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Type 2 diabetes beyond obesity

- additional risk markers in young women

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1. Abbreviations

GDM ..... *gestational diabetes mellitus*

HbA1c ..... *hemoglobin A1c*

oGTT ..... *oral glucose tolerance test*

PPSDiab..... *Prediction, Prevention and Subclassification of type 2 Diabetes*

T2D ..... *type 2 diabetes mellitus*

2. Introduction

2.1. Epidemiology of type 2 diabetes mellitus (T2D)

2.1.1. Prevalence of T2D

Worldwide, over 400 million people at the age of 20-79 years are estimated to live with diabetes mellitus [1, 2]. In industrialized countries, the proportion of patients with type 2 diabetes mellitus (T2D) is about 90% of all cases of diabetes [2]. T2D therefore causes both a great economic and, for the individuals affected, also a personal burden. Globally, the total health care expenditure in 2015 due to diabetes in 20-79 year-old patients has been estimated to be US $673 billion and is expected to rise to more than US $800 billion in 2040 [2]. The personal consequences for patients are a limitation in quality of life, disability [3-5], and increased mortality rates [2]. These burdens will increase further with the continuously rising prevalence. In 2040, the global prevalence of diabetes is expected to exceed 10% (642 million people) [2]. To reverse this trend, prevention must be improved and progression of the disease minimized.

2.1.2. Undiagnosed prediabetes and T2D

The worldwide proportion of adults with unknown diabetes is estimated to be 46.5% (192.8 million people) of all cases of diabetes [2]. Even in industrialized countries, screening studies show that the rate of unknown diabetes among the general population is high [6, 7]. For Europe, the amount of undiagnosed diabetes in adults was found to still be 39.3% (23.5 million people) [2]. Almost all cases of unknown diabetes are T2D, because the clinical presentation of other forms of diabetes is much clearer.

Oral glucose tolerance testing (oGTT) is the gold standard method to test for prediabetes and T2D. However, it is time consuming and expensive. Therefore, routine screening for T2D is usually conducted with fasting glucose and hemoglobin A1c (HbA1c) only [8]. This strategy misses subjects with the prediabetic stage of impaired glucose tolerance and also early forms of T2D [9, 10]. Diagnostics of prediabetes and early T2D are thus still suboptimal. Similarly, risk prediction for T2D is incomplete and rather simple-minded. Current risk scores in principle detect the metabolic syndrome, ask for a family history of diabetes, and older age [8, 11].

Consequently, the scores miss individuals without these characteristics, in particular young and lean patients, which certainly exist in rather large numbers.

2.1.3. Consequences of insufficient diagnostic tools

With the current screening methods, many individuals with prediabetes and early T2D are missed. This is problematic, because disease duration is a critical point for the success of lifestyle interventions: the longer glucose metabolism has been deranged, the lesser patients benefit from intervention programs [12]. Thus, healthy subjects at high risk for T2D and subjects in a prediabetic stage should be the key target groups for prevention programs. To identify more subjects at high risk, a reliable prediction of T2D through cost-effective biomarkers is needed.

2.2. Heterogeneity of T2D

Another problem, besides the lack of sufficient prediction and early detection, is that, after diagnosis, T2D therapy follows a “one size fits all” approach [8, 11]. This leads to heterogeneous responses to therapy, which is not surprising given the diversity of the patients’ phenotypes [13-15].

The general preconception of T2D as a disease of older age and obesity/the metabolic syndrome also has to be revisited. The metabolic syndrome is a cluster of changes that unquestionably put affected individuals at high risk for cardiovascular diseases and T2D [16]. Despite small differences [17-21], all definitions of the metabolic syndrome include the same clinical conditions: hyperglycemia, central obesity, hypertension, and dyslipidemia. However, the proportion of T2D cases that do not have the classical phenotype is noteworthy. For example, almost one third of incident T2D cases in the United Kingdom Prospective Diabetes Study cohort was lean with a BMI of less than 25kg/m^2 [22, 23].

Particularly this high proportion of lean T2D patients substantiates the need for research on T2D pathophysiology apart from the contribution of obesity. This could enable a better prediction of T2D risk in lean subjects, facilitate the development of novel, cost-effective biomarkers, and could lead to more tailored intervention programs. Thereby, the onset of T2D could be delayed or even prevented. Understanding pathophysiological differences between

different groups of patients would also permit individualized therapy guidelines. Finally, further research on specific subgroups of T2D might also lead to the discovery of alternative drug targets.

3. The PPSDiab study

Previous studies on T2D mostly included older subjects with incident or even long-standing diabetes [24-27]. This complicates research, as the subjects often have co-morbidities and medication affecting metabolism. An optimal study design should therefore be prospective, including young and healthy subjects with the development of T2D as primary endpoint.

Based on these criteria, the Prediction, Prevention and Subclassification of type 2 Diabetes (PPSDiab) study was designed. To pre-select for individuals at high risk for T2D, women after a pregnancy complicated by gestational diabetes mellitus (GDM) were recruited preferentially [28]. GDM serves as a risk-marker for T2D because pregnancy is a stress test for glucose metabolism [29]. Nevertheless, GDM is a transient condition, meaning that after delivery affected women usually return to normoglycemia [29, 30]. This allows for the inclusion of healthy subjects who are at high risk for T2D. Additionally, the inclusion of women after a recent pregnancy naturally results in a cohort of young adults.

The baseline visit of the PPSDiab study was conducted with 304 women 3-16 months after the index pregnancy. Women after a pregnancy complicated by GDM were included as cases and women after a normoglycemic pregnancy served as controls in a ratio of 2:1. The study has yearly follow ups, which include an oGTT, medical and family history, anthropometric measurements, and optionally additional tests, e.g. cardiopulmonary exercise testing (**Figure 1**).

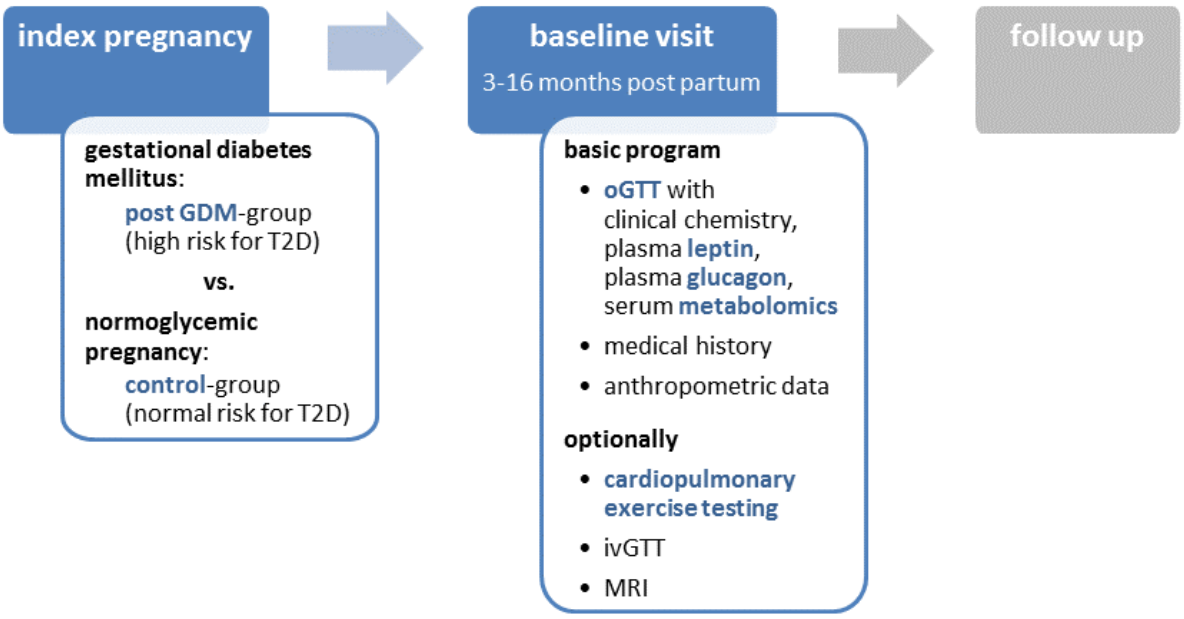

Figure 1: *PPSDiab study design.*

GDM: gestational diabetes mellitus, ivGTT: intravenous glucose tolerance test, MRI: magnetic resonance imaging, oGTT: oral glucose tolerance test, T2D: type 2 diabetes mellitus

All analyses for this thesis were done with data from the baseline visit of the PPSDiab study. Detailed information about the baseline characteristics of the PPSDiab study cohort is given in a publication by Rottenkolber et al. [31].

4. Specific aims of this thesis

4.1. Physical fitness, leptin, and T2D risk

Physical activity and fitness are known determinants of T2D risk and other cardiovascular diseases [32-34]. One possible reason for the reduction of T2D risk with increased physical activity could simply be an increased energy expenditure, counterbalancing excessive energy supply and preventing obesity. Furthermore, exercise increases the quantity of the glucose transporter GLUT-4 on the plasma membrane of contracting muscle cells, leading to an enhanced glucose uptake into the muscle [35, 36]. This positive effect of exercise continues even after cessation of activity: A meta-analysis by Way et al. confirmed improved insulin sensitivity following the last of at least 3 bouts of exercise [37]. Exercise also alters inter-organ communication via myokines (i.e. cytokines produced by skeletal muscle), which can also affect glucose metabolism in a positive manner [38].

Physical fitness covers cardiorespiratory and muscular endurance, muscular strength, body composition, and flexibility [39]. In T2D research, cardiorespiratory fitness (measured as maximal oxygen uptake in cardiopulmonary exercise testing) is most commonly used as a surrogate parameter also for the other domains of general physical fitness. Sui et al. have shown that high cardiorespiratory fitness independently reduces the risk for the development of T2D [40]. Besides inherited factors, the amount of regular physical activity is the main determinant of physical fitness [40]. Beyond the effects of regular physical activity (see above), a high physical fitness improves glycemia as it is positively associated with muscle mass [35, 36]. A high muscle mass improves postprandial glucose tolerance, because skeletal muscle is the main organ for glucose deposition in that situation [41].

Physical activity and fitness have been shown to be negatively associated with plasma leptin levels in humans [42-47]. The adipokine leptin is secreted by the adipose tissue in positive correlation to the amount of stored energy [48, 49]. It mainly acts centrally to control energy [48, 49] and glucose homeostasis [50-52]. Both leptin deficient and leptin resistant phenotypes are characterized by hyperinsulinemia, insulin resistance, and hyperglycemia [52]. Consequently, euglycemia requires adequate leptin action. Leptin resistance can be observed in overweight and obese individuals, where central leptin action is insufficient despite high

amounts of leptin being produced by adipose tissue [53]. Therefore, leptin resistance is one link from overweight/obesity to disturbed glucose homeostasis.

We examined the association of physical fitness with T2D risk and recent GDM on the one hand, and with plasma leptin levels on the other hand. We hypothesized that if physical fitness would associate with leptin levels, it might affect leptin resistance. Therefore, increasing physical fitness may potentially counterbalance some of the negative effects of obesity in our study population.

4.2. Plasma glucagon and prediabetes

In the pancreatic islet, alpha-cells secrete glucagon to counteract hypoglycemia [54]. This raises plasma glucose by an increase in gluconeogenesis and glycogenolysis and a reduction of glycolysis [54, 55]. Insulin serves as a negative feedback for alpha-cells and inhibits the secretion of glucagon [54]. Hence, rising insulin levels in an oGTT are expected to suppress the release of glucagon. Diminished insulin secretion, as seen in T2D, could therefore lead to differences in the glucagon response. High glucagon levels could also add to hyperglycemia independent of insulin levels [54].

The literature about glucagon levels in T2D is inconsistent. Some studies showed high fasting glucagon and insufficient glucagon suppression during an oGTT in prediabetes and T2D [56-59] while others found no alterations [60].

We examined the plasma glucagon dynamics during an oGTT in different metabolic subgroups (normoglycemic, prediabetes, T2D) of the PPSDiab study cohort.

4.3. Serum and plasma amino acids as risk markers for T2D

Because routine screening misses many cases of early T2D and especially prediabetes [9, 10], novel biomarkers are needed. Amino acids serve as precursors for gluconeogenesis [61] and derangements of glucose homeostasis are reflected by amino acid metabolism [62]. Therefore, amino acids possibly serve as biomarkers for T2D. However, blood amino acid levels are also altered by other factors like nutrition and comorbidities [61]. For example, blood amino acid profiles differ between lean and obese subjects [63] and also associate with

hypertension, dyslipidemia, and the metabolic syndrome [64, 65]. These data point to the question whether amino acids can reliably indicate glycemia besides being affected by many other conditions.

We reviewed the current literature on serum or plasma amino acids as biomarkers for prediabetes and T2D and also analyzed original data from the PPSDiab study. Here we examined, whether plasma amino acid levels reflect pathological glucose tolerance and other components of the metabolic syndrome in the PPSDiab cohort.

5. Conclusions

5.1. Physical fitness and leptin

In the PPSDiab study cohort, women post GDM had a lower physical fitness (measured as maximum oxygen uptake and maximum load in cardiopulmonary exercise testing) than control subjects after a normoglycemic pregnancy. This association remained significant after adjustment for BMI, age, and months post-delivery. The results suggest that reduced physical fitness is an independent risk factor for the increased T2D-risk in the post GDM group of women, and potentially also for the GDM itself.

Fasting plasma leptin levels associated inversely with physical fitness and the insulin sensitivity index, and positively with the BMI in our study. The negative association of leptin with physical fitness was confirmed by a linear regression analysis adjusted for BMI or body fat mass. These data confirm previous results [42-44, 47] and could mean that physical fitness influences insulin sensitivity via leptin signaling (**Figure 2**). This conclusion needs further examination, e.g. in intervention trials aiming at a weight stable improvement of physical fitness. Additionally, to address this and similar questions, we recently set up a new protocol for cardiopulmonary exercise testing that has a specific focus on glucose metabolism and hormonal signaling.

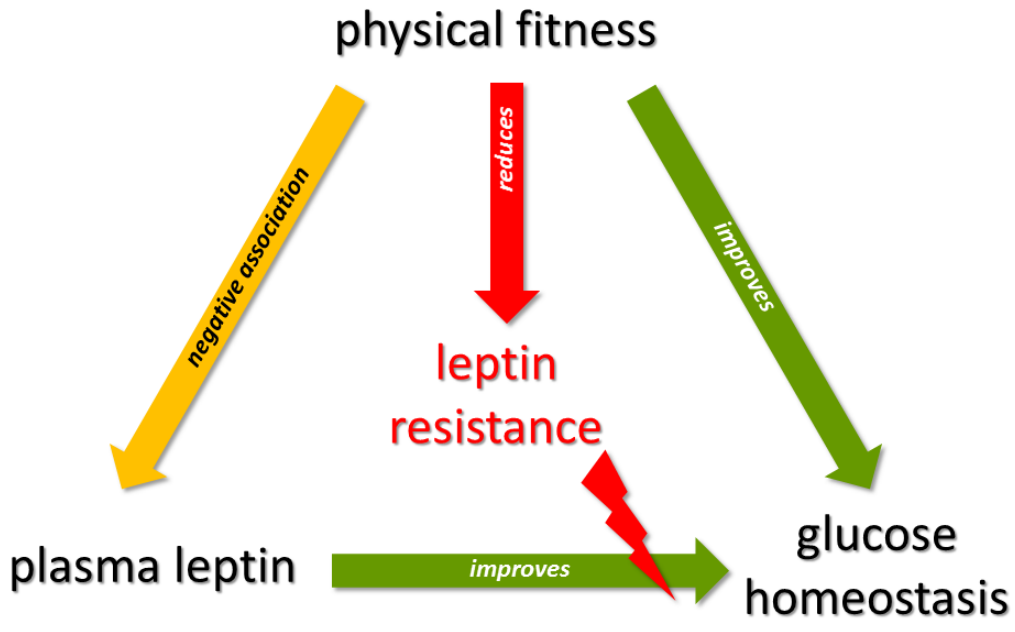

Figure 2: *Suggested interrelation of physical fitness, plasma leptin, and glucose homeostasis.*

5.2. Plasma glucagon

In our study, mean fasting glucagon levels were higher and glucagon suppression during oGTT was delayed in the prediabetes-/T2D-group. These were expected findings. However, the plasma glucagon dynamics were not homogeneous within the metabolic groups that we examined. Instead, an unsupervised cluster analysis showed 4 patterns of glucagon dynamics that did not match the predefined metabolic groups.

Clusters with high fasting glucagon and delayed glucagon suppression were enriched for subjects at high risk for T2D but also contained a large proportion of control subjects. Conversely, subjects from high-risk groups were also found in the "healthiest" glucagon cluster characterized by low fasting glucagon and appropriate glucagon suppression. We conclude that hyperglucagonemia may promote hyperglycemia, but it is neither a clear indicator nor a prerequisite for T2D.

With respect to the metabolic syndrome, the cluster with high fasting glucagon and poor glucagon suppression clearly differed from the other clusters. Despite their low mean age, the women in this cluster had the highest mean BMI, waist circumference, high-sensitivity c-reactive protein, liver fat, and intra-abdominal fat, and the worst insulin sensitivity. Hence, fasting plus postprandial hyperglucagonemia is closely linked to the metabolic syndrome.

5.3. Serum and plasma amino acids

Our systematic literature review revealed several candidates that are most consistently associated with pathological glucose metabolism: the branched chain amino acids (isoleucine, leucine, and valine), aromatic amino acids (alanine and proline), glycine, and glutamate. Our own data from the PPSDiab study showed that isoleucine, leucine, the sum of the branched chain amino acids, proline, glycine, and glutamate were significantly associated with pathological glucose tolerance. Except for glycine, all associations of amino acids with the glycemic state were lost with BMI-adjustment in a logistic regression analysis.

Furthermore, not only the glycemic state, but also other components of the metabolic syndrome (BMI, blood pressure, and lipids) associated with plasma amino acids. Classic risk models for the development of T2D already include these factors of the metabolic syndrome.

Thus, the addition of plasma amino acids does not substantially improve already established clinical models, except possibly for glycine.

5.4. T2D beyond obesity

This thesis aimed at the characterization of several factors, other than obesity, that are associated with T2D risk in young women. It reveals new aspects of T2D pathophysiology, highlights the heterogeneity of this disease, and critically evaluates a class of potential novel biomarkers. Together with other recent studies, our results show that there is still a lot to learn about T2D and that distinct disease subtypes likely exist but are still underappreciated. Future work will hopefully close these gaps in our knowledge and will provide means for personalized T2D prevention, which should be much more effective than current approaches.

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7. Published articles

7.1. Gar et al. PLOS ONE 2017

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Physical fitness and plasma leptin in women with recent gestational diabetes

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RESEARCH ARTICLE

Physical fitness and plasma leptin in women with recent gestational diabetes

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Abstract

Aims/Hypothesis

Low physical fitness (PF) is a risk factor for type 2 diabetes mellitus (T2D). Women with a history of gestational diabetes (GDM) are at risk for T2D at a young age, but the role of PF in this population is not clear. PF has also been found to correlate inversely with plasma leptin in previous studies. Here, we examine whether women who had GDM have lower PF than women after a normoglycemic pregnancy and, second, whether PF is associated with plasma leptin, independently of body fat mass.

Methods

Cross-sectional analysis of 236 participants in the PPSDiab Study (cohort study of women 3–16 months after delivery, 152 after gestational diabetes (pGDM), 84 after normoglycemic pregnancy (control subjects); consecutively recruited 2011–16); medical history, physical examination with bioelectrical impedance analysis (BIA), whole body magnetic resonance imaging (MRI) (n = 154), 5-point oral glucose tolerance test, cardiopulmonary exercise testing, clinical chemistry including fasting plasma leptin; statistical analysis with Mann–Whitney U and t -test, Spearman correlation coefficient, multiple linear regression.

Results

Women pGDM had lower maximally achieved oxygen uptake (VO_{2peak}/kg: 25.7(21.3–29.9) vs. 30.0(26.6–34.1)ml/min/kg; total VO_{2peak}: 1733(1552–2005) vs. 1970(1767–2238)ml/min; p<0.0001 for both), and maximum workload (122.5(105.5–136.5) vs. 141.0(128.5–159.5)W; p<0.0001). Fasting plasma leptin correlated inversely with PF (VO_{2peak}/kg $\rho = -0.72$ p<0.0001; VO_{2peak} $\rho = -0.16$ p = 0.015; max. load $\rho = -0.35$ p<0.0001). These associations remained significant with adjustment for body mass index, or for body fat mass (BIA and MRI).

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Abbreviations: BBB, blood–brain barrier; GDM, gestational diabetes; hs-CRP, high sensitivity c-reactive protein; ISI, insulin sensitivity index; PF, physical fitness; pGDM, previous gestational diabetes; RER, respiratory exchange ratio; T2D, type 2 diabetes mellitus; TG, triglycerides; VO_{2peak}, peak volume of oxygen uptake.

Conclusions/Interpretation

Women with a recent history of GDM were less fit than control subjects. Low PF may therefore contribute to the risk for T2D after GDM. This should be tested in intervention studies. Low PF also associated with increased leptin levels–independently of body fat. PF may therefore influence leptin levels and signaling. This hypothesis requires further investigation.

Introduction

Gestational diabetes (GDM) is a transient disturbance of glucose metabolism, with a prevalence ranging from 1.1% to 24.3%, depending on diagnostic criteria [1]. It is a strong risk marker for subsequent type 2 diabetes mellitus (T2D) [2, 3], and women with recent GDM already show many metabolic characteristics associated with T2D [4].

Physical fitness (PF) and activity are major determinants of diabetes risk [5]. Muscle glucose uptake is particularly important for postprandial glucose tolerance, and regular exercise increases the insulin sensitivity of skeletal muscle [6]. Additionally, glucose metabolism of trained individuals with high PF is also probably affected more indirectly by altered interorgan communication [7, 8]. The gold-standard methodology to quantify PF is cardiopulmonary exercise testing. To our knowledge, this has not been done in women with recent GDM.

The hormone leptin is produced by the adipose tissue as an indicator of the amount of stored energy (as fat) [9]. Its plasma level correlates closely with the quantity of fat tissue and therefore also the body mass index (BMI) [10]. Leptin mainly acts centrally, predominantly to control appetite [11]. It is also required for an adequate neuroendocrine function, i.e. secretion of sexual hormones, growth and thyroid hormone and has been shown to influence glucose metabolism by increasing insulin sensitivity [9, 11–14]. This mainly occurs through an activation of the sympathetic nervous system and by direct action on peripheral leptin receptors in skeletal muscle [13]. Surprisingly, however, high plasma leptin levels often coexist with insulin resistance in human subjects. We and other groups have even found a negative correlation between fasting plasma leptin and insulin sensitivity after adjustment for BMI [4, 5]. Given the insulin-sensitizing effect of leptin, this finding requires dysfunction or saturation of leptin signaling, phenomena often summarized under the term "leptin resistance".

An additional factor that may influence leptin sensitivity is physical fitness. Low PF has been found to be associated with high leptin levels in previous studies [5, 15–17] and exercise interventions can lead to a reduction in plasma leptin [18].

Based on these lines of evidence, we examined two research questions in an observational study of young women. First, whether women with a recent history of GDM have lower PF than appropriate control subjects. Second, whether PF is associated with the plasma leptin level after adjustment for BMI or body fat mass. Positive answers to both questions would provide initial evidence that increasing PF in women with recent GDM by an exercise intervention may have the double benefit of reducing both insulin and leptin resistance.

