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Metabolomic Characteristics of Fatty Pancreas

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ABSTRACT

Objective Pancreatic steatosis is associated with impaired beta cell function in patients with prediabetes. The pathomechanisms underlying this association still remain to be elucidated. Recent data show that adipocytes are situated within the pancreatic parenchyma and therefore give raise to hypothesize that pancreatic fat together with known and unknown metabolites such as hepatokines affect insulin secretion. Applying a targeted metabolomic approach we investigated possible circulating markers of pancreatic fat in order to better understand its role in the pathophysiology of impaired beta cell function.

Methods We included 361 Caucasians, at increased risk of type 2 diabetes, from the Tübingen Family Study. All participants underwent a frequently sampled oral glucose tolerance test to assess insulin secretion and a magnetic resonance imaging to quantify pancreatic fat content, total body fat and visceral fat. Among the 152 subjects with prediabetes (IFG and/or IGT), two groups each with 20 individuals, having the lowest and highest pancreatic fat content were selected. The groups were matched for sex, age, BMI, total fat content, visceral fat content, liver fat content and insulin sensitivity. Metabolites were analyzed using the AbsoluteIDQ® p400 HR Kit by Biocrates.

Results Pancreatic fat content of all 152 subjects with prediabetes was negatively associated with insulin secretion represented by $AUC_{C\text{-peptide }0-120}/AUC_{Glucose\ 0-120}$ ($p = 0.04$; $\beta = -3.24$). Furthermore, pancreatic fat content was positively associated with BMI, total body and visceral fat (all $p < 0.005$). Levels of aminoacids, biogenic amines and monosaccharides were similar between the groups with high/low pancreatic fat content ($p > 0.90$). Also, levels of polar lipids such as lysophosphatidylcholines, phosphatidylcholines, sphingomyelins and ceramides did not differ significantly between the groups ($p > 0.90$). Investigating the levels of neutral lipids such as acylcarnitines, diglycerides, triglycerides and cholesteryl esters also revealed no differences between the groups ($p > 0.90$).

Conclusion The amount of pancreatic fat is not associated with the metabolomic pattern in individuals with prediabetes. This might be due to the relatively low pancreatic fat content compared to the total amount of fat stored in other depots. The impact of pancreatic steatosis on insulin secretion might be mediated by paracrine effects which cannot be detected in the circulation.

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ABBREVIATIONS

AU	arbitrary unit
BMI	body mass index
NAFPD	non-alcoholic fatty pancreas disease
NAFLD	non-alcoholic fatty liver disease
VAT	visceral adipose tissue
CT	computer tomography
MRI	magnetic resonance imaging
^{1}H-MRS	proton magnetic resonance spectroscopy

Introduction

The amount of adipocytes in human pancreatic parenchyma varies largely [1]. Recently, pancreatic steatosis has attracted increasing attention in various fields of research since it has been shown to be associated with major diseases such as pancreatic cancer, non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes [2–7]. In this context it has been demonstrated that pancreatic fat content is elevated in subjects with type 2 diabetes and also increases with age [2, 8–10]. However these changes may be reversible [9, 11–13]. Several studies demonstrated that weight loss due to dietary energy restriction, increased physical activity or gastric bypass surgery may lead to a decrease of pancreatic fat [11, 14–16].

