

Markers of remodeling in subcutaneous adipose tissue are strongly associated with overweight and insulin sensitivity in healthy non-obese men

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Abstract

Background Alteration in extracellular matrix (ECM) in adipose tissues (AT) has been associated with insulin resistance, diabetes and obesity. Aim To investigate whether selected biomarkers of ECM remodeling in AT associate with the amount and distribution of abdominal AT and with insulin sensitivity and other glucometabolic variables.

Materials and Methods Subcutaneous AT and fasting blood samples were collected in 103 middle-aged healthy non-obese men. Gene expression in AT was determined by RT-PCR and distribution of AT by computed tomography, separated into subcutaneous, deep subcutaneous and visceral AT. Circulating levels were determined by ELISA, and insulin sensitivity was measured by glucose clamp technique, assessing glucose disposal rate (GDR).

Results Metalloproteinase (MMP)-9, tissue inhibitor of MMP (TIMP)-1 and plasminogen activator inhibitor (PAI)-1 expression in AT correlated significantly to the amount of AT in all compartments ($r_s = 0.41-0.53$, all $p \leq 0.01$), and to insulin sensitivity, insulin, C-peptide, waist circumference and body mass index (BMI) ($r_s = 0.25-0.57$, all $p \leq 0.05$). When dichotomizing the glucometabolic variables at median levels the expression of MMP-9 was 5.3 fold higher in subjects with insulin sensitivity below median ($GDR < 6.3 \text{ mg/kg/min}$) ($p = 0.002$) and 3.1 fold higher in subjects with BMI above median level ($> 23.5 \text{ kg/m}^2$) ($p = 0.013$). Galectin-3 did not associate with the amount of AT, but inversely with insulin and c-peptide ($p < 0.01$ both).

Conclusion In our healthy non-obese middle-aged population AT-expressed genes, central in remodeling of ECM, associated strongly with the amount of abdominal AT, overweight and insulin sensitivity, indicating AT-remodeling to be important also in non-obese individuals. The remodeling process seems furthermore to play a central role for glucometabolic disturbances.

Background

The adipose tissue (AT) is now recognized as an active endocrine organ in addition to play a central role in the energy homeostasis¹. Especially abdominal AT has been reported to be associated with atherosclerosis and type 2 diabetes (T2DM) and insulin resistance². It has been suggested that visceral AT (VAT), accumulates when the subcutaneous AT (SAT) loose its ability to expand and store excess energy³. The SAT is separated into superficial SAT (sSAT) and deep SAT (dSAT) layers, discussed to differ metabolically with regards to insulin resistance².

In the development of overweight and obesity, AT is modified with hypertrophic adipocytes, infiltration of macrophages and other pro-inflammatory immune cells. In addition, dynamic remodeling of the extracellular matrix (ECM) occurs⁴, crucial for the expansion of the AT to allow necessary and proper structural changes⁵.

A consequence of obesity is accumulation of ECM with subsequent impairment of adapting to metabolic changes, resulting in reduced glucose uptake⁶ and insulin sensitivity⁵. The ECM composition is modified by a number of ECM degrading enzymes, like matrix metalloproteinases (MMPs), of which MMP-9 is one that are produced and released by adipocytes⁷. The activity of MMPs including MMP-9, are regulated by tissue inhibitors of MMPs (TIMPs)⁸, and also the serine protease inhibitor, plasminogen activator inhibitor (PAI)-1

plays a role in remodeling by inhibiting urokinase-induced plasminogen activation, important for MMP-9 activation^{9,10}. PAI-1 is produced and secreted from AT, and seems to be higher expressed in VAT compared to SAT¹¹. There is a body of evidence that PAI-1 levels are increased with abdominal obesity, insulin resistance, metabolic syndrome and T2DM^{12,13}. GALECTIN 3 which is present both in adipocytes and infiltrating macrophages¹⁴ localized in the ECM¹⁵ seems to be involved in expansion and remodeling of AT by stimulating preadipocytes into mature adipocytes^{16,17}. GALECTIN 3 has also recently been related to progression of obesity, insulin resistance and T2DM in humans¹⁸⁻²⁰, however, conflicting results have been reported²¹.

