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ORIGINAL ARTICLE

Pancreatic stone protein/regenerating protein (PSP/reg): a novel secreted protein up-regulated in type 2 diabetes mellitus

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Abstract Type 2 diabetes mellitus (T2DM) has insulin resistance (IR) or reduced β-cell mass, partially due to an increased β-cell apoptosis rate. Pancreatic stone protein/regenerating protein (PSP/reg) is a secretory protein produced in the pancreas and up-regulated dramatically during pancreatic disease. Recent studies revealed that β-cells undergoing apoptosis induce PSP/reg expression in surviving neighboring cells. Further experiments demonstrated that PSP/reg was elevated during disease progression in type 1 diabetes mellitus (T1DM). However, the association between PSP/reg and T2DM patients is unknown. The aim of this pilot study was to investigate PSP/reg in different clinical stages of T2DM and evaluate its correlation with chronic complications of diabetes. A total of 1,121 participants (479 males, 642 females; age range 23–80 years) were enrolled in this study. PSP/reg serum values were measured by a newly developed enzyme-linked immunosorbent assay (ELISA). We analyzed its correlation with clinical and biochemical parameters in subjects with T2DM at different clinical phases. Statistical analyses were conducted using SPSS 17.0 software. Correlations of PSP/reg and clinical parameters were performed using Spearman's rank correlation coefficient. Differences between groups were determined by Nemenyi test. PSP/reg was elevated in high-risk and impaired glucose regulation (IGR) patients ($p < 0.05$). PSP/reg was significantly up-regulated in newly diagnosed T2DM patients and long-term diabetes patients with complications ($p < 0.001$). PSP/reg levels correlated with the duration of diabetes ($p < 0.001$). The area under the curve (AUC) for presence of diabetes-onset and its chronic complications was 0.640 and 0.754, respectively. PSP/reg is significantly up-regulated in T2DM patients, and PSP/reg levels are related to the duration of diabetes. Therefore, PSP/reg might be useful as a predictor of T2DM and disease progression.

Keywords Pancreatic stone protein · Type 2 diabetes mellitus · β-cell · Complications · Serum parameters

Introduction

Type 2 diabetes mellitus (T2DM) is an epidemic global health hazard with estimated 113.9 million adults in China and rapidly-rising incidence rates [1, 2]. This detrimental disease affects health, life expectancy, quality of life, and is a heavy burden to the health care systems for its continuing medical care requirements and self-management education [3]. It is also a leading cause of morbidity and disability, which results in macrovascular and microvascular complications [4]. Frequently, T2DM is only diagnosed when complications occur. The latest study of prediabetes among Chinese adults was more than 50 % [2]. The increasing prevalence, serious complications, and high-undiagnosed

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rate make T2DM an important field of research. Two major defects play a crucial role in disease progression of T2DM: insulin resistance (IR) and β-cell failure or eventual loss of β-cell mass [5]. Approximately, 80 % of the IR patients are able to compensate, at least for a period of time, due to the β-cell capability of compensating with hyperinsulinemia to overcome IR [6].

Pancreatic stone protein/regenerating protein (PSP/reg) was originally identified in pancreatic stones, as a 16 kDa polypeptide belonging to the superfamily of calcium-dependent lectin gene [7]. PSP/reg and its related homolog pancreatitis associated protein/regIII share highly conserved amino acid sequences. They contain sensitive N-terminal trypsin cleavage sites, as well as conserved functional responses in conditions of pancreatic stress. Activated PSP/reg polymerizes into highly organized fibrillar structures with helical configurations [8]. It has been studied mainly in the pancreas and is prominently up-regulated in acute or chronic pancreatitis [9]. In addition, the same protein has been associated with islet regeneration. PSP/reg appeared to boost β-cell growth and regeneration by induction of cellular proliferation [7, 10]. PSP/reg was also found to be altered in the condition of sepsis [11], ventilator associated pneumonia (VAP) [12], chronic obstructive pulmonary disease (COPD) exacerbation [13], and posttraumatic complications [14] which indicated that PSP/reg might serve as a reflection of infectious conditions and organ failure. Recently, PSP/reg has been suggested to be associated with diabetes. A study by Bonner et al. reported that β-cell apoptosis might stimulate the PSP/reg expression in surviving neighboring cells, which could facilitate the recovery of β-cell mass [15]. Moreover, elevated PSP/reg level was observed in both of the disease progression state of HNF1A–maturity onset diabetes of the young (HNF1A-MODY) and the type 1 diabetes mellitus (T1DM) human subjects [16]. However, the association of PSP/reg and T2DM has not been reported.

