
SJAAM JAINANDUNSING
THE FINAL LAUNCH
UNDERSTANDING A CHANGED BETA CELL DYNAMICS IN T2D
THROUGH INSULIN SYNTHESIS MEASUREMENTS *IN VIVO*

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COLOFON

The Final Launch: Understanding a Changed Beta Cell Dynamics in T2D through Insulin Synthesis Measurements *in Vivo*

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THE FINAL LAUNCH:
Understanding a Changed Beta Cell Dynamics in T2D through Insulin Synthesis Measurements *in Vivo*

DE FINALE LANCERING:
Het beter begrijpen van een veranderde bètacel dynamiek in DM type 2 middels insuline synthese metingen *in vivo*

T H E S I S

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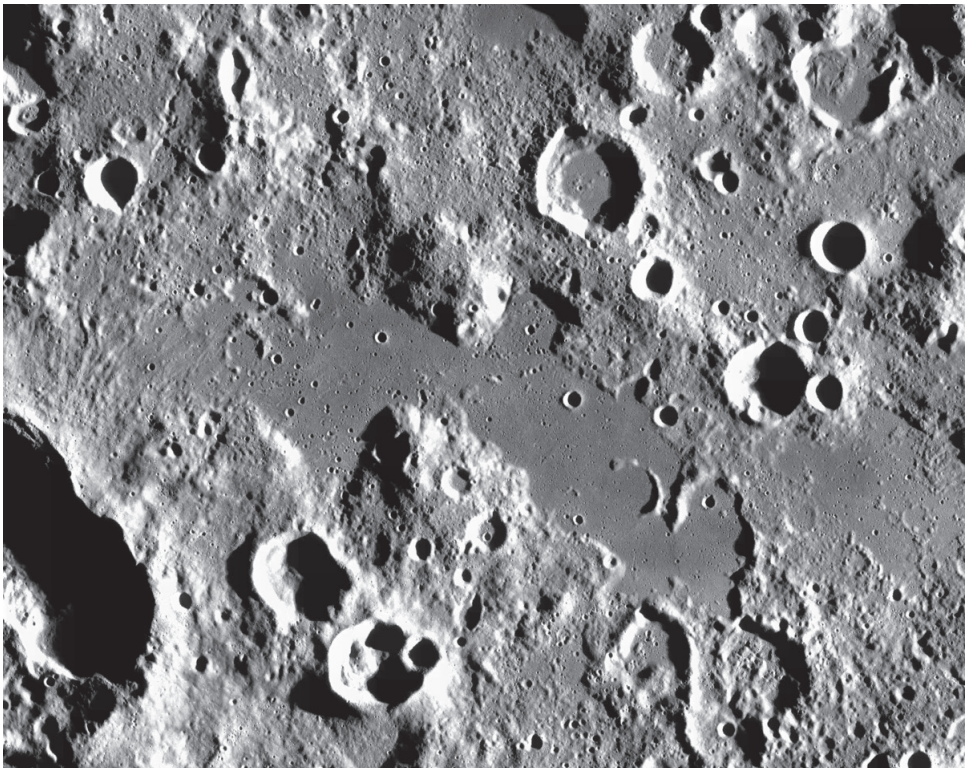

Moon Surface. Lacus Timoris

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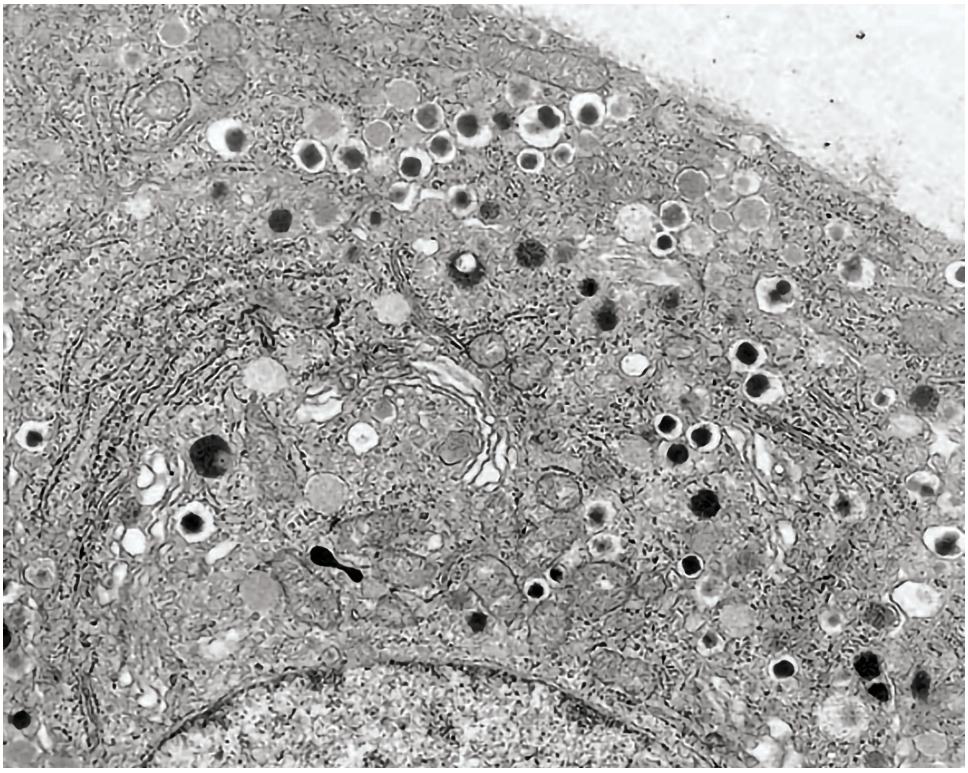
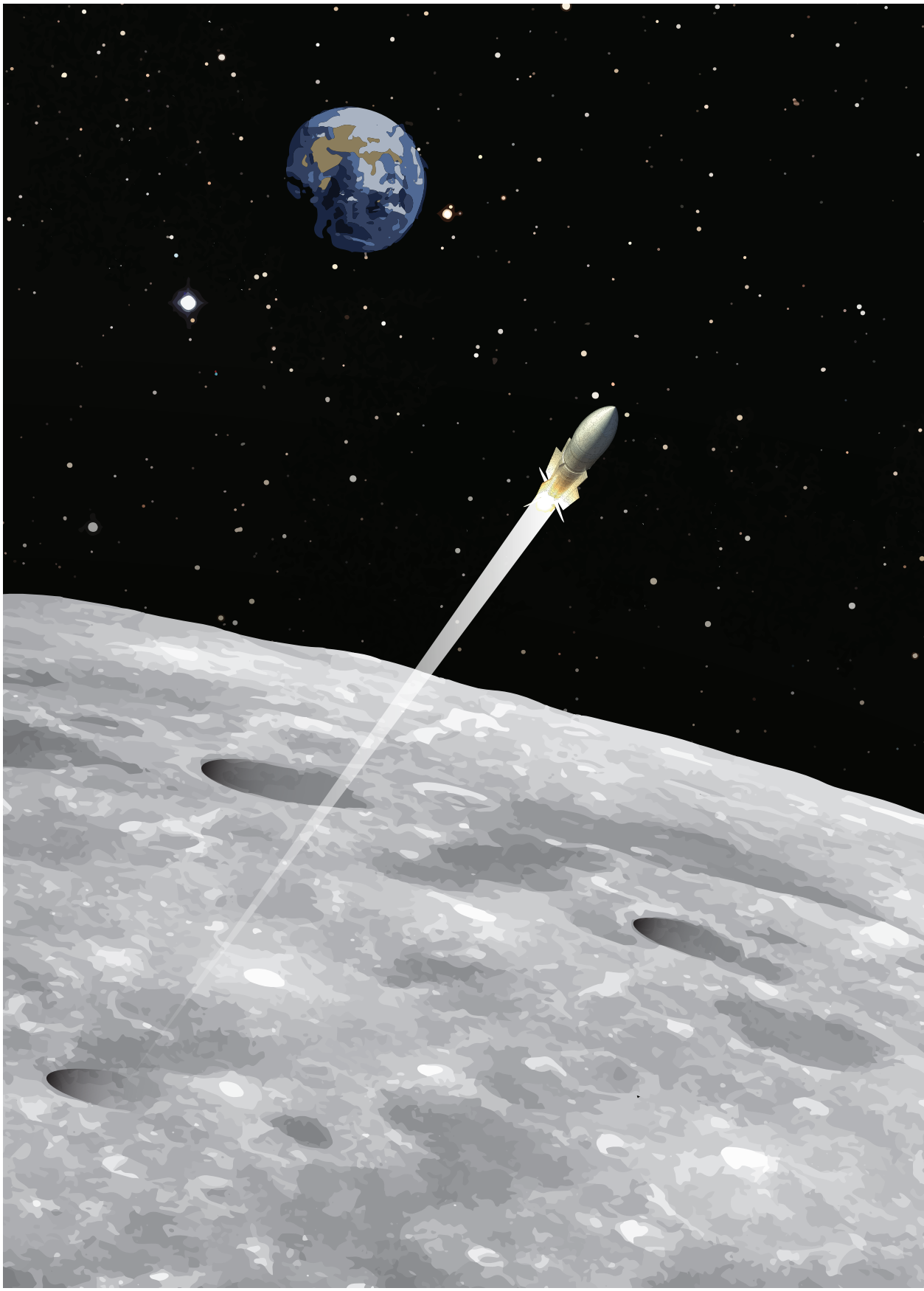


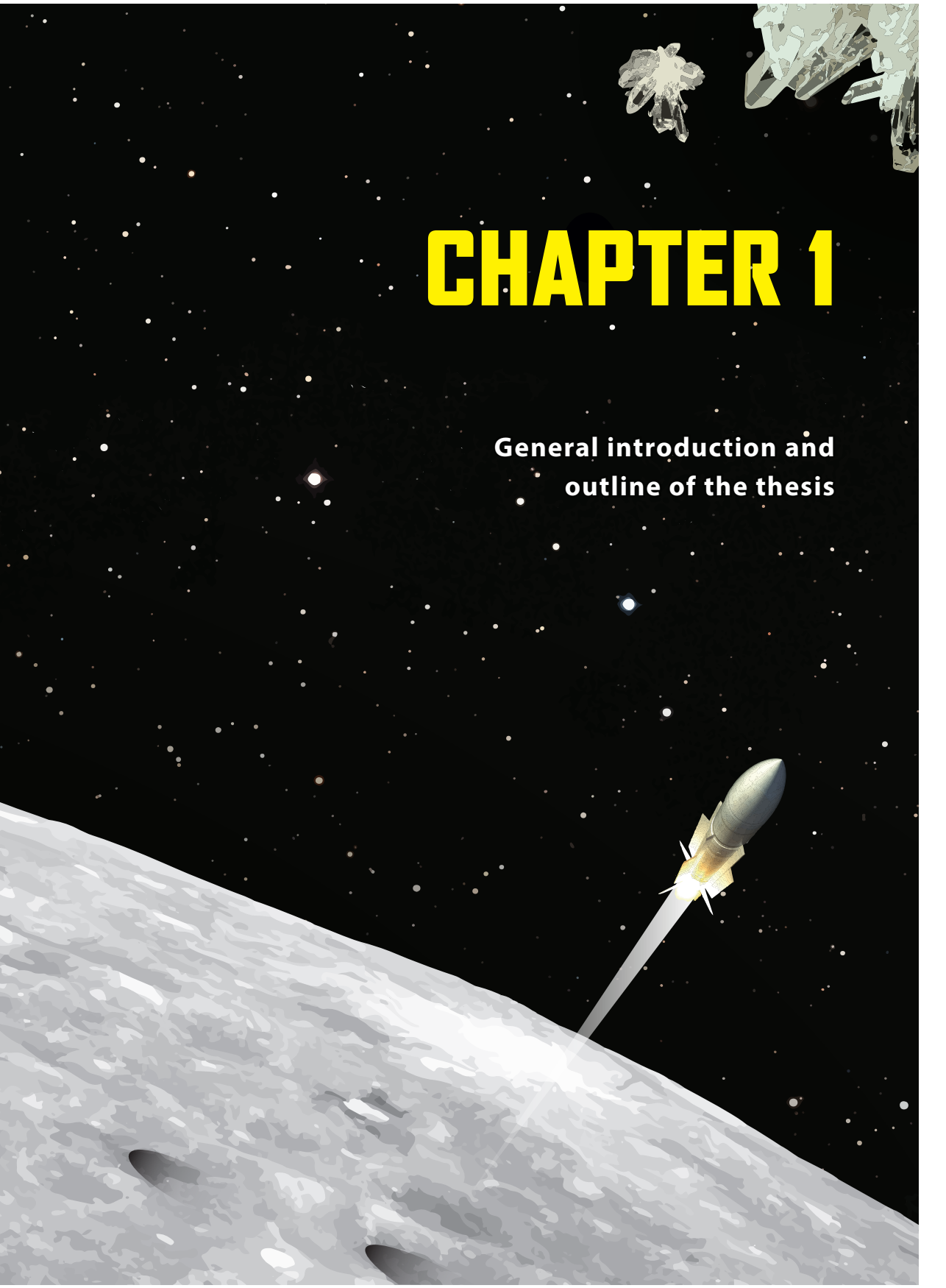
Electron micrograph of a beta cell

Source: © Current Medicine

"Let's swim to the moon
Let's climb through the tide
Surrender to the waiting worlds
That lap against our side."

— Jim Morrison





CHAPTER 1

General introduction and outline of the thesis

GENERAL INTRODUCTION

Type 2 diabetes mellitus (T2D) has become one of the main threats to human health in the 21st century[1]. Insulin resistance is an important characteristic, but abnormal function of the pancreatic beta cell is thought to play a crucial role in T2D as well[2, 3]. In this introduction, I summarize the research related to the normal physiology of insulin biosynthesis, the factors leading to beta cell dysfunction and the subsequent development of T2D. In addition, the currently available functional beta cell tests will be reviewed, and hereafter I will give the aims of this thesis, with the primary focus on my novel method based on labelling of newly synthesized insulin and C-peptide with stable isotopes, which can be used to assess *in vivo* human beta cell synthesis and secretion as part of function in more detail. Such tests are potentially valuable for research on the mechanisms causing T2D and for monitoring the effects of drugs on the beta cells. The end of this chapter is the outline of the thesis.

Regulatory mechanisms of insulin secretion

Of all circulating nutrients, variation in the concentration of glucose is the most important signal for beta cell response[3, 4]. Transport of glucose into the beta cell through the glucose transporter elevates intracellular glucose concentrations, which are sensed and phosphorylated by glucokinase with subsequent aerobic glycolysis. This increases the cellular ATP/ADP ratio, resulting in closure of ATP-dependent K+ channels and depolarization of the beta cell membrane. This results in opening of voltage dependent Ca2+ channels causing a rise in intracellular Ca2+ levels[5]. The latter triggers fusion of large insulin-containing granules with the cell membrane and subsequent secretion of insulin. This rapid insulin response is called the triggering pathway. When this triggering pathway is insufficient to achieve normal blood glucose concentrations, the insulin secretion continues by the amplifying pathway (figure 1) in which distant granules are transported toward the cell membrane for fusion and insulin release. This second insulin response lasts longer, and is mainly modulated by glucose. This glucose stimulated insulin secretion is potentiated by lipid signalling molecules derived from glycerolipid-fatty acid cycling[6] and other metabolic or neurohormonal signalling mechanisms also play a role in this amplifying pathway.

