Circulating plasma proteins and new-onset diabetes in a population-based study: proteomic and genomic insights from the STANISLAS cohort

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Abstract

Objective: Determining the factors associated with new-onset pre-diabetes and type 2 diabetes mellitus (T2D) is important for improving the current prevention strategies and for a better understanding of the disease.

Design: To study the factors (clinical, circulating protein and genetic) associated with new onset pre-diabetes and T2D in an initially healthy (without diabetes) populational familial cohort with a long follow-up (STANISLAS cohort).

Methods: A total of 1506 participants attended both the visit 1 and visit 4, separated by ≈20 years. Over 400 proteins, GWAS and genetic associations were studied using models adjusted for potential confounders. Both prospective (V1 to V4) and cross-sectional (V4) analyses were performed.

Results: People who developed pre-diabetes ($n = 555$) and/or T2D ($n = 73$) were older, had higher BMI, blood pressure, glucose, LDL cholesterol, and lower eGFR. After multivariable selection, PAPP-A (pappalysin-1) was the only circulating protein associated with the onset of both pre-diabetes and T2D with associations persisting at visit 4 (i.e. ≈20 years later). FGF-21 (fibroblast growth factor 21) was a strong prognosticator for incident T2D in the longitudinal analysis, but not in the cross-sectional analysis. The heritability of the circulating PAPP-A was estimated at 44%. In GWAS analysis, the SNP rs634737 was associated with PAPP-A both at V1 and V4. External replication also showed lower levels of PAPP-A in patients with T2D.

Conclusions: The risk of developing pre-diabetes and T2D increases with age and with features of the metabolic syndrome. Circulating PAPP-A, which has an important genetic component, was associated with both the development and presence of pre-diabetes and T2D.

European Journal of
Endocrinology
(2020) **183**, 285–295

Introduction

The number of people with type 2 diabetes mellitus (T2D) more than doubled in the past 20 years in both the developed and developing countries (1). The latest prediction from the International Diabetes Federation

is that over 380 million people are living with diabetes nowadays and that by 2035 this number may rise up to 600 million (2). Type 2 diabetes was traditionally considered as a disorder of middle-aged and elderly people; however,

the more recent 'type 2 diabetes epidemics' is particularly worrisome among adolescents and young adults, leading to tremendous costs, impairment in the quality of life and reduced life expectancy, mainly due to associated cardiovascular and renal complications (1, 3, 4).

Type 2 diabetes results from a complex interaction between genetic/epigenetic systems and multiple behavioral plus environmental factors (1). Despite the challenges linked to the gene-environment interplay, determining the factors associated with new-onset pre-diabetes and T2D is important not only for improving the current prevention strategies and treatments, but also for a better understanding of the disease (5, 6).

The STANISLAS (Suivi Temporaire Annuel Non-Invasif de la Santé des Lorrains Assurés Sociaux) cohort is a single-centre familial prospective cohort comprising 1006 initially healthy (including absence of diabetes) families (4295 participants) from the Nancy region of France, who were recruited from 1993 to 1995 to the visit 1 (V1). Participants were then followed each 5 to 10 years, and from the initial visit, 1705 participants returned for the fourth visit (V4), held from 2011 to 2016, which comprised a detailed clinical and laboratorial assessment (7). Beyond the clinical, imaging and routine laboratorial measurements, circulating levels of multiple proteins were also assessed at V1 and V4. Additionally, a genome wide association study (GWAS) was performed for the V4 participants. This design allows the assessment of the clinical factors, genetic variants and proteomic signatures associated with the new-onset of pre-diabetes and/or T2D almost 20 years in advance; the family structure also allows the study of heritability (8).

In the present analysis, we considered patients without diabetes at V1 (patients entering the familial STANISLAS study did not have chronic conditions) with the main aims of studying the factors associated with new onset pre-diabetes and T2D at V4, considering the clinical features, proteomic signatures and genetic variants that might help explaining the main proteomic findings.

Methods

Study population

A detailed description of the STANISLAS cohort has been previously published (7). The STANISLAS cohort was established with the primary objective of investigating gene-gene and gene-environment interactions in the field of cardiovascular diseases. In order to assess the effect of genetics on the variability of intermediate phenotypes

on the transition toward pathology, the families were deemed healthy and free of declared acute and/or chronic illness at V1. The implementation of the fourth visit (V4) enabled a follow-up of 18 to 23 years (mean of 18.5 years). The collected information was enriched with new circulating proteins and detailed clinical phenotyping. The study protocols for all examinations were reviewed and approved by the Local Ethics Committee of CPP-Est 3, France. All participants provided written informed consent to participate in the study.

In the present analysis we included 1506 participants that attended both the V1 and V4 and in whom we had information on fasting glucose, random glucose, glycated hemoglobin, treatments and/or previous diagnosis that allow the pre-diabetes and T2D classification. The study flow-chart is shown in the Fig. 1. The main objective is to assess the circulating proteins that are associated with the development of diabetes in a prospective analysis (V1 to V4) and to ascertain if the associations remained significant almost 20 years later at V4 in a cross-sectional analysis.

Figure 1
Study flow chart.

