Circulating PIIINP levels identify adipose tissue-associated inflammation in individuals with type 2 diabetes mellitus with and without non-alcoholic fatty liver disease.

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ABSTRACT

Aims. Procollagen-III peptide (PIIINP) is a marker of fibrosis associated with increased cardio-metabolic risk and progression of chronic liver diseases such as non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH); its association with type 2 diabetes mellitus has not been elucidated yet. Aim of this study was to investigate the relationship between circulating PIIINP levels, metabolic traits and body fat distribution in subjects with T2DM with or without NAFLD.

Materials and methods. Data from sixty-two T2DM subjects recruited in our Diabetes outpatient clinics at Sapienza University of Rome, Italy, were analysed. Participants underwent metabolic and inflammatory profiling (CRP, TNFα, IL-6, IL-8, WISP1, adiponectin) and magnetic resonance (MRI) for diagnosing NAFLD on the basis of hepatic fat fraction (HFF, ≥5.5%) and quantifying visceral and subcutaneous adipose tissue areas (VAT, SAT). Serum PIIINP was measured by Human-PIIINP ELISA Kits.

Results. Higher PIIINP levels correlated with greater BMI and VAT area and were associated with systemic signatures of AT-associated inflammation –i.e. higher WISP-1, IL-8, lower adiponectin levels-; conversely, PIIINP did not differ significantly between T2DM patients with or without NAFLD and were not associated with HFF, fatty liver index, FIB-4 or transaminases.

Conclusions. Greater circulating PIIINP levels specifically identify T2DM individuals with AT expansion and systemic pro-inflammatory profile suggestive for AT dysfunction; our results point towards a new role of PIIINP as a marker of fibro-inflammation in dysmetabolic conditions, likely related to AT expansion.

Introduction

The Procollagen type III aminoterminal Propeptide (PIIINP) is a circulating peptide resulting from biosynthesis and degradation of type III collagen[1] and represents an early marker of tissue remodelling and fibrosis in several conditions such as heart failure[2], atherosclerosis[3], interstitial lung disease[4] and pulmonary artery hypertension[5]. Serum PIIINP has been demonstrated to increase consistently with the hepatic damage progression in the course of chronic liver diseases [6-10] and, because of the tight correlation between circulating PIIINP concentration and tissue collagen production, it displays a role in risk stratification of subjects with advanced liver fibrosis.[8-11]

Within chronic hepatopathies, non-alcoholic fatty liver disease (NAFLD) is the most common cause of abnormal hepatic function in Western countries, with a prevalence ranging from 20-25% in general population up to 60-90% in patients with type 2 diabetes mellitus (T2DM) and obesity[12].

Due to the close association with insulin resistance, NAFLD is nowadays considered as the hepatic hallmark of metabolic syndrome. Furthermore, in presence of diabetes, an accelerated evolution from NAFLD to non-alcoholic steatohepatitis (NASH), severe fibrosis, cirrhosis and hepatocarcinoma has been widely demonstrated, along with the increased risk of liver-related mortality in diabetic individuals[13-16]. Conversely, the presence of NAFLD in T2DM patients is associated with poor metabolic control, higher rate of diabetes' micro- and macro-vascular complications[17-19] and represents an independent risk factor for cardiovascular mortality[20]. Of note, liver fibrosis, rather than other histological features of steatohepatitis, has been identified as the main determinant of overall-mortality and liver-related events in subjects with NAFLD[21]. Although so far liver biopsy has represented the gold standard for diagnosing NAFLD/NASH, the execution of this invasive procedure at a very high-scale level, i.e. in obese populations at high risk of hepatic steatosis, is rather questioned, even

considering the possibility of non-diagnostic results in case of local steatosis and the lack of medically approved treatment for NAFLD.