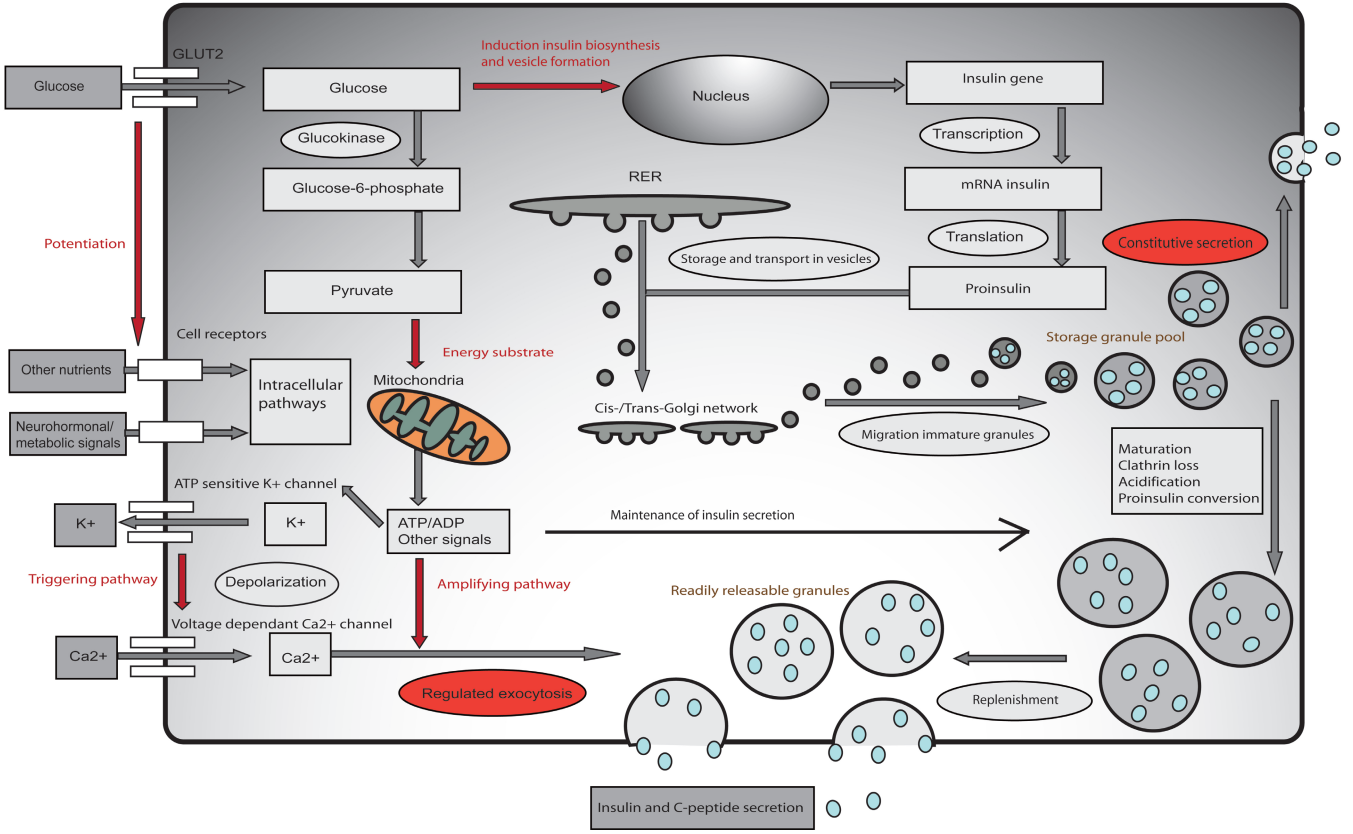


Figure 1 | Schematic overview of key intracellular mechanisms induced by glucose in pancreatic beta cells. Insulin is secreted predominantly through regulated exocytosis. Under conditions of enduring high glucose concentrations, a rapid insulin release from a ready releasable pool of granules is followed by a more sustained insulin release through release from a storage granule pool. *De novo* synthesis of (pro-)insulin replenishes the storage granule pool, and is eventually also secreted. More details about this process are mentioned in the text.

Finally, glucose and its intracellular metabolites induce *de novo* synthesis of insulin, which results in the maintenance of an intracellular insulin storage pool, and when blood glucose levels remain elevated, in immediate secretion of newly synthesized insulin from new granules. After transcription of the insulin gene, proinsulin is synthesized in the ER (figure 2). Proinsulin is then transported through the trans-Golgi network and stored in immature granules, where proteolytic enzymes convert proinsulin into intermediate insulin products and finally into insulin and C-peptide. Insulin consists of an A- and B-polypeptide chain connected by two disulfide bonds with an additional intra-molecular disulfide bond in the A chain. Cleavage efficiency is optimized by intragranular increase of calcium concentration and acidification. Simultaneously, the granules migrate towards and fuse with the cell membrane, lose their clathrin coat, and release the content of granules containing insulin and C-peptide into the blood. As a result insulin and C-peptide are secreted in a 1:1 ratio. When *de novo* synthesized insulin is secreted rapidly, insulin maturation may not have completed, and also insulin precursors including proinsulin are secreted and become detectable in the circulation [7].

In addition to glucose, human *in vivo* insulin secretion and biosynthesis of insulin by beta cells are regulated by complex extracellular mechanisms involving circulating neurohormonal and fuel signals, and parasympathetic and sympathetic innervation pathways[8]. Amino acids like leucine, lysine and arginine, enhance glucose stimulated insulin secretion [9-11]. Compared to these amino acids, the role of lipid signalling seems less potent[12]. Gastrointestinal hormones including Glucagon-Like Peptide-1 (GLP-1) and Glucose-dependent Insulinotropic Peptide (GIP), play an important role during oral food intake within the entero-hormonal axis and have a direct stimulatory effect on insulin secretion primarily through activation of cAMP signalling in beta cells[13] whereas acylated ghrelin reduces insulin secretion[14]. In addition, insulin itself and other hormones like growth factors affect insulin secretion[15, 16]. Somatostatin from delta-cells acts as a suppressor and glucagon from alpha-cells acts as a secretagogue of insulin [17, 18]. Under conditions of increased metabolic demand, not infrequently insulin resistance, insulin secretion is increased by both intra- and extracellular regulation mechanisms either through proliferation of beta cells or by enhanced function of individual beta cells. Worsening insulin sensitivity is initially compensated by increased insulin secretion. Genetic and acquired beta cell defects, however, preclude indefinite adaptation of beta cells, finally resulting in T2D. Better insights into beta cell function and regulatory mechanisms are therefore crucial for improving prevention and treatment of T2D.

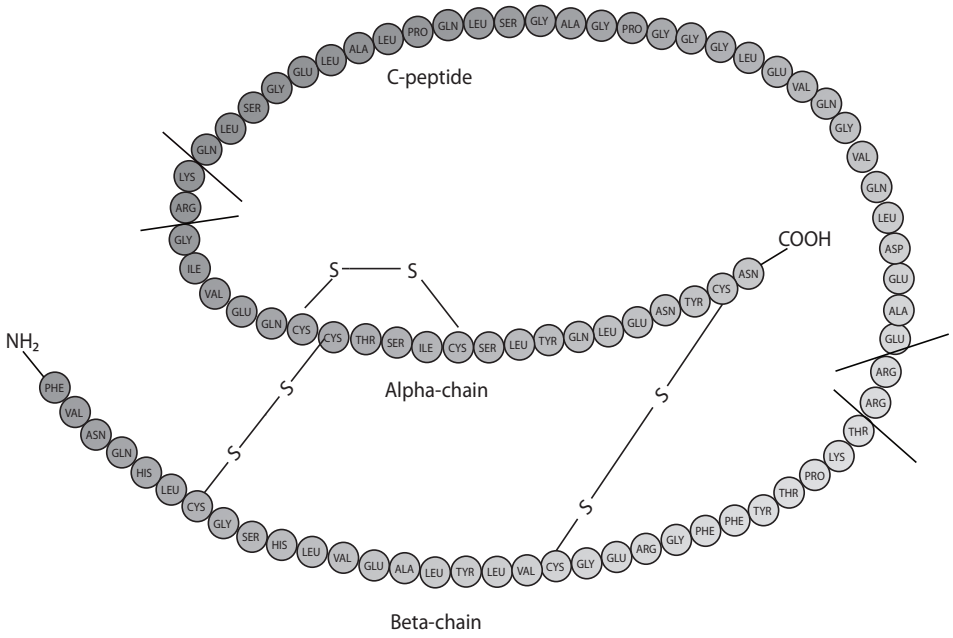


Figure 2 | Amino acid pattern of proinsulin, precursor of both insulin and C-peptide.

Biphasic pattern of insulin secretion in vivo and in vitro

One characteristic aspect of insulin secretion is its biphasic pattern in response to a challenge with glucose, observed both *in vivo* and *in vitro* (figure 3). This biphasic insulin secretion is pronounced in *in vitro* studies and *in vivo* during an intravenous glucose tolerance test, but hardly present during an oral glucose tolerance test[19]. The biphasic response is according to the storage-limited model thought to be the result of the triggering and amplifying pathways described above[5], involving the readily releasable pool (RRP) and the storage granule pool (SGP), respectively. The RRP consists of the granules located close to cell membrane, and is responsible for a rapid and transient first phase of insulin release. Granules from the SGP are subsequently recruited to the RRP, resulting in a sustained second phase of insulin release[20]. Distinct mechanisms are thought to be involved in granule kinetics, involving the actin cytoskeleton and related remodelling proteins that are responsible for granule translocation, and SNARE proteins, which are involved in the process of fusion of granules with the cell membrane.[21], The glucose-induced insulin secretion is much higher and faster during oral glucose tolerance tests than during an intravenously administered glucose load. The biphasic nature of insulin secretion is hardly discernable

during oral glucose tolerance tests. Due to neurohormonal signalling elicited among others by vagal stimulation and incretines released during oral glucose delivery, the amplification pathway is probably accelerated [22]. It should be emphasized that the reported mechanisms of insulin secretion by RRP and SGP are mainly based on *in vitro* studies of pancreatic beta cells involving island perfusion, capacitance measurement and internal reflection fluorescence microscopy. These studies provide crucial information on the intracellular mechanisms involved in insulin secretion. However, development of novel *in vivo* beta cell function tools is highly relevant, as insights derived from primary or clonal animal beta cell cultures as well as results from *in vitro* single beta cell experiments do not necessarily translate to human (patho)physiology.

Upon glucose induction, insulin *de novo* synthesis is upregulated in parallel with exocytosis and insulin secretion[4]. Upregulation of insulin synthesis occurs at a lower glucose threshold compared to that of insulin secretion, ensuring maintenance of insulin reserve in the granules [23, 24]. *In vitro*, newly synthesized insulin is secreted preferentially after stimulation by high glucose concentrations [25]. This suggests heterogeneity among the granules in the storage granule pool. *In vitro*, under conditions of high glucose concentration exposure of beta cells, secretion of newly synthesized insulin, was delayed with the time needed for vesicular transport from the ER to the cell membrane, which is estimated to be ~60 minutes[24, 26]. However, whether or not newly synthesized insulin is secreted under high glucose concentrations and/or alters in T2D pathogenesis in the *in vivo* situation in humans, is unknown.

Evidence for beta cell dysfunction during T2D development

T2D is characterized by the failure of beta cells to compensate for insulin resistance[2]. In the past decade, it has become clear that without this beta cell dysfunction T2D does not occur[27, 28]: beta cells undergo various alterations during T2D development and the role of beta cell dysfunction is substantiated by genetic studies. Initially, the pancreas compensates for insulin resistance with hyperinsulinemia to maintain normal glucose tolerance, both by increased beta cell mass and function of individual cells [29]. Eventually, the beta cells decompensate through multiple mechanisms, and this results in raised plasma glucose levels (figure 4). It is not fully known whether the failure is primarily due to a reduction in number of beta cells or to reduced function per cell. However, there are a number indications that a decrease in individual cell function, which is more relevant for short-term adaptation, is the main contributor to T2D[30].

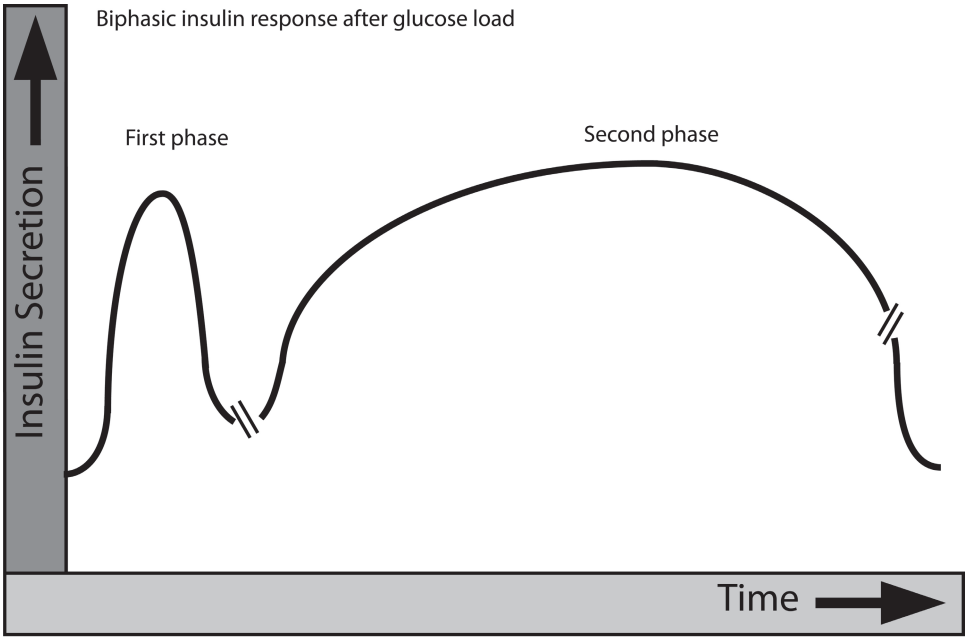


Figure 3 | Biphasic insulin release as observed in ***in vitro*** beta cell function studies and ***in vivo*** after intravenous glucose challenge.

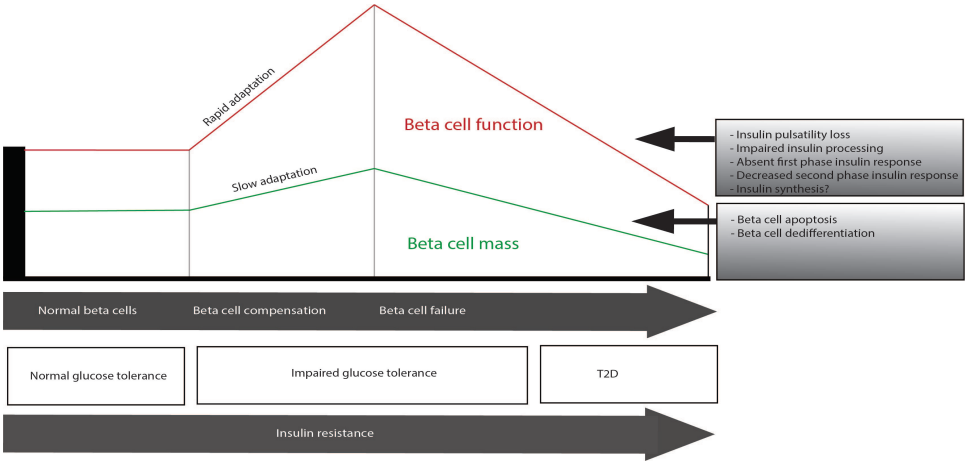


Figure 4 | Time-course of beta cell changes in relation to insulin resistance during T2D development. Characteristics of deterioration of either beta cell function or mass are listed. The fate of insulin synthesis during this time-course is unknown.

Signs of beta cell impairment have also been observed in the prediabetic stage. The main changes are relative loss of insulin pulsatility[31, 32] and impaired insulin maturation reflected by the increased concentrations of circulating insulin precursor metabolites. With regard to the loss of circulating insulin, in *in vivo* beta cell function tests impairment of the first phase insulin response is observed, eventually followed by a decrease of the second phase insulin response[31, 33, 34]. Histological examination of the pancreas of patients with T2D reveals decreased beta cell mass, due to increased dedifferentiation and/or apoptosis. In addition, higher levels of amyloid deposition compared to age-matched controls have been observed [29, 35, 36]. The amyloid deposition might result from increased insulin secretion and is thought to be cytotoxic to beta cells [37].