In this study, we investigate whether serum PSP/reg is associated with the presence of T2DM and its chronic complications in patients at different phases of diabetes, and to assess its predictive value of disease progression.

Materials and methods

Setting and study population

A total of 1121 participants (479 males, 642 females; age range 23–80 years) were enrolled in this study. Data and serum samples were collected within a multi-center trial. The subjects were divided into five groups, including patients with high-risk for diabetes ($n = 254$), clinically diagnosed impaired glucose regulation (IGR) patients ($n = 342$), newly diagnosed T2DM ($n = 213$), long-term T2DM (without complications: $n = 116$, with complications: $n = 79$), and 117 volunteers were also enrolled as a healthy control group in the study. The study was carried out at eight community hospitals in Nanjing, Jiangsu, China (Hongshan, Xuanwu, Tongrenjie, Mochou, Fuzimiao, Shuangtang, Zhonghuamen, and Xujiaxiang) and Zhongda Hospital, Southeast University, Nanjing from September 2010 to February 2012. PSP/reg was analyzed, retrospectively. The study was performed according to the principles of the Declaration of Helsinki and was approved by the institutional clinical research ethics committee in China. All subjects provided informed consent to participate in the study.

Diagnostic criteria

The diagnostic criteria for diabetes were according to the American Diabetes Association (ADA) criteria 2012 [17]. Impaired glucose regulation (IGR) was defined as having impaired fasting glucose (IFG) (the fasting plasma glucose [FPG] levels 5.6 mmol/L to 6.9 mmol/L), or impaired glucose tolerance (IGT) (2-h values in the OGTT of 7.8 mmol/L to 11.0 mmol/L) without a previous diabetes diagnosis.

High-risk group of T2DM was defined as : (1) HbA1c > = 5.7 %, IGT, or IFG on previous testing; (2) Adults who are overweight (BMI >=25 kg/m^2) and who have one or more additional risk factors: a. physical inactivity; b. first-degree relative with diabetes; c. women who delivered a baby weighing > 4,000 g or who were diagnosed with gestational diabetes mellitus (GDM), any degree of glucose intolerance with onset or first recognition during pregnancy; d. hypertension (blood pressure >=140/90 mmHg or on therapy for hypertension); e. HDL cholesterol level <35 mg/dl (0.90 mmol/L) and/or a triglyceride level >250 mg/dl (2.82 mmol/L); f. women with polycystic ovary syndrome (PCOS), a common hormonal disorder among women of reproductive age; g. other clinical conditions associated with insulin resistance; h. history of cardiovascular disease [3].

Chronic complications of diabetes were assessed: all patients with diagnosis diabetes were checked for chronic complications. (1) Macroangiopathy: Coronary heart disease was diagnosed by clinical or electrocardiographic criteria, or by positive ischemic stress tests. Patients with suggestive symptoms of cerebrovascular disease (dizziness, nausea, or vomiting; unusually severe headache; confusion, disorientation, or memory loss; numbness, weakness in an arm, leg, or the face, especially on one side; abnormal or slurred speech; difficulty with comprehension; loss of vision or difficulty seeing; loss of balance, coordination, or the ability to walk) were evaluated with a central nervous

