

Research Article

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A Comprehensive Analysis to Study the Histological and Immunohistochemical Profile of Diabetic and Non-Diabetic Human Pancreases in the Indian Population

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ABSTRACT

The pathogenesis of hyperglycemia observed in most forms of diabetes is intimately tied to the islet β cell. Impairments in pro-peptide processing and secretory function, along with the loss of these vital cells, is demonstrable not only in those in whom the diagnosis is established but typically also in individuals who are at increased risk of developing the disease. Due to ethical and practical difficulties, genomic or pathological data in Indian population are remarkably missing. Here we report the immunohistological observations for 21 pancreases. We analyzed the pathological differences between diabetic and non-diabetic samples along with pathological changes associated with beta cell density, expression, size, and diameter. This study provides an insight into the immunopathological and histochemical mechanisms that highlight a number of inflammatory, immunoregulatory and regenerative pathways, some of which have received relatively little attention so far.

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Introduction

In all vertebrates, maintenance of normal blood sugar levels is vital for life. The hormone, insulin, regulates glucose homeostasis at major metabolic sites like liver, muscle and adipose tissue. The pancreatic endocrine cells (α - and β -cells) predominantly regulate glucose homeostasis in an antagonistic manner. While α -cells respond to hypoglycemia with the release of glucagon resulting in the elevation of circulating glucose, β -cells secrete insulin at hyper-glycemic conditions leading to the reduction of plasma glucose levels. The efficiency of α - and β -cells to regulate plasma glucose levels relies on their ability of detecting changes in the extracellular glucose levels. The α - and β -cells have special glucose transporters which continuously monitor extracellular concentrations of glucose and aid in quick adaptation during hormone secretion [1-3]. A substantial insight on the regulation of insulin secretion and beta-cell mass through various signaling pathways is essential in developing novel approaches for type 1 diabetes. And such therapeutic interventions include beta cell maintenance and replacement, islet transplantation, and stem cell therapy.

Beta cells are unique endocrine cells that synthesize, store and secrete insulin under the control of multiple and integrated signals,

thus tightly regulating blood glucose concentrations. In the beta cell, insulin secretion stimulated by glucose is a multistep process. Direct assessment of expression of genes from freshly isolated islets in relation to some of the major underlying mechanisms and simultaneous examination of pancreatic tissue will provide us some clue for the future research for conceiving better prevention and treatment of diabetes by targeting pancreatic beta cells.

Evidence mounts in support of transplanting cadaveric human islets as an effective therapeutic mode for type 1 diabetes. Then again, evaluating the suitability of islet samples in a clinical setting is quite challenging. In this research paper, we propose to understand the expression, regulation and function of normal and diabetes beta-cell function and beta-cell mass regulation, and related signals in the beta-cell milieu. To interpret the pathology more clearly and to advance in our understanding of the processes that leads to expression and regulation, it is necessary to have an extensive description of every change at the molecular level *in situ* [4-7].

Materials and Methods

Twenty-one human pancreases were collected according to protocols approved by Apollo Hospital and Velammal Medical College and Hospital. Our protocol was in accordance with the Indian Council of Medical Research' guidelines, and it abided by the Indian organ procurement program. Human adult pancreases

were provided by Velammal Medical College and Hospital with consent to use for research purposes.

As soon as the tissue samples reached the laboratory they were divided into different portions depending on the weight of the pancreas received. Approximately three-quarters of each gland underwent digestion, while one-quarter was cut into small cubes in which one part was snap-frozen and kept in liquid nitrogen until RNA extraction. The other part was used for immunohistology studies.

The pancreata were harvested from the sacrificed rats after dissection, and were weighed and washed with saline. The specimens were stretched on filter paper and fixed in 10% buffered formalin (pH 7.4). The fixed specimens were sliced, processed, and embedded into paraffin blocks. The blocks were cut into 4 μ m paraffin sections by a rotator microtome. The sections were stained with Hematoxylin and Eosin (H&E) and with Masson trichrome stains. The pancreata were harvested from the sacrificed rats after dissection, and were weighed and washed with saline. The specimens were stretched on filter paper and fixed in 10% buffered formalin (pH 7.4). The fixed specimens were sliced, processed, and embedded into paraffin blocks. The blocks were cut into 4 μ m paraffin sections by a rotator microtome. The sections were stained with Hematoxylin and Eosin (H&E) and with Masson trichrome stains

Immunohistology

A surgically removed human tissue specimen was obtained and washed with saline. The specimens were fixed in 10% buffered formalin (pH 7.4). And then, the fixed specimens were subjected to slicing and processing, before being embedded in paraffin blocks. The blocks were cut into 4 μ m paraffin sections by a rotator microtome. Hematoxylin and Eosin (H&E) stain manufactured by Sigma-Aldrich Pty Ltd, an affiliate of Merck KGaA, Darmstadt, Germany) were used to stain these sections.