Recently, PIIINP measurement, alone or with other circulating markers in the context of serum diagnostic panels, has been validated as the one most constantly associated with NAFLD[8] and NASH[10]. The presence of a positive correlation between circulating PIIINP concentration and liver fibrosis has been identified in several studies carried out in patients with alcoholic liver disease[9] and NASH[10]; furthermore, baseline serum PIIINP levels were capable of predicting the response to anti-fibrotic treatments in NASH patients.[10] Thus, PIIINP concentration has been used as a marker of NASH and hepatic fibrogenesis activity in children[22-24], adolescents[23] and adult populations[8, 25] whereas the role of circulating PIIINP in improving the diagnostic accuracy of NAFLD has been recently disputed[26, 27]. Moreover, the evidence of increased PIIINP levels in presence of abdominal obesity[1], insulin resistance[28] and conditions associated with metabolic syndrome and T2DM, such as impaired arterial stiffness and atherosclerosis[29,30], may rise questions on the specificity of PIIINP in identifying NAFLD in dysmetabolic populations and T2DM. As far as we know, no study has investigated circulating PIIINP levels in diabetic individuals in relation to the presence of NAFLD. Therefore, aim of this study was to investigate the relationship between circulating PIIINP levels, metabolic traits and body fat distribution in subjects with T2DM, with and without NAFLD.

Methods

Population

For this study, we analysed data from sixty-two consecutive T2DM subjects referring to our Diabetes outpatient clinics at Sapienza University of Rome, Italy, and participating to the Eudract 2011-003010-17 trial (www.clinicaltrialsregister.eu)[31]. In brief, this is a monocentric,

randomized, double-blind, placebo-controlled trial investigating the efficacy and safety of 24-week oral high-dose vitamin D supplementation in T2DM patients with NAFLD evaluated by magnetic resonance imaging (MRI). All study participants underwent clinical work-up and fasting blood sampling for metabolic and inflammatory profiling. Weight and height were measured with patients wearing light clothing and no shoes. The body mass index (BMI, Kg/m^2) was calculated as weight in kilograms divided by the square of the height in meters; waist circumference was measured midway between the 12th rib and the iliac crest. Systolic and diastolic blood pressure (SBP, DBP; mmHg) was measured after 5 minutes rest by means of an electronic recorder with the patient sitting in the upright position. Three measurements were taken and the average of the second and third measurements was recorded and used in the analyses.

Laboratory assessments

Fasting glycaemia (FBG, mg/dl), glycosylated hemoglobin (HbA1c, % - mmol/mol), total cholesterol (mg/dl), high-density lipoprotein cholesterol (HDL, mg/dl), triglycerides (mg/dl), AST (IU/l), ALT (IU/l), gamma-glutamyl transpeptidase (γ-GT, IU/l) and C-reactive protein (CRP, mg/dl) were measured by centralized standard laboratory methods. Fasting blood insulin (FBI) was assessed by radio-immuno-assay (PANTEC s.r.l., Italy; intra- and inter-assay coefficients of variation <5%). Among circulating markers of AT inflammation, we measured serum levels of IL-6, IL-8, TNF-□ (pg/mL; Multiplex, BioRad) and adiponectin (mg/mL; enzyme-linked immunosorbent assay; Tema Ricerca srl, Italy), WISP1 concentration was detected in plasma samples by enzyme-linked immunosorbent assay commercial kits (ng/mL; RayBiotech Inc., Norcross, GA). As a biomarker of liver injury and apoptosis, we assessed serum CK18-M30 concentration by Human Cytokeratin 18-M30 ELISA kit (Cusabio®, intra- and inter-assay coefficients of variation <8%). Circulating PIIINP levels were measured on serum by Human PIIINP ELISA kit, Elabscience™ (pg/ml,

intra- and inter-assay coefficients of variation <10%). Low-density lipoprotein cholesterol (LDL, mg/dl) values were calculated using the Friedewald formula. Insulin resistance and secretion have been indirectly estimated by using the homeostasis model assessment of insulin resistance (HOMA-IR) and insulin secretion (HOMA-β%), respectively[32]. As additional indicators of hepatic damage in NAFLD, we calculated the Fatty Liver Index (FLI= ($e^{0.953*\log e(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e(\text{ggt}) + 0.053*\text{waist circumference} - 15.745}$) / ($1 + e^{0.953*\log e(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e(\text{ggt}) + 0.053*\text{waist circumference} - 15.745}$) * 100), a clinical predictor of fatty liver,[33] and the FIB-4 Index (FIB-4= age[years] x AST[U/L])/(platelet [10^9] x √ALT[U/L]) which represents a marker of hepatic fibrosis in chronic hepatopathies, such as HCV-related hepatitis and NASH [34,35].