system (CNS) computerized tomography. (2) Nephropathy: It requires at least two urinary albumin excretion ratios (UAE) > =30 mg/24 h or proteinuria > = 0.5 g/24 h, or confirmed reduction of the glomerular filtration rate (creatinine clearance <1 mL/s or serum creatinine >130 umol/L). (3) Retinopathy: A fundus eye examination was applied to evaluate and diagnose diabetic retinopathy. (4) Neuropathy: It was defined by an abnormal neurologic examination consistent with the presence of peripheral sensorimotor neuropathy. Clinical examination for diabetic neuropathy included inspection of the feet and evaluation of reflexes and sensory responses to vibration, light touch, and pinprick. In addition, some patients also underwent some special examination, such as magnetic resonance imaging (MRI) and electromyography [18].

Exclusion criteria were (1) enrolled in another trial; (2) T1DM; (3) unstable angina (unexpected chest pain and usually occurs while resting); (4) blood pressure >=200/100 mmHg; (5) active infection; (6) with active tumor and take radiotherapy or chemotherapy within 6 months; (7) on medicine affecting blood or urine glucose levels (hormone, anti-hypertensive drugs, antibiotics, or NSAID).

Baseline assessment

A full clinical assessment was taken at the time of enrollment, including medical history and physical examination. Anthropometric measurements including height, weight, waistline, hipline, body mass index (BMI), and waist hip rate (WHR) were obtained. Personal information such as age, gender, smoking, drinking, family history of diabetes, and duration of diabetes was also recorded. All subjects underwent a 75 g OGTT after a 12-h overnight fast with measurement of FBG, 2-h plasma glucose (2hPG), and hemoglobin A1c (HbA1c).

Preparation of recombinant PSP/reg

Human recombinant PSP/reg was expressed in the yeast pichia pastoris and purified from medium supernatants as described elsewhere [19]. Briefly, the coding region of PSP/reg was cloned into a transfer vector containing the signal sequence of the yeast α-mating factor to drive the protein into the secretory pathway. A sterile filtered stock solution was stored at −20 °C and protected from light [20].

PSP/reg enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) to quantify human PSP/reg was performed using anti-sera from rabbits and guinea pigs immunized with recombinant human PSP/reg protein as previously described [10, 15]. Serum was prepared by centrifugation, and the IgG were purified by affinity chromatography on protein A columns. Subsequently, a sandwich ELISA was designed on 96-well ELISA plates. Antibody of the first species (guinea pig) was coated to the bottom, blocked with BSA, and aliquots of serum were then incubated for 2 h. After washing, antibodies of the other species (rabbit) were incubated. Finally, a phosphatase-coupled anti-rabbit IgG was used [21]. The reaction of the phosphatase with a substrate was determined on a multiplate reader (Dynatech), and subjects' serum PSP/reg levels were compared with standard amounts of recombinant human PSP/reg protein.

Statistical analyses

Data are presented as median (interquartile range [IQR]). The statistical analyses were conducted using a SPSS 17.0 software. Correlations of PSP/reg and clinical parameters were performed using Spearman's rank correlation coefficient. Differences between groups were determined by Nemenyi test. Area under the curve (AUC) values were presented with 95 % confidence intervals (95 % CI). Cut-offs were identified by Youden's index. Sensitivity and specificity were used to calculate likelihood ratios. Correlations and differences were defined as significant at $p < 0.05$.

Results

Participants and baseline characteristics

1121 subjects (479 males, 642 females; age range 23–80 years) participated in the study. The baseline subject characteristics of the six enrolled populations are summarized in Table 1. These groups were age-matched except control. The control group in this study was younger than all the other groups. Young control individuals were chosen to rule out the coincidence with other chronic disease that may interfere with the levels of PSP/reg. Furthermore, an analysis of 117 healthy volunteers showed no correlation of PSP/reg with age ($R^2 = 0.002809$).

