PAPP-A, IGFBP-4 and IGF-II are secreted by human adipose tissue cultures in a depot-specific manner

Mette Faurholdt Gude¹, Rikke Hjortebjerg^{1,2}, Claus Oxvig³, Anne Anker Thyø⁴,
Nils Erik Magnusson¹, Mette Bjerre¹, Steen Bønløkke Pedersen^{1,5} and Jan Frystyk^{1,5}

¹Medical Research Laboratory, Department of Clinical Medicine, Faculty of Health, Aarhus University, Aarhus C, Denmark, ²Danish Diabetes Academy, Odense, Denmark, ³Department of Molecular Biology and Genetics, Faculty of Science and Technology, Aarhus University, Aarhus C, Denmark, ⁴Departments of Abdominal Surgery, and ⁵Endocrinology and Internal Medicine, Aarhus University Hospital, Aarhus C, Denmark

Correspondence
should be addressed
to J Frystyk
Email
Frystyk@clin.au.dk

Abstract

Objective: Adipose tissue secretes pregnancy-associated plasma protein-A (PAPP-A), which may increase local IGF action through cleavage of IGF-binding protein-4 (IGFBP-4). We tested whether this mechanism was operational in human visceral and subcutaneous adipose tissue (i.e. VAT and SAT).

Design: Explants of VAT and SAT from 26 obese subjects (hereof 17 women, BMI 39.5 (37.2; 42.8) kg/m² (median (25%; 75% confidence interval) and SAT from eight lean, age-matched women (BMI 23.6 (22.4; 24.9) kg/m²) were incubated with or without GH (100 µg/L) and the media were harvested.

Methods: Media were assessed for concentrations of PAPP-A, intact and PAPP-A-cleaved IGFBP-4, IGF-I and IGF-II, and IGF-I receptor (IGF-IR) activation by bioassay.

Results: In obese subjects, VAT media contained higher concentrations than SAT of PAPP-A (4.4-fold) and both PAPP-A-generated IGFBP-4 fragments (C-terminal: 3.3-fold, N-terminal: 1.5-fold) (all $P < 0.0005$). Intact IGFBP-4 levels were similar in SAT and VAT. VAT media contained elevated IGF-II (1.4-fold; $P < 0.005$), but similar IGF-I concentrations compared with SAT. Still, VAT media contained a 1.8-fold increased ability to stimulate the IGF-IR ($P < 0.005$). IGF-I protein concentration and IGF-IR activation increased more in VAT media than SAT media following GH stimulation (both $P < 0.05$). At baseline, SAT media protein levels from lean and obese women were similar, with the exception of PAPP-A being 1.8-fold elevated in VAT media ($P < 0.05$). GH induced a similar increase in IGF-I media levels in SAT from obese and lean women.

Conclusion: Human adipose tissue cultures secrete enzymatically active PAPP-A, IGFBP-4 and IGF-II in a depot-specific manner, suggesting differential regulation of IGF activity. Further, IGF-II appears to be more prominent than IGF-I. Finally, VAT appears more GH responsive than SAT.

European Journal of
Endocrinology
(2016) **175**, 509–519

Introduction

Adipose tissue synthesizes proteins belonging to the insulin-like growth factor (IGF) system. This includes the IGF-I receptor (IGF-IR), its two ligands, IGF-I and IGF-II, and many of the IGF-binding proteins (IGFBPs)

(1, 2, 3). The role of the IGF system in adipose tissue remains to be defined, but the expression and secretion of IGF-I appear to be important for adipocyte homeostasis. *In vitro*, IGF-I stimulates adipocyte growth and lipogenesis

Clinical Study	M F Gude and others	PAPP-A and IGFBP-4 in human adipose tissue	175:6	510
----------------	---------------------	--	-------	-----

(4) as well as the differentiation of pre-adipocytes into mature adipocytes, and IGF-I also controls anti-apoptotic signaling (5, 6, 7, 8).

The vast majority of the IGFs are complexed to IGFBPs, which block the growth factors from activating their primary target, the IGF-IR (9). Accordingly, to become receptor accessible the IGFs need to be liberated. This is achieved by enzymatic cleavage of the IGFBPs, causing release of bound IGF. Pregnancy-associated plasma protein-A (PAPP-A) is one of the most well-characterized IGFBP-degrading enzymes. The primary substrate for PAPP-A is IGFBP-4, which is cleaved in an IGF-dependent manner, and by this mechanism PAPP-A has been demonstrated to control growth, visceral fat accumulation and longevity in experimental animal models (10, 11, 12).

Adipose tissue from different regions is inherently distinct in regard to expression and secretion of pro- and anti-inflammatory proteins (13, 14). The regional differences may explain that accumulation of visceral adipose tissue (VAT) associates more strongly with an unfavorable metabolic risk profile than does subcutaneous adipose tissue (SAT) (15). Also PAPP-A, which holds pro-atherosclerotic properties (11), appears to be secreted in a site-specific manner. Studies in experimental animals have demonstrated that PAPP-A is highly expressed in and secreted from pre-adipocytes of visceral origin compared with subcutaneous pre-adipocytes, and the expression and secretion of IGFBP-4 follow the same regional distribution (10, 16, 17, 18). At present, human data are scanty and it remains unknown whether the site-specific secretion of PAPP-A and IGFBP-4 translates into regional differences in local IGF action.

Insulin stimulates the hepatic growth hormone (GH) sensitivity by increasing the GH receptor (GHR) synthesis (19). This explains why obese subjects, despite a reduced GH secretion (20), are able to maintain serum IGF-I levels within the normal range (21, 22, 23, 24), and it also explains why obese subjects respond with a higher increase in serum IGF-I following GH administration than lean subjects (25). Whether obesity also augments the sensitivity to GH in human adipose tissue remains unknown.

This study aimed to compare the expression and secretion of components of the IGF system in media from cultures of SAT and VAT from obese subjects, focusing on media levels of PAPP-A, intact IGFBP-4 and PAPP-A-generated IGFBP-4 fragments and their impact on IGF bioactivity. Furthermore, by stimulating SAT with GH *in vitro*, we tested the hypothesis that GH was able to

induce a higher IGF-I production in adipose tissue from obese than lean women.

