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

3

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28

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., ≈ 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.

47 *Conclusions:* The risk of developing pre-diabetes and T2D increases with age, and with features of the
48 metabolic syndrome. Circulating PAPP-A, which has an important genetic component, was associated
49 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**

59 The number of people with type 2 diabetes mellitus (T2D) more than doubled in the past 20 years in
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
62 number may rise up to 600 million². Type 2 diabetes was traditionally considered as a disorder of
63 middle-aged and elderly people; however, the more recent “type 2 diabetes epidemics” is particularly
64 worrisome among adolescents and young adults, leading to tremendous costs, impairment in the
65 quality of life and reduced life expectancy, mainly due to associated cardiovascular and renal
66 complications^{1, 3, 4}.

67 Type 2 diabetes results from a complex interaction between genetic/epigenetic systems and
68 multiple behavioural plus environmental factors¹. Despite the challenges linked to the gene-
69 environment interplay, determining the factors associated with new-onset pre-diabetes and T2D is
70 important not only for improving the current prevention strategies and treatments, but also for a better
71 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
77 2016, which comprised a detailed clinical and laboratorial assessment⁷. Beyond the clinical, imaging
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
81 signatures associated with the new-onset of pre-diabetes and/or T2D almost 20 years in advance; the
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**

90 A detailed description of the STANISLAS cohort has been previously published⁷. The STANISLAS
91 cohort was established with the primary objective of investigating gene-gene and gene-environment
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.

99 In the present analysis we included 1506 participants that attended both the V1 and V4, and in
100 whom we had information on fasting glucose, random glucose, glycated hemoglobin, treatments
101 and/or previous diagnosis that allow the pre-diabetes and T2D classification. The study flow-chart is
102 shown in the **Figure 1**. The main objective is to assess the circulating proteins that are associated with
103 the development of diabetes in a prospective analysis (V1 to V4) and to ascertain if the associations
104 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 \geq 126mg/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
123 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
126 Proseek® Multiplex cardiovascular (CVD) II, III, cardiometabolic (CM), organ-damage (OD) and
127 inflammatory (INF) panels, using a proximity extension assay (PEA) technology¹⁰, where 92
128 oligonucleotide-labelled antibody probe pairs per panel (total of 92×5 ; $n = 460$ 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
131 amplicons are subsequently quantified using a Fluidigm BioMark™ HD real-time PCR platform. The
132 platform provides \log_2 -normalized protein expression (NPX) data.

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

139 Our findings were replicated using the baseline data of the HOMAGE (Bioprofiling Response
140 to Mineralocorticoid Receptor Antagonists for the Prevention of Heart Failure; ClinicalTrials.gov

141 Identifier: NCT02556450) trial, a prospective trial that included 527 people at risk for developing
142 heart failure randomized to either spironolactone or standard of care for up to 9 months; the
143 description of this study and baseline characteristics of the participants have been published
144 elsewhere¹¹.

145 **Statistical, genetic and bioinformatical considerations**

146 For the baseline clinical characteristics, continuous variables are expressed as means and respective
147 standard deviation (SD) and compared using ANOVA or Kruskal-Wallis test. Categorical variables
148 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
158 circulating proteins with pre-diabetes and T2D was tested 1-by-1 on top of the clinical model (i.e.
159 adjusted for the clinical features) built in the previous step. The statistical significance level was fixed
160 at a $FDR_q < 0.05$, 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
162 multivariable stepwise forward selection model adjusted for the clinical variables. Since the
163 circulating proteins were measured using NPX values on a \log_2 scale, the odds ratio (OR) for each
164 protein estimates the increase/decrease in the odds of pre-diabetes/T2D associated with a doubling in
165 the protein concentration. As exploratory analysis, we have assessed the additive value of the
166 biomarkers on top of the clinical model for the “prediction” of pre-diabetes and T2D onset (from V1
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,
170 the statistical significance level was fixed at 10^{-7} . For heritability estimation, matrix of pedigree or
171 genetic relationship were used in a linear mixed model to estimate the variance captured by additive
172 genetic effects via average information restricted maximum likelihood analysis. To take into account
173 the genetic information at both V1 and V4, we have added two random effects in the model (one for
174 individuals and another for visits) in order to assess the variance component resulting from the
175 repeated measures. Both models were implemented in the R package “gaston”. For the top-SNP
176 association tests, age and sex were used as covariates and the statistical significance level was fixed at
177 0.05.

