PREDIABETES IS CHARACTERIZED BY AN INCREASE IN INFLAMMATION AND ISLET DYSFUNCTION

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INTRODUCTION

- Many individuals will be diagnosed first with prediabetes and then Type 2 Diabetes (T2D) later.
- Prediabetes is a condition in which the person does not have normoglycemia; however, their blood glucose levels are not high enough to be diagnosed with T2D.
- People with prediabetes have a high lifetime risk (70% frequency) of prediabetes converting into T2D.[1]
- Inflammation caused by the pro-inflammatory cytokines Interleukin-1β (IL-1β), Tumor necrosis factor-alpha (TNF-α), and Interleukin-6 (IL-6) impacts the development of T2D.[2]
- Studies have shown increases in these inflammatory markers, including TNF-α and IL-6, in serum samples from people with prediabetes.[3]
- People with prediabetes have been shown to have similar risk factors as those individuals with T2D including increased triglycerides and lipoproteins, hypertension, and obesity.[1,4]
- A link between adipose tissue, inflammation, and diabetes has been suggested because adipocytes can secrete adipokines, including Resistin.[5,6,7]
- Immune cells, including M1 and M2 macrophages, may be another source of inflammation and play a role in T2D development.
- M1 cells (or CD68+ cells) express pro-inflammatory cytokines IL-1β, TNF-α, IL-6, and Monocyte Chemoattractant Protein-1 (MCP-1) while M2 cells (or CD163+ cells) are anti-inflammatory or anti-diabetes mediators.[8,9]
- Several studies have found increases in islets infiltrated with CD68+ and CD163+ cells in T2D patients compared with non-diabetic patients.[9,10]

The objective of this study was to compare inflammatory gene and protein expression between normal donors and donors with prediabetes using pancreata and islets. Viability and glucose stimulated insulin secretion (GSIS) were also compared between the two types of donors.

METHODS

Culture of human islets

- Islets were isolated from donor tissue consented for research purposes (LifeNet Health, VA Beach, VA) or received from the Diabetes Research Institute (U of Miami Leonard M. Miller School of Medicine, Miami, FL) and cultured in CMRL-1066 medium supplemented with 10% fetal bovine serum and 1% ciproflaxin at 22ºC in 5% CO_2 overnight in order to recover from isolation or shipping.

Classification of Donors

- Donors were classified according to the American Diabetes Association (ADA) standards for having prediabetes. Donors having an A1C between 5.7% (39 mmol/mol) to 6.4% (47 mmol/mol) were classified as having prediabetes. Donors who had an A1C below 5.7% (39 mmol/mol) were considered normal.

TABLE 1. *Characteristics of normal donors and donors with Prediabetes.*

Donor #	Sex	Age (y/o)	BMI (kg/m²)	Ethnicity	COD	HbA1c value
Normal						
1	Male	42	24.31	Caucasian	Head Trauma	4.7
2	Male	35	23.27	African Am.	Trauma	5.1
3	Male	50	23.41	Caucasian	Anoxia	5.5
4	Female	46	33.91	Caucasian	Stroke	5.2
5	Male	39	29.9	Caucasian	Trauma	5.4
6	Male	48	24.4	Hispanic	Head Trauma	5.1
7	Female	53	20.8	Caucasian	Head Trauma	5.4
Avg		45 ± 6.4	25.7 ± 4.5			5.20 ± 0.27
With Prediabetes						
1	Male	49	21.82	African Am.	ICH	5.7
2	Female	44	24.27	Caucasian	DCD	6.3
3	Female	60	24.18	Caucasian	DCD	5.7
4	Female	50	24.7	Caucasian	Anoxia	5.8
5	Male	50	31.8	Caucasian	Stroke	5.8
Avg		51 ± 5.8***	25.4 ± 3.8			5.86 ± 0.25**

Real-Time PCR

- 100 islets per donor were collected and lysed in RLT buffer (Qiagen, Valencia, CA). Total RNA was isolated using the Rneasy Mini kit as per the manufacturer's instructions. cDNA was prepped, and RT-PCR reactions were prepared using the QuantiNova SYBR Green RT-PCR kit (Qiagen). PCR amplification was done on a StepOnePlus Real Time PCR System (Applied Biosystems, Foster City, CA), and analyzed with StepOne Software (Applied Biosystems). Data were normalized to GAPDH and analyzed using the $2^{-\Delta\Delta C}{}_T$ method.

