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Advancing Diabetes Research: a novel islet isolation method from living donors.

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Abstract: Pancreatic islet isolation is critical for type 2 diabetes research. Although -omics approaches have shed light on islet molecular profiles, inconsistencies persist; on the other side, functional studies are essential, but they require reliable and standardized isolation methods. Here, we propose a simplified protocol applied to very small-sized samples collected from partially pancreatectomized living donors. Islet isolation was performed digesting tissue specimens collected during surgery within a collagenase P solution, followed by a Lympholyte density gradient separation; finally, functional assays and a staining with dithizone were carried out. Isolated pancreatic islets exhibited functional responses to glucose and arginine stimulation mirroring donors' metabolic profiles, with insulin secretion significantly decreasing in diabetic islets compared to non-diabetic islets; conversely, proinsulin secretion showed an increasing trend from non-diabetic to diabetic islets. This novel islet isolation method from living patients undergoing partial pancreatectomy offers a valuable opportunity for targeted study of islet physiology, with the primary advantage of being time-effective and successfully preserving islet viability and functionality. It enables the generation of islet preparations closely reflecting donors' clinical profiles, simplifying the isolation process and eliminating the need for a Ricordi chamber. Thus, this method holds promises for advancing our understanding of diabetes and for new personalized pharmacological approaches.

Keywords: pancreatic islets; type 2 diabetes; islet isolation; insulin secretion.

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1. Introduction

Pancreatic islet isolation is a critical procedure in diabetes research and is of great importance for studying the pathophysiology of islet cells. Particularly, type 2 diabetes (T2D) has proven to be highly heterogeneous both in its clinical and molecular features [1], hence resulting in the lack of a comprehensive understanding of its underlying causes. Even though the -omics approaches are providing a thorough view of the islets' molecular footprints in health and disease [2–4], the results are not always matching [5] and consequently, a unique profiling of diabetes trajectory from its very beginning is still to be achieved. Furthermore, transcriptional and proteomic analyses -although very informative from the molecular side- cannot replace functional studies. Thus, developing a reliable and, ideally, standardized islet isolation method represents a crucial issue in the field of diabetes, both for a deeper understanding of the cellular and molecular basis of the disease and for clinical/therapeutic purposes [6–8].

Several protocols have been developed and improved over the years from the standardized Ricordi chamber method designed for human pancreata digestion [9,10]: the attention has been focused not only on samples from human derivation [11–15], but also on various preclinical models, such as rodents [16–19] and pigs [20–23]. Indeed, rodents have been variously used to model the islet pathophysiology in health and disease, despite the metabolic [24] and structural [25] differences with human islets; on the other side, there is a strong concern about pig islets as a promising therapeutic resource for xenotransplantation in type 1 diabetes treatment [26,27].

For research purposes, isolated human islets mostly derive from deceased organ donors [28–33], with the main advantage of having considerable starting material, standardized conditions, and an overall high purification yield. On the other side, clinical information and family history about brain-dead organ donors are limited [34], resulting in poorly characterized samples and the lack of clinical in vivo features to be correlated with functional or molecular assays.

In this work, we provide a novel method for isolating pancreatic islets from living patients undergoing a partial pancreatectomy. All individuals underwent a deep metabolic evaluation and functional studies before the surgery, thus allowing to distinguish different metabolic conditions and to correlate any in-vitro features revealed on the pancreas samples to a specific in vivo profile. The main advantage of this procedure is that we finally obtain samples enriched with living pancreatic islets that reflect the clinical profile of their donors, starting from a minimal amount (1-1.5 g) of healthy tissue. Lastly, we do not use a Ricordi chamber, considerably simplifying the whole procedure in terms of time- and cost-effectiveness.

2. Procedure and Results

2.1 Patient characterization and surgical procedure

2.1.1 Study Design and Experimental Procedures

Patient characterization and surgical procedures were performed as previously described [35–38]. Patients scheduled for pylorus-preserving pancreatoduodenectomy were recruited at the Digestive Surgery Unit and studied at the Centre for Endocrine and Metabolic Diseases unit, Agostino Gemelli University Hospital, Rome, Italy. Indications for surgery were: periampullary tumors, pancreatic intraductal papillary tumors, mucinous cystic neoplasm of the pancreas, non-functional pancreatic neuroendocrine tumors.

Each subject underwent an oral glucose tolerance test, a hyperinsulinemic-euglycemic clamp, a hyperglycemic clamp, and a mixed-meal test 1 week before the surgical procedure (table 1).

-Oral Glucose Tolerance Test

Normal glucose metabolism and glucose tolerance status were determined by a standard 75-g oral glucose tolerance test measuring glycemia, insulin, and C-peptide at 0, 30, 60, 90, 120 min after the glucose load.

-Hyperinsulinemic Euglycemic Clamp Procedure

The hyperinsulinemic euglycemic clamp test was performed after a 12-h overnight fast using insulin 40 mIU·m−2·min−1 of body surface according to DeFronzo et al. [39] A primed constant infusion of insulin was administered (Actrapid HM, 40 mIU·m−2·min−1; Novo Nordisk, Copenhagen, Denmark). The constant rate for the insulin infusion was reached within 10 min to achieve steady-state insulin levels. In the meantime, a variable infusion of 20% glucose was started with a separate infusion pump, and the rate was adjusted, on the basis of plasma glucose samples drawn every 5 min, to maintain the plasma glucose concentration at each participant's fasting plasma glucose level. During the last 20 min of the clamp procedure, plasma samples from blood drawn at 5- to 10-min intervals were used to determine glucose and insulin concentrations. Whole-body peripheral glucose utilization was calculated during the last 30-min period of the steady-state insulin infusion and was measured as the mean glucose infusion rate (mg·kg−1·min−1).

-Hyperglycemic Clamp Procedure

The plasma glucose was clamped at a stable level of 125 mg/dL above the fasting blood glucose concentration. The hyperglycemic clamp was started with a 200 mg/mL bolus dose of dextrose (150 mg/kg) administered into the antecubital vein. Blood was drawn from a cannulated dorsal hand vein on the opposite arm. Venous plasma glucose was analyzed every 5 min with a glucose analyzer, and the 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.

The first-phase insulin release, reflecting the early insulin peak secreted from the pancreatic β-cell in response to glucose stimulation, was calculated as the area under the curve (AUC) during the first 10 min of the clamp by using the trapezium rule. The second-phase insulin release, reflecting β-cell function under sustained elevated glucose levels, was calculated as the AUC from 10 to 120 min. Subsequently, a 5-g arginine bolus was administered to measure maximum C-peptide secretory capacity at a steady-state blood glucose concentration of 250 mg/dL. Combined hyperglycemia- and arginine-stimulated β-cell secretory capacity was calculated as the insulin AUC during the 30 min after the arginine bolus. Insulin and c-peptide AUC values are shown in table 1 and in Supplementary figure 1.

-Mixed-Meal Test

Patients were instructed to consume a meal of 830 kcal (107 kcal from protein, 353 kcal from fat, and 360 kcal from carbohydrates) within 15 min. Blood samples were drawn twice in the fasting state and at 30-min intervals over the following 240 min (sample time 0, 30, 60, 90, 120, 150, 180, 210, and 240 min) for the measurement of plasma glucose, insulin, C-peptide, glucagon, and glucagon-like peptide 1 (GLP-1) or glucose-dependent insulinotropic polypeptide (GIP) concentrations. Blood samples for glucagon, total GLP-1, or intact GIP were sampled in tubes containing EDTA and a dipeptidyl peptidase-4 inhibitor (Millipore, Billerica, MA); after centrifugation (1,000 rpm for 10 min at 4°C), samples were stored at −80°C until analyses. Insulin levels were determined using a commercial radioimmunoassay kit (Medical System, Immulite DPC, Los Angeles, CA). Plasma glucose

concentrations were determined by the glucose oxidase technique, using a glucose analyzer (Beckman Instruments, Palo Alto, CA). Plasma C-peptide was measured by Auto-DELPHIA automatic fluoroimmunoassay (Wallac, Turku, Finland), with a detection limit of 17 pmol/L.

