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ORIGINAL ARTICLE

Correlation of circulating full-length visfatin (PBEF/NAMPT) with metabolic parameters in subjects with and without diabetes: a cross-sectional study

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Summary

Objective Here we use a novel ELISA that is specific for full-length visfatin (PBEF/NAMPT), compare it with the existing C-terminal based assay and use it to investigate associations of visfatin with metabolic parameters.

Design, patients and measurements We established the specificity and effectiveness of the new ELISA and evaluated the associations of full-length visfatin with clinical, anthropometric and metabolic parameters in a cross-sectional study of 129 Thai subjects, consisting of 50 outpatients with type 2 diabetes and 79 healthy volunteers.

Results The new ELISA accurately recovered full-length recombinant visfatin and detected visfatin secreted by primary human and rat adipocytes. We found serum full-length visfatin was significantly higher in subjects with diabetes compared to their nondiabetic peers (median 2·75 *vs*. 2·22 ng/ml, $P = 0{\cdot}0142$). After adjustment for age, gender and traditional metabolic risk factors, adjusted mean visfatin remained significantly higher in the diabetes group (3·80 *vs*. 2·10 ng/ml, $P = 0{\cdot}0021$). On Spearman univariate correlation analysis, visfatin was significantly associated with resistin ($r = 0{\cdot}30$, $P = 0{\cdot}0011$), but not with any other anthropometric or metabolic variables, including adiponectin multimers. On multiple linear regression analysis, the only covariates independently associated with visfatin were diabetes ($t = 3{\cdot}11$, $P = 0{\cdot}0024$) and log resistin ($t = 2{\cdot}68$, $P = 0{\cdot}0086$).

Conclusions Circulating visfatin is independently associated with diabetes and resistin concentration, but is not related to adiponectin multimers or other metabolic covariates. These data are suggestive of a potential role of visfatin in subclinical inflammatory states.

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Introduction

Visfatin [also known as Pre-B cell colony-enhancing factor (PBEF) and nicotinamide phosphoribosyltransferase (NAMPT),[1,2]] is a novel adipokine which was initially described as being highly expressed in visceral fat and possessing insulin-mimetic bio-activity including promotion of glucose disposal (note this work was subsequently retracted) and more recently as a regulator of insulin secretion.[2,3] Given these findings, it was proposed as a potential factor in the relationship between intra-abdominal obesity and type 2 diabetes.[4,5] Subsequent clinical studies, however, have yielded markedly conflicting associations between visfatin and both diabetes and measures of adiposity.[3,6–18] Indeed, although circulating levels of visfatin were initially correlated with visceral fat mass and with central obesity[6] several studies have noted (i) no difference in visfatin expression between visceral and subcutaneous adipose tissue[12,13] and (ii) no correlation between circulating levels and measures of anthropometry and fat mass (including multidetector computer tomography-determined visceral and subcutaneous fat).[3,8,10–13] As such, it has become apparent that the physiologic relationships of this adipokine remain unclear at this time.[19]

Recently, a series of reports have noted associations between visfatin and the acute-phase bio-markers C-reactive protein (CRP) and IL-6, raising the possibility that this adipokine may play a role in subclinical inflammation.[16,18,20,21] In this context, the relationship of visfatin to other adipokines that are known to be associated with inflammation, such as resistin (pro-inflammatory) and adiponectin (anti-inflammatory), emerges as a question of interest. To date, however, there has been limited study of visfatin in relation to these

adipokines. Indeed, the few such studies of total adiponectin have yielded conflicting results and have not assessed the high-molecular-weight (HMW), middle-molecular-weight (MMW) and low-molecular-weight (LMW) multimeric forms of adiponectin.[6–8,10] Thus, our objective in the current analysis was to evaluate the correlations of visfatin with a broad range of metabolic variables, including resistin and adiponectin multimers, in a well-characterized clinical cohort.[22] To do this we first validated the specificity and accuracy of an assay designed by Adipogen to measure full-length visfatin and compared with the previously available C-terminal assay.