Study design

All participants were observed at the Centre d'Investigation Clinique Plurithématique Pierre Drouin at Nancy Hospital Center (CIC-P de Nancy) in the morning after a 12- to 14-h fast. Blood samples were drawn, and medical history, medications, anthropometric parameters, blood pressure, carotid-femoral pulse-wave velocity (PWV), and carotid intima-media thickness (cIMT) were recorded.

Pre-diabetes and diabetes definition

Pre-diabetes was defined as a fasting plasma glucose (FPG) between 100 mg/dL and 125 mg/dL or a glycated hemoglobin between 5.7% and 6.4%. Diabetes was defined as a FPG ≥ 126 mg/dL or a glycated hemoglobin $\geq 6.5\%$ or a random plasma glucose ≥ 200 mg/dL with symptoms of hypoglycemia or previous diagnosis of diabetes or anti-glycemic drug therapy (9).

Circulating proteins and gene-candidate analysis

All samples were collected at the CIC-P de Nancy with minimally traumatic venipuncture. Standardized sample handling procedures enabled the collection of serum and plasma (EDTA, heparin) as well as buffy coat fraction. Blood DNA of all the participants to the Stanislas V4 was extracted using Gentra Puregene Blood Kit (Qiagen) and stored at -20°C . Genotyping was conducted at CNRGH (Evry, France) using two chips: the Illumina Global Screening Array (GSA), which is composed of 687 572 intronic and exonic markers, and the Illumina Exome Array, which is constituted of 244 330 SNPs, mostly exonic. All blood-derived bio-samples are stored in a central biobank facility with temperatures between -80°C and -196°C (as required, e.g. -196°C for plasma and serum).

Baseline plasma samples were analysed for proteins by the TATAA-biocenter using the Olink Proseek® Multiplex cardiovascular (CVD) II, III, cardiometabolic (CM), organ-damage (OD) and inflammatory (INF) panels, using a proximity extension assay (PEA) technology (10), where 92 oligonucleotide-labelled antibody probe pairs per panel (total of 92×5 ; $n=460$ proteins) are allowed to bind to their respective targets in the sample in 96-well plate format. When binding to their correct targets, they give rise to new DNA amplicons with each ID-barcoding their respective antigens. The amplicons are subsequently quantified using a Fluidigm BioMark™ HD real-time PCR

platform. The platform provides $\log_2$ -normalized protein expression (NPX) data.

The strategy for the genetic analyses of the present study was the following. First, we conducted GWAS on diabetes occurrence, fasting glycemia and glycated hemoglobin. Secondly, GWAS was conducted on the plasma protein(s) associated with both pre-diabetes and T2D in both analysis (i.e. longitudinal and cross-sectional). Thereafter, the GWAS top-SNP was tested for association with study outcome (i.e. pre-diabetes, T2D, fasting glycemia, glycated hemoglobin and circulating PAPPA-A).

Our findings were replicated using the baseline data of the HOMAGE (Bioprofiling Response to Mineralocorticoid Receptor Antagonists for the Prevention of Heart Failure; ClinicalTrials.gov Identifier: NCT02556450) trial, a prospective trial that included 527 people at risk for developing heart failure randomized to either spironolactone or standard of care for up to 9 months; the description of this study and baseline characteristics of the participants have been published elsewhere (11).

Statistical, genetic and bioinformatical analysis

For the baseline clinical characteristics, continuous variables are expressed as means and respective s.d. and compared using ANOVA or Kruskal–Wallis test. Categorical variables are presented as frequencies and percentages and compared using Chi-squared test.

The main aim of this study was to test the association of multiple circulating proteins with incident pre-diabetes and T2D. Multinomial logistic regression models were performed (using no pre-diabetes/T2D as the referent category). First, a stepwise backward model including all the clinical variables with a $P < 0.1$ from Table 1 was performed, to select the clinical features with stronger association with pre-diabetes and T2D. Due to collinearity between BMI and waist circumference (correlation > 0.9 in both visits), we included only BMI in the models (including waist circumference provided the same results). The clinical features retained in the final model were age, BMI, LDL cholesterol and systolic blood pressure. These variables were used for adjustment in all the subsequent models. Secondly, the potential association of the multiple circulating proteins with pre-diabetes and T2D was tested one-by-one on top of the clinical model (i.e. adjusted for the clinical features) built in the previous step. The statistical significance level was fixed at a FDRq < 0.05 , after applying a Benjamini–Hochberg correction for multiple testing (i.e. false discovery rate (FDR) set at 5%).

Table 1 Patients' characteristics at V1 and V4 in whole population and by the development status of pre-diabetes and diabetes at V4.