Previously, multiple studies including some from our own group revealed that there are associations between pancreatic fat, impaired glucose metabolism [1, 17, 18] and also type 2 diabetes [5, 19–21]. Given these results, several groups have further attempted to examine whether specifically impaired insulin secretion or impaired insulin sensitivity, both hallmarks in the development of type 2 diabetes, are related to these observations [17, 19, 22–25]. Lee et al. examined 293 Caucasian subjects by CT-scans showing that pancreatic fat content correlated positively with insulin resistance represented by HOMA-IR [23, 24]. Furthermore, results of a study carried out by Wong et al., examining 685 healthy Chinese subjects corroborated these findings by showing that fatty pancreas was independently associated with insulin resistance [25]. Regarding the relation of insulin secretion and pancreatic fat content we have previously demonstrated that pancreatic fat content was inversely correlated with insulin secretion in subjects with prediabetes but not in subjects with normal glucose tolerance [17]. Examining a smaller group of subjects without type 2 diabetes, by proton magnetic resonance spectroscopy (^{1}H-MRS), Tushuizen et al. also showed that pancreatic fat is correlated with decreased insulin secretion [19]. Further, Wong et al. confirmed in healthy subjects that pancreatic fat content was inversely associated with insulin secretion [25]. However, this association disappeared after adjustment for BMI and liver fat content [25]. Moreover, other groups could not find any association of pancreatic fat with insulin secretion [18, 26, 27]. These divergent results might be due to differences in methodology. The application of various radiologic techniques such as CT, MRI or ^{1}H-MRS and the lack of evaluation standards regarding the assessment of pancreatic fat might have led to the apparently contradictory results [5, 17, 18, 27–29]. Among the imaging methods MRI currently is the preferable technique as is its quite precise and does not expose the subjects to radiation [30]. However, determining pancreatic fat accumulation via MRI is costly and time-consuming [29].

Other ways of characterizing pancreatic fat accumulation, for example by analyzing potential biomarkers, have yet to be fully established [31]. According to Singh et al. markers such as triglycerides, yGT and HbA1c showed the strongest weighted correlation with pancreatic fat [31]. However these parameters are neither sufficiently sensitive nor specific and therefore not appropriate for characterizing pancreatic fat.

Consequently, more sensitive and more specific circulating biomarkers might help to characterize pancreatic fat in larger cohorts in order to better understand its role in health and disease. Previously we demonstrated that a targeted metabolomic analysis is an appropriate approach to detect circulating biomarkers for characterizing non-alcoholic fatty liver (NAFL) [32]. In detail we showed that lyso-phosphatidylcholines are reliable circulating metabolites enabling one to separate metabolically benign (insulins sensitive) from malignant (insulin resistant) NAFL in humans [32]. Inspired by these findings we addressed the question whether there are also circulating metabolites which enable us to better characterize pancreatic fat since until now its role remains elusive. Therefore we investigated two well-matched groups of participants with prediabetes which differed only in the amount of pancreatic fat [17].

Methods

Study population

The basis for our present analysis are data from the Tübingen Family Study (TUEF) which includes individuals with increased risk of type 2 diabetes (n > 3000). Inclusion criteria for the TUEF study were: family history of type 2 diabetes, a BMI > 27 kg/m^2 and a previous diagnosis of glucose intolerance and/or gestational diabetes [33]. Individuals with a history of alcohol abuse and pancreatitis were excluded.

We first identified individuals with MRI measurement of pancreatic fat content in the TUEF database (n = 361). Within this heterogeneous group of participants we differentiated between subjects with normal glucose tolerance, prediabetes (impaired fasting glucose defined as a fasting blood glucose of > 5.6 mmol/l and < 7 mmol/l and/ or impaired glucose tolerance defined as a blood glucose of > 7.8 mmol/l and < 11.1mmol/l after a 2-hour oral glucose tolerance test) and type 2 diabetes. In total we identified 152 subjects with prediabetes. The subject characteristics of these 152 prediabetic individuals are shown in ▶ **Table 1**.

We further separated the study group by the median (median: 7.67 %, standard deviation: 4.15) in individuals with high and low pancreatic fat content. Then, 20 individuals with maximally high and low pancreatic fat content were matched for sex, age, BMI, total fat content, visceral fat content and liver fat content (▶ **Table 2**). Due to technical reasons one subject had to be excluded, leaving 39 subjects for the analysis.

Oral glucose tolerance test and biochemical analysis

All subjects underwent a frequently sampled 75g oral glucose tolerance test. Biochemical variables (glucose, insulin, C-peptide) were measured at 0, 30, 60, 90 and 120 min. Insulin secretion was

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assessed by the insulinogenic index (IGI: Insulin $_{30min}$ – Insulin $_{0min}$ (pmol/l)/Glucose $_{30min}$ – Glucose $_{0min}$ (mmol/l), C-peptide 30min (pmol/l), $AUC_{C\text{-}peptide\,(0–30min)}/AUC_{Glucose\,(0–30min)}$, $AUC_{C\text{-}peptide\,(0–120min)}/AUC_{Glucose\,(0–120min)}$. Insulin sensitivity was determined using the index of insulin sensitivity proposed by Matsuda and DeFronzo [34].