1.2 Surgical Procedures

Pancreatoduodenectomy was performed according to the pylorus-preserving technique (18,19). 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 invaginated pancreatojejunostomy. Further downstream, an end-to-side hepaticojejunostomy and side-to side gastroenterostomy or an end-to-side pylorus-jejunostomy was made. A pancreas sample was collected during the surgery from the downstream edge of the surgical cut.

Subject characteristics	**ND (*n* =14)**	**IGT (*n* =8)**	**DM (*n* =15)**	**P Value**
Mean age (y)	*58.1 ± 4.02*	*66 ± 4.38*	*73.4 ± 2.04*	
Gender (F/M)	*7/7*	*3/5*	*9/6*	
BMI (kg/m^2)	*24.67 ± 1.56*	*25.04 ± 1.55*	*23.70 ± 0.94*	*0,77*
Insulin sensitivity (mg·kg^{-1}·min^{-1})	*4.60 ± 1.36*	*3.20 ± 0.48*	*4.90 ± 1.37*	*0,64*
Fasting glucose (mg/dl)	*86.5 ± 2.29*	*95.6 ± 5.27*	*129.4 ± 12.63*	***0,003*** *
Fasting insulin (μUI/ml)	*4 ± 0.40*	*4.9 ± 0.53*	*4.92 ± 1.11*	*0,358*
Fasting C-peptide (ng/ml)	*1.54 ± 0.29*	*1.13 ± 0.10*	*2.05 ± 0.29*	*0,87*
AUC insulin (μUI/ml)	*11351,1 ± 1513,6*	*12921,5 ± 1730,4*	*5433 ± 1643*	***0.0129****
AUC c-peptide (ng/ml)	*1159,9 ± 107,8*	*1343,1 ± 112,5*	*703,7 ± 114,7*	***0,0036****

Table 1: clinical and metabolic characteristics of the enrolled patients, classified into ND, IGT and DM according to glucose tolerance before surgery. Data are represented as means ± SEM.

2.2 Samples collection and tissue digestion

Small pancreatic tissue specimens -sized about 1 cm^3- are collected in a 50 ml tube during the surgery and immediately soaked in 10-15 ml of cold physiological solution; to prevent tissue autolysis, the samples should be kept on ice or at +4°C and the processing time should be minimized as much as possible.

The specimens are transferred under a laminar flow hood, and all the further steps are performed in sterile working conditions. Before starting, a 50 ml tube with an adequate volume of pre-warmed Hank's Balanced Salts Solution (HBSS) should be prepared:

usually, a tissue sample of about 1-1.5 g of weight is digested in a final volume of 10 ml, comprising 8 ml HBSS + 2 ml of collagenase P (1.5 U/mg, used at a working concentration of 1.4 mg/ml). If more tissue is available, the volume of HBSS + collagenase should be adjusted accordingly.

The samples are minced into smaller pieces of 1-2 mm^3 within a 60 x 15 mm petri dish, using tweezers and a surgical blade. The surrounding fat and embedding matrix residues are discarded, since their enzymatic digestion creates an oily layer at the top of the final suspension that might interfere with the following isolation steps. Chopped tissue pieces are finally transferred into the tube containing the collagenase P solution in HBSS, and the entire mixture is vigorously hand-shaken. Enzymatic digestion is carried out at 37°C in a shaking bath for 1 hour, pipetting up and down or manually shaking the tube 2-3 times during the whole procedure to ensure the proper mixing and resuspension of the mixture.

2.3 Islets isolation: filtration and density gradient

Before proceeding with the isolation, the following two solutions have to be prepared: HBSS-FBS (90% HBSS-10% FBS, Fetal Bovine Serum) and of 80% Lympholyte (80% Lympholyte-20% HBSS-FBS). Lympholyte 100% and 80% will be used at room temperature to create a density separation gradient. HBSS-FBS should be ice-cold to preserve islet vitality as much as possible; serum is added to sustain cellular recovery.

After the end of the incubation period, the digestion mixture should look homogeneous and turbid, with released cells floating within the suspension and the residual undigested tissue at the bottom of the tube (optimal-quality pancreatic specimens are compact and tend to sediment). The tubes are removed from the water bath and cleaned with 70% ethanol on the external surface, then placed on ice to prevent over-digestion and moved again to sterile working conditions. Meanwhile, a centrifuge should be pre-chilled at 4°C.

The size range for human pancreatic islets is about 50-400 μm, with an average diameter of about 150 μm [40–42]. To retain the highest possible number of islets on the upper face of the mesh, we filter the cell suspension using cell strainers of two different sizes (100 μm and 40 μm mesh).

Before proceeding to the serial filtrations, a 100 μm mesh cell strainer is placed over a clean 50 ml tube. About 8-9 ml of supernatant from the digestion mixture (this is the largest volume that can be recollected without picking up tissue residues) are drawn with a serological pipette and gently passed through the 100 μm strainer. The strainer is then enclosed within a clean 60 x 15 mm petri dish and 1-2 ml of ice-cold HBSS-FBS are rapidly added over the mesh with a p1000 micropipette, to avoid air-drying. The process is repeated identically, passing the filtered suspension through a 40 μm mesh cell strainer. Both the upper and the reverse side of the meshes are thoroughly washed within their respective plates, using abundant ice-cold HBSS-FBS. Every 1-2 ml used, all the medium -enriched with pancreatic cells- is accurately recollected from the strainer mesh and from the plate with a p1000 pipette, then put in a 15 ml tube. Even the dish surface has to be washed a couple of times, to minimize the loss of the cellular material due to plastic adhesion.

After recollecting the cells in about 14-15 ml of total medium, the tube is centrifuged at 1800 rpm for 5 minutes at +4°C. In the meanwhile, a clean 15 ml tube containing 3 ml of pure Lympholyte should be gently overlaid with 3 ml of 80% Lympholyte, reclining the tube and pouring the medium over its wall. At the end of centrifugation, the supernatant is discarded, while the pellet is resuspended in cold HBSS in two steps (2+1 ml) to recover all the material, and slowly stratified over the 80% Lympholyte. The tube is centrifuged at 1800 rpm x 10 minutes at +4°C, with reduced acceleration/deceleration (or removing the centrifuge brake).

Lympholyte is a cell separation medium commonly used for lymphocyte isolation [43–45]. We use this reagent at two different percentages (100% and 80%) so as to create a

density gradient of three layers (with denser Lympholyte at the bottom and HBSS at the top) and two interfaces. This procedure allows a better separation of tissue debris and single cells and the achievement of a final cell pellet highly enriched in pancreatic islets.

Upon centrifugation, larger islets will form a floating ring at the superior interface (at approximately 6 ml), while the lower interface (at approximately 3 ml, sometimes barely visible if the starting material is scarce) will be enriched in smaller islets (figure 1). The cellular material at the two interfaces is entirely recollected with a Pasteur pipette (starting from the upper one and being very careful not to mix the phases while drawing the interfaces) and gently poured into a clean tube. Ice-cold HBSS-FBS is quickly added to the sample up to a final volume of 14-15 ml, to dilute the Lympholyte and minimize cellular stress. The suspension is inverted up and down a couple of times and centrifuged at 1800 rpm for 5 minutes at +4°C; the islet pellet is finally resuspended in 1 ml of DMEM(-)-BCS (DMEM without glucose-10% BCS (Bovine Calf Serum)- 1% P/S- 0,34% L-Glutamine- 0,1% Gentamicin- 1% Amphotericin B) supplemented with glucose 3.3 mM, then plated in a 24-well (Figure 2) and put in a humidified incubator at 37°C+5% CO2 from 30 min to overnight, to promote cell recovery.

A quick staining with 100 µg/ml dithizone (1:100 from a 10 mg/ml stock) [46] will confirm the presence of pancreatic islets (Figure 3).