Glucose Stimulated Insulin Secretion (GSIS)

- 100 islets per donor were incubated at 37ºC for 1 hour in serum-free Krebs-Ringer buffer. They were then placed in Krebs buffer plus 2.8mM glucose (low) for 1 hour at 37ºC and transferred to Krebs buffer plus 28mM glucose (high) for 1 hour at 37ºC. Samples of the media were collected at the 0 time point and the 1 hour time point.
- Insulin levels were measured by ELISA (Mercodia, Winston Salem, NC) per the manufacturer's instructions.

Immunohistochemistry

- Biopsies were taken from normal and prediabetic pancreata (LifeNet Health) then fixed, embedded in paraffin, and sectioned. These sections were then deparaffinized and heat based antigen retrieval was performed (Dako, Santa Clara, CA).
- Samples were blocked and primary antibody was added for incubation overnight at 4ºC. Samples were then washed, and DAB detection was done. Sections were counterstained with hematoxylin (Thermo Fisher Scientific, Waltham, MA).
- Samples were mounted and imaged using a BX41 microscope (Olympus, Tokyo, Japan).

Immunofluorescence

- Samples were obtained, embedded in paraffin, and sectioned. Heat based antigen retrieval was performed.
- Sections were blocked and primary antibody (Abcam, Cambridge, MA) was added overnight at 4ºC at either a 1:100 or 1:50 dilution.
- Samples were washed and secondary antibody (Thermo Fisher Scientific) was added at a 1:1000 dilution for 30 minutes at room temperature.
- DAPI was added at a 1:2000 dilution (Thermo Fisher Scientific), and then the sections were mounted.
- Visualization of sections occurred using a Zeiss Observer.Z1 fluorescent microscope. Images were acquired with Z-stack using a Zeiss ApoTome.2 on the 20X objective lens.

RESULTS

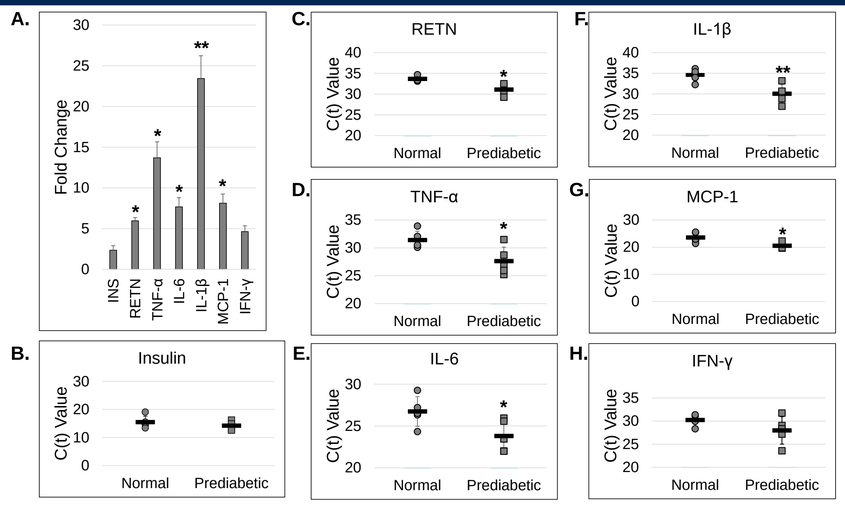

FIGURE 1. *(A) Graph represents fold change in gene expression relative to normal islets. Gene expression of (B) insulin, (C) resistin (RETN), (D) TNF-α, (E) IL-6, (F) IL-1β, (G) MCP-1, and (H) Interferon-γ (IFN-γ) from islets of normal donors and donors with prediabetes. Error bars represent standard deviation. *p<0.05, **p<0.01 to islets isolated from normal donors by student's t-test.*