Assessment of fat accumulation

Total body fat, visceral adipose tissue (VAT) and pancreatic fat were measured by MRI as described previously [29, 30]. In brief, a T1-weighted fast spin-echo technique was applied for assessment of total body fat and VAT [29] followed by a fuzzy-clustering algorithm for automatic segmentation [35]. Pancreatic fat was quantified applying a spectral-spatial fat selective MR-imaging technique that selectively excites protons bound to fat by a set of binomial RF-pulses whereas water containing/lean tissue is not influenced [29]. Post-processing was performed by manually drawing circular regions of interest in the caput, corpus and cauda of the pancreas, carefully avoiding inclusion of surrounding VAT. Nearby VAT served as a reference for 100 % fat and the signal intensities were corrected for noise as determined in an object-free part of the image [36]. Mean pancreatic fat content was calculated as the mean value of the three regions of interest and was indicated in %. Total adipose tissue was quantified in liters (l). Liver fat accumulation was assessed by localized magnetic resonance spectroscopy ^{1}H-MRS as shown previously [29]. Liver fat content was indicated in liters (l). All imaging techniques were performed by one single experienced researcher, who was not aware of the characteristics of the participants.

▶ **Table 1** Subject characteristics of 152 participants with prediabetes.

Trait	Mean	Median	Upper/lower 95 % confidence interval
Sex	93 female/59 male		
Age (years)	52	55	54/50
BMI (kg/m²)	30.40	30.22	31.1/29.6
VAT (l)	4.58	4.38	4.95/4.21
TAT (l)	37.81	37.81	39.8/35.8
Liver fat (%)	8.65	5.79	9.88/7.43
Mean pancreatic fat (%)	8.87	7.9	9.55/8.18
Triglycerides (mg/dl)	136.63	123	147.9/125.36
FFA (µmol/l)	663.43	649.5	699.49/627.37
Cholesterol (mg/dl)	204.56	205	210.17/198.94
HDL (mg/dl)	53.84	53	55.92/91.76
LDL (mg/dl)	130.89	127	136.21/125.56
$AUC_{C\text{-}Peptide\,(0–30)}$/ $AUC_{C\text{-}Peptide\,(0–30)}$ (AU)	171.94	172.51	182.11/161.77
$AUC_{C\text{-}Peptide\,(0–120)}$/ $AUC_{Glucose\,(0–120)}$ (AU)	285.22	282.22	299.63/270.8
IGI (AU)	165.38	121.48	187.9/142.86
Lipase (U/l)	40.96	32.64	54.03/27.89

Metabolite profiling

Metabolite profiling was performed by Biocrates® (Innsbruck, Austria) applying their commercial high resolution mass spectrometric-based metabolomic AbsoluteIDQ® p400 HR Kit. This quantitative kit covered 396 metabolites of several classes, including amino acids, biogenic amines, acylcarnitines, glycerophospholipids, sphingolipids, cholesterol esters, glycerides, and monosaccharides. Plasma samples were stored at − 80 °C. For metabolite quantification an aliquot of each sample was briefly centrifuged and the supernatant was used for further analysis. Liquid Chromatography based mass-spectrometry was used for quantifying amino acids and biogenic amines. Liquid chromatography was performed in a positive ion mode. Flow injection based mass-spectrometry was applied in order to assess hexoses, lipids, acylcarnitines and cholesterol esters. For the analysis high resolution, accurate mass (HRAM) OrbitrapTM mass spectrometers were used.

Statistics

ANOVA was performed analyzing the characteristics of the study population. Stepwise regression models using Pearson correlation were applied to analyze the relationship of continuous variables. Subjects with values > 2.5 standard deviations of the mean for the

▶ **Table 2** Group specific characteristics.