Magnetic Resonance Imaging

Abdominal MRIs were performed by the same operator, unaware of participants' clinical data, to assess hepatic fat fraction (HFF, %) and to measure total subcutaneous and visceral fat areas (SAT and VAT, cm^2) in all study participants. All MRIs were performed using a 1.5-T magnet (Magnetom Avanto, Siemens Medical Systems, Erlangen, Germany) equipped with a phased-array surface coil and a spine array coil. NAFLD was diagnosed in patients with HFF ≥ 5.5%; VAT and SAT were quantified by acquiring a three-dimensional gradient echo T1-weighted volumetric interpolated breath- hold examination sequence on an axial plane modified by Dixon [repetition time, 4.7 msec; echo time, 2.3 msec; flip angle, 10°; matrix, 256 3192 mm; section thickness, 5 mm (reconstructed 2.5 mm); intersection gap, 0] and analysing the fat-only data sets with specific software (Slice-O-Matic; Tomovision Inc., Montreal, QC, Canada). Data were calculated from AT area at L1–L2, L2–L3, L3–L4, and L4–L5 levels; free-form regions of interest and manual threshold were used to select fat tissue within VAT and SAT slides. More details on MRI acquisition have been provided elsewhere[31].

Statistical analysis

This is the first study evaluating serum PIIINP levels in T2DM patients in relation to the presence of NAFLD; power analysis has been performed based on data from Tanwar et al.[8] validating PIIINP as a marker of hepatic damage in subjects with fatty liver. Based on circulating PIIINP concentrations found in subgroups with or without NAFLD, we theoretically needed a minimum of 20 subjects per group to obtain a statistically significant observation, with $\alpha = 0.01$ and power= 0.90. Values are reported as mean ± standard deviation of the mean (SD) or as median (interquartile range) for continuous variables and as a percentage for categorical variables. Comparisons between two groups were performed by the non-parametric Kruskal-Wallis test for continuous variables and Pearson's χ^2 test for categorical variables. Bivariate correlation analyses were calculated by Pearson and Spearman's rank correlation tests; THE MULTIVARIATE REGRESSION MODEL INCLUDED AGE, SEX AND VARIABLES SIGNIFICANTLY ASSOCIATED WITH CIRCULATING PIIINP LEVELS AT THE BIVARIATE CORRELATION ANALYSES, AS WELL AS MARKERS OF NAFLD, I.E. HFF, AST/ALT AND FIB-4, SINCE THIS CONDITION REPRESENTS A POSSIBLE DETERMINANT OF INCREASED PIIINP CONCENTRATION.

All statistics have been performed by SPSS version 23; two-tailed p values < 0.05 were considered statistically significant, with a 95% confidence interval.

The study protocol was reviewed and approved by the Ethics Committee of Policlinico Umberto I, Sapienza University of Rome and the study was conducted in conformance with the Helsinki Declaration. Written consent was obtained from all patients before the study.

Results

In the total study population, median (interquartile range) serum PIIINP levels were 970.7 (103.7-1931) pg/ml and associated with greater BMI (r= 0.26, p=0.04), VAT area (r= 0.44, p= 0.03) and with a specific pattern of AT-associated inflammation characterized by higher

WISP-1 (r= 0.29, p= 0.02), IL-8 (r= 0.33, p= 0.01) and lower adiponectin levels (r= -0.31, p= 0.02). Since previous evidence identified height as a novel independent determinant of cardiometabolic risk[36], we investigated the presence of a possible correlation between circulating PIIINP concentration and height and body weight, finding NO SIGNIFICANT CORRELATION WITH EITHER HEIGHT (R= 0.052, P= 0.699) OR BODY WEIGHT, ALTHOUGH THE LATTER REVEALED A TREND TOWARDS POSITIVE ASSOCIATION NOT REACHING STATISTICAL SIGNIFICANCE (R= 0.25, P= 0.061).