Although dysfunction in AT and its ECM have been associated with metabolic disturbances, the precise mechanisms are not well understood. The aim of the present study was therefore to investigate whether markers of ECM remodeling, genetically expressed in AT and circulating levels, associate with the amount and distribution of abdominal AT, and with glucometabolic variables, in a cohort of healthy non-obese men. Whether the genetic expression in AT was reflected in the circulation levels were further explored.

Materials And Methods

The present investigation is a follow-up study on 103 middle-aged Caucasian men initially recruited 20 years earlier, from the yearly military draft in Oslo and Akershus, Norway, previously described in details²².

The study was approved by the Regional Committee for Medical and Health Research Ethics, and conducted in accordance with the Helsinki Declaration. Informed written consent was obtained from all the participants. Fasting venous blood samples were drawn between 8 and 11 AM at the time of follow-up. Conventional routine methods were used for determination of fasting glucose, HbA1c, insulin, C-peptide and serum lipids. Additional serum, obtained by centrifugation within one hour at 2500 x g for 10 min, and citrate blood, kept on ice until centrifugation within 1 hour, at 2500 x g for 20 min, were kept frozen at -80°C until analysis.

Subcutaneous AT was sampled from the gluteal region and immediately stored at -80°C until RNA extraction.

Laboratory analyses

Circulating levels of MMP-9, TIMP-1 and GALECTIN 3 (all R&D Systems, Inc., 614 McKinley Place NE, Minneapolis, US) were measured in serum and PAI-1 activity (BioMedica Diagnostics, Stamford, CT, US) in citrated plasma, all by commercially available enzyme-linked immunosorbent assays (ELISA). The inter-assay coefficients of variation (CV) were 5.1%, 1.0%, 0.8% and 13.8%, respectively.

Total RNA was isolated from subcutaneous AT by use of the RNeasy Lipid Tissue Mini Kit and QIAcube according to the manufacturer protocol (Qiagen, GmbH, Hilden, Germany). The quality and quantity (ng/μL) of RNA were determined by the NanoDrop™ 1000 Spectrophotometer (Saveen Werner, Sweden). cDNA was made from equal amount of RNA (5 ng /μl) with qScript™ cDNA superMix (Quanta Biosciences, Gaithersburg, Maryland, USA). Gene expression analyses of MMP-9 (Hs00234579_m1), TIMP-1 (Hs00171558_m1), GALECTIN 3 (Hs00173587_m1) and PAI-1 (Hs01126606_m1) (all Applied Biosystems, Foster City, CA, USA) were performed with real-time PCR on a ViiA™7 instrument (Applied Biosystems) using custom designed

TaqMan RT-PCR Arrays. The mRNA levels from the RT-PCR reaction were determined with the $\Delta\Delta\text{CT}$ method, normalized to $\beta 2$ -microglobulin (HS99999907_m1) (Applied Biosystems) and related to a reference sample giving relative quantification (RQ) ²³.

Glucose clamp

To assess insulin sensitivity, glucose disposal rate (GDR), was determined by a 120 min hyperinsulinemic isoglycemic glucose clamp, modified after DeFronzo ²⁴, and used as previously described in details ²⁵. In brief, fasting glucose value was calculated from the average of three blood glucose values (Accu-Chek Performa, Roche, Mannheim, Germany) in blood drawn from catheterization of the left antecubital vein. A human insulin analog (Insulin Lispro, Eli Lilly, Houten, The Netherlands) (30 IE) was infused at a constant infusion rate at 1 mU/min/ kg body weight. Glucose infusion was started 5 min later with 20 mL/h for 5 min, 30 mL/h the next 5 min and then adjusted according to the blood glucose values measured every 5 min to keep the participants at their fasting isoglycemic level. GDR was calculated from the average glucose infusion rate from the last 20 min of the clamp divided by body weight (mg/kg/min).

Computed tomography (CT)

The amount and distribution of AT were assessed by CT. The method has previously been described in details ²⁶. Briefly, the CT-scan was conducted at the level of L3/4, with a Siemens Somatom Sensation 64 CT Scanner (Erlangen, Germany) and the CT parameters were set to 120 kV, 200 mAs and slice thickness of 5 mm. The DICOM images were analyzed with the software program Osirix (Pixmo, Geneva, Switzerland). The amount, in square centimeter (cm²) of dSAT and sSAT was assessed by tracking the circumferences divided by a membranous layer (Scarpa`s fascia). The muscle compartments including the spine, were assessed by tracking the circumferences between the dSAT and the inner abdominal wall. VAT was calculated by highlighting the pixels containing fat (-30 to -190 Hounsfield Units) after assessing the muscle compartments including the spine, by tracking the circumferences between the dSAT and the inner abdominal wall. A radiologist blinded for the other study results read the CT-scans.

