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Identification of new disease mechanisms and treatments for type 2 diabetes based on genetic variants and gene expression networks

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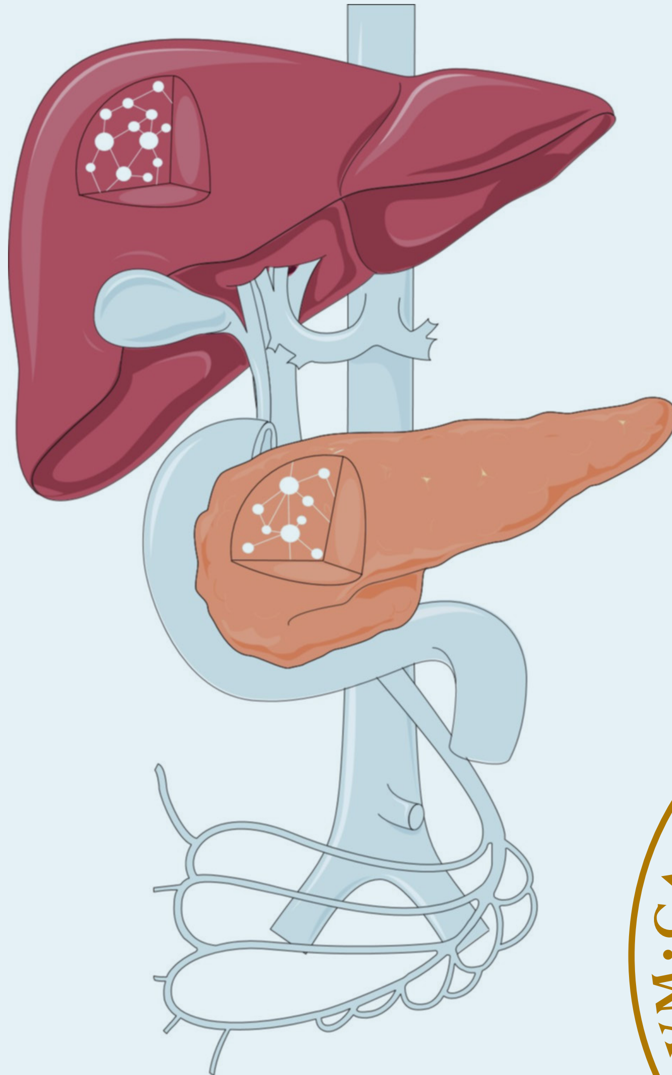

Identification of new disease mechanisms and treatments for type 2 diabetes based on genetic variants and gene expression networks

Annika Axelsson

DOCTORAL DISSERTATION
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Faculty opponent

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Abstract

Improved understanding of the disease mechanisms underlying type 2 diabetes (T2D) is needed, and so are new treatments.

A new T2D risk variant was recently identified in ADRA2A, which encodes the α2A-adrenergic receptor. The risk allele leads to receptor overexpression in β-cells that causes increased adrenergic signaling and impaired insulin secretion. We showed that the α2A-adrenergic receptor antagonist yohimbine normalized insulin secretion in risk allele carriers with T2D, whereas it was without effect in non-risk allele carriers. These findings suggest that individualized, genotype-based treatment for T2D is possible.

Next, in an attempt to identify new genes relevant for the pathogenesis of T2D and to identify new drugs for the treatment of T2D, we utilized microarray gene expression data to gain information about gene co-expression networks. Gene expression in human islets from T2D and non-diabetic donors, and gene expression in liver tissue from hyperglycemic and normoglycemic mice, was analyzed to find groups of co-expressed genes (modules) with disturbed expression in diabetes. "Disease signatures" derived from these modules were used to interrogate publically available microarray data sets. These data sets included gene expression profiles induced by a wide range of drugs and treatments. Data sets with an expression pattern similar to our islet disease signature gave clues to the underlying pathogenic process in β-cell failure, and data sets with a reverse expression pattern to our liver disease signature helped identify drug candidates for treatment of excessive hepatic glucose production.

The islet disease signature was associated with β-cell dedifferentiation and loss of a mature β-cell state. We identified the transcription factor *SOX5* as a regulator of the T2D-associated islet module. Overexpression of *SOX5* increased the expression of β-cell specific genes in human islets and improved secretory function in islets from donors with T2D.

The liver disease signature was used to rate compounds based on reverse expression compared with the disease signature. The rationale was that compounds with potential to reverse the disease signature might affect the pathophysiology. Sulforaphane, a sulfur-containing compound found naturally in e.g. broccoli, was identified as the top-rated compound. Sulforaphane reduced glucose production from hepatoma cells via a mechanism that involves reduced expression of gluconeogenic enzymes. Sulforaphane improved glucose tolerance in animal models of diabetes. Moreover, in a small clinical study, sulforaphane-rich broccoli sprout extract reduced fasting blood glucose and HbA1c levels in obese T2D patients with poor glycemic control.

Taken together, the data presented in this thesis demonstrate the opportunities of genotype-based treatment for T2D, and show the usefulness of gene network analysis to identify pathophysiological mechanisms and new potential therapies for T2D. By this approach, we have identified Sox5 as a new regulator of β-cell function, and sulforaphane as a liver-targeting therapy for T2D patients with poor glycemic control.

Key words: Type 2 diabetes, insulin, ADRA2A, genotype, gene network analysis, SOX5, drug repositioning, sulforaphane, clinical study

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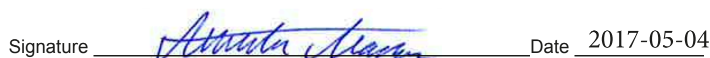
Signature  Date 2017-05-04

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Paper I

Genotype-based treatment of type 2 diabetes with an α2A-adrenergic receptor antagonist.

Tang Y*, **Axelsson AS***, Spégel P*, Andersson LE, Mulder H, Groop LC, Renström E, Rosengren AH

Science Translational Medicine. 2014 Oct 8;6(257):257ra139

*Equal contribution

Paper II

Sox5 regulates β-cell phenotype and is reduced in type 2 diabetes

Axelsson AS, Mahdi T, Nenonen HA, Singh T, Hänzelmann S, Wendt A, Bagge A, Reinbothe TM, Millstein J, Yang X, Zhang B, Gusmao EG, Shu L, Szabat M, Tang Y, Wang J, Salö S, Eliasson L, Artner I, Fex M, Johnson JD, Wollheim CB, Derry JMJ, Mecham B, Spégel P, Mulder H, Costa IG, Zhang E, Rosengren AH

Nature Communications, In press

Paper III

Sulforaphane reduces hepatic glucose production and improves glucose control in patients with type 2 diabetes

Annika S Axelsson, Emily Tubbs, Brig Mecham, Shaji Chacko, Hannah A Nenonen, Yunzhao Tang, Jed W Fahey, Jonathan MJ Derry, Claes B Wollheim, Nils Wierup, Morey W Haymond, Stephen H Friend, Hindrik Mulder, Anders H Rosengren

Science Translational Medicine, In press

Publications not included in the thesis

Thrombin stimulates insulin secretion via protease-activated receptor-3

Hänzelmann S, Wang J, Güney E, Tang Y, Zhang E, **Axelsson AS**, Nenonen H, Salehi AS, Wollheim CB, Zetterberg E, Berntorp E, Costa IG, Castelo R, Rosengren AH

Islets. 2015;7(4):e1118195

Optogenetic control of insulin secretion in intact pancreatic islets with β-cell-specific expression of Channelrhodopsin-2

Reinbothe TM, Safi F, **Axelsson AS**, Mollet IG, Rosengren AH

Islets. 2014;6(1):e28095

Eukaryotic translation initiation factor 3 subunit E controls intracellular calcium homeostasis by regulation of Cav1.2 surface expression

Buda P1, Reinbothe T, Nagaraj V, Mahdi T, Luan C, Tang Y, **Axelsson AS**, Li D, Rosengren AH, Renström E, Zhang E

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Secreted frizzled-related protein 4 reduces insulin secretion and is overexpressed in type 2 diabetes

Mahdi T, Hänzelmann S, Salehi A, Muhammed SJ, Reinbothe TM, Tang Y, **Axelsson AS**, Zhou Y, Jing X, Almgren P, Krus U, Taneera J, Blom AM, Lyssenko V, Esguerra JL, Hansson O, Eliasson L, Derry J, Zhang E, Wollheim CB, Groop L, Renström E, Rosengren AH

Cell Metabolism. 2012 Nov 7;16(5):625-33

Reduced insulin exocytosis in human pancreatic β-cells with gene variants linked to type 2 diabetes

Rosengren AH, Braun M, Mahdi T, Andersson SA, Travers ME, Shigeto M, Zhang E, Almgren P, Ladenvall C, **Axelsson AS**, Edlund A, Pedersen MG, Jonsson A, Ramracheya R, Tang Y, Walker JN, Barrett A, Johnson PR, Lyssenko V, McCarthy MI, Groop L, Salehi A, Gloyn AL, Renström E, Rorsman P, Eliasson L

Diabetes. 2012 Jul;61(7):1726-33

Approved patent:

Axelsson AS, Rosengren AH. Approved US patent, number 9,597,307 and EU patent, number 2919775: Sulforaphane for treating or reducing insulin resistance in the liver.

Abbreviations

α_{2A}AR	α2A-adrenergic receptor
ADA	American Diabetes Association
α-KIC	α-ketoisocaproic acid
AMPK	AMP-activated protein kinase
AUC	Area under the curve
BSE	Broccoli sprout extract
cAMP	Cyclic AMP (cyclic adenosine monophosphate)
$Ca_V1.2$	Calcium channel, voltage-dependent 1.2
$Ca_V1.3$	Calcium channel, voltage-dependent 1.3
CIR	Corrected insulin response
DPP-4	Dipeptidyl peptidase 4
EGP	Endogenous glucose production
FLI	Fatty liver index
FDA	U.S. Food and Drug Administration
GIR	Glucose infusion rate
GIP	Gastric inhibitory polypeptide
GAD	Glutamic acid decarboxylase
GLP-1	Glucagon-like peptide-1
GTT	Glucose tolerance test
HDAC	Histone deacetylase
HFD	High-fat diet
HFrD	High-fructose diet
HGP	Hepatic glucose production

HOMA-IR	Homeostatic model assessment of insulin resistance
HOMA-B	Homeostatic model assessment of β-cell function
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
Ins30	Insulin secretion at 30 min during an oral glucose tolerance test
i.p.	Intraperitoneal(-ly)
IPGTT	Intraperitoneal glucose tolerance test
IPITT	Intraperitoneal insulin tolerance test
ISI	Insulin sensitivity index
KD	Knockdown
NGT	Normal glucose tolerance
OCR	Oxygen consumption rate
OGTT	Oral glucose tolerance test
PC	Pyruvate carboxylase
PEPCK	Phosphoenolpyruvate carboxykinase
p.o.	Per os (oral)
R_a	Rate of appearance
R_d	Rate of disappearance
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SFN	Sulforaphane
SGLT2	Sodium-glucose co-transporter type 2
siRNA	Small interfering RNA or silencing RNA
SNP	Single nucleotide polymorphism
T2D	Type 2 diabetes
UKPDS	United Kingdom Prospective Diabetes Study
VPA	Valproic acid

Populärvetenskaplig sammanfattning

För att bättre kunna behandla typ 2-diabetes (T2D) behövs en större förståelse för vad som orsakar sjukdomen samt fler behandlingsmöjligheter.

Förmågan att frisätta insulin är nedsatt hos personer med T2D. Insulin behövs för att hålla blodsockret på en hälsosam nivå. Den nedsatta förmågan till insulinfrisättning hos personer med T2D beror delvis på genvariationer. Det visade sig nyligen att personer med en viss variant av genen *ADRA2A* har större risk att drabbas av diabetes. Personer med riskvarianten av *ADRA2A* har fler receptorer för stresshormoner i de insulinfrisättande betacellerna än vad personer utan riskvarianten har. Det leder till försämrad insulinfrisättning.

Vi ville i arbete 1 undersöka om det är möjligt att förbättra insulinfrisättningen hos patienter med T2D som bär på riskvarianten genom att blockera receptorn för stresshormoner. Vi rekryterade därför 50 patienter med T2D, varav ungefär hälften bar på riskvarianten. Patienterna fick först inta en dos av ett läkemedel, yohimbin, som blockerar receptorn för stresshormoner. De fick sedan genomgå en sockerbelastning där det är möjligt att se hur mycket insulin som utsöndras till blodet. När patienter som bar på riskvarianten fick en tablett yohimbin förbättrades deras insulinfrisättning och blev lika bra som hos patienterna utan riskvarianten. Hos patienterna utan riskvarianten hade yohimbin däremot ingen effekt. Effekten av yohimbin berodde alltså på patientens genuppsättning, och var specifikt gynnsam för patienterna som bar på riskvarianten. Yohimbin har kort verkningstid och även andra bieffekter som gör det olämpligt som läkemedel. Just yohimbin kan därför inte användas kliniskt för att behandla patienter som bär på riskvarianten av *ADRA2A*. Dock pekar vår studie på möjligheten att i framtiden kunna skapa individanpassade behandlingar baserade på patientens genuppsättning.

I ett nästa steg ville vi testa en ny strategi för att både identifiera nya gener med betydelse för T2D och hitta nya läkemedel för T2D. Strategin kallas *gennätverksanalys* och bygger på att gener samverkar i större grupper. Gener är bitar av vårt DNA som fungerar som ritningar för proteiner. Proteiner utför viktiga uppgifter i varje cell, och mängden av olika proteiner bestämmer hur cellen fungerar. Vid T2D innehåller de sviktande betacellerna mer av vissa proteiner och mindre av andra proteiner jämfört med friska betaceller. När cellen ska tillverka ett protein med en gen som ritning så gör den först en avskrift av genen som fungerar som mall. Denna mall kallas mRNA. Ju mer mRNA en cell innehåller, desto mer av

proteinet produceras. Hur mycket mRNA av varje sort som tillverkas beror på signaler utanför cellen – till exempel höga blodsockervärden. Det är en komplex reglering. Ett protein från en viss gen som påverkas av höga blodsockervärden kan exempelvis påverka hur mycket mRNA som produceras från flera andra gener. Nivåerna av mRNA från en del gener tenderar att vara kopplade. Det innebär att när nivån av ett mRNA ökar, så ökar även nivån av de kopplade generna. Det har visat sig att gener som på det här sättet är kopplade ofta har liknande uppgifter i cellen. Vårt mål med att använda gennätverksanalys var att hitta grupper av gener som samspelar med varandra, och som tillsammans kan tänkas påverka sjukdomsförloppet vid T2D.

I arbete 2 använde vi gennätverksanalys för att hitta nya gener med betydelse för T2D i de insulinfrisättande betacellerna. Vi identifierade först grupper av gener i betacellerna som är kopplade med varandra. Nivåerna av mRNA från gener i en av dessa grupper var lägre hos personer med T2D än hos friska personer. Vi såg även att ju lägre mRNA-nivåer personerna hade, desto sämre var förmågan hos deras betaceller att frisätta insulin, vilket tydde på att vi hittat en grupp av gener som påverkar sjukdomsförloppet vid T2D.

Genom att jämföra mRNA-nivåerna i betaceller från personer med T2D med tidigare publicerade data upptäckte vi att betacellerna hos dessa personer påminde mycket om omogna betaceller. Det skulle kunna betyda att betaceller delvis går tillbaka i utvecklingen när de utsätts för den stress som diabetes innebär. Vi identifierade sedan genom en rad försök *SOX5* som en gen med förmåga att positivt påverka mRNA-nivåerna i gruppen av gener kopplad till T2D. *SOX5* är känd för sin roll vid broskbildning, men har tidigare inte studerats i samband med T2D. Vi kunde visa att en ökning av mRNA-nivåerna av *SOX5* ökade nivåerna av de mRNA som är kännetecknande för mogna betaceller. Betacellerna blev alltså mer mogna. Ökade mRNA-nivåer av *SOX5* motverkade alltså omognaden av betacellerna och förbättrade även insulinsfrisättningen i betaceller från personer med T2D.

Personer med T2D har inte bara försämrad insulinfrisättning. Ett annat vanligt problem är att levern producerar och släpper ut för mycket socker till blodet. I arbete 3 använde vi därför gennätverksanalys för att försöka hitta nya läkemedel som skulle kunna förhindra överdriven sockerproduktion från levern. På så sätt identifierade vi först 50 gener som sannolikt är särskilt drivande i att orsaka överdriven sockerproduktion. Nivåerna av mRNA för dessa gener kan beskrivas som en *sjukdomssignatur*, ungefär som ett fingeravtryck av sjukdomsprocessen i levern. Vår hypotes var att substanser som ändrar mRNA-nivåerna från dessa gener i *motsatt* riktning jämfört med sjukdomssignaturen skulle kunna motverka sjukdomen och alltså utgöra nya möjliga läkemedel. Vi jämförde därför sjukdomssignaturen med publicerade data om hur olika substanser påverkar mRNA-nivåer. Totalt använde vi data för 3852 olika substanser. Av alla dessa substanser

fann vi att behandling med ämnet sulforafan bäst motverkade sjukdomssignaturen. Sulforafan är ett ämne som förekommer naturligt i bland annat broccoli. Vi testade sulforafan på odlade leverceller, och såg att det faktiskt minskade deras sockerproduktion. Sulforafan förbättrade även blodsockervärdena i råttor och möss med diabetes. Vi testade slutligen sulforafan på patienter med T2D i en liten klinisk studie. Eftersom sulforafan finns i hög mängd i broccoligroddar fick patienterna inta sulforafan i form av ett pulver gjort på broccoligroddar en gång dagligen under 12 veckor. Vi såg att sulforafan minskade blodsockernivåerna och långtidsblodsocker (HbA1c) i kraftigt överviktiga patienter (BMI > 30) med dålig blodsockerkontroll.

Sammantaget visar resultaten i denna avhandling på möjligheterna till individanpassad behandling av T2D, och demonstrerar nyttan med att använda gennätverksanalys för att identifiera viktiga gener och potentiella nya läkemedel för T2D. Vi har identifierat *SOX5* som en gen som påverkar betacellens funktion, och identifierat ämnet sulforafan som en potentiell ny behandling för patienter med T2D som har dålig blodsockerkontroll.Introduction

Introduction

Type 2 diabetes

Type 2 diabetes is an insidious disease. The high blood glucose levels that are characteristic of the disease are toxic in the long term but often without symptoms in the early stages. An affected person can live with the disease for years without knowing of it, while important pathophysiological and possibly irreversible changes take place in the body. Almost half of all persons with diabetes worldwide are undiagnosed and do therefore not receive treatment that could reduce the risk for complications and premature death (Beagley et al., 2014). Under-diagnosis of diabetes occurs in all parts of the world and in all socioeconomic groups. Even in Europe in high-income groups, the number of undiagnosed persons with diabetes is estimated to be as high as 37% (Beagley et al., 2014).

Although proper treatment can reduce diabetes-associated morbidity and mortality (UKPDS, 1998a; UKPDS, 1998b), individuals with diagnosed T2D receiving treatment are still more likely to develop cardiovascular disease and have higher overall mortality compared to non-diabetic individuals (Tancredi et al., 2015). The average life expectancy for a person with diabetes at age 55 is 5-6 years less compared to a person without diabetes (Loukine et al., 2012). That amounts to millions of life years lost due to T2D worldwide. Around 415 million people had diabetes 2015, and 642 million people are estimated to have diabetes 2040 according to the International Diabetes Federation (IDF, 2015). More measures are needed to prevent the increase in type 2 diabetes, and to improve treatment for those who are affected in order to avoid complications and premature death.