Methods

Subjects and measurements

A cross-sectional study was undertaken in 129 healthy volunteers and outpatients attending a diabetes clinic of the Burapha University Health Center in Thailand. The participants were (i) volunteers from a diabetes mellitus outpatient clinic of the Burapha University Health Center, Thailand, and (ii) healthy volunteers from the villages around the health centre, representing the same catchment area from which the diabetic subjects for this study were recruited. The subjects with diabetes all had type 2 diabetes diagnosed by their treating physician prior to their attendance at the diabetes clinic at which they were recruited for the current study. Exclusion criteria included current use of insulin and previous history of chronic disease, including kidney, liver and inflammatory disorders. Participants with diabetes on oral antidiabetic agents were receiving treatment with either metformin or sulphonylureas, with no use of thiazolidinediones, statins or antihypertensive medications. The study protocol was approved by the ethics committee of Burapha University and all participants provided written informed consent. On the day of assessment, participants underwent physical examination consisting of blood pressure measurement and a series of anthropometric measurements, including weight, height, waist circumference, hip circumference, mid-upper arm circumference and triceps skinfold thickness measurement. Body mass index (BMI) and waist : hip ratio were determined. Fasting venous blood samples were drawn for biochemical studies.

Measurement of circulating and recombinant visfatin by ELISA

We used a new ELISA kit (AdipoGen, Seoul, Korea/ALPCO Diagnostics, Salem, NH) designed to quantitatively measure visfatin in human serum, plasma or cell culture supernatants. A monoclonal antibody specific for human visfatin is precoated onto 96-well microplates and bound standards or samples are captured by antihuman visfatin polyclonal antibody which is then coupled to HRP-linked antirabbit IgG for detection. This new ELISA exhibits increased sensitivity and specificity for full-length visfatin. The sensitivity of this assay is 30 pg/ml; with a dynamic range of 0·0625–16 ng/ml; the mean intra-assay variation (CV, $n = 8$ samples tested 10 time between 7·47 and 18·92 ng/ml), was 4·32%; the mean interassay variation (CV, $n = 8$ samples tested 10 time between 8·08 and 18·10 ng/ml) was 7·58%. We also compared results obtained using this kit with an existing commercially available ELISA (Phoenix Pharmaceuticals, Belmont, CA) which is based upon detecting an epitope (amino acid residues 413–431) at the C-terminal domain of visfatin.

Differentiation of 3T3-L1 preadipocytes

3T3-L1 cells were grown and differentiated essentially as described previously.[23] In this case, 10 μg/ml recombinant FLAG tagged human visfatin or human insulin was added to 3T3-L1 cells that had been treated with 1 μm dexamethasone and 0·5 mM IBMX for 2 days. After a further 5 days, fat droplets were stained using oil-red O.

Isolation of human primary adipocytes and differentiation of primary human preadipocytes

Human preadipocytes were isolated from subcutaneous and omental adipose tissue lipoaspirate waste material or omental tissue biopsies as previously described.[24] Primary human preadipocytes were plated in preadipocyte media (DMEM/*F*12, 15 mM HEPES, 10% FBS, Penicillin, Streptomycin, Amphotericin B) at a density of 40 625 cells/cm^2 in 6-well plates (390 813 cells per well in 3 ml/preadipocyte media) and allowed to attach the plate overnight at 37 °C, 5% CO_2. The following day, adipocyte differentiation was induced by adding 3 ml of differentiation media [DMEM/Ham's F-10, 1 : 1, v/v; HEPES buffer (15 mM); fetal bovine serum (3%); biotin (33 μm), Pantothenate (17 μm), human insulin (100 nm), dexamethasone (0·5 μm), PPAR agonist (1 μm) and antibiotics] per well and incubating without further feeding for 7 days. After the differentiation period, 1·8 ml differentiation media was removed per well and replaced with 2·4 ml of the same media lacking IBMX and PPARγ agonist. Then the cells were allowed to mature without further feeding for an additional 7 days. Conditioned media was removed from each well and assessed for the presence of visfatin (50 μl in C-terminal and 100 μl in full-length assays).

Induction of diabetes in rats using streptozotocin and isolation of primary rat adipocytes

Diabetes was induced in 6–8 week-old male Wistar rats with a single i.p. injection of STZ (in 50 mM citrate buffer, pH 4·5) at a dose of 100 mg/kg body weight. Blood glucose levels were routinely measured with the OneTouch® Ultra® Meter glucometer (Lifescan, Burnaby, BC, Canada) and a glucose tolerance test (2 g glucose/kg body weight) was performed to confirm the induction of diabetes. Adipocytes were isolated as previously described[25] then the number of adipocytes counted and the cell suspension diluted to 1×10^6 cells/ml with 10% FBS α-MEM. Adipocytes were stabilized in a 25 cm^2 cell suspension flask for 3 h in humidified atmosphere (95% air and 5% CO_2) at 37 °C. After stabilization, media was removed from adipocytes and changed to serum free α-MEM media and then used to prepare conditioned media.