Statistics

Non-parametric methods were used throughout due to mostly skewed distributed data. Data are presented as median values (25, 75 percentiles) if not otherwise stated. Correlation analyses were performed with Spearman's rho (r_s), and multiple comparisons were adjusted for by Bonferroni correction, when appropriate. Mann-Whitney U test was used for comparing continuous data between two groups. The cohort consisted of men with similar age, and the investigated metabolic variables were closely inter-correlated (Supplementary Table), therefore no adjustments in group comparisons were performed. p-values less than 0.05 were considered statistically significant. SPSS version 23 (SPSS Inc., IL, USA) was used for all analyses.

Results

Characteristics of the study population are shown in Table 1, and as can be seen the mean age was 39.5 years and the measured glucometabolic variables were within normal ranges. As were also the serum lipid levels. Family history of diabetes was present in 20%, and 5% were smokers.

Correlations between AT gene expressions of the biomarkers and the amount of abdominal AT

Unadjusted coefficients of correlation are shown in Table 2. The expression of MMP-9, TIMP-1 and PAI-1 in AT was significantly correlated to the amount of abdominal AT in all compartments (sSAT, dSAT and VAT) (all $p < 0.001$), still significant after Bonferroni correction ($p < 0.004$ by 12 performed comparisons), whereas the expression of GALECTIN 3 did not correlate significantly to any of the AT compartments.

Correlations between the AT gene expression of the biomarkers and glucometabolic variables

The expression of MMP-9 and PAI-1 in AT correlated significantly to all the glucometabolic variables (all $p < 0.05$, (Table 3)), however, for fasting glucose, the significance was lost after correction ($p > 0.002$ by 28 comparisons). Also TIMP-1 expression correlated significantly to all ($p < 0.001$, corrected $p < 0.002$), except fasting glucose and HbA1c. The expression of GALECTIN 3 was inversely correlated to insulin and C-peptide ($p < 0.05$, both), but the significance was lost after correction ($p > 0.002$).

To further explore the results, glucometabolic variables were dichotomized at the median value (Figure 1). The group with GDR below median (< 6.3 mg/kg/min) (Figure 1a) had 5.3 fold higher expression of MMP-9, 1.5 fold higher TIMP-1 and 2.1 fold higher PAI-1 expression (all $p = 0.002$), but significantly lower expression of GALECTIN 3 (1.2 fold) ($p = 0.015$).

In subjects with fasting glucose levels above median (> 5.0 mmol/L), significantly higher expression of MMP-9 was observed ($p = 0.015$), (Figure 1B), and HbA1c separated by median (5.3 %) showed those with highest levels to have significantly higher expression of MMP-9, TIMP-1 and PAI-1 ($p < 0.05$, all) (Figure 1C). Insulin and C-peptide showed the same pattern with higher expression of MMP-9, TIMP-1 and PAI-1, and were also inversely related to GALECTIN 3 ($p < 0.05$, all) (Figure 1D and E, respectively).

When separating BMI by median (23.5 kg/m²) (Figure 1F), those with high BMI had 3.1-fold higher expression of MMP-9, 1.5-fold higher TIMP-1 and 2.1 fold higher PAI-1 expression (all $p \leq 0.01$), but no difference in the expression of GALECTIN 3. Waist circumference divided by median (93.8 cm), showed the same pattern as BMI (Figure 1G).

When combining groups with high/low BMI and low/high GDR (Figure 2), it revealed that MMP-9 AT expression was significantly higher in groups with low GDR, either high ($p = 0.001$) or low ($p = 0.017$) BMI compared to the group with high GDR and low BMI. The group with low GDR and high BMI had also significant higher expression compared to the group with high GDR and high BMI ($p = 0.009$). The lowest expression occurred in the group with the combination of both low BMI and high GDR, i.e. were most insulin sensitive.