Genetic and acquired factors make beta cells susceptible to failure, contributing to beta cell exhaustion and T2D development[3]. A genetic predisposition for T2D has been demonstrated by twin and family studies, as well as by genome wide association studies (GWAS) in the general population[38-41]. Interestingly, the majority of T2D-associated genes identified in GWAS are related to pathways involved in beta cell development and function[41]. The identified risk gene variants have been associated with reduced beta cell development, reduced glucose stimulated insulin secretion, impaired membrane depolarization and increased apoptosis, all resulting in increased risk for T2D development[42]. Acquired factors that may lead to reduced beta cell function or mass are related to diet and lifestyle, and include lack of physical exercise and increasing body weight. These factors contribute to the development of insulin resistance. With prolonged or worsening of the insulin resistance, beta cells fail to further compensate and eventually decompensate, resulting in dysfunction, failure and cell death by apoptosis. This process depends on beta cell modulating factors, which may start or enhance the progression of beta cell dysfunction. They may include intra-uterine and epigenetic factors up to chronic fuel excess leading to glucotoxicity and lipotoxicity [3, 29].

In vivo assessment of total beta cell function and whole body insulin sensitivity.

A number of function tests are available to examine total beta cell function, which is explained by the product of individual cell function and number of functional beta cells. A number of measures are available that reflect the steady state condition and with dynamic function tests beta cell secretion patterns in response to insulin secretagogues can be followed in time. They are performed with oral or intravenous beta cell stimuli (figure 5). Oral function tests are more physiological as they include

activation of the entero-insular axis [1]. Intravenous function tests are useful in assessing insulin secretion responses to stimuli without direct involvement of the cephalic or gastrointestinal signals.

Whole body insulin sensitivity can be measured in the fasting state, and in the dynamic state following a standard glucose load given either orally or intravenously[43]. The intravenous euglycemic hyperinsulinemic clamp has been accepted as the gold standard for whole body insulin sensitivity. In this method, glucose removal from the circulation during intravenous administration of exogenous insulin is taken as a measure of whole body insulin sensitivity. In contrast to insulin sensitivity, there is no gold standard for beta cell function. The disposition index, which is calculated as insulin secretion corrected for glucose concentration and insulin sensitivity, is probably the best estimate currently available for beta cell function[44]. Studies on the glucose potentiation of the first-phase insulin response induced by non-glucose nutrients currently provide the best estimate of beta cell mass[45].

For the assessment of beta cell function, C-peptide levels are a better proxy of insulin secretion than peripheral insulin concentrations, as insulin undergoes both hepatic and peripheral extraction [55]. In contrast, C-peptide is mainly cleared by renal extraction and has a much longer plasma half-time value than insulin. Deconvolution techniques based on plasma C-peptide concentrations have been used to estimate pancreatic insulin secretion rate (figure 6)[56]. Part of the cleared C-peptide appears unaltered in the urine. In this thesis, I provide literature and demonstrate that urinary C-peptide levels correspond well to estimated C-peptide clearance rates obtained from plasma-based beta cell kinetic models[57], and that urinary C-peptide can be used as a target peptide for stable isotope labelling techniques for detailed beta cell function analysis which is central to this thesis.

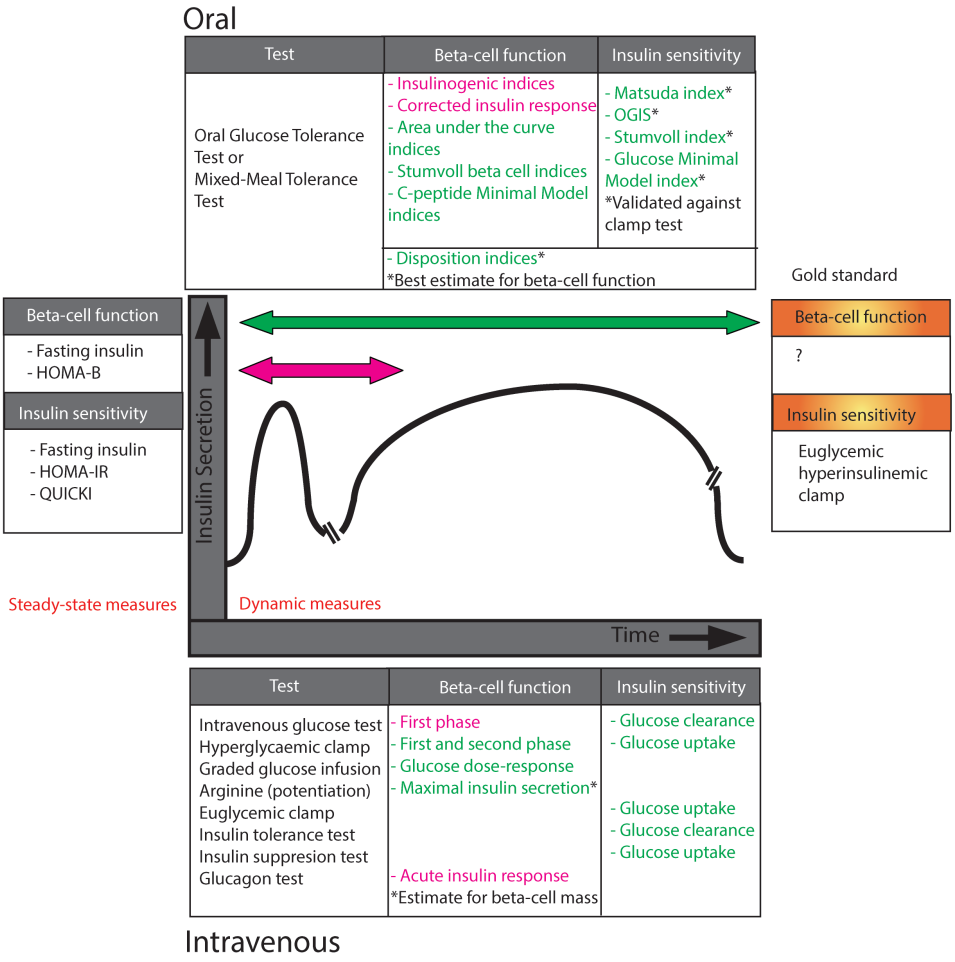


Figure 5 | Dynamic and steady-state methods for assessing beta cell function[46-53] and insulin sensitivity[43, 45, 46, 52, 54].

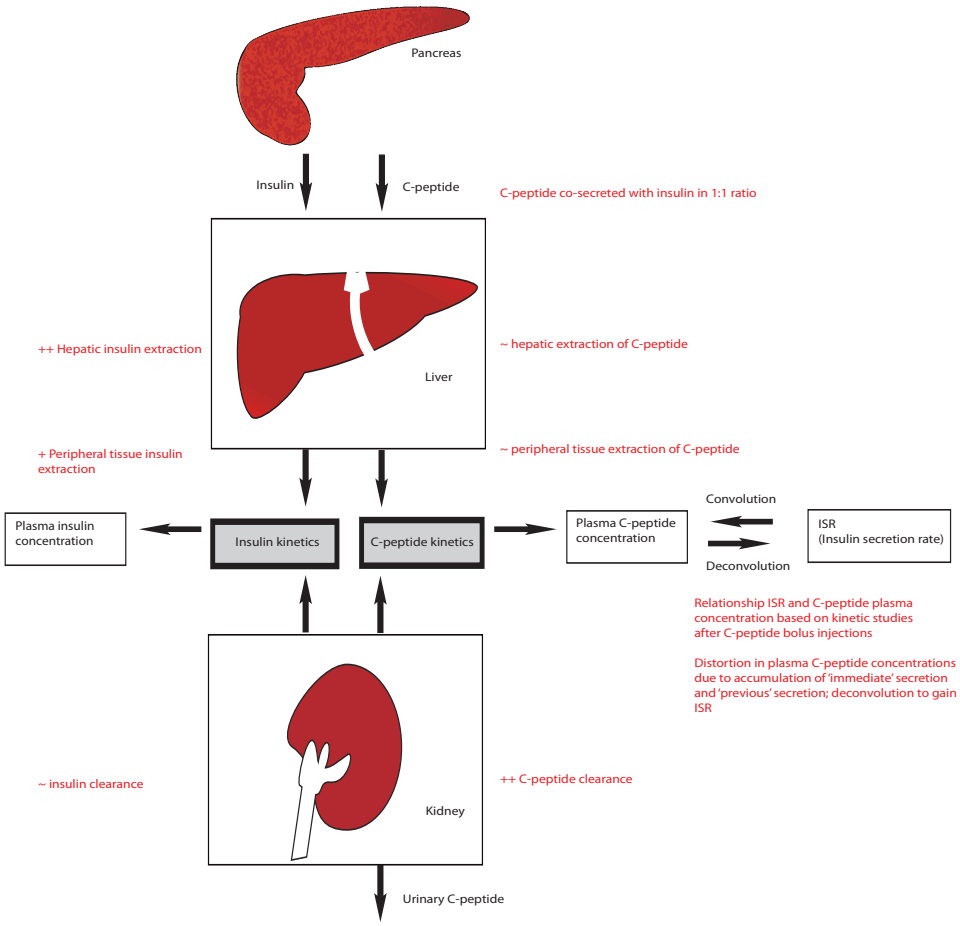


Figure 6 | Schematic overview of insulin and C-peptide extraction after pancreatic release with C-peptide kinetics as preferred choice for calculation of insulin secretion rate (ISR) and thus beta cell function.

AIMS AND OUTLINE OF THE THESIS

This thesis is aimed at exploring beta cell function in-depth in T2D high-risk families, this includes developing and applying a novel stable isotope based test to measure *in vivo* insulin synthesis. This setup enables us to explore pathogenetic effects of genes related to pathways involved in insulin synthesis and secretion, as family analyses offer the opportunity to analyse association while considering transmission of genetic variants, they enhance matching of cases and controls, and avoid confounders that frequently disturb case-control studies. Beta cell function comprises multiple aspects of glucose-

stimulated insulin secretion, including the amount of insulin secreted, which consists of already available insulin in different pools of granules, and the rate of secretion of newly synthesized insulin. Stable isotope labelling of proinsulin enables to determine the contribution of the secretion of stored insulin versus newly synthesized insulin. My method is based on a bolus dose method[58], using the stable isotope ^{13}C- labelled leucine administered prior to an oral glucose load. As the stable isotope is incorporated into proinsulin, both *de novo* synthesized insulin and C-peptide will be enriched with ^{13}C-leucine. Subsequently, the enrichment factor can be determined in plasma insulin and C-peptide at different times during the OGTT, and in urinary C-peptide. With both measurements and the changes in the plasma or the urinary concentrations, the relative contribution of insulin (and C-peptide) secreted from storage pools versus *de novo* synthesis can be estimated.

Before assessment with stable isotopes, I investigated beta cell function in relation to glucose disposal and insulin sensitivity with classical indices derived from prolonged oral glucose tolerance tests in family analyses and according to the stages of glucose tolerance in chapter 2. I also included South Asian families living in the Netherlands as they are heavily burdened by type 2 diabetes. Although lifestyle, social factors, diet and other environmental factors have been studied extensively, the role of beta cell function in T2D development has been relatively undervalued in this population. In chapter 3 I further phenotyped our families and determined their metabolic syndrome state, which is a major health problem contributing to type 2 diabetes and cardiovascular disease. The effects of metabolic syndrome parameters on insulin sensitivity and beta cell indices, derived from oral minimal modelling were assessed. As additional part of this metabolic profiling in chapter 4, I determined the effects of their fasting amino acid profiles, and also screened for the predictive capability of these profiles for determining glucose tolerance state. In chapter 5, as part of the work-up towards a novel stable isotope test, I explored C-peptide and glucose excretion in urine during OGTT. Surprisingly, data were not available in the literature on how urinary C-peptide and glucose secretion during OGTT might affect plasma based indices of insulin sensitivity and beta cell function, through various stages of glucose tolerance. This is highly relevant for interpretation of pharmacokinetic models in general that provide plasma based beta cell function and insulin sensitivity indices, as the role of renal extraction compared to hepatic and peripheral tissue extraction is relatively unknown. Apart from this, as urinary measurements are non-invasive, I also explored their correlation with plasma based indices and their predictive capability to detect glucose tolerance state. In chapter 6, I describe a novel stable isotope method to follow insulin biosynthesis and release by the pancreatic beta cells in detail during an oral glucose tolerance test, using urinary C- peptide as the target peptide. Stable isotopes

have not previously been used to examine insulin kinetics in man *in vivo*. In healthy volunteers, I show that it is possible to quantitate insulin synthesis and secretion *in vivo*. Furthermore, I used this method in individuals from T2D high-risk families, enabling us to investigate the mechanisms of beta cell dysfunction in type 2 diabetes in-depth as described in chapter 7. Also in this chapter, I determined if the T-allele of the rs7903146 in the transcription factor 7-like 2 gene, which is the main susceptibility gene for T2D, increases T2D risk based on insulin synthesis rate. Finally in chapter 8, I discuss my main findings, propose a pathophysiological model and make suggestions for future research options.

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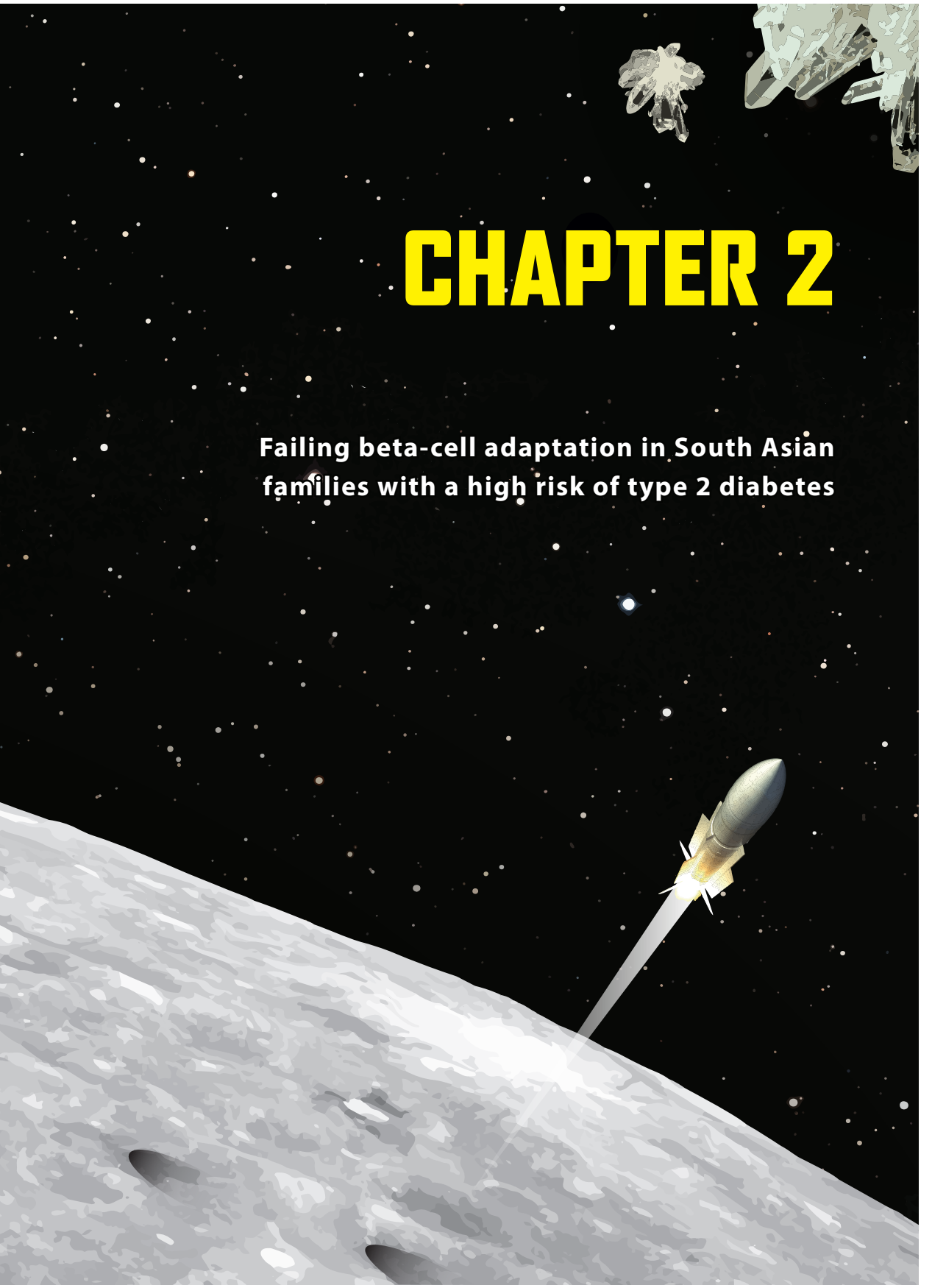

