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Karagiannopoulos, Alexandros

2023

Document Version:
Publisher's PDF, also known as Version of record

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ALEXANDROS KARAGIANNOPOULOS
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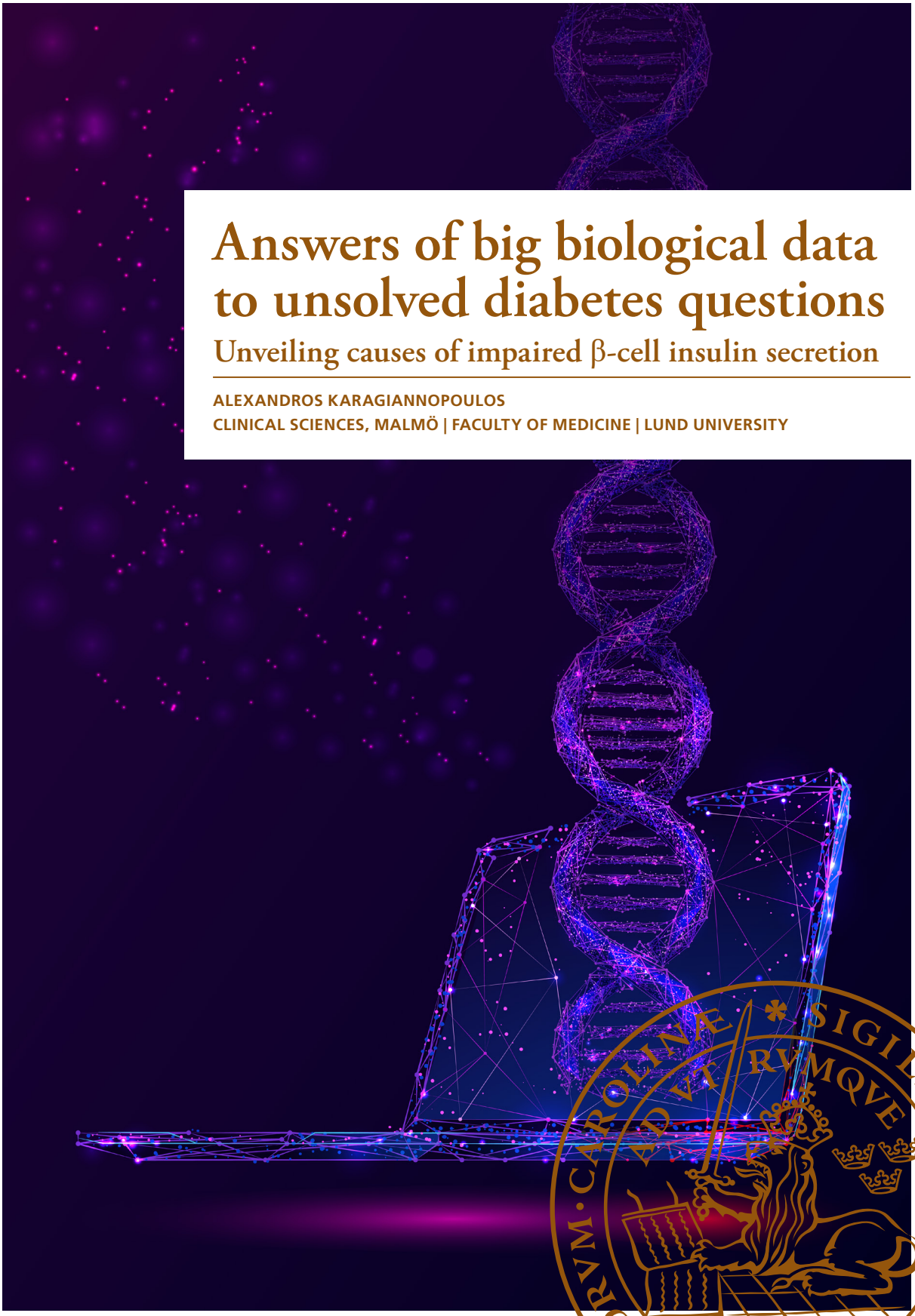

Printed by Media-Tryck, Lund 2023 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Clinical Sciences, Malmö

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2023:100
ISBN 978-91-8021-440-7
ISSN 1652-8220

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Alexandros Karagiannopoulos

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UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 15th of September 2023 at 09.00 in “Agardhsalen CRC”, Jan Waldenströms gata 35, 20502, Malmö

Faculty opponent
Dr Timothy Pullen

King's College London

Organization: LUND UNIVERSITY

Document name: Doctoral dissertation **Date of issue: 2023-09-15**

Author(s): Alexandros Karagiannopoulos **Sponsoring organization:**

Title and subtitle: Answers of big biological data to unsolved diabetes questions - Unveiling causes of impaired β-cell insulin secretion

Abstract: Pancreatic β-cells in the islets of Langerhans are indispensable for maintaining glucose homeostasis by secreting insulin. Chronically abnormal glucose levels, or hyperglycemia, can be triggered by genetic and/or environmental factors, leading to defective insulin secretion or action and eventually diabetes. While much is already known about the processes underlying β-cell function and insulin secretion, the complicated mechanisms governing β-cell dysfunction are waiting to be further explored.

Glucocorticoids (GCs) are widely prescribed anti-inflammatory and immunosuppressive drugs. It has been shown that prolonged or high-dose treatment can hinder β-cell function leading to GC-induced diabetes, but the exact mechanisms behind this process are unclear. In Paper I, the mode of GC action on specific genes and molecular pathways was revealed by integrating large-scale transcriptomic, genetic, and epigenetic data. Further analysis and functional validation assays showed that the transcription factor (TF) ZBTB16 is a direct GC target involved in a compensatory protective mechanism against the deleterious GC effects on β-cell function and insulin secretion.

Type 2 diabetes (T2D) is a multifactorial disease and, thus, multiple genes with smaller effects are expected to contribute to its onset. Nevertheless, the limited supply of islets undermines their discovery. In Paper II, a large human islet cohort was used to detect extensive transcriptome changes in T2D islets, which were associated with impaired β-cell function and T2D. Moreover, PAX5 was determined as a key novel TF that is induced in T2D islets and is implicated in the decreased insulin secretion output of β-cells.

MiRNAs are small, non-coding RNAs that regulate gene expression via mRNA targeting. In Papers III and IV global miRNA changes were investigated in human islets from hyperglycemic and T2D donors and dysregulated miRNAs were associated with T2D-related mRNA targets. In Paper III, it was shown that these mRNA targets form co-expression networks that are linked to islet-specific functions. SNPs associated with both the expression of dysregulated miRNAs (eQTLs) and T2D/insulin secretion traits revealed miRNAs that could contribute to the susceptibility to β-cell dysfunction and T2D. In Paper IV, the important role of upregulated miRNAs in T2D was highlighted, as their mRNA targets were grouped in a co-expression network related to insulin secretion. Several miRNAs, as well as their targets, were correlated with insulin release and, thus, could be involved in T2D pathogenesis by impairing insulin secretion.

This thesis provides new insights into the way different types of biological data can be integrated to unravel potential causes of β-cell impaired insulin secretion. Moreover, it reports on novel genes and miRNAs that affect β-cell function and are dysregulated in GC-induced/Type 2 diabetes, making them ideal therapeutic targets for diabetes in the future.

Key words: Type 2 Diabetes, Glucocorticoid-induced diabetes, pancreatic islets, β-cells, β-cell dysfunction, omics, data integration, insulin secretion, ZBTB16, PAX5

Classification system and/or index terms (if any) Supplementary bibliographical information

Language: English

ISSN and key title: **1652-8220** **ISBN: 978-91-8021-440-7**

Recipient's notes **Number of pages**: 95

Price Security classification

Signature Date 2023-08-03

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Orignial coverphoto by VectorStock®. Modified by Alexandros Karagiannopoulos.

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ISBN 978-91-8021-440-7

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University

Lund 2023

Ἓν οἶδα, ὅτι οὐδέν οἶδα
(The only true wisdom is in knowing you know nothing)
-Socrates

Table of Contents

Popular Summary

Type 2 diabetes (T2D) is the most common form of diabetes, affecting the lives of 10% of the adult population worldwide. Individuals with T2D have increased blood sugar (glucose) for an extensive period of time, which can have serious complications in different organs such as the nerves, the eyes, the kidneys, the heart and the blood vessels. The two main causes of T2D are the inability of the pancreas to produce and release enough insulin or the inefficient use of the produced insulin. Insulin is a hormone that makes sure that the glucose in the circulation, which is produced after the consumption of a meal, is transferred inside the different organs in order to be used as fuel and give them the necessary energy to work properly. The reasons behind the development of T2D include lifestyle choices, such as sedentary life, obesity and smoking, abnormal genes or the consumption of certain drugs, such as glucocorticoids.

As mentioned, among the causes of diabetes can be the consumption of glucocorticoid (GCs) drugs in high doses or for a long time. GCs are substances that belong to the corticosteroid family and these drugs are commonly used against inflammation and allergies, but they are also responsible for 2% of all new diabetes diagnoses. GCs act by entering the cells, which in turn modify several genes by turning them on (activate) or off (suppress). For example, GCs have the ability to activate genes that boost the immune system. However, we found that GCs can act directly on the pancreas, suppressing several genes that participate in the production and release of insulin. With the help of publicly available biological data, we identified genes that were affected by GCs and were implicated in insulin release. Moreover, we also detected one gene, ZBTB16, which acted as a shield protecting the pancreatic cells from the harmful effects of GCs. This gene can potentially be used to reduce the risk of diabetes development in patients that will need GC treatment in the future.

Our next goal was to discover all the genes that are turned on/off in the pancreas during the development of T2D. To do that, we compared pancreatic cells from healthy individuals and individuals with T2D and we found differences in 395 genes. Then, after using an extensive collection of biological data and performing a series of experiments, we confirmed that the function of many of these genes was connected to T2D. Moreover, we identified PAX5 as an important gene activated in

individuals with T2D. This gene can turn on/off many genes in a way that negatively affects the function of pancreatic cells and the release of insulin. Thus, new T2D therapies targeting the PAX5 gene hold great promise.

Finally, a big part of this thesis focused on the study of microRNAs (miRNAs). MiRNAs are small molecules that can turn off specific genes. Each miRNA can have multiple genes as targets and, consequently, miRNAs participate in many processes inside the cells. There is evidence that miRNAs contribute to the reduction of the produced insulin observed in T2D, but the exact roles of miRNAs are still not clearly defined. We managed to discover extensive differences between the miRNAs of healthy and T2D individuals with different technological methods. Furthermore, by analyzing data from publicly available sources we discovered many candidate genes that these miRNAs could target and, at the same time, were related to T2D. In that way, we uncovered complex networks consisting of miRNAs and target genes that control insulin release from the pancreas and are destabilized during T2D development. We suggest that concerted efforts to reveal and "fix" these networks will provide new ways to manage and treat T2D.

Overall, this thesis demonstrates how diverse types of biological data can be combined and used as a tool for exploring and revealing new causes of reduced insulin production in T2D. In this manner, we determine previously unexplored genes and miRNAs that can affect pancreatic function and T2D development, demonstrating that they can be ideal therapeutic candidates for this condition.

Popular Summary (Greek)-Περίληψη

Ο διαβήτης τύπου 2 (T2D) είναι ο πιο κοινός τύπος διαβήτη, επηρεάζοντας τη ζωή του 10% του ενήλικου πλυθησμού παγκοσμίως. Άτομα με T2D έχουν υψηλό σάκχαρο (γλυκόζη) για παρατεταμένο χρονικό διάστημα, το οποίο μπορεί να επιφέρει σοβαρές επιπλοκές σε διάφορα όργανα όπως τα νεύρα, τα μάτια, τα νεφρά, την καρδιά και τα αιμοφόρα αγγεία. Οι δύο κύριες αιτίες του T2D είναι η αδυναμία του παγκρέατος να παράξει αρκετή ινσουλίνη ή η παραγόμενη ινσουλίνη να μη χρησιμοποιείται αποτελεσματικά. Η ινσουλίνη είναι η ορμόνη που επιτρέπει στην γλυκόζη, που παράγεται μετά την κατανάλωση ενος γεύματος, να μεταφερθεί μέσα στα διαφορετικά όργανα για να χρησιμοποιηθεί ως καύσιμο και να τους δώσει την απαιτούμενη ενέργεια για τις απαραίτητες διεργασίες τους. Οι αιτίες που οδηγούν στην ανάπτυξη του T2D περιλαμβάνουν συνήθειες στην καθημερινή ζωή, όπως π.χ. καθιστική ζωή, παχυσαρκία και κάπνισμα, ανωμαλίες σε γονίδια ή πρόσληψη φαρμάκων, όπως τα γλυκοκορτικοστεροειδή.

Όπως αναφέρθηκε, ανάμεσα στις αιτίες ανάπτυξης διαβήτη είναι και η κατανάλωση γλυκοκορτικοστεροηδών (GCs), όταν αυτά προσλαμβάνονται σε μεγάλες δόσεις ή για παρατεταμένο χρονικό διάστημα. Τα GCs είναι ουσιές που ανήκουν στην οικογένεια των κορτικοστερεοειδών και τα φάρμακα αυτά χρησιμοποιούνται ευρέως κατά των φλεγμονών και των αλλεργιών, αλλά θεωρούνται και υπεύθυνα για το 2% των νέων περιστατικών T2D. Τα GCs δρουν μόλις μπουν στα κύτταρα και έχουν την ικανότητα να τροποποιούν γονίδια με το να τα «ανοίγουν»/ενεργοποιούν ή να τα «κλείνουν»/καταστέλλουν. Για παράδειγμα, τα GCs ενεργοποιούν γονίδια που ενισχύουν το ανοσοποιητικό σύστημα. Παρ'όλα αυτά, τα GCs δρουν απευθείας και στο πάγκρεας, καταστέλλοντας γονίδια που συμμετέχουν στην παραγωγή και την απελευθέρωση της ινσουλίνης. Με τη βοήθεια διαθέσιμων βιολογικών δεδομένων, ταυτοποιήσαμε τα γονίδια που επηρεάζονται από τα GCs και εμπλέκονται στην απελευθέρωση της ινσουλίνης. Επιπλέον, εντοπίσαμε ένα γονίδιο που ενεργοποιείται από τα GCs, το ZBTB16, το οποίο δρα σαν ασπίδα προστατεύοντας τα παγκρεατικά κύτταρα από την βλαβερή επίδραση των GCs. Το γονίδιο αυτό θα μπορούσε δυνητικά να χρησιμοποιηθεί για να μειώσει το ρίσκο εμφάνισης διαβήτη σε ασθενείς που χρήζουν θεραπείας με GCs στο μέλλον.

Ο επόμενος στόχος μας ήταν να ανακαλύψουμε ποια γονίδια «ανοίγουν» ή «κλείνουν» στο πάγκρεας κατά την εξέλιξη του T2D. Για αυτό το λόγο, συγκρίναμε παγκρεατικά κύτταρα από υγιή και διαβητικά άτομα και εντοπίσαμε διαφορές σε

395 γονίδια. Έπειτα, με τη χρήση ενός μεγάλου όγκου βιολογικών δεδομένων και μια σειρά πειραμάτων επιβεβαιώσαμε ότι η λειτουργία πολλών γονιδίων από αυτά συνδέεται με τον T2D. Επίσης, αναγνωρίσαμε το PAX5 ως ένα σημαντικό γονίδιο που ενεργοποιείται στα διαβητικά άτομα. Το γονίδο αυτό μπορεί με τη σειρά του να «ανοίξει» και να «κλείσει» γονίδια με τρόπο που να επηρεάζει αρνητικά την λειτουργία των παγκρεατικών κυττάρων και την απελευθέρωση της ινσουλίνης. Συνεπώς, νέες θεραπείες κατά του T2D που στοχεύουν το PAX5 είναι πολλά υποσχόμενες.

Τέλος, μεγάλο μέρος αυτής της διατριβής επικεντρώθηκε στη μελέτη των μικροRNA (miRNAs). Τα miRNAs είναι μικρά μόρια που μπορούν να «κλείσουν» συγκεκριμένα γονίδια. Το κάθε miRNA μπορεί να στοχεύσει πολλά γονίδια και, ως αποτέλεσμα, τα miRNAs συμμετέχουν σε πολλές διεργασίες μέσα στα κύτταρα. Έχει αποδειχτέι ότι τα miRNAs συνεισφέρουν στη μείωση της παραγώμενης ινσουλίνης που παρατηρείται στον T2D, αλλά οι επακριβείς ρόλοι τους δεν έχουν διασαφηνιστεί. Καταφέραμε να ανακαλύψουμε εκτενείς διαφορές μεταξύ των miRNA των υγιών και των διαβητικών ατόμων με διάφορες τεχνολογικές μεθόδους. Επιπλέον, μέσω της ανάλυσης διαθέσιμων βιολογικών δεδομένων ανακαλύψαμε πολλά υπόψια γονίδια που θα μπορούσαν να είναι στόχοι των miRNA και, ταυτόχρονα, σχετίζονται με τον T2D. Με αυτόν τον τρόπο ρίξαμε φως σε περίπλοκα δίκτυα αποτελούμενα απο miRNA και από γονίδια-στόχους που ελέγχουν την απελευθέρωση της ινσουλίνης και αποσταθεροποιούνται κατα την εξέλιξη του T2D. Συνεπώς, συντονισμένες προσπάθειες να διασαφηνιστούν και να αποκατασταθούν αυτά τα δίκτυα θα προσφέρουν καινούριους τρόπους να διαχειστούμε και να θεραπεύσουμε τον T2D.

Συνολικά, η διατριβή αυτή παρουσιάζει μεθόδους με τις οποίες διάφορα είδη βιολογικών δεδομένων μπορούν να ενσωματωθούν και να αποτελέσουν χρήσιμο εργαλείο ώστε να εξερευνηθούν και να αποκαλυφθούν νέες αιτίες της μειωμένης παραγωγής ινσουλίνης στον T2D. Έτσι, αποκαλύπτουμε ανεξερεύνητα μέχρι τώρα γονίδια και miRNA, που μπορούν να επηρεάσουν την παγκρεατική λειτουργία, και κατ'επέκταση, την εμφάνιση T2D, αποδεικνύοντας ότι είναι ιδανικοί υποψήφιοι για θεραπείες έναντι αυτής της νόσου.

