diabetesresearchandclinicalpractice 221 (2025) 112029

Contents lists available at ScienceDirect

Diabetes Research and Clinical Practice

journal homepage: www.journals.elsevier.com/diabetes-research-and-clinical-practice

In humans increase in intrapancreatic adipose tissue predicts beta-cell dedifferentiation score before diabetes onset: A pilot study

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ARTICLE INFO

Keywords:
Diabetes
Metabolism
Beta cell dedifferentiation
Adipose tissue
Precision medicine

ABSTRACT

Background: The role of intrapancreatic fat (WAT) in the development of T2D remains debated. In T2D, β-cell dedifferentiation is one of the mechanisms responsible for β-cell failure but its role in prediabetes is unknown. We aimed to investigate the relation between WAT and β-cell dedifferentiation prior to diabetes onset.
Methods: We evaluated pancreatic samples from patients without history of diabetes, who had previously undergone an oral glucose tolerance test and hyperglycemic clamp. Subjects were divided into 3 glucose tolerance groups: normal (NGT), altered (IGT) or newly diagnosed diabetes (nDM). Dedifferentiation and WAT% were morphologically assessed.
Results: WAT was higher in nDM patients compared to NGT and IGT (WAT nDM 43.79 ± 20.83 %, IGT 10.67 ± 8.5 %, NGT 4.43 ± 4.37 %). We observed a progressive increase in dedifferentiation score, in parallel with worsening glucose tolerance (from NGT to IGT to nDM; 4.8 ± 3.8; 32.37 ± 7.4; 40.38 ± 19 respectively). A strong linear regression established that WAT could statistically significantly predict dedifferentiated β-cells ($R = 0.86$, $p = 0.005$), and that the predicted increase in dedifferentiated β-cells was 1.25 points for every extra one-point change in WAT. Interestingly, the WAT and dedifferentiation score variable pair were significantly related to 1-hour post-load glycemia.
Conclusions: The accumulation of WAT might be responsible for dedifferentiation, making it a potential new target to curb diabetes onset.

1. Introduction

Type 2 diabetes (T2D) is a metabolic disorder clinically identified by chronic hyperglycemia, triggered by reduced beta cell functional mass and/or peripheral insulin resistance[1,2]. However, the pathophysiological mechanisms of the disease are still a matter of debate. It is well-known that obesity is associated with an increased risk of developing T2D[3–6] but, recently, clinical evidence has suggested that the pathogenesis of the disease is identical in people with normal or obese body mass index (BMI). Indeed, the significant reduction of

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https://doi.org/10.1016/j.diabres.2025.112029
Received 14 November 2024; Received in revised form 18 January 2025; Accepted 29 January 2025
Available online 10 February 2025
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intrapancreatic[7] fat tissue in T2D patients, obtained by dietary restriction, has been associated with diabetes remission regardless of the initial BMI[8]. These findings confirm the 'Personal fat threshold' hypothesis, which postulates an individual threshold for subcutaneous fat storage capacity. Once this threshold is exceeded, the organism starts to accumulate visceral ectopic fat (including intrahepatic and pancreas storage) with a detrimental effect on beta-cell function[8–11]. However, the role of intrapancreatic fat accumulation in the development of T2D (i.e., in prediabetes) is unknown, and even the definition of intrapancreatic fat accumulation (e.g. pancreatic steatosis? pancreatic lipomatosis?) is still being debated[12,13].

Beta cell dedifferentiation is recognized as one of the main mechanisms responsible for the disappearance of beta cells from the pancreatic islets of diabetic subjects[14–19]. Even though the underlying mechanisms of this process are still under investigation[20,21], its reversibility, associated with a remission of T2D, has been confirmed in rodent and in vitro models[22,23], suggesting the importance of focusing on this process to better understand the pathophysiology of the disease [23–25]. The role of beta cell dedifferentiation in prediabetes is still unexplored. While previous studies have reported dedifferentiation in the non-diabetic condition, the lack of metabolic characterization of the subjects makes it impossible to formulate a hypothesis to explain the role of dedifferentiation in T2D development. Since the heterogeneity of prediabetes has been widely acknowledged[26–28], it is important to thoroughly phenotype individuals metabolically before formulating conclusions. The possibility of correlating in vivo metabolic parameters (such as beta cell function and insulin resistance obtained with gold standard techniques) with ex vivo morphological data is a unique opportunity to formulate hypotheses on the pathogenesis of the disease in humans.