Subjects and methods

Study participants

VAT and SAT were collected peri-operatively during elective bariatric surgery from 26 obese subjects (age 38.0 (33.5; 43.5) (median (25%; 75% confidence interval)); BMI 39.5 (37.2; 42.8) kg/m²), with no differences in BMI and age between women ($n=17$, age 38.0 (29.0; 42.0) years, BMI 40.1 (35.4; 44.7) kg/m²) and men ($n=9$, age 40.0 (35.5; 46.0) years, BMI 39.3 (38.2; 41.8) kg/m²). SAT was collected from eight lean women (age 40.0 (30.0; 52.3) years, BMI 23.6 (22.4; 24.9) kg/m²), whose age was matched with that of the obese women. The latter tissue samples originated from cosmetic liposuctions performed on the abdomen and/or the hips.

All lean subjects were healthy and took no medication influencing adipose tissue metabolism. Among obese subjects some received anti-hypertensives ($n=7$), oral anti-diabetics ($n=5$), anti-psychotics ($n=2$), proton-pump inhibitors ($n=4$), thyroxine ($n=4$), anti-arrhythmics ($n=3$), anti-estrogen ($n=1$) or anti-thrombotics ($n=1$), or were treated with medications for asthma ($n=1$), osteoporosis ($n=1$) or depression ($n=6$). The project was approved by the local Ethical Committee and performed in accordance with the Helsinki Declaration.

Adipose tissue culturing

Immediately after collection, adipose tissue fragments were rinsed in sterile saline. SAT from lean women was incubated in fragments of 500 mg, floating freely in 5 mL incubation medium (consisting of Medium 199 (Sigma) supplemented with 25 mM HEPES, pH 7.4 and 1% human serum albumin, 1 nM insulin, antipain and leupeptin (each 20 mL/100 mL medium) and penicillin (100 IU/mL) and streptomycin (100 IU/mL) (each 1 mL/100 mL medium)) at 37°C, 5% CO₂. Due to practical circumstances, the SAT and VAT fragments obtained during bariatric surgery only resulted in 200 mg tissue fragments. These fragments were incubated in 2 mL incubation medium and consequently, the ratio between tissue and medium was similar for all fragments. After an equilibration period of 24 h, tissues were incubated for 48 h at 37°C before harvest of cell media. SAT from lean women was incubated with various concentrations of GH (0 (i.e. baseline), 1, 10 and 100 µg/L; Novo Nordisk A/S), whereas tissue samples from obese men and women were incubated

without GH (baseline) and with GH (100 µg/L). After harvest, the media were frozen at -20°C for later analysis of proteins, whereas tissue fragments were frozen in liquid nitrogen and kept at -80°C for mRNA extraction.

Bioactive IGF by kinase receptor activation assay (KIRA)

The ability of IGFs present in cell media to stimulate the IGF-IR *in vitro* (i.e. IGF bioactivity) was measured by an in-house kinase receptor activation (KIRA) assay (26) with modifications (27). Media were diluted 1:2 before assay and all samples from the same patient were measured in the same assay. IGF-IR phosphorylation was compared with that of a serial dilution of recombinant human IGF-I (WHO 02/254; the National Institute for Biological Standards and Control (NIBSC), Potters Bar, Hertfordshire, UK). Samples with readings below detection limit (zero calibrator (NSB) plus 3 s.d. of the NSB signal) were given the concentration of zero. As the IGF-IR may be stimulated by IGF-I as well as IGF-II, the output of the assay has been designated 'bioactive IGF'.

IGF-immunoassays

Media concentrations of total, immunoreactive IGF-I were measured by in-house time-resolved immunofluorometric assay (TR-IFMA) following sample acidification and IGF-II blockage of IGFBPs as previously outlined (3) with modifications (28). Media were acidified to pH <3 by a 1:10 dilution in acetic acid (200 mM, 0.5 g/L HSA, 9 g/L NaCl and 5 mL/L Tween 20) containing an excess of IGF-II (200 µg/L), and incubated for 2 h at 5°C . The samples (100 µL) were added directly to a 96-well microtiter plate, which before use had been coated with IGF-I antibody (MAb41, generously provided by Novo Nordisk A/S), blocked (10 mL/L Tween 20, 0.5 g/L NaN_3 in 40 mM PBS) and added 100 µL Tris buffer (400 mM, pH 8.5: 17.68 g/L Tris-HCl, 34.88 g/L Tris-base, 2.5 µM EDTA, 2 mL/L Tween 20, 9 g/L NaCl and 1 g/L HSA), which neutralized the acetic acid. After overnight incubation at 5°C , the wells were washed (50 mM Tris-HCl pH 8.0 with 9 g/L NaCl, 5 mL/L Tween 20 and 0.5 g/L NaN_3) and 200 µL of biotinylated IGF-I antibody (catalog # I-8773, Sigma) containing europium-labeled streptavidin (1:1000; Perkin Elmer Life Science) were added to the wells. After incubation at room temperature for 2 h, the wells were washed six times, enhancement solution (Perkin Elmer Life Science) was added to the wells and read using

time-resolved immunofluorometry. A serial dilution of rhIGF-I (WHO 02/254) served as calibrator and was treated as cell media. All samples were assayed in duplicates.

Cell media levels of immunoreactive, total IGF-II were assayed in a similar manner. However, IGFBPs were blocked with 200 µg/L IGF-I and different antibodies were used for coating (clone S1F2, catalog # 05-166, Merck Millipore) and detection (polyclonal antibody # I-7276, Sigma, tagged with europium), and rhIGF-II (WHO 96/538, obtained from NIBSC) served as calibrator. Otherwise, the procedures were as described for IGF-I.

IGFBP-4

We originally assessed IGFBP-4 by a commercial assay, which had been validated for measurements in cell culture media (catalog # DY804, Bio-Techne, Abingdon, UK), whereas its ability to recognize IGFBP-4 fragments generated by PAPP-A had not been explored. However, after having finalized all tissue incubations, we had the opportunity to establish three novel in-house IGFBP-4 assays that specifically targeted intact IGFBP-4, the PAPP-A-generated C-terminal (CT) IGFBP-4 fragment and the PAPP-A-generated N-terminal (NT) IGFBP-4 fragment respectively. The three assays were based on antibodies and calibrators generously donated by HyTest Ltd. (Turku, Finland) and they have recently been described in detail (29). In brief, culture media levels of IGFBP-4, CT-IGFBP-4 and NT-IGFBP-4 were measured in duplicate using TR-IFMAs based on monoclonal antibodies and recombinant human calibrators generously provided by HyTest Ltd. In each fragment assay, one of the antibodies specifically recognizes the proteolytic neo-epitope generated upon degradation of IGFBP-4 by PAPP-A. In all three assays, intra- and inter-assay CVs were below 10% and 15% respectively. Detection limits were 0.5 µg/L for IGFBP-4, 0.4 µg/L for CT-IGFBP-4 and 0.9 µg/L for NT-IGFBP-4. As the assays for intact and PAPP-A-fragmented IGFBP-4 are relatively new, these assays were the last to be performed. Hence, as some media vials were used, we were only able to measure intact and degraded IGFBP-4 in a limited number of samples.

