Università degli Studi di Siena

Dipartimento di Biotecnologie Mediche

Dottorato di ricerca in: Biotecnologie Mediche

Ciclo XXXIII

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Phenotyping of single pancreatic islets reveals a crosstalk between proinsulin intracellular alteration, ER stress and loss of β cell identity in impaired glucose tolerant and type 2 diabetic patients

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Anno accademico: 2019/2020

Table of contents

ABBREVIATIONS **4**

ABSTRACT **7**

1. INTRODUCTION **9**

1.1 PANCREATIC ISLETS: ARCHITECTURE AND FUNCTION 9
1.2 PANCREATIC BETA CELL IDENTITY 14
1.3 DIABETES MELLITUS 17
1.4 Type 1 diabetes mellitus *18*
1.4.1 Genetic factors of Type 1 diabetes *22*
1.4.2 Environmental factors of Type 1 diabetes *22*
1.5 Type 2 diabetes mellitus *23*
1.5.1 Genetic factors of Type 2 Diabetes *25*
1.5.2 Environmental factors of type 2 diabetes *27*
1.6 ER STRESS AND PROINSULIN MISFOLDING IN TYPE 2 DIABETES 28
1.7 MIRNA AND ER STRESS 30

2. AIMS OF PROJECT THESIS **34**

3. EXPERIMENTAL PROCEDURES **35**

3.1 STUDY POPULATION 35
3.2 SURGICAL PROCEDURE 35
3.3 SAMPLE COLLECTION AND CLINICAL ANALYSIS 36
3.4 PROINSULIN-INSULIN IMMUNOFLUORESCENCE ANALYSIS OF HUMAN PANCREATIC SECTION 36
3.5 IMAGE ACQUISITION AND ANALYSIS 37
3.6 LASER CAPTURE MICRODISSECTION (LCM) 38
3.7 RNA EXTRACTION FROM LCM ISOLATED HUMAN PANCREATIC ISLETS 39
3.8 GENE EXPRESSION ANALYSIS 39
3.9 STATISTICAL ANALYSIS 40
3.10 *IN-SITU* CHARACTERIZATION AND MOLECULAR ANALYSIS OF SINGLE PANCREATIC ISLETS 41

4. RESULTS **43**

4.1 METABOLIC PROFILE OF PATIENTS ANALYZED 43
4.2 PROINSULIN-INSULIN INTRACELLULAR DISTRIBUTION IN HUMAN PANCREATIC ISLETS OF NORMAL GLUCOSE TOLERANT (NGT), IMPAIRED GLUCOSE TOLERANT (IGT) AND TYPE 2 DIABETIC (T2D) PATIENTS 44
4.3 PROINSULIN-INSULIN COLOCALIZATION, AREA (μM^2) OF PROINSULIN POSITIVITY AND PROINSULIN-INSULIN RATIO GRADUALLY INCREASES FROM NGT TO IGT AND T2D PANCREATIC ISLETS 47
4.4 INCREASED PROINSULIN-INSULIN RATIO IS RELATED TO THE LOSS OF GLUCOSE TOLERANCE AND IMPAIRED BETA CELL FUNCTION 51
4.5 GRP78, XBP1 AND PDIA1 GENES INVOLVED IN UNFOLDED PROTEIN RESPONSE (UPR) ARE UPREGULATED IN LCM PANCREATIC ISLETS OF PATIENTS WITH IMPAIRED GLUCOSE TOLERANCE AND TYPE 2 DIABETES 53

4.6 THE UPR RESPONSE GENES GRP78 AND PDIA1 CORRELATE WITH THE INCREASE OF PI-INS RATIO AND COLOCALIZATION OF PI-INS, WITH THE LOSS OF GLUCOSE TOLERANCE AND B CELL FUNCTION 55
4.7 SINGLE ISLETS PHENOTYPING REVEALS A DIRECT ASSOCIATION BETWEEN PROINSULIN PROCESSING, ER STRESS AND LOSS OF BETA CELL IDENTITY DURING METABOLIC ALTERATIONS 58
4.8 THE *IN-SITU* STAINING MEASUREMENTS RELATED TO ALTERATION OF PROINSULIN ARE CORRELATES POSITIVELY WITH THE EXPRESSION OF PDIA1, GRP78 AND XBP1 AND NEGATIVELY WITH GENES ASSOCIATED TO BETA CELL FUNCTION 64
5. DISCUSSION AND CONCLUSIONS 68
6. BIBLIOGRAPHY 76

Abbreviations

AB+	Autoantibody positive
ABCC8	ATP Binding cassette subfamily c member 8
ADAMTS9	ADAM Metallopeptidase with thrombospondin type 1 motif 9
AGO	Argonaute
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
B-ACT	Beta actin
BAD	BCL2 Associated Agonist of cell death
BMI	Body mass index
c-MAF	c-Musculoaponeurotic fibrosarcoma oncogene homolog
Ca^{2+}	Calcium ion
CAMK1D	Calcium/Calmodulin Dependent Protein Kinase ID
CAR	Coxackie Adenovirus Receptor
CD9	Cluster 9 differentiation
CDC123	Cell Division Cycle 123
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CHGA	Chromogranin A
CHOP	C/EBP homologous protein
CPE	Carboxypeptidase
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
CPT1	Carnitine palmitoyltransferase-1
CVBs	Coxsackieviruses B-group
DALYs	Disability-adjusted life-years
DC	Dendritic cell
DGCR8	DiGeorge Syndrome critical Region 8
DiViD	Diabetic Virus Detection
DNAJC3	DNA J heat shock protein family
EMT	Epithelium-mesenchymal transition
EXP5	Exportin-5
FFA	Free fatty acids
FOXA2	Forkhead box protein A2
FOXO1	Forkhead box protein O1
FTO	Fat mass and obesity associated
GABA	Gamma aminobutyric acid
GAD65	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCG	Glucagon
GDM	Gestational diabetes mellitus
GHRL	Ghrelin
GIP	Gastric Inhibitor Polypeptide
GLP-1	Glucagon-like peptide 1
GLUT-2	Glucose Transporter 2
GRP78/HSPA5	Glucose-regulated protein
GSIS	Glucose-stimulated insulin secretion
GWAS	Genome-wide associated studies
hCAR	Coxsackie- and adenovirus receptor
HHEX	Haematopoietically expressed homeobox
HLA	Human leukocyte complex

HNF	Hepatic nuclear factor
HNF4A	Hepatocyte nuclear factor 4 alpha
HPA	Hypothalamic-pituitary-adrenal
HSP40	Heat shock protein 40
IA-2	Tyrosine phosphatase 2
IA-2β	Tyrosine phosphatase 2β
IAPP	Islets amyloid polypeptide
IFG	Fasting blood glucose
IFIH1	Interferon Induced with helicase C domain 1
IGF-1	Insulin-like growth factors 1
IGF-2	Insulin-like growth factors 2
IGF2BP2	Insulin-like growth factor two binding protein 2
IGT	Impaired glucose tolerance
IL18RAP	Interleukin 18 receptor accessory protein
IL2RA	Interleukin 2 receptor alpha
INS	Insulin
INSR:	Insulin receptor substrate 1
IRE1alfa/ERN1	Inositol-requiring enzyme 1α
IRS-2	Insulin receptor substrate 2
KCNJ11	Potassium Inwardly Rectifying Channel Subfamily J Member 11
LCM	Laser capture microdissection
MAFA	V-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MAPK14	Mitogen-activated protein kinase-14
MCT1	MonoCarboxylate Transporter 1
MDA5	Melanoma differentiation-associated gene 5
miRNAs	microRNAs
MODY	Maturity-Onset Diabetes of the Young
MTNR1B	Melatonin-receptor gene
NGN3	Neurogenin 3
NGT	Normal glucose tolerance
NKX6.1	NK6 Homeobox 1
NOD	Non-obese diabetic
NOTCH2	Notch Receptor 2
OCT4	Octamer-binding transcription factor 4
OGTT	Oral glucose tolerant test
P58PK	Proteins kinase inhibitor p58
PAMP	Pathogen associated molecular pattern
PC	Pyruvate Carboxylase
PC1	Protein convertase 1
PC2	Protein convertase 2
PC3	Protein convertase 3
PCSK1	Proprotein Convertase Subtilisin / Kexin Type 1
PDIA1	Protein disulfide isomerase
PDL1	Programmed death-ligand 1
PDX1	Pancreatic and duodenal homeobox 1
PERK	Pancreatic ER kinase
PFA	Paraformaldehyde
PI3-K	Phosphoinositol 3 kinase
PIK3R1	Phosphoinositide-3-kinase regulatory subunit 1
PKB	Protein-kinase B
PPARG2	Peroxisome proliferator-activated receptor gamma 2

PPI	Pre-proinsulin
PRDX4	Peroxiredoxin
PRR	Pattern recognition receptor
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
rER	Rough endoplasmic reticulum
RIG-I	Retinoic acid-inducible gene I
RLRs	RIG-I-like receptors
ROI	Region of interest
SG	Secretory granules
SLC16A11	Solute Carrier Family 16 Member 11
SOCS3	Suppressor of cytokine signaling 3
SOS1	SOS Ras/Rac Guanine Nucleotide Exchange Factor 1
SOX2	Sex determining region Y)-box 2
SOX9	Sex determining region Y)-box 9
SRP	Ribonucleoprotein signal recognition particles
SST	Somatostatin
ST8SIA1	Human Alpha-N-acetyl neuraminide alpha-2, 8-sialyltransferase
STXBP1	Syntaxin binding protein1
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TCF7L2	Transcription Factor 7 Like 2
TXNIP	Thioredoxin-interacting protein
UPR	Unfolded protein response
V-1/Mtpn	V-1/myotrophin
VEGFA	Vascular endothelial growth factor A
VNTR	Variable number tandem repeat
VP1	Viral Protein 1
VTI1a	Vesicle transport interaction with t-SNARE yeast homologue 1A
WFS1	Wolframin ER transmembrane glycoprotein
WNT	Wingless-related integration site
XBP1	Splicing of X-box binding protein 1
XIAP	X-linked inhibitor of apoptosis
Zeb1	Zing finger E-box-binding homeobox
ZnT8	Zinc transporter
βCGS	β-Cell Glucose Sensitivity

ABSTRACT

Type 2 diabetes mellitus is a heterogeneous group of metabolic diseases characterized by increased levels of blood glucose due to insulin resistance and alteration of insulin secretion by pancreatic β cells. Recent studies suggest that β cell loss in T2D results from endoplasmic reticulum stress which can cause an alteration in the processing of PI to INS. In particular, it has been reported that the increased circulating levels of PI and elevated PI/INS ratio are well-known abnormalities in type 2 diabetes. Several studies have hypothesized that an elevated PI/INS ratio was caused by increased secretory demand on β cells due to insulin resistance and hyperglycemia. However, an in-depth analysis of PI/INS expression pattern inside the pancreatic islets during metabolic alteration is not entirely clear. For this reason, the first aim of this work was: *(i)* to analyze PI and INS expression pattern in pancreatic islets of tissue biopsies of patients undergoing partial pancreatectomy (PP) with normal glucose tolerance (NGT), impaired glucose tolerance (IGT) and type 2 diabetes, in order to explore the *in-situ* alterations that occur in islets during metabolic stress. Given the enormous heterogeneity between pancreatic islets of different donors but also between islets belonging to the same donors, the second aim of project thesis was: (ii) to perform single islet phenotyping by characterizing histological and molecular aspects, in order to investigate the underlying molecular mechanisms driving β cell failure at single islet level with the aim to specifically determine the cues leading to intracellular alteration of PI and insulin during metabolic stress. In order to explore the alterations that occur in PI and INS staining pattern in pancreatic islets, OGTT (Oral Glucose Tolerant Test) was performed in n=19 patients scheduled for PP, classified into n=5 NGT, n=9 IGT and n=5 T2D. Frozen sections of pancreatic tissue biopsy were stained for INS and PI through double immunofluorescence staining. Image analysis was performed through Volocity software. *In-situ* staining measurements were correlated with patients' clinical parameters. Subsequently, we evaluated the expression of ER stress genes and β cell mature phenotype-related genes in microdissected pancreatic islets collected from n=4 NGT, n=7 IGT and n=4 T2D patients. Given the high heterogeneity among pancreatic islets we also performed a single islets phenotyping analysis in n=88 islets from n=3 NGT, n=3 IGT and n=3 T2D patients. Results showed that in pancreatic islets of IGT and T2D patients, PI intracellular localization was altered. The colocalization coefficient INS-PI as well as PI levels and PI/INS ratio gradually increased from NGT to IGT and T2D pancreatic islets and were related to the loss of glucose tolerance and impaired β-cell function. PDIA1, GRP78 and XBP1, genes involved in unfolded protein response (UPR) were significantly upregulated in pancreatic islets of IGT and T2D patients compared to NGT and were positively correlated with *in-situ*

PI/INS ratio and colocalization, with *in-vivo* measurements of glucose intolerance and β cell functional reduction. Single islets phenotyping approach revealed a progressively increased heterogeneity from NGT to IGT and T2D patients. Of note, *in-situ* PI/INS ratio and colocalization were positively correlated with the expression of UPR genes and negatively with those associated to β cell identity. In conclusion, we demonstrated that: (*i*) the *in-situ* expression patterns of proinsulin and insulin are altered in prediabetic and diabetic patients reflecting their metabolic profiles; (*ii*) the molecular mechanism behind this *in-situ* alteration involves the ER stress and the establishment of UPR within the β cell; (iii) single islets phenotyping analysis in T2D pancreas reveals a high heterogeneity among pancreatic islets in terms of ER stress and β cell differentiation profile.

1. Introduction

1.1 Pancreatic islets: Architecture and Function

The pancreas can be defined as the central engine for controlling energy consumption and metabolism. It is a voluminous elongated gland located in the abdominal-pelvic cavity, between the posterior wall of the abdomen, duodenum and spleen. From the anatomical point of view, the pancreas is divided into three portions: head, body and tail. The head is located in the concavity formed by the duodenum, the body represents the intermediate segment and the tail which represents the thinned tract with which this organ ends. It is a mixed gland consisting of an exocrine (80-85%) and an endocrine (2%) portion. The exocrine portion is characterized by pancreatic acinar cells that synthesize and secrete digestive enzymes (proteases, amylases, lipases, nucleases) in an inactive form, to pour them directly into the duodenum under the influence of physiological stimuli; on the other hand, the endocrine component, is responsible for the secretion of hormones that are secreted and released into the bloodstream with the function of controlling the metabolism of sugars, fats and proteins. The endocrine compartment is organized in structures known as islets of Langerhans, whose cells are specialized in the secretion of specific hormones responsible for maintaining homeostatic blood glucose levels. The islets of Langerhans are a heterogenous mixture of endocrine cells scattered through the pancreas, including insulin-secreting β-cells, glucagon-secreting α-cells, somatostatin-secreting δ-cells, pancreatic polypeptide-secreting PP-cells and ghrelin-secreting ε-cells, that function as mini-organs. In addition to metabolic function, the islets are highly innervated and vascularized, providing numerous signals that can modulate ad support cellular differentiation, survival and proliferation [1]. The architecture and distribution of pancreatic islets is species-specific. The murine pancreatic islets are characterized of a central core of β cells and α and δ cells located in the periphery of the islet, differentiating themselves from the human pancreatic islets in which the α- β- and δ- cells are heterogeneously distributed throughout the islet. There are also differences in the number of cells which characterize the islets of Langerhans between human and mouse: in human, β-cells represent the 50-70%, while in mice are 60-80%; α-cells in human represent the 20-40%, while in mice are 10-20%; δ-cells and PP cells are the least frequent cell types and are less than 10% in human and less than 5% in mice. The ε cells, on the other hand, are less than 1% **(Figure 1)**.

Studies on human islets suggest that these differences, probably, reach adult architecture about two years after birth (in fact, the replication rate of β-cells is greater during the first two years of age and then decreases in adolescence); on the other hand, in mice, the number of islets

increases in the first 3-4 weeks of life and then remains stable over time [2-3]. Comparative studies on pancreatic islets of different species have revealed surprising plasticity in the architecture of the islets and their composition. Physiological and pathological changes have been shown to lead to differences in the architecture, function and gene expression of islets, rather than species differences [4]. In a study, it has been performed a three-dimensional islet distribution map and it has been estimated that in the healthy human pancreas there are on average 3.2 million islets, with a mean islet diameter of 102.92 μm and a mean islet volume of 0.00068 mm^3 or 0.00069 μL. Moreover, the majority of islets had a surface area between 1000 and 10000 μm^2, all the others of more than 100000 μm^2. By comparing the 2D distribution of pancreatic sections, it was observed that islets are grouped in the head of the pancreas and are gradually dispersed throughout the pancreas up to the tail region. Islet density gradually increases from the head to the tail region of the pancreas. The smaller islets stretched to clusters around blood vessels [5]. The pancreatic islet shape confers a unique cellular arrangement. Recent data suggest that many changes in islet structure and function were associated with diabetes and were attributable to hyperglycaemia alone. In fact, these changes reversed when blood glucose is normalized. Thus, the uniqueness of this heterogeneous conformation has a deep functional implication [6,7].

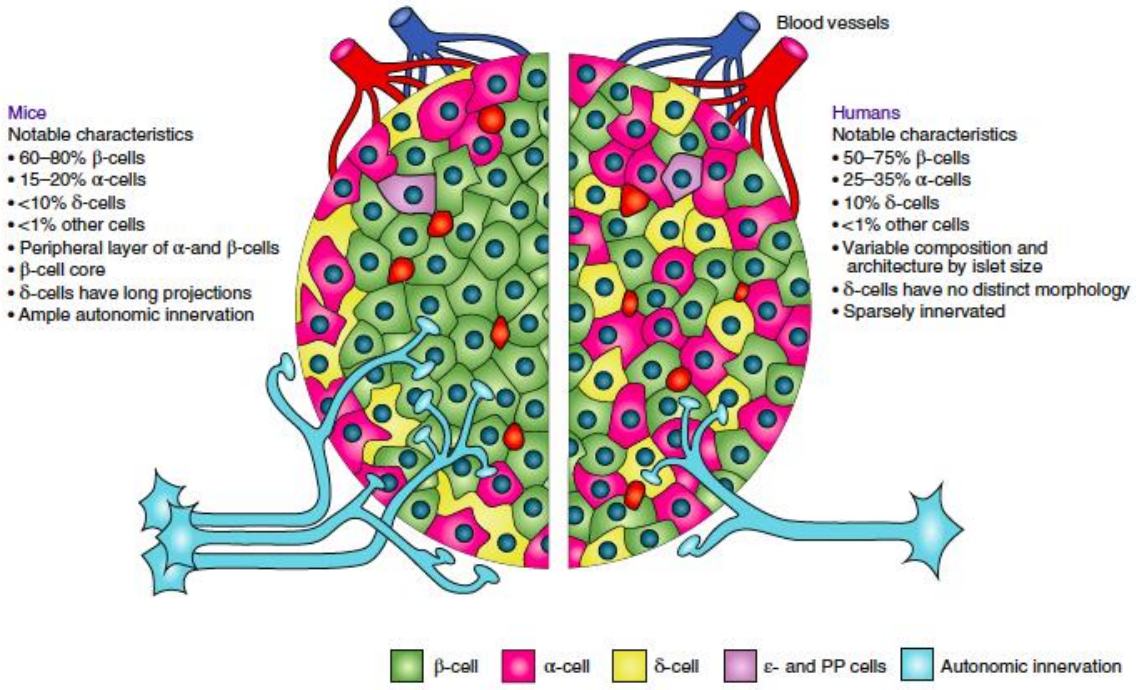


Figure 1: Comparative architecture of pancreatic islets of mice and humans. The architecture of the pancreatic islets of mice and humans differ greatly, but they also share many features in common. These shared characteristics make mouse islets useful experimental models for studying many aspects of human islet biology. The different types of endocrine cells in mice (left) and human islets (right) are similar between beta cells (β; green), which make up most of the cell mass of the islet, and alpha cells (α; magenta) and delta (δ; yellow). The pancreatic polypeptide and epsilon cells (PP and ε; purple) are more spread out. Human pancreatic islets are characterized by highly structured cells that can have different sizes and conformations. Murine pancreatic islets are more uniform, with α and δ cells on the periphery of the islet surrounding a nucleus of β cells. The islets of both species are vascularized (dark red) and innervated (light blue) for rapid detection of changing energy needs, although murine islets are more densely innervated than human ones. *Noguchi G.M. et al,* [8].

The endocrine cells scattered through the pancreas have different functions and communicate with each other to guarantee glucose homeostasis.

α-cells produce the glucagon in fasting conditions and its main function is to increase the amount of glucose in the blood by stimulating glycogenolysis and gluconeogenesis. In the pathophysiology of T2D, the glucagon has an important function, in fact, Unger and colleagues have demonstrated in the bihormonal hypothesis that both hypoinsulinaemia and hyperglucagonaemia can cause hyperglycaemia in T2D [9-10]. Glucagon-like peptide 1 (GLP-1) and Gastric Inhibitor Polypeptide (GIP) are involved in the regulation of glucagon secretion; indeed, the somatostatin produced by δ-cells, negatively regulate glucagon secretion. In a study has been proposed that GLP-1 indirectly inhibits glucagon secretion through somatostatin secretion, regardless of β cell secretory products [11]. Several studies have highlighted the ability of α-cells to transdifferentiate into β-cells, in particular, recently it has been demonstrated that the administration of gamma aminobutyric acid (GABA) induced the trans-differentiation of α-cells into β-cells maintaining a functional population of α cells, suggesting that this treatment is important to guarantee the total proportion of islet cell type [12].

β-cells are the most important and studied cells of the pancreatic islets. Insulin is the hormone released by β-cells in response to high glucose concentration, which translate to a decrease of blood sugar, promoting the synthesis of glycogen in the liver and muscle, increasing the use of glucose by cells and inhibiting the formation of new glucose.

Insulin is a peptide consisting of two amino acid chains (A and B) held together by two disulfide bonds (A7-B7, A20-B19). Chain A is made up of 21 amino acids, while chain B is made up of 30 amino acids. In humans, the gene responsible for insulin synthesis is located in the short arm of chromosome 11. The insulin gene encodes a precursor protein of 110-amino acid known as preproinsulin that contains a hydrophobic N-terminal signal precursor that interacts with cytosolic ribonucleoprotein signal recognition particles (SRP) [13]. The preproinsulin translocate through the rough endoplasmic reticulum (rER) membrane into the lumen where the signal peptide is cleaved by peptidases becoming proinsulin. In the ER proinsulin undergoes folding and maturation, then is transported from ER to the Golgi and trans-Golgi network apparatus where PC1/3 and PC2 (protein convertase 1-2-3) and CPE (carboxypeptidase) cleaved proinsulin to produce Insulin and C-peptide stored in secretory granules [14,15] (**Figure 2).** Recently, this canonical proinsulin processing pathway has been revisited more thoroughly. Recent evidence shows that in human β cells the endoproteolytic cleavage of proinsulin was performed preferentially by PC1 and PC3 enzyme but PC2 activity was not detected. Moreover, it has been demonstrated that the inhibition of PC2 rendered β cells able to secrete mature insulin while the suppression of PC1/3 enzyme inhibited proinsulin processing. In healthy human β cells, PC2 immunoreactivity was not detected but its expression increased in β cells of type 2 diabetes individuals, suggesting that the defect of PC2 expression in diabetic condition may contribute to alteration of proinsulin processing and disease [16]. This aspect represents mostly a debate and in the light of the latest published works, it is increasingly reported that β cell is characterized by a remarkable plasticity and ability to adapt to physiological and pathological conditions. Marchetti et al., showed the presence of GLP-1-like and PC1/PC3 immunoreactivity in subsets of α cells but in the β cells GLP-1 was not detected. Moreover, the release of GLP-1 as well as PC1/PC3 expression genes were upregulated in type 2 diabetic respect to control isolated islets, suggesting a strong dynamism of pancreatic islets cells following β cell dysfunction [17].

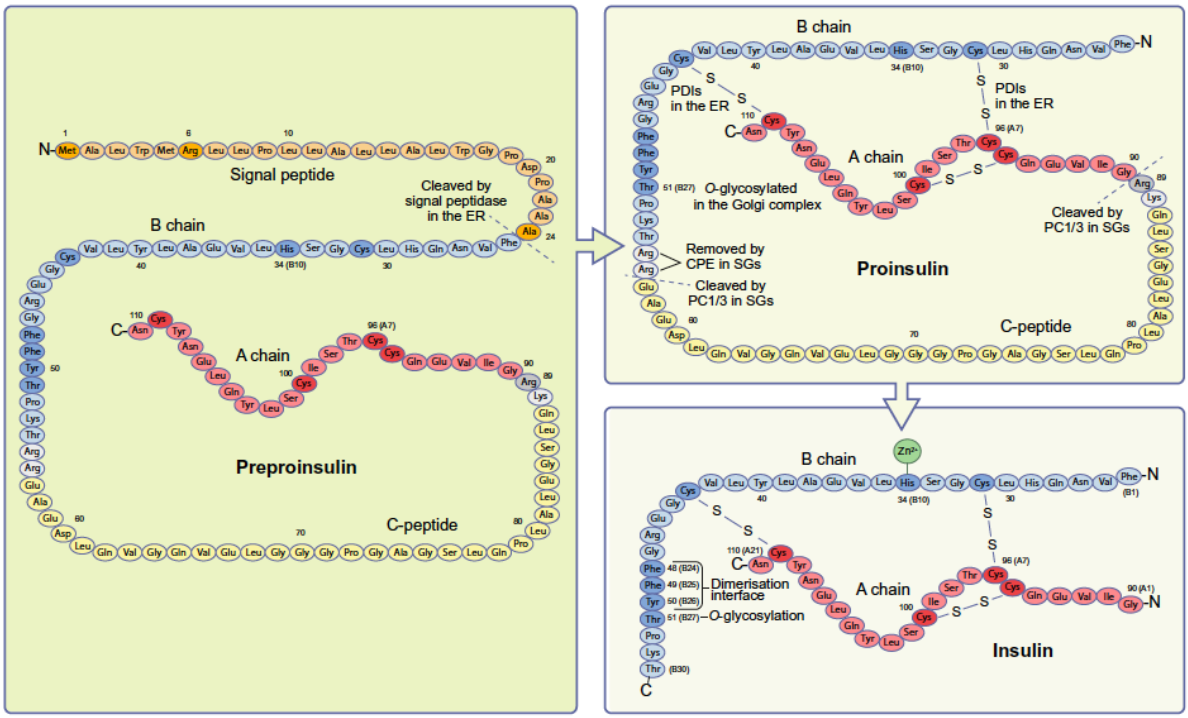


Figure 2: The insulin peptide. Insulin is synthesized from a precursor, the preproinsulin 110 amino acid long which includes a signal peptide (orange), a B chain (blue), a linking peptide (C peptide, yellow) and an A chain (red). The preproinsulin with the signal peptide translocates into the ER, where the peptide is cleaved by the signal peptidase and converted to proinsulin. In the ER, three disulfide bonds are formed between cysteine residues with the help of PDI. Proinsulin translocates from the ER to the Golgi apparatus and through the trans-Golgi network to the secretory granules (SG), where PC1 / 3 and CPE process dibasic (gray) residues to form mature insulin. *Vasiljević J. et al,* [18].

The function of insulin is to keep plasma glucose levels within physiological limits (80-100mg / dl); in fact, its secretion is controlled by the concentration of glucose in the blood. The increase of concentration of plasma glucose, leads to the entry, in small doses of glucose into the β cells thanks to the presence of the insulin-dependent transporter GLUT-2. An increase in cytosolic glucose causes an increase in the concentration of ATP, produced by the mitochondrial catabolism of sugar, which causes the closure of the ATP-dependent potassium channels, with depolarization of the membrane and opening of the voltage-dependent calcium channels. The intracellular increase in the calcium ion (Ca^{2+}), which acts as a messenger, induces the fusion of the insulin-containing vesicles with the plasma membrane. Insulin is thus released from the granules in which it is contained. The target organs of insulin are mainly skeletal muscle, adipose tissue and liver, with which it interacts by binding to its receptor located on the plasma membrane of their cells. In these organs it promotes glucose uptake, glycogenosynthesis,

stimulates exocytosis and activation of glucose transporters; it also favors the uptake of amino acids by increasing protein synthesis [19].

A recent work has found that β cells, considered as a homogeneous cell population, differentiate between them in four main antigenically distinct subtypes and defined as β1, β2, β3, β4. These cells are distinguished by differential expression of particular cellular markers: ST8SIA1 (Human Alpha-N-acetyl neuraminide alpha-2, 8-sialyltransferase) and CD9 (cluster 9 differentiation) and are characterized by different gene expression profiles and glucose-stimulated insulin secretion. In type 2 diabetes, the distribution of these β cells is altered [20]. These results open the way to new knowledge regarding the architecture of the pancreatic islets and the specific function of the cells they compose.

δ-cells secrete the somatostatin, which acts under conditions as a negative regulator of insulin, glucagon and pancreatic polypeptide [21]. In a study, it has been demonstrated that δ-cells can transdifferentiate in β-cells following acute depletion of β cell mass [22]. It has been reported that the gene *Hhex* is essential for the maintenance of δ-cells fate. In fact, the loss of Hhex gene led to a failure of paracrine regulation of insulin secretion contributing to T2D [23].

PP-cell or **F-cells** produce the pancreatic polypeptide, concentrated in particular in the head of pancreas. The regulation of the secretion of pancreatic polypeptide is done by vagal and enteric nervous input. This post-prandial hormone under low glucose, inhibit the release of glucagon. It is considered the satiety hormone [24,25].

The communication of the cells that characterize the pancreatic islet is extremely fragile, and the alteration of the mechanisms regulated by them can lead to pathological conditions known as Diabetes Mellitus.

1.2 Pancreatic beta cell identity

Pancreatic β cell identity can be defined as the result of gene expression which makes the β cell able to synthesize, to process and to secrete insulin efficiently in response to metabolic, hormonal and neurological stimuli. The maintenance of β cell identity is guaranteed by molecular mechanisms that involve specific receptors, transporters, processing enzymes, transcription factors and non-coding RNAs. Among the transcription factors (TF) essential for maintaining β cellular identity, PDX1 (pancreatic and duodenal homeobox 1) is the most

important. Indeed, it regulates the embryonic development of the pancreas and multiple aspects of the function of the mature β cells. It has been shown that the deletion of PDX1 in β cells led to a reduction of gene expression associated with β cellular function and an increase of genes typical of α cells, such as Glucagon (Gcg) [26].

FOXA2 is another transcription factor expressed in the pancreatic endoderm in the early stages of development, even though its expression persists until adulthood, where it is expressed both throughout the islets of Langerhans and in acinar cells. FOXA2 plays an important role in the regulation of PDX1 and MafA expression, directly or indirectly, in β-cells. It has been shown that in mature β cells, Foxa2 regulates insulin secretion, its deletion in fetal β cells causes an alteration of the morphology and secretory function of β cells, leading to the premature death due to hypoglycemia [27–29].

Another main TF for pancreatic differentiation and β cell function is NKX6.1 (NK6 Homeobox 1), which regulates the transcription of genes which encode for proteins involved in glucose metabolism and insulin biosynthesis. Several studies have shown that NKX6.1 is a critical regulator of insulin biosynthesis and secretion, as well as proliferative capacity and β cell identity. The loss of Nkx6.1 activity has an immediate and dramatic impact on the expression of the genes that give to the β-cells the ability to synthesize and release insulin and on those genes that control β-cell function and proliferation. NKX6.1 controls the activity of genes involved in insulin biosynthesis (Slc30a8 and Ero1lb), in the entry of glucose (Glut2) and in its metabolism (Pcx). In fact, it has been observed that the proliferative capacity of β cells lacking Nkx6.1 is limited by the reduced intracellular availability of glucose due to the loss of Glut2 expression. Therefore, it acts as a metabolic sensor modulating both insulin secretion and the ability to respond to metabolic stress. The inactivation of NKX6.1 is related to the development of type 2 diabetes (this observation would suggest that the restoration of NXK6.1 level could be a therapeutic strategy for type 2 diabetes) [30].

It has been demonstrated that the specific deletion of NKX6.1 in murine β cells is associated with some peculiar phenotypic characteristics of δ cells including the expression of somatostatin (Sst)[30]. Furthermore, the deletion of Pax6 in adult β cells determines a gradual loss of insulin expression and an increase of GCG, STT and fetal hormone Grelina (Ghrl) expression [31].