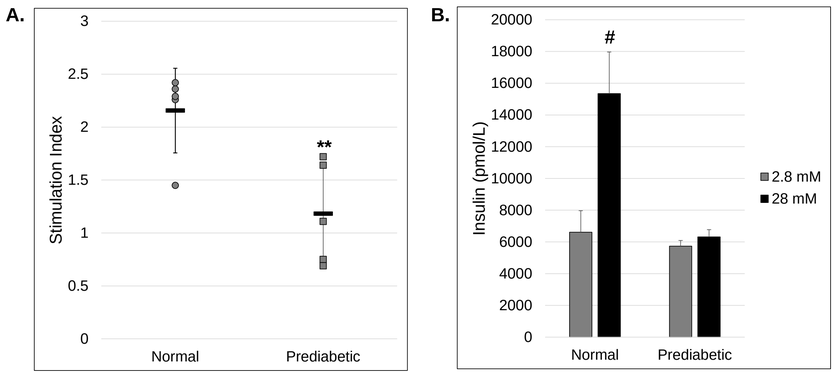

FIGURE 2. *(A) The Stimulation Index (SI) was calculated for islets from normal donors and donors with prediabetes using the ratio of insulin secretion in 28mM of glucose over insulin secretion in 2.8mM of glucose. (B) Graph showing insulin secretion in normal islets and prediabetic islets treated with low glucose (2.8mM, grey bars) and high glucose (28mM, black bars). Error bars represent standard deviation. **p<0.01 to islets isolated from normal donors; #p<0.05 compared to low glucose (2.8mM) by student's t-test.*

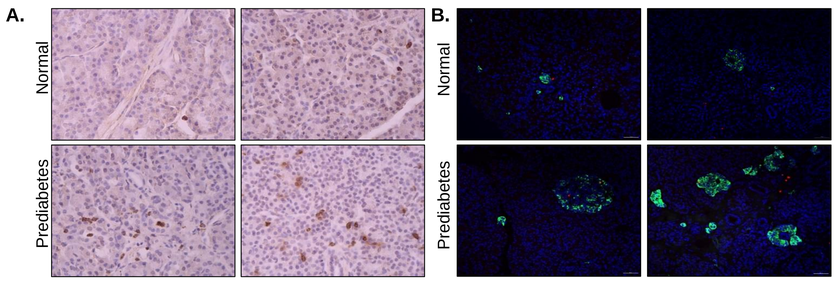

FIGURE 3. *(A) Resistin staining in pancreata from normal donors and donors with prediabetes. Positive expression is indicated by dark brown staining. No staining was seen when DAB only was added (data not shown). (B) IFC showing Resistin (red) and Insulin (green) expression in pancreata from normal donors and donors with prediabetes. Secondary antibody only staining was performed (data not shown). Graphs show quantification of (C) Resistin and (D) Insulin expressed as Relative Fluorescent Units (RFU) from normal and prediabetic pancreata. Error bars represent standard deviation. **p<0.01 to normal donors. Representative images are shown. All images are 20X.*

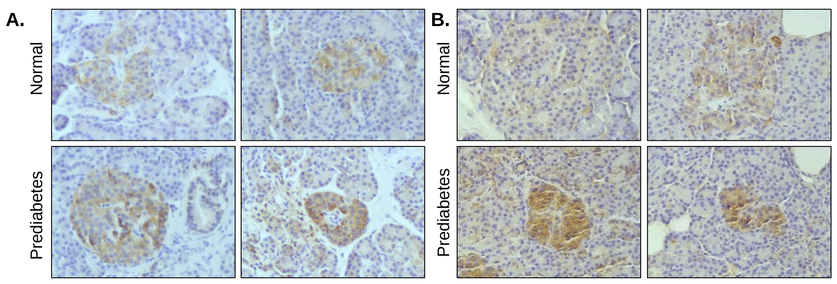

FIGURE 4. *Representative images of (A) TNF-α and (B) IL-6 staining in pancreata from normal donors and donors with prediabetes. All images are 20X.*