Papers included in the thesis

Paper I

Glucocorticoid-mediated induction of ZBTB16 affects insulin secretion in human islets and EndoC-βH1 β-cells. **Karagiannopoulos A**, Westholm E, Ofori JK, Cowan E, Esguerra JLS, Eliasson L. iScience. 2023;26(5):106555.

Paper II

Type 2 diabetes candidate genes, including PAX5, cause impaired insulin secretion in human pancreatic islets. Bacos K, Perfilyev A*, **Karagiannopoulos A***, Cowan E*, Ofori JK*, Bertonnier-Brouty L, Rönn T, Lindqvist A, Luan C, Ruhrmann S, Ngara M, Nilsson Å, Gheibi S, Lyons CL, Lagerstedt JO, Barghouth M, Esguerra JLS, Volkov P, Fex M, Mulder H, Wierup N, Krus U, Artner I, Eliasson L, Prasad RB, Cataldo LR and Ling C. J Clin Invest. 2023;133(4). *Contributed equally

Paper III

Human pancreatic islet miRNA-mRNA networks of altered miRNAs due to glycemic status. **Karagiannopoulos A**, Esguerra JLS, Pedersen MG, Wendt A, Prasad RB, Eliasson L. iScience. 2022;25(4):103995.

Paper IV

Human pancreatic islet miRNA-mRNA regulatory networks associated with insulin secretion and type 2 diabetes development. Cowan E*, **Karagiannopoulos A***, Maziarz M, Esguerra JLS, Eliasson L. Manuscript. *Contributed equally

Papers not included in the thesis

Original papers

I. Interleukin-4 reduces insulin secretion in human islets from healthy but not type-2 diabetic donors. Westholm E, Edlund A, **Karagiannopoulos A**, Wendt A, Eliasson L. Biochem Biophys Res Commun. 2023;649:87-92.

II. The T-type calcium channel Ca(V)3.2 regulates insulin secretion in the pancreatic β-cell. Barghouth M, Ye Y, **Karagiannopoulos A**, Ma Y, Cowan E, Wu R, Eliasson L, Renström E, Luan C and Zhang E. Cell Calcium. 2022;108:102669.

III. Glucocorticoids and glucolipotoxicity alter the DNA methylome and function of human EndoC-βH1 cells. Dos Santos C, **Karagiannopoulos A**, Rafacho A, Perfilyev A, Eliasson L, Ling C and Bacos K. Life Sci. 2022;307:120854.

IV. The highly expressed calcium-insensitive synaptotagmin-11 and synaptotagmin-13 modulate insulin secretion. Ofori JK, **Karagiannopoulos A**, Barghouth M, Nagao M, Andersson ME, Salunkhe VA, Zhang E, Wendt A and Eliasson L. Acta Physiol (Oxf). 2022;236(1):e13857.

V. A critical role of the mechanosensor PIEZO1 in glucose-induced insulin secretion in pancreatic β-cells. Ye Y, Barghouth M, Dou H, Luan C, Wang Y, **Karagiannopoulos A**, Jiang X, Krus U, Fex M, Zhang Q, Eliasson L, Rorsman P, Zhang E and Renström E. Nat Commun. 2022;13(1):4237.

VI. The Calcium Channel Subunit Gamma-4 as a Novel Regulator of MafA in Pancreatic Beta-Cell Controls Glucose Homeostasis. Wu R, **Karagiannopoulos A**, Eliasson L, Renström E, Luan C, Zhang E. Biomedicines. 2022;10(4).

VII. Human Islet MicroRNA-200c Is Elevated in Type 2 Diabetes and Targets the Transcription Factor ETV5 to Reduce Insulin Secretion. Ofori JK, **Karagiannopoulos A**, Nagao M, Westholm E, Ramadan S, Wendt A, Esguerra JLS and Eliasson L. Diabetes. 2022;71(2):275-84.

VIII. Ribosomal biogenesis regulator DIMT1 controls β-cell protein synthesis, mitochondrial function, and insulin secretion. Verma G, Bowen A, Gheibi S, Hamilton A, Muthukumar S, Cataldo LR, Asplund O, Esguerra JLS, **Karagiannopoulos A,** Lyons C, Cowan E, Bellodi C, Prasad R, Fex M and Mulder H. J Biol Chem. 2022;298(3):101692.

Reviews and book chapters

I. miRNAs in the Beta Cell—Friends or Foes? **Karagiannopoulos A**, Cowan E, Eliasson L. Endocrinology. 2023;164(5).

II. MicroRNAs in Type 2 Diabetes: Focus on MicroRNA Profiling in Islets of Langerhans. Cowan E, **Karagiannopoulos A**, Eliasson L. Methods Mol Biol. 2023;2592:113-42.

Abbreviations

ChIP	Chromatin Immunoprecipitation
DE	Differentially Expressed
EndoC	EndoC-βH1
ER	Endoplasmic Reticulum
GBS	GR Binding Motif Sequence
GO	Gene Ontology
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Receptor Elements
GSIS	Glucose-stimulated Insulin Secretion
GWAS	Genome-wide Association Studies
HbA1c	Hemoglobin A1C
IDF	International Diabetes Federation
IGT	Impaired Glucose Tolerance
INS1	INS-1 832/13 Rat Insulinoma cell line
LNA	Locked Nucleic Acid
lncRNA	long non-coding RNAs
LUDC	Lund University Diabetes Centre
miRNA	microRNA
NGS	Next-Generation Sequencing
NGT	Normal Glucose Tolerance
OCR	Oxygen Consumption Rate
PERK	PKR-like ER-associated Kinase
PP	Pancreatic Polypeptide
qPCR	Quantitative Real-time Polymerase Chain Reaction

RNA-seq	RNA sequencing
RT-PCR	Transcriptase Polymerase Chain Reaction
SYT	Synaptotagmin
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TF	Transcription Factor
UMI	Unique Molecular Identifier
WGCNA	Weighted Correlation Network Analysis

Introduction

Diabetes mellitus

Overview

Diabetes mellitus is the most frequent form of diabetes and is related to high blood glucose due to a metabolic disorder. *Diabetes* originates from the Greek word "pass-through" and *mellitus* from the Latin word "honey sweet" and the disease was first accurately described during the 2nd century AD by Aretaeus of Cappadocia as a chronic disease with excessive urination as a prominent symptom, affecting only a few people [1]. Today things have changed drastically since diabetes is a well-known chronic metabolic disorder with diverse complications attributed to elevated blood glucose levels (hyperglycaemia) [2]. According to the International Diabetes Federation (IDF) as of 2021 almost 10% of the adult population are living with diabetes worldwide with the number only expected to rise further by 2047 (12.2%) [3]. Diabetes mellitus and its complications cause one of the top 10 highest percentages of deaths from all causes globally with 6.7 adult million deaths in 2021 [4, 5]. This imposes a tremendous socioeconomic impact on a global scale including Sweden, where billions of Swedish kronor are provided in the healthcare system annually for direct or indirect costs related to diabetes treatment [6].

This high prevalence of diabetes is mainly the result of rapid economic development and a global nutritional and lifestyle transition. High-caloric diet and obesity, reduced physical activity, smoking, and increased psychological stress have all been described as key determinants in the development of diabetes [7]. However, equally important traits that can lead to the onset of the disorder are connected to genetic factors that characterize specific ethnic groups, particular families, or individuals with genetic predisposition [7, 8]. The vulnerability of a large proportion of the population to diabetes can be explained by the evolutionary history of our species and two main hypotheses have been suggested. The "thrifty *genotype* hypothesis" proposes that natural selection favoured individuals or groups that displayed superior metabolic efficiency and increased capability of energy storage in periods of food scarcity [9]. The "thrifty *phenotype* hypothesis", on the other hand, assumes that nutrition in early developmental stages defines structural and metabolic changes that affect glucose metabolism during adulthood [10]. For instance, foetal and infant

undernutrition can result in slower systemic glucose clearance, a trait that can be useful for survival early in life, but can lead to diabetes development in the future [10, 11]. The crosstalk between environmental conditions and developmental changes seems to be mediated by epigenetic changes, which can impact both insulin secretion and insulin action [12].

A regulatory feedback loop between insulin secretion by pancreatic β-cells and insulin action in target tissues such as liver, adipose, and muscle tissues tightly regulates blood glucose levels [13]. The combination of dysfunctional β-cells and suboptimal insulin sensitivity of target tissues is what causes hyperglycaemia with severe long-term complications. Chronic exposure to elevated blood glucose levels has been found to provoke microvascular and macrovascular structural alterations which disrupt the normal function of multiple organs including the kidneys, the nerves, the eyes, and the heart [14].

Despite its high prevalence and severity, in many cases, diabetes is often diagnosed long time after onset due to the absence of acute symptoms. According to the most recent IDF statistics, the majority of people with diabetes globally (55.3%) are undiagnosed [15]. This is important since lack of diagnosis and thus treatment poses a greater risk of suffering from diabetes-related complications [7]. Diagnosis takes place after intravenous measurement of blood glucose levels either in a fasting state (fasting plasma glucose), after an oral glucose tolerance test or after measurement of glycated haemoglobin A1C (HbA1c) levels in the blood. HbA1c is an indicator of the average blood glucose over a span of 2-3 months [13, 16]. In addition to diabetes, these tests can also demonstrate if an individual is prediabetic with a high risk of developing diabetes and its complications. Prediabetes and diabetes are both characterized by impaired fasting glucose, impaired glucose tolerance, and HbA1c over 6% (42 mmol/mol) [16]. In most cases lifestyle interventions are sufficient to prevent long-term complications or revert the prediabetes/diabetes status, however, other cases require temporary or life-long medication [17]. In most cases, this depends on the type of diabetes an individual is diagnosed with.

Diabetes classification

Diabetes mellitus is a heterogeneous disorder with different causes and phenotypic outcomes. The first World Health Organization (WHO) report in 1965 classified diabetes cases using the age of diagnosis and the amount of insulin necessary for survival as the main criteria, alongside gestational diabetes, described as hyperglycaemia during pregnancy and hyperglycaemia related to specific diseases or drugs [18]. Figure 1 shows the current widely accepted classification that divides diabetes into four categories: 1) type 1 diabetes (T1D) with severe insulin deficiency due to immunological destruction of β-cells, 2) type 2 diabetes (T2D) with progressive loss of β-cell insulin secretory capability usually combined with insulin resistance, 3) gestational diabetes mellitus and 4) diabetes due to other causes

including i) monogenic diabetes syndromes, ii) diabetes caused by diseases or iii) drug/chemically-induced diabetes [19], which includes, for instance, glucocorticoid-induced diabetes. Among the different categories, the most common is type 2 diabetes, accounting for 90% of all diabetes cases [20]. Given the substantial variability of susceptibility, progression, related complications, and response to therapeutic remedies of people with T2D, there have been recent attempts to further stratify T2D into specific subgroups.

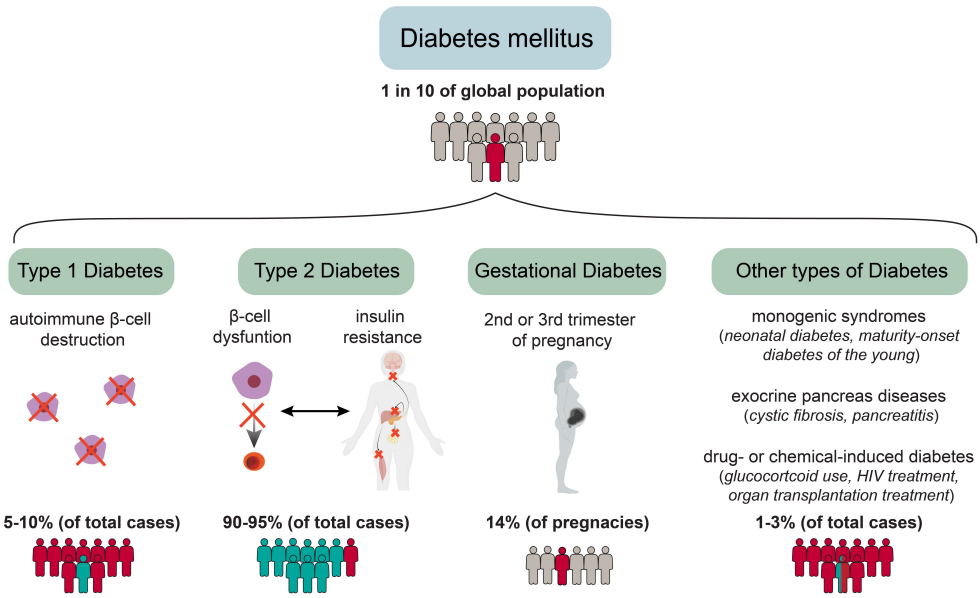

Figure 1. Classification and prevalence of Diabetes Mellitus.

T2D subgroups

By using high-dimensional clinical phenotypic data, Li and colleagues identified three subgroups of T2D patients based on their diabetes-related complications [21]. On the other hand, by performing cluster analysis on six anthropometric and clinical variables Ahlqvist et al. reported five distinct diabetes subgroups in the ANDiS (All New Diabetics in Scania) cohort, namely severe autoimmune diabetes (SAID), severe insulin-deficient diabetes (SIDD), insulin-resistant diabetes (SIRD), mild obesity-related diabetes (MOD) and mild age-related diabetes (MARD) [22]. The five subgroups have been robustly replicated in cohorts from different ethnic groups [23-26]. Interestingly, by using the same clustering method in an Asian Indian population two novel subgroups have emerged: Insulin Resistant Obese Diabetes (IROD) and Combined Insulin Resistant and Deficient Diabetes (CIRDD) [27],

while the inclusion of socioeconomic variables led to the identification of three low-risk clusters (very low-risk (VLR), low-risk low β-cell function (LRLB), low-risk high β-cell function (LRHB)) and three genome-wide clusters (high-risk high blood pressure (HRHBP), high-risk β-cell failure (HRBF), and high-risk insulin-resistant) in a combined set of Swedish and Mexican cohorts [28]. Contrary to the hard clustering methods applied above, a recent study performed soft clustering in T2D patients using 32 anthropometric, clinical, and biochemical variables to detect the baseline T2D aetiology and classify people into a phenotypic spectrum, rather than clearly defined subgroups, with four phenotype archetypes on the "extremes" [29]. Furthermore, other studies have focused on classifying individuals depending on how susceptible they were to T2D and their risk for developing serious related complications [28, 30, 31]. A robust and accurate clustering may be the key to personalized treatment of T2D in the future. However, there are still concerns about how this heterogeneous disease can be classified and how this knowledge can be transitioned into the clinic. In this regard, there are studies that are unable to replicate the aforementioned findings in other cohorts [32] or are skeptical towards the clinical applicability of these methods since they require hard-to-obtain clinical and biochemical data [25].

Glucocorticoid-induced diabetes

Glucocorticoids are steroid hormones naturally produced by the adrenal glands and are involved in diverse physiological processes including metabolism, immune response, growth, development, and behaviour [33, 34]. The therapeutic action of exogenous glucocorticoid supplementation was first discovered in patients with rheumatoid arthritis in 1949 [35]. Further research led to the development of synthetic glucocorticoids that were structurally similar to the endogenous hydrocortisone (cortisol) but more stable and potent [36].

Due to their anti-inflammatory, anti-allergic, and immunosuppressive properties glucocorticoid use is now essential in the clinical setting against a wide array of diseases [37-39], with oral glucocorticoids being prescribed to almost 1% of the global population [40, 41]. However, it is the same diversity in their mechanisms of action that can cause a wide range of adverse effects that are associated with the dose and duration of the glucocorticoid treatment [42, 43]. Hyperglycemia is a well-documented complication of high-dose or prolonged glucocorticoid treatment and can be attributed to various metabolic side effects including increased insulin resistance, dysregulated lipid metabolism, and overproduction of endogenous glucose from the liver [39, 44]. This can gradually lead to the development of glucocorticoid-induced (or steroid-induced) diabetes mellitus. On a global scale, 2% of newly diagnosed diabetes is a direct consequence of oral glucocorticoid use [45, 46]. As shown by a meta-analysis, long-term glucocorticoid treatment (>1 month) of non-diabetic individuals preceded glucocorticoid-induced hyperglycemia in 32%

of the cases and progressed into diabetes in 19% of them [47]. Glucocorticoid-induced hyperglycemia has more severe consequences in individuals with pre-existing insulin resistance [48] or impaired glucose-stimulated insulin secretion [49], as well as in obese females [50], first-degree relatives of individuals with T2D [51], those at a higher age [52, 53] or of specific races [54].

β-cell contribution to glucocorticoid-related diabetes

The mechanisms behind the induction of peripheral insulin resistance by glucocorticoids that contribute to hyperglycemia are well-described in the literature [39, 44]. In that context, glucocorticoid-induced diabetes can be mainly triggered by the inability of β-cells to sustain an increased insulin secretion rate to compensate for the elevated glucose levels [39]. However, evidence also suggests a direct negative effect of glucocorticoids in the β-cells. Administration of a single oral dose of prednisolone [55] or dexamethasone [56] in healthy volunteers results in impaired insulin secretion and/or a reduced insulinogenic index after a meal or oral glucose tolerance test. In a similar fashion, a single dose of glucocorticoid treatment in mice inhibits glucose-stimulated insulin secretion [57]. These findings are confirmed *in vitro* in rodent islets [58-60], in human islets, and in the human β-cell line EndoC-βH1 [61]. Nevertheless, the full set of genes affected by glucocorticoids in the β-cell is not disclosed, and neither are the mechanisms by which they act on insulin secretion.