Characteristic	n	Overall	n	Non-progressing	n	Progressing to pre-diabetes	n	Progressing to diabetes	P for trend
Visit 1 (V1)									
Female sex	1506	793 (52.7 %)	878	470 (53.5 %)	555	296 (53.3 %)	73	27 (37.0 %)	0.023
Age (years)	1506	38.0 (16.6–42.7)	878	21.0 (13.8–40.2)	555	40.7 (37.2–44.2)	73	42.3 (39.8–46.4)	<0.0001
BMI (kg/m ²)	1505	22.26 (19.78–25.08)	878	21.24 (18.62–23.94)	554	23.26 (21.23–25.87)	73	27.07 (25.21–29.90)	<0.0001
Waist circumference (cm)	1457	74.0 (67.0–85.0)	838	70.0 (64.0–80.0)	548	78.0 (71.0–87.0)	71	92.0 (85.0–99.0)	<0.0001
Female	778	70.0 (64.0–75.0)	448	67.0 (62.0–72.0)	293	72.0 (68.0–77.0)	27	84.0 (77.0–89.0)	<0.0001
Male	696	83.0 (74.0–90.0)	390	78.0 (68.0–86.0)	255	86.0 (79.0–93.0)	44	94.5 (91.0–102.0)	<0.0001
SBP office (mmHg)	1501	120.0 (110.0–128.0)	877	116.0 (110.0–124.0)	552	120.0 (112.0–130.0)	72	128.0 (122.0–140.0)	<0.0001
DBP office (mmHg)	1501	69.1 ± 12.6	877	66.0 ± 12.5	552	72.6 ± 11.3	72	79.6 ± 10.2	<0.0001
Heart rate (bpm)	1504	68.3 ± 11.6	877	69.8 ± 12.0	554	65.9 ± 10.4	73	68.0 ± 11.8	<0.0001
Glucose (g/L)	1464	0.89 ± 0.08	854	0.88 ± 0.07	540	0.90 ± 0.07	70	0.98 ± 0.13	<0.0001
LDLc (g/L)	1458	1.36 ± 0.37	850	1.28 ± 0.34	539	1.47 ± 0.38	69	1.55 ± 0.35	<0.0001
eGFR (mL/min/1.73 m ²)	1465	98.7 (86.2–117.3)	854	106.0 (89.7–130.9)	541	92.4 (82.6–105.4)	70	87.4 (81.7–101.1)	<0.0001
Visit 4 (V4)									
Female	1506	794 (52.7 %)	878	470 (53.5 %)	555	296 (53.3 %)	73	28 (38.4 %)	0.043
Age (years)	1506	56.0 (35.0–60.0)	878	39.0 (33.0–58.0)	555	59.0 (55.0–62.0)	73	60.0 (58.0–64.0)	<0.0001
Waist circumference (cm)	1496	90.0 (80.0–99.0)	872	86.0 (77.0–95.0)	551	92.0 (83.0–101.0)	73	103.0 (96.0–114.0)	<0.0001
Female	787	82.0 (74.0–93.0)	465	80.0 (73.0–90.0)	294	87.0 (77.0–95.0)	28	101.5 (84.5–107.0)	<0.0001
Male	709	95.0 (87.0–102.0)	407	91.0 (85.0–99.0)	257	98.0 (91.0–106.0)	45	104.0 (98.0–117.0)	<0.0001
BMI (kg/m ²)	1506	25.26 (22.63–28.50)	878	24.46 (22.13–27.38)	555	26.51 (23.47–29.67)	73	28.84 (26.57–34.12)	<0.0001
Current smoker	1502	308 (20.5 %)	874	191 (21.9 %)	555	107 (19.3 %)	73	10 (13.7 %)	0.17
Heart rate (bpm)	1505	63.3 (57.3–69.0)	878	63.3 (58.0–69.7)	555	62.3 (56.3–67.3)	73	67.0 (61.3–77.0)	<0.0001
SBP office (mmHg)	1505	123.3 (114.3–135.0)	878	121.8 (113.0–133.0)	554	125.0 (116.0–138.3)	73	138.7 (128.3–147.7)	<0.0001
DBP office (mmHg)	1505	72.6 ± 8.9	878	71.6 ± 8.8	554	73.6 ± 8.9	73	77.1 ± 9.4	<0.0001
24-h SBP (mm Hg)	1414	120.1 ± 10.4	812	119.1 ± 10.0	533	120.8 ± 10.5	69	127.0 ± 10.2	<0.0001
24-h DBP (mm Hg)	1414	74.3 ± 7.3	812	73.8 ± 7.2	533	74.6 ± 7.4	69	76.7 ± 6.9	0.002
Hypertension 24-h	1417	594 (41.9 %)	811	269 (33.2 %)	535	268 (50.1 %)	71	57 (80.3 %)	<0.0001
Hypertension office	1490	497 (33.4 %)	871	195 (22.4 %)	546	245 (44.9 %)	73	57 (78.1 %)	<0.0001
Hypertension treatment	1479	285 (19.3 %)	872	96 (11.0 %)	536	142 (26.5 %)	71	47 (66.2 %)	<0.0001
Glucose (g/L)	1506	0.88 (0.83–0.95)	878	0.86 (0.80–0.91)	555	0.92 (0.86–0.99)	73	1.30 (1.09–1.52)	<0.0001
Glycated hemoglobin (%)	1506	5.6 (5.4–5.8)	878	5.4 (5.2–5.5)	555	5.8 (5.7–6.0)	73	6.9 (6.5–7.8)	<0.0001
Total cholesterol (g/L)	1506	2.11 (1.86–2.41)	878	2.07 (1.82–2.36)	555	2.22 (2.00–2.49)	73	1.84 (1.61–2.08)	<0.0001
Triglycerids (g/L)	1506	0.92 (0.68–1.26)	878	0.84 (0.64–1.14)	555	1.02 (0.74–1.40)	73	1.28 (0.94–1.96)	<0.0001
HDLc (g/L)	1506	0.58 ± 0.14	878	0.59 ± 0.14	555	0.59 ± 0.14	73	0.49 ± 0.12	<0.0001
LDLc (g/L)	1502	1.35 ± 0.34	878	1.32 ± 0.32	552	1.43 ± 0.33	72	1.09 ± 0.35	<0.0001
eGFR (mL/min/1.73 m ²)	1506	95.8 (86.4–106.0)	878	99.0 (88.8–111.9)	555	92.7 (81.6–98.5)	73	93.1 (82.3–100.3)	<0.0001
Microalbuminuria (mg/L)	1497	6.1 (3.5–9.9)	874	5.8 (3.5–9.4)	550	6.1 (3.5–9.6)	73	10.2 (5.3–21.4)	<0.0001
PWV (m/s)	1476	8.2 (7.4–9.4)	862	7.9 (7.2–8.9)	542	8.6 (7.7–9.8)	72	9.9 (8.5–11.2)	<0.0001
cIMT >0.9 mm	1470	86 (5.9 %)	855	27 (3.2 %)	542	47 (8.7 %)	73	12 (16.4 %)	<0.0001
Carotid plaques	1469	192 (13.1 %)	855	78 (9.1 %)	541	96 (17.7 %)	73	18 (24.7 %)	<0.0001
LVMI (g/m ²)	1484	74.5 (63.0–87.9)	869	72.2 (61.1–85.1)	545	76.7 (65.7–90.9)	70	78.2 (68.2–92.6)	<0.0001
Lipid-lowering therapy	1506	242 (16.1 %)	878	73 (8.3 %)	555	128 (23.1 %)	73	41 (56.2 %)	<0.0001
Prior diagnosis of diabetes	1500	60 (4.0 %)	873	3 (0.3 %)	554	8 (1.4 %)	73	49 (67.1 %)	<0.0001