FOXO1 regulates β-cell function by inhibiting their proliferation during insulin resistance and β-cell differentiation. The main function of FOXO1 is to protect the β cells from oxidative stress. It has been shown that the translocation of FOXO1 from cytoplasm to nucleus is paralleled to the exclusion of PDX1 from the nucleus as a useful mechanism for the inhibition

of β cell proliferation. During metabolic stress, the phosphorylation mechanism that guarantee the cytoplasmic localization of FOXO1 is lost and its nuclear presence increases the acetylation mechanisms causing the increase of genes expression such as NEUROD1 and MAFA in order to increase the transcription of insulin gene in order to prevent the replication of β cells [32,33]. MAFA is another example of TF involved in the maintenance of mature β cell function and in the prevention of the expression of "disallowed genes", whose expression is inhibited in β cells because not necessary for β cells function. In fact, since the activation of "disallowed genes" hinders the maintenance of β cell identity, their expression was regulated at numerous levels. As a matter of fact, many of them are silenced by microRNAs (miRNAs), small non-coding endogenous RNAs that negatively regulate gene expression. In the murine β cell line MIN6 cells, it has been shown that the inhibition of miRNAs preferentially expressed by β cell (miR-200c, miR-182 and miR-125b) caused an increase in c-MAF levels (c-Musculoaponeurotic fibrosarcoma oncogene homolog), which regulates the production of GCG in α cells. On the contrary, in the murine α cell line αTC6 the overexpression of the same β-miRNA determined a reduction of this TF [34]. Furthermore, some "disallowed genes" which in physiological conditions are expressed at low level in β cells, encode for enzymes and mitochondrial proteins, and their activation in β cells regulated alternative metabolic pathways interfering with glucose sensitivity. Among these we find, for example, MCT1 (MonoCarboxylate Transporter 1), whose expression is ubiquitous in several cell types and tissues, except for β cells where it negatively regulates glucose-stimulated insulin secretion (GSIS). MCT1 was silenced by miR-29 and miR-124 then contributing to the maintenance of the GSIS [35]. The differentiated state of β cells is particularly sensible, in fact the alterations in glycemic control and metabolic homeostasis can lead to degranulation and / or β cell dysfunction, as well as the loss of mature phenotype through a mechanism of dedifferentiation [36] **(Figure 3)**.

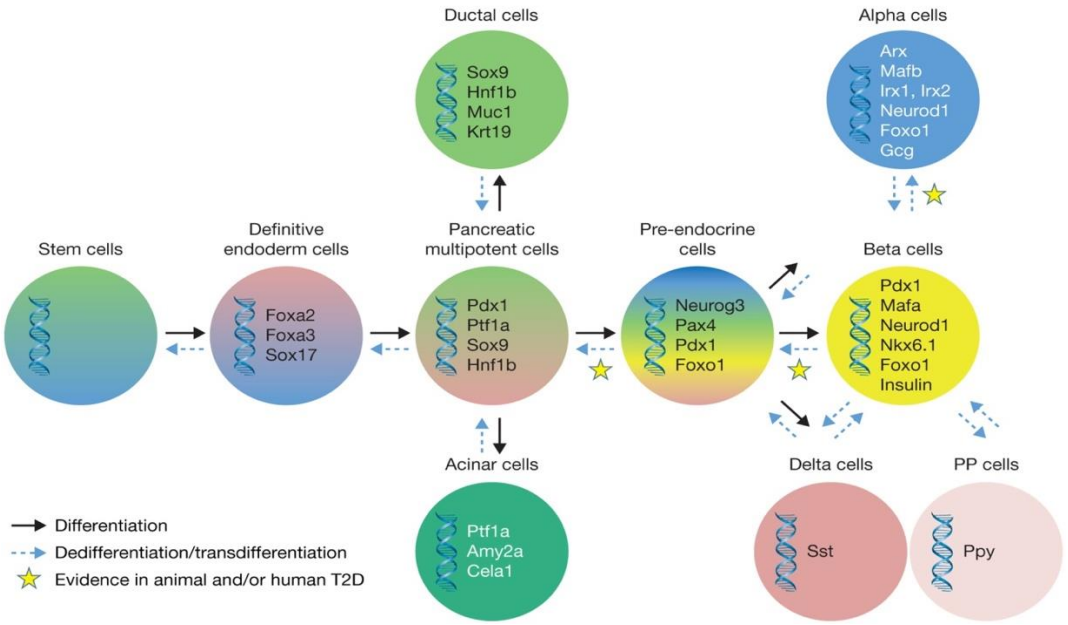


Figure 3: β cells differentiation. Differentiation process involves coordinated and tightly controlled activation/repression of specific transcription factors that drive the transition from a stem cell to a mature β cell during embryonic development and the postnatal period. Evidence from animal and human studies suggest that mature β cells could regress backwards to a precursor-like state and/or transdifferentiate into other islet cells in T2D. *Bensellam M. et al,* [36].

1.3 Diabetes Mellitus

Diabetes mellitus represents a family of metabolic disorders characterized by chronic hyperglycaemia caused by destruction or dysfunction of pancreatic β cells following a deficit of insulin secretion.

In 2017, global incidence, prevalence, death, and disability-adjusted life-years (DALYs) associated with diabetes were 22.9 million, 476.0 million, 1.37 million, and 67.9 million, with a projection to 26.6 million, 570.9 million, 1.59 million, and 79.3 million in 2025, respectively [37].

The most common forms of diabetes mellitus are Type 1 Diabetes (T1D), which represents 5–10% of diabetes cases and is caused by immune mediated destruction of β cells, and Type 2 Diabetes (T2D), which represents 90–95% of diabetes cases and is caused by insulin resistance and β cell dysfunction [38]. However, both T1D and T2D often lead to several types of chronic complications (e.g., retinopathy, neuropathy, nephropathy, and cardiovascular diseases) which affect different organs [39]. A third form of diabetes is gestational diabetes mellitus (GDM),

characterized by an alteration of glucose metabolism diagnosed in the second or third trimester of pregnancy. Another form of diabetes is MODY (Maturity-Onset Diabetes of the Young), caused by a β-cell dysfunction that leads to hyperglycaemia at an early age (usually under 25 years of age). This form of diabetes follows an autosomal dominant expression pattern, in fact there is a genetic alteration in at least six loci placed on different chromosomes, for example, the most common form (MODY 3) is associated with mutations of the HNF1-alpha gene located on chromosome 12 [40].

1.4 Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1D) is a chronic autoimmune disorder characterized by dysfunction and destruction of insulin-producing pancreatic beta cells. The clinical onset of T1D is variable, ranging from few weeks to many years, during which the cells of immune system starts to attack β cells [41]. During this preclinical phase, we can witness the gradual loss of functional β cell mass with the appearance of autoantibodies direct against specific β cell autoantigens [42].
The autoimmune reaction is characterized by the presence of autoreactive T cells directed against specific autoantigens deriving from β cells and their autoantibodies: proinsulin, GAD65 (glutamic acid decarboxylase) IA-2 and IA-2β (tyrosine phosphatase) and ZnT8 molecules (zinc transporter) [43]. The clinical manifestation of this form of diabetes occurs following the massive destruction of β cells (90-95%); in this context the islets of Langerhans are infiltrated by different immune cell populations (insulitis) thus causing β cell destruction through direct mechanisms or through the production of inflammatory cytokines. Several studies revealed that insulitis is extremely heterogenous both among T1D donors and between the islets of the same donor. The insulitis is characterized by $CD8^+$ cytotoxic T cells that represent the predominant immune cell population, followed by macrophages, $CD4^+$ T cells and B-cells. During T1D stages, islets inflammatory infiltrates are mainly composed by cytotoxic CD8+ T cells; macrophages are less abundant than CD8 cells and increase during progression of insulitis; $CD4^+$ cells are present but in smaller numbers; the B cells ($CD20^+$) can be observed in the early stages of insulitis and finally the neutrophils are localized in exocrine and endocrine region and increase during T1D progression [44] (**Figure 4).**
The inflammatory process contributes to: (i) the destruction of β cells, (ii) the prolonged suppression of β cell function, (iii) the inhibition or stimulation of β cell regeneration.

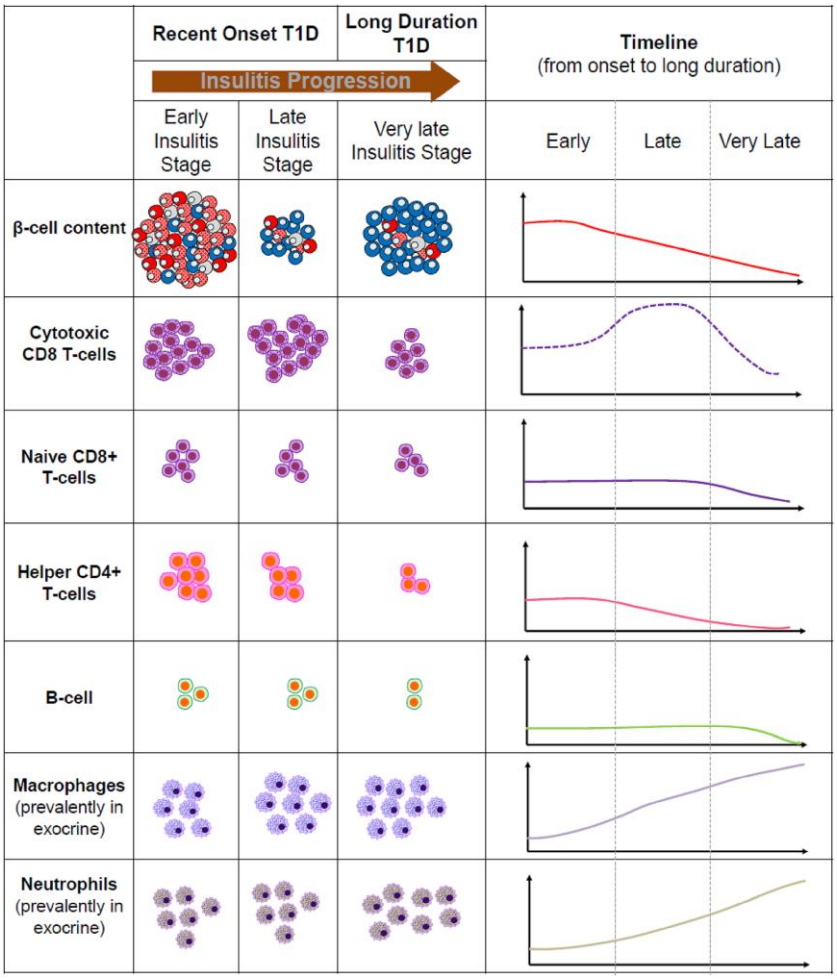


Figure 4: Progressive sequence of immune cells during insulitis and T1D progression. The scheme reports the dynamics of immune cells during insulitis and T1D progression. Insulitis described as a progressive phenomenon is characterized by several pseudo-stages (pseudostage 1: early insulitis; pseudostage 2 in T1D of recent onset: late insulitis; pseudostage 3 in T1D of long duration: insulitis in a very advanced stage. Dysfunctional (dedifferentiated) beta cells (narrow red) during the early stages of insulitis during the transition to destruction, replaced by α cells (dark blue), resulting in a reduction of fully functional β-cells (red). Insulitis is present in 30% of pancreatic islets rich in beta cells and decreases in the islets where there are fewer of them. Inflammatory islet infiltrates are mainly characterized by cytotoxic $CD8^+$ T (purple) lymphocytes, which increase in parallel with beta cell destruction, decrease dramatically when beta lymphocytes are no longer present within the islets. Macrophages are present in fewer numbers and increase during the progression of insulitis. $CD4^+$ T cells are less abundant than $CD8^+$ T cells and macrophages. B cells were seen in small numbers during the insulitis phases. Neutrophils are present in both the exocrine and insulitic areas and increase during the progression of T1D. *Nigi L. et al,* [44].

Recently it has been shown that preproinsulin (PPI), a key and crucial autoantigen in T1D, is recognized with high frequency by $CD8^+$ T cells even in healthy individuals. In fact, the relative frequencies of PPI-specific $CD8^+$ T cells were detected in the exocrine region of the pancreas regardless of the disease state. This observation challenges the dogma that T1D is caused by a defect in the thymic selection or dysregulation of the immune system [45]. However, the pathophysiological heterogeneity that characterize the pancreatic islets determine the complex scenario of T1D pathogenesis. Indeed, it has been observed that T1D patients show different residual β cell mass: some patients showed residual β cells several years after onset, while some others completely lack insulin-positive cells after a short period from the onset of the disease [46].

In 2017, Rui et al identified a population of β cells in non-obese diabetic (NOD) mice, able to survive the immune attack during the progression of T1D. In fact, in the pancreatic islets of NOD (non-obese diabetic) mice, during the progression of disease, it was observed the presence of two β cell subpopulations: the β "TOP" cells, fully mature and able to secrete insulin, and the β "BOTTOM" cells, characterized by reduced levels of genes associated to β cellular function and increased levels of endocrine progenitor markers (Ngn3) and stem (Oct4, Sox2, Sox9 and L-Myc), attributable to β cells dedifferentiated. Moreover, "BOTTOM" cells presented elevated levels of two factors associated with protection from attack of the immune system, Pdl-1 and Qa2. The authors shown that these dedifferentiated beta cells were more resistant and protected from stress-induced inflammation (cytokines or lymphocytic infiltrates) respect to mature β cells, suggesting that although beta-cell dedifferentiation leads to a loss of function by reducing the expression of beta cell specific genes, it can induce a protection from apoptosis [47].

In the same year, Wasserfall et al. showed that pancreatic islets of donors with recent-onset and long-standing T1D were characterized by low protein levels of insulin, but normal levels of insulin mRNA compared to non-diabetic donors. Furthermore, T1D pancreatic islets were characterized by a PCSK1 (Proprotein Convertase Subtilisin / Kexin Type 1) expression, but not of proinsulin, suggesting that the loss of beta cell mass in T1D may be partially due to dedifferentiation phenomena [48].

Rodriguez-Calvo et al., showed that in human pancreatic sections derived from Network of Pancreatic organ donors (nPOD), prediabetic donors (autoantibody positive) before disease onset, the beta cell mass was preserved and the levels of proinsulin and proinsulin to insulin area ratio (PI/INS area ratio) were increased, suggesting that PI-INS ratio value as an indicator of early beta cell dysfunction and a possible defect in proinsulin processing before the clinical

manifestation of disease. These data highlight that the beta cellular mass and function is maintained before the onset of T1D and the prevention in this stage could be important as therapeutic strategy [49].

A study on insulin and proinsulin in pancreas and serum have supported the presence of aetiopathological endotypes of T1D associated with age at diagnosis. Indeed, it has been demonstrated that different patterns of proinsulin localization may coexist in recent-onset T1D pancreatic islets. In particular, in the majority of insulin-containing islets of children diagnosed with T1D less than 7 years of age, proinsulin processing resulted aberrant compared to those with diagnosis after the age of 12 years and correlated with immune cell profiles defined based on lymphocyte composition of islets infiltrates (namely, CD20+cells). Moreover, in the same patients the circulating proinsulin-c-peptide ratio was increased in children diagnosed <7 years respect to those diagnosed > 13 years. These results suggest that the stratification of children and young people based on endotype could be important to design immunotherapies trial in T1D [50].

Environmental and genetic factors influence the timing and modalities of the inflammatory process in the progression of the disease, contributing to the wide heterogeneity found in subjects with T1D.

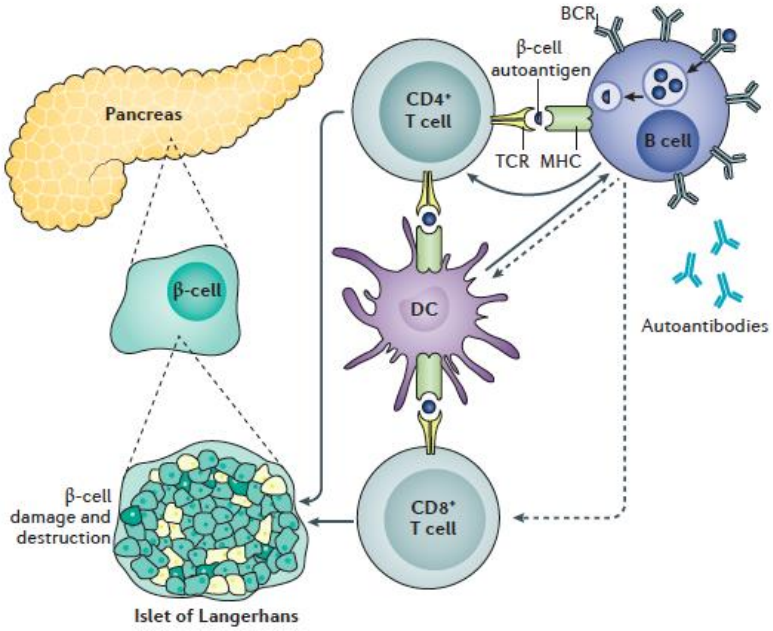


Figure 5: Pathogenesis of T1DM. Type 1 diabetes mellitus (T1DM) is an immune-mediated disease. Activated B cells interact with CD4+ and CD8+ T cells, as well as dendritic cells (DCs). Antigen presentation by B cells and DCs drives the activation of β cell-specific T cells. In addition, the exposure

of B cells to β cell autoantigens leads to the production of islet-targeting autoantibodies, which serve as biomarkers of asymptomatic disease. *Katsarou A. et al,* [51].

1.4.1 Genetic factors of Type 1 diabetes

The overall risk to develop T1D in general population is primarily associated to human leukocyte complex (HLA) region on chromosome 6, whose locus contains genes encoding for the MHC molecules and in particular is related to MHC class II. The most associated HLA class II haplotypes to disease onset and development are DR3-DQ2 and DR4-DQ8, while HLA class II haplotypes DRB1*1501 and DQA1*0102-DQB1*0602 confer disease protection in children younger than 12 years of age [52–55].

T1D can also be attributed to the approximately 50 non-HLA genes or loci identified through genome-wide association study approaches. The highest non-HLA genetic contribution is associated to INS, PTPN22, CTLA4 and IL2RA genes, which may also confer susceptibility to other autoimmune diseases [56]. Genetic variation can influence both immune regulation and the host response to environmental etiologies. Several studies have demonstrated that some non-HLA genes, including IL2, CD25, INS VNTR, IL18RAP, IL10, IFIH1 and PTPN22 are capable of influencing disease progression and even the alternative splicing of their product may probably be associated to immune system regulation and pro inflammatory cytokine production.

Moreover, HLA and non-HLA genetic risk is observed in relatives of individuals with T1D: the cumulative risk of developing the disease in monozygotic twins is reported to be as high as 30-70% with higher rates observed when the proband develops T1D at an earlier age [52].

The classification of HLA and non-HLA genetic polymorphisms and risk alleles important to stratify patients and risk for both developing islet autoantibodies and progressing from islet autoimmunity to symptomatic T1D, as well as to better understand the natural history of T1D in order to potentially prevent symptomatic disease.

1.4.2 Environmental factors of Type 1 diabetes

The incidence of type 1 diabetes can only be explained by change in environment or lifestyle. Potential environmental triggers include dietary factors during infancy: short duration of breastfeeding, cow's milk proteins assumption and other food, which could induce alteration

of intestinal microbiota, toxins, low serum concentration of vitamin D and viral and/or bacterial infections [57–59].

Special attention should be given to the Enterovirus infection, which have been historically associated to type 1 diabetes [60,61]. Enteroviruses appear to play a key role and may have the ability to infect β-cells and, in particular, the Coxsackieviruses B-group (CVBs) could trigger the damage of these cells [62]. In the blood of both mice and human patients with T1D at onset, the expression of CVB RNA, as well as Viral Protein 1 (VP1), the main component of CVB capsid protein, and Coxsackie Adenovirus Receptor (CAR) were increased in β cells, suggesting a particular tropism of CVBs for the insulin-producing cells [63]. The antiviral response could be modulated by the local expression of specific viral receptors such as hCAR (coxsackie- and adenovirus receptor) and viral intracellular sensors for the viral genome [64,65]. The melanoma differentiation-associated gene 5 (MDA5), encoded by IFIH1 (a particular polymorphism of which seems to regulate the interferon signature expressed by human pancreatic islets following a Coxsackievirus infection) [66] and the retinoic acid-inducible gene I (RIG-I) are well-established ubiquitous cytoplasmic viral dsRNA sensors, belonging to the retinoic acid-inducible gene 1 RIG-I-like receptors (RLRs), which are part of the innate immune pattern recognition receptors (PRRs) that recognize pathogen associated molecular patterns (PAMPs) [67].

1.5 Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2D) is the most common form of diabetes mellitus, which represents the 90-95% of all diabetic patients [68]. T2D is a metabolic disease characterized by a combination of genetic and environmental factors that result in decreased insulin function at sites of insulin action and a reduced ability of pancreatic beta cells to enhance insulin synthesis and secretion in response to increased blood glucose levels. The failure of islet beta cell compensation in response to the insulin resistance, may generate a progressive beta cell functional decline and, consequently, the progression from Normal Glucose Tolerance (NGT) status to Impaired Glucose Tolerance (IGT) and finally to established T2D [69] **(Figure 6).**

Pathophysiological changes are characterized by β-cell dysfunction, insulin resistance and chronic inflammation, which progressively hinders the control of blood glucose levels and the development of microvascular (retinopathy, neuropathy) or macrovascular (atherosclerosis) complications. Obesity and physical inactivity together with genetic predisposition, may lead to insulin resistance causing beta cell stress and the progressive loss of glucose-induced insulin

secretion. The development of type 2 diabetes has been divided into five stages, each of which is characterized by major changes in mass, phenotype and β-cell function:

(i) Stage-1: characterized by a compensation stage in which insulin secretion increases to restore normal glucose levels despite the augmented peripheral insulin resistance resulted from obesity, physical inactivity and genetic predisposition;

(ii) Stage-2: characterized by β-cell adaptation mechanisms in order to face the presence of elevated amount of glucose (glucotoxicity) in the blood;

(iii) Stage 3: is an unstable phase of non-compensation.

(iv) Stage 4: characterized by a stable and constant decompensation.

(v) Stage 5: characterized by a marked β-cell dysfunction of which can also lead to the development of ketoacidosis [70].

Obesity and insulin resistance lead to an increase of β cell mass following mechanisms of replication, neogenesis and cellular dimensions. The progression from an insulin resistance condition to diabetes is characterized by a decrease of β cell mass following an increase in apoptosis of β cells. The analysis of pancreatic tissue samples belonging to obese and non-obese patients, with or without alteration of fasting blood glucose (IFG) and with type 2 diabetes, showed that non-diabetic obese subjects were characterized by a substantial increase of β cell mass while obese subjects with IFG or diabetes showed a β cell volume reduction. These results have been reported and confirmed by other studies, suggesting that the reduction of cells number in patients with T2D is probably due to an increase of apoptosis rate or inadequate cell regeneration or neogenesis [71].

The stimulating factors involved in β cells compensatory processes, include an increased supply of nutrients in particular glucose and free fatty acids (FFA), insulin, growth factors and a greater sensitivity to incretins such as GLP-1 (glucagon-like- peptide) which promotes β cell proliferation and neogenesis to prevent apoptosis [72].

Several studies indicate an important role of growth factors in the signaling pathways triggered during the compensation process in β cells. Insulin and insulin growth factors 1 and 2 (IGF-1, IGF-2) bind to the tyrosine kinase (IR) receptor, which causes activation of IRS-1 / IRS-2 (Insulin Receptor Substrate), which activates the enzyme phosphoinositol 3 kinase (PI3-K) which activates the Akt / mTOR pathway. The signaling of IRS-2 through the phosphorylation of PKB (protein-kinase B) and the inactivation of the growth factor FOXO1, increases the expression levels of the PDX1 gene, an important factor of cell proliferation and β survival. The activation of PKB determines the protection from apoptosis through phosphorylation and the inhibition of pro-apoptotic proteins such as BAD [73].

Other phenomena that characterize insulin resistance and β cell failure are oxidative stress, endoplasmic reticulum stress, amyloid deposition in the pancreas, ectopic deposition of lipids in muscle, liver and pancreatic tissue and finally lipotoxicity and glucotoxicity.

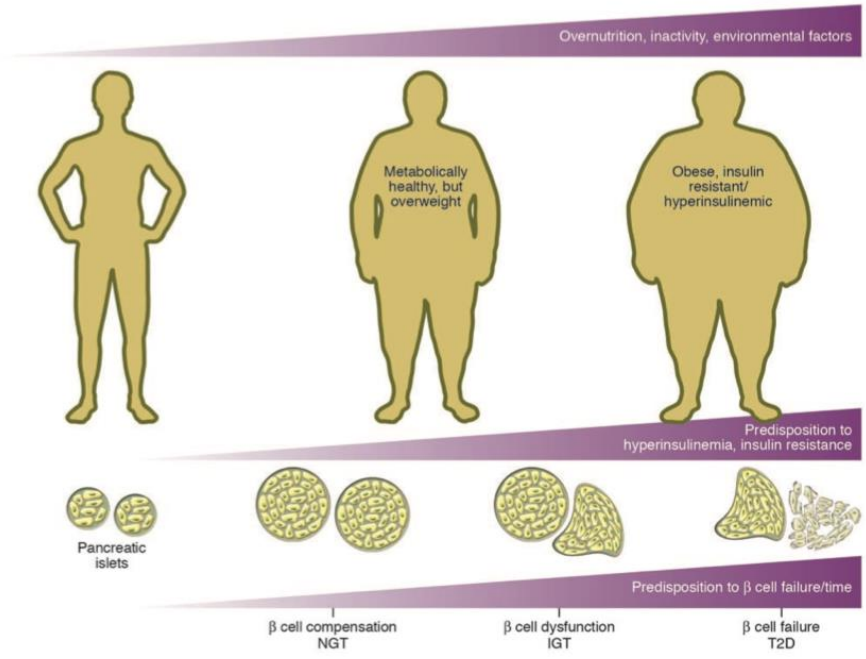


Figure 6: Islet beta cell failure and the natural history of T2D. T2D develops in response to overnutrition and lack of physical activity in individuals who have underlying genetic and acquired predispositions for both insulin resistance (and / or hyperinsulinemia) and beta cell dysfunction. Over time, pancreatic islet beta cell clearing fails due to insulin resistance, resulting in a progressive decline in beta cell function. As a result, subjects' transition from normal glucose tolerance (NGT) to IGT and eventually to T2D. Even after T2D is diagnosed, beta cell function may deteriorate, and people resort to oral hypoglycemic agents and possibly insulin to achieve adequate glycemic control. *Prentki M. et al,* [69].

1.5.1 Genetic factors of Type 2 Diabetes

Type 2 diabetes (T2D) is a polygenic disease that results from the interaction between genetic and environmental factors. The genetic risk to develop T2D derived from combination between alterations in multiple genes scattered all across the genome **(Figure 7)**.

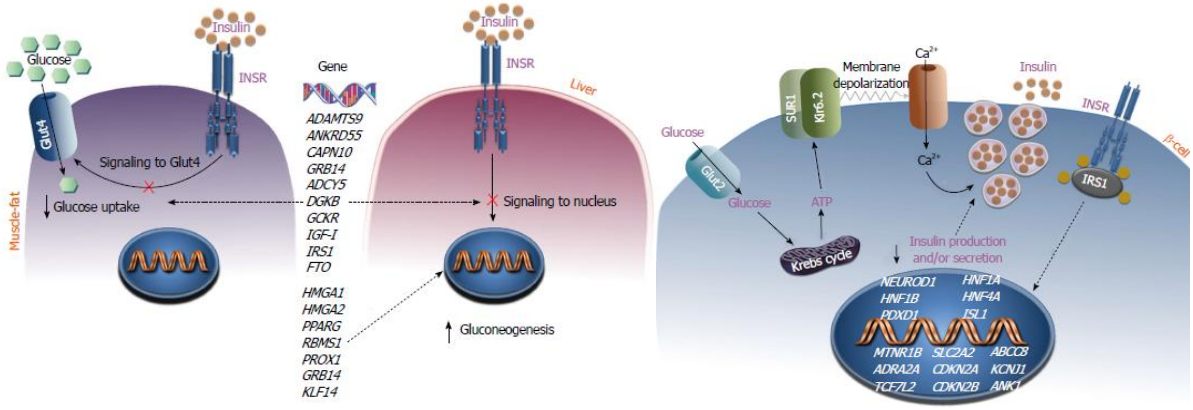


Figure 7: Mechanism of insulin resistance. The figure on the left shows the mechanisms by which gene variants can alter the action of insulin in the muscle, adipose and liver tissues of the target insulin. Peripheral insulin resistance in muscle and fat reduces cellular glucose uptake, while insulin resistance in the liver results in a failure to suppress glucose production and gluconeogenesis. Genes whose variations may affect the risk of developing insulin resistance and T2DM are indicated in black italics uppercase. The figure on the right shows a dysfunctional beta cell. Reduced insulin secretion is shown in beta cells with gene variants linked to T2DM. Genes associated with defects in beta cell mass and / or function are indicated in capital white italics. *Brunetti A. et al,* [74].

Evidence for a genetic component of T2D has come from in twins, first degree relatives of those with T2D and epidemiological studies have found to have an extremely high prevalence of T2D. Higher concordance rates are found among monozygotic twins (96%) than dizygotic twins; the 40% of first-degree relatives of T2D patients may develop diabetes, conversely the incident rate is only 6% in the general population[75–77].

Several single gene mutations have been identified as contributing of T2D. In fact, the mutations in hepatic nuclear factor (HNF) 1 or 4 are associated with severe impairment in insulin secretion and characterized a form of T2D called maturity onset diabetes of youth (MODY) and T2D patients with mutations in the insulin-receptor gene exhibit extreme insulin resistance [78,79]. In a study, 152 single polymorphisms have been examined in 71 candidate genes associated to the disease and found several genes which can cause insulin resistance such as ABCC8 (sulphonylurea receptor), KCNJ11 (KIR6.2), SLC2A2 (GLUT2), HNF4A (HNF4α), and INS; other genes are associated with insulin action (INSR, PIK3R1, SOS1).

Genome-wide associated studies (GWAS) have identified approximately 75 susceptibility loci correlated to T2D. Examples of candidate genes are KCNJ11 (potassium inwardly rectifying channel, subfamily J, member 11) that encodes for Kir6.2 ATP-sensitive potassium channel

playing an important role in the regulation of insulin secretion [80], TCF7L2 (transcription factor 7–like 2) which encodes for a member of Wnt signaling pathway active in the beta cell. The risk allele of the TCF7L2 gene is located in intron 3, and it has been demonstrated that the upregulation of TCF7L2 protein lead to impaired insulin secretion in beta cells, incretin effect and enhanced rate of hepatic glucose production [81]; IRS1 (insulin receptor substrate 1) has an effect on insulin action; MTNR1B (melatonin-receptor gene), PPARG2 (peroxisome proliferator-activated receptor gamma 2), IGF2BP2 (insulin-like growth factor two binding protein 2), CDKN2A (cyclin-dependent kinase inhibitor 2A), HHEX (hematopoietically expressed homeobox) and FTO (fat mass and obesity associated) gene [82].

1.5.2 Environmental factors of type 2 diabetes

There are several non-genetic factors directly or indirectly linked to Type 2 Diabetes, such as lifestyle, food and its components, persistent organic pollutants as well as the gut ecosystem that can lead obesity and oxidative stress. Lifestyle habits have a strong impact on a person's health and well-being [83]. Some lifestyle habits like alcohol intake, smoking, exercise, sedentariness as well as stress, insomnia and the use of clinical drugs are closely related to certain diseases such as type 2 diabetes. For example, the stress has an effect on glucose metabolism through hypothalamic-pituitary-adrenal (HPA) axis and its constant activation can have negative effects on insulin activities casing insulin resistance [84]. The diet is the most common environmental cause of T2D. It has been shown that a huge calorie restriction can revert diabetes in T2D patients [85]. Oxidative stress and inflammation are linked to T2D probably due to insufficient antioxidant defense. Several studies have demonstrated that low dietary intake of antioxidant such as beta-carotene, alfa-tocopherol and vitamin E can compromise the insulin action. In fact, diets rich of antioxidant, fruit and vegetables could prevent pancreatic beta cell dysfunction, reducing the risk of T2D [86].

