

Cardiovascular-related proteins and the abdominal visceral to subcutaneous adipose tissue ratio

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Abstract *Background and aims:* An increased amount of visceral adipose tissues has been related to atherosclerosis and future cardiovascular events. The present study aims to investigate how the abdominal fat distribution links to plasma levels of cardiovascular-related proteins.

Method and results: In the Prospective investigation of Obesity, Energy and Metabolism (POEM) study (n = 326, all aged 50 years), abdominal visceral (VAT) and subcutaneous (SAT) adipose tissue volumes were quantified by MRI. Eighty-six cardiovascular-related proteins were measured by the proximity extension assay (PEA). Similar investigations were carried out in the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study (n = 400, all aged 75 years).

In the discovery dataset (POEM), 10 proteins were related to the VAT/SAT-ratio using false discovery rate < .05. Of those, Cathepsin D (CTSD), Interleukin-1 receptor antagonist protein (IL-1RA) and Growth hormone (GH) (inversely) were related to the VAT/SAT-ratio in the validation in PIVUS following adjustment for sex, BMI, smoking, education level and exercise habits (p < 0.05). In a secondary analysis, a meta-analysis of the two samples suggested that 15 proteins could be linked to the VAT/SAT-ratio following adjustment as above and Bonferroni-correction of the p-value.

Conclusion: Three cardiovascular-related proteins, cathepsin D, IL-1RA and growth hormone, were being associated with the distribution of abdominal adipose tissue using a discovery/validation approach. A meta-analysis of the two samples suggested that also a number of other cardiovascular-related proteins could be associated with an unfavorable abdominal fat distribution. © 2020 The Italian Diabetes Society, the Italian Society for the Study of Atherosclerosis, the Italian Society of Human Nutrition and the Department of Clinical Medicine and Surgery, Federico II University. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Introduction

Visceral accumulation of fat, as evaluated by visceral adipose tissue volume (VAT) at computerized tomography (CT) or magnetic resonance imaging (MRI), is known to be related to risk factors for cardiovascular disease to a

greater extent than abdominal subcutaneous tissue (SAT) [1–5]. It has therefore been suggested to use the ratio between VAT and SAT as a marker for increased CV risk, since this measure is related to increased CV risk also beyond BMI [6].

In accordance with findings regarding CV risk factors, it was shown in the Dallas Heart study that VAT, but not SAT, was related to coronary calcium at scanning of the coronary arteries [7], and in other studies that the VAT/SAT ratio was associated with coronary atherosclerotic plaque

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[8], as well as to an increased intima-media thickness (IMT) in the carotid arteries [9]. Also two prospective studies have shown the VAT/SAT ratio to be related to future major cardiovascular events (MACE) [10,11].

In order to further characterize the mechanisms behind the associations between an increased VAT/SAT ratio and atherosclerosis and future CV events, we measured the VAT/SAT ratio and 86 cardiovascular-related proteins in two different population-based cohorts, Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study [12], and the Prospective investigation of Obesity, Energy and Metabolism (POEM) study [13]. We evaluated the hypothesis that some of these proteins were related to the VAT/SAT ratio independently of general obesity, as evaluated by BMI using a discovery/validation approach. As a secondary analysis, we also performed a meta-analysis of the two samples.

Table 1 Basic characteristics in the two samples. Mean and SD or proportions are given.

Variable	POEM (n = 326)	PIVUS (n = 400)
Age	50	75
Sex (% females)	51%	48%
BMI (kg/m ²)	26.4 (4.1)	26.8 (4.2)
VAT (L)	3.4 (2.1)	2.2 (1.1)
SAT (L)	6.6 (3.3)	3.6 (1.5)
VAT/SAT ratio	.54 (.31)	.63 (.31)
Liver fat (%)	4.1 (5.7)	Not measured
Education level	<10 years: 6% 10–12:44% >12:50%	<10 years: 58% 10–12:20% >12:22%
Exercise habits	Sedentary:13% Low:23% Medium:35% High:29%	Sedentary:11% Low:60% Medium:22% High:7%
Current smoker	9.9%	5.9%
Antihypertensive treatment	8.2%	48%
Myocardial infarction	0%	8.2%
Stroke	0%	7.4%
Heart failure	0%	5.1%
Diabetes	2.2%	15%
Statin use	3.4%	25%
Insulin treatment	1.2%	3.3%
Oral antidiabetics	2.3%	8.7
Antiarrhythmic drugs	0%	1.2%