Material and methods

Study design and participants

Women included in the present cross-sectional analysis were participants of the prospective, mono-center observational study PPSDiab ("Prediction, Prevention and Subclassification of type 2 diabetes") enrolled between November 2011 and May 2016. The study population

consists of women with GDM during their last pregnancy (pGDM) and women following a normoglycemic pregnancy (controls) in the ratio 2:1. The cohorts were recruited consecutively from the Diabetes Center and the obstetrics department of the University Hospital (Klinikum der Universität München) in Munich, Germany.

Eligible women were premenopausal and within 3 to 16 months after a singleton or twin (n = 9) pregnancy with live birth(s). The diagnosis of GDM was based on a 75g oral glucose tolerance test (OGTT) after the 23rd week of gestation. The cut-off values for GDM were 92/180/153 mg/dl plasma glucose following the International Association of the Diabetes and Pregnancy Study (IADPSG) recommendations [19]. Women were eligible to participate as controls if they had no history of GDM in any previous pregnancy and either a normal 75g OGTT or a normal 50g screening OGTT (<135 mg/dl plasma glucose after 1 hour, n = 10) after the 23rd week of gestation. We included controls with only a screening OGTT because in Germany a 2-Step approach of testing for GDM is widely used and women without known risk-factors for GDM may only receive the 50g test.

Exclusion criteria for this study were alcohol or substance abuse, pre-pregnancy diabetes, and chronic diseases requiring systemic medication (except for hypothyroidism (n = 52), mild hypertension (n = 4), gastroesophageal reflux (n = 2), and history of pulmonary embolism resulting in Rivaroxaban prophylaxis (n = 1)).

Written informed consent was obtained from all study participants and the protocol was approved by the ethical review committee of the Ludwig-Maximilians-Universität.

All data used in this analysis were collected at the baseline visit of the PPSDiab study, 3 to 16 months after the index pregnancy.

Study procedures

After an overnight fast, the women underwent a 5-point 75-g oral glucose tolerance test with measurement of plasma glucose (Glucose HK Gen.3, Roche Diagnostics, Mannheim, Germany), serum insulin (CLIA, DiaSorin LIASON systems, Saluggia, Italy), plasma leptin (ELISA "Dual Range", Merck Millipore, Darmstadt, Germany), high sensitivity c-reactive protein (hs-CRP; wide-range CRP, Siemens Healthcare Diagnostics, Erlangen, Germany) and blood lipids (LDL and HDL cholesterol, triglycerides; enzymatic caloric test, Roche Diagnostics, Mannheim, Germany). Insulin sensitivity index (ISI) was calculated according to Matsuda and DeFronzo (ISI = 10,000/square root of [fasting glucose x fasting insulin] x [mean glucose x mean insulin during OGTT]) [20]. Anthropometric data included body mass, body fat mass (determined by bioelectrical impedance analysis (Tanita BC-418; Tanita Corporation) [21, 22]), height, waist and hip circumference. A detailed description of the study design, anthropometric and clinical measurements as well as methodologies of blood sampling and analysis can be found elsewhere [4].

For determination of PF, cardiopulmonary exercise testing was performed on a bicycle ergometer using the cardiopulmonary exercise testing system MasterScreen CPX (Care Fusion, Höchberg, Germany). Prior to this test, cardiopulmonary health was ascertained from the medical history and clinical examination including auscultation and measurement of resting blood pressure. Due to the heterogeneity of the study cohort regarding physical fitness levels, we used a standardized stepwise ramp protocol for all participants. It consisted of stepwise increments of 25 W every 3 minutes, starting with a reference phase without load. In order to reach a plateau phase of the oxygen curve (levelling-off effect), which is required for determining the maximal possible oxygen uptake of the cardiopulmonary system (VO_{2max}), an individualized, steep ramp protocol and a reasonable baseline fitness of the study participant, who also has to be familiar with the test procedure, would have been needed. This was not possible

in our study and we therefore determined the peak oxygen uptake before termination of workload (VO_{2peak}), a close approximation of VO_{2max} [23]. 12-channel ECG, oxygen uptake, and carbon dioxide exhalation were monitored continuously, while at the end of each increment, capillary lactate was measured using a SuperGL Analyser (Hitado, Möhnesee, Germany), and participants were asked to rate their perceived exertion by pointing to a BORG scale [24]. The test was terminated when the participant was exhausted. A maximal respiratory exchange ratio (RER) of at least 1.05 was required for a valid exercise test.

Study participants were invited to undergo a whole-body magnetic resonance imaging (MRI) measurement (3 Tesla system, Ingenia or Achieva; Philips Healthcare) with determination of total adipose tissue volumes. Three days before MRI study, participants were advised to refrain from heavy exercise. The MRI study protocol has been described previously [4].

Statistical analysis

All metric and normally distributed variables are reported as mean ± standard deviation; non-normally distributed variables are presented as median (first quartile–third quartile). For group comparisons, the t-test was used for normally and the Mann–Whitney U-test was used for non-normally distributed metric variables. P-values <0.05 were considered to be statistically significant. Spearman correlation coefficient (ρ) was calculated for correlation analysis. Linear regression models (raw and with adjustment for BMI or body fat mass (BIA and MRI), age, months post-delivery) were conducted with the dependent variables (all logarithmized) peak oxygen uptake ("VO_{2peak}"), peak oxygen uptake per body weight ("VO_{2peak}/kg") and maximum workload in cardiopulmonary exercise testing ("Max. load") and "pGDM/control-status" as independent variable. We also calculated raw and adjusted (BMI or body fat mass (BIA and MRI), age, months post-delivery, pGDM/control status) linear regression models with "leptin" (logarithmized) as dependent and "VO_{2peak}", "VO_{2peak}/kg" and "Max. load" as independent variables. All statistical calculations were performed using the SAS statistical software package, version 9.3 (SAS Institute Inc., Cary, NC, USA) or R version 3.0.2 (http://www.R-project.org).

Results

From November 2011 to May 2016, 304 women were recruited into the PPSDiab study cohort. This analysis focuses on the baseline visit, which was 3 to 16 months after delivery (Fig 1). We excluded five women from this analysis, two because of type 1 diabetes diagnosed during follow-up, two because of overt hyperthyroidism and one because of an acute upper respiratory infection at baseline (Fig 1). 58 women declined to participate in cardiopulmonary exercise testing and 5 were excluded from the analysis due to an invalid exercise test (technical failure in measurement of O_2/CO_2 curves: n = 3; failure in measurement of O_2/CO_2 curves due to leaky mask: n = 1; unmet exhaustion criteria (low RER): n = 1). Consequently, the final sample consisted of 236 women, 152 women pGDM and 84 control subjects (Fig 1). Women with a valid exercise test were slightly older and less overweight than those without (S1 Table). The proportion of participants with a valid test was comparable in women pGDM and controls.

The baseline characteristics of the final study sample are shown in Table 1. Women pGDM had larger waist circumference, higher values for BMI, fat mass measured in BIA and MRI, blood pressure, hs-CRP, HDL cholesterol, triglycerides, as well as plasma leptin compared with the control group. Fasting and 2-hour plasma glucose were higher and insulin sensitivity index (ISI) was lower in the pGDM group.

With respect to our first research question (differences in PF between pGDM and control subjects), women pGDM had lower VO_{2peak}/kg body weight, total VO_{2peak}, and maximum

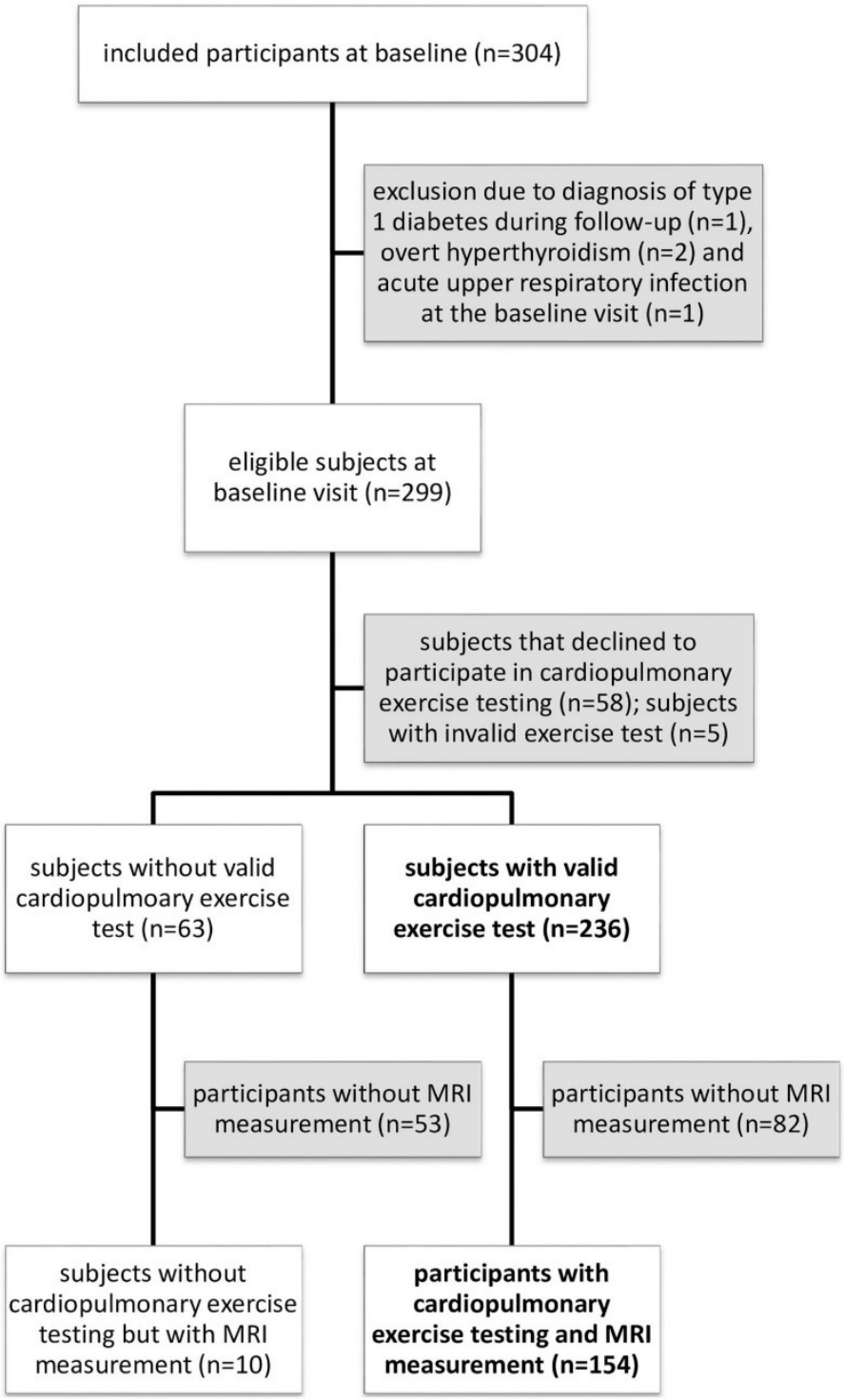

Fig 1. Recruitment flow chart. Cohorts analyzed in this manuscript are shown in bold type. MRI: magnetic resonance imaging.

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Table 1. Characteristics of the study sample. BIA: bioelectrical impedance analysis; BMI: body mass index; ISI: insulin sensitivity index; Max. load: maximum workload in cardiopulmonary exercise testing; MRI: magnetic resonance imaging; VO_{2peak}: peak oxygen uptake; VO_{2peak}/kg: peak oxygen uptake per body mass.

		Total	pGDM	Control subjects	p-value
Clinical parameter	**N**	236	152	84	
	Age [years]	35.9±4.1	36.2±4.1	35.4±3.9	0.1438
	Waist circumference [cm]	80.6±11.3	82.4±12.1	77.4±9.0	0.0004
	BMI [kg/m^2] (missing n = 2)	25.0±5.4	25.9±5.9	23.4±3.9	0.0001
	Fat mass in BIA (missing n = 2)	23.1±10.5	24.6±11.5	20.2±7.8	0.0007
	Total fat mass in MRI (n = 154)	25.5±10.8	27.1±11.7	22.6±8.3	0.0059
	Systolic blood pressure [mmHg]	117.2±11.4	118.8±11.3	114.1±11.0	0.0020
	Diastolic blood pressure [mmHg]	73.4±9.1	74.6±8.9	71.3±9.1	0.0081
	Months post-delivery	9.3±2.8	9.3±2.9	9.2±2.6	0.8764
Laboratory parameter	**Leptin [ng/ml]**	10.1 (4.9–15.7)	11.5 (6.7–18.8)	6.4 (3.6–11.6)	<0.0001
	Adiponectin [ng/ml]	11.6 (8.0–14.9)	10.7 (7.7–15.2)	11.7 (9.1–14.8)	0.3437
	hs-CRP [mg/dl]	0.1 (0.0–0.1)	0.1 (0.0–0.3)	0.0 (0.0–0.1)	0.0044
	LDL cholesterol [mg/dl]	104.0 (86.0–120.0)	104.0 (86.5–120.0)	104.5 (84.5–118.0)	0.7842
	HDL cholesterol [mg/dl]	62.0 (55.0–73.0)	61.0 (52.0–71.0)	64.0 (57.0–73.5)	0.0368
	Triglycerides [mg/dl]	67.0 (53.0–89.5)	71.5 (54.5–97.5)	60.0 (50.0–77.5)	0.0041
Glucose parameter	**Fasting plasma glucose [mg/dl]**	91.0 (87.0–97.0)	94.0 (89.0–99.0)	89.5 (84.0–92.5)	<0.0001
	Plasma glucose 2h [mg/dl]	109.0 (90.0–125.5)	117.5 (101.0–133.5)	91.0 (80.0–108.0)	<0.0001
	ISI	5.4 (3.6–7.6)	4.6 (3.0–6.7)	6.9 (5.2–8.7)	<0.0001
Cardiopulmonary exercise testing parameter	**VO_{2peak}/kg [ml/min/kg]**	27.6 (22.6–31.3)	25.7 (21.3–29.9)	30.0 (26.6–34.1)	<0.0001
	VO_{2peak} [ml/min]	1828 (1608–2092)	1733 (1552–2005)	1970 (1767–2238)	<0.0001
	Max. load [W]	129.0 (110.0–149.5)	122.5 (105.5–136.5)	141.0 (128.5–159.5)	<0.0001

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workload compared with the control group (VO_{2peak}/kg: 25.7 (21.3–29.9) vs. 30.0 (26.6–34.1), p<0.0001; VO_{2peak}: 1733 (1552–2005) vs. 1970 (1767–2238), p<0.0001; max. load: 122.5 (105.5–136.5) vs. 141.0 (128.5–159.5), p<0.0001; Table 1). The associations of group status with VO_{2peak}/kg, total VO_{2peak} and maximum workload remained significant after adjustment for BMI. This association was independent of BMI, age and months since delivery as shown by linear regression analyses (Table 2). Substituting body fat mass determined by BIA or MRI for BMI in these models gave comparable results (S2 and S3 Tables).

Table 2. Linear regression analysis - dependent variable VO_{2peak}/kg (ml/min/kg), VO_{2peak} (ml/min) or Max. load (all logarithmized), independent variable pGDM/control status. BMI: body mass index; CI: confidence interval; Max. load: maximum workload in cardiopulmonary exercise testing; VO_{2peak}: peak oxygen uptake; VO_{2peak}/kg: peak oxygen uptake per body mass.

VO_{2peak}/kg (ml/min/kg)			VO_{2peak}			Max. load		
Regression coefficient (95% CI)	**p-value**	**Adjusted R^2**	**Regression coefficient (95% CI)**	**p-value**	**Adjusted R^2**	**Regression coefficient (95% CI)**	**p-value**	**Adjusted R^2**
No adjustment								
0.18 (0.11–0.24)	<0.0001	0.11	0.12 (0.07–0.17)	<0.0001	0.08	0.16 (0.11–0.21)	<0.0001	0.13
Adjustment for BMI								
0.10 (0.05–0.15)	<0.0001	0.50	0.13 (0.07–0.18)	<0.0001	0.08	0.14 (0.09–0.20)	<0.0001	0.15
Adjustment for BMI, age, and months post-delivery								
0.10 (0.05–0.15)	<0.0001	0.51	0.13 (0.08–0.18)	<0.0001	0.09	0.15 (0.10–0.20)	<0.0001	0.16

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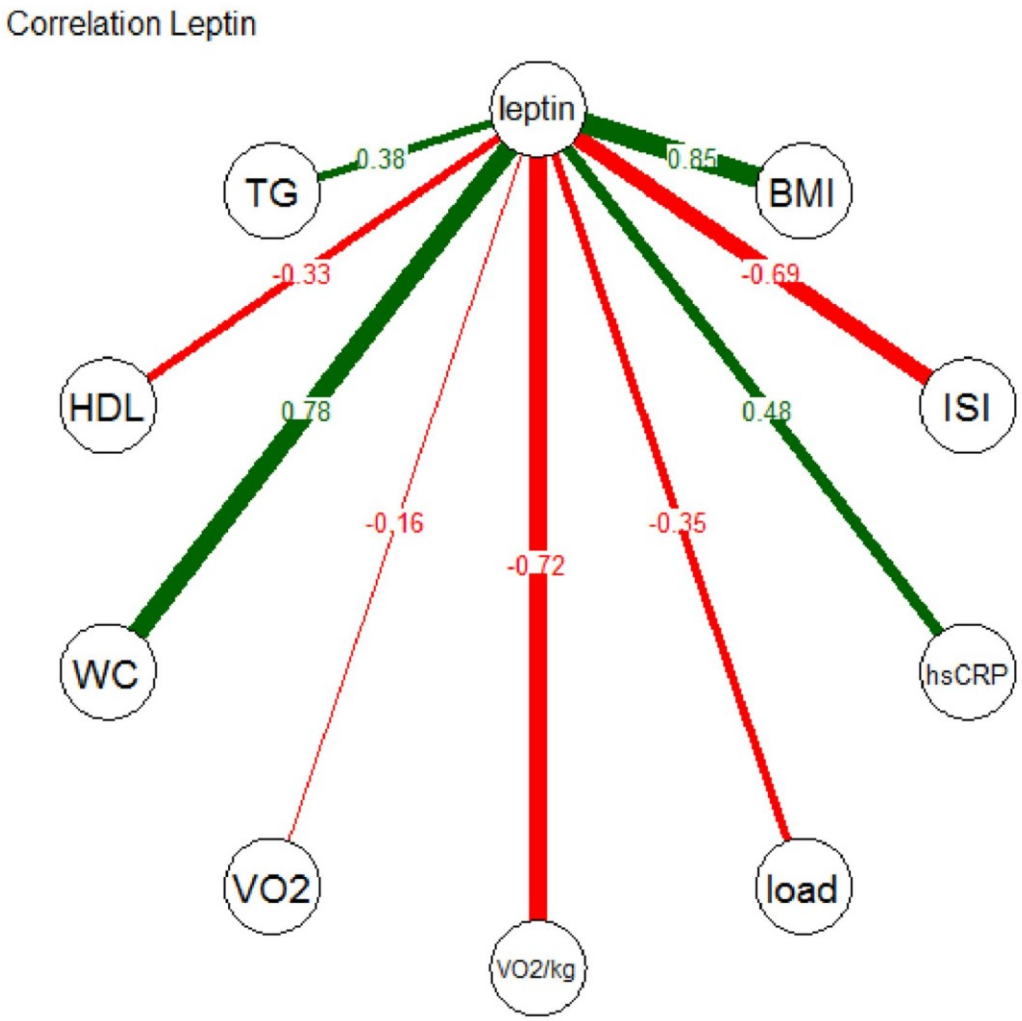

Fig 2. Spearman correlation coefficients for leptin and selected other variables. BMI: body mass index; TG: triglycerides; HDL: HDL cholesterol (mg/dl); ISI: insulin sensitivity index; load: maximum workload in cardiopulmonary exercise testing; VO2: peak oxygen uptake; VO2/kg: peak oxygen uptake per whole body mass; WC: waist circumference (cm); p-value <0.0001 for all, except for leptin with VO2 (p = 0.015).

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Concerning the second research question (association of PF and plasma leptin), we found negative correlations between plasma leptin and VO_{2peak}/kg, VO_{2peak}, and maximum load (Fig 2, VO_{2peak}/kg: $\rho = -0.72$, $p<0.0001$; VO_{2peak}: -0.16, $p = 0.015$; max. load: $\rho = -0.35$, $p<0.0001$).

In order to specifically examine the association of plasma leptin with PF, we calculated multiple linear regression models with adjustment for BMI, or for BMI, pGDM/control status, age, and months post-delivery (Table 3). These analyses confirmed negative associations between plasma leptin and VO_{2peak}/kg, VO_{2peak}, and maximum workload, which were independent of BMI and the other covariables. Similar models with body fat mass (measured with BIA and in MRI) instead of BMI gave comparable results (S4 and S5 Tables).

Table 3. Linear regression analysis - dependent variable leptin (logarithmized). BMI: body mass index; CI: confidence interval; pGDM: previous gestational diabetes; Max. load: maximum workload in cardiopulmonary exercise testing; VO_{2peak}: peak oxygen uptake; VO_{2peak}/kg: peak oxygen uptake per body mass.

VO_{2peak}/kg KG (ml/min/kg KG)			VO_{2peak}			Max. load		
Regression coefficient (95% CI)	**p-value**	**Adjusted R^2**	**Regression coefficient (95% CI)**	**p-value**	**Adjusted R^2**	**Regression coefficient (95% CI)**	**p-value**	**Adjusted R^2**
No adjustment								
-0.09 (-0.10/-0.08)	<0.0001	0.52	-0.001 (-0.001/-0.0002)	0.0007	0.04	-0.01 (-0.02/-0.01)	<0.0001	0.15
Adjustment for BMI								
-0.05 (-0.07/-0.04)	<0.0001	0.64	-0.0005 (-0.0007/-0.0003)	<0.0001	0.58	-0.01 (-0.01/-0.01)	<0.0001	0.60
Adjustment for BMI, pGDM/control status, age, and months post-delivery								
-0.05 (-0.07/-0.04)	<0.0001	0.64	-0.0004 (-0.0007/-0.0002)	<0.0001	0.58	-0.01 (-0.01/-0.004)	<0.0001	0.59

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Discussion

We measured PF (VO_{2peak}/kg, VO_{2peak}, and maximum workload during cardiopulmonary exercise testing) in women after gestational diabetes and in women who had a normoglycemic pregnancy. The women after gestational diabetes were less fit, independent of adiposity. We also examined the association of PF and plasma leptin in the whole study cohort. PF was negatively associated with fasting plasma leptin, also after adjustment for BMI or body fat mass.

Women pGDM carry an about 20% risk of developing T2D within 10 years of the index pregnancy [2], and our data suggest that low PF may be one modifiable risk factor contributing to this situation—although our cross-sectional analysis cannot show this directly. Our finding is in line with results from other types of at risk cohorts, e.g., older subjects with impaired glucose tolerance [25]. To our knowledge, PF has not been examined previously by objective measures, such as cardiopulmonary exercise testing, in women with recent GDM. The mean maximum oxygen uptake in women pGDM was over 200 ml/min lower than in control subjects. This represents a clinically meaningful difference. Exercise intervention programs can increase physical fitness [18] and may therefore also be valuable for women pGDM. This should be tested in a study setting.