178 The complex network analysis was performed using the FHF-GKB (Fight Heart Failure -
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
181 by the EdgeLeap company (available from: <https://www.edgeleap.com>). FHF-GKB data is extracted
182 from public data sources resulting in 20,431 protein nodes imported from Uniprot¹³, including all
183 proteins involved in this study and 2,272 pathway nodes from Reactome (v69)¹ and 47,162 disease
184 nodes from Disease Ontology and Human Phenotype Ontology¹². Protein - protein relationships were
185 retrieved from STRING (v11)¹⁵ or Reactome or WikiPathways¹⁶ or Mentha¹⁷ or BioGrid¹⁸, protein –
186 pathway relationships from Reactome and protein – disease relationships from DisGeNET¹³. The
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
191 between proteins, pathways, and disease (i.e. T2D [HP:0005978]).

192

193 **Results**

194 **Patient characteristics**

195 The patients` characteristics at V1 overall (n =1506) and by the pre-diabetes (n =555; 37%) and T2D
196 (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
215 protease 4), SERPINA-12 (serpin family A member 12), and VSIG-2 (V-set and immunoglobulin
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
218 the **Table 3.** In the external replication analysis, patients with diabetes prospectively recruited in the
219 HOMAGE clinical trial also presented lower levels of PAPP-A. **Supplemental Table 1.**

220 In the exploratory “predictive” analysis, the clinical model for prediabetes presented an AUC
221 of 0.74, adding PAPP-A to this model improved its predictive capacity to 0.75 (p-value for AUC
222 comparison =0.003). The clinical model performed even better for diabetes with an AUC of 0.90,
223 adding PAPP-A to this model did not improve its predictive capacity as the AUC remained at 0.90 (p-
224 value for AUC comparison =0.32). However, adding FGF-21 (that had a strong association with new-

225 onset diabetes) improved this model to an AUC of 0.92 (p-value for AUC comparison =0.020).

226 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
229 circulating PAPP-A, the heritability was estimated at 44% for subjects with measurements at both
230 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
232 SNP and study outcome are shown in the **Table 5**, and suggests that subjects with mutation at
233 rs634737 are more susceptible to develop diabetes, have higher level of fasting glycemia and glycated
234 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
239 features of the metabolic syndrome. Furthermore, several circulating proteins were associated with
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
243 this familial cohort, the PAPP-A protein has an important heritability (\approx 44%) and acts via proteins
244 and a pathway linked to IGFs. These data were externally replicated in the HOMAGE trial cohort, and
245 provide novel insights on the pathophysiology of T2D, generating hypothesis for future research in
246 the field.

