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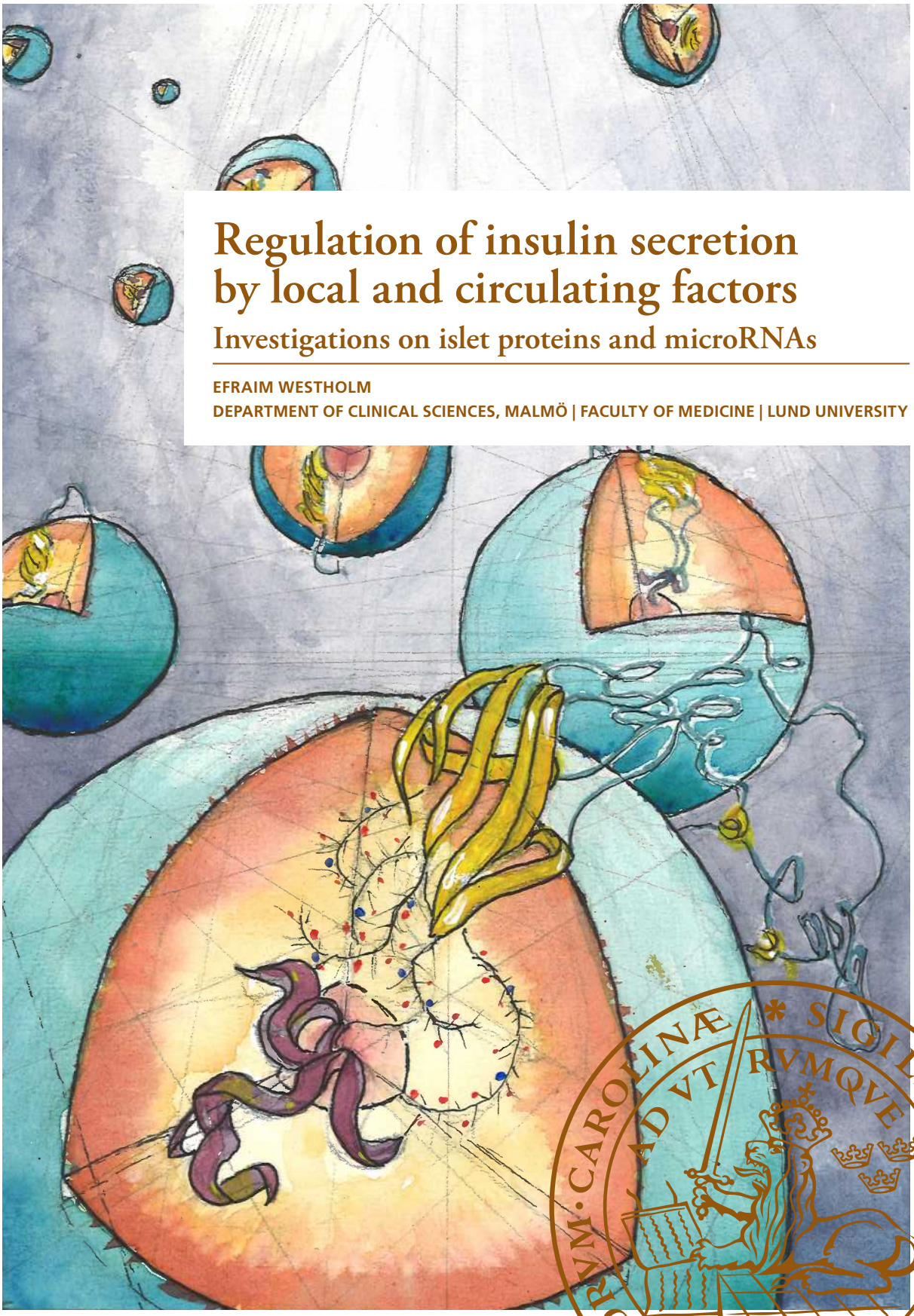

# Regulation of insulin secretion by local and circulating factors

## Investigations on islet proteins and microRNAs

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## Investigations on islet proteins and microRNAs

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Efraim Westholm

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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**Abstract:** Impaired insulin secretion is a key step in the development of type 2 diabetes mellitus (T2D), the most common metabolic disease in the world. Insulin secretion from β-cells in the pancreatic islets is affected by several extrinsic processes, such as insulin resistance influencing the islet, as well as islet-intrinsic factors. Dysregulated insulin secretion caused by aberrant intra-islet crosstalk and microRNA (miRNA) expression has been implicated in T2D pathogenesis.

Insulin-like growth factor binding protein 7 (IGFBP7) binds insulin and is upregulated in several tissues in cardiometabolic diseases. In Paper I, IGFBP7 expression in human pancreatic islets from healthy and T2D donors is investigated. Using published data on whole-islet and single-cell RNA-sequencing, IGFBP7 was found to be upregulated in T2D. *In vitro* experiments revealed IGFBP7 reduced insulin secretion in human islets, whereas siRNA-mediated IGFBP7-knockdown improved insulin secretion in islets from IGT/T2D human donors. This opens up to the possibility that IGFBP7 works as negative autocrine/paracrine regulator of β-cell function.

In Paper II, interleukin-4 (IL-4) reduced insulin secretion in islets from healthy donors. Islets from T2D donors were unaffected by the IL-4 treatment. IL-4 induced genes in the peroxisome proliferator-activator receptor γ (PPARγ) pathway which has been shown to impair insulin secretion. This was interpreted as IL-4-mediated anti-inflammatory responses may come at the expense of reduced insulin secretion.

To gain a deeper understanding of miRNA regulation in islet cells in health and disease, miRNA-sequencing of sorted α- and β-cells was performed in Paper III. Although the most abundant miRNAs were common between the cell types, the global miRNA expression profile differed substantially. In β-cells from T2D donors, miR-551b-3p was robustly upregulated. Overexpression of this miR-551b-3p in a clonal β-cell line increased insulin secretion, indicating a compensatory role for this miRNA in T2D.

In cystic fibrosis (CF), development of CF-related diabetes mellitus (CFRD) is a serious challenge for afflicted individuals. Development of CFRD is also due to impaired insulin secretion. In Paper IV, circulating miRNAs were studied in CF during an oral glucose tolerance test (OGTT). Two miRNAs differentially regulated at baseline in CFRD were identified: miR-34a-5p and miR-122-5p. These miRNAs associated with hepatobiliary damage markers, implying involvement of liver dysfunction in CFRD. MiR-223-3p showed dynamic changes throughout the OGTT in CFRD. Overexpression of miR-122-5p and miR-223-3p increased insulin secretion, suggesting a potential compensatory role in CFRD.

In summary, this thesis provides new findings in regulation of insulin secretion by IGFBP7, IL-4 and miRNAs with cell-specific expression in the islet. Additionally, circulating miRNAs in CFRD have been identified and assessed with regards to effects on insulin secretion.

**Key words:** type 2 diabetes mellitus, cystic fibrosis-related diabetes mellitus, pancreatic islet, β-cell, insulin secretion, insulin-like growth factor binding proteins, interleukin-4, microRNA,

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*Till min familj*

*"Du låter min lampa lysa. Herren, min Gud, gör mitt mörker till ljus."*
*Psaltaren 18:29*

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# Preface

There are several layers of understanding in studying diabetes mellitus. This disease can be approached from various perspectives: epidemiological, sociological and psychological, genetic, endocrinological and cell biological to name a few. In the case of type 2 diabetes mellitus, arguments have been made for the best treatment of the wider population being the use of political initiatives. Helping people make healthier lifestyle choices, for example via healthcare subsidies, more physical education in school and a greater variety of plant-based food options are essential steps in improving the metabolic health of the general population.

Yet, it is well-established that diabetes mellitus is not caused by a single environmental factor, such as overnutrition, nor by a single genetic variant, but rather the interplay between genes and the environment in which they reside. In this context, it becomes evident that further understanding the biology of the intrinsic physiological mechanisms maintaining our blood glucose homeostasis is of importance to finding new treatment strategies for diabetes mellitus. Treatment strategies to go alongside wider societal approaches in helping people achieve better metabolic health.

As a medical doctor, I have been trained in thinking about diseases from a holistic perspective. In my PhD studies, I have focused on understanding intricate cell biological mechanisms governing the insulin-secreting β-cells and how these are regulated in different forms of diabetes mellitus. Here, I lay my thesis. Still, I recognize that this is only one layer of understanding diabetes mellitus and this line of thinking has remained with me throughout my studies. I acknowledge the research on which it is built, and I hope that it may help in building a better understanding of diabetes mellitus for future scientists. One layer at a time.

# Popular science summary

Type 2 diabetes mellitus (T2D) is one of the most common diseases in Sweden and internationally. Being diagnosed with T2D is very serious, and numerous complications as a result of elevated blood glucose levels have been described. Obstruction of blood vessels in the leg can lead to amputation. Kidney damage may contribute to kidney failure and the need for dialysis. Cystic fibrosis (CF), although not a very common condition, is among the most frequently diagnosed heritable diseases in Sweden. A very common co-morbidity in CF is CF-related diabetes mellitus (CFRD). Individuals with CFRD have a similar risk of developing diabetic complications. At the core of T2D and CFRD is the hormone insulin. Insulin is a hormone which aid the tissues of the body to take up glucose and other nutrients from the bloodstream. Insulin is released from hormone producing cells in the pancreas. These insulin cells, also known as beta cells, are situated together with other hormone producing cells in clusters called pancreatic islets.

Common for both T2D and CFRD is the failure of insulin secretion which contribute to elevated blood glucose levels. Regulation of insulin is a multifaceted process and occurs in several different layers in the body. The most obvious regulation is by food intake. Elevated blood glucose will stimulate secretion of insulin from the beta cells. Insulin secretion is also regulated by hormones from the gut and by signals from the neighbouring cells in the pancreatic islets. The second most common cell type is the alpha cell. These cells secrete the hormone glucagon which raises the blood glucose. Glucagon and numerous other locally secreted molecules in the islet also regulate insulin secretion. MicroRNAs belong to a class of RNA molecules which modulate cellular functions by binding to messengerRNA. MessengerRNAs can be translated into proteins, and microRNAs binding to messengerRNA inhibit protein expression and regulate cellular processes such as insulin secretion. MicroRNAs are also present in the circulation and can govern cellular functions in a fashion similar to that of traditional hormones. In this thesis, the focus has been on deeper understanding of how insulin secretion is regulated by two proteins, IGFBP7 and IL-4, and by microRNAs in both alpha and beta cells. The role of circulating microRNAs in CFRD has also been investigated.

In the first paper IGFBP7 (insulin-like growth factor binding protein 7) was studied. This protein has several interesting properties. Firstly, IGFBP7 can bind to insulin. Secondly, elevated levels of IGFBP7 in other organs affected by T2D and in metabolic diseases have been described in previous studies. High levels of IGFBP7

have been linked to fatty liver, heart failure and kidney failure. However, the expression of IGFBP7 in human pancreatic islets in T2D has not been researched. In this work, IGFBP7 was found to be upregulated, both on a gene level and a protein level, in the pancreatic islets in T2D. This increase occurs mainly in the alpha cells and to a lesser extent in beta cells. IGFBP7 could negatively regulate insulin secretion, i.e., beta cells treated with the protein displayed reduced insulin secretion. And vice versa: when the levels of IGFBP7 were lowered, insulin secretion was increased. Altogether, these results suggest that IGFBP7 is a potential local regulator of insulin secretion in the pancreatic islets and manipulation of this protein may be a putative treatment strategy for T2D.

In the second article, IL-4 (interleukin-4) was investigated. IL-4 is a protein that participates in signaling between cells of the immune system to dampen inflammation. IL-4-treatment of pancreatic islets from healthy donors resulted in reduced insulin secretion. Islets from donors with T2D showed an impaired insulin secretion which was neither worsened nor improved by IL-4. This indicates that signaling by IL-4 is intact in islets from non-diabetic donors, which was supported by the finding of upregulation of genes and microRNAs regulated by IL-4. An overarching interpretation of these results is that even though IL-4 has protective and anti-inflammatory effects in the islets, this may come at the expense of reduced insulin secretion.

Expression of microRNAs in alpha- and beta cells is the focus of the third project. The most abundant miRNAs are similar between the two cell types; however, the global microRNA expression profile differs quite significantly. Several microRNAs enriched in alpha cells targeted genes important in beta cells, which suggest a role for these microRNAs in hindering the alpha cells from transforming into beta cells. Dysfunctional regulation of microRNAs in islets have previously been linked to T2D. Here, in beta cells from T2D donors, microRNA-551b-3p, was increased. This microRNA improved insulin secretion, indicative of a compensatory mechanism.

In the fourth and final work, circulating microRNAs in people with CF were investigated. Three microRNAs, microRNA-34a-5p, microRNA-122-5p and microRNA-223-3p were associated with CFRD. MicroRNA-34a-5p and microRNA-122-5p showed higher levels in the blood in people with CFRD than people with only CF. These two microRNAs could be linked to liver damage markers, suggesting involvement of the liver in CFRD development. MicroRNA-223-3p rose after intake of a glucose solution in people with CFRD. Interestingly, microRNA-122-5p and microRNA-223-3p improved insulin secretion, suggesting a compensatory role for these microRNAs in CFRD.

Altogether, this thesis shows that insulin secretion can be regulated by the investigated factors. The role of IGFBP7 as a local negative regulator of insulin secretion merits further research. Levels of circulating microRNAs should be assessed in longitudinal studies to ascertain prognostic capacity for CFRD.

# Populärvetenskaplig sammanfattning

Diabetes typ 2 (T2D) är en av de vanligaste folksjukdomarna i Sverige och internationellt. Att drabbas av T2D är mycket allvarligt och ett flertal komplikationer som följd av förhöjda blodsockernivåer finns beskrivna. Förträngningar i blodkärl i benen kan leda till amputation. Skador på njurarna kan ge njursvikt och behov av dialys. Cystisk fibros (CF), förvisso en ovanlig sjukdom, är likväl en av de vanligaste ärftliga sjukdomarna i Sverige. En mycket vanlig följdsjukdom av CF är CF-relaterad diabetes (CFRD) och dessa individer drabbas också av diabeteskomplikationer. Grunden till utvecklingen av både T2D och CFRD finns i hormonet insulin. Insulin är ett hormon som hjälper kroppens vävnader att ta upp socker, oftast sockermolekylen glukos, och andra näringsämnen från blodcirkulationen. Insulin utsöndras från hormonproducerande celler i pankreas. Dessa insulinceller, även kända som betaceller, ligger tillsammans med andra hormonproducerande celler i små kluster som kallas för de pankreatiska öarna.

Gemensamt för både T2D och CFRD är att utsöndringen av insulin blir bristfällig vilket höjer blodsockernivåerna. Reglering av insulinsekretion är mångfacetterad och sker på flera olika nivåer i kroppen. Den främsta regleringen sker av matintag och förhöjda nivåer av glukos i blodet ger utsöndring av insulin från betacellerna. Insulinsekretion regleras även av hormoner från tarmen och av signaler från intilliggande celler i de pankreatiska öarna. Den andra mest vanliga celltypen i öarna är alfacellerna. Dessa celler utsöndrar hormonet glukagon som höjer blodsockret. Glukagon och ett flertal andra molekyler som utsöndras mellan ö-cellerna kan även reglera insulinsekretionen. MicroRNA är en klass av RNA-molekyler som kan modulera cellfunktioner genom att binda till messengerRNA. MessengerRNA översätts till protein, och microRNA som binder till messengerRNA hämmar proteinuttryck och därmed regleras cellprocesser såsom insulinsekretion. MicroRNA finns även i blodcirkulationen och kan styra cellfunktioner på liknande sätt som hormoner i blodbanan. I den här avhandlingen har fokus legat på att djupare förstå hur insulinsekretion påverkas av två proteiner, IGFBP7 och IL-4, samt av microRNA inuti både alfa- och betaceller. Betydelsen av cirkulerande microRNA i CFRD har även undersökts.

I det första arbetet studeras IGFBP7 (insulin-like growth factor binding protein 7). Detta protein har mycket intressanta egenskaper. IGFBP7 kan binda till insulin och förhöjda nivåer av IGFBP7 i andra organ som drabbas i T2D och sjukdomar i ämnesomsättningen har beskrivits i tidigare studier. Höga nivåer av IGFBP7 har

kopplats till fettlever, hjärtsvikt och njursvikt. Men, IGFBP7 har inte undersökts i de pankreatiska öarna i T2D. I det här arbetet visas att nivåerna av IGFBP7, både uttrycket av genen samt proteinet, stiger i de pankreatiska öarna i T2D. Den här ökningen äger främst rum i alfacellerna men till viss del även i betacellerna. IGFBP7 är en negativ regulator av insulinsekretion, dvs att betaceller som behandlas med proteinet utsöndrar mindre insulin. Vice versa: när nivåerna av IGFBP7 minskas, ökar insulinsekretionen. Sammantaget visar dessa resultat att IGFBP7 är en potentiell lokal regulator av insulinsekretion i de pankreatiska öarna och manipulation av nivåerna är en tänkbar behandlingsstrategi för T2D.