This thesis aims at identifying new mechanisms of disease and finding new treatments for T2D to help address the issues above. T2D is a complex disease that involves multiple organs, including the pancreatic islets, the liver, adipose tissue, muscle, gut, and brain (Defronzo, 2009). The focus of this thesis is on the role of β-cells and the liver in T2D. Their role in regulating plasma glucose levels will be presented below, followed by a description of the pathogenic series of events leading to T2D, possible causes, current treatments for T2D, and strategies to find new treatments options.

Role of the β-cell in regulating plasma glucose

β-cells reside in the islets of Langerhans

The high glucose levels in T2D result from a relative lack of insulin, meaning that insulin levels are not sufficient to mediate glucose removal from the blood. Insulin is secreted from endocrine mini-organs in the pancreas called islets of Langerhans, or pancreatic islets. A healthy human pancreas contains 3.2-14.8 million islets (Ionescu-Tirgoviste et al., 2015; Saito et al., 1978). The islets are spherical clusters of around 1500 cells interspersed in the exocrine pancreas (Pisania et al., 2010). Each islet contains a mixture of endocrine cell types: α-cells secreting glucagon, β-cells secreting insulin and amylin, δ-cells secreting somatostatin, PP-cells secreting pancreatic polypeptide and ε-cells secreting ghrelin. Human islets contain approximately 50-60% β-cells, 30-40% α-cells, 10% δ-cells and a small number of PP- and ε-cells (Brissova et al., 2005; Cabrera et al., 2006).

The islet composition and degree of variation in composition differ between species, which may be of importance when considering results from rodent models in T2D research. Human islets contain a lower proportion of β-cells compared to mouse islets, and show a larger variation in cell type composition (Brissova et al., 2005). Mouse islets have β-cells at the core, with α- and δ-cells localized at the periphery (Brissova et al., 2006). It has been suggested that endocrine cells are distributed throughout human islets without specific clustering (Brissova et al., 2005; Cabrera et al., 2006), but this appears to be the case only for cultured human islet. In human islets with preserved cell architecture, islets are organized as folded trilaminar sheets, where each sheet is made up of a central bulk of β-cells lined at both sides with α-cells and other islet cells (Bosco et al., 2010; Weir et al., 2009) (Figure 1). Capillaries are found in the folds of the trilaminar sheet structure, juxtaposed to the α-cells and never crossing though the β-cell core (Bosco et al., 2010). Islets are highly vascularized in order to respond rapidly to changes in plasma glucose levels (Brissova et al., 2006).

In addition to endocrine cells and capillary-forming endothelial cells, the islets also contain neurons, smooth skeletal muscle cells, and fibroblasts (Beattie et al., 1991; Rodriguez-Diaz et al., 2011). A thin capsule of connective tissue fibers surrounds each islet (Ohtani, 1987).

Insulin processing in the β-cells

Insulin is the most abundant transcript in β-cells, amounting to 10-15% of the total mRNA (Goodge and Hutton, 2000). During translation of insulin mRNA, the

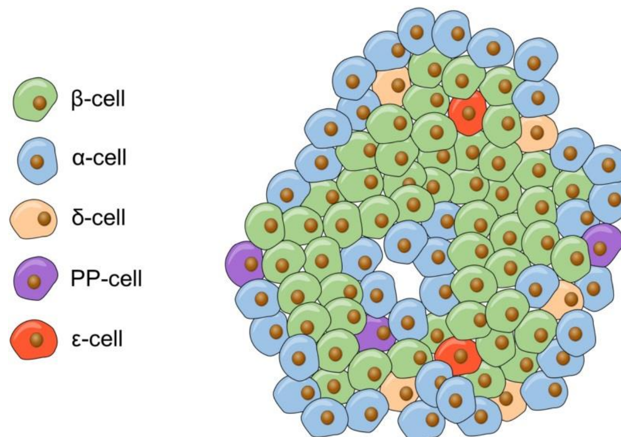

Figure 1. Schematic picture of a human islet.

emergence of a signaling peptide directs the protein to the rough endoplasmic reticulum (ER). The signaling peptide of preproinsulin (110 amino acids) is cleaved off, resulting in proinsulin (81 amino acids). In the ER, three disulfide bonds are formed within the proinsulin protein: two bonds between what will be the A and B chain, and one bond within the A chain, stabilizing the protein structure (Chang et al., 2003).

Proinsulin is directed to the trans-Golgi network for assembly into secretory granules (vesicles). The newly formed immature granules also contain endo- and exopeptidases required for insulin processing. Prohormone convertase 1/3 and 2 (PC1/3 and PC2) cleave off a middle part of the protein, the C-peptide (31 amino acids). In addition, carboxypeptidase E (CPE) removes C-terminal basic residues (Davidson and Hutton, 1987) (Figure 2).

In order to become release-competent, secretory granules must go through an ATP-dependent process termed priming. This involves acidification of the granules via an ATP-dependent proton pump. The influx of positively charged protons is coupled to influx of negatively charged chloride ions through ClC-3 chloride channels, which prevents the build-up of a large electrical gradient over the granule membrane (Barg et al., 2001a).

In the granules, insulin is stored in a crystalline form bound to zinc, as a 2-zinc insulin hexamer. The granules are transported to the plasma membrane along a microtubule network. In order to release their content, the granules must first tether to the plasma membrane in a process termed docking. To enable docking, the dense actin web situated just below the plasma membrane needs to be rearranged (Eliasson et al., 2008).

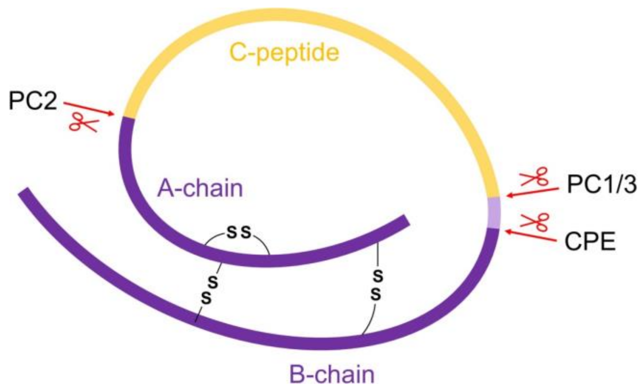

Figure 2. Insulin processing by the prohormone convertases (PC1/3 and PC2) and carboxypeptidase E (CPE) in the secretory granules.

Mechanism for insulin release from β-cells in response to glucose

Glucose enters the β-cell through facilitated diffusion via GLUT1 (glucose transporter 1, encoded by *SLC2A1*) and GLUT2 (encoded by *SLC2A2*). GLUT1 is a high affinity-low capacity glucose transporter responsible for basal glucose uptake in most tissues, whereas GLUT2 is a low affinity-high capacity glucose transporter responsible for "glucose sensing". The β-cell secrete insulin in response to glucose in a process called stimulus-secretion coupling. The cornerstone of stimulus-secretion coupling is the *triggering pathway* (Figure 3) summarized below:

1) Glucose metabolism leading to ATP production via
   (a) Glycolysis (in the cytosol)
   (b) Citric acid cycle (in the mitochondrion)
   (c) Oxidative phosphorylation (in the mitochondrion)
2) Plasma membrane depolarization through closure of K_{ATP} channels
3) Calcium influx through voltage-gated calcium channels
4) Calcium-dependent exocytosis

Glucose metabolism leading to ATP production

In the β-cell, glucose is phosphorylated by the enzyme glucokinase, and is thereby prevented from leaving the cell. The product, glucose-6-phosphate, enters glycolysis and is eventually converted to pyruvate. Pyruvate is actively transported into the mitochondrion via pyruvate translocase and is converted to acetyl coenzyme A (acetyl-CoA) by the pyruvate dehydrogenase complex. Acetyl-CoA enters the

citric acid cycle through conjugation with oxaloacetate to form citric acid. Pyruvate can also be carboxylated by pyruvate carboxylase (PC) to generate oxaloacetate, and the fact that pyruvate enters both of these pathways in similar proportions suggest that they are both important for β-cell function (Schuit et al., 1997).

The main products of the citric acid cycle, the reduced co-factors NADH and $FADH_2$, are electron donors for the electron transport chain. Complexes I-V of the electron transport chain are localized to the inner mitochondrial membrane, and electrons move through the complexes via a series of redox reactions. The redox reactions are coupled to the transfer of protons into the mitochondrial intermembrane space, creating a proton gradient over the inner mitochondrial membrane. The proton gradient is used to drive production of ATP from ADP and inorganic phosphate via the enzyme ATP synthase (complex V). In the process, oxygen accepts electrons from the electron transport chain and protons from the ATP synthase to form H_2O; this is the final step of oxidative phosphorylation. ATP-ADP translocase in the inner mitochondrial membrane couples transport of ATP out from the mitochondrial matrix with transport of ADP into the matrix.

Plasma membrane depolarization through closure of K_{ATP} channels

ATP, or rather the ratio of ATP to ADP, is a measure of the energy status of the cell. Binding of ATP to the regulatory sulfonylurea receptor 1 (SUR1) subunit of the ATP-sensitive potassium channel (K_{ATP} channel) alters the confirmation of the pore-forming Kir6.2 subunits and leads to closure of the channel (Ashcroft, 1988). The constant activity of the Na^{2+}/K^+ pump at the plasma membrane maintains a negative membrane resting potential with high intracellular concentration of potassium. Consequently, the electrochemical gradient for potassium over the plasma membrane favors potassium flow out of the cell through the K_{ATP} channel. When the K_{ATP} channel closes due to increased levels of cytosolic ATP, potassium is prevented from leaving the cell, leading to an increase in membrane potential.

Calcium influx through voltage-gated calcium channels

This triggers the opening of voltage-gated calcium channels and enables calcium to enter the cell. Two types of L-type (long-lasting) calcium channels, $Ca_V1.2$ and $Ca_V1.3$ (encoded by *CACNA1C* and *CACNA1D*, respectively) are primarily responsible for calcium-dependent insulin secretion. Which type that predominates varies between species (Barg et al., 2001b; Reinbothe et al., 2013). Insulin secretion in humans is also dependent on P/Q-type calcium channels (Rorsman et al., 2012).

Calcium-dependent exocytosis

Several membrane-associated proteins are critical for exocytosis. Fusion of the secretory vesicle with the plasma membrane requires SNARE (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) proteins on both the

vesicle and plasma membrane. The v-SNARE synaprobrevin/VAMP2 on the vesicle intertwines with the t-SNAREs SNAP25 and syntaxin 1 on the plasma membrane to form a complex that bring the membranes together and catalyzes membrane fusion. Formation of the SNARE complex is calcium-dependent (Chen et al., 1999). Accessory proteins are also needed for vesicle fusion, for example munc-18, Rab GTPases and synaptotagmins (Hong and Lev, 2014; Sudhof and Rizo, 2011). Synaptotagmins (Syt) have proven to be the calcium sensors that couple intracellular calcium to exocytosis, and SYT VII and SYT IV seem to be of greatest importance in β-cells (Gauthier and Wollheim, 2008).

Cyclic AMP potentiates the effect of glucose on insulin secretion

Cyclic AMP (cAMP) is a second messenger generated from ATP by the action of adenylate cyclase, a transmembrane enzyme localized to the plasma membrane. Adenylyl cyclase is in turn activated by stimulatory G-protein (G_s) coupled receptors. These include receptors for the incretin hormones, gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1; see Figure 3). Cyclic AMP alone cannot stimulate insulin secretion, but potentiates the effect of glucose and calcium on exocytosis (Tengholm, 2012). Its effects are mediated by cAMP-binding protein kinase A (PKA) and Epac2, a guanine nucleotide exchange factor for the Rap family of small GTPases. PKA phosphorylates the K_{ATP} channel and voltage-gated receptors, thereby modulating their activity (Ammala et al., 1993; Light et al., 2002). Epac2 has been shown to interact with SUR1 and SNAP25, and its effect on insulin secretion seems to be dependent on the small GTPase Rap1, possibly via mobilization of calcium from intracellular stores (Tengholm, 2012).

The action of cAMP is regulated via production by adenylyl cyclase and via degradation by phosphodiesterases (PDEs). For *in vitro* experiments, the effect of cAMP is often augmented by stimulating adenylyl cyclase with forskolin or by inhibiting PDEs with the nonselective PDE inhibitor 3-isobutyl-1-methylxanthine (IMBX).

The amplifying pathway increases the effect of calcium

In the triggering pathway, ATP produced by β-cell metabolism closes K_{ATP} channels, which leads to depolarization of the cell membrane potential and calcium influx. Besides ATP, metabolism of glucose gives rise to other metabolites that act to increase the effect of calcium on insulin secretion. This additional effect of glucose stimulation has been termed *the amplifying pathway* (Gembal et al., 1992), and is thought to depend on metabolites that couple metabolism to insulin secretion (Wiederkehr and Wollheim, 2012). Glutamate has been shown to be one such

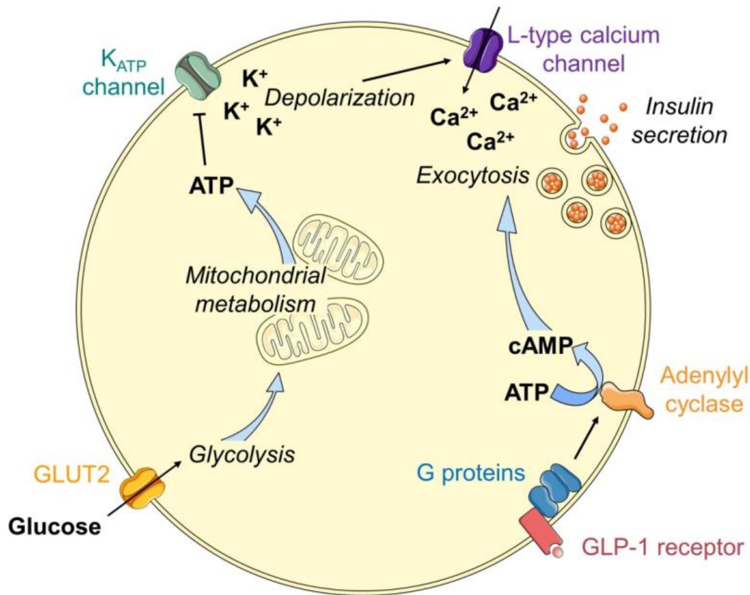

Figure 3. Stimulus-secretion coupling in the β-cell. The triggering pathway and the mechanism for cAMP generation are shown.

coupling factor (Maechler and Wollheim, 1999). It is produced during glucose metabolism and stimulates insulin exocytosis under conditions of fixed cytosolic calcium, thus amplifying the effect of glucose (Maechler and Wollheim, 1999). Other metabolites, such as NADPH and mitochondrial GTP, have also been shown to act as coupling factors (Wiederkehr and Wollheim, 2012).

Insulin is secreted in a biphasic pattern

Insulin secretion in response to glucose occurs in a biphasic manner (Del Prato, 2003). Following glucose stimulation, insulin secretion rapidly increases and peaks within 5-7 min. This distinct first phase is followed by slow but sustained second phase (Del Prato, 2003). The underlying mechanism for this biphasic pattern has not yet been satisfyingly explained (Wang and Thurmond, 2009).

When exocytosis is studied in individual β-cells in response intracellular calcium, a rapid initial increase in membrane capacitance (reflecting exocytosis) is followed by a slow, sustained increase (Rorsman et al., 2000). This phasic exocytosis pattern of β-cells studied *in vitro* is likely to reflect physiologically relevant processes; however, the exact nature of its relationship with human biphasic insulin secretion is yet to be determined.

Role of the liver in regulating plasma glucose

Another tissue that plays a central role in the regulation of plasma glucose is the liver. It is the body's main source of endogenous glucose production and is responsible for maintaining euglycemia between meals. Like β-cells, hepatocytes express the low affinity-high capacity glucose transporter GLUT2 and the glucose-sequestering enzyme glucokinase (a low-affinity hexokinase). This allows them to take up glucose in a concentration-dependent manner. Glucose can be stored in the liver in a readily mobilized form, as glycogen. For glycogen synthesis, glucose-6-phosphate is isomerized to glucose-1-phosphate and converted to UPD-glucose, which is the building block used by the enzyme glycogen synthase for glycogen synthesis.

Hepatocytes are also capable of gluconeogenesis, the *de novo* generation of glucose from 3-carbon metabolites such as pyruvate, lactate and glycerol, and from amino acids such as alanine and glutamine. In contrast to cells of other organs (the kidney and possibly the intestine excluded), hepatocytes express the enzyme glucose-6-phosphatase (G6pase), which performs the reverse reaction of glucokinase by dephosphorylating glucose and allowing it to leave the cell. In this way, glucose produced by glycogen breakdown (glycogenolysis) or via gluconeogenesis can be released for use by peripheral organs. The sum of gluconeogenesis and glycogenolysis determines the total hepatic glucose production (HGP). The HGP constitutes the majority of the endogenous glucose production (EGP), approximately 80% in the fasted (post-absorptive) state, while the remaining part comes from the kidney (20%) and possibly the intestine (Battezzati et al., 2004; Cano, 2002).

Gluconeogenesis – *de novo* production of glucose

Lactate, glycerol, alanine and glutamine are the most important substrates for gluconeogenesis, together accounting for >90% of gluconeogenesis (Gerich et al., 2001). These substrates are produced during metabolism in other organs and released to the blood. Glycerol, the backbone of triglycerides, is released upon breakdown of triglycerides to free fatty acids. Lactate is produced by anaerobic metabolism in skeletal muscle in order to replenish NAD^+ needed for glycolysis. The metabolic pathway that involves reduction of pyruvate to lactate in the muscle, and conversion of lactate to glucose via gluconeogenesis in the liver, is known as the Cori cycle. Transport of lactate and pyruvate across the plasma membrane is mediated by monocarboxylate transporter 1 (Halestrap and Meredith, 2004).

The steps of gluconeogenesis are the reversal of glycolysis, with three important exceptions listed below:

1) Pyruvate → oxaloacetate (in the mitochondrion)
2) Oxaloacetate → phosphoenolpyruvate (in the cytosol)
3) Fructose-1,6-bisphosphate → fructose 6-phosphate (in the cytosol)

Pyruvate → oxaloacetate

Lactate is converted to pyruvate by the enzyme lactate dehydrogenase in the cytosol, and is then actively transported into the mitochondrion where it is converted to oxaloacetate by the enzyme *pyruvate carboxylase* (PC) in a reaction that requires ATP and acetyl-CoA. Glucogenic amino acids such as alanine and glutamate enter the gluconeogenetic pathway as pyruvate or oxaloacetate.

Oxaloacetate → phosphoenolpyruvate

Oxaloacetate is transported out from the mitochondrion (in an indirect way involving reduction of oxaloacetate to malate by mitochondrial malate dehydrogenase, export of malate to the cytoplasm, and oxidation of malate by cytoplasmic malate dehydrogenase to regenerate oxaloacetate) and is converted to phosphoenolpyruvate by the enzyme *phosphoenolpyruvate carboxykinase 1* (PCK1 or cytosolic PEPCK) in a GTP-dependent reaction.

Fructose-1,6-bisphosphate → fructose 6-phosphate

The third enzyme specific for gluconeogenesis is *fructose‑1,6‑bisphosphatase* (FBP1), which converts fructose-1,6-bisphosphate to fructose 6-phosphate. Glycerol enters the gluconeogenetic pathway prior to this reaction, as dihydroxyacetone phosphate (DHAP). Formation of DHAP occurs in two steps. First, glycerol is phosphorylated to glycerol 3-phosphate by glycerol kinase. Second, glycerol 3-phosphate is oxidized to DHAP in a reaction catalyzed by glycerol 3-phosphate dehydrogenase. DHAP and glyceraldehyde 3-phosphate (G3P) are the substrates for fructose-bisphosphate aldolase, which convert them to fructose-1,6-bisphosphate.

