The IGF system in patients with type 2 diabetes: associations with markers of cardiovascular target organ damage

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Abstract

Objective: Perturbations in the insulin-like growth factor (IGF) system may contribute to the accelerated cardiovascular disease (CVD) that occurs in patients with type 2 diabetes (T2D). However, it remains unknown whether the IGF system is also involved in the development of early, subclinical CVD. We characterised the IGF system in T2D patients and matched controls and examined the associations with markers of subclinical target organ damage.

Methods: The study included 99 patients with recently diagnosed T2D and 99 age- and sex-matched controls. IGF-1 and IGFBP-1 to -4 were measured by immunoassays, as were pregnancy-associated plasma protein-A (PAPP-A) and the PAPP-A-generated N-terminal (NT) and C-terminal (CT) IGFBP-4 fragments, which are novel CVD risk markers. Arterial stiffness was evaluated by carotid-femoral pulse wave velocity (PWV). Cerebral white matter lesions (WMLs) and carotid artery remodelling were determined by MRI.

Results: After multivariate adjustments, patients with T2D had lower concentrations of IGFBP-2, IGFBP-4, NT- and CT-IGFBP-4, when compared with controls. IGFBP-2 was inversely correlated to PWV in all subjects in multivariate analysis ($P < 0.05$), and IGFBP-3 was inversely associated with severity of WMLs ($P < 0.05$). The NT-IGFBP-4 fragment was associated with the degree of carotid artery remodelling among all subjects (regression coefficient (95% CI): 2.95 (0.70, 5.16), $P = 0.011$). Levels of NT- and CT-IGFBP-4 were reduced in T2D patients receiving metformin compared to those in controls and patients not receiving metformin.

Conclusions: Even in recently diagnosed and well-controlled T2D patients, IGF protein levels are altered and associated with CVD risk factors.

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Introduction

Patients with type 2 diabetes (T2D) carry an increased risk of cardiovascular disease (CVD), which cannot solely be explained by traditional risk factors (1, 2). Several prospective epidemiological studies have indicated that serum levels of insulin-like growth factor (IGF)-1 (3, 4, 5, 6),

IGF-2 (7, 8), IGF-binding protein (IGFBP)-1 (5, 9, 10, 11), IGFBP-2 (5, 12, 13) and IGFBP-3 (3, 5) are associated with the development of T2D and the cardiovascular complications ensuing T2D. Accordingly, many members of the IGF system have emerged as attractive disease

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markers, but causal relationships between IGF system perturbations and the development of CVD remain to be firmly identified.

One of the most promising candidates linking the IGF system to CVD is the enzyme pregnancy-associated plasma protein-A (PAPP-A), which is a metalloproteinase that amplifies local IGF signalling. By proteolytic cleavage of IGFBP-4, resulting in specific IGFBP-4 fragments, IGF-1 is liberated and available for IGF-1 receptor (IGF-1R) activation (14, 15). Studies have shown that increased circulating levels of PAPP-A correlate with the degree of atherosclerosis and that PAPP-A is highly expressed in vulnerable plaques and exerts proatherogenic effects (16, 17, 18). Consequently, it has been suggested that quantification of PAPP-A-generated IGFBP-4 fragments in the circulation may reflect PAPP-A activity in the vascular wall and hence provide novel cardiac risk markers (19, 20). We have recently demonstrated that IGFBP-4 fragment levels are indeed associated with future CVD in patients with type 1 diabetes (21). To what extent PAPP-A is involved in the pathophysiological processes underlying CVD in T2D remains to be determined.

Established risk factors such as BMI, blood pressure, cholesterol and triglyceride levels are used to evaluate the risk of T2D and CVD (6). However, the prognostic information carried by these classical markers is challenged by their propensity to fluctuate over time and by individual variation in susceptibility to their detrimental effects. Target organ damage (TOD) represents a mediating step between risk factors and CVD that integrates the cumulative effect of risk factors and the individual susceptibility, and within recent years, novel markers of TOD have emerged (22). Increased arterial stiffness, as assessed by carotid-femoral pulse wave velocity (PWV), is associated with the degree of atherosclerosis and predicts cardiovascular mortality, and the number of cerebral white matter lesions (WML) is associated with the risk of stroke (23, 24). Finally, the extent of carotid artery remodelling, as estimated by a normalised wall index (NWI), is associated with accelerated atherosclerosis (25, 26).

The primary aim of our study was to investigate if the PAPP-A-generated IGFBP-4 fragments associate with early, subclinical markers of CVD in patients with recently diagnosed, well-controlled T2D and controls matched individually for age and sex. If so, this would further strengthen the link between PAPP-A, IGFBP-4 and CVD. The second aim was to provide a hypothesis-generating description of the relationship between markers of TOD and other components of the IGF system.

Subjects and methods

Study population and blood samples

The study population consisted of 99 patients with T2D and 99 controls matched individually for sex and age. Details of the observational study population have been described elsewhere (Clinical Trial no: NCT00674271) (27). Patients were consecutively recruited from the outpatient clinic at Aarhus University Hospital, Denmark. Patients were diagnosed with diabetes according to World Health Organization criteria and known duration of diabetes was <5 years. The control subjects were recruited by advertising in the local press, and undiagnosed diabetes was excluded by fasting plasma glucose and oral glucose tolerance test. Non-diabetic subjects with impaired fasting glucose or impaired glucose tolerance were accepted as controls. Exclusion criteria included pregnancy, infectious disease, prior or present cancer and end-stage renal failure.

The study was approved by the Research Ethics Committee of Central Region, Denmark, and by the Danish Data Protection Agency. Written, informed consent was obtained from all participants.

Fasting serum and EDTA-plasma were collected from patients and controls. Samples were centrifuged for 15 min, aliquoted and stored at -80°C until analysis. The original study cohort consisted of 100 patients and 100 controls, but due to limited amounts of EDTA-plasma, one patient sample and the matched control sample were omitted. Information on medications was missing in one T2D patient.

Magnetic resonance imaging and pulse wave velocity measurements

The investigation of the brain and morphology of atherosclerosis in the carotid arteries as well as the PWV measurements have been thoroughly described elsewhere (27). In brief, all subjects underwent magnetic resonance imaging (MRI) of the brain and carotid arteries bilaterally in a 1.5 Tesla Phillips Intera MRI scanner (Achieva, Philips). Brain scans were performed with a slice thickness of 5 mm, and a radiologist blinded to patient data evaluated the images. Cerebral WMLs were defined as areas of brain parenchyma with an increased signal on T2-weighted scans but without significant volume loss. WMLs in the subjects were graded qualitatively on a scale from 0 to 2 according to the scale introduced by Breteler *et al.* (28), with 0–4 punctate WMLs=0, >4 punctate WMLs without confluent lesions=1 and the presence of confluent WMLs regardless of number of lesions=2.