We have previously been involved in the selection of proteins on a multiplex protein analysis chip. 92 proteins were selected that in experimental and clinical studies have shown a relationship with atherosclerosis and other cardiovascular diseases. Some proteins with an obvious link to cardiovascular diseases, such as CRP or troponins, could not be used in this multiplex system due to lack of available, valid commercial antibodies, levels being severalfold different than the majority of the other proteins or other technical reasons.

Methods

Samples

In the Prospective investigation of Obesity, Energy and Metabolism (POEM) study, subjects aged 50 years living in the city of Uppsala, Sweden, were invited to a health screening survey in 2011–2017. Of the 503 investigated individuals, 326 performed an examination with MRI of the abdomen. The details have previously been described [13].

In the In the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study, subjects aged 70 years living in the city of Uppsala, Sweden, were invited to a health screening survey in 2001–2004. 1,016 individuals participated (50% women). The details have previously been described [12]. A reinvestigation was performed after 5 years (n = 826), including an examination with MRI of the abdomen in 400 individuals.

All subjects gave their informed consent and the study was approved by the Ethics Committee of Uppsala University.

All subjects in both samples were investigated in the morning after an overnight fast. Blood was drawn and plasma was put in –80C for later analysis of proteins. Height and weight were recorded and BMI was calculated. In a questionnaire, the participants were asked about current smoking, education level (years in school <10, 10–12, >12) and exercise habits (sedentary, light exercise, moderate and athlete).

Proteomics analysis

Using the proximity extension assay (PEA) technique 92 proteins were measured by the OLINK CVD-1 chip (Olink,

Table 2 Discovery phase: Relationships between cardiovascular proteins and the VAT/SAT ratio in the POEM study when adjusted for sex only (age same in all subjects). Only proteins with a false discover rate (FDR) < 5% are shown. Results for all proteins in the POEM sample are given in Fig. 1.

Protein	Beta	95%CI lower	95%CI higher	p-value
Interleukin-1 receptor antagonist protein (IL-1RA)	.051	.031	.07	8.18e-07
Tissue-type plasminogen activator (t-PA)	.051	.031	.071	1.35e-06
Growth hormone (GH)	-.053	-.077	-.03	8.82e-06
Cathepsin D (CTSD)	.046	.024	.067	.000033
E-selectin (SELE)	.043	.022	.063	.000047
Vascular endothelial growth factor D (VEGF-D)	-.036	-.057	-.015	.00068
Pentraxin-related protein PTX3 (PTX3)	-.034	-.053	-.014	.00077
Endothelial cell-specific molecule 1 (ESM-1)	-.035	-.056	-.015	.00088
Interleukin-18 (IL-18)	.034	.013	.055	.0015
SIR2-like protein (SIRT2)	.033	.011	.056	.0038