The gut microbiota is vital to maintain individual health and it has been demonstrated to be associated with several metabolic disease including obesity and T2D. Several studies have shown that patients with T2D are characterized by increased abundance of Lactobacillus and lower abundance of butyrate-producing microbes, suggesting a strong correlation between Proteobacteria and Gram-negative Bacteroidetes and T2D [87].

A meta-analysis study on coffee consumption, has demonstrated that both caffeinated coffee and decaffeinated coffee are associated with reduced diabetes risk, with a 25-30% lower risk

for drinking three or more cups per day [88]. Furthermore, there is a strong correlation between cigarette smoke and increased risk of T2D compared to non-smokers [89]. Other environmental factors related to T2D are stress, depression or anxiety, all factors that increase the risk to develop T2D [90].

1.6 ER stress and proinsulin misfolding in Type 2 Diabetes

Beta cell failure in type 2 diabetes mellitus can be caused by hyperglycemia, hyperlipidemia, glucolipotoxicity, proinflammatory cytokines, accumulation of islet amyloid polypeptide as well as by endoplasmic reticulum (ER) stress. During beta cell compensation following insulin resistance there is an expansion of β cell mass and an increase in β cell insulin output. The chronic insulin demand and the decrease of insulin production may cause the depletion of β cell insulin stores leading to impaired insulin secretion and to "β cell-exhaustion" [91]. Inadequate production of insulin is thought to be a consequence of ER dysfunction. Several studies have been reported that β cell during the development and progression of T2D are characterized by elevated ER stress markers both in human and in *db/db* diabetic mouse [92,93]. Marchetti et al. have demonstrated that the ER volume density and β cell apoptosis increase in T2D. Moreover, isolated islets from T2D patients treated with high glucose were characterized by high levels of ER stress markers such as BiP, XBP1 and C/EBP homologous protein (also known as CHOP), suggesting an increased susceptibility of β cells to high glucose induced by ER stress [94]. A key factor that could contribute to ER stress and subsequently to β cell failure is the protein overload. In particular, the proinsulin biosynthesis that represents the primary driver of ER protein load, can rise up to 50-fold in response to insulin resistance [95].

Increased rate of proinsulin synthesis with alteration in the ER environment can cause accumulation of misfolding proinsulin and an accelerated proinsulin synthesis stimulates the unfolded protein response (UPR) "physiological UPR" in order to re-establish ER homeostasis. Emerging evidence showed that proinsulin misfolding is not only the molecular basis of mutant insulin-gene induced diabetes (MIDY) but may play an important role in the progression of type 2 diabetes. In Akita mice in which mutant proinsulin causes diabetes, it has been found that misfolded proinsulin interacted incorrectly with the precursor of insulin receptor (ProIR) in the ER causing an impaired insulin secretion in beta cells. In *db/db* insulin resistance mice, the overproduction of proinsulin led to an increase of proinsulin misfolding following the aberrant interaction between proinsulin and ProIR in the ER with defective intracellular

processing of insulin [96]. This stress condition leads to failure of normal proinsulin processing and regulated insulin secretion with a decrease of mature insulin granule formation and abnormal basal release of poorly functioning insulin characterized by a high proinsulin/insulin ratio (PI/INS).

Disproportionate PI secretion has been reported as a marker for β cell dysfunction in both diabetic patients and individuals at risk of diabetes. Several studies have shown that PI levels and PI/INS ratio are increased in plasma and serum of patients with metabolic alterations and in particular in Type 2 diabetes [97–99]. It has been hypothesized that an elevated PI/INS ratio is caused by increased secretory demand on β cells due to insulin resistance and hyperglycaemia, which promotes the release of immature granules with a higher relative content of PI and its conversion intermediates. Moreover, another study has demonstrated that in non-diabetic patients after partial pancreatectomy, the increase of β cell workload can modulate proinsulin-insulin ratio suggesting that whole-body insulin resistance is associated with alteration in proinsulin secretion, detectable only in the presence of increased insulin secretion demand [100].

Initially, the beta cell ER has the ability to adapt to accumulation of misfolded protein through the activation of chaperones and oxidoreductase such as protein disulphide isomerase (PDI) and ER oxireductin 1 (ERO1) that contribute to oxidative folding. In a study, it has been demonstrated that knockout of ERO1β in mutant mice, led to alteration of oxidative folding of proinsulin promoting glucose intolerance. UPR activation is mediated by 3 ER transmembrane kinases: PERK (protein kinase RNA-like endoplasmic reticulum kinase); IRE1α (endoribonuclease inositol-requiring protein 1α) and ATF-6α (activating transcription factor 6α) which have several functions such as: to repair protein misfolding, to activate chaperones, to degrade misfolded protein, to inhibit protein synthesis and to reduce the cargo load in the ER. When ER stress persists, the prolonged UPR activation "terminal UPR" may induces apoptosis and diabetes [101–103].

Genome-Wide-Association Studies (GWAS) have found several type 2 diabetes-associated genes involved in ER homeostasis and ER stress response, including *CAMK1D, CDC123, NOTCH2, THADA, VEGFA, IRS1, WFS1, ADAMTS9, and SLC16A11*. Of interest, *WFS1* (Wolfram syndrome 1) encodes an ER transmembrane protein is an important gene for normal ER function and protein folding. It has been shown that WFS1 deficiency caused ER stress and beta cell apoptosis in mice; the mutation of this gene in humans is associated with Wolfram Syndrome, including early onset insulin-dependent diabetes [104,105].

Although these observations explain the direct association between ER stress and type 2 diabetes, no study has explicitly focused on why there is an increase of proinsulin misfolding

in beta cells of type 2 diabetes patients or animal models and it would be important to investigate more thoroughly.

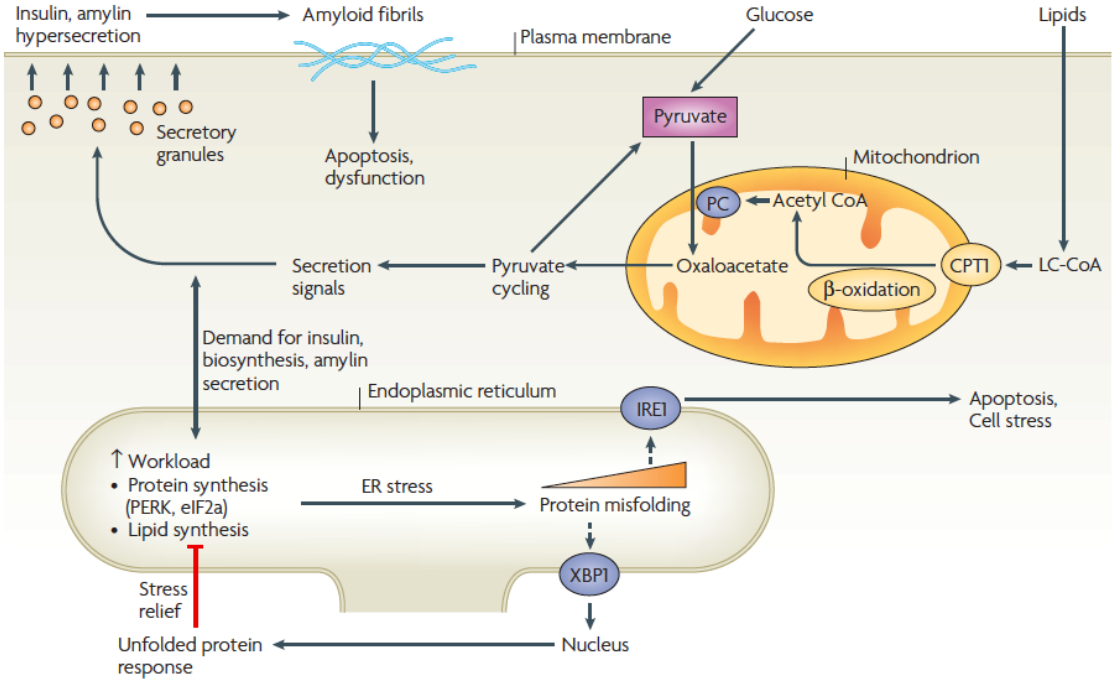


Figure 8. Mechanisms of β-cell failure in type 2 diabetes. The figure shows metabolic overload (mitochondria), endoplasmic reticulum (ER) stress and deposition of harmful amyloid fibrils. Overnutrition and elevated lipid supply cause enzymes of β-oxidation, such as carnitine palmitoyltransferase-1 (CPT1), resulting in increased acetyl CoA levels, allosteric activation of pyruvate carboxylase (PC) and constitutive upregulation of pyruvate cycling. This causes hypersecretion of basal insulin and loss of glucose-stimulated increment in pyruvate cycling flux, resulting in decrease of glucose stimulated insulin secretion. The elevated of insulin demand increases demand (workload) in the ER leading to ER stress and increased protein misfolding. ER stress is initially alleviated by the unfolded protein response (UPR), mediated by the transcription factor XBP1. Over time, UPR becomes less effective and the deleterious effects of ER stress cause cell death, mediated by IRE1. Finally, insulin hypersecretion induces amylin secretion, which in humans can form amyloid fibrils that accumulate at the surface of β-cells inducing dysfunction and apoptotic death. *Muoio D.M. et al,* [106].

1.7 MiRNA and ER stress

Pancreatic beta cells result particularly susceptible to oxidative stress due to their deficiency in antioxidant defense mechanism [107]. In diabetes, oxidative stress is principally caused by an excessive increase of glucose, Free Fatty Acids (FFA), and/or inflammatory mediators which,

in turn, cause mitochondrial dysfunction and/or ER stress with consequent insulin resistance and beta cell dysfunction. ER is the most important subcellular membrane organelle implicated in protein synthesis and folding, as well as in the control of cellular calcium concentrations. The alteration of protein synthesis and folding, automatically triggering an UPR as defense mechanism. Among several molecules and factor involved in the development and exacerbation of oxidative stress, microRNAs (miRNAs) have been identified as potent modulators of multiple genes composing such complex molecular signaling machinery [108]. MiRNAs are small endogenous non coding RNAs of 19–24 nucleotides which negatively regulate gene expression. The direct binding of the miRNA seed sequence on the 3'Untranslated Region (3' UTR) of target gene leads to degradation of mRNA or repression of protein translation (**Figure 9**).

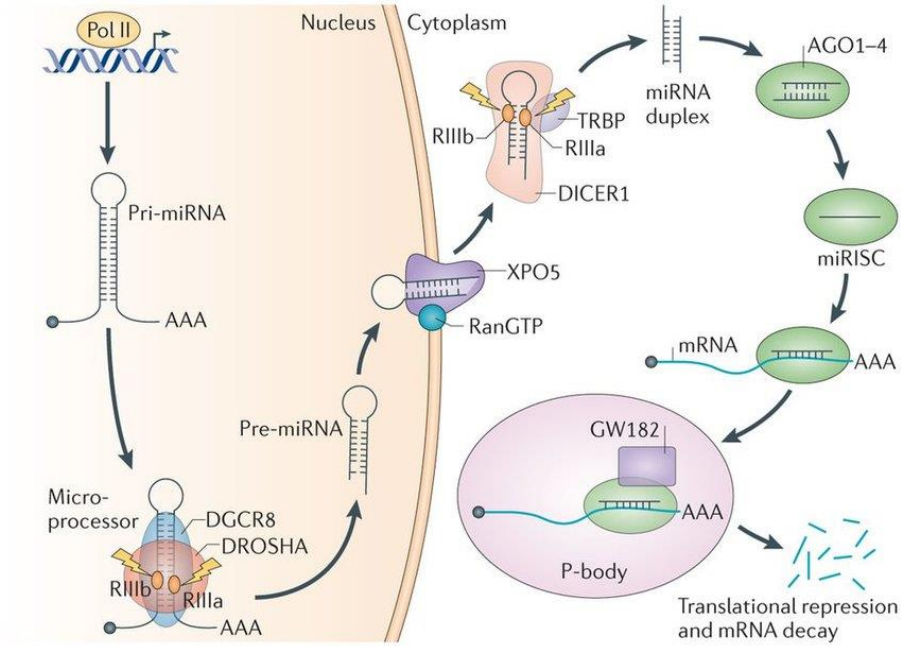


Figure 9: Overview of miRNA biogenesis pathway. MicroRNA are transcribed as primary miRNAs (pri-miRNAs) by RNA polymerase II (Pol II) in the nucleus. The long pri-miRNAs are cleaved by Microprocessor, which includes DROSHA and DiGeorge syndrome critical region 8 (DGCR8), to produce the 60–70-nucleotide precursor miRNAs (pre-miRNAs). The pre-miRNAs are then exported from the nucleus to the cytoplasm by exportin 5 (XPO5) and further processed by DICER1, a ribonuclease III (RIII) enzyme that produces the mature miRNAs. One strand of the mature miRNA (the guide strand) is loaded into the miRNA-induced silencing complex (miRISC), which contains DICER1 and Argonaute (AGO) proteins, directs the miRISC to target mRNAs by sequence complementary binding and mediates gene suppression by targeted mRNA degradation and translational repression in processing bodies (P-bodies). *Lin S. et al,* [109].

MiRNAs are involved in many cellular processes such as development, cell proliferation and survival, differentiation and apoptosis [110]. Their altered expression may contribute to several human diseases, including metabolic and cardiovascular disorders, cancer and neurological diseases [111]. The alterations of specific miRNA levels can induce oxidative stress and damage and consequent development of different diseases. Several studies showed that in human and murine pancreatic islets cultured at high glucose concentration the expression of miR-708 was elevated. Treatment with thapsigargin, chemical a chemical inducer of Endoplasmic Reticulum (ER) stress, led to the upregulation of miR-708 in human islets as well as in *ob/ob* mouse islets; the treatment with chemical chaperone 4-phenylbutyrate, a molecule known to improve ER folding capacity caused the inhibition of this miRNA, suggesting the involvement of ER stress folding in this response. Moreover, Neuronatin, previously shown to be involved in the modulation of the secretory function of beta cells, has been identified as a potential target of miR-708. In fact, the expression of Neuronatin was reduced following miR-708 hyperexpression and inversely correlated with miR-708 levels in human and murine islets exposed to different glucose concentrations. Of note, overexpression of Neuronatin restored the Glucose-Stimulated Insulin Secretion (GSIS) in murine and human islets cultured at low glucose levels. These results demonstrated that miR-708 could be used as potential target to restore beta cell function under stress conditions [112,113].

Recent studies have linked miRNAs to the UPR pathway (**Figure 10**) [114]. It has been demonstrated that miR-30d, miR-181a and miR-199a involve in obesity-induced endothelial dysfunction were potentially regulators of GRP78/BIP, a major ER chaperone protein [115–118]. MiR-204 was involved in ER stress in human cells by directly targeting PERK signaling causing ER-stress-induced apoptosis [119]. The transcription factor ATF4, located downstream of PERK signaling was targeted and inhibited by miR-204 whose expression was altered in obesity [120,121]. In the downstream signaling cascade, XBP1 was a potential target of miR-214 and miR-30c, both involve in the process of obesity-induced endothelial damage [122]. Moreover, the regulation of PERK and IRE1alfa from miR-23a-27a-24 cluster lead to perturbation in intracellular calcium levels and mitochondrial function [123].

A recent study demonstrated the anti-proliferative effect of miR-204 in the islets of db/db mice. The authors showed that upon genetic deletion of miR-204 improved cell proliferation decreasing islet ER stress through the reduction of CHOP [124]. Of note, it has been reported that additional miRNAs may play a role in the induction of protection from metabolic stresses. Indeed, the deficiency of miR-200 in murine islets, led to a protection of beta cells from

oxidative stress through the repression of pro-apoptotic genes, indicating a critical role for this miRNA in the pathophysiology of diabetes. Furthermore, it has been reported that the miR-200 family was hyperexpressed in pancreatic islets of diabetic mice, thus inducing beta cell apoptosis. MiR-200 negatively modulates the expression of DNAJ heat shock protein family (Hsp40) Member C3 (Dnajc3) [an essential beta cell chaperone known as Protein Kinase Inhibitor p58 (p58IPK)] and the caspase inhibitor X-Linked Inhibitor of Apoptosis Protein (Xiap), thus confirming its essential role in the regulation of beta cell survival during oxidative stress conditions [125].

In diabetic setting, microRNA miR-24 was hyperexpressed inducing beta cell dysfunction and replicative deficiency. In a study, it has been shown that overexpression of miR-24 protected beta cell from thapsigargin-induced apoptosis. Moreover, the increase of this miRNA enhanced the expression of markers associated with dedifferentiation by inhibiting effectors of ER stress (XBP1, ATF4 and IRE1alfa) suggesting a potential protective role of miR-24 from apoptosis during metabolic stress [126].

2. Aims of project thesis

Type 2 diabetes mellitus is a heterogeneous group of metabolic diseases characterized by increased levels of blood glucose due to insulin resistance and alteration of insulin secretion by pancreatic β cells. Recent studies suggest that β cell loss in T2D results from endoplasmic reticulum stress which can cause an alteration in the processing of PI to INS. In particular, it has been reported that the increased circulating levels of PI and elevated PI/INS ratio are well-known abnormalities in type 2 diabetes. Several studies have hypothesized that an elevated PI/INS ratio is caused by increased secretory demand on β cells due to insulin resistance and hyperglycemia. However, an in-depth analysis of PI/INS expression pattern inside the pancreatic islets during metabolic alteration is not entirely clear.
For this reason, the first aim of this work is:

(i) to analyze PI and INS expression pattern in pancreatic islets of tissue biopsies of patients undergoing partial pancreatectomy (PP) with normal glucose tolerance (NGT), impaired glucose tolerance (IGT) and type 2 diabetes, in order to explore the *in-situ* alterations that occur in islets during metabolic stress.

Pancreatic islets of Langerhans are heterogenous tissues consisting of different types of cells that work together to regulate blood glucose homeostasis. Given the enormous heterogeneity between pancreatic islets of different donors but also between islets belonging to the same donors, the second aim of project thesis is:

(ii) to perform single islet phenotyping by characterizing histological and molecular aspects, in order to investigate the underlying molecular mechanisms driving beta cell failure at single islet level with the aim to specifically determine the cues leading to intracellular alteration of PI and insulin during metabolic stress.

3. Experimental procedures

3.1 Study population

For this study we have analyzed pancreatic tissue biopsy of n=19 patients classified following OGTT (oral glucose tolerant test) into n=5 NGT (normal glucose tolerance), n=9 IGT (impaired glucose tolerance) and n=5 T2D (type 2 diabetes), on the waiting list for partial pancreatectomy recruited from the Digestive Surgery Unit and studied in the Center for Endocrine and Metabolic Diseases unit (both at the Agostino 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, which was followed by a comprehensive medical evaluation. Indication for surgery was tumor of the ampulla of Vater. All patients had an accurate metabolic profiling which included evaluation of glucose tolerance and insulin secretion. Patients selected for this study were required a normal cardiopulmonary and kidney function, as determined by medical history, physical examination, electrocardiography, estimated glomerular filtration rate and urinalysis. Patients with altered serum lipase and amylase levels, as well as morphological criteria for pancreatitis, severe obesity (BMI>40), uncontrolled hypertension and/or hypercholesterolemia were considered exclusion criteria. Clinical and metabolic characteristics are shown in **Table 1**.

3.2 Surgical procedure

The surgical procedure of pancreatoduodenectomy was performed according to the pylorus preserving technique [127]. Briefly, the pancreatic head, the entire duodenum, common bile duct, and gallbladder were removed in 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 pancreato-jejunostomy. In addition, it was performed downstream an end-to-side hepaticojejunostomy and an end-to-side pylorojejunostomy. We took advantage of this operation to collect a small sample of pancreatic tissue at the edge of the surgical cut. The volume of pancreas removed during the surgery is constant (~50%), as previously reported by Schrader et al. [128].

3.3 Sample collection and clinical analysis

All patients underwent a 2-hour euglycemic clamp (insulin infusion rate: 40 mIU.min-1.m-2). Hyperglycemic clamp with arginine stimulation and a 4-hour mixed meal test to evaluate insulin secretion (from C-peptide deconvolution) [129]. During the mixed meal test, insulin secretion was originated from C-peptide levels by deconvolution. Moreover it has been calculated the β-Cell Glucose Sensitivity (βCGS), which represents the slope of the relationship between insulin secretion and glucose concentration and was estimated from the mixed meal by modeling, as previously described [130,131].

Table 1

Cases	*Age*	*Diabetes duration (years)*	*BMI (kg/m^2)*	*Sex*	*Glucose (mg/dl)*					*Insulin (µUI/ml)*					*C-peptide (ng/ml)*					*Basal glucose (mg/dl)*	*Basal insulin (µUI/ml)*	*Glucose sensitivity ($pmol \cdot min^{-1} m^{-2} \cdot mM^{-1}$)*
					0'	30'	60'	90'	120'	0'	30'	60'	90'	120'	0'	30'	60'	90'	120'			
NGT (n=5)																						
O7-MF	79	0	30,1	M	104	202	217	171	87	5	52	90,8	137	33,1	1,7	4,8	7,4	11	7,9	5,77304	30	75,75
19-BMA2	78	0	24,3	F	96	145	167	153	115	8,3	77,8	126	51,2	53	2,4	10	12	18	15,3	5,32896	49,8	189,36
21-TRA	32	0	24,4	F	58	128	168	153	109	2,6	16,2	21,7	24,2	15,2	0,6	2,9	3,6	4,4	3,3	3,21958	15,6	30,55
22-GO	71	0	28,6	M	92	210	210	144	120	7,1	82,8	17,7	82,4	64	1,7	6,2	9,1	11	10,9	5,10692	42,6	83,25
59-PM	76	0	24,1		80	154	129	114	122	5,2	85,8	55,2	46,7	65,3	1,2	5,9	6,6	6,4	8,4	4,44080	31,2	92,41
IGT (n=9)																						
03-GP	60	0	26,3	F	93	190	231	185	168	23,1	116	216	226	255	2	5,9	8,8	8,7	11,2	5,16243	138,6	54,33
14-LA	70	0	27,3	F	85	180	144	161	157	8,9	11	78,7	154	112	1,7	5,4	7,2	10	9,7	4,71835	53,4	85,45
16-OMG1					85	125	164	163	158	4	10	27,8	40,4	49,8								
20-RA	71	0	29,7	M	96	166	172	175	176	16,3	121	129	139	186	2,9	10,7	12	14	16,9	5,32896	97,8	123,11
23-CQ	78	0	27,7	F	107	136	142	136	142	15,7	34,6	41,5	49,5	46,3	1	2,6	3,5	4,4	5,9	5,93957	94,2	91,93
24-PS	66	0	29,0	M	87	192	226	195	151	6,6	84,1	140	175	135	1,8	7	13	12	12,9	4,82937	39,6	93,96
51-CA	75	0	23,5	F	83	194	248	241	154	8,4	31,4	46,6	62,2	61,6	1,2	2,8	4,5	7,8	9	4,60733	50,4	38,12
52-LR	78	0	22,7	F	100	191	205	169	133	13,6	33,6	171	122	83,2	2,2	6,2	14	15	15,6	5,55100	81,6	134,99
58-FP	59	0	20,8	F						3,1	2,2	24,5	27,2	32	0,9	2,5	4,1	7,8	7	5,38447	90	33,56
T2D (n=5)																						
01-CC	74	2	28,8	F	195	367	334	319	202	29,7	54,6	101	79,7	57,3	7	10,7	15	15	10,6	10,82445	178,2	43,07
04-GML	70	2	28,1	F	162	214	276	287	317	26,3	28,9	30,6	30,4	30	2,7	3,3	3,5	4,1	5,3	8,99262	157,8	12,17
05-RR	73	0	21,4	F	115	183	220	218	186	9,1	18,5	16,2	42,9	60,2	4	5,3	6,2	9,2	12,4	6,38365	54,6	32,94
65-MQ	62	11	20.8	M	76										0,1					4,22000	206,4	8,85
71-DL	79	2	26,4	F	200					8,4					1,7					8,33000	49,8	28,50

Table 1. Clinical and metabolic characteristics of NGT, IGT and T2D patients. ID case**,** Age, Diabetes disease (years), BMI (kg/m^2), Sex, Glucose (mg/dl), Insulin /µUI/ml), C-peptide (ng/ml), Basal glucose (mg/dl), Basal insulin (µUI/ml) and Glucose sensitivity ($pmol \cdot min^{-1} m^{-2} \cdot mM^{-1}$) are reported.

3.4 Proinsulin-Insulin Immunofluorescence analysis of human pancreatic section

Frozen pancreatic sections were analysed through double immunofluorescence in order to evaluate the expression pattern of proinsulin and insulin. Briefly, after one hour of drying at room temperature (RT), the sections were fixed in paraformaldehyde (PFA) 4% for 20 minutes

at RT. After, the sections were washed three times in 0,1% PBS-tween w/Ca^{2+} Mg^{2+} 5 min each at RT. In order to unmasking the antigens, pancreatic sections were subjected to antigen retrieval using 10 mM citrate buffer pH-9 for 40 minutes at 70°C. Sections were washed three times in 0,1% PBS-tween w/Ca^{2+} Mg^{2+} 5 min each at RT. Subsequently, sections were incubated with PBS 1X supplemented with 1% Bovine Serum Albumin (BSA, cat. A1470-25G, Sigma Aldrich, St. Louis, MO, USA) to reduce non-specific reactions. Then, sections were incubated with primary antibodies, polyclonal Guinea Pig anti-human Insulin (cat. A0564 - Agilent Technologies, Santa Clara, CA, USA) diluted 1:2000 and Mouse monoclonal anti-human Proinsulin (cat. GS9A8 – DSHB epitope: B-C junction of proinsulin spanning aa 26-37) [132–134] diluted 1:100 in PBS 1X supplemented with 1% BSA overnight in damp chamber at 4°C. The next day, sections were washed three times in 0,1% PBS-tween w/Ca^{2+} Mg^{2+} 5 min each at RT and subsequently incubated with secondary antibodies, goat anti-guinea pig Alexa-Fluor 488 conjugate (cat. A11073, Molecular Probe, Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:500 in PBS 1X and goat anti-mouse Alexa-Fluor 594 conjugate (cat. A11032, Molecular Probe, Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:500 in PBS 1X for 1h at RT. Sections after the washes were counterstained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, cat. D8517, Sigma-Aldrich) diluted 1:3000 in PBS 1X for 5 minutes, and then mounted with Vectashield antifade medium (cat. H-1000, Vector Laboratories, Burlingame, CA, USA) and analysed immediately or stored at +4°C until ready for confocal image analysis.

3.5 Image acquisition and analysis

Images were acquired using Leica TCS SP5 confocal laser scanning microscope system (Leica Microsystems, Wetzlar, Germany). Images were acquired as a single stack focal plane or in z-stack mode capturing multiple focal planes. Sections were scanned and images acquired at 40× magnification. The same confocal microscope setting parameters (laser power, photomultiplier voltage gain and offset values, pinhole value) were applied to all stained sections before image acquisition in order to uniformly collect detected signal related to each channel. Image analysis was performed using Volocity 6.3 software (Perkin Elmer, Waltham, MA, USA) on each individual islet to measure colocalization coefficient (M_1), islet area (μm^2), PI and INS positivity area. The islet area (μm^2) was calculated taking into consideration the value (sum area) which represents the total number of objects that identify the total area of islet on the basis of the region of interest (ROI). A background threshold filter was uniformly applied to all

processed images before the evaluation of specific parameters. After setting manual thresholds based on the positivity of the signals to be analyzed, the PI and INS positivity area was calculated, taking into consideration (sum area) which represents the total of objects identifying the positivity area of each channel. The PI/INS ratio was calculated by dividing the PI signal positive area and the INS positive area, normalized by the total area of islet.

The percentage of colocalization coefficient between PI and INS was calculated through the Colocalization Coefficient M_1 (Mander's coefficient) [135] that considers the percentage of pixels (or voxels in case of volume) of a given channel, in our case the INS, which overlaps to total pixels (or voxels) related to the other channel, in our case the PIβ. Of note, Mander's coefficient is independent of absolute signal as it measures the fraction of one protein that colocalizes with a second protein where M represents the fraction (reported in percentage) of colocalizing pixels channel-1/channel-2 on total channel-1 pixels.

3.6 Laser capture microdissection (LCM)

Pancreatic human tissue samples n=4 NGT, n=7 IGT and n=4 T2D from living donors (**Table 1**) were frozen in Tissue-Tek OCT compound and then 7-μm thick sections were cut from frozen O.C.T. blocks. Sections were fixed in 70% ethanol for 30 s, dehydrated in 100% ethanol for 1 min, in 100% ethanol for 1 min, in xylene for 5 min and finally air-dried for 5 min. Laser capture microdissection (LCM) was performed using an Arcturus XT Laser-Capture Microdissection system (Arcturus Engineering, Mountain View, CA, USA) by melting thermoplastic films mounted on transparent LCM caps (cat. LCM0214 - ThermoFisher Scientific, Waltham, MA, USA) on specific islet areas. Human pancreatic islets were subsequently visualized through islet autofluorescence for LCM procedure. Thermoplastic films containing microdissected cells were incubated with 10 μl of extraction buffer (cat. kit0204 - ThermoFisher Scientific, Waltham, MA, USA) for 30 min at 42 ◦C and kept at −80°C until RNA extraction. Each microdissection was performed within 30 min from the staining procedure. Overall n=50 microdissected pancreatic islets from each case were analysed for the global molecular analysis.

3.7 RNA extraction from LCM isolated human pancreatic islets

Total RNA was extracted from each LCM sample using PicoPure RNA isolation kit Arcturus (cat. kit0204 - ThermoFisher Scientific, Waltham, MA, USA) following manufacturer's procedure. Briefly, the cellular extracts were mixed with 12.5 µl of Ethanol (100%) and transferred to the purification column filter membrane. DNase treatment was performed using RNase-Free DNase Set (cat. 79254 - Qiagen, Hilden, Germany). Total RNA was eluted in 11 µl of DNase/RNase-Free Water and LCM captures deriving from human sample were pooled and subjected to a subsequent concentration through Savant SpeedVac SC100 centrifugal evaporator. Agilent 2100 Bioanalyzer technology with RNA Pico chips (cat. 5067-1513 Agilent Technologies, Santa Clara, CA, USA) was performed for each RNA sample, in order to analyse RNA integrity (RIN) and concentration, by excluding samples with RIN<5.0.

3.8 Gene expression analysis

In order to analyze gene expression associated to ER stress, folding, processing of proinsulin as well as to β cell function and dysfunction, a reverse transcriptase reaction was performed on each RNA sample extracted (500pg) from microdissected islet endocrine cells from each donor using SuperScript™ VILO™ cDNA Synthesis Kit (cat. 11754050- ThermoFisher Scientific, Waltham, MA, USA). cDNA deriving was then amplified using TaqMan PreAmp Master Mix (cat. 4488593, ThermoFisher Scientific, Waltham, MA, USA) following manufacturer's instructions. Real-Time PCR analysis was performed using TaqMan gene expression assays using the primers (**Table 2**) and SensiFast Probe Lo-ROX Kit (cat.# BIO-84020, Bioline) following manufacturer's recommendation. Data were collected and analysed through Expression Suite software 1.0.1 (ThermoFisher Scientific, Waltham, MA, USA) using $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ method. Data analysis was performed using ViiA7™ RUO software to collect data and Expression Suite 2.1 software (both Thermo Fisher Scientific) to evaluate amplification plot efficiency and to export Ct values. Analysis was performed using $2^{-\Delta CT}$ following normalization with β-actin and GAPDH.