G6Pase is not exclusive for gluconeogenesis, but is required and rate-limiting for glucose output from the liver (Rui, 2014).

Regulation of hepatic glucose production

Liver glucose production is regulated by both nutrients and hormones, primarily insulin and glucagon. Blood from the pancreas is delivered directly to the liver through the portal vein, which allows rapid responses to changes in hormone levels. In addition, the concentration of hormones is higher in the portal vein than in the peripheral blood; for insulin, the concentration in the portal vein is approximately 3-fold higher (Roden and Bernroider, 2003).

The glucagon receptor is linked to G-protein complexes containing G_s-proteins. Glucagon binding to the glucagon receptor leads to activation of adenylyl cyclase and production of cAMP.

The insulin receptor (IR) is a tyrosine kinase receptor which upon binding of insulin autophosphorylates itself. The phosphorylated receptor contains binding sites for the scaffold protein insulin receptor substrate (IRS). Three members have been identified in human cells: IRS-1, IRS-2, and IRS-4 (Boucher et al., 2014). Docked IRS proteins are phosphorylated on multiple sites by the insulin receptor, and in turn act as binding sites for downstream proteins. Phosphoinositide 3-kinase (PI3K) is an IRS-binding kinase that is critical for mediating the metabolic effects of insulin (Boucher et al., 2014). Binding to phosphorylated IRS activates PI3K, and makes it phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP_2) in the plasma membrane to create phosphatidylinositol-3,4,5-triphosphate (PIP_3). PIP_3 acts as a docking site for the kinase Akt, and enables the activating phosphorylation of Akt by phosphoinositide dependent kinase 1 (PDPK1) on Thr308. PIP_3 binding is also necessary for Akt Ser473 phosphorylation, which together with Thr308 phosphorylation leads to full activity of the kinase (Scheid et al., 2002).

Insulin and glucagon have opposite effects on hepatic glucose production. Insulin promotes glucose storage and prevents glucose production, whereas glucagon promotes glucose production. The key enzymes for glycogen synthesis and breakdown – glycogen synthase and glycogen phosphorylase, respectively – are largely regulated by their phosphorylation state. The phosphorylation state is in turn reciprocally regulated by insulin and glucagon. Insulin stimulates the activity of glycogen synthase through Akt, which phosphorylates and inhibits the negative regulator glycogen synthase kinase-3 (GSK3) (Cross et al., 1995). Glucagon activates glycogen phosphorylase via PKA-mediated phosphorylation of phosphorylase kinase. The hormones also have opposite effect on gluconeogenesis. Glucagon promotes gluconeogenesis by increasing the expression of PEPCK and G6Pase, whereas insulin downregulates the expression of these enzymes (Li et al., 2007; Puigserver et al., 2003) (Figure 4).

The relative contribution of glycogenolysis and gluconeogenesis to HGP varies with the duration of fasting. As the liver glycogen stores are depleted, the body gets

increasingly dependent on gluconeogenesis for glucose production. Landau and colleagues showed that in healthy subjects, the contribution of gluconeogenesis to glucose production was 47% after 14 h, 67% after 22 h and 93% after 42 h of fasting (Landau et al., 1996). After a regular overnight fast, approximately half of the endogenous glucose production comes from gluconeogenesis and half from glycogenolysis.

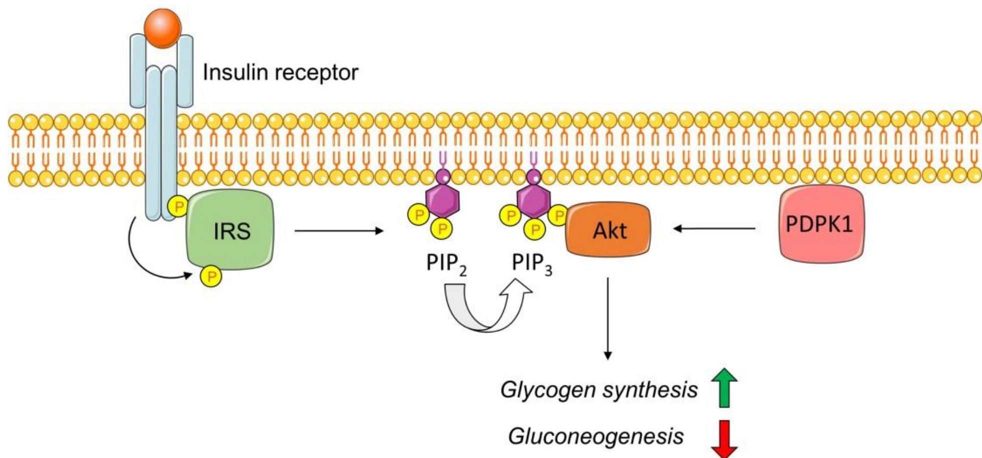

Figure 4. Insulin signaling in the liver. IRS: Insulin receptor substrate; PIP_2: phosphatidylinositol-4,5-bisphosphate; PIP_3: phosphatidylinositol-3,4,5-bisphosphate; PDPK1: phosphoinositide dependent kinase 1

The course of T2D

Definition of T2D

Diabetes is diagnosed based on either of three clinical tests: fasting plasma glucose, glucose levels at 2 h during an oral glucose tolerance test (OGTT) or levels of glycated hemoglobin (HbA1c) (ADA, 2016a). HbA1c reflects average plasma glucose levels during the last 3 months. A fasting glucose above 7 mM, glucose levels at 2 h during an OGTT above or equal to 11.1 mM, or HbA1c above 6.5% (48 mmol/mol) are the criteria for diabetes according to the American Diabetes Association (ADA) (ADA, 2016a). A fasting glucose between 5.6-6.9 mM is defined as impaired fasting glucose (IFG), and glucose levels at 2 h during an OGTT

between 7.8-11.1 mM is defined as impaired glucose tolerance (IGT). IFG, IGT or HbA1c between 5.7-6.4% (39-46 mmol/mol) is defined as prediabetes (ADA, 2016a).

Pathophysiological changes are related to plasma glucose levels

A series of changes takes place in the metabolism as a person goes from having normal glucose tolerance (NGT) to prediabetes and to manifest T2D. These changes can be largely related to the fasting plasma glucose level (Weir and Bonner-Weir, 2004). Important pathogenic changes take place early in the disease process at fasting glucose levels not generally considered impaired (<5.6 mM) (Weir and Bonner-Weir, 2004). Even before the development of IGT, insulin resistance is well-established, and the progression from NGT to IGT is associated with development of severe insulin resistance (DeFronzo et al., 1992).

The first phase of insulin secretion is disrupted early in the disease process. In T2D patients with fasting plasma glucose up to 7.8 mM, the total insulin response to glucose is similar or even greater than in non-diabetic individuals (Del Prato, 2003), with fasting plasma insulin levels typically elevated 2-3 fold (DeFronzo et al., 1992). However, despite an increase in second phase secretion, first phase insulin secretion is almost always blunted (Del Prato, 2003).

First phase insulin secretion declines quickly as fasting glucose rises beyond 5.6 mM, and is totally lost above 6.4 mM (Weir and Bonner-Weir, 2004). First phase insulin secretion is critical for controlling postprandial glucose levels, because it rapidly inhibits hepatic glucose production (Del Prato, 2003). Mitrakou and colleagues showed that in individuals with IGT and reduced first phase insulin secretion, the total appearance of glucose in the plasma after ingestion of a meal was higher compared to individuals with NGT. This increase was caused entirely by impaired inhibition of HGP (28% inhibition in IGT compared to 48% in NGT) (Mitrakou et al., 1992). Loss of first phase insulin secretion occurs early in the disease process, at a time when basal insulin secretion is maintained or even elevated. It therefore makes sense that in patients with mild or moderate hyperglycemia, postprandial hyperglycemia plays a larger role in overall diurnal hyperglycemia compared to fasting glucose (Monnier et al., 2003). In patients with more severe hyperglycemia, fasting glucose is the most important contributor to diurnal hyperglycemia (Monnier et al., 2003).

Insulin resistance leads to a reduction in the uptake of glucose by peripheral organs (glucose clearance). In patients with T2D, most of the reduction, around 90%, can be explained by reduced muscle glucose uptake (DeFronzo, 1992; DeFronzo et al., 1985). As fasting glucose increases from 5.8 to 7.8 mM (mild diabetes), glucose clearance declines linearly to plateau around 7.8-10 mM (DeFronzo et al., 1992). In

contrast, basal HGP remains unchanged up to 7.8 mM glucose (DeFronzo et al., 1992). However, concomitant with the increase in in fasting glucose, there is a 2-3 fold increase in basal insulin levels. The fact that HGP does not *decrease* as a consequence of increased insulin levels reveals hepatic insulin resistance (DeFronzo et al., 1992).

At fasting glucose levels above 7.8 mM the inhibiting effect of hyperinsulinemia on the liver is lost and basal HGP increases progressively, correlating strongly with fasting glucose (DeFronzo et al., 1992). This increase in basal HGP is specifically caused by an increase in gluconeogenesis (Defronzo, 2009; Wajngot et al., 2001). Above fasting glucose of 7.8 mM, the compensatory hypersecretion of insulin from the β-cells (measured by basal insulin levels) also starts to decline (DeFronzo et al., 1992). However, as shown by Ferrannini and colleagues, β-cell glucose sensitivity declines drastically with increasing glucose intolerance, and the decline is accelerated in the prediabetic range (Ferrannini et al., 2005). Thus, there is much support for the idea that severe pathogenic changes take place early in the course of the disease and that prediabetic individuals should in fact be considered to have diabetes (Defronzo, 2009).

Dedifferentiation as a potential mechanism of β-cell failure

Both β-cell mass and function are affected in T2D

Which mechanisms contribute to β-cell failure has been debated. Especially the question whether β-cell death (loss of β-cell mass) or functional defects of the β-cell contribute the most to β-cell failure has been of intense interest. There is ample evidence for reduced β-cell mass in individuals with T2D, but the estimates of β-cell reduction vary depending on the methodology.

β-cell mass assessed by insulin immunohistochemistry is decreased by 40-60% in subjects with T2D compared to non-diabetic control subjects (Butler et al., 2003; Rahier et al., 2008). This decrease reflects a reduction both in total pancreatic weight and in islet density in subjects with T2D (Butler et al., 2003; Rahier et al., 2008). The decrease in β-cells relative to all islet cells was estimated to 19-27% (Butler et al., 2003) and 24% (Marselli et al., 2014). In contrast, islet β-cell fraction as assessed by transmission electron microscopy was decreased by merely 7% (Del Guerra et al., 2005; Marselli et al., 2014). This difference probably reflects the sensitivity of electron microscopy to detect β-cells that contain only a small amount of insulin

granules, since highly degranulated cells may not stain positive for insulin using immunohistochemistry (Marselli et al., 2014).

Although the estimates vary, it has been demonstrated beyond doubt that subjects with T2D have decreased β-cell mass. However, the observed decrease in β-cell mass alone is not sufficient to explain the reduced insulin response. Removal of half the pancreas in humans has surprisingly little effect on diabetes incidence (Slezak and Andersen, 2001). Moreover, 30-100% of patients who go through bariatric surgery show complete remission of diabetes within a few days after surgery, accompanied by only 1-2% weight loss (Bradley et al., 2012). The short time span alone makes it unlikely that an increase in β-cell mass would be responsible for the diabetes remission, and the low regeneration rate of human β-cells (Menge et al., 2008) even more so. Taken together, current data suggest that functional defects of the β-cells are relatively more important than reduced β-cell mass for causing insulin insufficiency in T2D.

Dedifferentiation of β-cells causes diabetes in mice

Mechanisms involving oxidative stress, endoplasmic reticulum stress and mitochondrial dysfunction have been linked to impaired β-cell function (Del Guerra et al., 2005; Laybutt et al., 2007; Mulder and Ling, 2009). In addition, an intriguing new mechanism for β-cell dysfunction was recently demonstrated in diabetic mice. Talchai and colleagues showed that mice lacking the β-cell transcription factor *Foxo1* develop diabetes when exposed to physiological stress such as multiple pregnancies or aging (Talchai et al., 2012). There was a loss of β-cells (~30%) and a concomitant increase in α-cells (~50%) in islets from these mice (assessed by insulin and glucagon staining), together with an impairment in insulin secretion.

To determine whether the β-cell defect was due to β-cell death or functional impairment, Talchai and colleagues performed linage tracing. Surprisingly, the linage tracing showed that the apparent loss of β-cells was not due to cell death, but to loss of insulin expression. Some cells were simply highly degranulated, while others had lost not only insulin expression but also expression of the β-cell transcription factors MAFA and PDX1. These cells of β-cell linage stained negative for SOX9 (a pre-endocrine marker) but positive for chromogranin A (an endocrine marker). They also expressed high levels of the endocrine progenitor markers NGN3, OCT4, L-MYC and NANOG, suggesting that they were β-cells that had reverted – dedifferentiated – to a progenitor-like state (Talchai et al., 2012). The phenotype described is convincing evidence for dedifferentiation.

Some former β-cells also started to express glucagon and the transcription factor MAFB, which in rodents is expressed only in α-cells (Nishimura et al., 2006). The apparent loss of β-cells and increase in α-cells (glucagon-positive cells) could be

explained almost completely by β-cells that dedifferentiated and started to express other islet hormones, such as glucagon. Moreover, these findings proved not to be unique for *Foxo1*-knockout mice, but applied to *db/db* mice and insulin-resistant diabetic GIRKO mice as well. Thus, dedifferentiation seems to be a common feature in several diabetic mouse models. Brereton and colleagues employed linage tracing in mice with chronic hyperglycemia, caused by a gain-of-function K_{ATP} channel mutation (Brereton et al., 2014). Similarly to Talchai and colleagues, they found that 24% of the cells of β-cell linage expressed neither insulin nor glucagon, and 8% had commenced to express glucagon. In contrast to Talchai and colleagues, they found that 7% of the cells of β-cell linage expressed *both* insulin and glucagon. The double-positive (bihormonal) cells retained the expression of PDX1 and GLUT2, but also expressed MAFB, thus exhibiting a phenotype somewhere between β- and α-cells. At the islet level, gene expression of the β-cell markers *Pdx1*, *Nkx6.1*, *Mafa* and *Slc2a2* (encoding GLUT2) was reduced in these mice, as would be expected considering the loss of functional β-cells.

Evidence for dedifferentiation in humans

Could dedifferentiation explain β-cell failure in humans? Researchers have explored the possibility, but come to different conclusions. Definite evidence is hard to provide, since linage tracing cannot be done in humans, at least not *in vivo*. A starting point is the notion that β-cell loss in T2D may have been overestimated. As mentioned above, β-cell loss assessed by electron microscopy gives a lower estimate than immunostaining (Del Guerra et al., 2005; Marselli et al., 2014). The cells identified as β-cells by electron microscopy but not by insulin staining are likely to be dysfunctional. But have they become dedifferentiated?

Cinti and colleagues found that the number of endocrine cells (identified by synaptophysin or chromogranin A staining) from human islets that did not express any of the four major islet hormones (insulin, glucagon, somatostatin or pancreatic polypeptide) increased from 6.5% in non-diabetic subjects to 16.8% in subjects with T2D (Cinti et al., 2016). This loss of hormone expression in endocrine cells is similar to the loss seen in diabetic mice, where the cause is dedifferentiation (Talchai et al., 2012). However, when Butler and colleagues performed similar immunostaining experiments, they found much lower numbers of hormone-negative endocrine cells. In obese non-diabetic donors, hormone-negative cells represented only 0.17% of all endocrine cells, or 0.03 cells/islet (Butler et al., 2016). In obese donors with T2D the number was significantly higher, but still not more than 0.11 cells/islet, which is not sufficient to explain the apparent loss of β-cells in T2D.

Ectopic expression of transcription factors in diabetic islets

Besides the presence of hormone-negative endocrine cells, a characteristic feature in diabetic mouse models that exhibit dedifferentiation is the mismatched expression of transcription factors relative to islet hormones. Expression of glucagon or somatostatin in former β-cells was accompanied by ectopic lingering expression of MAFA, PDX1 or NKX6.1 in diabetic mice (Talchai et al., 2012). Similarly, in islets from donors with T2D, Cinti and colleagues found ectopic expression of β-cell transcription factors. Of the glucagon-expressing cells, 15% expressed both the α-cell transcription factor ARX and FOXO1, which was seven times more than in the non-diabetic donors (Cinti et al., 2016). In addition, 7.5% of somatostatin-positive cells expressed ectopic, cytosolic NKX6.1 (Cinti et al., 2016). The cellular localization of MAFA and NKX6.1 in β-cells shifted from being exclusively nuclear in non-diabetic donors, to being both nuclear and cytoplasmic in donors with T2D. Cytosolic localization of MAFA and NKX6.1 in T2D has also been demonstrated also by Spijker and colleagues (Spijker et al., 2015).

Bihormonal cells are more common in diabetic islets

The presence of cells expressing both insulin and glucagon – bihormonal cells – was observed by Brereton and colleagues in diabetic mice that also harbored hormone-negative former β-cells (Brereton et al., 2014). Bihormonal cells constituted 7% of the former β-cells, an approximately 20-fold increase compared to control mice (Brereton et al., 2014). In a small case-report study with pancreases from three donors with T2D and three non-diabetic donors, White and colleagues found co-expression of insulin and glucagon in ~1% of the islet cells in islets from T2D donors, whereas no bihormonal cells were found in islets from non-diabetic donors (White et al., 2013). Interestingly, ~5% of the insulin-positive cells and ~1% of the glucagon-positive cells in diabetic islets also expressed the mesenchymal marker vimentin, a co-expression which was not seen in non-diabetic islets (White et al., 2013). This is similar to findings by Talchai and colleagues, where vimentin was shown to be expressed in some glucagon-positive cells in animals that also harbored dedifferentiated β-cells (Talchai et al., 2012). Based on the findings by Talchai and colleagues, White and colleagues suggested that ectopic expression of vimentin is circumstantial evidence for β-cell dedifferentiation.

Using lineage tracing, Spijker and colleagues recently saw that human β-cells spontaneously converted to glucagon-producing *ex vivo* when islets were dispersed and then re-aggregated (Spijker et al., 2013). Although the *ex vivo* milieu is very different from *in vivo*, this spontaneous cell type conversion points to a plasticity of adult β-cells. In a follow-up study, Spijker and colleagues demonstrated the presence of cells co-expressing insulin and glucagon in human islets. Bihormonal

cells were significantly increased in T2D donors (~4% and ~0.5% of the insulin-positive cells in donors with and without T2D, respectively) (Spijker et al., 2015). Around 50% of these cells did not express NKX6.1. Some, but not all, bihormonal cells expressed ARX. The progenitor marker NGN3 was not found in bihormonal cells (Spijker et al., 2015).

Loss of β-cell identity

Do the findings summarized above provide evidence for dedifferentiation in human β-cells? Cinti and colleagues defined dedifferentiated cells as endocrine cells (identified by synaptophysin or chromogranin A staining) that ceased to express islet hormones. This was the fate of ~25% of β-cells in diabetic mice, where many such cells displayed a progenitor-like phenotype characterized by loss of MAFA and PDX1, and acquisition of NGN3, OCT4, L-MYC and NANOG (Talchai et al., 2012). The phenotype described by Talchai and colleagues provides convincing evidence for dedifferentiation. In contrast, Cinti and colleagues did not report expression of any of the above progenitor markers in the supposedly dedifferentiated human β-cells. They also acknowledge the possibility that hormone-negative cells (as assessed by immunostaining) may still express low levels of hormones – a plausible case considering the discrepancy in β-cell identification between immunostaining and electron microscopy analysis (Marselli et al., 2014). Difference in hormone detection sensitivity or donor characteristics (e.g. BMI) may explain the vastly different estimates of hormone-negative cells between Cinti and colleagues and Butler and colleagues.