PAPP-A assay

Media levels of PAPP-A were determined using a commercial ELISA, generously provided by Ansh Labs (Webster, TX, USA). The assay was performed according to protocol procedures. All samples were determined in single determination.

Real-time RT-PCR

This was performed as outlined previously (30). Total RNA was extracted from frozen adipose tissue using TRIzol reagents (Gibco BRL, Life Technologies). The RNA concentration was determined by absorbance at 260 nm, and purity and quality were checked by 260/280 ratio and by gel electrophoresis respectively. Reverse transcription polymerase chain reaction (RT-PCR) was performed with random hexamer primers as described by the GeneAmp PCR Kit (Perkin Elmer Cetus). Real-time PCR was performed with a SYBR Green real-time PCR assay using an iCycler PCR machine from Bio-Rad Laboratories. The primers for target genes are shown in Table 1. Quantitative results obtained by real-time RT-PCR were expressed as cycle threshold (CT). The relative gene expression of target gene to β -actin was calculated by the formula $1/((CT_{\text{target}} - CT_{\beta\text{-actin}})^2)$ as described previously (30).

Statistics

The vast majority of data did not comply with a parametric distribution, and log transformation did not change this. Therefore, we used Mann–Whitney rank sum test, Wilcoxon signed rank sum test or Friedman repeated-measures analysis of variance on ranks as appropriate. A significant Friedman test was followed by the Student–Newman–Keul *post hoc* test, which is suitable for multiple comparisons. The only exception was IGFBP-4 data from obese subjects (when comparing SAT and VAT before and after GH stimulation), which complied with a normal distribution. In this case we used repeated-measures ANOVA followed by Student–Newman–Keul *post hoc* test. Spearman rank order correlation was used to study the associations between variables. *P*-values <0.05 were considered significant. All data are stated as median and interquartile range.

Results

Protein levels in culture media

GH stimulation of SAT from lean women

SAT from lean women were available in large amounts and therefore used to study the effect of increasing doses of GH, comparing baseline (zero) vs 1, 10 and 100 $\mu\text{g/L}$. GH stimulation of SAT dose-dependently increased concentrations of IGF-I ($P=0.003$; Fig. 1A), IGF-II ($P=0.003$; Fig. 1B) and bioactive IGF ($P=0.003$; Fig. 1C). IGFBP-4 also increased following GH stimulation ($P=0.002$; Fig. 1D), whereas its effects on PAPP-A were less clear-cut, as we only observed a difference when comparing GH concentrations of 1 and 100 $\mu\text{g/L}$ ($P=0.02$; Fig. 1E).

SAT media from lean vs obese women

SAT media from lean women were compared with SAT media from obese women, focusing on the conditions at baseline and after GH stimulation (100 $\mu\text{g/L}$). The most notable differences included IGFBP-4 (Fig. 1D) and PAPP-A (Fig. 1E), which at baseline were 2.0-fold and 1.8-fold higher, respectively, in SAT media from obese than those from lean women ($P<0.001$). Upon GH treatment, the difference in IGFBP-4 persisted (Fig. 1D), whereas media levels of PAPP-A remained numerically, but insignificantly, higher in obese SAT media (Fig. 1E).

GH stimulation increased media concentrations of IGF-I ($P<0.05$; Fig. 1A) similarly in SAT from lean and obese women. By contrast, IGF-II decreased ($P<0.05$) in obese SAT media, but increased ($P<0.05$) in lean SAT media (Fig. 1B). The opposing responses of IGF-II to GH stimulation may explain that bioactive IGF increased less in obese than in lean SAT media ($P<0.05$; Fig. 1C). A few other differences in the response to GH stimulation were noticed. GH stimulation caused a small, but significant

Table 1 Primers used in the study.

Target	Sense	Antisense	Product size (base pairs)
IGF-I	GACAGGGGCTTTTATTTCAAC	CTCCAGCCTCCTTAGATCAC	117
IGF-II	AGCCGTGGCATCGTTGAG	GGTCTTGGGTGGGTAGAGCAATC	310
PAPP-A	CAGTCAGCTGCTCAACGGAA	GCAGGCTCTGGGACTGCAC	194
GH receptor	ATTTCTAAACAGCAAAGGA	CACTGTGGAATTCGGGTTTA	158
IGF-I receptor	AAAAGGAATGAAGTCTGGCTC	CACAATGTAGTTATTGGACAC	517
IGF-II receptor	CCCGGAGCACTACCTCA	ACATAAGTCGCCATCAACGTA	187
IGFBP-4	GCCACTTGCGCCCTGGGCTT	GTCAGCCAGCTGGTGGCAGTCCAGCTC	594

Figure 1

IGF-related peptides in culture medium from SAT obtained from lean and obese women. White boxes show media levels of IGF-related peptides from cultures of SAT obtained from eight lean women. Each SAT was incubated with increasing concentrations of GH (0 (baseline), 1, 10 and 100 µg/L) added to the incubation medium. Dark gray boxes show media levels in cultures from 17 obese women treated with 0 (baseline) and 100 µg/L GH. The two tissue donor groups were matched according to age. All data are stated as median (interquartile range). Among lean women, the effect of SAT stimulation with increasing concentrations of GH was tested using the Friedman repeated-measures analysis of variance on ranks, which if significant was followed by the Student–Newman–Keul test. Differences between SAT from lean and obese women were found using the Mann–Whitney test, and differences within obese women were found using the Wilcoxon test. * $P < 0.05$.

increase in IGFBP-4 levels in SAT media from lean but not obese women ($P < 0.05$; Fig. 1D), whereas GH stimulation decreased PAPP-A levels in SAT media from obese but not lean women ($P < 0.05$; Fig. 1E).

VAT media vs SAT media from obese subjects

The paired experiment compared SAT and VAT media before and after GH stimulation (100 µg/L). As we did not observe clear gender differences (data not shown), it was decided to pool data. At baseline, IGF-I concentrations (Fig. 2A) did not differ between unstimulated VAT and SAT media, whereas bioactive IGF levels were 1.8-fold higher in VAT than those in SAT media ($P < 0.005$). Stimulation by GH increased IGF-I concentrations (Fig. 2A) and bioactive IGF (Fig. 2C) in both media ($P < 0.05$), with both levels being higher in VAT than those in SAT ($P < 0.05$). In contrast to IGF-I, GH altered neither IGF-II concentrations in SAT nor VAT media. Noteworthy, VAT media contained significantly higher IGF-II concentrations than SAT media at baseline (1.4-fold) as well as after GH stimulation (2.1-fold) ($P < 0.005$) (Fig. 2B). This observation may explain that VAT media contained significantly higher levels of bioactive IGF (Fig. 2C) than SAT media at baseline ($P < 0.05$) as well as after GH stimulation ($P < 0.05$).