V1 is the baseline visit that took place 18.5 years before V4 and where all patients were deemed healthy and without diabetes.

cIMT, carotid intima-media thickness; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate (CKD-EPI formula); HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; LVEF, left ventricular ejection fraction; LVMI, left ventricular mass indexed; PWV, pulse-wave velocity; SBP, systolic blood pressure.

Subsequently, the proteins selected at the 5%FDR went through a multivariable stepwise forward selection model adjusted for the clinical variables. Since the circulating proteins were measured using NPX values on a $\log_2$ scale, the odds ratio (OR) for each protein estimates the increase/decrease in the odds of pre-diabetes/T2D associated with a doubling in the protein concentration. As exploratory analysis, we have assessed the additive value of the biomarkers on top of the clinical model for the 'prediction' of pre-diabetes and T2D onset (from V1 to V4). These analyses were performed using SAS version 9.1.

The genetic analyses were performed using R (version 3.4.1). GWAS were run using a linear mixed model, in order to take into account for the pedigree data, with age and sex used as covariates, the statistical significance level was fixed at 10^{-7} . For heritability estimation, matrix of pedigree or genetic relationship were used in a linear mixed model to estimate the variance captured by additive genetic effects via average information restricted maximum likelihood analysis. To take into account the genetic information at both V1 and V4, we have added two random effects in the model (one for individuals and another for visits) in order to assess the variance component resulting from the repeated measures. Both models were implemented in the R package 'gaston'. For the top-SNP association tests, age and sex were used as covariates and the statistical significance level was fixed at 0.05.

The complex network analysis was performed using the FHF-GKB (Fight Heart Failure - Graph Knowledge Box) resource, representing most available public knowledge about human protein-protein and protein-pathway relationships in a customized upgraded version of the EdgeBox provided by the EdgeLeap company (available from: <https://www.edgeleap.com>). FHF-GKB data is extracted from public data sources resulting in 20 431 protein nodes imported from Uniprot (13), including all proteins involved in this study and 2272 pathway nodes from Reactome (v69)1 and 47 162 disease nodes from Disease Ontology and Human Phenotype Ontology (12). Protein-protein relationships were retrieved from STRING (v11) (15) or Reactome or WikiPathways (16) or Menta (17) or BioGrid (18), protein-pathway relationships from Reactome and protein-disease relationships from DisGeNET (13). The FHF-GKB complex network was queried in order to explore pathways and proteins that connect the circulating proteins to disease through common mechanistic pathways. Queries were expressed according to patterns defining a path structure between two nodes, such as 'protein-disease' and

'pathway-protein-disease'. The resulting graph is displayed in figure illustrating the main connection between proteins, pathways, and disease (i.e. T2D (HP:0005978)).