The studies summarized above present evidence for loss of β-cell identity in T2D, characterized by loss of or ectopic cytoplasmic expression of β-cell transcription factors, or by co-expression of insulin with glucagon and mesenchymal markers. Whether such loss of identity is a sign of dedifferentiation is still an open question. In diabetic mouse models, cells with ectopic expression of transcription factors seem to accompany hormone-negative, presumably dedifferentiated cells, but whether this is the case in humans is not clear. The presence of bihormonal cells in some studies (Spijker et al., 2015; White et al., 2013; Yoneda et al., 2013) points at direct transdifferentiation rather than dedifferentiation, but does not preclude that dedifferentiation could occur in other cells.

A common finding in both of the mouse studies mentioned here, where dedifferentiation was convincingly demonstrated through lineage tracing (Brereton et al., 2014; Talchai et al., 2012), was the considerable downregulation of β-cell markers such as Pdx1, Nkx6.1, Mafa and Slc2a2. Thus, even if downregulation of β-cell markers in itself is not a proof of dedifferentiation, it is at least a prerequisite. Taken together, only few publications to date have addressed dedifferentiation in

humans, and their conclusions vary. Further investigation into this topic is therefore needed. It is possible that other methods than immunohistochemistry could give clues as to whether dedifferentiation is a mechanism of β-cell failure in humans.

Current drug treatments for T2D

Patients who are newly diagnosed with T2D are encouraged to change their lifestyle (including healthy eating, reducing stress and increasing physical activity) as a first step to promote weight loss and reach glycemic goals (ADA, 2016b). However, when lifestyle changes alone are not sufficient to achieve target glycemic control, drugs are needed. Today, several drugs are available for treatment of T2D in addition to insulin. Some of them, metformin and sulfonylureas, have been available since the 1950s (at least in Europe), whereas the GLP-1 analogues, DPP-4 inhibitors and sodium-glucose co-transporter type 2 (SGLT2) inhibitors are additions after 2005.

Metformin

Metformin is recommended by the ADA as the first-line treatment for patients with T2D. It is safe and generally well tolerated, although gastrointestinal side effects may prevent its use in approximately 5% of patients (Garber et al., 1997). The primary mechanism of action is to reduce glucose production from the liver (Hundal et al., 2000), and specifically to gluconeogenesis (Hundal et al., 2000).

In patients with severely dysregulated T2D, the rate of gluconeogenesis is three times higher than in non-diabetic controls, and metformin treatment proved to reduce gluconeogenetic rate by 36% (Hundal et al., 2000). The effect of metformin on insulin-stimulated glucose uptake in peripheral tissue is modest or nonexistent (Natali and Ferrannini, 2006). Metformin seems to affect gluconeogenesis through several mechanisms. Perhaps the most well-documented effect is the slight inhibition of the electron transport chain by specific binding to mitochondrial complex 1 (Foretz et al., 2014). The resulting relative increase in AMP and ADP leads to activation of AMP-activated protein kinase (AMPK). AMPK inhibits acetyl CoA carboxylase, and the resulting changes in hepatic lipid homeostasis improve insulin action (Fullerton et al., 2013). AMPK signaling also reduces the expression of gluconeogenetic genes (Foretz et al., 2014). In addition, metformin has AMPK-independent effects that include direct inhibitory effects of AMP on adenylyl cyclase (which mediates the effects of glucagon) (Miller et al., 2013) and on the gluconeogenetic enzyme fructose 1,6-bisphosphatase through allosteric regulation (Foretz et al., 2014).

Metformin has positive effects on cardiovascular health and life expectancy. In the United Kingdom Prospective Diabetes Study (UKPDS), metformin given to newly diagnosed T2D patients proved to reduce the risk for myocardial infarction by 39% and the risk for all-cause death by 36% compared to conventional therapy (diet) (UKPDS, 1998a). Although no efforts were made to maintain the allocated therapy after the end of the study, the reductions in risk for myocardial infarction and all-cause death were still significant 10 years later (Holman et al., 2008), which encourages early treatment with metformin. Similarly, the prevalence of cardiovascular disease in a large Italian study was lower in participants treated with metformin (20.2%) than in those treated with other drugs (32.4%) (Solini et al., 2013).

Metformin used to be contraindicated in individuals with impaired renal function due to fear for lactic acidosis. Lactic acidosis is a rare but life-threating condition characterized by low blood pH and elevated blood lactate levels (Brown et al., 1998). The association of the family member phenformin with lactic acidosis, together with early pharmacokinetic data suggesting reduced clearance in patients with impaired kidney function (Inzucchi et al., 2014), has for a long time restricted prescription of metformin to patients normal kidney function. Only recently did ADA change its guidelines to support metformin use in patients with moderately reduced kidney function (impairments down to a glomerular filtration of 30 mL/min/1.73 m^2) (ADA, 2015; ADA, 2016b). This change was based on findings from several studies reporting that metformin does not significantly increase blood lactate concentrations in patients with mild to moderate chronic kidney disease (Inzucchi et al., 2014), and accumulated data showing that metformin does not increase the risk for lactic acidosis above the background rate in the diabetic population (Inzucchi et al., 2014).

Sulfonylureas

Sulfonylureas increase insulin secretion from the β-cells by binding to the sulfonylurea receptor SUR1 and thereby closing the K_{ATP} channel. The majority of sulfonylureas in clinical use today belong to the second generation of sulfonylureas and include glibenclamide (glyburide), glipizide, gliclazide and glimepiride. The second-generation sulfonylureas have around 1000 times higher affinity for SUR1 than first-generation compounds and are therefore given at lower doses (Melander, 2004). Sulfonylureas are effective in lowering plasma glucose levels and HbA1c, but have a number of side effects, including weight gain and increased risk for hypoglycemia (UKPDS, 1998b). The risk for hypoglycemia seems to be higher with glibenclamide than with other sulfonylureas (Gangji et al., 2007).

Sulfonylureas have been suspected of causing β-cell "exhaustion" by apoptosis (Maedler et al., 2005), but findings related to this topic have varied. Results from the UKPDS showed that after an initial marked decrease in plasma glucose and HbA1c with sulfonylurea, glycemic control deteriorated at the same rate seen in patients with diet-controlled T2D; deterioration was not worse with sulfonylurea, and the significant improvement in glycemic control compared to diet therapy remained over time (Holman, 2006). However, another study, specifically designed to analyze β-cell dysfunction, found that a longer treatment duration with sulfonylurea was associated with a more rapid decline in β-cell function (Shin et al., 2012).

Meglitinides

Like sulfonylureas, meglitinides stimulate insulin secretion by binding to the SUR1 subunit and thereby blocking the K_{ATP} channel. However, they have a much shorter duration of action, with plasma half-lives of only 1-2 h (Guardado-Mendoza et al., 2013). The meglitinides were introduced 1995 with repaglinide. Repaglinide and nateglinide are the approved meglitinides in Europe and the USA. In addition to shorter half-lives for meglitinides compared to sulfonylureas, the dissociation rate from SUR1 is also faster; only around 1 s for nateglinide and 2 min for meglitinide compared to over 60 min for glibenclamide (Hu et al., 2000). Their short duration of action makes them suitable for targeted treatment of post-prandial hyperglycemia (Stein et al., 2013), and they are preferable to sulfonylureas in T2D patients with irregular meal schedules (ADA, 2016b). In contrast to sulfonylureas, they have the advantage of causing no or only minor weight gain (Guardado-Mendoza et al., 2013). When repaglinide and nateglinide were compared in a trial with 150 T2D patients, the glucose-lowering effect of repaglinide was greater than that of nateglinide (HbA1c -1.6% versus -1.0%) (Rosenstock et al., 2004). However, although no patients experienced major hypoglycemic events requiring assistance from another person, 7% of patients in the repaglinide group had minor hypoglycemic events compared to none in the nateglinide group (Rosenstock et al., 2004).

Thiazolidinediones

Thiazolidinediones act mainly by improving peripheral insulin sensitivity (Natali and Ferrannini, 2006), and are currently the only type of T2D drug with that effect. The members of the class that are in use today were introduced 1999, and comprise rosiglitazone and pioglitazone. Thiazolidinediones bind to and activate the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ), which is expressed

in several tissue types but most abundant in adipose tissue (Soccio et al., 2014). Activated PPARγ induces transcription of genes with PPARγ binding sites, including genes needed for fat storage and adipocyte differentiation (Soccio et al., 2014).

Increased adipogenesis and adipose tissue lipid storage contribute to weight gain in patients using thiazolidinediones. However, the fat gained is mainly subcutaneous fat (Bray et al., 2013), and seems to be part of the mechanism by which thiazolidinediones exert their insulin-sensitizing effect (Soccio et al., 2014). Adipose tissue is the metabolically safe place to store fat. Studies in animal models have shown that rosiglitazone promotes fat uptake in adipose tissue, while reducing uptake in liver and muscle (Ye et al., 2004). In addition, rosiglitazone actively redistributes fat away from skeletal muscle (Kim et al., 2003).

Thiazolidinediones have a protective effect on β-cells, leading to slower deterioration of glycemic control compared to metformin and glibenclamide (Kahn et al., 2006). Thiazolidinediones may even improve β-cell function in patients with T2D, at least short-term (Gastaldelli et al., 2007). Side effects, apart from weight gain, include an increased risk for bone fractures, and edema caused by fluid retention (Colhoun et al., 2012; Soccio et al., 2014). The fluid retention significantly increases the prevalence of congestive heart failure; however, heart failure following pioglitazone therapy does not increase mortality or the risk for cardiovascular events (Dormandy et al., 2005). In contrast, a meta-analysis from 2007 showed a that rosiglitazone increased the risk for myocardial infarction with 43% (Nissen and Wolski, 2007), and rosiglitazone was therefore withdrawn from the market in Europe.

GLP-1 receptor agonists and DPP-4 inhibitors

The incretin hormones GLP-1 and GIP are secreted by intestinal L- and K-cells, respectively, in response to nutrients (Campbell and Drucker, 2013). They potentiate GSIS from the β-cells by binding to their respective G_S-protein-coupled receptors and stimulating cAMP production. GLP-1 and GIP are rapidly degraded by dipeptidyl peptidase 4 (DPP-4), leading to a half-life of only 1-2 min (Holst, 2007). Two types of drugs are available that increase incretin receptor signaling: GLP-1 receptor agonists and DPP-4 inhibitors.

DPP-4 inhibitors prevent the degradation of incretin hormones, thereby allowing higher concentrations and more long-lasting effects (Holst, 2007). The first DPP-4 inhibitor, sitagliptin, was introduced in 2006. Sitagliptin, alogliptin, and linagliptin inhibit DPP-4 by non-covalent binding, whereas saxagliptin and vildagliptin form a covalent bond with serine in the active site (Aroda et al., 2012).

Exogenous GIP fails to stimulate insulin secretion in most hyperglycemic subjects with T2D (Campbell and Drucker, 2013), and thus focus has been on enhancing the GLP-1 effect. The first GLP-1 receptor agonists, exenatide, was introduced in 2005. GLP-1 receptor agonists currently available are exenatide, liraglutide, lixisenatide, albiglutide and dulaglutide.

GLP-1 receptor agonists and DPP-4 inhibitors improve glycemic control by increasing glucose-stimulated insulin secretion. In addition, they also suppress inappropriate glucagon secretion, slow gastric emptying (which reduces the rate of glucose appearance in the circulation) and increase satiety (Aroda et al., 2012). Since GLP-1 receptor agonists and DPP-4 inhibitors potentiate insulin secretion in a glucose-dependent manner, the risk for hypoglycemia is minimal. GLP-1 receptor agonists are more effective than DPP-4 inhibitors in lowering plasma glucose, and they lead to substantial weight loss, while DPP-4 inhibitors are weight neutral or lead to minor weight loss (Aroda et al., 2012). However, DPP-4 inhibitors have the advantage of oral administration, whereas GLP-1 receptor agonists need to be injected (although dulaglutide and exenatide are available as weekly injections).

DPP-4 inhibitors could possibly have a preserving effect on β-cell function, as suggested by a reduced deterioration of glycemic control during two 2-year trials compared to sulfonylureas (Scheen, 2012).

SGLT2 inhibitors

SGLT2 inhibitors are the newest of the T2D drugs, introduced 2011 with dapagliflozin. Other SGLT2 inhibitors currently available in Europe and the USA are empagliflozin and canagliflozin. They differ from previous T2D drugs by exerting their effect on the kidneys, preventing reabsorption of glucose from the proximal tubules via SGLT2 (Scheen, 2015). This leads to a decrease in plasma glucose independent of insulin, and also to significant weight loss (Scheen, 2015). In addition, the osmotic effect of glucose excretion leads to a decrease in blood pressure (Scheen, 2015). SGLT2 inhibitors also induce a modest increase in HDL cholesterol (Monami et al., 2014). By reducing hyperglycemia, SGLT2 inhibitors improve peripheral glucose sensitivity, and may also improve β-cell function (Scheen and Paquot, 2014). SGLT2 inhibitors have proven to have similar glucose-lowering effects as metformin, sulphonylureas or the DPP-4 inhibitor sitagliptin in comparative trials (Scheen, 2015). Moreover, exciting data from the EMPA-REG OUTCOME trial showed that empagliflozin reduced death from cardiovascular causes with 38%, and all-cause death with 32%, in T2D patients with established cardiovascular disease (Zinman et al., 2015).

Disadvantages with SGLT2 inhibitors are related to glycosuria. Besides leading to polyuria (ADA, 2016b), the risk for genital infections is increased approximately 4-

fold, and the risk for urinary tract infections is modestly (~20-40%) but significantly increased (Monami et al., 2014; Scheen, 2015). Interestingly, although the overall glycemic effect is favorable, SGLT2 inhibitors increase glucagon levels and hepatic glucose production (Ferrannini et al., 2014). Moreover, the U.S. Food and Drug Administration (FDA) recently issued warnings about potential increased risk of ketoacidosis and acute kidney failure with SGLT2 inhibitors (Drug Safety Communications from 12-4-2015 and 6-14-2016).

Other drugs for T2D

The drugs described above, plus insulin, are the drugs recommended by ADA as second-line therapies, or as first-line therapies if metformin is not tolerated or contraindicated (ADA, 2016b). Other drugs exist, and may be tried in specific situations, but are generally not preferred by ADA or the European Association for the Study of Diabetes (EASD) due to modest efficacy, frequency of administration, or side effects (ADA, 2016b; Inzucchi et al., 2015). These include a-glucosidase inhibitors, which slow intestinal carbohydrate absorption; colesevelam, which binds bile acids in the intestinal tract (the reason for the glucose-lowering action is unclear); bromocriptine, a dopamine agonist that modulates hypothalamic regulation of metabolism; and pramlintide, an amylin analogue that delays gastric emptying (ADA, 2016b).

Drug repositioning

An attractive strategy for acquiring new T2D therapies is drug repositioning, also known as drug repurposing. Drug repositioning implies finding new indications for existing drugs. A single drug can act on multiple targets, and a drug target may be shared by different biological processes relevant for disease (Dudley et al., 2011a). There are several notable examples of successful drugs that were developed for a different indication than the one of major use today. Sildenafil (Viagra), used for erectile dysfunction, was originally developed for treatment of angina, and minoxidil, used for prevention of hair loss (marketed as Rogaine or Regaine in many countries), was originally developed for treatment of high blood pressure (Dudley et al., 2011a). The antidepressant bupropion proved to be an effective aid for quitting smoking (Hurt et al., 1997), and the cancer drug methotrexate has been widely used for treating rheumatoid arthritis (Alfonso-Cristancho et al., 2017). One of the T2D drugs mentioned above, colesevelam, was originally used for reducing LDL cholesterol in patients with hyperlipidemia (Chen et al., 2015), but proved to

significantly decrease plasma glucose and HbA1c and was approved by the FDA for treatment of T2D (Chen et al., 2015; Elkeles, 2014).

A major advantage of repositioning existing drugs is that they already went through clinical trials and passed safety test in humans. Since drug safety and bioavailability is already known, the road to clinical implementation can be considerably shorter compared to *de novo* drug development (Dudley et al., 2011a). Moreover, compounds that have proved to be safe, but fail in late development due to poor efficacy in phase III clinical trials, might be successfully used for other implications (Novac, 2013). Considering drugs that fail late phase trials for other implications maximizes the use of patient information and research efforts. Drug repositioning has particular value for rare diseases, where treatment options are few and prescription costs for *de novo* developed drugs are typically high (Novac, 2013; Power et al., 2014).

Drug repositioning guided by gene expression profiles

Historically successful examples of drug repositioning have been serendipitous, often based on observations of side effects (Dudley et al., 2011a). The availability of large-scale data such as genome-wide mRNA expression data, together with novel bioinformatics tools, make it possible to use a systemic approach for drug repositioning (Lamb et al., 2006; Power et al., 2014). The potential of using gene expression data as a means for drug repositioning was demonstrated by Lamb and colleagues with the Connectivity Map project (Lamb et al., 2006). They constructed a library containing gene expression data from four human cell lines exposed to 164 different compound, including drugs approved for human use. They then constructed *query signatures* consisting of differentially expressed genes (44-324 genes) in tissues of interest: adipose tissue from rats with diet-induced obesity, brain tissue from patients with Alzheimer's disease, and leukemic cells from patients exhibiting resistance to drug treatment. Searching the compound library with these query signatures (using Gene Set Enrichment Analysis), they could identify compounds that induced a gene expression profile similar or opposite to the query signature. The compound that showed the highest anti-correlation to the Alzheimer query signature was a compound recently shown to reverse the formation of amyloid fibers (Lamb et al., 2006). The findings suggested that gene expression profiles could be used to identify new drugs – even when the query (disease) signature is from tissue and the drug profiles from simple cell lines (Figure 5).

The Connectivity Map library of drug gene expression profiles, together with the approach for matching a query signature of interest with drug profiles (Gene Set Enrichment Analysis), has been used in several studies since to identify potential new therapies for various diseases. Validating the findings in animal models – but

so far not in humans – these studies have shown that the anticonvulsant drug topiramate can be used to treat inflammatory bowel disease (Dudley et al., 2011b), and the PPARα agonist bezafibrate and the histamine H_2 receptor antagonist cimetidine to treat lung adenocarcinoma (Liu et al., 2015; Sirota et al., 2011). Drug repositioning guided by a gene expression signature that is representative for the disease seems to have promising prospects. The prerequisite is access to drug gene expression profiles.

To date, the Connectivity Map library contains gene expression profiles from ~5,000 small-molecule compounds and ~3,000 genetic reagents. The Connectivity Map project is a great example of how making data publically available can aid the discovery of new treatments. In addition, most scientific journals require that published microarray data is deposited in a public repository (e.g. Gene Expression Omnibus), from which gene expression profiles can be acquired. The efforts of the research community to make large-scale data publically available are essential to accelerate both basic research and drug discovery (Friend and Norman, 2013). It allows data to be analyzed in different contexts, from new angles, and thus ensures maximal use of the data.