Further differences between the pGDM and the control group in our study were a higher BMI and worse lipid profiles in the pGDM group. These findings are not surprising and indicate a higher prevalence of the metabolic syndrome in pGDM subjects.

We also saw an inverse association between plasma leptin and PF. Body fat mass remains the main determinant of fasting plasma leptin, but our finding was consistent for three measures of PF, as well as after adjustment for covariates.

Our results regarding an association of leptin with physical fitness are in agreement with work by Cicchella et al. [15], Chu et al. [26] and Miyatake et al. [17]. Chu et al. [26] studied a cohort of 268 male health professionals (age: 47–83 years; mean BMI: normal weight 23.2 kg/m^2; overweight 27.7 kg/m^2) but only relied on a questionnaire to estimate physical activity. Cicchella et al. [15] measured VO_{2peak}, as we did in this work, but the study cohort consisted of 10- to 12-year old boys. Miyatake et al. [17] found a BMI-independent negative association between leptin and PF in men and between leptin and physical activity in women in a middle aged, healthy Japanese cohort. Only in a 1996 study by Ostlund et al. [10] was the reverse association of leptin and VO_{2peak} lost after adjustment for percent body fat. However, only individuals between 60 and 70 years of age were included in that analysis, which suggests that these

results are not representative for the general population. Additionally, correcting for percent body fat may underestimate the role of PF, because of the positive association of PF and muscle mass (with the same fat mass, lower muscle mass leads to a higher percentage of body fat. Adjustment for percent body fat will then result in an over-adjustment of plasma leptin in those with lower muscle mass.). Taken together, an inverse and fat mass-independent correlation of plasma leptin and PF is supported by several studies from the literature and also our own data. Additionally, exercise interventions that increase physical fitness have been shown to also reduce plasma leptin [18].

Poorly trained muscle has a reduced insulin-mediated glucose uptake [27], but hormonal signaling also links PF to insulin sensitivity and glucose homeostasis. This involves myokines but also other hormones like epinephrine, glucocorticoids and, potentially, leptin [6, 28, 29]. An interesting hypothesis, which would be in agreement with our findings, is that PF affects "leptin resistance" where high plasma leptin levels coexist with late satiety and insulin resistance [9, 11, 30]. Several mechanisms have been implicated in this phenomenon [9, 11, 12], most prominently the saturation of the leptin transport system across the blood–brain barrier (BBB) and impaired intracellular signaling downstream of the leptin receptor [11, 31]. One possibility that could link PF to a reduction in "leptin resistance" is the finding that the transport of leptin across the BBB is increased by epinephrine [32–34]. Achieving and maintaining fitness requires regular exercise, which acutely increases plasma epinephrine with each workout [35]. As a consequence, leptin transport across the BBB and its central effects would be enhanced [34].

However, several alternative explanations can be found for the observed reverse association between PF and plasma leptin, e.g., a direct muscle–adipose tissue interaction or an influence of leptin resistance on central rewarding systems that promote voluntary physical activity [36]. Further studies of the effects of changes in PF and of acute and chronic exercise on plasma leptin levels, leptin transport across the BBB and central responses to leptin will be necessary to clarify this issue.

Strengths of this study include its homogeneous, all-female cohort with a small age range and very little concomitant disease and medication. Additionally, PF was measured by the gold standard method of cardiopulmonary exercise testing.

The homogeneous cohort in this study is also one of its weaknesses, as it precludes the generalization of our findings to other populations. The cross-sectional observational design of this analysis does not permit the investigation of cause–effect relationships. In our cohort, women with a valid exercise test were leaner than those who declined to participate in or did not complete exercise testing. This was true for both study groups and therefore probably did not bias our results. The cohort with a valid test also still covered a BMI-range from 18 to 44 kg/m^2.

In conclusion, our findings suggest that poor PF may contribute to the T2D risk of women with recent GDM. Additionally, our results support the hypothesis of a link between PF and leptin signaling. Specific studies on this issue in humans and animal models are certainly needed to confirm this assumption and, if true, elucidate the relevant pathways. Such studies seem warranted because leptin resistance is probably involved in the pathophysiology of obesity as well as of impaired glucose metabolism [37].

Supporting information

S1 Table. Characteristics of the study sample. BIA: bioelectrical impedance analysis; BMI: body mass index; ISI: insulin sensitivity index; MRI: magnetic resonance imaging.
(TIF)

S2 Table. Linear regression analysis - dependent variable VO_{2peak}/kg (ml/min/kg), VO_{2peak} or Max. load (all logarithmized), independent variable pGDM/control status. BIA:

bioelectrical impedance analysis; CI: confidence interval; Max. load: maximum workload in cardiopulmonary exercise testing; VO_{2peak}: peak oxygen uptake; VO_{2peak}/kg: peak oxygen uptake per body mass.
(TIF)

S3 Table. Linear regression analysis - dependent variable VO_{2peak}/kg (ml/min/kg), VO_{2peak} or Max. load (all logarithmized), independent variable pGDM/control status. Analysis of participants with MRI data (n = 154). CI: confidence interval; Max. load: maximum workload in cardiopulmonary exercise testing; MRI: magnetic resonance imaging; VO_{2peak}: peak oxygen uptake; VO_{2peak}/kg: peak oxygen uptake per body mass.
(TIF)

S4 Table. Linear regression analysis - dependent variable leptin (logarithmized), independent variable VO_{2peak}/kg (ml/min/kg), VO_{2peak} or Max. load. BIA: bioelectrical impedance analysis; CI: confidence interval; pGDM: previous gestational diabetes; Max. load: maximum workload in cardiopulmonary exercise testing; VO_{2peak}: peak oxygen uptake; VO_{2peak}/kg: peak oxygen uptake per body mass.
(TIF)

S5 Table. Linear regression analysis - dependent variable leptin (logarithmized), independent variable VO_{2peak}/kg (ml/min/kg), VO_{2peak} (logarithmized) or Max. load (logarithmized). Analysis of participants with MRI data (n = 154). CI: confidence interval; Max. load: maximum workload in cardiopulmonary exercise testing; MRI: magnetic resonance imaging; pGDM: previous gestational diabetes; VO_{2peak}: peak oxygen uptake; VO_{2peak}/kg: peak oxygen uptake per body mass.
(TIF)

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Supplemental Material

S1 Table. Characteristics of the study sample. BIA: bioelectrical impedance analysis; BMI: body mass index; ISI: insulin sensitivity index; MRI: magnetic resonance imaging.

		With cardiopulmonary exercise testing	Without cardiopulmonary exercise testing	p-value
	N	236	63	
	pGDM	152 (64.4%)	45 (71.4%)	0.2963
Clinical parameter	**Age [years]**	35.9±4.1	33.7±5.2	0.0021
	Waist circumference [cm]	80.6±11.3	84.1±12.1	0.0373
	BMI [kg/m²] (missing=2)	25.0±5.4	27.2±7.5	0.0352
	Fat mass in BIA (missing=5)	23.1±10.5	27.7±14.9	0.0266
	Total fat mass in MRI (missing=135)	25.5±10.8	28.1±23.8 (n=10)	0.5340
	Systolic blood pressure [mmHg]	117.2±11.4	118.8±12.6	0.3219
	Diastolic blood pressure [mmHg]	73.4±9.1	75.2±10.0	0.1959
	Months post-delivery	9.3±2.8	9.6±2.7	0.4821
Laboratory	**Leptin [ng/ml]**	10.1 (4.9-15.7)	11.2 (7.3-16.9)	0.0574
parameter	**Adiponectin [ng/ml]**	11.6 (8.0-14.9)	9.8 (6.4-14.5)	0.0953
	hs-CRP [mg/dl]	0.1 (0.0-0.1)	0.1 (0.0-0.3)	0.0408
	LDL cholesterol [mg/dl]	104.0 (86.0-120.0)	109.0 (82.0-124.0)	0.5922
	HDL cholesterol [mg/dl]	62.0 (55.0-73.0)	62.0 (46.0-69.0)	0.1333
	Triglycerides [mg/dl]	67.0 (53.0-89.5)	69.0 (59.0-100.0)	0.1672
Glucose parameter	**Fasting plasma glucose [mg/dl]**	91.0 (87.0-97.0)	94.0 (87.0-100.0)	0.1899
	Plasma glucose 2h [mg/dl]	109.0 (90.0-125.5)	113.0 (94.0-134.0)	0.2598
	ISI	5.4 (3.6-7.6)	4.7 (2.9-7.2)	0.1080

S2 Table. Linear regression analysis - dependent variable VO_{2peak}/kg (ml/min/kg), VO_{2peak} or Max. load (all logarithmized), independent variable pGDM/control status. BIA: bioelectrical impedance analysis; CI: confidence interval; Max. load: maximum workload in cardiopulmonary exercise testing; VO_{2peak}: peak oxygen uptake; VO_{2peak}/kg: peak oxygen uptake per body mass.

	VO_{2peak}/kg (ml/min/kg)			**VO_{2peak}**			**Max. load**		
	Regression coefficient (95% CI)	p-value	Adjusted R^2	Regression coefficient (95% CI)	p-value	Adjusted R^2	Regression coefficient (95% CI)	p-value	Adjusted R^2
Adjustment for fat mass in BIA									
	0.11 (0.06-0.15)	<0.0001	0.54	0.13 (0.07-0.18)	<0.0001	0.08	0.15 (0.09-0.20)	<0.0001	0.14
Adjustment for fat mass in BIA, age, and months post-delivery									
	0.11 (0.06-0.16)	<0.0001	0.55	0.13 (0.08-0.18)	<0.0001	0.09	0.15 (0.10-0.20)	<0.0001	0.15

S3 Table. Linear regression analysis - dependent variable VO_{2peak}/kg (ml/min/kg), VO_{2peak} or Max. load (all logarithmized), independent variable pGDM/control status. Analysis of participants with MRI data (n=154). CI: confidence interval; Max. load: maximum workload in cardiopulmonary exercise testing; MRI: magnetic resonance imaging; VO_{2peak}: peak oxygen uptake; VO_{2peak}/kg: peak oxygen uptake per body mass.

	VO_{2peak}/kg (ml/min/kg)			**VO_{2peak}**			**Max. load**		
	Regression coefficient (95% CI)	p-value	Adjusted R^2	Regression coefficient (95% CI)	p-value	Adjusted R^2	Regression coefficient (95% CI)	p-value	Adjusted R^2
Adjustment for total fat mass in MRI									
	0.08 (0.02-0.14)	0.0092	0.56	0.09 (0.03-0.16)	0.0045	0.04	0.11 (0.05-0.17)	0.0007	0.14
Adjustment for total fat mass in MRI, age, and months post-delivery									
	0.08 (0.02-0.14)	0.0065	0.56	0.10 (0.04-0.17)	0.0027	0.05	0.11 (0.05-0.18)	0.0004	0.14

S4 Table. Linear regression analysis – dependent variable leptin (logarithmized), independent variable VO_{2peak}/kg (ml/min/kg), VO_{2peak} or Max. load. BIA: bioelectrical impedance analysis; CI: confidence interval; pGDM: previous gestational diabetes; Max. load: maximum workload in cardiopulmonary exercise testing; VO_{2peak}: peak oxygen uptake; VO_{2peak}/kg: peak oxygen uptake per body mass.

	VO_{2peak}/kg (ml/min/kg)			**VO_{2peak}**			**Max. load**		
	Regression coefficient (95% CI)	p-value	Adjusted R^2	Regression coefficient (95% CI)	p-value	Adjusted R^2	Regression coefficient (95% CI)	p-value	Adjusted R^2
Adjustment for total fat mass in BIA									
	-0.05 (-0.06/-0.03)	<0.0001	0.67	-0.001 (-0.001/-0.0003)	<0.0001	0.64	-0.01 (-0.01/-0.01)	<0.0001	0.65
Adjustment for total fat mass in BIA, pGDM/control status, age, and months post-delivery									
	-0.05 (-0.06/-0.03)	<0.0001	0.67	-0.0005 (-0.001/-0.0003)	<0.0001	0.64	-0.01 (.0,01/-0.005)	<0.0001	0.65

S5 Table. Linear regression analysis – dependent variable leptin (logarithmized), independent variable VO_{2peak}/kg (ml/min/kg), VO_{2peak} (logarithmized) or Max. load (logarithmized). Analysis of participants with MRI data (n=154). CI: confidence interval; Max. load: maximum workload in cardiopulmonary exercise testing; MRI: magnetic resonance imaging; pGDM: previous gestational diabetes; VO_{2peak}: peak oxygen uptake; VO_{2peak}/kg: peak oxygen uptake per body mass.

	VO_{2peak}/kg (ml/min/kg)			**VO_{2peak}**			**Max. load**		
	Regression coefficient (95% CI)	p-value	Adjusted R^2	Regression coefficient (95% CI)	p-value	Adjusted R^2	Regression coefficient (95% CI)	p-value	Adjusted R^2
Adjustment for total fat mass in MRI									
	-0.05 (-0.06/-0.03)	<0.0001	0.70	-0.76 (-1.19/-0.33)	0.0006	0.67	-0.67 (-1.11/-0.23)	0.0034	0.66
Adjustment for total fat mass in MRI, pGDM/control status, age, and months post-delivery									
	-0.04 (-0.06/-0.03)	<0.0001	0.70	-0.69 (-1.14/-0.25)	0.0024	0.67	-0.58 (-1.04/-0.12)	0.0147	0.66

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Christina Gar, Marietta Rottenkolber, Vanessa Sacco, Sarah Moschko, Friederike Banning, Nina Hesse, Daniel Popp, Christoph Hübener, Jochen Seissler, Andreas Lechner

Patterns of Plasma Glucagon Dynamics Do Not Match Metabolic Phenotypes in Young Women

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Patterns of Plasma Glucagon Dynamics Do Not Match Metabolic Phenotypes in Young Women

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Context: The role of hyperglucagonemia in type 2 diabetes is still debated.

Objective: We analyzed glucagon dynamics during oral glucose tolerance tests (oGTTs) in young women with one out of three metabolic phenotypes: healthy control (normoglycemic after a normoglycemic pregnancy), normoglycemic high-risk (normoglycemic after a pregnancy complicated by gestational diabetes), and prediabetes/screening-diagnosed type 2 diabetes. We asked if glucagon patterns were homogeneous within the metabolic phenotypes.

Design and Setting: Five-point oGTT, sandwich enzyme-linked immunosorbent assay for glucagon, and functional data analysis with unsupervised clustering.

Participants: Cross-sectional analysis of 285 women from the monocenter observational study Prediction, Prevention, and Subclassification of gestational and type 2 Diabetes, recruited between November 2011 and May 2016.

Results: We found four patterns of glucagon dynamics that did not match the metabolic phenotypes. Elevated fasting glucagon and delayed glucagon suppression was overrepresented with prediabetes/diabetes, but this was only detected in 21% of this group. It also occurred in 8% of the control group.

Conclusions: We conclude that hyperglucagonemia may contribute to type 2 diabetes in a subgroup of affected individuals but that it is not a *sine qua non* for the disease. This should be considered in future pathophysiological studies and when testing pharmacotherapies addressing glucagon signaling. (***J Clin Endocrinol Metab*** **103: 972–982, 2018)**

Glucagon is the main antagonist of insulin. It raises plasma glucose by reducing glycolysis and increasing gluconeogenesis and glycogenolysis (1, 2). Glucagon secretion from α cells is triggered by hypoglycemia and inhibited by insulin from neighboring β cells. In turn, glucagon inhibits insulin secretion (2).

Unger *et al.* (3, 4) first postulated that elevated glucagon is a *sine qua non* in the development of diabetes. This marked the departure from an *insulinocentric* concept of type 2 diabetes pathogenesis to a *bihormonal* or even *glucagonocentric* model (4). In a *glucagonocentric* model, most metabolic derangements of diabetes

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Abbreviations: DI, disposition index; ELISA, enzyme-linked immunosorbent assay; GDM, gestational diabetes; HDL, high-density lipoprotein; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; ISI, insulin sensitivity index; ivGTT, intravenous glucose tolerance test; MRI, magnetic resonance imaging; oGTT, oral glucose tolerance test; PPSDiab, Prediction, Prevention, and Subclassification of gestational and type 2 Diabetes; RIA, radioimmunoassay.

are caused by the disinhibition of glucagon secretion (resulting from insulin-resistant α cells or impaired insulin release), but not directly by insufficient insulin action in other tissues (4, 5).

The issue of the different pathophysiologic models remains unresolved, at least in part due to technical difficulties: glucagon is unstable, difficult to measure because of many similar peptides in plasma (2, 6), and its concentration is very low (7). Furthermore, α cells are harder to isolate than their insulin-producing neighbors (8). This impedes cellular studies.

Current data on plasma glucagon levels in (pre)diabetic human subjects are also inconsistent. Several studies have found impaired glucagon suppression during an oral glucose tolerance test (oGTT) in prediabetic and diabetic individuals when compared with healthy controls (9–11). Other studies reported on increased fasting glucagon levels (12, 13). In contrast, Ahrén and Larsson (14) saw no differences between impaired glucose tolerance (IGT) and normoglycemic subjects, and Wagner *et al.* (15) observed rising glucagon values during an oGTT in 21% to 34% of healthy, insulin-sensitive individuals. These authors even found that this pattern predicted future metabolic health.

New sandwich enzyme-linked immunosorbent assays (ELISAs) with improved specificity for glucagon became available recently, and this prompted us to re-examine the issue in a postpregnancy cohort of young women. We compared three groups of study participants with different metabolic phenotypes: a control group (normoglycemic women, who had recently completed a normoglycemic pregnancy), a normoglycemic high-risk group for type 2 diabetes [normoglycemic women after a recent pregnancy complicated by gestational diabetes (GDM)] (16, 17), and a prediabetes/diabetes group (women with prediabetes or screening-diagnosed type 2 diabetes after GDM).

We first confirmed that average fasting plasma glucagon was higher and glucagon suppression during an oGTT was impaired in the normoglycemic high-risk and the prediabetes/diabetes groups, similar to what was seen in the majority of previous studies. However, our main research goal was to determine whether glucagon dynamics within each metabolic group were homogeneous or followed heterogeneous patterns. We used functional data analysis and unsupervised clustering to address this question.

Research Design and Methods

Study cohort

Study participants were women enrolled in the prospective, monocenter observational study Prediction, Prevention, and Subclassification of gestational and type 2 Diabetes (PPSDiab) between November 2011 and May 2016 (18). The cohort includes women with GDM during their last pregnancy and women following a normoglycemic pregnancy in a 2:1 ratio, recruited consecutively from the diabetes center and the obstetrics department of the University Hospital (Klinikum der Universität München) in Munich, Germany.

Premenopausal women 3 to 16 months after a singleton (n = 295) or twin (n = 9) pregnancy with live birth(s) were eligible to participate. The GDM diagnosis was based on a 75-g oGTT with cut-off values for GDM according to the International Association of the Diabetes and Pregnancy Study Groups recommendations (plasma glucose: fasting 92 mg/dL, 1 hour 180 mg/dL, and 2 hours 153 mg/dL). Women without a history of GDM and either a normal 75-g oGTT (n = 294) or a normal 50-g screening oGTT (<135 mg/dL plasma glucose after 1 hour, n = 10) after the 23rd week of gestation were included in the normoglycemic group.

Exclusion criteria for this study were alcohol or substance abuse, prepregnancy diabetes, and chronic diseases requiring continuous medication, except for hypothyroidism (n = 52), bronchial asthma (n = 8), mild hypertension (n = 4), gastroesophageal reflux (n = 2), and history of pulmonary embolism resulting in rivaroxaban prophylaxis (n = 1).

Written informed consent was obtained from all study participants, and the protocol was approved by the ethical review committee of the Ludwig-Maximilians-Universität (study ID 300-11).

Data used in this analysis were collected at the baseline visit of the PPSDiab study, 3 to 16 months after the index pregnancy. In addition to the baseline visit, post-GDM women also attended yearly follow-up visits with a 75-g oGTT.

Groups

We compared three groups of women: a control group (women normoglycemic at the baseline visit and after a normoglycemic pregnancy), a normoglycemic high-risk group (women normoglycemic at the baseline visit but with GDM during the preceding pregnancy), and a prediabetes/diabetes group [women with impaired fasting glucose (IFG), IGT, combined IFG plus IGT, or screening-diagnosed type 2 diabetes at the baseline visit and with GDM during the preceding pregnancy]. IFG [fasting plasma glucose ≥100 mg/dL (5.6 mmol/L)], IGT [2-hour plasma glucose ≥140 mg/dL (7.8 mmol/L)], and diabetes [fasting plasma glucose ≥126 mg/dL (7.0 mmol/L) or 2-hour plasma glucose ≥200 mg/dL (11.0 mmol/L)] were defined according to the criteria of the American Diabetes Association (19).

Measurements

We conducted a five-point 75-g oGTT with measurement of plasma glucose (Glucose HK Gen.3; Roche Diagnostics, Mannheim, Germany), serum insulin (chemiluminescent immunoassay; DiaSorin LIASON Systems, Saluggia, Italy), high-sensitivity C-reactive protein (wide-range C-reactive protein; Siemens Health Care Diagnostics, Erlangen, Germany), and blood lipids [low-density lipoprotein and high-density lipoprotein (HDL) cholesterol and triglycerides] (enzymatic caloric test; Roche Diagnostics, Mannheim, Germany) after an overnight fast.

Plasma glucagon was measured at all five time points of oGTT with an ELISA (Glucagon ELISA; Mercodia, Uppsala, Sweden; catalog no: 10-1271-01) and also a radioimmunoassay (RIA) (Merck Millipore, Darmstadt, Germany; catalog no: GL-32K) for 283 subjects. ELISA and RIA measurements gave different results (Supplemental Table 1; Supplemental Fig. 1). In particular, suppression of plasma glucagon during the oGTT was insufficiently represented in the RIA measurement. Sensitivity and specificity of ELISA for pancreatic glucagon (amino acids 33 to 61) have been proven to be superior to RIA (20, 21). Thus, for this analysis, we exclusively used glucagon data measured by ELISA (n = 299). Plasma for glucagon measurements was collected in BD p800 tubes (BD Biosciences, San Jose, CA), which contain specific proteinase inhibitors to stabilize glucagon and other metabolically important hormones. Plasma was immediately separated by centrifugation and directly frozen in aliquots on dry ice, before being transferred to a –80°C freezer within 1 hour from completion of the oGTT. Glucagon measurements were done in one batch and only from aliquots that had not been thawed previously.

Height and waist circumference were measured to the nearest 1 cm. Body mass and body fat mass were determined by a bioelectrical impedance analysis scale (Tanita BC-418; Tanita Corporation, Tokyo, Japan) (22, 23). Blood pressure was calculated as the mean out of two measurements in a resting seated position.

In addition to these basic tests, all study subjects were asked to participate in a magnetic resonance imaging (MRI) measurement and an intravenous glucose tolerance test (ivGTT) on a voluntary basis.

MRI (3 Tesla System, Ingenia, or Achieva; Philips Health Care, Hamburg, Germany) included determination of abdominal visceral adipose tissue volumes and liver fat content, using an mDixon low-fat fraction map. In the ivGTT, a glucose bolus of 0.3 g/kg body weight was injected over 1 minute with subsequent frequent blood sampling at 0, 2, 4, 6, 8, 10, 20, 30, 45, and 60 minutes. The measurements were used for the calculation of first-phase insulin response.