Taking advantage of the possibility of examining patients undergoing pancreatic surgery, we aimed to investigate the presence of intrapancreatic fat and beta-cell dedifferentiation in metabolically well-characterized patients without a known history of diabetes.

2. Research design and Methods

This is an observational, prospective, pilot study where eight patients with indication for surgery for low grade malignancy periampullary tumor, scheduled for pylorus-preserving pancreatoduodenectomy, were recruited at the Digestive Surgery Unit and studied at the Center for Endocrine and Metabolic Diseases (both at the A. Gemelli University Hospital, Rome, Italy). The study protocol (ClinicalTrials.gov Identifier: NCT02175459) was approved by the local ethics committee (P/656/CE2010 and 22573/14) and all participants provided written informed consent, after which a comprehensive medical evaluation was carried out. None of the patients enrolled had a known history of diabetes. Altered serum lipase and amylase levels prior to surgery, as well as morphologic criteria for pancreatitis, were considered exclusion criteria. Patients with severe obesity (BMI $>$ 40), uncontrolled hypertension and/or hypercholesterolemia were also excluded[29]. All subjects were administered an oral glucose tolerance test (OGTT) (75 g glucose) and a hyperglycemic clamp (HC) with arginine stimulation to evaluate beta cell function (from C-peptide deconvolution) and insulin sensitivity, as described below. [30–33].

3. Metabolic evaluation

3.1. Oral glucose tolerance test

A standard 75 g oral glucose tolerance test was performed with measurements of glucose and insulin at 0, 30, 60, 90, 120 min after glucose load. Glycemia and insulinemia were used to calculate the Matsuda Index as previously described and detailed below[31].

3.2. Hyperglycemic clamp procedure

Plasma glucose was clamped at a stable level of 125 mg/dl above fasting blood glucose concentration. The hyperglycemic clamp was started with a bolus dose of dextrose 200 mg/ml (150 mg/kg) administered into the antecubital vein. Blood was drawn from a cannulated dorsal hand vein on the opposite arm. Every 5 min, venous plasma glucose was analyzed with a glucose analyzer and an infusion of 20 % glucose was adjusted to achieve a stable glucose level of 125 mg/dl above the fasting value. Serum samples for insulin and C-peptide were drawn at 0, 2.5, 5, 7.5, 10, 15, 30, 60, 90, 120, 130, 140, and 150 min. At 120 min, a 5 g arginine bolus (SALF s.p.a. Cenate Sotto, BG, Italy) was administered to measure maximum C-peptide secretory capacity at a steady-state blood glucose concentration of 250 mg/dl[30].

3.3. Calculations

During the hyperglycemic clamp, insulin secretion rate was derived from C-peptide levels by deconvolution[32]. The first-phase insulin secretion response was calculated as the mean incremental insulin secretion between 0 and 5 min, when insulin secretion rate had fallen from the initial peak to a nadir in all subjects. Second-phase insulin secretion was calculated as the increment in insulin secretion above basal insulin secretion during the last 20 min of the hyperglycemic clamp. Beta-cell Glucose Sensitivity (βCGS), i.e. the slope of the relationship between insulin secretion and glucose concentration, was estimated from the hyperglycemic clamp as the ratio of the increments in insulin secretion and glucose concentration from baseline [33]. Matsuda indexes were calculated as indexes of whole-body insulin sensitivity based on insulin and glucose values obtained from the OGTT, while β cell function was evaluated by calculating the insulinogenic index as the change in insulin over the first 30 min divided by the change in glucose over the first 30 min[30].We also measured the oral disposition index, which provides an assessment of insulin secretion in relation to insulin sensitivity, calculated as the product of the insulinogenic index and the Matsuda index[34].

3.4. Surgical procedures

Pancreatoduodenectomy was performed according to the pylorus-preserving technique. Briefly, the pancreatic head, the entire duodenum, common bile duct, and gallbladder were removed en bloc, leaving a functioning pylorus intact at the gastric outlet. All adjacent lymph nodes were carefully removed. The continuity of the gastrointestinal tract was restored by an end-to-side pancreatojejunostomy. Further downstream, an end-to-side hepaticojejunostomy and an end-to-side pylorojejunostomy were performed. The volume of pancreas removed during the surgery is constant (~50 %), as previously reported by Schrader et al[35].