	Group 0 = low pancreatic fat		Group 1 = high pancreatic fat		
Trait	**mean**	**SEM**	**mean**	**SEM**	**p-value**
Gender	19 women/1 man		15 women/4 men		
BMI (kg/m²)	30.75	1.12	29.64	0.90	0.45
Age (years)	48.75	3.09	53.89	1.83	0.16
Visceral fat (l)	3.69	0.21	3.97	0.17	0.30
Liver fat (%)	9.03	1.59	9.72	1.97	0.81
Total adipose tissue (l)	41.30	2.56	37.79	2.99	0.38
Mean pancreatic fat (%)	5.29 *	0.16	12.21 *	1.01	< 0.0001
IGI (AU)	183.32	20.38	219.68	59.94	0.57
$AUC_{C\text{-}peptide\,(0–30)}/AUC_{Glucose\,(0–30)}$ (AU)	191.11	12.01	178.31	16.72	0.54
C-peptide$_{30}$ (pmol/l)	2310.1	180.40	2076.4	203.41	0.39
Fasting Glucose (mmol/l)	5.61	0.09	5.89	0.12	0.08
ISI-Matsuda (AU)	5.28	0.65	6.73	0.94	0.21

Shown are the characteristics of the groups with low vs. high pancreatic fat. The two groups differed only significantly for mean pancreatic fat while anthropometric parameters as well as parameters of insulin secretion and insulin sensitivity were similiar. Depicted are means, SEM and p-values;
* denotes significant differences ($p < 0.05$) between the two groups applying ANOVA.

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▶ **Table 3** Correlation of pancreatic fat content with metabolic parameters:

	Pancreatic fat content (%)			
Trait	**Estimate**	**Std. beta**	**p-value**	**adjusted for**
TAT (l)	0.69	0.24	< 0.005	sex, age,
VAT (l)	0.10	0.18	< 0.001	sex, age, TAT
Liver fat (%)	0.11	0.06	0.48	sex, age, TAT
BMI (kg/m²)	0.33	0.29	< 0.001	sex, age
Trigylcerides (mg/dl)	2.96	0.18	0.0445	sex, age, TAT
Fasting Glucose (mmol/l)	0.02	0.17	0.05	sex, age, BMI
$Glucose_{120}$ (mmol/l)	0.09	−0.02	0.84	sex, age, BMI
$AUC_{Glucose\ (0–120)}$ (AU)	0.11	0.16	0.06	sex, age, BMI
$AUC_{C\text{-}peptide\ (0–120)}$/ $AUC_{Glucose\ (0–120)}$ (AU)	−3.24	−0.16	0.0424	sex, age, ISI-Matsuda
ISI-Matsuda (AU)	−0.07	−0.06	0.46	sex, age, BMI

Given are the adjusted correlations of pancreatic fat of all subjects with prediabetes with assessed metabolic parameters. Depicted are estimates, std. beta, p-values and adjustments.

assessed parameters were excluded (n = 15) in order to exclude the outliers and improve the power of the study. Effect size estimates from linear regression models are provided as standardized coefficients (β). Due to technical reasons one subject had to be excluded after matching. Adjustments were performed as demonstrated in ▶ **Table 3**.

Multiple hypothesis testing was performed applying ANOVA and the model of Benjamini and Hochberg controlling the false discovery rate and reducing probability for type 1 error. A false detection rate (adjusted p-value using the Benjamini Hochberg method) of 0.05 or lower was considered statistically significant. Statistical analysis was performed using the statistical software package JMP 13.0 (SAS Institute, Cary, NC, USA).

Power analysis was performed using G * Power 3.1.9.4 (Heinrich-Heine Universität Düsseldorf, Germany). Employing an alpha level of 0.00013 as statistical significant after correction for multiple testing (0.05/396) and assuming that there is a 40 % difference with a standard deviation of 20 % in a metabolite level between two groups, one would need 15 subjects with low and 15 subjects with high pancreatic fat in order to reject the null hypothesis with a power of 80 %.