Circulating levels

Measured in the circulation, only PAI-1 correlated significantly to the amount of abdominal AT, in all compartments (all $p \leq 0.001$) (Table 2), still significant after correction ($p < 0.004$). PAI-1 correlated also significantly to glucometabolic variables (all $p < 0.05$), still significant after correction ($p < 0.002$), except for HbA1c and fasting glucose, whereas MMP-9, TIMP-1 and GALECTIN 3 did not correlate significantly to any of the glucometabolic variables (Table 3). And as shown in Table 4, PAI-1 was also the only marker found to correlate significantly to its corresponding AT gene expression ($p < 0.001$, corrected $p = 0.004$).

Discussion

In this study on healthy men without metabolic disease we observed that: 1) gene expression in subcutaneous AT of MMP-9, TIMP-1 and PAI-1, reflecting ECM remodeling, strongly associated with the amount of abdominal AT, assessed by CT, 2) the expression of these remodeling markers was strongly associated with glucometabolic variables including insulin sensitivity and BMI, 3) measured in the circulation, only PAI-1 correlated to the amount of AT and to the corresponding gene expression, and 4) PAI-1 was also the only marker measured in the circulation which correlated significantly to any of the glucometabolic variables.

Although the genes were measured in subcutaneous AT, similar associations were observed in all compartments i.e. sSAT, dSAT and VAT, suggesting the genes measured in subcutaneous AT to reflect an ongoing overall remodeling of the AT. MMP-9 has previously, in a small study, been shown to be higher expressed in VAT in obese compared to lean individuals²⁷, but also to be reduced in subcutaneous AT after weight loss in obese patient with metabolic syndrome²⁸. We have recently published data from the present population, where we observed strong associations between the amount of AT and gene expression of the pro-inflammatory cytokine interleukin-18 and the NLR family pyrin domain containing-3 inflammasome (NLRP3) and further between these inflammatory markers and the glucometabolic state²⁹. Taken together with the present findings, it seems that elevated amount of AT in non-obese healthy individuals, is accompanied by increased upregulation of inflammation and remodeling, and further reflected in the glucometabolic state, as also shown in obese subjects³⁰.

Gene expression of MMP-9 and PAI-1 correlated significantly to all the measured glucometabolic variables, including insulin sensitivity assessed by glucose-clamp, waist circumference and BMI. A similar pattern was observed for TIMP-1. The results were strengthened when dichotomizing the glucometabolic levels at median values, showing MMP-9 expression especially, to be significantly higher with less favorable values for all the glucometabolic variables. Our results are supportive to a study on non-diabetic subject, which also showed reduction in subcutaneous AT MMP-9 expression with improved insulin sensitivity after treatment with insulin-sensitizing drugs³⁰. In line with our results, it was recently also shown that expression of MMP-9 and TIMP-1 in AT from healthy overweight and obese individuals were associated with BMI, and especially MMP-9 was also strongly related to glucose clamp assessed insulin sensitivity, independent of BMI³¹. The authors discuss and concluded that the remodeling process together with adipogenesis in AT are the initial processes leading to reduced insulin sensitivity. This hypothesis is even strengthened by our additional findings of the strong associations with the amount of AT assessed by CT and further, that MMP-9 expression was high in individuals with poor insulin sensitivity, either high or low BMI. GALECTIN 3 expression did not correlate to BMI, but was inversely related to insulin sensitivity, insulin and C-peptide, although vanished after Bonferroni correction. This is to some degree in line with results from an animal model showing that GALECTIN 3 deficiency mice fed on a high fat diet develop increased VAT and increased fasting glucose, HbA1c and insulin resistance, indicating GALECTIN 3 to be protective against T2DM and obesity³². In another study increased accumulation of advanced glycation end products (AGEs) in the pancreatic islets was shown with GALECTIN 3 deficiency, suggesting GALECTIN 3 also to play a protective role against AGE related injury³³.

The only significant association found between the measured markers in circulation and the corresponding gene expression in AT was for PAI-1, which is in line with others studies, suggesting AT to be a major source of

circulating PAI-1^{34,35}. These findings are further supported in our study by PAI-1 being the only circulatory marker that also correlated to the amount of abdominal AT, in all compartments. PAI-1 was also the only circulatory marker that associated significantly to the glucometabolic variables, which is in line with accepted knowledge³⁶.