3.5. Immunohistochemical and morphometric analyses

We fixed and processed tissue for immunohistochemistry as previously described[36]. Paraffin sections, each 3 μm-thick, were obtained for hematoxylin and eosin staining, and for immunohistochemistry. Tissue sections were imaged with a Nikon Eclipse E800 light microscope using a 40 × lens and digital images were captured with a Nikon DXM 1200 camera. We performed histochemical reactions for the entire cohort at the same time. We controlled each reaction by omitting primary or secondary antibodies to determine signal specificity. We used Alexa conjugated donkey secondary antibodies (Jackson Immunoresearch Laboratories and Molecular Probes), as described[14]. We used confocal microscopy and Laser Scanning Microscope Software (LEICA) to study dedifferentiation and capture images. We performed quantification in a blinded fashion using ImageJ software to analyze individual cells in whole-slide fluorescent and optical images. To perform

quantitative analyses, we scored at least one random section per donor and 10 random islets. We scored dedifferentiation as the percentage of cells positive for the endocrine marker synaptophysin but negative for the 4 major endocrine hormones (insulin, glucagon, somatostatin and pancreatic polypeptide) as previously described[14]. The intraparenchymal white adipose tissue (WAT) score was calculated as the percentage of pancreatic tissue occupied by white adipose tissue identified using a Nikon Eclipse E800 light microscope with a 40 × lens.

3.6. Statistical analysis

We used descriptive statistical measures to describe the clinical and demographic characteristics of the sample: categorical variables were summarized with absolute frequencies and percentages, while mean ± standard deviation or median and interquartile range (IQR), and sometimes for clarity both, were used for continuous variables. Maintaining the descriptive perspective of the study, the following clinical measurements were evaluated for the groups derived from OGTT classification: BMI, % of dedifferentiated beta cell, % WAT, Disposition, Insulinogenic Index, HOMA-IR AND Matsuda Index. A linear regression procedure was applied to evaluate the relation between WAT (%) and dedifferentiated beta cells (%), between glycemia during OGTT and dedifferentiated beta cells (%), between glycemia during OGTT and WAT (%).

4. Results

4.1. Clinical data

The study sample included a total of N = 8 patients, 4 men and 4 women, undergoing pylorus-preserving pancreatoduodenectomy. Indications for surgery were periampullary tumors, pancreatic intraductal papillary tumors, mucinous cystic neoplasm of the pancreas, and nonfunctional pancreatic neuroendocrine tumors. None of the patients had a history of diabetes. Based on the results of the OGTT performed prior to surgery, we classified the patients as normal glucose tolerant (NGT = 3), impaired glucose tolerant (IGT = 3) and newly diagnosed diabetes (nDM = 2). The median age was 72 years with (IQR: 59.8 – 73.7) and the median BMI was 25.9 (IQR: 23.8 – 28.4). *See* Table 1 for the clinical and demographic description of the sample and Table S1 for details on each patient (supplementary material).

4.2. Increase in intraparenchymal white adipose tissue parallels worsening glucose tolerance

We measured the percentage of pancreatic tissue occupied by white adipose tissue and observed a progressive increase in intraparenchymal white adipose tissue from normal glucose tolerant to impaired glucose tolerant to newly diagnosed type 2 diabetes (5.81 %± 1.66 in NGT; 10.67 % ± 4.71 in IGT; 32.2 %±14.71 in nDM;) (Fig. 1 A). Moreover, dedifferentiated beta cells (%) increased as glucose tolerance worsened (from a mean of 4.8 in NGT to a mean of 28.9 in nDM) (Fig. 1 B).

Table 1
Clinical and demographic description of the sample (N = 8).