Clinical study – biochemical analyses

Circulating visfatin concentration was measured using the Adipogen/ALPCO full-length visfatin ELISA. Total, HMW, MMW and LMW

adiponectin levels were quantified by ELISA (ALPCO Diagnostics). Resistin was measured by radioimmunoassay (RIA) from LINCO Research (St. Charles, MO). Total cholesterol (TC) was determined by a colorimetric method. Triglyceride (TG) levels were determined by enzymatic colorimetric test with lipid clearing factor (GPO-PAP method). Chylomicrons, very-low-density-lipoprotein cholesterol, and low-density-lipoprotein (LDL) cholesterol were removed from plasma by precipitation using phosphotungstic acid and magnesium chloride. After centrifugation of the remaining plasma, high-density-lipoprotein (HDL) cholesterol concentration in the supernatant was measured in the same way as TC. LDL was calculated according to the formula LDL = TC – HDL – (TG/5) mg/dl. Fasting plasma glucose was measured by the enzymatic colorimetric method of Ware and Marbach. Fasting insulin was measured by RIA from LINCO Research. The Homeostasis Model of Assessment of insulin resistance (HOMA-IR)[26] was calculated as fasting insulin (μU/ml) multiplied by fasting glucose (mmol/l) divided by 22·5. The HOMA of β-cell function (HOMA-B)[26] was calculated as (20 × fasting insulin)/(fasting glucose – 3·5).

Statistical analysis

All statistical analyses for clinical studies were conducted using the Statistical Analysis System (SAS, Version 9·1, SAS Institute, Cary, NC). In Table 2, continuous variables are presented as mean followed by SD and categorical variables are presented as percentages. The distributions of ALT, TG, fasting glucose, HOMA-IR, HOMA-B, resistin, visfatin and total, HMW, MMW and LMW adiponectin were skewed and thus medians and interquartile ranges are presented for these variables in Table 2. The natural logarithmic transformations of these skewed variables were used in subsequent multivariate analyses, with back-transformed values presented where applicable. In Table 2, differences between the diabetic and nondiabetic groups were determined by *t*-test and χ^2-test for continuous and categorical variables, respectively. Analysis of covariance (ANCOVA) was used to assess differences in mean visfatin between study participants with and without diabetes, after adjustment for age, gender, BMI, waist circumference, systolic blood pressure, LDL, HDL, TGs, fasting glucose and HOMA-IR and diabetes. Spearman correlation analysis was performed to assess the univariate associations of visfatin with anthropometric and metabolic variables, with subsequent adjustment for age, gender and diabetes (Table 3). Multiple linear regression analysis was used to determine factors independently associated with dependent variable log visfatin (Table 4). Covariates in this model were demographic factors (age, gender), traditional metabolic/vascular risk factors (BMI, waist circumference, systolic blood pressure, LDL, TGs, HOMA-IR, diabetes) and variables found to be significantly associated with visfatin on univariate analysis (resistin). Finally, ANCOVA was used to assess differences in mean visfatin between study participants stratified by tertiles of resistin, after adjustment for age, gender, diabetes, HOMA-IR and three distinct elements of adiposity (BMI, waist circumference, triceps skinfold). In all other cases, data are expressed as means ± SEM and statistical analysis was undertaken using paired Student's *t*-test.

Results

We first established the specificity and sensitivity of the new full-length visfatin assay. Using Western blotting, we confirmed that the antibodies used in this assay were highly specific for recombinant human and rodent visfatin (data not shown). Unlike the previous commercially available assay which uses a C-terminal fragment (413–431) of visfatin as standard, this new assay uses recombinant FLAG-tagged full-length visfatin (1–491) as standard and indeed did not detect the C-terminal peptide as efficiently (data not shown). Fig. 1a shows that this assay accurately recovers serial dilutions of recombinant FLAG-tagged protein. In contrast, Table 1 shows the limited specificity of the C-terminal ELISA for various forms of recombinant visfatin. In this analysis, three visfatin recombinant proteins were analysed with the C-terminal visfatin ELISA: C-terminal peptide (413–431), FLAG-tagged full-length human visfatin and His-tagged full-length human visfatin. C-terminus visfatin concentrations were recognized, but were significantly decreased and did not vary as would be expected with concentrations added. FLAG- and His-tagged full-length visfatin recombinant proteins were 100-fold decreased in terms of the actual values detected, albeit in parallel