CHAPTER 2
Failing beta-cell adaptation in South Asian families with a high risk of type 2 diabetes

ABSTRACT

We performed an extended oral glucose tolerance test (OGTT) to investigate the relationship between early and late beta-cell response and type 2 diabetes (T2D) in families of South Asian origin and indigenous Dutch, burdened by T2D. Based on the OGTT, 22 individuals were normoglycemic, 12 glucose intolerant and 23 had T2D in the South Asian families; these numbers were 34, 12 and 18 in the Caucasian families, respectively. The OGTT had 11 blood samplings in 3.5 h for glucose, insulin and C-peptide measurements. Through early and late insulin secretion rate (ISR), the above basal glucose area-under-the-curve after glucose load (glucose disposal) and insulin sensitivity index (ISI), we obtained early and late disposition indices (DI). South Asians on average had lower ISI than Caucasians (3.8±2.9 vs 6.5±4.7, respectively $P < 0.001$), with rapid decline of their early and late DI between normal glucose tolerance versus impaired fasting glucose/impaired glucose tolerance (late DI; $P < 0.0001$). Adjusted for ISI, age, gender and waist-to-hip ratio, early ISR was significantly associated with glucose disposal in South Asians (β=0.55[0.186; 0.920]), but not in Caucasians (β=0.09[-0.257; 0.441]). Similarly, early ISR was strongly associated with late ISR (β=0.71[0.291; 1.123];R^2=45.5%) in South Asians, but not in Caucasians (β=0.27, [-0.035; 0.576];R^2=17.4%), with significant interaction between ethnicity and early ISR (β=0.341, [0.018; 0.664]). Ordinal regression analyses confirmed that all South Asian OGTT subgroups were homogenously resistant to insulin and solely predicted by early ISR(β=-0.782[-1.922; 0.359], β=-0.020 [-0.037; -0.002], respectively), while in Caucasian families both ISI and early ISR were related to glucose tolerance state(β=-0.603[-1.105; -0.101], β=-0.066 [-0.105; -0.027] respectively).

In South Asian individuals, rapid beta-cell deterioration might occur under insulin resistant conditions. As their early insulin response correlates strongly with both glucose disposal and late insulin response, alterations in beta-cell dynamics may give an explanation to their extreme early onset of T2D, although larger prospective studies are required.

INTRODUCTION

Dutch citizens of South Asian origin have a nearly fivefold higher prevalence of type 2 diabetes (T2D) than the indigenous Dutch population (further described as Caucasian) [1, 2]. The increased susceptibility to T2D is also evident from the early onset of the disorder at relatively low body mass and the remarkably high incidence of cardiovascular and microvascular damage among the South Asians [2, 3]. A number of factors have been proposed to account for this strikingly high risk in South Asians, including a high prevalence of metabolic syndrome, impaired maternal lipid profile conditions, low birth weight causing central obesity later in life, dysfunction of adipocytes, as well as educational, social and economic inequalities [4-14]. These factors all enhance insulin resistance and promote hyperinsulinemia [14, 15]. In addition, T2D is characterized by beta-cell dysfunction. Genetic loci predisposing individuals to T2D affect both beta-cell function and insulin action [16, 17].

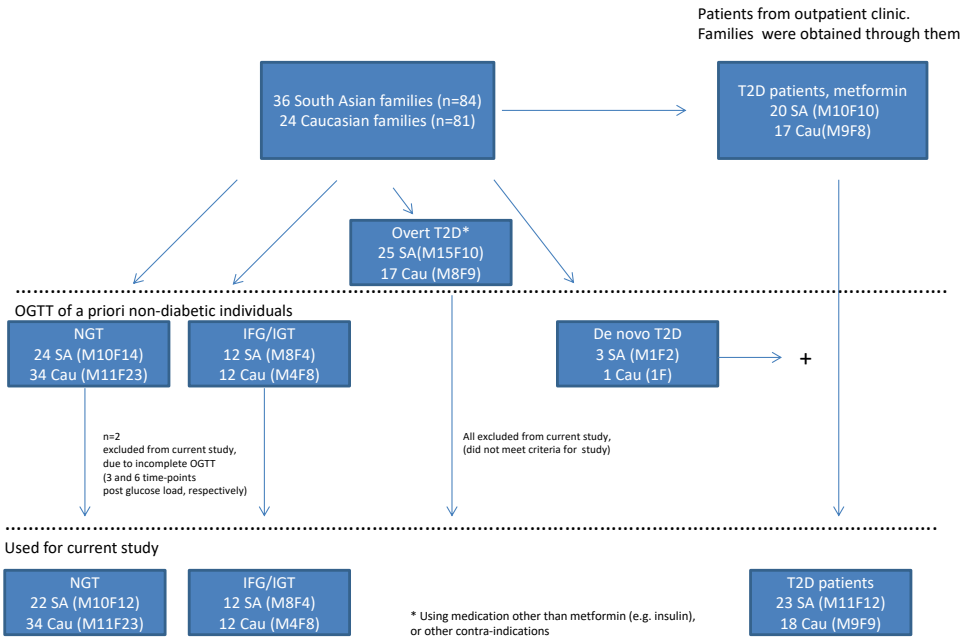
The ancestors of South Asian families in the Netherlands moved from a circumscribed region in India to Surinam. During the past 150 years, these South Asian families lived largely in genetic isolation before arriving in the Netherlands. The conservation of susceptibility loci may have contributed to the strong aggregation of T2D in these families. We hypothesized that, in addition to severe resistance to insulin, these South Asian families are also predisposed to develop beta-cell dysfunction. Therefore, we investigated beta-cell function and insulin sensitivity simultaneously in South Asian and Caucasian patients with T2D and first-degree relatives. In effect, we assessed the contribution to the risk of T2D of changes in early and late insulin secretion rates (ISR) and insulin sensitivity during an extended oral glucose tolerance test (OGTT) with insulin and C-peptide measurements.

METHODS

Subjects

The study was conducted during a time period between August 2007 and January 2011. Patients with T2D and first-degree relatives without T2D were recruited from 36 South Asian families and 24 Caucasian families (Scheme 1). Power calculation was performed with Quanto version 1.0 [31] and was based on differences in early phase ISR (described further on in Methods section) between healthy South Asian and Caucasian performed in a pilot phase of the study among, with alpha 0.05 and power 80%. All probands were attending the outpatient clinic of the Department of Internal Medicine of the Erasmus Medical Center in Rotterdam. T2D was diagnosed according to

World Health Organization (WHO) criteria [18]: plasma glucose level ≥7.0 mmol/L in a fasting state and/or ≥11.1 mmol/L in a non-fasting state. Inclusion criteria for probands were age of 18 years or older and T2D in at least 1 sibling. Both parents of the South Asian probands were of South Asian origin and Caucasian probands were born in The Netherlands with both parents of Caucasian Dutch origin. Exclusion criteria were insulin-dependent diabetes mellitus, using medication other than metformin, a history of pancreatitis, insulinoma or other reasons that made participation impossible. Written informed consent was obtained from all participants. The study protocol was approved by the Erasmus Medical Center Medical Ethics Review Board.



Scheme 1 | Inclusion flow chart of individuals from South Asian and Caucasian families.

Physical examination

Body height and weight were measured to the nearest 0.1 cm and 0.1 kg for the determination of body mass index (BMI). Waist circumference was measured in cm halfway between the lowest rib and the iliac crest, the maximum circumference of the hips was measured in the standing position in cm, and, from these measurements, the

waist-to-hip ratio (W/H) was calculated. Systolic and diastolic blood pressures were measured with an electronic blood pressure monitor (Datascope Accutorr Plus Inc., Montvale, NJ) after 5 min rest in the sitting position.

Oral glucose tolerance test (OGTT)

Glucose, 75 g dissolved in 200 ml H_2O, was administered orally after a 10 h overnight fast. Venous blood was drawn via an intravenous canula, 60 min and 15 min before the glucose load and 15, 30, 45, 60, 90, 120, 150, 180 and 210 min after glucose loading. WHO criteria based on OGTT were used to define family members with normal glucose tolerance (NGT), impaired fasting glucose/impaired glucose tolerance (IFG/IGT) and T2D [18].

Assays

Plasma glucose was measured by a hexokinase-based method (Gluco-quant; Roche Diagnostics, Mannheim, Germany). Plasma insulin and C-peptide were measured separately by a competitive chemiluminescent immunoassay, supplied by Euro/DPC (Diagnostic Product Corporation, Los Angeles, CA). The assay was performed on a DPC Immulite 2000 analyzer (Euro/DPC) according to the manufacturer's recommended protocol.

Calculation of indices

Beta-cell function indices

For the assessment of early, late and overall beta-cell function, we calculated incremental ISR area-under-the-curves (AUCs); ISR t0-30 and ISR t60-210 and ISR t0-210 respectively based on plasma C-peptide concentrations with ISEC software [19]. The ISR reflects the prehepatic secretion rate, as C-peptide has negligible hepatic clearance. Hereafter, we investigated early, late and overall beta-cell function in relation to glucose concentrations and insulin sensitivity to obtain early, late, and overall disposition indices (DI), respectively.

Insulin sensitivity

The insulin sensitivity index (ISI) was determined according to [20, 21]

$$ISI = 10.000 / (G_0 \times I_0 \times meanG_{0\text{-}30\text{-}60\text{-}90\text{-}120} \times meanI_{0\text{-}30\text{-}60\text{-}90\text{-}120})^{½}$$

Early, late and overall Disposition Indices

Early, late and overall DI were calculated as follows: ISR t0-30/ glucose disposal t0-30 ×ISI, ISR t60-210/ glucose disposal t60-210 × ISI, and ISR t0-210/ glucose disposal t0-210 × ISI, respectively.[22] In addition, we calculated the ratio of late phase DI to early phase DI, based on earlier observations marking their relationship [23].

To improve comparison with previous studies, we also added a large number of classical indices to online supplemental Table 1 and supplemental Figure 1. All formulae are described below the online supplemental Table 1. All OGTT indices were derived from insulin and C-peptide concentrations in pmol/L and glucose concentrations in mmol/L, with the exception that insulin concentrations were converted to μU/mL for the calculation of HOMA and ISI and, subsequently, DIs were both calculated with glucose in mg/dL. All AUCs were calculated according to the trapezoid method, and incremental AUCs were calculated by subtracting basal values from total calculated AUC values between given time points [24].

Statistical analyses

We performed family-based analyses with the SOLAR software package [25]. Comparison between ethnicities was performed with variance component analyses adjusted for a number of covariates within SOLAR. For the prediction of NGT, IFG/IGT or T2D stage (WHO OGTT subgroup) in both ethnicities we used ordinal regression analyses with SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA), adjusted for family ties, using a variable grouping each family with their own distinct number in SPSS. Data are expressed as mean ± SD, unless otherwise indicated. ANOVA were used for differences within given WHO OGTT subgroups and performed with SPSS; for each WHO OGTT subgroup, three comparisons were performed with ANOVA (unless otherwise stated); with the other two WHO OGTT subgroups of same ethnicity and with the corresponding other ethnic WHO OGTT subgroup. Inverse or log transformations were used when normality or equal variance assumptions were not met. P value < 0.05 was considered significant, unless otherwise stated.

RESULTS

In 36 South Asian families, 15 out of 37 (41%) apparently healthy first-degree relatives were classified as IFG (n=4), IGT (n=5), the combination of IFG and IGT (n=3) or newly diagnosed T2D (n=3). In 24 Caucasian families, 13 (28%) out of 47 apparently healthy first-degree relatives were classified as IFG (n=3), IGT (n=5), the combination of IFG and IGT (n=4), while one was newly identified as T2D. For both ethnic groups, individuals

with IFG and/or IGT were combined into one group of intermediate phenotypes and newly identified individuals with T2D were included with the original T2D cases in the diabetes group. The general characteristics of the three groups according to ethnicity are shown in Table 1. Waist circumference and W/H were lower in the groups with NGT compared with the other groups in both ethnicities. However, the relation of increasing W/H with glucose intolerance appeared to be less clear in South Asians when compared to Caucasians Notably, the South Asians with T2D were on average 10 years younger than the Caucasians with T2D and they already had a substantial prevalence of macrovascular disorders. Results from the OGTT demonstrated the following results; in both ethnicities with increasing glucose intolerance, glucose disposal increased, while both ISR t0-30 min and ISI decreased. Both ISR t0-210 min and ISR t60-210min increased from NGT toward IFG/IGT, but decreased from IFG/IGT towards T2D. In general, ISR derived parameters in South Asians were markedly higher compared with the Caucasians, while ISI was lower with an overall between-ethnicity difference of 3.8±2.9 vs 6.5±4.7, respectively ($P < 0.001$).

Disposition indices, first/second phase beta-cell function

The unadjusted relationships between glucose disposal t0-210 min, ISR t0-30 min and ISR t60-210 min, are shown in ternary plots (Supplementary Fig. 1a, c). We determined the relationship between ISR t0-30 and ISR t60-210 with glucose disposal t0-210 using variance component analyses; after adjustment for ISI, age, W/H and gender the effect of early beta-cell function on glucose disposal t0-210 remained present in the South Asians but disappeared in the Caucasian families, explaining the variance of glucose disposal t0-210 in our final model by 22.7 and 8.9% in South Asian and Caucasian families, respectively (Table 2, Model 1). We also explored the effect of ISR t0-30 and ISI on ISR t60-210. The unadjusted relationships between ISI, ISR t0-30 and ISR t60-210 are shown in the ternary plots of Supplementary Figure 1b, d. After adjustment for age, W/H,WHO OGTT subgroup and gender, ISR t0-30 in South Asians had an effect on ISR t60-210, but such effects were not observed in Caucasian families, explaining 45.5 and 17.4% of the variance of ISR t60-210 in our final model in South Asian and Caucasian families, respectively (Table 2, Model 2). We combined both ethnicities into an overall group and applied both Model 1 and 2, and tested for interaction between ethnicity and ISR t0-30 to glucose disposal t0-210 or ISR t60-210, respectively; only in Model 2 this interaction was significant (β=0.341, [0.018;0.664]).

Table 1 | Clinical characteristics of the NGT, IGT and/or IFG and T2D subgroups.