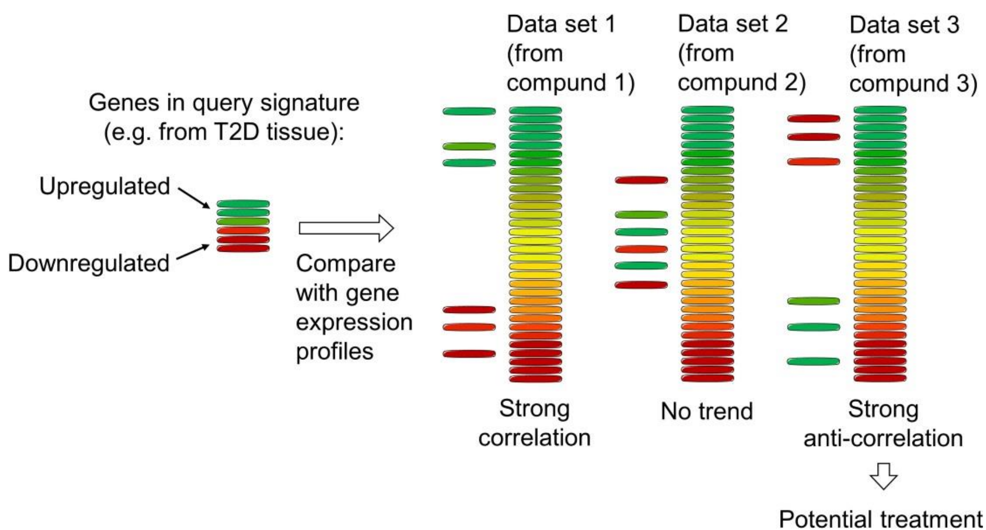

Figure 5. The principle for using gene expression data to find new treatments for a disease.

Aims

T2D occurs when β-cells fail to compensate for increased systemic insulin resistance by maintaining elevated insulin secretion over time. Progressive deterioration of β-cell function in combination with insulin resistance and excessive glucose production from the liver causes plasma glucose levels to rise to harmful levels. Improving β-cell function, reducing insulin resistance, and normalizing hepatic glucose production are all means to achieve better glycemic control. In this work, we aim to gain information about the β-cell disease process, and to test strategies for improving insulin secretion and reducing excessive hepatic glucose production.

The mechanisms leading to β-cell failure are not fully understood. The complexity of the pathophysiological processes is underscored by the hundreds of genes that are involved. We aim to use gene co-expression network analysis to gain information about genes driving the disease processes and find new treatments strategies. Although a large number of β-cell risk alleles have been identified, so far, few risk alleles have been linked to genes or disease mechanisms, and the genetic information has not yet led therapeutic improvements. A new T2D risk allele was recently found in *ADRA2A*, which encodes the α2A-adrenergic receptor. This risk allele leads to receptor overexpression in β-cells that causes increased adrenergic signaling and impaired insulin secretion. Blocking the receptor could possibly improve insulin secretion in risk allele carriers with T2D.

Specifically, we aim to:

I Investigate whether blocking the alpha 2-adrenergic receptor with yohimbine improves insulin secretion in risk allele carriers with T2D.

II Identify new genes with importance for T2D by first identifying gene co-expression networks that are perturbed in islets from T2D donors, and then identifying and examining potential regulators of the networks.

III Employ gene co-expression network analysis to identify new drugs that can be used to treat excessive glucose production from the liver.

Methods

Cell lines

In several experiments in this thesis, we used cell lines to model the function of β-cells and hepatocytes. Cell lines have a number of advantages. They are often easier to manipulate than primary cells, and they are easier to obtain and expand for large experiments. Moreover, they reduce the number of animals that need to be used, which is advantageous from an ethical perspective. Cell lines also have a number of disadvantages in that their molecular and functional phenotypes differ from those of primary cells, as discussed in detail below. Hence, we have aimed to validate all key findings from cell lines in primary tissues from animals and/or humans.

INS-1 832/13

INS-1 832/13 cells were used for most experiments in paper II. They provide a good model for stimulus-secretion coupling (Hohmeier et al., 2000), and their short doubling time make them efficient to work with. In contrast to intact islets, which consist of several endocrine cell types, they provide a pure population of insulin-secreting cells as a model to study β-cell-specific effects.

The parental cells of the INS-1 832/13 cell line, the INS-1 insulinoma cells, were isolated by Asfari and colleagues by dispersion of a radiation-induced INS tumor from NEDH rats into a tissue culture medium containing the reducing compound β-mercaptoethanol (Asfari et al., 1992). As a consequence, these cells and their derivatives have an unusual dependence on β-mercaptoethanol for propagation and phenotype retention. The INS-1 832/13 clone was created by Hohmeier and colleagues from INS-1 cells stably transfected with the gene encoding human proinsulin (Hohmeier et al., 2000). The INS-1 832/13 cells have greater insulin secretion in response to glucose than the parental INS-1 cells (8-11 fold compared to 2-4 fold, respectively, measured at 3 and 15 mM glucose) (Hohmeier et al., 2000). They are also sensitive to secretagogues such as palmitate/oleate and GLP-1, and to IBMX. The glucose response (fold-change at high compared to low glucose) is similar to that of primary β-cells.

Although useful, cell lines never recapitulate all traits of primary cells, and they have features that primary cells do not have. This is exemplified by an experiment in paper II where we measured mitochondrial oxygen consumption in INS-1 832/13 cells in response to pyruvate. Such an experiment would make no sense in primary β-cells, since they express the pyruvate/lactate transporter monocarboxylate transporter 1 (MCT1) at levels that are too low to enable pyruvate uptake. It is important that expression of MTCs *in vivo* is low, since pyruvate and lactate would otherwise stimulate insulin secretion during exercise, leading to hypoglycemia. However, INS-1 832/13 cells express MCT1 at levels that enable exogenous pyruvate to stimulate metabolism and insulin secretion (Andersson et al., 2015).

H4IIE

The rat H4IIE hepatoma cell line was used for several experiments in paper III to assess effects on gene expression and glucose production. The cell line was derived from Reuber Hepatoma H-35 by Pitot et al. (Pitot et al., 1964). HII4E cells are considered well-differentiated (Darlington, 1987; Schamhart et al., 1979), and have many characteristics of primary hepatocytes, including hormone-regulated expression of phosphoenolpyruvate carboxykinase (PEPCK) and hormone-regulated glucose production (Duong et al., 2002). In addition, glucose production from H4IIE cells is regulated by physiological concentrations of insulin (IC_{50} around 100 pM) (de Raemy-Schenk et al., 2006). H4IIE cells are easy to culture and have been extensively used for mechanistic studies of glucose metabolism and insulin signaling.

H4IIE cells feature some notable differences compared to primary hepatocytes. Glucose uptake in H4IIE cells occurs through GLUT1 since they lack expression of GLUT2 (Tal et al., 1992). Moreover, H4IIE cells do not express glucokinase (hexokinase 4) (Schamhart et al., 1979). They do express a high-affinity hexokinase (hexokinase 1) at levels higher than observed primary hepatocytes (Schamhart et al., 1979), but the lack of glucokinase still has implications for glucose metabolism. Glycogen content in H4IIE cells is low, and the fact that vector-mediated expression of glucokinase greatly increases the glycogen content proves that lack of glucokinase causes the aberrantly low glycogen levels (Valera and Bosch, 1994). Regulation of the key gluconeogenetic enzyme PEPCK also differs in H4IIE cells. The expression of PEPCK is not inhibited by glucose, since glucose flux through hexokinase 1 is not sufficient to generate an inhibitory signal (Scott et al., 1998). However, for the applications presented in this thesis, we consider H4IIE to be a good model of primary hepatocytes, despite the above-mentioned differences. The intrinsically low levels of glycogen suggest that gluconeogenesis is of relatively greater importance than glycolysis for glucose production in H4IIE cells.

Human islets

Human islets were used in paper I and II. The islets were obtained from brain-dead cadaveric multiorgan donors with informed consent either from the organ donor registry or from relatives (Goto et al., 2004).

Pancreases from organ donors in Sweden, Norway and Finland are handled by the Nordic Network for Clinical Islet Transplantation (NNCIT). The NNCIT is a collaboration between Nordic transplantation units, and its main objective is to transplant islets to type 1 diabetes (T1D) patients as a therapy to achieve better glycemic control. Islets from all donor pancreases in the NNCIT are isolated and tested for glucose responsiveness at the islet isolation laboratory at Uppsala University (Goto et al., 2004). The islets that cannot be used for transplantation are used for research, provided that there is consent for research use, and are distributed to research centers in Scandinavia. Before being shipped to these research centers, the islets are usually cultured for 1-5 days in Uppsala.

In the papers included in this thesis, donors diagnosed with T2D or HbA1c >6.0% (42 mmol/mol) and no GAD antibodies were defined as having T2D. According to the American Diabetes Association, HbA1c >6.5% is defined as diabetes, and 6.0% falls within the prediabetic range (5.7-6.4%). However, the severity of metabolic alterations and the risk for diabetic complications is continuous over the prediabetes-diabetes range. For example, the prevalence of retinopathy was shown to be similar in prediabetic and newly diagnosed diabetic patients (7.9% and 12.6%, respectively) (DPP, 2007), and it has been argued that individuals with prediabetes should in fact be considered to have T2D, based on their high degree of insulin resistance and impairment in β-cell glucose response (Defronzo, 2009). We therefore find it justifiable to include donors with HbA1c >6.0% in the T2D category.

Diabetic animal models

Wistar rats and C57BL/6J mice on diabetogenic diet

Male Wistar rats and male C57BL/6J mice were used for several experiments in paper III, and C57BL/6J mice were used for gene expression analysis in paper II. Wistar is an outbred rat strain, meaning that the animals are genetically diverse, and breeding is designed to maintain the genetic variation in the population. In contrast, C57BL/6J is an inbred mouse strain with minimal genetic variability, but due to spontaneous mutations and genetic drift, they genotype does differ slightly between

populations that have been separated for several generations, and therefore such populations form different substrains. Two different substrains were used in paper III; C57BL/6J from the Jackson laboratory ("J" for Jackson) and the C57BL/6JBomTac from Taconic. The BomTac substrain was established from a batch of C57BL/6J mice separated from the parental colony in 1971 (Zurita et al., 2011).

It was revealed 2005 that the C57BL/6J mice contain a deletion in the gene Nnt encoding mitochondrial NAD(P) transhydrogenase (Huang et al., 2006; Toye et al., 2005), an enzyme that increases mitochondrial NADPH concentrations and plays a role in the defense against reactive oxygen species (Hoek and Rydstrom, 1988). This mutation is likely to affect insulin secretion and thus glucose tolerance (Toye et al., 2005). The C57BL/6JBomTac mice do not carry this mutation; however it was recently discovered that they have a large deletion on the Y-chromosome, with 40% of the Y-chromosome missing (Fischer et al., 2017). These genetic mutations and deletions could have important phenotypic consequences. Mice of different substrains should therefore not be mixed in an experiment unless the purpose is to identify substrain-specific effects. Heeding such precautions, C57BL/6J and C57BL/6JBomTac substrains are both useful as diabetic models after feeding with a diabetogenic diet.

High-fat feeding induces obesity and leads to hyperglycemia and hyperinsulinemia in susceptible mouse strains, as first shown by Surwit and colleagues (Surwit et al., 1988). In their study, obesity induced by high-fat feeding for 16 weeks led to severe fasting hyperinsulinemia and fasting glucose levels > 11 mM in C57BL/6J mice, whereas A/J mice developed only moderately elevated fasting glucose and insulin levels (Surwit et al., 1988). High-fat feeding of C57BL/6 mice is now a well-established model of T2D (King, 2012). Diabetes in this model is induced by environmental impact (diet) but requires genetic susceptibility to the disease, which resembles the situation in humans (King, 2012). Depending on the classification of diabetes in mouse models, one might consider these mice prediabetic with severe IGT rather than diabetic (Leiter, 2009), since they present compensatory insulin hypersecretion and do not progress to absolute β-cell failure (Sims et al., 2013).

In Wistar rats, a high-fat diet (HFD) causes fat accumulation and insulin resistance in liver and peripheral organs, but does not affect basal glucose or plasma insulin levels (Storlien et al., 1986). Similarly, a diet high in fructose (high-fructose diet; HFrD) induces insulin resistance without affecting basal glucose (Thorburn et al., 1989). Basal insulin levels are either unchanged or increased, and plasma triglyceride are elevated (Robbez Masson et al., 2008; Thorburn et al., 1989; Thresher et al., 2000). In the study by Thorburn and colleagues, triglycerides had a strong negative correlation with total body glucose uptake during hyperinsulinemic-euglycemic clamps (which measure insulin sensitivity) (Thorburn et al., 1989).

Unlike glucose, which is metabolized by most cells in the body, fructose is mainly metabolized by the liver (Douard and Ferraris, 2008), and HFrD leads to the development of hepatic steatosis in rats (Bizeau and Pagliassotti, 2005). Thus, Wistar rats on a HFD or HFrD diet exhibit traits of T2D, especially insulin resistance, but do not develop overt diabetes.

Ob/ob and *db/db* mice

Ob/ob and *db/db* mice of different ages were used for gene expression analysis in paper II. These mouse strains are monogenic models of obesity and diabetes that arose because of spontaneous mutations in mice at the Jackson Laboratory. The genetic cause for the obese and diabetic phenotypes was later characterized and shown to involve the satiety hormone leptin.

In the *ob/ob* mouse ($Lep^{ob/ob}$), a stop codon mutation in the leptin gene prevents expression of leptin protein (Zhang et al., 1994). Loss of leptin expression causes hyperphagia in *ob/ob* mice and leads to severe obesity, hyperglycermia and insulin resistance. However, hyperglycemia in these mice is dependent on overexpression of neuropeptide Y in the hypothalamus, and is partly independent of obesity (Schwartz et al., 1996), unlike hyperglycemia in humans. Also contrary to the situation in T2D, the hyperglycemia is transient and starts to slowly decline after around 3 months. The β-cells from *ob/ob* mice are able to compensate for the increased secretory demand by hypersecretion of insulin, and do not progress to β-cell failure (Wang et al., 2014). *Ob/ob* mice can therefore be considered to model the early phase of T2D (Lindstrom, 2007).

The *db/db* mouse ($Lepr^{db/db}$) carries a mutation in the leptin receptor gene that introduces a new splice site, resulting in a dysfunctional receptor (Chen et al., 1996). Like the *ob/ob* mice, *db/db* mice are hyperphagic, obese and insulin resistant, but they gradually lose β-cell function and develop severe diabetes. Thus, the phenotype of *ob/ob* and *db/db* mice is similar except that *ob/ob* mice have islets that are capable of compensation whereas *db/db* mice have islets prone to β-cell failure.

In vivo tests

Glucose tolerance test

Glucose tolerance tests were employed in paper II and III. The glucose tolerance test (GTT) assesses the ability of an animal to clear glucose from the circulation

when given glucose as a bolus dose. This corresponds to the postprandial glucose response (the glucose response after ingestion of a meal), a response which is typically disturbed early in the course of T2D and manifested in elevated postprandial plasma glucose levels. The removal of glucose from the circulation is dependent both on insulin secretion from the β-cells, and on insulin sensitivity of the target organs. The degree of insulin sensitivity determines the magnitude of inhibition of glucose production from the liver and insulin-stimulated glucose uptake by skeletal muscle and adipose tissue. Glucose can be given either as an oral bolus dose by gavage, or as an intraperitoneal (*i.p.*) or intravenous (*i.v.*) injection. When administering glucose orally, incretin hormones also contribute to the response, an effect that is bypassed when using an *i.p.* or *i.v.* injection. Before the GTT, the animals are fasted to ensure stable baseline glucose levels. Plasma glucose is measured at predetermined time points during the test, in our studies at 0, 15, 30, 60 and 120 min after glucose injection. Typically, plasma glucose levels peak after 10-30 min (faster with an *i.v.* test) and then decline at variable rate. Insulin levels are analyzed from blood sampled at selected time points during the GTT.

Glucose tolerance is commonly reported as "area under the curve", AUC. For AUC calculation, plasma glucose at time 0 is sometimes used as an individual baseline for each animal. However, presenting data in this way means that the contribution of fasting glucose is ignored, and the data therefore do not represent overall glucose tolerance.

The GTT is an informative and relatively simple test to perform. For the experiments in paper III, I have used *i.p.* GTT (IPGTT) for mice and IPGTT or oral GTT (OGTT) for rats. Plasma glucose was measured from the tail vein using a hand-held glucose meter, and blood for insulin analysis was drawn from the saphenous vein. The animals were fasted for 5 h with start in the morning, as opposed to fasted overnight. Rodents are nocturnal animals having most of their food intake during the dark period, and overnight fast is a major metabolic stress for mice (McGuinness et al., 2009). Effects on metabolism include increased insulin action, and findings from overnight-fasted mice may therefore be less relevant to the free-living state compared to a shorter fast (McGuinness et al., 2009).

We used a fixed dose of glucose for all animals in a single experiment, as opposed to the body weight-adjusted dose that is commonly used. The rationale for this is that animals of the same strain and age are likely to have the same lean mass (muscle, liver and brain), and lean mass is responsible for most of glucose uptake (McGuinness et al., 2009). Body weight difference in rodents is caused mainly by fat (which is relatively inert), and increasing glucose dose based on weight gives heavy animals a disproportional disadvantage (McGuinness et al., 2009). Similarly, for OGTTs performed on patients in the clinic, a fixed dose of glucose is used.

Insulin tolerance test

Insulin tolerance tests were employed in paper III. The insulin tolerance test (ITT) assesses whole body insulin sensitivity. The test involves administering a bolus dose of exogenous insulin (given *i.v.* or *i.p*) and monitoring the changes in plasma glucose levels. The effect of exogenous insulin on the rate and magnitude of plasma glucose decline depends on the insulin sensitivity of target organs; insulin-sensitive animals exhibit a swifter and greater decline in plasma glucose levels. Since the half-life of insulin in the circulation is only a few minutes, the observed effects are initiated during the very first minutes. Prolonged effects on plasma glucose levels are due to long-lasting effects of insulin signaling and (lack of) influence of counter-regulatory mechanisms that act to raise plasma glucose levels (Cryer, 2004).

For the experiments in paper III, animals were fasted for 5 h in the morning, insulin (human) was injected intraperitoneally, and plasma glucose was measured from the tail vein using a hand-held glucose meter. We used a body weight-based insulin dose since this is the more conservative choice; heavier animals are likely to be more insulin resistant, and insulin resistance trumps a slight increase in insulin dose.

Hyperinsulinemic-euglycemic clamp and gluconeogenesis measurements

In paper III, hyperinsulinemic-euglycemic clamps were performed on HFD-fed C57BL/6J mice to measure endogenous glucose production (EGP) and whole body insulin sensitivity. The clamp was combined with measurements of the fractional (percent) contribution of gluconeogenesis to HGP to enable calculation of absolute gluconegenetic rate.