Table 1. Ability of C-terminal visfatin assay to detect full-length and C-terminal fragments of the protein. Serial dilutions of recombinant proteins (500–62·5 ng/ml) were analyzed and recovered values shown are mean ± SD of duplicate samples from two independent experiments

	Visfatin standards Concentration (ng/ml)	Recovered value (ng/ml)
C-term visfatin peptide (human visfatin amino acids 413–431)	500	197·89 ± 20·07
	250	194·25 ± 23·71
	125	152·90 ± 17·64
	62·5	190·35 ± 12·53
FLAG-visfatin peptide (full-length human visfatin (1–491) with FLAG at N-terminus)	500	6·21 ± 0·57
	250	4·27 ± 0·10
	125	2·44 ± 0·31
	62·5	0·77 ± 0·14
His-visfatin peptide (full-length human visfatin 1–491) with 6x His at N-terminus)	500	4·56 ± 0·44
	250	2·40 ± 0·54
	125	0·79 ± 0·06
	62·5	0·49 ± 0·14

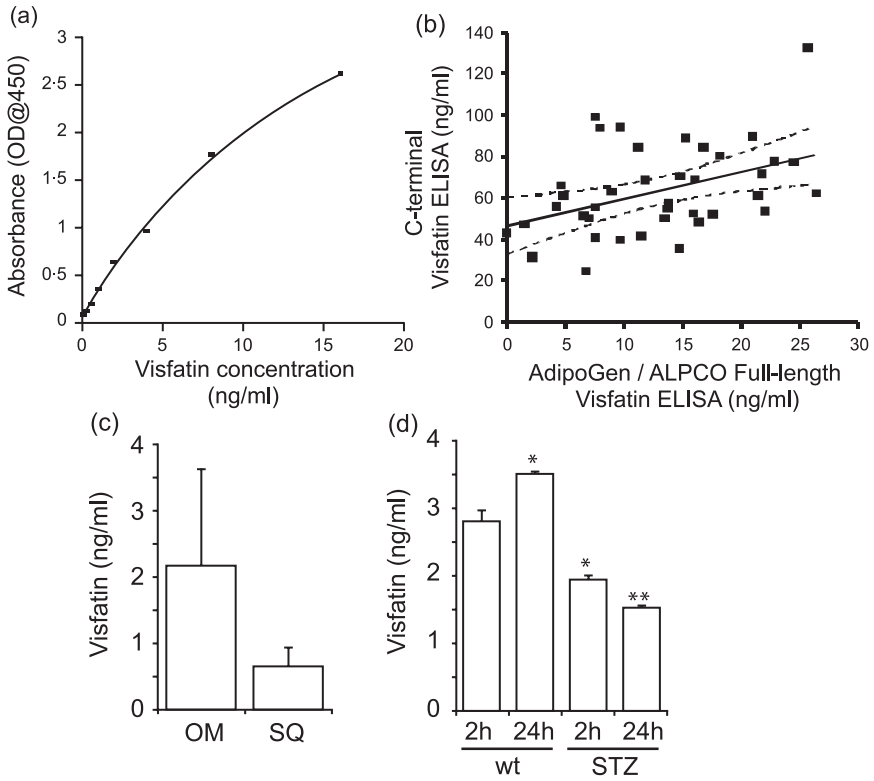

Fig. 1 Characteristics and validation of full-length visfatin ELISA and comparison with C-terminal assay. (a) Shows a standard curve of serial dilutions (0·0625–16 ng/ml) of recombinant human FLAG-tagged visfatin using this new assay. (b) Shows that upon analysis of human serum there was a modest correlation between the two assays ($r = 0{\cdot}4121$, $P = 0{\cdot}0091$), indicating that the new full-length visfatin assay only explains 17·56% of the variance in the C-terminal assay. Solid line indicates a regression line with the equation: [C-terminal visfatin] = 1·291 [full-length visfatin] + 46·65 ng/ml. The dotted lines indicate the 95% confidence interval for the regression line. This figure also demonstrates that visfatin is secreted by both human (c) and rat (d) primary adipocytes. In the human studies, cells from both omental (OM) and subcutaneous (SQ) depots were studied with higher visfatin levels being apparent in the omental-derived adipocytes. For all analyses after being drawn from the subjects, blood was allowed to clot for 15–30 mins, then centrifuged for 10 mins at 1500 ***g***. Supernatant was frozen at –80 °C until assays were performed. Visfatin was measured in fresh (never defrosted) human serum that had been stored at –80 °C. Measurements were performed in each assay using the same sample on the same day. Adipocytes from both wild-type (wt) and streptozotocin-induced diabetic (stz) rats were studied and visfatin was secreted in a time-dependent manner and at reduced levels by adipocytes from diabetic animals. All values are mean ± SEM of $n > 3$ and * signifies $P < 0{\cdot}05$ *vs.* wt 2 h and ** $P < 0{\cdot}05$ *vs.* wt 24 h.

