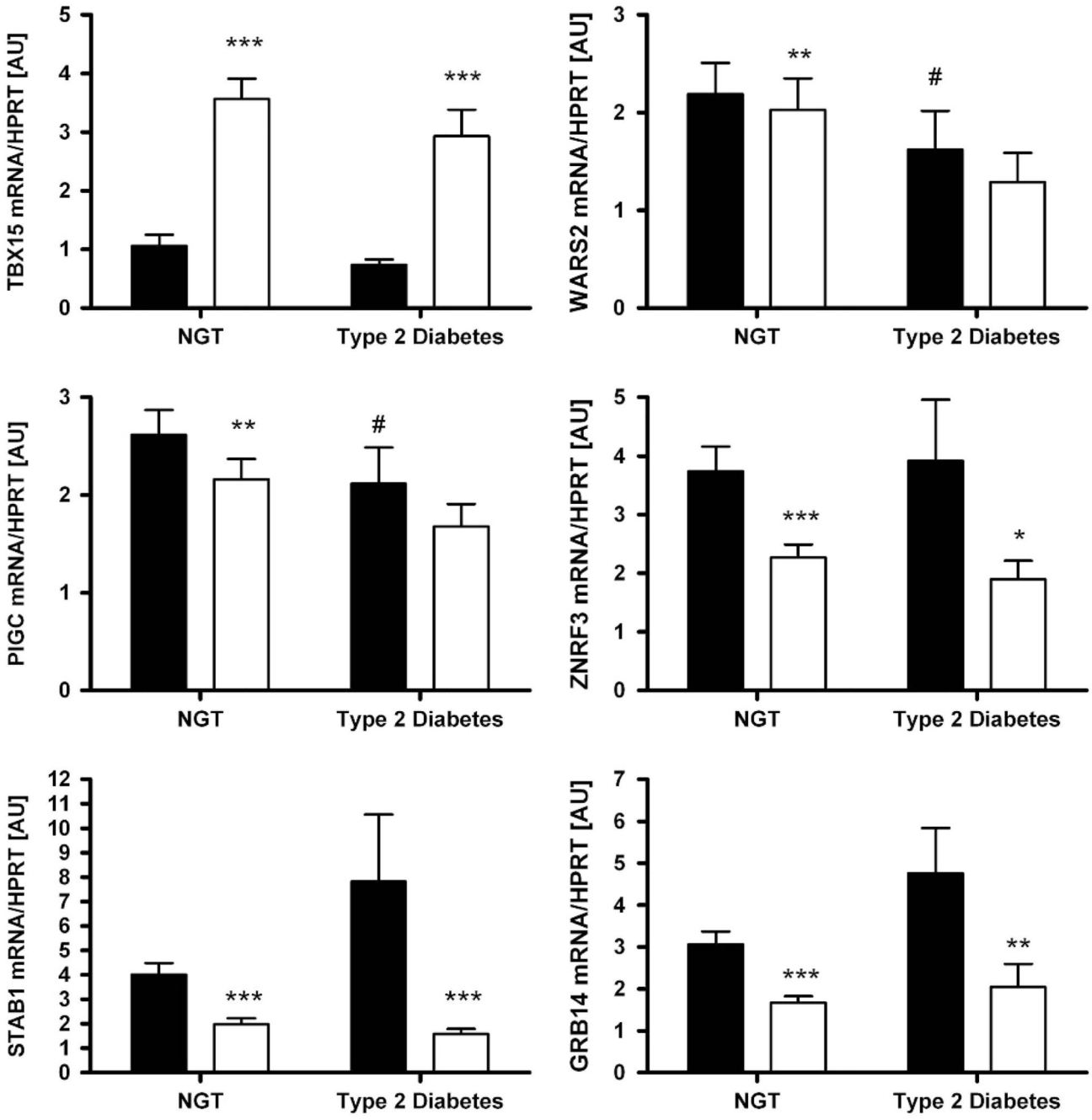

Figure 3.

Expression of *TBX15, WARS2, PIGC, ZNRF3, STAB1* and *GRB14* in paired human samples of visceral (vis) and subcutaneous (sc) adipose tissue (AT) grouped by type 2 diabetes status (NGT—subjects with normal glucose tolerance, N = 145; subjects with diabetes N = 71). Mean±s.e.m. Black bars represent vis and white bars sc AT. *vis vs sc AT depot; #vs NGT depot. *,#P < 0.05; **,##P < 0.01; ***/###P < 0.001.