The pancreata were harvested from the sacrificed rats after dissection, and were weighed and washed with saline. The specimens were stretched on filter paper and fixed in 10% buffered formalin (pH 7.4). The fixed specimens were sliced, processed, and embedded into paraffin blocks. The blocks were cut into 4 μ m paraffin sections by a rotator microtome. The sections were stained with Hematoxylin and Eosin (H&E) and with Masson trichrome stains [8]. The pancreata were harvested from the sacrificed rats after dissection, and were weighed and washed with saline. The specimens were stretched on filter paper and fixed in 10% buffered formalin (pH 7.4). The fixed specimens were sliced, processed, and embedded into paraffin blocks. The blocks were cut into 4 μ m paraffin sections by a rotator microtome. The sections were stained with Hematoxylin and Eosin (H&E) and with Masson trichrome stains [8].

Immunohistochemistry

Three-micrometer-thick paraffin sections were mounted on positively charged slides and subjected to the immunohistochemical (IHC) procedure using a direct detection system (BioScience Bio SB, CA, USA). Sections were incubated with insulin antibody (manufactured by Cell Signaling Technology, Inc. Alexa Fluor, a registered trademark of Life Technologies Corporation, headquartered in Danvers, Massachusetts, USA) for 30 min at room temperature followed by secondary HRP antibody. The IHC procedure was performed by manual method following the manufacturer's instructions. A light microscope (BX51, Olympus,

Tokyo, Japan) with an in-built 'Olympus' digital camera was used to examine and photograph the slides.

Real Time RT-PCR

Total RNA from the tissue was extracted and isolated with TRI Reagent (Sigma). We assessed RNA quantity and purity by using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and RNA was measured. We synthesized cDNA templates from RNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time Polymerase Chain Reaction/PCR was performed with TaqMan Fast Advanced Master Mix (Applied Biosystems), where in each well 10-ng cDNA and 1 μ L of TaqMan Gene Expression Assay (Applied Biosystems) were used. 100 ng of RNA was used to perform RT-PCR on a Bio-Rad Gene Cycler. For each sample, we performed triplicate amplifications and used average measurements for data analysis.

Statistics

All the statistical analysis was performed using Graph-pad prism (Version 6). All the data that followed normality were expressed as Mean \pm standard deviation, while Median was also considered for analysis where data deviated from normality. To determine the statistical significance between two unrelated groups (diabetic and non-diabetic), inferential tests (both parametric and non-parametric) like Independent T test, Mann-Whitney U test, Fishers' test and Linear by linear association test were used. The p-value has been chosen at 0.05 to test for significance in all tests, which means we established the 95% confidence intervals and 5% level of significance. Pearson correlation was done between the measured variables with $p < 0.05$ being considered as statistically significant. Rstudio version 2023.03.0+386 'Cherry Blossom' was used for the analysis.

Results

General Profile of the Study Population

In the study population, non-diabetic subjects out-numbered their counterparts, and the following table (Table 1) and figure (Figure 1) support this statement.

Table 1: Distribution of Diabetic and Non-Diabetic Subjects in the Study Population

| Category | Non-Diabetic | Diabetic | Total |
|----------------|--------------|----------|-------|
| Number | 12 | 9 | 21 |
| Percentage (%) | 57.1 | 42.9 | 100 |

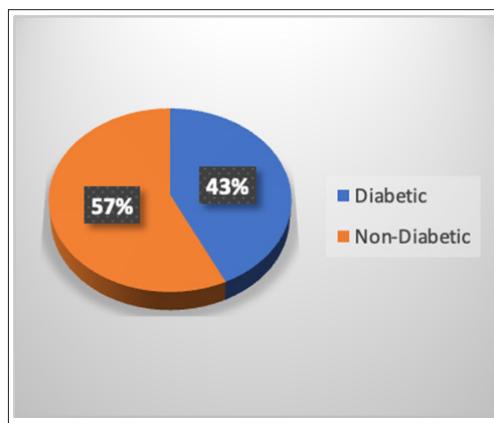


Figure 1: Distribution of Diabetic and Non-Diabetic Subjects in the Study Population

As shown in Table 2, a majority of the study population (38%) were in the age group of '46 to 60' years with second-in-line being the 'less than 30' age group (33.3%). With regard to gender, males were predominant in the study population (71.4%).