A detailed description of the study design, anthropometric, clinical, and MRI measurements, and methodologies of blood sampling and analysis can be found elsewhere (24).

Calculations

$$\text{Mean blood pressure} = (\text{diastolic value}*2 + \text{systolic value})/3$$

The insulin sensitivity index (ISI) according to Matsuda and DeFronzo (25) was calculated from the oGTT:

$$ISI = 10000 \Big/ \sqrt{[(glucose\,0' * insulin\,0') * (glucose\,0' + 2*(glucose\,30' + 60' + 90') + glucose120') / 8 * (insulin\,0' + 2*(insulin\,30' + 60' + 90') + insulin\,120')/8]}$$

The disposition index (DI) was calculated as (26):

$$DI = ISI * IR30$$

with

$$IR30 = insulin\,30' - insulin\,0'$$

ISI and insulin release 0' to 30' in the oGTT were previously validated with data from ivGTT-euglycemic clamp tests in this cohort (24).

Glucagon suppression indices were calculated as (27):

$$Early\ suppression = (1\text{-}[glucagon\,30'/glucagon\,0'])*100\%$$

$$Late\ suppression = (1\text{-}[glucagon\,120'/glucagon\,30'])*100\%$$

$$Overall\ suppression = (1\text{-}[glucagon\,120'/glucagon\,0'])*100\%$$

Area under the glucagon curve was calculated using the trapezoidal rule.

First-phase insulin response in the ivGTT test was calculated as the incremental area under the insulin curve from 0 to 10 minutes.

Statistical analysis

All metric and normally distributed variables are reported as mean ± standard deviation; nonnormally distributed variables are presented as median (first quartile to third quartile). Categorical variables are presented as frequency and percentage. The Kruskal-Wallis test was used to compare metric variables, and the χ^2 or Fisher's exact test was used to compare categorical variables. For *post hoc* analysis, Dunn's test was used. P values <0.05 were considered statistically significant.

Functional data analysis methods were used for the analysis of the oGTT measurements (28). In the first step, the five-point oGTT measurements were converted into continuous, smooth curves based on B-spline basis functions (29). Afterward, a functional principal component analysis was performed based on the fitted curves to analyze the temporal variation (28). In the next step, a cluster analysis was conducted to identify patients with similar plasma glucagon dynamics. Hierarchical clustering was performed on the first three principal components of the functional principal component analysis via the Hierarchical Clustering on Principal Components function of Husson *et al.* (30). Hierarchical clustering was performed using the Ward's criterion on the selected principal components. The number of clusters was chosen based on the growth of between-inertia. For the final partitioning, the k-means algorithm was performed with the partition obtained from the hierarchical tree as the initial partition. All statistical calculations were performed using SAS statistical software package version 9.3 (SAS Institute, Inc., Cary, NC) or R version 3.1.3 (www.r-project.org).

Results

Mean glucagon curves differ between metabolic groups

We recruited 304 women into the PPSDiab study cohort but excluded 19 from this analysis. Two women were excluded because of type 1 diabetes diagnosed during follow-up, two because of overt hyperthyroidism, and one because of an acute upper respiratory infection at baseline. Eight women were excluded from the control group due to pathological glucose tolerance at the baseline visit, and six women were excluded due to missing glucagon values.

Our final sample consisted of 285 study participants: 93 normoglycemic women after a normoglycemic pregnancy (control group), 121 normoglycemic women who had GDM (normoglycemic high-risk group), and 71

women with IFG, IGT, or newly diagnosed type 2 diabetes (prediabetes/diabetes group).

Baseline characteristics of the study cohort are shown in Table 1. Mean age and low-density lipoprotein cholesterol were comparable, but mean blood pressure, waist circumference, triglycerides, c-reactive protein, liver fat content, intra-abdominal fat, and fasting and 2-hour plasma glucose increased and HDL cholesterol and insulin sensitivity decreased from the control over the normoglycemic high-risk to the prediabetes/diabetes groups (all significant over the three groups; results of pairwise *post hoc* tests shown in Table 1).

We next compared plasma glucagon levels during the oGTT in the three groups (Table 1). Fasting plasma glucagon was significantly elevated, and early glucagon suppression was diminished in the prediabetes/diabetes group compared with the control group [median (Q1 to Q3) for fasting plasma glucagon: 6.0 (4.6 to 8.2) (pmol/L) vs 7.7 (5.6 to 11.2) (pmol/L); early glucagon suppression: 47.6 (32.8 to 57.9) (pmol/L) vs 32.0 (14.5 to 51.3) (pmol/L), respectively]. The normoglycemic high-risk group lay in between for these variables, but closer to the control group and not statistically different from it [median (Q1 to Q3) for fasting plasma glucagon: 6.6 (4.5 to 8.4) (pmol/L); early glucagon suppression: 41.3 (22.9 to 58.3) (pmol/L)] (Fig. 1; Table 1). Total glucagon suppression was similar in all three groups.

Similar to a recent publication by Faerch *et al.* (27), we further examined fasting glucagon values and glucagon suppression indices in women with isolated IFG compared with those with isolated IGT and combined IFG + IGT (Supplemental Fig. 2; Supplemental Table 2). Late and overall glucagon suppression was smaller in women with isolated IFG compared with both other groups [median (Q1 to Q3) late suppression: 41.8 (16.5 to 50.4) (%) vs 58.1 (43.1 to 71.3) (%) vs 58.9 (46.1 to 69.6) (%) and overall suppression: 58.9 (39.8 to 70.2) (%) vs 71.2 (68.4 to 81.0) (%) vs 73.7 (63.8 to 81.0) (%) in IFG vs IGT vs IFG + IGT,

Table 1. Baseline Characteristics of the PPSDiab Study Sample

	Control	Normoglycemic High-Risk	Prediabetes/Diabetes	*P* Value
n	93	121	71	
Glucose status				
NGT	93 (100.0%)	121 (100.0%)	—	
IFG	—	—	31 (43.7%)	
IGT	—	—	22 (31.0%)	
IFG + IGT	—	—	12 (16.9%)	
Type 2 diabetes	—	—	6 (8.5%)	
Age (y)	35.3 ± 4.2	35.2 ± 4.5	35.9 ± 4.5	0.6204
Mean blood pressure (mm Hg) (missing = 1)	85.8 ± 9.0	89.0 ± 8.6[a]	90.9 ± 10.3[a]	0.0026
BMI (kg/m^2) (missing = 4)	23.7 ± 4.0	25.2 ± 5.8	28.2 ± 7.1[a,b]	0.0001
Waist circumference (cm) (missing = 5)	78.1 ± 8.9	80.7 ± 11.2	86.6 ± 13.2[a,b]	0.0002
hsCRP (mg/dL)	0.04 (0.01–0.08)	0.06 (0.02–0.25)[a]	0.09 (0.02–0.30)[a]	0.0030
Triglycerides (mg/dL)	61.0 (51.0–77.0)	65.0 (50.0–87.0)	81.0 (62.0–130.0)[a,b]	<0.0001
HDL cholesterol (mg/dL)	64.0 (57.0–73.0)	63.0 (56.0–73.0)	56.0 (46.0–65.0)[a,b]	<0.0001
LDL cholesterol (mg/dL)	104.0 (88.0–118.0)	105.0 (89.0–120.0)	104.0 (85.0–124.0)	0.9035
Plasma glucose 0 min (mg/dL)	89.0 (83.0–92.0)	91.0 (87.0–95.0)	102.0 (97.0–106.0)[a,b]	<0.0001
Plasma glucose 120 min (mg/dL)	93.0 (81.0–108.0)	114.0 (96.0–122.0)[a]	141.0 (113.0–165.0)[a,b]	<0.0001
ISI (missing = 1)	6.8 (5.2–8.6)	5.5 (3.7–7.5)[a]	3.3 (2.1–4.6)[a,b]	<0.0001
DI (missing = 1)	297.4 (221.4–363.1)	246.6 (179.7–322.0)	160.0 (111.4–207.6)[a,b]	<0.0001
FPIR (missing = 152)	2.2 (1.4–3.5)	2.2 (1.6–3.5)	2.3 (1.5–3.9)	0.8218
Liver fat content (%) (missing = 132)	0.2 (0.0–0.8)	0.5 (0.0–1.1)	1.7 (0.0–4.1)[a,b]	0.0122
Intra-abdominal fat (L) (missing = 124)	1.4 (0.9–2.1)	1.8 (1.1–2.9)[a]	2.3 (1.3–3.2)[a]	0.0046
Glucagon 0 min (pmol/L)	6.0 (4.6–8.2)	6.6 (4.5–8.4)	7.7 (5.6–11.2)[a,b]	0.0069
Glucagon 30 min (pmol/L)	3.0 (2.4–4.7)	3.7 (2.5–4.9)	5.0 (3.0–7.6)[a,b]	<0.0001
Glucagon 60 min (pmol/L)	1.9 (1.4–3.1)	2.6 (1.8–3.7)	2.9 (2.0–4.4)[a]	0.0009
Glucagon 90 min (pmol/L)	2.1 (1.3–3.0)	2.1 (1.6–3.2)	2.5 (1.8–3.9)	0.0527
Glucagon 120 min (pmol/L)	2.3 (1.4–3.5)	2.2 (1.5–3.3)	2.3 (1.6–3.5)	0.5239
AUC glucagon	339.4 (248.5–473.6)	392.1 (283.5–518.2)	511.5 (353.4–615.2)[a,b]	0.0006
Early-suppression glucagon (0–30) (%)	47.6 (32.8–57.9)	41.3 (22.9–58.3)	32.0 (14.5–51.3)[a]	0.0055
Late-suppression glucagon (30–120) (%)	31.8 (8.9–49.6)	40.9 (14.9–56.7)	47.4 (33.3–63.6)[a,b]	<0.0001
Suppression glucagon (0–120) (%)	61.2 (48.2–76.9)	64.1 (49.5–74.4)	68.5 (57.3–75.0)	0.3130

Abbreviations: AUC, area under the curve; BMI, body mass index; FPIR, first-phase insulin response; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; NGT, normal glucose tolerance.

[a]Significant *post hoc* tests vs control.

[b]Significant *post hoc* tests vs normoglycemic high-risk.

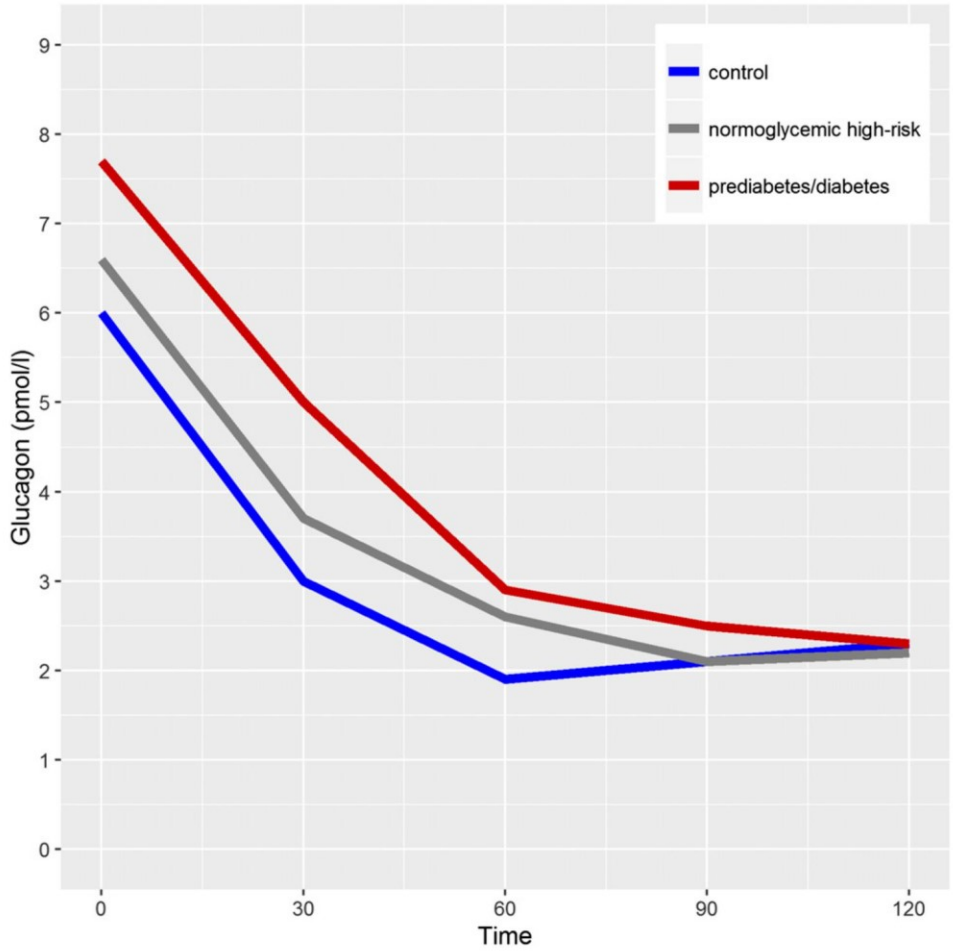

Figure 1. Glucagon during oGTT stratified by risk groups (blue = controls, gray = normoglycemic high-risk, red = prediabetes/diabetes).

respectively). We observed no significant differences in early glucagon suppression and fasting glucagon.

Plasma glucagon patterns are heterogeneous within each metabolic group

The five-point glucagon curves in response to oral glucose were heterogeneous between individuals (Fig. 2a). To examine this further, we calculated continuous, smooth curves from the five measurements during the oGTT based on B-spline basis functions (Fig. 2a). Stratified by group, these curves confirmed within-group heterogeneity of plasma glucagon dynamics (Supplemental Fig. 3). To permit pattern identification, we added a principal component analysis of the curves. The first three principal component factors explained 79%, 17%, and 3% of curve variance, respectively (Fig. 2b). We used these three principal components as input for an unsupervised cluster analysis (Fig. 2c). This identified four clusters corresponding to four distinct patterns of plasma glucagon dynamics (Fig. 2d).

Cluster 3 was the largest (n = 188; Table 2) and showed low mean fasting glucagon and rapid suppression during the oGTT (Figs. 2d and 3a). Cluster 2, the second largest (n = 62), had higher mean fasting glucagon but equally rapid suppression. Cluster 1 (n = 21) had high mean fasting glucagon and delayed suppression, and cluster 4 (n = 7) had low mean fasting glucagon and a rising curve after glucose ingestion (Fig. 3a; Table 2).

Cluster 1 contained the highest proportion of women from the prediabetes/diabetes group (53%), followed by cluster 2, cluster 3, and cluster 4. Women in cluster 1 had significantly higher body mass index, waist circumference, triglycerides, liver fat content, and intra-abdominal fat and lower HDL cholesterol and ISI than those in the other three clusters. The DI of cluster 1 was significantly lower than those of clusters 2 and 3 (Table 2). Cluster 4 included lean, insulin-sensitive women with a tendency toward low glucose values (Fig. 3b and 3c; Table 2).

Discussion

In our first analysis, we found that women with prediabetes/screening-diagnosed type 2 diabetes had higher fasting glucagon and delayed glucagon suppression during an oGTT compared with healthy control subjects (normoglycemic women after a normoglycemic pregnancy). Normoglycemic women after GDM, a high-risk group for type 2 diabetes (16, 17), lay in between,

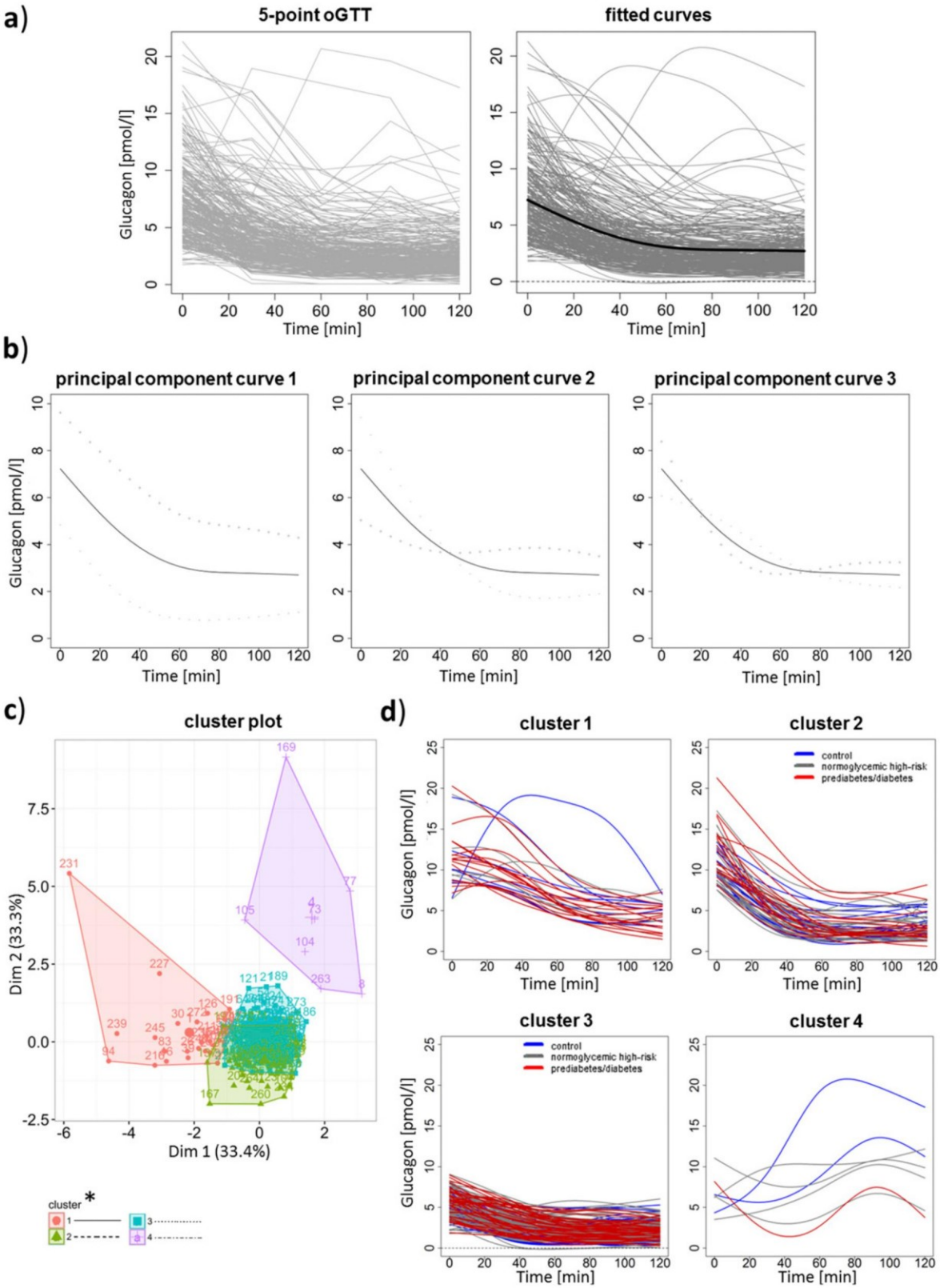

Figure 2. Process of functional data analysis. (a) Based on the five-point oGTT data curves, continuous, smooth curves were calculated (median indicated by black line). (b) Then, a principal component analysis of the curves was conducted (median indicated by solid line; extremes indicated by dotted lines). (c) The three principal components were used as input for an unsupervised cluster analysis (asterisk indicates line types used to represent the clusters in Fig. 3). (d) Fitted glucagon curves during oGTT stratified by the four clusters (colors: original risk groups as used in Table 1 and Fig. 1; blue = controls, gray = normoglycemic high-risk, red = prediabetes/diabetes).

Table 2. Baseline Characteristics of the PPSDiab Study Sample, Stratified by Clusters of Glucagon Dynamics

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	*P* Value
n	28	62	188	7	
Risk group					
Control	7 (25.0%)	19 (30.7%)	65 (34.6%)	2 (28.6%)	0.0279
Normoglycemic high-risk	6 (21.4%)	27 (43.6%)	84 (44.7%)	4 (57.1%)	
Prediabetes/diabetes	15 (53.6%)	16 (25.8%)	39 (20.7%)	1 (14.3%)	
Glucose status					
NGT	13 (46.4%)	46 (74.2%)	149 (79.3%)	6 (85.7%)	0.0099
IFG	5 (17.9%)	6 (9.7%)	19 (10.1%)	1 (14.3%)	
IGT	3 (10.7%)	7 (11.3%)	12 (6.4%)	0	
IFG + IGT	3 (10.7%)	3 (4.8%)	6 (3.2%)	0	
Type 2 diabetes	4 (14.3%)	0	2 (1.1%)	0	
Age (y)	33.5 ± 4.8	35.5 ± 4.4	35.7 ± 4.3[a]	35.0 ± 4.0	0.0315
Mean blood pressure (mm Hg) (missing = 1)	96.2 ± 8.6	89.4 ± 9.2	87.0 ± 9.0	85.6 ± 7.4	<0.0001
BMI (kg/m^2) (missing = 4)	33.3 ± 6.1	26.5 ± 6.4[a]	24.0 ± 4.6[a]	21.6 ± 1.5[a]	<0.0001
Waist circumference (cm) (missing = 5)	96.0 ± 11.9	83.8 ± 12.3[a]	78.6 ± 9.3[a]	73.5 ± 4.1[a]	<0.0001
hsCRP (mg/dL)	0.19 (0.07–0.47)	0.05 (0.01–0.17)[a]	0.04 (0.01–0.12)[a]	0.12 (0.05–0.38)	0.0004
Triglycerides (mg/dL)	91.5 (58.5–132.0)	62.5 (53.0–83.0)	67.5 (53.0–88.5)	63.0 (58.0–91.0)	0.0898
HDL cholesterol (mg/dL)	49.0 (44.5–61.5)	62.0 (51.0–73.0)	63.0 (56.0–73.0)	65.0 (56.0–70.0)	0.0012
Plasma glucose 0 min (mg/dL)	97.5 (90.5–106.0)	91.0 (88.0–97.0)	91.0 (86.0–97.0)	87.0 (82.0–92.0)	0.0078
Plasma glucose 120 min (mg/dL)	127.0 (115.5–154.5)	113.5 (95.0–130.0)[a]	106.5 (90.0–121.5)[a]	80.0 (74.0–92.0)[a,b]	<0.0001
ISI (missing = 1)	2.5 (1.9–4.3)	5.0 (3.3–6.9)[a]	5.8 (4.2–8.1)[a]	7.9 (5.6–8.3)[a]	<0.0001
DI (missing = 1)	152.0 (96.5–247.8)	230.2 (165.3–392.0)[a]	252.8 (176.7–324.4)[a]	232.9 (156.2–276.4)	0.0007
IR30 (missing = 1)	55.7 (37.1–82.2)	50.3 (36.4–86.1)	41.6 (30.9–60.1)	28.7 (26.2–41.3)[a,b]	0.0023
FPIR (missing = 152)[c]	3.9 (2.2–6.2)	3.3 (2.2–4.3)	2.1 (1.4–3.1)	2.1 (1.0–2.7) (n = 3)	0.0140
Liver fat content (%) (missing = 131)	2.4 (1.1–6.4)	0.7 (0.0–1.7)[a]	0.3 (0.0–0.8)[a]	0.1 (0.0–0.5)[a]	<0.0001
Intra-abdominal fat (L) (missing = 124)	3.4 (2.9–4.4)	2.0 (1.5–3.0)[a]	1.5 (1.0–2.3)[a,b]	1.1 (0.9–1.6)[a,b]	<0.0001

Abbreviations: BMI, body mass index; FPIR, first-phase insulin response; hsCRP, high-sensitivity C-reactive protein; IR30, insulin release 0′ to 30′ in the oGTT; NGT, normal glucose tolerance.