Results

Patient characteristics

The patients' characteristics at V1 overall ($n=1506$) and by the pre-diabetes ($n=555$; 37%) and T2D ($n=73$; 4.8%) at V4 are depicted in the Table 1. Patients who developed pre-diabetes and/or T2D were older, had higher BMI, blood pressure, glucose levels, LDL cholesterol, and lower estimated glomerular filtration rate (eGFR).

Clinical factors associated with pre-diabetes and type 2 diabetes mellitus

The clinical factors independently associated with pre-diabetes onset were older age and higher LDL cholesterol and with T2D were older age, higher BMI, higher systolic blood pressure, and higher LDL cholesterol (tendency) (Table 2).

Circulating proteins associated with diabetes

The full list of proteins, their respective associations with pre-diabetes and T2D, and details on the Olink® technology are depicted in the Supplementary material (see section on [supplementary materials](#) given at the end of this article).

After multivariable selection adjusted for the clinical features, PAPP-A (pappalysin-1) was the only circulating protein that was associated with lower odds of both pre-diabetes and T2D onset in the V1 to V4 (prospective) analysis and also with the presence of pre-diabetes and T2D at V4 (cross-sectional analysis). CALC-A (calcitonin related polypeptide alpha) and VCAM-1 (vascular cell adhesion molecule 1) were also associated with lower odds of developing pre-diabetes (but not diabetes) in the V1 to V4 prospective analysis, but the associations were no longer present at the V4 cross-sectional analysis. On the other hand, FABP2 (fatty acid binding protein 2), LOX-1 (lysyl oxidase), and THPO (thrombopoietin) were associated with higher odds of developing pre-diabetes (but not diabetes) from V1 to V4, whereas FGF-21 (fibroblast growth factor 21), MCP-4 (mast-cell protease 4), SERPINA-12 (serpin family A member 12), and VSIG-2 (V-set and immunoglobulin domain containing 2) were associated with higher odds of developing diabetes from

V1 to V4. None of these associations remained significant at V4. The details of the associations are fully depicted in the Table 3. In the external replication analysis, patients with diabetes prospectively recruited in the HOMAGE clinical trial also presented lower levels of PAPP-A (Supplementary Table 1).

In the exploratory 'predictive' analysis, the clinical model for prediabetes presented an AUC of 0.74, and adding PAPP-A to this model improved its predictive capacity to 0.75 (P -value for AUC comparison=0.003). The clinical model performed even better for diabetes with an AUC of 0.90, and adding PAPP-A to this model did not improve its predictive capacity as the AUC remained at 0.90 (P -value for AUC comparison=0.32). However, adding FGF-21 (that had a strong association with new-onset diabetes) improved this model to an AUC of 0.92 (P -value for AUC comparison=0.020). Adding

more biomarkers lead only to marginal improvements in the models.

Genetic associations, heritability estimation, and complex network analyses

No signal was found for GWAS with diabetes, fasting glycemia or glycated hemoglobin. For circulating PAPP-A, the heritability was estimated at 44% for subjects with measurements at both visits (V1 and V4) (Supplementary Table 2). Only one SNP, rs634737, was associated with the protein PAPP-A both at V1 and V4 at the GWAS significance level (Table 4). Association tests between this SNP and study outcome are shown in the Table 5 and suggests that subjects with mutation at rs634737 are more susceptible to develop diabetes, have higher level of fasting glycemia and glycated hemoglobin.

Network analysis results are presented in the Fig. 2.

Table 2 Multinomial logistic regression: clinical predictors at visit 1 (V1) of pre-diabetes (Pre-DM) and diabetes (DM) at visit 4 (V4) (prospective analysis), and association of V4 clinical variables for Pre-DM and DM (cross-sectional analysis).

Risk factor/Status	OR (95%CI)	P-value
V1 clinical predictors at V4 (prospective)		
Age per 10 years		
Pre-DM	1.87 (1.67–2.10)	<0.0001
DM	2.07 (1.45–2.95)	<0.0001
BMI per 1 kg/m ²		
Pre-DM	1.03 (0.99–1.07)	0.12
DM	1.24 (1.15–1.33)	<0.0001
LDL $\geq$ 1.6 g/L		
Pre-DM	1.45 (1.09–1.92)	0.011
DM	1.69 (0.97–2.96)	0.065
SBP per 10 mmHg		
Pre-DM	0.99 (0.89–1.10)	0.84
DM	1.44 (1.15–1.80)	0.001
V4 clinical variables (cross-sectional)		
Age per 10 years		
Pre-DM	2.03 (1.82–2.25)	<0.0001
DM	2.62 (1.91–3.60)	<0.0001
HR $\geq$ 80 bpm		
Pre-DM	0.79 (0.42–1.47)	0.46
DM	3.35 (1.49–7.57)	0.004
BMI per 1 kg/m ²		
Pre-DM	1.07 (1.04–1.10)	<0.0001
DM	1.19 (1.13–1.25)	<0.0001
LDLc $\geq$ 1.6 g/L		
Pre-DM	1.30 (0.99–1.70)	0.057
DM	0.26 (0.11–0.63)	0.003
SBP per 10 mmHg		
Pre-DM	0.94 (0.86–1.02)	0.13
DM	1.19 (1.01–1.40)	0.036

BMI, body mass index; HR, heart rate; LDLc, low density lipoprotein cholesterol; OR, odds ratio; SBP, systolic blood pressure (office).