Table 2

ID	Gene name	ID assay
INS	Insulin	Hs02741908_m1
GCG	Glucagon	Hs00174967_m1
STT	Somatostatin	Hs00174949_m1
PDX1	Pancreas/duodenum homeobox protein 1	Hs00236830_m1
FOXO1	Forkhead box protein O1	Hs01054576_m1
MAFA	V-maf musculoaponeurotic fibrosarcoma oncogene homolog A	Hs01651425_s1
NKX6.1	Homeobox protein NK-6 homolog A	Hs00232355_m1
CRTC1	CREB-regulated transcription coactivator 1	Hs00993064_m1
GLI1	Glioma-associated oncogene	Hs00171790_m1
ALDH1A3	Aldehyde dehydrogenase family 1 member A3	Hs00167476_m1
CHGA	Chromogranin-A	Hs00900375_m1
NANOG	Homeobox protein NANOG	Hs02387400_g1
SLC2A1	Solute Carrier Family 2 Member 1	Hs00892581_m1
AMY2A	Pancreatic alpha-amylase	Hs00420710_g1
FOXA2	Forkhead box protein A2	Hs00232764_m1
GCK	Glucokinase	Hs00175951_m1
CDH1	Cadherini-1	Hs01023895_m1
CADM1	Cell adhesion molecule 1	Hs00942509_m1
PAX4	Paired box gene 4	Hs00173014_m1
DICER	Endoribonuclease Dicer	Hs00229023_m1
DROSHA	Ribonuclease Drosha	Hs00203008_m1
ONECUT2	One cut domain family member 2	Hs00191477_m1
VIMENTIN	Vimentin	Hs00185584_m1
BETA ACTIN	Beta actin	4332645
B2M	Beta 2 microglobulin	4333766
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	4333764
SEL1L	Protein sel-1 homolog 1	Hs01071406_m1
HRD1/SYVN1	ERAD-associated E3 ubiquitin-protein ligase HRD1	Hs00381211_m1
ABCC8	ATP Binding Cassette Subfamily C Member 8	Hs01093752_m1
ERP27	Endoplasmic reticulum resident 27	Hs00929901_m1
UCN3	Urocortin-3	Hs07289310_m1
SLC2A1	Solute Carrier Family 2 Member 2	Hs01096908_m1
ACLY	ATP citrate lyase	Hs00982738_m1
IAPP	Islet amyloid polypeptide	Hs00169095_m1
PC1/PCSK1	Prohormone convertase 1	Hs01026107_m1
PC2/PCSK2	Prohormone convertase 2	Hs00159922_m1
CPE	Carboxypeptidase E	Hs00175676_m1
ERO1B	ER oxidoreductin 1β	Hs00219877_m1
CHOP/DDIT3	C/EBP homologous protein	Hs01090850_m1
GRP78/HSPA5	Glucose-regulated protein	Hs99999174_m1
XBP1	Splicing of X-box binding protein 1	Hs00231936_m1
PERK/EIF2AK3	Pancreatic ER kinase	Hs00984003_m1
IRE1α/ ERN1	Inositol-requiring enzyme 1α	Hs00980095_m1
ATF6	Activating transcription factor 6	Hs00232586_m1
CHGB /CgB	Chromogranin B	Hs01084631_m1
LMNB1	Lamin B1	Hs01059210_m1
PDIA1/P4HB	Protein disulfide isomerase	Hs01050257_m1
RO60/TROVE2	RNA binding protein	Hs01567394_m1
TCP1/CCT4	Chaperonin containing TCP1 subunit 4	Hs00272345_m1
S100A9	S100 Calcium-Binding Protein A9	Hs00610058_m1

Table 2. List of primer used for qRT Real-Time PCR. List of Taqman primers used to evaluate gene expression in LCM pancreatic islets of NGT, IGT and T2D living donors.

3.9 Statistical analysis

Results were expressed as mean ± SD. Statistical analyses were performed using Graph Pad Prism 8 software. Comparisons between two groups were carried out using Mann-Whitney U test (for non-parametric data) or Wilcoxon matched signed rank test. Multiple comparisons were analysed using ordinary one-way ANOVA and Kruskal Wallis test. The correlation analysis was evaluated by Pearson and Spearman non-parametric test. Differences were considered as statistically significant with p values less than 0.05. GraphPad 6 and 8 was adopted for statistical analysis of the data.

3.10 I*n-situ* characterization and molecular analysis of single pancreatic islets

The phenotypic characterization of individual pancreatic islets was performed on two frozen serial pancreatic sections for each donor. On the first section we performed an INS-PI staining (as previously described) in order to characterize the morphological profile of single islets in order to evaluate the *in-situ* expression levels of PI and INS. After carrying out the image analysis, we mapped the single pancreatic islets using the fluorescence microscope and photographing the entire section at low magnification (4X). Subsequently, on the second serial section mounted on a slide for LCM, we isolated the same single islets previously mapped for gene expression analysis in order to investigate what the molecular alteration was associated with a high PI/INS ratio. The laser microdissection isolation procedure was performed as described above, isolating a single islet at a time (**Figure 10**).

The total volume of RNA extracted (8.8 µl) using PicoPure RNA isolation kit Arcturus (cat. kit0204 - ThermoFisher Scientific, Waltham, MA, USA) from microdissected single pancreatic islets was subjected to a reverse transcriptase reaction using SuperScript™ VILO™ cDNA Synthesis Kit (cat. 11754050- ThermoFisher Scientific, Waltham, MA, USA) in order to analyse gene expression in single pancreatic islets. The total volume of cDNA deriving was then amplified using TaqMan PreAmp Master Mix (cat. 4488593, ThermoFisher Scientific, Waltham, MA, USA) by preparing the preamplification pool using Taqman primers (**table 3**) by diluting each 20X TaqMan gene expression Assays to obtain a final concentration of each assay equals 0.5×. The preamplification product was diluted 1:5 in 0.1X Tris-EDTA pH 8.0. Real-Time PCR analysis was performed using TaqMan gene expression assays and SensiFast Probe Lo-ROX Kit (cat.# BIO-84020, Bioline) following manufacturer's recommendation.

Figure 10

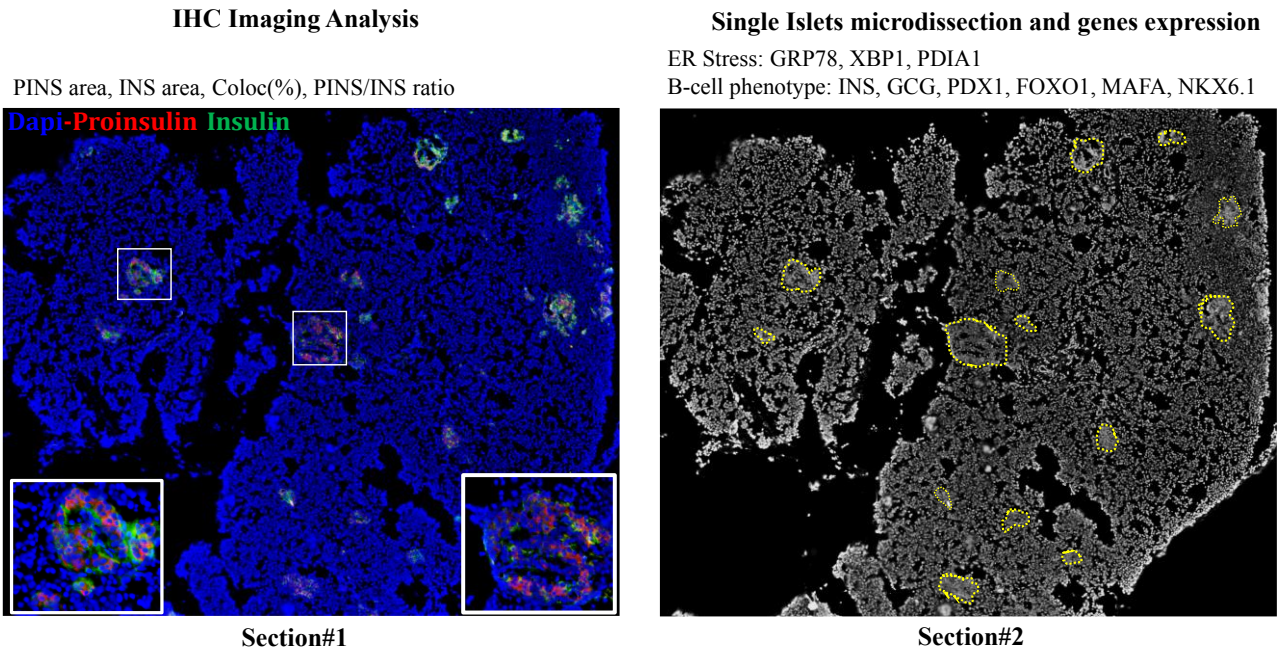


Figure 10. Experimental procedure of i*n-situ* characterization and molecular analysis of single pancreatic islets. In the first section (left) we performed an INS-PI staining to evaluate the *in-situ* expression levels of PI and INS and we mapped the single pancreatic islets. On the second serial section mounted on a slide for LCM (right), we isolated the same single islets previously mapped for gene expression analysis.

4. Results

4.1 Metabolic profile of patients analyzed

Oral glucose tolerance test (OGTT) was performed in all patients who underwent partial pancreatectomy in order to evaluate the concentration of glucose (mg/dl), insulin (µUI/ml) and C-peptide (ng/ml). Following this, the patients were classified into n=5 with normal glucose tolerance (NGT), n=9 with impaired glucose tolerance (IGT) and n=5 with Type 2 diabetes (T2D) (**Figure 11. a, b, c**).

Figure 11

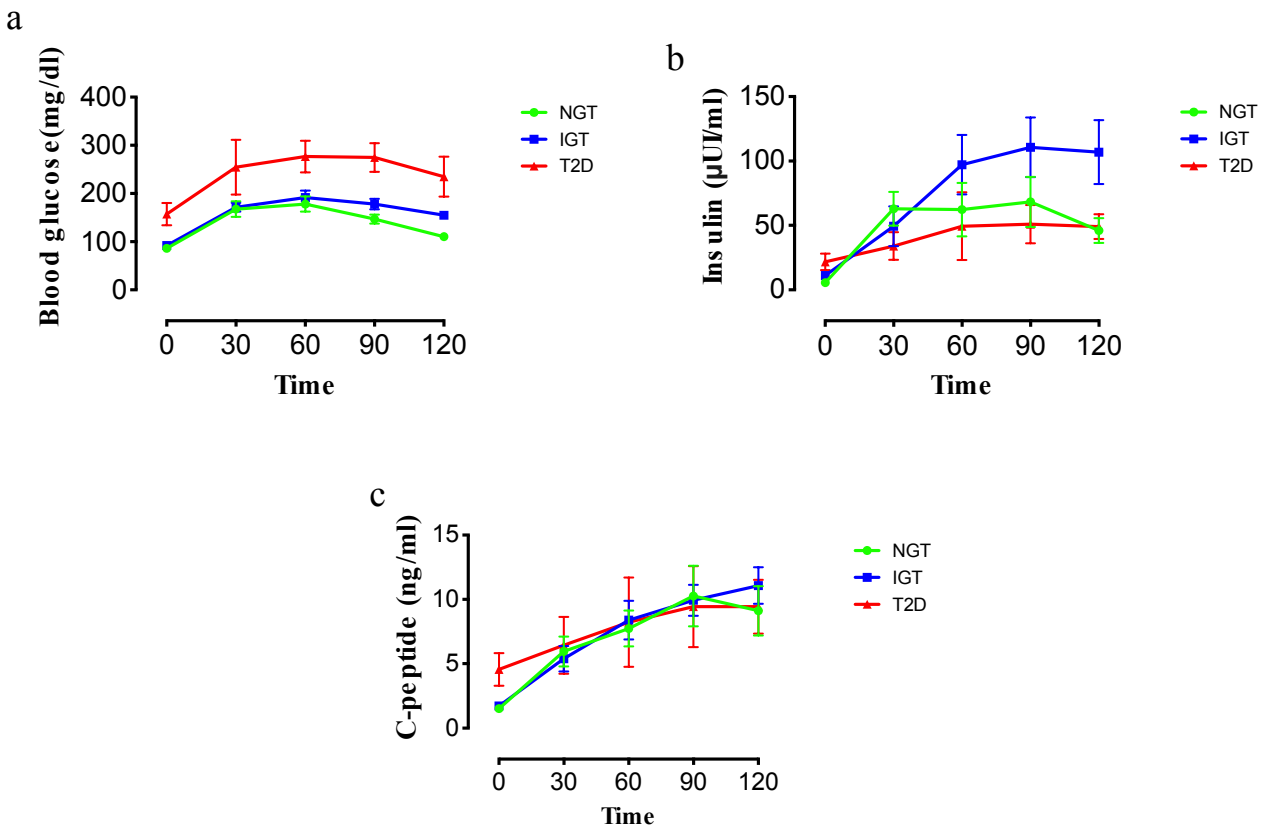


Figure 11. Metabolic profile of patients analyzed. Oral glucose tolerant test (OGTT) of n=19 patients analyzed. This figure shows the levels of blood glucose (mg/dl) (a), insulin (µUI/ml) (b) and c-peptide (ng/ml) (c) following OGTT.

4.2 Proinsulin-Insulin intracellular distribution in human pancreatic islets of Normal glucose tolerant (NGT), Impaired glucose tolerant (IGT) and Type 2 diabetic (T2D) patients

In order to explore the alteration that occur in PI and INS staining pattern in pancreatic islets, we performed an immunofluorescence analysis in n=19 patients on the waiting list for partial pancreatectomy, classified into n=5 NGT (normal glucose tolerance), n=9 IGT (impaired glucose tolerance) and n=5 T2D (type 2 diabetes) to detect the intracellular distribution of PI and INS on frozen pancreatic section. From image analysis, we observed that in most of the NGT pancreatic islets, the PI expression pattern was mainly distributed within a perinuclear compartment that was largely depleted of INS, while mature INS was more widely localized within the cytoplasm (**Figure 12a**; **Figure 13. a-l**). In IGT pancreatic islets, the intracellular distribution of PI is localized both in the perinuclear region and at the cytoplasmic level where INS resides (**Figure 12b**; **Figure 14. a-l**). Of interest, in the diabetic pancreatic islets most of the PI is localized in the cytoplasmic compartment by colocalizing with INS in the β cells (**Figure 12c**; **Figure 15. a-l**). These data suggest that following metabolic alterations, PI processing is aberrant, and part of PI accumulates in the cytoplasm without being converted into INS.

Figure 12

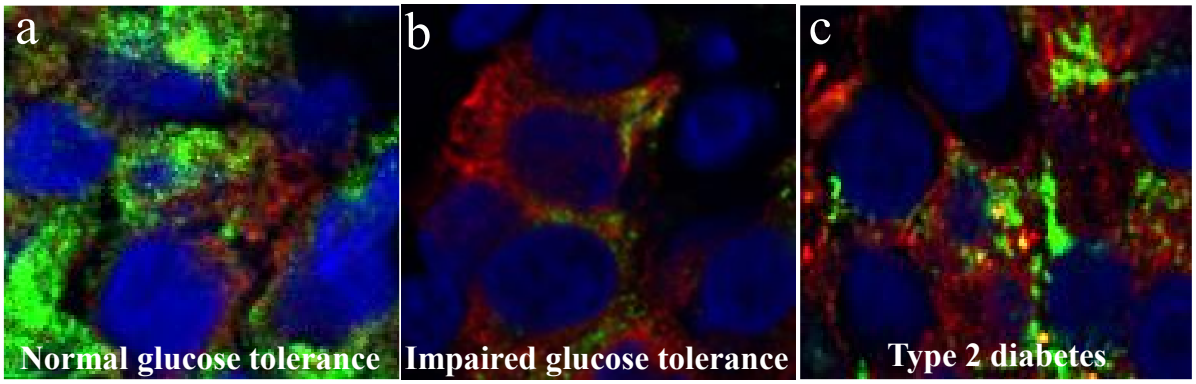


Figure 12: Proinsulin-Insulin intracellular distribution in NGT, IGT and T2D pancreatic islets. Figure shows the representative digital zoom of double immunofluorescence images related to the intracellular distribution of proinsulin (red) and insulin (green) in NGT (a), IGT (b) and T2D (c) pancreatic islets.

Figure 13

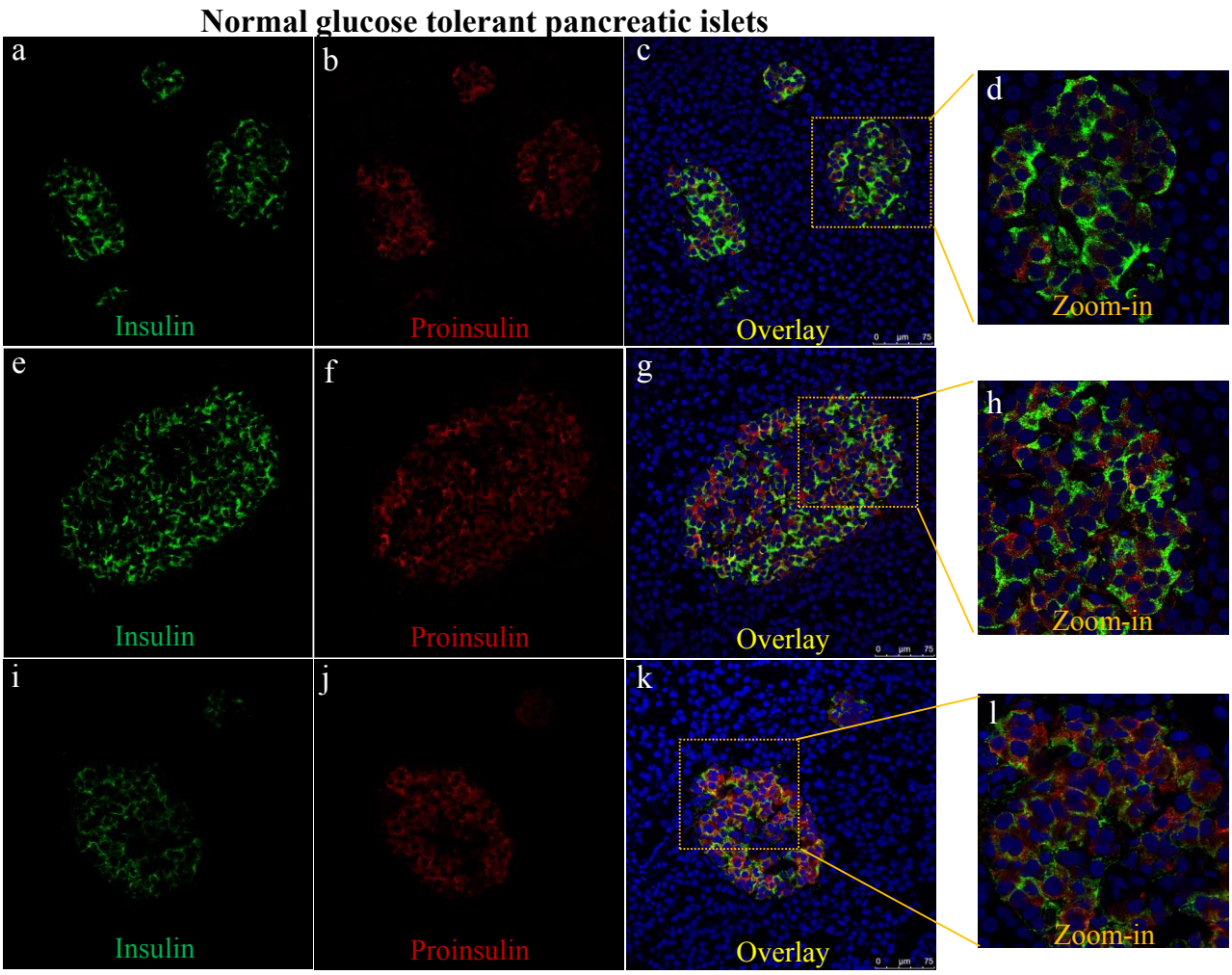


Figure 13: Proinsulin-Insulin intracellular distribution in Normal Glucose Tolerant pancreatic islets. Double immunofluorescence imaging showing the expression of INS (green) (a, e, i) using Dako-A0564 antibody, PI (red) (b, f, j) using DSHB GS9A8 antibody, and overlay channels (yellow) (c, g, k) in frozen pancreatic tissue sections of normal glucose tolerant. Scale bar 75 µm. Digital zoom-in overlay images are reported in panel -d, -h and -l.

Figure 14

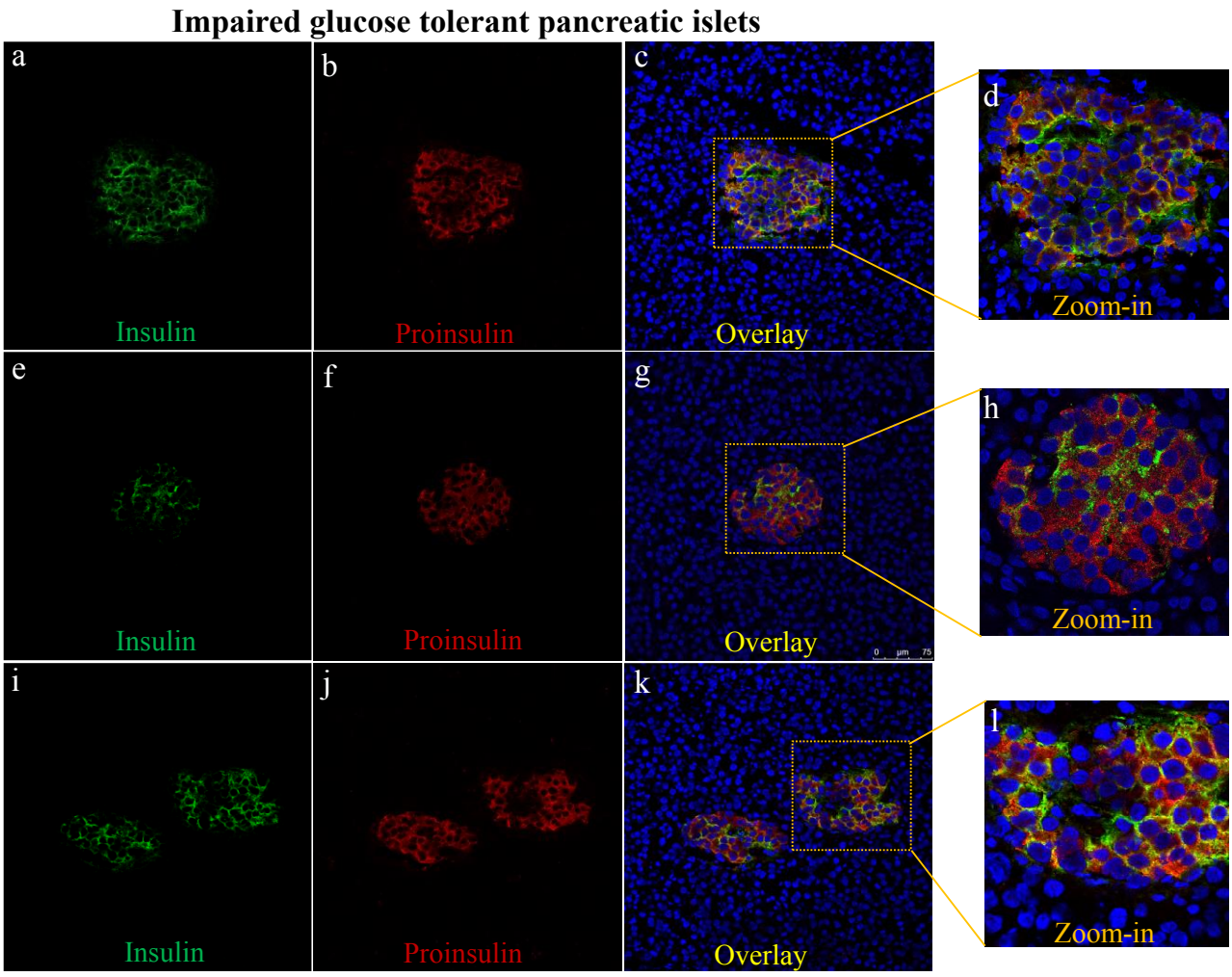


Figure 14: Proinsulin-Insulin intracellular distribution in Impaired Glucose Tolerant pancreatic islets. Double immunofluorescence imaging showing the expression of INS (green) (a, e, i) using Dako-A0564 antibody, PI (red) (b, f, j) using DSHB GS9A8 antibody, and overlay channels (yellow) (c, g, k) in frozen pancreatic tissue sections of impaired glucose tolerant. Scale bar 75 µm. Digital zoom-in overlay images are reported in panel -d, -h and -l.

Figure 15

Type 2 diabetic pancreatic islets

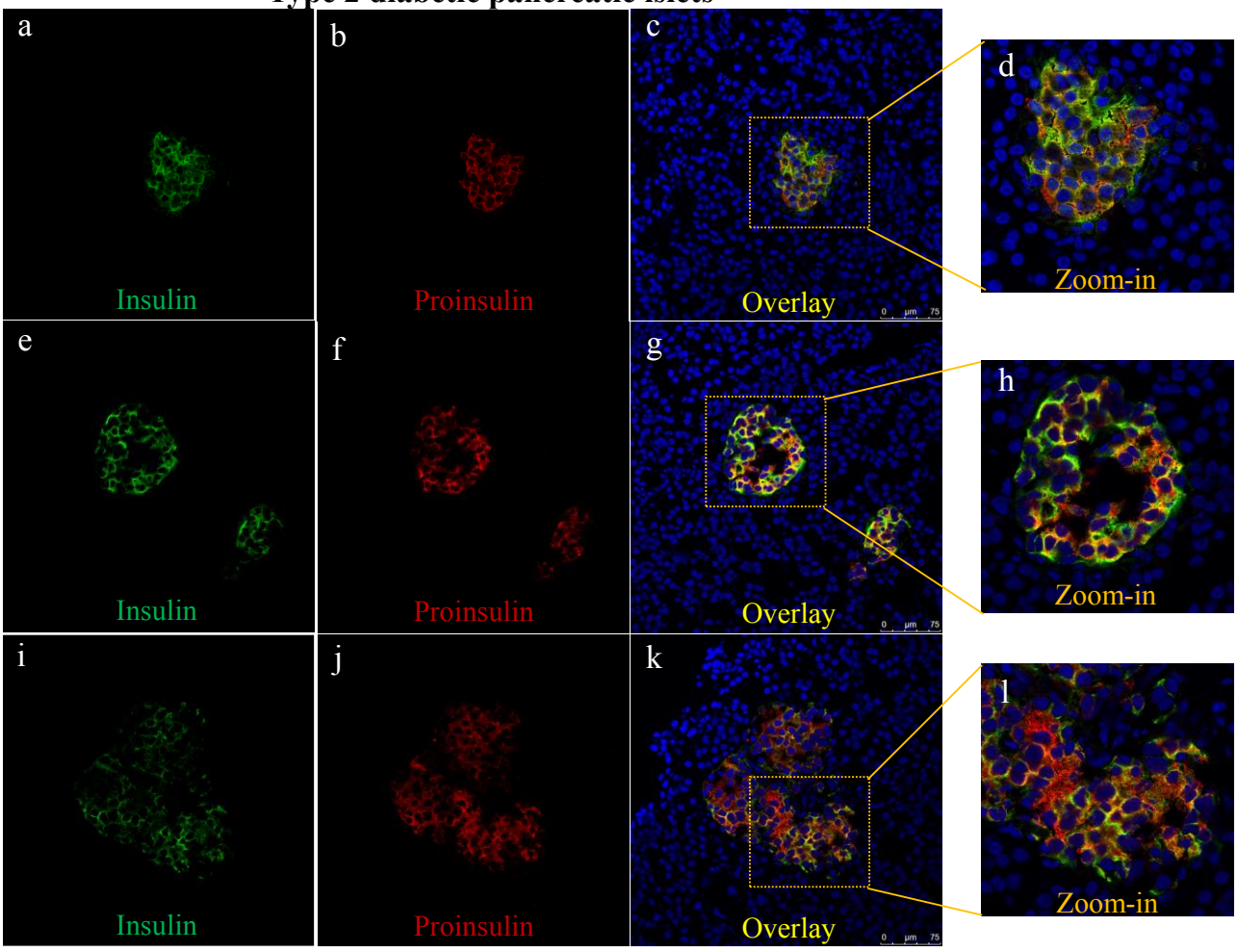


Figure 15: Proinsulin-Insulin intracellular distribution in Type 2 Diabetic pancreatic islets. Double immunofluorescence imaging showing the expression of INS (green) (a, e, i) using Dako-A0564 antibody, PI (red) (b, f, j) using DSHB GS9A8 antibody, and overlay channels (yellow) (c, g, k) in frozen pancreatic tissue sections of type 2 diabetic. Scale bar 75 µm. Digital zoom-in overlay images are reported in panel -d, -h and -l.

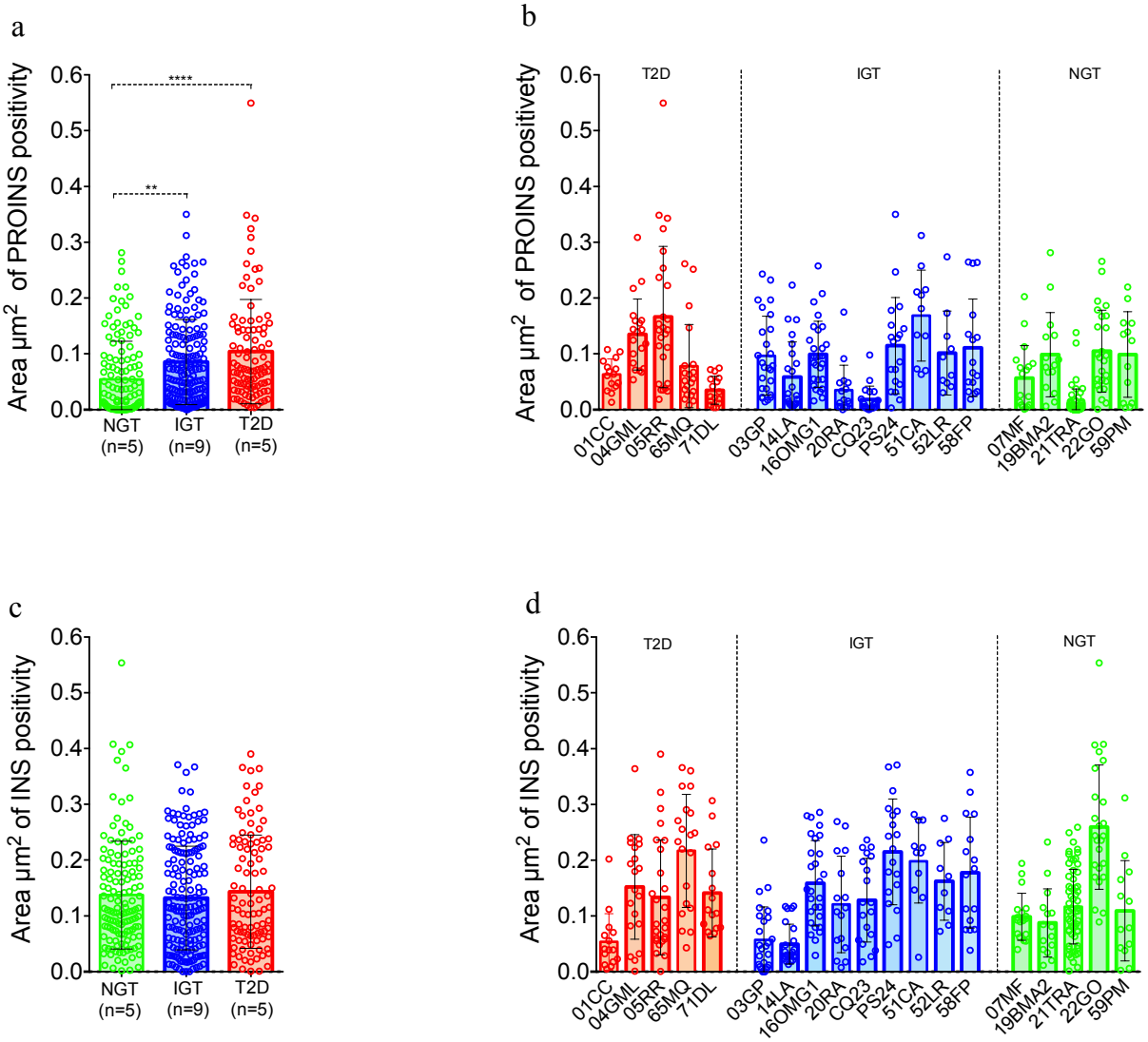
4.3 Proinsulin-Insulin colocalization, area (µm^2) of proinsulin positivity and Proinsulin-Insulin ratio gradually increases from NGT to IGT and T2D pancreatic islets

In order to analyse the intracellular distribution of PI-INS staining pattern, we performed an image analysis using Volocity software on each single islet of all cases examined, specifically we analysed n=131 islets from five NGT patients; n=168 from nine IGT patients and n=97 islets

from five T2D patients. Image analysis showed that PI-INS colocalization gradually increases from NGT to IGT and T2D pancreatic islets ($p<0.0001$) (**Figure 16. e-f**), indicating an alteration of PI processing due to the localization of PI and INS in the same compartment. Moreover, in the same islets we measured the PI positivity area (μm^2) and observed that the PI positivity was progressively increased from NGT to T2D pancreatic islets ($p<0.0001$) (**Figure 16. a-b**). We also evaluated the ratio of PI/INS in the same islets normalizing both intensity values of the two channels for the total area of the islet, thus demonstrating that PI/INS ratio was significantly increased in T2D respect to NGT pancreatic islets (p: 0.03) (**Figure 16. g-h**). We did not observe any statistically significant difference in the area of INS positivity in the three groups of pancreatic islets analyzed (**Figure 16. c-d**). Detailed values are reported in **Table 3**.