Glucose homeostasis and human pancreatic islets

Glucose homeostasis is crucial for the survival of mammals. Therefore glucose levels need to be tightly regulated to avoid disease pathogenesis and should remain within a range of 4–6 mM [62]. Increased blood glucose levels after feeding stimulate the pancreatic islets to release insulin into the bloodstream (Figure 2). Insulin binds to receptors on the skeletal muscles, liver, and adipose tissue to promote glucose uptake, utilization, and storage in those tissues and inhibits endogenous glucose production by the liver at the same time [63]. This leads to the reduction of blood glucose levels, which also removes the stimulus for insulin release. Conversely, during prolonged fasting in between meals or during sleep when glucose levels are decreased, islets secrete glucagon, which targets receptors in the liver that induce endogenous glucose production and release into the bloodstream [64]. This fine-tuned system is controlled by the counter-acting hormones insulin and glucagon secreted by the endocrine pancreas.

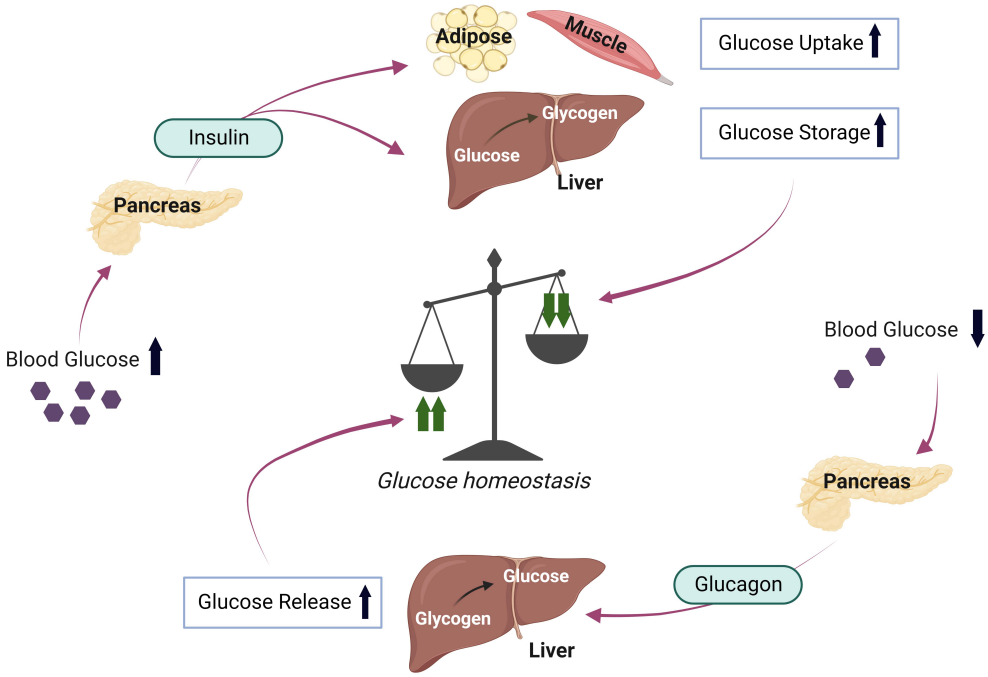

Figure 2. Regulation of glucose homeostasis by the pancreas and peripheral tissues.

Pancreas is both an exocrine and endocrine organ. The exocrine part of the pancreas is dominant and mainly consists of acinar cells responsible for the production and secretion of digestive enzymes such as amylase, pancreatic lipase, and trypsinogen, into the ducts and straight to the duodenum [65]. The endocrine part consists of groups of clustered cells scattered across the pancreas called pancreatic islets, or islets of Langerhans, and comprise only 1-2% of the entire pancreas [66]. Each islet consists of an intermixed population of five distinct cell types: α-cells that secrete glucagon (15-46% of the total islet), β-cells that secrete insulin (48–80% of the total islet), PP- or γ-cells that secrete pancreatic polypeptide (PP) (3-12% of the total islet), δ-cells that secrete somatostatin (3-10% of the total islet) and ε-cells that secrete ghrelin (<1% of the total islet) [62, 67]. Insulin reduces blood glucose and glucagon induces blood glucose increase. However, glucose homeostasis is also affected by the other endocrine cell types as well, as both PP [68] and somatostatin [69] have been shown to exert inhibitory effects on insulin and glucagon secretion. Due to the central role of insulin in the modulation of metabolism, the insulin-secreting β-cells have been extensively studied.

β-cell stimulus-secretion coupling and exocytosis

Stimulus-secretion coupling refers to the molecular mechanisms involved in the transition from a stimulus to the eventual hormonal release. In the case of β-cells, the term glucose-stimulated insulin secretion (GSIS) is used since elevated blood glucose levels are the main stimulus for insulin release [70]. This molecular pathway which has been described several times before [71-73] starts when circulating glucose is taken up by β-cells through the glucose transporter GLUT2 (SLC2A2) and undergoes glycolysis generating pyruvate. Pyruvate is then used by the mitochondria as a substrate for the tricarboxylic acid (TCA) cycle that leads to the generation of ATP and the subsequent increase of the cellular ATP/ADP ratio. This causes the closure of ATP-sensitive potassium (K_{ATP}) channels, which inhibits the flow of positively charged K^+ ions out of the cell. Under low glucose conditions, the open K_{ATP} channel and the outward flow of K^+ ions maintain the resting membrane potential under non-stimulatory conditions. The closure of the K_{ATP} channel at high glucose causes the generation of β-cell action potentials and depolarization of the cell membrane [73]. Nevertheless, a recent study suggested that the closure of the K_{ATP} channel is not enough for generating action potentials and that a depolarizing current through the mechanosensitive ion channel PIEZO1 is also required [74]. The depolarization of the membrane causes voltage-gated Ca^{2+} channels (VGCCs) to be activated and results in the consequent transportation of Ca^{2+} ions into the cell. This sharp rise of intracellular Ca^{2+} concentration triggers the exocytosis of insulin granules.

Insulin exocytosis requires the prior microtubule-based transportation of insulin granules from the Golgi apparatus towards the plasma membrane, followed by the docking, priming and fusion of the granules with the membrane. Priming is the process that prepares the granules for fusion with the plasma membrane and the subsequent release of insulin. This process is ATP- and cAMP-dependent [75-77]. The primed insulin granules form the readily releasable pool of granules that comprise only 1% of the total granules in the β-cell [78]. The final fusion requires the formation of a SNARE-complex consisting of the vesicular VAMP2 and the membrane-bound SNAP25 and syntaxin-1A (Stx1A) proteins. The docking, priming and fusion of the insulin granules are also modulated by other proteins such as STXBP1 (MUNC18), RAB3A and/or RAB27 [75]. The SNARE complex undergoes several conformational changes before the granules are fused with the membrane and, subsequently, release their content. The Ca^{2+}-sensing synaptotagmin (SYT) proteins transfer the Ca^{2+} signal, which is necessary to trigger insulin secretion, to the SNARE complex [73].

A constant increase in blood glucose results in the release of insulin in two phases, a first rapid phase lasting 5-15 minutes followed by a second slower phase. The first phase can be stimulated in the absence of glucose, e.g., through membrane depolarization using an extracellular solution with a high concentration of K^+. On a

cellular level, the pool of readily release granules can also be released in an ATP-independent way [77], which is why it has been suggested that this pool of granules is released during the first phase of insulin secretion, whereas the second phase requires glucose for the mobilization of new granules to the membrane [75, 78].

The process mentioned above, which describes the β-cell stimulus-secretion, is summarized in Figure 3 and defines the "triggering pathway" of insulin release [72, 73]. However, sustained glucose stimulation after a meal can lead the β-cells to eventually secrete a higher amount of insulin through exposure to other stimuli such as incretin hormones (e.g. GLP-1, GIP) through a pathway that is independent of the K_{ATP} channel closure, but requires the depolarization of the plasma membrane and an increase in Ca^{2+} levels. This characterizes the "amplifying" pathway [71]. The amplifying pathway of insulin secretion cannot substitute, but has an additive effect, on the triggering pathway and is mediated by other intracellular signals such as cAMP, glutamate, NADPH, long-chain acyl-CoA derivatives, and superoxides [79].

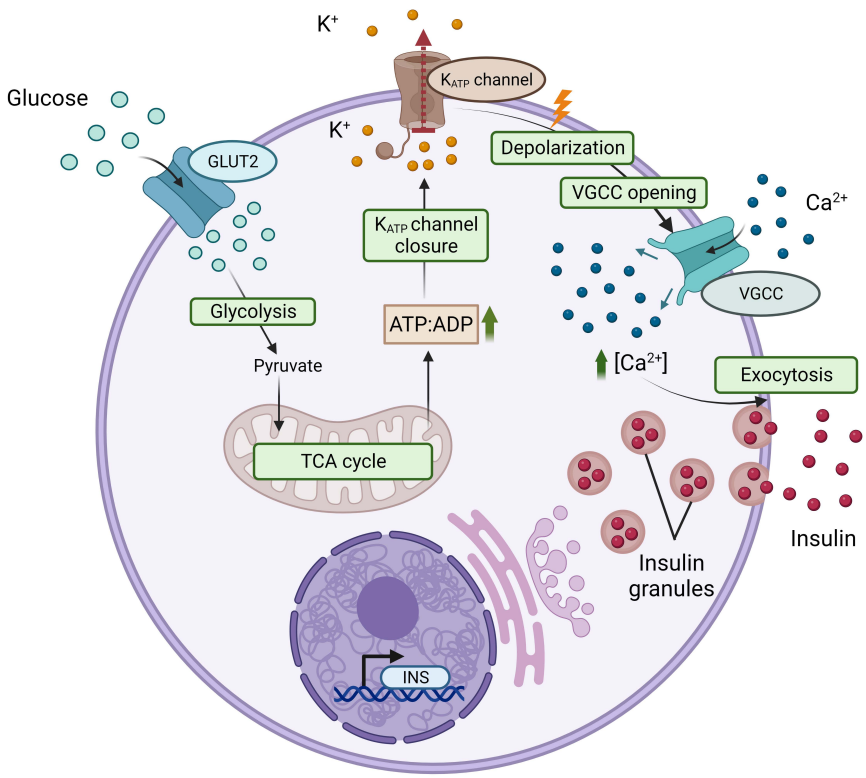

Figure 3. Schematic representation of the stimulus-secretion coupling and secretory granule exocytosis in the pancreatic β-cell

β-cell dysfunction in type 2 diabetes

As described above, insulin deficiency is a major driver of T2D development, which is the result of both the reduction of β-cell mass and the deterioration of β-cell function [80]. Considering the substantial reduction of β-cell function [81, 82], but the limited decrease of β-cell mass at T2D onset [83], we could assume that the two processes are independent and β-cell function impairment comes at an earlier stage during T2D pathogenesis.

Recent studies suggest that the loss of β-cell mass and functionality is the result of a de-differentiation process in which mature β-cells revert to endocrine progenitor-like cells [84, 85]. This mechanism was first demonstrated in a β-cell specific FoxO1-knockout mouse model with reduced pancreatic insulin content, whose β-cells were found to be expressing progenitor markers such as *Neurog3*, *Pou5f1*(*Oct4*), *Nanog,* and *Mycl* [86]. This has been later supported by findings in the β-cells from diabetic mouse models or individuals with T2D, in which lineage-defining transcription factors, such as PDX-1 and MAFA, were suppressed [87]. Some studies also demonstrated the transition of β-cells to other endocrine cell types, a process referred to as trans-differentiation. For instance, β-cell-specific removal of FoxO1 and Pdx-1 in mice led to the transition of a proportion of β-cells into cells that strongly resemble glucagon-secreting α-cells [86, 88]. The β-cell loss of identity can be induced under conditions of prolonged hyperglycemia [89]. Several studies have also described increased de-differentiation in T2D, as defined by the existence of endocrine cells with a lack of hormone expression. While some studies report a smaller proportion of de-differentiated cells compared to the total islet cell number [90, 91], others have calculated a ≈3-4 fold increase of de-differentiated cells in islets from T2D individuals [92, 93]. However, the relevance of de-differentiation on the reduction of functional β-cell mass that leads to T2D is still unclear.

T2D and the associated β-cell dysfunction have been also tightly linked to chronic exposure to excess lipids (lipotoxicity), usually in the presence of elevated glucose levels (glucolipotoxicity) [94]. This can explain why individuals with high BMI display increased fat accumulation in their islets [95]. Glucolipotoxicity was shown to impair GSIS, an effect partly attributed to the increased physical distance of Ca^{2+} channels and insulin granules [96, 97]. Glucolipotoxicity was shown to diminish insulin transcription through the reduction of MafA expression and the impairment of the Pdx-1 translocation into the nucleus in isolated rat islets [98]. The same effect has been also observed after the suppression of NeuroD [99] and the induction of C/EBPB expression [100] under glucolipotoxicity conditions.

Metabolic cellular alterations can also have a significant impact on β-cell impairment. Pancreatic β-cells can gradually lose their "metabolic flexibility" which under normal conditions allows mitochondria to utilize efficiently different substrates for energy production other than pyruvate, such as fatty acids and amino

acids [101]. Evidence suggests that glucolipotoxicity can cause mitochondria to "overwork" to the point that substantial accumulation of oxidation byproducts, such as NADH and reactive oxygen species (ROS), contributes to β-cell dysfunction related to T2D [102, 103]. Interestingly, changes in mitochondrial morphology (fragmentation) and number (reduction) are also observable in T2D [103, 104]. Elevated nutrient levels also lead to increased demand for insulin biosynthesis and processing, a process which is undertaken by ER. ER stress can be the result of sustained overnutrition, which activates the unfolded protein response (UPR) and the consequent inhibition of protein translation through regulation of the PKR-like ER-associated kinase (PERK). Absence of PERK has been associated with impaired β-cell function and severe diabetes in both humans and mice [105]. Consistent with these findings, individuals with T2D were found to have both altered expression of several ER stress-sensing markers [106] and enlarged ERs [107]. However, the mechanism seems to be much more complex, since mitochondrial dysfunction and ER stress can be mutually sustained through a feedback loop where increased mitochondrial oxidation induces Ca^{2+} release from the ER though activation of pro-apoptotic regulators of the Bcl-2 family [108].

Furthermore, defects in the exocytotic process are apparent in T2D. Individuals with T2D display reduced or absence of first-phase GSIS [109, 110], which has been associated with a reduced number of readily releasable insulin granules [111]. Exocytotic proteins related to the SNARE complex, namely STX1A, SNAP25 and VAMP2, have been shown to have reduced expression in human and rodent T2D β-cells [112-115]. Moreover, the expression of several members of the Ca^{2+}-sensing SYT protein family are decreased in T2D individuals [95, 112].

Regulation of islet/β-cell gene expression and type 2 diabetes

Genetics

Even though T2D is generally considered a multi-factorial and polygenic disease, in some cases, diabetes can be attributed to defects in a single gene. So far almost 40 genes have been linked to cases of monogenic diabetes, which can be mainly categorized into maturity-onset diabetes of the young (MODY), neonatal diabetes mellitus (NDM), and syndromic diabetes [116]. Contrary to the well-defined genetic background of monogenic diabetes, in the polygenic T2D cases genome-wide association studies (GWAS) are necessary for the discovery of genes that are related to the disease. A recent large-scale, multi-ancestry meta-analysis has revealed almost 700 genetic risk loci that are related to T2D, which can collectively explain 19% of the heritability of T2D [117]. This shows that single nucleotide

polymorphisms (SNPs) that comprise these loci have moderate, but cumulative, effects on disease risk [118].

As the aim of GWAS is to identify and validate specific genes and molecular pathways that may contribute to T2D pathogenesis, the fact that the majority of the risk alleles reside in non-protein coding genomic regions introduces more complexity to the analysis [119]. In such cases, risk variants are linked to the most proximal gene. Alternatively, the casual risk variant can be located within a certain gene. One such case is the transcription factor *TCF7L2*, which harbours an intronic variant (rs7903146) that has been displaying the strongest and most consistent signal of association with T2D in multiple studies [120-122]. Further investigation showed that *TCF7L2*, which is involved in Wnt signalling, is a regulator of several pathways involved in the regulation of β-cell mass and development, as well as the regulation of insulin synthesis and processing [123, 124]. Similarly, T2D risk variants have been associated with more genes that regulate β-cell function and development, as well as insulin transcription, secretion and exocytosis across different studies [95, 125-127]. Notably, when T2D-risk loci were classified according to their association with other phenotypes relevant to T2D pathophysiology, the majority of them were found to be related to insulin secretion rather than insulin resistance traits [128]. As the systematic functional validation of the high number of T2D-risk variants and the potential casual genes remains a technical challenge, high-throughput β-cell dysfunction screens in human β-cell lines could be an effective way forward [129].

Intriguingly, recent research on islet 3D chromatin structure suggests that the linear proximity approach applied to GWAS analyses may not always be optimal [130]. That is why SNPs that can explain part of the expression variation of specific genes, or expression quantitative trait loci (eQTLs), are usually used to complement the outcomes of GWAS studies. If a specific genetic variation is correlated with the expression levels of a gene and at the same time it is detected as a disease risk locus, while the gene itself displays altered expression in the disease state, then a strong causal relationship can be established between this allele and the disease pathogenesis [131]. Large-scale studies have managed to reveal the colocalisation of around 50 variants that are identified as both islet-specific eQTLs and GWAS T2D risk loci, which are expected to play crucial roles in the pathogenesis of the disease [132, 133].

Genetic variant composition is only one component of a complex network of molecular interactions that can lead to T2D pathogenesis.

Transcriptomics

Gene transcription is a highly dynamic and tissue-specific process that is dependent on a genetic and an environmental component. In that way, certain conditions can

trigger distinct expression patterns that can affect the phenotype. However, there are several genes whose expression needs to be tightly controlled for the establishment and maintenance of the mature β-cell identity such as *NEUROD1, NKX6.1, PAX4, PAX6, FOXA2, PDX-1, NKX2.2, MAFA* and *ISL1* [134, 135]. Equally important to the expression of β-cell specific master regulators is the constant repression of genes whose action is not consistent with the requirements of β-cell function, but are highly abundant in most tissues [136, 137]. The list of nearly 70 "disallowed" genes includes genes that are involved in the transportation or metabolism of alternative glycolysis products that would result in suboptimal ATP production compared to glucose [138, 139]. Dysfunctional β-cells are characterized by either decreased expression of β-cell markers or abrupt expression of disallowed genes [140, 141].