Sex	
F	4 (50.0 %)
M	4 (50.0 %)
Age	72.0 (59.8–73.7)
BMI	25.9 (23.8–28.4)
OGTT based classification	
IGT	3 (37.5 %)
NGT	3 (37.5 %)
nDM	2 (25.0 %)
Hypertension	
NO	5 (66.7 %)
YES	3 (33.3 %)
Dyslipidemia	
NO	5 (66.7 %)
YES	3 (33.3 %)
Waist circumference (WC)	89.0 (72.0–90.0)
Hip circumference (HC)	100.0 (93.0–100.0)

A strong, positive correlation was observed between WAT (%) and dedifferentiated beta cells (%) (R = 0.868). WAT (%) accounted for 75.3 % of the explained variability of dedifferentiated beta cells (%): in other words, if WAT (%) is causally linked to dedifferentiated beta cells (%), then approximately 75.3 % of the variation in dedifferentiated beta cells (%) is justified by the value of WAT (%). A linear regression established that WAT (%) could statistically significantly predict the percentage of dedifferentiated beta cells, F (1, 6) = 18,4; p = 0.005. This means that there is a predicted increase in dedifferentiated beta cells (%) of 1.25 points for every extra one-point change in WAT (%). The 95 % confidence interval for B is [0.535; 1.962], p = 0.005; which means that the coefficient is statistically significantly different to zero. (Fig. 1 C). Consequently, the value of the dependent variable, dedifferentiated beta cells (%), can be approximately predicted from the value of the independent variable WAT (%).

We observed a clear, but not significant, negative correlation between beta cell function, (measured as beta cell glucose sensitivity with either OGTT or HC), the percentage of dedifferentiation and the percentage of intraparenchymal white adipose tissue (data not shown). Furthermore, we observed a numerical increase in the percentage of WAT and dedifferentiated beta cells in the more insulin resistant group (data not shown).

A different trend was observed for the following measures: mean and median disposition decreased as glucose tolerance worsened (from NGT subjects with a mean disposition of 3.9 to nDM subjects with a mean disposition of 0.8); mean and median Insulinogenic Index decreased as glucose tolerance worsened (from an NGT subject with a mean Insulinogenic Index of 1.0 to a nDM subject with a mean Insulinogenic Index of 0.2). Lastly, we found that mean and median BMI increased with worsening glucose tolerance (from NGT subject with a mean BMI of 24.1 ± 1.1 to a nDM subject with a mean BMI of 28.8 ± 0.8). See Table 2 for details.

4.3. 1-hour post load glucose level predicts intraparenchymal white adipose tissue and dedifferentiation score

We performed a linear regression analysis for the following pairs of variables: glycemia during OGTT and dedifferentiation score, and glycemia during OGTT and WAT score. Dedifferentiation score and 1-hour post-load glycemia were strongly associated as indicated by the multiple correlation coefficient R = 0.72. Moreover R^2 = 0.512 highlights that glycemia 60′ can be explained by 51.2 % variance in dedifferentiation score. The regression model is statistically significant (p = 0.046) hence glycemia 60′ statistically significantly predicted the dedifferentiation score. The slope coefficient (β_1 = 0.2) highlights that there is a predicted increase in dedifferentiated beta cells of 0.2 points for every extra one point (mg/dl) in glycemia 60′ (Fig. 2 A). The linear regression analysis between glycemia during OGTT and dedifferentiation score was not significant when considering glycemia at baseline and glycemia at 120′.

Indeed, glycemia during OGTT and WAT were strongly associated when considering glycemia at 60′, as indicated by the multiple correlation coefficient R = 0.74. Moreover, R^2 = 0.55 highlights that glycemia 60′ can be explained by 55 % variance in WAT. The regression model is statistically significant (p = 0.035), hence glycemia 60′ statistically significantly predicted WAT. The slope coefficient (β_1 = 0.143 highlights that there is a predicted increase in WAT of 0.143 points for every extra one point (mg/dl) in glycemia 60′ (Fig. 2 B).