Table 2: Age and Gender Distribution of the Study Population

| Category | Age (years) | | | | | Gender | | |
|---------------------|-------------|----------|----------|------|-------|--------|--------|-------|
| | < 30 | 31 to 45 | 46 to 60 | > 60 | Total | Male | Female | Total |
| Number (total = 21) | 7 | 4 | 8 | 2 | 21 | 15 | 6 | 21 |
| Percentage (%) | 33.3 | 19 | 38.1 | 9.5 | 100 | 71.4 | 28.6 | 100 |

With regard to blood group, it is explicit with Table 3, that, a majority (42.9%) were B+ve followed by O+ve (28.6 %) and A+ve (23.8%). The following table (Table 3) and figure (Figure 2) reveal that a majority of the study population (47.6 %) were having HbA1C levels between 5.6% to 8.0%; followed by 38% having a value of less than 5.5%; while 14.4 % had the levels in the range of 8.1% to 10.0%.

Table 3: Characteristics Like Blood Group and HbA1c of the Study Population

| Category | Blood group | | | | HbA1c (%) | | | |
|---------------------|-------------|------|------|-------|-----------|-----------|------------|-------|
| | A+ve | B+ve | O+ve | Total | < 5.5 | 5.6 - 8.0 | 8.1 - 10.0 | Total |
| Number (total = 21) | 5 | 9 | 7 | 21 | 8 | 10 | 3 | 21 |
| Percentage (%) | 23.8 | 42.9 | 33.4 | 100 | 38 | 47.6 | 14.4 | 100 |

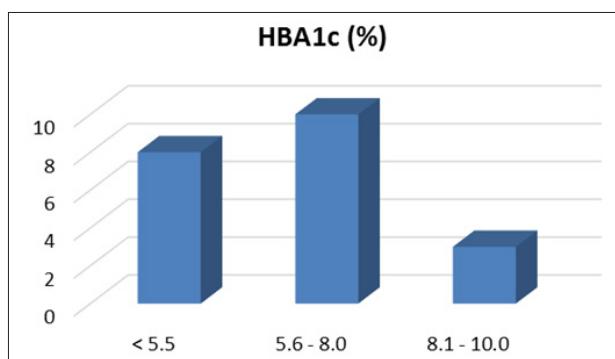


Figure 2: HbA1c Levels of the Study Population

Comparison between the Groups for General Profile

There was no statistically significant difference between the diabetic and non-diabetic groups with regard to parameters like, age, gender and blood group. This observation is supported by the following table (Table 4).

Table 4: Comparison between the Groups for General Profile

| Variables | | Diabetic | Non-Diabetic | P-Value |
|-------------|---------------------|--------------|--------------|---------|
| Age | Mean (SD) | 42.1 (16.07) | 42.8 (15.64) | .92@ |
| | Median (Min, Max) | 40 (18, 67) | 41 (24, 76) | |
| | Standard Error (SE) | 5.08 | 4.18 | |
| Gender | Male | 6 | 9 | .393* |
| | Female | 4 | 2 | |
| Blood Group | O+ve | 5 | 2 | .176# |
| | A+ve | 2 | 3 | |
| | AB-ve | 0 | 0 | |
| | B+ve | 3 | 6 | |

@ Independent t-test

* Fishers' test

Linear by linear association test

Findings on Immunohistology & Immunohistochemistry

Studies on immunohistology were done to identify pancreatic beta cells morphology, location, characteristics, while related insulin expression was done using immunohistochemistry. We also assessed mean islet size, beta cell number, density, insulin positive area, and percentage of insulin positive area/islet cell. Figure 3 is a non-diabetic pancreata, which shows normal morphology of the acini and ducts. The Islets of Langerhans appear adequate and show normal morphology and seen dispersed among the pancreatic acini.