Another marked difference between SAT and VAT was their secretion of IGFBP-4 and PAPP-A. When measured with the commercial IGFBP-4 assay, VAT media contained approximately twice as much IGFBP-4 (Fig. 2D) and four times as much PAPP-A ($P < 0.001$) as SAT at baseline, and this difference remained unchanged after GH stimulation (Fig. 2E). GH did not change PAPP-A levels in media from VAT, but caused a small yet significant reduction in PAPP-A levels from SAT.

IGFBP-4 normally inhibits IGF-mediated IGF-IR activation *in vitro*. Hence, the combination of high levels of IGFBP-4 and bioactive IGF in VAT media was counterintuitive compared with SAT media. However, we learned that the commercial IGFBP-4 assay detected both intact IGFBP-4 and NT-IGFBP-4 generated upon PAPP-A cleavage (data not shown). Accordingly, baseline media IGFBP-4 levels were re-measured using our IGFBP-4 fragment-specific assays (29). By these assays we could demonstrate that intact IGFBP-4 levels were identical in VAT and SAT media, whereas levels of NT and CT fragments were 1.5-fold and 3.3-fold respectively higher in VAT than in SAT ($P < 0.01$; Fig. 3A). Based on these findings, we returned to SAT media from lean women. We had only access to media from SAT exposed to 1.0 µg/L GH, but still these measurements (Fig. 3B) showed similar

Figure 2

Culture media levels of IGF-related peptides from SAT and VAT from obese subjects. White and gray boxes show media levels of IGF-related peptides from cultures of VAT and SAT respectively. Data are based on paired observations of VAT and SAT from 26 obese subjects. All data are stated as median (interquartile range). Differences between protein levels were analyzed using the Friedman repeated-measures analysis of variance (ANOVA) on ranks, which if significant was followed by the Student–Newman–Keul method. The only exception was IGFBP-4 levels, which complied with the normal distribution and therefore were compared by repeated-measures ANOVA, and subsequently the Student–Newman–Keul test. * $P < 0.05$.

levels of intact as well as NT- and CT-IGFBP-4, i.e. as observed in SAT media from obese women (Fig. 3A).

Correlations between levels of bioactive IGF, IGFBP-4 and PAPP-A are shown in Table 2. The three distinct measures of IGFBP-4 were positively correlated in both SAT and VAT media. In VAT, bioactive IGF correlated positively with PAPP-A, and inversely with NT-IGFBP-4. No such correlations were observed in SAT.

Correlations between BMI and GH induced increments in bioactive IGF and IGF-I

To study the impact of BMI on the GH responsiveness of SAT, data from lean and obese women were analyzed using Spearman rank order correlations between BMI and increments in bioactive IGF and IGF-I concentrations, following GH stimulation (i.e. concentrations after GH stimulation minus concentrations at baseline). BMI correlated inversely with the increase in bioactive IGF

($r = -0.54$; $P = 0.005$; $n = 25$), but not with the increase in IGF-I concentration ($r = -0.16$; $P = \text{NS}$; $n = 25$).

mRNA levels in adipose tissue cultures

GH stimulation of SAT from lean women

Upon GH treatment, the expression of IGF-I increased, but only at the highest GH dose ($P < 0.05$; Table 3). Otherwise, no significant effects of GH were detected.

SAT from lean vs obese women

SAT from obese women contained higher baseline expression of IGF-IR, IGF-IIR, IGF-II and IGFBP-4 and lower expression of GHR and PAPP-A, compared with SAT from lean women, whereas the expression of IGF-I was similar (all $P < 0.05$; Table 3). The differences in expression patterns were slightly altered following GH stimulation, as GHR and IGF-II were no longer different

Figure 3

Culture media levels of intact IGFBP-4 and IGFBP-4 fragments. Intact IGFBP-4 and IGFBP-4 fragments were measured in culture media from VAT and SAT from obese women (A) and SAT from lean women (B). The two fragments were determined by assays that are specific for the PAPP-A-generated neo-epitopes on the IGFBP-4 fragments. As we had only limited media available for the assays, measurements on obese women (A) were restricted to the baseline condition only. In lean women (B), measurements were performed on SAT media following stimulation with the lowest GH dose (1 µg/L). No difference between the concentrations of intact IGFBP-4 and the two IGFBP-4 fragments was observed. All data are stated as median (interquartile range). Differences between protein levels were performed using the Friedman repeated-measures analysis of variance on ranks, which if significant was followed by the Student–Newman–Keul method. * $P < 0.05$.

between SAT from obese and lean women, whereas the other differences observed at baseline persisted ($P < 0.05$; Table 3). Finally, SAT from lean and obese women responded similarly to GH as only the expression of IGF-I increased ($P < 0.05$).

VAT vs SAT from obese subjects

At baseline, SAT showed a higher expression of GHR and a lower expression of PAPP-A than VAT ($P < 0.05$; Table 4), but apart from this no differences were observed. GH stimulation increased IGF-I expression similarly in SAT and VAT, whereas the expression of GHR was no longer different between the two tissues. Otherwise, GH stimulation caused no changes in the expression pattern observed at baseline.

Discussion

This study compared the expression, secretion and GH responsiveness of proteins belonging to the IGF system in cultures of VAT and SAT from lean and obese subjects. A central observation was that adipose tissue cultures secrete substantial amounts of enzymatically active PAPP-A, IGFBP-4 and IGF-II in a depot-specific manner. Thus, VAT media contained substantially more IGF-II, PAPP-A and PAPP-A-degraded IGFBP-4 fragments than SAT media. Consequently, VAT media possessed an increased IGF-IR activation potential *in vitro*. Another central observation was that in obese subjects, GH stimulation resulted in a higher secretion of IGF-I from VAT than SAT, whereas SAT from obese and lean women responded with similar increases in IGF-I following GH stimulation.