Results

We first analyzed the correlation of pancreatic fat with anthropometric characteristics, parameters of glucose metabolism and body fat compartments in all 152 subjects with IFG and/or IGT. Pancreatic fat content correlated significantly with BMI ($p < 0.001$, $\beta = 0.33$) and total adipose tissue mass ($p < 0.005$, $\beta = 0.69$) after adjustment for gender and age (▶ **Fig. 1**, ▶ **Table 3**). Further, pancreatic fat associated significantly with visceral fat ($p < 0.001$, $\beta = 0.10$) but not with liver fat ($p = 0.48$) after adjusting for gender, age and total adipose tissue. Moreover pancreatic fat correlated independently with levels of serum triglycerides ($p = 0.04$, $\beta = 0.18$) after adjustment for gender, age and total adipose tissue (▶ **Fig. 1**, ▶ **Table 3**). Examining glucose metabolism, pancreatic fat correlated significantly with fasting glucose ($p = 0.05$, $\beta = 0.02$) and $AUC_{C\text{-}peptide\ (0–120min)}$/ $AUC_{Glucose\ (0–120min)}$ ($p = 0.04$, $\beta = -3.24$) after adjustment for gender, age and BMI (▶ **Table 3**). Further, there was a trend for an association with $AUC_{Glucose\ (0–120min)}$ ($p = 0.06$) after adjustment for gender, age and BMI. Pancreatic fat was not significantly associated with glucose levels at 120 min of the 75g OGTT ($p = 0.84$) and there was no correlation between pancreatic fat and insulin sensitivity represented by ISI-Matsuda ($p = 0.46$) after adjustment for gender, age and BMI.

Next, we analyzed matched groups with extremes of pancreatic fat content. As presented in ▶ **Table 2**, the groups were comparable in gender, age, BMI, visceral fat content, liver fat content, total adipose tissue, fasting glucose, insulinogenic index and insulin sensitivity. The groups only differed significantly in mean pancreatic fat content ($p < 0.0001$). The mean pancreatic fat content in the low pancreas fat group was 5.3 % ± 0.16 (SEM), whereas in the other group it amounted to 12.2 % ± 1.01 (SEM).

Levels of aminoacids, biogenic amines and monosaccharides showed no significant difference between the groups with high and with low pancreatic fat content ($p > 0.90$; (**Supplementary** ▶ **Table 1S**). Also levels of polar lipids such as lysophosphatidylcholines, phosphatidylcholines, sphingomyelins and ceramides did not differ significantly between the groups ($p > 0.90$; (**Supplementary** ▶ **Table 1S**). Furthermore, the levels of acylcarnitines, diglycerides, triglycerides, cholesteryl esters were also not different between the group with high and with low pancreatic fat content ($p > 0.90$; (**Supplementary** ▶ **Table 1S**).

Discussion

In the present study, we investigated possible associations of circulating metabolites with pancreatic fat by searching for potential new biomarkers for pancreatic steatosis in individuals with prediabetes. We could confirm several associations of pancreatic fat with glucose metabolism, insulin secretion, and body fat distribution. However, our broad targeted metabolomic approach did not reveal new circulating biomarkers for pancreatic steatosis.

We demonstrated that total and visceral fat content are positively associated with pancreatic fat content. These findings are in line with several previous studies which show that pancreatic fat is positively correlated to BMI [6, 10, 18] and the metabolic syndrome [23]. Taking into account that NAFLD is known to be associated with the