[a]Significant *post hoc* test: significant vs cluster 1.

[b]Significant *post hoc* test: significant vs cluster 2.

[c]The *post hoc* test for FPIR was conducted both including cluster 4 and after exclusion of cluster 4 (due to the small group size in cluster 4); in any case, the *post hoc* test has not reached significance.

with values closer to and not statistically different from the control group.

These results are in line with most previous studies that saw the highest fasting glucagon and most impaired glucagon suppression in subjects with diabetes, followed by those with prediabetes, and, at the low end, normoglycemic individuals (10–13, 27). In several nondiabetic cohorts, fasting glucagon was higher in insulin-resistant than in insulin-sensitive subjects (31–33). A majority of studies also found a positive association of plasma glucagon with obesity in groups with similar glucose tolerance (11, 13, 31). Some earlier studies had different findings. Ahrén and Larsson (14) reported that fasting and postprandial glucagon did not differ between IGT and normoglycemic subjects in 84 postmenopausal women. Wagner *et al.* (15) analyzed cohorts of nondiabetic individuals and found that, in 21% to 34% of subjects, glucagon was not suppressed until 120 minutes into the oGTT. These individuals were lean and insulin-sensitive, and also had a favorable prognosis of insulin sensitivity over time.

In their recent study, Faerch *et al.* (27) described that glucagon curves differed between individuals with IFG and those with IGT. They found a smaller overall decrease in glucagon during an oGTT in the group with isolated IFG compared with isolated IGT and combined IFG + IGT. Our analysis confirms this result, with the difference in overall glucagon suppression mainly caused by the late phase of the oGTT (Supplemental Fig. 2; Supplemental Table 2).

In our second analysis, we saw that plasma glucagon dynamics in the study cohort followed four different patterns, based on an unsupervised cluster analysis. The clusters detected did not fully or even closely match the predefined metabolic groups. We consider this the main finding of this paper. Subjects from the prediabetes/

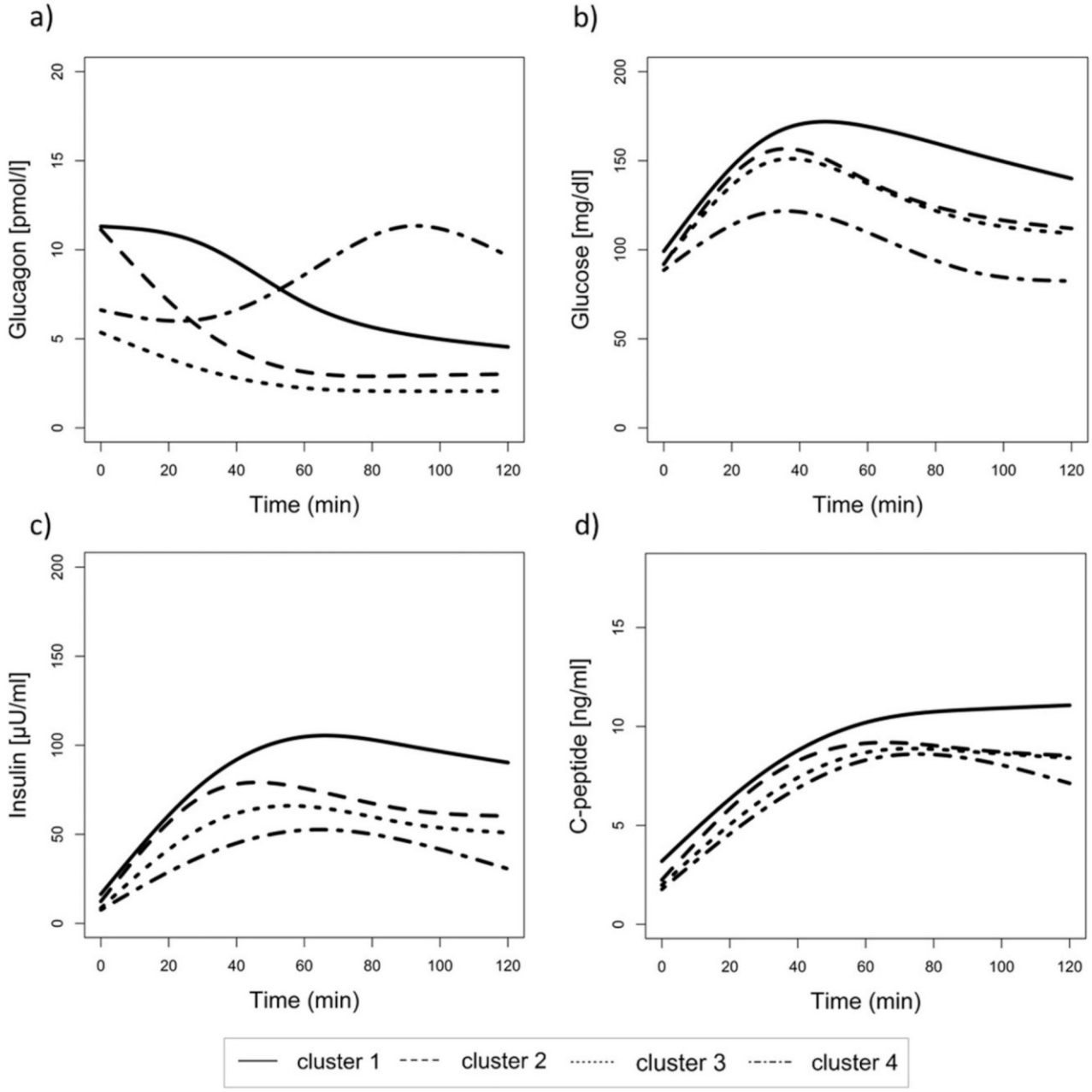

Figure 3. Means of (a) glucagon, (b) glucose, (c) insulin, and (d) c-peptide curves during oGTT stratified by the four clusters derived from the glucagon curves (Fig. 2).

diabetes group were overrepresented in cluster 1 (with high fasting glucagon and diminished suppression), but still only made up 50% of this cluster, which also contained 25% control subjects. Conversely, the majority of women from the prediabetes/diabetes group (n = 39; 55%) fell into cluster 3, the "most normal" cluster (with low fasting glucagon and rapid suppression). Therefore, hyperglucagonemia was not a universal prerequisite for impaired glucose metabolism or early type 2 diabetes. It only affected a subgroup of individuals.

Delayed glucagon suppression was clearly associated with obesity and metabolic syndrome markers in our study. This is evident from the clinical characteristics (*e.g.*, waist circumference, blood lipids, plasma glucose, and intra-abdominal and liver fat) of the subjects in cluster 1 compared with the other clusters (Table 2).

Hepatic steatosis may even be a cause of hyperglucagonemia, as it disrupts hepatic glucagon sensitivity and probably leads to reactive hypersecretion of the hormone (34). The association of liver fat and hyperglucagonemia was found independent of the presence of disrupted glucose metabolism (34, 35).

Impaired early insulin secretion could be another cause of delayed postprandial glucagon suppression, but we do not find evidence for this relationship. Early insulin and c-peptide levels during the oGTT and first-phase insulin

secretion in the ivGTT were not reduced in the women in cluster 1. The reduced DI results from lower insulin sensitivity (ISI) in this cluster, but not from reduced early insulin secretion (insulin release 0' to 30' in the oGTT) (Table 2). The α cell resistance to inhibition by insulin or a reactive glucagon hypersecretion due to a resistance of the liver is therefore the most likely explanations for our findings.

.Another noteworthy observation was the small cluster 4 (n = 7; 2.5% of participants), with low fasting glucagon, but rising glucagon levels during the oGTT. The women in this cluster were lean and insulin-sensitive and had low glucose levels. In this group, the rising glucagon probably is a physiologic response to avoid postchallenge hypoglycemia as a result of an overactive insulin response, which is not uncommon in lean, young women (36). Wagner *et al.* (15) associated rising glucagon during an oGTT with a favorable metabolic prognosis. Our small and probably not representative sample does not confirm this finding. Five of the 7 women in cluster 4 had had GDM (Table 2), and all of these 5 women developed prediabetes or diabetes during the follow-up of this study (mean duration of follow-up was 38.2 months; data not shown). In our cohort, this phenotype is also much less common than reported in the previous publication. However, given the small number of subjects in cluster 4, we find these observations interesting and worth following up on, but we do not claim that they constitute scientific evidence by themselves.

Finally, we believe it is important to use highly specific glucagon assays, in particular to study postprandial glucagon dynamics. We initially used a standard RIA, which strongly underestimated glucagon suppression (Supplemental Fig. 1). This was probably due to cross-reactivity with other peptides cleaved from proglucagon, such as oxyntomodulin, glicentin 1-61 (N-terminally elongated glucagon), and miniglucagon. Intestinal secretion of these peptides increases in the postprandial state, masking glucagon suppression (21, 37–39). Sandwich ELISAs, with antibodies against the N- and the C-terminal end of the glucagon molecule, circumvent this problem.

Strengths of this study are optimal preanalytic and analytic techniques plus a cohort homogeneous for age and sex and with little medication and concomitant diseases. We used functional data analysis to interpret glucagon dynamics and also consider this a strength of our work. This method can extract more of the information contained in a function than classic multivariate statistical techniques (40–42). Together with a subsequent cluster analysis, it permits the grouping of data sets according to their curve shapes. Using a recent history of GDM to identify a high-risk cohort early in the process of type 2 diabetes development should have limited secondary metabolic abnormalities to the minimal extent possible in a human study. At the same time, the study cohort can also be interpreted as a weakness, because results may not apply to the general population. Another limitation of this analysis is its cross-sectional design, which precludes the clarification of cause-effect relationships.

We conclude that fasting hyperglucagonemia and delayed postprandial glucagon suppression associate with insulin resistance, prediabetes, and diabetes, but are, in reality, only present in subgroups of individuals. Dysglycemia can develop without elevated plasma glucagon, and elevated glucagon does not preclude normoglycemia. Fasting hyperglucagonemia and delayed suppression are strongly linked to obesity and metabolic syndrome. Rising glucagon during an oGTT may be a rare phenomenon. It occurs in insulin-sensitive individuals with a tendency toward hypoglycemia, but does not necessarily indicate metabolic health.

Our results have consequences for the pathophysiologic understanding of type 2 diabetes and for the development of precision treatments. At present, glucagon agonists and antagonists are evaluated for diabetes therapy (1, 2, 43, 44). Based on our findings, patients should probably be stratified by glucagon values for such treatments. For those patients with hyperglucagonemia, glucagon antagonists could be an appropriate therapy, whereas for others, agonists may be useful to induce beneficial effects mediated through the glucagon receptor, such as weight loss (2, 44).

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Supplemental Material

Patterns of plasma glucagon dynamics do not match metabolic phenotypes in young women

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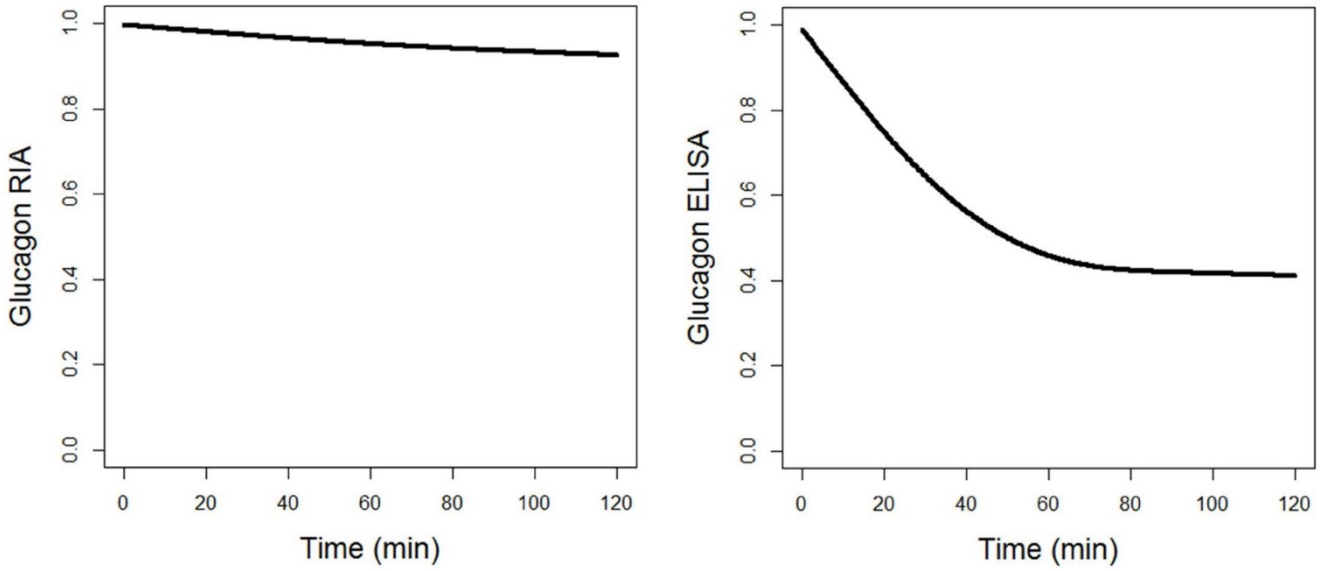
Supplemental Table 1: Comparison of glucagon values measured with RIA vs. ELISA (n=283); RIA: radioimmunoassay, ELISA: Enzyme-linked Immunosorbent Assay, GDM-NG: normoglycemic subjects at baseline after a pregnancy complicated by gestational diabetes; GDM-PGM: subjects after a pregnancy complicated by gestational diabetes with pathological glucose tolerance at baseline

	RIA				ELISA			
	total	**control**	**normoglycemic high-risk**	**prediabetes/diabetes**	**total**	**control**	**normoglycemic high-risk**	**prediabetes/diabetes**
Glucagon 0 min	1	1	1	1	1	1	1	1
Glucagon 30 min	0.95 (0.87-1.05)	0.94 (0.86-1.03)	0.95 (0.85-1.05)	0.98 (0.89-1.07)	0.58 (0.43-0.77)	0.53 (0.42-0.68)	0.59 (0.42-0.77)	0.68 (0.49-0.85)
Glucagon 60 min	0.90 (0.81-1.06)	0.90 (0.82-1.06)	0.91 (0.81-1.07)	0.90 (0.81-1.05)	0.38 (0.26-0.52)	0.34 (0.21-0.50)	0.40 (0.28-0.54)	0.43 (0.28-0.52)
Glucagon 90 min	0.90 (0.79-1.04)	0.91 (0.82-1.06)	0.89 (0.77-1.03)	0.89 (0.74-1.03)	0.36 (0.24-0.50)	0.33 (0.22-0.54)	0.38 (0.24-0.50)	0.36 (0.25-0.49)
Glucagon 120 min	0.89 (0.78-1.02)	0.94 (0.82-1.07)	0.89 (0.78-1.01)	0.87 (0.73-0.95)	0.35 (0.25-0.49)	0.39 (0.23-0.53)	0.36 (0.26-0.50)	0.32 (0.25-0.43)

Supplemental Fig. 1: Comparison of glucagon curves measured with RIA vs. ELISA (n=283); RIA: radioimmunoassay, ELISA: enzyme-linked immunosorbent assay.

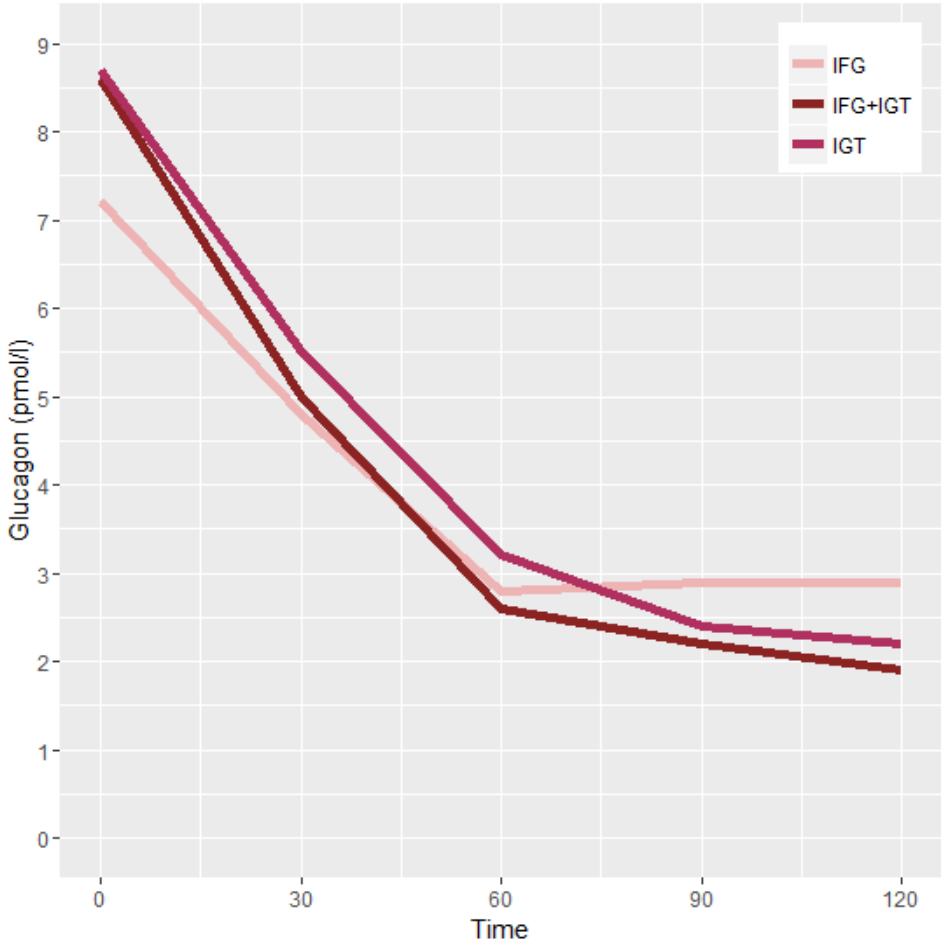


Supplemental Table 2: Fasting plasma glucagon and glucagon suppression indices during oGTT in different groups of prediabetes (isolated IFG, IGT, and combined IFG+IGT). IFG: impaired fasting glucose, IGT: impaired glucose tolerance.

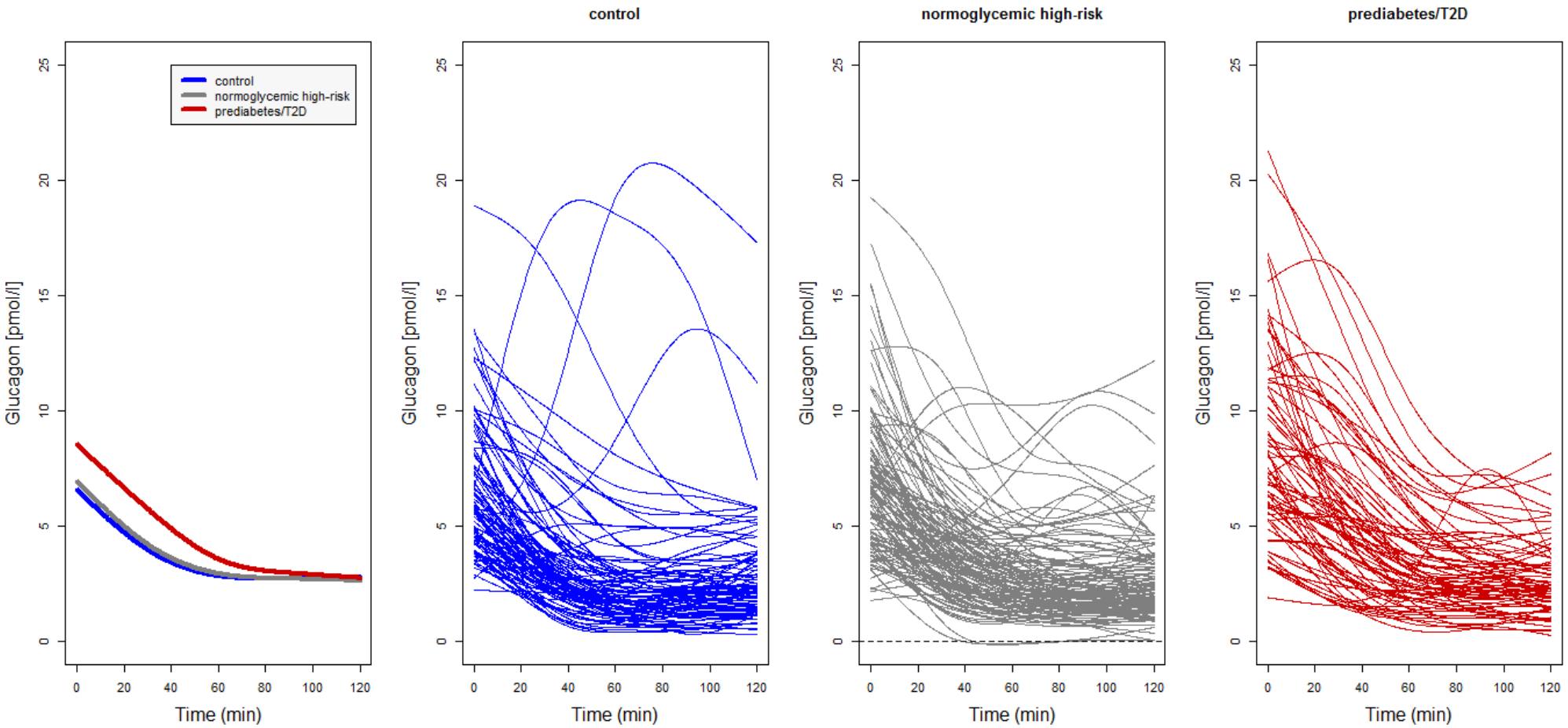
	IFG	IGT	IFG+IGT	p-value
n	31	22	12	
Glucagon 0 min [pmol/l]	7.2 (5.3-9.6)	8.7 (6.6-11.7)	8.6 (5.8-11.1)	0.2503
Early suppression glucagon (0-30) [%]	28.0 (11.9-65.5)	34.0 (17.4-51.7)	38.4 (17.6-52.1)	0.7273
Late suppression glucagon (30-120) [%]	41.8 (16.5-50.4)	58.1 (43.1-71.3)†	58.9 (46.1-69.6)†	0.0004
Suppression glucagon (0-120) [%]	58.9 (39.8-70.2)	71.2 (68.4-81.0)†	73.7 (63.8-81.0)†	0.0009

† in post hoc test significant vs. IFG

Supplemental Fig. 2: Plasma glucagon during oGTT in different groups of prediabetes (isolated IFG: light pink; isolated IGT: red; combined IFG+IGT: brown).
IFG: impaired fasting glucose, IGT: impaired glucose tolerance.



Supplemental Fig. 3: Glucagon curves stratified by risk group (blue = controls, gray = normoglycemic high-risk, red = prediabetes/diabetes) and subjects (each curve indicates one subject).