Collectively these results suggest that the increased *in-situ* of PI/INS ratio is related to an ongoing metabolic alteration probably due to the release of immature granules with a higher relative content of PI and its conversion intermediates, in line with previously demonstrated for *in-vivo* circulating levels.

Figure 16


a
b
c
d
Area µm2 of PROINS positivity
Area µm2 of PROINS positivety
Area µm2 of INS positivity
0.6
0.5
0.4
0.3
0.2
0.1
0.0
****
**
NGT
(n=5)
IGT
(n=9)
T2D
(n=5)
T2D
IGT
NGT
01CC
04GML
05RR
65MQ
71DL
03GP
14LA
16OMG1
20RA
CQ23
PS24
51CA
52LR
58FP
07MF
19BMA2
21TRA
22GO
59PM

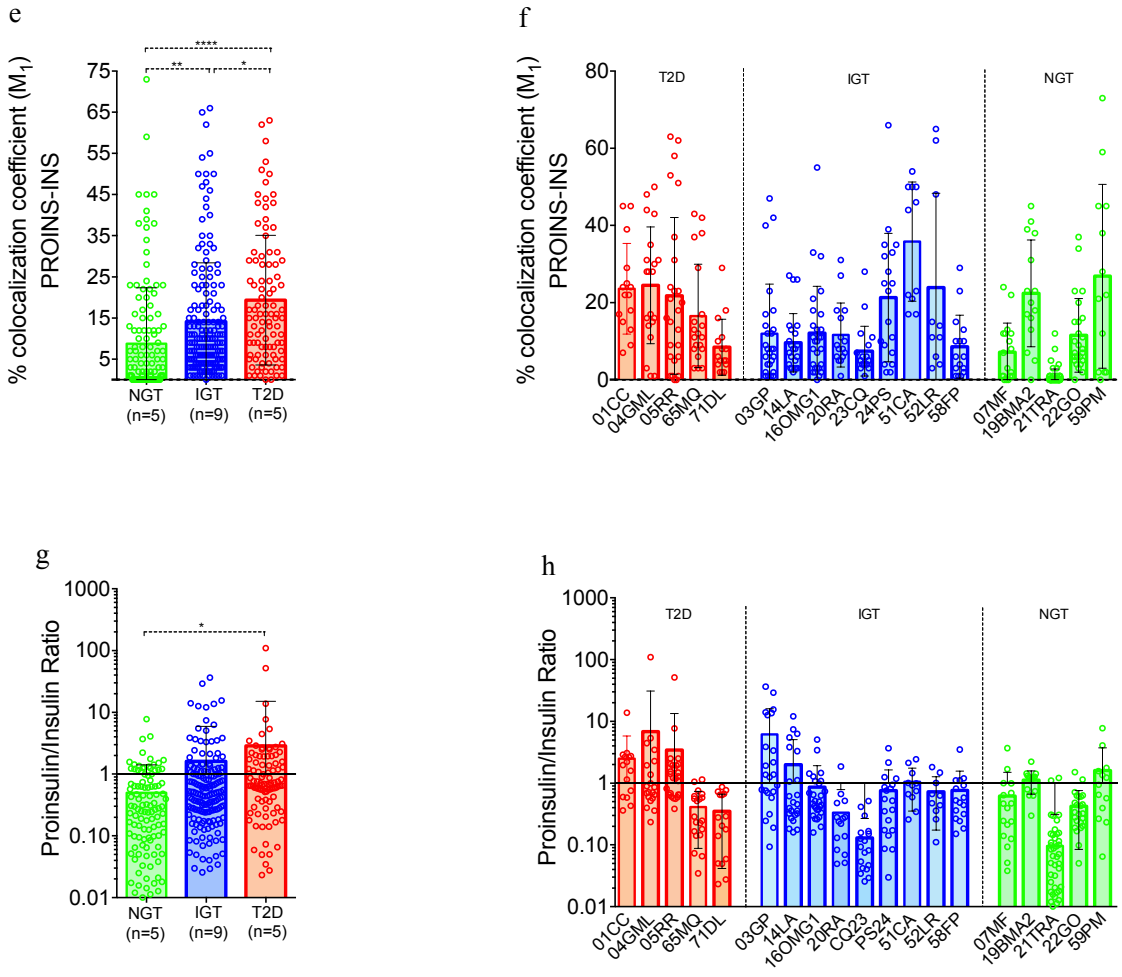


Figure 16. Percentage of colocalization coefficient PROIN-INS, area of PROINS positivity and PROINS-INS ratio are increased in IGT and T2D pancreatic islets. Dot plot graphs showing the area (µm^2) of PI positivity (a), the PI/INS ratio (g) in n=5 NGT, n=9 IGT and n=5 T2D pancreatic islets. Dot plot graphs showing the area (µm^2) of PI positivity (b), the area (µm^2) of INS positivity (d), the % of colocalization coefficient (M_1) INS-PI (f) and PI/INS ratio (h) in all cases analysed. * p-values ($p<0.01$) were obtained using One-way Anova and Tukey's multiple comparisons post-test.

Table 3

CASE ID	TYPE	% COLOC INS-PI	AREA INS	AREA PROINS	PROINS-INS RATIO
07MF	NGT	7,167	0,09864	0,05676	0,6206
19BMA2	NGT	22,4	0,08757	0,09863	1,119
21TRA	NGT	0,7667	0,1168	0,01173	0,09602
22GO	NGT	11,52	0,2593	0,1048	0,4218
59PM	NGT	28,85	0,1093	0,0988	1,606
03GP	IGT	11,96	0,05732	0,09655	6,182

14LA	IGT	9,609	0,04949	0,05823	1,997
160MG1	IGT	12,11	0,1588	0,09925	0,8648
20RA	IGT	11,63	0,1209	0,03507	0,3341
23CQ	IGT	7,421	0,1284	0,01816	0,1299
24PS	IGT	21,32	0,2151	0,1147	0,7556
51CA	IGT	34,17	0,1985	0,1686	1,05
52LR	IGT	23,9	0,1622	0,1015	0,7235
58FP	IGT	8,563	0,1776	0,1107	0,7638
01CC	T2D	23,6	0,05358	0,06312	2,495
04GML	T2D	24,5	0,1522	0,1348	6,842
05RR	T2D	21,77	0,1335	0,1659	3,447
65MQ	T2D	16,55	0,2168	0,07819	0,4117
71DL	T2D	8,438	0,1413	0,03473	0,352

Table 3. Image analysis. % of colocalization coefficient proinsulin-insulin, area of insulin positivity, area of proinsulin positivity and proinsulin-insulin ratio are reported as mean value among all the pancreatic islets analysed.

4.4 Increased Proinsulin-Insulin ratio is related to the loss of glucose tolerance and impaired beta cell function

After pancreatic islets image analysis, we sought to determine whether pancreatic *in-situ* staining measurements were associated with clinical parameters, in order to link *in-situ* defects to *in-vivo* metabolic alterations.

We observed that *in-situ* PI/INS ratio was positively correlated with basal glucose (mg/dl) (r: 0.47, p: 0.05) (**Figure 17a**), basal insulin (μUI/ml) (r: 0.65, p: 0.0032) (**Figure 17b**) and 2h glucose levels following OGTT (mg/dl) (r: 0.75, p: 0.0008) (**Figure 17c**). Furthermore, we also found that the increased *in-situ* levels of PI-INS ratio are not significantly correlated with an increase of the percentage of PI-INS colocalization. As a matter of fact, we observed several islets characterized by elevated PROINS-INS ratio but low levels of INS-PI colocalization. Interestingly, we report for the first time that the increase of PI/INS ratio in pancreatic islets of patients analyzed is associated with reduced β cell function (r: -0.64, p-value: 0.02) (**Figure 17d**). Collectively, these results highlight that the alteration of PI-INS staining pattern during metabolic alteration might mirror *in-vivo* the increased INS secretion rate and β cell function reduction in the same patients.

Figure 17

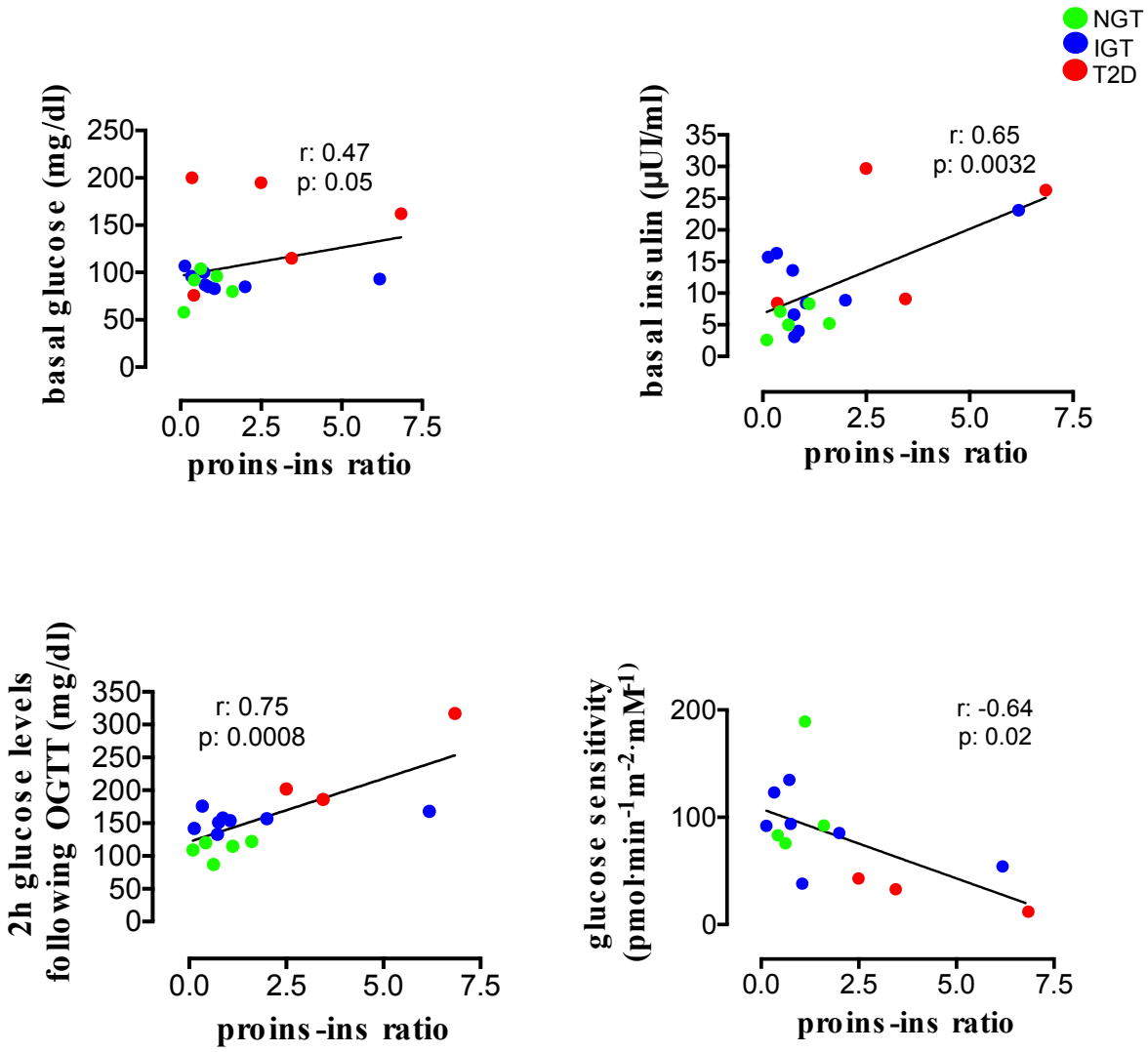


Figure 17. Increased Proinsulin-Insulin ratio is related to the loss of glucose tolerance and impaired beta cell function. Correlation analysis between *in-situ* PI-INS ratio with basal glucose (mg/dl) (r: 0.47, p-value: 0.05) (a), basal insulin (µUI/ml) (r: 0.65, p-value: 0.0032) (b), glucose levels 2-hours following OGTT (mg/dl) (r: 0.75, p-value: 0.0008) (c) and glucose sensitivity ($pmol \cdot min^{-1}m^{-2} \cdot mM^{-1}$) (r: -0.64, p-value: 0.02) (d). Green circles are indicated the NGT patients; blue circles are shown the IGT patients and red circles are indicated the T2D patients. P-value and r-value were obtained using Pearson correlation test.

4.5 GRP78, XBP1 and PDIA1 genes involved in unfolded protein response (UPR) are upregulated in LCM pancreatic islets of patients with impaired glucose tolerance and type 2 diabetes

Subsequently, in order to uncover the molecular mechanisms underlying the failure of β cells associated to the intracellular alteration of PI and INS during metabolic stress, we analyzed the expression of a panel of genes associated with ER stress, PI processing, unfolded protein response (UPR) and the maintenance of β cell phenotype (**Table 2**) in pancreatic islets isolated by laser capture microdissection (LCM). We selected the RNA samples extracted from islets pools (composed of n=50-60 pancreatic islets/donor)) of the patients analyzed *in-situ* on the basis of RNA integrity number (RIN), considering the samples with a RIN>5. In total, we analyzed pancreatic islets from n=4 NGT, n=7 IGT and n=4 T2D RNA samples. Genes expression analysis, revealed that several genes, involved in the activation of unfolded protein response (UPR), were upregulated in LCM pancreatic islets of IGT and T2D patients (**Figure 18a**). Of relevance, we observed that the expression levels of PDIA1 (protein disulfide isomerase), also known as propyl 4-hydroxylase (P4HB), and XBP1 (splicing of x-box binding protein) were upregulated in T2D pancreatic islets compared to NGT (p: 0.02). Moreover, the expression of GRP78 (glucose regulated protein 78) was increased in T2D pancreatic islets respect to IGT and NGT pancreatic islets (p: 0.004).

Moreover, we did not observe any statistically significant difference regarding the expression of genes associated with the maintenance of the β cell phenotype (**Figure 18b**), probably because, as already mentioned above, by analyzing pool of pancreatic islets belonging to different donors, given the elevated heterogeneity, a lot of information and molecular differences are not detected.

Figure 18

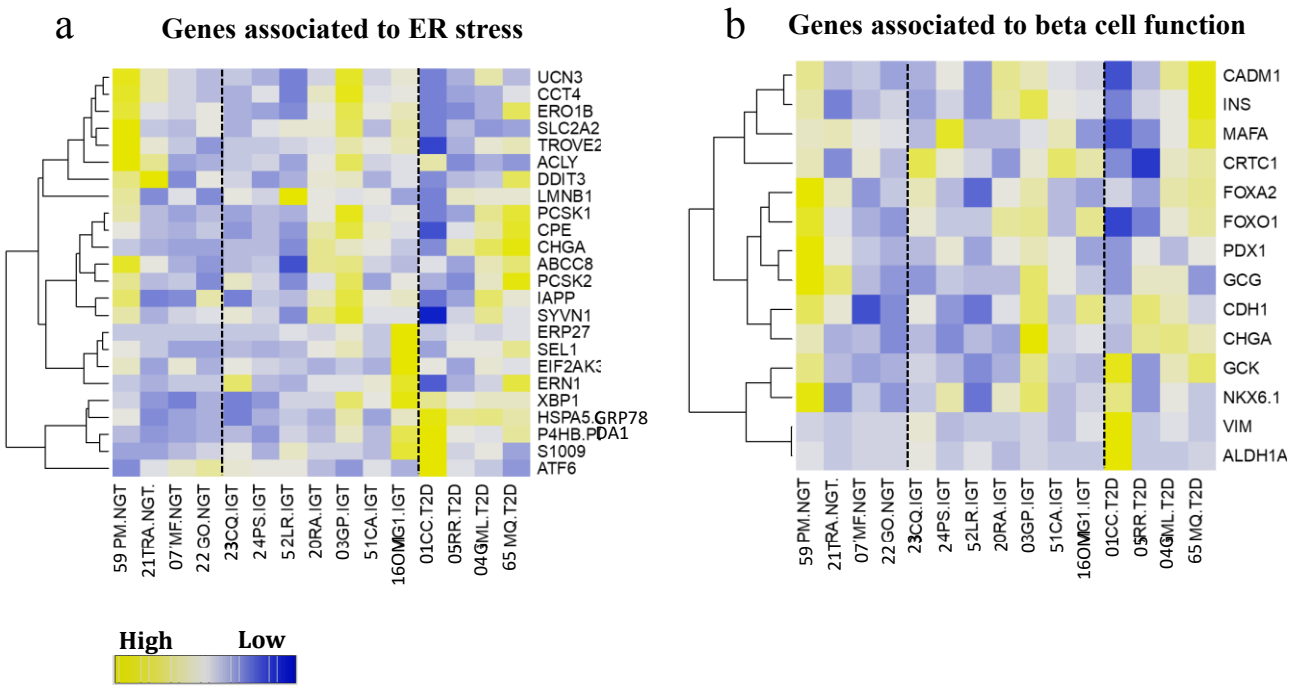


Figure 18. Hierarchical Heatmap clustering analysis based on the expression of genes associated to ER stress and beta cell phenotype. Hierarchical clustering and heatmap based on the relative expression (2^-DCT) of genes associated to ER stress (a) and β cell function (b) in pancreatic islets of n=4 NGT, n=7 IGT and n=4 T2D isolated trough LCM. Color key indicates the relative expression values from the less expressed (light yellow) to the most expressed (dark blue).

Figure 19

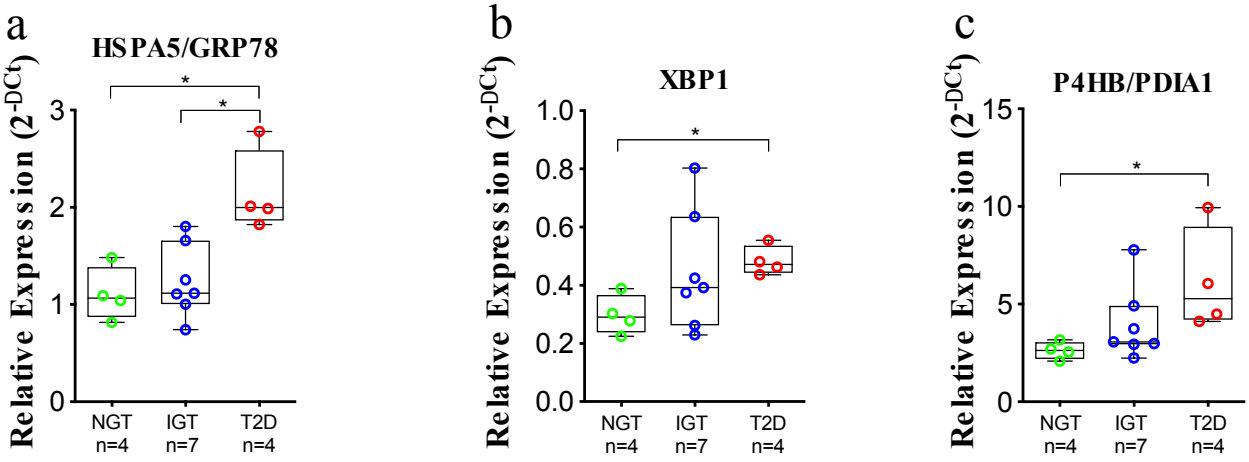


Figure 19. GRP78, XBP1 and PDIA1 are upregulated in LCM pancreatic islets of IGT and T2D patients. Box plot graphs show the expression of mRNAs HSPA5/GRP78, XBP1 and P4HB/PDIA1 evaluated through RT-Real-Time PCR in n=4 NGT, n=7 IGT and n=4 T2D pancreatic islets isolated with LCM. Statistically significant has been evaluated by Kruskal Wallis multiple comparison test *p<0.05.

4.6 The UPR response genes GRP78 and PDIA1 correlate with the increase of PI-INS ratio and colocalization of PI-INS, with the loss of glucose tolerance and β cell function

The expression of genes involved in the activation of unfolded protein response (UPR) were correlated to the clinical metabolic parameters and *in-situ* staining measurements of patients analysed in order to understand the association between ER stress and β cell dysfunctional defects observed in impaired glucose tolerant and type 2 diabetic pancreatic islets. The correlation analysis showed that GRP78 expression is positively correlated with *in-situ* PI/INS ratio (r: 0.6, p: 0.01) (**Figure 20a**) and colocalization of INS-PI (r: 0.5, p: 0.05) (**Figure 20b**) as well as basal glucose (mg/dl) (r: 0.8, p: 0.0007) (**Figure 20d**) and insulin (pmol/L) (r: 0.76, p: 0.002) (**Figure 20e**) and also at 2 hours glucose levels following oral glucose tolerance test (OGTT) (r: 0.6, p: 0.02) (**Figure 20f**). Moreover, we observed an inverse correlation between GRP78 expression and glucose sensitivity ($pmol \cdot min^{-1}m^{-2} \cdot mM^{-1}$) (r: -0.6, p: 0.04) (**Figure 20c**). The levels of PDIA1 expression are positively correlated with the percentage of colocalization

coefficient PI-INS (r: 0.5, p: 0.09) (**Figure 20g**) as well as basal glucose (mg/dl) (r: 0.8, p: 0.005) (**Figure 20h**) and insulin (pmol/L) (r: 0.76, p: 0.002) (**Figure 20i**) and also at 2 hours glucose levels following oral glucose tolerant test (OGTT) (r: 0.7, p: 0.009) (**Figure 20j**). While we found a trend in inverse correlation between PDIA1 expression with glucose sensitivity ($pmol \cdot min^{-1} m^{-2} \cdot mM^{-1}$) (r: -0.4, p: 0.1) (**Figure 20k**). These results suggest that the activation of UPR genes as an adaptive mechanism, starts from the prediabetic phase and is linked to defect of PI folding, thus determining the deterioration of glucose tolerance and β cell function.

Figure 20

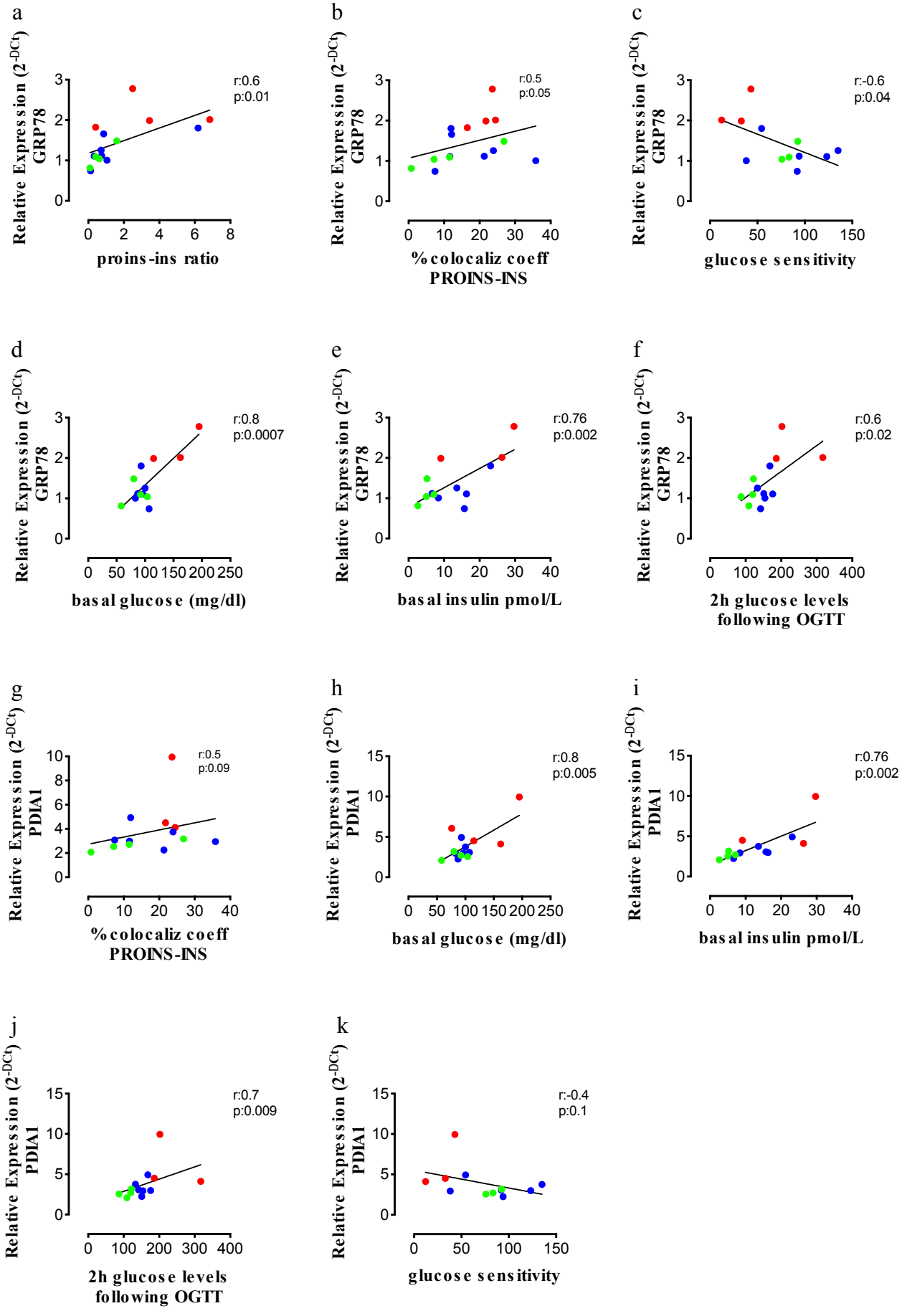

a
Relative Expression (2-DCt) GRP78
r:0.6
p:0.01
proins-ins ratio
b
Relative Expression (2-DCt) GRP78
r:0.5
p:0.05
%colocaliz coeff PROINS-INS
c
Relative Expression (2-DCt) GRP78
r:-0.6
p:0.04
glucose sensitivity
d
Relative Expression (2-DCt) GRP78
r:0.8
p:0.0007
basal glucose (mg/dl)
e
Relative Expression (2-DCt) GRP78
r:0.76
p:0.002
basal insulin pmol/L
f
Relative Expression (2-DCt) GRP78
r:0.6
p:0.02
2h glucose levels following OGTT
gg
Relative Expression (2-DCt) PDIA1
r:0.5
p:0.09
%colocaliz coeff PROINS-INS
h
Relative Expression (2-DCt) PDIA1
r:0.8
p:0.005
basal glucose (mg/dl)
i
Relative Expression (2-DCt) PDIA1
r:0.76
p:0.002
basal insulin pmol/L
j
Relative Expression (2-DCt) PDIA1
r:0.7
p:0.009
2h glucose levels following OGTT
k
Relative Expression (2-DCt) PDIA1
r:-0.4
p:0.1
glucose sensitivity

Figure 20. The expression of GRP78 and PDIA1 correlate with the increase of PI-INS ratio and colocalization of PI-INS, with the loss of glucose tolerance and β cell function. Correlation analysis between the relative expression of GRP78 with *in-situ* PI-INS ratio (r: 0.6, p: 0.01) (a), % of colocalization coefficient INS-PI (r: 0.5, p: 0.05) (b), glucose sensitivity (pmol·min^{-1}m^{-2}·mM^{-1}) (r: -0.6, p: 0.04) (c), basal glucose (mg/dl) (r: 0.8, p: 0.0007) (d), basal insulin (μUI/ml) (r: 0.76, p: 0.002) (e) and glucose levels 2-hours following OGTT (mg/dl) (r: 0.6, p: 0.02) (f) in pancreatic islets of NGT, IGT and T2D patients. Correlation analysis between the relative expression of PDIA1 with % of colocalization coefficient INS-PI (r: 0.5, p: 0.09) (g), basal glucose (mg/dl) (r: 0.8, p: 0.005) (h), basal insulin (μUI/ml) (r: 0.76, p: 0.002) (i), glucose levels 2-hours following OGTT (mg/dl) (r: 0.7, p: 0.009) (j) and glucose sensitivity (pmol·min^{-1}m^{-2}·mM^{-1}) (k) in pancreatic islets of NGT, IGT and T2D patients. Green circles are indicated the NGT patients; blue circles are shown the IGT patients and red circles are indicated the T2D patients. P-value and r-value were obtained using Pearson and Spearman correlation test.

4.7 Single islets phenotyping reveals a direct association between proinsulin processing, ER stress and loss of beta cell identity during metabolic alterations

Given the heterogeneity among pancreatic islets of different donors and of the same donor, even if from a pathophysiological point of view it is important to understand globally what is the molecular alteration that causes an erroneous processing of PI leading to endoplasmic reticulum stress that results in metabolic alterations, much information is lost because each pancreatic islet is different of other and can be more or less stressed and dysfunctional at the level of morphology and expression of genes associated with the maintenance of β cell identity.

In light of this, we carried out a phenotypic characterization of individual pancreatic islets in the same donors in which we carried out the global *in-situ* and molecular analysis (**Figure 24**). In order to uncover a direct link between PI intracellular alteration, ER stress and β cell identity, we analysed single pancreatic islets of n=3 NGT (cases: 21TRA, 59PM, 07MF), n=3 IGT (cases: 03GP, 24PS, 20RA) and n=3 T2D (cases: 05RR, 65MQ, 04GML).

Firstly, we performed an INS-PI immunofluorescence staining analysis on the first pancreatic frozen section; then, we evaluated the image analysis parameters as previously described and mapped each single pancreatic islet from each donor in order to perform the laser capture microdissection of the same islet on the subsequent serial section.

The image analysis confirmed that the INS-PI colocalization, PI positivity area (μm^2) and PI/INS ratio were increased in single pancreatic islets of IGT and T2D respect to NGT donors

($p<0.01$) (**Figure 22 a-c**). As previously observed from global *in-situ* analysis, also in single islets phenotyping we found that the increased *in-situ* levels of PI-INS ratio are not significantly correlated with an increase of the percentage of PI-INS colocalization. In effect, we observed several pancreatic islets characterized by elevated PI/INS ratio but low levels of INS-PI colocalization.

In the subsequent serial section, we LCM-isolated single pancreatic islets, previously histologically characterized, in order to perform a molecular analysis to evaluate the expression of n=3 genes associated to ER stress (GRP78, PDIA1, XBP1) and n=4 genes associated beta cell function (NKX6.1, MAFA, PDX1, FOXO1) (see Table 2 for details on Taqman primers used to evaluate gene expression in LCM pancreatic islets)

Using this single islet characterization approach, we observed a quite high heterogeneity also among the islets of the same donor suggesting that each single pancreatic islet was characterized by a unique phenotype and molecular profile. Of interest, heterogeneity among the pancreatic islets was gradually increasing from NGT to IGT and T2D islets. In the three groups of single pancreatic islets analyzed, we also observe that there are some control islets with a phenotype and molecular profile similar to IGT and T2D islets, there are several IGT and T2D pancreatic islets more stressed and dedifferentiated, other still characterized by a mature β cell phenotype. Specifically, from hierarchical clustering heatmap analysis we observed that most of NGT single pancreatic islets of n=3 cases were characterized by a low percentage of colocalization INS-PROINS, low PI-INS ratio and a reduced area of PI positivity **(Figure 21; Figure 22 a-c)**. Moreover, NGT pancreatic islets had reduced expression levels of UPR genes such as GRP78, XBP1 and PDIA1 and elevated levels of genes associated with the maintenance of β cell phenotype **(Figure 21; Figure 23 a-g)**. In single prediabetic pancreatic islets of IGT cases, we observed an increase of INS-PI colocalization as well as PI/INS ratio and area of PI positivity **(Figure 21; Figure 22 a-c)** and an increase of GRP78, PDIA1 and XBP1 expression **(Figure 21; Figure 23 a-g)**. Interestingly, in IGT single pancreatic islets analysed the expression of FOXO1, in particular in 03GP and 20RA cases, was overexpressed as compensatory role in response to metabolic stress to preserve β-cell function **(Figure 21; Figure 23e)**. The expression of PDX1, NKX6.1 and MAFA genes was reduced in single IGT pancreatic islets compared to the NGT islets **(Figure 21; Figure 23 a-g)**. We analysed single pancreatic islets from n=2 recent onset and n=1 long standing T2D cases. The pancreatic islets of T2D cases at onset were characterized by elevated expression of GRP78, XBP1 and PDIA1, and by increased INS-PI colocalization, PI/INS ratio and area of PI positivity. Interestingly, we observed low levels of gene expression associated to the maintenance of the β cell phenotype **(Figure 21;**

Figure 22 a-c; Figure 23 a-g). While, in the only T2D case analysed with a long disease duration, the expression of genes associated to UPR was reduced respect to recent onset pancreatic islets **(Figure 21; Figure 23 a-c)**. Some pancreatic islets characterized by reduced expression of genes such as NXK6.1, MAFA and PDX1, could undergo dedifferentiation as a protective mechanism against β cell death. On the other hand, in pancreatic islets with a long disease duration, the expression of UPR genes is reduced, probably because the β cells undergo a phase of exhaustion due to chronic ER stress which could activate molecular pathways that lead to β cell death because ER homeostasis is not restored, and proteins such as PI not folded correctly undergoing degradation.