to changes in concentration of visfatin added. Indeed, as shown in (Fig. 1b), the correlation between the full-length and C-terminal assays is surprisingly modest for two assays that are supposed to measure the same analyte (nonparametric Spearman correlation coefficient $r = 0{\cdot}4121$ and $P = 0{\cdot}0091$). Finally, the new full-length visfatin assay was tested for cross reactivity with a variety of targets and showed 100% cross reactivity with both human and rat visfatin and no cross reactivity with human adiponectin, resistin, RELM-β, leptin, GPX3, ANGPTL4, FABP4, AGF and PAI-1 (data not shown).

We have demonstrated that the recombinant FLAG-tagged full-length human visfatin used as standard in the new assay exhibits potent biological activity. This was confirmed via stimulation of differentiation of 3T3-L1 fibroblasts to adipocytes with insulin used as comparison for positive control (data not shown). As visfatin mRNA has previously been demonstrated in adipocytes[27,28] here we examined whether primary human or rat adipocytes secreted the full-length protein. Figure 1c shows that visfatin is indeed secreted by human primary adipocytes, with a tendency towards higher levels in omental-derived adipocytes compared to adipocytes obtained from the subcutaneous depot. We also observed a time-dependent secretion of visfatin into culture medium by rat adipocytes, higher at 24 h than at 2 h (Fig. 1d). Comparing adipocytes from both wild-type and streptozotocin-induced diabetic rats, a model of pancreatic β-cell destruction and hypoinsulinaemia, we also demonstrated that visfatin is secreted at reduced levels by adipocytes from streptozotocin-induced diabetic rats (Fig. 1d).

We next examined the associations of visfatin with a broad range of metabolic parameters in a clinical study of 129 subjects using the new full-length assay. Table 2 shows demographic, clinical and metabolic characteristics of the 129 study participants stratified by the presence ($n = 50$) or absence ($n = 79$) of diabetes. Subjects with diabetes were older than their nondiabetic peers. As expected, the diabetic participants exhibited greater adiposity (BMI, waist circumference, waist : hip ratio, triceps skinfold, mid-upper arm circumference), higher fasting glucose, greater insulin resistance (HOMA-IR) and poorer β-cell function (HOMA-B). In addition, the diabetic group had higher serum creatinine, ALT, TGs, LDL cholesterol, TC and resistin levels Importantly, serum visfatin was

Table 2. Demographic, clinical and metabolic characteristics of study participants, stratified by presence or absence of diabetes

	No diabetes ($n = 79$)		Diabetes ($n = 50$)		P
Demographics					
Age (year)	52·9	(10·7)	57·5	(8·7)	**0·0122**
Gender (M/F)	16%/84%		12%/88%		0·4883
Anthropometry					
BMI (kg/m^2)	24·9	(4·0)	26·6	(4·9)	**0·0331**
Waist (cm)	83·2	(9·4)	93·0	(9·3)	**< 0·0001**
Waist : hip ratio	0·87	(0·04)	0·93	(0·05)	**< 0·0001**
Triceps skinfold	28·3	(8·8)	24·7	(5·9)	**0·0175**
Mid-upper arm circumference	29·8	(3·2)	31·6	(3·8)	**0·0097**
Liver/renal					
Creatinine (mg/dl)	0·82	(0·21)	1·05	(0·38)	**< 0·0001**
ALT	22	(16–30)	26	(19–36)	**0·0478**
Traditional risk factors					
Systolic BP (mmHg)	129·1	(25·0)	137·0	(16·8)	0·0661
Diastolic BP (mmHg)	80·0	(14·2)	84·7	(10·6)	0·0574
Total cholesterol (mg/dl)	220·5	(48·2)	263·6	(78·5)	**0·0002**
LDL (mg/dl)	149·2	(45·7)	184·0	(78·9)	**0·0033**
HDL (mg/dl)	47·1	(12·3)	44·3	(10·1)	0·1793
Triglycerides (mg/dl)	116·0	(74·0–169·0)	169·0	(107·0–226·0)	**0·0004**
Fasting glucose (mmol/l)	4·6	(4·2–5·4)	6·7	(5·6–8·4)	**< 0·0001**
HOMA-IR	3·0	(2·1–4·1)	5·8	(3·6–7·4)	**< 0·0001**
HOMA-B	248·3	(138·7–420·3)	103·1	(64·9–201·6)	**0·0002**
Non-traditional risk factors					
Total adiponectin (μg/ml)	6·01	(4·77–8·33)	5·38	(4·40–6·23)	0·1326
HMW adiponectin (μg/ml)	3·91	(2·60–5·49)	3·36	(2·45–3·97)	0·1697
MMW adiponectin (μg/ml)	1·15	(0·74–1·53)	1·25	(0·78–1·59)	0·5127
LMW adiponectin (μg/ml)	1·18	(0·84–1·81)	1·01	(0·69–1·45)	0·1735
Resistin	1·89	(1·15–2·95)	2·88	(1·85–4·60)	**0·0187**
Visfatin (ng/ml)	2·22	(1·36–4·08)	2·75	(1·83–4·61)	**0·0142**

