

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

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29 Abstract

30 *Objective*: Determining the factors associated with new-onset pre-diabetes and type 2 diabetes

31 mellitus (T2D) is important for improving the current prevention strategies and for a better

32 understanding of the disease.

33 Design: To study the factors (clinical, circulating protein and genetic) associated with new onset pre-

34 diabetes and T2D in an initially healthy (without diabetes) populational familial cohort with a long

35 follow-up (STANISLAS cohort).

36 *Methods*: 1506 participants that attended both the visit-1 and visit-4, separated by ≈ 20 years. Over 400

37 proteins, GWAS and genetic associations were studied using models adjusted for potential

38 confounders. Both prospective (V1 to V4) and cross-sectional (V4) analyses were performed.

39 *Results*: People who developed pre-diabetes (n =555) and/or T2D (n =73) were older, had higher

40 BMI, blood pressure, glucose, LDL cholesterol, and lower eGFR. After multivariable selection,

41 PAPP-A (pappalysin-1) was the only circulating protein associated with the onset of both pre-diabetes

42 and T2D with associations persisting at visit-4 (i.e., \approx 20 years later). FGF-21 (fibroblast growth factor

43 21) was a strong prognosticator for incident T2D in the longitudinal, but not in the cross-sectional

44 analysis. The heritability of the circulating PAPP-A was estimated at 44%. In GWAS analysis the

45 SNP rs634737 was associated with PAPP-A both at V1 and V4. External replication also showed

46 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.

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51 *Key-words*: diabetes mellitus; pre-diabetes; cohort study; pappalysin-1; genetic variants.

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58 Introduction

The number of people with type 2 diabetes mellitus (T2D) more than doubled in the past 20 years in 59 60 both the developed and developing countries¹. The latest prediction from the International Diabetes 61 Federation is that over 380 million people are living with diabetes nowadays, and that by 2035 this number may rise up to 600 million². Type 2 diabetes was traditionally considered as a disorder of 62 middle-aged and elderly people; however, the more recent "type 2 diabetes epidemics" is particularly 63 worrisome among adolescents and young adults, leading to tremendous costs, impairment in the 64 quality of life and reduced life expectancy, mainly due to associated cardiovascular and renal 65 complications^{1, 3, 4}. 66

Type 2 diabetes results from a complex interaction between genetic/epigenetic systems and
multiple behavioural plus environmental factors¹. Despite the challenges linked to the geneenvironment 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}.

72 The STANISLAS (Suivi Temporaire Annuel Non-Invasif de la Santé des Lorrains Assurés 73 Sociaux) cohort is a single-centre familial prospective cohort comprising 1006 initially healthy 74 (including absence of diabetes) families (4295 participants) from the Nancy region of France, who 75 were recruited from 1993 to 1995 to the visit 1 (V1). Participants were then followed each 5 to 10 76 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 assessment⁷. Beyond the clinical, imaging 77 78 and routine laboratorial measurements, circulating levels of multiple proteins were also assessed at V1 79 and V4. Additionally, a genome wide association study (GWAS) was performed for the V4 80 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 81 82 family structure also allows the study of heritability⁸.

83 In the present analysis, we considered patients without diabetes at V1 (patients entering the
84 familial STANISLAS study did not have chronic conditions) with the main aims of studying the

85 factors associated with new onset pre-diabetes and T2D at V4, considering the clinical features,

86 proteomic signatures and genetic variants that might help explaining the main proteomic findings.

87

88 Methods

89 Study population

A detailed description of the STANISLAS cohort has been previously published⁷. The STANISLAS 90 cohort was established with the primary objective of investigating gene-gene and gene-environment 91 92 interactions in the field of cardiovascular diseases. In order to assess the effect of genetics on the 93 variability of intermediate phenotypes on the transition toward pathology, the families were deemed 94 healthy and free of declared acute and/or chronic illness at V1. The implementation of the fourth visit 95 (V4) enabled a follow-up of 18 to 23 years (mean of 18.5 years). The collected information was 96 enriched with new circulating proteins and detailed clinical phenotyping. The study protocols for all 97 examinations were reviewed and approved by the Local Ethics Committee of CPP-Est 3, France. All 98 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 **Figure 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.