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The degree of carotid vascular remodelling was evaluated by a NWI that represents the area of the wall compared to total vessel area (wall area/(wall area+lumen area), with wall area defined as the area between lumen and outer wall) (25, 26). Scans were accomplished with four different contrast weightings and analysed in a software tool to calculate NWI. NWI was determined in the right and left internal carotid artery, and the maximal NWI was used for statistical analyses.

For assessment of arterial stiffness, carotid-femoral PWV was determined by an applanation tonometer (SPT-301B, Millar, Houston, Texas, USA) and SphygmoCor equipment (AtCor Medical, Sydney, Australia). Measurements were performed from 09:00 h until 13:00 h, and the mean of two measurements was used. Intra-subject variations averaged 5.1%.

In 18 subjects, PWV measurements were not obtained due to obesity or atrial fibrillation. WML and NWI data were lacking in four and 31 subjects respectively, due to insufficient data quality, claustrophobia, magnetic material in the body or body weight >120 kg. In the paired statistical analyses (Table 1), the matched subjects were also excluded whenever patients or controls were excluded due to missing data.

Immunoassays

IGF-1, IGFBP-1, IGFBP-2 and IGFBP-3

Serum total IGF-1 and IGFBP-3 levels were measured using the IDS-iSYS Multi-Discipline Automated Analyser (Immunodiagnostic Systems Nordic SA, Denmark) as recently published (29, 30). Limit of detection for total

Table 1 Baseline characteristics. Data represent mean $\pm$ s.d. or median (25th percentile; 75th percentile). Categorical variables are indicated as numbers (n).

Characteristics	Controls	Type 2 diabetes patients	P
Age (years)	58 $\pm$ 0	58 $\pm$ 10	0.89
Males/females	51/48	51/48	1.00
BMI (kg/m ²)	26 $\pm$ 4	30 $\pm$ 5	<0.001
Diabetes duration (years)	–	1.8 (0.7; 3.2)	
HbA _{1c} (%)	5.6 (5.4; 5.8)	6.5 (6.2; 6.9)	<0.001
HOMA-IR	0.9 (0.6; 1.5)	1.3 (1.0; 2.1)	<0.001
HOMA- β	84 (65; 106)	69 (50; 92)	<0.001
Fasting insulin (pmol/L)	42 (27; 65)	58 (43; 95)	<0.001
Fasting glucose (mmol/L)	5.2 (5.0; 5.6)	6.7 (6.0; 7.5)	<0.001
High-sensitive CRP (mg/L)	0.8 (0.5; 1.2)	1.8 (0.9; 3.8)	<0.001
Smoking (n) (current/previous/never)	21/32/46	21/36/41	0.77
Statin use (%)	18.2	76.5	<0.001
Antihypertensive treatment (%)	25.7	64.6	<0.001
Previous cardiovascular disease (%)	13.3	20.2	<0.001
Cholesterol (mmol/L)			
Total	5.7 $\pm$ 1.0	4.4 $\pm$ 0.8	<0.001
LDL	3.4 $\pm$ 1.0	2.3 $\pm$ 0.7	<0.001
HDL	1.7 $\pm$ 0.6	1.4 $\pm$ 0.3	<0.001
Triglycerides (mmol/L)	1.2 (0.9; 1.6)	1.4 (1.1; 1.9)	<0.01
24-h ambulatory blood pressure (mmHg)			
Systolic	125 $\pm$ 13	126 $\pm$ 11	0.62
Diastolic	76 $\pm$ 8	74 $\pm$ 7	0.19
Mean arterial pressure	93 $\pm$ 9	92 $\pm$ 8	0.68
24-h pulse pressure (mmHg)	49 $\pm$ 9	51 $\pm$ 8	0.07
24-h heart rate (beats/min)	68 $\pm$ 9	74 $\pm$ 10	<0.001
eGFR (mL/min/1.73 m ²)	81 (72; 89)	87 (76; 98)	<0.01
Creatinine (μ mol/L)	76 $\pm$ 14	72 $\pm$ 15	<0.05
Urine albumin/creatinine ratio (mg/mmol)	0.25 (0.17; 0.40)	0.39 (0.28; 0.98)	<0.001
PWV (m/s)	7.7 (6.7; 9.0)	9.0 (7.9; 10.3)	<0.001
NWI (wall area/(wall+lumen area))	54 $\pm$ 6.3	56 $\pm$ 6.8	0.10
WML (Breteler score: 0/1/2) (n)	52/30/13	53/31/11	0.82

P values: unpaired t-tests or χ^2 -tests between control subjects and patients with type 2 diabetes. PWV, NWI and WML were compared using paired t-tests or signed rank test. Significant P values are highlighted in bold.

β , β -cell function; BMI, body mass index; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; HbA_{1c}, glycated haemoglobin; HDL, high-density lipoprotein; HOMA, homeostasis model assessment; IR, insulin resistance; LDL, low-density lipoprotein; NWI, normalized wall index; PWV, pulse wave velocity; WML, white matter lesion.

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IGF-1 and IGFBP-3 were 4.4 and 50 µg/L respectively. An IGF-1/IGFBP-3 ratio was calculated to provide a rough index of IGF-1 bioavailability (31).

IGFBP-1 was measured using an in-house time-resolved immunofluorometric assay (TR-IFMA) as previously described (32). The IGFBP-1 assay had intra- and inter-assay coefficients of variations (CVs) of 5% and 8.1% respectively. IGFBP-2 was determined by an in-house TR-IFMA as previously described, with intra- and inter-assay CVs of 5% and 12% respectively (32).

IGFBP-4, NT-IGFBP-4 and CT-IGFBP-4

EDTA-plasma levels of IGFBP-4, CT-IGFBP-4 and NT-IGFBP-4 were determined by newly established TR-IFMAs based on monoclonal antibodies (MAb) and corresponding recombinant human (rh) calibrators, generously provided by HyTest Ltd. (Turku, Finland). Full-length IGFBP-4 was determined using coating MAb IBP182 (Cat# 4IGF4 IBP182) and detection MAb IBP144 (Cat# 4IGF4EU IBP144). MAb IBP3 (Cat# 4IGF4 IBP3) and MAb IBP180 (Cat# 4IGF4EU IBP180) were used for the determination of NT-IGFBP-4, and MAb IBP182 and MAb IBP163 (Cat# 4IGF4EU IBP163) were used for the determination of CT-IGFBP-4. Full-length rhIGFBP-4 (Cat# 8IGF4), rhCT-IGFBP-4 (Cat# 8CIG4) and rhNT-IGFBP-4 (Cat# 8NFB4) were used as calibrators. Detection limits were 0.5, 0.4 and 0.9 µg/L for the IGFBP-4, CT-IGFBP-4 and NT-IGFBP-4 immunoassays respectively. In all three assays, inter- and intra-assay CVs were less than 15% and 10% respectively. The assays were performed as recently described (33).

PAPP-A

Total PAPP-A levels were determined by a commercial, ultrasensitive PAPP-A sandwich ELISA (Cat# AL-101) generously provided by Ansh Labs (Webster, TX, USA). Assay procedures were as described by the manufacturer. The antibody pair used in the assay kit detects both dimeric PAPP-A and PAPP-A/proMBP complexes in equimolar concentrations with an analytical range of 0.1–10 µg/L and a sensitivity of 0.025 µg/L. The assay behaved linearly within the analytical range.