SAT from obese women secreted almost twice as much PAPP-A as SAT from lean women. Furthermore, VAT from obese subjects secreted more than four times as much PAPP-A as SAT, which confirm in a human setting what has previously been documented pre-clinically (17). In addition, IGFBP-4 was highly abundant in culture media. However, using highly specific IGFBP-4 fragment assays

Table 2 Spearman rank order correlation between media levels of bioactive IGF, intact IGFBP-4, N-terminal IGFBP-4, C-terminal IGFBP-4 and PAPP-A. All correlations are based on 26 VAT and SAT media, analyzed separately. Only data from unstimulated tissues (baseline condition) are available. Data are stated as *r*-values and *P*-values. For clarity, only data with correlations with *P*-values below 0.05 have been included.

SAT	VAT				
	IGF bioactivity	Intact IGFBP-4	N-terminal IGFBP-4	C-terminal IGFBP-4	PAPP-A
IGF bioactivity		NS	-0.50; $P < 0.009$	NS	0.45; $P < 0.03$
Intact IGFBP-4	NS		0.53; $P < 0.006$	0.80; $P < 0.0001$	NS
N-terminal IGFBP-4	NS	0.50; $P < 0.01$		0.43; $P < 0.03$	NS
C-terminal IGFBP-4	NS	0.84; $P < 0.0001$	0.53; $P < 0.006$		NS
PAPP-A	NS	NS	NS	NS	

All significant correlations are in bold. NS, not significant.

Table 3 SAT mRNA densities of GH and IGF-related peptides and receptors. The four left-sided data columns show mRNA levels in SAT obtained from eight lean women. The two right-sided data columns show mRNA levels in SAT from 17 obese women. Each SAT from lean women was incubated with either 0 (baseline), 1, 10 or 100 µg/L, whereas SAT from obese women was only stimulated with 0 (baseline) and 100 µg/L GH. Lean and obese women were matched according to age. All data are stated as median (interquartile range). Among lean women, the effect of SAT stimulation with increasing concentrations of GH was tested using the Friedman repeated-measures analysis of variance on ranks, which if significant was followed by the Student–Newman–Keul test. Differences between SAT from lean and obese women were tested using the Mann–Whitney test, and differences between SAT among obese women were tested using the Wilcoxon test.

mRNA	Lean baseline	Lean GH stimulation (1 µg/L)	Lean GH stimulation (10 µg/L)	Lean GH stimulation (100 µg/L)	Obese baseline	Obese GH stimulation (100 µg/L)
GHR	0.087 (0.044–0.111)	0.048 (0.020–0.079)	0.025 (0.019–0.042)	0.051 (0.027–0.069)	0.032 (0.020–0.075) ^d	0.031 (0.021–0.049)
IGF-IR	0.003 (0.001–0.004)	0.003 (0.002–0.004)	0.001 (0.000–0.004)	0.003 (0.002–0.006)	0.024 (0.018–0.041) ^d	0.026 (0.020–0.042) ^e
IGF-IR	0.156 (0.053–0.207)	0.067 (0.047–0.090)	0.056 (0.035–0.071)	0.073 (0.045–0.091)	0.265 (0.143–0.595) ^d	0.244 (0.157–0.508) ^e
IGF-I	0.043 (0.025–0.110)	0.071 (0.034–0.107)	0.065 (0.034–0.102)	0.151 (0.076–0.209) ^{a,b,c}	0.030 (0.018–0.078)	0.113 (0.084–0.168) ^a
IGF-II	0.335 (0.245–0.624)	0.251 (0.142–0.512)	0.221 (0.151–0.423)	0.301 (0.240–0.447)	0.572 (0.392–2.281) ^d	0.611 (0.285–1.746)
IGFBP-4	0.009 (0.007–0.013)	0.008 (0.005–0.013)	0.007 (0.005–0.014)	0.011 (0.009–0.014)	0.321 (0.126–0.587) ^d	0.268 (0.126–0.528) ^e
PAPP-A**	0.072 (0.051–0.098)	0.047 (0.036–0.100)	0.053 (0.033–0.070)	0.077 (0.059–0.089)	0.007 (0.054–0.093) ^d	0.012 (0.001–0.026) ^e

SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

^a*P* < 0.05 compared with baseline media levels in SAT tissues from the same subject; ^b*P* < 0.05 compared with media from lean SAT tissue stimulated by 1 µg/L GH; ^c*P* < 0.05 compared with media from lean SAT tissue stimulated by 10 µg/L GH; ^d*P* < 0.05 when comparing baseline SAT media levels from lean and obese women; ^e*P* < 0.05 when comparing 100 µg/L GH-stimulated SAT media levels from lean and obese women.

**Data are multiplied by 10.

(29) we could demonstrate that the apparently higher concentration of IGFBP-4 in VAT media was in fact due to elevated levels of PAPP-A-generated CT- and NT-IGFBP-4 fragments rather than intact IGFBP-4. Furthermore, the apparent difference in IGFBP-4 immunoreactivity between SAT media from obese and lean women vanished when using specific IGFBP-4 assays. In our opinion these findings highlight that the interpretation of IGFBP data may be grossly biased unless the presence of IGFBP-fragments is taken into account.

The primary function of PAPP-A is to cleave IGFBP-4 in an IGF-dependent manner, and thereby to release bound IGF for receptor activation (11, 12). Accordingly, it is tempting to speculate that the increased ability of VAT media to activate the IGF-IR *in vitro* (i.e. bioactive IGF) is secondary to an upregulated PAPP-A-mediated release of IGFBP-4-complexed IGF. This view is supported by the positive relationship between PAPP-A and bioactive IGF as demonstrated in VAT media. On the other hand, we would also have predicted positive correlations between bioactive IGF levels and the two IGFBP-4 fragments, which are generated by PAPP-A in a 1:1 molar ratio. However, media concentrations of the CT- and NT-fragments were significantly distinct, in particular in VAT. Accordingly, we speculate that the clearance of the two fragments, whether this involves cellular uptake and/or further enzymatic degradation in the incubation media, may be responsible for the lack of relationship between the two PAPP-A-generated IGFBP-4 fragment levels and bioactive IGF.

Our IGF bioassay cannot discriminate between IGF-I- and IGF-II-mediated IGF-IR activation. Given that IGF-II cross-reacts with the affinity being 12% of that of IGF-I (26) and that media IGF-II was considerably higher than IGF-I, locally produced IGF-II may contribute substantially to IGF-IR activation within adipose tissues. Noteworthy, obese subjects have higher serum IGF-II than lean subjects (31). Therefore, we hypothesize that a significant part of circulating IGF-II originates from adipose tissue.