7.3. Gar et al. Critical reviews in clinical laboratory sciences 2017

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Serum and Plasma Amino Acids as Markers of Prediabetes, Insulin Resistance, and Incident Diabetes

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REVIEW ARTICLE

Serum and plasma amino acids as markers of prediabetes, insulin resistance, and incident diabetes

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ABSTRACT

Presently, routine screening misses many cases of prediabetes and early type 2 diabetes (T2D). Therefore, better biomarkers are needed for a simple and early detection of abnormalities of glucose metabolism and prediction of future T2D. Possible candidates for this include plasma or serum amino acids because glucose and amino acid metabolism are closely connected. This review presents the available evidence of this connectivity and discusses its clinical implications. First, we examine the underlying physiological, pre-analytical, and analytical issues. Then, we summarize results of human studies that evaluate amino acid levels as markers for insulin resistance, prediabetes, and future incident T2D. Finally, we illustrate the interconnection of amino acid levels and metabolic syndrome with our own data from a deeply phenotyped human cohort. We also discuss how amino acids may contribute to the pathophysiology of T2D. We conclude that elevated branched-chain amino acids and reduced glycine are currently the most robust and consistent amino acid markers for prediabetes, insulin resistance, and future T2D. Yet, we are cautious regarding the clinical potential even of these parameters because their discriminatory power is insufficient and their levels depend not only on glycemia, but also on other components of the metabolic syndrome. The identification of more precise intermediates of amino acid metabolism or combinations with other biomarkers will, therefore, be necessary to obtain in order to develop laboratory tests that can improve T2D screening.

Abbreviations: T2D: type 2 diabetes mellitus; NGT: normal glucose tolerance; PGT: pathological glucose tolerance; BMI: body mass index; BCAAs: branched-chain amino acids; AAAs: aromatic amino acids; oGTT: oral glucose tolerance test

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Introduction

The need for novel biomarkers for type 2 diabetes risk

A chronic and progressive disease, diabetes mellitus often yields secondary complications like neuro-, nephro-, and retinopathy [1]. The global prevalence of diabetes almost quadrupled from 108 to 422 million people from 1980 to 2014 [2]. Worldwide, the direct annual costs of type 2 diabetes (T2D) have been estimated to be more than US $825 billion [2–4]. Over 90% of the diabetes cases globally are defined as type 2 [5,6].

Currently, routine screening for T2D is usually performed with fasting plasma glucose or HbA1c measurements [7–9]. An oral glucose tolerance test (oGTT), the gold-standard for the diagnosis of prediabetes and T2D, is used infrequently, because it is time consuming and difficult to reproduce [10]. Screening with HbA1c or fasting plasma glucose misses many cases of prediabetes and early T2D. Measurement of HbA1c reliably detects later disease stages [8], but at that point, remission of the disease is unlikely [11,12]. However, if detections occur in the prediabetic stage, lifestyle, and pharmacologic interventions can often delay T2D manifestation [11,13–15]. Hence a new, simple, sensitive, and reliable laboratory test for prediabetes and early T2D would be desirable. In this respect, amino acids and related metabolites in the blood have been intensely studied in recent years as some of the most promising biomarker candidates.

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Supplemental data for this article can be accessed here.

Why amino acids? The interrelation of glucose and amino acid metabolism

Protein provides the most important structural and functional components of the human body. Muscle protein in particular also serves as an energy store. Protein-derived amino acids are constantly turned over and transported between organs and the blood stream. In anabolic phases, dietary amino acids are added to the body's protein pool. These phases alternate with catabolic states, which occur with energy deprivation or when dietary protein is available in excess of structural requirements. Then energy is provided by the breakdown of endogenous protein and amino acids can be used for gluconeogenesis [16]. Over-activation of gluconeogenesis occurs in most cases of prediabetes and T2D [17,18]. Glucagon stimulates this process in the liver and, to a lower extent, in the kidneys [16]. After deamination, amino acids form keto acids like acetyl-CoA (derived from leucine, isoleucine, lysine, and tryptophan), alpha-ketoglutarate (derived from glutamate, glutamine, arginine, proline, and histidine), succinyl-CoA (derived from valine), and fumarate (derived from aspartate, asparagine, tyrosine, and phenylalanine), which are further metabolized to oxaloacetate in the Krebs-cycle (Figure 1) [16,19]. Deamination of asparagine and aspartate directly forms oxaloacetate and alanine; the deamination of cysteine, glycine, serine, and tryptophan form pyruvate. Oxaloacetate and pyruvate feed gluconeogenesis [16,19]. Among the amino acids, alanine and glutamine are the most important gluconeogenic precursors in liver (major site of gluconeogenesis) [20–22].

In turn, non-essential amino acids can be synthetized *de novo* from glucose. Phosphoglycerate (an intermediate of glycolysis) and pyruvate are carboxylic acids. The addition of an amine group to these carboxylic acids results in amino acids. Thereby serine is gained from phosphoglycerate and alanine is gained from pyruvate [23]. Carboxylic acid-intermediates of the Krebs-cycle also form amino acids by transamination, e.g. aspartate from oxaloacetate and glutamate from alpha-ketoglutarate [23].

Free amino acids also modulate glucose metabolism by stimulating insulin secretion. For example, oral ingestion of amino acids simultaneously raises insulin and glucagon [24,25] without changes in the plasma glucose [26,27]. This hormonal response is dependent on the type of amino acid ingested [24].

In conclusion, amino acid and glucose metabolism are closely linked. Amino acids represent a main reservoir for gluconeogenesis and influence insulin and glucagon secretion. Both processes are altered early in the pathogenesis of T2D, which, at least in theory, makes amino acids good candidates for biomarkers in this area.

Pre-analytics – plasma or serum, fasting or non-fasting, stability

Studies on how pre-analytic procedures affect the amino acid content in serum and plasma yield inconsistent results. Many studies have found differences in amino acid concentrations between serum and plasma [28–35]. These differences range from a few to more than 100%, depending on the specific amino acid [28,31]. One possible reason for differences between serum and plasma is the delay until sample processing. Concentrations of amino acids in plasma or serum alter rapidly if analysis (or freezing) is delayed after the blood draw, even after centrifugation [30]. The concentrations of some amino acids rise, e.g. when amino acids are released from blood cells during clotting in a serum tube, while other amino acids are degraded when stored at room temperature [28,31,35]. Hence, time and sample temperature affect the results and stability varies for each amino acid [30,35,36]. Normally, one freeze-thaw cycle seems acceptable, but more than one may cause problems [36]. Different concentrations of serum and plasma metabolites can also result from matrix effects during analysis that occur due to ion suppression. In electrospray ionization mass spectrometry, competition of molecules for ionization can reduce the detection rate [29,34]. Hence, Denery et al. suggest enhanced sensitivity for small molecules like amino acids in serum, because serum contains less protein than plasma [29].

Other studies find comparable amino acid concentrations in serum and plasma [26,27] and Wedge et al. demonstrate that inter-individual differences in amino acid concentrations clearly outweigh possible differences between serum and plasma [34].

Another factor that profoundly affects the amino acid levels is the fasted/non-fasted state. After a protein containing meal, amino acid concentrations rise substantially and, depending on the food consumed, their composition in plasma and serum differs [16,37]. Therefore, blood sampling in the fasted state should be preferred in order to gain robust results.

In summary, plasma or serum amino acids should be measured in the fasted state for most research questions. Neither plasma nor serum has a consistent advantage. However, the processing time should generally be brief and rapid cooling of the samples should be attempted.

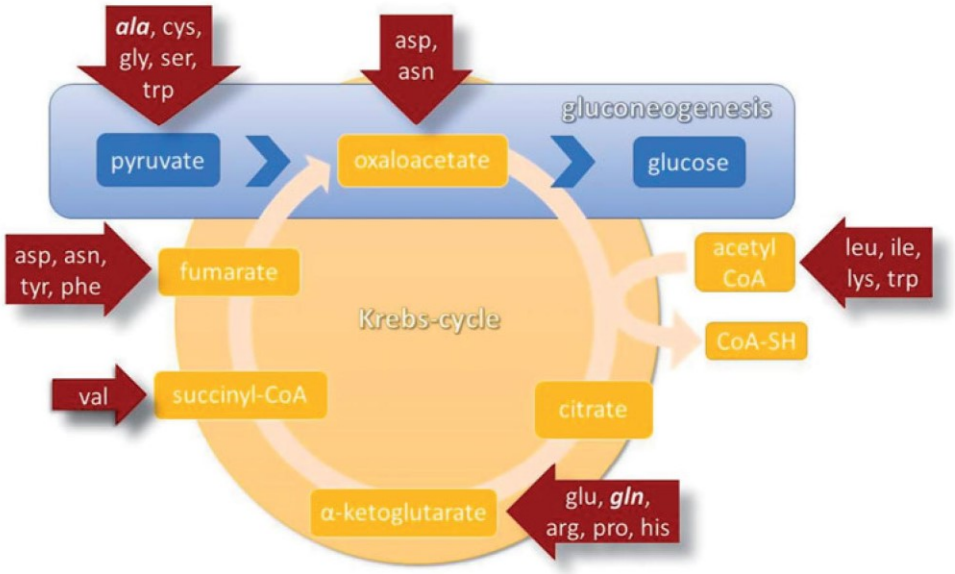

Figure 1. Schematic interrelation of amino acids and gluconeogenesis. Amino acids printed in bold italic represent the major gluconeogenic precursors from liver and intestine.

Analytic approaches to quantify amino acids

Building on the introduction of starch columns by Elsden and Synge [38], W. H. Stein and S. Moore have developed the first method for the chromatography of amino acids [39–42]. At this time, amino acids were detected via UV–Vis-spectroscopy with post-column derivatization using ninhydrin [43,44]. Later, ion-exchange columns replaced the starch columns [45,46]. Finally, analysis time had shortened with the "automatic recording apparatus", invented by D. H. Spackman et al. [47]. Instead of a derivatization after separation, pre-column derivatization with aromatic reagents subsequently permitted reversed-phase chromatography as small, polar molecules were converted into hydrophobic ones [48].

Modern gas and liquid chromatography of amino acids are still conducted primarily on ion-exchange and reverse-phase columns [49,50], but now usually coupled with high resolution mass spectrometry (MS) to analyze the effluent [49,51–55]. Gas chromatography is used less frequently today because it is only suitable for volatile yet thermo-stable molecules. Instead, high performance liquid chromatography (HPLC) is used widely in the metabolomics field. It maintains high sensitivity and quantitative reproducibility without a mandatory need for chemical derivatization [51]. Stationary phases can now be manufactured from very small particles of about 2 µm diameter that enhance specificity and sensitivity with a higher peak capacity compared to standard HPLC columns. This technique is usually recognized as ultra-high performance liquid chromatography (UHPLC) [56–59].

An alternative to chromatography, mass spectrometry (MS) can also directly separate most amino acids (separation of metabolites with different molecular weights or different fragmentation patterns) [60]. This method is often combined with direct sample injection, e.g. the widely used flow injection analysis (FIA). Even if the direct injection lowers the ionization efficiency, this combination analyzes rapidly and covers a wide range of metabolites with high selectivity and a very low limit of detection [51,61]. One disadvantage of direct MS for metabolite separation is the need for a sample preparation, which probably causes a loss of metabolites by a reaction with added substrates or a degradation when physical conditions are changed [62]. Additionally, ionization effects impair the quantification of compounds [51,62,63]. Hence, MS is primarily used for detection in combination with other separation methods.

Nuclear magnetic resonance (NMR) spectroscopy represents another key method for amino acid analysis. Compared to MS, NMR spectroscopy implies a lower sensitivity (micro-molar range) [58,64], yet sample preparation is easier to conduct and NMR signals are inherently semi-quantitative [58,63]. Also, NMR provides some information about molecule structure that is especially useful for non-targeted metabolomics [65]. The signal's integral reflects the relative concentration of a compound and the addition of an internal or external standard can make the method fully quantitative. To some degree, an electronic reference also permits the calculation of absolute concentrations [65].

In summary, neither method ideally separates and detects all metabolites [66]. For simultaneous

determination of various amino acids, NMR and (UHP)LC-MS are currently the most widely used techniques [51,58]. NMR provides more detailed information about molecular structure and the samples remain intact, while LC-MS has higher sensitivity and throughput [51,58,67].

Results

Plasma or serum amino acids as biomarkers for prediabetes and insulin resistance

In the previous paragraphs, we showed that in theory amino acids could be good biomarkers for prediabetes and T2D and that efficient methodologies exist that can quantify them in human serum or plasma. But, how effective are amino acids in identifying individuals with prediabetes or insulin resistance?

In cross-sectional studies, the three branched-chain amino acids (BCAAs) valine, leucine, and isoleucine [68–85], and the aromatic amino acids (AAAs) tyrosine [69,71,75,81,82] and phenylalanine [69,71,74,75,78,81,82] show the most consistent positive associations with prediabetes and insulin resistance. An equally robust, but negative, association is found for glycine [73,76,80,82,85–88].

A less consistent finding is the association of prediabetes or T2D with higher levels of alanine [69,71,75,76,78,86], serine [73,76,80,81], proline [71,76,78,84], glutamine [68,75,76,78], the glutamine/glutamate ratio [71,80], glutamate [69,76,81,85], histidine [69,72,76], and lysine [69,76,80].

More detailed information on the current literature is provided in the Online Resource 1, where we update a previous systematic review [89] on the subject.

Novel cross-sectional data from a deeply phenotyped human cohort

We used original data obtained from our own study, PPSDiab, to illustrate how plasma amino acid concentrations also correlate with other components of the metabolic syndrome, beyond glucose metabolism. PPSDiab is a prospective cohort study of young women after a recent pregnancy. It was enriched for T2D at-risk individuals by recruiting women after gestational diabetes and women after a normoglycemic pregnancy in a 2:1 ratio. In a cross-sectional analysis of the baseline visit of the study, 8.9 ± 2.8 months after delivery, we measured 29 amino acids and related biogenic amines in fasting plasma from 153 women. Baseline characteristics are shown in Table 1. Detailed information about the study procedures are provided in the Online Resource 1.

Between subjects with normal glucose tolerance (NGT) and those with pathologic glucose tolerance (PGT) glycine (fold change (FC) = 1.21, Benjamini Hochberg corrected $p = .01$; NGT median 272.6 (95% CI: 228.1–340.7) µmol/l; PGT 224.6 (186.9–258.3) µmol/l) and glutamate (FC =0.76, $p = .01$; NGT 40.1 (28.2–50.3) µmol/l; PGT 53.3 (37.8–65.5) µmol/l) showed the largest differences. Other significant amino acids (after correction) were proline, the sum of the BCAAs, and isoleucine and leucine by themselves (Figure 2). All values of the group comparison are provided in the Online Resource 2.

Only glutamate and glycine were significantly (but weakly) associated with glucose status in a logistic regression analysis with the amino acids that significantly differed between the NGT and PGT group. In the BMI-adjusted model, solely glycine remained significant (Table 2).

Figure 3 depicts a heatmap of correlations between plasma amino acids and the main components of the metabolic syndrome. Details for this analysis are provided in the Online Resource 2. The pictured associations illustrate that some plasma amino acids are indeed markers for all aspects of this syndrome, not only disturbed glucose metabolism. This finding corresponds with several previous studies [90–95].

We believe these data illustrate the problems of amino acids as biomarkers for prediabetes. The overlap between normoglycemic and prediabetic individuals is high and associations with other components of the metabolic syndrome obscure the connections between amino acid levels and glycemia.

Plasma or serum amino acids to predict future incident T2D in prospective studies

Amino acid levels not only associate with current metabolic disease but, to a certain extent, also predict future T2D. In prospective studies, high levels of the BCAAs [75,83,96–104] [RR (95% CI) for T2D isoleucine: 1.36 (1.24–1.48); leucine: 1.36 (1.17–1.58); valine: 1.35 (1.19–1.53) in the meta-analysis by Guasch-Ferre et al. [89]] and the AAAs tyrosine [75,83,96,98,102–104] [RR (95% CI) = 1.36 (1.19–1.55) [89]] and phenylalanine [75,96,98,101–104] [RR (95% CI) = 1.26 (1.10–1.44) [89]], as well as low levels of glycine [83,99,100,101,103] [RR (95% CI) = 0.89 (0.81–0.96) [89]], were seen most consistently.

Some studies also report associations of incident prediabetes or T2D with higher levels of alanine [98,100,104] and the glutamine/glutamate ratio

Table 1. Baseline characteristics of the PPSDiab study cohort.

	Total	Glucose status		p value
		NGT	PGT	
n	151	113	38	
Post GDM	100 (66.2%)	65 (57.5%)	35 (92.1%)	<.0001
Clinical parameter (mean ± SD)				
Age [years]	35.6 ± 3.9	35.3 ± 3.8	36.5 ± 4.1	.1199
Waist circumference [cm] [missing =6]	81.5 ± 12.0	79.8 ± 11.3	86.5 ± 12.7	.0016
BMI [kg/m^2] [missing =1]	25.4 ± 5.7	24.5 ± 5.2	27.9 ± 6.5	.0008
Systolic blood pressure [mmHg] [missing =1]	119.2 ± 11.0	118.3 ± 10.6	121.9 ± 12.0	.1687
Diastolic blood pressure [mmHg] [missing =1]	74.9 ± 9.1	74.3 ± 8.6	76.8 ± 10.4	.3145
Months post delivery	8.9 ± 2.8	9.1 ± 2.6	8.2 ± 3.1	.0580
Laboratory parameter [median (Q1–Q3)]				
LDL cholesterol [mg/dl]	105.0 (89.0–122.0)	105.0 (92.0–119.0)	102.5 (83.0–125.0)	.7624
HDL cholesterol [mg/dl]	62.0 (52.0–71.0)	64.0 (56.0–73.0)	58.5 (46.0–64.0)	.0015
Triglycerides [mg/dl]	68.0 (54.0–91.0)	65.0 (51.0–88.0)	74.5 (60.0–110.0)	0217
Gamma glutamyl-transferase [U/l]	14.0 (12.0–20.0)	14.0 (11.0–18.0)	16.5 (13.0–23.0)	.0212
hsCRP (missing =18)	0.1 (0.0–0.3)	0.1 (0.0–0.2)	0.2 (0.0–0.3)	.0189
Glucose parameter [median (Q1–Q3)]				
Fasting plasma glucose [mg/dl]	91.0 (87.0–97.0)	90.0 (86.0–93.0)	102.0 (96.0–105.0)	<.0001
Plasma glucose 2 h [mg/dl]	109.0 (91.0–123.0)	104.0 (87.0–118.0)	131.0 (99.0–161.0)	<.0001
ISI [missing =2]	5.3 (3.5–8.0)	6.0 (4.4–8.4)	3.4 (2.3–4.9)	<.0001
HOMA-IR [missing =1]	1.7 (1.0–2.7)	1.3 (0.9–2.1)	2.8 (1.7–3.6)	<.0001
HbA1c [%] [missing =1]	5.4 (5.2–5.6)	5.4 (5.2–5.6)	5.5 (5.3–5.8)	.0085
HbA1c [mmol/mol]	36 (33–38)	36 (33–38)	37 (34–40)	.0085

NGT: normoglycemic glucose tolerance; PGT: pathological glucose tolerance; GDM: gestational diabetes mellitus; BMI: body mass index; LDL: low density lipoprotein; HDL: high density lipoprotein; hsCRP: high-sensitivity C-reactive protein; ISI: insulin sensitivity index; HOMA-IR: homeostatic model assessment-estimated insulin resistance; HbA1c: glycated hemoglobin.

[88,103], plus glutamine [88,98] and glutamate on their own [88,100], but these findings are less consistent.

Discussion

The clinical potential of plasma or serum amino acids as T2D biomarkers

The strongest differences in plasma amino acids prevail between individuals with prevalent and often long-standing T2D, and healthy individuals. In this setting, however, additional disease biomarkers are superfluous. With less extreme phenotypes, such as prediabetes or normoglycemic insulin resistance, the differences become smaller. Classic clinical risk markers, such as BMI, blood pressure, and lipids, correlate with the most promising amino acids in the blood (Figure 3). This correlation reduces their diagnostic potential for diabetes. The measurement of plasma amino acids, therefore, does not substantially improve clinical models [88,95,96,101]. Nevertheless, the available data are too consistent to fully neglect them, in particular for glycine and the BCAAs. Examining related intermediates of amino acid metabolism or combining these amino acids with other metabolite classes in diagnostic panels may, therefore, still foster clinically useful tests.

Possible pathophysiological roles of amino acids

Given the consistent associations of several amino acids with various stages of T2D development, it is also warranted to consider potential contributions of these metabolites to the pathogenesis of this disease.

Glycine

The strongest evidence for pathophysiologic involvement exists for glycine. Yan-Do et al. [105] have identified glycine receptors on pancreatic beta cells that activate chloride currents and promote membrane depolarization. The depolarization opens voltage dependent Ca^{2+}channels, followed by the subsequent secretion of insulin (Figure 4). In a positive feedback loop, insulin augments the glycine-induced current. Low levels of glycine could therefore impair pancreatic insulin release. Consistent with Yan-Do, Gonzalez-Ortis et al. [105,106] have shown that oral glycine supplementation can increase insulin secretion without affecting insulin sensitivity and Cochrane et al. have observed a decrease in blood glucose after glycine ingestion [107]. In our opinion, these results warrant further investigation regarding therapeutic applications.

Other possible links between a low blood-glycine concentration and a metabolic risk are increased glycine utilization for the formation of glutathione to counteract oxidative stress [108], an increased uptake rate of glycine for gluconeogenesis in insulin-resistant tissues [95], or an inverse correlation of glycine with visceral and subcutaneous adipose tissue mass [109]. Our data supports this inverse correlation, which can be seen in the negative association of glycine with

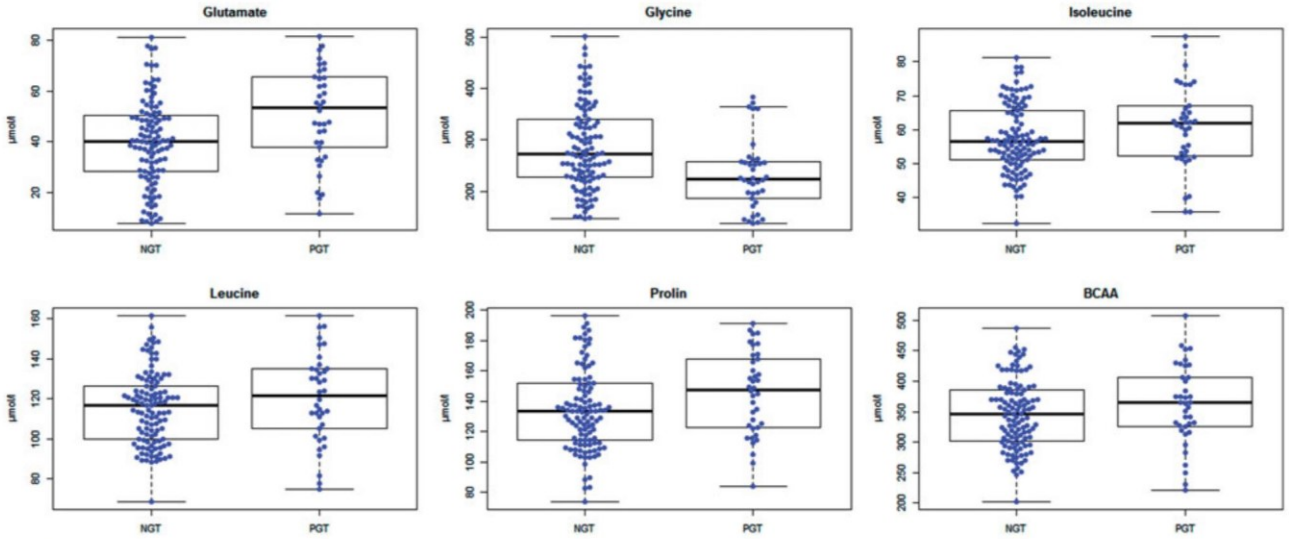

Figure 2. Beeswarm box plots for significant amino acids in NGT vs. PGT group comparison stratified for glucose status. NGT: normal glucose tolerance; PGT: pathological glucose tolerance.