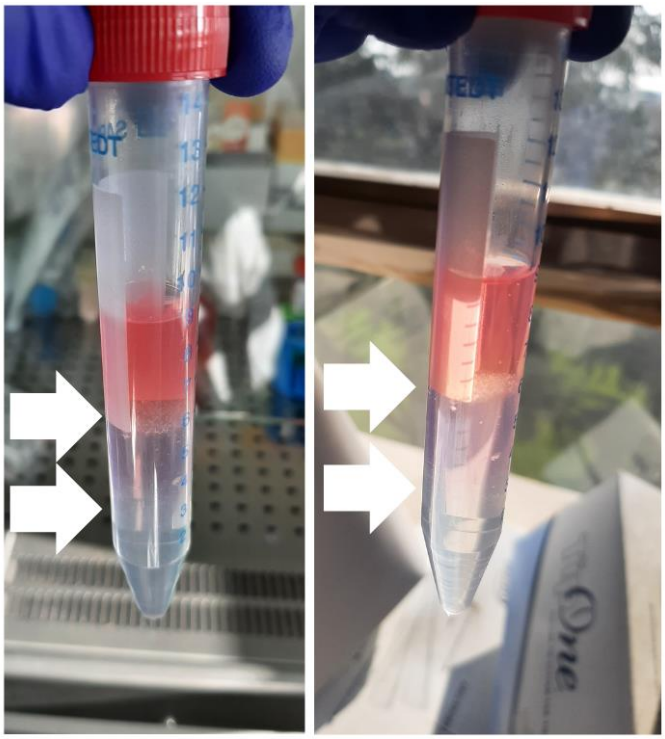
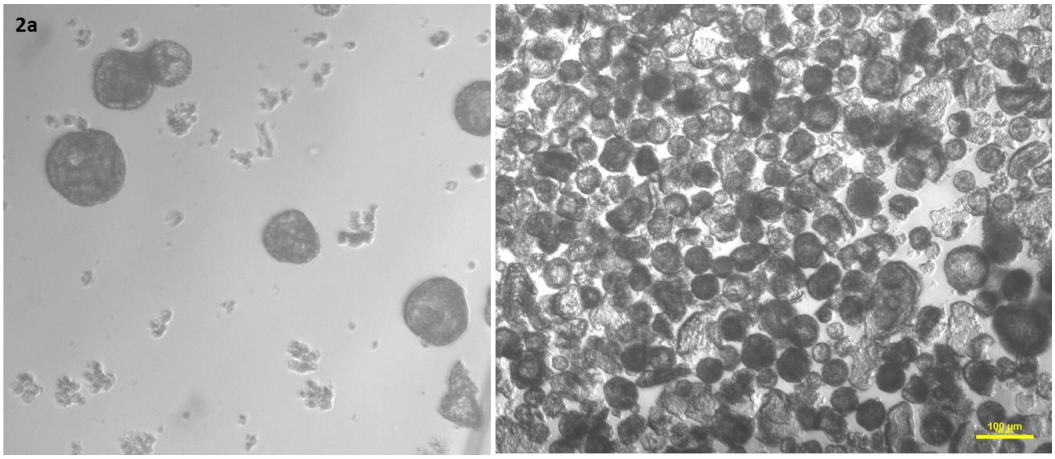


Figure 1: **Density separation gradient**. Representative pictures of the three-phase density gradient created by layering the HBSS-resuspended sample over two different percentages of Lympholyte (80%-HBSS and 100%) from the top to the bottom of the tube. The white arrows point out the floating interphases (at 6 and 3 ml, approximately) enriched with pancreatic islets.



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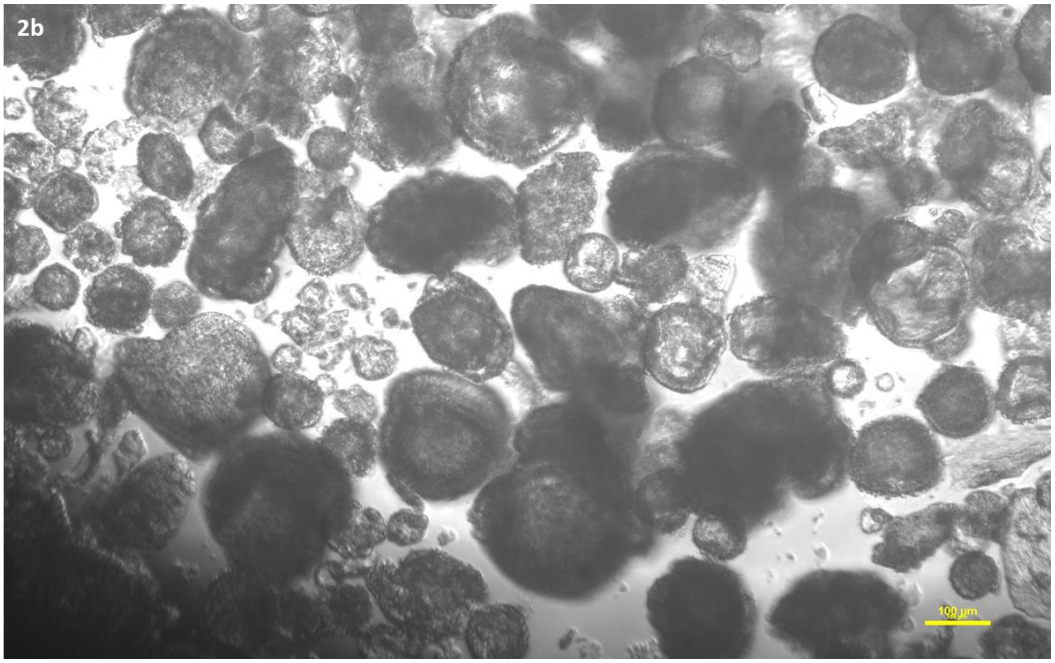

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Figure 2: **Brightfield images of purified human pancreatic islets after the isolation and a recovery period**. Ffigure 2a 10x magnification;figure 2b 20x magnification.

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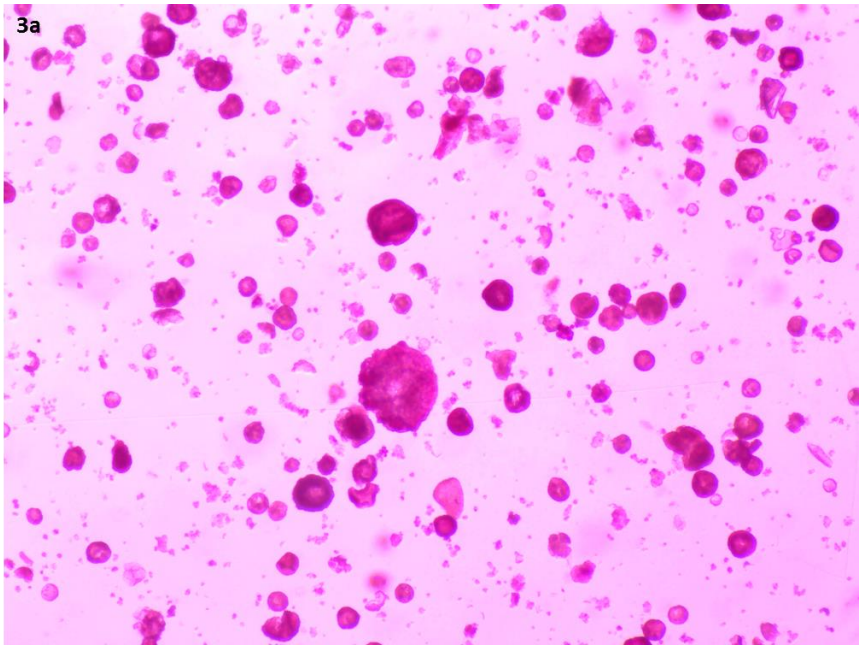