	NGT SA	NGT Cau	IFG/IGT SA	IFG/IGT Cau	T2D SA	T2D Cau
n	22	34	12	12	23	18
Sex(male/female), n%(male)	10/12(45.5)	11/23(32.4)	8/4(66.7)	4/8(33.3)	11/12(47.8)	9/9(50.0)
Age (years)	39.6±11.6¶	38.9±9.4‡	46.3±8.8	44.5±11.4‡	52.3±8.8‡§	63.2±7.6*†¶
Weight (kg)	78.7±13.8	81.1±15.7	78.7±14.5	94.1±30.4	74.4±12.4	90.5±15.0¶
Length (cm)	1.69±0.1	1.75±0.1	1.67±0.1	1.75±0.1	1.61±0.1‡	1.76±0.1¶
BMI (kg/m2)	27.6±4.1	26.3±4.1	27.9±2.9	30.4±8.5	28.6±4.1	29.3±4.9
Waist (cm)	94±10	91±15‡	98±13	105±20	97±11	105±14*
Hip (cm)	105±5	108±8	103±6	116±20	104±9	112±10
W/H	0.90±0.07	0.84±0.09‡	0.95±0.10	0.90±0.07	0.93±0.08	0.94±0.08*
RR systolic (mmHg)	122.6±14.1	123.2±12.5	125.9±15.0	129.6±20.2	132.4±15.1	135.6±13.0
RR diastolic (mmHg)	77.0±9.7	76.3±9.1	85.0±10.8	79.4±10.3	80.4±8.4	83.4±12.2
Smoking, n%[D]	2(11.8)	13(40.6)	(5)55.6	(5)50.0	8(50.0)	5(50.0)
Antihypertensives, n%	2(9.1)	0‡	2(16.7)	3(25.0)	9(39.1)	9(50.0)*
Lipid treatment, n%	3(13.6)	1(2.9)‡	3(25.0)	0‡	11(47.8)	11(61.1)*†
Macrovascular history,n%	4.5	3.0	16.7	0	8.7	12.5*
Microvascular history,n%					13.0[B]	25.0[B]
Metformin usage					20(87%)	17(94.4%)
Age of diagnosis					44.3±7.3[A,B]	56.1±7.2[A,B]
Period of having T2D					9.9±7.3[A,C]	8.5±8.4[A,C]
Fasting glucose(mmol/L)	5.3±0.4¶	5.2±0.3†‡	6.0±0.5¶	6.0±0.6*‡	7.2±1.1§‖	8.0±1.1*†
120min glucose (mmol/L)	5.4±1.1¶	5.5±1.1‡	7.8±1.1¶	7.7±2.3*‡	12.5±4.4§‖	14.1±3.6*†
ISI	5.0±3.9	8.2±5.1‡	3.2±1.8	5.0±3.2	2.9±1.6	4.2±3.3*
ISR t0-210	1647±852	1153±385	1873±862	1369±561	1645±513‡	1060±478
ISR t0-30	297±122*¶	208±82‡	254±158¶	166±69	116±64§‖	124±75
ISR t60-210	921±632	595±296	1217±699	900±524	1225±430‡	744±360
Glucose disposal t0-210	169±95¶	211±126‡	394±138¶	446±176‡	830±438§‖	1035±445*†
Glucose disposal 0-30	33±17	35±14‡	40±14	44±27‡	51±22§	70±23*†
Glucose disposal 60-210	73±60¶	99±81‡	239±107¶	281±162‡	611±383§‖	764±405*†

Data are means± SD, *n* or *n*(%). *P* values are from ANOVA, *P* values between subgroups in post hoc Bonferroni analysis denoting statistical significance ($P < 0.0125$) are shown with symbols; *=versus Cau NGT, † =versus Cau IFG/IGT, ‡ = versus Cau T2D, § = versus SA NGT, || = versus SA IFG/IGT, ¶ = versus SA T2D. [A]newly identified individuals with T2D excluded , [B] significance, [C]non-significance with Student's *t* test or χ^2 test $P < 0.05$, [D]incomplete data, however with a >75% response rate

Table 2 | SOLAR multiple regression analysis within family matrices.

		beta	se	wald test	95% CI	*P* value
Model 1						
SA	ISR t0-30	0.553	0.187	8.745	[0.186; 0.920]	**0.003**
	ISRt60-210	-0.211	0.197	1.147	[-0.597; 0.175]	0.284
	ISI	-0.101	0.217	0.217	[-0.526; 0.324]	0.642
	Age	-0.677	4.641	0.021	[-9,773; 8.419]	0.884
	W/H	-0.00001	0.0002	0.003	[-0.0005; 0.0004]	0.956
	Gender	6.649	111.197	0.0036	[-211,297; 224,595]	0.952
	R^2 (%)	22.7				
Cau	ISRt0-30	0.092	0.178	0.267	[-0.257; 0.441]	0.605
	ISRt60-210	-0.057	0.188	0.092	[-0.425; 0.311]	0.762
	ISI	-0.040	0.056	0.510	[-0.150; 0.070]	0.475
	Age	2.909	4.182	0.484	[-5.288; 11.106]	0.487
	W/H	0.0003	0.0003	1.778	[-0.0002; 0.0008]	0.182
	Gender	-46.69	120.990	0.149	[-283.830; 190.450]	0.700
	R^2 (%)	8.9				
Model 2						
SA	ISRt0-30	0.707	0.212	11.122	[0.291; 1.123]	**<0.001**
	ISI	-0.245	0.190	1.663	[-0.617; 0.127]	0.197
	age	-2.338	4.595	0.259	[-11.344; 6.668]	0.611
	W/H	-0.0002	0.0002	1.000	[-0.0006; 0.0002]	0.317
	WHO OGTT	0.402	1.872	0.046	[-3.267; 4.071]	0.830
	Gender	-4.134	117.477	0.001	[-234.389; 226.121]	0.972
	R^2 (%)	45.5				
Cau	ISRt0-30	0.270	0.156	2.996	[-0.035; 0.576]	0.083
	ISI	-0.055	0.045	1.494	[-0.143; 0.033]	0.222
	age	-2.080	3.701	0.316	[-9.334; 5.173]	0.574
	W/H	0.0003	0.0003	1.000	[-0.0003; 0.001]	0.317
	WHO OGTT	-0.020	0.030	0.444	[-0.079; 0.039]	0.505
	Gender	-125.358	93.631	1.793	[-308.975; 58.159]	0.181
	R^2 (%)	17.4				
Model 3						
SA	DIratio	0.316	0.115	7.551	[0.091; 0.541]	**0.006**
	age	1.989	5.480	0.132	[-8.752; 12.730]	0.717
	W/H	-0.001	0.001	2.778	[-0.002; 0.0001]	0.096
	WHO OGTT	2.456	2.240	1.202	[-1.934; 6.846]	0.273
	Gender	12.557	115.011	0.012	[-212.865; 237.979]	0.913
	R^2 (%)	19.2				

		beta	se	wald test	95% CI	*P* value
Cau	DIratio	0.090	0.040	5.063	[0.012; 0.168]	**0.024**
	age	0.866	3.643	0.057	[-6.274; 8.006]	0.812
	W/H	0.0002	0.0003	0.444	[-0.0004; 0.001]	0.505
	WHO OGTT	-0.030	0.030	1.000	[-0.089; 0.029]	0.317
	Gender	-192.943	95.45	4.086	[-380.025; -5.861]	**0.043**
	R^2 (%)	27.1				

Model 1) Trait glucose disposal t0-210, covariates ISR t0-30, ISR t60-210, ISI, age, W/H, gender Model 2) Trait ISR t60-210, covariates ISR t0-30, ISI, age, W/H, WHO OGTT subgroup, gender. Model 3) Trait glucose disposal t0-210, covariates DI ratio, age, W/H, WHO OGTT subgroup, gender. Bold values indicate the significance of P values

The overall DIs after logarithmic transformation of the three WHO OGTT subgroups according to ethnicity are shown in Fig. 1a. In both ethnicities, the overall DI decreased from the NGT to the IFG/IGT and further to the T2D groups. Illustrated in Fig. 1b, the early and late DIs of the South Asian WHO OGTT subgroups were higher than those of the equivalent Caucasian WHO OGTT subgroups, although a more rapid decline could be observed between South Asian NGT toward the IFG/IGT subgroup. In both ethnicities, early as well as late DI of both NGT and IFG/IGT subgroups differed significantly from their T2D subgroup. We examined the ratio of early and late phase DI in the WHO OGTT subgroups (Fig. 1b). In both the South Asian and the Caucasian families, the IFG/IGT group had lower ratios compared with the NGT group. This ratio was substantially higher in the South Asians with T2D compared with their relatives with IFG/IGT, whereas in the Caucasian families, a very low late phase DI resulted in the lowest ratio.

Adjusted for age, W/H, WHO OGTT subgroup and gender, DI ratio had an effect on glucose disposal t0-210 in both ethnicities, with the explained variances of glucose disposal t0-210 in our final model of 19.2 and 27.1% in South Asian and Caucasian families, respectively (Table 2, Model 3). However, gender also played a role in Caucasian families, but not in South Asian families.

Finally, to explore the differences in glucose handling in the WHO OGTT subgroups within the ethnicities, ternaries based on early DI are shown in Fig. 2a-b. (for total overview, three ternaries based on overall, early and late DI are shown in Supplementary Fig. 2a-f). We used ordinal regression analyses to examine the nature of the components forming early and late DI. The ordinal analyses based on early DI parameters to predict WHO OGTT subgroups, adjusted for family ties, are given in Table 3. In contrast to Caucasians, there was an exclusive role for early beta-cell function,

and not ISI, in predicting glucose tolerance in the South Asian families. Even when including early glucose disposal (glucose disposal t0-30) as an additional covariate in the analysis, ISR t0-30 remained the single significant predictor. In the Caucasian families, both ISR t0-30 and ISI contributed significantly. For both ethnicities, similar ordinal analyses based on late DI parameters did not show significant effects with the exception of late glucose disposal t60-210 (data not shown). Figure 2a-b suggest that the groups with T2D occupy a more distinct area toward the left corner of the ternaries, whereas the other two groups overlap more in the center. Therefore, we also performed logistic regression, adjusted for family ties, with the T2D groups versus the other relatives, the results can be found in Table 3; again, ISR t0-30 remained the most important discriminating variable in South Asians, even when glucose disposal t0-30 was included. For both ethnicities, similar binary logistic regression analyses based on late DI parameters demonstrated glucose disposal t60-210 as the most discriminating variable (data not shown).

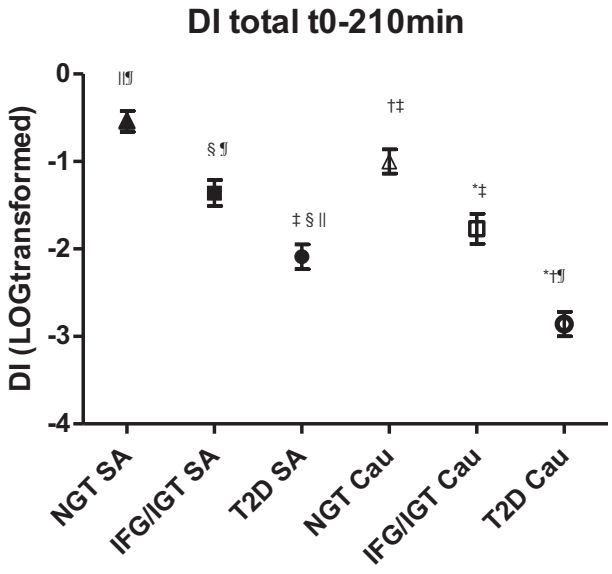


Figure 1a | Overall DI (DI t0-210) for all WHO OGTT subgroups (mean ±SEM) of both South Asian (*closed*) and Caucasian (*open*) families (*triangle* represents NGT, *square* IFG/IGT and *circle* T2D for both ethnicities). *P* values between subgroups in post hoc Bonferroni analysis denoting statistical significance ($P < 0.0125$) are shown with symbols; *=versus Cau NGT, † =versus Cau IFG/IGT, ‡ = versus Cau T2D, § = versus SA NGT, || = versus SA IFG/IGT, ¶ = versus SA T2D

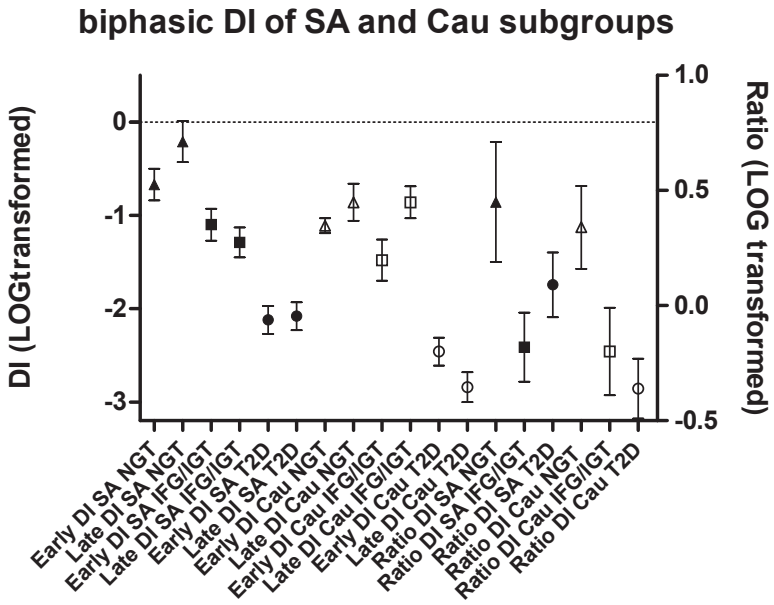


Figure 1b | early DI (DI t0-30) and late DI (t60-210) on *left* Y-axis in mean±SEM , and ratio of late phase/early phase DI (*right* Y-axis, mean±SEM) for NGT, IFG/IGT and T2D of both South Asian (*closed*) and Caucasian (*open*) families (*triangle* represents NGT, *square* IFG/IGT and *circle* T2D for both ethnicities). South Asians: In early DI there was a significant difference between NGT versus T2D and IFG/IGT versus T2D ($P < 0.0001$). In late DI, there was a significant difference between NGT versus IFG/IGT, NGT versus T2D and IFG/IGT versus T2D ($P < 0.0001$). In DI ratio, no significant differences were found ($P = 0.14$). Caucasians: In both early and late DI, there was a significant difference between NGT versus T2D and IFG/IGT versus T2D (both $P <$ 0.0001, respectively). In DI ratio, there was a significant difference between NGT vs T2D ($P = 0.016$).

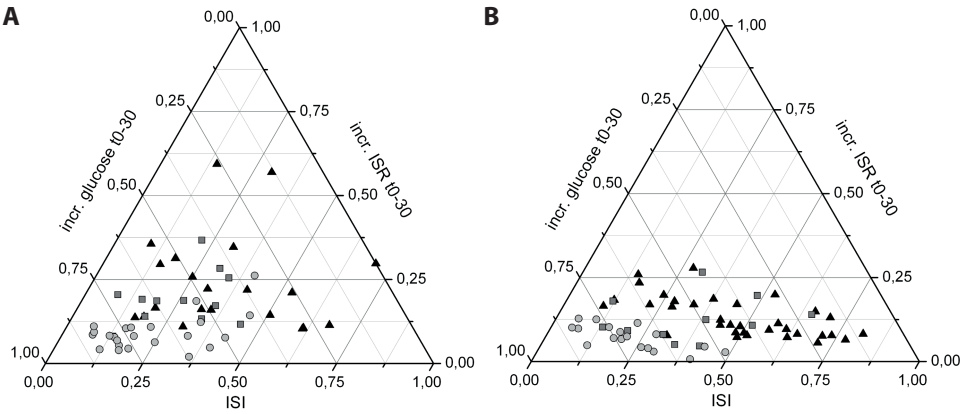


Figure 2 | Ternary plot of relationship between insulin sensitivity (ISI), early phase beta-cell function (ISR t0-30) and glucose disposal (glucose AUC t0-30) based on OGTT from South Asian (*figures left*) and Caucasian families (*triangle* represents NGT, *square* IFG/IGT and *circle* T2D for both ethnicities).