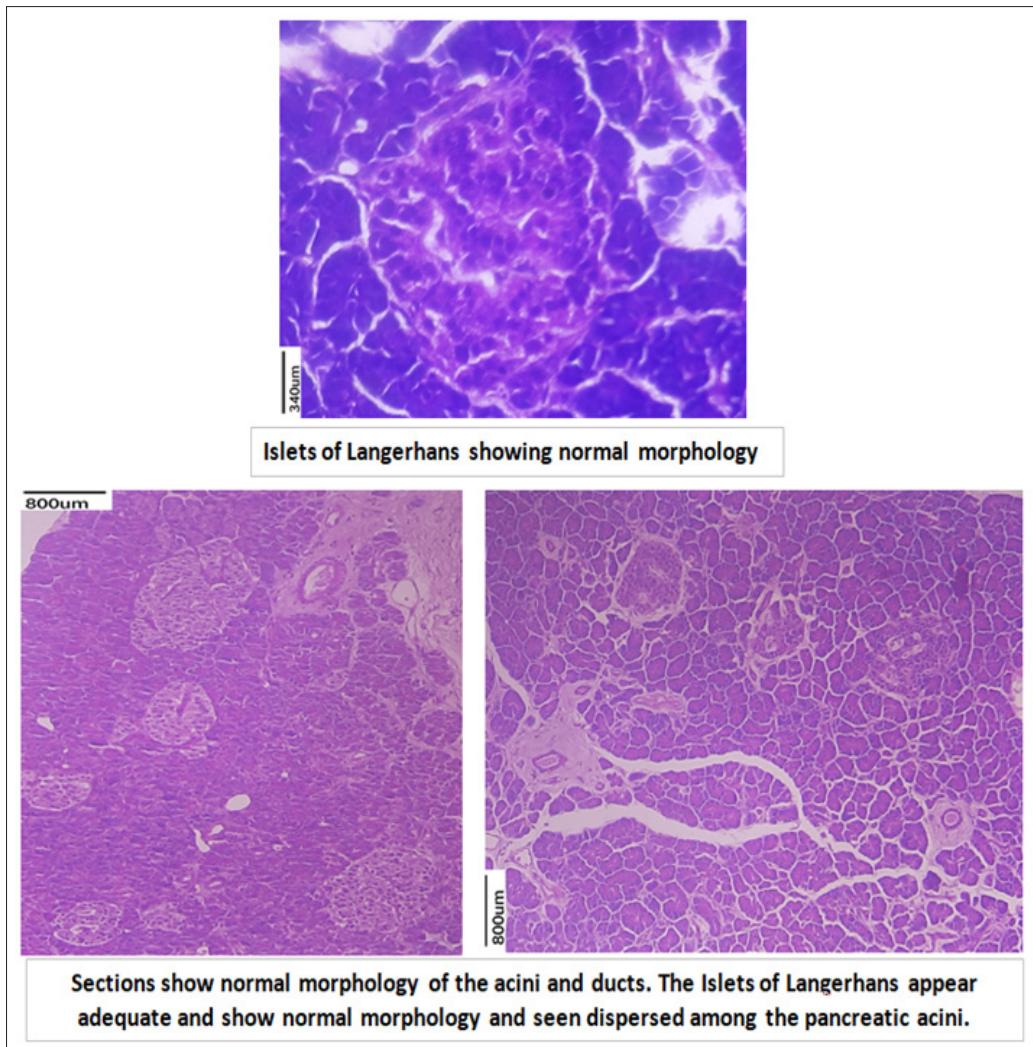


Figure 3: Islet Characteristics, And Morphology of the Acini and Ducts in Non-Diabetic Pancreata

The following figure (Figure 4) is a diabetic pancreata, showing normal morphology of the pancreatic acini and ducts, few ducts are dilated. The Islets of Langerhans are reduced in number and size. Only scattered Islets are seen.