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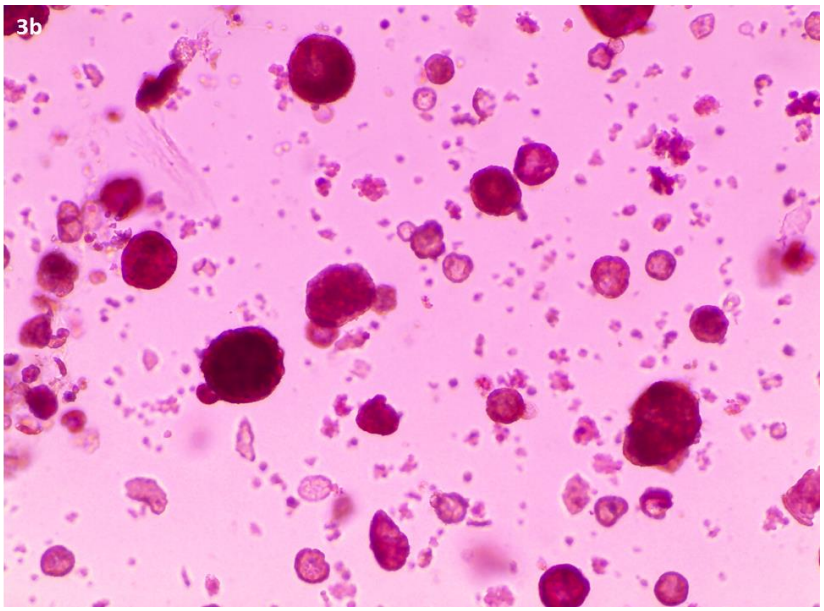

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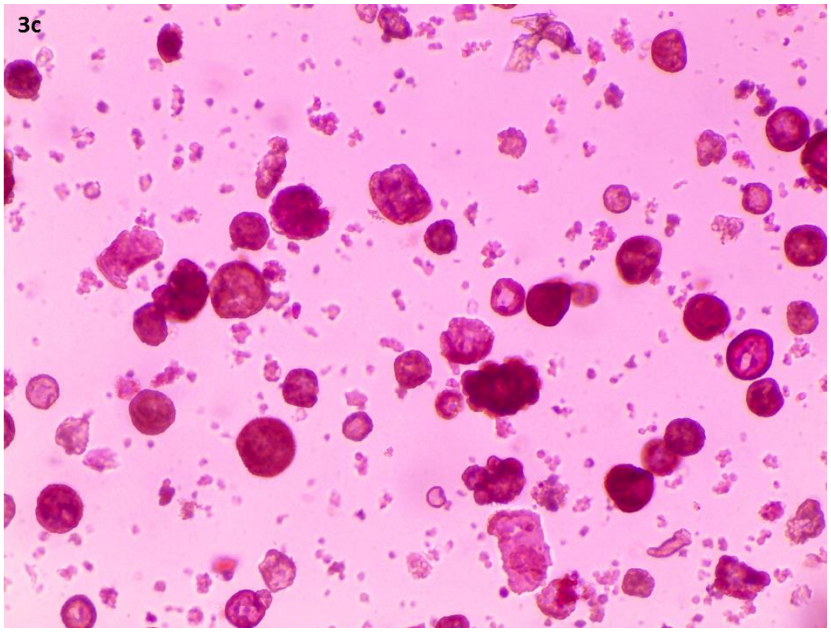


Figure 3: **Dithizone staining confirming the presence of pancreatic islets (in purple).** The images have been acquired through EVOS™ XL Core Imaging System #AMEX1000. Figure 3a has been captured at a 4x magnification, figures 3b and 3c are 20x enlargements.

2.4 Glucose stimulation and insulin secretion

2.4.1 Islets stimulation

To confirm the presence of a physiological β-cell response (i.e. glucose-induced insulin release), the islets were exposed, after the recovery period, to different glucose concentrations. The cells were first transferred in a 1.5 ml tube, gently resuspended, and centrifuged at 1800 rpm for 5 min at +4°C, using a pre-chilled bench centrifuge. The supernatant was fully discarded (without disturbing the cell pellet) to remove the insulin released into the medium during the recovery time. The islet pellet was resuspended in DMEM-BCS and divided equally into three 1.5 ml tubes, distributing 150 µl for each tube. Normalization was achieved by using consistently comparable samples in terms of size: this principle was applied by roughly estimating the islet pellet dimensions and adjusting accordingly the amount of pellet to be assayed. Glucose and arginine were added to the cell suspensions from previously reconstituted stocks (glucose= 334 mM, 1:100 or 1:20; arginine= 400 mM, 1:20) to reach the following concentrations in a final volume of 200 µl/sample:

-basal glucose, 3.3 mM;

-high glucose, 16.7 mM;

-basal glucose 3.3 mM+ arginine 20 mM (referred to as arginine 20 mM).

The prepare stocks of glucose and arginine, the powders were resuspended in DMEM(-)-BCS, filtered (0,22 µM) and stored at +4°C.

Cells were stimulated at 37°C+5% CO2 for one hour. The samples were then centrifuged at 1800-2000 rpm x 5 minutes at +4°C, and the supernatants were separately collected on ice and immediately stored at -80°C.

2.4.2 Assessment of insulin and proinsulin secretion

Supernatants from non diabetic- (ND), pre-diabetic- (IGT) and diabetic- (DM) derived islets were thawed on ice and human insulin and proinsulin were measured by commercial ELISA kits, following the manufacturer's recommendations. Insulin and proinsulin

content was reported as OD (optical density) values; stimulation index (SI) for insulin and proinsulin was calculated as follows:

SI (Glucose 16.7 mM) = (OD insulin (or proinsulin) at glucose 16.7 mM)/ (OD insulin (or proinsulin) at glucose 3.3 mM)

SI (Arginine 20 mM) = (OD insulin (or proinsulin) at arginine 20 mM)/ (OD insulin (or proinsulin) at glucose 3.3 mM)

Mean differences among the three groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's HSD multiple comparison test.

As shown in figure 4a, insulin SI decreases significantly in DM islets versus ND islets in response to both high glucose and arginine 20 mM, and in IGT compared to ND islets for stimulation with arginine 20 mM.

Proinsulin SI, conversely, displayed an increasing trend from ND- to DM-derived islets (with a significant difference between ND and DM), especially under high glucose stimulation (figure 4b).

To estimate cell death in parallel with insulin and proinsulin secretion, we assayed ~~the presence of~~ LDH enzyme activity released in~~within~~ the islets supernatants at all the timepoints. LDH detection was well below the threshold of the positive control (dotted line, CTRL 100% death) for all three groups, thus reasonably ensuring our samples' acceptable levels of viability (Supplementary figure S2A). Furthermore, we investigated any possible correlation between LDH values and SI (Supplementary figure S2B); however, none of the linear regressions proved statistically significant, highlighting the apparent independence of the variables considered.

We also investigated whether a single centrifugation step after filtration, before density gradient separation, was enough to ensure islet enrichment in the final sample (refer to the procedure described in section 2.3). To address this question, the supernatant that would otherwise be discarded from two distinct samples underwent serial centrifugations. Subsequently, two separate glucose stimulation assays were conducted on the resulting pellets, following the previously described protocol. Overall, insulin levels were found to be exceedingly low and scarcely detectable within the supernatants (data not shown), thus confirming that viable islets are mostly pulled down during the first centrifugation step after filtration.

Thus, collectively, data from stimulation experiments confirm that a) islet preparations are competent for beta cell response to physiological stimuli; and b) their responses in vitro reflect the metabolic and glycemic profile of the donor as assessed in the clinical setting.