Figure 21

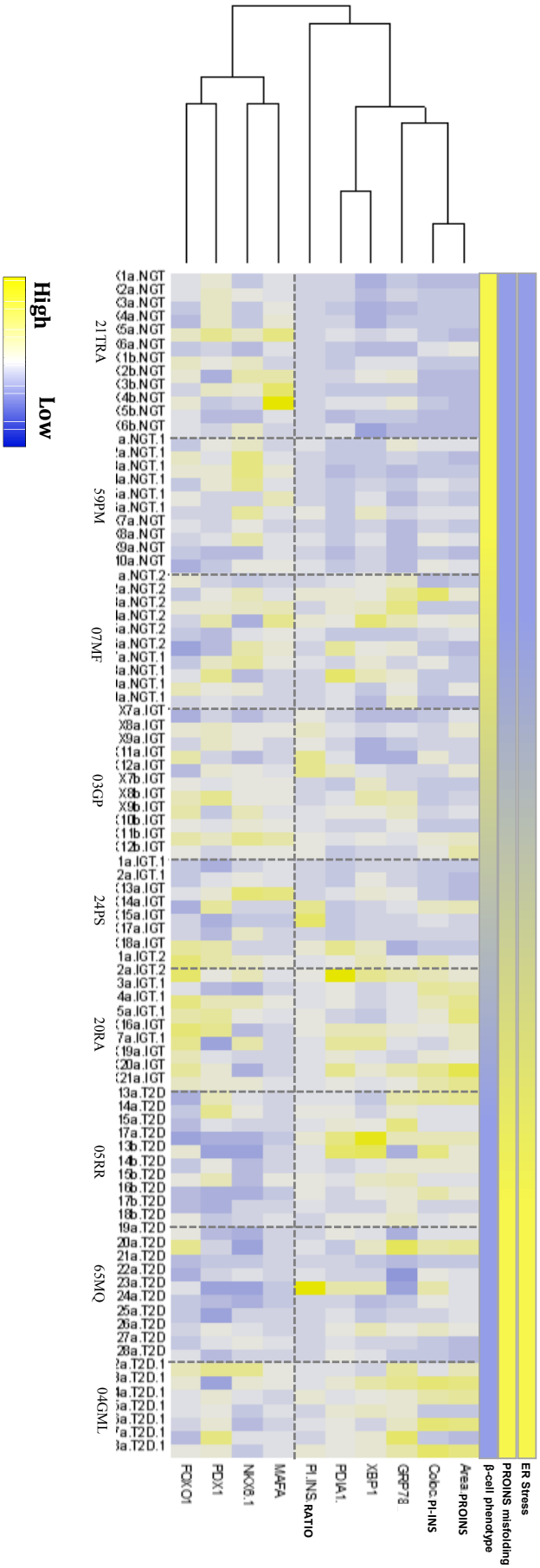

High
Low
21TRA
59PM
07MF
03GP
24PS
20RA
05RR
65MQ
04GML
FOXO1
PDX1
NKX6.1
MAFA
PI.INS.RATIO
PDIA1.
XBP1
GRP78
Cobic.PI-INS
Area.PROINS
β-cell phenotype
PROINS misfolding
ER Stress

Figure 21: Hierarchical clustering and heat map based on the expression of genes associated to ER stress, beta cell phenotype and *in-situ* parameters associated to intracellular alteration of proinsulin in single pancreatic islets. Hierarchical clustering and heatmap based on the relative expression (2^-DCT) of genes associated to ER stress, β cell function and *in-situ* parameters associated to intracellular alteration of PI in single pancreatic islets of n=3 NGT (21TRA, 59PM, 07MG), n=3 IGT (03GP, 24PS, 20RA) and n=3 T2D (05RR, 65MQ, 04GML) isolated trough LCM. Color key indicates the relative expression values from the less expressed (light yellow) to the most expressed (dark blue).

Figure 22

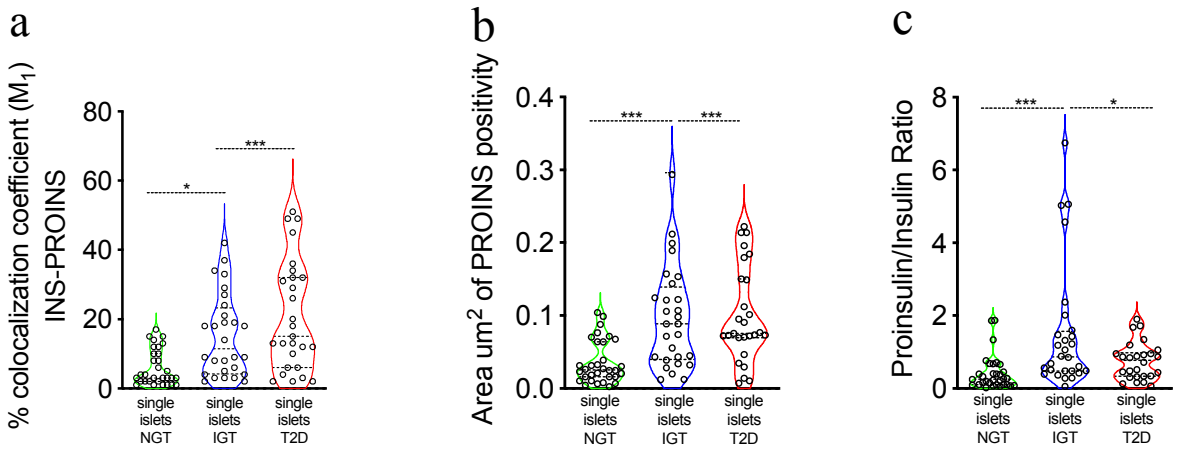


Figure 22. Proinsulin-Insulin colocalization, area (µm^2) of proinsulin positivity and Proinsulin-Insulin ratio was increased in single pancreatic islets of IGT and T2D respect to NGT donors. Violin plot showing the % of colocalization coefficient (M_1) INS-PI (a) the area (µm^2) of PI positivity (b) and PI/INS ratio (c) in n=32 single islets NGT, n=29 single islets IGT and n=28 single islets T2D evaluated trough Volocity software. * p-values ($p<0.05$) were obtained using One-way Anova and Tukey's multiple comparisons post-test.

Figure 23

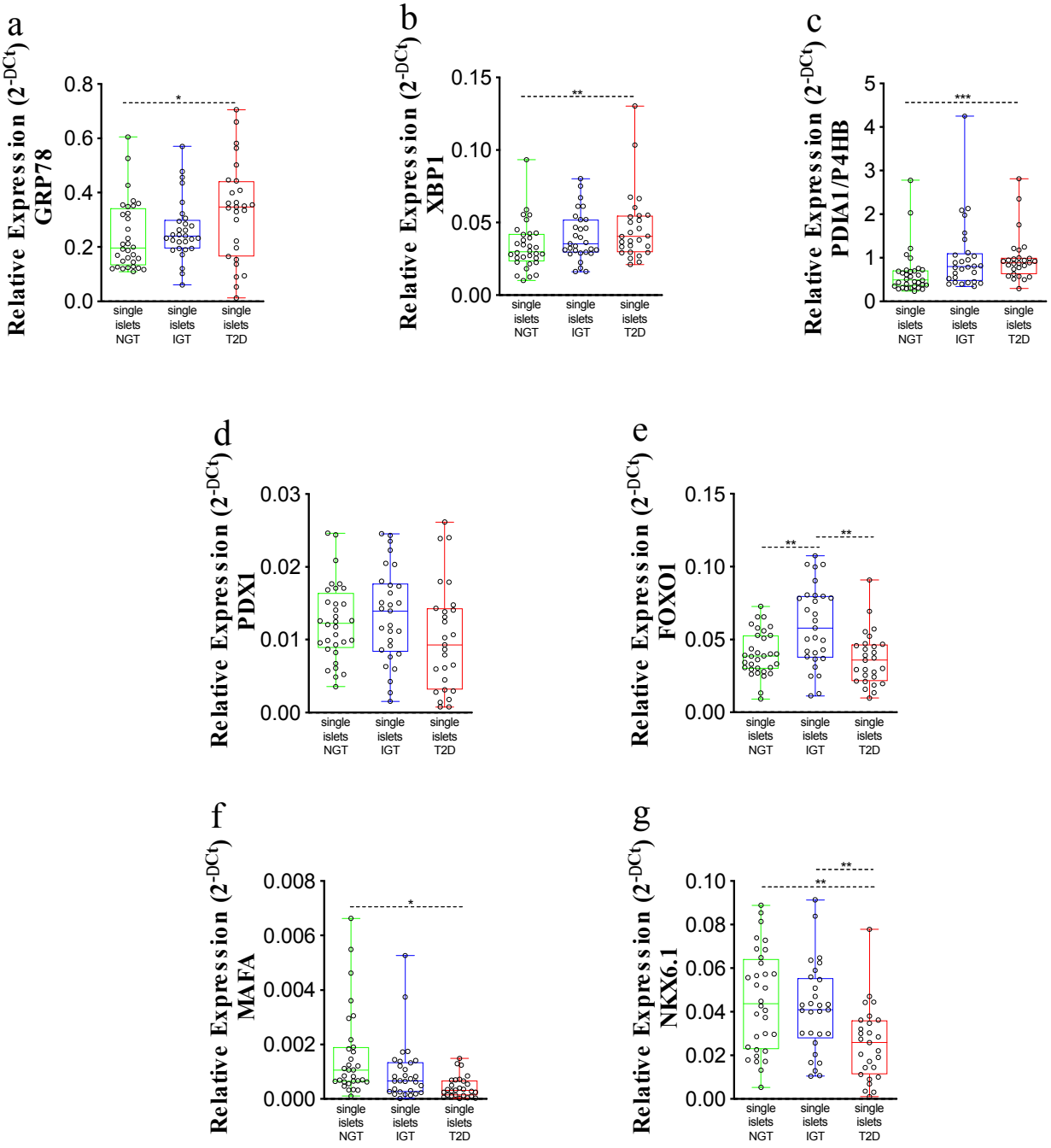


Figure 23. Single pancreatic islets of T2D patients are characterized from an upregulation of GRP78, PDIA1 and XBP1 and reduced expression levels of PDX1, FOXO1, MAFA and NKX6.1. Box plot graphs show the expression of mRNAs HSPA5/GRP78 (a), XBP1 (b), P4HB/PDIA1 (c), PDX1 (d), FOXO1 (e), MAFA (f) and NKX6.1 (g) evaluated through RT-Real-Time PCR in n=32 single islets NGT, n=29 single islets IGT and n=28 single islets T2D isolated with LCM. Statistically significant has been evaluated by Kruskal Wallis multiple comparison and Mann Whitney test *p<0.05.

Figure 24

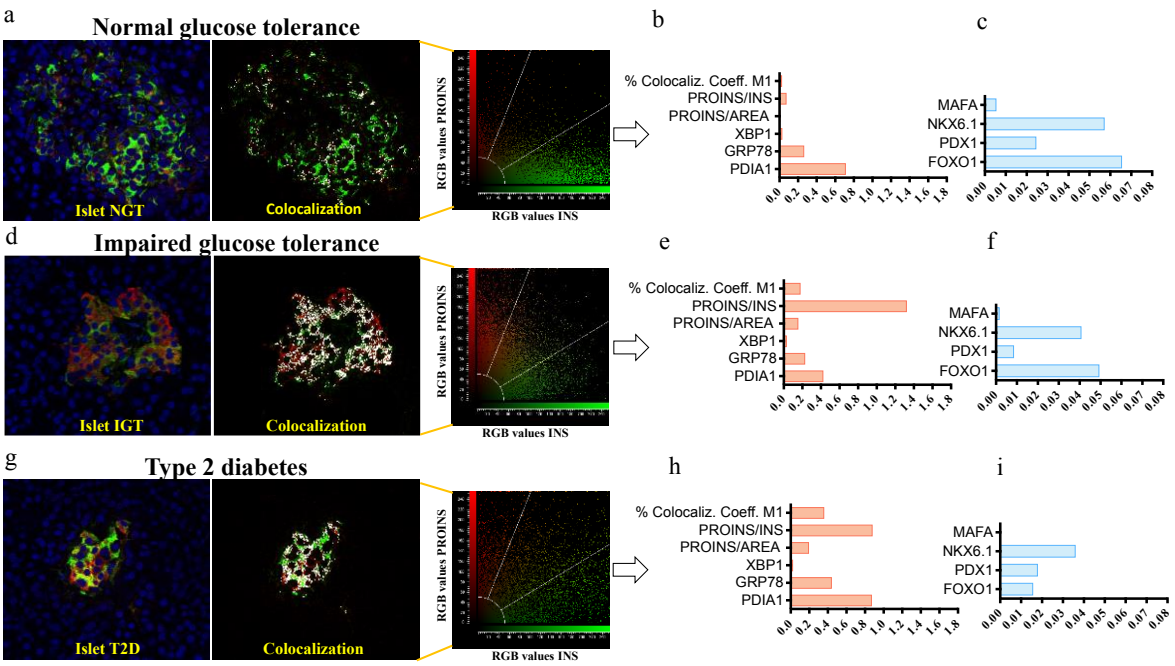


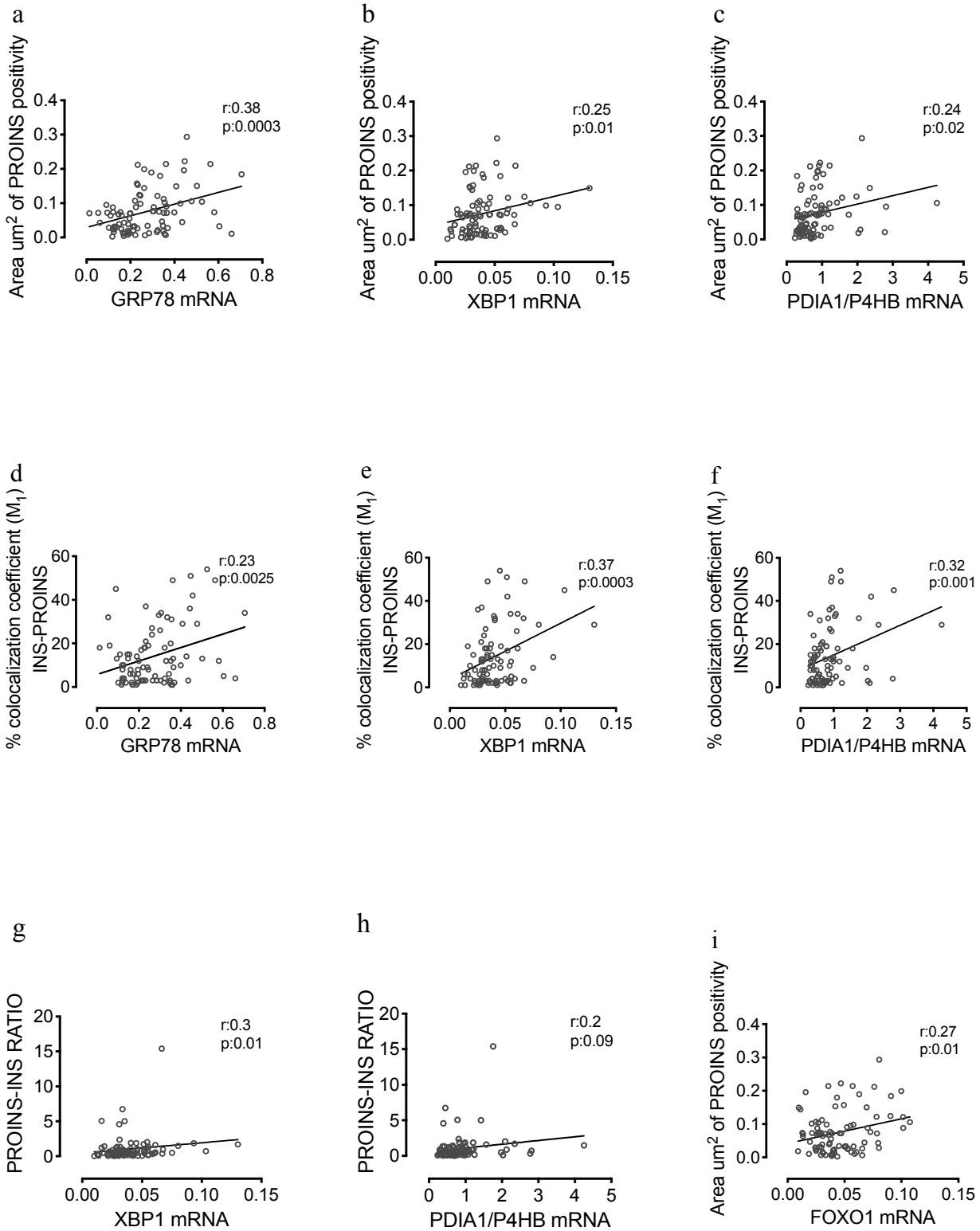
Figure 24. Single islet phenotyping in single pancreatic islet NGT, IGT and T2D. The figure shows the gradual increase of intracellular alteration of PI (% coloc coeff INS-PI, PI-INS ratio, PROINS area) (a, b, d, e, g, h), gradual increase of UPR genes expression (GRP78, XBP1 and PDIA1) (b, e, h) and gradual decreases of expression of gene associated to β cell function (MAFA, NKX6.1, PDX1. FOXO1) (c, f, i) from NGT to IGT and T2D single pancreatic islet.

4.8 The *in-situ* staining measurements related to alteration of proinsulin are correlate positively with the expression of PDIA1, GRP78 and XBP1 and negatively with genes associated to beta cell function

The expression of genes associated to the activation UPR and β cell identity has been correlated with *in-situ* staining measurements related to the alteration of PI and INS in single pancreatic islets with impaired glucose tolerance and type 2 diabetes. Specifically, we showed a direct association between the expression of PDIA1, GRP78 and XBP1 with the increase of colocalization coefficient as well as with the area of PI positivity and PI/INS ratio (**Figure 25 a-h**). In the same single pancreatic islets, we have also observed that the increase of intracellular alteration of PI leads to a reduction of gene expression essential for the maintenance of β cell identity such as NKX6.1 and MAFA (**Figure 25 j-n**). In fact, we found a negative correlation

between NKX6.1 expression with INS-PI colocalization coefficient (r:-0.2 p: 0.05) (**Figure 25j**) and between MAFA expression with area of PI positivity (r:-0.22 p: 0.04) as well as with colocalization coefficient (r:-0.23 p: 0.03) and PI/INS ratio (r:-0.25 p: 0.02) (**Figure 25 k-m**). Furthermore, we also observed that the expression of FOXO1 was directly associated with the expression of PDIA1 (r:0.31 p: 0.003) and XBP1 (r:0.22 p: 0.03) (**Figure 25n, Figure 25o**) and with the increase of area of PI positivity (r:0.27 p: 0.01) (**Figure 25i**). Collectively data confirm our hypothesis and revealed a crosstalk between ER stress, PI intracellular alteration and β cell function reduction during metabolic alteration.

Figure 25


a
Area um2 of PROINS positivity
r:0.38
p:0.0003
GRP78 mRNA
b
Area um2 of PROINS positivity
r:0.25
p:0.01
XBP1 mRNA
c
Area um2 of PROINS positivity
r:0.24
p:0.02
PDIA1/P4HB mRNA
d
% colocalization coefficient (M1)
INS-PROINS
r:0.23
p:0.0025
GRP78 mRNA
e
% colocalization coefficient (M1)
INS-PROINS
r:0.37
p:0.0003
XBP1 mRNA
f
% colocalization coefficient (M1)
INS-PROINS
r:0.32
p:0.001
PDIA1/P4HB mRNA
g
PROINS-INS RATIO
r:0.3
p:0.01
XBP1 mRNA
h
PROINS-INS RATIO
r:0.2
p:0.09
PDIA1/P4HB mRNA
i
Area um2 of PROINS positivity
r:0.27
p:0.01
FOXO1 mRNA

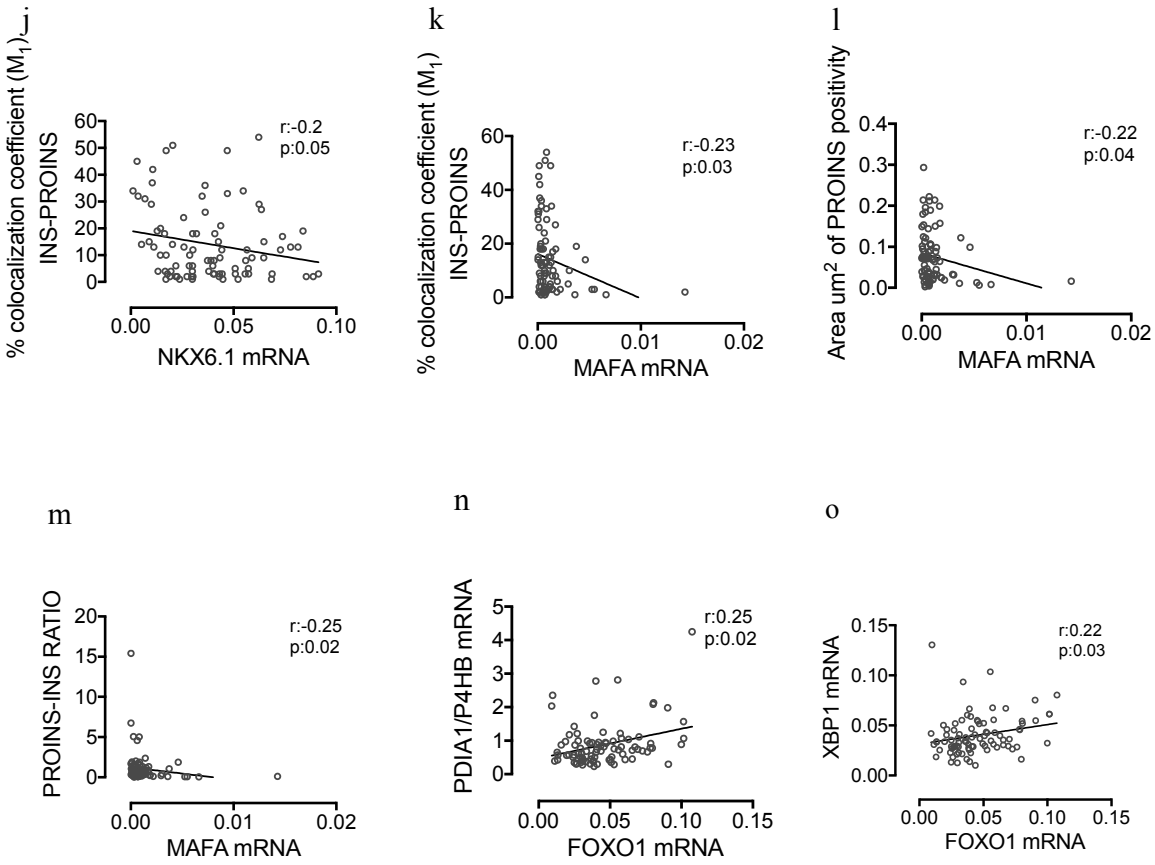


Figure 25. ***In-situ*** **staining measurements related to alteration of proinsulin are correlates positively with the expression of PDIA1, GRP78 and XBP1 and negatively with genes associated to beta cell function.** Correlation analysis between area of PI positivity with expression of GRP78 (r: 0.38, p: 0.003) (a), XBP1 (r: 0.25, p: 0.01) (b) PDIA1 (r: 0.24, p: 0.02) (c); correlation analysis between % of colocalization coefficient with expression of GRP78 (r: 0.23, p: 0.0025) (d), XBP1 (r: 0.37, p: 0.0003) (e) PDIA1 (r: 0.32, p: 0.001) (f); correlation analysis between PI/INS ratio with expression of XBP1 (r: 0.3, p: 0.01) (g) PDIA1 (r: 0.2, p: 0.09) (h) in single pancreatic islets of NGT, IGT and T2D patients. Correlation analysis between % of colocalization coefficient with expression of NKX6.1 (r: -0.2, p: 0.05) (j); correlation analysis between expression of MAFA with % of colocalization coefficient (r: -0.23, p: 0.03) (k), area of PI positivity (r: -0.22, p: 0.04) (l) and with PI/INS ratio (r: -0.25, p: 0.02) (m). Positive correlation between FOXO1 expression and area of PI positivity (r: 0.27, p: 0.01) (i), PDIA1 expression (r: 0.25, p: 0.02) (n) and XBP1 expression (r: 0.22, p: 0.03) (o) in single pancreatic islets of NGT, IGT and T2D patients. P-value and r-value were obtained using Pearson and Spearman correlation test.

5. Discussion and Conclusions

It is a commonly held view that alteration of PI synthesis contributes to dysfunction and β cell failure. Our findings represent the link between typical metabolic alterations of type 2 diabetes and molecular alterations taking place within the β cells. Our study was conducted in human pancreatic islets of patients with an accurate metabolic profiling which included evaluation of glucose tolerance and insulin secretion. For this study we have analysed pancreatic tissue biopsy from n=5 NGT (normal glucose tolerance), n=9 IGT (impaired glucose tolerance) and n=5 T2D (type 2 diabetes) patients on the waiting list for partial pancreatectomy. The reason for the surgery was tumor of the ampulla of Vater for all patients analysed. We observed an alteration of PI expression pattern in human pancreatic islets with IGT and T2D. Specifically, we demonstrated that in most of the normal glucose tolerant pancreatic islets, the PI expression pattern was mainly distributed within a perinuclear compartment respect to insulin more widely localized within the cytoplasm. The same intracellular distribution of INS and PI immunostaining pattern has been observed in human pancreatic islets of children at different age of diagnosis of type 1 diabetes; in those with a diagnosis <7 years the proinsulin colocalized with mature insulin respect to those with diagnosis after 12 years and control individuals where PI was preferentially distributed in the perinuclear compartment, suggesting the presence of distinct endotypes based on intracellular alteration of PI that correlated with age at diagnosis [50].

As a matter of fact, we observed an alteration of intracellular distribution of PI in impaired glucose tolerant and type 2 diabetic pancreatic islets. In fact, image analysis showed an increase of colocalization coefficient INS-PI, as well as area of PI positivity and PI/INS ratio in patients with altered metabolic profile, suggesting an abnormal PI processing which leads to the release of immature granules with a higher relative content of PI and its conversion intermediates.

Several studies have previously observed these phenomena in animal models of type 2 diabetes. In particular, it has been demonstrated an increase of PI biosynthesis together with expansion of ER and Golgi apparatus in obese diabetic mice in order to overcome the high metabolic demand. Moreover, the same study highlighted an adaptive plasticity of β cells, in fact when physiological condition are recovered, β cell were able to restore normal insulin secretory function [136]. In several unusual conditions (mutant proinsulins or with insulinoma cells) it has been shown that PI could be poorly targeted to constitutive secretory pathway causing a 40% of unprocessed proinsulin [137,138], despite the normal process results > 99% efficient [139]. In another work performed in *db/db mice,* a sub-population of "extreme" β cells was identified.

They are characterized by high ribosomal and PI content, low levels of mature INS protein and high levels of genes such as insulin (Ins1), islets amyloid polypeptide (Iapp), chromogranin A (Chga) and PC2, suggesting that extreme β cells are more predisposed to secrete basal insulin rather than insulin storage [140]. Our results showed for the first time that altered glucose sensitivity in β cells is related to the increase of PI/INS ratio in human pancreatic islets with metabolic defects; the increase of PI/INS ratio *in-situ* was positively correlated with basal glucose, basal insulin and 2h glucose levels following oral glucose tolerant test (OGTT); this probably happens because the β cells are overstimulated following insulin resistance and then diabetes due to an increase in insulin demand by organism and which is not correctly manageable by dysfunctional β cells.

These results highlighted that INS-PI staining pattern alterations during metabolic defects might mirror the *in-vivo* t increased insulin secretion rate and β cell functional reduction in the same patients. Furthermore, both from the global analysis and from the analysis on single islets we observed that the increased *in-situ* levels of PI/INS ratio are not significantly correlated with an increase of the percentage of INS-PI colocalization. This probably occur because the enhance of PI levels leads to its accumulation in the ER causing an increase of PI/INS ratio, instead when misfolded PI is degraded, the rest could accumulate in the same compartment of insulin leading to increase of colocalization.

Mezza et al., demonstrated that in non-diabetic patients undergoing to partial pancreatectomy, circulating PI-INS ratio was increased during upon a high β cell workload (i.e. during/after a meal), suggesting an alteration of PI secretion when the demand for INS was elevated. The same patients were characterized by defects of β cell insulin secretion rate and by reduced glucose sensitivity, suggesting that the remaining β cell mass was stressed when the workload was increased [100]. These data are in line with our results; indeed, we can speculate that the increased β cell demand typical of prediabetic and recent onset type 2 diabetic patients, lead to an alteration of PI expression pattern that cause an impaired PI conversion to insulin. This mechanism has been well studied in type 1 diabetes mellitus. However, in type 2 diabetes mellitus there are still several unclear mechanisms that should be investigated. Rodriguez-Calvo et al., showed that human pancreatic islets of islets-autoantibody positive (Ab+) patients were characterized by elevated PI and PI/INS ratio suggesting a defect in PI processing with an accumulation of immature vesicles prior to disease onset [49]. It has also been demonstrated an increase of Proinsulin-C-peptide (PI/C) ratio in serum of subjects antibody-positive in whom there was progression to diabetes compared to control serum. These data suggested that an elevation of PI/C ratio as a biomarker of β cell ER dysfunction could be important to predict

the onset of type 1 diabetes [141]. Our hypothesis is that the impaired PI synthesis in IGT and T2D pancreatic islets was caused by increased PI misfolding which leads to the abnormal basal release of poorly functioning insulin characterized by a high PI/INS ratio. A recent study has demonstrated that in Akita mice in which mutant PI causes diabetes, the alteration of INS secretion was mainly generated by an incorrect interaction between misfolded PI and INS receptor (ProIR) precursor in the ER. The same result has been confirmed in *db/db* diabetic mice in which the over synthesis of PI leads to an increase of PI misfolding following the alteration of INS processing [96]. According to a study in which was analyzed the biosynthetic interaction network of PI in human islets, it has been observed that UPR was the most enriched pathway involved in PI biosynthesis. Among the ER localized interactors, we found that the ER-localized peroxiredoxin (PRDX4) represented a prominent PI interacting protein and its silencing rendered the PI more susceptible to misfolding following oxidative stress. Moreover, we demonstrated that PRDX4 was predisposed to sulfonylation which caused its inactivation following high glucose. Interestingly, pancreatic islets of patients with T2D were characterized by high levels of sulfonylated PRDX4 compared to control islets, suggesting the importance of studying the human PI interaction network in order to identify the critical factors that control insulin biosynthesis, β cell function and their relevance in diabetes T2D [142].