Although β-cells are considered a group of cells with high functional similarity, multiple studies investigating single-cell transcriptomic differences between human islet cell types have suggested that β-cells display diverse properties and are clustered into different functional groups with various methods [142]. This is in line with observations that show important β-cell markers to be only expressed in a fraction of β-cells [143]. The transcriptome heterogeneity also confirms previous findings regarding functional heterogeneity related to glucose response and insulin secretory capacity [144-146]. A pioneering study managed to prove β-cell heterogeneity related to ion channel activity and exocytosis by combining electrophysiological measurements with single-cell RNA sequencing data, a technique referred to as patch-seq [147]. However, the use of single-cell RNA sequencing methods comes with drawbacks, as the probability of inflated false-negative values due to the systematic bias towards highly abundant transcripts is high [148]. This is why it is important to also consider studies investigating the transcriptome in the whole islet using bulk RNA sequencing techniques.

In the pursuit of discovering genes important for the pathogenesis of T2D, several studies have compared the transcriptomic profiles of human islets from donors with and without T2D and are included in Table 1. A recent review that compared β-cell T2D DE genes between several single-cell sequencing studies found only *FXYD2*, a gene encoding a regulatory subunit of the Na^+,K^+-ATPase, to be replicated across studies [142]. Moreover, although reduced insulin biosynthesis is widely observed under T2D conditions, only three studies have reported decreased insulin transcription in islets from T2D donors [149, 153, 154]. This can be explained by the fact that insulin content is not dependent on the number of insulin transcripts, but rather the mechanisms underlying the translation and processing of pro-insulin into insulin [72]. However, the fact that other well-defined T2D genes have not been identified with high-throughput sequencing approaches raises concerns about the reliability and reproducibility of the methods. The low number of samples and cells that result in low statistical power, the individual donor variability and the islet heterogeneity, as well as the variety of statistical methods used, can be potential reasons for the divergent outcomes. In any case, large-scale studies that combine

multiple levels of genetic, epigenetic, and transcriptome data in order to capture the T2D pathophysiology are missing.

Table 1. Summary of published studies in which comparisons between the human islet transcriptomes of control and individuals with T2D were performed.

Transcriptomic type	Detection method	Control/ T2D cases	Reference
Bulk (Microarray) sequencing	Affymetrix HG U133A	7/6	Bugliani et. al [149]
	Affymetrix HG U133A and B	7/5	Gunton et. al [150]
	Affymetrix HG U133Plus2.0	116/55	Solimena et. al [151]
	GeneChip Human Gene 1.0 ST	54/9	Taneera et. al [152]
Single-cell sequencing	Smart-seq2	6/4	Segerstople et. al [153]
	Fluidigm C1	5/3	Lawlor et. al [154]
	Fluidigm C1	12/6	Xin et. al [155]
	Fluidigm C1	3/2	Wang et. al [156]
	Smart-seq2	18/7	Camunas-Soler et. al [147]
	Drop-seq	6/3	Fang et. al [157]
	Fluidigm C1	4/10	Avrahami et. al [158]

Epigenetics

An important layer of gene expression regulation depends on heritable changes in gene function or activity without alteration of the DNA sequence. Contrary to the genome, the epigenome is dynamic and highly variable between tissues and cell types, across different environmental conditions and over time [159]. DNA methylation and histone modifications are the two widely accepted epigenetic mechanisms. Despite the lack of evidence regarding their heritability, miRNAs can also be considered epigenetic regulators. The fact that miRNAs are able to both orchestrate and be regulated by epigenetic modifications [160] and that they are largely affected by environmental stimuli [161], validates their substantial epigenetic role. This is further supported by findings demonstrating the heritability of miRNA abundance in mice [162].

Molecular tags

Epigenetic modifications include the methylation of specific DNA nucleotides (DNA methylation) and the methylation, acetylation, phosphorylation, and ubiquitination of specific histone residues (histone modifications) [163]. Both these changes affect the accessibility of gene regulatory elements on the DNA, which can be facilitated or hindered by the local chromatin formation [164]. Depending on the pattern of these alterations, transcription factors (TFs) can bind on certain DNA

sequences by relocating or removing nucleosomes generating, in that way, ”open chromatin” [165].

Open chromatin regions are widely used to determine regulatory active loci across different species and cell types [166]. In the human pancreatic islet, thousands of conserved and islet-specific open chromatin sites have been identified [167]. Integration of histone modification pattern data showed that these regions overlap with promoters and distal enhancer clusters (active or quiescent) which are targeted by transcription factors in order to activate the expression of genes responsible for maintaining the islet identity [168]. The distal enhancers and promoters can interact in 3D space and form regulatory hubs, which control the activation of genes implicated in islet differentiation and function [130]. Thus, it is no surprise that T2D risk variants have been consistently shown to reside in open chromatin/enhancer regions [130, 167-170]. Findings from single-cell islet chromatin accessibility analysis revealed lineage (between cell types) and state-specific (within the same cell type) differences between the endocrine islet cells [171]. For instance, β-cells were clustered into high and low insulin producers based on their open chromatin profile and, interestingly, genetic variants linked with fasting glucose were associated only with the high insulin producers, while T2D-related variants were linked to β-cells in both states [171]. The same pattern was observed in a recent study where two β-cells groups were identified based on the dosage of a specific epigenetic histone modification, in which higher levels were accompanied by increased metabolic activity and insulin secretion capability [172]. Furthermore, the comparison of the human islet genome-wide methylome in human islets from T2D and non-diabetic donors revealed almost 26,000 differentially methylated regions in T2D islets, which overlapped with genes with altered expression in T2D that are involved in important islet functions [173]. Interestingly, changes in methylation levels were observed together with altered histone markers, suggesting that they might be part of a common epigenetic mechanism of gene expression regulation that drives β-cell dysfunction in T2D [173]. However, further studies are required to conclude if these changes can lead to the development of T2D or are just a consequence of the disease.

miRNAs

MicroRNAs (miRNAs) are a group of short non-coding RNAs (≈19–23nt), responsible for post-transcriptional control of gene expression. Their main function is to mediate gene silencing by triggering poly(A)-deadenylation or translational inhibition followed by degradation of their target mRNAs [174, 175]. However, under certain conditions, miRNAs can induce mRNA translation [176, 177].

The canonical miRNA biogenesis pathway, as illustrated in Figure 4, starts with the transcription, splicing, and poly-adenylation of primary miRNAs (pri-miRNAs, ≈1000nt) from the corresponding genes, which form a characteristic hairpin structure [178]. The duplex at the hairpin base is then cleaved by Drosha in the

nucleus, generating the precursor miRNA (pre-miRNA, ≈70nt), which is then exported to the cytoplasm by Exportin-5 (XPO5) [179, 180]. The pre-miRNA is further cleaved by Dicer in its terminal loop to produce a mature ≈22nt double-stranded miRNA [181]. The mature miRNA is then loaded to members of the AGO family of proteins, forming the RNA-induced silencing complex (RISC), where it is unwound, one strand is discarded ("passenger" strand), while the other ("guide" strand) establishes an active RISC that can bind on target mRNAs [182]. The binding involves complementary base pairing of the miRNA sequence at positions 2–8, which are referred to as the seed sequence, to nucleotides within the 3′ UTRs of the target mRNAs [183].

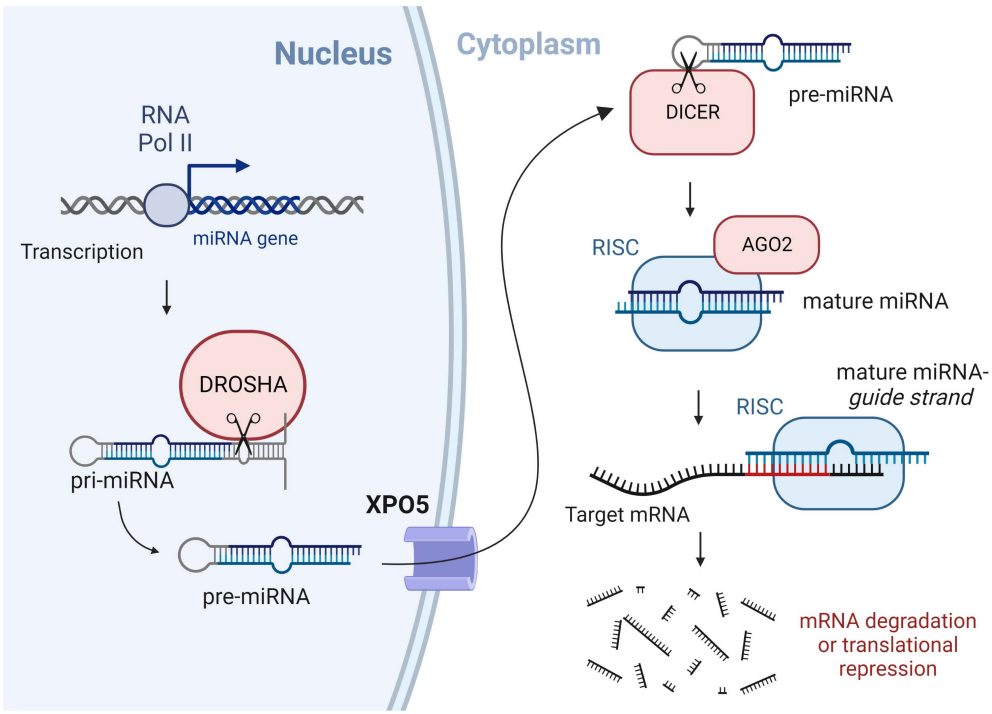

Figure 4. The canonical pathway of miRNA biogenesis.

Members of the same miRNA family are defined by the similarity of their seed sequences and, even though they are evolutionary related, they may differ in their primary or secondary structure [184]. Moreover, members of the same miRNA family display heterogeneity between their mRNA targets due to pairing involving sites beyond the seed sequence [185]. The small size of the seed sequence is the reason that one miRNA can have multiple targets and why a single gene can be targeted by different miRNAs. In reality, the impact of miRNA action is even larger considering that non-canonical binding, which involves sites outside the miRNA seed sequence or the mRNA 3' UTR, is an extensive phenomenon [183]. Moreover, miRNA action can be affected by genetic polymorphisms or epigenetic

modifications, such as RNA editing, as well as by interactions with RNA binding proteins (RBPs) or other non-coding RNAs. Overall it is estimated that ≈60% of all human protein-coding genes are conserved targets of miRNAs [186].

The importance of the global miRNA impact on β-cell identity and function has been shown in β-cell specific Dicer1 knockout mouse models. These mice displayed diminished insulin transcription, reduced granule docking, and impaired insulin secretion [187]. The first miRNA that was described in the pancreatic islets is the highly abundant miR-375 [188]. Further investigation of this miRNA revealed its implication in multiple key mechanisms in the β-cells including insulin biosynthesis, β-cell development and differentiation, and insulin exocytosis [189]. Since then many miRNAs have been associated with the regulation of all aspects of GSIS such as glucose transport and metabolism, insulin biosynthesis and transcription of important β-cell transcription factors, electrical activity and Ca^{2+} influx, as well as exocytosis [190, 191].

Although most of the findings were reported in rodent islets/cell lines, due to the difficulty in obtaining human islets or the limited number of functional human β-cell lines, some miRNAs have been replicated in human islets/cell lines. For instance, suppression of miR-7, miR-125b, or miR-335 improved GSIS, while overexpression of miR-200 and miR-375 decreased GSIS [192-196]. Furthermore, two studies that independently investigated the effects of miRNAs on insulin transcription in human islets showed that miR-204 and a group of 8 miRNAs, including miR-375 and miR-7, can regulate the levels of insulin abundance in the β-cell [197, 198]. While a higher number of β-cell-specific miRNAs have been identified with microarrays or small RNA sequencing in sorted β-cells from human islets [199-201], their significance in β-cell function is still not known.

As an element of epigenetic regulation miRNAs are mediators between environmental stimuli and protein expression. In the context of β-cell function, the expression of miRNAs has been shown to be modified by glucose or fatty acids usually in the form of palmitate. Specifically in human islets, glucose or palmitate treatment can induce the expression of miR-25, miR-455, miR-24, and miR-125b, which exert negative downstream effects on insulin secretion or β-cell proliferation/apoptosis [193, 202-204]. This is a type of condition-specific miRNA regulation where alterations of miRNA expression levels under certain conditions disrupt the standard gene expression motifs [205, 206]. MiRNAs can also act as constitutive gene expression suppressors that are essential in preserving the functionality of specific cell types by constantly repressing gene targets that could hinder their function, referred to as “disallowed” genes. In other cases, miRNAs are responsible for maintaining the protein output within an optimal range to ensure an ideal cellular phenotype through a feedback mechanism [205]. For example, both increased or decreased expression of miR-375 can have a deleterious effect on the β-cells [189]. This miRNA mode of action can be mediated by positive and negative regulatory feedback loops with specific transcription factors [207]. Lastly, changes

in the miRNA expression levels can have a neutral impact on the phenotypic output of a cell as its targeting may lack biological function, or may be tolerated via compensation by other cellular processes and molecular programs [208].

Based on the above, it is not surprising that islets derived from individuals with T2D display distinct global miRNA profiles compared to controls [191]. However, such data are not available for the different islet cell types due to the low availability of human islets and the technical challenges of islet cell sorting and high-throughput small RNA sequencing [209]. Interestingly, on the islet level, miRNA differential regulation in T2D and abundance do not seem to coincide.

The expression of highly abundant miRNAs, such as miR-375, seems to be unaffected in T2D, possibly because of its implication in islet "housekeeping" processes and the need for its expression to be maintained at an optimal level, as mentioned before. On the other hand, significant changes in low-abundant miRNAs may be the result of small changes in the absolute copy number of the miRNAs that have insignificant biological relevance [190, 191]. This is why functional validation of the findings suggested by large-scale studies is necessary. As seen in Figure 5, several studies that experimentally validated differentially expressed miRNAs in T2D found that these miRNAs can either compensate for the adverse effects of the disease by increasing the proliferation or insulin secretion of the β-cells or contribute to T2D pathogenesis by promoting the functional impairment or apoptosis of the β-cells [209].

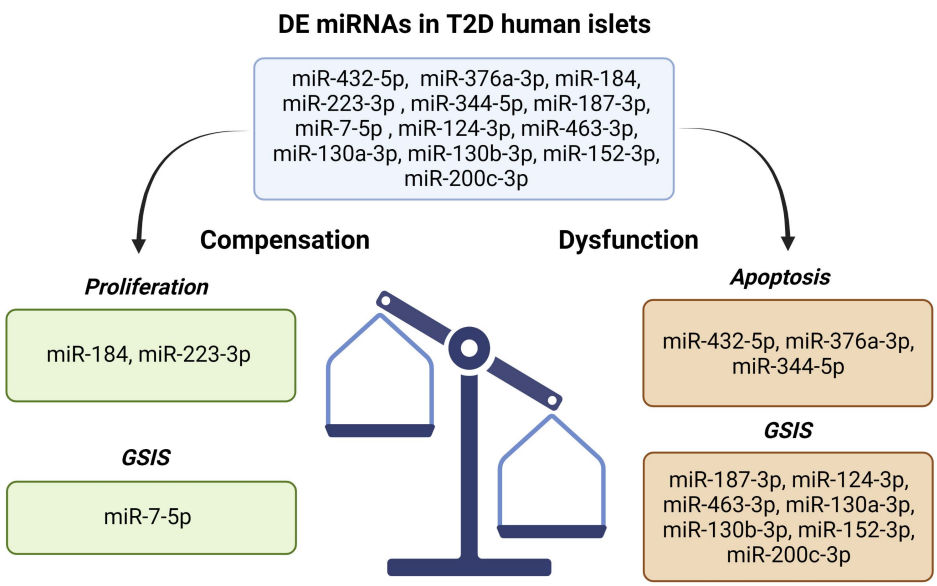

Figure 5. The dual role of experimentally validated differentially expressed (DE) miRNAs in T2D. Adapted from [209]. GSIS; glucose-stimulated insulin secretion.

Epitranscriptomics

Another field of epigenetic control studies epitranscriptomics, which encompasses the investigation of reversible chemical modifications in diverse positions on the RNA (tRNA, mRNA, rRNA, miRNA, and other non-coding RNAs) [210]. These changes can affect the transcript splicing, translation, and RNA-protein interactions leading to functional and metabolic RNA alterations [211]. N6-methyladenosine (m^6A), which is the most abundant modification of the mRNA, was found to be a mediator between glucose sensing and β-cell function [212]. High glucose caused a reduction of m^6A methylation in human islets, which subsequently affected the expression of genes important for β-cell identity and insulin secretion [212]. In agreement with these findings, human islets from T2D donors displayed reduced levels of m^6A methylation on the mRNA of key genes involved in cell cycle regulation, β-cell identity, and function [213]. Although this is still an emerging topic in the context of T2D pathogenesis, it holds great promise for potential therapies for the disease.

Aims

This thesis aims to investigate the molecular mechanisms underlying the pathogenesis of glucocorticoid-induced and type 2 diabetes in human islets and β-cells by utilizing different bioinformatics methods and types of data.

Glucocorticoid-induced diabetes is a type of diabetes with high prevalence due to the wide usage of glucocorticoids. The effect of glucocorticoids on the induction of peripheral insulin resistance is well-described, however many studies also suggest a direct deleterious effect on the β-cell function. While glucocorticoid gene targets important for the β-cell action have been detected, the molecular pathways behind the glucocorticoid-mediated human β-cell dysfunction remain largely uncovered.

Type 2 diabetes is a multi-factorial disease whose aetiology should be sought among both genetic and environmental factors. The recent advancement of high-throughput molecular methods has led to an explosive increase of available data covering multiple biological layers including genetics, epigenetics, and transcriptomics. Despite impressive efforts to integrate data from various sources, the high complexity behind the mechanisms involved in type 2 diabetes pathogenesis makes the investigation of additional regulatory networks in a higher number of individuals necessary.