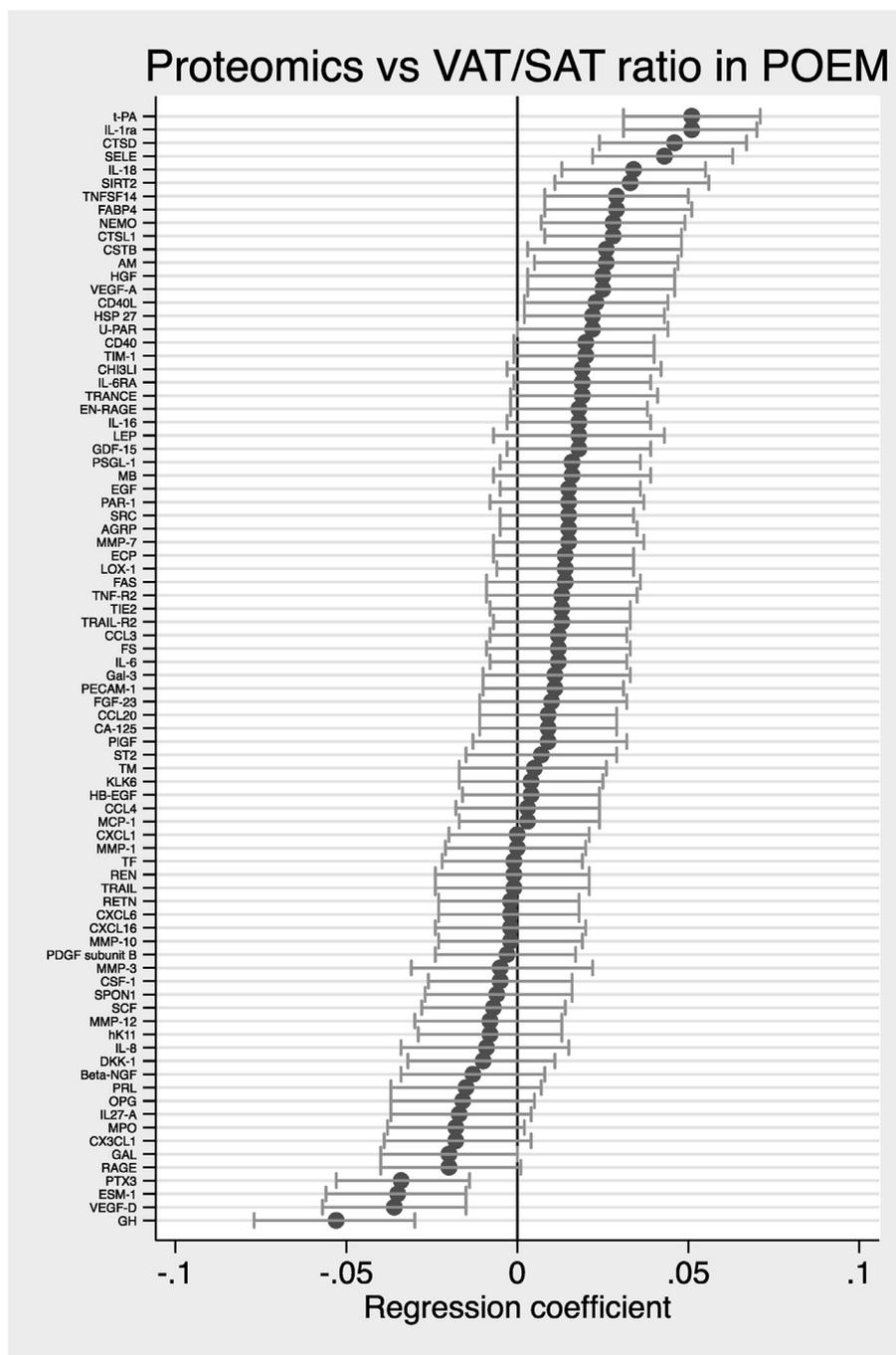


Figure 1 Discovery phase: Relationships between cardiovascular proteins and the VAT/SAT ratio in the POEM study when adjusted for sex only (all subjects of the same age).

Uppsala, Sweden). 86 proteins with a call-rate $>75\%$ were further evaluated. Details on measurements, QC etc have been given previously [14].

Magnetic resonance imaging (MRI)

All subjects were imaged on a 1.5T clinical MR system (Philips Achieva, Philips Healthcare, Best, Netherlands).