It is remarkable that 200 mg adipose tissue, incubated in 2 mL medium for 48 h is able to secrete PAPP-A in concentrations exceeding what is usually measured in the circulation (29, 32). Since obese subjects do not have elevated serum PAPP-A (33), our observation supports the notion that PAPP-A is primarily active within the tissues (11, 12). It was beyond the scope of this study to explore the mechanisms responsible for the excessive secretion of PAPP-A, but mRNA data indicate different possibilities. When comparing SAT from lean and obese subjects, the latter group presented a higher secretion

Clinical Study	M F Gude and others	PAPP-A and IGFBP-4 in human adipose tissue	175:6	517
----------------	---------------------	--	-------	-----

Table 4 Adipose tissue mRNA densities of GH and IGF-related peptides and receptors in SAT and VAT. Each group consists of 26 mRNA measurements. Each study participant served as a donor of VAT and SAT. Thus, data are based on paired observations. All data are stated as median (interquartile range). Differences between mRNA levels were performed using the Friedman repeated-measures analysis of variance on ranks, which if significant was followed by the Student–Newman–Keul method.

mRNA	VAT baseline	VAT GH stimulation (100 µg/L)	SAT baseline	SAT GH stimulation (100 µg/L)
GHR*	0.014 (0.001–0.045)	0.014 (0.011–0.053)	0.036 (0.020–0.087)*	0.031 (0.018–0.050)
IGF-IR	0.031 (0.024–0.084)	0.030 (0.025–0.079)	0.028 (0.021–0.050)	0.027 (0.017–0.046)
IGF-IIR	0.264 (0.107–0.668)	0.256 (0.138–1.026)	0.311 (0.158–0.786)	0.313 (0.190–0.557)
IGF-I	0.051 (0.025–0.114)	0.106 (0.036–0.283) ^a	0.031 (0.019–0.100)	0.108 (0.083–0.174) ^a
IGF-II	1.122 (0.423–3.242)	0.933 (0.399–5.596)	0.683 (0.420–2.769)	0.647 (0.341–1.810)
IGFBP-4	0.300 (0.150–0.925)	0.289 (0.194–1.292)	0.358 (0.180–0.724)	0.289 (0.126–0.551)
PAPP-A**	0.032 (0.010–0.071)	0.021 (0.011–0.244)	0.007 (0.004–0.039) ^b	0.013 (0.006–0.031) ^c

R, receptor; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

^a $P < 0.05$ when comparing baseline and GH-stimulated tissue of similar localization; ^b $P < 0.05$ when comparing baseline SAT and VAT; ^c $P < 0.05$ when comparing GH-stimulated SAT and VAT.

*Friedman's test was significant, but the *post hoc* test could not identify differences between groups. However, a paired test (Wilcoxon) showed that levels of GHR was significantly higher in SAT than those in VAT at baseline ($P < 0.02$), but not after GH stimulation ($P = \text{NS}$); **Data are multiplied by 10.

of PAPP-A protein, despite a lower mRNA expression. In obese subjects, on the other hand, the mRNA expression followed the secretion, being high in VAT and low in SAT. In summary, this indicates that transcriptional as well as post-translational mechanisms may be in play.

In this study we focused on IGFBP-4, but adipose tissue is indeed reported to express all IGFBPs (2). We were able to detect the mRNA expression of IGFBP-2, -3, -5 and -6 (data not shown), but the incubation media contained no detectable IGFBP-2 or -3 protein, as previously reported by us (3), and we had no assay for IGFBP-5. Therefore these data were not included. The only other IGFBP present in the incubation media in detectable concentrations was IGFBP-6 (data not shown). Similar to IGFBP-4, levels of IGFBP-6 were higher in VAT media than SAT media (median levels being ≈ 12 and ≈ 6 µg/L respectively), with no difference between SAT from lean and obese women. Thus, IGFBP-6 may also play a role in adipose tissue and indeed this tissue may be used as a model to improve our incomplete understanding of the role of IGFBP-6 in human biology.

Both VAT and SAT responded to GH stimulation by increasing IGF-I production, but VAT appeared to be more GH sensitive than SAT. This is in line with clinical studies showing that treatment of GH-deficient patients results in a relatively more pronounced reduction in VAT than that in SAT (34, 35, 36). On the other hand, neither the expression of IGF-IR nor the expression of IGF-I differed between the two fat depots; if anything, the expression of GHR was lower in VAT than that in SAT. Thus, the ability of VAT to secrete more IGF-I than SAT may be caused by post-transcriptional mechanisms. In this context it is tempting to focus on insulin, which shares overlapping

intercellular signaling cascades with GH (37). However, our tissue culture system requires insulin to be added to the incubation medium; therefore, it is not possible to study the distinct effects of GH in the absence of insulin.

The liver is the major source of circulating IGF-I (28) and obese subjects respond with larger increases in IGF-I following GH stimulation than lean subjects (25), indicative of an augmented hepatic GH sensitivity (24). On this basis, we found it of interest to study whether the increased GH responsiveness in obesity also included adipose tissue. However, data suggested the opposite as the baseline expression of GHR was lower in SAT from obese than that from lean women. Nevertheless, the two tissues responded with similar increases in IGF-I mRNA expression and protein secretion following GH stimulation, whereas the levels of bioactive IGF correlated inversely with BMI. Thus, obesity does not appear to increase the SAT synthesis of IGF-I following GH stimulation; indeed, the overall IGF bioactivity may be lower following GH exposure.

Our study has limitations. We compared whole tissue fragments and accordingly, differences in cellular composition and number of adipocytes in VAT and SAT were not taken into consideration (14, 36). We acknowledge that visceral adipocytes are in general smaller than subcutaneous adipocytes, and that the observed differences in GH response may in part relate to adipocyte numbers. However, from a biological point of view, we and others believe that studies of whole tissue fragments (including all cell types and extracellular matrix) are more representative of the physiology of adipose tissue *in vivo* than the studies of isolated adipocytes (36). It is also a limitation that we for practical reasons had to compare 200 mg of SAT and VAT from obese women vs 500 mg of

Clinical Study	M F Gude and others	PAPP-A and IGFBP-4 in human adipose tissue	175:6	518
----------------	---------------------	--	-------	-----

SAT from lean women, as a comparison of similar tissue sizes would have been ideal. Further, depending on where the liposuction was performed, the lean SAT originated from hips as well as abdomen; although we do not believe so, this may have influenced our results. In addition, our study did not include details about diseases among the obese tissue donors. Drug lists from the participants revealed that some of the obese donors received anti-diabetic medication and no doubt, it would be of interest to investigate the consequences of diabetes and its treatment on the IGF system in adipose tissue. Finally, as our data are obtained *in vitro*, one has to be cautious when extending these findings to the *in vivo* situation.