The lack of correlation between circulating MMP-9, TIMP-1 and GALECTIN 3 and their levels of gene expression in AT as well as the amount of AT, indicates that AT is not the main source for these circulatory levels, which also has been suggested by others²⁸, and enlargement of AT per se seems thus not to affect the levels of these biomarkers in the circulation. It should also be emphasized that although the remodeling markers were upregulated at the gene level, not all proteins undergo the post-transcriptional changes mandatory to function as an active protein³⁷. Nevertheless, these results, together with the lack of associations between these circulating markers and the glucometabolic variables, may indicate that dysregulation in AT, shown by genetic upregulation of the remodeling markers, results in a non-beneficial glucometabolic state in our healthy non-obese individuals. This hypothesis might be due to reduced storage capacity and reduced/ dysfunctional glucose receptors, as also suggested by others³⁸⁻⁴¹.

Although some previous studies have shown associations between obesity, remodeling of AT, inflammation and systemic insulin resistance, our results contribute to elucidate early changes in AT at the onset of overweight in association with the glucometabolic state. Although our study is limited by its cross-sectional design, it supports experimental studies in this field⁴². Strengths in our study are CT-measurement of AT amount in different compartments and insulin sensitivity measured by glucose clamp technique.

Conclusion

In our healthy non-obese population, AT-expressed genes central in remodeling of ECM, were strongly associated with overweight, insulin sensitivity and the amount of abdominal AT, indicative of remodeling to be important also in adipose tissue in non-obese individuals. The remodeling process seems furthermore to play a central role for glucometabolic disturbances.

Declarations

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Declaration

Ethics approval and consent to participate: The study was approved by the Regional Ethics Committee and written informed consent to participate was collected from all patients. The study was conducted in accordance to the Declaration of Helsinki.

Consent for publication: Not applicable

Availability of data and materials: On request

Competing interests: The authors declare that they have no competing interest.

Author`s contributions: SÅ was responsible for conduction of the study, the laboratory analysis, statistical analysis and drafting the manuscript. TAA contributed in the planning of the study and to the intellectual content and discussion of the manuscript. AF, TBO and IUN contributed to the intellectual content and discussion of the manuscript. HBE interpreted the abdominal adipose tissue images and contributed to the discussion of the manuscript. IS was a major contributor in the planning and conduction of the study, statistical analysis, interpretation of the results and the intellectual content. The manuscript have been read and approved by all authors.

Declaration of interest: none

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Tables

Table 1. Demographic characteristics, laboratory variables, and adipose tissue measures of the total study population (n=103)

Age (years)*	39.5 (3.5)
Smoking	5 %
Family history of diabetes	20 %
GDR (mg/kg/min)	6.3 (3.8, 9.0)
Fasting Glucose (mmol/L)	5.0 (4.8, 5.4)
HbA1c (%)	5.3 (5.1, 5.5)
Insulin (pmol/L)	44 (28, 64)
C-peptide (pmol/L)	641 (511, 850)
BMI (kg/m ²)	23.5 (21.8, 26.3)
Waist circumference (cm)	93.8 (87.7, 102.6)
Total cholesterol (mmol/L)	5.1 (4.5, 5.7)
HDL cholesterol (mmol/L)	1.3 (1.1, 1.6)
LDL cholesterol (mmol/L)	3.4 (2.7, 3.8)
SBP (mmHg)	118.0 (112.2, 125.0)
DBP (mmHg)	74.5 (69.5, 78.0)
MMP-9 (ng/mL)	247 (185, 320)
TIMP-1 (pg/mL)	143 (131, 162)
PAI-1 (IU/mL)	24.5 (18.2, 34.1)
Galectin-3 (ng/mL)	8.0 (6.3, 10.1)
sSAT, cm ²	94.5 (67.3, 124.8)
dSAT, cm ²	84.5 (50.5, 128.8)
VAT, cm ²	92.6 (61.2, 149.3)

The data are given as proportions or median values (25, 75 percentiles), if not otherwise stated. *mean \pm SD. For abbreviations: see text.

Table 2. Unadjusted coefficients of correlations (Spearman's rho) between genes expressed in subcutaneous adipose tissue (AT) and markers measured in the circulation and the different abdominal adipose tissue compartments assessed by axial CT scan.