Elevated PSP/reg levels were detected in T2DM

We measured fasting serum PSP/reg levels in the subjects with T2DM at different clinical phases using a newly developed ELISA assay. PSP/reg levels were elevated in high-risk group patients as compared to healthy controls (18.7 ng/ml [15.0–26.4] versus 16.4 ng/ml [13.9–20.8)], $p = 0.014$). We also found that PSP/reg levels were significantly higher in the IGR (18.5 ng/ml [13.7–26.3] versus 16.4 ng/ml [13.9–20.8], $p < 0.05$) and newly diagnosed group (21.0 ng/ml [14.8–29.4] versus 16.4 ng/ml [13.9–20.8], $p < 0.001$). Furthermore, PSP/reg levels were clearly elevated in long-term

Table 1 Clinical characteristics of subjects

Subject group	HC	HRG	IGR	Onset	LD without C	LD with C
Number of subjects	117	254	342	213	116	79
Age (yrs)	28.0 (25.0–33.0)	61.0 (56.0–68.0)	62.0 (56.0–69.3)	62.0 (57.0-69.0)	63.0 (59.0–69.8)	68.0 (57.0–75.0)
Gender male (%)	68 (58.1)	86 (33.9)	114 (33.3)	101 (47.4)	65 (56.0)	45 (57.0)
BMI (kg/m^2)	21.7 (19.6–23.9)	24.1 (21.9–25.8)	24.7 (22.9–27.1)	25.3 (23.5–27.5)	23.8 (21.8–25.6)	23.9 (22.0–25.8)
Family history, yes (%)	21 (17.9)	36 (14.2)	64 (18.7)	32 (15.0)	40 (34.5)	28 (35.4)
Smoking, yes (%)	26 (22.2)	46 (18.1)	45 (13.2)	51 (24.1)	29 (25.0)	7 (8.9)
Fasting plasma glucose (mmol/l)	4.7 (4.5–5.0)	4.9 (4.6–5.3)	5.8 (5.4–6.2)	7.2 (6.4-8.2)	6.0 (5.2–7.5)	7.2 (5.8–9.5)
2-h plasma glucose (mmol/l)	5.0 (4.8–5.6)	6.1 (5.2–6.7)	7.9 (6.4–9.0)	13.2 (11.6–15.8)	10.2 (8.2–12.6)	10.7 (8.8–13.2)
HbA1c (%)	5.2 (5.1–5.5)	5.7 (5.5–5.9)	6.0 (5.8–6.3)	7.0 (6.5–7.8)	7.1 (6.3–8.3)	8.7 (7.5–10.5)

Clinical characteristics of different groups of subjects, *HC* Healthy Control, *HRG* High-Risk Group, *IGR* Impaired Glucose Regulation, *LD without C* Long-term Diabetes Patients without Complications, *LD with C* Long-term Diabetes Patients with Complications

Data are given as median (IQR)

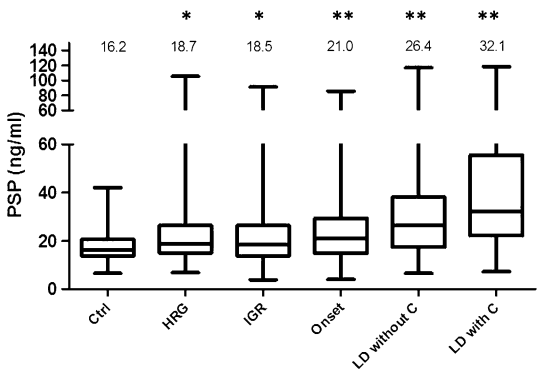

Fig. 1 Serum levels of PSP/reg in different groups of the study population. Median PSP/reg serum levels differed significantly in patients of high-risk ($p < 0.05$), impaired glucose regulation ($p < 0.05$), incipient diabetic ($p < 0.001$), long-term diabetes without complications ($p < 0.001$), long-term diabetes with complications ($p < 0.001$) as compared to healthy controls. *$p < 0.05$, **$p < 0.001$. *Ctrl* Healthy Control, *HRG* High-Risk Group, *IGR* Impaired Glucose Regulation, *LD without C* Long-term Diabetes Patients without Complications, *LD with C* Long-term Diabetes Patients with Complications

patients with T2DM (without complications: 26.4 ng/ml [17.4–38.2] versus 16.4 ng/ml [13.9–20.8], $p < 0.001$; with complications: 32.1 ng/ml [22.1–55.5] versus 16.4 ng/ml [13.9–20.8], $p < 0.001$) (Fig. 1).