Other measurements

C-reactive protein (CRP) was measured by a high-sensitive CRP TR-IFMA with a detection limit of 0.1 µg/L (34). Fasting plasma glucose was analysed by glucose oxidase method, and fasting insulin was measured by ELISA.

Statistical analysis

Homeostasis model assessment (HOMA) indexes were calculated as described by Levy *et al.* (35). Assumptions of normal distributions were tested by histograms and QQ-plots. Skewed data (IGFBP-1, -2 and -4, triglycerides, HbA1c, HOMA insulin resistance (IR), HOMA β -cell function (β), fasting insulin, fasting glucose, high-sensitive CRP, alcohol consumption, urine albumin/creatinine ratio, estimated glomerular filtration rate (eGFR) and PWV) were transformed using the natural logarithm prior to all statistical analyses. Differences in baseline characteristics between groups were determined by unpaired *t*-tests or χ^2 test. Differences in PWV, NWI and mean IGF system protein concentrations between patients with T2D and controls were assessed by paired *t*-tests, as patients and controls were individually matched. Differences in Breteler score between groups were tested using Wilcoxon matched-pairs signed-rank tests. Correlations between baseline variables and IGF systems parameters were assessed by Pearson's correlation coefficient. Unadjusted correlations are reported, as the standard methods for correction for multiple comparisons are considered far too conservative with large correlation matrices. Significant correlations were further investigated by univariate and multivariate regression analyses. Associations with Breteler score were investigated using ordinal logistic regression analysis with adjustment for confounders.

Potential confounder variables comprised all variables in Table 1. First, we used clinical judgement, as is recommended, to select pre-specified confounder. These included age, sex, BMI and, when analysing all subjects, the dichotomous diabetes group variable. In addition, potential confounder variables were evaluated by examining their association with risk factors and outcome variables. To avoid over-adjustment, the direction of selected influences on outcome variables was evaluated using directed acyclic graphs. Analyses of IGFBP-2, IGFBP-4 and the NT- and CT-IGFBP-4 fragments were adjusted for use of metformin. No other antidiabetic treatment was associated with the IGF proteins and outcome. As previous studies have shown strong associations between IGFBP-4 fragment levels and kidney function, the NT- and CT-IGFBP-4 fragment levels were also adjusted for eGFR (21, 33, 36). Adjustments were made for statin intake in regression analyses including cholesterol levels. When analysing PWV, NWI and WML, adjustments were made for 24-h ambulatory blood pressure (AMBP), 24-h heart rate and use of statins and antihypertensive medication. Results are presented

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as mean $\pm$ s.d. for normally distributed data or median (25th percentile; 75th percentile) for skewed data. A two-tailed *P* value of less than 0.05 was considered statistically significant. Regression coefficients are reported with 95% confidence intervals (CI). Data were analysed using Stata, version 13 (StataCorp LP).

Results

Baseline characteristics

A total of 99 patients with T2D and 99 controls matched individually for sex and age were analysed. Patient characteristics are presented in Table 1. Patients with diabetes were recently diagnosed and had good glycaemic control. However, as expected, significant differences were observed between patients and controls with regard to HbA1c, HOMA-IR and HOMA- β . Patients were obese compared with the controls, but cholesterol levels were significantly lower in patients, probably due to a higher proportion of subjects receiving cholesterol-lowering treatment. Similarly, a higher number of patients were taking antihypertensive medications. Patients presented with a higher eGFR and lower creatinine levels compared with those in controls. In the diabetic group, 96 patients received one or more medical treatments. As antidiabetic treatment, patients received metformin ($n=61$), sulfonylureas ($n=13$), glucagon-like peptide 1 (GLP-1) ($n=2$), dipeptidyl peptidase-4 inhibitors ($n=3$) or long- ($n=8$) or short- ($n=3$) acting insulin. In some patients, antidiabetic medications were used in combination. Carotid-femoral PWV was significantly higher in patients

compared with that in controls, whereas no difference was seen in WML or NWI between the two groups.

The IGF system in recently diagnosed T2D

Protein levels are presented in Table 2. Pearson's correlation analysis of all the members of the IGF system with various variables in control subjects and patients with type 2 diabetes are provided in Supplementary Table 1 (see section on supplementary data given at the end of this article). No differences in circulating levels of total IGF-1 and IGFBP-3 were observed between patients with T2D and controls. However, the IGF-1/IGFBP-3 ratio was lower in patients. Serum levels of IGFBP-1 and -2 were significantly lower in patients. However, in regression analyses with adjustments for age, sex, BMI, triglycerides and fasting insulin levels, only IGFBP-2 levels differed between groups. Diabetic patients had lower levels of IGFBP-4 and NT-IGFBP-4 compared with controls, and the differences remained significant in a regression analysis after adjustment for age, sex, BMI, triglycerides and, for NT-IGFBP-4 and CT-IGFBP-4, eGFR. CT-IGFBP-4 and PAPP-A levels were similar in the two groups. After adjustments, CT-IGFBP-4 was significantly lower in the diabetic patients.

Associations of IGF system proteins with traditional metabolic and cardiovascular risk factors

IGF-1 levels were negatively associated with age in both groups. Circulating IGFBP-1 and -2 were negatively associated with BMI and strongly associated with

Table 2 Circulating IGF protein levels. Protein levels of IGF-1, IGFBP1-4, NT-IGFBP-4, CT-IGFBP-4 and PAPP-A in control subjects and patients with type 2 diabetes. Values are mean $\pm$ s.d. or median (25th percentile; 75th percentile).

	Controls	Type 2 diabetes patients	<i>P</i> value	Adjusted <i>P</i> value
IGF-1 (μ g/L)	142 $\pm$ 40	136 $\pm$ 39	0.246	0.859
IGFBP-1 (μ g/L)	50 (29; 66)	42 (25; 54)	<0.05	0.735
IGFBP-2 (μ g/L)	182 (132; 240)	127 (81; 176)	<0.001	0.001
IGFBP-3 (μ g/L)	3731 $\pm$ 768	3929 $\pm$ 950	0.113	0.296
IGF-1/IGFBP-3 ratio	0.039 $\pm$ 0.009	0.036 $\pm$ 0.010	<0.05	0.714
IGFBP-4 (μ g/L)	179 $\pm$ 43	143 $\pm$ 60	<0.001	<0.001
NT-IGFBP-4 (μ g/L)	178 (145; 242)	153 (103; 203)	<0.001	<0.001
NT-IGFBP-4/IGFBP-4 ratio	1.06 (0.80; 1.48)	1.14 (0.81; 1.51)	0.815	0.181
CT-IGFBP-4 (μ g/L)	101 $\pm$ 25	93 $\pm$ 43	0.112	0.013
CT-IGFBP-4/IGFBP-4 ratio	0.59 $\pm$ 0.18	0.68 $\pm$ 0.26	0.008	0.006
PAPP-A (μ g/L)	0.81 $\pm$ 0.27	0.88 $\pm$ 0.27	0.628	0.794

P values: paired *t*-tests between controls and patients with type 2 diabetes. Adjusted *P* values: multivariate regression analysis with adjustments for age, sex, BMI, triglycerides, and insulin. Analyses of IGFBP-4, NT- and CT-IGFBP-4, and PAPP-A were adjusted for age, sex, BMI, and triglycerides. Further, NT- and CT-IGFBP-4 were adjusted for eGFR. Significant *P* values are highlighted in bold.