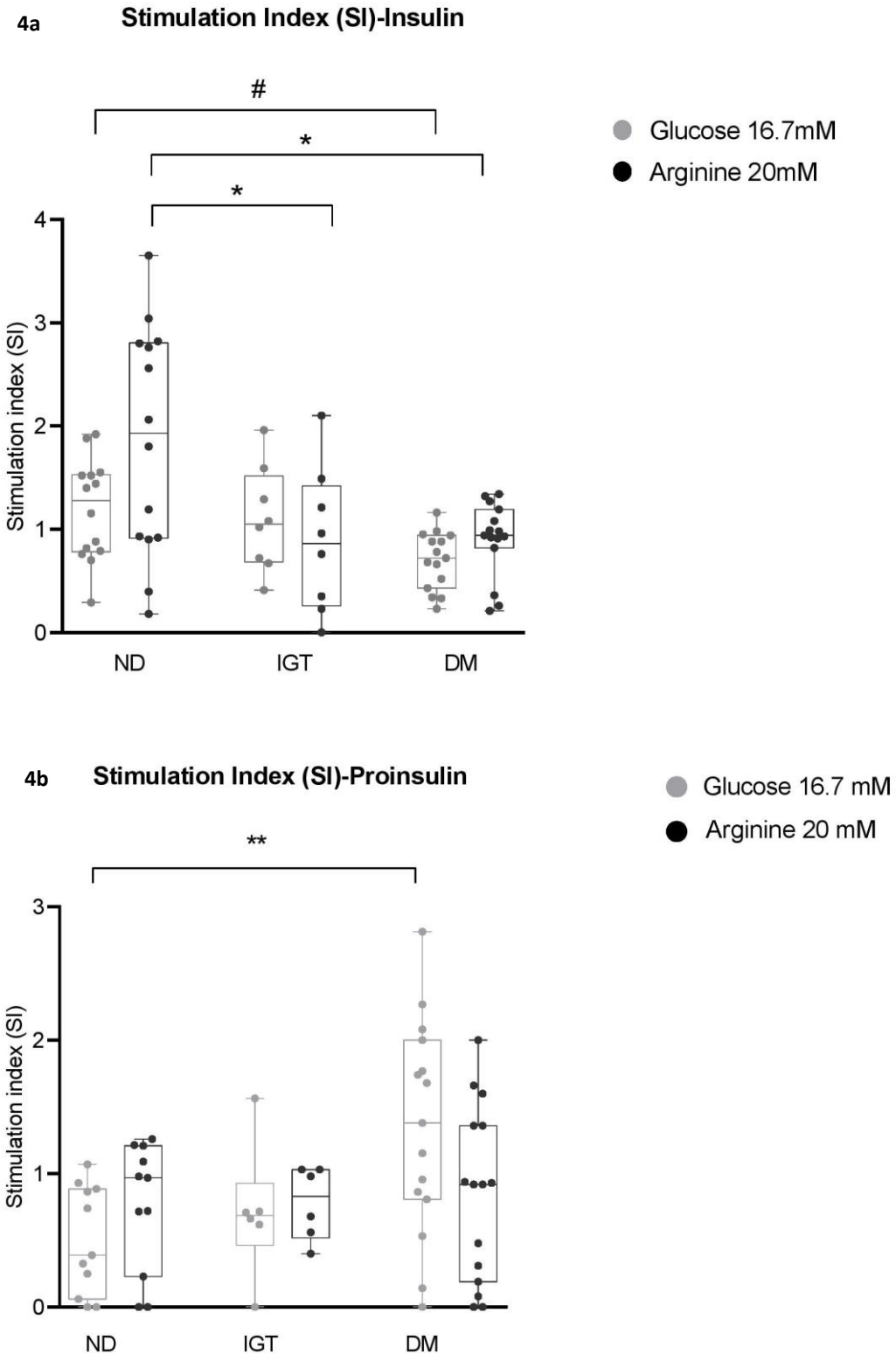


Figure 4: **Insulin and proinsulin Stimulation Index (SI) in islet preparations from non-diabetic (NGT), impaired glucose tolerant (IGT) and diabetic (DM) patients (4a, 4b).** Islets were exposed in vitro to high glucose (Glucose 16.7 mM, in white) or basal glucose + arginine (Arginine 20 mM, in grey); insulin (panel 4a) and proinsulin (Panel 4b) released within the medium were assessed through an ELISA assay. Note that insulin SI is significantly lower in IGT and DM islets compared to ND, especially after arginine stimulation. Conversely, proinsulin levels tend to increase from ND to DM , with a significant increment in the DM group in response to high glucose. One-way ANOVA was applied for the statistics (# $p<0.01$;* $p< 0.05$ in 4a; ** $p<0.01$ in 4b), followed by

Tukey's HSD multiple comparison test. All data are presented as mean and 'min to max' bars. Human insulin in DMEM-BCS alone was undetectable.

3. Discussion

Pancreatic islet isolation plays a crucial role in diabetes research by providing a controlled environment to study β-cell function, investigate disease mechanisms, test potential treatments and develop therapeutic strategies. Optimizing the whole procedure can be challenging due to the endless variants that can affect the outcome of the protocol and the final product yield and quality; these include the clinical state of the donor and the conditions of tissue preservation [47], the source of the specimen -since islets are apparently differently distributed in humans between head, body and tail [48]- but also the enzymes used for digestion [49]. Of note, the clinical and family history of brain-dead organ donors is very limited, and, within the specific context of diabetes research, a deep assessment of the patients' glycemic profile is impossible, just as the post-surgery follow-up of their metabolic state [5].

On the other side, several studies have been conducted on pancreatic tissue slices [38,50–53] and also on purified islets derived from surgical specimens or pancreatectomized patients, generally referred to as living donors [12,54–57]. The greatest advantage of this model is that it allows an extensive metabolic characterization of the patient, before and potentially even after the surgery: a series of clinical and β-cell functional parameters can be assessed or inferred through different kinds of tests (OGTT, euglycemic/hyperglycemic clamp, mixed meal test), having in the end a comprehensive patient-specific overlook of the endocrine compartment functionality [37].

In this work, we describe an improved protocol for the isolation of the islets from living, partially pancreatectomized donors. The procedure has been optimized for a minimal amount of starting material (about 1.5 g of pancreatic tissue); while this quantity is similar to what described by Bötticher et al. [12], their reported sample weight range (2-15 g) is still moderately larger than ours, which features our starting conditions as particularly challenging. Indeed, the initial amount of tissue and the histological features of the sample have a great impact on the outcome of the whole process, since the final yield can be highly variable depending both on the non-cancerous tissue available from the surgery and on the overall disease status of the patient. As an example, it is known that extensive fibrosis and inflammation are a hallmark of pancreata from subjects affected by chronic pancreatitis [58], but they can also occur in the exocrine compartment of T2D (Type 2 Diabetes) patients [59]: for our purposes, an altered or disrupted tissue morphology represents a fundamental issue to be taken into account during the islets purification process, due to the fact that an inflammatory milieu negatively affects islets functionality [60] and also because the increased stiffness of fibrotic tissue [61] could critically impair the efficiency of the digestion step. All these variables, together with a series of technical issues and the nature of reagents used (FBS /BCS of animal derivation, Lympholyte® specifically designed for research purposes), contribute to making this specific protocol unsuitable for clinical and translational applications (e.g. islets transplantation). Of note, isolation protocols aimed at auto- or allotransplantation importantly require a tissue digestion step performed through pancreatic perfusion/intraductal cannulation, to ensure the highest possible release of islets from parenchyma; moreover, the whole procedure must be strictly conducted according current good tissue practice (cGTP) or current good manufacturing practice (cGMP) protocols, and adequate standards of number and purity must be reached for the graft to be safe and effective [62,63]. An upgrade to GMP standards could indeed represent an important future development of the procedure here described.

Functionally, our islets display a physiological insulin response to low/high glucose, (which mimicks *in vitro* the physiological glycemic fluctuations occurring within the human body) and to arginine, a powerful secretagogue that elicits a massive release of insulin β-cells granules. What is more, the insulin secretory response of β-cells -calculated in terms of SI at higher stimuli over basal glycemic conditions- is differentially regulated among the three study groups, particularly during arginine administration. This implies that the purified islets not only are viable and functional, but also recapitulate in vitro the metabolic endocrine features profiled within their respective donors at a clinical level.