5. Discussion

The pathogenesis of type 2 diabetes is multifaceted, and the scientific

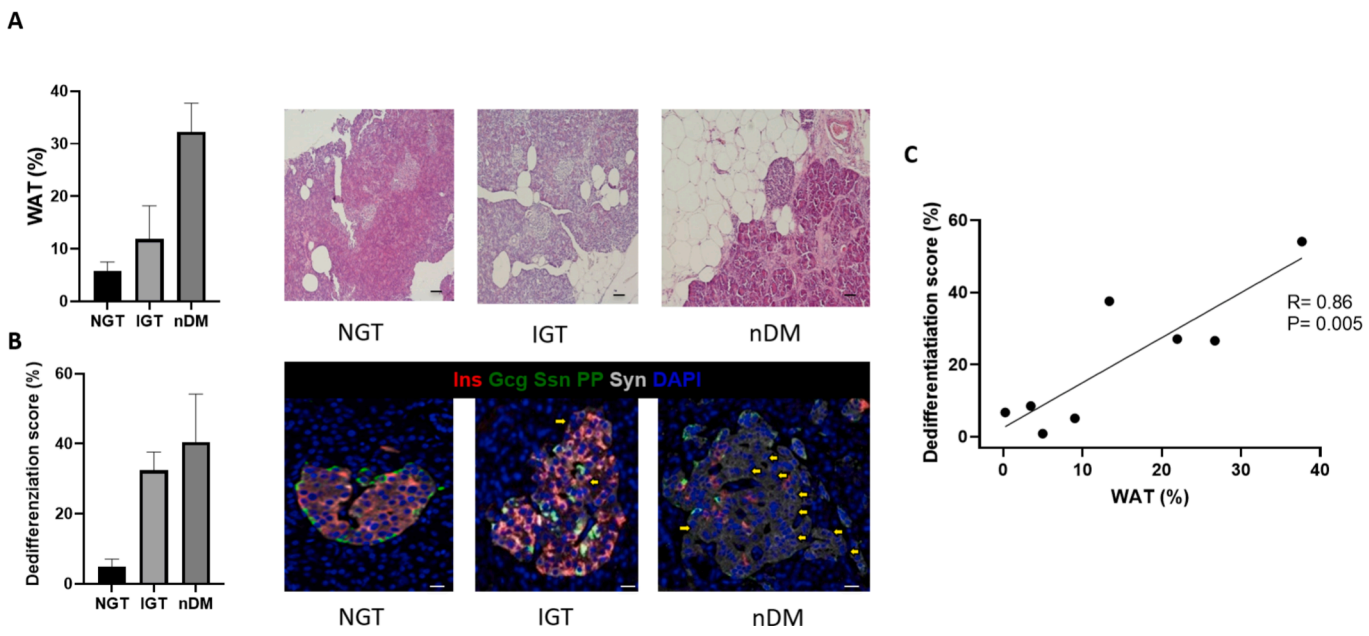


Fig. 1. Intraparenchymal white adipose tissue and beta cell dedifferentiation in different glucose tolerance conditions. A) intraparenchymal white adipose tissue score (%) in normal glucose tolerant (NGT), impaired glucose tolerant (IGT) and newly diagnosed type 2 diabetes (nDM); B) beta cell dedifferentiation score (%) in NGT, IGT and nDM; insulin positive cells (red), hormone cocktail (glucagon – somatostatin – pancreatic polypeptide in green), synaptophysin positive cells (gray), DAPI nuclei in blue; yellow arrows indicate dedifferentiated beta cell (i.e. synaptophysin positive 4 hormone negative cells); C) intraparenchymal WAT and dedifferentiation score correlation; (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Results by glucose tolerance group.

	NGT (N = 3)	
	Mean ± SD	**Median (IQR)**
% of dedifferentiated beta cells	4.8 ± 3.8	5.12 (2.9–6.8)
BMI	24.1 ± 1.1	24.1 (23.5 – 24.6)
% WAT	5.8 ± 2.9	4.96 (4.2–6.9)
Oral Disposition Index	3.9 ± 3.7	3.44 (1.9–5.6)
Insulinogenic Index	1.0 ± 1.0	0.87 (0.5–1.5)
HOMA-IR	2.0 ± 0.4	2.06 (1.8–2.1)
Matsuda Index	4.2 ± 0.6	3.93 (3.8–4.4)
	IGT (N = 3)	
	Mean ± SD	**Median (IQR)**
% of dedifferentiated beta cells	23.8 ± 15.6	27.1 (16.9–32.4)
BMI	25.5 ± 3.7	26.6 (24.0–27.6)
% WAT	11.8 ± 10.9	13.4 (6.8–17.6)
Oral Disposition Index	2.1 ± 1	2.2 (1.6–2.6)
Insulinogenic Index	0.7 ± 0.6	0.6 (0.4–0.9)
HOMA-IR	1.9 ± 0.7	1.5 (1.5–2.1)
Matsuda Index	5.7 ± 1.6	5.8 (4.9–6.6)
	nDM (N = 2)	
	Mean ± SD	**Median (IQR)**
% of dedifferentiated beta cells	40.3 ± 19.4	40.4 (33.5–47.3)
BMI	28.8 ± 0.8	28.8 (28.6 – 29.1)
% WAT	32.2 ± 7.7	32.2 (29.4–34.9)
Oral Disposition Index	0.8 ± 0.8	0.87 (0.57–1.2)
Insulinogenic Index	0.2 ± 0.2	0.27 (0.2–0.355)
HOMA-IR	2.1 ± 0.3	2.2 (2.05–2.3)
Matsuda Index	3 ± 0.3	3.1 (2.9–3.2)