247 Beyond the genetic susceptibility, that may be particularly important in specific ethnic
248 groups¹⁴⁻¹⁶, T2D is essentially caused by an interplay between environmental and behavioural factors,
249 that include sedentary lifestyle, nutritional habits rich in sugars, carbohydrates and processed foods,
250 and obesity¹⁷. The lack of robust genetic associations with diabetes in our study also supports this
251 notion. Importantly, it has been suggested that the risk of dysglycemia-associated complications
252 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 pre-
254 diabetes, moreover the proteomic profiles of these patients are altered^{17, 19-21}. Landmark clinical trials
255 have shown that lifestyle modification programs focused on weight loss and/or the use of metformin
256 (although the lifestyle interventions aiming at significant weight loss were the most effective measure)
257 can delay the onset of T2D²²⁻²⁴. Unfortunately, no study has shown that diabetes can be delayed or
258 prevented in a community setting¹⁹. This should not demotivate us from continuing the efforts, and
259 possibly aiming the preventive strategies to individuals at higher risk for developing the disease may
260 be more effective than an one-size-fits-all strategy²⁵. In this regard, people who developed pre-
261 diabetes and T2D had lower levels of circulating PAPP-A (compared to those who did not developed
262 pre-diabetes nor diabetes) and continued expressing lower levels of this protein after the disease was
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
265 bioavailability and stimulates IGF-mediated growth through cleavage of the insulin-like growth factor
266 binding protein-4 (IGFBP-4)²⁶. The two central components of the IGF system, IGF-I and IGF-II,
267 serve as ligands for the ubiquitously expressed IGF-I receptor, through which they facilitate cellular
268 proliferation and differentiation as well as metabolic effects mimicking those conveyed by insulin²⁷.
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
271 pregnancy, but latter PAPP-A was found to be the primary IGFBP-4 protease in humans and many
272 other species²⁸⁻³⁰. Under normal circumstances, IGFBPs bind IGFs with high affinity, stabilize the
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
275 within the tissues, and the direct liberation of IGF is a local event. PAPP-A could be a modulator of
276 the local action of IGF-1, and increased PAPP-A levels may reflect a repair mechanism against
277 vascular damage³¹. Collectively, whether is glucose, insulin or both that affect PAPP-A/IGFBP-4
278 levels, and the clinical meaning of the circulating levels of PAPP-A remain speculative^{32, 33}. The
279 association of PAPP-A with diabetes in clinical studies has also been inconsistent³³⁻³⁵. It has been
280 reported that genetic deletion of PAPP-A in mice confers resistance to the development of diabetic

281 nephropathy and induces an overall reduction in age-related diseases³⁶⁻³⁸. The human PAPP-A gene is
282 located on chromosome 9³⁹. In the present study, the SNP rs634737 was strongly associated with the
283 expression of PAPP-A both at V1 and V4 and may provide valuable information for future studies on
284 the role of PAPP-A. Moreover, this SNP was also associated with T2D, fasting glycemia, and
285 glycosylated hemoglobin; thus, reinforcing the strength of our findings. Collectively, given that
286 experimental and clinical data on PAPP-A have been rather inconclusive so far, much remains to be
287 elucidated about their roles in metabolic diseases and complications²⁶.

288 Other proteins were also associated with the onset and presence of pre-diabetes and T2D,
289 these may represent several pathways associated with obesity, glucose intolerance, inflammation,
290 apoptosis, angiogenesis and oxidative stress, for example⁴⁰⁻⁴³. Particularly, FGF-21 had a strong
291 association with new-onset diabetes later in life. FGF-21 is an endocrine hormone that regulates
292 energy homeostasis and insulin sensitivity⁴⁴. Patients with T2D may present higher circulating levels
293 of FGF-21, which may be influenced by other metabolic factors such as obesity and cholesterol⁴⁵. In
294 our study, adding FGF-21 to the clinical model for predicting diabetes, improved this model which
295 already had a very good performance with the clinical variables alone. However, a causal link
296 between the levels of FGF-21 and T2D has not been established⁴⁶. The proteins (e.g., EPHB4,
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
303 section), hence no direct conversion to the standard values is possible. Fourth, the SNP rs634737 is
304 located in a non-coding region of the genome; therefore, no conclusion can be drawn about its mode
305 of action and no replication could be performed and these results should be regarded with caution.
306 Fifth, Mendelian randomization analyses could not be performed due to our “small” sample size
307 for this type of analysis.

308

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
313 development and presence of pre-diabetes and T2D. Our data provide novel insights on the
314 pathophysiology of T2D and generate hypothesis for future research in the field, including the study
315 of PAPP-A as a potential biotarget for the prevention and/or early treatment of diabetes.