I den andra artikeln undersöks IL-4 (interleukin-4). IL-4 är ett protein som är delaktigt i signalering mellan celler i immunsystemet för att dämpa inflammation. Behandling med IL4 av pankreatiska öar från donatorer utan T2D ledde till en försämring av insulinutsöndringen. Öar från donatorer med T2D hade redan en försämrad insulinsekretion, och behandling med IL-4 varken försämrade eller förbättrade insulinsekretionen. Detta talar för att signalvägarna för IL-4 är intakta i öar från icke-diabetiska donatorer vilket visades med att nivåer av gener och microRNA som regleras av IL-4 steg i dessa öar. En övergripande tolkning av dessa resultat är att även om IL-4 har skyddande och anti-inflammatoriska egenskaper i öarna, så sker detta på bekostnad av försämrad insulinsekretion.

Uttryck av microRNA i alfa- och betaceller är fokus i det tredje arbetet. De mest förkommande microRNA är gemensamma för båda celltyperna, men, den globala microRNA-profilen skiljer sig mellan alfa- och betaceller. Ett flertal microRNA i alfaceller undertrycker gener som är viktiga i betaceller, vilket kan tala för att dessa microRNA hindrar alfaceller från att förvandlas till betaceller. Dysfunktionell reglering av microRNA i hela öar har tidigare kopplats till utveckling av T2D. I betaceller steg nivåerna av microRNA-551b-3p i T2D. Överuttryck av detta microRNA gav höjd insulinsekretion, talande för en kompensatorisk mekanism.

I det fjärde och sista arbetet studerades cirkulerande microRNA hos personer med CF. Tre microRNA, microRNA-34a-5p, microRNA-122-5p samt microRNA-223-3p kunde kopplas till CFRD. MicroRNA-34a-5p och microRNA-122-5p visade högre nivåer i blodet hos personer med CFRD än hos personer med CF men utan CFRD. Dessa två microRNA var associerade med markörer för leverskada, vilket talar för att levern är involverad i CFRD. MicroRNA-223-3p steg hos personer med CFRD efter intag av en glukoslösning. Intressant nog förbättrade microRNA-122-5p och microRNA-223-3p insulinsekretion, vilket kan tyda på en kompensatorisk mekanism i CFRD för dessa microRNA.

Sammantaget visar den här avhandlingen att insulinsekretion kan regleras av ovan nämnda faktorer. Betydelsen av IGFBP7 som en lokal regulator av insulinsekretion i de pankreatiska öarna är värd att undersöka vidare. Nivåerna av cirkulerande microRNA hos personer med CF bör följas i långtidsstudier för att utröna prognostisk förmåga av CFRD för dessa microRNA.

# Papers included in the thesis

***Paper I***

IGFBP7 is upregulated in islets from T2D donors and reduces insulin secretion. **Efraim Westholm**, Alexandros Karagiannopoulos*, Nicole Kattner*, Yara Al-Selwi, George Merces, James AM Shaw, Anna Wendt, Lena Eliasson. *iScience*, accepted August 15th, 2024, DOI: 10.1016/j.isci.2024.110767. *: equal contribution.

***Paper II***

Interleukin-4 reduces insulin secretion in human islets from healthy but not type-2 diabetic donors. **Efraim Westholm**, Anna Edlund, Alexandros Karagiannopoulos, Anna Wendt, Lena Eliasson. *Biomedical and Biophysical Research Communications*, 2023 Mar 15:649:87-92. DOI: 10.1016/j.bbrc.2023.01.092.

***Paper III***

MicroRNA profiling in sorted human pancreatic alpha and beta cells from healthy and type 2 diabetic donors. Alexandros Karagiannopoulos, **Efraim Westholm**, Jones K Ofori, Karl Bacos, Anna Wendt, Charlotte Ling, Lena Eliasson. *Manuscript.*

***Paper IV***

Identification of differentially expressed circulating microRNAs following an oral glucose challenge in CFRD. **Efraim Westholm**, Alexandros Karagiannopoulos*, Bibi Uhre Nielsen*, James AM Shaw, Anna Wendt, Daniel Faurholt-Jepsen, Lena Eliasson. *Submitted manuscript.* *: equal contribution.

## Author's contribution to the papers

***Paper I***

EW together with AW and LE designed the study. EW performed the *in vitro* experiments. AK performed the bioinformatic analyses. NK, YS and GM performed imaging experiments. EW wrote the manuscript together with AW and LE. All authors participated in data review and editing of the manuscript.

***Paper II***

EW, AE, AW and LE designed the study. EW performed the gene and miRNA expression analyses. AE performed the *in vitro* islet experiments. AK performed the bioinformatic analysis. EW, AW and LE wrote the manuscript. All authors participated in data analysis and review of the manuscript.

***Paper III***

AK, EW, CL and LE designed the study. AK performed the bioinformatic analyses. EW performed preparation of libraries for miRNA-sequencing and assisted in cell line experiments. AK, EW, AW and LE wrote the manuscript. All authors participated in data analysis and review of the manuscript.

***Paper IV***

EW, AW and LE designed the study. EW performed measurements of miRNAs. AK performed the bioinformatic analyses. BUN and DFJ provided samples from the patient cohort. EW and LE wrote the manuscript. All authors participated in data analysis and review of the manuscript.

# Papers not included in the thesis

## Original papers

I. MiR-205 is up-regulated in islets of diabetes-susceptible mice and targets the diabetes gene Tcf7l2. Meriem Ouni, Pascal Gottmann, **Efraim Westholm**, Kristin Schwerbel, Markus Jähnert, Mandy Stadion, Kilian Rittig, Heike Vogel, Annette Schürmann. *Acta Physiologica*, 2021 Aug; DOI: 10.1111/apha.13693.

II: Human Islet MicroRNA-200c Is Elevated in Type 2 Diabetes and Targets the Transcription Factor ETV5 to Reduce Insulin Secretion. Jones K Ofori, Alexandros Karagiannopoulos, Mototsugu Nagao, **Efraim Westholm**, Shaima Ramadan, Anna Wendt, Jonathan L S Esguerra, Lena Eliasson. *Diabetes*, 2022 Feb 1;71(2):275-284. DOI: 10.2337/db21-0077

III: Glucocorticoid-mediated induction of ZBTB16 affects insulin secretion in human islets and EndoC-βH1 β-cells. Alexandros Karagiannopoulos, **Efraim Westholm**, Jones K Ofori, Elaine Cowan, Jonathan LS Esguerra, Lena Eliasson. *iScience*, 2023 Apr; DOI: 10.1016/j.isci.2023.106555.

IV: MicroRNA 29 modulates β-cell mitochondrial metabolism and insulin secretion via underlying miR-29-OXPHOS complex pathways. Elaine Cowan, Jianming Sun, Alexander Hamilton, Sabrina Ruhrmann, Alexandros Karagiannopoulos, **Efraim Westholm**, Jones K Ofori, Cheng Luan, Enming Zhang, Hindrik Mulder, Lena Eliasson. *Acta Physiologica*, 2024 Aug; DOI: 10.1111/apha.14180.

## Review

I: Islet Function in the Pathogenesis of Cystic Fibrosis-Related Diabetes Mellitus. **Efraim Westholm**, Anna Wendt, Lena Eliasson. Clin Med Insights Endocrinol Diabetes, 2021 Jul; DOI: 10.1177/11795514211031204.

# Abbreviations

| | |
|---|---|
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| cAMP | cyclic adenosine monophosphate |
| CF | cystic fibrosis |
| CFRD | cystic fibrosis-related diabetes mellitus |
| CFTR | cystic fibrosis transmembrane conductance regulator |
| F508del | *CFTR* genetic variant, deletion of phenylalanine at position 508 |
| GABA | γ-aminobutyric acid |
| GCK | glucokinase |
| GLP-1 | glucagon-like peptide 1 |
| GLUT | glucose transporter |
| GSIS | glucose-stimulated insulin secretion |
| HbA1C | hemoglobin A1C, glycated hemoglobin |
| IGF | insulin-like growth factor |
| IGF1R | IGF1 receptor |
| IGFBP | insulin-like growth factor binding protein |
| IGT | impaired glucose tolerance |
| IL | interleukin |
| INDET | indeterminate glucose tolerance |
| INSR | insulin receptor |
| LNA | locked nucleic acid |
| mRNA | messengerRNA |
| miRNA, miR | microRNA |
| ND | non-diabetic |

| | |
|---|---|
| NGT | normal glucose tolerance |
| OGTT | oral glucose tolerance test |
| PGC1B | peroxisome proliferator-activated receptor γ co-activator 1B |
| PP | pancreatic polypeptide |
| PPARγ | peroxisome proliferator-activated receptor γ |
| RT-qPCR | real-time quantitative polymerase chain reaction |
| SNP | single nucleotide polymorphism |
| T1D | type 1 diabetes mellitus |
| T2D | type 2 diabetes mellitus |
| UCN3 | urocortin 3 |
| VGCC | voltage gate $Ca^{2+}$ channel |

# Introduction

## Diabetes mellitus

### Overview

Diabetes mellitus is the most common metabolic disease in the world. The prevalence is rapidly increasing worldwide with the global patient population in 2021 estimated to be 536 million people, rising to 783 million people in 2045 (1). Clearly posing a strain on healthcare systems in Sweden and internationally (2), finding new ways for characterizing and treating diabetes mellitus is key in managing this global health concern.

Diabetes mellitus is linked to several environmental risk factors. These include age, obesity, high calorie diet, low physical activity, smoking and low socioeconomic status, all of which are well characterized contributors to poor metabolic health (2, 3). There is also a well-recognized genetic component in T2D risk (4, 5). Developing diabetes mellitus confers a multitude of complications. These include, but are not limited to, diabetic retinopathy, diabetic nephropathy, diabetic neuropathy, diabetic cardiomyopathy and fatty liver disease (6). Together, these complications result in a significant decrease in quality of life and increased morbidity. Diagnosis of diabetes mellitus is made upon discovery of elevated blood glucose levels. This can be done either with a fasting blood sample, in a time series measurement of blood glucose after ingestion of a glucose load termed oral glucose tolerance test (OGTT) or by measuring glycated hemoglobin A1C (HbA1C) which reflect long-term glucose levels in a patient over the last 2-3 months (7).

Elevated blood glucose levels will eventually result in glucose passing through the kidneys, leading to polyuria. In fact, the name *diabetes mellitus* comes from the Greek word *diabetes* meaning “passing through, siphoning” referring to the polyuria, and *mellitus* meaning “sweet, honey” referring to the urinary glucose. Ancient medical texts recommended tasting the urine to diagnose diabetes mellitus (8). However, elevated blood glucose, while still being a central diagnostic test, is today rather viewed as an end stage result of several metabolic derangements or autoimmune processes present in a patient with diabetes mellitus (9, 10). The two main types of diabetes are type 1 diabetes (T1D) and type 2 diabetes (T2D). In T1D, elevated blood glucose levels are due to the autoimmune destruction of insulin

secreting pancreatic β-cells (10). Insulin is secreted post-prandially to facilitate the uptake of nutrients, e.g. glucose, from the blood stream that have been absorbed by the gut (11). Insulin deficiency in T1D leads to elevated blood glucose levels, and diagnosis of this disease is often made with the identification of blood autoantibodies targeting the pancreatic islets or proteins in the pancreatic β-cells (10).

In T2D, diagnosis is often accompanied by measurement of high blood lipids, hypertension diagnosis, general vascular disease and fatty liver. A collective term for describing these, often co-occurring, symptoms is "metabolic syndrome" (9, 12). The pathophysiology is thought to be the result of two parallel processes: insulin resistance and impairment of insulin secretion (13). Insulin resistance is when target tissues for the actions of insulin become resistant to insulin signaling resulting in failure to take up glucose and other nutrients. This process reciprocally leads to a demand for increased insulin secretion (14). Initially, β-cells can respond with increased insulin production and secretion (12). However, a critical step in T2D pathophysiology is when the β-cells no longer adequately secrete insulin in response to glucose (15), which leads to and contributes to the aforementioned metabolic derangements (12).

## Classification

As previously stated, the two main types of diabetes mellitus are T1D and T2D, constituting 5-10% and 90-95% of the global diabetes mellitus patient population respectively (1, 7). Both the American Diabetes Association (ADA) and the World Health Organization (WHO) have provided recommended classification systems with specified diagnostic criteria (7, 16). The ADA classification lists general types of diabetes mellitus: T1D, T2D, specific types of diabetes mellitus due to other causes, and gestational diabetes mellitus (GDM) (7). The WHO classification divides the disease into similar groups to the ADA system: T1D, T2D, hybrid forms of diabetes mellitus, other specific types of diabetes mellitus, unclassifiable forms of diabetes mellitus, and hyperglycemia detected during pregnancy (16). The category "specific types of diabetes mellitus due to other causes" from the ADA system overlaps with "other specific types of diabetes mellitus" in the WHO system. In Table 1, the classification of diabetes mellitus as proposed by the ADA is presented.

### *Subgroups of T2D and implications for disease development*

As evident by Table 1, T2D is by far the most common type of diabetes mellitus. As previously alluded to, this disease is heterogeneous in its causes and can be viewed as a result of several pathological processes ultimately affecting glucose metabolism (8, 9, 12). To date, two of the most established risk factors for developing T2D are age and being overweight or obese (6).

**Table 1:** Classification of diabetes mellitus. Adapted from the ADA (7).

| ***Form of diabetes mellitus*** | **Type 1 Diabetes** | **Type 2 Diabetes** | **Other types of diabetes mellitus** | **Gestational Diabetes Mellitus** |
|---|---|---|---|---|
| *Percentage of patients* | 5-10% | 90-95% | <1% | Often transitory. |
| *Etiology* | Autoimmune destruction of pancreatic islets. Leads to insulin deficiency and hyperglycemia. | Several environmental and genetic risk factors affecting both insulin resistance and insulin secretion. | Exocrine pancreas disease, cystic fibrosis, monogenic causes, drug-induced syndromes. | Physiological stress during pregnancy. Overlapping genetic risk with T2D |
| *Diagnosis* | Elevated fasting glucose and detection of autoantibodies against pancreatic islet antigens. | Elevated fasting glucose, elevated HbA1C, pathological OGTT. Exclusion diagnosis. Often family history of T2D, previous GDM. | Varies depending on subtype. Genetic testing is often necessary. | Standard procedure is OGTT at start of 3rd trimester. |

Overweight and obesity are defined as having a body mass index (BMI) between 25-30 kg/m$^2$ or above 30 kg/m$^2$ respectively (17). Attempts to identify genes associated with T2D pathogenesis have been conducted in genome-wide association studies (GWAS). Early GWAS initiatives showed that the strongest signal for T2D incidence resides in a single nucleotide polymorphism (SNP) in the *transcription factor 7-like 2, TCF7L2,* gene, located on chromosome 10q (18-20). However, while common in several populations, the effect of *TCF7L2* on T2D risk cannot alone fully explain the high prevalence of the disease (21). The current view of genetic causes for T2D is that it is a polygenic disorder, where variants of several genetic loci determine a person's overall genetic risk for developing T2D (4, 5). However, it is also abundantly clear that T2D develops in the context of several environmental factors, such as obesity, interacting with genetic factors (22). There is no generally accepted subdivision of T2D patients based on either environmental risk factors or genetic risk factors.

In order to categorize T2D patients from a clinically useful perspective, and further understand T2D in a more granular perspective, Ahlqvist *et al* performed a study with the aim of classifying newly diagnosed T2D patients (23). In this study, All New Diabetics In Scania (ANDIS), patients newly diagnosed with diabetes mellitus, were divided into five different subgroups using unsupervised hierarchical clustering. Three of the groups, representing 75-80% of all newly diagnosed diabetes mellitus patients were characterized by impaired insulin secretion. Genetic studies in the ANDIS cohort also showed that variants in *TCF7L2* only associated

with T2D in the three subgroups characterized primarily by loss of insulin secretion. This also held true for another gene, *potassium inwardly rectifying channel, subfamily J, member 11, KNCJ11*, which codes for a $K^+$ channel important for regulating insulin secretion from the β-cells in the pancreatic islets.

As mentioned before, T2D can be viewed as a spectrum of several metabolic derangements together with dysglycemia. This occurs prior to fully developing T2D (9). Clinical studies assessing insulin secretory capacity in patients with pre-diabetic glucose levels show that initially, the insulin secretion from β-cells in the pancreatic islets is increased, likely as a compensation for whole-body insulin resistance. However, the pivotal step in the final development of hyperglycemia diagnostic for T2D is when the islet function decreases, as shown by impaired insulin secretion (24, 25). Importantly, insulin is secreted in a biphasic manner with an initial rapid first-phase within the first 10 minutes after a glucose challenge, and a sustained second phase in the hours thereafter. Specific loss of the first-phase insulin secretion is considered a hallmark of T2D (26, 27).