In this study we present novel data on the production of IGF components in human cultures of VAT and SAT. Given the demonstrated pro-atherogenic role of PAPP-A (11, 12, 38), our findings are compatible with the notion that VAT via a dysfunctional IGF system may be pathogenically linked to the development of cardiovascular disease, which is frequent in subjects suffering from visceral obesity (39).

Declaration of interest

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

Funding

This work was supported by the Danish Medical Research Council and the Department of Clinical Medicine at Aarhus University, Denmark. R H was funded by a research grant from the Danish Diabetes Academy supported by the Novo Nordisk Foundation.

Acknowledgements

The authors thankfully acknowledge the technicians at the Medical Research Laboratory for technical assistance. The authors are grateful to HyTest Ltd. for providing antigens and antibodies for the IGFBP-4 and the two IGFBP-4 fragment assays. The authors also would like to thank Ansh Labs for providing kits for PAPP-A.

References

- Wabitsch M, Heinze E, Debatin KM & Blum WF. IGF-I- and IGFBP-3-expression in cultured human preadipocytes and adipocytes. *Hormone and Metabolic Research* 2000 **32** 555–559. (doi:10.1055/s-2007-978685)
- Baxter RC & Twigg SM. Actions of IGF binding proteins and related proteins in adipose tissue. *Trends in Endocrinology and Metabolism* 2009 **20** 499–505. (doi:10.1016/j.tem.2009.07.002)
- Gude MF, Frystyk J, Flyvbjerg A, Bruun JM, Richelsen B & Pedersen SB. The production and regulation of IGF and IGFBPs in human adipose tissue cultures. *Growth Hormone and IGF Research* 2012 **22** 200–205. (doi:10.1016/j.gthr.2012.09.004)
- Scavo LM, Karas M, Murray M & LeRoith D. Insulin-like growth factor-I stimulates both cell growth and lipogenesis during differentiation of human mesenchymal stem cells into adipocytes. *Journal of Clinical Endocrinology and Metabolism* 2004 **89** 3543–3553. (doi:10.1210/jc.2003-031682)
- Boney CM, Moats-Staats BM, Stiles AD & D'Ercole AJ. Expression of insulin-like growth factor-I (IGF-I) and IGF-binding proteins during adipogenesis. *Endocrinology* 1994 **135** 1863–1868. (doi:10.1210/en.135.5.1863)
- Smith PJ, Wise LS, Berkowitz R, Wan C & Rubin CS. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. *Journal of Biological Chemistry* 1988 **263** 9402–9408.
- Chan SS, Twigg SM, Firth SM & Baxter RC. Insulin-like growth factor binding protein-3 leads to insulin resistance in adipocytes. *Journal of Clinical Endocrinology and Metabolism* 2005 **90** 6588–6595. (doi:10.1210/jc.2005-0595)
- Fischer-Posovszky P, Tornqvist H, Debatin KM & Wabitsch M. Inhibition of death-receptor mediated apoptosis in human adipocytes by the insulin-like growth factor I (IGF-I)/IGF-I receptor autocrine circuit. *Endocrinology* 2004 **145** 1849–1859. (doi:10.1210/en.2003-0985)
- Frystyk J. Free insulin-like growth factors – measurements and relationships to growth hormone secretion and glucose homeostasis. *Growth Hormone and IGF Research* 2004 **14** 337–375. (doi:10.1016/j.gthr.2004.06.001)
- Conover CA, Harstad SL, Tchkonja T & Kirkland JL. Preferential impact of pregnancy-associated plasma protein-A deficiency on visceral fat in mice on high-fat diet. *American Journal of Physiology: Endocrinology and Metabolism* 2013 **305** E1145–E1153. (doi:10.1152/ajpendo.00405.2013)
- Conover CA. Key questions and answers about pregnancy-associated plasma protein-A. *Trends in Endocrinology and Metabolism* 2012 **23** 242–249. (doi:10.1016/j.tem.2012.02.008)
- Oxvig C. The role of PAPP-A in the IGF system: location, location, location. *Journal of Cell Communication and Signaling* 2015 **9** 177–187. (doi:10.1007/s12079-015-0259-9)
- Motoshima H, Wu X, Sinha MK, Hardy VE, Rosato EL, Barbot DJ, Rosato FE & Goldstein BJ. Differential regulation of adiponectin secretion from cultured human omental and subcutaneous adipocytes: effects of insulin and rosiglitazone. *Journal of Clinical Endocrinology and Metabolism* 2002 **87** 5662–5667. (doi:10.1210/jc.2002-020635)
- Ibrahim MM. Subcutaneous and visceral adipose tissue: structural and functional differences. *Obesity Reviews* 2010 **11** 11–18. (doi:10.1111/j.1467-789X.2009.00623.x)
- Fox CS, Massaro JM, Hoffmann U, Pou KM, Maurovich-Horvat P, Liu CY, Vasan RS, Murabito JM, Meigs JB, Cupples LA *et al.* Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. *Circulation* 2007 **116** 39–48. (doi:10.1161/CIRCULATIONAHA.106.675355)
- Davidge-Pitts C, Escande CJ & Conover CA. Preferential expression of PAPPA in human preadipocytes from omental fat. *Journal of Endocrinology* 2014 **222** 87–97. (doi:10.1530/JOE-13-0610)
- Tchoukalova YD, Nathanielsz PW, Conover CA, Smith SR & Ravussin E. Regional variation in adipogenesis and IGF regulatory proteins in the fetal baboon. *Biochemical and Biophysical Research Communications* 2009 **380** 679–683. (doi:10.1016/j.bbrc.2009.01.149)
- Tchkonja T, Lenburg M, Thomou T, Giorgadze N, Frampton G, Pirtskhalava T, Cartwright A, Cartwright M, Flanagan J, Karagiannides I *et al.* Identification of depot-specific human fat cell progenitors through distinct expression profiles and developmental gene patterns. *American Journal of Physiology: Endocrinology and Metabolism* 2007 **292** E298–307. (doi:10.1152/ajpendo.00202.2006)
- Leung KC, Doyle N, Ballesteros M, Waters MJ & Ho KK. Insulin regulation of human hepatic growth hormone receptors: divergent effects on biosynthesis and surface translocation. *Journal of Clinical Endocrinol and Metabolism* 2000 **85** 4712–4720. (doi:10.1210/jc.85.12.4712)