IGF-1, insulin-like growth factor I; IGFBP, insulin-like growth factor binding protein; PAPP-A, pregnancy-associated plasma protein-A.

Figure 1

Scatter plot and linear prediction of IGFBP-2 and PWV in controls (black) and patients with type 2 diabetes (white). r = Pearson's unadjusted correlation coefficient (all subjects). IGFBP-2, insulin-like growth factor-binding protein 2; PWV, pulse wave velocity.

high-density lipoprotein (HDL) cholesterol, both overall ($r=0.433$, $P<0.001$, and $r=0.446$, $P<0.001$ respectively) and in the control and diabetic groups (Supplementary Table 1). The overall associations remained significant in regression analyses after adjustment for age, sex, BMI, diabetes, triglycerides, fasting insulin and statin use. In contrast, IGFBP-3 did not associate with the traditional risk factors.

IGFBP-4, NT-IGFBP-4 and CT-IGFBP-4 levels were all pairwise associated ($P<0.001$). IGFBP-4 levels did not differ between males and females (154 ± 53 vs $168\pm57\mu\text{g/L}$ respectively, $P=0.077$), and were age independent. As previously shown, NT- and CT-IGFBP-4 were negatively associated with eGFR in all subjects ($P<0.05$) (21, 33). Levels of NT-IGFBP-4 and CT-IGFBP-4 were lower in T2D patients receiving metformin ($n=61$) than those in T2D patients not receiving metformin ($n=37$) (NT-IGFBP-4: 137 (96; 177) vs 189 (134; 219) $\mu\text{g/L}$, $P<0.01$, and CT-IGFBP-4: 85 ± 45 vs $108\pm37\mu\text{g/L}$, $P<0.01$ respectively). This difference was not observed for intact

IGFBP-4 (135 ± 65 vs 156 ± 50 , $P=0.096$). Interestingly, when comparing controls to patients with T2D without metformin treatment, no differences were seen in NT- or CT-IGFBP-4 levels. However, when comparing controls to metformin-treated patients with T2D, levels were significantly different for both NT-IGFBP-4 (175 (144; 239) vs 137 (96; 177) $\mu\text{g/L}$, $P<0.0001$) and CT-IGFBP-4 (98 ± 25 vs $85\pm44\mu\text{g/L}$, $P=0.028$).

PAPP-A levels were lower in females compared to those in males (0.76 ± 0.25 vs $0.87\pm0.28\mu\text{g/L}$, $P<0.01$) when analysing the entire population, but did not associate with other baseline variables. PAPP-A associated positively with the NT-IGFBP-4 fragment and the NT-IGFBP-4/IGFBP-4 ratio in a regression analysis adjusted for age, sex, BMI, diabetes and eGFR (both $P<0.05$).

Associations between the IGF system and PWV, NWI and WML

PWV was inversely associated with IGF-1 ($r=-0.306$, $P<0.001$) and IGFBP-2 ($r=-0.195$, $P<0.01$) (Fig. 1) among all participants. However, when adjusted for age, sex,

Figure 2

Association between IGFBP-3 and Breteler score among all subjects. Boxes represent median with 25th and 75th percentiles, and the whiskers represent the smallest and largest values that lie within 1.5 times the interquartile range away from the nearer quartile. * $P<0.05$, Breteler score 0 vs 2. IGFBP, insulin-like growth factor-binding protein.

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Table 3 Association of NT-IGFBP-4 and NWI in controls and patients with type 2 diabetes. The multivariate regression analyses were adjusted for age, sex, BMI, eGFR, 24-h ambulatory blood pressure, 24-h heart rate and use of metformin, statins and antihypertensive medication. Analyses on all subjects were also adjusted for the group variable.

		All (n=167)		Controls (n=90)		Type 2 diabetics (n=77)	
NWI		Coefficient (95% CI)	P	Coefficient (95% CI)	P	Coefficient (95% CI)	P
NT-IGFBP-4	Unadjusted	2.52 (0.29; 4.74)	0.027*	3.89 (0.39; 7.37)	0.030*	2.75 (−0.40; 5.90)	0.086
	Adjusted	2.93 (0.70; 5.16)	0.011*	2.67 (−1.14; 6.48)	0.168	3.20 (−0.39; 6.79)	0.070
NT-IGFBP-4/ IGFBP-4 ratio	Unadjusted	2.95 (0.55; 5.35)	0.016*	2.18 (−1.38; 5.75)	0.227	3.44 (0.12; 6.75)	0.042*
	Adjusted	2.18 (0.19; 4.17)	0.041*	1.72 (−1.67; 5.11)	0.315	3.34 (−1.62; 8.30)	0.183

Statistically significant predictions are marked by asterisk (* $P < 0.05$).

NT-IGFBP-4, N-terminal insulin-like growth factor-binding protein 4; NWI, normalized wall index; SE, standard error.

BMI, diabetes, triglycerides, fasting insulin, 24-h AMBP, 24-h heart rate and use of statins and antihypertensives, only the association between PWV and IGFBP-2 remained significant ($P < 0.05$).

IGFBP-2 levels increased with qualitative grading of cerebral WMLs (Breteler score 0–2) with concentrations of 138 (95; 200), 151 (104; 215) and 186 (123; 290) $\mu\text{g/L}$ respectively. By contrast, IGFBP-3 levels decreased with Breteler score (Fig. 2). Breteler score associated with both IGFBP-2 ($P < 0.01$) and IGFBP-3 ($P < 0.001$) in unadjusted ordinal logistic regression analyses of the entire population, and the association remained significant for IGFBP-3 after adjustment for the aforementioned variables ($P < 0.05$).

In unadjusted analyses of the total study cohort, NT-IGFBP-4 and the NT-IGFBP-4/IGFBP-4 ratio were associated with the NWI among all subjects (Table 3). The associations remained significant in an adjusted model including age, sex, BMI, eGFR, diabetes, 24-h AMBP, 24-h heart rate and use of statins, antihypertensives and metformin. When analysing the control group and patient group separately, the associations were no longer statistically significant.