Overall, genetic studies (21) as well as clinical studies (24) support that a critical process in the development of T2D is failure of insulin secretion in response to nutrient intake, such as glucose. It is important to recognize that this often occurs in the context of increased insulin demand following insulin resistance. Distinct subgroups comprising the majority of T2D cases, show evidence that dysfunction of the pancreatic islet is a central contributing process in developing T2D (23).

## Cystic fibrosis-related diabetes mellitus

Cystic fibrosis (CF) is among the most common monogenetic diseases and affects nearly 100 000 people globally (28) and circa 700 individuals in Sweden (29). CF is caused by loss-of-function variants in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene (30). The CFTR protein is mainly expressed in epithelial cells and is important in regulating luminal secretions in several organs. The organs mainly affected are the bronchial tree and the lungs, gastrointestinal tract, pancreatic and biliary ducts, sweat glands in the skin and female and male reproductive tracts respectively, and the liver (30, 31).

The CFTR protein works as channel transporting chloride across cell membranes to regulate mucosal viscosity (32). Dysfunction of this protein leads to aberrant $Cl^-$ secretion, resulting in thickened mucosal linings. Clinically, the most apparent and severe manifestation in CF is lung disease involving bronchial obstruction and build-up of phlegm which is often colonised by airway pathogens (33). In recent years, highly effective CFTR modulator therapy has been developed and has greatly improved disease outcomes for CF patients (34).

The most common co-morbidity in CF is development of CFRD , the prevalence of which, increases with age. Nearly half of all individuals with CF have been diagnosed with CFRD by the age of 30 (35). Diagnosis of CFRD is usually made with an OGTT, and patients are followed regularly for the development of worsened glucose tolerance (36). Improvement in CF care through better drug therapies and overall disease management, has led to an increased lifespan and better quality of life for these individuals (34, 37). However, the impact of CFTR modulator therapy on glycemic control and CFRD symptoms has been modest (38), with some studies showing promising improvements in insulin secretion for CF individuals with IGT (39). Still, studies evaluating incidence of CFRD after long-term highly effective CFTR modulator therapy are yet to be concluded, and currently one of the most urgent research questions in the CF field is to understand and hopefully of prevent CFRD development (38).

The longstanding hypothesis for CFRD pathogenesis was that dysfunctional CFTR in the pancreatic duct resulted in primarily an exocrine pancreatic disease, entailing pancreatic insufficiency from duct blockage contributing to malabsorption, pancreatic fibrosis and pancreatic steatosis (40). There are ~2000 variants of *CFTR* described to cause dysfunctional CFTR protein products and these are divided into five classes (I-V) based on the cellular effect on CFTR (41). Variants with more detrimental effects leading to failed synthesis of CFTR (class I) or failed processing and delivery to the plasma membrane (class II) confer higher risk of developing both pancreatic insufficiency and CFRD, further implicating connection between exocrine pancreatic disease and CFRD (42, 43). By far the most common disease causing variant for CF is the F508del (deletion of a phenylalanine at position 508 in CFTR) and is a class II variant (30).

Investigations challenging the hypothesis of exocrine pancreatic disease creating a suboptimal milieu for the endocrine pancreatic islets, have primarily argued that CFTR is expressed in pancreatic islet cells and directly affects hormone secretion *per se* (44). CFTR expression has been identified in insulin-secreting β-cells (45-48) although it is clear that it is only present in a minority of β-cells (46). Dysfunctional islet CFTR, studied in a mouse model harbouring the F508del CFTR variant, resulted in reduced insulin secretion (49). However, researchers in this field are currently debating this issue, and some still regard exocrine pancreatic disease as the sole cause of CFRD (50). In a broader view, these two processes of intrinsic islet dysfunction and islet damage, are not necessarily mutually exclusive but may rather interact with each other. Dysfunctional CFTR in β-cells may initially lead to reduced glucose tolerance, which is exacerbated over time with exocrine pancreatic disease (51). In Figure 1, the current understanding of changes occurring in the pancreas in CFRD are outlined.

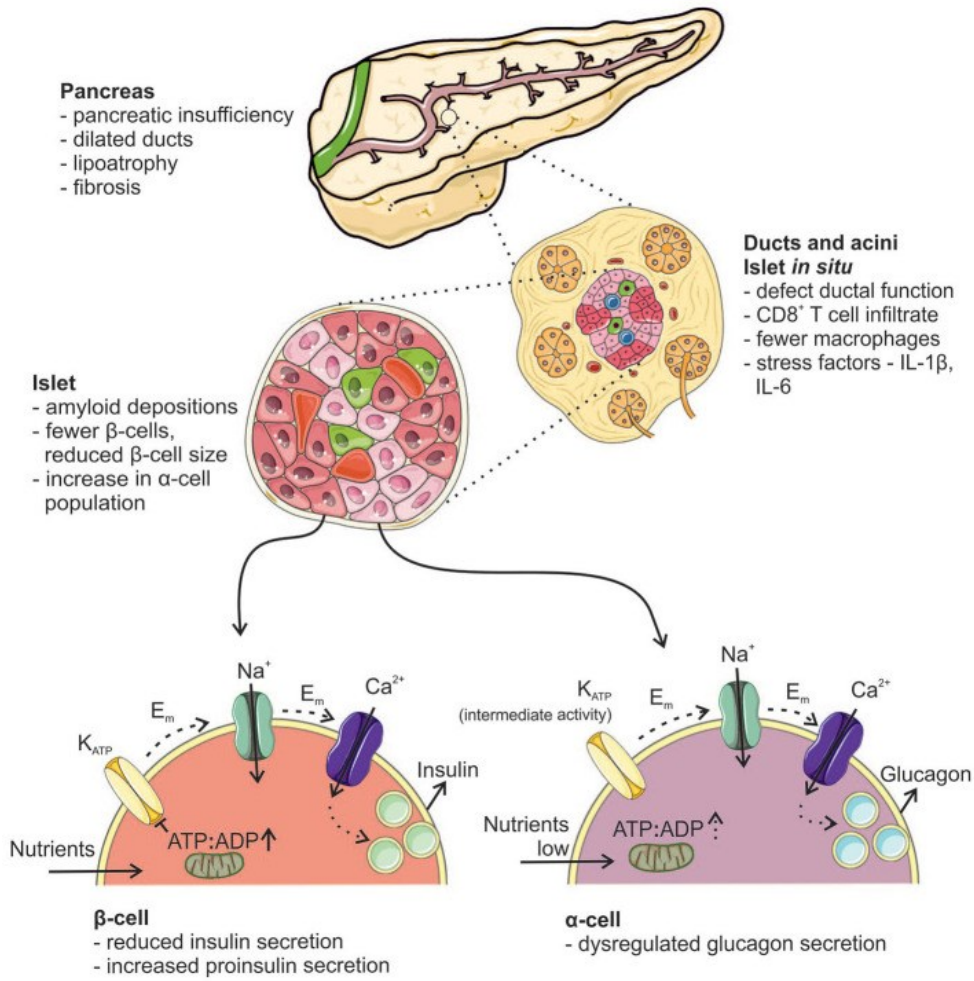


**Figure 1:** Overview of pancreas changes in CFRD. $CD8^+$: cluster of differentiation 8 expressing T lymphocytes. IL-1β: interleukin-1β. IL-6: interleukin-6. $K_{ATP}$: $K_{ATP}$ channel. Image from (51).

# Pancreatic islet biology

## Overview

The pancreatic islets are endocrine micro-organs located within the exocrine pancreatic tissue. Comprising only 1-2% of the total pancreas weight, or about one to two grams of cells, the pancreatic islets intricately control the blood glucose homeostasis of the entire body (8, 11). The entire pancreas works in concert to regulate whole body metabolism by modulating nutrient uptake, distribution and usage. The exocrine pancreas consists of acinar cells, which are mainly responsible for producing pancreatic juices containing digestive enzymes such as trypsinogen, chymotrypsinogen and procarboxypeptidases; and ductal cells, responsible for regulating fluidity and pH of the pancreatic juices (52). The exocrine pancreatic juices are secreted into the duodenum where they aid in digestion and food uptake from the gut. Once absorbed, endocrine pancreatic islet cells sense nutrients in the bloodstream and will dictate further nutrient usage by secretion of islet hormones (52).

The pancreatic islets work in conjunction with other organs to regulate metabolism. Hormones secreted from the gut, upon nutrient uptake such as glucagon-like peptide 1 (GLP-1) from intestinal L-cells, can signal to the islet cells and modulate hormone secretion. GLP-1 works in conjunction with β-cells to amplify insulin secretion whereas it inhibits glucagon secretion in α-cells (53). This post-prandial process is called the incretin effect and other hormones such as gastric inhibitory polypeptide (GIP) also contribute to this (53). In addition, the pancreatic islets receive numerous other regulatory inputs. There are considerable effects from both branches of the autonomic nervous system. Sympathetic activity, either through direct release of noradrenalin from pancreatic nerve endings or indirectly through circulating adrenaline, will inhibit insulin secretion by binding to α2-adrenergic receptor. In opposing fashion, signaling through β2-adrenergic receptors on α-cells will stimulate glucagon secretion (54). Effects of parasympathetic activity are thought to be part of the cephalic phase of insulin secretion, and acetylcholine signaling through muscarinic receptors generally stimulates insulin secretion (54, 55). Circulatory hormones also influence glucose levels and glucocorticoids (56), thyroid hormones (57), and growth hormone (GH) (58) all provide regulatory inputs. Furthermore, direct effects of glucocorticoids can have a negative impact on β-cell function (59). Many of these factors are in this context termed *counterregulatory hormones* for their ability to increase blood glucose. This is important, as insulin is the only hypoglycemic hormone. Life-threatening hypoglycemic episodes can cause irreversible brain damage. Therefore, it is appropriate to have several hyperglycemic regulators in place for protection (60).

The two main hormones secreted from the pancreatic islets are insulin from β-cells and glucagon from α-cells. Insulin, as previously mentioned, is secreted post-prandially and the main secretion stimulant is elevated blood glucose levels (11). Insulin will signal for uptake and storage of glucose and other nutrient in target tissues such as the liver, muscle tissue and adipose tissue and promote anabolic processes throughout the body (61). Glucagon from α-cells will promote opposing processes such as hepatic gluconeogenesis and glycogenolysis to produce glucose for release into the bloodstream. Glucagon is the primary *counterregulatory hormone*. This is important after sleep when the body has fasted over-night (62). The main trigger for glucagon release from α-cells is thought to be prolonged periods of fasting, as the highest levels of glucagon in the circulation is measured after a three day fast (63). Studies in recent years have nuanced the regulation of glucagon release. Fatty acid oxidation is thought to be required for proper glucagon release, but glucose shuts down this process to inhibit glucagon secretion (64). Overall, the primary islet hormones generally work in opposite fashions to regulate blood glucose, and they serve different roles with regards to recency of meal intake and how they regulate the liver.

The β-cells and the α-cells are not the only endocrine cells in the pancreatic islets. In addition, three other neighbouring hormone secreting cell types have been described. These are the somatostatin secreting δ-cells, the pancreatic polypeptide secreting PP-cells (also referred to as γ-cells) and the ghrelin secreting ε-cells (65). Human islets have a considerable difference in composition of the islet cell types when compared to rodent islets, with the clearest difference being a lower proportion of β-cells in human islets (66). The morphology and organization of the human islet is also markedly different from rodent islets. In rodents, the α-, δ and PP-cells form a periphery around a core of mainly β-cells, providing an anatomical basis for local regulation of islet hormone secretion through the microvasculature (65). In humans however, the endocrine cells are intermingled, directly interconnected and paracrine regulation between cell types is well described (65, 67, 68). In Figure 2, a schematic representation of a human islet with approximate endocrine cell type proportions are shown. Of note, a recent study on human islet morphology and distribution, suggests that there are considerable differences in islet composition. Larger islets are predominantly composed of a mix of α- and β-cells, whereas smaller islets mainly contain β-cells (69).

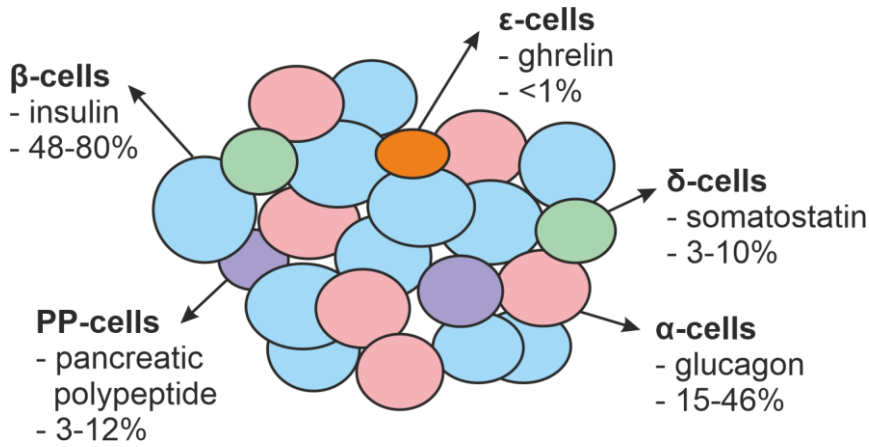


**Figure 2:** Schematic illustration of a human pancreatic islet. Endocrine cell types and their main hormones are shown. Relative cell type composition from (65, 66, 68).

## Islet cell development

Pancreatic islet cells develop from pancreatic progenitor cells present within the developing gland. The first key transcription factor determining either exocrine or pancreatic cell fate is the *pancreatic duodenal homeobox-1* (*PDX-1*). Expression of *neurogenin-3* (*NGN3*) determines commitment to endocrine cell lineage. From endocrine progenitor cells, the first division is into either *aristaless-related homeobox* (*ARX*) or *paired box 4* (*PAX4*) positive cells, corresponding to the development of α- or β-cells, respectively (70). In β-cells, continued expression of *PDX-1* as well as induction of expression of the transcription factor *MAFA*, which interacts with *PDX-1* to maintain insulin gene expression and expression of exocytotic genes (70, 71). From the same group of transcription factors, *MAFB*, generally considered to have a more prominent role in α-cell identity, still has a crucial role in β-cell migration in the developing gland (72). However, a few transcription factors do not paint the complete picture. β-cell development is more intricately regulated, with a recent study using single-cell RNA sequencing on islets generated from stem cells and adult islets has unveiled a complex network with a myriad of transcription factors and proteins involved in β-cell development (73).

## Regulation of intra-islet physiology

The primary goal of a functioning and a fully developed β-cell is to adequately secrete insulin as a response to nutrient uptake. Within the islet, there is considerable paracrine regulation and the β-cells work within a context of responses to the other islet hormones (55). The two most well-stablished paracrine regulators of insulin

secretion are the α-cells and the δ-cells. Glucagon from α-cells stimulates the release of insulin from β-cells (62, 74, 75). This effect works through signaling of glucagon both on the glucagon receptor (GCGR) and the GLP-1 receptor (GLP1R) (75). Glucagon secretion is stimulated both by both amino acids (62) and by fatty acids (64, 76). This form of intra-islet regulation likely becomes important post-prandially to ensure that ingestion of all meal constituents, proteins and fats alike, are handled properly (77). The pancreatic δ-cells also play a key role in regulating insulin secretion. Somatostatin has an overall dampening effect on hormone secretion by inhibiting glucagon secretion (67) and insulin secretion (55).

The β-cells in turn affect their neighbouring islet cells. Insulin secretion will exert local effects on neighbouring α- and δ-cells and regulate their secretion. Insulin inhibits α-cell glucagon secretion via insulin receptor (INSR) signaling (60, 78). Urocortin 3 (UCN3), which is co-released with insulin, stimulates δ-cell somatostatin secretion (79). This later mechanism serves as a local negative feedback loop, likely to protect from hypoglycemic episodes (60, 80). UCN3 also has substantial effects on δ-cell development as such. Reduction of UCN3 expression occurs in T2D (79). Another example of a β-cell-derived local islet regulator is γ-amino-butyric acid (GABA). GABA is released from microvesicles separate from insulin vesicles as well as from insulin vesicles. GABA release is increased in a glucose-dependent manner (81, 82). GABA inhibits glucagon secretion from α-cells by signaling via $GABA_A$ receptors, initiating a $Cl^-$current which subsequently dictates α-cell membrane potential and glucagon release (83)

Insulin also regulates β-cells in an autocrine fashion. However, whether insulin stimulates further release of insulin or inhibits insulin release, is under debate and is likely dependent on the nutritional status as well as presence or absence of stress put on the β-cell in T2D (84). In general, however, insulin activating the INSR on β-cells is presumed to inhibit its own secretion (85, 86). In addition to insulin and the aforementioned UCN3 and GABA, the β-cells also secrete a number of small factors which include but are not limited to: amylin (also known as islet amyloidogenic polypeptide), $Zn^{2+}$, and serotonin (60). Overall, several paracrine regulators of hormone secretion have been described alongside direct regulation of the primary islet hormones.