Data presented as mean followed by SD in parentheses except for (i) gender (presented as percentages) and (ii) ALT, triglycerides, fasting glucose, HOMA-IR, HOMA-B, adiponectin (total, HMW, MMW, LMW), resistin and visfatin (presented as median followed by interquartile range in parentheses). *P*-values refer to differences between groups as determined by *t*-test and χ^2 test for continuous and categorical variables, respectively.
Note adiponectin was measured in 96 subjects (41 with diabetes).
Bold value implies $P < 0·05$.

significantly higher in participants with diabetes compared to their nondiabetic peers (median 2·75 *vs.* 2·22 ng/ml, $P = 0·0142$). After adjustment for age, gender and traditional metabolic risk factors (BMI, waist circumference, systolic blood pressure, LDL, HDL, TGs, fasting glucose and HOMA-IR), adjusted mean visfatin remained significantly higher in the diabetes group (3·80 *vs.* 2·10 ng/ml, $P = 0·0021$).

On Spearman univariate correlation analysis (Table 3), the only metabolic covariate with which visfatin was significantly associated was resistin ($r = 0·30$, $P = 0·0011$). This relationship remained significant after further adjustment for age, gender and diabetes. In contrast, visfatin exhibited no significant univariate correlations with any anthropometric measurements, creatinine, ALT, lipid measures, fasting glucose, insulin resistance (HOMA-IR), β-cell function (HOMA-B), total adiponectin or individual adiponectin multimeric forms. Furthermore, whereas a multiple linear regression model consisting of age, gender, BMI, waist circumference, systolic blood pressure, LDL, TGs, HOMA-IR, diabetes and resistin explained 19·3% of the variance in dependent variable log visfatin (Table 4), the only covariates independently associated with visfatin were diabetes ($t = 3·11$, $P = 0·0024$) and log resistin ($t = 2·68$, $P = 0·0086$).

Having identified an association between the adipokines visfatin and resistin, we sought to further evaluate this relationship. Indeed, after adjustment for age, gender, diabetes, insulin resistance (HOMA-IR) and three distinct elements of adiposity (BMI, waist circumference, triceps skinfold), mean visfatin was lowest in subjects in the lowest tertile of resistin concentration (2·27 ng/ml) and rose progressively over the middle (2·74 ng/ml) and highest tertiles (3·18 ng/ml), respectively (trend $P = 0·0392$), consistent with an independent association between visfatin and resistin.

Discussion

In this report, we have used a new ELISA which specifically and accurately measures full-length visfatin to evaluate the clinical and metabolic correlates of circulating visfatin. The data reported herein

Table 3. Spearman univariate correlations of visfatin: (i) unadjusted (ii) adjusted for age and gender, and (iii) adjusted for age, gender and diabetes