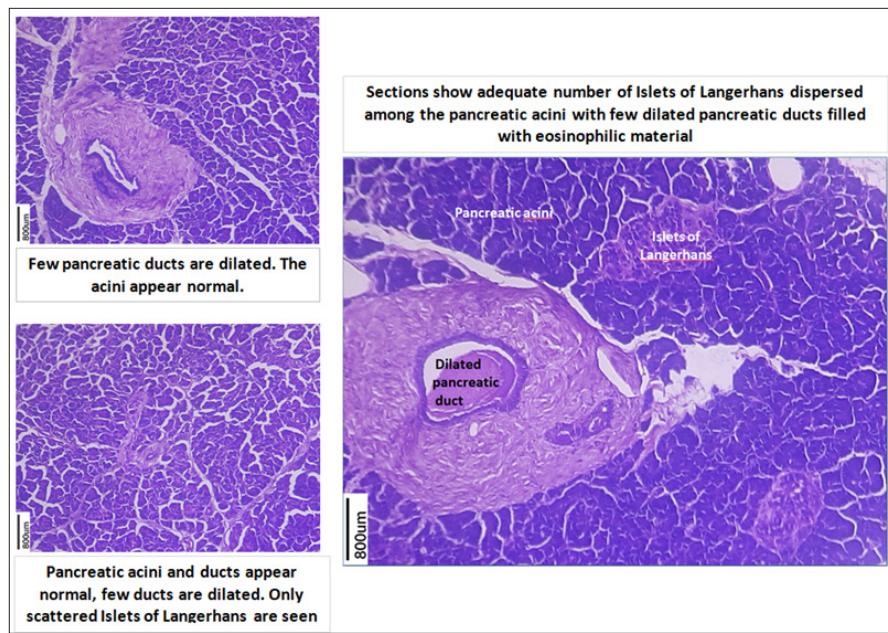


Figure 4: Islet Characteristics and Morphology of the Acini and Ducts in Diabetic Pancreata

Insulin expression on the diabetic and non-diabetic pancreases was done by immunohistochemistry.

Figure 5 explains on the increased percentage of positive cells in the non-diabetic samples.

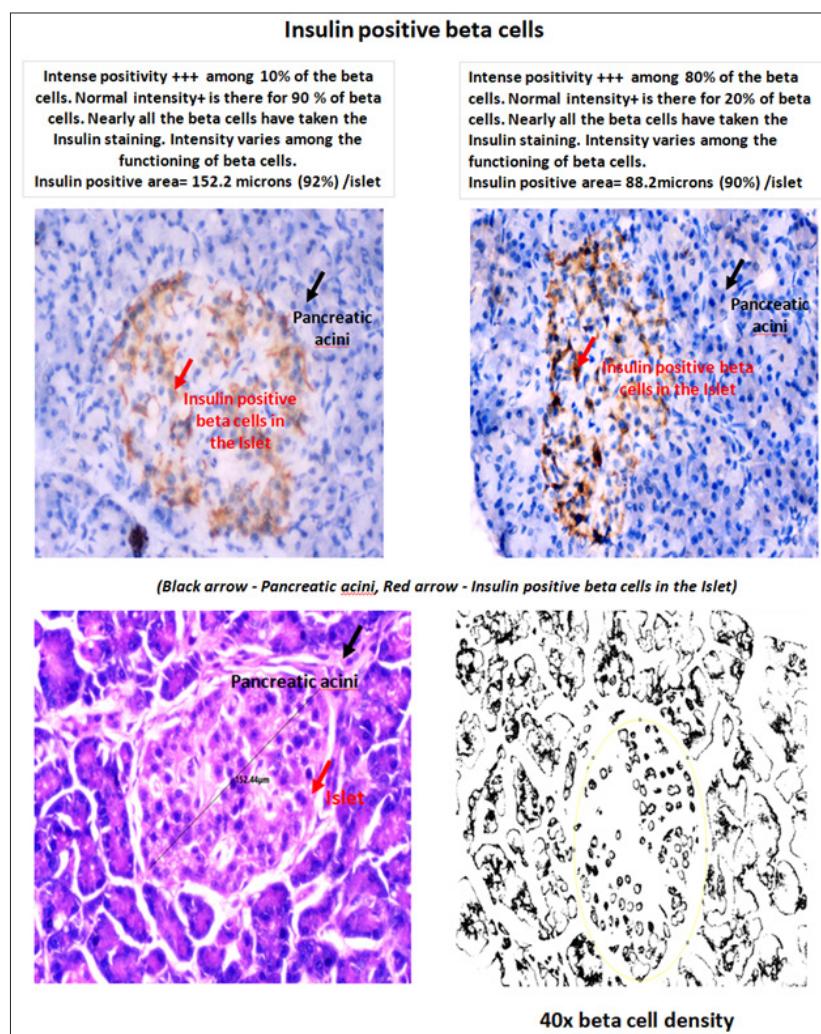


Figure 5: Increased Percentage of Insulin Positive Cells in the Non-Diabetic Samples

The following figure, (Figure 6) shows the decreased percentage of positive cells in the diabetic population. It was interesting to note as the diameter of islet cell size increases the percentage of insulin positive area was decreased.