105 Study design

106 All participants were observed at the Centre d'Investigation Clinique Plurithématique Pierre Drouin at

107 Nancy Hospital Center (CIC-P de Nancy) in the morning after a 12- to 14-hour fast. Blood samples

- 108 were drawn, medical history, medications, anthropometric parameters, blood pressure, carotid-
- 109 femoral pulse-wave velocity (PWV), and carotid intima-media thickness (cIMT) were recorded.

110 Pre-diabetes and diabetes definition

111 Pre-diabetes was defined as a fasting plasma glucose (FPG) between 100mg/dL and 125mg/dL or a

112 glycated hemoglobin between 5.7% and 6.4%. Diabetes was defined as a FPG ≥ 126 mg/dL or a

113 glycated hemoglobin $\geq 6.5\%$ or a random plasma glucose $\geq 200 \text{ mg/dL}$ with symptoms of

114 hypoglycemia or previous diagnosis of diabetes or anti-glycemic drug therapy⁹.

115 Circulating proteins and gene-candidate analysis

116 All samples were collected at the CIC-P de Nancy with minimally traumatic venipuncture.

117 Standardized sample handling procedures enabled the collection of serum and plasma (EDTA,

118 heparin) as well as buffy coat fraction. Blood DNA of all the participants to the Stanislas V4 was

119 extracted using Gentra Puregene Blood Kit (Qiagen, Hilden Germany) and stored at -20°C.

120 Genotyping was conducted at CNRGH (Evry, France) using two chips: the Illumina Global Screening

121 Array (GSA) which is composed of 687 572 intronic and exonic markers and the Illumina Exome

122 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

124 plasma and serum).

125 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 126 inflammatory (INF) panels, using a proximity extension assay (PEA) technology¹⁰, where 92 127 128 oligonucleotide-labelled antibody probe pairs per panel (total of 92 x 5; n =460 proteins) are allowed 129 to bind to their respective targets in the sample in 96-well plate format. When binding to their correct 130 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 131 platform provides log₂-normalized protein expression (NPX) data. 132

The strategy for the genetic analyses of the present study was the following. Firstly, we conducted GWAS on diabetes occurrence, fasting glycemia and glycated haemoglobin. 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 haemoglobin 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
elsewhere¹¹.

145 Statistical, genetic and bioinformatical considerations

For the baseline clinical characteristics, continuous variables are expressed as means and respective
standard deviation (SD) and compared using ANOVA or Kruskal-Wallis test. Categorical variables
are presented as frequencies and percentages and compared using Chi-2 test.

149 The main aim of this study was to test the association of multiple circulating proteins with 150 incident pre-diabetes and T2D. Multinomial logistic regression models were performed (using no pre-151 diabetes/T2D as the referent category). Firstly, a stepwise backward model including all the clinical 152 variables with a p<0.1 from Table 1 was performed, to select the clinical features with stronger 153 association with pre-diabetes and T2D. Due to collinearity between body mass index and waist 154 circumference (correlation >0.9 in both visits) we included only body mass index in the models 155 (including waist circumference provided the same results). The clinical features retained in the final 156 model were age, body mass index, LDL cholesterol and systolic blood pressure. These variables were 157 used for adjustment in all the subsequent models. Secondly, the potential association of the multiple circulating proteins with pre-diabetes and T2D was tested 1-by-1 on top of the clinical model (i.e. 158 adjusted for the clinical features) built in the previous step. The statistical significance level was fixed 159 160 at a FDRq <0.05, after applying a Benjamini-Hochberg correction for multiple testing (i.e. false 161 discovery rate [FDR] set at 5%). Subsequently, the proteins selected at the 5% FDR went through a multivariable stepwise forward selection model adjusted for the clinical variables. Since the 162 circulating proteins were measured using NPX values on a log₂ scale, the odds ratio (OR) for each 163 protein estimates the increase/decrease in the odds of pre-diabetes/T2D associated with a doubling in 164 the protein concentration. As exploratory analysis, we have assessed the additive value of the 165 biomarkers on top of the clinical model for the "prediction" of pre-diabetes and T2D onset (from V1 166 167 to V4). These analyses were performed using SAS version 9.1.