	Unadjusted		Adjusted for age and gender		Adjusted for age, gender and diabetes	
	r	*P*	*r*	*P*	*r*	*P*
Age	–0·13	0·1410				
BMI	0·14	0·1047	0·15	0·1198	0·11	0·2516
Waist circumference	0·15	0·1212	0·18	0·0590	0·06	0·5359
Triceps skinfold	–0·11	0·2511	–0·13	0·2002	–0·08	0·4271
Mid-upper arm circumference	0·07	0·4767	0·05	0·5805	–0·02	0·8075
Creatinine	0	0·9579	0·03	0·7620	–0·11	0·2749
ALT	0·15	0·0898	0·17	0·0844	0·12	0·2268
Systolic BP	0·05	0·6291	0·12	0·2284	0·07	0·4975
Diastolic BP	0·02	0·8132	0·02	0·8399	–0·05	0·5856
LDL	–0·01	0·9328	0	0·9606	–0·07	0·4821
HDL	–0·10	0·2632	–0·12	0·2396	–0·08	0·4405
Triglycerides	–0·03	0·7480	0	0·9636	–0·11	0·2654
Fasting glucose	0·03	0·7328	0·07	0·4746	–0·11	0·2885
HOMA-IR	0·03	0·7622	0·02	0·8782	–0·13	0·1919
HOMA-B	–0·02	0·8690	–0·08	0·4153	0·05	0·6333
Total adiponectin	–0·13	0·2040	–0·12	0·2524	–0·08	0·4331
HMW adiponectin	–0·16	0·1238	–0·14	0·1710	–0·10	0·3200
HMW : total adiponectin	–0·07	0·4761	–0·05	0·6303	–0·02	0·8431
MMW adiponectin	–0·07	0·5088	–0·07	0·5156	–0·07	0·4774
MMW : total adiponectin	0·00	0·9851	–0·02	0·8651	–0·05	0·6508
LMW adiponectin	0·01	0·9286	0	0·9799	0·02	0·8687
LMW : total adiponectin	0·09	0·3992	0·07	0·4907	0·07	0·5035
Resistin	**0·30**	**0·0011**	**0·28**	**0·0041**	**0·21**	**0·0287**

Bold values imply $P < 0·05$.

Table 4. Multiple linear regression analysis of dependent variable log visfatin

	β-estimate	*t*-value	*P*
Age	–0·0073	–0·81	0·4192
Gender	–0·1912	–0·69	0·4913
Body mass index	0·0111	0·47	0·6377
Waist circumference	–0·0043	–0·39	0·6984
Systolic blood pressure	0·0013	0·37	0·7157
LDL	–0·0023	–1·89	0·0618
Log triglycerides	–0·1046	–0·70	0·4838
Log HOMA-IR	–0·1571	–1·51	0·1337
Diabetes	0·5362	3·11	0·0024
Log resistin	0·2917	2·68	0·0086

Model $r^2 = 19·3\%$.

support earlier observations of an independent relationship between visfatin and diabetes. However, there was no correlation of visfatin with other metabolic covariates, including anthropometric measurements, lipid measures, fasting glucose, insulin resistance and β-cell function. Instead, full-length visfatin is found to be independently associated with circulating resistin concentration but not related to adiponectin or its multimers. As such, the biology of visfatin in relation to other adipokines and inflammatory proteins emerges as an area that warrants further study.

It must be noted that the existing literature has produced many paradoxical observations regarding the clinical correlates of circulating visfatin.[3,6–18] It is now becoming apparent that at least part of the reason for the discrepancies reported lies in the assays used to measure visfatin. To date, the majority of clinical studies have utilized a commercial competitive assay that recognizes the carboxy-terminal of the visfatin protein. In these studies, the reported concentrations of circulating visfatin have shown marked variability, ranging from about 1–3 ng/ml in some studies[10,29] to between 14 and 50 ng/ml in most reports.[19] The physiologic associations reported have also been inconsistent. Indeed, although circulating levels of visfatin were initially thought to be correlated with visceral fat mass and with central obesity[6,30] several studies have noted (i) no difference in visfatin expression between visceral and subcutaneous adipose tissue[12,13] and (ii) no correlation between circulating levels and measures of anthropometry and fat mass (including multidetector computer tomography-determined visceral and subcutaneous fat).[3,8,10–13] The fact that the physiologically relevant full-length visfatin was not detected by the carboxy-terminal assay has been proposed as a potential explanation for these inconsistent results. Indeed, our data suggest that the C-terminal assay measures recombinant full-length human visfatin around 100–200-fold lower than the true values. This data supports recent suggestions that this assay is compromised by interference with a nonspecific HMW compound.[28] Thus, we believe that it is important to document that

the newly developed assay used in our study accurately and specifically detects circulating human full-length visfatin and this in itself may help to clarify the physiological role of this protein.