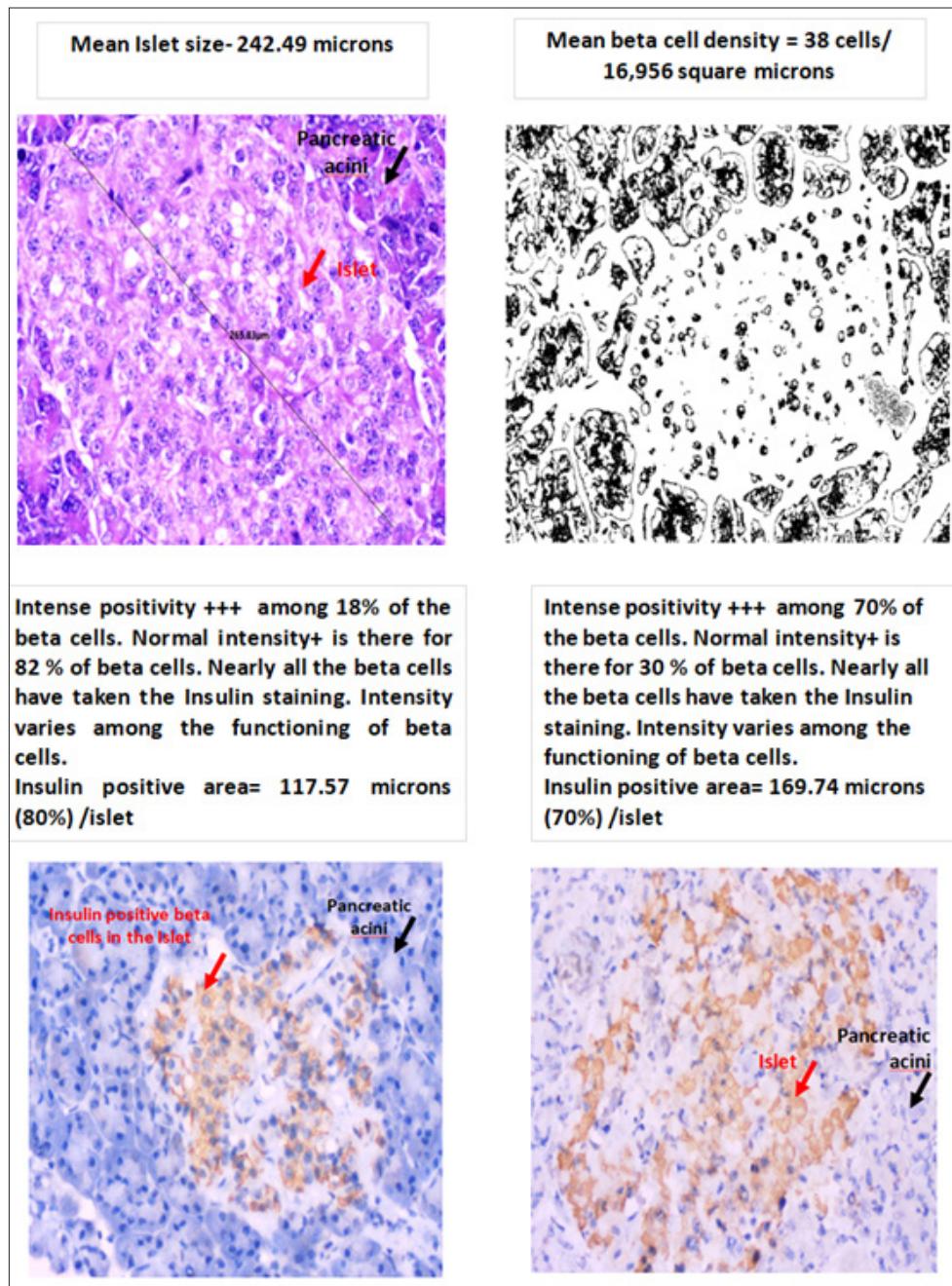


Figure 6: Decreased Percentage of Insulin Positive Cells in the Diabetic Samples

Comparison between the Groups on Immunohistology & Immunohistochemistry

Table 5, supported by Figure 7, shows the mean islet size, beta cell number, density, insulin positive area, and percentage of insulin positive area/islet cell in both diabetic and non-diabetic population. Though mean islet size/diameter was increased in the diabetic group, the rest of the parameters, including beta cell number, insulin positive area and percentage of insulin positive area/islet cell were higher in the non-diabetic group. This could be explained by the fact that, the insulin positive area decreases as islet cell size increases; Figure 8 supports this negative correlation. A comparison between the groups on mean Islet size/diameter and percentage of insulin positive area/islet cell showed statistical significance ($p<0.01$).