168

The genetic analyses were performed using R (version 3.4.1). GWAS were run using a linear

169 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 170 genetic relationship were used in a linear mixed model to estimate the variance captured by additive 171 genetic effects via average information restricted maximum likelihood analysis. To take into account 172 173 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 174 repeated measures. Both models were implemented in the R package "gaston". For the top-SNP 175 176 association tests, age and sex were used as covariates and the statistical significance level was fixed at 0.05. 177

The complex network analysis was performed using the FHF-GKB (Fight Heart Failure -178 179 Graph Knowledge Box) resource, representing most available public knowledge about human protein-180 protein and protein-pathway relationships is a customized upgraded version of the EdgeBox provided by the EdgeLeap company (available from: https://www.edgeleap.com). FHF-GKB data is extracted 181 from public data sources resulting in 20,431 protein nodes imported from Uniprot¹³, including all 182 proteins involved in this study and 2,272 pathway nodes from Reactome (v69)1 and 47,162 disease 183 nodes from Disease Ontology and Human Phenotype Ontology¹². Protein - protein relationships were 184 retrieved from STRING (v11)¹⁵ or Reactome or WikiPathways¹⁶ or Mentha¹⁷ or BioGrid¹⁸, protein – 185 pathway relationships from Reactome and protein – disease relationships from DisGeNET¹³. The 186 187 FHF-GKB complex network was queried in order to explore pathways and proteins that connect the 188 circulating proteins to disease through common mechanistic pathways. Queries were expressed 189 according to patterns defining a path structure between two nodes, such as "protein-disease" and 190 "pathway-protein-disease". The resulting graph is displayed in figure illustrating the main connection between proteins, pathways, and disease (i.e. T2D [HP:0005978]). 191

192

193 **Results**

194 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

197 were older, had higher body mass index (BMI), blood pressure, glucose levels, LDL cholesterol, and

198 lower estimated glomerular filtration rate (eGFR).

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

200 The clinical factors independently associated with pre-diabetes onset were older age and higher LDL

201 cholesterol, and with T2D were older age, higher BMI, higher systolic blood pressure, and higher

202 LDL cholesterol (tendency). **Table 2**.

203 Circulating proteins associated with diabetes

204 The full list of proteins, their respective associations with pre-diabetes and T2D, and details on the

205 Olink® technology are depicted in the **Supplemental Material**.

206 After multivariable selection adjusted for the clinical features, PAPP-A (pappalysin-1) was 207 the only circulating protein that was associated with lower odds of both pre-diabetes and T2D onset in 208 the V1 to V4 (prospective) analysis, and also with the presence of pre-diabetes and T2D at V4 (cross-209 sectional analysis). CALC-A (calcitonin related polypeptide alpha) and VCAM-1 (vascular cell 210 adhesion molecule 1) were also associated with lower odds of developing pre-diabetes (but not 211 diabetes) in the V1 to V4 prospective analysis, but the associations were no longer present at the V4 212 cross-sectional analysis. On the other hand, FABP2 (fatty acid binding protein 2), LOX-1 (lysyl 213 oxidase), and THPO (thrombopoietin) were associated with higher odds of developing pre-diabetes 214 (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 215 216 domain containing 2) were associated with higher odds of developing diabetes from V1 to V4. None 217 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 218 HOMAGE clinical trial also presented lower levels of PAPP-A. Supplemental Table 1. 219 In the exploratory "predictive" analysis, the clinical model for prediabetes presented an AUC 220 of 0.74, adding PAPP-A to this model improved its predictive capacity to 0.75 (p-value for AUC 221

comparison =0.003). The clinical model performed even better for diabetes with an AUC of 0.90,

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.

227 Genetic associations, heritability estimation, and complex network analyses

- 228 No signal was found for GWAS with diabetes, fasting glycemia or glycated haemoglobin. For
- circulating PAPP-A, the heritability was estimated at 44% for subjects with measurements at both
- visits (V1 & V4) (Supplemental Table 2). Only one SNP, rs634737, was associated with the protein
- 231 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.
- 235 Network analysis results are presented in the **Figure 2**.
- 236

237 Discussion

238 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 239 240 pre-diabetes and T2D; particularly PAPP-A was associated with the development of both pre-diabetes 241 and T2D (i.e. in initially healthy young adults without diabetes, at V1 \approx 19 years before V4), and 242 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 243 244 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 245 the field. 246

Beyond the genetic susceptibility, that may be particularly important in specific ethnic groups¹⁴⁻¹⁶, T2D is essentially caused by an interplay between environmental and behavioural factors, that include sedentary lifestyle, nutritional habits rich in sugars, carbohydrates and processed foods, and obesity¹⁷. 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 diabetes¹⁸. Our findings also support that the