The hyperinsulinemic-euglycemic clamp was first applied to human subjects (DeFronzo et al., 1979) with the purpose of studying insulin sensitivity separate from the effect of endogenous insulin production. It is now considered the gold standard for assessing insulin sensitivity. The technique has been successfully applied to mice, but the need for cannulation of blood vessels in a small animal makes it a demanding technique to master. The clamp can be performed on conscious animals where cannulas have been permanently attached or in sedated mice directly after insertion of the cannulas. In paper III, we used sedated mice. The overall procedure for clamp on sedated mice is as follows. Fasted mice are sedated and cannulas/catheters are inserted into the carotid artery for administration of glucose and insulin and into the jugular vein for blood sampling. During a basal period, a glucose tracer is infused at a low rate to reach steady-state. Commonly used tracers are tritiated glucose (radioactive) and deuterium-labeled glucose (stable isotope); we used 6,6-$^{2}H_2$ glucose. When steady-state is reached, the concentration

of tracer glucose in the circulation is stable, since the tracer infusion rate is equal to the rate of tracer uptake to tissues. The only sources of glucose in the circulation are EGP (mainly from the liver), and tracer glucose infusion. Hence, from values of tracer enrichment (percent glucose tracer compared to non-labeled glucose) in the plasma, the total rate of glucose appearance (R_a) and basal EGP can be calculated as

R_a (mg/kg/min) = infusion rate of glucose tracer (mg/kg/min) / enrichment of tracer in plasma

EGP (mg/kg/min) = R_a (mg/kg/min) – infusion rate of glucose tracer (mg/kg/min)

In our work, we added steps to the standard clamp protocol to enable measurement of fractional gluconeogenesis during the basal period. The mice were given an *i.p.* dose of deuterium water (^2H_2O) 2 h into the fast to create an approximate 0.5% enrichment of deuterium in the body water, and this deuterium enrichment level was maintained by addition of 0.5% deuterium water to the drinking water and all infusion solutions. Because of reactions during gluconeogenesis, hydrogen (or deuterium) from water will end up on the carbons of any newly synthesized glucose molecule. Enrichment of deuterium on all carbons of glucose except carbon 2 will reflect gluconeogenesis (Landau et al., 1995; Rognstad et al., 1974), and will depend on the deuterium enrichment in the body water. Chacko and colleagues have developed a method for measuring the enrichment of deuterium on all glucose carbons except carbon 2 using gas chromatography – mass spectrometry (GCMS) (Chacko et al., 2008). In this way, fractional gluconeogenesis can be calculated as the average deuterium enrichment on the gluconeogenetic carbons relative to deuterium enrichment in body water (Chacko et al., 2008) such that

Fractional gluconeogenesis (%) = (average enrichment of deuterium on carbons 1,3,4,5,6,6) / (enrichment of deuterium in body water)

Gluconeogenetic rate (mg/kg/min) = fractional gluconeogenesis (%) $\times$ EGP (mg/kg/min)

Following the basal period (where we made some additions to the protocol), infusion of glucose tracer at a fixed rate continues. In addition, insulin is infused at a fixed rate (creating hyperinsulinemia), and unlabeled glucose is infused at a variable rate to maintain plasma glucose at a fixed, predetermined level close to the fasting plasma glucose concentration. Plasma glucose is measured at regular intervals (every 10 min), and the glucose infusion rate (GIR) is adjusted to maintain euglycemia. In insulin sensitive individuals, a higher infusion rate is required to maintain euglycemia in the face of hyperinsulinemia. When steady-state is reached, the total rate of glucose appearance (R_a) is equal to the rate of disappearance (R_d), which is the insulin-stimulated whole body glucose uptake. Glucose in the circulation derives from EGP, glucose tracer and unlabeled glucose. In the insulin-

stimulated state, the contribution of EGP to glucose appearance is reduced, and GIR is the major contributor to R_a/R_d. Accordingly, there is a strong correlation between GIR and R_d.

Microarray gene expression analysis

Microarray analysis of gene expression was employed in all papers included in this thesis. Microarray technology enables measurement of gene expression from thousands of genes simultaneously. This is made possible by the construction of a vast library of oligonucleotides (25-60 base pairs long) matching sequences in the transcripts to be analyzed. The oligonucleotides, referred to as probes, are attached to or synthesized directly onto a solid surface in a precise array pattern. A prerequisite for microarray analysis is that the sequence of the transcripts that are to be analyzed is known, since probes have to be designed beforehand. The microarrays used in this thesis were from two major manufacturers, Affymetrix and Agilent Technologies, and though the exact format and test procedures differ somewhat, the same general principle applies.

Intact RNA from tissue or cultured cells is reverse-transcribed to complementary DNA (cDNA) using a reverse transcriptase enzyme and oligo(dT) or random hexamer primers. The primers are linked at the 5' end to a promoter for the T7 RNA polymerase. Upon second strand synthesis following reverse transcription, the T7 promoter locates to the 3' end of the second strand and is in the right orientation for initiating transcription. In vitro transcription with T7 RNA polymerase generates anti-sense cRNA (i.e. RNA complementary to the input RNA). During this transcription step, cRNA is linearly amplified to more than 100 times the amount of input RNA. For the Agilent arrays, cRNA is fluorescently labeled with cyanin 3-conjugated nucleotides, fragmented into shorter cRNA stretches, and used directly for the hybridization.

The Affymetrix GeneChip Gene ST arrays introduce an additional step where cRNA is reverse-transcribed to sense DNA before hybridization. During this second reverse transcription, dUTP at a specific concentration is added together with dTTP to ensure incorporation at a specific ratio. The resulting sense DNA is treated with uracil DNA glycosylase and apurinic/apyrimidinic endonuclease 1, enzymes that together recognize and cut DNA at the dUTP sites to create fragmented DNA. The DNA fragments are labeled with biotin before hybridization, and biotin-targeted fluorescent staining of DNA is performed after hybridization. Following hybridization (and in the case of Affymetrix arrays, staining) where DNA fragments are allowed to bind to complementary probes on the array, the array is washed to

remove all reagents and DNA fragments except fragments specifically bound to the probes.

The arrays are scanned to record fluorescence, and the image is analyzed using specific software to determine signal intensity for each probe. The signal intensity reflects transcript abundance; however, all the steps involved in the procedure – RNA preparation, transcription, amplification, sample labeling, hybridization and more – introduce variation that will affect the outcome. The same sample analyzed on two different array slides will yield different result due to this technical variation. In order to compare samples from different arrays the results need to be normalized, which means processing the data to reduce the effect of non-biological variation. For the Affymetrix arrays, this is generally performed using Robust Multi-array Average (RMA). As a part of the RMA, quantile normalization is performed, which was also employed for the Agilent microarrays used in this thesis. In quantile normalization, probes with the same signal rank order (the nth strongest signal) will be given a new signal value that is the average value of probes with that rank order from all microarrays. Thus, the final signal distribution will be the same across all arrays. As a final step, log ratio transformation is generally applied to convert data to a linear scale.

A limitation with microarrays is the relatively low dynamic range. Gene expression levels are measured by fluorescent signal intensity, and the measurements are limited by background signal at the low end, and by signal saturation at the high end of the expression range. Reliable detection of low-abundance genes has been difficult to achieve, and microarrays tend to underestimate fold-changes in expression (Draghici et al., 2006). However, above their sensitivity threshold, microarray measurements accurately reflect the existence and direction of expression changes in approximately 70−90% of the genes (Draghici et al., 2006). Microarrays measure ratios more accurately than absolute expression levels (Draghici et al., 2006). However, an Agilent microarray with labeling reagent modification has proven capable of measuring fold-changes of the same magnitude as RT-qPCR (Yu et al., 2015), suggesting that Agilent microarrays exhibit less of a problem with dynamic range compared to arrays from other major manufacturers.

Gene co-expression network analysis

Properties of biological networks

Biological systems can be described as networks of macromolecules that interact. For example, the products of genes may be transcription factors that directly

regulate the expression of other genes, or may be proteins in a signaling cascade that ultimately regulates the expression of certain genes. In the case of gene expression, genes can be visualized as nodes connected by edges, where the edges represent co-regulation.

It has been demonstrated that biological networks of many different types – gene expression networks, protein-protein interaction networks, metabolic networks etc. – follow the same organizing principles (Carter et al., 2004; Jeong et al., 2000; Ravasz et al., 2002). These networks exhibit scale-free properties, which implies that the nodes are connected in a specific pattern; the absolute majority of nodes (genes, in co-expression networks) are connected to only a few other nodes, while a small number of nodes are connected to a large number of other nodes. "Scale-free" stands in contrast to random, exponential networks in which connections between nodes are randomly distributed, resulting in a similar number of connection for all nodes (Jeong et al., 2000). Scale-free networks follow a power law where the probability that a nod has k connections, $p(k)$, is $k^{-\gamma}$, γ being a constant. To give an example, for $\gamma=3$, the distribution of connections will look like this: all nodes will have at least one connection, but only 12.5% will have at least two connections. 3.7% of nodes will have three connections, 1.6% will have four connections, and as few as 0.1% will have 10 connections or more.

The wide prevalence of scale-free networks in nature suggests that their uneven organization is beneficial from an evolutionary point of view (Jeong et al., 2000). Indeed, scale-free networks have proven very robust, in the sense that random removal of nodes is unlikely to affect the function of the network (Albert et al., 2000). However, they are sensitive to targeted removal of the rare, highly connected nodes (Albert et al., 2000). Such highly connected nodes, termed hubs (Figure 6), are more likely to be essential than less connected genes (Carter et al., 2004).

Identifying gene modules of importance for T2D

Another property of biological networks is the clustering of nodes in modules (sub-networks; see Figure 6). The co-expressed genes in a module tend to participate in the same cellular processes, and modules are therefore enriched for genes with similar functions (Eisen et al., 1998; Stuart et al., 2003). Thus, identifying modules relevant to disease can help inform about the cellular processes that are disturbed.

In this thesis, we have used microarray gene expression data to identify gene modules of importance for T2D. In paper II, we identified a module in human islets related to T2D, and in paper III, we identified modules in liver related to hyperglycemia. The mathematical/statistical analysis (performed in R) was not part of this thesis work, but will be explained here in general terms.

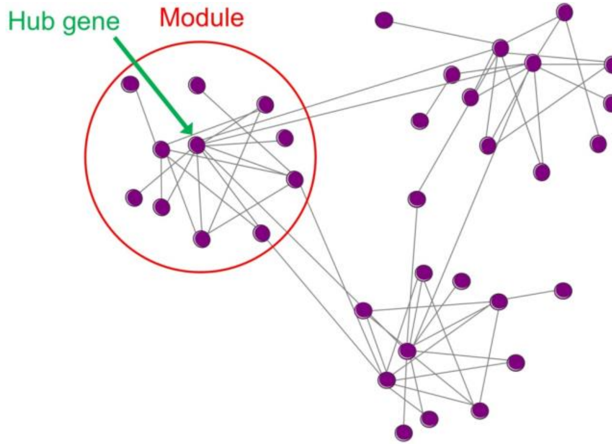

Figure 6. Schematic picture of the structure of a gene network, where the nodes represent genes and the connections indicate that the genes are co-expressed. A gene module and a highly connected hub gene are highlighted.

In gene co-expression networks, genes that exhibit similar expression pattern across samples are considered connected. The samples might reflect different time points, different treatments or, as in our case, different disease states. Co-expression was analyzed for all gene pairs in the microarrays using the absolute value of the Pearson correlation coefficient, which gives a correlation value between 0 and 1 (this is referred to as a *similarity measure*). All samples, diabetic and non-diabetic, were used for each gene pair comparison.

In typical networks, the connection between nodes is binary, meaning that a connection between two nodes either exists or does not exist (on or off). However, in gene co-expression networks, connections are not binary. Even though they can be made binary by selecting a threshold for correlation (Carter et al., 2004), such cut-off values are arbitrary, and the connection between a pair of genes with a correlation just below the threshold are likely to be of similar importance as connections just above the threshold. We have therefore used a weighted gene co-expression analysis, in which all gene pairs are assigned a *correlation weight* (Zhang and Horvath, 2005).

The correlation weight (referred to as *adjacency measure*) was calculated from the Pearson correlation coefficient using the power function $a_{i,j} = |\mathrm{cor}(x_i,x_j)|^{\beta}$, where $a_{i,j}$ is the adjacency measure for the connection between genes *i* and *j*, $|\mathrm{cor}(x_i,x_j)|$ is the absolute Pearson correlation coefficient between *i* and *j*, and β is the exponent. The value of β was determined so that the criterion of a scale-free network was met, as will be described below. The sum of all adjacency values for gene *i* is the *connectivity*, *k*, for gene *i* (Ravasz et al., 2002; Zhang and Horvath, 2005). In gene

networks with binary connectivity values, k represents the number of connections that a gene has with other genes, and that intuitive interpretation is valid also for weighted networks (Zhang and Horvath, 2005). For the model to be considered biologically relevant, it should give rise to a scale-free network where the connectivity distribution, p(k), follows a power law. This is tested by plotting, log-transformed p(k) against log-transformed k and evaluate the fit (R^2) of a linear trend line. Traditionally, R^2 is considered sufficient to assume scale-free properties (Zhang and Horvath, 2005). The value of parameter β in the adjacency function was consequently adjusted upward until a fit of R^2>0.8 was achieved.

Next, the adjacency values were used to calculate the *topological overlap*, *ω*, between each gene pair. Just like the adjacency measure, the topological overlap is a measure of connectivity, but in addition to measuring the connectivity between gene *i* and *j* ($a_{i,j}$ highly influences ω), it also takes into account the number of common neighbors that gene *i* and *j* have. The topological overlap was then converted into a *dissimilarity measure*, 1-ω, which was used for identification of modules. Using a topological overlap-based measure for module identification has proven to generate robust modules (Langfelder and Horvath, 2007). Modules were identified by performing *hierarchical clustering*. Hierarchical clustering groups genes based on distance (the dissimilarity measure is a type of distance), and the result can be visualized by a tree-graph where branches of the tree represent groups of genes, i.e. modules (Zhang and Horvath, 2005).

We next correlated gene expression of the identified modules with T2D status or phenotype traits such as hyperglycemia or insulin secretion. For that purpose, the *eigengene* of the modules was determined. The eigengene is the *first principal component* of the expression values for all genes in the module. By definition, the eigengene captures most of the variability in the data, and it can intuitively be understood as an imaginary gene that represents the expression of all genes in the module (Langfelder and Horvath, 2007). The module eigengene for all identified modules was correlated with T2D status and phenotype traits to find modules with relevance for T2D.

Calculations from clinical studies

Paper I and III include clinical studies with T2D patients. Insulin and glucose values from OGTT data were used to calculate the following indexes related to β-cell function and insulin sensitivity: the corrected insulin response (CIR), insulinogenic index (IGI), disposition index (DI), insulin sensitivity index (ISI), homeostatic model assessment of insulin resistance (HOMA-IR) and homeostatic model assessment of β-cell function (HOMA-B). In addition, waist circumference, BMI,

triglycerides and gamma-glutamyl transferase (GGT) levels were used to calculate fatty liver index (FLI) in paper III. The formulas used are listed below, with glucose and insulin values from time point 0 min (fasting) and 30 min during the OGTT. Glucose (G) is in mmol/L and insulin (I) in mIE/L.

The corrected insulin response (CIR):

$100 \times I_{30} / (G_{30}(G_{30} - 3.89))$

CIR measures first phase insulin secretion adjusted for plasma glucose values. This index proved to be efficient in predicting SNP-dependent effects on insulin release in individuals at increased risk for T2D (Herzberg-Schafer et al., 2010)

Insulinogenic index (IGI):

$(I_{30} - I_0) / (G_{30} - G_0)$

Like CIR, IGI measures first phase insulin secretion adjusted for plasma glucose values, but in contrast to CIR, it reflects the relative increase of insulin to glucose above baseline levels. IGI proved to be a strong predictor of progression of impaired glucose tolerance to T2D (Kadowaki et al., 1984).

Disposition index (DI):

$((I_{30} - I_0) / (G_{30} - G_0)) \times 1 / I_0$

The DI measures first phase insulin secretion (as IGI) corrected for insulin sensitivity. Low fasting insulin levels (I_0) is a measure of insulin sensitivity in individuals with normal β-cell function (Kahn et al., 1993). However, a weak insulin response in combination with reduced insulin sensitivity gives a low disposition index value, and is an early marker of inadequate β-cell compensation (Utzschneider et al., 2009).

Insulin sensitivity index (ISI):

$10\,000 / (G_0 \times I_0 \times G_{mean} \times I_{mean})^{½}$

The ISI was developed by Matsuda and DeFronzo as a measurement of whole body insulin sensitivity derived from OGTT data. It is highly correlated (r = 0.73) with the rate of whole-body glucose disposal during hyperinsulinemic-euglycemic clamp (Matsuda and DeFronzo, 1999).

HOMA-IR:

$G_0 \times I_0 / 22.5$

(The formula was originally presented as $I_0 \times (22.5 \times e^{-\ln(G0)})$.)

HOMA-IR assesses insulin resistance based on fasting insulin and glucose values. The formula above is derived from a computer-solved model that has been used to

predict the glucose and insulin concentrations resulting from varying degrees of β-cell deficiency and insulin resistance (Matthews et al., 1985). HOMA-IR correlated well with estimates of insulin resistance derived from hyperinsulinemic-euglycaemic clamp (R=0.88) (Matthews et al., 1985). However, whereas clamp IR mainly reflects peripheral insulin resistance, HOMA-IR mainly reflects hepatic insulin resistance (Katsuki et al., 2001), which is not surprising since it is based on fasting glucose levels, and impairments in fasting glucose mainly reflect elevated hepatic glucose production at higher fasting glucose levels (DeFronzo et al., 1992).

HOMA-B:

$(20 \times I_0) / (G_0 - 3.5)$

HOMA-B assesses percent remaining β-cell function based on fasting insulin and glucose values. The formula above is derived from the model by Matthews and collegues mentioned above (Matthews et al., 1985).

Fatty liver index (FLI):

$(e^{0.953 \times \text{log-e (triglycerides)} + 0.139 \times \text{BMI} + 0.718 \times \text{log-e (GGT)} + 0.053 \times \text{waist circumference} - 15.745}) /$

$(1 + e^{0.953 \times \text{log-e (triglycerides)} + 0.139 \times \text{BMI} + 0.718 \times \text{log-e (GGT)} + 0.053 \times \text{waist circumference} - 15.745}) \times 100$

Based on based on BMI, waist circumference, triglycerides and GGT, Bedogni and collegues developed an index which detects fatty liver (hepatic steatosis) with high accuracy (0.84, 95% CI 0.81–0.87). The index varies between 0-100, and a FLI ≥ 60 rules in the presence of fatty liver (Bedogni et al., 2006).

Key findings

Paper 1

The α2A-adrenergic receptor is overexpressed in islets from T2D patients carrying the risk allele of a single-nucleotide polymorphism, rs553668, in *ADRA2A*. Overexpression leads to reduced insulin secretion. We show that the α2A-adrenergic receptor antagonist yohimbine normalizes insulin secretion in risk allele carriers with T2D, whereas it is without effect in non-risk allele carriers. This is a proof-of-concept finding which shows that individualized, genotype-based treatment for T2D is possible.

Paper II

We identify a group of co-expressed genes (module) whose average expression is reduced in islets from T2D donors. The expression of the T2D-associated module also correlates with islet insulin secretion. Core genes of the T2D module has reduced expression in T2D patients, and the gene expression pattern is reminiscent of immature β-cells. We identify the transcription factor *SOX5* as a regulator of the T2D-associated module and show that Sox5 is important for β-cell function, including effects on *Mafa* expression, insulin processing, mitochondrial metabolism and L-type calcium channel expression. *SOX5* overexpression in human islets restores insulin secretion to levels of non-diabetic donors and increases the expression of β-cell differentiation markers.

Paper III

We employ gene co-expression network analysis to find new liver-targeting T2D drugs among already characterized compounds, and identify sulforaphane as a potential new drug for treating excessive glucose production from the liver. We show that sulforaphane reduces glucose production from H4IIE hepatoma cells via a mechanism that involves the transcription factor Nrf2 and reduced expression of gluconeogenetic enzymes. Sulforaphane improves glucose tolerance in animal models of diabetes and reduces gluconeogenetic rate in obese mice. In a small clinical study, we show that sulforaphane-rich broccoli sprout extract reduces fasting plasma glucose and HbA1c levels in obese patients with T2D and poor glycemic control.