In the POEM study the subjects were examined in supine position using the body coil and a whole-body water-fat imaging protocol that used a spoiled 3D multi-echo

gradient sequence. Scan parameters and water-fat reconstruction and quantification of VAT, SAT and liver fat has previously been described in detail [13]. In brief, the VAT and SAT depots were quantified by deforming manually defined depots in a male and female reference subject to all other subjects by utilizing image registration. The deformed regions were further processed by thresholding operations, removing voxels with fat content $<50\%$. Liver fat was quantified by manual delineation of the volume of interest using the software ImageJ (version 1.45s, National Institute of Mental Health, Bethesda, Maryland, USA) [15].

Table 3 Validation phase: Relationships between the cardiovascular proteins with $FDR < .05$ in the discovery phase and the VAT/SAT ratio in the PIVUS study when adjusted for sex only (age same in all subjects) and following further adjustment for BMI and some life-style factors, education level, smoking and exercise habits. Results for all proteins in the PIVUS sample are given in Fig. 2.

Protein	Sex-adjusted				Adjusted for sex, BMI, education level, smoking and exercise habits			
	Beta	95%CI lower	95%CI higher	p-value	Beta	95%CI lower	95%CI higher	p-value
Cathepsin D (CTSD)	.056	.032	.079	5.71e-06	.048	.023	.074	.00026
Interleukin-1 receptor antagonist protein (IL-1RA)	.046	.021	.071	.00039	.043	.013	.072	.0052
Tissue-type plasminogen activator (t-PA)	.036	.008	.064	.012	.028	-.002	.057	.069
Growth hormone (GH)	-.03	-.055	-.005	.021	-.031	-.057	-.005	.019
Pentraxin-related protein PTX3 (PTX3)	-.024	-.05	.002	.075	-.026	-.053	.002	.067
E-selectin (SELE)	.021	-.002	.044	.080	.015	-.01	.04	.23
Vascular endothelial growth factor D (VEGF-D)	-.022	-.049	.004	.099	-.019	-.046	.008	.16
Endothelial cell-specific molecule 1 (ESM-1)	-.019	-.045	.008	.16	-.013	-.041	.015	.36
Interleukin-18 (IL-18)	.017	-.007	.042	.17	.016	-.01	.041	.22

In PIVUS the abdominal imaging was performed as previously described [16] using the body coil. In brief, 16 contiguous axial T1-weighted slices, centred at the L4-L5 level, were acquired in one breath hold. The abdominal MRI data was analysed using in-house developed automated software [17]. The coefficients of variation (defined as standard deviation divided by mean) from test retest measurements have been determined to 2.81% and 1.45% for VAT and SAT, respectively.

Statistics

Protein levels were log₂-transformed to achieve normal distributions. They were thereafter transformed to a SD-scale to allow direct comparison between the proteins.

Discovery step: In the POEM study, one linear regression model was run for each protein. The VAT/SAT ratio was the dependent variable and sex and BMI were used as a confounders. Proteins showing a false discovery rate ($FDR < 5\%$) was taken further to the validation step.

Validation step: In the PIVUS study, two linear regression models were run for each protein with $FDR < 5\%$ in the discovery step. In the first model, the VAT/SAT ratio was the dependent variable and sex and BMI were used as a confounders. In the second model, the life-style factors smoking, education level and exercise habits were added to sex and BMI as confounders. In this step $p < 0.05$ was considered as significant.

To investigate possible interactions with sex, in the PIVUS study, a separate model including an interaction term between sex and the protein was evaluated for each protein.

To investigate if the proteins found to be related to the VAT/SAT ratio in the validation step were associated with VAT, SAT and liver fat, we performed linear regression models with sex, BMI and the life-style factors smoking,

education level and exercise habits as confounders (performed in the POEM study only).

Secondary analysis: Results from the fully adjusted model in POEM and PIVUS with the VAT/SAT ratio as the dependent variable were meta-analyzed using inverse-variance weighted fixed effect models. In this secondary analysis, Bonferroni-adjustment of the p-value for 86 tests was applied ($p < 0.00059$).