## Stimulus-secretion pathway for insulin secretion

The stimulus-secretion pathway for insulin release couples the uptake and metabolization of nutrients from the bloodstream to an electrical signal at the β-cell plasma membrane. This electrical signal leads to the influx of $Ca^{2+}$ into the cells triggering the exocytosis of vesicles containing the insulin hormone. Below, the major steps of this process are described.

The trigger for insulin secretion in β-cells is elevation of plasma glucose. In humans, the GLUT1 glucose transporter is the primary route of glucose uptake into the β-cell whereas GLUT2 is the primary transporter in rodent β-cells (87). However, GLUT2 is also expressed in human β-cells and knockdown of this gene impairs insulin secretion (88). Glucose is then phosphorylated by glucokinase (GCK). GCK activity is the rate limiting step in the stimulus-secretion pathway and loss-of-function mutations in GCK can lead to neonatal diabetes mellitus (11, 89). In β-cells, almost all pyruvate generated in glycolysis will undergo further metabolization in mitochondria (90). ATP generated from tricarboxylic acid (TCA) cycle and electron transport chain activity will result in an increase of the cytoplasmic ratio of ATP:ADP.

At rest, the baseline plasma membrane potential of human β-cells is hyperpolarized at around -70 mV. The most important ion channel for maintaining this hyperpolarized resting potential is the $K_{ATP}$ channel (11). The $K_{ATP}$ channel is a complex consisting of a central tetrameric ion channel, Kir6.2 transcribed by the *KCNJ11* gene (91), surrounded by four sulfonylurea receptor 1 (SUR1) subunits. As noted, genetic variants of *KCNJ11* associate with distinct subgroups of T2D predominated by dysregulated insulin secretion (23, 92, 93). The SUR1 subunits are equipped with an ATP binding cassette, and upon increased ATP:ADP ratio, SUR1 will regulate Kir6.2 and decrease its open probability (91). The closure of the $K_{ATP}$ channel hinders the outflow of $K^+$ and provides an initial depolarization of the β-cell plasma membrane. This is the step where the metabolic processes occurring prior to the $K_{ATP}$ channel are coupled to elicit an electrical signal in the plasma membrane. In humans, voltage gated $Na^+$ channels are likely important in furthering the depolarization and these channels are opened at around -40 mV, letting $Na^+$ enter the cell (94). Additionally, osmotic swelling of the β-cell triggered by glucose uptake can result in the opening of pressure-sensitive Piezo1 channels. Piezo1 is a non-selective cation channel and will promote the influx of both $Na^+$ and $Ca^{2+}$ and may thus contribute to the stimulus secretion coupling pathway (95).

Voltage gated $Ca^{2+}$ channels (VGCCs) are activated at slightly different membrane potentials and serve different roles in regulating $Ca^{2+}$ influx to the cell (11). T-type $Ca^{2+}$ channels will open at around -55 mV and transiently let $Ca^{2+}$ into the cell, contributing to the depolarization (96). Interestingly, T-type channels are inhibited by $Zn^{2+}$ and this mechanism, whereby $Zn^{2+}$ in insulin vesicles inhibits channel activity, likely serves as a local negative feedback loop in insulin secretion (94). L-type and P/Q-type $Ca^{2+}$ channels are the most important $Ca^{2+}$ channels to depolarize in the β-cell action potential and the L-type channel has the highest open probability at high glucose of all VGCCs (94). L-type channel activity has also been shown to be associated with T-type channels, serving as an example of crosstalk between $Ca^{2+}$ channels in the regulation of insulin secretion (97). Influx of $Ca^{2+}$ near the insulin vesicles is the triggering signal for exocytosis and the release of insulin (98, 99).

Production and processing of insulin itself is promoted by glucose and involves regulation of PDX-1 through mitogen activated protein kinase signaling pathways and direct control of the translation of insulin mRNA (100, 101). The hormone is packed into vesicles near the plasma membrane and the exocytotic release machinery bears striking resemblance to that of vesicles with neurotransmitters near synaptic terminals (102, 103). Another example of regulation within the stimulus-secretion coupling pathway, is that SUR1 of the $K_{ATP}$ channel also participates in the priming of insulin vesicles in a cAMP-dependent manner (104). Figure 3 outlines the aforementioned steps of the stimulus-secretion pathway for insulin in β-cells. Omitted steps in this pathway, include, but are not limited to: repolarizing $K^+$ channels (94) and potentiator pathways such as elevation of intracellular cAMP by for example GLP-1 (74).

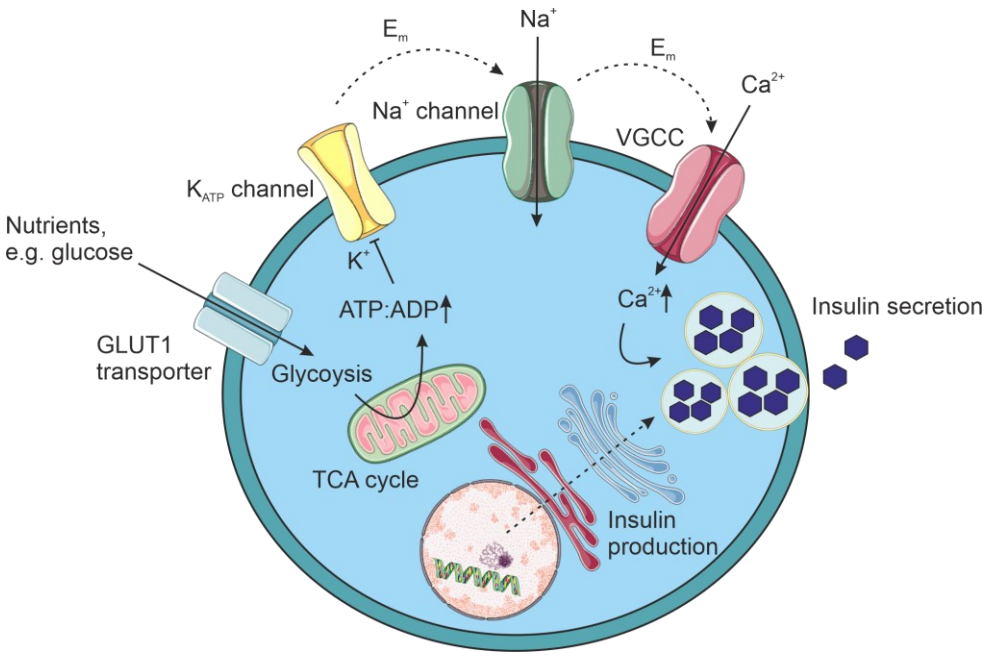


**Figure 3:** Outline of the stimulus-secretion pathway in β-cells. Glucose is taken up by glucose transporter 1 (GLUT1) and its subsequent metabolization in glycolysis and the mitochondrial tricarboxylic acid cycle (TCA) leads to an increase in intracellular ATP:ADP ratio. Closure of the $K_{ATP}$ channel depolarizes the plasma membrane and voltage gated $Na^+$ channels and voltage gated $Ca^{2+}$ channels (VGCC) are opened. Influx of $Ca^{2+}$ leads to exocytosis of insulin vesicles. Insulin production and processing is stimulated by glucose and other nutrients. Figure created using generic cellular structure images from Servier Medical templates, licenced under a Creative Commons Attribution 3.0 Unported Licence; https://smart.servier.com.

## Impairment of islet cell function

The topic of how islets as a whole, and β-cells specifically, dysfunction in development of different forms of diabetes mellitus has been a point of focused research for many decades. Many layers of understanding have uncovered presence of dysfunctional crosstalk between islets and other tissues in the body. Intra-islet pathological processes include dysregulated paracrine signaling, impairments in islet cell development and direct molecular alterations to the constituent parts of the stimulus secretion pathway. To cover every facet of this extensive field, is beyond the scope of this text. Here, some examples of how dysregulated islet cell function develops are presented.

### *Perturbed mitochondrial function*

Mitochondria are vital in β-cell function. Altered expression of electron transport chain proteins in β-cells and concomitantly reduced glucose-stimulated insulin secretion is reported in islets from T2D donors (105). Mitochondrial function is also impaired by accumulation of reactive oxygen species (106) and this accumulation increases with aging (107). A common variant in the transcription factor *transcription factor B1 mitochondrial* (*TFB1M*) alter the gene expression. TFB1M is important for mitochondrial function and its reduction leads to reduced insulin secretion and increased risk for T2D (108). Taken together, alterations in mitochondria, which are specifically associated with aging, can in part explain impaired insulin secretion in T2D.

### *Dysregulated $Ca^{2+}$ channels*

The final step of insulin secretion requires entry of $Ca^{2+}$ into the cell to drive membrane fusion of exocytotic proteins and release vesicle content. The L-type $Ca^{2+}$ channels are specifically located in plasma membrane lipid rafts near insulin vesicles (109) and the local elevation of $Ca^{2+}$ is required for proper exocytosis (98). Chronic exposure to palmitate results in dissociation of L-type $Ca^{2+}$ channels from secretory vesicles and impairment of insulin secretion (110). In β-cells from donors with T2D, the intricate organization of L-type $Ca^{2+}$ channels and insulin vesicles is lost, providing a basis for disturbed insulin secretion kinetics in T2D (111). Palmitoylation of subunits after fatty acid overload results in aberrant $Ca^{2+}$ and can induce β-cell apoptosis (112). Additionally, the lipid rafts can be disturbed in dyslipidemia and by high glucose treatment (109). Polymorphisms in the L-type $Ca^{2+}$ channel Cav1.3 (encoded by the gene *CACNA1D*) confer lower gene expression and confer a higher risk of developing T2D (113). Of note, several subunits VGCCs are under the transcriptional control of *TCF7L2* which may contribute to impaired insulin secretion in T2D (114). In all, disturbed $Ca^{2+}$ channel function has been described as both a result of environmental factors influencing the β-cell and genetic risk variants, highlighting the importance of these channels in insulin secretion.

***Dysregulated exocytosis and insulin vesicle priming***

The intrinsic regulation of insulin vesicle exocytosis is dependent on maintaining functional domains in the plasma membrane together with $Ca^{2+}$ channels. Insulin vesicle exocytosis is often measured as an increase in membrane capacitance upon $Ca^{2+}$ entry into the β-cell (115) or direct visualization of fusing vesicles using recently developed techniques such as total internal reflection fluorescence microscopy (111). Lower local levels of cholesterol, important for membrane stabilization, around these lipid raft domains have been shown to impair insulin secretion and membrane enlargement upon $Ca^{2+}$ entry into the β-cell (116, 117). These findings point to the importance of a functional relationship between $Ca^{2+}$ channels and docked insulin vesicles.

As loss of first-phase insulin secretion is indicative of T2D (27), experimental studies have been conducted to explain the biphasic nature of insulin release on a vesicle level. Investigations have shown that an initial burst of insulin secretion occurs when primed vesicles near the plasma membrane, termed the readily releasable pool, fuse after a glucose stimulus. This is followed by a slower release of vesicles in a reserve pool (118). In the readily releasable pool hypothesis for biphasic insulin secretion, impaired exocytosis is described as incomplete vesicle fusion (119). This is termed "kiss-and-run" exocytosis. The insulin vesicle docks to the inside of the plasma membrane but fails to undergo complete fusion, likely contributing to insulin secretion defects in pathophysiological states such as hyperglycemia (118). Support for the readily releasable pool hypothesis comes in the form of lower gene and protein expression of protein key parts in the secretory complex surrounding insulin vesicles in islets from T2D donors. The SNARE complex (soluble NSF-attachment protein receptor) contains proteins such as syntaxin-1, SNAP25 (synaptosomal associated protein 25 kDa) and VAMP2 (vesicle-associated membrane protein 2), all of which are decreased in islets in T2D (15, 120, 121). Furthermore, assessment of gene variants linked to T2D showed that the primary effect on islets in individuals with risk variants was decreased insulin vesicle exocytosis (122). Notably, this view has recently been challenged. Biphasic insulin response is proposed to be the result of functional heterogeneity among β-cells. This explanation for biphasic insulin secretion hypothesizes that a subpopulation of β-cells display a very tight coupling between $Ca^{2+}$ influx and insulin secretion and respond more readily to small elevations in glucose. In β-cells from diabetic mice, the tight coupling between $Ca^{2+}$ influx and exocytosis is lost (123).

The priming of insulin vesicles entails the lowering of vesicular pH for prohormone convertases to cleave proinsulin to insulin and C-peptide. CFTR is proposed to be involved in this process by interacting with the $Cl^-$ channel anoctamin 1 (ANO1) (124). In β-cells with the F508del variant, insulin vesicle priming is impaired resulting in an increase in secreted proinsulin (45, 49). This corresponds to higher levels of circulating proinsulin in individuals with CFRD (125, 126). Additionally,

dysfunctional CFTR *per se* also reduces insulin secretion (49). Taken together, there is considerable evidence indicating dysregulation of exocytotic processes in β-cells in T2D and CFRD.

### *Endoplasmic reticulum stress*

Increased endoplasmic reticulum (ER) stress has received particular attention in relation to the increased demand for insulin production put on the β-cell in the context of insulin resistance present in T2D. The ER stress hypothesis states that a possible cause for β-cell dysfunction in T2D arises when increased insulin production, due to insulin resistance, evokes the unfolded protein response in the ER (127). This is suggested to lead to increased β-cell apoptosis and loss of functional β-cell mass, although, this is only likely present in long-standing T2D (128).

### *Dysfunctional α-cells and impaired intra-islet crosstalk*

Hyperglucagonemia as a contributor to high blood glucose in T2D has attracted significant attention in the last decades (62, 129). Elevated glucagon levels lead to stimulation of hepatic glucose production through gluconeogenesis and glycogenolysis. Fasting levels of glucagon are higher in T2D and are positively associated with plasma insulin levels (130). In OGTT studies of T2D, there is dysfunctional secretion of glucagon in response to glucose (131) and this has been investigated on the cellular level. Electrophysiological studies in α-cells from islets from T2D donors have shown that the secretion signature and identity markers of α-cells shift toward a more β-cell like state and this could contribute to the aberrant glucagon secretion seen in T2D (132). Of note, dysfunctional CFTR has been proposed to play a role in deregulation of glucagon secretion in CF. Studies on α-cells indicate that the F508del variant causes hypersecretion of glucagon, which could contribute to hyperglycemia in CF (133). However, presence of hyperglucagonemia in CF is only moderate with one study indicating slightly elevated hepatic gluconeogenesis (interpreted as a proxy for elevated glucagon levels) (134). One small study also suggests failure of suppression of glucagon secretion following a glucose challenge in patients with CFRD (135).

In T2D, dysregulated glucagon secretion has also been hypothesized as a disturbed paracrine loop. In short, β-cells and δ-cells are electrically coupled (67) and via UCN3 (60). Morphological evidence supports fewer connections between β- and δ-cells in T2D (136). Here, impaired β-cell function is proposed to lead to failure of suppression of glucagon secretion both via lower local insulin levels and through loss of δ-cell somatostatin secretion (67). Overall, disturbed glucagon secretion and intra-islet paracrine signaling as part of the explanation for T2D development is an emerging area in the pancreatic islet biology research field.

***Hyperinsulinemia as a dysfunctional islet response***

The previous sections have focused on different processes leading to decreased insulin secretion or increased glucagon secretion. However, while decreased insulin secretion resulting in relative insulin deficiency and hyperglycemia are accepted as the final stages in developing T2D, increased fasting levels of insulin, which are observable several years before formal diagnosis (137), have been postulated to be a driver of the disease. In this view, hypersecretion of insulin is not due to whole-body insulin resistance with increased insulin demand, but rather initial hyperinsulinemia results in overweight and obesity, which in turn drive insulin resistance and further islet dysfunction (138). Long-term hyperinsulinemia can also contribute to fatty liver disease, contributing to T2D development (139). However, it is still clear that in order for overt T2D to develop, a decrease in β-cell function must occur (11). It is intriguing to envision that early hyperfunction of β-cells, in response to environmental stressors, leading to fat accumulation, insulin resistance and eventually pushing β-cells to failure causes T2D. However, conclusive long-term follow-up studies in humans elucidating the order of events are lacking.

# Insulin-like growth factor binding proteins

The insulin-like growth factor binding protein (IGFBP) family consists of seven proteins, IGFBP1-7, and have been investigated for their roles in regulating the hormones insulin-like growth factor (IGF) 1 and 2 (140). The IGFBPs are a part of the GH-IGF system and are present in the circulation and in several tissues throughout the body (141). IGFBP3 and IGFBP5 are the predominant proteins present in the circulation. These proteins bind to both IGF1 and 2 and regulate IGF bioavailability. IGFBPs can also bind to the IGF1 receptor (IGF1R) and modulate its activation (142). The concentrations of IGFBP3 and IGFBP5 greatly exceed that of IGF1 and IGF2, and both IGFBP3 and IGFBP5 are present in the circulation in the form of protein complexes which release the IGF hormones to their bioactive free state (143, 144). A large part of their regulation arises from the cleavage of IGFBP proteins by IGFBP proteases which are present in the circulation as well as on cell surfaces. IGFBP protease expression on cell membranes provide an additional layer of IGF-IGFBP regulation near the IGF1R (141, 142).