Results summary and discussion

Paper 1

Genotype-based treatment of type 2 diabetes with an α_{2A}-adrenergic receptor antagonist

A genetic variant in *ADRA2A*, which encodes the α2A-adrenergic receptor (α_{2A}AR), was recently associated with T2D (Rosengren et al.). The A allele for a single-nucleotide polymorphism (rs553668) in *ADRA2A* is associated with reduced glucose-stimulated insulin secretion and increased risk of T2D. This risk allele is present in 30% of the normal population and in 40% of patients with T2D. Pancreatic islets from donors carrying the *ADRA2A* risk allele display an overexpression of α_{2A}AR and impaired insulin secretion. *In vitro*, the α_2AR antagonist yohimbine was shown to enhance insulin secretion in islets from risk allele carriers to levels similar to those in islets from non-risk allele carriers. We therefore hypothesized that blocking α_{2A}AR pharmacologically using yohimbine could improve insulin secretion in T2D patients carrying the risk allele.

Increased *ADRA2A* expression caused impaired insulin secretion in human islets from risk allele carriers with T2D

First, we aimed to determine if islet *ADRA2A* expression levels are increased in diabetic risk allele carriers, and if so, its effect on insulin secretion. The expression of *ADRA2A* in human islets from donors with T2D was determined by microarray, and glucose-stimulated insulin secretion was measured in islets from the corresponding diabetic donors. In islets from risk allele carriers, there was a trend towards overexpression of *ADRA2A* and also a trend towards decreased insulin secretion. *ADRA2A* expression correlated with insulin secretion, and analysis of causal relationship using a Causal Inference Test (Millstein et al., 2009) suggested that the level of insulin secretion in these diabetic islets was determined by the expression level of *ADRA2A*.

Design of the clinical study

Patients were recruited through ANDIS (All New Diabetics in Scania) (http://andis.ludc.med.lu.se).We aimed at achieving an equal distribution between rs553668 risk and non-risk carriers. Of the 47 patients who completed the study, 20 were non-risk, 20 were heterozygous risk, and 7 were homozygous risk allele carriers. The study was randomized and double-blind, involving three visits with two weeks in between. At each visit, the patients were given an acute dose of yohimbine (10 or 20 mg) or placebo orally 1 h before undergoing an OGTT. Based on analysis of 4681 individuals from the Botnia cohort where the *ADRA2A* risk variant was associated with impaired insulin secretion at 30 min during an OGTT (Ins30), we used Ins30 as the primary study variable. Since each patient received all three treatments (placebo, 10 mg yohimbine and 20 mg yohimbine), each patient was its own control in the analysis of the data.

Yohimbine increased insulin secretion in risk allele carriers but not in non-risk allele carriers

At baseline, the risk allele carriers (heterozygous and homozygous grouped) had a 25% reduction of glucose-stimulated insulin secretion (Ins30) compared to non-risk allele carriers. However, following yohimbine intake, Ins30 increased by 20% (10 mg) and 29% (20 mg) in the risk allele carriers. In contrast, yohimbine had no significant affect in the non-risk allele carriers.

The effect of yohimbine was dependent on the number of risk alleles

We next investigated whether there was any difference in yohimbine effect between heterozygous and homozygous risk allele carriers. Indeed, there proved to be a linear relationship between number of risk alleles and effect of yohimbine on Ins30, where a yohimbine dose of 20 mg increased Ins30 by 16% per risk allele. Similarly, intake of 10 mg of yohimbine increased Ins30 by 14% per risk allele. Thus, the stimulatory effect on Ins30 was dependent on both yohimbine dose and the number of *ADRA2A* risk alleles.

Yohimbine decreased glucose levels independent of *ADRA2A* genotype without affecting insulin sensitivity

Yohimbine (20 mg) significantly decreased fasting plasma glucose and glucose at 30 min during the OGTT in both risk allele and non-risk allele carriers. There was no difference in the effect of yohimbine between the genotype groups. Moreover, insulin sensitivity was not affected by yohimbine in either of the genotype groups. We also analyzed the yohimbine-mediated improved insulin response (Ins30) in relation to plasma glucose levels and insulin sensitivity using validated indices to assess β-cell function. We found that the corrected insulin response (CIR) was increased by yohimbine (20 mg) in both risk groups, while the insulinogenic index (IGI) and disposition index (DI) were increased specifically in risk allele carriers.

Yohimbine induced a stress response and affected levels of free fatty acids and branched chain amino acids

Yohimbine is known to induce a stress response, which has been suggested to involve α_{2A}AR in central brainstem neurons (MacMillan et al., 1996). Accordingly, yohimbine significantly increased noradrenaline levels in both genotype groups (86% increase in risk allele carriers and 105% increase in non-risk allele carriers). There was no difference in basal noradrenaline concentrations between patients with and without risk genotype. In addition, yohimbine (20 mg) increased systolic blood pressure by 7-8 mmHg in both risk and non-risk allele carriers.

We also analyzed plasma levels of free fatty acids and observed an increase in free fatty acids after yohimbine intake in both risk groups (57% increase in risk allele carriers and 74% increase in non-risk allele carriers). The α_{2A}AR inhibits lipolysis (Lafontan and Berlan, 1980), and the stimulating effect of yohimbine on free fatty acids levels is in agreement with its antagonistic effect on α_{2A}AR. Analysis of serum metabolites showed that fasting plasma levels of branched chain amino acids were decreased by on average 21% in risk allele carriers compared to non-risk allele carriers. These low levels of branched chain amino acids in the risk group were normalized after yohimbine (20 mg) intake.

Summary

a) Overexpression of the α2A-adrenergic receptor in islets from T2D patients carrying the risk allele rs553668 in *ADRA2A* leads to reduced insulin secretion

b) The α2A-adrenergic receptor antagonist yohimbine normalizes insulin secretion in risk allele carriers with T2D whereas it is without effect in non-risk allele carriers

c) The findings suggest that individualized, genotype-based treatment for T2D is possible

Discussion

We showed that blocking the α_{2A}-AR improves insulin secretion in T2D patients carrying the risk allele (overexpressing *ADRA2A*), but not in patients carrying the non-risk allele. The insulinogenic index (IGI), which measures first-phase insulin secretion, and the disposition index (DI), which measures first-phase insulin secretion corrected for insulin sensitivity, were both selectively improved in risk allele carriers. DI is considered the gold standard for measuring β-cell function (Defronzo, 2009), and hence these index values confirm the positive effect of yohimbine on islet function.

Yohimbine reduced plasma glucose levels in both risk allele and non-risk allele carriers. There was no difference in the glucose-lowering effect between the genotype groups, despite normalization of insulin secretion in the risk allele carriers. Interestingly, the decrease in plasma glucose occurred concomitantly with increased

levels of noradrenaline, a hormone that acts to mobilize glucose from body stores. The decrease in plasma glucose cannot be explained by the actions of insulin or glucagon, since insulin levels were unaffected in non-risk allele carriers and glucagon levels were unaffected in both risk groups. Future studies on yohimbine might bring clarity about the cause for these complex effects.

Taken together, the findings presented in this paper indicate that individualized, genotype-based treatment for T2D is possible. The prospects of genotype-based T2D are exciting, but genotype-targeting treatment of *ADRA2A* risk allele carriers in the clinic is not yet practically feasible. The side effects of yohimbine, such as a noradrenaline stress response, increased systolic blood pressure, elevations in circulating free fatty and feelings of anxiety are too severe. In addition, yohimbine has a short half-life (0.6±0.3 hours) which makes it less suitable for continuous α_{2A}-AR blockage. Considering that not only Ins30 (reflecting the situation after a meal) but also fasting insulin was reduced in risk allele carriers, long-lasting blockage of the α_{2A}-AR is probably to prefer. For these reasons, yohimbine is not the ideal α_{2A}-AR antagonist for T2D treatment. Future studies will have to determine whether an α_{2A}-AR antagonist with greater specificity, longer half-life, and less adverse effects can have clinically beneficial effects.

Paper II

Sox5 regulates β-cell phenotype and is reduced in type 2 diabetes

In an attempt to find new genes of relevance for T2D, we analyzed gene expression in islets from non-diabetic and T2D donors using gene co-expression network analysis. Network methods have proven valuable for studying how larger groups of genes interact and cause disease (Chen et al., 2008; Zhang and Horvath, 2005). We first analyzed microarray gene expression data from islets from 64 human donors to identify groups of co-expressed genes (“modules”). We then used the module eigengene (the first principal component of the module gene expression), where the eigengene represents the gene expression of the entire module, to correlate modules with T2D traits.

A gene co-expression module (“T2D module”) correlated with T2D and insulin secretion

The eigengene of one of the modules, representing a group of 3032 genes, was downregulated in T2D islets. Moreover, this disease-associated module (“T2D module”) correlated negatively with donor HbA1c and positively with insulin secretion from the donor islets.

Genes with islet-specific open chromatin ("T2D signature") were at the center of the T2D module

The T2D module contained many genes with known β-cell function. Gaulton and colleagues recently published data over genes with islet-specific open chromatin (Gaulton et al., 2010), suggesting that these genes have tissue-specific functions and may be especially important for β-cell function. Interestingly, the T2D module was highly enriched for genes with islet-specific open chromatin, to the degree that 168 of the 340 genes with islet-specific open chromatin were found in the T2D module. These 168 genes were at the center of the T2D module (based on connectivity), and correlated even more strongly to T2D status and insulin secretion than the average gene in the module. We decided to use these 168 genes as a "T2D signature" to gain information about T2D pathophysiology.

The T2D signature was reminiscent of immature β-cells

We compared the T2D signature to >8100 gene expression data sets that were publically available at the time. The data set with gene expression changes that most closely resembled the T2D signature were from an attempt to dedifferentiate human islet cells. Moreover, sorted human β-cells (identified by *PDX1* expression) with low insulin expression compared to high insulin expression (considered less mature β-cells (Szabat et al., 2011)) had a gene expression pattern similar to the T2D signature. This suggested that the T2D signature, and by extension the T2D module, resembles the gene expression profile in immature β-cells.

The transcription factor *SOX5* – a potential regulator of the T2D module

We hypothesized that regulators of the T2D module could be important for β-cell function. We therefore attempted to find such regulators, and found nine candidate genes by focusing on:

a) Genes with transcription factor binding sites in the T2D signature genes

b) Genes that cause expression changes similar to the T2D signature when knocked down (publically available microarray data)

c) Genes with single nucleotide polymorphisms (SNPs) associated with the module eigengene

d) Genes most highly correlated to the T2D signature

The expression of seven of these nine candidate genes was reduced in rat islets incubated for 48 h with high palmitate or high glucose to simulate a diabetic milieu. Next, we knocked down the candidate genes in INS-1 832/13 cells using siRNA and assessed the effect on glucose-stimulated insulin secretion. Knockdown of the transcription factor *Sox5* decrease glucose-stimulated insulin secretion by 50%. We

also found that the expression of Sox5 mRNA and protein was reduced in islets from human T2D donors compared to non-diabetic donors.

Sox5 knockdown in INS-1 832/13 cells impaired insulin secretion, altered metabolism, and reduced the expression of L-type calcium channels

We decided to further examine the effects of *Sox5* knockdown in INS-1 832/13 cells to gain information about its mechanism of action. *Sox5* knockdown led to a wide range of effects (see Figure 7), including:

- Reduced expression of *Mafa* and *Pdx1* mRNA and reduced expression of MAFA protein
- Reduced glucose-stimulated insulin secretion in the range of 5-16.7 mM glucose
- Increased ratio of secreted proinsulin relative to insulin
- Reduced secretion in response to the mitochondrial substrates L-leucine and α-ketoisocaproic acid, indicating that the insulin secretion defect is downstream of glycolysis
- Reduced insulin secretion in response to tolbutamide and high potassium (both of which cause depolarization of the β-cell), pointing to an insulin secretion defect downstream of the K_{ATP} channel
- Reduced oxygen consumption rate in response to both glucose and pyruvate (a mitochondrial substrate), indicating a problem of metabolism at the mitochondrial level
- Accumulation of early glycolytic intermediates and a reduction of the Krebs cycle intermediate fumarate, suggesting a perturbation in the mitochondrial shuttles and a shift in the balance between aerobic and anaerobic metabolism
- Reduced early exocytosis and reduced charge entry upon depolarization (measured by patch-clamp)
- Reduced calcium entry (measured by Fluo-5F fluorescence)
- Reduced L-type calcium currents (currents not blocked by isradipine)
- Reduced expression of *Cacna1c* mRNA, and reduced expression of the L-type calcium channels $Ca_V1.2$ and $Ca_V1.3$ on the protein level

Overexpression of _Sox5_ improved insulin secretion

Having analyzed the effects of *Sox5* knockdown, we also overexpressed *Sox5* in INS-1 832/13 cells using plasmid transfection and observed increased protein expression of $Ca_V1.2$ and increased exocytosis in these cells. Moreover,

overexpression of *Sox5* in rat islets using lentivirus led to increased glucose-stimulated insulin secretion.

Opposite effects of *Sox5* knockdown and *Sox5* overexpression on the T2D module

We analyzed gene expression in INS-1 832/13 cells with *Sox5* knockdown or overexpression by microarray, and found that both treatments significantly affected genes in the T2D module (1.4-1.5-fold more genes significantly changed than expected by chance). Gene expression in *Sox5*-kd cells resembled the changes seen in the T2D module in diabetic patients. Notably, the changes induced by *Sox5* knockdown and *Sox5* overexpression were in opposite directions.

Sox5 expression was perturbed in animal models of diabetes

To explore the role of *Sox5* in the pathophysiology of diabetes, we examined the expression of *Sox5* and its relation to the T2D module in islets from mouse models of diabetes with different stages or degrees of diabetes. *Sox5* expression was reduced already in 4 weeks old *db/db* mice that had not yet developed hyperglycemia, suggesting that *Sox5* is involved in the pathophysiology of diabetes. *Sox5* expression was not affected in 6 weeks old *ob/ob* mice, but was reduced in 13 weeks old *ob/ob* mice. At this age, the mice are still normoglycemic but insulin resistant and have increased β-cell demand (Chan et al., 2013). In the models where *Sox5* expression was decreased, a significant fraction of the T2D module genes were perturbed.

Sox5 expression was regulated by the transcription factor *Yy1* and was increased by the drug valproic acid

We next aimed to gain information about the regulation of *SOX5*, and therefore analyzed transcription factor binding sites in the vicinity of *SOX5*. We identified 39 putative regulators, and silenced each of these 39 genes in INS-1 832/13 cells. Silencing of the transcription factor Yin Yang 1 (*Yy1*) led to reduced *Sox5* expression and reduced insulin secretion, suggesting that *Sox5* expression in INS-1 832/13 cells is in part regulated by *Yy1*. *Yy1* expression was in turn significantly reduced by palmitate treatment.

Since the expression of *SOX5* and genes with islet-selective open chromatin was reduced in the T2D state, we wanted to examine the effect of a modulator of open chromatin on gene expression. For this purpose, we used the histone deacetylase (HDAC) inhibitor valproic acid (VPA), as it has been well characterized in terms of dosing and kinetics in other tissues. VPA dose-dependently increased *Sox5* expression and increased expression of genes in the T2D module. Moreover, VPA dose-dependently increased glucose-stimulated insulin secretion in INS-1 832/13 cells. A Casual Inference Test (Millstein et al., 2009) suggested that the stimulating effect of VPA on insulin secretion was dependent on *Sox5* expression. VPA also increased *Sox5* expression and insulin secretion in mouse islets.

SOX5 knockdown in the human β-cell line EndoC-BH1 reduced insulin secretion, and *SOX5* overexpression in human islets increased insulin secretion

We silenced *SOX5* in the human β-cell line EndoC-BH1 and observed a 29% decrease in glucose-stimulated insulin secretion. Finally, we overexpressed *SOX5* in human islets using lentivirus, and assessed the effects on insulin secretion and gene expression. *SOX5* overexpression increased glucose- and potassium-stimulated insulin secretion, and the increase was most pronounced in islets from T2D donors. In the non-diabetic islets, insulin secretion was not significantly increased, whereas in the diabetic islets, insulin secretion was increased by 18% and restored to the levels of non-diabetic donors. The expression of several markers of differentiated human β-cells (*GAD2, GLP1R, MAFA, PCSK1, PDX1* and *SLC2A2*) was upregulated following *SOX5* overexpression, suggesting that *SOX5* promotes a differentiated (mature) β-cell phenotype (Figure 7).

Summary

a) A group of co-expressed genes ("T2D module") is reduced in islets from T2D donors and correlates with HbA1c and insulin secretion

b) The expression of genes at the core of the T2D module is reminiscent of the gene expression pattern in immature β-cells

c) The transcription factor *SOX5* is a regulator of the T2D module and is important for β-cell function, including effects on Mafa expression, mitochondrial metabolism, L-type calcium channel expression and insulin processing

d) *SOX5* overexpression in human islets restores insulin secretion to the levels of non-diabetic donors and increases the expression of β-cell differentiation markers

Discussion

We identified the transcription factor *SOX5* as new gene with relevance for β-cell phenotype and function based on it being a possible regulator of the T2D module. Of our nine initial candidates for regulating the T2D module, only *Sox5* had a significant effect on insulin secretion in INS-1 832/13 cells when silenced. This does not necessarily mean that the other candidate genes are without importance in *human* β-cells. It is also possible that the importance for β-cell function is only evident under conditions of metabolic stress, as in the case of Foxo1 (Talchai et al., 2012). Most likely, there are several important regulators of the T2D module, and *SOX5* is one of those.

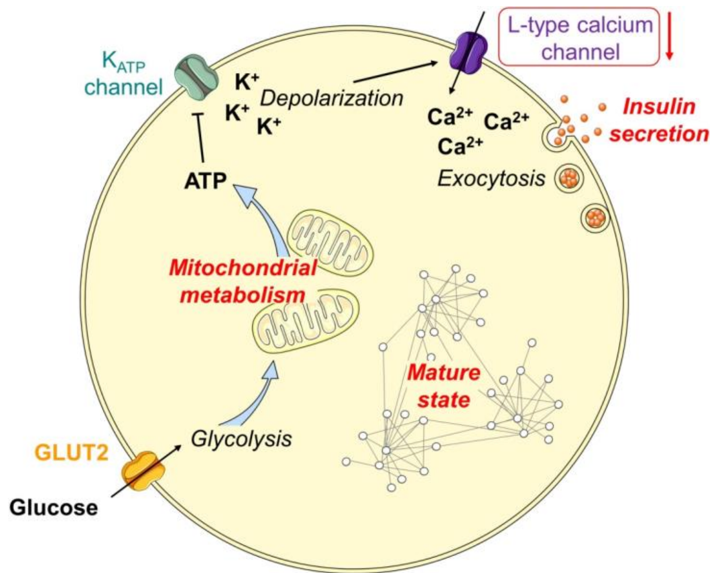

Figure 7. Summary graphics of how *Sox5/SOX5* expression affects the β-cell. The cellular processes that are disturbed upon *Sox5* knockdown or improved upon SOX5 overexpression are marked in red.

SOX5 has not previously been implicated in the pathophysiology of T2D, but is known to be involved in chondrogenesis and neurogenesis (Lefebvre, 2010). A highly related protein belonging to the same group of SoxD transcription factors, Sox6, has been shown to negatively affect insulin secretion and β-cell proliferation in β-cell lines (Iguchi et al., 2005; Iguchi et al., 2007). This is in marked contrast to the function of Sox5 in β-cells, and our study thus adds an important piece of knowledge about the diverse roles of Sox transcription factors, of which several are involved in the development of the pancreas (Lioubinski et al., 2003).