Specific aims of the thesis

I. Investigate the mode of action and impact of glucocorticoids on human islets and β-cells and determine genes implicated in the glucocorticoid-mediated β-cell dysfunction

II. Identify and associate human islet genes with multiple signals of association with T2D that could contribute to the disease pathophysiology

III. Discover genomic sites and molecular pathways associated with islet miRNAs with altered expression due to glycaemic status that could play a role in T2D onset

IV. Uncover differentially expressed miRNAs in T2D human islets that are implicated in the insulin secretory mechanism and promote the development of T2D

Materials and Methods

Various functional laboratory methods, bioinformatics workflows, and types of data have been employed during this thesis. This section will provide an overview of the biological data types used in the different projects and will focus on the methods used for transcriptome analysis of human islets and cell lines, which have been extensively applied in this thesis. Last, a brief description of the laboratory methods utilized in the projects will be presented.

Overview of biological data types

Table 2 presents an overview of the different types of large-scale biological data sets used in this thesis. It should be noted that this list is not exhaustive and additional information from various databases, e.g., miRNA-mRNA target, gene function and miRNA-disease data, were also utilised.

Table 2. Overview of biological data types used in each project included in the thesis. Abbreviations: SNP; single nucleotide polymorphism, eQTL; expression quantitative trait loci, mQTL; methylation quantitative trait loci, TF; transcription factor, OCR; open chromatin region, DMR; differentially methylated region, ChIP; chromatin immunoprecipitation

Data category	Data type	Paper I	Paper II	Paper III	Paper IV
Transcriptomics	Total RNA sequencing	X	X	X	X
	Total RNA microarrays		X		
	Single-cell sequencing	X	X		
	small RNA sequencing				X
	small RNA microarray			X	
Genetics	SNPs		X	X	
	eQTL		X	X	
	mQTL		X		
	TF binding motifs	X	X		
Epigenetics	OCRs	X	X		
	DMRs		X		
	ChIP sequencing	X			
	Enhancer-Gene interaction scores	X			
	miRNA-mRNA targets			X	X

Methods of transcriptome analysis

The methods of transcriptome characterization that were applied in this thesis include microarrays, quantitative real-time polymerase chain reaction (qPCR), and next-generation sequencing (NGS). These methods were applied to identify both mRNA and miRNA expression levels.

All procedures were initiated by extracting the RNA of the samples of interest. By using the miRNeasy isolation kit (Qiagen) the isolation of both mRNAs and small RNAs was ensured. Controlling the quality of the extracted RNA is essential for the accuracy and reproducibility of the experimental outputs. Moreover, since downstream assays require very small amounts of RNA, the accurate determination of the RNA quantity is also important.

Quantitative real-time polymerase chain reaction (qPCR)

qPCR is considered the gold standard for assessing nucleic acid levels in biological samples due to its high specificity and sensitivity [214]. Recently, the efficacy of qPCR was demonstrated to the general public when it was used as the most accurate diagnostic test against SARS-CoV-2 [215].

Throughout the projects in this thesis, TaqMan® reagents (ThermoFisher) were utilized for qPCR. The procedure starts when RNA molecules are reverse-transcribed to cDNA through a transcriptase polymerase chain reaction (RT-PCR). While RT-PCR is a one-step procedure, a different set of primers are suitable for cDNA synthesis when either mRNA or miRNA molecules are used as substrate. In the case of mRNAs, a set of random primers consisting of oligodeoxyribonucleotides (mostly hexamers) is used. This facilitates the initiation of the reverse transcription in potentially every mRNA in the sample, including the mRNA(s) under study. On the other hand, the problem of the small size of miRNAs (≈19-23nt) is addressed with a stem-loop reverse transcription (RT) primer [216]. This primer is specific for the reverse transcription of the mature miRNA under study, ensuring the extension of the miRNA 3' end which leads up to the formation of an RT primer/mature miRNA chimaera. In both cases, both the pool of transcribed cDNAs and the RT/miRNA chimeric cDNA can be used as input for real-time PCR.

Taqman® qPCR technology is based on a set of primers and a labelled probe, which binds specifically to the target sequence. The primers are then extended by the polymerase until they reach the labelled probe, which is degraded, and a fluorescent signal is emitted [217]. The sample undergoes multiple cycles of PCR, with more dye molecules being released in each cycle, which results in an increase in fluorescence intensity in proportion to the amount of synthesized amplicon. In the end, a Ct (cycle threshold) value is given to the target cDNA, representing the

number of cycles for the fluorescent signal to be detectable by exceeding the background threshold. The method is quantitative and as such only the relative expression of the target molecule compared to an appropriate control is of interest.

To analyze the data first a ΔCt value is calculated, which represents the difference between the Ct values of the target gene/miRNA and those of appropriate housekeeping genes/miRNAs in each sample. Next, the difference of ΔCt values between samples of interest and control samples is calculated (ΔΔCt). Finally, the relative fold change of gene/miRNA expression compared to the control condition is determined by the $2^{-\Delta\Delta Ct}$ value [218].

Despite the robustness of qPCR, a labelled probe is required for every region of interest, thus raising the cost in case multiple targets need to be assessed. Another limitation is the fact that the sequence of the targets needs to be known, limiting the possibility of identifying novel targets under certain experimental conditions/treatments.

Microarrays

Microarray-based mRNA and miRNA expression detection has been one of the most widely used methodologies of large-scale nucleic acid screening in a large number of samples simultaneously. Although different microarray technologies have been developed, they are all based on the hybridization of specific probes on the surface of a microarray plate [219]. The RNA undergoes a reverse transcription and an amplification step, in which cDNA is generated. The cDNA is subsequently stained with fluorescent dyes. The labelled cDNA is then hybridized with the available probes and, after a washing step to remove unbound molecules, the plate is stained. Finally, scanning and image analysis takes place using specialised software. Downstream analysis usually includes background correction, normalization, probe-specific background correction, and probe-level information summarization [220]. In that way, raw image data are translated into RNA expression data.

As seen in Table 1, in the context of T2D investigation, distinct versions of GeneChip® Human Genome (HG) Assays (former Affymetrix, currently ThermoFisher) were used to identify the mRNA expression profile of human islets from non-diabetic and T2D donors. Despite utilizing the same technology, these assays were different in the number and composition of their probes, with newer versions consisting of denser probe sets and, consequently, providing coverage of a higher number of transcripts with increased sensitivity and specificity. According to ThermoFisher, these assays cover ≈18,000-38,000 genes, which is enough to cover a substantial proportion of the currently identified protein-coding genes (total: ≈20,000, GENCODE v43, 2023), as well as a proportion of long non-coding RNAs (lncRNAs) (total: ≈20,000 GENCODE v43, 2023) [221]. However, recent advances

in the discovery of lncRNA and small non-coding RNA genes set the total number of genes even higher (total: ≈62,000, GENCODE v43, 2023).

The identification of miRNAs is technically more challenging than that of mRNAs and lncRNAs due to their smaller size. To overcome this limitation, technologies such as locked nucleic acid (LNA)-based microarrays have been developed. LNAs are RNA analogues with a "locked" ribose ring, which optimises the stability of base pairing. These high-affinity LNA-modified capture probes can be hybridized with the short-sized miRNAs with high specificity and sensitivity [222]. The LNA-based microarray used in Paper III (former Exiqon, currently Qiagen) allowed the identification of 840 miRNAs (total: ≈2,880, miRBase v22, 2023).

Microarrays are a well-established and cost-effective method to reveal the level of thousands of genes and RNA molecules in multiple samples. However, their use comes with certain disadvantages such as the inability to identify novel transcripts, the lack of reproducibility across platforms, and the complicated data processing and normalization protocols [223, 224].

Next-generation sequencing (NGS)

NGS is also a method for large-scale identification of transcript levels in multiple samples in a parallel fashion. However, it does not require prior knowledge of specific targets of interest, but it captures a screenshot of the whole transcriptome profile in a given sample [225]. NGS methods differ in the principles of library preparation depending on whether total RNA or small RNA sequencing is used as substrate.

The aim of total RNA sequencing is to detect protein-coding mRNAs and specific categories of large non-coding RNAs. In the projects of this thesis, the TruSeq Stranded Total RNA Library Prep with Ribo-Zero kit (Illumina) for library preparation was used. The first step of library preparation is to fragment the RNA molecules and remove rRNA fragments which may introduce noise due to their high abundance when sequencing is performed. Next, strand-specific cDNA is created through reverse transcription. Strand specificity is important for correctly identifying overlapping or partly overlapping genes located in opposite strands. After 3'end adenylation, adapters are ligated in both the 3' and 5' ends of cDNA fragments, allowing their hybridization to the flow cell during the sequencing step. These adapters are specific to the sequencing system and act as both sample- and molecule-specificity molecular barcodes. They are also targeted by amplification primers used in the sequencing step.

The aim of small RNA sequencing is to retain and detect only the short non-coding proportion of the total isolated RNA. In the projects of this thesis, the QIAseq

miRNA Library Kit (Qiagen) was used. The first step includes the ligation of a pre-adenylated 3' and a 5' adapter to the corresponding ends of the small RNA. This procedure requires a T4 RNA ligase that recognizes the hydroxyl group on the 3′-end and the phosphate group on the 5′-end of RNAs and, thus, it leads to selective profiling of RNAs with these properties such as miRNAs and excludes other RNA classes such as protein-coding mRNAs. Similarly to RNA sequencing, the ligands indicate sample specificity and facilitate the hybridization of the miRNAs on the flow cell. During the subsequent reverse transcription step, Unique Molecular Identifiers (UMIs) are incorporated in the primers and are attached to each small RNA molecule. This step allows the detection and elimination of sequencing errors introduced during the amplification and sequencing processes, providing a more accurate representation of the initial miRNA number in the sample [226]. The generated cDNA is then cleaned up with a bead-based method that filters out sources of background noise, such as adapter dimers and other RNA species, leading to miRNA enrichment. In both mRNA and miRNA sequencing processes, the final cDNA product is enriched through a polymerase reaction and is subsequently sequenced, after a quality control step. Throughout this thesis, the sequencing was performed in an Illumina NextSeq 500 Sequencing System.

In the end, bioinformatics analysis is performed on the generated sequence reads. This includes another round of quality control with several pre-processing steps [227]. Reads from mRNA and miRNA samples are similar except the latter encompass the short UMI sequences that need to be stored before they are eventually filtered out. After removing the adapter sequences, mRNAs and miRNAs can be mapped to a reference transcriptome (e.g., GENCODE) or miRNAome (e.g., miRBase) respectively, before they are quantified and analysed further. It should be mentioned that more established pipelines and sophisticated software are available for total RNA-seq read mapping and quantification compared to the most recently introduced small RNA-seq.

Similarly to microarrays, NGS can reveal novel transcripts and miRNAs and, importantly, is ideal for high-resolution transcriptomic analyses, such as the identification of alternative splicing isoforms or the detection of canonical miRNAs and their isomiRs, both shown to play important roles in various diseases [228, 229]. However, the high cost per sequencing run and the labour-intensive processes of generating sequencing libraries, performing the sequencing and analysing the sequencing data, still make microarray-based sequencing attractive in certain circumstances.

More details on the bioinformatics workflows and tools applied in this thesis can be found in the original papers.

Laboratory methods

Experimental models

Human pancreatic islets

Human islets derived from deceased donors were obtained from the Human Tissue Lab EXODIAB/LUDC via the Nordic Network for Islet Transplantation (http://www.nordicislets.org). Ethical permits issued by the Uppsala and Lund University Ethics committees defined the frame for the processing of human islets. Preparation of human islets involved enzymatic digestion and density gradient separation before they were handpicked in cold Hank's buffer and transferred to appropriately supplemented RPMI 1640 medium under a stereomicroscope. Islets were incubated in a humidified atmosphere with 5% CO2 at 37°C.

EndoC-βH1 cells

EndoC-βH1 cells are one of the most widely used human β-cell lines. They originate from human fetal pancreatic tissue that was subjected to targeted oncogenesis. EndoC-βH1 cells have been validated as human pancreatic β cell models with glucose-stimulated insulin secretion and gene marker assays [230-232]. The cells were grown in Matrigel fibronectin-coated culture vessels in appropriately supplemented DMEM medium and were preserved in a humidified atmosphere with 5% CO2 at 37°C.

INS1 832/13

INS1 832/13 cells derive from INS1 cells, a rat insulinoma cell line, and display robust expression of both human and rat insulin [233, 234]. INS1 cells were preserved in appropriately supplemented RPMI 1640 medium in a humidified atmosphere with 5% CO2 at 37°C.

Functional assays

Transfection

In order to overexpress or suppress the expression of specific genes in the cells, delivery of siRNAs and DNA plasmids was performed via lipid-based transfection, respectively. For that, Lipofectamine® RNAiMAX (siRNA delivery) and Lipofectamine® LTX (plasmid DNA delivery) were used (Thermo Fisher). These reagents are broadly compatible with different cell lines and display low toxicity. Cells were transfected for 72h with the corresponding siRNAs and plasmids and were previously seeded in antibiotic-free medium until they reached 100% confluence.

Insulin secretion

Insulin secretion assay was performed in both EndoC and INS1 cells. Before the assay, the cells were seeded in appropriate plates until they reached 100% confluence. A 2h pre-incubation step in freshly prepared Secretion Assay Buffer (SAB) followed by a 1h stimulation step with high glucose concentration was applied before collection of the secreted insulin. EndoC and INS1 cells were pre-incubated in 1- and 2.8-mM glucose and were stimulated with 16.7- and 20-mM glucose, respectively.

Mitochondrial function

Mitochondrial function, as evaluated by measurement of the mitochondrial oxygen consumption rate (OCR), was assessed in EndoC and INS1 cells with the Seahorse XF analyser system (Agilent Technologies). Starvation of the cells in SAB buffer with 1- or 2.8-mM glucose, respectively, was followed by OCR measurements every 3 minutes over the span of 90 minutes. OCR was measured at basal glucose and after the addition of 10 mM pyruvate, 5μM oligomycin, 4μM carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP) and 1 μM rotenone. The specified online tool for the Analyzer system (URL: seahorseanalytics.agilent.com) was used to analyse OCR, after normalising to total protein content quantified with BCA assay.

More details on the aforementioned techniques, as well as other laboratory techniques employed in this thesis such as Western blot, Chromatin Immunoprecipitation followed by PCR (ChIP-PCR), and cell viability measurements, can be found in the original papers.

Results and Discussion

Paper I

Glucocorticoid treatment induces transcriptomic changes important for the β-cell function in human islets and EndoC-βH1 cells

To explore the direct effects of glucocorticoids in human islets and β-cells, islets and human insulin-secreting EndoC-βH1 (EndoC) cells were treated with or without dexamethasone, a synthetic glucocorticoid. EndoC cells display extensive similarity with human β-cells in regards to their transcriptomes, proteomes, and secretomes and, therefore, they can be used as a translational model of human β-cells [235]. Global transcriptome analysis revealed 1473 and 3147 differentially expressed (DE) genes in human islets and EndoC cells, respectively ($q < 0.05$). Interestingly, the DE genes included key genes involved in β-cell development and function such as *PDX-1, NKX1-1*, and *MAFA* [61, 236]. Of note, 581 DE genes overlapped between human islets and EndoC cells with the same directionality of expression change (309 upregulated and 272 downregulated genes), indicating robust transcriptomic changes in the human β-cells.

Functional annotation showed the involvement of DE genes in β-cell specific processes, for instance, insulin processing and secretion, glucose homeostasis, regulation of β-cell gene expression, and β-cell proliferation and differentiation. Several of these pathways have been shown to be dysregulated in rodent islets and cell lines with glucocorticoid-induced insulin secretion impairment. These include glucose metabolism [237], potassium channel activity [60], calcium transport and insulin secretion [58, 59, 237], β-cell proliferation, and differentiation [238, 239].

Glucocorticoid action is exerted in a distal manner and is dependent on auxiliary transcription factors in human islets and EndoC-βH1 cells

The properties of Glucocorticoid Receptor (GR) action were further investigated in human islets and EndoC cells, as glucocorticoids exert their effects by binding and activating the GR, which translocates into the nucleus and acts as a transcription factor. To do this, first a comprehensive list of GR binding regions derived from many ChIP-seq experiments (GR-ChIP sites) was obtained from the GTRD

database [240]. GR-ChIP sites were then associated with genomic sites close to the DE genes after dexamethasone treatment in order to yield Glucocorticoid Receptor Elements (GREs). GREs were located closer to DE genes compared to non-DE genes and were equally distributed around upregulated and downregulated genes. This validates the role of GR as both an activator and a repressor of gene expression in the β-cell, similar to what has been described in other cell types [241, 242]. Furthermore, ≈95% of GREs resided more than 3 kb of the transcription start site of their associated DE genes in both human islets and EndoC-βH1 cells, suggesting that glucocorticoids affect their target genes in a distal manner. This also corroborates previous findings about the non-typical promoter-proximal binding pattern of GR [243, 244].

De novo motif discovery on the DNA sequences of the GREs led to the discovery of over-represented binding motifs belonging to the AP-1, ETS/TEAD, and FOX transcription factor families, other than the GR binding motif. Analysis of the GR Binding motif Sequence (GBS) showed that only 77% of GREs encompass a canonical 15-bp GBS [245], consistent with a similar proportion (≈60-80%) in other human cell types [243, 246, 247]. Strikingly, the vast majority of GREs (≈95%) were composite elements consisting of at least one alternative AP-1, ETS/TEAD, or FOX motif sequence besides a GBS. Indeed, a study from Starick et al. demonstrated co-occupation of FOX factors with GR on GREs possibly to preserve an open chromatin conformation allowing GR to bind to the target DNA sequence. In the same study, AP-1 was shown to facilitate GR DNA binding without directly interacting with the DNA sequence, while members of the ETS/TEAD family appeared to directly interact with GR to promote tethering to the DNA [248]. The enrichment of the motif binding sequences of these transcription factors in GREs has been replicated in other cell types [247-250], highlighting their regulatory significance on the effects mediated by GR. Taken together, GR binding on GREs and, consequently, the regulation of GR action seems to be heavily relying on the binding of auxiliary transcription factors [251].