Results

Basic characteristics for the two samples are given in Table 1. In the discovery phase in the POEM study, 10 proteins showed $FDR < 5\%$ vs the VAT/SAT ratio (see Table 2 and Fig. 1 for details).

When these 10 proteins were analyzed in the PIVUS sample as validation, four proteins showed $p < 0.05$ vs the VAT/SAT ratio when adjusted for sex only Cathepsin D (CTSD), Interleukin-1 receptor antagonist protein (IL-1RA), Tissue-type plasminogen activator (t-PA) and Growth hormone (GH). Following further adjustment for BMI and some life-style factors, all, but t-PA ($P = 0.069$), still showed $p < 0.05$ (see Table 3 and Fig. 2 for details).

An analysis of possible sex interactions in the PIVUS study did not disclose any interactions between sex and a protein regarding the VAT/SAT ratio, using $p < 0.00059$ as significance.

When the validated proteins being associated with the VAT/SAT ratio were related to VAT, SAT and liver fat in the POEM study, CTSD showed highly significant relationships vs VAT and liver fat, but the p-value for SAT was of borderline significance ($p = 0.02$). This pattern is in contrast with IL-1RA which showed highly significant relationships vs VAT and liver fat, not to SAT ($p = 0.11$). GH, on the other hand, was mainly related to liver fat in an