Our current findings confirm that type 2 diabetes is associated with increased circulating visfatin, as has been reported in most[3,6–9] but not all[10,11] previous studies. Furthermore, again in agreement with the majority of previous studies[3,12,14] there was no association between visfatin and insulin sensitivity/resistance. In contrast, although increased serum visfatin has been linked to β-cell dysfunction in one study[3] no correlation was noted with HOMA-B in the current analysis. This discrepancy may be due to the limitations of HOMA-B as a marker of β-cell function and hence further study of full-length visfatin with more sensitive β-cell measures is needed. Overall, our findings suggest that full-length visfatin is associated with type 2 diabetes but not with other metabolic variables, including anthropometric measurements, lipid measures, fasting glucose and insulin resistance.

There has been a great deal of controversy recently regarding the validity of initial suggestions that visfatin was an insulin-mimetic hormone.[1,2,5,28,31] Indeed, it has been suggested that the cytokine-like function of PBEF and the insulin-mimetic function of visfatin could be explained by the NAD biosynthetic activity of this protein as NAMPT.[1,2] It has therefore been suggested that promotion of systemic NAD biosynthesis represents one mechanism via which the body might attempt to maintain β-cell function in obesity and type 2 diabetes. Indeed, circulating PBEF/NAMPT/visfatin level was recently proposed as an indicator of beneficial lipid profile.[32] This idea is consistent with our data which demonstrated an elevation in circulating levels of this protein in individuals with type 2 diabetes compared to their nondiabetic peers, even after adjustment for age, gender and traditional metabolic risk factors. Furthermore, recent published work has strengthened the link between visfatin and inflammatory signal modulation.[18,21,33]

A novel finding in this report is the independent association between circulating visfatin and resistin. While the role of resistin in human obesity, insulin resistance, and type 2 diabetes remains controversial[34] increased resistin has been proposed as a mediator of inflammation in humans.[34,35] Taken together with the emerging recognition of obesity as a state of low-grade inflammation associated with increased macrophage infiltration of adipose compartments,[36,37] the demonstration that macrophages are the predominant source of both resistin and visfatin in human visceral adipose tissue raised the possibility that both of these adipokines may be pro-inflammatory factors in obese individuals.[38] Indeed, visfatin has been characterized as an inflammatory cytokine in neutrophils[39] and as pro-inflammatory in atherosclerosis.[40] In addition, visfatin has been positively associated with CRP and IL-6 in patients with chronic kidney disease[20] and a visfatin promoter polymorphism has been linked to increased plasma levels of CRP and fibrinogen[41] consistent with an inflammatory role of the protein.[42] While an earlier study found no correlation between serum visfatin and resistin,[6] Li *et al*. recently reported a positive univariate association that did not persist on multivariate adjustment.[43] The current study thus extends this literature by demonstrating an independent association between full-length visfatin and resistin. Further study of full-length visfatin in relation to inflammatory markers is warranted at this time.

Previous studies have yielded conflicting results regarding the relationship between visfatin and adiponectin. While Chen *et al*. reported an inverse association[6] subsequent studies have found no correlation between visfatin and total adiponectin.[7,8,10] It should be noted that adiponectin circulates as LMW, MMW and HMW multimeric forms,[44] which are believed to mediate distinct aspects of the pleiotropic bio-activity of the protein.[45,46] The HMW isoform has been linked to insulin-sensitizing and vascular-protective effects[47,48] while LMW adiponectin has been correlated with anti-inflammatory activity.[49] We thus speculated that differences in multimer distribution may underlie the conflicting associations reported to date between total adiponectin and visfatin. While the current analysis extends the literature by addressing the impact of adiponectin multimeric forms in this context, no correlation was detected between full-length visfatin and total, HMW, MMW and LMW adiponectin, respectively.

Certain limitations should be noted in this study. Firstly, the cross-sectional nature of the protocol precludes commentary on causality in the relationships under study. Secondly, the study population was modest in size ($n = 129$) and consisted of a single ethnic group (Thai). Thirdly, subjects with diabetes were assessed without stopping their oral antidiabetic agents, which theoretically could affect the relationships under study. It should be noted, however, that metformin and sulphonylureas (the only agents used by participants) have not been associated with changes in visfatin levels in earlier reports.[3,12]

In summary, circulating full-length visfatin measured by this newly developed assay is independently associated with diabetes but not with other metabolic covariates, including anthropometric measurements, lipid profile, fasting glucose, insulin resistance and β-cell function. Instead, visfatin is independently associated with circulating resistin concentration, while not related to total adiponectin or its individual multimeric forms. These data are in keeping with the emerging consensus of opinion and suggest that further study of the relationships of full-length visfatin with other adipokines and inflammatory proteins may help to elucidate the complex physiology of this protein.

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