	Subcutaneous AT expression				Circulating levels			
	MMP-9	TIMP-1	PAI-1	GALECTIN 3	MMP-9	TIMP-1	PAI-1	GALECTIN 3
sSAT	0.41***	0.37**	0.53***	-0.08	0.19	0.11	0.39***	0.16
dSAT	0.42***	0.47***	0.52***	-0.14	0.20	0.07	0.55***	0.18
VAT	0.51***	0.53***	0.50***	-0.05	0.13	0.12	0.56***	0.03

** $p < 0.01$, *** $p \leq 0.001$

Table 3. Unadjusted coefficients of correlations (Spearman's rho) between the glucometabolic variables and gene expression in subcutaneous adipose tissue (AT) and levels in circulation (s).

	Subcutaneous AT expression				Circulating levels			
	AT MMP-9	AT TIMP-1	AT PAI- 1	AT GALECTIN 3	sMMP- 9	sTIMP- 1	sPAI-1	sGALECTIN 3
GDR	-0.51***	-0.47***	-0.50***	0.18	-0.18	-0.11	-0.59***	0.05
Fasting glucose	0.33**	0.18	0.25*	-0.16	0.12	0.13	0.28**	-0.01
HbA1c	0.46***	0.22	0.38***	-0.23	0.17	0.09	0.18	-0.11
Insulin	0.43***	0.42***	0.39***	-0.28*	0.17	0.15	0.55***	0.03
C- peptide	0.47***	0.41***	0.39***	-0.30*	0.13	0.16	0.59***	-0.05
BMI	0.45***	0.48***	0.50***	-0.20	0.11	-0.01	0.55***	0.04
Waist	0.52***	0.57***	0.56***	-0.12	0.13	0.05	0.58***	0.11

* $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$

Table 4. Unadjusted coefficients of correlations (Spearman's rho) between gene expression in subcutaneous adipose tissue (AT) and levels in circulation (s).

		Circulating levels			
		sMMP-9	sTIMP-1	sPAI-1	sGALECTIN 3
Gene expression	AT MMP-9	-0.037	0.072	0.354**	0.051
	AT TIMP-1	0.039	0.099	0.534***	0.030
	AT PAI-1	-0.023	0.247*	0.425***	0.044
	AT GALECTIN 3	-0.051	-0.260*	-0.056	0.084

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figures

Figure 1.

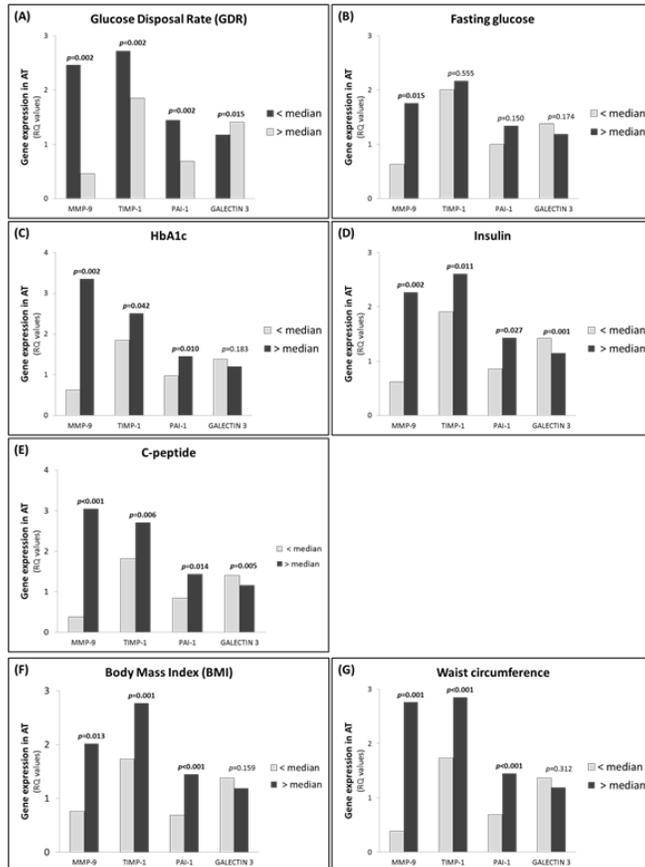


Figure 1

Gene expression of remodeling biomarkers as related to glucometabolic variables dichotomized by median values. p-values refer to Mann-Whitney U Test.