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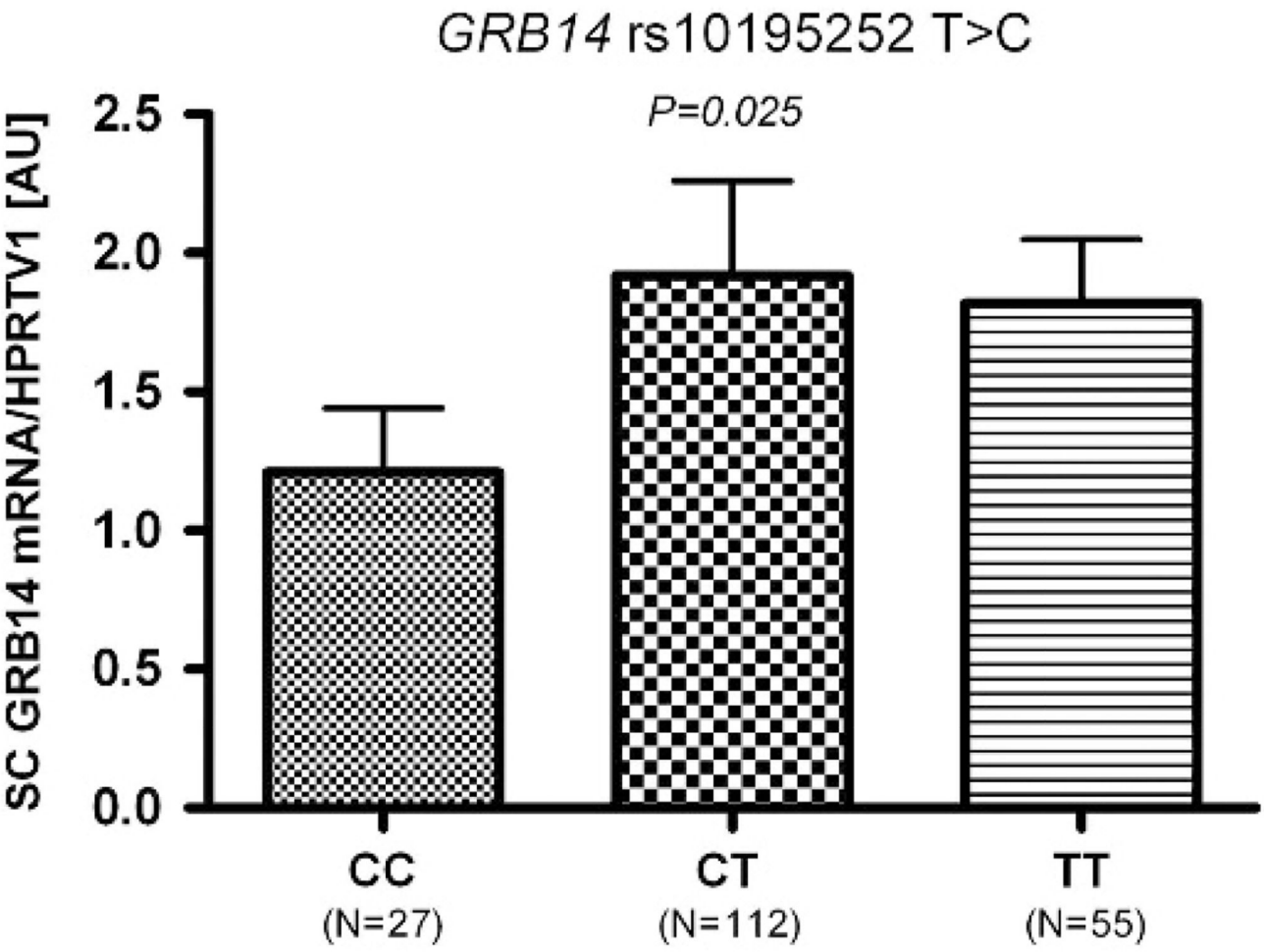

Figure 4.

Association of rs10195252 with *GRB14* mRNA expression in subcutaneous (sc) adipose tissue. Data are given as arithmetic means±s.e.m. $P < 0.05$ in additive mode of inheritance; adjusted for age, sex and body mass index.

Table 1
Correlations between gene expression and obesity-related parameters

mRNA expression	*vis fat area* (r; P-*value*)	*sc fat area* (r; P -*value*)	*BMI* (r; *P-value*)	*WHR* (r; P-*value*)
TBX15				
vis	0.02; 0.920	0.099; 0.146	0.125; 0.094	− 0.008; 0.691
sc	**− 0.137; 0.054**	− 0.089; 0.189	− 0.094; 0.133	− 0.113; 0.092
WARS				
vis	**− 0.345; 8.3 × 10 − 5**	**− 0.344; 2.8 × 10 − 4**	**− 0.248; 0.001**	− 0.089; 0.106
sc	**− 0.403; 4.0 × 10 − 6**	**− 0.414; 3.0 × 10 − 5**	**0.339; 9.2 × 10 − 5**	− 0.065; 0.056
PIGC				
vis	− 0.230; 0.003	− 0.148; 0.055	− 0.072; 0.121	− 0.118; 0.095
sc	**− 0.305; 0.001**	**− 0.256; 0.035**	**− 0.233; 0.021**	**− 0.115; 0.021**
ZNFR3				
vis	− 0.056; 0.371	− 0.014; 0.719	0.043; 0.956	− 0.071; 0.336
sc	**− 0.199; 0.009**	− 0.095; 0.248	− 0.082; 0.293	**− 0.147; 0.020**
STAB1				
vis	− 0.111; 0.238	− 0.135; 0.240	− 0.104; 0.243	0.015; 0.748
sc	**− 0.243; 0.015**	− 0.242; 0.077	− 0.214; 0.168	**− 0.089; 0.014**
GRB14				
vis	0.197; 0.056	**0.256; 0.005**	**0.284; 0.001**	0.066; 0.489
sc	0.004; 0.516	0.006; 0.173	0.036; 0.071	0.042; 0.956

Abbreviations: AT, adipose tissue; sc, subcutaneous; vis, visceral adipose tissue. Correlations with $P \leq 0.05$ are indicated in bold. All *P*-values adjusted for age and sex in linear regression models.