Circulating PIIINP did not differ significantly between T2DM patients with or without MRI-assessed NAFLD (568.7 (143.3 – 2061) vs 1021 (96.8 – 1418.5) pg/ml, respectively; p=0.98, Table 1); accordingly, no correlation was found between serum PIIINP levels and transaminases, FIB-4, FLI and HFF, whereas a trend towards an association was observed with CK-18 (r= 0.26, p= 0.051). Results from all the correlation analyses are shown in table 2. The lack of an association between circulating PIIINP levels and NAFLD persisted across different BMI categories (normal-, over-weight, obesity) at the partial correlation analyses (data not shown).

All patients underwent cardiovascular subclinical and clinical evaluations, such as intima-media thickness, flow-mediated dilatation and ankle-brachial index measurement, finding no association between serum PIIINP levels and these parameters (data not shown).

The multivariate stepwise linear regression analysis showed that higher BMI represented the main determinant of increased PIIINP levels after adjusting for sex, age, HFF, FIB-4, AST/ALT and systemic inflammatory pattern (Table 3a). Furthermore, in order to investigate whether the association between greater circulating PIIINP levels and BMI persisted throughout different BMI categories, we further adjusted the multivariate regression model for classes of weight (normal-weight, overweight, obesity), confirming that BMI represented

the clinical variable most associated with greater PIIINP concentration across these different phenotypes and independently from all the other confounders (Table 3b).

Discussion

Our study demonstrates that in subjects with T2DM increased PIIINP levels are associated with greater adiposity and features of AT-associated inflammation whereas they do not correlate with hepatic fat content and markers of liver damage.

This is the first study investigating the relationship between circulating PIIINP, body fat distribution and signatures of dysmetabolism in presence of T2DM, with and without NAFLD. Younossi et al.[37] examined the performance of a panel of circulating markers of hepatic damage in detecting NAFLD and NASH in obese subjects; among them, PIIINP levels were capable of distinguishing patients with NASH and fibrosis from those with simple steatosis without fibrosis. However, no data were available on obese subjects without fatty liver disease.[37] In line with our findings on the existence of an association between PIIINP levels and body adiposity, other groups reported higher serum PIIINP levels in obese patients compared to healthy volunteers[1,28,38] which linearly decreased after weight loss in a report exploring the relationship between obesity, fat distribution and markers of collagen turnover[1]. Similarly, circulating PIIINP correlated with markers of insulin resistance and signs of impaired ventricular dysfunction in non-diabetic obese subjects[38]. Besides, no information on hepatic fat content and/or liver-related complications have been provided in these studies.

In a small intervention trial aiming to investigate the efficacy of the glucagon-like peptide 1 receptor agonist liraglutide in improving markers of liver fibrosis in obese women with polycystic ovary syndrome[25], the authors observed a significant correlation between weight reduction and PIIINP decrease, whereas no correlation with other markers of liver damage, such as AST and ALT, was found[25]. These results support our hypothesis of the existence of

an association between increased PIIINP concentration and AT expansion and inflammation, beside NAFLD, in subjects with dysmetabolic conditions without severe fibrotic liver disease.

As a peptide derived from collagen synthesis and degradation, PIIINP has been investigated in different chronic diseases sharing as a common pathophysiology chronic inflammatory damage and aberrant collagen deposition[2-5,9,10]. Some studies described the correlation between higher PIIINP and the occurrence of peripheral arterial disease[29] or, in general, atherosclerosis.[30] In the large "Cardiovascular Health Study" cohort, Agarwal et al. demonstrated that higher blood PIIINP levels were associated with greater carotid intima-media thickness and impaired brachial artery reactivity, mostly in patients with concurrent higher CRP levels[3]. Moreover, in agreement with our results, they observed increased PIIINP levels throughout different classes of obesity[3]. An independent association between PIIINP and several markers of insulin resistance, such as higher fasting insulin, BMI, non-esterified fatty acids, 2-hour glucose and insulin, has been reported in cross-sectional observations conducted on the same cohort.[28,39] Again, no data on the presence of NAFLD/NASH were available in any of these studies. Although Wang TJ et al.[40] reported the existence of an association between higher PIIINP and presence of obesity, in their study conducted on the Framingham Heart Study cohort, no correlation between the circulating levels of this peptide and indexes of structural heart disease was found.[40] Similarly, in our study PIIINP levels were not related to any parameters of either sub-clinical or clinical cardiovascular impairment.