Moreover, the analysis of proinsulin SI shows an inverse trend with respect to insulin, i.e. is higher in the DM group compared to ND. Proinsulin is physiologically secreted at lower concentrations than insulin [64] and increased circulating proinsulin levels have been reported to be higher in T2D patients [65,66], so that this condition has been considered as a marker of β-cell dysfunction in diabetic and pre-diabetic subjects [67,68]. Our results are perfectly aligned with these earlier findings, although more samples are needed to validate the significance of the analysis. On the other side, our method does not detect changes in glucose-stimulate proinsulin secretion in islets from IGT patients. While this limitation may depend on insufficient sampling, the results could also reveal a better-preserved islet function in this patient subgroup compared to DM. Interestingly, we could not detect impaired GSIS in IGT individuals either (Fig. 4a), suggesting that peripheral insulin resistance may predominate in these dysmetabolic subjects, although this aspect deserves further investigation.

As discussed above, one of the main challenges of our method is the high variability of the experimental outcome, partially depending on the amount and quality of each specimen. The most relevant source of variability, however, relies on the inter-individual islet heterogeneity involving both genetic and epigenetic features, which reflect the unique α- and β- functional asset of each donor, and the intra-islet diversity within each subject, defined by different endocrine subpopulations, β-cell hubs and different insulin-secretion rates according to islet size and IR signaling [5,41,69–74]; s~~]~~. o, it is likely that not all the islets from the same sample equally respond to glucose. In our specific case, since a relatively limited number of islets was randomly distributed in three tubes for stimulation, an uneven proportion of responsive and non responsive islets may have contributed to increase the overall variability of the insulin SI for each sample. For these reasons, it can be even more challenging to obtain consistent results from functional evaluations performed on a reduced-sized sample, which we assume to be representative of the whole pancreatic endocrine complexity. Furthermore, future improvements are needed to standardize the procedure better and control the quality of the samples, e.g., for islet viability (using Fluorescein Diacetate (FDA) or Propidium Iodide (PI) ~~FDA/PI)~~ and purity, before functional analyses.

In conclusion, we believe that the present study has significant novelty compared to the reported publications. In particular, our protocol is applied to extremely low amounts of pancreatic specimens, often less than 2 g (to the authors' knowledge, the smallest amount used for islets isolation) and does not employ any digestion chamber or cell separator (Ricordi chamber, COBE processor), which makes it much more affordable to most laboratories. In addition, before undergoing surgery, the subjects recruited in this study performed a deep metabolic evaluation, including OGTT, MMT, Hyperinsulinemic Euglycemic clamp and Hyperglycemic clamp. This metabolic evaluation and the additional mathematical modelling on C-peptide curve allow to determine specific β-cell functional parameters. Further, these results can be related to results obtained from *ex vivo* functional experiments and can allow define the changes in secretory pattern *ex vivo* in different metabolic conditions, ranging from normal glucose tolerant to type 2 diabetic subjects. Such

deep *in vivo* characterization has not been reported in other islet isolation studies, especially from brain-dead donors.

Notwithstanding the above limitations, our results highlight the potential of our method and its broad applications in a future perspective: having a patient-specific model of islets/β-cellular function may represent a breakthrough in T2D research, firstly because the functional state of the endocrine compartment (or at least of part of it) can be characterized in a fine-tuned setting, making it possible to match the secretion data with the subject's metabolic profile obtained prior surgery, and possibly predict the metabolic trajectory of the donor over time. In the second instance, integrating the functional data with the molecular signature disclosed by the -omics approaches may help unravel the complexity of the molecular and cellular basis of T2D. Finally, this kind of model could be of great importance in the drug testing field, particularly in view of optimizing new patient-tailored therapies or validating the existing ones.

4. Materials (List of reagents)

- HBSS (Hank's Balanced Salts Solution w/ Calcium w/ Magnesium w/ Phenol Red; Euroclone, Cat. #ECB4006L)
- 96-Well, Cell Culture-Treated (#353072, Falcon)
- 24-Well, Cell Culture-Treated (#3524, Corning)
- Fetal Bovine Serum (Merck-Millipore, Sigma-Aldrich, Cat. #F7524)
- Collagenase P, 1.5 U/mg (Merck-Millipore, Sigma-Aldrich, Cat. #11249002001)
- Lympholyte®-H, sterile liquid (Euroclone, Cat. #DVCL5020)
- DMEM, no glucose, no glutamine, no phenol red (Gibco™, Cat. #A1443001)
- Bovine Calf Serum (BCS), US Origin (Cytiva, Cat. #SH30073.03)
- L-Arginine Minimum 98% (Merck-Millipore, Sigma-Aldrich, Cat.#A-5006)
- D(+)-Glucose Anhydrous (Merck-Millipore, Sigma-Aldrich, Cat.#G-5767)
- Amphotericin B (Fungizone→) 250 ug/ml (100 ml) (Euroclone, Cat. #ECM0009D)
- Penicillin-Streptomycin 10.000 U-10mg (Merck-Millipore, Sigma-Aldrich, Cat.#P0781)
- L-Glutamine Solution 200 mM (Merck-Millipore, Sigma-Aldrich)
- HEPES buffer 1M (Eurobio, Cat.# CSTHEP00-0U)
- Gentamicin solution 50 mg/ml (Merck-Millipore, Sigma-Aldrich, Cat.#G1397)
- Tweezers
- Single-use stainless surgical blades (Paragon, Cat. #P301)
- Polypropylene 15 ml-50 ml Graduated Tubes (Sarstedt, Cat. #62 554502, #62 547254)
- Petri dish, 60 x 15 mm, transparent, with ventilation cams (Sarstedt, Cat. #82.1194.500)
- Corning® Cell strainer, pore size 100 μm and 40 μm (Cat. #431752, #431750)
- Pipettes and tips (2-100 μl)
- MACROMAN Pipette controller (Gilson, Cat. #F110120), serological pipettes
- Shaking water bath

- SL 16 Centrifuge Series (Thermo Scientific™, Cat. #75004031)
- Series 8000 Direct-Heat CO2 Incubator (Thermo Scientific™, Cat. #3540-MAR)
- Ethanol 70%
- 3,5 ml Transferpipette (Sarstedt, Cat.# 86.1171.001)
- Human Insulin ELISA kit (Merk-Millipore Sigma-Aldrich #EZHI-14K)
- Human Total Proinsulin ELISA kit (Merk-Millipore Sigma-Aldrich #EZHPI-15K)
- Dithizone (Sigma-Aldrich, Cat.#D5130)
- CytoScan™ LDH Cytotoxicity Assay (G-Biosciences #786-324)

5. Tips section:

1. Minimize the sample processing time as much as possible, always using ice-cold buffers/media (except for Lympholyte). Prepare HBSS-FBS fresh aliquots before starting.

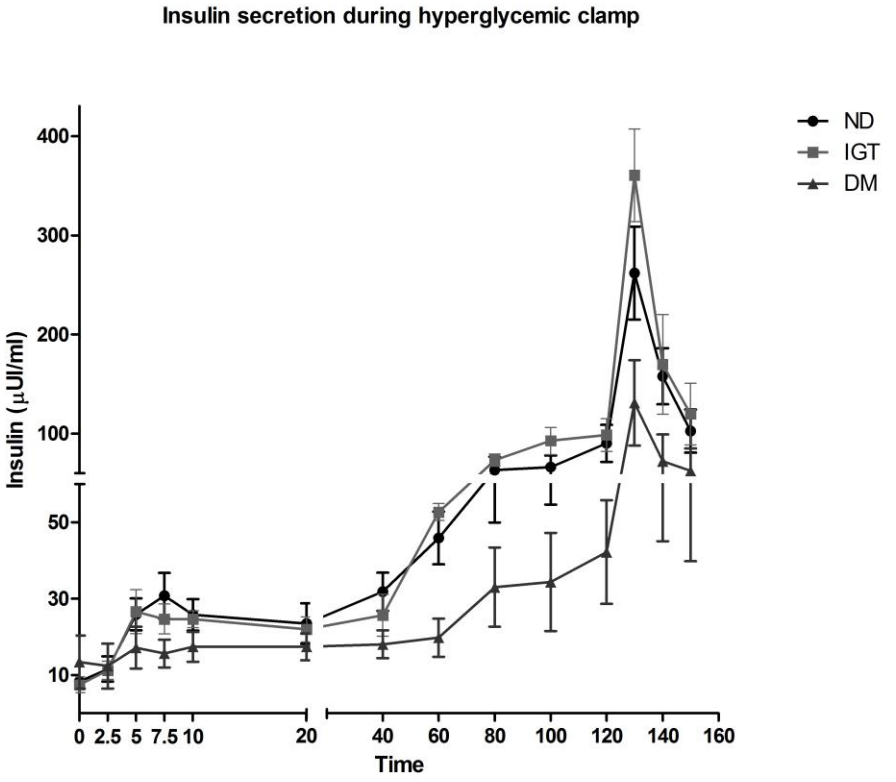
2. If the tissue is very fatty or fibrotic, discard the unsuitable parts during cutting. This will improve the overall quality of digestion -especially in the case of fat, which tends to form a superficial oily layer that significantly lowers the final yield.