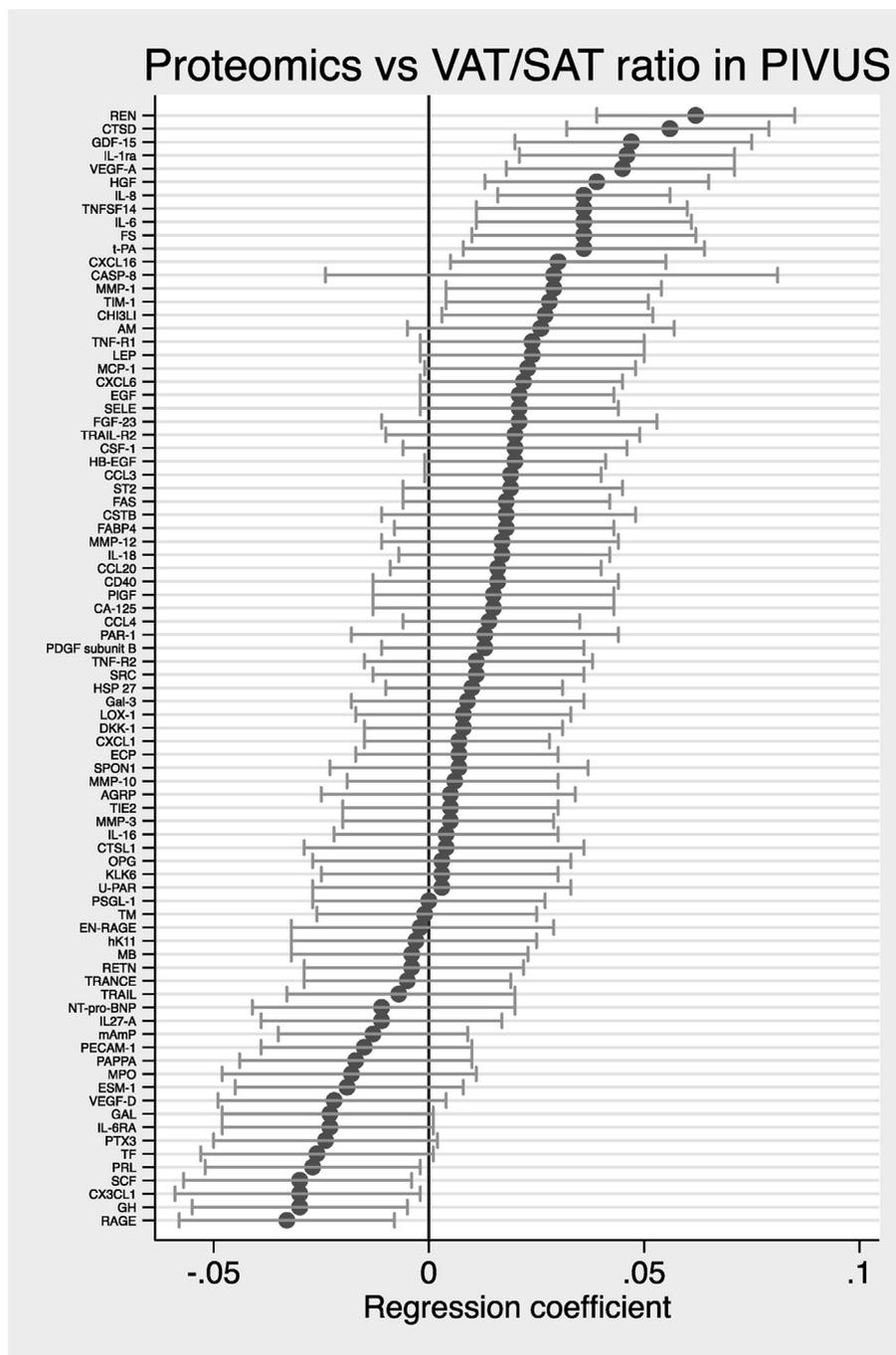


Figure 2 Validation phase: Relationships between cardiovascular proteins and the VAT/SAT ratio in the PIVUS study when adjusted for sex only (all subjects of the same age).

inverse was, while the inverse relationship vs VAT was of borderline significance (see [Table 4](#)).

In the secondary analysis in which the POEM and PIVUS samples were meta-analyzed, fifteen proteins showed $p < 0.00059$ following adjustment for BMI, smoking and some other life-style factors (Bonferroni-adjustment) (see [Table 5](#)).

When we further added information on myocardial infarction, stroke, heart failure, antihypertensive medication,

statin use, diabetes, insulin and oral antidiabetic treatment and antiarrhythmic drug use to the models, the point estimates were essentially unchanged with only a marginally increase in the p-values corresponding to the expected rise due to adding 9 additional co-variates to the model.

To evaluate if the relationships between the proteins and VAT/SAT are influenced by BMI, we added an interaction term between BMI and the proteins to the adjusted models. However, the p-value was not significant for any

Table 4 Relationships between the three proteins being related to the VAT/SAT ratio and VAT, SAT and liver fat in the POEM study. Relationships were adjusted for BMI, education level, smoking and exercise habits.

	Cathepsin D (CTSD)	Growth hormone (GH)	Interleukin-1 receptor antagonist (IL-1RA)
VAT			
Beta	.20	-.086	.21
95%CI lower	.12	-.166	.13
95%CI higher	.27	-.0056	.28
p-value	6.41e-08	.035	2.43e-08
SAT			
Beta	.067	.009	.047
95%CI lower	.010	-.053	-.011
95%CI higher	.12	.071	.105
p-value	.020	.76	.11
Liver fat			
Beta	.24	-.14	.21
95%CI lower	.14	-.26	.11
95%CI higher	.33	-.038	.30
p-value	6.72e-07	.0086	.000016

Table 5 Relationships between the cardiovascular proteins and the VAT/SAT ratio in a meta-analysis of the PIVUS and POEM studies when adjusted for sex, BMI and some life-style factors, education level, smoking and exercise habits. Only proteins with $p < 0.00059$ are shown (Bonferroni-adjustment). SE = standard error.