Figure 2.

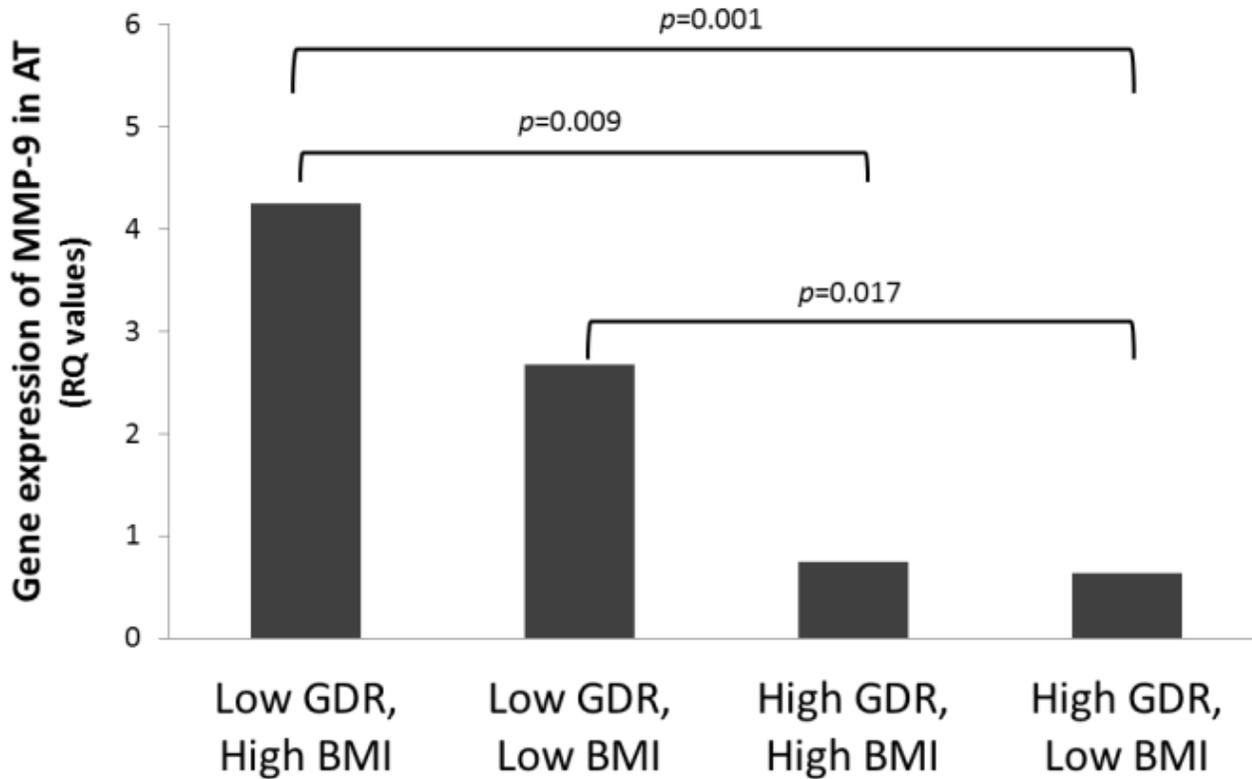


Figure 2

AT gene expression of MMP-9 related to groups combining subjects with low/high GDR and high/low BMI. Low GDR/ High BMI: Subjects in the lowest quartile of GDR (< 3.8 mg/kg/min) i.e. with poorest insulin sensitivity and the highest quartile of BMI (>26.3 kg/m²), (n=11) Low GDR/ Low BMI: Subjects in the lowest quartile of GDR and the three lowest quartiles of BMI, (n=14) High GDR/ High BMI: Subjects in the three upper quartiles of GDR (> 3.8 mg/kg/min) and highest quartile of BMI (>26.3 kg/m²), (n=14) High GDR/ Low BMI: Subjects in the three upper quartiles of GDR and the three lowest quartiles of BMI, (n=59)

Supplementary Files

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- [SupplementaryTable.docx](#)