Discussion

The primary findings in the present study were that IGF protein levels were associated with conventional as well as newly established risk factors for CVD and that significant differences in the IGF protein levels were seen in recently diagnosed and well-controlled T2D patients compared to non-diabetic controls. CVD is one of the major concerns in T2D, and conventional cardiovascular risk factors only partially explain the excessive risk of developing cardiovascular complications. IGF-1 levels tend to decline in poorly controlled T2D patients (37), and both IGF-1 deficiency and excess have been associated with poor cardiovascular outcomes (38, 39, 40). Consequently, the

IGF system has been postulated to partly explain the increased risk of CVD in T2D, and the findings in this study add to the growing body of evidence linking the IGF system to T2D and cardiovascular risk. However, as this is a cross-sectional study, our findings can at best be interpreted as hypothesis generating or supporting.

Association of the IGF system with metabolic and cardiovascular risk factors

Both IGFBP-1 and -2 have been proposed as markers for the metabolic syndrome (12, 41, 42, 43), and accumulating evidence suggests that IGFBP-1 levels outside the normal range as well as low IGFBP-2 levels are associated with an increased risk of CVD (11, 13), a condition that is accelerated by T2D. IGFBP-1 is acutely regulated by insulin, whereas obese and pre-diabetic subjects display low fasting IGFBP-1 levels equivalent to the relatively high ambient insulin concentrations, severely insulin-resistant patients present with increased IGFBP-1 levels (4, 9, 43). IGFBP-2 is also metabolically regulated, albeit not as rapidly as IGFBP-1 and reflects more chronic and long-term alterations in hepatic exposure to insulin (44). Indeed, previous publications report low levels of IGFBP-1 and -2 in patients with T2D (45, 46). However, this study demonstrates that the differences are pronounced even in recently diagnosed diabetic patients. IGFBP-1 and -2 levels were inversely associated with BMI, and levels were significantly decreased in the diabetic patients, whereas IGF-1 and IGFBP-3 levels were unaltered. It is of interest that IGF-1 and IGFBP-3 levels were similar between the two groups. Generally, IGF-1 is found to be slightly lowered in patients with T2D, whereas high IGFBP-3 levels are associated with insulin resistance (43, 47). The lack of association in this study suggests that the alterations in IGF-1 and IGFBP-3 that are normally observed in T2D may not have emerged this early in the time course of the diabetic state.

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After adjustments, IGFBP-4, NT- and CT-IGFBP-4 levels were lower in patients with T2D compared with those in controls, whereas no differences were observed in PAPP-A levels. Thus, the decreased level of intact IGFBP-4 in patients does not appear to be caused by an altered proteolytic activity of PAPP-A. So far, few studies have investigated possible changes in the expression of IGFBP-4 and PAPP-A in diabetic patients, and no correlation between IGFBP-4 and the diabetic phenotype has been confirmed (48). By contrast, experimental investigations suggest that IGFBP-4 and PAPP-A levels are affected by glucose homeostasis (49, 50, 51). Clearly, more in-depth research is needed to elucidate the mechanisms. Interestingly, we found an association between low IGFBP-4 fragment levels and treatment with metformin in patients with T2D. Thus, patients with T2D, who did not receive metformin, presented with fragment levels similar to controls. These findings suggest that established T2D *per se* is not associated with reduced IGFBP-4 levels, whereas metformin treatment may be. It is beyond doubt that metformin protects against CVD (52), but the mechanisms remain unclear. We have previously shown that the IGFBP-4 fragments are predictors of future CVD (21, 33). The findings may have several explanations. First, it is theoretically possible that the use of metformin improves renal function in T2D patients, and thus, the effects of metformin on IGFBP-4 fragment levels are mediated through a more limited decrease in eGFR. However, we found no association between eGFR and metformin treatment in the T2D patients, suggesting that this mechanism cannot explain our observations. Second, we speculate that our findings relate to the anti-inflammatory actions of metformin. Studies have shown that metformin suppresses levels of pro-inflammatory cytokines, which are stimulators of PAPP-A (17, 53). Thus, metformin may reduce IGFBP-4 proteolysis by reducing inflammation-mediated PAPP-A activity. Collectively, the association between metformin treatment and IGFBP-4 is of interest and requires further investigations.

Increased arterial stiffness assessed by PWV was inversely associated with IGFBP-2 levels. Vessel stiffness and PWV increase due to alterations in the media layers of the vessels, and this process is pronounced in T2D patients. PWV independently predicts CVD, and a low PWV is considered favourable. Our study also demonstrated that low IGFBP-2 levels were associated with other cardiovascular risk factors (low HDL, high insulin and high BMI) in both patients with T2D and in control subjects. Leptin and insulin are known to potently suppress IGFBP-2, and thus, it is difficult to distinguish between the effects of obesity and insulin resistance on the regulation

of IGFBP-2 (43). However, despite adjustments for all these confounding factors, IGFBP-2 remained associated with PWV. This association warrants further investigations.

IGFBP-3 concentrations associated negatively with WMLs, and this association remained significant in the adjusted model. The number of WMLs is associated with the risk of stroke, and our observation is in agreement with a previous publication, in which low levels of IGFBP-3 predicted an increased CVD mortality (54). However, the role of IGFBP-3 in T2D and CVD is still to be elucidated, and there are large discrepancies in the literature regarding this matter (55, 56).

IGF-1 and IGFBP-3 display opposing mechanistic effects, and the molar ratio has often been used as a proxy of IGF-1 bioavailability. A previous study demonstrated that a 12-week lasting fitness program reduced arterial PWV and that PWV was inversely associated with the IGF-1/IGFBP-3 ratio (57). Also, a decreased IGF-1/IGFBP-3 ratio can be found in both obese subjects and T2D patients (31). In the present study, we observed similar levels of IGF-1 and IGFBP-3 in the two groups, whereas the molar ratio was reduced in patients with T2D. We failed to observe any relationship between the IGF-1/IGFBP-3 ratio and PWV. However, it should be noted that the IGF-1/IGFBP-3 ratio is merely a surrogate for free IGF-1, and actual IGF bioavailability should be estimated by other means (58).

IGFBP-4, PAPP-A and cardiovascular disease

An important finding of this study was a significant association between circulating NT-IGFBP-4 levels and the amount of carotid vascular remodelling as estimated by the NWI. When the control group and patient group were analysed separately, the same direction of associations was seen. Yet, this subgroup analysis did not reach the level of significance, perhaps due to lack of power. Additionally, concentrations of PAPP-A correlated positively with NT-IGFBP-4 and the NT-IGFBP-4/IGFBP-4 ratio. These observations support the view that PAPP-A and IGFBP-4 may be involved in and exacerbate the pathophysiological processes underlying atherosclerosis (17). Most commercial PAPP-A assays are unable to differentiate between active and inactive PAPP-A (17, 19, 20), and therefore, NT-IGFBP-4 levels, which are likely to reflect the degree of IGFBP-4 proteolysis, may provide a better measure of the *in vivo* PAPP-A activity than PAPP-A levels *per se* (19, 33). To the best of our knowledge, we are the first to demonstrate the direct association between carotid artery remodelling and IGFBP-4 fragment concentrations. However, our findings also indicate that

the absolute concentration of NT-IGFBP-4 cannot be interpreted as a simple marker of CVD, as the controls presented with higher levels of NT-IGFBP-4, but a generally better cardiovascular risk profile as compared to the patients. It is likely that the finding should be interpreted in the context of possible confounding effects. Especially differences in antidiabetic and dyslipidaemic treatments are likely to account for the discrepancies. Finally, as shown in previous studies, both NT- and CT-IGFBP-4 were negatively associated with eGFR, suggesting a renal clearance of the IGFBP-4 fragments (21).