- 20 Rasmussen MH. Obesity, growth hormone and weight loss. *Molecular and Cellular Endocrinology* 2010 **316** 147–153. (doi:10.1016/j.mce.2009.08.017)
- 21 Stanley TL & Grinspoon SK. Effects of growth hormone-releasing hormone on visceral fat, metabolic, and cardiovascular indices in human studies. *Growth Hormone and IGF Research* 2015 **25** 59–65. (doi:10.1016/j.ghir.2014.12.005)
- 22 Frystyk J, Brick DJ, Gerweck AV, Utz AL & Miller KK. Bioactive insulin-like growth factor-I in obesity. *Journal of Clinical Endocrinology and Metabolism* 2009 **94** 3093–3097. (doi:10.1210/jc.2009-0614)
- 23 Friedrich N, Jørgensen T, Juul A, Spielhagen C, Nauck M, Wallaschofski H & Linneberg A. Insulin-like growth factor I and anthropometric parameters in a Danish population. *Experimental and Clinical Endocrinology and Diabetes* 2012 **120** 171–174. (doi:10.1055/s-0031-1301289)
- 24 Savastano S, Di Somma C, Barrea L & Colao A. The complex relationship between obesity and the somatotropic axis: the long and winding road. *Growth Hormone and IGF Research* 2014 **24** 221–226. (doi:10.1016/j.ghir.2014.09.002)
- 25 Gleeson HK, Lissett CA & Shalet SM. Insulin-like growth factor-I response to a single bolus of growth hormone is increased in obesity. *Journal of Clinical Endocrinology and Metabolism* 2005 **90** 1061–1067. (doi:10.1210/jc.2004-0501)
- 26 Chen JW, Ledet T, Ørskov H, Jessen N, Lund S, Whittaker J, De Meyts P, Larsen MB, Christiansen JS & Frystyk J. A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum. *American Journal of Physiology: Endocrinology and Metabolism* 2003 **284** E1149–E1155. (doi:10.1152/ajpendo.00410.2002)
- 27 Reinhard M, Frystyk J, Jespersen B, Bjerre M, Christiansen J, Flyvbjerg A & Ivarsen P. Effect of hyperinsulinemia during hemodialysis on the insulin-like growth factor system and inflammatory biomarkers: a randomized open-label crossover study. *BMC Nephrology* 2013 **14** 80. (doi:10.1186/1471-2369-14-80)
- 28 Espelund U, Grønbaek H, Villadsen GE, Simonsen K, Vestergaard PF, Jørgensen JO, Flyvbjerg A, Vilstrup H & Frystyk J. The circulating IGF system in hepatocellular carcinoma: the impact of liver status and treatment. *Growth Hormone and IGF Research* 2015 **25** 174–181. (doi:10.1016/j.ghir.2015.05.002)
- 29 Hjortebjerg R, Lindberg S, Hoffmann S, Jensen JS, Oxvig C, Bjerre M & Frystyk J. PAPP-A and IGFBP-4 fragment levels in patients with ST-elevation myocardial infarction treated with heparin and PCI. *Clinical Biochemistry* 2015 **48** 322–328. (doi:10.1016/j.clinbiochem.2014.11.022)
- 30 Bruun JM, Pedersen SB & Richelsen B. Interleukin-8 production in human adipose tissue: inhibitory effects of anti-diabetic compounds, the thiazolidinedione cglitazone and the biguanide metformin. *Hormone and Metabolic Research* 2000 **32** 537–541. (doi:10.1055/s-2007-978682)
- 31 Frystyk J, Skjærbaek C, Vestbo E, Fisker S & Ørskov H. Circulating levels of free insulin-like growth factors in obese subjects: the impact of type 2 diabetes. *Diabetes/Metabolism Research and Reviews* 1999 **15** 314–322. (doi:10.1002/(SICI)1520-7560(199909/10)15:5<314::AID-DMRR56>3.0.CO;2-E)
- 32 Terkelsen CJ, Oxvig C, Norgaard BL, Glerup S, Poulsen TS, Lassen JF, Møller HJ, Thuesen L, Falk E, Nielsen TT *et al.* Temporal course of pregnancy-associated plasma protein-A in angioplasty-treated ST-elevation myocardial infarction patients and potential significance of concomitant heparin administration. *American Journal of Cardiology* 2009 **103** 29–35. (doi:10.1016/j.amjcard.2008.08.027)
- 33 Woelfle J, Roth CL, Wunsch R & Reinehr T. Pregnancy-associated plasma protein A in obese children: relationship to markers and risk factors of atherosclerosis and members of the IGF system. *European Journal of Endocrinology* 2011 **165** 613–622. (doi:10.1530/EJE-11-0423)
- 34 Bengtsson BA, Eden S, Lonn L, Kvist H, Stokland A, Lindstedt G, Bosaeus I, Tolli J, Sjöström L & Isaksson OG. Treatment of adults with growth hormone (GH) deficiency with recombinant human GH. *Journal of Clinical Endocrinology and Metabolism* 1993 **76** 309–317. (doi:10.1210/jc.76.2.309)
- 35 Johannsson G, Marin P, Lonn L, Ottosson M, Stenlof K, Björntorp P, Sjöström L & Bengtsson BA. Growth hormone treatment of abdominally obese men reduces abdominal fat mass, improves glucose and lipoprotein metabolism, and reduces diastolic blood pressure. *Journal of Clinical Endocrinology and Metabolism* 1997 **82** 727–734. (doi:10.1210/jc.82.3.727)
- 36 Berryman DE, List EO, Sackmann-Sala L, Lubbers E, Munn R & Kopchick JJ. Growth hormone and adipose tissue: beyond the adipocyte. *Growth Hormone and IGF Research* 2011 **21** 113–123. (doi:10.1016/j.ghir.2011.03.002)
- 37 Olarescu NC & Bollerslev J. The impact of adipose tissue on insulin resistance in acromegaly. *Trends in Endocrinology and Metabolism* 2016 **27** 226–237. (doi:10.1016/j.tem.2016.02.005)
- 38 Hjortebjerg R, Tarnow L, Jorsal A, Parving HH, Rossing P, Bjerre M & Frystyk J. IGFBP-4 fragments as markers of cardiovascular mortality in type 1 diabetes patients with and without nephropathy. *Journal of Clinical Endocrinology and Metabolism* 2015 **100** 3032–3040. (doi:10.1210/jc.2015-2196)
- 39 Yusuf S, Hawken S, Öunpuu S, Bautista L, Franzosi MG, Commerford P, Lang CC, Rumboldt Z, Onen CL, Lisheng L *et al.* Obesity and the risk of myocardial infarction in 27 000 participants from 52 countries: a case-control study. *Lancet* 2005 **366** 1640–1649. (doi:10.1016/s0140-6736(05)67663-5)

Received 1 July 2016

Revised version received 16 August 2016

Accepted 1 September 2016