Table 2. Logistic regression, dependent variable NGT/PGT.

	p value	Odds ratio	R^2
BMI	.0032	1.10 (1.03–1.17)	0.09
glutamate	.0071	3.29 (1.28–7.82)	0.08
glycine	.0005	0.09 (0.02–0.35)	0.13
isoleucine	.1143	5.19 (0.67–39.98)	0.02
leucine	.2207	4.10 (0.43–39.25)	0.02
proline	.1260	3.95 (0.68–22.89)	0.02
BCAA	.2184	4.02 (0.44–36.80)	0.02
BMI/glutamate			
BMI	.0488	1.07 (1.00–1.15)	0.11
glutamate	.1003	2.16 (0.86–5.43)	
BMI/glycine			
BMI	.0856	1.06 (0.99–1.14)	0.15
glycine	.0087	0.14 (0.03–0.61)	
BMI/isoleucine			
BMI	.0091	1.09 (1.02–1.17)	0.09
isoleucine	.6255	1.74 (0.19–15.90)	
BMI/leucine			
BMI	.0061	1.10 (1.03–1.17)	0.09
leucine	.8143	1.34 (0.12–15.12)	
BMI/proline			
BMI	.0045	1.10 (1.03–1.17)	0.10
proline	.2350	3.05 (0.48–19.28)	
BMI/BCAA			
BMI	.0062	1.10 (1.03–1.17)	0.09
BCAA	.8605	1.24 (0.11–13.65)	

BMI: body mass index; NGT: normoglycemic glucose tolerance; PGT: pathological glucose tolerance.

triglycerides and BMI and the positive association with HDL cholesterol (Figure 3). However, findings regarding the causal connection of glycine and obesity are contradictory. Kamaura et al. have observed a normalization of glycine levels after a life style intervention that decreased body weight and waist circumference [92]. In contrast, El Hafidi et al. find that oral glycine supplementation reduces visceral obesity in an animal study, via enhanced oxidation of fatty acids in the adipose tissue [110].

The branched-chain amino acids

For the BCAAs, their role in the pathogenesis of T2D remains debatable. Higher BCAAs with insulin resistance may result from muscle tissue that does not adequately respond to the anti-catabolic effect of insulin in this situation. This dysregulation would lead to an increased proteolysis of skeletal muscle and, because BCAAs are the most prominent class of amino acids in this tissue, their plasma concentration would subsequently rise [70,111,112]. This association is supported by a recent Mendelian randomization study by Mahendran et al., which suggests that insulin resistance drives high fasting BCAA levels and not the other way around [113].

An alternative explanation for higher BCAAs may be the disruption of BCAA-catabolism (Figure 5). The branched-chain aminotransferase (BCAT), most notably present in muscle tissue, metabolizes BCAAs to their analogous branched-chain keto acids [91,114–116]. Usually, the branched-chain keto acid dehydrogenase (BCKD) further oxidizes these keto acids to acetyl- or succinyl-CoA (depending on the initial BCAA) that are then used as substrates in the Krebs-cycle [115]. Several studies suggest a defect of BCKD as the cause for high BCAA levels in T2D [115,117,118]. This defect has been ascribed to occur due to three possible reasons: genetic alteration of BCKDH (the gene encoding for BCKD) in T2D and obesity [115], the disruption of enzyme activity by high fatty acids [115,118], and high insulin levels in insulin resistant and obese subjects [117].

A defect of BCKD may directly contribute to T2D development because of the accumulation of

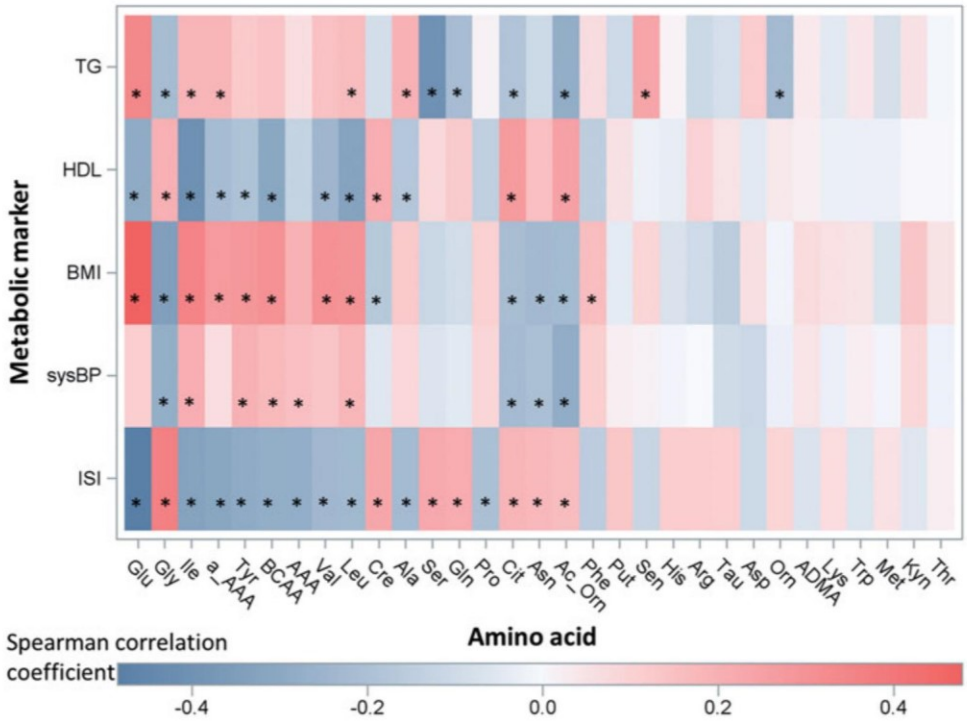

Figure 3. Spearman correlation coefficient heatmap between components of the metabolic syndrome and amino acids with related biogenic amines. $^{*}p < .05$; metabolites are sorted by strength of association with ISI (strongest association on the left). BMI: body mass index; HDL: high density lipoprotein; ISI: insulin sensitivity index; sysBP: systolic blood pressure; TG: triglycerides; ala: alanine; arg: arginine; asn: asparagine; asp: aspartate; cit: citrulline; gln: glutamine; glu: glutamate; gly: glycine; his: histidine; ile: isoleucine; leu: leucine; lys: lysine; met: methionine; orn: ornithine; phe: phenylalanine; pro: proline; ser: serine; thr: threonine; trp: tryptophan; tyr: tyrosine; val: valine; ac-orn: acetylornithine; ADMA: asymmetric dimethylarginine, alpha AAA; a_AAA: alpha-aminoadipic acid, creatinine, kynurenine, putrescine, serotonin, taurine; AAAs: the sum of the aromatic amino acids; BCAAs: the sum of the branched-chain amino acids. Details for this analysis are provided in the Online Resource 2.

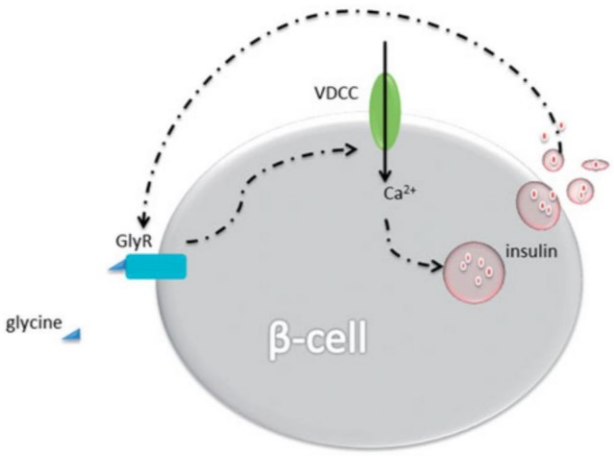

Figure 4. Scheme of suggested role of glycine receptors in beta-cell activation. Activation of the glycine receptor (GlyR) on the beta-cell induces Cl^{-}currents that open voltage dependent Ca^{2+}-channels followed by insulin secretion. Insulin itself enhances the activation of GlyR. Taken together, this constitutes a positive feedback loop between glycine and insulin.

branched-chain keto acids [91]. Branched-chain keto acids are degraded to C3- and C5-acylcarnitines and, in excess, could overwhelm the β-oxidation machinery. This would result in their degradation to toxic lipids, like diacylglycerol and ceramide, that probably contribute to insulin resistance [119–121] and beta-cell failure [91]. Similarly, by preventing the overproduction of branched-chain keto acids, e.g. by disruption of BCAT, it has been observed that glucose regulation, insulin sensitivity, and lipid profiles improve [115]. There are findings that contradict the idea that enzymatic malfunction in few or even single tissues could cause an increase in whole body BCAA concentrations. Burrage et al. have found that liver transplantation from BCKD defective individuals into healthy humans does not disrupt BCAA homeostasis [116]. The authors conclude that BCAA catabolism in other tissues, like skeletal muscle and adipose tissue, can compensate for the defect in the liver. Furthermore, transplantation of intact liver or adipose tissue into metabolically impaired mice or patients with maple syrup urine disease (a genetic defect of BCAA breakdown) normalized BCAA levels [115]. Nevertheless, these findings do not preclude the aforementioned effects of ubiquitous disrupted enzymes of the BCAA catabolism.

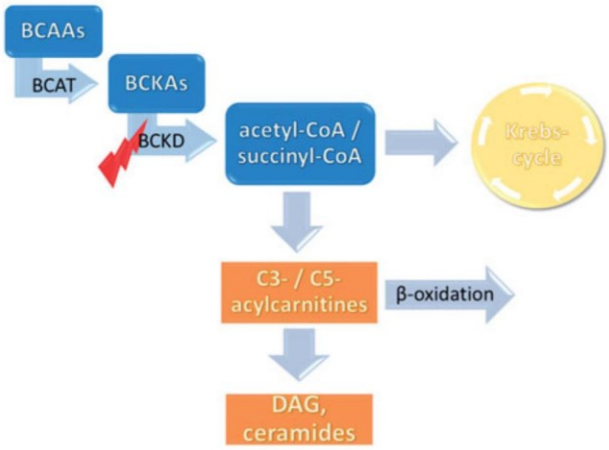

Figure 5. A model of perturbation in branched-chain amino acid catabolism. Branched-chain amino acids (BCAAs) are catabolized to branched-chain keto acids (BCKAs) by the branched-chain amino transferase (BCAT) and further oxidized to acetyl- or succinyl-CoA by the branched-chain keto acid dehydrogenase (BCKD). Acetyl- and succinyl-CoA will then serve as intermediates in the Krebs-cycle. A defect in BCKD leads to the accumulation of BCKAs, followed by the accumulation of BCAAs. An overflow of BCKAs will be compensated by alternative degradation of BCKAs to C3- and C5-acylcarnitines, which are degraded by beta-oxidation. If acylcarnitines exceed the capacity of beta-oxidation, diacylglycerol (DAG) and ceramides are formed, which promote insulin resistance.

Glutamate

The breakdown of BCAAs to branched-chain keto acids releases ammonia that is used for the synthesis of glutamate from alpha-ketoglutarate [111,122]. Increased breakdown of BCAAs to their keto acids will increase the synthesis of glutamate, thus, linking elevated BCAA with elevated glutamate levels.

In beta cells, leucine allosterically activates the glutamate dehydrogenase [123]. This enzyme catalyzes the conversion of glutamate to alpha-ketoglutarate with a glucose independent release of ATP. Thereby, the ATP/ADP-ratio increases; this increase primarily causes insulin release [123,124]. Glutamate synthesized in the beta-cell is transported into and stored in insulin secreting granules [124]. Subsequently, it is released together with insulin, linking hyperinsulinemia to high levels of glutamate.

In contrast, glutamate decreases insulin and increases glucagon secretion via activation of NMDA receptors on the beta cell and AMPA/Kainate receptors on the alpha cell, respectively [124,125]. This can worsen insulin resistance.

The aromatic amino acids

For the AAAs, it has been suggested that tyrosine and phenylalanine are increased because of a decreased activity of the tyrosine aminotransferase, caused by insulin resistance [126]. Additionally, an enhanced breakdown of methionine to cysteine/cystine, and finally to alpha-hydroxybutyrate, which has been reported to be elevated in T2D, possibly inhibits the tyrosine aminotransferase [126]. As phenylalanine is directly converted into tyrosine, alterations in one of the two amino acids will probably cause an alteration in the other.

Conclusions

The need for better biomarkers for prediabetes and future T2D remain and selected plasma or serum amino acids, in particular glycine and the BCAAs, are possible candidates for such biomarkers. For reproducible results of an amino-acid analysis, we recommend fasting samples, rapid pre-analytical processes, and a maximum of one freeze-thaw cycle. High-quality analytic methods are available. Besides their association to diabetes, amino acids are connected to other markers of the metabolic syndrome. Given this and the limited strength of the observed associations in cross-sectional and prospective studies, amino acids cannot yet serve as clinically relevant biomarkers by themselves. Therefore, future research should focus on broader metabolomics analyses, including other metabolite classes, and also examine metabolite interactions and additional intermediates of amino acid metabolism. The therapeutic potential of the effect of glycine on insulin secretion should be further investigated in human studies.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with

the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Data availability

The datasets generated during and/or analyzed during this study are not publicly available due to restricted consent obtained from study participants but are available from the corresponding author on reasonable request.

Disclosure statement

The authors report no declarations of interest.

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Supplementary data

Serum and Plasma Amino Acids as Markers of Prediabetes, Insulin Resistance, and Incident Diabetes—Systematic Literature Review and Results from a Cohort Study of Young Women.

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Online Resource 1: Materials and methods

Systematic review

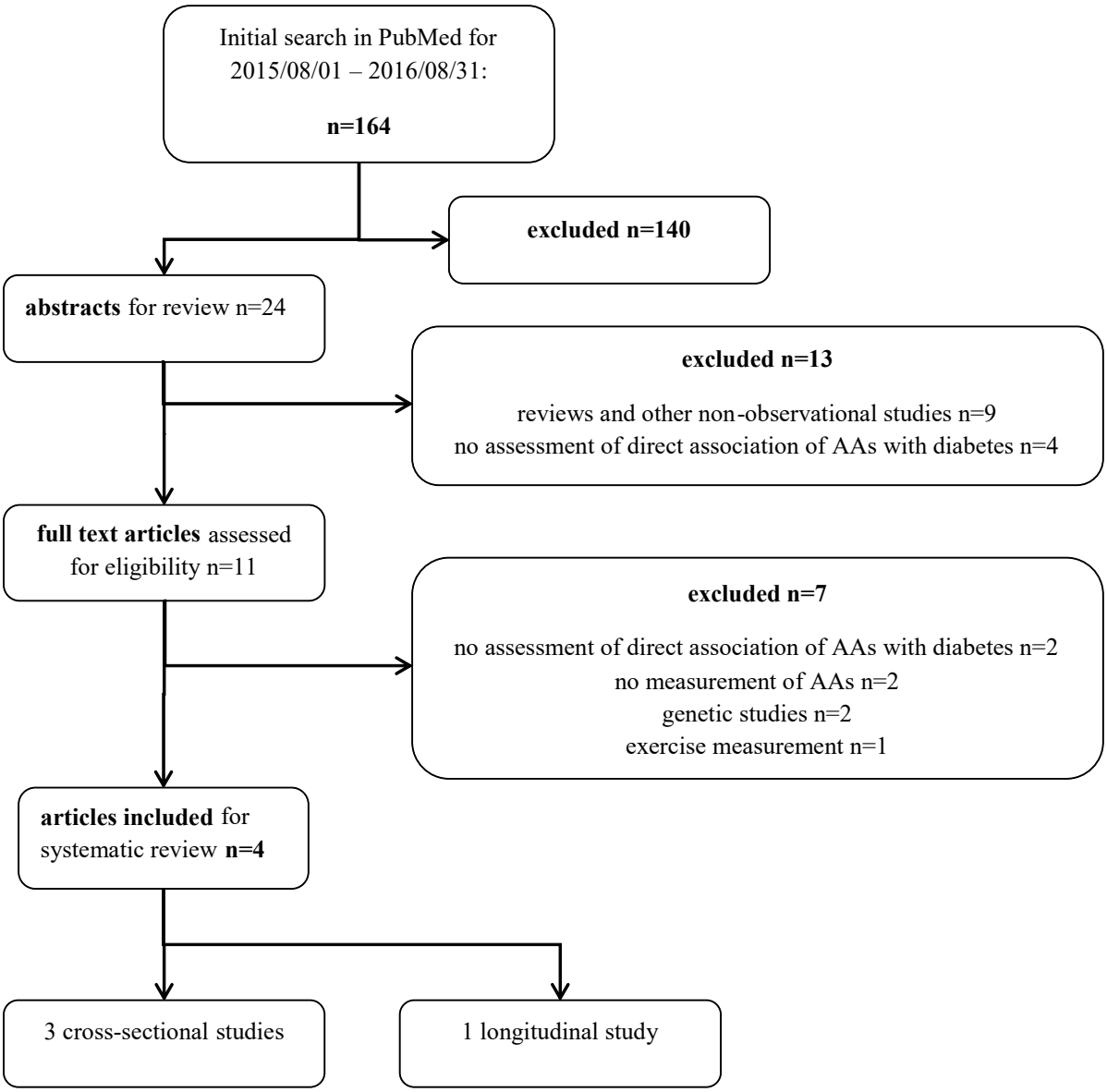
Our search in MEDLINE for previous systematic reviews on the subject produced/yielded a 2015, high-quality publication by Guasch-Ferre et al. [68]. We used this publication as the starting point for our literature search and conducted a qualitative review of observational human studies published subsequently that analyzed plasma amino acids and their relationship to prediabetes and T2D. Our search strategy mirrored Guasch-Ferre et al. [68] and focused on MEDLINE for publications from August 1, 2015 until May 10, 2017, because this period is not covered by Guasch-Ferre et al.

Search strategy:

MEDLINE was searched for articles published between 08/01/2015 and 05/10/2016 in English using the following search term:

((“metabolomics”[MeSH Terms] OR “metabolomics”[All Fields] OR “metabolome”[MeSH Terms] OR “metabolome”[All Fields]) AND (“diabetes mellitus”[MeSH Terms] OR (“diabetes”[All Fields] AND “mellitus”[All Fields]) OR “diabetes mellitus”[All Fields] OR “diabetes”[All Fields] OR “type 2 diabetes”[All Fields] OR “metabolic diseases”[MeSH Terms] OR “insulin resistance”[All Fields] OR “insulin sensitivity”[All fields] OR “metabolic syndrome x”[MeSH Terms] OR “metabolic syndrome”[All Fields] OR “HOMA-IR”[All Fields] OR “HOMA- β”[All Fields] OR “impaired glucose”[All Fields] OR “impaired fasting insulin”[All Fields]) AND “humans”[MeSH Terms])

All stages of publications (early view, in press, published) in English were considered relevant. Inclusion criteria were also adapted from Guasch-Ferre et al. [68] (observational human studies on prediabetes or T2D plus the measurement of a plasma or serum amino acid panel; in contrast to Guasch-Ferre et al., we did not include studies on urinary amino acids). Study quality was rated following the STROBE checklist [69].



Flow chart of literature search and selection process.

PPSDiab study design and participants

The observational, prospective cohort study PPSDiab [70] assessed women 3–16 months after delivery with either gestational diabetes or normoglycemia during pregnancy at the Medical Center of the University of Munich ("Klinikum der Universität München"), Germany, starting in November 2011. Recruitment for the sample analyzed here concluded in December 2013. Gestational diabetes mellitus was diagnosed with a 75g oral glucose tolerance test (oGTT) and according to the IADPSG criteria [71]. Exclusion criteria were alcohol or substance abuse and chronic diseases requiring systemic medication (except for hypothyroidism (n=23), mild hypertension (n=1), bronchial asthma (n=3), and seasonal allergies treated with desloratadine (n=1)). Four women included in this analysis without a clear medical diagnosis were also taking daily medication at the time of the study visit (proton-pump inhibitors (n=3) and dehydroepiandrosterone (n=1)).

Written informed consent was obtained from all study participants, and the study protocol was approved by the ethics review committee of the Ludwig-Maximilians-Universität in Munich, Germany.

Two participants were excluded from the analysis because of a diagnosis of hyperthyroidism (n=1) and positivity for GAD65 and IA2 antibodies (n=1) respectively. The final sample included 151 women.

PPSDiab study procedures

Participants underwent a five-point, 75g oGTT with measurement of plasma glucose and serum insulin (after an overnight fast) and were divided into normoglycemic subjects [NGT; fasting plasma glucose 5.6 mmol/l (<100 mg/dl) and 2h plasma glucose 7.8 mmol/l (<140 mg/dl)) and subjects with pathological glucose tolerance (PGT; fasting plasma glucose ≥5.6 mmol/l (≥100 mg/dl) and/or 2h plasma glucose ≥7.8 mmol/l (≥140 mg/dl)]. The oGTT data were also used to calculate the insulin sensitivity index according to Matsuda and DeFronzo [72] as well as the homeostasis model assessment insulin resistance index (HOMA-IR) [73].

Metabolite quantification out of fasted plasma samples was conducted using the targeted metabolomics assay Absolute*IDQ*™ p180 Kit (Biocrates Life Sciences AG, Innsbruck, Austria) and liquid chromatography-triple quadrupole mass spectrometry (LC-MS/MS). The assay procedures of the Absolute*IDQ*™ p180 Kit have been described in detail previously [74]. Concentrations of all metabolites were calculated using internal standards and reported in µM. Amino acids and related biogenic amines, as represented in the kit, were included in this analysis. These were: alanine (ala), arginine (arg), asparagine (asn), aspartate (asp), citrulline, glutamine (gln), glutamate (glu), glycine (gly), histidine (his), isoleucine (ile), leucine (leu), lysine (lys), methionine (met), ornithine (orn), phenylalanine (phe), proline (pro), serine (ser), threonine (thr), tryptophan (trp), tyrosine (tyr), valine (val), acetylornithine, asymmetric dimethylarginine, alpha-aminoadipic acid, creatinine, kynurenine, putrescine, serotonin, taurine, the sum of the aromatic amino acids (AAAs), and the sum of the branched-chain amino acids (BCAAs).

Anthropometric data and body fat mass, as determined by a bioimpedance measurement, were also obtained. A detailed description of the study design, anthropometric and clinical measurements as well as methodologies of blood sampling and analysis has been published previously [70]. In brief, fasting plasma samples were collected in protease inhibitor containing tubes (P800 vacutainer, BD Biosciences, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). After blood sampling, plasma was immediately processed and subsequently stored at -80°C until analysis.

Statistical analysis

All numerical values are presented as mean ± standard deviation (SD) or median (first - third quartile). Proportions were compared using chi-squared test or Fisher's exact test. Different groups were compared using a t-test or Mann–Whitney U-test. The Benjamini–Hochberg method was used to correct for multiple testing. To assess whether a variable is normally distributed or not, the Shapiro–Wilk test was used. Logistic regression models with NGT/PGT status as dependent and plasma concentrations and/or BMI as independent variables were performed for selected metabolites. All statistical analyses were performed using SAS statistical software package, version 9.3 (SAS Institute Inc., Cary, North Carolina, USA) and R version 3.1.3 (R Development Core Team, Vienna, Austria).