Table 5: Comparison between the Groups on Immunohistology and Immunohistochemistry

| Diabetic | Diabetic | Non-Diabetic | P-value |
|--|---------------------|------------------------|---------|
| Mean Islet Size/Diameter | Mean (SD) | 191.22 (48.6) | <0.01@ |
| | Median [min, max] | 176.7 [145.98, 289.32] | |
| | Standard Error (SE) | 15.37 | |
| Beta Cell Number | Mean (SD) | 0.018 (0.016) | 0.92\$ |
| | Median [min, max] | 0.015 [0.001, 0.044] | |
| | Standard Error (SE) | 0.004 | |
| Insulin Positive Area | Mean (SD) | 152.78 (20.4) | 0.29@ |
| | Median [min, max] | 152.76 [117.6, 178.6] | |
| | Standard Error (SE) | 6.79 | |
| Percentage of Insulin Positive Area/Islet Cell | Mean (SD) | 78.0 (9.3) | <0.01@ |
| | Median [min, max] | 80 [65, 95] | |
| | Standard Error (SE) | 3.1 | |

@ Independent t-test

\$ Mann-Whitney U test

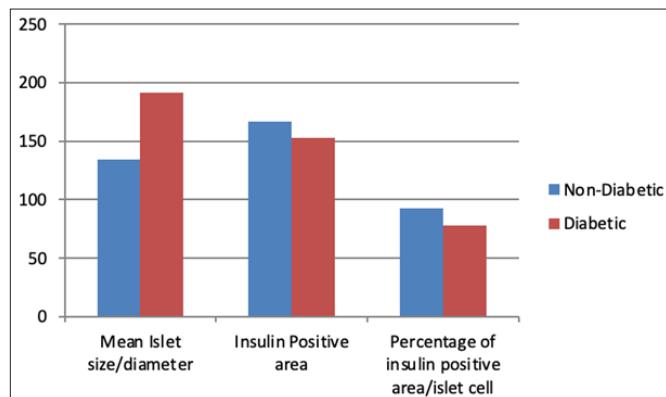


Figure 7: Comparison between the Groups on Immunohistology and Immunohistochemistry

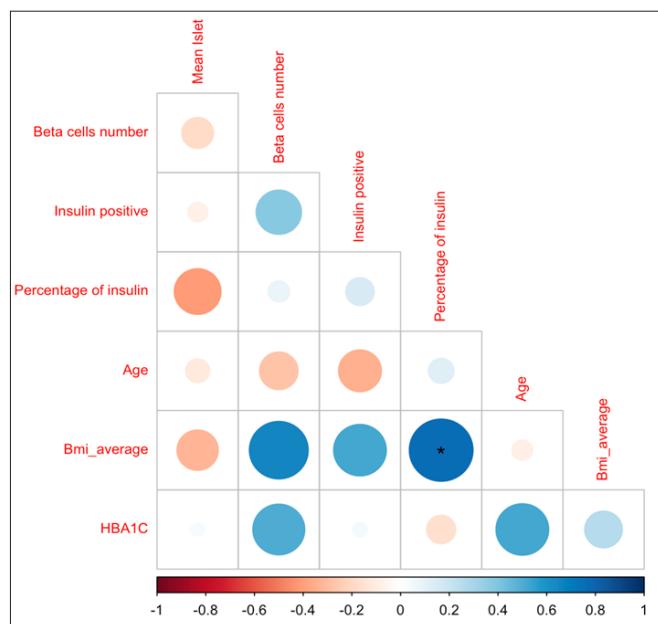


Figure 8: Negative Correlation between Mean Islet Cell Diameter and Insulin Positive Area (Shades of Red Show Negative Correlation)

Discussion

Our current knowledge regarding the transcriptional changes in the regulation of human pancreas and the changes associated with morphological events are unavailable in both diabetic and non-diabetic conditions for the Indian population. It is unanimously accepted that, there is a lot of variability in islet preparation, which in turn affects the islet quality. Islet quality consequently gets reflected in the expression of certain genes. Currently there is paucity in published research to explain about the basal gene expression of glucose homeostasis in the human pancreatic tissues [9-14].

We hypothesized that beta cell functionality and the interaction of graft with host determine the effectiveness of islet graft, and these are governed by specific gene expressions in islet cells. Therefore, gene expression in the human pancreases was analyzed on functional basis so that this can be correlated for their functions with the clinical characteristics in our future studies. We have aimed to build a diagnostic tool to assess the quality of cell preparations prior to their use in clinical islet transplantation. To create islet gene signature it was also necessary to know about the gene expression even in the tissue levels before the isolation process. Thus, we have evaluated the gene expression by RT-PCR analysis with a subset of specific genes involved in the glucose homeostasis. Equally it is important to know the pathological conditions of this tissue and expression of insulin by immunohistochemistry during the diabetic and non-diabetic conditions.

The expression of functional and regulatory genes by islet cells is a key determinant for the success of islet transplantation.