Table 3 | Ordinal and binary logistic regression analysis in both ethnicities predicting WHO OGTT subgroups, adjusted for family ties

	Independent	B	SE	Wald	95% CI	*P* value
Ordinal regression analysis with NGT, IFG/IGT and T2D as dependent variables						
SA	ISR t0-30	-0.020	0.009	5.246	[-0.037;-0.002]	**0.022**
	ISI	-0.782	0.582	1.806	[-1.922;0.359]	0.179
Cau	ISR t0-30	-0.066	0.020	11.128	[-0.105;-0.027]	**0.001**
	ISI	-0.603	0.256	5.556	[-1.105;-0.101]	**0.018**
Binary logistic regression analysis with T2D/non T2D as dependent variable						
SA	ISR t0-30	-0.029	0.009	11.350	[-0.047;-0.011]	**0.001**
	ISI	-0.338	0.275	1.516	[-0.877;0.201]	0.218
Cau	ISR t0-30	-0.017	0.006	7.859	[-0.029;-0.005]	**0.005**
	ISI	-0.319	0.124	6.630	[-0.562;-0.076]	**0.01**

Bold values indicate the significance of *P* values

DISCUSSION

Across WHO OGTT subgroups from South Asian families, including the NGT group, we observed more insulin resistance, with more rapid decline of both early and late DI in NGT toward IFG/IGT, suggestive of early onset beta-cell failure. Across the WHO OGTT subgroups in Caucasian families, we observed a clear trend from normal insulin sensitivity to insulin resistance, while the DI decreased. Among the South Asians, the early insulin response explained at least partly the late insulin response as well as the overall glucose disposal. The ratio of the late over early DI decreased in both ethnicities from NTG to IFG/IGT, but waxed in the South Asian T2D and waned in the Caucasian T2D group, resulting in significant, but opposing effects in both ethnicities on the overall glucose disposal. The South Asians developed overt T2D at young age, while they still had a relatively high DI ratios. As a result of the lack of variance between the South Asian WHO OGTT subgroups in insulin sensitivity, only the early ISR predicted glucose tolerance state. Taken together, our findings confirm that changes in beta-cell dynamics play a prominent role in the development of T2D in South Asians. In Caucasians, more gradual processes of increasing resistance to insulin and decreasing overall insulin secretion seem to take place.

Our data confirm that - without adjustment for insulin sensitivity - South Asian individuals wrongly seem to have enough beta-cell capacity (Supplementary Table 1) with an above-average ability to secrete insulin[15]. Unfortunately, this compensatory

beta-cell function is insufficient, leading to very early onset of T2D, as shown by the young age of manifest T2D in South Asians. These observations underline the important role of changes in beta-cell function, which have been reported to be the main contributor to abnormal glucose tolerance among a wide range of ethnicities, and are in line with increasing genetic evidence for beta-cell defects as an important predisposing factor for T2D [16, 17]. Hypersecretion of insulin may reflect beta-cell responses to different signals or a combination of an increased potentiating effect of glucose on beta-cells, long-lasting adaptation to severe insulin resistance and/or problems with the processing of insulin.

Clamp studies have demonstrated decreased insulin sensitivity among healthy South Asians when compared to other healthy controls [26-29]. We also found a decreased insulin sensitivity in the South Asians compared with the Caucasians. In contrast to the Caucasians, the degree of insulin sensitivity did not change between the three South Asian WHO OGTT subgroups. This very strong familial aggregation of insulin resistance suggests a strong contribution of environmental factors. However, we cannot infer from our data whether lifestyle, type of food, microbiome or other factors are involved. Among our families with high-risk of T2D, the South Asians had much earlier onset of signs and symptoms of T2D compared with the Caucasians. Notably, the burden from macrovascular disease was larger in our South Asian families, even in relatives who did not have T2D. This suggests that the severe insulin resistance of the South Asians contributes strongly to atherogenesis.

In addition to a demanding insulin resistant environment, failing beta-cell capacity is a major susceptibility factor to T2D in South Asian families, as was supported by recent genome wide association studies (GWAS). These studies show greater effects of SNP variants in beta-cell related genes in South Asians than in other populations [30].

The strength of the present study is that it was a family-based approach and analysis of two ethnic groups, among various stages of glucose tolerance. Moreover, beta-cell and insulin sensitivity indices were based on multiple sampled prolonged OGTT's. The relatively small numbers within the WHO OGTT subgroups of the families are a potential weakness, but a characteristic of both extensive phenotyping and family analyses is that it can be performed in relatively small populations. In line, we were able to observe beta-cell function alterations in a very consistent way.

CONCLUSION

Based on extended OGTT measurements, we found that insulin sensitivity is already lower in South Asian than in Caucasian people with NGT. Insulin resistance in the South Asians does not change much during progression of glucose intolerance and beta-cell dysfunction might play a dominant role in the early development of T2D among South Asian families in the Netherlands.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1 | Measures from the OGTT in persons with NGT, IGT and/or IFG and T2D

	South Asian families			Caucasian families		
	NGT	**IFG/IGT**	**T2D**	**NGT**	**IFG/IGT**	**T2D**
n	22	12	23	34	12	18
I_0/G_0 *(pmol/mmol)*	12.5 ± 1.7	12.0 ± 2.5	13.2 ± 3.1	8.4 ± 1.1	8.7 ± 1.4	9.5 ± 2.0
$Cpep_0/G_0$ *(pmol/mmol)*	143.8 ± 12.2	157.8 ± 20.0	129.2 ± 7.4	106.3 ± 6.5	114.2 ± 10.5	120.4 ± 16.7
I_{30}/G_{30} *(pmol/mmol)*	86.0 ± 12.5*¶	64.0 ± 13.5	30.4 ± 4.7§	43.9 ± 4.7‡§	33.5 ± 5.5	19.8 ± 3.3*
$Cpep_{30}/G_{30}$ *(pmol/mmol)*	467.0 ± 38.4*¶	351.2 ± 36.1¶	196.3 ± 20.6§‖	327.0 ± 21.0‡§	243.8 ± 23.3	157.1 ± 18.7*
HOMA-B	109.5 ± 15.4	86.8 ± 20.2	80.7 ± 22.2	73.4 ± 9.7	59.3 ± 8.6	50.7 ± 11.0
$\Delta I_{0\text{-}30}/\Delta G_{0\text{-}30}$ *(pmol/mmol)*	177.7 ± 93.9	168.0 ± 40.3	69.5 ± 15.4	144.8 ± 23.8‡	89.0 ± 20.7	41.2 ± 8.1*
$\Delta Cpep_{0\text{-}30}/\Delta G_{0\text{-}30}$ *(pmol/mmol)*	978.4 ± 368.6	764.3 ± 115.1	352.4 ± 77.9	1043.0 ± 162.7‡	569.3 ± 111.8	235.9 ± 29.1*
$\Delta I_{0\text{-}30}/G_{30}$ *(pmol/mmol)*	76.9 ± 11.9*¶	56.1 ± 13.5	21.7 ± 3.8§	38.3 ± 4.4‡§	27.7 ± 5.5	13.8 ± 2.5*
$Cpep_{0\text{-}30}/G_{30}$ *(pmol/mmol)*	364.1 ± 32.3*¶	246.8 ± 34.2¶	112.6 ± 19.0§‖	252.9 ± 18.2‡§	168.1 ± 21.4	81.6 ± 10.5*
CIR	2646.1 ± 470.5*¶	1301.2 ± 298.8	538.9 ± 136.0§	1254.3 ± 142.4‡§	676.0 ± 129.4	252.1 ± 44.7*
$incrAUC_{ins0\text{-}30}/incrAUC_{gluc0\text{-}30}$ *(pmol/mmol)*	298.0 ± 87.9*¶	172.0 ± 32.9	66.3 ± 14.9§	117.6 ± 11.8‡§	90.8 ± 18.8	47.4 ± 7.8*
$incrAUC_{Cpep0\text{-}30}/incrAUC_{gluc0\text{-}30}$ *(pmol/mmol)*	1654.2 ± 476.6¶	812.4 ± 114.7	351.3 ± 83.4§	817.0 ± 70.3‡	582.7 ± 114.4	224.4 ± 25.6*
$incrAUC_{ins0\text{-}120}/incrAUC_{gluc0\text{-}120}$ *(pmol/mmol)*	587.3 ± 129.3*¶	224.3 ± 26.7	111.3 ± 28.4§	268.0 ± 41.0‡§	124.7 ± 20.9	48.2 ± 8.6*
$incrAUC_{Cpep0\text{-}120}/incrAUC_{gluc0\text{-}120}$ *(pmol/mmol)*	2859.8 ± 468.2‖¶	1051.6 ± 131.6§	580.9 ± 131.2§	2075.7 ± 339.1‡	794.6 ± 117.3	271.1 ± 40.3*
$incrAUC_{ins120\text{-}210}/incrAUC_{gluc120\text{-}210}$ *(pmol/mmol)*	1971.0 ± 615.2¶	527.9 ± 83.7	265.2 ± 77.0§	1161.5 ± 665.8	847.1 ± 514.1	128.8 ± 37.0
$incrAUC_{Cpep120\text{-}210}/incrAUC_{gluc120\text{-}210}$ *(pmol/mmol)*	12601.8 ± 3049.7¶	2754.7 ± 306.8	1882.4 ± 477.1§	7484.9 ± 2588.1	5917.7 ± 3193.7	889.8 ± 174.2
1st phase Stumvoll (pmol/l)	1801.6 ± 171.4¶	1362.2 ± 158.8	724.1 ± 177.5§	1242.5 ± 110.8‡	1061.5 ± 182.2‡	252.2 ± 157.8*†
2d phase Stumvoll (pmol/l)	464.1 ± 43.0¶	364.7 ± 39.5	218.9 ± 42.1§	328.4 ± 27.5‡	287.9 ± 43.4	108.5 ± 41.5*
MCRi (l/min)	1.8 ± 0.2*	1.6 ± 0.2	2.0 ± 0.1	2.5 ± 0.2§	2.4 ± 0.2	2.8 ± 0.4

Data are mean±SEM. *P* values between subgroups in ANOVA post-hoc Bonferroni analysis denoting statistical significance ($P < 0.0125$) are shown with symbols; *=versus Cau NGT, † =versus Cau IFG/IGT, ‡ = versus Cau T2D, § = versus SA NGT, ‖ = versus SA IFG/IGT, ¶ = versus SA T2D

Indices of Beta-cell function

We calculated the fasting insulin/glucose ratio (I_0 / G_0), fasting C-peptide/glucose ratio ($Cpep_0 / G_0$) and homeostatic model assessment for beta-cell function (HOMA-B) using $20 \times I_0 / (G_0 - 3.5)$[1].

Early insulin response indices in OGTT were the insulin/glucose ratio at t=30min (I_{30} / G_{30}), C-peptide/glucose ratio at t=30min ($Cpep_{30} / G_{30}$);[2, 3]the insulinogenic indices (IGI) for the increase in insulin and C-peptide as response to the increment in glucose within first 30 min after the glucose-load: $\Delta I_{0-30} / \Delta G_{0-30}$, $\Delta Cpep_{0-30} / \Delta G_{0-30}$, $\Delta Cpep_{0-30} / G_{30}$ and $\Delta I_{0-30} / G_{30}$[4] the corrected insulin response (CIR) was calculated as $100 \times I_{30} \left[G_{30} \left(G_{30} - 3.89 \right) \right]$[5] and the incremental area under curve (AUC) of insulin and C-peptide concentrations in response to the increment in glucose during the OGTT as: $incrAUC_{ins0-30} / incrAUC_{gluc0-30}$ and $incrAUC_{Cpep0-30} / incrAUC_{gluc0-30}$[6].

Late insulin response indices in OGTT were calculated as: $incrAUC_{ins120-210} / incrAUC_{gluc120-210}$, $incrAUC_{Cpep120-210} / incrAUC_{gluc120-210}$, and overall insulin response indices as $incrAUC_{ins0-120} / incrAUC_{gluc0-120}$ and $incrAUC_{Cpep0-120} / incrAUC_{gluc0-120}$[6].

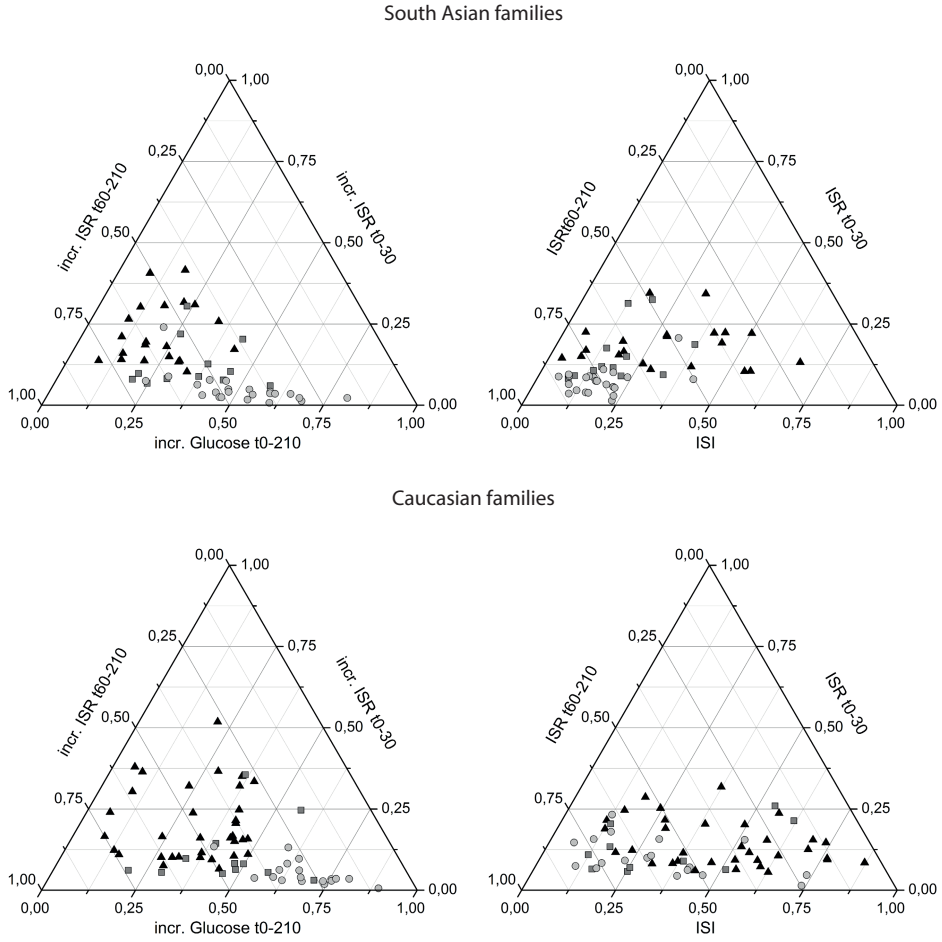
Estimates of beta-cell function according to Stumvoll et al. for first phase and second phase insulin release among NGT and IGT individuals were calculated with the following formulas:

$1^{st}\ phase = 728 + 3.537 \times Ins_0 - 120.3 \times Gluc_{60} + 1.341 \times Ins_{60} + 21.27 \times BMI$,