In order to understand whether the PI processing alterations observed *in-situ* in IGT and T2D pancreatic islets could be associated to ER stress or β-cell phenotype, we analyzed a panel of genes associated to ER stress, PI misfolding and processing as well as of genes associated to β cell function in LCM-human pancreatic islets from n=4 NGT, n=7 IGT and n=4 T2D. We observed an upregulation of GRP78, PDIA1 and XBP1 in IGT and T2D respect to NGT pancreatic islets. These results showed a link between *in-situ* PI intracellular distribution alteration and increased ER stress alongside with UPR activation.

GRP78 is an ER-resident molecular chaperon which belongs to the heat-shock-protein (HSP) family that allows the correct folding of de-novo proteins in the ER. It is a master regulator of the UPR that regulates ER stress signaling pathway leading to UPR survival or apoptosis responses [143]. XBP1 is a transcription factor that undergoes unconventional splicing in response to ER stress signals leading to the activation of ER chaperone genes as PDI required for the folding and trafficking of secretory cargo proteins [144]. It has been shown that IRE1alfa-XBP1 pathway represented a key regulator of oxidative PI folding in the ER trough the activation of specific PDI family proteins in pancreatic beta cells [145]. More importantly, it has been shown that the overexpression of PDIA1 led to an accumulation of PI that forms covalent mixed disulfides with PDI itself increasing the levels of misfolded PI in the ER [146,147]. The

overexpression of GRP78 under hyperglycemia led to an increase of INS levels and secretion; while the overexpression of PDIA1 caused a reduction of INS secretion leading to the accumulation of misfolded PI in the ER [146,147]. In contrast, a recent study demonstrated that in young high-fat diet fed mice or aged mice the deletion of Pdia1 led to worsen of glucose intolerance enhancing circulating and *in-situ* PI/INS ratio compared to wild type mice. Moreover, Pdia1 deletion increased the accumulation of disulfide-linked high molecular weight PI complexes and ER stress [146,147]. Other evidence demonstrate that PDIA1 could also reduce improper PI disulfide bonds supporting its role as a reductase for degradation of mutant Akita proinsulin [148]. In light of these results, we hypothesized that the *in-situ* alteration of expression pattern of PI and INS in impaired glucose tolerant and type 2 diabetic pancreatic islets may be caused by an upregulation of PDIA1 which represent an early β cell functional defect during the compensation when the β cells increase insulin output. This can cause the activation of UPR in order to reestablish the ER homeostasis.

Our results also shown that GRP78 positively correlated with PI/INS ratio, INS-PI colocalization as well as basal glucose (mg/dl), basal insulin (pmol/L) and 2 hours glucose levels following OGTT. We also found an inverse correlation between GRP78 expression and glucose sensitivity. The expression of PDIA1 was positively associated with INS-PI colocalization coefficient as well as basal glucose (mg/dl) and insulin (pmol/L) and also at 2 hours glucose levels following OGTT. These findings highlight an early activation of UPR genes during prediabetic phase following PI misfolding which may cause a deterioration of glucose tolerance. We did not observe any statistically significant difference regarding the expression of genes associated with the maintenance of the β cell identity, probably because, by analyzing pool of pancreatic islets belonging to different donors, given the elevated heterogeneity, we found islets with altered β cell phenotype and others characterized by a mature β cell phenotype, for this, a lot of information and molecular differences are not detected. Regarding this last point, we performed a single pancreatic islets phenotyping in NGT, IGT and T2D in order to investigate whether the *in-situ* alterations of PI processing was actually related to the increased ER stress and β cell function reduction at a single islet level. We observed a high heterogeneity between pancreatic islets of different donors but also belonging to the same donor, highlighting a different behavior among pancreatic islets. Of interest, the heterogeneity among the pancreatic islets was gradually increasing from NGT to IGT and T2D pancreatic islets.

Hodish et al., showed that in diabetic mouse models there was an elevated β cellular heterogeneity and subsets of β cells could react to misfolding PI on the basis on the quantity of

accumulated mutant PI, suggesting the high capacity for β cells to adapt to PI misfolding [149]. In human we observed that the most control single pancreatic islets were characterized by low levels of INS-PI colocalization as well as PI/INS ratio, PI positivity and reduced expression of UPR genes, but high levels of genes associated with the maintenance of β cell phenotype. Moreover, we also observed that several single islets of NGT cases, in particular in 07MF NGT donor, showed a histological and molecular profile comparable to IGT and T2D islets. The phenotyping characterization of single islets with impaired glucose tolerant have were characterized by an increase of INS-PI colocalization, PI/INS ratio and area of PI positivity; the expression of GRP78, PDIA1 and XBP1 was elevated. In particular, in the same islets, FOXO1 was overexpressed as compensatory role following metabolic stress in order to restore β cell identity but PDX1, NKX6.1 and MAFA expression was reduced. We analysed single pancreatic islets from n=2 recent onset type 2 diabetic and n=1 long standing type 2 diabetic. The histological and molecular profile of single pancreatic islets T2D at onset presented an elevated expression of GRP78, XBP1 and PDIA1, an increase of INS-PI colocalization, PI/INS ratio and area of PI positivity but low levels of gene expression associated to β cell function and differentiation. While pancreatic islets with long standing type 2 diabetic were characterized from a progressive reduction of UPR genes respect to recent type 2 diabetic pancreatic islets. These results suggest different stages of the diabetic disease; at first, PDIA1, GRP78 and XBP1 genes were activated in an early phase of the disease following an overstimulation of β cells consequent to the increase of peripheral insulin demand in an acute phase of ER stress in order to restore ER homeostasis and to avoid β cell apoptosis as a mechanism of compensation and adaptation. During this first phase, some pancreatic islets may dedifferentiate to escape cell death by apoptosis. In the second stage of the diabetic disease, in the pancreatic islets with a long disease duration, UPR expression genes (GRP78, PDIA1 and XBP1) were reduced, probably because following chronic stress β cells undergo a phase that we can define "exhaustion" which could activate molecular pathway that cause β cellular apoptosis. Moreover, from phenotyping characterization approach of single islets we also observe that several NGT islets showed a phenotype and molecular profile similar to IGT and T2D islets, some single IGT and T2D pancreatic islets were more stressed and dedifferentiated, other still were characterized by a mature β cell phenotype.

This approach also highlights the importance to analyse individual pancreatic islets given that this specific analysis revealed that a certain amount of IGT and T2D pancreatic islets show a similar molecular profile to control islets, some others particularly stressed are characterized by a loss of β cell phenotype determining β cell failure typical of diabetes.

According to our molecular results, in a study it has been examined in Nile rat (NR) fed standard chow at 2 months as a model of β cell compensation and at 6 months as decompensation model the change of β cell mass during the progression of T2D. The authors observed an increase of PI secretion from compensation to decompensation phase suggesting an altered of PI processing. Moreover, the β cells during the transition phase were characterized by an adaptive UPR response with the increase of chaperonins such as PDI. Furthermore, non-proliferative neogenic β cells were elevated during the compensation phase as a mechanism of adaptation; conversely, during the decompensation phase the mechanisms of neogenesis and dedifferentiation of β cells were reduced [150].

Arunagiri et al., have demonstrated in a recent study that an elevated misfolding of PI via disulphide-linked complexes was an early event related with prediabetic phase that worsened with β cell dysfunction in type 2 diabetes. In fact, we observed an improperly folded PI in the ER during the compensation stage but the levels of insulin were kept, while in the decompensation stage increased the PI misfolding levels and insulin [151].

Our results also showed that in single pancreatic islets *in-situ* staining measurements related to alteration of PI were positively correlated with expression of PDIA1, GRP78 and XBP1 and negatively with genes associated to β cell function such as NKX6.1 and MAFA. The expression of FOXO1 was positively correlated with the increase of area of PI and its expression was directly associated with XBP1 and PDIA1 expression levels, suggesting that the activation of FOXO1 together with UPR genes could have a compensatory role in order to restore β cell function and ER homeostasis.

This result may be related to another work in which they demonstrated a regulation of XBP1 on FOXO1. In fact, the authors observed that XBP1 can bind to FOXO1 promoting its degradation through the 26S proteasome pathway [152]. Following obesity, the migration of XBP1 into the nucleus is impaired and its defect may help accumulate FOXO1 in the nucleus. This data could partly explain the positive correlation between the expression of FOXO1 and XBP1.

Subsequently, we asked ourselves what might be the molecular mechanisms that upstream regulate the expression of GRP78, XBP1 AND PDIA1. Among several molecules and factors controlling gene expression in physiological and pathological mechanisms, miRNAs have been identified as potent modulators of multiple genes. For this reason, we have investigated through bioinformatic analysis using Target Scan that predicts biological targets of miRNAs, we found that miR-33a-5p/miR-33b-5p, miR-181a/b/d/c/5p and miR-204-5p are potentially regulators of genes XBP1, GRP78 and PDIA1 respectively. From literature studies, it has been shown that miR-33-dificient mice predisposed to the development of obesity and insulin resistance and the

loss of this miRNA resulted in impaired responsiveness to insulin in several metabolic organs such as liver, adipose tissue and skeletal muscle [153]. Thus, the predicted regulation of miR-33 on XBP1 expression could partly explain the upregulation of XBP1 in pancreatic islets of IGT and T2D patients and it would be interesting to deepen the molecular analysis that involve miR-33-XBP1 pathway in our study. It has been reported that in prediabetic subjects at baseline the circulating levels of miR-181a-5p were decreased compared to control individuals, suggesting an important prognostic role of this miRNA [154]. The potential regulation of GRP78 from miR-181a-5p should be further investigated in order to understand if the upregulation of GRP78 in impaired glucose tolerant patients could have an improving effect on insulin resistance before the onset of diabetes. PDIA1 is a predicted target of miR-204-5p a miRNA involves in the regulation of insulin secretion. Guanlan Xu et al. demonstrated an upregulation of miR-204 in INS-1 beta cells treated with high levels of thioredoxin-interacting protein (TXNIP), suggesting that TXNIP induced the expression of miR-204 which in turn caused a suppression of insulin production by directly targeting and downregulating of MafA [155]. Accordingly, to our study we might speculate that following acute ER stress the upregulation of miR-204 could indirectly regulate the expression of PDIA1 leading to its overexpression, while following acute ER stress it could directly regulated PDIA1 expression causing its downregulation and β cell death.

In conclusion, our data show that: (*i*) the PI and INS expression pattern is altered in IGT and T2D patients; (*ii*) PI-INS *in-situ* ratio reflects β cellular dysfunction *in-vivo*; (*iii*) the intracellular alteration of PI is directly associated with increased ER stress following the overexpression of genes involved in the activation of UPR; (*iv*) single islets phenotyping analysis in T2D pancreas reveals a high heterogeneity among pancreatic islets in terms of ER stress and β cell differentiation profile. Collectively results, highlight the importance of correct PI processing and folding in the maintenance of glucose homeostasis. These data reveal a crosstalk between ER stress, proinsulin processing and β cell identity during metabolic alterations and understanding these mechanisms will help us in the future to develop new potential therapeutic strategies to prevent/delay β cell failure in type 2 diabetes mellitus.

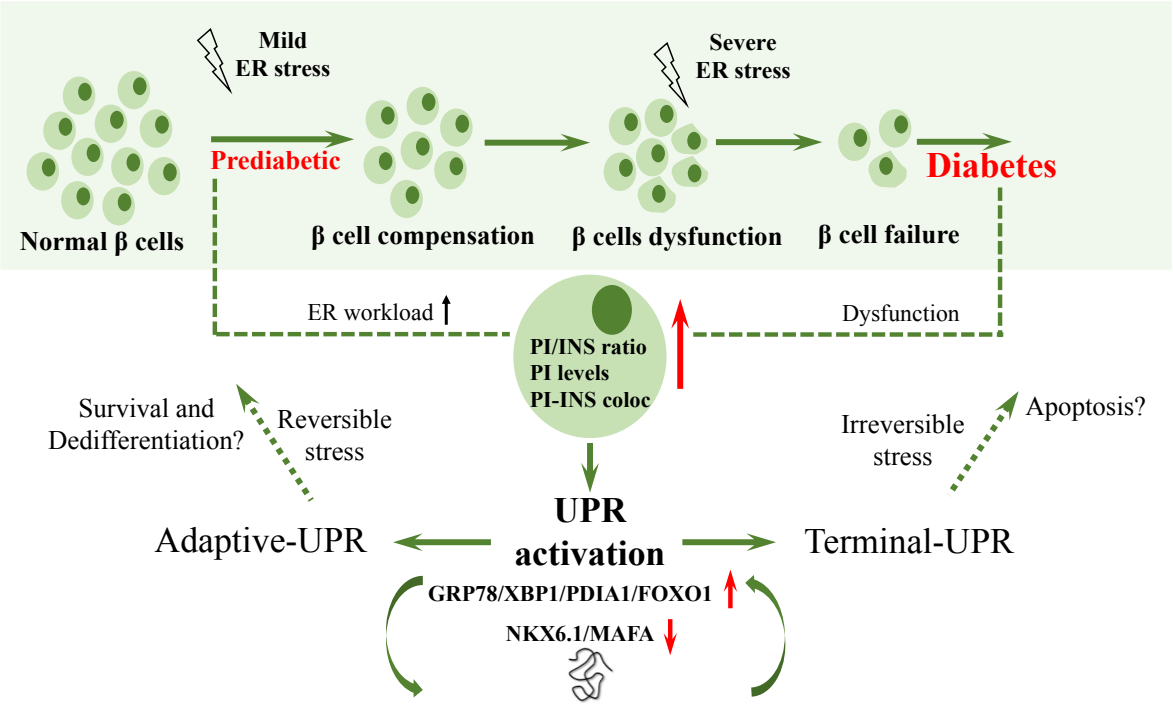


Figure 25. Graphical abstract highlights the crosstalk between ER stress, proinsulin intracellular alteration and beta cell identity during the transition from a pre-diabetic state to an overt diabetic state.

6. Bibliography

1. Aamodt, K.I., and Powers, A.C. (2017). Signals in the pancreatic islet microenvironment influence β-cell proliferation. Diabetes Obes. Metab. *19 Suppl 1*, 124–136.
2. Dolenšek, J., Rupnik, M.S., and Stožer, A. (2015). Structural similarities and differences between the human and the mouse pancreas. Islets *7*, e1024405.
3. Brissova, M., Fowler, M.J., Nicholson, W.E., Chu, A., Hirshberg, B., Harlan, D.M., and Powers, A.C. (2005). Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. J. Histochem. Cytochem. *53*, 1087–1097.
4. Kim, A., Miller, K., Jo, J., Kilimnik, G., Wojcik, P., and Hara, M. (2009). Islet architecture: A comparative study. Islets *1*, 129–136.
5. Ionescu-Tirgoviste, C., Gagniuc, P.A., Gubceac, E., Mardare, L., Popescu, I., Dima, S., and Militaru, M. (2015). A 3D map of the islet routes throughout the healthy human pancreas. Sci. Rep. *5*, 14634.
6. Cabrera, O., Berman, D.M., Kenyon, N.S., Ricordi, C., Berggren, P.-O., and Caicedo, A. (2006). The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. Proc. Natl. Acad. Sci. USA *103*, 2334–2339.
7. Brereton, M.F., Iberl, M., Shimomura, K., Zhang, Q., Adriaenssens, A.E., Proks, P., Spiliotis, I.I., Dace, W., Mattis, K.K., Ramracheya, R., et al. (2014). Reversible changes in pancreatic islet structure and function produced by elevated blood glucose. Nat. Commun. *5*, 4639.
8. Noguchi, G.M., and Huising, M.O. (2019). Integrating the inputs that shape pancreatic islet hormone release. Nat. Metab. *1*, 1189–1201.
9. Unger, R.H., and Orci, L. (1975). The essential role of glucagon in the pathogenesis of diabetes mellitus. Lancet *1*, 14–16.
10. Cryer, P.E. (2012). Minireview: Glucagon in the pathogenesis of hypoglycemia and hyperglycemia in diabetes. Endocrinology *153*, 1039–1048.
11. de Heer, J., Rasmussen, C., Coy, D.H., and Holst, J.J. (2008). Glucagon-like peptide-1, but not glucose-dependent insulinotropic peptide, inhibits glucagon secretion via somatostatin (receptor subtype 2) in the perfused rat pancreas. Diabetologia *51*, 2263–2270.
12. Ben-Othman, N., Vieira, A., Courtney, M., Record, F., Gjernes, E., Avolio, F., Hadzic, B., Druelle, N., Napolitano, T., Navarro-Sanz, S., et al. (2017). Long-Term GABA Administration Induces Alpha Cell-Mediated Beta-like Cell Neogenesis. Cell *168*, 73–85.e11.
13. Egea, P.F., Stroud, R.M., and Walter, P. (2005). Targeting proteins to membranes: structure of the signal recognition particle. Curr. Opin. Struct. Biol. *15*, 213–220.
14. Patzelt, C., Labrecque, A.D., Duguid, J.R., Carroll, R.J., Keim, P.S., Heinrikson, R.L., and Steiner, D.F. (1978). Detection and kinetic behavior of preproinsulin in pancreatic islets. Proc. Natl. Acad. Sci. USA *75*, 1260–1264.
15. Huang, X.F., and Arvan, P. (1995). Intracellular transport of proinsulin in pancreatic beta-cells. Structural maturation probed by disulfide accessibility. J. Biol. Chem. *270*, 20417–20423.
16. Ramzy, A., Asadi, A., and Kieffer, T.J. (2020). Revisiting Proinsulin Processing: Evidence That Human β-Cells Process Proinsulin With Prohormone Convertase (PC) 1/3 but Not PC2. Diabetes *69*, 1451–1462.
17. Marchetti, P., Lupi, R., Bugliani, M., Kirkpatrick, C.L., Sebastiani, G., Grieco, F.A., Del Guerra, S., D'Aleo, V., Piro, S., Marselli, L., et al. (2012). A local glucagon-like peptide 1 (GLP-1) system in human pancreatic islets. Diabetologia *55*, 3262–3272.
18. Vasiljević, J., Torkko, J.M., Knoch, K.-P., and Solimena, M. (2020). The making of insulin in health and disease. Diabetologia *63*, 1981–1989.
19. Zhuo, F., Elizabeth R, G., and Dongmin, L. (2013). Regulation of insulin synthesis and

secretion and pancreatic Beta-cell dysfunction in diabetes. Curr. Diabetes Rev. *9*, 25–53.
20. Dorrell, C., Schug, J., Canaday, P.S., Russ, H.A., Tarlow, B.D., Grompe, M.T., Horton, T., Hebrok, M., Streeter, P.R., Kaestner, K.H., et al. (2016). Human islets contain four distinct subtypes of β cells. Nat. Commun. *7*, 11756.
21. Kailey, B., van de Bunt, M., Cheley, S., Johnson, P.R., MacDonald, P.E., Gloyn, A.L., Rorsman, P., and Braun, M. (2012). SSTR2 is the functionally dominant somatostatin receptor in human pancreatic β- and α-cells. Am. J. Physiol. Endocrinol. Metab. *303*, E1107-16.
22. Chera, S., Baronnier, D., Ghila, L., Cigliola, V., Jensen, J.N., Gu, G., Furuyama, K., Thorel, F., Gribble, F.M., Reimann, F., et al. (2014). Diabetes recovery by age-dependent conversion of pancreatic δ-cells into insulin producers. Nature *514*, 503–507.
23. Zhang, J., McKenna, L.B., Bogue, C.W., and Kaestner, K.H. (2014). The diabetes gene Hhex maintains δ-cell differentiation and islet function. Genes Dev. *28*, 829–834.
24. Rahier, J., Wallon, J., Loozen, S., Lefevre, A., Gepts, W., and Haot, J. (1983). The pancreatic polypeptide cells in the human pancreas: the effects of age and diabetes. J. Clin. Endocrinol. Metab. *56*, 441–444.
25. Aragón, F., Karaca, M., Novials, A., Maldonado, R., Maechler, P., and Rubí, B. (2015). Pancreatic polypeptide regulates glucagon release through PPYR1 receptors expressed in mouse and human alpha-cells. Biochim. Biophys. Acta *1850*, 343–351.
26. Gao, T., McKenna, B., Li, C., Reichert, M., Nguyen, J., Singh, T., Yang, C., Pannikar, A., Doliba, N., Zhang, T., et al. (2014). Pdx1 maintains β cell identity and function by repressing an α cell program. Cell Metab. *19*, 259–271.
27. Lee, C.S., Sund, N.J., Vatamaniuk, M.Z., Matschinsky, F.M., Stoffers, D.A., and Kaestner, K.H. (2002). Foxa2 controls Pdx1 gene expression in pancreatic beta-cells in vivo. Diabetes *51*, 2546–2551.
28. Raum, J.C., Gerrish, K., Artner, I., Henderson, E., Guo, M., Sussel, L., Schisler, J.C., Newgard, C.B., and Stein, R. (2006). FoxA2, Nkx2.2, and PDX-1 regulate islet beta-cell-specific mafA expression through conserved sequences located between base pairs -8118 and -7750 upstream from the transcription start site. Mol. Cell. Biol. *26*, 5735–5743.
29. Sund, N.J., Vatamaniuk, M.Z., Casey, M., Ang, S.L., Magnuson, M.A., Stoffers, D.A., Matschinsky, F.M., and Kaestner, K.H. (2001). Tissue-specific deletion of Foxa2 in pancreatic beta cells results in hyperinsulinemic hypoglycemia. Genes Dev. *15*, 1706–1715.
30. Taylor, B.L., Liu, F.-F., and Sander, M. (2013). Nkx6.1 is essential for maintaining the functional state of pancreatic beta cells. Cell Rep. *4*, 1262–1275.
31. Swisa, A., Avrahami, D., Eden, N., Zhang, J., Feleke, E., Dahan, T., Cohen-Tayar, Y., Stolovich-Rain, M., Kaestner, K.H., Glaser, B., et al. (2017). PAX6 maintains β cell identity by repressing genes of alternative islet cell types. J. Clin. Invest. *127*, 230–243.
32. Kitamura, T., Nakae, J., Kitamura, Y., Kido, Y., Biggs, W.H., Wright, C.V.E., White, M.F., Arden, K.C., and Accili, D. (2002). The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. J. Clin. Invest. *110*, 1839–1847.
33. Kitamura, Y.I., Kitamura, T., Kruse, J.-P., Raum, J.C., Stein, R., Gu, W., and Accili, D. (2005). FoxO1 protects against pancreatic beta cell failure through NeuroD and MafA induction. Cell Metab. *2*, 153–163.
34. Klein, D., Misawa, R., Bravo-Egana, V., Vargas, N., Rosero, S., Piroso, J., Ichii, H., Umland, O., Zhijie, J., Tsinoremas, N., et al. (2013). MicroRNA expression in alpha and beta cells of human pancreatic islets. PLoS One *8*, e55064.
35. Lemaire, K., Thorrez, L., and Schuit, F. (2016). Disallowed and allowed gene expression: two faces of mature islet beta cells. Annu. Rev. Nutr. *36*, 45–71.
36. Bensellam, M., Jonas, J.-C., and Laybutt, D.R. (2018). Mechanisms of β-cell dedifferentiation in diabetes: recent findings and future research directions. J. Endocrinol.

236, R109–R143.
37. Lin, X., Xu, Y., Pan, X., Xu, J., Ding, Y., Sun, X., Song, X., Ren, Y., and Shan, P.-F. (2020). Global, regional, and national burden and trend of diabetes in 195 countries and territories: an analysis from 1990 to 2025. Sci. Rep. *10*, 14790.
38. Zheng, Y., Ley, S.H., and Hu, F.B. (2018). Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. Nat. Rev. Endocrinol. *14*, 88–98.
39. Lotfy, M., Adeghate, J., Kalasz, H., Singh, J., and Adeghate, E. (2017). Chronic complications of diabetes mellitus: A mini review. Curr. Diabetes Rev. *13*, 3–10.
40. Jang, K.M. (2020). Maturity-onset diabetes of the young: update and perspectives on diagnosis and treatment. Yeungnam Univ. J. Med. *37*, 13–21.
41. American Diabetes Association (2018). 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2018. Diabetes Care *41*, S13–S27.
42. Skyler, J.S., Bakris, G.L., Bonifacio, E., Darsow, T., Eckel, R.H., Groop, L., Groop, P.-H., Handelsman, Y., Insel, R.A., Mathieu, C., et al. (2017). Differentiation of diabetes by pathophysiology, natural history, and prognosis. Diabetes *66*, 241–255.
43. Taplin, C.E., and Barker, J.M. (2008). Autoantibodies in type 1 diabetes. Autoimmunity *41*, 11–18.
44. Nigi, L., Maccora, C., Dotta, F., and Sebastiani, G. (2020). From immunohistological to anatomical alterations of human pancreas in type 1 diabetes: New concepts on the stage. Diabetes Metab Res Rev *36*, e3264.
45. Bender, C., Rodriguez-Calvo, T., Amirian, N., Coppieters, K.T., and von Herrath, M.G. (2020). The healthy exocrine pancreas contains preproinsulin-specific CD8 T cells that attack islets in type 1 diabetes. Sci. Adv. *6*,.
46. Oram, R.A., Sims, E.K., and Evans-Molina, C. (2019). Beta cells in type 1 diabetes: mass and function; sleeping or dead? Diabetologia *62*, 567–577.
47. Rui, J., Deng, S., Arazi, A., Perdigoto, A.L., Liu, Z., and Herold, K.C. (2017). β Cells that Resist Immunological Attack Develop during Progression of Autoimmune Diabetes in NOD Mice. Cell Metab. *25*, 727–738.
48. Wasserfall, C., Nick, H.S., Campbell-Thompson, M., Beachy, D., Haataja, L., Kusmartseva, I., Posgai, A., Beery, M., Rhodes, C., Bonifacio, E., et al. (2017). Persistence of Pancreatic Insulin mRNA Expression and Proinsulin Protein in Type 1 Diabetes Pancreata. Cell Metab. *26*, 568–575.e3.
49. Rodriguez-Calvo, T., Zapardiel-Gonzalo, J., Amirian, N., Castillo, E., Lajevardi, Y., Krogvold, L., Dahl-Jørgensen, K., and von Herrath, M.G. (2017). Increase in Pancreatic Proinsulin and Preservation of β-Cell Mass in Autoantibody-Positive Donors Prior to Type 1 Diabetes Onset. Diabetes *66*, 1334–1345.
50. Leete, P., Oram, R.A., McDonald, T.J., Shields, B.M., Ziller, C., TIGI study team, Hattersley, A.T., Richardson, S.J., and Morgan, N.G. (2020). Studies of insulin and proinsulin in pancreas and serum support the existence of aetiopathological endotypes of type 1 diabetes associated with age at diagnosis. Diabetologia *63*, 1258–1267.
51. Katsarou, A., Gudbjörnsdottir, S., Rawshani, A., Dabelea, D., Bonifacio, E., Anderson, B.J., Jacobsen, L.M., Schatz, D.A., and Lernmark, Å. (2017). Type 1 diabetes mellitus. Nat. Rev. Dis. Primers *3*, 17016.
52. Insel, R.A., Dunne, J.L., Atkinson, M.A., Chiang, J.L., Dabelea, D., Gottlieb, P.A., Greenbaum, C.J., Herold, K.C., Krischer, J.P., Lernmark, Å., et al. (2015). Staging presymptomatic type 1 diabetes: a scientific statement of JDRF, the Endocrine Society, and the American Diabetes Association. Diabetes Care *38*, 1964–1974.
53. Noble, J.A., and Erlich, H.A. (2012). Genetics of type 1 diabetes. Cold Spring Harb. Perspect. Med. *2*, a007732.
54. Müller, D., Telieps, T., Eugster, A., Weinzierl, C., Jolink, M., Ziegler, A.-G., and Bonifacio, E. (2018). Novel minor HLA DR associated antigens in type 1 diabetes. Clin.

Immunol. *194*, 87–91.
55. Manczinger, M., and Kemény, L. (2018). Peptide presentation by HLA-DQ molecules is associated with the development of immune tolerance. PeerJ *6*, e5118.
56. Concannon, P., Rich, S.S., and Nepom, G.T. (2009). Genetics of type 1A diabetes. N. Engl. J. Med. *360*, 1646–1654.
57. Vaarala, O. (2012). Is the origin of type 1 diabetes in the gut? Immunol. Cell Biol. *90*, 271–276.
58. Endesfelder, D., zu Castell, W., Ardissone, A., Davis-Richardson, A.G., Achenbach, P., Hagen, M., Pflueger, M., Gano, K.A., Fagen, J.R., Drew, J.C., et al. (2014). Compromised gut microbiota networks in children with anti-islet cell autoimmunity. Diabetes *63*, 2006–2014.
59. Grammatiki, M., Karras, S., and Kotsa, K. (2019). The role of vitamin D in the pathogenesis and treatment of diabetes mellitus: a narrative review. Hormones (Athens) *18*, 37–48.
60. Filippi, C., and von Herrath, M. (2005). How viral infections affect the autoimmune process leading to type 1 diabetes. Cell Immunol. *233*, 125–132.
61. Rodriguez-Calvo, T. (2019). Enterovirus infection and type 1 diabetes: unraveling the crime scene. Clin. Exp. Immunol. *195*, 15–24.
62. Dotta, F., Censini, S., van Halteren, A.G.S., Marselli, L., Masini, M., Dionisi, S., Mosca, F., Boggi, U., Muda, A.O., Del Prato, S., et al. (2007). Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. Proc. Natl. Acad. Sci. USA *104*, 5115–5120.
63. Hober, D., Chehadeh, W., Weill, J., Hober, C., Vantyghem, M.-C., Gronnier, P., and Wattré, P. (2002). Circulating and cell-bound antibodies increase coxsackievirus B4-induced production of IFN-alpha by peripheral blood mononuclear cells from patients with type 1 diabetes. J. Gen. Virol. *83*, 2169–2176.
64. Chehadeh, W., Kerr-Conte, J., Pattou, F., Alm, G., Lefebvre, J., Wattré, P., and Hober, D. (2000). Persistent infection of human pancreatic islets by coxsackievirus B is associated with alpha interferon synthesis in beta cells. J. Virol. *74*, 10153–10164.
65. Spagnuolo, I., Patti, A., Sebastiani, G., Nigi, L., and Dotta, F. (2013). The case for virus-induced type 1 diabetes. Curr Opin Endocrinol Diabetes Obes *20*, 292–298.
66. Domsgen, E., Lind, K., Kong, L., Hühn, M.H., Rasool, O., van Kuppeveld, F., Korsgren, O., Lahesmaa, R., and Flodström-Tullberg, M. (2016). An IFIH1 gene polymorphism associated with risk for autoimmunity regulates canonical antiviral defence pathways in Coxsackievirus infected human pancreatic islets. Sci. Rep. *6*, 39378.
67. Morse, Z.J., and Horwitz, M.S. (2017). Innate viral receptor signaling determines type 1 diabetes onset. Front. Endocrinol. (Lausanne) *8*, 249.
68. Tripathi, B.K., and Srivastava, A.K. (2006). Diabetes mellitus: complications and therapeutics. Med. Sci. Monit. *12*, RA130-47.
69. Prentki, M., and Nolan, C.J. (2006). Islet beta cell failure in type 2 diabetes. J. Clin. Invest. *116*, 1802–1812.
70. Weir, G.C., and Bonner-Weir, S. (2004). Five stages of evolving beta-cell dysfunction during progression to diabetes. Diabetes *53 Suppl 3*, S16-21.
71. Butler, A.E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R.A., and Butler, P.C. (2003). Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes *52*, 102–110.
72. Donath, M.Y., and Shoelson, S.E. (2011). Type 2 diabetes as an inflammatory disease. Nat. Rev. Immunol. *11*, 98–107.
73. Nigi, L., Grieco, G.E., Ventriglia, G., Brusco, N., Mancarella, F., Formichi, C., Dotta, F., and Sebastiani, G. (2018). Micrornas as regulators of insulin signaling: research updates and potential therapeutic perspectives in type 2 diabetes. Int. J. Mol. Sci. *19*,.
74. Brunetti, A., Chiefari, E., and Foti, D. (2014). Recent advances in the molecular genetics

of type 2 diabetes mellitus. World J. Diabetes *5*, 128–140.
75. Poulsen, P., Grunnet, L.G., Pilgaard, K., Storgaard, H., Alibegovic, A., Sonne, M.P., Carstensen, B., Beck-Nielsen, H., and Vaag, A. (2009). Increased risk of type 2 diabetes in elderly twins. Diabetes *58*, 1350–1355.
76. Poulsen, P., Kyvik, K.O., Vaag, A., and Beck-Nielsen, H. (1999). Heritability of type II (non-insulin-dependent) diabetes mellitus and abnormal glucose tolerance--a population-based twin study. Diabetologia *42*, 139–145.
77. Kobberling, J. (1982). Empirical risk figures for first degree relatives of non-insulin dependent diabetes. The Genetics of Diabetes Mellitus.
78. Shih, D.Q., and Stoffel, M. (2002). Molecular etiologies of MODY and other early-onset forms of diabetes. Curr Diab Rep *2*, 125–134.
79. Taylor, S.I., Kadowaki, T., Kadowaki, H., Accili, D., Cama, A., and McKeon, C. (1990). Mutations in insulin-receptor gene in insulin-resistant patients. Diabetes Care *13*, 257–279.
80. Hani, E.H., Boutin, P., Durand, E., Inoue, H., Permutt, M.A., Velho, G., and Froguel, P. (1998). Missense mutations in the pancreatic islet beta cell inwardly rectifying K+ channel gene (KIR6.2/BIR): a meta-analysis suggests a role in the polygenic basis of Type II diabetes mellitus in Caucasians. Diabetologia *41*, 1511–1515.
81. Lyssenko, V., Lupi, R., Marchetti, P., Del Guerra, S., Orho-Melander, M., Almgren, P., Sjögren, M., Ling, C., Eriksson, K.-F., Lethagen, A.-L., et al. (2007). Mechanisms by which common variants in the TCF7L2 gene increase risk of type 2 diabetes. J. Clin. Invest. *117*, 2155–2163.
82. Mambiya, M., Shang, M., Wang, Y., Li, Q., Liu, S., Yang, L., Zhang, Q., Zhang, K., Liu, M., Nie, F., et al. (2019). The Play of Genes and Non-genetic Factors on Type 2 Diabetes. Front Public Health *7*, 349.
83. Marseglia, L., Manti, S., D'Angelo, G., Nicotera, A., Parisi, E., Di Rosa, G., Gitto, E., and Arrigo, T. (2014). Oxidative stress in obesity: a critical component in human diseases. Int. J. Mol. Sci. *16*, 378–400.
84. Spruijt-Metz, D., O'Reilly, G.A., Cook, L., Page, K.A., and Quinn, C. (2014). Behavioral contributions to the pathogenesis of type 2 diabetes. Curr Diab Rep *14*, 475.
85. Lean, M.E., Leslie, W.S., Barnes, A.C., Brosnahan, N., Thom, G., McCombie, L., Peters, C., Zhyzhneuskaya, S., Al-Mrabeh, A., Hollingsworth, K.G., et al. (2018). Primary care-led weight management for remission of type 2 diabetes (DiRECT): an open-label, cluster-randomised trial. Lancet *391*, 541–551.
86. Quansah, D.Y., Ha, K., Jun, S., Kim, S.-A., Shin, S., Wie, G.-A., and Joung, H. (2017). Associations of Dietary Antioxidants and Risk of Type 2 Diabetes: Data from the 2007-2012 Korea National Health and Nutrition Examination Survey. Molecules *22*,.
87. Larsen, N., Vogensen, F.K., van den Berg, F.W.J., Nielsen, D.S., Andreasen, A.S., Pedersen, B.K., Al-Soud, W.A., Sørensen, S.J., Hansen, L.H., and Jakobsen, M. (2010). Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. PLoS One *5*, e9085.
88. Ding, M., Bhupathiraju, S.N., Chen, M., van Dam, R.M., and Hu, F.B. (2014). Caffeinated and decaffeinated coffee consumption and risk of type 2 diabetes: a systematic review and a dose-response meta-analysis. Diabetes Care *37*, 569–586.
89. Zhang, L., Curhan, G.C., Hu, F.B., Rimm, E.B., and Forman, J.P. (2011). Association between passive and active smoking and incident type 2 diabetes in women. Diabetes Care *34*, 892–897.
90. Hasan, S.S., Clavarino, A.M., Mamun, A.A., and Kairuz, T. (2014). Incidence and risk of diabetes mellitus associated with depressive symptoms in adults: evidence from longitudinal studies. Diabetes Metab Syndr *8*, 82–87.
91. Cerf, M.E. (2013). Beta cell dysfunction and insulin resistance. Front. Endocrinol. (Lausanne) *4*, 37.