Protein	Beta	SE	p-value
Interleukin-1 receptor antagonist protein (IL-1RA)	.06	.0079	4.04e-14
Tissue-type plasminogen activator (t-PA)	.059	.0082	1.16e-12
Growth hormone (GH)	-.05	.0086	7.71e-09
Endothelial cell-specific molecule 1 (ESM-1)	-.038	.0075	5.28e-07
Cathepsin D (CTSD)	.041	.0082	7.77e-07
E-selectin (SELE)	.037	.0075	1.04e-06
Vascular endothelial growth factor D (VEGF-D)	-.036	.0075	2.02e-06
Pentraxin-related protein PTX3 (PTX3)	-.033	.0072	4.80e-06
SIR2-like protein (SIRT2)	.036	.0082	.000014
NF-kappa-B essential modulator (NEMO)	.031	.0075	.000042
Fatty acid-binding protein 4 (FABP4)	.038	.010	.00016
Interleukin-18 (IL-18)	.028	.0075	.00021
Cathepsin L1 (CTSL1)	.026	.0072	.00031
Heat shock 27 kDa protein (HSP 27)	.026	.0072	.00031
CD40 ligand (CD40L)	.027	.0075	.00036

of these interactions, suggesting that the relationships between the proteins and VAT/SAT are not influenced by general obesity to a major degree.

Discussion

The present study found three replicated proteins to be related to the VAT/SAT ratio following adjustment for BMI, Cathepsin D (CTSD), Interleukin-1 receptor antagonist protein (IL-1RA), and Growth hormone (GH). A meta-analysis suggested that also another 12 proteins might be of interest in this respect. It was furthermore found that CTSD, e-selectin (SELE) and Galanin peptides (GAL) were related to VAT, but not SAT.

Comparison with literature

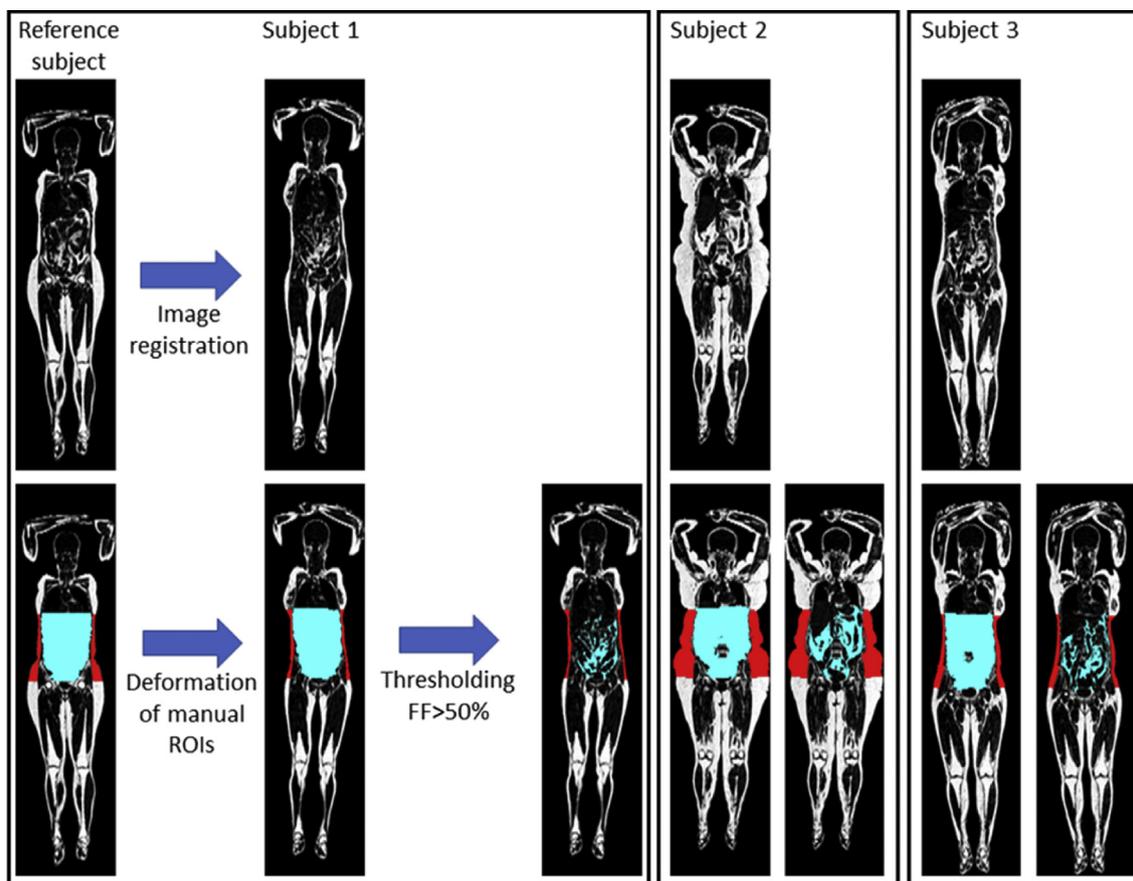
It is well known that CRP and a number of proinflammatory cytokines, such as IL-6, are increased in obese subjects, as reviewed by Fain [18]. Also a relationship between CRP levels and the VAT/SAT ratio has previously been described [19]. In the present study, using a