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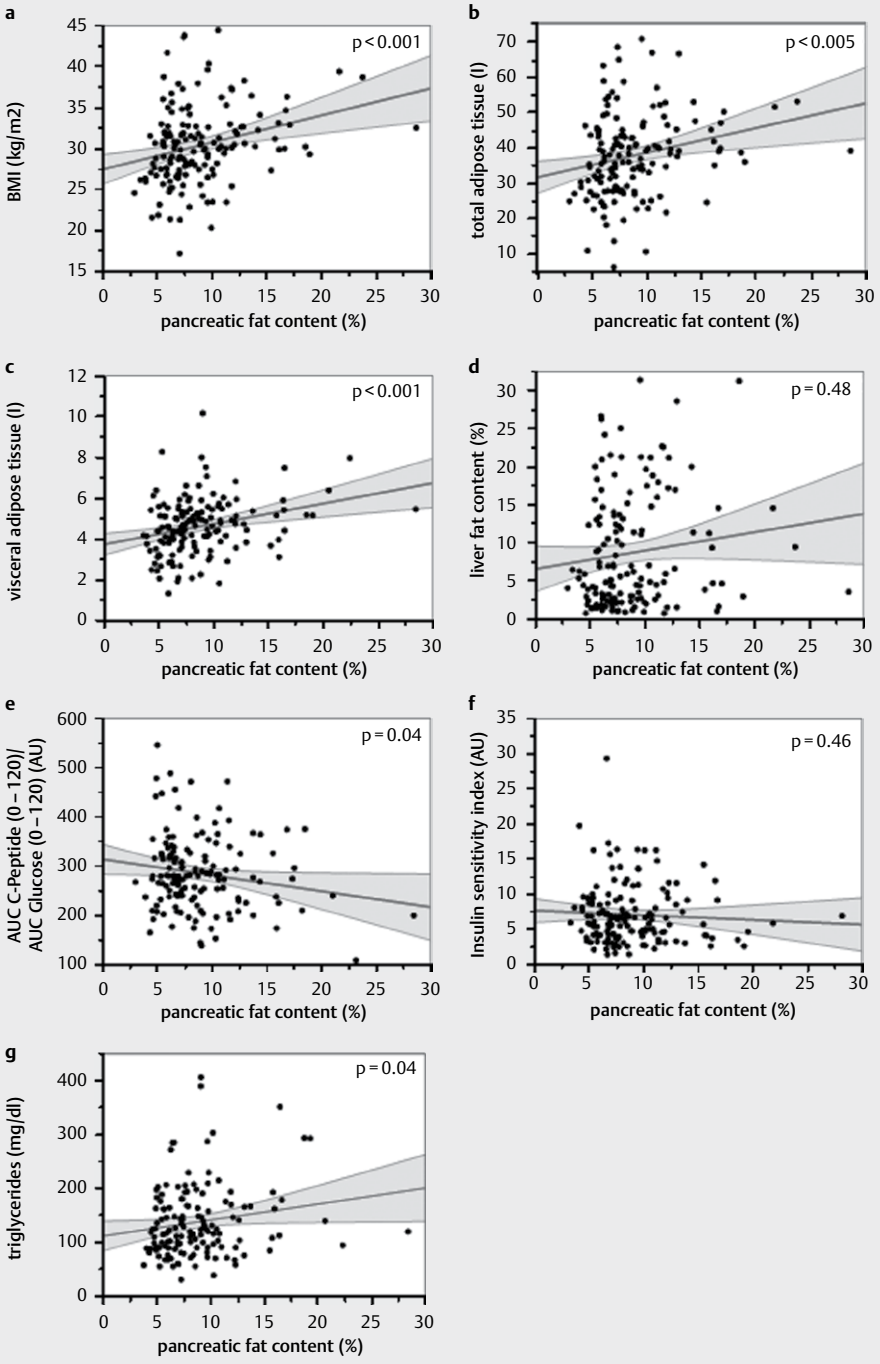

▶ **Fig. 1** Association of pancreatic fat content with anthropometric and metabolic parameters in 152 subjects with prediabetes: Plots show correlation of pancreatic fat with BMI *, total adipose tissue content *, visceral adipose tissue content **, liver fat content **, $AUC_{C\text{-peptide }(0–120)}/AUC_{Glucose\ (0–120)}$ ***, insulin sensitivity index (ISI-Matsuda) **** and triglycerides **. P-values are shown. Lines represent model correlation, red areas are CI. N = 152. * adjusted for sex, age; ** adjusted for sex, age, TAT; *** adjusted for sex, age, ISI-Matsuda; **** adjusted for sex, age, BMI.

metabolic syndrome [37, 38] we also examined the association of liver fat accumulation and pancreatic fat. After adjustment for sex, age and total visceral adipose tissue there was no association between liver fat and pancreatic fat in our cohort which is, however, contradictory to other studies [39, 40]. Our results therefore suggest that pancreatic steatosis might play an independant role in the pathogenesis of impaired insulin secretion and type 2 diabetes.