Here we report whole genome transcript analysis validated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) along with the immunohistological observations for these pancreases. We have found the basal level of expression in the given diabetic and non-diabetic pancreases. It was also interesting to see the pathological conditions and insulin expression in these pancreases. It was evident that insulin expression in diabetic samples has a direct role and there were some changes both in the mRNA and protein levels. The morphology, count of beta cells, percentage of insulin positive area in the diabetic sample varied significantly when compared to the non-diabetic sample. Our insight on beta cells' ability to rebound from an inflammatory

damage reasons out cautious optimism. Noteworthy is a finding that, in many patients at least a fraction of their functional beta-cell population is maintained, emphasizing the existence of potent beta-cell survival pathways. Some of the studies have shown results similar to our study mentioning that the gene expression of glucose transporters and glucokinase has been found to be lower in human type 2 diabetic islets than in control preparations [8, 15-18].

Gene expressions underlie molecular mechanisms, which in turn govern the harmony of cellular functions. A number of interlocked transcriptional and posttranslational feedback loops are responsible for generation and maintenance of rhythms. The expression of functional and regulatory genes by islet cells is a key determinant for the success of islet transplantation. The aim with which the study was initiated is twofold: first, to characterize the cluster of genes expressed in human pancreases; and second in the future, to validate the capability of same gene panel to assess with accuracy and expression of various transcripts before the islet transplantation. In future, this data will highlight the importance of understanding the molecular and physiological basis governing islet functionality, immediately after isolation as well as before transplantation. For the clinician, the basal expression levels of relevant molecular parameters will notify on the islet functionality prior to its transplantation. Nevertheless, post-transplantation, these levels would vary based on the success of transplant surgery and also the circadian regulation.

There are several studies that highlights that beta cell compartments, functions, structures and molecules involved in the mechanisms leading to glucose-stimulated insulin release are impaired in human diabetes which is likely due to the interplay between the genetic architecture of the β cells and the role of environmental factors [8, 16-23]. The pathology of type 2 diabetes in humans and certain features of beta cell dysfunction have shown how major in vivo insulin secretion defects can be recapitulated ex vivo through direct study of isolated islets. Islet study also gives the scope for assessing crucial biochemical and molecular traits which relate with functional impairment. Pathology studies have revealed fundamental beta cell characteristics in type 2 diabetes, such as alterations of β cell quantity and turn over as well as the presence of inflammatory traits. These are suggestive of unexpected β cell plasticity (comprising of trans- and de-differentiation) as a possible attempt for adaptation to metabolic stress. However, it has to be kept in mind that type 2 diabetes clinically shows heterogeneity as several variables may impact the quality of the pancreatic tissue and its isolated islet cells. Considering pancreatic tissue as the source, heterogeneity can arise from autoptic, surgical, bioptic variables from organ donors. Notably, there is a biological variation in isolation of human islets from donors with and without diabetes. While for isolated islet cells, the islet preparation itself can show variability with parameters such as, isolation stress, culture conditions and duration. [19-26].

Noteworthy is a fact that, the outcome of islet transplantation in humans is significantly affected by collagenases as they decide the yield and quality of isolated pancreatic islet cells. A study conducted by Nithyakalyani et al., 2022 concluded that the post-purification of islets in the Indian population, yielded better results with Serva collagenase NB1 compared to Vitacyte and Roche liberase. Islet cells' morphology (both free and intact), the islet yield and proportion per gram of pancreas were higher using serva collagenase compared to the other two enzyme blends [27-28]. Therefore, correct reporting of islet data may be challenging leading to potential inconsistencies in the experimental results. These

unavoidable limitations, provides pathophysiological evidences and clues to conceive new strategies for better prevention and treatment of diabetes by targeting the β cells.

This study provides an insight into the complex pattern of the immune gene expression disturbances in addition to confirming the predicted immunopathological mechanisms that highlight a number of natural immunity-inflamatory, immunoregulatory and regenerative pathways, some of which have received relatively little attention so far. In conclusion, the markers presented here may also serve as a potential target in the management and seem to be playing a role in diabetes as well as in clear-cell pathogenesis. As such, it seems to be a good diagnostic and perhaps even a prognostic marker in the future islet cell isolation, function before the clinical transplantation, but warrants further investigation [29].

Conflict of Interest

We hereby declare that we have no conflict of interest of any form pertaining to our research study titled, 'A comprehensive analysis to study the Histological and Immunohistochemical Profile of Diabetic and Non-Diabetic Human Pancreases in the Indian population'.

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