It is interesting that IGFBP-2, IGFBP-3 and NT-IGFBP-4 show distinct correlations with specific measures of vascular TOD. NWI, PWV and WMLs are all measures of related pathological processes associated with CVD. However, studies investigating their direct associations do not provide unequivocal answers. For instance, PWV has been shown to associate with the severity of WMLs, but the magnitude of the association was limited (27). Also, neither PWV nor WMLs associate with the morning blood pressure surge, which is suggested as a new CVD risk marker (59). Accordingly, the lack of associations with certain IGF proteins suggests that NWI, PWV and WMLs reflect other aspects of cardiovascular risk than these parameters or that a possible association has not emerged this early in the time course of the diabetic state. Finally, it is possible that the underlying pathophysiology associating markers of TOD with the IGF system may be different and perhaps showing a closer association in patients with T2D compared with healthy subjects. Such differences may not be revealed by this cross-sectional study. Further clarification of the differences must await future studies.

The present study has some limitations that should be taken into consideration when interpreting the results. Due to the cross-sectional study design and as most baseline variables and IGF proteins are highly influenced by diet, glycaemic control and so forth, causality cannot be inferred for the reported associations, and it remains elusive to what extent the IGF system exerts causal effects on the metabolic regulation. Adding to this, it is very likely that the IGFBPs are able to functionally compensate for the lack of other IGFBPs and that the dysregulation to some extent is due to compensatory mechanisms. Furthermore, we suspect that the sample size may be too limited to verify relevant differences. Especially the lack of the statistical difference in the subgroups may result from low statistical power. We also acknowledge that the risk of false-positive results is increased by the high number of analyses. To the best of our ability, we tried to account for this by performing thorough multivariate analyses.

Yet, the multivariate analysis is prone to over-fitting and can at best be interpreted as hypothesis generating. Finally, patients with overt T2D usually receive medical therapy that affect components of the IGF system and its regulation. Thus, cautious interpretation of data is indeed necessary, as our findings require further support by longitudinal as well as mechanistic studies.

In conclusion, the present study demonstrates distinct differences in the regulation of the IGF system in patients with T2D and in non-diabetic controls. The patients were recently diagnosed; yet, pronounced differences were observed with regard to associations between the IGF system and metabolic and CVD risk factors. These findings add to the growing body of evidence linking the IGF system to T2D and CVD. However, the clinical and pathophysiological importance of this dysregulation, and whether it may partake in the onset and progression of a dysfunctional metabolic profile, remains to be elucidated.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-16-0940>.

Declaration of interest

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

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References

- 1 Haffner SM, Lehto S, Rönnemaa T, Pyörälä K & Laakso M. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *New England Journal of Medicine* 1998 **339** 229–234. (doi:10.1056/NEJM199807233390404)
- 2 WHO methods and data sources for global causes of death 2000–2011. *Global Health Estimates Technical Paper WHO* 2013 **2013** 1–72.
- 3 Drogan D, Schulze MB, Boeing H & Pischon T. Insulin-like growth factor 1 and insulin-like growth factor-binding protein 3 in relation to the risk of type 2 diabetes mellitus: results from the EPIC–Potsdam study. *American Journal of Epidemiology* 2016 **183** 553–560. (doi:10.1093/aje/kwv188)
- 4 Sandhu MS, Heald AH, Gibson JM, Cruickshank JK, Dunger DB & Wareham NJ. Circulating concentrations of insulin-like growth