We discovered that an attempt to dedifferentiate human islets cells showed a similar expression pattern as the T2D signature. A similar pattern was also seen in human β-cells with low insulin expression, indicating an immature phenotype. These observations introduced us to the idea of β-cell dedifferentiation as a mechanism behind β-cell failure. The loss of a differentiated state is related to impaired β-cell function, a well-known feature in T2D, but an exact definition of dedifferentiation has not yet been established. A phenotype described as dedifferentiated by Talchai and colleagues was the loss of expression of insulin, PDX11, MAFA and FOXO1, combined with gain of expression of NGN3, NEUROG, OCT4 and L-MYC (Talchai et al., 2012). There was no induction of *NGN3*, *NANOG* or other developmental progenitor markers in the T2D islets, although we cannot exclude

that these markers could be expressed in a small subset of cells. However, there was reduced expression of key β-cell differentiation markers such as *GAD2*, *GLP1R*, *MAFA*, *NKX6-1*, *PCSK1*, *PDX1*, *SLC2A2* and *SLC30A8*. The state of the T2D islets in our study may therefore be described as a state of β-cell immaturity or partial loss of β-cell identity.

Care should be taken when investigating mechanisms related to dedifferentiation in cell lines. However, knockdown of *Sox5* in INS-1 832/13 cells replicated a significant part of the gene expression changes seen in human T2D islets, suggesting that this model can provide information relevant to human disease. We confirmed the relevance of *SOX5* for β-cell-specific gene expression and insulin secretion in human islets. To explore the effect of open chromatin modification, we used the HDAC inhibitor valproic acid (VPA). As assessed by a Casual Inference Test, the effect of VPA was dependent on *Sox5* in the INS-1 832/13 cells. However, the *Sox5*-mediated effect of VPA may be highly dose-dependent or restricted to rodent β-cells, because we did not see any change in *SOX5* expression in human islets treated with VPA (data not shown).

Although *SOX5* currently cannot be directly targeted for T2D therapy, the demonstrated relevance of this transcription factor for the pathophysiology of T2D is a finding that may aid future diabetes research. Moreover, our results corroborate the usefulness of gene co-expression network analysis as a means to identify genes of relevance for disease, and to gain information about underlying disease mechanisms. An increasing amount of data, including the ones presented in this work, support the view of "loss of differentiation" as a cause of β-cell failure (Cinti et al., 2016; Spijker et al., 2015), which has exciting implications for the development of new T2D therapies.

Paper III

Sulforaphane reduces hepatic glucose production and improves glucose control in patients with type 2 diabetes

We here aimed to identify compounds to treat excessive hepatic glucose production in patients with type 2 diabetes (T2D), since this is a severe clinical problem. It is possible that compounds already exist that could possibly be used for this purpose but have not yet been identified. Drug repositioning implies using existing drugs for new implications. In this work, we employed gene co-expression network analysis as a strategy for drug repositioning in an attempt to find new T2D drugs targeting the liver among already characterized compounds.

We first used gene expression data from livers from a large mouse cross characterized for metabolic traits (Chen et al., 2008; Wang et al., 2006) to identify groups of co-regulated genes ("gene modules") associated with hyperglycemia. We used 1720 genes from four such gene modules to construct a 50-gene "disease signature". The hepatic disease signature was used to interrogate publically available microarray data covering 3852 treatments with various compounds. The gene expression profiles in the microarray data sets (i.e. the gene expression patterns induced by the compounds) were compared to the hepatic disease signature. We hypothesized that compounds that induced gene expression changes in the opposite direction to the hepatic disease signature could potentially affect the clinical phenotype. The compounds were therefore ranked based on their dissimilarity to the hepatic disease signature. The highest ranked compound was sulforaphane (SFN). It is a naturally occurring isothiocyanate found in cruciferous vegetables such as broccoli. We next proceeded to evaluate the biological effects of SFN.

Sulforaphane reduced glucose production in the liver cell line H4IIE and in mouse hepatocytes

We first wanted to test the effect of SFN on glucose production. As a model for examining liver glucose production, we used the rat liver cell line H4IIE. Preincubation with SFN for 24 h decreased glucose production from H4IIE cells in a dose-dependent manner with an effect size similar to that of metformin. Moreover, in both H4IIE cells and primary mouse hepatocytes, SFN prevented palmitate-induced increase in glucose production. We showed that SFN induces nuclear translocation of the transcription factor *Nrf2* in H4IIE cells, and by silencing *Nrf2* we demonstrated that a significant part of the effect of SFN on glucose production is mediated by *Nrf2*. Since insulin is one of the main regulators of hepatic glucose production, we analyzed insulin-mediated phosphorylation of IRS-1 and Akt1. However, SFN did not affect phosphorylation of these enzymes. SFN also did not affect mitochondrial oxygen consumption.

Sulforaphane reduced the expression of enzymes involved in gluconeogenesis

Gluconeogenesis is one of two major pathways for glucose production from the liver. In microarray data from H4IIE cells, we noticed that three out of the four key enzymes involved in gluconeogenesis were downregulated in response to SFN (*Pck1*, *Fbp1* and *G6pc*, but not *Pc*). These findings were confirmed by RT-qPCR. PCK1 protein levels were reduced by 60% in H4IIE cells after treatment with SFN. This reduction was largely prevented by *Nrf2* knockdown, consistent with the importance of Nrf2 in mediating the effects of SFN. Silencing *Pck1* with siRNA in H4IIE cells resulted in a 38% reduction in glucose production and diminished the inhibiting effect of SFN on glucose production. Based on these results, we suggest that an important mechanism for SFN-mediated reduction of glucose production is downregulation of key gluconeogenetic enzymes via Nrf2.

Sulforaphane prevented the development of glucose intolerance in rats

We next wanted to assess the ability of SFN to prevent the development of glucose intolerance *in vivo*. Male Wistar rats on 45% high-fat diet (HFD) were injected with SFN (2.5 mg/kg *i.p.* three times per week) or vehicle for 15 weeks. After 15 weeks, the vehicle-treated rats had significantly increased fasting plasma glucose, whereas plasma glucose in the SFN-treated rats remained unchanged. Insulin sensitivity measured by an IPITT was also higher in the SFN-treated rats.

The rats were then divided into new groups and challenged with 60% HFD versus 60% high-fructose diet (HFrD). After 5 weeks, the SFN-treated rats on 60% HFD showed no difference in glucose tolerance (assessed by an IPGTT) compared to vehicle-treated controls, but had better insulin sensitivity as assessed by an *i.p.* ITT. The SFN-treated rats on 60% HFrD showed improved glucose tolerance compared to vehicle-treated controls.

The hepatic disease signature was reversed by sulforaphane

Analysis of liver gene expression in the rats treated with SFN for 20 weeks confirmed that a significant fraction of genes in the hepatic disease signature was changed by SFN.

Sulforaphane improved glucose tolerance and reduced hepatic glucose production in rats to the same degree as metformin

To test whether SFN could improve glycemic control in rats that had already developed glucose intolerance, male Wistar rats were fed 60% HFD for 11 months and then treated with 5 mg/kg SFN *i.p.* daily for 14 days. Treatment with SFN improved glucose tolerance, assessed by an OGTT.

We next compared the effects of SFN and metformin on glucose tolerance and hepatic glucose production *in vivo*. Male Wistar rats that had been fed a 60% HFrD for 6 months were treated with either 10 mg/kg SFN *i.p.* or 300 mg/kg metformin *p.o.* for 9-12 days. Glucose tolerance, assessed by an OGTT, was significantly improved by both SFN and metformin.

Hepatic glucose production was predicted to be decreased in both SFN- and metformin-treated rats, and was assessed by an *i.p.* pyruvate tolerance test (IPPTT). SFN reduced plasma glucose during the IPITT (AUC 30-120 min) by 20% and metformin by 25%. These results show that SFN improves glucose tolerance and reduces hepatic glucose production in rats to the same degree as metformin.

Sulforaphane improved glucose tolerance in mice with diet-induced diabetes

To test the effect of SFN in a more severe model of diabetes, we used C57BL/6J mice, which develop hyperglycemia and severe glucose intolerance when challenged with a high-fat diet. Male C57BL/6JBomTac mice fed 60% HFD for 10

weeks were treated with either 0.5 or 10 mg/kg SFN *i.p.* for 4 weeks. The high dose of SFN improved both fasting glucose and glucose tolerance as measured by an IPGTT, whereas the low dose was without effect. SFN did not affect insulin sensitivity in this animal model.

Sulforaphane reduced gluconeogenesis in obese mice

We next performed measurements of absolute gluconeogenetic rate combined with hyperinsulinemic-euglycemic clamp in SFN-treated male C57BL/6J mice on 60% HFD. There was no difference in gluconeogenetic rate when considering the results for all the mice. However, when analyzing the animals with the highest body weight in each group, there was a significant decrease in gluconeogenetic rate in the SFN-treated mice compared to vehicle-treated controls. Insulin sensitivity, as measured by total body insulin-stimulated glucose uptake (Rd clamp), was similar between the groups, which parallels our findings in H4IIE cells that SFN does not influence insulin signaling.

Sulforaphane-rich broccoli sprout extract improved fasting glucose and HbA1c in obese patients with dysregulated T2D

Finally, we tested the effects of SFN on glucose control in T2D patients in a randomized, double blind study. For this purpose, we used broccoli sprout extract (BSE), which contains high amounts of a precursor of SFN, glucoraphanin. Totally 103 patients with T2D were recruited and given either BSE (containing 150 µmole SFN per dose) or placebo to consume daily for 12 weeks. Glucose tolerance was assessed by an OGTT before and after the treatment period, and metabolic parameters were analyzed. The primary study variables were fasting plasma glucose and HbA1c. Of the included patients, 97 completed the study. In obese patients (BMI>30 kg/m^2) with poor glycemic control ("dysregulated T2D"; HbA1c>50 mmol/mol), in total 17 patients, BSE-treatment significantly decreased fasting plasma glucose and HbA1c. BSE had no effect on non-obese patients or patients with well-regulated T2D. Moreover, in these patients, the effect of BSE on fasting plasma glucose was strongly correlated to the plasma concentration of SFN.

Summary

a) Using gene co-expression network analysis and a method for gene expression profile similarity detection, we identify sulforaphane as a potential new drug for treating excessive hepatic glucose production

b) Sulforaphane reduces glucose production from the H4IIE liver cell line in a mechanism that involves the transcription factor Nrf2 and reduced expression of gluconeogenetic enzymes

c) Sulforaphane improves glucose tolerance in animal models of diabetes and reduces gluconeogenetic rate in obese mice

d) Sulforaphane-rich broccoli sprout extract reduces fasting plasma glucose and HbA1c in obese T2D patients with poor glycemic control

Discussion

Excessive glucose production from the liver is one of the characteristics of T2D, and its contribution to disease is particularly significant in obese T2D patients (DeFronzo et al., 1989; Gastaldelli et al., 2000; Gastaldelli et al., 2004). Hepatic glucose production is strongly correlated with fasting plasma glucose, and is derived entirely from gluconeogenesis in the fasted state (Defronzo, 2009). A compound affecting hepatic glucose production would therefore be expected to primarily benefit obese T2D patients with elevated fasting plasma glucose. That proved to be the case in our study; BSE as a source of SFN improved HbA1c and fasting glucose, but only in a subset of patients with obesity and poor glycemic control.

Today, metformin is the first-line drug for treatment of T2D, and one of its primary effects is reduction of hepatic glucose production (Hundal et al., 2000). However, many patients treated with metformin develop nausea or digestive disturbances, and around 5% of the patients are therefore unable to continue with metformin (Garber et al., 1997). SFN is considered non-toxic and few side effects have been reported from BSE intake (Brown et al., 2015; Egner et al., 2014; Kensler et al., 2005). However, the total number of patients treated with BSE is too small to determine its effect on rare, severe adverse events.

In the study presented here, all patients except three (well-regulated) were on metformin treatment. The fact that we saw improved glycemic control in obese patients with dysregulated T2D on top of metformin suggests that the mechanism whereby SFN regulates hepatic glucose production differs from that of metformin. If SFN were to be used in the clinic, it would most likely be as a second-line or third-line treatment on top of metformin. It is therefore relevant to compare the effects of we observed with SFN with effects of other T2D drugs on metformin background.

It is well documented that the effect of glucose-lowering treatment increases with the severity of dysregulation (Ferrannini et al., 2009; Flory et al., 2014; Nauck et al., 2007) so that a higher baseline HbA1c is associated with a larger response. Flory and colleagues showed that a 1% increase in baseline HbA1c (percentage units) translated to approximately 0.5% greater decrease when sulfonylurea, thiazolidinediones or DPP-4 inhibitors were added to metformin therapy (Flory et al., 2014). We saw a 0.34% reduction in HbA1c (from 7.37% to 7.04%) with BSE treatment in the obese patients with dysregulated T2D. This effect should be compared to that of T2D drugs in metformin add-on studies with similar patient baseline characteristics, and preferably of similar duration (12 weeks). A summary of studies, where reduction of HbA1c three months after a second-line treatment

had been added to metformin therapy was reported, is listed in the NICE guidelines (NG28) 2015, appendix J. A subset of these studies had patients with baseline characteristics similar to the obese patients with dysregulated T2D in or study. The DPP-4 inhibitor vildagliptin (100 mg daily) gave an HbA1c reduction of 0.53% at 3 months in patients with a baseline HbA1c of 7.31% (Ferrannini et al., 2009), and a reduction of 0.51% in a 24-week study with baseline HbA1c 7.4% (Filozof et al., 2010). After 3 months of treatment with another DPP-4 inhibitor, sitagliptin (100 mg daily), HbA1c was reduced with 0.57% (from 7.48% at baseline) in one study (Nauck et al., 2007) and with 0.43% (from 7.5% at baseline) in another study (Arechavaleta et al., 2011). The HbA1c reduction achieved with DPP-4 inhibitors in these studies is comparable to the effect we saw with BSE.

In the T2D patients, we did not detect any difference in insulin sensitivity (measured by HOMA-IR and Insulin Sensitivity Index values from the OGTT) following BSE treatment, suggesting that the effect on fasting plasma glucose and HbA1c in obese patients with dysregulated T2D was entirely due reduced hepatic glucose production. Similarly, in C57BL/6JBomTac mice on HFD, SFN improved fasting glucose and glucose tolerance (measured by an IPGTT) without significant effects on insulin sensitivity (measured by an IPITT). In the group of C57BL/6J mice on HFD subjected to hyperinsulinemic-euglycemic clamp combined with measurement of gluconeogenetic rate, we saw a significant effect of SFN on gluconeogenetic rate only in the animals with the highest body weight. The mice subjected to clamp had a lower average body weight compared to the mice subjected to the IPGTT, which would explain why we only saw an effect in the heaviest subgroup - similar to the situation in the T2D patients. However, in Wistar rats, SFN was able to prevent the development of insulin resistance (measured by an IPITT), and thus it seems that SFN may have additional effects on insulin sensitivity in target organs such as skeletal muscle or adipose tissue, and that these effects could be species-dependent.

Taken together, SFN is a promising treatment option, especially for obese patients with dysregulated T2D, and could potentially be used as a complement to metformin therapy to improve glycemic control. Our study also suggests that functional food based on broccoli sprout extract (BSE) is an effective way to raise SFN plasma levels to therapeutic concentrations.

Future perspectives

In this thesis, we have explored ways to improve the treatment for T2D patients and tried to attain greater understanding of the pathophysiologic mechanisms underlying T2D. We showed that an antagonist to the adrenergic receptor 2α, yohimbine, improved insulin secretion specifically in patients with a risk variant of the *ADRA2A* gene. Even though yohimbine is not an option for treatment of T2D patients in the clinic, the result of the study is still of great interest since it points to the possibility of individualized, genotype-based medicine in the future. For a small group of patients with monogenic diabetes, genotype-based treatment is already a reality. Individuals with activating mutations in *KCNJ11*, encoding the K_{ATP} channel subunit Kir6.2, can be effectively treated with sulfonylureas (Pearson et al., 2006). However, T2D is a polygenic disease and presents different challenges. While T2D has a clear genetic component, the contribution of each individual risk variant is typically small (Drong et al., 2012). Future genotype-based T2D treatment may therefore consist of drugs that have a generally favorable effect on one or several of the aspects of T2D (β-cell function, β-cell preservation, insulin resistance etc.) but in addition are especially effective for a subgroup of patients with a certain genotype. As an example, ‘t Hart and colleagues showed that T2D patients with the G allele of the single nucleotide polymorphism rs7202877 responded less effectively to DPP-4 inhibitors compared to non-G allele carriers; the difference in glucose-lowering effect was 0.51% HbA1c (t Hart et al., 2013). Clinical trials of T2D drugs coupled with genotype scanning could help reveal genotype-based variations in drug effects. However, individualized treatment does not necessarily mean genotype-based treatment. Focus on pathophysiological mechanisms may be more effective. Blocking α_{2A}AR could be helpful for all patients with increased adrenergic tone, not just ADRA2A risk allele carriers.

The topic of expanding the drug arsenal for T2D is relevant, since we here present data suggesting that the naturally occurring compound sulforaphane could be used to treat T2D. We acknowledge that we could only see an effect of SFN in a subgroup of patients – those who were obese and had an HbA1c values above 50 mmol/mol (6.7%). However, this is not a rare phenotype in T2D patients. It is our hope that this small clinical study will be follow by other, larger trials, perhaps consisting of head-to-head comparisons of SFN with established glucose-lowering drugs. We showed that supplement with BSE is an effective way to raise plasma levels of SFN

to therapeutic concentrations. The use of pure sulforaphane is another alterative that would allow intake of smaller volumes of drug. The possibility to take SFN as a food supplement is exciting. SFN has several benefits as a T2D therapy. Its antioxidative effects have prompted studies on its effect on vascular health, where it has shown to reduce inflammatory markers (Kim et al., 2012; Wu and Juurlink, 2001) and prevent nephropathy in animal models (Zheng et al., 2011). Accordingly, in addition to the glucose-lowering effects demonstrated in this thesis, SFN may also prevent complications of T2D, which would make it a potent future therapy.

Another interesting finding was the similarity of the T2D signature to immature β-cells. To date it is still unclear whether proper β-cell dedifferentiation takes place in humans or not, but the results presented in this thesis and by others point to loss of a differentiated state in T2D. Treatments that promote a differentiated β-cell state could therefore provide important additions to the future therapeutic arsenal. The attempt to dedifferentiate human islet cells, which led to a similar gene expression profile as the T2D signature, was part of an effort by Kutlu and colleagues to expand human islet cells for islet transplantations (Kutlu et al., 2009). The problem they faced was that when they managed to successfully expand β-cells, i.e. make them proliferate, the cells also lost their mature state as shown by strongly reduced expression of the islet-enriched transcription factors *ISL1*, *NEUROD1*, *NKX2–2*, and *PAX6*. Kutlu and colleagues tried differentiation protocol to make the immature cells regain maturity, but were unsuccessful (Kutlu et al., 2009). However, such efforts may eventually succeed, and may be of great value not only for islet transplantations but also for the development of “differentiation drugs” for T2D patients.

Conflict of interest

Annika Axelsson and Anders Rosengren (SE1251306-5, US-patent no US9,597,307 B2) are inventors on patent applications (SE1251306-5, US9,597,307B2, and EU2919775) submitted by Lund University that cover the use of sulforaphane to treat exaggerated hepatic glucose production. The rights to use this patent have been licensed to Lantmännen AB, an agricultural cooperative owned by Swedish farmers. Lantmännen AB provided the BSE and placebo for the clinical study presented in paper III and Lantmännen Research Fund financed part of the study. However, this academic thesis and the corresponding paper was sponsored by Lund University, and Lantmännen AB had no influence whatsoever on study procedures, data analysis or the interpretation of the data in the manuscript.

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