PSP/reg levels increase with the duration of T2DM

A significant correlation between PSP/reg level and duration of T2DM (Spearman's rank correlation coefficient 0.319, $p < 0.001$, Fig. 2a) was noted. A correlation between PSP/reg and HbA1c in all the participants (Spearman's rank correlation coefficient 0.188, $p < 0.001$ Fig. 2b) was also found. Meanwhile, FBG and 2hPG correlated positively with PSP/reg levels. A correlation between PSP/reg and pulse pressure (systolic pressure-diastolic pressure) (Spearman's rank correlation coefficient 0.130, $p < 0.001$) and smoking (Spearman's rank correlation coefficient 0.10, $p < 0.001$) were also found. However, there was no association between PSP/reg and age, gender, and BMI.

Relationship between PSP/reg and T2DM progression

We set out to assess the predictive value of PSP/reg in T2DM by receiver operating characteristic (ROC) analysis. The AUC of PSP/reg for presence of diabetes-onset and diabetes with chronic complications was 0.640 and 0.754 (95 %CI:0.605–0.674 and 0.694–0.813), respectively. We identified a PSP/reg cut-off of 22 ng/ml in the nondiabetes group. Subjects with high PSP/reg ($\geq$ 22 ng/ml) were more likely to experience diabetes in future than those with low PSP/reg levels ($<$ 22 ng/ml), (Fig. 3a). The sensitivity was 54 % and specificity was 65 % (positive likelihood ratio: 1.54, negative likelihood ratio: 0.70). On the contrary, a PSP/reg threshold of 27 ng/ml in subjects with T2DM was the most significant parameter for the presence of chronic complications of diabetes (Fig. 3b). The sensitivity and specificity were 65 and 74 % (positive likelihood ratio: 2.5, negative likelihood ratio: 0.47) respectively.

Discussion

The present study analyzed PSP/reg levels in patients with different stages of T2DM. We revealed three interesting new findings. First, patients with diabetes have significantly elevated PSP/reg levels, and it is worth noting that this trend is already observed in the high-risk groups. Secondly, there is a positive correlation between PSP/reg

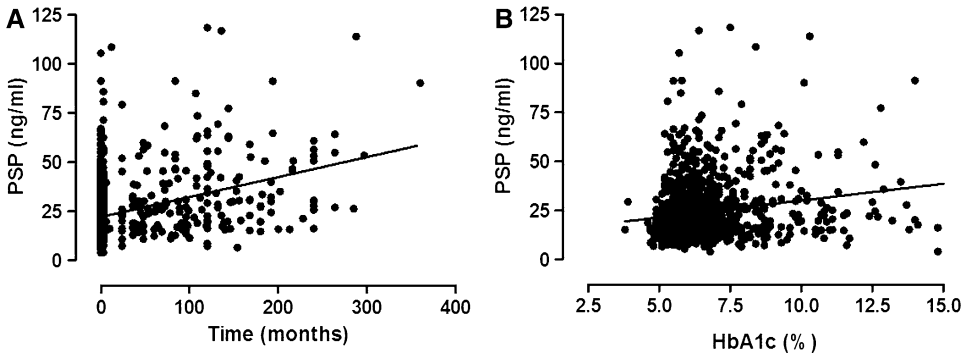

Fig. 2 **a/b** Correlation analysis of PSP/reg in all subjects. **a** Correlation of PSP/reg with duration of type 2 diabetes (Spearman $r = 0.319$, $p < 0.001$). **b** Correlation of PSP/reg with HbA1c (Spearman $r = 0.188$, $p < 0.001$). Lines represent the trend calculated by linear regression