ZBTB16 demonstrates the strongest signal of direct glucocorticoid targeting in human islets and EndoC-βH1 cells

A bioinformatics workflow was established to explore which of the DE genes after dexamethasone treatment were direct targets of glucocorticoids and GR. The overlap of the gene-associated GREs, as defined in the previous section, with human/EndoC-specific open chromatin regions, enhancer-gene association scores and GR binding sequence motifs led to the generation of a Normalized Annotation Score. Subsequently, the combination of the Normalized Annotation Score and the gene expression fold-change resulted in a gene Rank Product, which indicated the probability of the DE gene being directly targeted by glucocorticoids. Among the most highly ranked genes are well-characterized direct glucocorticoid targets such

as *FKBP5* [252] and *VIPR1* [253, 254], which supports the robustness of the analysis. *ZBTB16* was the top-ranked gene in both human islets and EndoC cells and was strongly induced after dexamethasone treatment both at the RNA and protein level. *ZBTB16* is an established glucocorticoid target in non-pancreatic cells [255-257] and is involved in various molecular pathways including stem cell self-renewal and differentiation [258], maintenance of spermatogenesis [259], limb development [260], and hematopoietic development [261]. Recently, *ZBTB16* was found to be displaying substantial methylation changes after dexamethasone treatment in insulin-secreting cells [236]. *ZBTB16* has also been associated with systemic glucose homeostasis in the skeletal muscle and liver, as it negatively regulates adipogenesis [262] and insulin sensitivity [263, 264].

ZBTB16 displays intronic and conserved Glucocorticoid Responsive Elements in EndoC-βH1 cells

Even though DE genes were linked to GREs within a 150 kb window of their transcription start site (TSS), ZBTB16-associated GREs were located exclusively in intronic regions of the gene. Despite the fact that regulatory elements typically reside in proximal upstream or downstream sites of their target genes, a significant proportion of GREs (25-30%) has been recorded in introns [243, 250]. Luciferase assays also suggested a functional role of the sequences comprising intronic GREs [265, 266]. Interestingly, out of the 10 GREs linked to ZBTB16, only 4 encompassed a GBS. This highlights the important role of other factors and auxiliary TFs in the glucocorticoid-GR signalling pathway. Moreover, 5 out of 10 GREs were located in highly evolutionary conserved regions and contained at least one GBS. A piece of evidence that supports the significance of these sites in GR-mediated regulation is the significant induction of open chromatin signal in these regions in human islet samples after dexamethasone treatment [253]. ChIP followed by PCR confirmed the GR binding in all *ZBTB16* GREs after dexamethasone treatment in EndoC cells. GR binding signal seemed to be consistently stronger in dexamethasone-treated cells, however, there were big fluctuations between the replicates, similar to those demonstrated in the islet open chromatin profiles of dexamethasone-treated islets [253]. Taken together, GR seems to dynamically regulate the expression of *ZBTB16* via intronic binding, which is mediated by additional transcription factors.

Dexamethasone activates *ZBTB16* in a dose- and time-dependent manner and decreases insulin secretion in EndoC-βH1 cells

The expression of *ZBTB16* was assessed after dexamethasone treatment with different dosages and times under treatment. Two known glucocorticoid targets, the activated *SGK1,* and the repressed *GR,* were used as positive and negative controls,

respectively. EndoC cells were treated with different doses of dexamethasone (0.1–2000 nM) which led to a dose-dependent induction of *ZBTB16* expression. *ZBTB16* activation was stronger than that of *SGK1* in all conditions and it reached a plateau after 1 μM. Increasing dexamethasone concentrations also led to an increased proliferation rate in EndoC cells. The duration of the treatment was also important as *ZBTB16* demonstrated its highest upregulation after 48h of treatment, which was, once more, higher than the expression of *SGK1*.

Dexamethasone treatment was found to significantly reduce insulin secretion after 48h. However, insulin content measurements also displayed an apparent and consistent reduction 8h post-treatment, which can partly account for the inhibitory effect on insulin secretion. Insulin secretion decrease, together with the observed increase in cell proliferation rate under the same conditions, may indicate that cells undergo a gradual de-differentiation process as shown in c-Myc-expressing INS1 cells [267]. This is supported by the fact that the *β-cell pancreatic cell differentiation* pathway was significantly enriched during functional annotation of the DE genes after dexamethasone treatment. Strikingly, since *ER stress* is the highest over-represented term in the EndoC-exclusive subset of DE genes, it is rational to propose that insulin content reduction may form part of a compensatory mechanism against elevated ER stress levels in EndoC cells [268].

ZBTB16 activation is protective against glucocorticoid-induced β-cell dysfunction

Since *ZBTB16* is highly induced after dexamethasone treatment, an insulin secretion assay was performed in dexamethasone-treated control and *ZBTB16*-overexpressing EndoC cells. Insulin secretion under stimulating glucose concentration and insulin content, both of which were impaired upon dexamethasone treatment, were moderately rescued after *ZBTB16* overexpression. In addition, partial repression of the dexamethasone-induced *ZBTB16* expression led to elevated insulin secretion under basal glucose conditions, a phenotype that resembles T2D [269]. The partial suppression of *ZBTB16* also resulted in reduced transcription of the insulin (INS) gene. Moreover, the reduction of *ZBTB16* expression inhibited the dexamethasone-induced SGK1 activation, which has been linked to insulin release and T2D [60, 270]. Overall, *ZBTB16* expression induction triggered by dexamethasone seems to exert a protective effect by partially rescuing β-cells from the negative consequences of glucocorticoid exposure.

To further assess the function of ZBTB16, the bioinformatics workflow described earlier was utilized to identify potential direct gene targets of ZBTB16. The analysis led to the discovery of 1093 predicted targets. Integration of single-cell islet transcriptome data showed that 751 (68%) of the targets are expressed in the human β-cells. Functional annotation of the ZBTB16 gene targets in islet and β-cell

uncovered similar enriched pathways belonging to processes related to the regulation of translation, ER protein targeting, cell cycle/division, and mitochondrial membrane/biogenesis.

The potential effects of *ZBTB16* on the cell cycle were examined in EndoC cells, as ZBTB16 has been described as both a positive and negative regulator of cell cycle in other cell types [271-275]. *ZBTB16* overexpression did not demonstrate any additive effects in the increased proliferation rate of EndoC cells caused by dexamethasone. Next, the association of *ZBTB16* with mitochondrial function was studied as *ZBTB16* has been shown to affect mitochondrial number and function in brown adipocytes [276]. To do that, mitochondrial oxygen consumption rate (OCR) measurements were performed in control and *ZBTB16*-overexpressing EndoC cells after dexamethasone treatment. Dexamethasone had a significantly negative impact on mitochondrial metabolism, as assessed by measurements of basal, acute, and maximal respiration, as well as ATP production. The deleterious impact of glucocorticoids on mitochondrial function has been described in insulin-secreting, as well as other cell lines [277, 278]. Strikingly, induction of *ZBTB16* expression could compensate for these negative effects and restore mitochondrial function. As mitochondrial respiration is tightly connected to insulin secretion, this can also explain the mechanism behind the restoration of the glucocorticoid-mediated impairment of insulin secretion after *ZBTB16* overexpression, demonstrating the protective action of *ZBTB16* in the β-cell.

Key findings

- Dexamethasone treatment triggers extensive transcriptomic changes in human islets and EndoC cells that are related to β-cell development and function
- Glucocorticoid action is mediated by auxiliary transcription factors
- *ZBTB16* is a direct dexamethasone target in human islets and EndoC cells
- Dexamethasone perturbs insulin secretion and is a potent activator of *ZBTB16* expression in EndoC cells
- ZBTB16 has a protective role against glucocorticoid-induced β-cell dysfunction

Paper II

Large-scale human islet transcriptome analysis reveals genes with altered expression in T2D

Total RNA sequencing (RNA-seq) was performed in human islets to identify genes with distinct profiles in islets derived from non-diabetic (ND) donors compared to donors with T2D. For that, the Lund University Diabetes Centre (LUDC) pancreatic islet cohort was used, consisting of islets from 309 donors of which 283 have available sequenced transcriptomes. After RNA-seq data quality control and application of specific donor filtering criteria, the gene expression profiles between 138 ND and 33 T2D individuals were compared. Differential expression analysis, after taking into account donor sex, age, as well as islet purity and days in culture (DIC), showed 395 genes to be differentially expressed (DE) (q-value < 0.05) with 228 and 167 being upregulated and downregulated in T2D, respectively. Functional annotation as performed with Gene Ontology (GO) term enrichment analysis revealed that DE genes are involved in several pathways that have been associated with islet function, development, and T2D such as growth factor binding [279], regulation of lipid transport [280], negative regulation of Wnt signalling [281, 282], regulation of inflammatory response [283], inorganic cation homeostasis [284] and hormone secretion [72]. To evaluate the gene expression of DE genes separately for α and β cells, the LUDC sorted α/β cell cohort consisting of fluorescence-activated cell-sorted (FACS) islets from 13 ND individuals, 2 individuals with T2D and 3 individuals with prediabetes was utilized. Out of 395 DE genes, 366 and 368 were expressed in α and β cells, respectively. However, 174 genes displayed higher expression in β cells, as opposed to 90 genes with higher expression in α cells.

Identifying the shift in the islet transcriptomic profile in T2D is not indicative of whether these changes lead to T2D progression or are induced after disease onset. For that reason, gene expression was studied in a subset of the LUDC islet HbA1c cohort, comprising individuals not previously diagnosed with T2D. Strikingly, the expression of 142 out of the 395 DE genes was linearly correlated with HbA1c levels with the same directionality (positive/negative) as the expression changes in T2D (induction/repression). This shows that a proportion of gene expression alterations may be present before T2D onset and may contribute to the disease predisposition.

Overlap of differentially expressed genes in T2D between studies

To check the reproducibility of the RNA-seq results of the present study, data from studies that also investigated the human islet transcriptomic changes in T2D using bulk (whole islet) [149-152] or single-cell sequencing [153-155] methods were

compiled. Out of 395 total DE genes, 75 were also present in at least one of the previous bulk sequencing studies and 28 in at least one of the previous single-cell sequencing studies. In total, 94 DE genes were previously detected, including known T2D-associated genes such as *SLC2A2 (GLUT2)* [285], *IAPP* [286], *PPP1R1A* [287], and SYT13 [112, 288], demonstrating the robustness of the analysis. Notably, the majority of DE genes in the current study (301/395), including *PAX5, OPRD1,* or *PCOLCE2*, were not previously detected.

Interestingly, there were only minor overlaps in the DE genes identified between different bulk- or single-cell studies, while no gene was found to be commonly DE among all studies. This divergence may be the result of inconsistencies regarding experimental methods, donor characteristics, or statistical power/approaches. Specifically, differences in islet isolation and culture protocols, library preparation, and sequencing systems (see also Table 1), as well as the distinct pathophysiological and demographic backgrounds of the donors under study can greatly affect the analysis outcome. Nevertheless, an even greater limitation, particularly among single-cell studies, is the low sample size which results in poor statistical power and does not allow correction for the aforementioned batch effects. Moreover, variables that are taken into account during DE analysis greatly differ between studies as there are studies that do not account for any variable [149, 150], account only for sex [153, 154], account for sex and age or ethnicity [151, 155] or account only for islet purity [152].

DE genes are associated with genetic signals related to T2D

To investigate the relationship between the expression of DE genes and genetic variability, DE genes were first associated with expression quantitative trait loci (eQTL) using data from the INSPIRE (Integrated Network for Systematic analysis of Pancreatic Islet RNA Expression) study [132] and the T2D Knowledge Portal (https://t2d.hugeamp.org/). In this way, the expression of 120 DE genes was linked to 148 eQTLs. Interestingly, some of these eQTLs have also been correlated with phenotypic traits relevant to T2D. Specifically, 2 eQTLs associated with islet expression of *FXYD2* and *RPL39L* have been linked to T2D risk, 2 eQTLs associated with islet expression of *FOXE1* and *ENTR1* to glucose measurements, 4 eQTLs associated with islet expression of *ARPC1B*, *COMP*, *DIXDC1*, and *HSD3B7* to BMI or waist/hip ratio and 6 eQTLs associated with islet expression of *ACP2*, *CBLC*, *CD5*, *HSD3B7*, *PCOLCE2*, and *TMED6* to triglyceride or LDL levels [289-295].

To define the broader spectrum of genetic influence on T2D DE genes, associations between SNPs related to T2D phenotypic traits and the 395 DE genes were performed based on close proximity. For that, a list of SNPs linked to T2D and/or glycemic traits in GWAS, including fasting glucose, fasting insulin, HbA1c, homeostatic model assessment of β cell function [HOMA-B], disposition index

[DI], and corrected insulin response [118], was acquired from the Common Metabolic Diseases Knowledge Portal (hugeamp.org). In total, 106 DE genes were associated with 149 SNPs that satisfied the criteria. Out of the 149 unique SNPs, 131 were associated with T2D, 38 with HbA1c, 10 with fasting glucose, 4 with DI, and 1 with fasting insulin and CIR. It is worth mentioning that several genes were associated with both eQTL and SNPs of interest such as *FXYD2, RPL39L, ENTR1, COMP, ACP2, CBLC,* and *TMED6*. The fact that these genes were also significantly correlated with HbA1c levels suggests their potential contribution to T2D pathogenesis.

DE genes are associated with T2D epigenetic signals related to T2D

To investigate regions with potential epigenetic activity in the vicinity of DE genes, publicly available human islet open chromatin sites, as determined by ATAC-seq, were obtained and were associated with the DE genes [296]. The vast majority of DE genes (346/395) were assigned to at least one open chromatin site. Interestingly, a subset of islet open chromatin regions that additionally displays altered conformation in T2D was assigned to 24 DE genes, of which 23 showed increased expression in islets from T2D donors. On the whole, the expression changes of a subset of DE genes seem to be driven by changes in the open chromatin state observed under T2D conditions.

Moreover, publicly available differentially methylated regions (DMRs) in T2D were assigned to DE genes as an additional factor of epigenetic control [173]. In total, 262 DE genes were marked with 732 DMRs. Taking a step further, SNPs associated with methylation around DE genes, known as methylation quantitative trait loci (mQTLs), were also investigated [297]. Overall, 90 DEGs were annotated to 490 SNPs associated with DNA methylation of 176 unique sites. Taking everything into account, there is enough evidence to suggest a degree of epigenetic influence on the differential expression of genes related to T2D.

Changes in DE gene expression impair β cell function

In order to functionally validate a subgroup of the 395 identified DE genes, a series of filtering steps were applied. First, genes with fold-change higher than 2 (80 genes), which were associated with HbA1c (31 genes) were retained. Subsequently, genes that were expressed in endocrine cells were assessed based on the lowest DE q-values, generating a final list of 9 genes, including *BARX1, ELFN1, FAIM2, HHATL, NEFL, OPRD1, PAX5, PCOLCE2,* and *SFRP1*. Two additional genes were selectively added to the list. *CHL1* served as a positive control, as β-cell silencing of this gene results in impaired insulin secretion [298] and *SLC2A2* was selected due to its debated role in β-cell function [299, 300]. Notably, 10 of 11 genes were

associated with at least 1 genetic or epigenetic signal including islet eQTLs, GWAS SNPs, T2D DMRs, or mQTLs.

To appropriately imitate the directionality of the DE gene expression changes in T2D, expression suppression of T2D downregulated genes, e.g., *CHL1, HHATL, OPRD1,* and *SLC2A2*, was performed in insulin-secreting rat insulinoma 832/13 INS1 (INS1) cells. Insulin secretion measurements showed a tendency towards reduced secretion under basal glucose conditions (2.8 mM) upon *CHL1* and *SLC2A2* knockdown and displayed reduced insulin secretion after glucose stimulation (16.7 mM) upon *OPRD1* knockdown.

In the same way, overexpression of T2D upregulated genes, e.g., *BARX1, ELFN1, FAIM2, NEFL, PAX5, PCOLCE2,* and *SFRP1* was performed using lentiviral vectors. *Pax5* overexpression was found to increase insulin secretion under basal glucose concentration and reduce insulin secretion under stimulated glucose concentration, with no change in insulin content. This resulted in a substantial decrease in the induction of insulin secretion between basal and stimulatory glucose conditions. *Nefl* and *Pcolce2* overexpression led to reduced insulin secretion under basal glucose concentration and increased insulin content, while *Pcolce2* overexpression also reduced secretion under stimulatory glucose concentration.

Overall, these analyses revealed previously unidentified genes in an islet or β-cell context that can modulate β-cell function by acting on insulin secretion, namely *OPRD1, NEFL, PCOLCE2,* and *PAX5*. *OPRD1* is a G-protein-coupled receptor for endogenous enkephalins, which have been shown to both trigger and suppress insulin secretion [301-303]. *NEFL*, which encodes for the light chain of neurofilament, was shown to be expressed in higher levels in the serum of T2D individuals and has been correlated with fasting glucose [304]. *PCOLCE2* is a collagen-binding protein that has been found to be downregulated in the skin of T2D individuals. *PAX5,* a member of the paired box (PAX) family of transcription factors, is important for the differentiation and development of B lymphocytes, acting as both an activator and repressor of gene expression [305]. Hence, loss of *PAX5* has been implicated in the development of human B cell malignancies such as acute lymphoblastic leukaemia [306]. It should be mentioned that other members of the PAX family including *PAX2*, *PAX4*, and *PAX6* are considered crucial regulators of human islet development, differentiation, and function [307].

Pax5 impairs insulin secretion through mitochondrial impairment and induces β-cell apoptosis

Manipulation of *Pax5* caused the most severe effects on β-cell function and this is the reason this gene was further investigated. First, immunohistochemical staining of human pancreas sections confirmed the RNA-seq findings regarding the low expression of PAX5 in islets from ND donors compared to the increased expression

in islets from T2D donors. Single-cell transcriptome data from β-cells derived from ND donors also demonstrated that *PAX5* is expressed in low levels in adult β-cells [308].