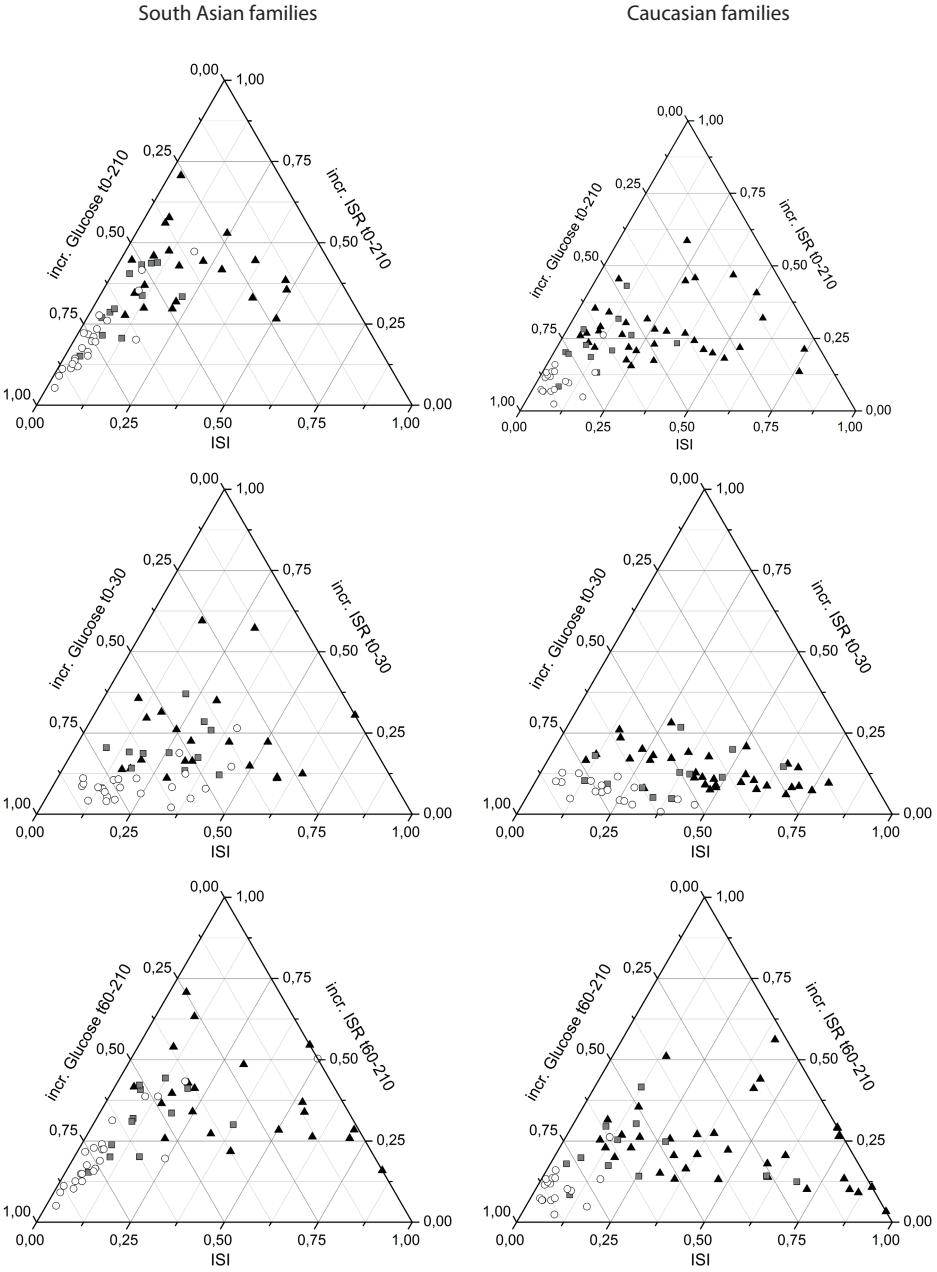
$2^{d}\ phase = 208 + 0.335 \times Ins_{60} - 26.33 \times Gluc_{60} + 0.887 \times Ins_0 + 3.933 \times BMI$ [7].

Metabolic clearance rate

The metabolic clearance rate of insulin (MCri) was calculated from the ratio between the total AUC of the ISR and the AUC of total insulin measured in plasma: $AUC_{ISR0-210} / AUC_{ins0-210}$[8]



Supplementary figure 1 | Ternary plot of ISR t0-30 and ISR t60-210 with glucose disposal (*left*) or ISI (*right*) in South Asian (top panel) and Caucasian(*below*) families(*triangle* NGT, *square* IFG/IGT and *circle* T2D subgroup, respectively).



Supplementary figure 2 | Ternary plot of ISI, ISR t0-210 and glucose disposal t0-210 based on OGTT in South Asian and Caucasian families (*left side* and *right side,* respectively). Also, ternary plots of relationship between ISI, ISR t0-30 and glucose disposal t0-30 are depicted in the *middle* and ternary plots of ISI, ISR t60-210 and glucose disposal t60-210 are depicted *below* (*triangle* NGT, *square* IFG/IGT and *circle* T2D subgroup, respectively)

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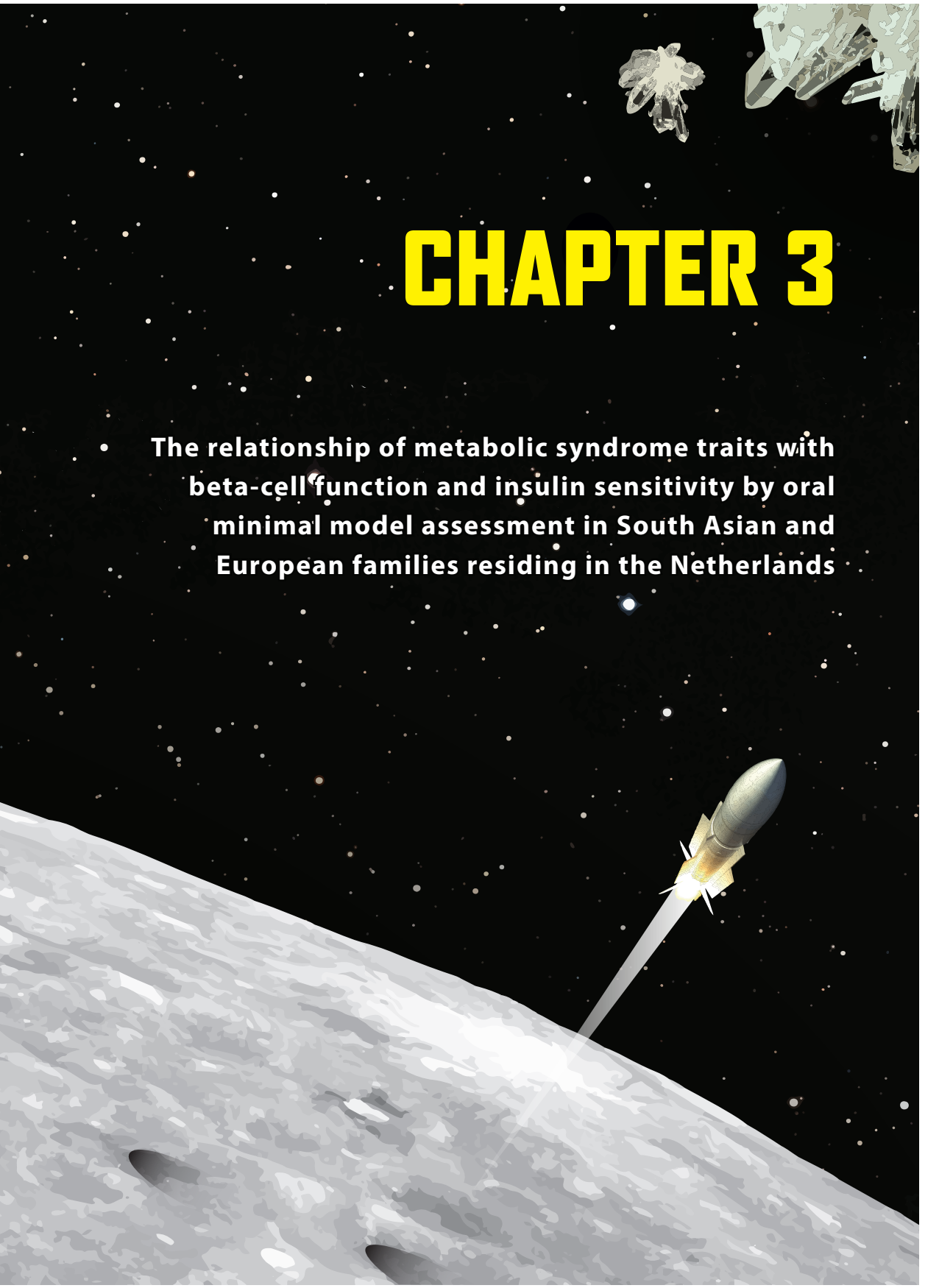

CHAPTER 3

- **The relationship of metabolic syndrome traits with beta-cell function and insulin sensitivity by oral minimal model assessment in South Asian and European families residing in the Netherlands**

ABSTRACT

Background. There are different metabolic syndrome traits among patients with different ethnicities.

Methods. We investigated this by studying 44 South Asians and 54 Europeans and classified them in three groups according to the occurrence of metabolic syndrome (MetS) and Type 2 Diabetes (T2D). Insulin sensitivity index (ISI), static, dynamic and total beta-cell responsivity indices (Φ), and disposition indices (DIs) were calculated with the use of oral minimal model (OMM).

Results. In both ethnicities, ISI was lower in the subgroup with MetS and T2D as compared to the subgroup without MetS nor T2D ($P < 0.004$). South Asians without MetS were more insulin resistant than Europeans without MetS ($P = 0.033$). In the South Asians, ISI, dynamic DI, and static DI were associated significantly ($P < 0.006$) with high-density lipoprotein cholesterol and triglycerides. In the Europeans, ISI was associated with waist-to-hip ratio ($P = 0.005$) and systolic and diastolic blood pressure ($P < 0.005$), while static DI was related to the systolic blood pressure ($P = 0.005$).

Conclusions. MetS was linked with insulin resistance and reduced capacity to handle glucose regardless of ethnicity. ISI and DIs were associated with lipid traits in South Asians and with blood pressure in Europeans suggesting that insulin resistance enhances different metabolic syndrome traits among different ethnicities.

INTRODUCTION

Overweight and physical inactivity enhance each other and decline the sensitivity to insulin. Resistance to insulin is characteristic of metabolic syndrome (MetS), which is defined as a cluster of the following cardiovascular risk factors: central obesity, impaired glucose tolerance, dyslipidemia and hypertension. MetS constitutes a major health problem, as it is strongly associated with type 2 diabetes mellitus (T2D) and cardiovascular disease [1-3]. Furthermore, insulin resistance is a consistent finding in T2D and appears to contribute to the development of T2D. However, T2D develops only if there is dysfunction of beta-cells [4]. In the absence of beta-cell dysfunction individuals can compensate indefinitely for resistance to insulin action with the appropriate hyperinsulinemia. Therefore, many people with remarkable resistance to insulin may never develop T2D [5, 6].

Lifestyle factors clearly underlie MetS incidence, but genetic susceptibility may be important as well [7]. For example, specific ethnic groups are more susceptible to MetS than others [8, 9]. In particular, South Asians are predisposed to develop MetS and subsequently T2D and cardiovascular disease at a younger age [10-12]. They also have a higher prevalence of abdominal obesity, are less sensitive to insulin, and have a lower glucose disposal rate than Europeans [12-14]. In addition, South Asians have lower plasma levels of HDL and adiponectin and higher levels of glucose, insulin, leptin, complement C3, plasminogen activator inhibitor-1, fibrinogen and tissue plasminogen activator compared to Europeans [15-20]. However, traditional risk factors such as smoking, hypertension, and dyslipidemia do not explain the increased risk for cardiovascular disease in South Asians [21, 22]. Insulin resistance itself has been held responsible for the high rates of T2D and cardiovascular disease in this ethnic group [10, 12].

Oral minimal modeling is a pharmacokinetic/pharmacodynamic algorithm developed to estimate beta-cell function and insulin sensitivity index (ISI) from dynamic data [23]. In the present study, we used this oral minimal model (OMM) to investigate the relationship between MetS traits and beta-cell function in South Asian and European families with prevalent T2D.

MATERIALS AND METHODS

Subjects

The recruitment of patients with T2D and their relatives at our university outpatient clinic has been described in detail previously [24]. In brief, 48 South Asians and 54 Europeans that are residing in the Netherlands were initially recruited for the present study and we used an oral glucose tolerance test (OGTT) to group the subjects in T2D or noT2D according to the WHO criteria. In addition, the International Diabetes Federation (IDF) criteria were used to define MetS [25]. Four South Asians had T2D but not MetS and were excluded from the study as the number of subjects was too small for meaningful analyses and there were no European counterparts for comparison. Hence, 98 subjects were included in our analyses (44 South Asians and 54 Europeans) from 25 families (25 patients with T2D but not on insulin therapy and 73 relatives) and they were distributed among 3 groups; no metabolic syndrome/no type 2 diabetes mellitus (noMetS/noT2D), metabolic syndrome/ no type 2 diabetes mellitus (MetS/noT2D) and metabolic syndrome/ type 2 diabetes mellitus (MetS/T2D).

Written informed consent was obtained from all participants. The Erasmus Medical Ethics Review Board approved the study protocol.

Physical Examination

Body height and weight were measured in light clothing without shoes and were used to estimate body mass index. Waist circumference was measured halfway between the lowest rib and the iliac crest while the maximum circumference of the hips was measured in the standing position; from these measurements the waist-to-hip ratio was calculated. Systolic and diastolic blood pressures were measured in the sitting position with an electronic blood pressure monitor (Datascope Accutorr Plus Inc., Montvale, NJ), after five minutes' rest.

Samples and Measurements

All participants underwent a 210 min OGTT. A 75g glucose load was administered (t=0), after an overnight fast and 11 venous blood samples were acquired at pre-specified time intervals (-60min, -15min, 15min, 30min, 45min, 60min, 90min, 120min, 150min, 180min and 210min) for the measurement of plasma glucose, insulin and C-peptide levels. Baseline blood samples were obtained in order to estimate glucose, insulin, C-peptide plasma concentrations and the lipid profile.

Plasma glucose was estimated using a hexokinase-based method (Gluco-quant; Roche Diagnostics, Mannheim, Germany). Plasma insulin and C-peptide were measured separately by a competitive chemiluminescent immunoassay, supplied by Euro/DPC. The assay was performed on a DPC Immulite 2000 analyzer (Euro/DPC) according to the manufacturer's recommended protocol. Serum total cholesterol, HDL, LDL and triglycerides were determined with an automatic enzymatic procedure by Roche Diagnostics (Mannheim, Germany).

Oral Minimal Model Calculations

The OMM, which consists of the glucose OMM and the C-peptide OMM, was used to describe changes of plasma glucose, insulin and C-peptide concentrations during an oral glucose stimulus [26-28]. Glucose, C-peptide and insulin concentrations were measured at 11 time points before and after intake of 75g glucose. The glucose OMM estimated ISI with plasma glucose and insulin concentrations measured during the OGTT. In addition the C-peptide OMM indices were calculated during the oral glucose tolerance test and in terms of insulin secretion can be interpreted as follows: (1) the basal (Φ_{basal}), that gives a basal nonstimulated measurement of insulin secretion (2) the dynamic ($\Phi_{dynamic}$), that provides the amount of insulin secreted during the dynamic phase (first phase secretion by beta-cells) (3) the static (Φ_{static}), that assesses the release of insulin that occurs after a time delay (second phase secretion by beta-cells) and represents a beta-cell response according to the prevailing glucose concentration and (4) the total (Φ_{total}) overall secretion which is the sum of the dynamic and static phase release of insulin from beta-cells. The parameters of glucose OMM and C-peptide OMM were multiplied to obtain the disposition indices (DIs), which is a measure for beta-cell function corrected for insulin sensitivity. It can be considered a measure of the functionality of the pancreas in the intact individual: $DI_{basal} = \Phi_{basal} \times ISI$, $DI_{dynamic} = \Phi_{dynamic} \times ISI$, $DI_{static} = \Phi_{static} \times ISI$ *and* $DI_{total} = \Phi_{total} \times ISI$. We performed the analyses with SAAMII software [29].

Statistical Analysis

Continuous variables are expressed as mean ± SEM, unless indicated otherwise. ANOVA test was used to compare the mean of raw data presented in Table 1 and the figures of different subgroups within the two ethnicities. Adjusted analyses were performed with multiple regression models in which all covariables were entered synchronously.

A $P<0.05$ was considered statistically significant. All statistical tests were conducted with SPSS software, version 20 (SPSS Inc., Chicago, IL).

RESULTS

Baseline Characteristics

32 out of 44 South Asians (72.7%, 95% CI 58.0 to 83.8%) and 23 out of 54 Europeans (42.6%, 95% CI 30.3 to 55.9%) had MetS according to the IDF criteria. In Table 1, the general characteristics of the 3 subgroups are shown according to ethnicity. The systolic blood pressure, fasting and 2 h plasma glucose levels were increased in South Asians with MetS and T2D compared to the noMetS/noT2D group. South Asians with MetS and T2D were on average 10.4 years younger than Europeans with MetS and T2D. Compared to the analyses of the South Asian subgroups, we observed a larger heterogeneity among the 3 subgroups of the Europeans in clinical and biochemical characteristics with significant differences in age, weight, body mass index, waist, waist-to-hip ratio, blood pressure and fasting and 2 h plasma glucose levels.