The primary source of circulating IGFBPs is the liver (145). However, assessments using *in situ* hybridisation of liver tissue, differential expression of IGFBPs has been demonstrated. IGFBP1 is primarily expressed in parenchymal hepatocytes whereas IGFBP3 is mainly located in resident liver macrophages (146). Interestingly, the levels of IGFBP1 secreted from hepatocytes are negatively regulated by insulin, and this is postulated to provide a nutrient sensing dimension to the IGF system. Low

insulin levels signal low food availability, which increases IGFBP1 levels and restricts growth when food is scarce (141, 147).

IGFBP7 stands apart from the other members of the IGFBP family. It is the most recently discovered of the seven proteins and was initially described in many tissues and named several times before consensus was reached that it was indeed an IGFBP protein (148). Old nomenclature for IGFBP7 reflects this, and names include mac25, prostacyclin stimulating factor and IGFBP-related protein 1 (149-151). IGFBP7 is different from IGFBP1-6 in that it preferentially binds insulin over IGF1 or IGF2 (151). Furthermore, IGFBP7 can bind to and modulate the actions of both the IGF1R and the INSR (152, 153).

The circulating levels of IGFB7 are in the low nanomolar range (154, 155), substantially lower than those of IGFBP3 and IGFBP5 which are between 10-100-fold higher (142, 156). The expression of IGFBP7 has been explored in several tissues in the healthy adult showing modest to weak staining intensity in liver and pancreatic islet cells (150). IGFBP7 has been shown to be upregulated in resident liver macrophages in obesity, and locally promote fat accumulation and hepatic gluconeogenesis in hepatocytes. In the same study, IGFBP7 mRNA editing was shown to occur which increased the binding affinity of IGFBP7 to INSR and promoted an obesity-associated metabolic profile in the liver. Knockdown of IGFBP7 reversed this phenotype (153). Moreover, IGFBP7 has been linked to development of senescence in cardiac myocytes, resulting in outcomes such as heart failure and fibrosis. Neutralising antibodies against IGFBP7 reversed the senescence profile (157). IGFBP7 has in recent years attracted significant attention as a novel biomarker for heart failure (158-161). In addition, urinary IGFBP7 together with tissue inhibitor metalloproteinase 2 (TIMP2), have been investigated as markers of kidney injury in several different settings (162, 163). Signaling through the transforming growth factor β1 (TGFβ1) pathway induces IGFBP7 expression in tubular cells and is proposed play a causative role in kidney injury in diabetic nephropathy (164).

Taken together, there is a growing body of research showing the involvement of IGFBP7 dysregulation in tissues relevant in T2D and in cardiometabolic diseases. However, very little is known about IGFBP7 in pancreatic islets. As noted, there is weak and variable staining intensity of IGFBP7 in islet cells (150) and there is a considerable upregulation of the *IGFBP7* gene in maturing α-and β-cells in developing islets (73). Intriguingly, one study in CFTR knockout ferrets has suggested that upregulation of IGFBP7 by TGFβ1 in exocrine tissue surrounding pancreatic islet may mediate pathological intrapancreatic crosstalk and lead to reduced insulin secretion, contributing to CFRD (165). Given the biochemical properties of IGFBP7, insulin binding and modulation of INSR and IGF1R, combined with its pathological dysregulation in several metabolically active tissues in T2D and CFRD, it is of the utmost interest to study this protein in pancreatic islets.

# Interleukin-4 in pancreatic islets

The pathogenesis of T2D is also characterized by systemic inflammation arising in adipose tissue (166) as well as local inflammation in the pancreatic islets (167). In this way, inflammatory processes mediated by innate and humoral facets of the immune system contribute to the main drivers of T2D development: impaired insulin secretion and insulin resistance. Interleukin-4 (IL-4) has been mainly studied as a cytokine secreted from T helper 2 cells in allergy and asthma (168), but is also important in signaling to monocytes to differentiate into M2-polarized (anti-inflammatory) macrophages (169). In T2D, pancreatic islets display altered composition of macrophages (170-172). M1-polarized macrophages secrete IL-1β, one the most potent pro-inflammatory cytokines (169). The role of IL-1β in islet inflammation and its blockade as potential therapeutic approach in T2D has been extensively investigated (167).

Altogether, the stage is set for altered innate immune cells and local secretions of cytokines to regulate β-cell function. Co-treatment experiments with IL-1β and IL-4 in human islets showed protective effects of IL-4 to the impaired secretion caused by IL-1β (173) and, in line with this, clonal rat β-cells co-treated with IL-1β and IL-4 showed the same protective effects (174). Human β-cells express the IL-4 receptor (IL4R) (175), opening up to the possibility that not only innate immune cells but also β-cells in the human pancreatic islet are regulated by IL-4 signaling. However, very little is known about IL-4 working in isolation on human islets in T2D, and whether IL-4 can affect insulin secretion in this disease.

# MicroRNAs in diabetes mellitus

MicroRNAs (miRNAs) are a collection of short, ~20-25 nucleotide (nt) long, single-stranded non-coding RNA molecules. After their discovery in 1993, knowledge and insight into how miRNAs shape cellular function has grown rapidly (176). With the ability of miRNAs to directly bind and regulate mRNA transcripts, their role in regulating cell function in numerous diseases, including T2D, has been extensively investigated (177, 178).

## General miRNA biology

MiRNAs are transcribed in the nucleus from either intronic or intergenic regions by RNA polymerase II. The transcript forms a characteristic hairpin loop, pri-miRNA, which is subsequently cleaved and shorted by the Drosha enzyme to form a ~60-70 nt long transcript, pre-miRNA, with the hairpin in the middle (179). Pre-miRNAs are exported from the nucleus to the cytoplasm via Exportin-5 (180) where they

undergo further cleavage by the Dicer enzyme, which removes the hairpin and results in the formation of a miRNA duplex (181). From here, one strand of the miRNA duplex, usually the 5' strand, is loaded on the Argonaute protein which together with a group of supporting proteins form the RNA-induced silencing complex (RISC) (182). The RISC guides the miRNA to 3' UTRs of target mRNAs, resulting in mRNA degradation or removal of the poly-A tail from the 3'UTR, both processes leading to repression of the mRNA expression (179). Importantly, in specific instances, this miRNA-mRNA interaction has oppositely been shown to result in improved mRNA stability and increased expression (183). Figure 4 presents the canonical miRNA biogenesis pathway.

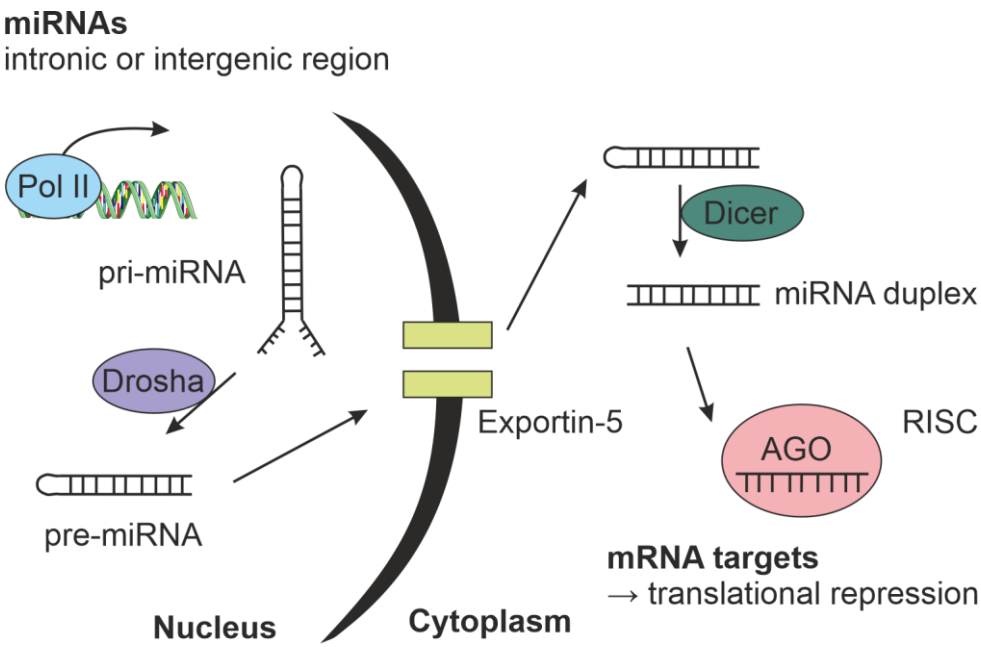


**Figure 4:** Outline of the canonical miRNA biogenesis pathway. RNA polymerase II (Pol II) transcribes a pri-miRNA hairpin loop. Drosha cleaves and shortens both the 5' and 3' ends. Exportin-5 translocates the pre-miRNA to the cytoplasm, where Dicer removes the hairpin. One strand of the miRNA duplex is loaded onto the Argonaute (AGO) protein, forming the RNA-induced silencing complex (RISC). Adapted from (179).

## MiRNAs in islet cells

The first miRNA discovered in pancreatic islets was miR-375-3p (184). Overexpression of this miRNA in a clonal mouse β-cell line resulted in reduced insulin secretion that was not due to reduced $Ca^{2+}$ influx (184). Subsequent investigations in human islets revealed that miR-375-3p expression correlated with insulin expression and that this association was lost in islets from hyperglycemic

donors (185). In a clonal rat β-cell line, overexpression miR-375-3p was found to inactivate voltage-gated $Na^+$ channels, however, no clear effects on insulin secretion were observed (186). MiR-375-3p has been associated with several other β-cell functions and is viewed as a miRNA with a high abundance in all islet cells, laying the foundations for this miRNA being part of a regulatory network in islet cells (185, 187). Several miRNAs have since then been described to be dysregulated in pancreatic islets in T2D (188). Examples include miR-130a-3p, miR-130b-3p and miR-152-3p which are collectively upregulated in islets from T2D donors. Overexpression of these miRNAs perturbed intracellular ATP:ADP ratios, with decreased insulin secretion observed after overexpression of miR-130a-3p and miR-152-3p (189). In a similar study, miR-200c-3p was found to also decrease insulin secretion through downregulation of the transcription factor ETS variant transcription factor 5 (ETV5) (190).

Importantly, deregulated miRNAs can be regarded as both causative of β-cell dysfunction in T2D, as outlined above, or to serve compensatory purposes in β-cells in T2D (191). One such miRNA is miR-223-3p which is elevated in islets in obesity and T2D and increases insulin content and β-cell proliferation (192). However, inherent in the biology of miRNAs is their ability to target several mRNAs and act as regulatory nodes in transcriptional networks. And, as such, it is important to assess miRNAs and mRNAs together and view changes in expression as part of a finely tuned system which is disrupted in several ways in dysglycemic states (193).

All of the above-mentioned studies have investigated whole-islet miRNA expression. Still, given the properties of miRNAs to dictate cell specific development fates and functions, it is reasonable to assume that there is differential expression of miRNAs between islet cells. However, there is currently a knowledge gap in our understanding of miRNA expression profiles between α- and β-cells and how these change in T2D.

## Circulatory miRNAs as biomarkers

Despite their role as intracellular regulators, miRNAs can also be found circulating in the blood and in the extracellular space in tissues. MiRNAs stored in exosomes can be released from cells upon the exocytosis of multivesicular bodies with the plasma membrane. Additionally, small microvesicles packed with miRNAs can bud and branch off from the plasma membrane. Lastly, miRNAs can simply be released together with miRNA binding proteins such as AGO or HDL into the circulation (194). This opens up the possibility that extracellular miRNAs can influence target cells and mediate regulation of cellular function distally (195).

In T2D and obesity, circulating miRNAs have been studied as possible disease mediators and evaluated as biomarkers (196). For example, miR-99b in exosomes released from adipose tissue can target and decrease liver production of fibroblast

growth factor 21 (FGF21). FGF21 in turn serves important functions in regulating glucose metabolism. Mice deficient in the Dicer enzyme in adipose tissue, resulting in less exosomal release of miR-99b, have higher levels of circulating FGF21 (197). Several cross-sectional studies have been undertaken investigating the profiles of circulating miRNAs in normoglycemic individuals and in people with T2D. MiR-34a-5p is elevated in patients with established T2D (198, 199) and is linked to dysregulation of circadian rhythm genes in the liver (200). Exosomes containing miR-142-3p, miR-142-5p and miR-155 are released from T lymphocytes in pancreatic islets and are taken up by β-cells resulting in apoptosis. This process has been shown to occur in T1D (201). Furthermore, miR-375-3p has also been investigated as a biomarker for T1D and T2D (187)

In summary, in both T1D and T2D, several studies have shown alterations in the circulating pool of miRNAs and that these may influence disease pathogenesis. In CF however, no studies have been conducted investigating the miRNA profiles of CF individuals with varying glucose tolerance status. CF is a multi-organ disease, and pathological release of miRNAs from affected tissues may play a role in CFRD.

# Aims of this thesis

Regulation of insulin secretion is tightly controlled by several factors. This regulation occurs on several levels. From circulating incretin hormones to direct neuronal input to the islet, via intra-islet mechanisms and intricate regulation within the stimulus-secretion pathway. All levels have their role in adjusting insulin release. In all forms of diabetes mellitus, insulin secretion is dysregulated and this is a pivotal step in the development of dysglycemia.

The aim of this thesis is to investigate the roles of two proteins, IGFBP7 and IL-4, miRNAs in α- and β-cells and circulating miRNAs in CF as potential regulators of insulin secretion.

## Specific aims of the thesis

I: Investigate the gene and protein expression of IGFBP7 in human islets and assess changes in T2D. Manipulate levels of IGFBP7 and measure insulin secretion to unravel if IGFBP7 affects β-cell function.

II: Investigate the impact of IL-4 in islets from non-diabetic and T2D donors and assess differences in insulin secretion, gene expression and miRNA expression.

III: Perform miRNA-sequencing of α- and β-cells respectively in sorted cell fractions from pancreatic islets. Assess differences in miRNA expression between the cell types and examine how miRNA expression changes in T2D.

IV: Identify circulating miRNAs potentially involved in the pathogenesis of CFRD and assess their effects on insulin secretion.

# Ethical considerations

In this thesis, in Paper I-III, we present research conducted on human pancreatic islets obtained from individuals who have been declared brain-dead. Consent to use their organs for transplantation or biomedical research was acquired from firstly: the donor's expressed consent if it was known prior to death, or secondly: written consent from the donor's family. A very common approach in studying T2D is to model the disease in animals, either through genetic manipulations or diet and lifestyle alterations. However, animal studies inherently have issues and questions regarding translatability to the human disease, as well as causing animal suffering. In this thesis, no animal experiments were conducted. Even though we cannot perform precise modifications in a live individual, we have assessed that it is superior to use human islets from brain-dead donors as compared to islets from animals.

There are clear benefits to study human tissues, both islets from non-diabetic human donors to use as control and islets from donors with T2D, as analyses of these islets directly reflect the healthy and the diseased state. Importantly, anonymity for islet donors is guaranteed by storage of personal data on secure servers where only a few assigned researchers have access. For islet batches delivered to research groups, only donor IDs become available to the experimenters, ensuring linked personal data is not disseminated. As guiding principle for publication, only group level statistics are presented.

In each study, Paper I-III, the number of islet batches we use for *in vitro* experiments is fairly small. Thus, to complement our findings in Paper I-III, we have used bioinformatic analysis of previously published findings from larger cohorts of human islets to put our results into context and strengthen our conclusions. In Paper IV, we used previously published data on gene expression in islets to assess putative targets of selected miRNAs. Altogether, we have successfully combined big data sets with carefully conducted experiments on human islets to build our understanding. The permit to handle human islets for biomedical research was granted by Etikprövningsmyndigheten, 2019–00357. Each islet batch was handled with care and respect, and we ensured to use all the material we obtained.

In Paper IV, we studied circulating miRNAs in individuals with CF. Even though there are animal models available to study differences between CF and CFRD, there are, as outlined above, still clear advantages in investigating the disease in afflicted

individuals directly. The permit to perform OGTT study as such and collect samples was granted by the Scientific Ethics Committees for the Capital Region of Denmark, Copenhagen, Denmark, permit number H-19085530. This study was conducted only in adult individuals with CF, and written consent was acquired from each participant. The study followed all conventions stated in the Helsinki Declaration for research in human subjects. As we also generated individual level data in Sweden with the measurement of serum miRNAs, we applied for and were granted permit to conduct the blood sample miRNA analysis and handle the patient data. Etikprövningsmyndigheten permit number 2021-04670 applies.