proteomics approach, a validated relationship between the VAT/SAT ratio and IL-1RA was seen, and in the meta-analysis, IL-18 and CD40L, two other proinflammatory-related proteins, were related to the VAT/SAT ratio, further supporting the role of inflammation in visceral adipose tissue accumulation. A recent Mendelian randomization analysis showed that obesity (BMI) was causally linked to increased levels of a number of proinflammatory proteins, including IL-1RA (SCALLOP).

Genome-wide association studies have identified three genetic loci being related to the VAT/SAT ratio, with closest genes being *UBE2E2*, *LYPLAL1* and *LY86* [20], but the published genome-wide association studies on VAT and SAT are too small to give a good power to evaluate the causal effect of the VAT/SAT ratio on these proteins.

Cathepsin D is a protease previously shown to be associated with the Metabolic Syndrome [21], diabetes and insulin resistance [22], and endothelial dysfunction [23].

A recent Mendelian randomization analysis showed that obesity (BMI), as well as to the waist/hip ratio, was causally linked to increased levels of also this protein



Supplementary figure The abdominal VAT and SAT segmentation process for three randomly chosen subjects in the POEM cohort: Manually defined ROIs (regions of interest) for the reference subject is deformed to all other subjects in the cohort. The final volumes are obtained by removing voxels with a fat fraction (FF) lower than 50%. Coronal slices of the volume images are shown.

(SCALOP). In the present study, Cathepsin D was related to the VAT/SAT ratio independently of BMI, suggesting that cathepsin D levels are influenced by the distribution of abdominal fat, not only to the total fat mass.

As reviewed by Berryman and List [24], reduced GH levels have been noticed in obese subjects. However, a meta-analysis of treatment trials of GH does not support a role of administration of GH to reduce body weight [25].

CTSD was in the POEM study related to VAT and liver fat, but showed also a borderline significance vs SAT. IL-1RA was also related to VAT and liver fat, but did not show a significant relationship vs SAT, when adjusted for BMI. Thus, of these two proteins, IL-1RA seems to be the most specific biomarker for ectopic fat accumulation.

Strength and limitations

The strength of the present study is that we have two independent samples including measurements of the same proteins as well as volumetric MRI measurements of VAT and abdominal SAT. It should be noticed however that different protocols were used for the abdominal MRI with a more extensive coverage of the abdominal cavity in the

POEM study. Although the absolute level of VAT and SAT is different in PIVUS and POEM, the VAT/SAT ratio is as expected slightly higher in the older PIVUS sample. This difference in absolute levels for VAT and SAT in the two samples is the reason for the transformation to the SD scale when the meta-analysis was performed for the proteins vs VAT and SAT in separate models. If this difference in protocols used for the abdominal MRI would influence the results, it could only be towards the null hypothesis, producing false negative findings.

In conclusion, three cardiovascular-related proteins, cathepsin D, IL-1RA and growth hormone, were being associated with the distribution of abdominal adipose tissue using a discovery/validation approach. A meta-analysis of the two samples suggested that also a number of other cardiovascular-related proteins could be associated with an unfavorable abdominal fat distribution.

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