253 metabolic alterations start many years before the onset of T2D and are also associated with prediabetes, moreover the proteomic profiles of these patients are altered^{17, 19-21}. Landmark clinical trials 254 have shown that lifestyle modification programs focused on weight loss and/or the use of metformin 255 256 (although the lifestyle interventions aiming at significant weight loss were the most effective measure) can delay the onset of $T2D^{22-24}$. Unfortunately, no study has shown that diabetes can be delayed or 257 prevented in a community setting¹⁹. This should not demotivate us from continuing the efforts, and 258 possibly aiming the preventive strategies to individuals at higher risk for developing the disease may 259 be more effective than an one-size-fits-all strategy²⁵. In this regard, people who developed pre-260 diabetes and T2D had lower levels of circulating PAPP-A (compared to those who did not developed 261 pre-diabetes nor diabetes) and continued expressing lower levels of this protein after the disease was 262 263 already installed. These findings were replicated in patients prospectively recruited for the HOMAGE 264 trial, were 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 265 binding protein-4 (IGFBP-4)²⁶. The two central components of the IGF system, IGF-I and IGF-II, 266 serve as ligands for the ubiquitously expressed IGF-I receptor, through which they facilitate cellular 267 proliferation and differentiation as well as metabolic effects mimicking those conveyed by insulin²⁷. 268 269 Our complex network analyses support that PAPP-A interacts directly with IGF I and II through a 270 pathway linked to IGFs. High concentrations of PAPP-A were initially found in women during pregnancy, but latter PAPP-A was found to be the primary IGFBP-4 protease in humans and many 271 other species²⁸⁻³⁰. Under normal circumstances, IGFBPs bind IGFs with high affinity, stabilize the 272 273 IGFs and transport them to peripheral tissues, where proteases, such as PAPP-A, cleave IGFBPs into 274 low binding-affinity fragments to allow release and action of IGFs. Thus, PAPP-A actions occur within the tissues, and the direct liberation of IGF is a local event. PAPP-A could be a modulator of 275 276 the local action of IGF-1, and increased PAPP-A levels may reflect a repair mechanism against vascular damage³¹. Collectively, whether is glucose, insulin or both that affect PAPP-A/IGFBP-4 277 levels, and the clinical meaning of the circulating levels of PAPP-A remain speculative^{32, 33}. The 278 association of PAPP-A with diabetes in clinical studies has also been inconsistent³³⁻³⁵. It has been 279 280 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 diseases³⁶⁻³⁸. The human PAPP-A gene is
located on chromosome 9³⁹. 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 complications²⁶.

Other proteins were also associated with the onset and presence of pre-diabetes and T2D, 288 these may represent several pathways associated with obesity, glucose intolerance, inflammation, 289 apoptosis, angiogenesis and oxidative stress, for example⁴⁰⁻⁴³. Particularly, FGF-21 had a strong 290 association with new-onset diabetes later in life. FGF-21 is an endocrine hormone that regulates 291 energy homeostasis and insulin sensitivity⁴⁴. Patients with T2D may present higher circulating levels 292 of FGF-21, which may be influence by other metabolic factors such as obesity and cholesterol⁴⁵. In 293 our study, adding FGF-21 to the clinical model for predicting diabetes, improved this model which 294 295 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 established⁴⁶. The proteins (e.g., EPHB4, 296 297 VCAM1, CES2) were only present in either one of the visits.

298 Limitations

299 Some limitations and caution in the interpretation of the results should be acknowledged in the present 300 study. First, this is an observational study, therefore no causality can be established and these findings 301 should be regarded as hypothesis generating. Second, PAPP-A and other circulating proteins were 302 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 303 located in a non-coding region of the genome; therefore, no conclusion can be drawn about its mode 304 of action and no replication could be performed and these results should be regarded with caution. 305 Fifth, Mendelian randomization analyses could not be performed due to the our "small" sample size 306 307 for this type of analysis.

309 Conclusion

- 310 The risk of developing pre-diabetes and T2D increases with age, and with features of the metabolic
- 311 syndrome. Several circulating proteins were associated with pre-diabetes and T2D. Particularly
- 312 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 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.
- 316
- 317 Disclosures
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- 319
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- 322

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Figure 1. Study flow chart

Figure 2. Pathway (in green) and proteins (grey circles) linking PAPP-A (red circle) to diabetes (blue square).

Type II dia<mark>bet</mark>es mellitus Insulin-like growthfactor Insulin-likegrowth factor I Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs) Pappalysin-1