We performed experiments on clonal β-cell lines in all four studies. In Paper I-II, we used the human foetal β-cell line EndoC-βH1 and in Paper III-IV we used the rat insulinoma cell line INS-1 832/13. The usage of cell lines is preferable over primary cells from animals or animal models in accordance with the 3R principle for animal research. With cell lines, animal experiments can be Replaced, number of animals used Reduced, and experimental models can be tested and Refined prior to testing in animals. In our work, this specifically applies to Paper I where several experiments were conducted and refined in EndoC-βH1 cells as proof of concept prior to being tested on human islets. For Paper II-IV, cell line experiments were primarily used for follow-up of specific hypotheses based on findings from human islets or circulating miRNAs from serum of individuals with CF. Primary cells from rodent pancreatic islets could have been used for these follow-up experiments, but we determined that these could be replaced with cell lines in order to reduce animal use and suffering. Lastly, a special note on the EndoC-βH1 cell line. This cell line was generated from the pancreas of a human foetus and immortalized for use in biomedical research. Written and informed consent to use the pancreas tissue for this purpose was given by women upon elective termination of pregnancy. The generation of the cell line was approved by the French Biomedical Agency in accordance with French bioethics legislation (202). In line with this, we consider all the steps in the making of this cell line to be ethically defensible. A central purpose of generating a human β-cell line was to be able to study diabetes mellitus in a model system closely resembling a primary human β-cell. Subsequent studies on the EndoC-βH1 cell line have shown that it shares many characteristics of adult human β-cells (203, 204), making it a very suitable model for studying β-cell function. We as biomedical researchers are very grateful to be able to use this cell line as a model to study diabetes mellitus.

# Materials and methods

Several experimental, bioinformatic and image analysis approaches have been used in this thesis. This chapter will highlight and discuss the main models and methods. Limitations, as well as how the different methodological approaches used can strengthen each other will be examined. For full and detailed descriptions of each method, please see the appendaged articles.

## Experimental models

### Human pancreatic islets

Pancreatic islet from human donors were obtained from the Nordic Network for Islet Transplantation, led by Prof. Olle Korsgren at the isolation centre Uppsala. Islets were received through collaboration with Excellence of Diabetes Research in Sweden and Lund University Diabetes Centre and distributed by Human Tissue Lab at Clinical Research Centre, Malmö, Sweden.

Islets were purified and delivered in CMRL-1066 medium supplemented with 10% human serum. For full details on purification protocols and CMRL-1066 medium constituents, see (205). Prior to experiments, islets were handpicked to KREB's buffer for washing. For culture, islets were transferred to RPMI-1640 medium supplemented with 5 mM glucose, 200 mM L-glutamine, 10% FBS, penicillin and streptomycin 100 U/mL and 100 μg/mL, respectively. In Paper I-II, islets were treated with the investigated proteins IGFBP7 and IL-4 and thereafter assayed for insulin secretion and harvest of RNA and protein samples. SiRNA-mediated knockdown of the *IGFBP7* gene was also performed in Paper I. In Paper III, islets were not cultured but instead upon receival washed in PBS and dissociated into single cells prior to glucagon and insulin staining.

### Cell lines

#### *EndoC-βH1 cells*

EndoC-βH1 cells were cultured based on protocols described in (202). Cells were kept in a humidified atmosphere, temperature 37 °C and 5% $CO_2$ and cultured in matrigel and fibronectin-coated 48-well plates. The cell line was grown in DMEM medium with the following additions: 5.6 mM glucose, 2% BSA, 50 μM β-mercaptoethanol, 5.5 μg/mL transferrin, 6.7 ng/mL sodium selenite, 10 mM nicotinamide, and antibiotics penicillin and streptomycin 100 U/mL and 100 μg/mL. In all experiments described in Paper I and II, cells were seeded at a concentration of 900 000 cells/mL, 180 000 cells/well in order to reach ~100% confluence at 4 days of culture when endpoint experiments were conducted.

#### *INS-1 832/13 cells*

The rat insulinoma INS-1 832/13 cell line was specifically developed and selected for its secretory response signature which is sensitive to several known stimulators, potentiators and inhibitors of insulin secretion. The cell line also expresses the human insulin gene (206). INS-1 832/13 cells were cultured in 37 °C and 5% $CO_2$ in RPMI-1640 medium 11.1 mM glucose supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol and antibiotic mix of 100 IU/ml of penicillin and 100 μg/ml of streptomycin. In Paper III-IV, INS-1 832/13 cells were seeded at 240 000 cells/mL, 120 000 cells/well in 48-well plates. Cells were grown 24 hours prior to transfection with pre-miRNAs, and thereafter grown for 72 hours at which point cells were tested for insulin secretion and RNA and protein samples collected.

## Descriptive approaches: transcriptomics and image analysis

In order to describe differences in gene expression, either as a result of a treatment or between different biological groups, transcriptomics data shown as Normalized Counts is presented in Paper I, III-IV. The method used is DESeq2, which is characterized by accurate correction for variability in transcriptomic profiles, supports complex experimental designs and can effectively deal with small sample sizes. By using the negative binomial distribution, it can reliably assess differential expression estimates and identify potential genes of interest (207). In Paper I, DESeq2 was used to detect the differential expression of the *IGFBP7* gene in previously published RNA-sequencing (RNA-seq) data sets (88, 208) as well as additional RNA-seq data generated from islets treated with IGFBP7.

In Paper I, previously published single-cell RNA-seq (scRNA-seq) data (208) of the *IGFBP7* in different islet cell types is presented. To validate these transcriptomics level findings, an image analysis tool using guided machine learning was developed. Images of stained pancreata were annotated for islets and for identification of individual islet cells. After training, the program was run on the full image set to identify single islet cells. In these cells, staining intensity of the individual hormones glucagon and insulin and the IGFBP7 protein was assessed, generating a cell-level data set of protein expression. Here, based on hormone expression, α- and β-cells could be annotated and IGFBP7 expression in these individual cells could be compared between groups in a similar fashion as for RNA-seq data. These two techniques, scRNA-seq and cell-level image analysis, were used to complement each other in describing IGFBP7 expression on gene level and protein level.

## Functional approaches: insulin secretion assays, oxygen consumption rate measurement and viability assay

The primary endpoint for all islet and cell culture experiments in this thesis is assessment of glucose-stimulated insulin secretion (GSIS). In GSIS, the full stimulus secretion coupling pathway for insulin vesicle exocytosis is tested and changes occurring in any step of the pathway can result in altered insulin secretion. For both islets and cell lines we used static batch incubation with a low glucose level (2.8 mM glucose for islets and INS-1 832/13 cells, 1 mM glucose For EndoC-βH1 cells) and high stimulatory glucose level (16.7 mM glucose for islets and INS-1 832/13 cells, 20 mM glucose For EndoC-βH1 cells). In islet experiments, the islets were incubated serially first at low glucose and then at high glucose level. In cell lines, the glucose levels were added to separate replicate wells. Part of the reason for the usage of supraphysiological glucose levels is to test the insulin secretory systems fully and observe treatment effects more readily. These are also conventional glucose concentrations used by several research groups in the field, making comparisons between different laboratories easier. The usage of these high glucose concentrations has been questioned (209). However, both EndoC-βH1 and INS-1 832/13 cells in ramp experiments show similar GSIS at concentrations closer to upper physiological ranges as at 20 and 16.7 mM, respectively (202, 206). Therefore, we consider the glucose levels used throughout Paper I-IV reasonable in our model systems. Another test for insulin secretion is depolarization-induced insulin secretion. In Paper I, we test this with the addition of a high concentration of extracellular $K^+$, offsetting the electrochemical gradient for $K^+$ over the plasma membrane.

Insulin secretion is closely linked to the metabolic state of the β-cell. As such, in order to complement our findings in Paper I, we used the Seahorse assay in EndoC-

βH1 cells to measure oxygen consumption rate (OCR) as a proxy for mitochondrial function. With this method, OCR is measured after injections of pyruvate, oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and rotenone. These injections test addition of a direct mitochondrial fuel (pyruvate), ATP-linked respiration by blocking ATP-synthase (oligomycin), maximal respiratory capacity by the addition of protonophore of the inner mitochondrial membrane (FCCP) and the non-mitochondrial OCR by blocking complex I (rotenone). Of note, EndoC-βH1 cells, while comparable to human islets (203, 204), express the monocarboxylate transporter 1 (MCT1) (210) allowing them to take up pyruvate. However, this is considered a "disallowed gene" in β-cells, as pyruvate stimulating insulin secretion could lead to exercise-induced hyperinsulinemia (211). This probably reflects that EndoC-βH1 cells in some aspects still display an immature gene expression profile. Nonetheless, for our purposes to test mitochondrial function in Paper I, the presence of MCT1 in the plasma membrane bears less relevance. Additionally, we tested cell viability using an MTS assay. The MTS assay measures the reductive capacity of live cells, serving as a proxy for viability/proliferation. Reductive capacity is linked to mitochondrial metabolism, and the MTS assay experiments used in Paper I furthermore reflect the results of the GSIS and the OCR measurements.

# Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) methods are used in all articles of this thesis. In Paper I-II, conventional RT-qPCR for measuring gene expression is used. In Paper II-IV, different methods of RT-qPCR are utilized for miRNA detection. Regardless of type of transcript or detection method, the principles of analysing RT-qPCR are the same. In conventional RT-qPCR protocols used in Paper I-II, isolated transcriptomic RNA is reverse transcribed to cDNA using random primers. Using primers for genes of interest and appropriate endogenous controls, the cDNA pool undergoes RT-qPCR whereby the primers specifically amplify their respective genes. This reaction is run in several cycles with a doubling of the probed gene each cycle. Fluorescent reporter systems are used to determine when the emitted fluorescent signal from the amplified product is above the background threshold fluorescence. The cycle at which a cDNA/primer combination reaches this threshold is called Ct (cycle threshold) value. To determine differences in gene expression between a group of samples, gene levels are first normalized to in-sample endogenous controls. Firstly, the Ct values of the endogenous controls are subtracted from the Ct value of the gene of interest. Thereafter, the mean Ct value of the gene of interest in the control group is subtracted from the normalized Ct value in each sample. This calculation results in a -ΔΔCt value, which is the normalized difference between groups to reach cycle

threshold. Exponentiating this value with base 2 results in the $2^{-\Delta\Delta Ct}$ value, which is the relative gene expression difference between studied groups (212). It is important within each experiment to select appropriate controls that do not change with the tested treatment or are inherently different between tested groups (212).

Follow-up of miRNAs of interest identified in the miRNA-sequencing (miRNA-seq) pilot study in Paper IV was conducted using a locked nucleic acid (LNA) RT-qPCR assay. A problem encountered when analysing miRNAs with RT-qPCR is that the short transcript length renders binding of primers and specific amplification of miRNAs of interest difficult. LNA based primers are short RNA analogues where the ribose rings are locked in a conformation optimized for hybridization with a matching oligonucleotide strand (213). This technology allows for the design of shorter primers suitable for miRNA detection. In Paper IV, a reaction for general miRNA reverse transcription was used. The cDNA product was assayed with LNA primers for miRNAs of interest and serum miRNA controls identified in the pilot study. SYBR Green fluorescent dye, which binds to double stranded DNA, was used as reporter for Ct value acquisition.

Other systems for miRNA detection in biological samples, such as the TaqMan system, use specific reverse transcription stem-loop primers for cDNA generation and fluorescently labelled probes in the RT-qPCR (214). A drawback of this protocol is the necessity of specific primers in the reverse transcription step, making it unsuitable for high-throughput experiments. However, the ease of use and high specificity when few miRNAs are studied makes the TaqMan system eminent in testing the expression of specific miRNAs. For analysis of miRNAs human islets in Paper II, and miRNA overexpression follow-up experiments on cell lines presented in Paper II-IV, the TaqMan assays are used.

## Paper IV pilot study design, miRNA library preparation and miRNA-seq

For identifying differentially expressed serum miRNAs in CF after an OGTT, a pilot study was designed in Paper IV. A cohort of 93 individuals with CF (126) was utilized and the participants were subdivided into four glucose tolerance categories based on capillary blood glucose measurements from OGTT. The four categories were: normal glucose tolerance (NGT), indeterminate glucose tolerance (INDET), impaired glucose tolerance (IGT) and CFRD. The full cohort is described in (126). For the pilot study, three individuals from each of the four glucose tolerance categories were selected. The 12 individuals were matched for genotype (homozygous for F508del) and were at the time of the trial not receiving highly effective triple CFTR modulator therapy. RNA was extracted from baseline samples (blood draw from 1 min before glucose ingestion) and 60 min samples. This study

design allowed for evaluation of differences in fasting circulating serum miRNAs between the four groups and how miRNAs change in response to glucose intake. Another goal of the pilot study was to identify stable endogenous serum miRNA controls that could be used for normalization of miRNA expression throughout the OGTT. The approach of utilizing a subset of individuals from the cohort for discovery of miRNAs of interest has been successfully performed in our group previously (215). Serum was used over plasma because plasma contains coagulation proteins, which can potentially obfuscate analysis of the total pool of non-cellular miRNA in the blood. Comparisons between serum and plasma miRNAs in paired samples show greater variability in the plasma miRNA profile (216).

Preparation of cDNA libraries for miRNA-seq was used in Paper III-IV. This method offers unbiased detection of all miRNAs instead of only a subset of pre-determined miRNAs which is the case in, for example, miRNA-microarray based methods. In Paper III, miRNA-seq was conducted on sorted α- and β-cell fractions from islets of both non-diabetic donors and donors with T2D. In Paper IV, miRNA-seq was used on serum samples selected in the pilot study. MiRNAs are unique among small RNAs in their biochemical properties with a 3' hydroxyl group and a 5' phosphate group (217). This allows for the specific ligation of adapters to either end of the miRNA strand, a necessary step as longer reads are more stable. In the protocols used in Paper III-IV, Qiagen's QIAseq miRNA library kit is used to ligate 3' and 5' adapters to purified RNA from islets or serum. The miRNA-adapter products underwent reverse transcription using primers tagged with unique molecular indices (UMI), enabling accurate counting of sequenced reads. Libraries were amplified using primers with specific sequences serving as indices for each sample allowing for multiplexing of libraries in miRNA-seq. Sequenced reads were trimmed from adapter sequences, assigned to the original sample based on the index sequence and counted using the UMIs. As in Paper I, DESeq2 was also employed in Paper III-IV to normalize and analyse sequencing data.

# Additional materials and methods

In this thesis data from several published cohorts are used to complement the new findings. Apart from the generated RNA-seq data generated in Paper I, previously published islet RNA-seq and scRNA-seq cohorts utilized can be found in (88, 208). In Paper II, data from a cohort (218) which partially overlaps with Bacos *et al* (88) is used. The CF cohort used in Paper IV is described by Nielsen *et al* (126). For information on additional methods used in this thesis such as Western blot, RNA isolation, immunostaining, transfection assays, analysis of secreted insulin and fluorescence activated cell sorting, see the original articles.

# Results and Discussion

## Paper I

### *The IGFBP7 gene is upregulated in islets from T2D donors and does not correlate with age or HbA1c levels*

To examine the expression of *IGFBP7* in human islets, a previously published data set of whole-islet RNA-seq was utilized (88). In total, data from 219 donors grouped as non-diabetic (ND: 149 donors, HbA1c < 42 or unavailable), impaired glucose tolerance (IGT: 37 donors, 42 ≤ HbA1c < 48) and T2D (33 donors, diagnosed with T2D). Islet *IGFBP7* was upregulated in T2D donors in comparison to ND donors. This upregulation was present in both male and female donors, although male donors had an overall higher *IGFBP7* expression. Spearman correlation revealed that *IGFBP7* was not associated with age, the most common risk factor for T2D. Furthermore, HbA1c levels did not associate with *IGFBP7*. The latter association was tested in ND donors with available HbA1c values. The reason only ND donors were used in this analysis, is because T2D or IGT donors in some cases were prescribed glucose-lowering treatment at the time of death, obscuring the interpretation of correlative results between *IGFBP7* and long-term glucose levels.

Taken together, this data shows that *IGFBP7* upregulation is neither linked to long-term glucose levels nor age. The data suggests that upregulation of *IGFBP7* is inherent to the T2D disease state. To explore potential regulators of *IGFBP7*, a search for transcription factors in the Pscan database (219) was performed. The search yielded several predicted transcription factors, among them *Runt-related transcription factor 1* (*RUNX1*) and *paired box 5* (*PAX5*). *RUNX1* is induced by TGFβ1 in renal fibrosis (220), and the IGFBP7 protein has also been shown to be upregulated by TGFβ1 in diabetic nephropathy (164). Furthermore, the *PAX5* transcription factor was identified as a T2D candidate gene in the original study from which RNA-seq data was compiled (88). In all, *IGFBP7* is upregulated in islets from T2D donors and may be regulated by *RUNX1* and *PAX5*. However, the exact regulation of *IGFBP7* remains to be elucidated and further research is required.