Next, insulin secretion was performed in INS1 cells under stimulation with both glucose and a depolarizing K^+ concentration after *Pax5* overexpression. Once again, insulin secretion was significantly impaired after incubation in basal and stimulatory glucose concentrations, whereas K^+-stimulated cells displayed increased levels of insulin secretion. This indicated that defective processes in the insulin secretory pathway upon *Pax5* overexpression may concern mechanisms prior to depolarization of the K_{ATP} channels, such as mitochondrial metabolism. Indeed, measurements of oxygen consumption rate (OCR) with a Seahorse XF analyzer showed a reduction in mitochondrial respiration, as outlined by a decrease in both acute respiration after glucose stimulation and in maximal respiration after the addition of an uncoupler of the inner mitochondrial membrane (FCCP). Mitochondrial dysfunction was also validated by a significant reduction of intracellular ATP/ADP ratio of *Pax5*-overexpressing cells after glucose stimulation, as measured by the fluorescent biosensor PercevalHR.

Pax5 overexpression induces large-scale transcriptomic changes in β-cells

As PAX5 is a transcription factor, global transcriptomic changes were investigated after *Pax5*-overexpression in INS1 β cells. Interestingly, 3069 genes were found to be differentially expressed ($q < 0.05$), including 75 of the 395 T2D DE genes in human islets. Functional annotation through GO term enrichment analysis revealed that these genes are involved in several pathways, with the top enriched ones including insulin secretion, glucose homeostasis, regulation of lipid transport, and cell death. These findings are in line with the experimental results observed in this study.

Furthermore, to identify putative gene targets of PAX5 in human islets, a PAX5-binding motif analysis was performed across the DE genes. Interestingly, 196 out of 395 DE genes displayed a PAX5 binding motif in their promoter region and literature review showed that 25% of them have been previously related to β-cell function or mass, as well as T2D-related genetic variants. Finally, weighted correlation network analysis (WGCNA) showed *PAX5* to demonstrate a similar expression pattern with 86 other DEGs, forming a co-expression network. Overall, *PAX5* overexpression seems to be capable of inducing transcriptome-wide changes that lead to β-cell dysfunction.

Key findings

- Transcriptome analysis of human islets from T2D and ND donors revealed 395 differentially expressed (DE) genes
- Transcriptomic changes related to T2D can impair β-cell function
- PAX5 is overexpressed in T2D islets
- Elevated Pax5 expression induces β-cell dysfunction by perturbing mitochondrial function and insulin secretion
- PAX5 overexpression triggers transcriptome-wide alterations in the β-cell, contributing to the dysregulation of several T2D DE genes

Paper III

Microarray-based profile of human pancreatic islet miRNAs

The expression profile of 840 miRNAs in human islets derived from 18 donors was evaluated with an LNA (locked nucleic acid)-based array platform from Exiqon. Analysis showed that 470 miRNAs were expressed in the human islet samples. A filtering step including the removal of probes that corresponded to more than 1 miRNA and of probes that were not captured by all samples, led to the inclusion of 269 miRNAs for downstream analysis.

Abundance analysis showed that miRNAs display a wide range of expression levels, with only a few miRNAs being highly expressed. Investigation of the 15 miRNAs with the highest expression levels revealed several miRNAs previously defined as highly abundant in islets and/or β-cells, including miR-375-3p, miR-7-5p, and members of miR-200 (miR-200c/miR-141) and miR-29 (miR-29a/b/c) families [199], validating the robustness of the analysis. The high expression of these miRNAs has facilitated their functional characterization in β-cells. For instance, suboptimal expression levels of miR-375 have been found to have a negative impact on β-cell physiology, as they affect insulin biosynthesis, processing, secretion, and cell proliferation [189]. Different studies have also demonstrated that miRNAs belonging to the miR-200 and miR-29 families have important roles in insulin secretion and β-cell apoptosis, and miR-7 function has been linked to insulin secretion and β-cell development [191, 309]. The list of highly expressed miRNAs also included miRNAs not described as abundant before such as miR-1246, miR-

1290, and miR-1908-5p. Of interest, miR-1246 was the miRNA with the highest expression, even though its function is unknown in the islets. However, miR-1246 has been identified as a serum biomarker for diabetes [310] and a prognostic biomarker for pancreatic cancer [311].

To check if there are differences in the highly abundant miRNAs between donors with distinct glycaemic status, human islet samples were stratified into three groups depending on their glucose tolerance status, as defined by their HbA1c levels. Out of 18 donors, seven had normal glucose tolerance (NGT) with HbA1c<6% (42 mmol/mol), six had impaired glucose tolerance (IGT) with an HbA1c range of 6.0%–6.4% (42 to 47 mmol/mol), and five of them were diagnosed with T2D with an HbA1c range of 6.2%–7.0% (44–53 mmol/mol). Interestingly, NGT, IGT, and T2D donors shared 11 out of the 15 top-expressed miRNAs, suggesting that glycaemic status does not influence the order of expression of abundant miRNAs.

The miRNA profile is changed in islets from donors with different glycaemic status

Differential miRNA expression analysis between normoglycemic (NGT) and donors with elevated blood glucose levels (IGT & T2D) was performed with the Significance Analysis of Microarrays (SAM) method, after ensuring donors were matched for age, gender, and BMI. In total, 63 miRNAs were differentially expressed (DE), 37 up- and 26 downregulated (FDR < 0.1). Interestingly, a higher number of upregulated miRNAs was also shown to characterize diabetic/obese human, mouse, and rat models [312-314]. To further assess which miRNAs were affected by long-term exposure to high glucose and can potentially contribute to T2D predisposition, miRNA expression was correlated with HbA1c levels using a linear regression model adjusted for sex, age, BMI, and diabetic status of the donors, as well as days of *in vitro* islet culture. The analysis showed a significant correlation of HbA1c with 93 miRNAs. These included miR-7, miR-23b, and miR-484, whose expression has been shown to be regulated by glucose [315].

To produce a set of miRNAs that are both associated with HbA1c and display altered expression due to glycaemic status, DE miRNAs were overlapped with HbA1c-correlated miRNAs depending on the directionality of the change/correlation. In that way, a set of 9 miRNAs that were upregulated in IGT/T2D islets and positively correlated with HbA1c levels comprised the *Up-Pos* set and a set of 22 miRNAs that were downregulated in IGT/T2D islets and negatively correlated with HbA1c levels comprised the *Down-Neg* set. The *Up-Pos* consisted of miR-1275, miR-513a-5p, miR-32-3p, miR-1827, miR-509-5p, miR-1236-3p, miR-130b-3p, miR-629-5p, and miR-130b-5p, while the *Down-Neg* set included miRNAs such as miR-7-5p, miR-23b-3p, miR-29b-3p, miR-200a-3p, miR-200b-3p, miR-19b-3p, and miR-126-3p. Of note, only 5 out of the 31 DE miRNAs in the two sets are among the most

abundant islet miRNAs, including miR-7-5p, miR-29b-3p, miR-141-3p, miR-21-5p, and miR-24-3p, all of which were downregulated. This suggests that regulation and abundance in a miRNA context do not necessarily coincide.

miRNAs associated with changes in glycaemic status are linked to islet gene targets related to T2D

To discover mRNA targets of the DE miRNAs, experimentally validated miRNA gene target data from Tarbase [316] and miRTarBase [317] were identified, compiled, and associated with the miRNAs belonging to the *Up-Pos* and *Down-Neg* sets. The 9 *Up-Pos* miRNAs were linked to 4,046 mRNAs generating 5,232 miRNA-mRNA interactions, while the 22 *Down-Neg* miRNAs were associated with 12,289 mRNAs forming 41,950 miRNA-mRNA interactions. The discrepancy between the low number of miRNAs and the high number of their targets is reasonable considering that one miRNA can target several mRNAs and one mRNA can be targeted by multiple miRNAs [309]. Interestingly miR-23b-3p, miR-7-5p, and miR-26a-5p were associated with more than 3,000 mRNAs. This is a nice illustration of how a few thousand miRNAs can control over 60% of protein-coding genes [186].

Nevertheless, the publicly available validated miRNA-mRNA target data used in this analysis was derived from experiments in non-pancreatic cell types. To improve the accuracy of the results, only targets expressed in human islets were used for downstream analysis. Integration of islet transcriptomic data from 188 donors, which showed confident expression of 11,689 genes, demonstrated that a large proportion of miRNA gene targets were expressed in the islets (68% - 89% of the total number of targets per miRNA). To explore if targets have been associated with T2D, a comprehensive set of differentially expressed genes in T2D was obtained and compiled from several bulk- and single-cell RNA-seq studies [149-155, 318]. Overlaps between the 3365 identified DE T2D genes and the miRNA mRNA targets expressed in islets showed that nearly 30% of the targets were related to T2D in at least one study. Of note, miR-130b-3p in the *Up-Pos* set and miR-23b-3p in the *Down-Neg* set displayed the highest number of validated targets and demonstrated the most extensive overlap with genes expressed in the islet and T2D-related genes.

Targets of differentially regulated miRNAs are clustered in islet-specific networks associated with β-cell function

As the focus of this study was not to investigate the role of individual miRNAs, but the impact of miRNA-mRNA networks on β-cell function, mRNA targets of miRNAs associated with changes in glycaemic status were grouped based on their expression pattern in highly-correlated clusters. In that way, 39 and 43 co-

expression clusters consisting of targets of the *Up-Pos* and *Down-Neg* miRNA sets, respectively, were generated by performing weighted gene correlation network analysis (WGCNA). Next, by summarizing the properties of each cluster into a single eigenvalue, the clusters could be correlated with specific variables of interest including HbA1c, islet purity, and mRNA expression of insulin and glucagon. The analysis was combined with functional annotation of the clusters to identify enriched biological pathways associated with the genes of each cluster.

Two clusters, one in the Up-Pos set (Up-Pos_Cluster 3) and one in the Down-Neg set (Down-Neg_Cluster 1) were of interest. The two clusters appeared to be islet-specific as both of them were positively correlated with islet purity, as well as with the expression levels of insulin and glucagon. Furthermore, the two clusters seemed to be involved in key β-cell functions, as demonstrated by the enrichment of pathways related to insulin processing and secretion, exocytosis, β-cell development, and gene expression regulation. Up-Pos_Cluster 3 and Down-Neg_Cluster 1 consisted of 301 and 1019 miRNA gene targets, respectively. Among those genes, several have been shown to have a pivotal role in β-cell identity and function, e.g., *NKX2*-2 [319], *NEUROD1* [320], *FOXO1* [321], and insulin exocytosis, e.g., *SNAP25* [322] and *SYT11* [288]. Lastly, overlaps between the T2D-related genes, as defined in the previous section, and the genes that comprised the clusters in each set showed that the two clusters, Up-Pos_Cluster 3 and Down-Neg_Cluster 1, displayed the highest proportion of genes associated with T2D (≈40%) when compared with the rest of the clusters in the *Up-Pos* sets and *Down-Neg* sets, respectively.

The expression of abundant and DE miRNAs can be affected by genetic elements

To determine how genetic variation affects the expression of miRNAs of interest, *cis*-eQTLs were mapped to the DE miRNAs belonging to the *Up-Pos* and *Down-Neg* sets, as well as the 15 most abundant miRNAs, in a 1 Mb window of the starting position of each miRNA. Nevertheless, no eQTL-miRNA correlation reached statistical significance when subjected to multiple testing correction, possibly due to low statistical power (n=18). This was also observed in studies with a higher number of subjects and, thus, superior statistical power [323, 324]. This can be attributed to the fact that compared to *cis*-mRNA eQTLs, which were found to account for 33%–53% of the variance in the expression levels of their related mRNAs in a large-scale study, *cis*-miRNA eQTLs could only explain a small percentage (1.3%) of the expression variability of their associated miRNAs [325]. However, as multiple nominal eQTL signals around a miRNA may imply a relationship between genetic variability and the expression of this miRNA, nominally correlated eQTLs ($p < 0.05$) were considered for downstream analysis.

The next step was to infer a causal relationship between the DE miRNAs and T2D, by taking into account SNPs that are related to glycaemic control, insulin secretion, and T2D from various GWAS [326-328]. If genetic variants were associated with the expression of dysregulated miRNAs under hyperglycaemic conditions and these variants were also linked to T2D-related traits, then multiple pieces of evidence would support the implication of these miRNAs in T2D pathogenesis. Indeed miR-7-5p, miR-126-3p, and miR-1236 demonstrated suggestive signals of association with T2D, miR-1275 and 130b-5p suggestive signals of association with traits related to insulin secretion and miR-194-5p was associated with both T2D and insulin secretion traits. Some of these miRNAs have been linked to diabetes before. For example, miR-126-3p was shown to be a confident prognostic biomarker for diabetes mellitus [329] and protective against the vascular complications of diabetes [330]. Interestingly, miR-1236, which has been described as a prognostic biomarker of latent autoimmune diabetes in adults (LADA) [331], was associated with 15 distinct variants related to "insulin-dependent diabetes" [327]. Moreover, the function of miR-130b was related to insulin secretion through the regulation of intracellular ATP levels in β cells [332]. Last but not least, miR-194 has been suggested as a circulating biomarker of diabetes mellitus incidence [333] and has been associated with the regulation of glucose metabolism in skeletal muscle [334]. Further validation of the functional roles of these miRNAs in human islets and β-cells could be necessary in order to better comprehend T2D aetiology.

Key findings

- The miRNA profile of human islets is altered in donors with poor glycaemic control
- Differentially expressed (DE) miRNAs can potentially target many T2D-related genes in the human islets
- mRNA targets of DE miRNAs are clustered in islet-specific co-expression networks that control islet function
- eQTLs associated with miRNAs coincide with genetic variants related to T2D and insulin secretion traits
- Analysis of dysregulated human islet miRNA-mRNA networks could elucidate underlying causes of T2D development

Paper IV

Global sequencing-based miRNA profiling of human pancreatic islets

Small RNA sequencing was performed in order to characterize the miRNA profile of human islets from 51 non-diabetic controls (ND) and 9 individuals with T2D. Overall, 715 miRNAs were found to be expressed in the islets, almost double the amount of the miRNAs identified by the microarray-based study in Paper III.

i) Abundant miRNAs

Among the most highly expressed miRNAs, several miRNAs such as members of the let-7 family, miR-375-3p, miR-200c-3p and miR-30d-5p have been replicated in several studies comparing human ND and T2D islets using microarray [335, 336] or small RNA-sequencing [199, 337] approaches. Moreover, similarly to the microarray-based study in Paper III, there were no major differences between the order of the top abundant miRNAs in islets from ND and T2D donors. Specifically, miR-30d-5p and miR-103a-3p were among the top 15 most abundant miRNAs in ND, but not T2D, donors which, conversely, showed high expression of miR-141-3p and miR-200a-3p.

ii) Endocrine and exocrine miRNAs

Uncovering the place of action of miRNAs can lead to better comprehension of their exact modes of action in the pancreas. By comparing the islet miRNA profile with the previously characterized whole pancreas miRNA profile in miRNATissueAtlas2 (n=6 donors) [338], miRNAs enriched in the endocrine and exocrine parts of the pancreas were identified. The whole pancreas profile was considered mainly of exocrine origin since it constitutes 98%–99% of the total pancreas mass [66]. The miRNA expression pattern between the two sets was significantly correlated as demonstrated by a regression model ($r^2 = 0.43$, $p<2.2x10^{-16}$). Focusing on the most highly expressed miRNAs, the analysis revealed several commonly abundant miRNAs in the islet and whole pancreas samples, e.g., miR-375-3p and members of the let-7 family, as previously demonstrated in a previous study performing a similar comparison [200]. Highly expressed, islet-enriched miRNAs included miR-16-5p, miR-7-5p, miR-30d-5p, miR-103a-3p, let-7e-5p, miR-125b-5p, miR-125a-5p. Notably, some of these miRNAs, including miR-16, miR-7, miR-30d, and miR-125b were found to be essential for β-cell function and development [192, 193, 339, 340].

iii) A-to-I modifications on miRNAs

Furthermore, miRNAs were scanned for A-to-I modifications, an additional epigenetic layer of miRNA biogenesis and function control in diverse tissues and organisms [341]. Overall, 34 miRNAs encompassed A-to-I modifications, with 20

of them being distributed uniformly across all donors and 14 of them being exclusive to either the ND or the T2D donors. Of interest, modifications in five of these miRNAs (miR-376c, miR-379, miR-381, miR-411, miR-497) were associated with ageing in a previous study describing six ageing-related miRNAs in total [342]. This can be explained by the increased age of the donors included in this study (median age: 65). The role of the detected modifications on the miR-381 and miR-497 sequences has been investigated in the brain, where the authors demonstrated that the modified miRNAs can affect brain development and function [343, 344]. Moreover, modifications on miR-27a-5p and miR-200b-3p have been linked to hypoxia [345]. It is worth mentioning that three different A-to-I modifications were identified on miR-335 only in the islets of T2D donors, a miRNA that is a modulator of insulin secretion [196].

miRNA expression is correlated with HbA1c, BMI and sex

To reveal associations of miRNA expression with specific donor characteristics, correlations of islet miRNA expression with HbA1c, BMI and sex were performed after accounting for batch effects and islet purity. The results showed that the expression of 113, 55, and 5 miRNAs was significantly correlated with HbA1c, BMI, and sex, respectively. Notably, around 30% of the HbA1c-related miRNAs were also DE in T2D, potentially preceding the onset of the disease. Although most of these miRNAs have no known function in islets, miR-21 was found to be regulated by glucose [346] and miR-30d was described as a mediator of insulin transcription in response to glucose [340]. Among the five sex-enriched miRNAs, four belong to the miR-181 family. Recently, a study demonstrated that elevated levels of miR-181a were only detectable in the plasma of older male individuals, and not females, indicating expression differentiation of this miRNA in a sex-specific manner [347]. Moreover, miR-122, which was associated with BMI both in the current and a recent study [337], was identified in higher levels in the serum of obese individuals and was shown to be implicated in T2D development [348, 349].