Online Resource 2

Study characteristics and main findings for the systematic review

Systematic review of the association of amino acids and biogenic amines with prediabetes and type 2 diabetes.

Reference	Study, population location	Study design	Number of participants	Technique and metabolite targets	Biological sample	Outcome	Covariates in fully adjusted model	Key findings	Other substudies included	PRISMA/ STROBE score*
[68]	Systematic review 06/2015	27 cross-sectional or case–control studies, 19 prospective studies	Participants: n=20–n=7098	Mainly LC- or GC-MS (24 studies); Targeted/ untargeted: 20/7	plasma / serum	**Cross-sectional or case–control:** 7 prediabetes-related measures (2h glucose, HOMA-IR); 9 studies prediabetes and T2D; 11 studies T2D **Prospective:** 3 prediabetes-related measures (fasting-/2h glucose, HOMA, oGTT glucose AUC); 8 studies prediabetes and T2D; 8 studies T2D	mainly: age, sex, BMI	**case-control and non-prospective population-based studies:** association with prediabetes: (↑) BCAAs, AAAs, ala, pro, gln/glu ratio (↓) gly, ser, gln; association with T2D: (↑) BCAAs, AAAs, ala, glu, lys, alpha-/beta-HB (↓) gly, his **Meta-analysis†**: ile RR=1.36 (1.24–1.48), leu RR=1.36 (1.17–1.58), val RR=1.35 (1.19–1.53), tyr RR=1.36 (1.19–1.55), phe RR=1.26 (1.10–1.44), gly (RR=0.89 (0.81–0.96), gln (RR=0.85 (0.82–0.89), ala (RR=1.19 (0.99–1.42), his (RR=0.98 (0.91–1.06), arg (RR=1.19 (1.14–1.25), orn (RR=1.10 (1.05–1.15), met (RR=1.45 (1.38–1.52)	–	1.0
[71]	Netherlands Epidemiology of Obesity (NEO) study, Netherlands	cross-sectional	n=533 (165 newly diagnosed T2D / 174 control subjects)	ESI-FIA-MS/ MS	Blood (no information about plasma or serum)	T2D	sex, age, and BMI	(↑) phe, tyr, sum of ile+leu (↓) gly	Cross-sectional analysis of postprandial changes in metabolites	0.81

[69]	Framingham Heart Study Offspring Study, US	cohort , prospective, population-based	n=2383 (154 DM), from baseline examination (1991–1995) to either one of the following examinations: exam 6 (1995–1998), 7 (1998–2001), or 8 (2005–2008)	LC with tandem MS	plasma	HOMA-IR; fasting glucose	age, sex, batch, BMI, log-triglycerides	association with HOMA-IR: (↑) ala, alpha-ketoglutarate, aminoadipic acid, ile, kynurenine, leu, pro, tyr, val (↓) asn, gly, SDMA association with fasting glucose: (↑) alpha-HB, beta-HB, isocitrate (↓) creatinine, gly, his, kynurenine, symmetric dimethylarginine, taurine, threonine	Cross-sectional sub-study of obesity and related cardiometabolic traits; longitudinal analysis of metabolites	0.84
[72]	Diabetes Risk Assessment Study, Canada	cross-sectional	n=30 (10 healthy-lean; 10 metabolically healthy-obese; 10 metabolically unhealthy-obese)	CE-MS	plasma	Prediabetes (fasting glucose, fasting insulin, HOMA-IR)	age, sex, and BMI	association with HOMA-IR and fasting insulin: (↑) pro, leu association with fasting glucose: (↑) creatine, pro	Analysis of fatty acids, postprandial analysis of metabolites	0.73
[70]	Qatar Metabolomics Study on Diabetes (QMDiab), Arab and Asian	cross-sectional	n=369 (188 T2D/181 control subjects)	Ultra-HPLC/GC-MS	plasma	T2D	age, sex, ethnicity, and BMI	(↑) 2-HB, alpha-ketobutyrate, alpha-hydroxypyruvate, 3-hydroxyisobutyrate (↓) pyroglutamine, 1-methylhistidine, 3-methoxytyrosine, citrulline	Analysis of metabolites in saliva and urine	0.76
[73]	Subjects recruited at the Virgen de laVictoria University Hospital and Carlos Haya Hospital (Málaga, Spain)	Cross-sectional	n=64 (31 non-prediabetic insulin sensitive; 33 prediabetic insulin resistant)	ESI-MS/MS	serum	Prediabetes (fasting insulin, HOMA-IR)	age (all), BMI (only val)	association with HOMA-IR and fasting insulin: (↑) val, glu (↓) gly	Analysis of association of BMI with metabolites	0.79

* Score calculated as sum of criteria fulfilled/sum of criteria requested.

†prospective association of amino acids with T2D; pooled RR for incident T2D per study-specific SD difference in the amino acid analyzed;

(↑), positive association (e.g., higher metabolite, higher risk); (↓), inverse association (e.g., lower metabolite, lower risk) with prediabetes traits or type 2 diabetes.

AAAs: aromatic amino acids; BCAAs: branched-chain amino acids; DM: diabetes mellitus, T2D: type 2 diabetes; HB: hydroxybutyrate

Comparison of plasma amino acids in NGT and PGT group (PPSDiab study)

	Fold change NGT vs. PGT-group	p value
alanine	0.963784208822498	0.449158005020214
arginine	1.02143595351558	0.830169269640774
asparagine	0.990206117990152	0.981519806304174
aspartate	0.937309545274435	0.449158005020214
citrulline	0.969422343700731	0.981519806304174
glutamine	1.06320978860949	0.0759106163604412
glutamate	0.758478365193794	0.0147770300563038
glycine	1.20764320964134	0.0147770300563038
histidine	1.00357421130837	0.947178590532944
isoleucine	0.92254202298691	0.0147770300563038
leucine	0.938875699691918	0.0355730504363148
lysine	1.00042060030229	0.981519806304174
methionine	1.0102707042081	0.853221343178087
ornithine	1.04449593578243	0.471775515093756
phenylalanine	0.949272721372389	0.0632558716223262
proline	0.922549074179386	0.0147770300563038
serine	1.05164897135846	0.471775515093756
threonine	1.01159818360666	0.981519806304174
tryptophane	1.00151202879749	0.981519806304174
tyrosine	0.964809232223344	0.418921357490307
valine	0.958299576211642	0.247730477421767
Ac-Orn	1.19792481428624	0.549542664740572
ADMA	1.00078011187636	0.982895333686895
alpha AAA	0.836062916498834	0.0577518126920937
creatinine	0.995972176258046	0.981519806304174
kynurenine	0.989914692206711	0.981519806304174
putrescine	1.05291533711337	0.549542664740572
serotonine	0.937303630463963	0.549542664740572
taurine	1.01876587858866	0.853221343178087
AAAs	0.946260539389602	0.0858961121448052
BCAAs	0.939418360444861	0.0355730504363148

Spearman correlation of amino acids and selected biogenic amines with components of the metabolic syndrome (PPSDiab study)

	correlation coefficient					p-value					number of participants (n)				
Variable	**ISI**	**BMI**	**sysBP**	**TG**	**HDL**	**ISI**	**BMI**	**sysBP**	**TG**	**HDL**	**ISI**	**BMI**	**sysBP**	**TG**	**HDL**
alanine	-0.2295	0.1292	0.0860	0.1956	-0.1784	0.0049	0.1152	0.2956	0.0161	0.0284	149	150	150	151	151
arginine	0.1175	-0.1044	-0.0027	-0.1140	0.1016	0.1535	0.2035	0.9742	0.1633	0.2145	149	150	150	151	151
asparagine	0.1767	-0.2308	-0.2051	-0.1101	0.1550	0.0311	0.0045	0.0118	0.1782	0.0575	149	150	150	151	151
aspartate	-0.1102	0.0618	-0.1151	0.1188	-0.0483	0.1810	0.4525	0.1607	0.1463	0.5557	149	150	150	151	151
citrulline	0.1872	-0.2155	-0.2232	-0.1881	0.2721	0.0223	0.0081	0.0060	0.0207	0.0007	149	150	150	151	151
glutamine	0.2127	-0.0946	-0.0507	-0.2198	0.1230	0.0092	0.2497	0.5381	0.0067	0.1325	149	150	150	151	151
glutamate	-0.4853	0.4787	0.1144	0.3392	-0.2941	0.0000	0.0000	0.1635	0.0000	0.0002	149	150	150	151	151
glycine	0.3680	-0.3436	-0.2792	-0.2212	0.2007	0.0000	0.0000	0.0005	0.0063	0.0135	149	150	150	151	151
histidine	0.1186	-0.0760	-0.0132	0.0111	-0.0378	0.1498	0.3555	0.8730	0.8920	0.6453	149	150	150	151	151
isoleucine	-0.3294	0.3558	0.2065	0.1862	-0.4107	0.0000	0.0000	0.0112	0.0221	0.0000	149	150	150	151	151
leucine	-0.2333	0.2992	0.1804	0.1654	-0.3299	0.0042	0.0002	0.0272	0.0424	0.0000	149	150	150	151	151
lysine	0.0711	0.0558	-0.0307	-0.0502	-0.0282	0.3890	0.4978	0.7093	0.5401	0.7308	149	150	150	151	151
methionine	0.0566	-0.0702	-0.0140	-0.0851	-0.0308	0.4930	0.3931	0.8648	0.2991	0.7070	149	150	150	151	151
ornithine	0.0934	-0.0151	-0.0307	-0.2279	0.0391	0.2572	0.8541	0.7096	0.0049	0.6340	149	150	150	151	151
phenylalanine	-0.1576	0.1630	0.1155	0.0721	-0.1532	0.0549	0.0463	0.1595	0.3789	0.0604	149	150	150	151	151
proline	-0.2063	0.1087	0.0916	0.0140	-0.1455	0.0116	0.1855	0.2647	0.8641	0.0746	149	150	150	151	151
serine	0.2255	-0.1168	-0.0681	-0.4068	0.0839	0.0057	0.1545	0.4079	0.0000	0.3056	149	150	150	151	151
threonine	0.0248	0.0515	-0.0259	-0.0113	-0.0001	0.7641	0.5315	0.7535	0.8904	0.9991	149	150	150	151	151
tryptophane	-0.0638	0.0487	0.0230	0.0441	-0.0292	0.4395	0.5537	0.7801	0.5907	0.7219	149	150	150	151	151
tyrosine	-0.2963	0.2858	0.1973	0.1289	-0.1939	0.0002	0.0004	0.0155	0.1148	0.0171	149	150	150	151	151
valine	-0.2386	0.3035	0.1408	0.1491	-0.2469	0.0034	0.0002	0.0857	0.0676	0.0022	149	150	150	151	151
ac-Orn	0.1638	-0.2264	-0.2856	-0.2837	0.2494	0.0459	0.0053	0.0004	0.0004	0.0020	149	150	150	151	151
ADMA	-0.0727	0.0743	0.0483	0.0362	0.0203	0.3782	0.3660	0.5576	0.6590	0.8044	149	150	150	151	151
alpha AAA	-0.3120	0.2754	0.0687	0.1847	-0.2205	0.0001	0.0006	0.4038	0.0232	0.0065	149	150	150	151	151
creatinine	0.2322	-0.1682	-0.0561	-0.0870	0.2070	0.0044	0.0396	0.4956	0.2883	0.0108	149	150	150	151	151
kynurenine	-0.0561	0.1423	0.0884	0.0528	-0.0017	0.4969	0.0825	0.2821	0.5194	0.9837	149	150	150	151	151
putrescine	0.1350	-0.0460	0.0246	-0.1074	0.0530	0.1007	0.5762	0.7647	0.1895	0.5178	149	150	150	151	151
serotonine	-0.1244	0.0923	0.0146	0.2384	-0.0274	0.1307	0.2612	0.8597	0.0032	0.7380	149	150	150	151	151
taurine	0.1144	-0.1572	-0.1017	-0.0837	0.0499	0.1648	0.0547	0.2154	0.3067	0.5427	149	150	150	151	151
AAAs	-0.2824	0.1981	0.1572	0.0657	-0.1324	0.0005	0.0151	0.0548	0.4230	0.1051	149	150	150	151	151
BCAAs	-0.2840	0.3032	0.1750	0.1441	-0.3078	0.0004	0.0002	0.0322	0.0775	0.0001	149	150	150	151	151

ISI: insulin sensitivity index, BMI: body mass index, sysBP: systolic blood pressure, TG: triglycerides, HDL: high density lipoprotein cholesterol, ac-Orn: acetylornithine, ADMA: asymmetric dimethylarginine, alpha AAA; alpha AAA: alpha-aminoadipic acid, AAAs: the sum of the aromatic amino acids, BCAAs: the sum of the branched-chain amino acids.

8. Summary

8.1. Summary of this thesis

Type 2 diabetes mellitus (T2D) is a global health burden with an increasing prevalence. Prevention of this disease is still insufficient, mainly due to an incomplete understanding of its pathophysiology and a lack of suitable tests for early diagnosis. To contribute to the understanding of early T2D pathophysiology, this thesis examines the role of physical fitness and the hormones leptin and glucagon in women with a recent history of gestational diabetes (GDM), a young, high-risk population for T2D. It also looks at plasma amino acids as potential early biomarkers for prediabetes and T2D.

All analyses were conducted in the Prediction, Prevention and Subclassification of type 2 Diabetes (PPSDiab) study, which prospectively follows women after a pregnancy complicated by gestational diabetes and also women after a normoglycemic pregnancy as control subjects.

The first manuscript of this thesis revealed differences in physical fitness between women post GDM and controls. T2D-risk (post GDM-status) was associated with lower physical fitness, independent of the BMI. Additionally, physical fitness correlated inversely with fasting plasma leptin, independent of the BMI. Therefore, high physical fitness could potentially reduce elevated leptin levels or counteract other mechanisms leading to leptin resistance. This may alleviate insulin resistance, hyperinsulinemia, and hyperglycemia.

In a second manuscript, we examined plasma glucagon dynamics in the oral glucose tolerance test in three different metabolic groups. Mean glucagon values (fasting and postprandial) were higher in the groups at higher risk for T2D, but this was not an ubiquitous finding. An unsupervised cluster analysis revealed four clusters of glucagon dynamics that only partially overlapped with the metabolic groups. Therefore, we concluded that hyperglucagonemia may contribute to hyperglycemia in some individuals, but that it is not a sine qua non for T2D development.

In the third publication of this thesis, we examined whether plasma amino acids can serve as biomarkers for pathological glucose tolerance. A systematic literature review and original data from the PPSDiab study showed that glutamate, glycine, isoleucine, leucine, proline, and the

sum of the branched chain amino acids were associated with pathological glucose tolerance. In the PPSDiab study however, only glycine remained significantly associated with glucose tolerance after adjustment for BMI. Additionally, besides insulin sensitivity, most amino acids also associated with other components of the metabolic syndrome (hypertension, dyslipidemia, high BMI, and visceral obesity). Because the metabolic syndrome is already the basis for most current T2D risk prediction models, the addition of plasma amino acids does not improve prediction. An exception may be glycine, which showed BMI-independent associations.

In sum, this thesis evaluated risk factors and novel biomarker-candidates for T2D in a prospective cohort study of young women. It shows novel aspects of the pathophysiology of T2D and highlights the heterogeneity of this disease. Personalized approaches to prevention will certainly be necessary to limit the current T2D epidemic.

8.2. Zusammenfassung dieser Arbeit

Diabetes mellitus Typ 2 (T2D) stellt ein globales Gesundheitsproblem mit steigender Prävalenz dar. Die Prävention der Krankheit ist immer noch unzureichend, vor allem aufgrund des unvollständigen Verständnisses ihrer Pathophysiologie und des Mangels an geeigneten Tests zur frühen Diagnose. Als Beitrag zur Aufklärung der frühen T2D-Pathophysiologie untersucht die vorliegende Dissertation die Rolle von körperlicher Fitness und der Hormone Leptin und Glucagon in Frauen nach einer kürzlichen Schwangerschaft mit Gestationsdiabetes (GDM), einer jungen hoch-Risiko Population für T2D. Außerdem beschäftigt sie sich mit Plasma-Aminosäuren als mögliche frühe Biomarker für Prädiabetes und T2D.

Alle Analysen wurden in der Prädiktion, Prävention und Subklassifikation von Typ 2 Diabetes (PPSDiab) Studie durchgeführt, welche prospektiv Frauen nach einer mit Schwangerschaft mit Gestationsdiabetes sowie Frauen nach einer normoglykämen Schwangerschaft als Kontrollen beobachtet.

Die erste Auswertung ergab Unterschiede in der körperlichen Fitness zwischen Frauen post GDM und Kontrollen. Das T2D-Risiko (post GDM-Status) war BMI-unabhängig mit einer geringeren körperlichen Fitness assoziiert. Des Weiteren korrelierte körperliche Fitness BMI-unabhängig invers mit Nüchtern Plasma-Leptin. Somit könnte eine hohe körperliche Fitness möglicherweise erhöhte Leptin-Spiegel reduzieren oder anderen Mechanismen, die zu Leptinresistenz führen, entgegenwirken. Das könnte Insulinresistenz, Hyperinsulinämie und Hyperglykämie verringern.

In einer zweiten Analyse wurde in drei verschiedenen metabolischen Gruppen der Verlauf von Plasma-Glucagon im oralen Glucosetoleranztest untersucht. Die mittleren Glucagonwerte (nüchtern und postprandial) waren höher in Gruppen mit höherem T2D-Risiko, jedoch war dieses Ergebnis nicht ubiquitär. Eine unbeaufsichtigte Clusteranalyse ergab vier Cluster von Glucagonverläufen, welche nur partiell mit den metabolischen Gruppen überein stimmten. Somit kamen wir zu dem Schluss, dass Hyperglucagonämie möglicherweise in manchen Individuen zu einer Hyperglykämie beiträgt, dass es aber keine unabdingbare Voraussetzung für die Entwicklung von T2D ist.

In der dritten Publikation dieser Dissertation untersuchten wir, ob Aminosäuren als Biomarker für pathologische Glucosetoleranz dienen können. Ein systematischer Literaturreview sowie

die Originaldaten aus der PPSDiab-Studie zeigten, dass Glutamat, Glycin, Isoleucin, Leucin, Prolin und die Summe der verzweigtkettigen Aminosäuren mit pathologischer Glucosetoleranz assoziiert waren. In der PPSDiab Studie blieb nach Adjustieren für BMI jedoch lediglich Glycin signifikant mit der Glucosetoleranz assoziiert. Außerdem assoziierten die meisten Aminosäuren neben der Insulinsensitivität auch mit anderen Charakteristika des Metabolischen Syndroms (Hypertonus, Dyslipidämie, hoher BMI und viszerale Adipositas). Da die Charakteristika des Metabolischen Syndroms die Basis für die meisten klassischen T2D-Risikomodelle bilden, verbessert die Hinzunahme von Aminosäuren die Prädiktion nicht. Eine Ausnahme könnte Glycin sein, welches BMI-unabhängige Assoziationen aufwies.

Zusammengefasst untersuchte diese Dissertation Risikofaktoren und neue Biomarker für T2D in einer prospektiven Kohortenstudie junger Frauen. Sie zeigt neue Aspekte der Pathophysiologie des T2D auf und hebt die Heterogenität der Erkrankung hervor. Sicherlich werden personalisierte Präventionsansätze nötig sein, um die derzeitige T2D-Epidemie einzudämmen.

9. List of publications

9.1. Original articles

Gar C, Rottenkolber M, Grallert H, Banning F, Freibothe I, Sacco V, Wichmann C, Reif S, Potzel A, Dauber V, Schendell C, Sommer NN, Wolfarth B, Seissler J, Lechner A, Ferrari U.

Physical fitness and plasma leptin in women with recent gestational diabetes

PLOS ONE. 2017 Jun 13;12(6):e0179128.

Gar C, Rottenkolber M, Sacco V, Moschko S, Banning F, Hesse N, Popp D, Hübener C, Seissler J, Lechner A.

Patterns of Plasma Glucagon Dynamics Do Not Match Metabolic Phenotypes in Young Women

The Journal of clinical endocrinology and metabolism. 2018;103(3):972-82.

Gar C, Rottenkolber M, Prehn C, Adamski J, Seissler J, Lechner A.

Serum and plasma amino acids as markers of prediabetes, insulin resistance, and incident diabetes

Critical reviews in clinical laboratory sciences. 2017 Dec 14:1-12.

Ferrari U, Banning F, Freibothe I, Tröndle K, Sacco V, Wichmann C, Reif S, Moschko S, Potzel A, **Gar C**, Sommer NN, Popp D, Seissler J, Lechner A, Künzel H.

Depressive symptoms, impaired glucose metabolism, high visceral fat, and high systolic blood pressure in a subgroup of women with recent gestational diabetes

Journal of Psychiatric Research. 2018 Feb;97:89-93.

9.2. Abstracts

Hawlitschek C, Rottenkolber M, Grallert H, Banning F, Freibothe I, Sacco V, Wichmann C, Reif S, Potzel A, Dauber V, Schendell C, Wolfarth B, Seißler J, Lechner A, Ferrari U.

Plasmaleptin und körperliche Fitness bei jungen Frauen mit und ohne erhöhtem Risiko für T2D

Diabetes Kongress, 2016. Published in: Diabetologie und Stoffwechsel 2016;11-P184.

Hawlitschek C, Ferrari U, Banning F, Freibothe I, Sacco V, Wichmann C, Reif S, Potzel A, Prehn C, Sommer NN, Hetterich H, Adamski J, Seissler J, Lechner A.

Elevated glutamate and branched chain amino acid levels in overweight/obese individuals: links to insulin resistance and visceral adiposity

EASD Annual Meeting, 2016. Published in: Diabetologia 2016;59(Suppl 1):S1-S581.

Gar C, Rottenkolber M, Banning F, Freibothe I, Sacco V, Grallert H, Prehn C, Adamski J, Wichmann C, Potzel A, Seissler J, Ferrari U, Lechner A.

Plasma-Aminosäuren als Marker für Prädiabetes, Insulinresistenz und Typ 2 Diabetes – Systematischer Review und Ergebnisse der PPSDiab-Studie

Diabetes Kongress, 2017. Published in: Diabetologie und Stoffwechsel 2017;12(S 01):S1-S84.

Reif S, Rottenkolber M, Ferrari U, Banning F, Freibothe I, Sacco V, Wichmann C, **Hawlitschek C**, Potzel A, Hetterich H, Sommer N, Grallert H, Lehmann R, Seißler J, Lechner A.

Hohe Fetuin-A-Werte korrelieren mit geringer körperlicher Fitness und niedrigerer Insulinempfindlichkeit bei Frauen nach Gestationsdiabetes

Diabetes Kongress, 2016. Published in: Diabetologie und Stoffwechsel 2016;11-P177.

Moschko S, **Gar C**, Potzel A, Sacco V, Wichmann C, Ferrari U, Banning F, Freibothe I, Seissler J, Lechner A.

Niedriges Adiponectin bei adipösen, nicht aber bei normalgewichtigen Frauen nach Gestationsdiabetes

Diabetes Kongress, 2017. Published in: Diabetologie und Stoffwechsel 2017;12(S 01):S1-S84.