### *IGFBP7 is upregulated in both α- and β-cells*

To deepen the characterization of *IGFBP7*, a published data set of scRNA-seq from sorted islet cells was accessed (208). This revealed an upregulation of *IGFBP7* in α-cells from T2D donors with no differences in the other endocrine cell types. A

guided machine-learning tool for image analysis of stained human pancreas was developed to corroborate these findings on protein level. IGFBP7 was, in line with the gene level results, elevated in α-cells in T2D. Additionally, a slight increase in IGFBP7 protein in β-cells in T2D was also observed. Altogether, the single cell level data of IGFBP7 gene and protein expression show that there is upregulation in mainly α-cells and to a smaller degree β-cells in T2D. These findings as a whole go in line with the islet RNA-seq showing *IGFBP7* upregulation in T2D. For determination of subcellular location, staining of dispersed islet cells for glucagon, insulin and IGFBP7 was performed. This revealed co-localization of IGFBP7 together with the two hormones in the respective cell types, opening up for the possibility that IGFBP7 is locally released from hormone vesicles. Collectively, there is basis for upregulation of IGFBP7 in both α- and β-cells in T2D with the upregulation in α-cells being predominant.

***IGFBP7 reduces GSIS through perturbation of mitochondrial function***

To explore the effects of IGFBP7 on GSIS, human islets from ND donors were treated for 72-h with IGFBP7. This resulted in a decrease of GSIS by 25%. RNA-seq revealed downregulation of two genes after IGFBP7-treatment: *p21-activated kinase 1* (*PAK1*) and *inositol polyphosphate 5-phosphatase B* (*INPP5B*). *INPP5B* is implicated in regulating the cytoskeleton in both endocytic and exocytotic pathways (221, 222) as well AKT signaling (221). *PAK1* has been extensively studied in β-cells and is well known to play a role in mitochondrial function. In islets from T2D donors, PAK1 is downregulated (223) and knockdown of PAK1 in EndoC-βH1 cells reduces mitochondrial OCR (224). Adenoviral-mediated overexpression of PAK1 in islets from T2D donors improved mitochondrial function (224). Taken together, the downregulation of these two genes after IGFBP7-treatment could have a significant impact on β-cell function.

Given its clearly defined roles and established expression in islets, PAK1 was followed up. A reduction of ~15% was confirmed on protein level in islet lysates from non-diabetic donors. To further investigate the role of IGFBP7 in β-cell insulin secretion, the EndoC-βH1 cell line was used. This cell line is comparable to primary human β-cells (203, 204) and displayed a similar decrease in GSIS after 72-h incubation of IGFBP7 as human islets. Depolarization-induced insulin secretion was unaffected by IGFBP7, indicating that the defect in GSIS was prior to the $K_{ATP}$ channel. This was in line with the reduction of PAK1, which implied perturbed mitochondrial function. Seahorse assay was used to test mitochondrial function EndoC-βH1 cells treated with IGFBP7. The cells showed an overall reduced OCR, and decreased ATP production and maximal respiration. In addition, EndoC-βH1 cells treated with IGFBP7 had reduced cell viability. These findings, using assays for measuring cellular functions in a cell line, support the whole-islet data.

***Knockdown of IGFBP7 improves GSIS: implications for intra-islet crosstalk***

To investigate a potential role for endogenous IGFBP7 secretion in β-cells, siRNA-mediated knockdown of *IGFBP7* was performed in EndoC-βH1 cells. A knockdown efficiency of ~90% was achieved, and this was reflected in a reduction the intracellular IGFBP7 content. Furthermore, the amount of secreted IGFBP7 in the culture medium was decreased. In these cells, GSIS was improved by ~25% without affecting insulin content. In experiments assessing potential mechanisms of action for IGFBP7, exogenous addition of IGFBP7 in combination with insulin strengthened Ser473AKT phosphorylation. This indicated that IGFBP7 together with insulin activated receptor tyrosine kinases (RTKs), likely INSR and/or IGF1R on the β-cells. Activation of the INSR in β-cells is thought to lead to decreased insulin secretion (86). Insulin secretion assays with IGFBP7 present in the stimulation buffer led to decreased C-peptide secretion.

Collectively, this provides support for secreted IGFBP7 acting together with insulin as a local negative regulator of insulin secretion. IGFBP7 is expressed in both α- and β-cells and co-localizes with glucagon and insulin in these cells, suggesting that IGFBP7 may be secreted by islet cells and play a role in autocrine and/or paracrine signaling. Locally secreted IGFBP7 between the islet cells can likely reach significantly higher concentrations than those seen in circulation (155) and the most likely source of IGFBP7-associated regulation is therefore the islet itself. Upregulation of IGFBP7 has also been observed in several other tissues in cardiometabolic disease (153, 157, 164). Treatments aimed at reducing IGFBP7 in liver improved blood glucose and lessened fat storage in hepatocytes (153) and blocking IGFBP7 with a neutralizing antibody reduced cardiac fibrosis in a mouse heart failure model (157). In line with this, knockdown of IGFBP7 in islets from donors with impaired glycemic control, like in EndoC-βH1 cells, improved GSIS. This is a desired treatment outcome for potential anti-diabetic therapeutics. Given the potential involvement of IGFBP7 in several conditions related to T2D, targeting IGFBP7 systemically may be a future pathway for treating metabolic diseases.

***Key findings***

- *IGFBP7* is upregulated in islets from T2D donors.
- In sorted cells scRNA-seq and image analysis of stained islets revealed IGFBP7 upregulation in primarily α-cells.
- IGFBP7-treatment of islets and EndoC-βH1 cells reduce insulin secretion through perturbed mitochondrial function.
- Knockdown of IGFBP7 improved insulin secretion providing support for a role of this protein as a local negative regulator of insulin secretion.

# Paper II

### *IL-4 reduces GSIS in islets from ND donors but not in T2D donors*

To investigate effects of the anti-inflammatory cytokine IL-4 on insulin secretion, islets from ND donors and T2D donors were incubated with 10 nM IL-4 for 48 hours before endpoint analyses. Secretion assays with glucose stimulation showed a decreased insulin secretion response to high glucose in islets from ND donors but not in T2D donors. T2D donors had an overall lower GSIS, recapitulating the disease state as such. Although not strictly comparable, preincubation with IL-4 was in another study unable to protect human islets exposed to proinflammatory cytokines (225). And here, in the absence of other cytokines, IL-4 had a surprisingly detrimental effect on GSIS in islets from ND donors.

The IL-4 receptor (IL4R) is present in both α- and β-cells, making IL-4-mediated regulation in these cells possible (175). A reason for the different responses to IL-4 between the groups could be differences in receptor expression. Using a previously published RNA-seq data set from whole-islets (218), the expression of *IL4R* was assessed. Albeit with a slightly higher expression in T2D, there was no difference in *IL4R* expression in islets from ND donors and T2D donors. IL-4 itself did not alter the expression of *IL4R*.

### *IL-4 increases the expression of miR-378a-3p in islets from ND donors*

Even though there was no difference in the receptor expression between ND and T2D islets, there may still be differences in signaling pathway effector proteins distal of the IL4R although this was not investigated here. The most important pathway for IL-4 signaling in β-cell is the activation of signal transducer and activator of transcription 6 (STAT6) transcription factor (226). In pancreas sections from T1D patients, STAT6 is downregulated in β-cells (227). Feasibly, IL-4 could therefore have differential effects on miRNA and gene expression regulation between the ND and T2D groups.

Three miRNAs were selected for investigation. MiR-130a and miR-375-3p are well known miRNAs in β-cells (228). Upregulation of islet miR-130a has been described in T2D and this miRNA has negative effects on ATP production in clonal β-cell lines (189). MiR-378a-3p was chosen as previous studies have shown upregulation of this miRNA in macrophages after IL-4 incubation (229). In this study, the increased expression of miR-130a in islets from T2D donors was replicated. IL-4 upregulated the expression of miR-378a-3p in islets from ND donors but not in T2D. There were no differences for the miR-130a or miR-375-3p with IL-4-treatment in any of the groups.

### *PGC1B and PPARG are induced by IL-4 in islets from ND donors*

MiR-378a-3p is transcribed from the first intron of the *peroxisome proliferator-activated receptor gamma* (*PPARG*) *coactivator 1 beta* (*PGC1B*) gene (230). The protein product PGC-1β is a co-activator of PPARγ transcription factor and together these protein regulate cellular processes such as fatty acid oxidation (231). PGC-1β can induce transcription of mitochondrial genes through interactions with nuclear respiratory factor 1 (NRF1) and promote mitochondrial biogenesis (232). IL-4 upregulates both PGC-1β and PPARγ in a STAT6-dependent manner in macrophages (233). Treatment of IL-4 in human donor islets resulted in upregulation of *PGC1B* and *PPARG*. However, as for miR-378a-3p, this upregulation was only observed in islets from ND donors. Induction of PPARγ in primary rat islets suppresses insulin secretion through uncoupling mechanisms in the mitochondria (234). Intact IL-4 signaling in islets from ND donors resulting in upregulation of genes in the PPARγ pathway and a shift in mitochondrial function is a likely explanation for the reduced GSIS. An overarching hypothesis generated from this finding is that IL-4 has anti-inflammatory effects and shifts gene expression in β-cells for survival, but this comes at the cost of less efficient GSIS. In individuals with obesity elevated circulating levels of IL-4 and proinflammatory cytokines IL-1β and IL-6 have been observed (235). Intriguingly, IL-4 together with proinflammatory cytokines can upregulate the expression of IL4R in β-cells in human islets (225). Although not significant in our analyses, there is a trend towards higher *IL4R* in islets from T2D donors. A second hypothesis generated from this, is that IL-4, in conjunction with other cytokines over time, contributes to decreased GSIS. This mechanism could be through aberrant IL4R signaling.

### *Overexpression of miR-378a-3p does not affect GSIS in EndoC-βH1 cells*

As the effect of overactivation of the PPARγ-pathway on insulin secretion is already established, the specific effects of miR-378a-3p remained to be tested. MiR-378a-3p has several gene targets expressed in β-cells with roles in insulin secretion. These include targets in the AKT-signaling pathway and NRF1 (236). Opposite regulation of NRF1 by miR-378a-3p and PGC-1β is likely part of a phenomenon whereby intragenic miRNAs fine tune cellular functions together with the host gene (237). However, overexpression of miR-378a-3p in EndoC-βH1 cells showed no changes in GSIS. The primary reason for the reduced GSIS in isles from ND donors is therefore likely not mediated through effects of miR-378a-3p but rather through the PPARγ pathway.

### *Key findings*

- IL-4 reduces GSIS in islets from ND donors with no effects in islets from T2D donors.
- There is an upregulation of *PPARG* and *PCG1B* with intronic miR-378a-3p in ND.

- The likely cause for reduced GSIS in islets from ND donors is activation of the PPARγ pathway.
- Overexpression of miR-378a-3p does not affect GSIS in a clonal β-cell line.

# Paper III

***Differential miRNA expression profiles in α- and β-cells***

In the third project, sorted islet cell fractions of α- and β-cells were investigated. Isolated RNA was subjugated to miRNA-seq to assess miRNA expression in the two different cell types. The first analyses of the miRNA-seq data entailed quality control, mapping of the reads to known small RNA transcripts and assessing separation of the samples using principal component analysis (PCA). A clear majority of sequenced transcripts aligned with known miRNAs. PCA revealed a clear separation of samples primarily based on cell-type (43% of the variation) and on sequencing batch (23% of the variation). Sequencing batch effect was corrected for in all subsequent analyses. PCA also identified a β-cell fraction in one ND donor as an outlier, and this donor was also excluded from further analysis.

In total 599 miRNAs were identified in the samples. Overall, among the top 20 most abundant miRNAs there was large overlap between α- and β-cells. One exception was miR-127-3p which was significantly higher in β-cell and has previously been described as an islet-enriched miRNA correlating positively with insulin gene expression (185). Its enrichment in the β-cell fraction is in line with the previous findings. Differential miRNA expression analysis was performed only in samples from ND donors. Out of 599 miRNA, 226 were robustly differentially expressed defined as adjusted *P*-value <0.05 and a 2-fold or 0.5-fold difference. In α-cells, 142 miRNAs were enriched and in β-cells 84 were enriched. Top miRNAs in α-cell were miR-1287-5p, miR-532-5p, miR-192-3p, miR-362-5p and miR-222-3p. Interestingly, target analysis of these 5 α-cell enriched miRNAs revealed several transcription factors important in maintaining β-cell identity. One interpretation is that these miRNAs suppress β-cell transcription factors in order to uphold an α-cell phenotype. Top β-cell enriched miRNAs were miR-411-5p, miR-431-5p, miR-337-5p, miR-379-5p, and miR-323-3p. Manual target analysis did not reveal any evident processes affected by these miRNAs.

***Correlations of cell-type specific miRNAs with donor traits***

Next, correlations with miRNA expression and donor traits in ND donors were performed. The donor traits were: HbA1c, BMI, age and sex. After adjustment for multiple comparisons, three miRNAs showed significant correlation with sex in α-cells: miR-130b-5p, miR-3613-3p and miR-4516. In β-cells several significant correlations were discovered: 15 miRNAs correlated with HbA1c, 13 with sex and

two with BMI. Although only associative, these findings could be interpreted as a more stable miRNA profile in α-cells, and α cells are less affected by stressors such as high BMI or long-term elevation of blood glucose levels. Associations with miRNAs and sex was found in both α- and β-cells. Islets from female donors have higher insulin secretion and more β-cells (238, 239). Differential expression of miR-532-5p and miR-660, with close to twofold higher expression in females than males, has been reported previously in human islets (238). Interestingly, miR-532-5p is enriched in α-cells in ND donors but was not associated with sex in this study. Still, this is an intriguing finding and the potential role of miR-532-5p as potential sex-specific and α-cell enriched miRNA remains to be investigated. Future studies would ideally be conducted with larger donor cohorts. This is a requirement for better in-depth characterization of how miRNAs may correlate with donor traits.

### *MiRNA expression profiles overall do not change overall in T2D; miR-551b-3p is upregulated in β-cells in T2D*

In the next series of analyses, changes in miRNA expression with the hyperglycemic state were explored. Donors with IGT and T2D were grouped and compared to ND donors. In PCA, the overall expression profile of miRNAs in neither α-cells nor β-cells showed significant separation of the donor groups. In assessment of miRNAs individually in volcano plots, very few miRNAs were robustly differentially expressed (assessed with adjusted *P*-value). In α-cells, miR-8485 was downregulated in IGT/T2D. In β-cells, miR-7704 was downregulated and miR-551b-3p was upregulated. When using nominal *P*-values, several previously studied miRNAs in islets in T2D were listed. Among these were miR-205-5p, which is upregulated in an aging and carbohydrate-overfeeding mouse model for T2D. This miRNA is conserved in humans however and was shown to target *TCF7L2* and dysregulate insulin secretion in INS-1 cells (240). MiR-130b-3p was also identified, and this miRNA can decrease ATP production in β-cells (189). The inability of this study to find robust expression differences between ND and T2D is likely a lack of statistical power and considerable inter-donor variability. For example, due to very few donors with diagnosed T2D, IGT donors needed to be pooled in this group. Of note, patients with IGT still display considerable differences in glucose levels and insulin secretion prior to T2D development (24). Previous studies have also investigated whole-islet miRNA expression in T2D development and not in sorted α- and β-cell fractions. This makes comparisons between previous research and the few present study more difficult to assess. Sample size limitation, heterogenous donors, pooled groups and incomplete comparability are all probable explanations for why very few miRNAs were detected in this analysis.

### *Overexpression of miR-551b-3p in INS-1832/13 cells*

The only robustly upregulated miRNA in β-cells in T2D was miR-551-b-3p. This miRNA was selected for functional follow-up in the clonal β-cell line INS-1 832/13. Overexpression of this miRNA would recapitulate the T2D disease state in the β-

cell, making this an interesting model to study. INS-1 832/13 cells overexpressing miR-551b-3p displayed increased GSIS with no changes in insulin content. This can be interpreted as a compensatory mechanism to improve insulin secretion driven by miR-551b-3p in the diabetic condition. A strongly predicted mRNA target for miR-551b-3p is *cAMP-specific 3',5'-cyclic phosphodiesterase 4C* (*PDE4C*). PDE4C is the major isoform of PDE4 proteins in human islets (241). PDE3B has been well described in islets and clonal β-cells in regulating insulin secretion by hydrolysis of cAMP, thus inhibiting cAMP-regulated pathways in insulin secretion (242). Given its expression in human islets, a similar role for PDE4C as for PDE3B is possible in β-cells. MiRNA-mediated knockdown of this *PDE4C* could lead to increased insulin expression, which is in line with the results obtained with overexpression of miR-551b-3p. Future validation experiments for this interaction could strengthen this hypothesis.