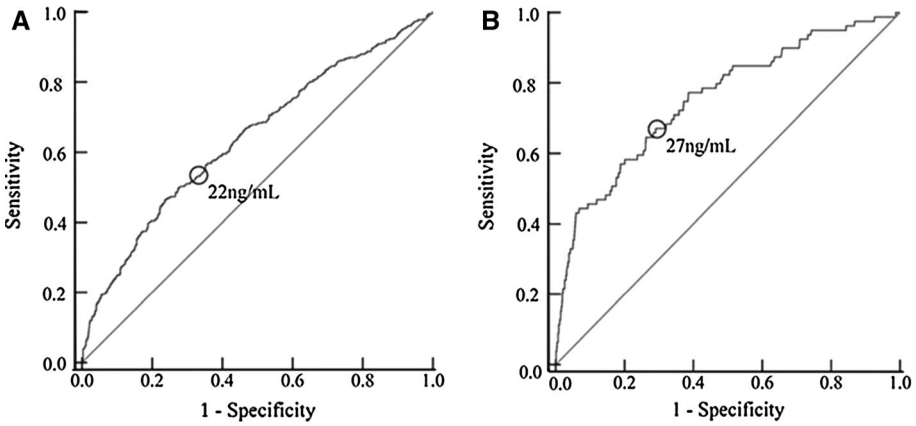

Fig. 3 **a/b** ROC curve analysis: PSP/reg predicts the incidence of type 2 diabetes (**a**), and of type 2 diabetes chronic complications (**b**). *Circles* highlight the most accurate cut-offs. Using the 22 ng/mL cut-off reveals a sensitivity of 54 % and a specificity of 65 % to predict the incidence of type 2 diabetes (**a**). 27 ng/mL among patients with type 2 diabetes was the best threshold to predict the incidence of type 2 diabetes chronic complications (sensitivity: 65 %, specificity: 74 %, **b**)

levels and the duration of diabetes, which was identified by a time dependent increase of PSP/reg levels in subjects with diabetes. Thirdly, PSP/reg level might reflect chronic complications of diabetes, and may potentially stratify patient's outcomes.

Under normal situations, the most predominant origin of PSP/reg is pancreatic acinar cells, while other tissues, intestines and islets for instance, appear to produce PSP/reg predominantly under pathologic conditions [22–25]. Initially, PSP/reg was suggested as a sensitive marker of pancreatic injuries, however, other conditions might activate the secretion of PSP/reg [26, 27]. Recent data revealed that PSP/reg could discriminate patients with infection, infection with sepsis and no infection, which might be a superior marker for posttraumatic complications [14]. Recent studies reported significantly elevated PSP/reg levels in blood of patients with sepsis [11], VAP [12], COPD exacerbation [13] and peritonitis [21].

In diabetes, islets are infiltrated by inflammatory cells. To counteract such attacks, high levels of PSP/reg were secreted to boost islet cell proliferation [7, 10, 28], as strong PSP/reg immunoreactivity in peri-vascular and peri-insular areas were observed [23]. Recent study in insulinoma cell lines and mouse models by Bonner et al. provided biological evidence that β-cells undergoing apoptosis induce the PSP/reg expression in surviving neighbor β-cells [15]. A following study by Bacon et al. [16] revealed that PSP/reg levels are prominently elevated in human subjects with HNF1A-MODY and TIDM, suggesting a role for pancreatic injury and β-cells apoptosis as a source for elevated PSP/reg serum levels. Our findings provided new evidence that PSP/reg levels were significantly higher in T2DM patients, especially in patients with chronic complications. The role of inflammation in the pathogenesis of T2DM and associated complications has been well established [29], and PSP/reg was a protein mainly reflect the progress of inflammation [11–13, 21]. These results further confirmed that PSP/reg levels show close associations with diabetes and imply that PSP/reg might be a promising sensitive marker of diabetes in future. Interestingly, no statistical differences were found among the high-risk, IGR and newly diagnosed diabetes patients. It might indicate

that diabetes progression might remain at a stable level during those periods. As a result, this may provide a strategic period to intervene and hence postpone or even prevent the onset of T2DM.