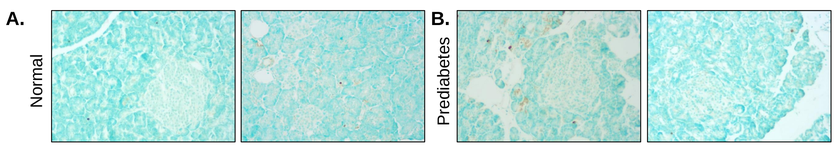

FIGURE 5. *TUNEL staining was performed on sections of pancreata from (A) normal donors and (B) donors with prediabetes in order to detect any apoptotic nuclei (indicated by dark brown staining). Representative images are shown. All images are 10X. The TUNEL Assay Kit-HRP-DAB (Abcam) was used according to the manufacturer's instructions.*

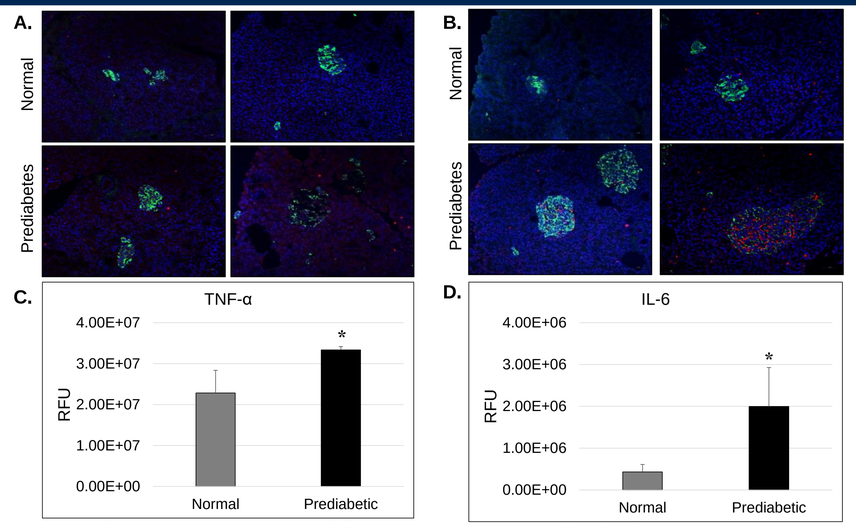

FIGURE 6. *Representative IFC images of (A) TNF-α (red) and Insulin (green) and (B) IL-6 (red) and Insulin (green) in normal and prediabetic pancreata. Graphs represent quantification of (C) TNF-α and (D) IL-6 expression shown as Representative Fluorescent Units (RFU). Error bars represent standard deviation. *p<0.05 to normal pancreata. All images are 20X.*