***Key findings***

- The most abundant miRNAs in each cell type have similar expression, however the complete miRNA expression profiles between the cell types are very different.
- MiRNAs in α-cells likely suppress β-cell transcription factors and maintain the α-cell phenotype.
- MiRNAs upregulated in β-cells (assessed with nominal *P*-value) in hyperglycemic donors have been associated with impaired β-cell function.
- MiR-551b-3p was robustly upregulated in β-cells in islets from hyperglycemic donors. This miRNA improved insulin secretion, suggesting involvement in a compensatory mechanism.

# Paper IV

***MiRNA-seq pilot study identifies serum controls and differentially expressed miRNAs in INDET at baseline***

In the fourth project, individuals with CF underwent an OGTT in order to assess miRNA levels at baseline and after glucose ingestion. With miRNA-seq, 275 miRNAs were robustly detected in serum samples of 12 individuals of varying glucose tolerance. As samples were extracted from complete serum using a kit for total RNA isolation, this data reflects the total miRNA pool in the serum. MiRNAs in microvesicles and miRNAs bound to RNA-binding proteins were therefore included in this analysis. Firstly, miR-16-5p, miR-486-5p and miR-122-5p were the three most abundant miRNAs across all samples. Additionally, the levels of these three miRNAs did not significantly change between baseline and the 60-min

timepoint, making them suitable as endogenous serum controls. Previous studies on miRNAs in blood have found miR-16-5p and miR-486-5p as abundant miRNAs in serum (243).

Paired groupwise comparisons between NGT, INDET, IGT and CFRD at baseline showed several miRNAs with differential expression by nominal *P*-value. In INDET, three miRNAs showed significant upregulation with adjusted *P*-value. These were: miR-34a-5p, miR-122-5p and miR-885-3p. Additionally, the corresponding 5' miRNA of miR-885-3p, miR-885-5p, was significantly upregulated by nominal *P*-value in INDET. The upregulation of miR-122-5p in one of the groups made it unsuitable for use as an endogenous serum control. In all, two appropriate serum controls (miR-16-5p, miR-486-5p) and four miRNAs with differential expression at baseline in INDET were selected (miR-34a-5p, miR-122-5p, miR-885-3p and miR-885-5p) for LNA RT-qPCR follow-up.

### *Differential response analysis identifies dysregulation of several miRNAs in CFRD*

To investigate dynamic expression changes with glucose ingestion, the fold change of each miRNA from baseline to 60-min timepoint was assessed in each individual and then compared on a groupwide level. This differential response analysis revealed that several miRNAs had opposite responses in NGT *vs* CFRD. E.g., miR-134-5p had a negative response in NGT (lower at 60-min timepoint than baseline) whereas the same miRNA in CFRD had a positive response (higher at 60-min timepoint than baseline). In CFRD, 10 miRNAs showed the same positive response to glucose (miR-28-3p, miR-127-3p, miR-134-5p, miR-223-3p, miR-223-5p, miR-224-5p, miR-382-5p, miR-409-3p, miR-432-5p and miR-1301-3p) and two showed a negative response (miR-363-3p and miR-451a). The most abundant miRNA identified in differential response analysis was miR-223-3p. In comparisons between NGT and INDET/IGT respectively, no miRNAs with differential response were found. This could reflect the small sample size in each group (3 individuals) as well as the inherent severity of the CFRD disease state leading to an aberrant serum miRNA profile. Overall, these results also show that miRNA levels in blood change with intake of glucose. The 12 miRNAs with differential response in CFRD compared to NGT were selected for validation in LNA RT-qPCR follow-up.

### *LNA RT-qPCR assay follow-up validates eight serum miRNAs*

Follow-up of selected miRNAs was performed in all samples from the cohort (126) using a custom-made LNA RT-qPCR assay. Out of the four miRNAs elevated in INDET in miRNA-seq, three, miR-34a-5p, miR-122-5p and miR-885-5p, were validated at baseline in LNA RT-qPCR. Interestingly, these three miRNAs were also upregulated in CFRD at baseline although not to the same extent as in INDET. Throughout the OGTT, miR-34a-5p, miR-122-5p, miR-885-5p and at some time points miR-885-3p, showed higher expression in INDET and CFRD. The fact that

these miRNAs in the two groups do not change throughout the OGTT, suggests that the serum levels of these miRNAs are stable and not affected by glucose intake. The only timepoint in IGT with differential expression of miRNAs was the 30-min timepoint. Here, miR miR-28-3p, miR-223-3p, miR-223-5p, miR-885-5p and miR-1301-5p were upregulated. In CFRD at 60-min, miR-223-3p ($P$=0.056) and miR-1301-5p also showed upregulation. In total, eight miRNAs from the miRNA-seq pilot study were validated at different timepoints and in different groups in the LNA RT-qPCR follow-up study to be differentially expressed.

A general overview of these eight miRNAs including total serum abundance and a literature survey resulted in the selection of miR-34a-5p, miR-122-5p and miR-223-3p for further follow-up. MiR-122-5p and miR-223-3p were among the two most abundant miRNAs measured in the LNA RT-qPCR. The high serum levels of these make uptake of sufficient amount of miRNA in target tissues more plausible. The ability of these two circulatory miRNAs to regulate cellular processes in for example β-cells was deemed more likely than for miR-885-3p which displayed very low serum levels. Overall, the two methods, miRNA-seq and LNA RT-qPCR reported similar results in abundance rankings of the miRNAs. In miRNA-seq, miR-122-5p was ranked higher than miR-223-3p whereas in LNA-RT-qPCR miR-223-3p ranked higher. Discrepancies in measured levels can be due to differences in RNA secondary structure (244) highlighting the importance of confirming findings with two different methods. MiR-34a-5p was primarily selected as this miRNA is elevated in the circulation of patients with T2D (198, 199). MiR-122-5p is a well described miRNA in hepatocytes and is associated with liver damage (245) and is elevated in individuals with CF-related liver disease (246). Elevated serum levels of miR-223-3p has been used to predict progression from pre-diabetes to T2D (247). Furthermore, miR-223-3p is upregulated in islets of T2D donors and is suggested to maintain β-cell functional mass (192). Additionally, miR-223-3p is upregulated in CF airway epithelium and targets *CFTR* in bronchial cell lines (248). Downregulation of mRNA silencing capability by mutating the miR-223-3p target sequence restores CFTR activity (249). Taken together, the three selected miRNAs are implicated in T2D, a metabolic disease related to CFRD and one of the miRNAs, miR-223-3p, can negatively regulate CFTR activity.

### *MiR-34a-5p and miR-122-5p associate with hepatobiliary damage markers and miR-122-5p increases insulin secretion*

Normalization of the expression values of miR-34a-5p, miR-122-5p and miR-223-3p across all groups to the NGT group confirmed that miR-34a-5p and miR-122-5p are specifically elevated at baseline in INDET and CFRD. MiR-122-5p is associated with liver damage in CF and non-CF individuals (245, 246) and miR-34a-5p regulates genes in the liver related to the circadian rhythm (200). Associations of these two miRNAs with established hepatic damage markers (alanine aminotransferase: ALT, aspartate aminotransferase: AST) and biliary damage

markers (alkaline phosphatase: ALP, γ-glutamyl transferase: γGT) showed several strong positive correlations in CFRD for primarily the liver damage markers and the two miRNAs. These associations were not found to the same extent in any of the other glucose tolerance groups.

To test if miR-34a-5p and miR-122-5p, putatively secreted from the hepatocytes, affects GSIS the two miRNAs were overexpressed in the INS-1 832/13 cell line. Here, miR-122-5p increased insulin secretion by ~60%. Collectively, these results suggest liver damage in CFRD and miR-122-5p may act compensatorily to increase insulin secretion. There is indication of hepatic insulin resistance in CFRD (250-252). In all, it can be hypothesized that secretion of miR-122-5p is a compensatory mechanism for stressed liver cells to stimulate insulin secretion as a mean to rescue ongoing tissue damage. However, initial portal hyperinsulinemia may contribute to and in the long-term exacerbate hepatic insulin resistance (139). The presented data give only evidence for disturbed inter-organ crosstalk between pancreatic islets and the liver in CFRD. However, testing of these intricate questions are beyond the scope of the present study. These questions merit future research in for example longitudinal studies.

### *MiR-223-3p displays dynamic expression changes in OGTT and affects insulin secretion*

MiR-223-3p was not differentially expressed at baseline but showed dynamic expression changes throughout the OGTT in the IGT and CFRD groups. This miRNA displayed a positive association with insulin levels in NGT at baseline in Spearman correlation analysis, in line with a role in maintaining β-cell functional mass (192). Transfection assays overexpressing miR-223-3p in INS-1 832/13 cells resulted in improved GSIS. This was in line with the correlation analyses showing a positive association between miR-223-3p and insulin in NGT at baseline. This suggests a compensatory role for miR-223-3p in CFRD. However, the intrinsic properties of this miRNA to target *CFTR* could inherently contribute to an overall worsening of the CF disease as such. Decline in lung function is precedes development of CFRD (253). This opens up to the possibility that miR-223-3p in a compensatory fashion improves GSIS but that this may come at the cost of a further reduction in CFTR function throughput the body. And, over time, this could lead to worsening of CF and contribute in CFRD pathogenesis.

### *Key findings*

- Serum levels of miRNAs change in CF depending on glucose tolerance and with glucose ingestion. MiR-16-5p and miR-486-5p were identified as endogenous serum controls.
- Eight miRNAs were validated in LNA RT-qPCR follow-up. Three miRNAs were selected for further follow-up: miR-34a-5p, miR-122-5p and miR-223-3p. All miRNAs previously shown to be relevant in T2D.

- MiR-34a-5p and miR-122-5p associated with hepatobiliary markers. MiR-223-3p showed dynamic changes with glucose intake in IGT and CFRD.
- MiR-122-5p and miR-223-3p increased GSIS, indicating a possible compensatory role for these miRNAs.

# Concluding remarks

The overarching aim of this thesis was to investigate regulation of insulin secretion by two proteins, IGFBP7 and IL-4, and miRNAs in development of T2D and CFRD. Primary human islets as well as β-cell line models were employed in functional experiments. Bioinformatic analyses, image analysis tool and structured follow-up of candidate miRNAs were combined with results from the functional assays to answer the set aims. From the presented articles, the following conclusions can be made:

In Paper I, IGFBP7 was found to be upregulated on both RNA and protein levels in islets from T2D donors. Treatment of islets from non-diabetic donors and the EndoC-βH1 cell lines with IGFBP7 reduced insulin secretion through perturbing mitochondrial function. Knockdown of IGFBP7 in EndoC-βH1 cells and islets from dysglycemic donors improved insulin secretion. In conclusion, this supports release of IGFBP7 in the islet and that this protein functions as an autocrine/paracrine negative regulator of β-cell function.

In Paper II, IL-4 reduced insulin secretion in islets from non-diabetic but not T2D donors. This effect is likely through activation of the PPARγ pathway. MiR-378a-3p, an intronic miRNA in *PGC1B*, does not affect insulin secretion. Taken together, this shows that beneficial effects of IL-4 may come at the cost of decreased insulin secretion.

In Paper III, miRNA-seq revealed that the most abundant miRNAs are shared for α- and β-cells. However, the global miRNA profiles between the cell types differ substantially. Several cell-type specific miRNAs are associated with donor traits, showing differential miRNA regulation in α- and β-cells. MiR-551b-3p is robustly upregulated in β-cells in T2D and increases insulin secretion, suggesting a possible compensatory mechanism.

In Paper IV, three miRNAs with differential levels, miR-34a-5p, miR-122-5p and miR-223-3p miRNAs were identified in serum of CF individuals. MiR-34a-5p and miR-122-5p showed higher levels in INDET and CFRD and are associated with hepatobiliary markers. MiR-122-5p and miR-223-3p improved insulin secretion, suggesting a compensatory role for these miRNAs. MiR-223-3p is upregulated in IGT and CFRD with glucose intake. Altogether, the miRNA profile in CFRD is changed. There is also evidence for dysregulated miRNA release upon glucose intake.

# Future perspectives

Diabetes mellitus is a global problem requiring the utmost attention of societies, healthcare professionals and scientists alike. Understanding development of diabetes mellitus, particularly T2D, is critical in developing new diagnostic tools and treatment strategies to offer better help for affected individuals. Impairment of insulin secretion is a critical step in the development of all types of diabetes mellitus. This thesis has investigated regulation of insulin secretion by factors local to the islet and circulating miRNAs. The presented articles have awakened further research questions, and here is an account of steps, both in the immediate term and later in the future, that could be taken to further these discoveries.

The suggested role of IGFBP7 as a paracrine negative regulator of insulin secretion secreted locally by the islet cells themselves is intriguing. The first steps in establishing this protein as a definitive autocrine/paracrine regulator would be to investigate how gene expression of *IGFBP7* is regulated, elucidate exact stimuli of IGFBP7 release, investigate effects on insulin secretion after neutralization of released IGFBP7, deeper assessment of receptor interactions and disentangle potential signaling pathways involved. It would also be interesting to study effects on metabolism in islet-specific *IGFBP7*-knockout animal models. Given the described negative effects of IGFBP7 upregulation in other tissues involved in cardiometabolic diseases, systemic modulation of IGFBP7 could prove to be a fruitful endeavour in finding new targets for treating these diseases. IGFBP7 could putatively be targeted by regulation of gene transcription, targeting IGFBP proteases, targeting direct receptor interactions or use of IGFBP7-neutralizing compounds. If taken to clinical studies, aside from studying insulin secretion, assessing whole-body effects in organs such as liver, heart and kidney would be of great interest.

IL-4, considered an anti-inflammatory cytokine, proved to impair insulin secretion in islets from non-diabetic donors without any effects in T2D. Treatment with IL-4 resulted in upregulation of genes in the PPARγ pathway, suggesting intact IL4R signaling in these islets from non-diabetic donors. Yet, PPARγ activation, while protective in other cells, also reduces insulin secretion in β-cells. There is evidence for elevated levels of circulating IL-4 in metabolic disease. A hypothesis arising from this, is that elevated IL-4, both circulating and from sources localized to the islet, could possibly contribute to decreased insulin secretion. A short-term adaptive response from an anti-inflammatory cytokine, may prove to be maladaptive in the

long-term. Future studies could investigate local sources of IL-4 in pancreatic islets and investigate if this process contributes to T2D development.

Examining differential expression of miRNAs in α- and β-cells will lead to a better understanding of regulation of intra-cellular processes in the respective cell types. An immediate next step in this project is to perform correlations of miRNA and gene expression to assess how miRNAs affect transcriptional networks. From this, putative gene targets and their potential effects on insulin and glucagon secretion can be derived. Specifically, miR-551b-3p and its regulation could also be the target of further investigation. Another path for this project would be to perform deeper characterization of the miRNA expression correlation with donor traits and to, for example, elucidate effects of miRNAs that are associated with BMI.

Understanding circulating miRNAs in CFRD pathogenesis may prove a viable path for developing personalized treatment strategies. A clear next step in this project would be to perform a longitudinal study to answer if the identified miRNAs hold predictive value in CFRD development. To follow in a longitudinal study how individuals with CF move from one glucose tolerance category to the next is also of interest. From this cross-sectional study, it can be gleaned that liver damage is involved in INDET and CFRD. Specific steps in projects birthed from this article would also need to answer what are the specific stimuli of release of miR-34a-5p and miR-122-5p from liver in CF, as well as tissue of release for miR-223-3p and how this is glucose-dependent. Interestingly, miR-122-5p and miR-223-3p increased insulin secretion, which was interpreted as a compensatory response. Future investigations must also ascertain if these miRNAs are taken up in β-cells. However, as noted in T2D, recent hypotheses put forth that initial hyperinsulinism may be a causative step in T2D pathogenesis. Whether this is true in T2D, remains to be seen. Speculatively, this process could occur in CFRD as well in which case increased insulin secretion would be seen in a different light. Overall, the interplay between the liver and endocrine pancreas has been postulated to play a significant role in the development of metabolic disease (254).

In summary, the articles presented in this thesis pave the way for several new research ideas. T2D and CFRD are complex metabolic diseases and untangling these complexities, how they lead to dysregulated insulin secretion and ultimately elevated blood glucose levels, is a vast undertaking. Here, some findings are presented which I hope will help future scientists in understanding T2D and CFRD and hopefully aid in developing better treatments for these diseases. Biomedical science requires patience, collaboration and openness to new ideas. It truly takes a village. And, most of all, scientific work requires humility to the myriads of ways in which the biological world organizes itself. It is there for us to see. As long as we keep looking.

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**EFRAIM WESTHOLM** earned his medical degree at Lund University. In his PhD studies at Lund University Diabetes Centre, Unit of Islet Cell Exocytosis, he has focused on understanding changes in pancreatic islets in diabetes mellitus development. In this thesis, novel findings regarding autocrine and paracrine regulation of insulin secretion as well as circulating miRNAs in cystic fibrosis are presented.

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