316

317 **Disclosures**

318 None.

319

320 **Acknowledgments**

321 Please see the supplement.

322

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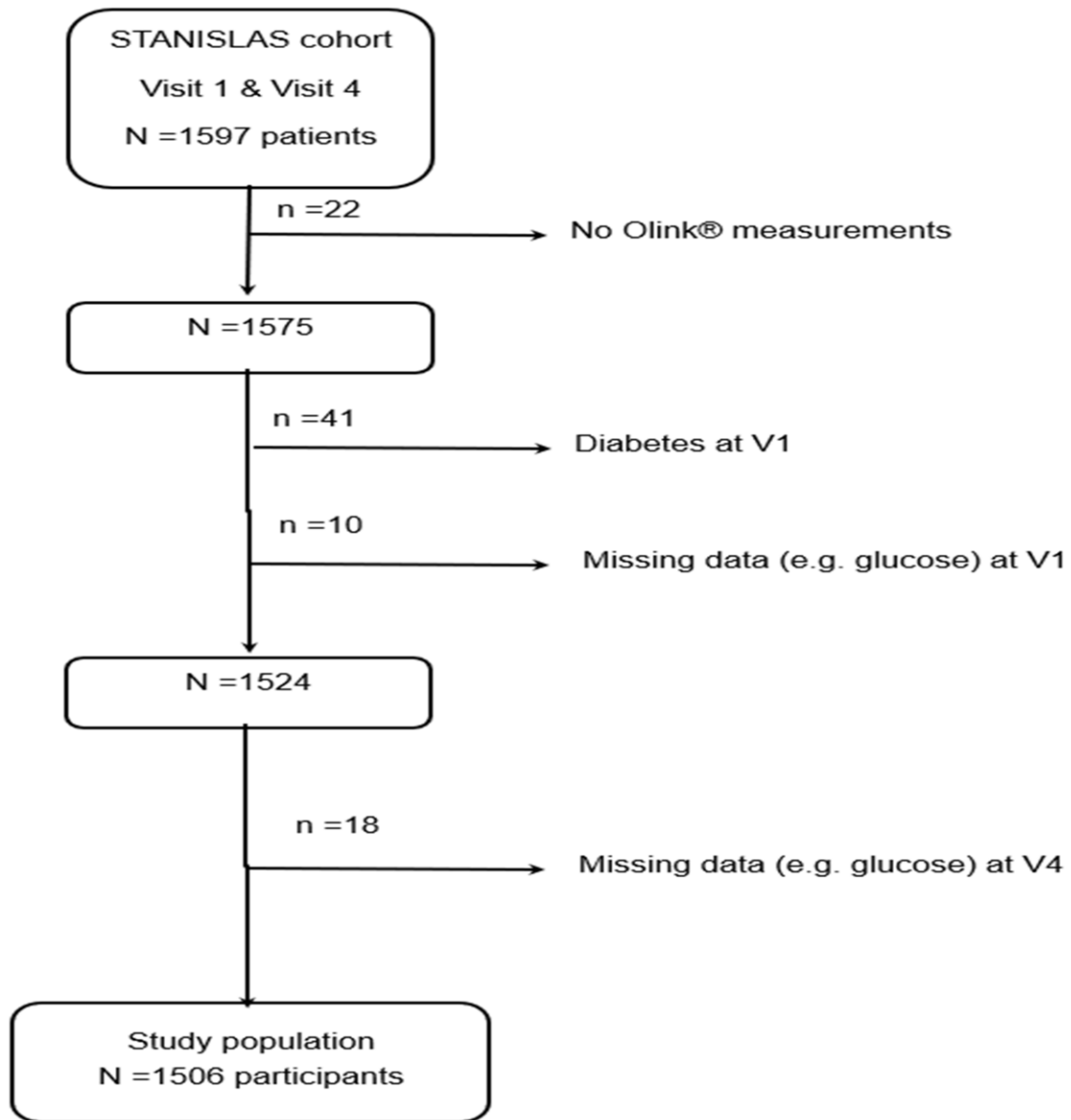


Figure 1. Study flow chart

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