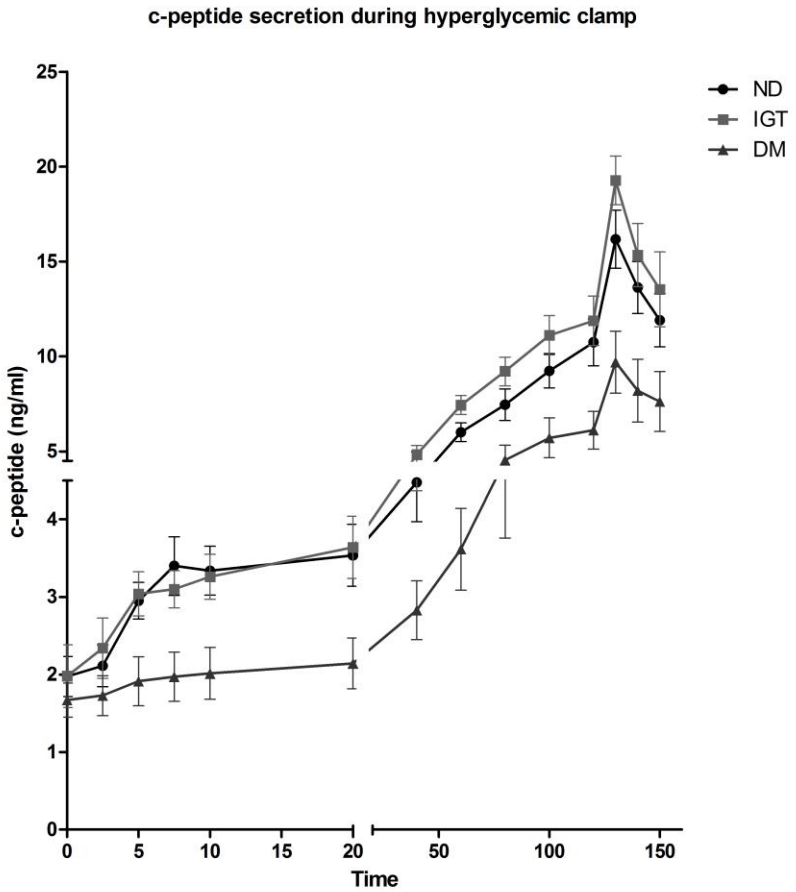
3. If the starting specimen is particularly small in size, repeat the filtration step through the 40 μm cell strainer to minimize the loss of islets, and thoroughly wash the mesh of the strainers with higher volumes of ice-cold HBSS-FBS.

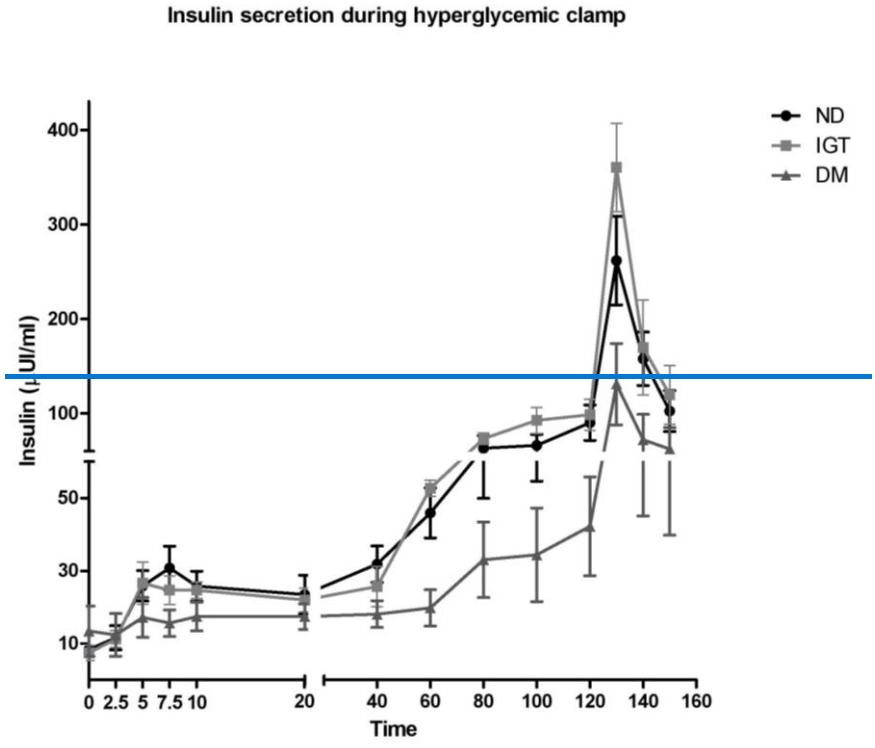
4. Glucose stimulation experiments are performed dividing the sample equally in three tubes. This requires a homogeneous cell suspension, that can be achieved pipetting 1/3 of the sample volume from the bottom to the top at least 3-4 times; then, draw the desired volume from the center of the suspension.

5. It's uncommon to see the 'cell ring' at the lower interface (3 ml), but it's crucial to proceed nonetheless to improve the yield.
It is necessary to handle the density gradient with extreme care, as the interfaces are delicate and prone to easy remixing, resulting in material loss. A 3.5 ml transfer pipette can be very helpful in recollecting almost the whole 'cell ring' at the interfaces; however, using a p1000 pipette can be more manageable for beginners.

S1


Insulin secretion during hyperglycemic clamp
ND
IGT
DM
Insulin (μUl/ml)
400
300
200
100
50
30
10
0 2.5 5 7.5 10 20 40 60 80 100 120 140 160
Time


c-peptide secretion during hyperglycemic clamp
ND
IGT
DM
c-peptide (ng/ml)
25
20
15
10
5
4
3
2
1
0
0 5 10 15 20 50 100 150
Time


Insulin secretion during hyperglycemic clamp
ND
IGT
DM
Insulin (μUI/ml)
400
300
200
100
50
30
10
0 2.5 5 7.5 10 20 40 60 80 100 120 140 160
Time