Indeed, our findings suggest that, in presence of dysmetabolic conditions, such as T2DM and overweight/obesity, circulating PIIINP levels are mostly associated with systemic pro-inflammatory profile likely associated with AT expansion rather than by fat deposition into the hepatic parenchyma. Notably, these results applied to different BMI categories,

identifying a phenotype at increased cardiometabolic risk likely correlated with visceral fat and AT inflammation, as also suggested by other reports.[41,42]

Thus, in absence of clinical advanced liver damage, PIIINP may not represent a useful tool for NAFLD detection and risk stratification, particularly.

Furthermore, it is important to consider that our cohort was selected among T2DM patients referring to our Diabetes outpatients' clinic for routine diabetes care, likely leading to recruit subjects with milder NAFLD levels than those detectable in patients referring to Hepatology setting specifically for treatment of liver diseases. Conversely, data on the role of serum PIIINP concentration as a marker of progression from NAFLD to NASH and advanced fibrosis are already largely available in literature[8-10,37,43] and our investigation is beyond the purpose of these studies.

To the best of our knowledge, this is the first study aiming to evaluate PIIINP levels in T2DM subjects in relation to the presence of NAFLD and has been carried out in an extensively phenotyped population, allowing to detect possible determinants of increased PIIINP among a large number of metabolic and inflammatory variables. In conclusion, in T2DM patients, greater circulating PIIINP levels identify AT expansion and correlate with systemic features of AT inflammation, whereas are not associated with hepatic fat content or liver damage in NAFLD. Our results point out a new role of PIIINP as a marker of fibro-inflammation in dysmetabolic conditions, likely related to adipose tissue dysfunction rather than hepatic damage.

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Competing Financial Interests

The authors have no competing interests to declare.

Tables

Table 1. Characteristics of study population according to the presence or absence of NAFLD. Mann-Whitney U test, *χ^2 Pearson's test applied.

	NAFLD (n= 35)	No NAFLD (n= 27)	P-value
Age (years)	**56.2 ± 9.7**	**62.1 ± 8.6**	**0.01**
Gender (% M)	72	72	0.08
T2D duration (years)	6.0 ± 5.0	8.3 ± 8.0	0.15
BMI (kg/m^2)	30.5 ± 4.4	29.4 ± 4.2	0.31
Waist Circumference (cm)	106.2 ± 14.2	100.4 ± 10.1	0.058
PAS (mmHg)	127.5 ± 16.3	133.9 ± 16.6	0.12
PAD (mmHg)	81.0 ± 9.6	81.8 ± 10.4	0.74
SAT area (cm^2)	295.2 ± 117.6	262.6 ± 86.1	0.24
VAT area (cm^2)	132.1 ± 52.9	118.9 ± 46.7	0.55
FBG (mg/dl)	130.4 ± 32.3	134.8 ± 45.5	0.66
HbA1c (mmol/mol)	6.7 ± 1.0	6.5 ± 0.9	0.81
HOMA-IR	**4.4 ± 1.6**	**3.2 ± 1.8**	**0.02**
HOMA-B	103.3 ± 72.2	70.8 ± 48.8	0.054
AST (IU/l)	**26.9 ± 13.3**	**20.8 ± 11.1**	**0.05**
ALT (IU/l)	**39.6 ± 25.2**	**24.3 ± 12.1**	**0.002**
γ-GT (IU/l)	51.2 ± 61.7	32.0 ± 33.2	0.14
FLI	70.2 ± 23.2	60.4 ± 25.6	0.11
PIIINP (pg/ml)	1102.3±1030.7	1175±1178.6	0.73
HFF (%)	**13.2 ± 7.4**	**2.7 ± 2.0**	**< 0.001**
CK-18	219.9 ± 142.8	207.4 ± 139.8	0.73
FIB-4	1.11 ± 0.53	1.28 ± 0.56	0.21
Total Cholesterol (mg/dl)	176.2 ± 36.9	176.4 ± 38.1	0.99
HDL (mg/dl)	48.4 ± 15.1	49.9 ± 13.0	0.68
LDL (mg/dl)	100.7 ± 35.1	98.9 ± 31.4	0.87
Triglycerides (mg/dl)	135.0 ± 65.8	137.3 ± 59.1	0.88
IL-8 (pg/ml)	78.6 ± 125.3	67.9 ± 121.4	0.73
WISP1 (pg/ml)	552.9 ± 2047.6	297.8 ± 699.9	0.52
Adiponectin (μg/ml)	6.5 ± 3.0	6.1 ± 3.8	0.64