Differentially expressed miRNAs in T2D

By using a generalized linear model, islet miRNA expression was compared between T2D and ND donors. The analysis showed 70 miRNAs to be DE ($q < 0.05$) after correcting for batch effects, islet purity and donor sex, of which 34 displayed higher expression and 36 lower expression in T2D donors. Of these, nine and seven miRNAs, respectively, have been investigated in relation to β-cell function and T2D in the last ten years [209], supporting the validity of the analysis and the potential importance of the novel findings. Examples of downregulated miRNAs include miR-183-3p and miR-185-5p, knockdown of which leads to inhibition of insulin transcription [198], miR-299-5p, whose reduced expression causes increased β-cell

apoptosis and viability [350], as well as miR-132 and miR-212, both of which improve GSIS upon overexpression [351]. Regarding upregulated miRNAs, overexpression of miR-200 and miR-130a reduces GSIS [332, 352], increased expression of miR-21 causes both decreased GSIS and loss of β-cell identity [353], while overexpression of miR-199a results in reduced insulin content and increased islet apoptosis [314]. Nevertheless, the majority of these functional validation assays were performed in rat/mouse islets and cell lines, thus the consequences of miRNA action may differ in human islets. It is worth mentioning that DE miRNAs do not coincide with highly abundant miRNAs, supporting a clear separation between miRNA expression levels and regulation [191]. Furthermore, miRNA-disease enrichment analysis using public data from two databases revealed a strong association of only the upregulated miRNAs with T2D, which in most cases have been reported as T2D circulatory biomarkers [354, 355].

To examine the reproducibility of the results of the current analysis, differentially regulated miRNAs in T2D human islets were compared with those reported in four previous studies with similar experimental design [198, 199, 337, 356]. The studies showed high heterogeneity probably due to differences in sample sizes, islet isolation and culture protocols, large-scale miRNA identification technologies and bioinformatics workflows. Nonetheless, one upregulated miRNA, miR-187-3p, was common among all studies and two upregulated miRNAs, miR-30a-5p and miR-9-5p were shared between the current and two other studies [198, 199].

miRNA-mRNA targets of dysregulated miRNAs in T2D human islets

To identify miRNA targets, correlations between miRNA and mRNA expression levels available for a subset of the 61 donors subjected to miRNA-seq (n=24) were performed. Only negatively correlated miRNA-mRNA pairs were considered (nominal $p < 0.05$) since miRNAs mainly act by repressing the expression of their targets. To provide further support to the analysis, only miRNA-mRNA targets that were shown to be either validated or predicted in public databases were retained [316, 317, 357, 358]. In that way, the 70 DE miRNAs were associated with 4951 mRNA targets. The upregulated miRNAs displayed a significantly higher number of targets (3159 genes) compared to the downregulated miRNAs (1800 genes) with minimum overlap between them, suggesting distinct regulatory roles and involvement in diverse cellular processes. Notably, genes with consistently high expression, such as those transcribing the key endocrine hormones insulin, glucagon, and somatostatin, or the gene *GAPDH,* which is one of the most commonly used housekeeping genes*,* are subjected to no or limited direct miRNA control. The miRNA with the highest number of mRNA targets was miR-101-3p, which was associated with 766 targets. On the other hand, the mRNA targeted by the highest number of miRNAs belongs to the *CHD9* gene, a chromatin remodeler that is involved in cell cycle regulation [359]. Interestingly, the key miRNA

biogenesis enzyme *DICER1* was also among the most targeted genes by miRNAs, supporting the existence of an intricate miRNA regulatory network modulated by feedback loops. Furthermore, by integrating a comprehensive list of DE genes in T2D from multiple sources [149-155, 360], ≈25% of miRNA targets, on average, were found to be related to T2D.

A co-expression miRNA-mRNA network is associated with insulin secretion

In order to connect miRNAs with specific molecular pathways, their mRNA targets were assigned to co-expression clusters based on a weighted correlation network analysis (WGCNA). The analysis resulted in 12 mRNA clusters, which were functionally annotated with Gene Ontology term enrichment analysis. Interestingly, only Cluster 6 displayed enrichment for insulin secretion-related terms, namely "insulin secretion", "vesicle-mediated transport", "synapse organization", and "regulation of membrane potential". These pathways were also among the top 5 enriched terms in this cluster regarding their statistical significance (FDR < 0.01). Cluster 6 included 238 genes with 67 of them being dysregulated in T2D according to the comprehensive list used in the previous section. Examples of Cluster 6 genes associated with insulin secretion are *CLOCK* [361], *RFX3* [362], and *KCNB*1 [363]. Strikingly, the mRNAs in Cluster 6 were targeted exclusively by the 34 upregulated miRNAs, several of which have been reported as insulin secretion regulators, e.g., miR-29, miR-9, miR-141, miR-30d, miR-200c, miR-130a, and miR-497 [332, 352, 364]. This again highlights the potentially crucial role of the upregulated miRNAs in islet function and T2D pathogenesis.

Association of dysregulated miRNAs with insulin secretion

In order to establish associations of miRNAs with insulin secretion, miRNA expression was correlated with first- and second-phase insulin secretion. For that purpose, insulin release curves of islets from ND and T2D donors were generated in response to dynamic (low; 1.76 mmol/L, high; 20 mmol/L) glucose perfusion. As expected, both first- and second-phase insulin release were decreased in T2D islets. Correlations between miRNA expression and AUC values extracted from the first- and second-phase insulin secretion curves were performed using two methods: 1) a likelihood ratio test and 2) a linear regression analysis with robust standard errors. Significant correlations were considered those indicated by both methods (nominal $p < 0.05$). Overall, 41 miRNAs were correlated with first-phase secretion and 53 with second-phase secretion. Of interest, out of the 16 miRNAs associated with both phases, 8 displayed differential regulation in T2D (5 up- and 3 downregulated). The five upregulated miRNAs, including miR-141-3p, miR-101-3p, miR-29c-3p, miR-9-5p and miR-153-3p, have well-described roles in the regulation of insulin

secretion [364-366], indicating that dysfunction of the insulin secretory mechanism in T2D may be the result of downregulation of gene targets that would normally support and promote insulin production and secretion.

Three of these miRNAs were studied further; miR-9-5p which had a known association with insulin secretion [367, 368], miR-187-3p, which showed universal dysregulation in T2D across all studies and miR-101-3p, which had the highest number of mRNA targets. Subsequently, the mRNA targets of these miRNAs were collected and those that were significantly correlated with first- and/or second-phase insulin secretion were considered. In total 84, 33, and 2 mRNA targets were associated with miR-101, miR-9 and miR-187, respectively. Almost all of these targets are expressed in human β-cells based on public single-cell transcriptome data (GEO: GSE153855). Of interest, nine mRNA targets were shared between miR-101 and miR-9, including *STARD13,* which affects GSIS through regulation of the cytoskeletal organization [369], *PDE7B,* which is differentially methylated and expressed in T2D and is shown to modulate GSIS [360, 370], and *ANK3,* which impacts GSIS via direct interaction with several voltage-dependent channels and vesicle trafficking proteins important for insulin secretion, e.g., *SYT13* [288, 371].

Key findings

- Small RNA sequencing in human islets from T2D and ND donors uncovered 70 DE miRNAs
- Donor BMI, HbA1c and sex are linked to changes in global miRNA expression
- Upregulated miRNAs form a miRNA-mRNA network that could contribute to insulin secretion defects in T2D
- Several dysregulated miRNAs and their target mRNAs are associated with first- and second-phase insulin secretion
- Increased expression of miR-101-3p and miR-9-5p could contribute to T2D pathogenesis by silencing target genes that promote insulin secretion

Concluding Remarks

This thesis displays different cases in which various types of biological data were combined and harmonized in order to further explore and uncover the mechanisms behind the impairment of β-cell function and insulin secretion in glucocorticoid-induced/type 2 diabetes. From the data presented, the following conclusions can be made:

In Paper I, the genes and the molecular mechanisms behind β-cell dysfunction caused by glucocorticoids were disclosed by integrating large-scale transcriptomic, genetic and epigenetic data. The transcription factor *ZBTB16* was discovered as a novel direct glucocorticoid target in β-cells and functional assays demonstrated its protective role against glucocorticoid-induced insulin secretion impairment via mitochondrial action.

In Paper II, the comparison of the transcriptome profiles of a large cohort of ND and T2D human islets revealed known and novel dysregulated genes in T2D. Multiple signals of association with high-throughput transcriptomic, genetic and epigenetic markers, as well as functional validation assays, coupled these changes to β-cell dysfunction and T2D. For the first time, it was shown that overexpression of the transcription factor PAX5 in T2D triggers extensive transcriptomic changes that can hinder mitochondrial function and insulin secretion.

In Paper III, human islet microarray-based miRNA profiling revealed changes in the miRNome of donors with altered glycaemic status. DE miRNAs were associated with several genes associated with T2D after analysing publicly available data on miRNA-mRNA targets and T2D-related genes. An mRNA co-expression network targeted by DE miRNAs revealed two islet-specific networks associated with islet function. Integration of public SNP data indicated eQTLs that are correlated with both dysregulated miRNA expression and T2D/insulin secretion traits, thus associating specific miRNAs with the genetic predisposition to β-cell dysfunction and T2D.

In Paper IV, changes in the miRNA profile of T2D islets were detected using small RNA-sequencing, hence using a more sensitive technology and also a larger cohort compared to Paper III. The mRNA targets of miRNAs were identified by making use of RNA-sequencing data of a subset of donors and were supported by publicly available data, increasing the robustness of the results. This led to the identification of a co-expression mRNA network associated with insulin secretion, which was targeted exclusively by upregulated miRNAs in T2D. Subsequent association of miRNAs and their targets with first- and/or second-phase insulin release provided miRNA candidates that could play a key role in insulin secretion deficiency during T2D onset.

Future Perspectives

T2D is a huge socioeconomic burden in most parts of the world due to its high and increasing prevalence. GC-induced diabetes also poses a serious threat since 2% of newly diagnosed diabetes is connected to the wide use of glucocorticoid medication. In both cases, defective insulin secretion from the pancreatic β-cells or diminished response to insulin from its target tissues leads to prolonged elevated blood glucose levels, which can cause severe health consequences. The findings of this thesis focus on deciphering the molecular mechanisms behind insulin secretion impairment in GC-induced/type 2 diabetes and suggest new therapeutic targets using a data-driven approach.

With the help of in-house and publicly available large-scale data, we identified direct GC gene targets in the β-cell and the molecular pathways associated with them. *ZBTB16* was the most confident direct target and functional validation demonstrated its strong induction upon GC treatment, as well as its protective effects on β-cell mitochondrial function and insulin secretion. Indisputably, further studies on *in vivo* systems are necessary to determine if this gene can be used to reverse the negative effects of GC treatment. Moreover, other direct GC targets reported in this thesis should be followed up in the same way to extract more meaningful conclusions about the overall GC impact on β-cells. It is also worth mentioning that the bioinformatics workflow suggested here, which allows the integration of big data to identify direct transcription factor gene targets, is a useful tool that can be utilized in different contexts and diseases.

The availability of a large in-house cohort of human islets from normal controls and T2D donors allowed the identification of dysregulated genes and miRNAs in T2D. While the big list of differentially expressed genes and miRNAs is by itself invaluable for a more complete comprehension of T2D aetiology, a series of further analyses that involved the integration of different types of high-throughput data highlighted the extensive impact of some genes and miRNAs on β-cell function, which could be among the main drivers of the development of the disease. A special focus was given to dissecting the relationship between miRNAs and their gene targets and trying to infer networks that could be affected in T2D. Once again, a series of *in vitro* and *in vivo* experiments are necessary to validate the importance of these findings. Since miRNAs can affect many pathways and genes at the same time, they hold great promise for future therapies. However, there are still concerns

regarding the specificity of their target cells, the efficiency of their delivery and uptake, and potential off-target effects.

During this thesis, a high degree of heterogeneity between studies investigating changes in control and T2D human islets was observed. Undoubtedly, a proportion of these differences can be attributed to different demographic and pathophysiological characteristics between individual donors, as well as innate cellular diversity, even between cells of the same type. However, there are also external factors responsible for these discrepancies such as differences in sample size and experimental protocols of islet handling, use of distinct high-throughput technologies, and diverse statistical and bioinformatics workflows. This leads to non-reproducible and, sometimes, contradictory results that hinder the discovery of new therapeutic targets. This is why there is an urgent need for universal and optimised protocols for processing and generation of study outputs. Fortunately, the promotion of "open science" and its benefits by the research society, including universities and funding bodies, is also a big step forward in this direction. Moreover, the problem of limited availability of human islets can be tackled if clinical centres and institutes all around the world combine their resources and increase the islet sample sizes, thus generating more robust studies.

This thesis focused on the dysfunction of β-cells. However, the regulation of glucose levels is also dependent both on the insulin sensitivity and action of other tissues, including the liver, muscle, and adipose tissue. In the future, attempts to compare data derived from all tissues involved in glucose homeostasis should be prioritized in order to discover similar patterns of molecular alterations in T2D. Furthermore, most studies that investigate transcriptomic changes in islets capture mere snapshots of gene expression. This is why such information should be complemented with spatial and temporal gene expression data, which would unravel how truly dynamic these molecular processes are.

The projects presented here, as well as an increasing number of recent studies, are currently trying to integrate additional layers of omics data in a harmonized manner to provide a more complete understanding of how cellular systems are modulated and, thus, which cellular pathways are affected in T2D. The generation of a high number of large-scale datasets, more sophisticated algorithms and increased computer power are going to support this endeavour.

T2D is manifested differently in every individual, potentially affecting multiple and diverse cellular mechanisms. To comprehend all aspects of the disease, a plethora of individuals with T2D need to be properly investigated and common disease patterns need to be identified. Only then we will be able to fill the gaps and gain a deeper insight into how the disease is progressing, how the disease can be successfully predicted and diagnosed or how to effectively treat the disease. This is the only path to personalised management of T2D. We need to see the forest for the trees.

Acknowledgements

I would like to thank all the people who have contributed to this thesis and have been part of my PhD journey.

First, I would like to thank my main supervisor, **Lena Eliasson**. Lena, I feel grateful for trusting me and giving me the chance to be a member of your group. During all these years, your guidance was outstanding and every time I needed advice you were there despite your busy schedule. Your work ethic is truly inspiring and you certainly know how to bring out the best in every member of the group. A good leader is one capable of keeping the team's morale high when it is necessary and you have certainly done that many times. Your support has been invaluable during work hours and off the clock.

I also feel grateful to my two co-supervisors, **Jonathan Esguerra** and **Rashmi Prasad**. Jonathan, you are admirable both as a scientist and as a person. Thank you for showing me your trust from the very beginning and for giving me a warm welcome to the group. You are very insightful and always seem to have an answer to every question. Also, your sense of humour and cordiality made it a joy to work with you. I believe both you and Lena were the ones that laid my research foundations and I will be forever grateful for that. Rashmi, I want to thank you for taking over in a challenging period and for our nice collaboration. You seem to master multi-tasking as nobody else and your high expertise and kind personality also made me better at what I am doing.

I am thankful to all the current and past members of the ICE and LER group. Thank you **Efraim Westholm**, I was lucky to be your colleague, your roommate and your friend. I am grateful for all our deep discussions, for honestly supporting me through failures and successes and for sharing with me your broad and abundant knowledge, especially regarding Swedish culture and golf. **Elaine Cowan**, I want to thank you for all our serious and fun conversations (in English and Swedish) and all the help you have given me inside and outside the lab throughout this time. You are a truly pleasant, compassionate and supportive person. **Anna Wendt**, I want to thank you for our nice collaboration and the knowledge you have shared with me always in your pleasant and calm way. **Jones Ofori**, thank you for bringing your optimistic attitude with you every day and for inspiring me with your work discipline. I was very lucky to learn a lot from you during my days in the lab and I appreciate all your tremendous help. A special thanks to the other two PIs of the LER group, **Enming**

Zhang and **Albert Salehi**, for the constructive discussions and the interesting projects I had the chance to be around or be a part of. I would also like to express my sincere gratitude to **Charlotte Ling** and **Karl Bacos** for giving me the chance to contribute to several of their projects and allowing me to include one of them in my thesis. Charlotte and Karl, your commitment and your high-quality work are truly motivating.

This journey would not be the same without the presence of more amazing and capable people I had the chance to cross paths and work with. My most sincere thanks to **Anna-Maria Veljanovska Ramsay**, to which I own a lot. Her willingness to always help, her genuine openness and her high competence are what drive the LER group forward and define its unique personality. I am also thankful to **Eugenia Cordero Concha**, the fellow pillar of the lab, for all her help, our interesting discussions and her support. Thank you **Mohammad Barghouth** for all your kind help and your guidance; I really enjoyed our everyday scientific and non-scientific discussions in the office. Thank you **Cheng Luan**, for all the nice collaborations, from which I learned a lot, and your help; your sense of humour made every fika and spex shine. I also want to thank my current roommate, **Alessio Pollastri**, as well as my old roommates, **Alexander Hamilton**, **Marie Sjögren**, **Anna Edlund**, and **Mototsugu Nagao** for creating such a special working environment every day in the office. Thank you, **Mohammad Tariq**, **Ruchi Jain**, **Rui Wu**, **Ye Yingying** and **Britt-Marie S Nilsson** for all the fun times during our group activities and our weekly fikas.

I would also like to thank all my friends in Sweden and Greece for their constant support and encouragement and for being there for me throughout my PhD studies.

To my biggest supporter, the one who has been there all along the way and makes me a better person every day in all aspects of my life. Thank you **Elena Mourati**! Your kindness, your understanding, and your constant encouragement are what guide me and motivate me to pursue my aspirations.

Eventually, my most sincere appreciation to my family, to which I owe everything to this day. I would like to thank my parents, **Christos** and **Marianna**, and my brother **Paris**! Thank you for always believing in my goals and for all your sacrifices along this long journey. Thank you for your constant encouragement, moral support and unconditional love. Ευχαριστώ για όλα!

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