The association of pancreatic fat with impaired glucose metabolism still remains controversial. Among current studies there are some pointing out that pancreatic fat may be related to type 2 diabetes [5, 11, 19, 21]. However, other studies could not confirm

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such an independent association [8, 10]. Further, the underlying mechanisms behind that possible relationship remain obscure since the studies are purely observational. In order to further elucidate the situation, several studies investigated the effects of pancreatic fat content on insulin sensitivity and insulin secretion, both hallmarks in the pathophysiology of type 2 diabetes. Regarding insulin sensitivity some of those studies detected a significant negative correlation between insulin sensitivity and pancreatic fat in cohorts without diabetes [23, 25]. In contrast, investigating the role of pancreatic fat in obese Hispanics and Afro-Americans without diabetes, Lê et al. did not show a relation to insulin sensitivity [26]. This is in accordance to our present and previous findings which also demonstrated no association of pancreatic fat with insulin sensitivity measured during a 5-point OGTT [17]. Regarding these divergent results, they might be due to the different ethnicities, different subject characteristics and varying radiographic techniques applied to assess pancreatic fat content.

Concerning potential implications on insulin secretion, we previously demonstrated that there is an inverse correlation between pancreatic fat and insulin secretion in subjects with prediabetes but not in subjects with normal glucose tolerance [17]. The present study corroborates this finding within a different prediabetic cohort. In accord, Tushuizen et al. also found an inverse relationship of pancreatic fat and insulin secretion, but within a group of non-diabetic subjects [19]. This leads to the speculation that during development of type 2 diabetes, pancreatic fat might play a role in the state of prediabetes, but that later on other factors could become more important superimposing the role of pancreatic fat so that this association is no longer seen in overt type 2 diabetes anymore. On the contrary, some groups did not detect any association of pancreatic fat with insulin secretion by investigating subjects with prediabetes or normal glucose tolerance [6, 18, 26, 27]. Among the studies which confirmed or negated a relationship between pancreatic fat content and insulin secretion, there is a big heterogeneity concerning the ethnicities of the individuals and radiological techniques which both make an objective comparison difficult.

Inspired by our previous findings which suggested that pancreatic fat affects beta-cell function in subjects with prediabetes we now specifically investigated the influence of pancreatic fat content on the metabolome in prediabetic subjects. Application of targeted metabolomics represents an appropriate method to detect circulating biomarkers for certain specific adipose tissue as we previously showed in subjects with fatty liver [32]. Hence we formed two groups with low versus high pancreas fat content and matched the two groups precisely for sex, age, BMI, total fat content, visceral fat content, liver fat content and insulin sensitivity in order to rule out possible confounding variables. Our data demonstrate that pancreatic fat is not associated with the various levels of the investigated metabolites such as sphingomyelins, lysophosphatidylcholines, phosphatidylcholines, ceramides, diglycerides, specific triglycerides, cholesteryl esters, acylcarnitines, monosaccharides, aminoacids, and biogenic amines in individuals with prediabetes. One explanation for missing differences in the metabolome is that the amount of pancreatic fat in relation to the total body fat may be too low to have an impact on the systemic plasma metabolites measured in the current study.

Moreover, other factors may influence insulin secretion in subjects with impaired fasting glucose and/or glucose tolerance, which are not assessed with a targeted metabolomic approach. Previously we showed that adipocytes are situated within the human pancreatic parenchyma [1]. We further demonstrated in vitro that there might be a metabolic crosstalk between liver fat and pancreatic fat related to the hepatokine Fetuin A which leads to impaired insulin secretion [1]. This potential paracrine signaling between pancreatic fat cells and beta cells in islets is probably not mirrored by plasma metabolites. Lastly, it has to be mentioned that we applied a targeted metabolomic investigation. By performing an untargeted metabolomic approach one might find unexpected biomarkers which could help to better understand the role of pancreatic fat.

In conclusion, we did not detect plasma metabolites predicting pancreatic fat content as it is possible in the case of liver fat. However, investigating the role of pancreatic fat is still important since there is sufficient data that let one hypothesize that pancreatic fat plays a relevant role in the development of insulin secretion failure and type 2 diabetes. Therefore, clarifying the part of pancreatic steatosis in impaired glucose metabolism could also bring up new potential therapeutical targets for the treatment of type 2 diabetes.

Author Contributions Statement

B.A.J. and A.F. analyzed the data and wrote the manuscript. M.H., R.W., J.M., R.L. and N.S. contributed to the interpretation of data and edited the manuscript. H-U.H. edited and reviewed the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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