Discussion

Our study shows that the risk of developing pre-diabetes and T2D increases with age and with features of the metabolic syndrome. Furthermore, several circulating proteins were associated with pre-diabetes and T2D; particularly, PAPP-A was associated with the development of both pre-diabetes and T2D (i.e. in initially healthy young adults without diabetes, at V1 $\approx$ 19 years before V4) and remained associated with both pre-diabetes and T2D once the disease had been installed (at V4). In this familial cohort, the PAPP-A protein has an important heritability ($\approx$ 44%) and acts via proteins and a pathway linked to IGFs. These data were externally replicated in the HOMAGE trial cohort and provide novel insights on the pathophysiology of T2D, generating hypothesis for future research in the field.

Beyond the genetic susceptibility, that may be particularly important in specific ethnic groups (14, 15, 16), T2D is essentially caused by an interplay between environmental and behavioral factors that include sedentary lifestyle, nutritional habits rich in sugars, carbohydrates and processed foods, and obesity (17). The lack of robust genetic associations with diabetes in our study also supports this notion. Importantly, it has been suggested that the risk of dysglycemia-associated complications commences many years before the onset of clinical diabetes (18). Our findings also support that the metabolic alterations start many years before the onset of T2D and are also associated with pre-diabetes, moreover

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Table 3 Final protein selection at V1 for the prediction of pre-diabetes and diabetes at V4 (prospective analysis) and V4 final protein selection for the prediction of pre-diabetes and diabetes at V4 (cross-sectional analysis).

Variable	Status	Adjusted *OR (95% CI)	P-value
Visit 1 (V1) proteomic predictors of pre-diabetes and diabetes at V4 (prospective)			
PAPP-A	Pre-DM	0.71 (0.55–0.92)	0.008
	DM	0.54 (0.30–0.96)	0.036
CALCA	Pre-DM	0.79 (0.66–0.94)	0.007
	DM	1.20 (0.82–1.76)	0.36
VCAM1	Pre-DM	0.60 (0.42–0.87)	0.007
	DM	0.77 (0.35–1.70)	0.52
FABP2	Pre-DM	1.25 (1.05–1.50)	0.013
	DM	0.82 (0.53–1.27)	0.37
LOX-1	Pre-DM	1.49 (1.15–1.93)	0.002
	DM	1.06 (0.56–2.02)	0.86
THPO	Pre-DM	1.88 (1.16–3.04)	0.01
	DM	0.73 (0.24–2.21)	0.58
FGF-21	Pre-DM	1.07 (0.97–1.18)	0.19
	DM	1.83 (1.47–2.30)	<0.0001
MCP-4	Pre-DM	1.08 (0.84–1.39)	0.54
	DM	2.28 (1.31–3.99)	0.004
SERPINA12	Pre-DM	1.01 (0.92–1.12)	0.81
	DM	1.39 (1.09–1.76)	0.008
VSIG2	Pre-DM	1.16 (0.83–1.63)	0.38
	DM	2.68 (1.27–5.65)	0.009
Visit 4 (V4) proteomic associations with pre-diabetes and diabetes (cross-sectional)			
PAPP-A	Pre-DM	0.57 (0.44–0.74)	<0.0001
	DM	0.50 (0.25–0.99)	0.048
EPHB4	Pre-DM	0.39 (0.19–0.78)	0.008
	DM	0.15 (0.02–0.97)	0.046
CES2	Pre-DM	3.27 (1.78–6.00)	0.0001
	DM	3.12 (1.12–8.64)	0.029
CD5	Pre-DM	1.99 (1.26–3.14)	0.003
	DM	3.47 (1.19–10.1)	0.023
GDNF	Pre-DM	1.51 (1.11–2.05)	0.008
	DM	2.56 (1.51–4.35)	0.0005
REN	Pre-DM	1.21 (0.99–1.48)	0.057
	DM	1.70 (1.06–2.75)	0.028
SCGB3A2	Pre-DM	1.35 (1.12–1.62)	0.001
	DM	1.54 (1.1–2.178)	0.012
CD8A	Pre-DM	0.68 (0.55–0.85)	0.0008
	DM	0.65 (0.36–1.16)	0.15
CX3CL1	Pre-DM	0.41 (0.26–0.66)	0.0002
	DM	0.36 (0.09–1.35)	0.13
PRSS8	Pre-DM	2.12 (1.40–3.22)	0.0004
	DM	2.52 (0.75–8.44)	0.13
TRAIL	Pre-DM	2.19 (1.30–3.68)	0.003
	DM	2.74 (0.70–10.6)	0.14
ENTPD2	Pre-DM	1.20 (0.70–2.06)	0.5
	DM	0.09 (0.01–0.44)	0.003
KLK6	Pre-DM	0.65 (0.36–1.16)	0.15
	DM	0.02 (0.01–0.10)	<0.0001
MCP2	Pre-DM	1.19 (0.95–1.41)	0.12
	DM	0.40 (0.22–0.73)	0.003
AIFM1	Pre-DM	1.00 (0.87–1.15)	0.97
	DM	1.63 (1.14–2.32)	0.007
AOC3	Pre-DM	1.38 (0.96–1.96)	0.074
	DM	2.77 (1.08–7.07)	0.033
DPP6	Pre-DM	1.49 (0.95–2.32)	0.079
	DM	12.4 (3.67–42.3)	<0.0001

(Continued)

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Table 3 Continued.