In our results, circulating PSP/reg values correlate with diabetes duration which implies that there is a positive correlation between PSP/reg levels and the course of diabetes. This was identified by a time dependent increase of PSP/reg levels in subjects with T2DM. Hence, diabetes duration might be an important risk factor for its complications. We also found a correlation between PSP/reg levels and HbA1c, FBG and 2hPG which indicates that the elevation of PSP/reg levels might play an important role in the development of abnormal glucose tolerance. This observation is in agreement with recent data indicating that increased extracellular glucose concentrations potentiated PSP/reg gene expression, which may represent an important physiological feedback loop for the regulation of β-cell mass [15]. Moreover, correlation between PSP/reg and smoking was observed as well. Cigarette smoking induces an inflammatory reaction in the lung and is a major risk factor for a number of lung diseases such as COPD. It leads to elevation of soluble markers of systemic inflammation in circulation [30, 31]. PSP/reg is a protein mainly reflecting infectious conditions and organ failure. Therefore, the inflammation in the air spaces of smokers might lead to elevated PSP/reg level in blood of smokers. Another correlation between pulse pressure (PP) and PSP/reg was also found. PP is useful in predicting risk for atherosclerotic diseases, which are inflammatory in nature [32, 33]. Thus, this might explain the correlation between PSP/reg and pulse pressure and its relevance may be explored in further studies.

For future risk assessment, we calculated the AUC of PSP/reg for presence of diabetes-onset and diabetes with chronic complications which were 0.640 and 0.754 (95 %CI:0.605–0.674 and 0.694–0.813), respectively. In the process of this analysis, we identified two cut-off values, and found a PSP/reg cut-off of 22 ng/ml in the nondiabetes group as the most valuable threshold for indicating incidence. On the contrary, a PSP/reg threshold of 27 ng/ml in subjects of T2DM was the most significant parameter associated with the occurrence of diabetes chronic complications. Both cut-offs are characterized by a high specificity. While these cut-offs have a high positive likelihood ratio, they have a low negative likelihood ratio. Thus, negative results (nondiabetes: <22 ng/ml, T2DM: <27 ng/ml) need to be explained cautiously.

The main strength of this multi-center study is the large number of T2DM subjects and the grouping according to the different stages of T2DM. However, this study also has a few limitations. First, these new findings need to be considered hypothesis-generating due to their observational nature. Hence, the exact performance of PSP/reg in the developing progress of T2DM remains unclear. Although the study was performed in several centers, external validation of our results is essential. Therefore, PSP/reg measurements cannot yet be recommended. Second, owing to its observational characteristic, this hypothesis requires a randomized, controlled trial to definitively determine the role of PSP/reg in patients with T2DM. Finally, the exact origin of elevated PSP/reg in T2DM patients is unknown. Pancreatic acinar cells are considered as the most important source of PSP/reg, while other releasing mechanisms and origins are also possible, but require further research.

Our preliminary data suggest that PSP/reg could be of clinical use in the stratification of T2DM patients, and may help to detect patients at high risk of T2DM and identify chronic complications of diabetes. Thus, PSP/reg might serve as a potential biomarker in T2DM.

Conclusion

PSP/reg is significantly up-regulated in T2DM patients, and the PSP/reg level is related to the duration of diabetes. Therefore, PSP/reg might be useful as a predictor of T2DM and disease progression. Future trials are required to reassess performance and evaluate the impact of PSP/reg measurements.

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Conflict of interest Rolf Graf is inventor of PSP in sepsis (Method for assaying sepsis in humans) for which the University of Zurich holds a patent.

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