Table 2. PIIINP- Bivariate correlation analyses (Spearman's coefficient, continuous variable)

	Correlation coefficient	***p-value***
Age	-0.09	0.47
Gender (M/F)	-0.02	0.82
T2DM duration	0.09	0.42
BMI	**0.26**	**0.04**
Waist Circumference	0.13	0.33
PAS	0.04	0.79
PAD	-0.17	0.20
SAT area	0.08	0.60
VAT area	**0.44**	**0.03**
FBG	0.04	0.74
HbA1c	0.03	0.82
AST	-0.16	0.24
ALT	-0.13	0.33
γ-GT	-0.03	0.82
FLI	0.13	0.33
HFF	0.03	0.82
NAFLD yes/no (NMR)	0.02	0.73
CK-18	0.26	0.051
Total Cholesterol	-0.16	0.23
HDL	-0.12	037
LDL	-0.19	0.16
Triglycerides	0.06	0.67
IL-8	**0.33**	**0.001**
WISP1	**0.29**	**0.02**
Adiponectin	**-0.31**	**0.02**
HOMA-IR	0.06	0.65
HOMA-B	-0.06	0.67

Table 3. Multivariate stepwise regression analysis (a), after further adjustment for BMI categories (b). Circulating PIIINP concentration is the dependent variable.

Coefficients

	Non-standardized Coefficients		Standardized Coefficients	t	Sig.
	B	Standard Deviation Error	Beta		
(Constant)	-3371.419	988.781	1174.351	2.871	0.008
BMI	**143.070**	**37.790**	**0.575**	**3.786**	**0.001**

a. Excluded variables

Parameter	Beta	t	P-value	Partial Correlations	Collinearity Statistics
					Tolerance
Age	0.010	0.067	0.947	0.013	0.989
Gender	-0.060	-0.375	0.711	-0.71	0.921
IL-8	0.273	1.802	0.082	0.322	0.934
WISP1	0.073	0.462	0.648	0.087	0.938
Adiponectin	-0.270	1.762	0.089	-0.316	0.9188
HFF	0.013	0.083	0.934	0.016	0.990
FIB-4	-0.66	-0.429	0.671	-0.81	0.990
AST/ALT	-0.25	-0.163	0.872	-0.031	0.972

b. Excluded variables

Parameter	Beta	t	P-value	Partial Correlations	Collinearity Statistics
					Tolerance
Age	0.010	0.067	0.947	0.013	0.989
Gender	-0.060	-0.375	0.711	-0.71	0.921
IL-8	0.273	1.802	0.082	0.322	0.934
WISP1	0.073	0.462	0.648	0.087	0.938
Adiponectin	-0.270	1.762	0.089	-0.316	0.9188
HFF	0.013	0.083	0.934	0.016	0.990
FIB-4	-0.66	-0.429	0.671	-0.81	0.990
AST/ALT	-0.25	-0.163	0.872	-0.031	0.972
BMI categories	-0.024	0.095	0.925	0.018	0.372