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- factor-I and development of glucose intolerance: a prospective observational study. *Lancet* 2002 **359** 1740–1745. (doi:10.1016/S0140-6736(02)08655-5)
- 5 Rajpathak SN, He M, Sun Q, Kaplan RC, Muzumdar R, Rohan TE, Gunter MJ, Pollak M, Kim M, Pessin JE *et al.* Insulin-like growth factor axis and risk of type 2 diabetes in women. *Diabetes* 2012 **61** 2248–2254. (doi:10.2337/db11-1488)
 - 6 Laughlin GA, Barrett-Connor E, Ciriqli MH & Kritz-Silverstein D. The prospective association of serum insulin-like growth factor I (IGF-I) and IGF-binding protein-1 levels with all cause and cardiovascular disease mortality in older adults: the rancho Bernardo study. *Journal of Clinical Endocrinology and Metabolism* 2004 **89** 114–120. (doi:10.1210/jc.2003-030967)
 - 7 Sandhu MS, Gibson JM, Heald AH, Dunger DB & Wareham NJ. Low circulating IGF-II concentrations predict weight gain and obesity in humans. *Diabetes* 2003 **52** 1403–1408. (doi:10.2337/diabetes.52.6.1403)
 - 8 Heald AH, Karvestedt L, Anderson SG, McLaughlin J, Knowles A, Wong L, Grill V, Cruickshank JK, White A, Gibson JM *et al.* Low insulin-like growth factor-II levels predict weight gain in normal weight subjects with type 2 diabetes. *American Journal of Medicine* 2006 **119** 167.e9–167.e15. (doi:10.1016/j.amjmed.2005.08.001)
 - 9 Heald AH, Cruickshank JK, Riste LK, Cade JE, Anderson S, Greenhalgh A, Sampayo J, Taylor W, Fraser W, White A *et al.* Close relation of fasting insulin-like growth factor binding protein-1 (IGFBP-1) with glucose tolerance and cardiovascular risk in two populations. *Diabetologia* 2001 **44** 333–339. (doi:10.1007/s001250051623)
 - 10 Heald AH, Siddals KW, Fraser W, Taylor W, Kaushal K, Morris J, Young RJ, White A & Gibson JM. Low circulating levels of insulin-like growth factor binding protein-1 (IGFBP-1) are closely associated with the presence of macrovascular disease and hypertension in type 2 diabetes. *Diabetes* 2002 **51** 2629–2636. (doi:10.2337/diabetes.51.8.2629)
 - 11 Wallander M, Norhammar A, Malmberg K, Öhrvik J, Rydén L & Brismar K. IGF binding protein 1 predicts cardiovascular morbidity and mortality in patients with acute myocardial infarction and type 2 diabetes. *Diabetes Care* 2007 **30** 2343–2348. (doi:10.2337/dc07-0825)
 - 12 Heald AH, Kaushal K, Siddals KW, Rudenski AS, Anderson SG & Gibson JM. Insulin-like growth factor binding protein-2 (IGFBP-2) is a marker for the metabolic syndrome. *Experimental and Clinical Endocrinology and Diabetes* 2006 **114** 371–376. (doi:10.1055/s-2006-924320)
 - 13 Urbonaviciene G, Frystyk J, Urbonavicius S & Lindholt JS. IGF-I and IGFBP2 in peripheral artery disease: results of a prospective study. *Scandinavian Cardiovascular Journal* 2014 **48** 99–105. (doi:10.3109/14017431.2014.891760)
 - 14 Mazerbourg S, Callebaut I, Zapf J, Mohan S, Overgaard M & Monget P. Up date on IGFBP-4: regulation of IGFBP-4 levels and functions, in vitro and in vivo. *Growth Hormone and IGF Research* 2004 **14** 71–84. (doi:10.1016/j.ghir.2003.10.002)
 - 15 Boldt HB & Conover CA. Pregnancy-associated plasma protein-A (PAPP-A): a local regulator of IGF bioavailability through cleavage of IGFBPs. *Growth Hormone and IGF Research* 2007 **17** 10–18. (doi:10.1016/j.ghir.2006.11.003)
 - 16 Bayes-Genis A, Conover CA, Overgaard MT, Bailey KR, Christiansen M, Holmes DR, Virmani R, Oxvig C & Schwartz RS. Pregnancy-associated plasma protein a as a marker of acute coronary syndromes. *New England Journal of Medicine* 2001 **345** 1022–1029. (doi:10.1056/NEJMoa003147)
 - 17 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)
 - 18 Conover CA. Discrepancies in insulin-like growth factor signaling? No, not really. *Growth Hormone and IGF Research* 2016 **30–31** 42–44. (doi:10.1016/j.ghir.2016.10.005)
 - 19 Postnikov AB, Smolyanova TI, Kharitonov AV, Serebryanaya DV, Kozlovsky SV, Tryshina YA, Malanicev RV, Arutyunov AG, Murakami MM, Apple FS *et al.* N-terminal and C-terminal fragments of IGFBP-4 as novel biomarkers for short-term risk assessment of major adverse cardiac events in patients presenting with ischemia. *Clinical Biochemistry* 2012 **45** 519–524. (doi:10.1016/j.clinbiochem.2011.12.030)
 - 20 Schulz O, Postnikov AB, Smolyanova TI, Katrukha AG, Schimke I & Jaffe AS. Clinical differences between total PAPP-A and measurements specific for the products of free PAPP-A activity in patients with stable cardiovascular disease. *Clinical Biochemistry* 2014 **47** 177–183. (doi:10.1016/j.clinbiochem.2013.10.027)
 - 21 Hjortebjerg R, Tarnow L, Jorsal A, Parving H-H, 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)
 - 22 Nilsson PM, Boutouyrie P & Laurent S. Vascular aging: a tale of EVA and ADAM in cardiovascular risk assessment and prevention. *Hypertension* 2009 **54** 3–10. (doi:10.1161/HYPERTENSIONAHA.109.129114)
 - 23 Debette S & Markus HS. The clinical importance of white matter hyperintensities on brain magnetic resonance imaging: systematic review and meta-analysis. *British Medical Journal* 2010 **341** c3666. (doi:10.1136/bmj.c3666)
 - 24 van Popele NM, Grobbee DE, Bots ML, Asmar R, Topouchian J, Reneman RS, Hoeks APG, van der Kuip DAM, Hofman A & Witteman JCM. Association between arterial stiffness and atherosclerosis. The Rotterdam Study. *Stroke* 2001 **32** 454–460. (doi:10.1161/01.STR.32.2.454)
 - 25 Zhu C, Teng Z, Sadat U, Young VE, Graves MJ, Li ZY & Gillard JH. Normalized wall index specific and MRI-based stress analysis of atherosclerotic carotid plaques: a study comparing acutely symptomatic and asymptomatic patients. *Circulation Journal* 2010 **74** 2360–2364. (doi:10.1253/circ.CJ-10-0305)
 - 26 Saam T, Yuan C, Chu B, Takaya N, Underhill H, Cai J, Tran N, Polissar NL, Neradilek B, Jarvik GP *et al.* Predictors of carotid atherosclerotic plaque progression as measured by noninvasive magnetic resonance imaging: saam “predictors of carotid plaque progression”. *Atherosclerosis* 2007 **194** e34–e42. (doi:10.1016/j.atherosclerosis.2006.08.016)
 - 27 Laugesen E, Høyem P, Stausbøl-Grøn B, Mikkelsen A, Thyrsoe S, Erlandsen M, Christiansen JS, Knudsen ST, Hansen KW, Kim WY *et al.* Carotid-femoral pulse wave velocity is associated with cerebral white matter lesions in type 2 diabetes. *Diabetes Care* 2012 **36** 722–728. (doi:10.2337/dc12-0942)
 - 28 Breteler MM, van Amerongen NM, van Swieten JC, Claus JJ, Grobbee DE, van Gijn J, Hofman A & van Harskamp F. Cognitive correlates of ventricular enlargement and cerebral white matter lesions on magnetic resonance imaging. The Rotterdam Study. *Stroke* 1994 **25** 1109–1115. (doi:10.1161/01.STR.25.6.1109)
 - 29 Bidlingmaier M, Friedrich N, Emery RT, Spranger J, Wolthers OD, Roswall J, Koerner A, Obermayer-Pietsch B, Hübener C, Dahlgren J *et al.* Reference intervals for insulin-like growth factor-1 (IGF-1) from birth to senescence: results from a multicenter study using a new automated chemiluminescence IGF-1 immunoassay conforming to recent international recommendations. *Journal of Clinical Endocrinology and Metabolism* 2014 **99** 1712–1721. (doi:10.1210/jc.2013-3059)
 - 30 Friedrich N, Wolthers OD, Arafat AM, Emery RT, Spranger J, Roswall J, Kratzsch J, Grabe HJ, Hübener C, Pfeiffer AFH *et al.* Age- and sex-specific reference intervals across life span for insulin-like growth factor binding protein 3 (IGFBP-3) and the IGF-I to IGFBP-3 ratio measured by new automated chemiluminescence assays. *Journal of Clinical Endocrinology and Metabolism* 2014 **99** 1675–1686. (doi:10.1210/jc.2013-3060)
 - 31 Sierra-Johnson J, Romero-Corral A, Somers VK, Lopez-Jimenez F, Malarstig A, Brismar K, Hamsten A, Fisher RM & Hellenius ML. IGF-I/IGFBP-3 ratio: a mechanistic insight into the metabolic syndrome. *Clinical Science* 2009 **116** 507–512. (doi:10.1042/CS20080382)