community has long focused on the impact of insulin resistance and beta cell dysfunction, comparing subjects with a long-term history of diabetes to subjects without diabetes.

Taking advantage of the availability of pancreas samples from metabolically well-characterized patients without a history of diabetes, we conducted a functional and morphological analysis to determine what happens before metabolic deterioration. We found that, with the worsening of glucose tolerance, there is a progressive increase in intrapancreatic WAT and in the dedifferentiation score; importantly, the increases in the dedifferentiation score and WAT are positively correlated, and the percentage of WAT can strongly predict the percentage of dedifferentiated beta cells before diabetes onset. Interestingly, 1-hour post-load glycemia is related to the percentage of WAT and dedifferentiation score, thus enhancing the translational relevance of our results.

Recent discoveries highlighting the complexity and heterogeneity of the state of prediabetes have shifted attention to attempting to understand what happens prior to diabetes onset, in a bid to prevent and curb the disease. Our data confirm the heterogeneity of the "without diabetes" state and show how the dedifferentiation process could influence the natural history of the disease. In a previous article, we reported 15 % of dedifferentiated beta cells in non-diabetes samples compared to diabetes samples[14], which has been confirmed in other studies[17,21]. In this study, we observed 4.8 % and 32 % of dedifferentiated beta cells in NGT and IGT group respectively, with an average of 18 % when combining the two groups. These data confirm our previous observations, but also underline the importance of subclassifying the prediabetes state.

Our data show that 1-hour post-load glycemia significantly predicts the WAT and dedifferentiation score, and recently 1-hour glycemia during OGTT has been proposed as a robust predictor for determining the future risk of type 2 diabetes [34,37,38], thus our data support the clinical importance of considering even glycemia at 60′ prior to diabetes onset. In terms of clinical implications, our results indicate that IGT and 1-hour altered post-load glycemia patients should become the main clinical target for the prevention of diabetes onset by attempting to reverse the dedifferentiation process.

As stated in the results, the percentage of dedifferentiation parallels the percentage of intrapancreatic WAT. The findings of the ReTUNE study, which recruited subjects with type 2 diabetes who had an average BMI of 24.8 kg/m^2, show that losing a substantial amount of weight can reverse type 2 diabetes even in subjects who are only mildly overweight [8]. The ReTUNE study was specifically designed to demonstrate Taylor's threshold hypothesis: namely, that there is a limit to the amount of fat individuals can accumulate around their liver and pancreas before health issues, including insulin resistance and type 2 diabetes, arise. For this reason, the focus is currently also on pancreatic adipose tissue and