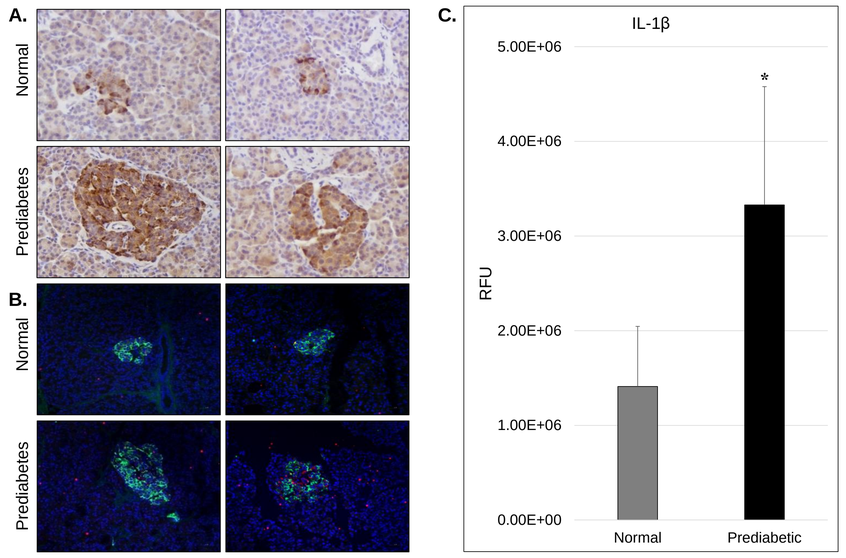

FIGURE 7. *Representative images of (A) IL-1β staining in pancreata from normal donors and donors with prediabetes. (B) IFC images of IL-1β (red) co-localized with Insulin (green) in normal and prediabetic islets. (C) Graph shows quantification of IL-1β expression shown as Relative Fluorescent Units (RFU). Error bars represent standard deviation. *p<0.05 to normal pancreata. All images are 20X.*

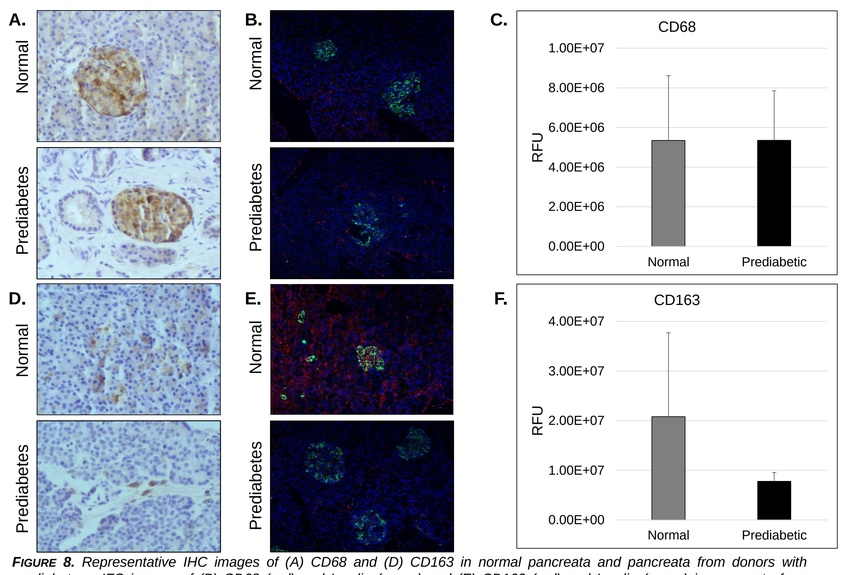

FIGURE 8. *Representative IHC images of (A) CD68 and (D) CD163 in normal pancreata and pancreata from donors with prediabetes. IFC images of (B) CD68 (red) and Insulin (green) and (E) CD163 (red) and Insulin (green) in pancreata from normal donors and donors with prediabetes. Quantification of (C) CD68 and (F) CD163 shown as Relative Fluorescent Units (RFU). All images are 20X.*

CONCLUSIONS

- **Gene and protein expression of Resistin, IL-1β, TNF-α, and IL-6 significantly increased in donors with prediabetes when compared to normal donors. However, there was no difference in viability between the two types of donors.**
- **Although there is no difference in insulin gene expression, GSIS was significantly decreased in islets from prediabetic donors.**
- **Expression of CD163+ but not CD68+ cells decreased in donors with prediabetes. This suggests a decrease in the level of anti-inflammation protection which may then allow an excess of inflammation to occur.**
- **Inflammation and islet dysfunction may be more significant than originally thought in people with prediabetes. Further research needs to be done to determine the trigger for inflammation which can lead to better intervention of prediabetes and prevention of T2D.**

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