Variable	Status	Adjusted *OR (95% CI)	P-value
GDF-15	Pre-DM	1.02 (0.69–1.51)	0.89
	DM	12.3 (5.46–27.8)	<0.0001
IL-1RT2	Pre-DM	1.04 (0.65–1.66)	0.86
	DM	6.13 (1.94–19.4)	0.002
MMP-10	Pre-DM	1.01 (0.79–1.26)	0.96
	DM	2.98 (1.65–5.39)	0.0003

Proteins ordered from top to bottom by their association with both pre-diabetes and diabetes (first), then pre-diabetes alone (second), and finally diabetes alone (third); following this order proteins that are associated with a lower risk of diabetes appear first.

*Adjusted for age, BMI, LDL cholesterol and systolic blood pressure in a stepwise forward model (see also the Table 2).

PAPP-A, pappalysin 1 (the only common protein between V1 and V4 and that is associated with lower risk of pre-diabetes and diabetes at both V1 and V4); EPHB4, EPH receptor B4; CES2, carboxylesterase 2; CD5, CD5 T-cell surface glycoprotein; GDNF, glial cell derived neurotrophic factor; REN, renin; SCGB3A2, secretoglobulin family 3A member 2; CD8A, T-cell surface glycoprotein CD8 alpha chain; CX3CL1, C-X3-C motif chemokine ligand 1; PRSS8, serine protease 8; TRAIL, TNF superfamily member 10; ENTPD2, ectonucleoside triphosphate diphosphohydrolase 2; KLK6, kallikrein related peptidase 6; MCP2, macrophage cationic peptide 2; AIFM1, apoptosis inducing factor mitochondria associated 1; AOC3, amine oxidase copper containing 3; DPP6, dipeptidyl peptidase like 6; GDF-15, growth differentiation factor 15; IL-1RT2, interleukin 1 receptor type 2; MMP-10, matrix metalloproteinase 10; CALCA, calcitonin related polypeptide alpha; VCAM1, vascular cell adhesion molecule 1; FABP2, fatty acid binding protein 2; LOX-1, lysyl oxidase; THPO, thrombopoietin; FGF-21, fibroblast growth factor 21; MCP-4, mast-cell protease 4; SERPINA12, serpin family A member 12; VSIG2, V-set and immunoglobulin domain containing 2.

the proteomic profiles of these patients are altered (17, 19, 20, 21). Landmark clinical trials have shown that lifestyle modification programs focused on weight loss and/or the use of metformin (although the lifestyle interventions aiming at significant weight loss were the most effective measure) can delay the onset of T2D (22, 23, 24). Unfortunately, no study has shown that diabetes can be delayed or prevented in a community setting (19). This should not demotivate us from continuing the efforts and possibly aiming the preventive strategies to individuals at higher risk for developing the disease may be more effective than an one-size-fits-all strategy (25). In this regard, people who developed pre-diabetes and T2D had lower levels of circulating PAPP-A (compared to those who did not developed pre-diabetes nor diabetes) and continued expressing lower levels of this protein after the disease was already installed. These findings were replicated in patients prospectively recruited for the HOMAGE trial, where those with diabetes also

expressed lower levels of PAPP-A. PAPP-A increases the IGFs bioavailability and stimulates IGF-mediated growth through cleavage of the insulin-like growth factor binding protein-4 (IGFBP-4) (26). The two central components of the IGF system, IGF-I and IGF-II, serve as ligands for the ubiquitously expressed IGF-I receptor, through which they facilitate cellular proliferation and differentiation as well as metabolic effects mimicking those conveyed by insulin (27). Our complex network analyses support that PAPP-A interacts directly with IGF I and II through a pathway linked to IGFs. High concentrations of PAPP-A were initially found in women during pregnancy, but later PAPP-A was found to be the primary IGFBP-4 protease in humans and many other species (28, 29, 30). Under normal circumstances, IGFBPs bind IGFs with high affinity, stabilize the IGFs and transport them to peripheral tissues, where proteases, such as PAPP-A, cleave IGFBPs into low binding-affinity fragments to allow release and action of IGFs. Thus, PAPP-A actions occur within the tissues, and

Table 4 GWAS associations with circulating levels of PAPP-A.

SNP id	A1	A2	freqA2	beta	s.d.	P-value*	chr
Visit 1 (V1)							
rs634737	A	G	0.732	0.147	0.024	9.02E-10	11
rs61806064	A	G	0.990	−0.588	0.102	8.37E-09	1
rs75242093	A	G	0.970	−0.342	0.060	1.53E-08	4
rs62412362	A	G	0.915	−0.199	0.037	5.19E-08	6
rs72630775	A	G	0.992	−0.630	0.116	6.12E-08	7
Visit 4 (V4)							
rs634737	A	G	0.734	0.160	0.022	9.14E-13	11
rs11606592	C	T	0.898	−0.204	0.032	2.03E-10	11
rs10400305	G	A	0.843	−0.149	0.027	2.18E-08	11

*Age and sex are used as covariates; statistical significance has been set at 10^{-7} .