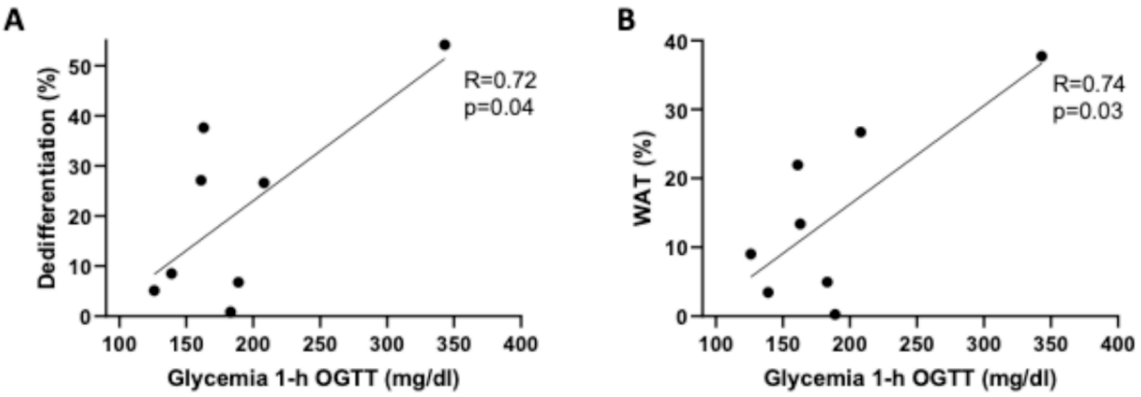


Fig. 2. 1-hour post load glucose level correlation with intraparenchymal white adipose tissue and dedifferentiation score: linear regression analysis between dedifferentiation score (%) and 1-hour post load glycemia (A) and linear regression analysis between intraparenchymal white adipose tissue % (WAT) and 1-hour post load glycemia (B).

its role in the pathogenesis of the disease. Our data show a correlation between intrapancreatic WAT accumulation and beta cell dedifferentiation, suggesting a pathophysiological correlation between the two phenomena. Rodent data have already demonstrated that caloric restriction induces redifferentiation of beta cells[23,39], unfortunately, to date, this cannot be reproduced in humans.

As any other study in humans, our results have limitations. The very small sample size is a limit of our study, but the unique advantage of performing morphology on pancreas samples derived from metabolically well-characterized patients, makes the results obtained reliable, despite the small sample size. We emphasize that this is a pilot study with exploratory aims, and that additional research with larger sample sizes is necessary. Lastly, to our knowledge, there is no evidence of a direct role of adipose tissue in islet cell plasticity, whereas many studies have demonstrated the deleterious effect of visceral fat accumulation in human metabolism [5,40]. Our experiments cannot provide mechanistic insights; however, a targeted in vitro co-culture study would be valuable in addressing this important gap.

6. Conclusions

Considering all the limitations that our small sample size entails, our data demonstrate that the accumulation of intrapancreatic WAT parallels beta cell dedifferentiation even in the prediabetic state, suggesting a role of intrapancreatic WAT in the pathogenesis of type 2 diabetes and, potentially, a new target to curb diabetes onset. The results of this study certainly indicate promising research topics to evaluate with a larger sample.

Funding

The research project was funded through grants received by FC from EFSD/Astrazeneca Future leader Mentorship Programme 2018 and EFSD/Lilly Young Investigator Award 2019.

Authors' contributions

CM, SG, GDG, UC, SM, LS, MB, AA, ECN, performed the metabolic procedures. GQ, VT performed surgeries from which donor samples were obtained. IS, MS and FC performed the morphological analysis. RM performed the statistical analysis. SA, AP, A Gasbarrini, SC and A Giaccari contributed to the discussion and edited the manuscript. F. Cinti and T. Mezza gave the major contribution in analyzing data and writing the manuscript. All authors have read and approved the final manuscript.

CRediT authorship contribution statement

Francesca Cinti: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Teresa Mezza:** Methodology, Investigation, Conceptualization. **Ilenia Severi:** Methodology, Investigation, Data curation. **Simona Moffa:** Methodology, Investigation. **Gianfranco Di Giuseppe:** Methodology, Investigation. **Umberto Capece:** Methodology, Investigation. **Gea Ciccarelli:** Methodology, Investigation. **Laura Soldovieri:** Methodology, Investigation. **Michela Brunetti:** Methodology, Investigation. **Cassandra Morciano:** Methodology, Investigation. **Shawn Gugliandolo:** Methodology, Investigation. **Martina Senzacqua:** Methodology, Investigation. **Adriana Avolio:** Methodology, Investigation. **Giuseppe Quero:** Resources. **Vincenzo Tondolo:** Resources. **Enrico Celestino Nista:** Resources. **Rossana Moroni:** Supervision, Methodology, Data curation. **Saverio Cinti:** Supervision, Conceptualization. **Sergio Alfieri:** Resources. **Antonio Gasbarrini:** Supervision. **Alfredo Pontecorvi:** Supervision. **Andrea Giaccari:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Serena Rotunno (Università Cattolica del Sacro Cuore) for editorial assistance with the writing of this manuscript. We would like to acknowledge the contribution of the Multispecialistic Biobank Research Core Facility G-STeP, Fondazione Policlinico Universitario "A. Gemelli" IRCCS (Biobank-FPG) which provided the bioresources.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diabres.2025.112029.

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