92. Huang, C., Lin, C., Haataja, L., Gurlo, T., Butler, A.E., Rizza, R.A., and Butler, P.C. (2007). High expression rates of human islet amyloid polypeptide induce endoplasmic reticulum stress mediated beta-cell apoptosis, a characteristic of humans with type 2 but not type 1 diabetes. Diabetes *56*, 2016–2027.
93. Laybutt, D.R., Preston, A.M., Akerfeldt, M.C., Kench, J.G., Busch, A.K., Biankin, A.V., and Biden, T.J. (2007). Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. Diabetologia *50*, 752–763.
94. Marchetti, P., Bugliani, M., Lupi, R., Marselli, L., Masini, M., Boggi, U., Filipponi, F., Weir, G.C., Eizirik, D.L., and Cnop, M. (2007). The endoplasmic reticulum in pancreatic beta cells of type 2 diabetes patients. Diabetologia *50*, 2486–2494.
95. Goodge, K.A., and Hutton, J.C. (2000). Translational regulation of proinsulin biosynthesis and proinsulin conversion in the pancreatic beta-cell. Semin. Cell Dev. Biol. *11*, 235–242.
96. Liu, S., Li, X., Yang, J., Zhu, R., Fan, Z., Xu, X., Feng, W., Cui, J., Sun, J., and Liu, M. (2019). Misfolded proinsulin impairs processing of precursor of insulin receptor and insulin signaling in β cells. FASEB J. *33*, 11338–11348.
97. Li, M., Feng, D., Zhang, K., Gao, S., and Lu, J. (2016). Disproportionately Elevated Proinsulin Levels as an Early Indicator of β-Cell Dysfunction in Nondiabetic Offspring of Chinese Diabetic Patients. Int J Endocrinol *2016*, 4740678.
98. Pfützner, A., Hermanns, I., Ramljak, S., Demircik, F., Pfützner, A.H., Kann, P.H., and Weber, M.M. (2015). Elevated Intact Proinsulin Levels During an Oral Glucose Challenge Indicate Progressive ß-Cell Dysfunction and May Be Predictive for Development of Type 2 Diabetes. J. Diabetes Sci. Technol. *9*, 1307–1312.
99. Pradhan, A.D., Manson, J.E., Meigs, J.B., Rifai, N., Buring, J.E., Liu, S., and Ridker, P.M. (2003). Insulin, proinsulin, proinsulin:insulin ratio, and the risk of developing type 2 diabetes mellitus in women. Am. J. Med. *114*, 438–444.
100. Mezza, T., Ferraro, P.M., Sun, V.A., Moffa, S., Cefalo, C.M.A., Quero, G., Cinti, F., Sorice, G.P., Pontecorvi, A., Folli, F., et al. (2018). Increased β-Cell Workload Modulates Proinsulin-to-Insulin Ratio in Humans. Diabetes *67*, 2389–2396.
101. Zito, E., Chin, K.-T., Blais, J., Harding, H.P., and Ron, D. (2010). ERO1-beta, a pancreas-specific disulfide oxidase, promotes insulin biogenesis and glucose homeostasis. J. Cell Biol. *188*, 821–832.
102. Riahi, Y., Israeli, T., Cerasi, E., and Leibowitz, G. (2018). Effects of proinsulin misfolding on β-cell dynamics, differentiation and function in diabetes. Diabetes Obes. Metab. *20 Suppl 2*, 95–103.
103. Bonnefond, A., Froguel, P., and Vaxillaire, M. (2010). The emerging genetics of type 2 diabetes. Trends Mol. Med. *16*, 407–416.
104. Riggs, A.C., Bernal-Mizrachi, E., Ohsugi, M., Wasson, J., Fatrai, S., Welling, C., Murray, J., Schmidt, R.E., Herrera, P.L., and Permutt, M.A. (2005). Mice conditionally lacking the Wolfram gene in pancreatic islet beta cells exhibit diabetes as a result of enhanced endoplasmic reticulum stress and apoptosis. Diabetologia *48*, 2313–2321.
105. Rigoli, L., and Di Bella, C. (2012). Wolfram syndrome 1 and Wolfram syndrome 2. Curr. Opin. Pediatr. *24*, 512–517.
106. Muoio, D.M., and Newgard, C.B. (2008). Mechanisms of disease:Molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. Nat. Rev. Mol. Cell Biol. *9*, 193–205.
107. Lenzen, S. (2017). Chemistry and biology of reactive species with special reference to the antioxidative defence status in pancreatic β-cells. Biochim. Biophys. Acta Gen. Subj. *1861*, 1929–1942.
108. Grieco, G.E., Brusco, N., Licata, G., Nigi, L., Formichi, C., Dotta, F., and Sebastiani, G. (2019). Targeting microRNAs as a Therapeutic Strategy to Reduce Oxidative Stress in Diabetes. Int. J. Mol. Sci. *20*,.

109. Lin, S., and Gregory, R.I. (2015). MicroRNA biogenesis pathways in cancer. Nat. Rev. Cancer *15*, 321–333.
110. Mendell, J.T., and Olson, E.N. (2012). MicroRNAs in stress signaling and human disease. Cell *148*, 1172–1187.
111. Lai, X., Wolkenhauer, O., and Vera, J. (2016). Understanding microRNA-mediated gene regulatory networks through mathematical modelling. Nucleic Acids Res. *44*, 6019–6035.
112. Chu, K., and Tsai, M.-J. (2005). Neuronatin, a downstream target of BETA2/NeuroD1 in the pancreas, is involved in glucose-mediated insulin secretion. Diabetes *54*, 1064–1073.
113. Rodríguez-Comas, J., Moreno-Asso, A., Moreno-Vedia, J., Martín, M., Castaño, C., Marzà-Florensa, A., Bofill-De Ros, X., Mir-Coll, J., Montané, J., Fillat, C., et al. (2017). Stress-Induced MicroRNA-708 Impairs β-Cell Function and Growth. Diabetes *66*, 3029–3040.
114. Malhi, H. (2014). Micrornas IN ER STRESS: DIVERGENT ROLES IN CELL FATE DECISIONS. Curr. Pathobiol. Rep. *2*, 117–122.
115. Nunez Lopez, Y.O., Garufi, G., Pasarica, M., and Seyhan, A.A. (2018). Elevated and Correlated Expressions of miR-24, miR-30d, miR-146a, and SFRP-4 in Human Abdominal Adipose Tissue Play a Role in Adiposity and Insulin Resistance. Int J Endocrinol *2018*, 7351902.
116. Mentzel, C.M.J., Anthon, C., Jacobsen, M.J., Karlskov-Mortensen, P., Bruun, C.S., Jørgensen, C.B., Gorodkin, J., Cirera, S., and Fredholm, M. (2015). Gender and Obesity Specific MicroRNA Expression in Adipose Tissue from Lean and Obese Pigs. PLoS One *10*, e0131650.
117. Liu, G., Li, Y., and Gao, X.G. (2016). microRNA-181a is upregulated in human atherosclerosis plaques and involves in the oxidative stress-induced endothelial cell dysfunction through direct targeting Bcl-2. Eur Rev Med Pharmacol Sci *20*, 3092–3100.
118. Su, S.F., Chang, Y.W., Andreu-Vieyra, C., Fang, J.Y., Yang, Z., Han, B., Lee, A.S., and Liang, G. (2013). miR-30d, miR-181a and miR-199a-5p cooperatively suppress the endoplasmic reticulum chaperone and signaling regulator GRP78 in cancer. Oncogene *32*, 4694–4701.
119. Xu, G., Chen, J., Jing, G., Grayson, T.B., and Shalev, A. (2016). miR-204 Targets PERK and Regulates UPR Signaling and β-Cell Apoptosis. Mol. Endocrinol. *30*, 917–924.
120. Wang, X., Guo, B., Li, Q., Peng, J., Yang, Z., Wang, A., Li, D., Hou, Z., Lv, K., Kan, G., et al. (2013). miR-214 targets ATF4 to inhibit bone formation. Nat. Med. *19*, 93–100.
121. He, L., Tang, M., Xiao, T., Liu, H., Liu, W., Li, G., Zhang, F., Xiao, Y., Zhou, Z., Liu, F., et al. (2018). Obesity-Associated miR-199a/214 Cluster Inhibits Adipose Browning via PRDM16-PGC-1α Transcriptional Network. Diabetes *67*, 2585–2600.
122. Byrd, A.E., Aragon, I.V., and Brewer, J.W. (2012). MicroRNA-30c-2* limits expression of proadaptive factor XBP1 in the unfolded protein response. J. Cell Biol. *196*, 689–698.
123. Chhabra, R., Dubey, R., and Saini, N. (2011). Gene expression profiling indicate role of ER stress in miR-23a~27a~24-2 cluster induced apoptosis in HEK293T cells. RNA Biol. *8*, 648–664.
124. Gaddam, R.R., Kim, Y.-R., Li, Q., Jacobs, J.S., Gabani, M., Mishra, A., Promes, J.A., Imai, Y., Irani, K., and Vikram, A. (2020). Genetic deletion of miR-204 improves glycemic control despite obesity in db/db mice. Biochem. Biophys. Res. Commun. *532*, 167–172.
125. Belgardt, B.-F., Ahmed, K., Spranger, M., Latreille, M., Denzler, R., Kondratiuk, N., von Meyenn, F., Villena, F.N., Herrmanns, K., Bosco, D., et al. (2015). The microRNA-200 family regulates pancreatic beta cell survival in type 2 diabetes. Nat. Med. *21*, 619–627.
126. Zhu, Y., Sun, Y., Zhou, Y., Zhang, Y., Zhang, T., Li, Y., You, W., Chang, X., Yuan, L., and Han, X. (2019). MicroRNA-24 promotes pancreatic beta cells toward dedifferentiation to avoid endoplasmic reticulum stress-induced apoptosis. J. Mol. Cell Biol. *11*, 747–760.
127. Traverso, L.W., and Longmire, W.P. (1980). Preservation of the pylorus in

pancreaticoduodenectomy a follow-up evaluation. Ann. Surg. *192*, 306–310.
128. Schrader, H., Menge, B.A., Breuer, T.G.K., Ritter, P.R., Uhl, W., Schmidt, W.E., Holst, J.J., and Meier, J.J. (2009). Impaired glucose-induced glucagon suppression after partial pancreatectomy. J. Clin. Endocrinol. Metab. *94*, 2857–2863.
129. Van Cauter, E., Mestrez, F., Sturis, J., and Polonsky, K.S. (1992). Estimation of insulin secretion rates from C-peptide levels. Comparison of individual and standard kinetic parameters for C-peptide clearance. Diabetes *41*, 368–377.
130. Mezza, T., Sorice, G.P., Conte, C., Sun, V.A., Cefalo, C.M.A., Moffa, S., Pontecorvi, A., Mari, A., Kulkarni, R.N., and Giaccari, A. (2016). β-Cell Glucose Sensitivity Is Linked to Insulin/Glucagon Bihormonal Cells in Nondiabetic Humans. J. Clin. Endocrinol. Metab. *101*, 470–475.
131. Mari, A., Tura, A., Natali, A., Laville, M., Laakso, M., Gabriel, R., Beck-Nielsen, H., Ferrannini, E., and RISC Investigators (2010). Impaired beta cell glucose sensitivity rather than inadequate compensation for insulin resistance is the dominant defect in glucose intolerance. Diabetologia *53*, 749–756.
132. Madsen, O.D. (1987). Proinsulin-specific monoclonal antibodies. Immunocytochemical application as beta-cell markers and as probes for conversion. Diabetes *36*, 1203–1211.
133. Asadi, A., Bruin, J.E., and Kieffer, T.J. (2015). Characterization of antibodies to products of proinsulin processing using immunofluorescence staining of pancreas in multiple species. J. Histochem. Cytochem. *63*, 646–662.
134. Rodriguez-Calvo, T., Zapardiel-Gonzalo, J., Amirian, N., Castillo, E., Lajevardi, Y., Krogvold, L., Dahl-Jørgensen, K., and von Herrath, M.G. (2017). Increase in Pancreatic Proinsulin and Preservation of β-Cell Mass in Autoantibody-Positive Donors Prior to Type 1 Diabetes Onset. Diabetes *66*, 1334–1345.
135. Zinchuk, V., Zinchuk, O., and Okada, T. (2007). Quantitative colocalization analysis of multicolor confocal immunofluorescence microscopy images: pushing pixels to explore biological phenomena. Acta Histochem. Cytochem. *40*, 101–111.
136. Alarcon, C., Boland, B.B., Uchizono, Y., Moore, P.C., Peterson, B., Rajan, S., Rhodes, O.S., Noske, A.B., Haataja, L., Arvan, P., et al. (2016). Pancreatic β-Cell Adaptive Plasticity in Obesity Increases Insulin Production but Adversely Affects Secretory Function. Diabetes *65*, 438–450.
137. Gross, D.J., Halban, P.A., Kahn, C.R., Weir, G.C., and Villa-Komaroff, L. (1989). Partial diversion of a mutant proinsulin (B10 aspartic acid) from the regulated to the constitutive secretory pathway in transfected AtT-20 cells. Proc. Natl. Acad. Sci. USA *86*, 4107–4111.
138. Gold, G., Gishizky, M.L., Chick, W.L., and Grodsky, G.M. (1984). Contrasting patterns of insulin biosynthesis, compartmental storage, and secretion. Rat tumor versus islet cells. Diabetes *33*, 556–561.
139. Rhodes, C.J., and Halban, P.A. (1987). Newly synthesized proinsulin/insulin and stored insulin are released from pancreatic B cells predominantly via a regulated, rather than a constitutive, pathway. J. Cell Biol. *105*, 145–153.
140. Farack, L., Golan, M., Egozi, A., Dezorella, N., Bahar Halpern, K., Ben-Moshe, S., Garzilli, I., Tóth, B., Roitman, L., Krizhanovsky, V., et al. (2019). Transcriptional heterogeneity of beta cells in the intact pancreas. Dev. Cell *48*, 115–125.e4.
141. Sims, E.K., Chaudhry, Z., Watkins, R., Syed, F., Blum, J., Ouyang, F., Perkins, S.M., Mirmira, R.G., Sosenko, J., DiMeglio, L.A., et al. (2016). Elevations in the Fasting Serum Proinsulin-to-C-Peptide Ratio Precede the Onset of Type 1 Diabetes. Diabetes Care *39*, 1519–1526.
142. Tran, D.T., Pottekat, A., Mir, S.A., Loguercio, S., Jang, I., Campos, A.R., Scully, K.M., Lahmy, R., Liu, M., Arvan, P., et al. (2020). Unbiased profiling of the human proinsulin biosynthetic interaction network reveals a role for peroxiredoxin 4 in proinsulin folding.

Diabetes *69*, 1723–1734.
143. Gonzalez-Gronow, M., Selim, M.A., Papalas, J., and Pizzo, S.V. (2009). GRP78: a multifunctional receptor on the cell surface. Antioxid. Redox Signal. *11*, 2299–2306.
144. Lee, A.-H., Heidtman, K., Hotamisligil, G.S., and Glimcher, L.H. (2011). Dual and opposing roles of the unfolded protein response regulated by IRE1alpha and XBP1 in proinsulin processing and insulin secretion. Proc. Natl. Acad. Sci. USA *108*, 8885–8890.
145. Tsuchiya, Y., Saito, M., Kadokura, H., Miyazaki, J.-I., Tashiro, F., Imagawa, Y., Iwawaki, T., and Kohno, K. (2018). IRE1-XBP1 pathway regulates oxidative proinsulin folding in pancreatic β cells. J. Cell Biol. *217*, 1287–1301.
146. Zhang, L., Lai, E., Teodoro, T., and Volchuk, A. (2009). GRP78, but Not Protein-disulfide Isomerase, Partially Reverses Hyperglycemia-induced Inhibition of Insulin Synthesis and Secretion in Pancreatic {beta}-Cells. J. Biol. Chem. *284*, 5289–5298.
147. Jang, I., Pottekat, A., Poothong, J., Yong, J., Lagunas-Acosta, J., Charbono, A., Chen, Z., Scheuner, D.L., Liu, M., Itkin-Ansari, P., et al. (2019). PDIA1/P4HB is required for efficient proinsulin maturation and ß cell health in response to diet induced obesity. Elife *8*,.
148. He, K., Cunningham, C.N., Manickam, N., Liu, M., Arvan, P., and Tsai, B. (2015). PDI reductase acts on Akita mutant proinsulin to initiate retrotranslocation along the Hrd1/Sel1L-p97 axis. Mol. Biol. Cell *26*, 3413–3423.
149. Hodish, I., Absood, A., Liu, L., Liu, M., Haataja, L., Larkin, D., Al-Khafaji, A., Zaki, A., and Arvan, P. (2011). In vivo misfolding of proinsulin below the threshold of frank diabetes. Diabetes *60*, 2092–2101.
150. Huang, H., Yang, K., Wang, R., Han, W.H., Kuny, S., Zelmanovitz, P.H., Sauvé, Y., and Chan, C.B. (2019). β-Cell compensation concomitant with adaptive endoplasmic reticulum stress and β-cell neogenesis in a diet-induced type 2 diabetes model. Appl Physiol Nutr Metab *44*, 1355–1366.
151. Arunagiri, A., Haataja, L., Pottekat, A., Pamenan, F., Kim, S., Zeltser, L.M., Paton, A.W., Paton, J.C., Tsai, B., Itkin-Ansari, P., et al. (2019). Proinsulin misfolding is an early event in the progression to type 2 diabetes. Elife *8*,.
152. Zhou, Y., Lee, J., Reno, C.M., Sun, C., Park, S.W., Chung, J., Lee, J., Fisher, S.J., White, M.F., Biddinger, S.B., et al. (2011). Regulation of glucose homeostasis through a XBP-1-FoxO1 interaction. Nat. Med. *17*, 356–365.
153. Price, N.L., Singh, A.K., Rotllan, N., Goedeke, L., Wing, A., Canfrán-Duque, A., Diaz-Ruiz, A., Araldi, E., Baldán, Á., Camporez, J.-P., et al. (2018). Genetic Ablation of miR-33 Increases Food Intake, Enhances Adipose Tissue Expansion, and Promotes Obesity and Insulin Resistance. Cell Rep. *22*, 2133–2145.
154. Le, A., Llaurad, G., and Altuna, A. Altered Expression of miR-181a-5p and miR-23a-3p Is Associated With Obesity and TNF a-Induced Insulin Resistance. Coy.
155. Xu, G., Chen, J., Jing, G., and Shalev, A. (2013). Thioredoxin-interacting protein regulates insulin transcription through microRNA-204. Nat. Med. *19*, 1141–1146.

Curriculum Vitae

PERSONAL INFORMATIONS

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PROFESSIONAL EXPERIENCE

01/10/2017 - to the current date: PhD student in Medical Biotechnologies; University of Siena, Siena (Italy)

Research experience:

- Study of the role of microRNA differentially expressed in human pancreatic islets isolated through laser microdissection (LCM) from multiorgan donors with type 2 diabetes mellitus and type 1 diabetes mellitus.
- Identification and characterization of beta-cell dedifferentiation-associated microRNAs in diabetes.
- To investigate the molecular mechanisms that regulate the transcription of microRNAs in beta cells under both physiological and pathophysiological conditions, involved in the function and in the maintenance of beta cell phenotype.
- Histological and molecular analysis related to intracellular alteration of proinsulin *in-situ* and metabolic dysfunction typical of type 2 diabetes.
- Processing and collection of blood samples and data from newly diagnosed patients with type 1 diabetes and their first-degree relatives in order to identify microRNAs as diagnostic and prognostic biomarkers of disease.

EDUCATION AND TRAINING

- Qualification as a Professional Biologist, University of Siena, II session 2018

- 3/11/2014–17/02/2017: Master's Degree in Molecular and Cellular Biology; University of Siena, Siena (Italy). Degree thesis entitled: “Regulation of miR-184 mediated by NKX6.1: a new beta cell protection mechanism in type 2 diabetes”. Vote 110/110

- 01/10/2010–11/04/2014: Bachelor's Degree in Biology; University of Siena, Siena (Italy). Thesis title: “Analysis of the expression profile of endocrine-pancreatic genes and microRNAs in pancreatic islets isolated trough laser capture microdissection (LCM)”. Vote: 98/110

MEMBERSHIP ASSOCIATIONS

- Italian Society of Diabetology (SID), from 2018
- YoSID (Young of SID), from 2018

SCIENTIFIC PUBLICATIONS

h-index: 4
Tot. Citations: 46
Source: Scopus (Updated 4/02/2021)

Grieco, G.E., **Brusco, N.**, Nigi, L., Formichi, C., Fignani, D., Licata, G., Marselli, L., Marchetti, P., Salvini, L., Tinti, L., Po, A., Ferretti, E., Sebastiani, G., Dotta, F. Reduced miR-184-3p expression occurring in Type 2 diabetic pancreatic islets protects β-cells from lipotoxic and proinflammatory apoptosis via a CRTC1-dependent mechanism. BioRxiv. 2021 January

Grieco, G.E., **Brusco, N.**, Licata, G., Fignani, D., Formichi, C., Nigi, L., Sebastiani, G., and Dotta, F. (2021). The Landscape of microRNAs in βCell: Between Phenotype Maintenance and Protection. Ijms 22, 803.

Marie Eliane Azoury, Mijke Buitinga, Fatoumata Samassa, Laura Nigi, **Noemi Brusco**, Matthieu Giraud, Aïsha Callebaut, Ana Ines Lalanne, Alexia Carré, Zhicheng Zhou, Barbara Brandao, Maikel L. Colli, Guido Sebastiani, Francesco Dotta, Maki Nakayama, Decio L. Eizirik, Sylvaine You1, Sheena Pinto, Mark J. Mamula, Yann Verdier, Joelle Vinh, Soren Buus, Chantal Mathieu, Lut Overbergh and Roberto Mallone. CD8+ T cells variably recognize native versus citrullinated GRP78 islet epitopes in type 1 diabetes" (our reference 145593-JCI-RG-1). In revision to the Journal of Clinical Investigation. 2020 November

Fignani, D., Licata, G., **Brusco, N.**, Nigi, L., Grieco, G.E., Marselli, L., Overbergh, L., Gysemans, C., Colli, M.L., Marchetti, P., et al. (2020). SARS-CoV-2 Receptor Angiotensin I-Converting Enzyme Type 2 (ACE2) Is Expressed in Human Pancreatic β-Cells and in the Human Pancreas Microvasculature. Front. Endocrinol. (Lausanne) 11, 596898.

Grieco, G.E., Sebastiani, G., Eandi, C.M., Neri, G., Nigi, L., **Brusco, N.**, D'Aurizio, R., Posarelli, M., Bacci, T., Benedetto, E.D., et al. (2020). MicroRNA Expression in the Aqueous Humor of Patients with Diabetic Macular Edema. Int. J. Mol. Sci. 21.

Nigi, L., **Brusco, N.**, Grieco, G.E., Licata, G., Krogvold, L., Marselli, L., Gysemans, C., Overbergh, L., Marchetti, P., Mathieu, C., et al. (2020). Pancreatic Alpha-Cells Contribute Together With Beta-Cells to CXCL10 Expression in Type 1 Diabetes. Front. Endocrinol. (Lausanne) 11, 630.
Grieco, G.E., **Brusco, N**., Licata, G., Nigi, L., Formichi, C., Dotta, F., and Sebastiani, G. (2019). Targeting microRNAs as a Therapeutic Strategy to Reduce Oxidative Stress in Diabetes. Int. J. Mol. Sci. 20.

Grieco, G.E., **Brusco N**., Pellegrini S. (2019). Dedifferentiation and cell β differentiation: molecular mechanisms and strategies for restoring β cells in diabetes. The Diabetes 31.

Grieco, G.E., Cataldo, D., Ceccarelli, E., Nigi, L., Catalano, G., **Brusco, N**., Mancarella, F., Ventriglia, G., Fondelli, C., Guarino, E., et al. (2018). Serum Levels of miR-148a and miR-21-5p Are Increased in Type 1 Diabetic Patients and Correlated with Markers of Bone Strength and Metabolism. Noncoding RNA 4.

Nigi, L., Grieco, G.E., Ventriglia, G., **Brusco, N.**, Mancarella, F., Formichi, C., Dotta, F., and Sebastiani, G. (2018). Micrornas as regulators of insulin signaling: research updates and potential therapeutic perspectives in type 2 diabetes. Int. J. Mol. Sci. 19.

Sebastiani, G., Grieco, G.E., **Brusco, N.**, Ventriglia, G., Formichi, C., Marselli, L., Marchetti, P., and Dotta, F. (2018). MicroRNA Expression Analysis of In Vitro Dedifferentiated Human Pancreatic Islet Cells Reveals the Activation of the Pluripotency-Related MicroRNA Cluster miR-302s. Int. J. Mol. Sci. 19.

CONFERENCES

N.Brusco, G.Licata, G.E.Grieco, D.Fignani, L.Nigi, E.Aiello, F.Cinti, G.P.Sorice, S.Moffa, C.M.A.Cefalo, A.Mari, A.Giaccari, T.Mezza, G.Sebastiani, F.Dotta. Altered *in-situ* expression of proinsulin-insulin in pancreatic islets reflects metabolic and molecular defects in Type 2 Diabetic and Glucose Intolerant living donors. nPOD 22-24 February 2021 (Poster).

Brusco N., Sebastiani G., Licata G., Grieco G.E., Nigi L., Cinti F., Sorice G.P., Moffa S., Cefalo C.M.A, Mari A., Dotta F., Giaccari A., Mezza T. L'espressione *in-situ* di proinsulina e insulina nelle isole pancreatiche correla con i difetti metabolici nei donatori con diabete di tipo 2 e intolleranti al glucosio. SID, 2-5 Dicember 2020 Rimini, Italy. (Poster)

Brusco N., Sebastiani G., Licata G., Grieco G.E., Nigi L., Fignani D., Moffa S., Sorice G.P., G.Di Giuseppe., Cefalo C.M.A, Mari A., Dotta F., Giaccari A. and Mezza T. Proinsulin-Insulin pancreatic islets *in-situ* expression mirrors metabolic defects observed in type 2 diabetic and glucose intolerant living donors. ADA 2020. (Poster)

Brusco N., Sebastiani G., Licata G., Grieco G.E., Nigi L., Cinti F., Sorice G.P., Moffa S., Cefalo C.M.A, Mari A., Dotta F., Giaccari A., Mezza T. Proinsulin-Insulin pancreatic islets in-situ

expression mirrors metabolic defects observed in Type 2 diabetic and glucose intolerant living donors. EASD, 16-20 September 2019 Barcelona, Spain. (Oral presentation)

Brusco N., Grieco G.E., Ventriglia G., Nigi L., Mancarella F., Marselli L., Marchetti P., Sebastiani G., Dotta F**.** Immunofluorescence imaging analysis and Laser Capture Microdissection of pancreatic islets from T2D donors reveal beta-cell NKX6.1 nucleus to cytoplasm translocation and miR-184-3p reduced expression. SIE, 29-31 May 1 June 2019, Roma, Italy. (Oral presentation)

Brusco N., Grieco G.E., Ventriglia G., Nigi L., Mancarella F., Marselli L., Marchetti P., Sebastiani G., Dotta F. Pancreatic islets from T2D donors are characterized by NKX6.1 nucleus-to-cytoplasm translocation and miR-184-3p reduced expression possibly contributing to β-cell dedifferentiation. JDRF-nPOD, 19-22 February 2019, Hollywood, Florida. (Poster)

Brusco N., Grieco G.E., Mancarella F., Nigi L., Ventriglia G., Marselli L., Marchetti P., Sebastiani G., Dotta F. Trascrizione del miR-184 mediata da NKX6.1: meccanismo di protezione β-cellulare nel DM2. SID 16-19 May 2018, Rimini, Italy. (Poster)

SCIENTIFIC AWARDS

- Italian Society of Diabetology (SID): EASD-2019 Travel Grant

Award description: travel grant awarded by the Italian Diabetes Society (SID) for participation in the European EASD congress (European Association for the Study of Diabetes) in which Dr. N Brusco's research work was selected for oral communication " Proinsulin-Insulin pancreatic islets in-situ expression mirrors metabolic defects observed in Type 2 diabetic and glucose intolerant living donors”. EASD, 16-20 September 2019 Barcelona, Spain.