Table 5 Association test between rs634737 and diabetes, pre-diabetes and associated continuous outcomes.

	Beta ^a ($\pm$ S.D.)	P value
Diabetes	0.056 (0.027)	0.04
Pre-diabetes	0.014 (0.019)	0.45
Fasting glycemia	0.015 (0.006)	0.02
Glycated hemoglobin	0.056 (0.022)	0.01

^aRisk per minor allele, adjustment for sex and age.

the direct liberation of IGF is a local event. PAPP-A could be a modulator of the local action of IGF-1, and increased PAPP-A levels may reflect a repair mechanism against vascular damage (31). Collectively, whether glucose, insulin or both that affect PAPP-A/IGFBP-4 levels and the clinical meaning of the circulating levels of PAPP-A remain speculative (32, 33). The association of PAPP-A with diabetes in clinical studies has also been inconsistent (33, 34, 35). It has been reported that genetic deletion of PAPP-A in mice confers resistance to the development of diabetic nephropathy and induces an overall reduction in age-related diseases (36, 37, 38). The human PAPP-A gene is located on chromosome 9 (39). In the present study, the SNP rs634737 was strongly associated with the expression of PAPP-A both at V1 and V4 and may provide valuable information for future studies on the role of PAPP-A. Moreover, this SNP was also associated with T2D, fasting glycemia, and glycated hemoglobin; thus, reinforcing the strength of our findings. Collectively, given that experimental and clinical data on PAPP-A

have been rather inconclusive so far, much remains to be elucidated about their roles in metabolic diseases and complications (26).

Other proteins were also associated with the onset and presence of pre-diabetes and T2D, and these may represent several pathways associated with obesity, glucose intolerance, inflammation, apoptosis, angiogenesis and oxidative stress (40, 41, 42, 43). Particularly, FGF-21 had a strong association with new-onset diabetes later in life. FGF-21 is an endocrine hormone that regulates energy homeostasis and insulin sensitivity (44). Patients with T2D may present higher circulating levels of FGF-21, which may be influenced by other metabolic factors such as obesity and cholesterol (45). In our study, adding FGF-21 to the clinical model for predicting diabetes improved this model which already had a very good performance with the clinical variables alone. However, a causal link between the levels of FGF-21 and T2D has not been established (46). The proteins (e.g. EPHB4, VCAM1, CES2) were only present in either one of the visits.

Limitations

Some limitations and caution in the interpretation of the results should be acknowledged in the present study. First, this is an observational study, therefore no causality can be established and these findings should be regarded as hypothesis generating. Second, PAPP-A and other circulating proteins were measured with the Olink® technology standardized log₂ NPX values (as described in the methods section), hence no direct conversion to the standard values is possible. Fourth, the SNP rs634737 is located in a non-coding region of the genome; therefore, no conclusion can be drawn about its mode of action and no replication could be performed and these results should be regarded with caution. Fifth, Mendelian randomization analyses could not be performed due to the our 'small' sample size for this type of analysis.

Conclusion

The risk of developing pre-diabetes and T2D increases with age and with features of the metabolic syndrome. Several circulating proteins were associated with pre-diabetes and T2D. Particularly, circulating PAPP-A, which has an important genetic component, was associated with both the development and presence of pre-diabetes and T2D. Our data provide novel insights on the pathophysiology

Figure 2

Pathway (in green) and proteins (grey circles) linking PAPP-A (red circle) to diabetes (blue square). A full colour version of this figure is available at <https://doi.org/10.1530/EJE-20-0246>.

of T2D and generate hypothesis for future research in the field, including the study of PAPP-A as a potential biotarget for the prevention and/or early treatment of diabetes.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-20-0246>.

Declaration of interest

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

Funding

The STANISLAS study is sponsored by the CHRU de NANCY. This work is supported by the French Ministry of Health 'Programme Hospitalier de Recherche Clinique inter regional 2013', by the Contrat de Plan Etat-Lorraine, and a public grant overseen by the French National Research Agency (ANR) as part of the second 'Investissements d'Avenir' program (reference: ANR-15-RHUS-0004). It is also supported by the European Fibro-Targets Project (grant agreement No. SP7#602904), European HOMAGE project (grant agreement N° Heart 'Omics' in Ageing, 7th Framework Program grant # 305507), the MEDIA project (Européen 'Cooperation' – Theme 'Health' / FP7-HEALTH-2010-single-stage (reference: 261409), FIGHT-HF (reference: ANR-15-RHU-0004), FOCUS-MR (reference: ANR-15-CE14-0032-01), ERA-CVD EXPERT (reference: ANR-16-ECVD-0002-02) and la Fondation de Recherche en Hypertension Artérielle.

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Received 18 March 2020

Revised version received 4 June 2020

Accepted 18 June 2020