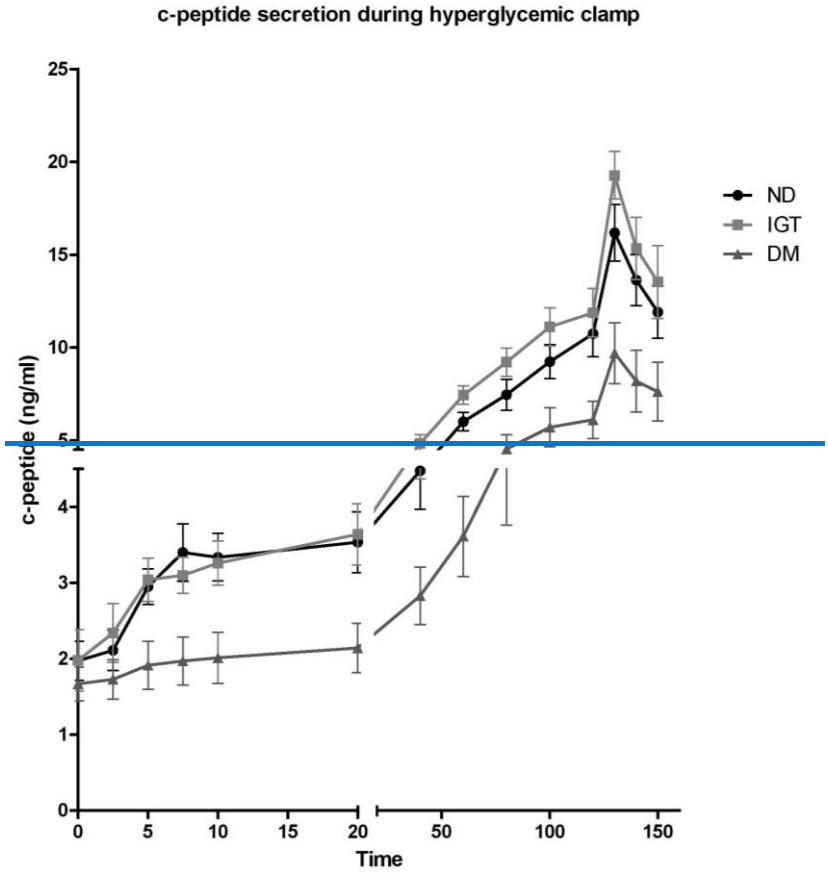
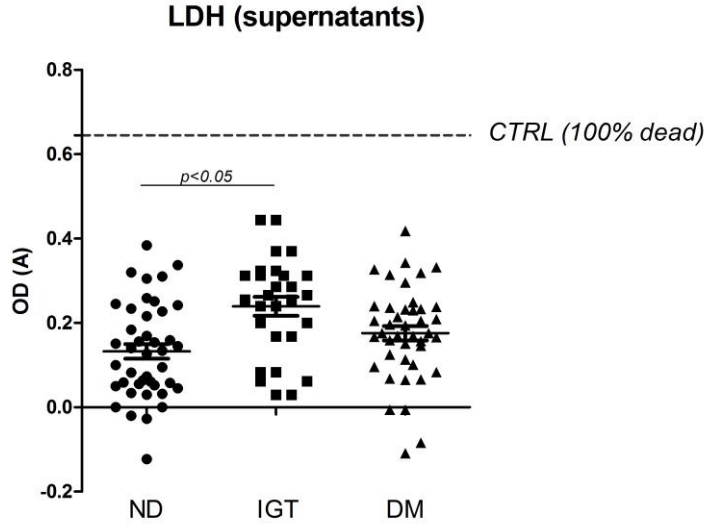


Figure S1: AUC of Insulin and c-peptide secretion monitored during hyperglycemic clamp in NGT (n=8), IGT (n=5), DM (n=7) patients.

S2A



S2B

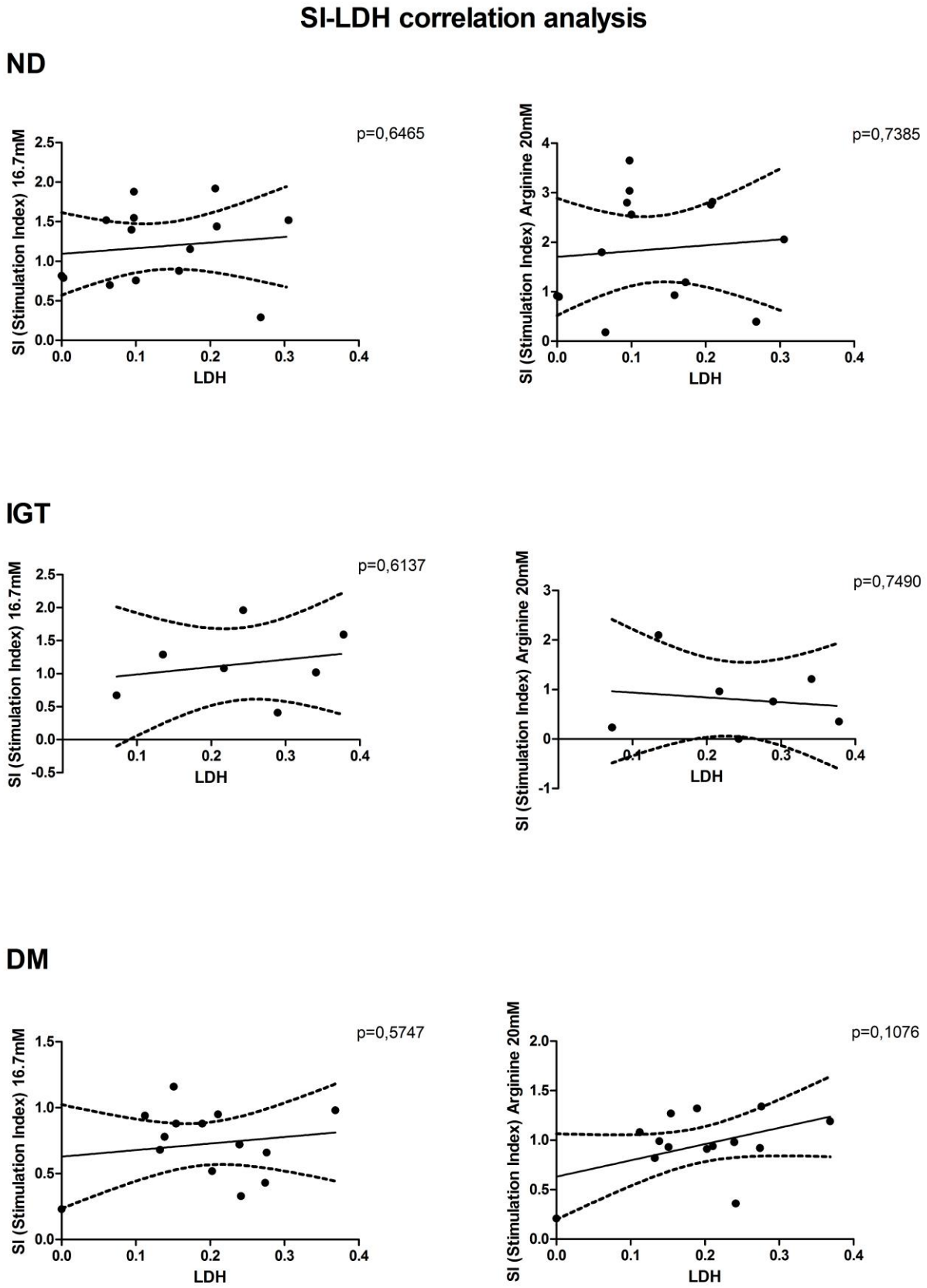

SI-LDH correlation analysis
ND
p=0,6465
SI (Stimulation Index) 16.7mM
LDH
p=0,7385
SI (Stimulation Index) Arginine 20mM
LDH
IGT
p=0,6137
SI (Stimulation Index) 16.7mM
LDH
p=0,7490
SI (Stimulation Index) Arginine 20mM
LDH
DM
p=0,5747
SI (Stimulation Index) 16.7mM
LDH
p=0,1076
SI (Stimulation Index) Arginine 20mM
LDH

Figure S2: In **S2A**, islets supernatants were assayed for LDH at all timepoints. Despite significantly higher detection in IGT islets compared to ND islets, LDH was well below the threshold of the positive control (100% death) for all three groups. One-way ANOVA was applied for the statistics ($p < 0.05$), followed by Tukey's HSD multiple comparison test. Data are presented as mean +/- SEM for each group. In **S2B**, several linear regression analysis were performed to evaluate any possible correlation between LDH values and SI considering each sample's average LDH value (calculated for 'Glucose 16.7 mM' and 'Arginine 20 mM' supernatants) and its relative SI at each timepoint: neither positive or negative correlations were identified. GraphPad Prism v.8 was used for this analysis.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Insulin and c-peptide secretion during hyperglycemic clamp. Figure S2: Cytotoxicity ~~assessment~~ assay and correlation analysis with SI.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by Ethics Committee of Fondazione Policlinico A.Gemelli of Rome (Italy), protocol code 2616.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest: The authors declare no conflicts of interest.

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