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- 32 Reinhard M, Frystyk J, Jespersen B, Bjerre M, Christiansen JS, 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)
- 33 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)
- 34 Ozbay LA, Moller N, Juhl C, Bjerre M, Carstens J, Rungby J & Jorgensen KA. Calcineurin inhibitors acutely improve insulin sensitivity without affecting insulin secretion in healthy human volunteers. *British Journal of Clinical Pharmacology* 2012 **73** 536–545. (doi:10.1111/j.1365-2125.2011.04118.x)
- 35 Levy JC, Matthews DR & Hermans MP. Correct homeostasis model assessment (HOMA) evaluation uses the computer program. *Diabetes Care* 1998 **21** 2191–2192. (doi:10.2337/diacare.21.12.2191)
- 36 Hjortebjerg R & Frystyk J. Determination of IGFs and their binding proteins. *Best Practice and Research Clinical Endocrinology and Metabolism* 2014 **27** 771–781. (doi:10.1016/j.beem.2013.08.010)
- 37 Frystyk J, Skjærbæk 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)
- 38 Juul A, Scheike T, Davidsen M, Gyllenborg J & Jørgensen T. Low serum insulin-like growth factor I is associated with increased risk of ischemic heart disease. *Circulation* 2002 **106** 939–944. (doi:10.1161/01.CIR.0000027563.44593.CC)
- 39 Clemmons DR. Metabolic actions of insulin-like growth factor-I in normal physiology and diabetes. *Endocrinology and Metabolism Clinics of North America* 2012 **41** 425–443, vii–viii. (doi:10.1016/j.ecl.2012.04.017)
- 40 Clemmons DR. Modifying IGF1 activity: an approach to treat endocrine disorders, atherosclerosis and cancer. *Nature Reviews Drug Discovery* 2007 **6** 821–833. (doi:10.1038/nrd2359)
- 41 Clemmons D, Snyder D & Busby W. Variables controlling the secretion of insulin-like growth factor binding protein-2 in normal human subjects. *Journal of Clinical Endocrinology and Metabolism* 1991 **73** 727–733. (doi:10.1210/jcem-73-4-727)
- 42 Lewitt MS, Hilding A, Östenson CG, Efendic S, Brismar K & Hall K. Insulin-like growth factor-binding protein-1 in the prediction and development of type 2 diabetes in middle-aged Swedish men. *Diabetologia* 2008 **51** 1135–1145. (doi:10.1007/s00125-008-1016-x)
- 43 Hjortebjerg R, Flyvbjerg A & Frystyk J. Insulin growth factor binding proteins as therapeutic targets in type 2 diabetes. *Expert Opinion on Therapeutic Targets* 2014 **18** 209–224. (doi:10.1517/14728222.2014.858698)
- 44 Skov J, Frystyk J & Christiansen JS. GLP-1 infusion reduces IGFBP-1 serum level in humans. *Growth Hormone and IGF Research* 2014 **24** 67–70. (doi:10.1016/j.ghir.2014.01.003)
- 45 Wheatcroft SB & Kearney MT. IGF-dependent and IGF-independent actions of IGF-binding protein-1 and -2: implications for metabolic homeostasis. *Trends in Endocrinology and Metabolism* 2009 **20** 153–162. (doi:10.1016/j.tem.2009.01.002)
- 46 Clauson PG, Brismar K, Hall K, Linnarsson R & Grill V. Insulin-like growth factor-I and insulin-like growth factor binding protein-1 in a representative population of type 2 diabetic patients in Sweden. *Scandinavian Journal of Clinical and Laboratory Investigation* 1998 **58** 353–360. (doi:10.1080/00365519850186544)
- 47 Ezzat VA, Duncan ER, Wheatcroft SB & Kearney MT. The role of IGF-I and its binding proteins in the development of type 2 diabetes and cardiovascular disease. *Diabetes, Obesity and Metabolism* 2008 **10** 198–211. (doi:10.1111/j.1463-1326.2007.00709.x)
- 48 Jehle P, Jehle D, Mohan S & Bohm B. Serum levels of insulin-like growth factor system components and relationship to bone metabolism in Type 1 and Type 2 diabetes mellitus patients. *Journal of Endocrinology* 1998 **159** 297–306. (doi:10.1677/joe.0.1590297)
- 49 Jacot TA & Clemmons DR. Effect of glucose on insulin-like growth factor binding protein-4 proteolysis. *Endocrinology* 1998 **139** 44–50. (doi.org/10.1210/en.139.1.44)
- 50 Cohick WS, Gockerman A & Clemmons DR. Vascular smooth muscle cells synthesize two forms of insulin-like growth factor binding proteins which are regulated differently by the insulin-like growth factors. *Journal of Cellular Physiology* 1993 **157** 52–60. (doi:10.1002/jcp.1041570107)
- 51 Aso Y, Okumura K-i, Wakabayashi S, Takebayashi K, Taki S & Inukai T. Elevated pregnancy-associated plasma protein-A in sera from type 2 diabetic patients with hypercholesterolemia: associations with carotid atherosclerosis and toe-brachial index. *Journal of Clinical Endocrinology and Metabolism* 2004 **89** 5713–5717. (doi:10.1210/jc.2004-0787)
- 52 Pryor R & Cabreiro F. Repurposing metformin: an old drug with new tricks in its binding pockets. *Biochemical Journal* 2015 **471** 307–322. (doi:10.1042/BJ20150497)
- 53 Hyun B, Shin S, Lee A, Lee S, Song Y, Ha NJ, Cho KH & Kim K. Metformin down-regulates TNF-alpha secretion via suppression of scavenger receptors in macrophages. *Immune Network* 2013 **13** 123–132. (doi:10.4110/in.2013.13.4.123)
- 54 Yeap BB, Chubb SAP, McCaul KA, Ho KKY, Hankey GJ, Norman PE & Flicker L. Associations of IGF1 and IGFBPs 1 and 3 with all-cause and cardiovascular mortality in older men: the Health In Men Study. *European Journal of Endocrinology* 2011 **164** 715–723. (doi:10.1530/EJE-11-0059)
- 55 Eggert M-L, Wallaschofski H, Grotevendt A, Nauck M, Völzke H, Samietz S & Friedrich N. Cross-sectional and longitudinal relation of IGF1 and IGF-binding protein 3 with lipid metabolism. *European Journal of Endocrinology* 2014 **171** 9–19. (doi:10.1530/EJE-13-1017)
- 56 Müller C, Wallaschofski H, Brabant G, Wahnschaffe U, Samietz S, Nauck M & Friedrich N. The association between IGF-I/IGFBP-3 and subclinical end points: epidemiology faces the limits. *Journal of Clinical Endocrinology and Metabolism* 2014 **99** 2804–2812. (doi.org/10.1210/jc.2013-3746)
- 57 Ohta M, Hirao N, Mori Y, Takigami C, Eguchi M, Tanaka H, Ikeda M & Yamato H. Effects of bench step exercise on arterial stiffness in post-menopausal women: contribution of IGF-1 bioactivity and nitric oxide production. *Growth Hormone and IGF Research* 2012 **22** 36–41. (doi:10.1016/j.ghir.2011.12.004)
- 58 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/ajpcell.00487.2002)
- 59 Lyhne JM, Laugesen E, Høyem P, Cichosz S, Christiansen JS, Knudsen ST, Hansen KW, Hansen TK & Poulsen PL. Morning blood pressure surge and target organ damage in newly diagnosed type 2 diabetic patients: a cross sectional study. *BMC Endocrine Disorders* 2015 **15** 77. (doi:10.1186/s12902-015-0068-4)

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