Insulin Sensitivity

The ISIs of the three subgroups according to ethnicity are shown in Figure 1. Subjects without MetS had higher ISI than those with MetS. On average and adjusted for sex and age, the Europeans had 7.18 x 10^{-4} dL/kg/min per µU/mL (95% CI 0.58 to 13.78, P = 0.033) higher ISI than the South Asians. This was fully explained by the differences between the two subgroups without MetS (after excluding this subgroup to the analysis the effect of ethnicity disappeared, P = 0.367).

Beta-cell Responsivity Indices

The fitting of both C-peptide and glucose OMM was satisfactory as the average weighted residuals did not deviate systematically from the zero value (Supplementary Figures 1 and 2). Figure 2 shows the beta-cell responsivity indices estimated by the C-peptide OMM during the OGTT. Adjusted for sex and age, ethnicity was significantly related to the basal responsivity index: β = 0.086 x 10^{-9}/min, 95% CI 0.17 to 0.155, P = 0.016; but not to the other responsivity indices, P > 0.7. Within the South Asian group adjusted for sex and age, the MetS/noT2D group had 0.164 x 10^{-9}/min (95% CI 0.045 to 0.282, P = 0.008) higher basal responsivity index compared to the other subgroups. The basal responsivity index was not significantly different between the European subgroups. The dynamic, the static and the total responsivity indices showed all the same trend of a lower beta-cell response (in effect less insulin secretion) in the MetS/T2D groups in both ethnicities: -191.9 x 10^{-9} (95% CI -282.2 to -101.6, P=0.001), -12.1 x 10^{-9}/min (95% CI -16.1 to -8.0, P=0.001) and -14.3 x 10^{-9}/min (95% CI -18.9 to -9.6, P=0.001), respectively. The South Asian noMetS/noT2D and MetS/noT2D had similar

values in the dynamic, the static and the total responsivity indices, whereas in the Europeans there was a clear trend to gradually lower values over these groups in the direction of the MetS/T2D subgroup: $P_{for\ trend} = 0.005$, 0.001 and 0.001, respectively.

Disposition Indices

The DIs according to subgroup and ethnicity are shown in Figure 3. Adjusted for sex and age the four DIs did not differ significantly between the ethnicities ($P > 0.11$). Both ethnicities showed the same gradual course of the different DI from high to intermediate and low over the noMetS/noT2D, MetS/noT2D and MetS/T2D subgroups, respectively ($P_{for\ trend} < 0.002$). There were no clear differences between the ethnicities, although the European noMetS/noT2D subgroup had relatively high DI compared to the same South Asian subgroup.

MetS Traits and OMM Indices

The relationships between the MetS traits and OMM indices adjusted for sex and age according to ethnicity are shown in Table 2. The MetS traits were not used in a large "holistic" multiple linear regression model since they are correlated. Therefore, each trait was analyzed separately in a multiple linear regression model adjusted for sex and age. Waist-to-hip-ratio was strongly associated with ISI in both ethnicities, but it may have a more detrimental effect of the glucose disposition in Europeans than in the South Asians. The dyslipidemia characterized by high triglycerides and low HDL was related to ISI and the DIs solely in the South Asians, whereas blood pressure, both systolic and diastolic, was solely related to ISI and the DIs in the Europeans. As expected, fasting glucose was associated with ISI and DIs as these indices are calculated with glucose values.

Table 1 | General characteristics of the study subjects.

	South Asians (n=44)			Europeans (n=54)		
	noMetS/noT2D	**MetS/noT2D**	**MetS/T2D**	**noMetS/noT2D**	**MetS/noT2D**	**MetS/T2D**
N	12	14	18	31	12	11
Male/Female	6/6	9/5	10/8	9/22	5/7	6/5
Age (yrs)	33.9±8.0	48.2±10.1‡	51.4±10.4‡	39.6±10.2	40.3±10.2	61.8±8.7*†
Height (m)	1.67±0.12	1.68±0.12	1.63±0.08	1.75±0.10	1.78±0.08	1.77±0.07
Weight (kg)	74.1±16.3	78.8±9.6	76.3±12.4	77.8±12.2	103.5±28.7*	98.3±11.8*
Body mass index (kg/m^2)	26.3±3.3	28±2.7	28.7±4.1	25.4±3.1	32.6±8.2*	31.4±4.3*
Waist (cm)	93±12	98±9	97±7	88±12	110±19*	110±13*
Hip (cm)	105±7	104±4	103±8	106±8	120±16*	116±8
Waist-to-hip ratio	0.89±0.08	0.94±0.07	0.95±0.08	0.83±0.09	0.92±0.08*	0.94±0.07*
Systolic blood pressure (mmHg)	114±11	130±16	136±15‡	120±12	137±14*	137±14*
Diastolic blood pressure (mmHg)	76±9	83±12	82±9	74±8	84±9*	84±11*
Total cholesterol (mmol/L)	4.7±0.7	5±0.9	4.6±1.2	4.8±1	4.9±0.9	4.5±1.1
Triglycerides (mmol/L)	0.97±0.37	1.43±0.65	1.55±0.65	0.97±0.36	1.39±0.77	1.38±0.55
HDL (mmol/L)	1.19±0.37	1.01±0.21	1.05±0.22	1.47±0.40	1.11±0.19*	1.17±0.26
LDL (mmol/L)	3.02±0.63	3.30±0.76	2.80±1.08	2.77±0.93	3.01±0.80	2.55±0.90
Ratio HDL-Cholesterol	4.2±1.3	5.2±1.4	4.4±1.2	3.5±1.1	4.5±1.2*	3.9±0.9
Fasting plasma glucose (mmol/L)	5.3±0.3	5.8±0.5	7.4±1.5§‡	5.3±0.5	5.7±0.5	8.1±1.1*†
2 h glucose (mmol/L)	5.5±1.7	6.8±1.2	13.8±5.2§‡	5.7±1.5	6.7±1.8	13.9±2.8*†
Insulin (pmol/L)	53.5±46.8	73.8±49.2	58.3±38.4	35.8±29	73.7±35.6	78.5±72.2

HDL: high-density lipoprotein; LDL: low-density lipoprotein; MetS: metabolic syndrome; T2D: type 2 diabetes mellitus.
* Indicates statistically significant differences between the noMetS/noT2D and the other 2 subgroups within Europeans; † Indicates statistically significant differences between the MetS/noT2D and the Met/T2D subgroup within Europeans; ‡ Indicates statistically significant differences between noMetS/noT2D and the other 2 subgroups within South Asians; § Indicates statistically significant differences between MetS/noT2D and the Met/T2D subgroup within South Asians.

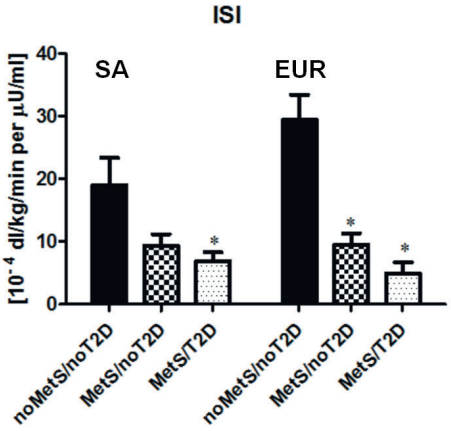

Figure 1 | Insulin sensitivity index (ISI) assessed by the OMM in South Asian (SA) and European (EUR) subgroups. * indicates a statistically significant difference between noMetS/noT2D and other subgroups.

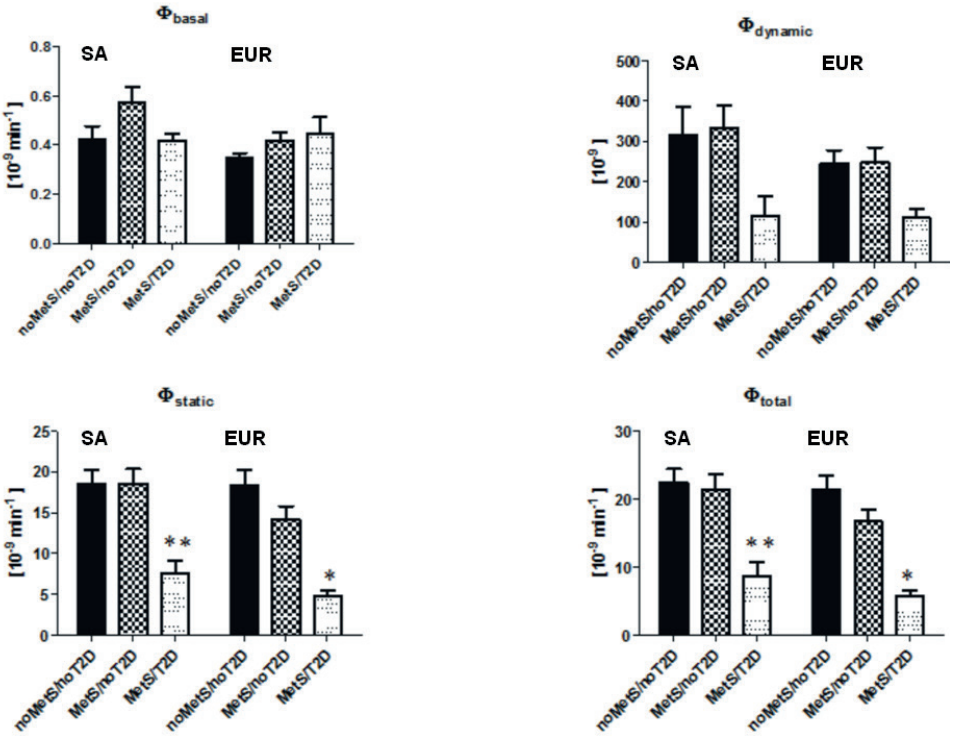

Figure 2 | beta-cell insulin responsivity indices in South Asian (SA) and European (EUR) subgroups.* indicates a statistically significant difference in comparison with the noMetS/noT2D subgroup; ** indicates a statistically significant difference between the MetS/noT2D and MetS/T2D subgroup.

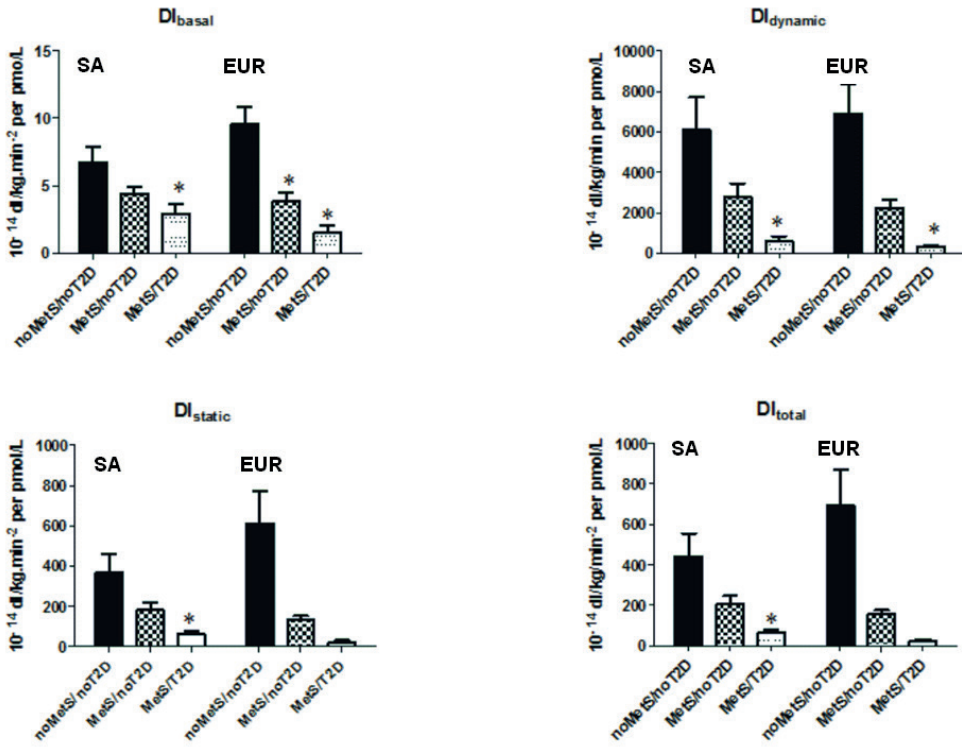

Figure 3 | Disposition indices in South Asian (SA) and European (EUR) subgroups.
* indicates a statistically significant difference in comparison with the noMetS/noT2D subgroup.

Table 2 | Relationship between metabolic syndrome traits and insulin sensitivity index (ISI), dynamic disposition index ($DI_{dynamic}$) and static disposition index ((DI_{static}) adjusted for sex and age according to ethnicity.

	ISI	95% CI	P	$DI_{dynamic}$	95% CI	P	DI_{static}	95% CI	P
Metabolic syndrome trait	**$x10^{-4}$ dL/kg/min per µU/mL**			**$x10^{-10}$ dL/kg/min per µU/mL**			**$x10^{-10}$ dL/kg/min per µU/mL**		
South Asians									
Waist-to-hip ratio	-61	-109 to -13	0.015	-9.4	-27.0 to 8.1	0.284	-0.7	-1.8 to 0.3	0.181
Triglycerides (mmol/L)	-7.1	-11.9 to -2.3	0.005	-2.0	-3.7 to -0.3	0.022	-0.1	-0.2 to -0.04	0.007
HDL (mmol/L)	25.7	15.8 to 35.7	0.001	5.9	1.9 to 9.9	0.005	0.5	0.2 to 0.7	0.001
Systolic blood pressure (mmHg)	-0.2	-0.4 to -0.03	0.028	0.06	-0.1 to 0.02	0.129	-0.005	-0.009 to -0.0006	0.026
Diastolic blood pressure (mmHg)	-0.2	-0.5 to 0.1	0.169	-0.04	-0.1 to 0.07	0.487	-0.003	-0.01 to 0.003	0.281
Fasting plasma glucose (mmol/L)	-3.0	-5.3 to -0.6	0.014	-1.2	-2.0 to -0.4	0.003	-0.07	-0.1 to -0.003	0.002
Europeans									
Waist-to-hip ratio	-95	-159 to -31	0.005	-25.6	-49.6 to -1.7	0.036	-22.9	-4.8 to -0.2	0.075
Triglycerides (mmol/L)	-7.9	-18 to 2.4	0.131	-0.3	-4.0 to 3.5	0.888	-0.1	-0.5 to 0.3	0.518
HDL (mmol/L)	10.6	-5.9 to 27.1	0.204	1.3	-4.7 to 7.3	0.668	0.3	-0.3 to 1.0	0.266
Systolic blood pressure (mmHg)	-0.6	-0.9 to -0.3	0.001	-0.1	-0.3 to -0.02	0.026	-0.02	-0.03 to -0.006	0.005
Diastolic blood pressure (mmHg)	-0.8	-1.3 to -0.3	0.004	-0.1	-0.3 to 0.05	0.144	-0.02	-0.04 to 0.004	0.111
Fasting plasma glucose (mmol/L)	-5.2	-10.3 to -0.09	0.046	-2.3	-4.1 to -0.5	0.01	-0.2	-0.4 to 0.005	0.056

HDL: high-density lipoprotein; ISI: insulin sensitivity index; $DI_{dynamic}$: dynamic disposition index; DI_{static}: static disposition index.

DISCUSSION

In the present study, we found a clear trend for increasing insulin resistance from noMetS/noT2D to MetS/noT2D and to MetS/T2D. We also found that South Asians without MetS were more resistant to insulin than a corresponding group of Europeans. In the European families, the dynamic, static and total beta-cell responsivity indices followed the trend of ISI in the same direction over the three subgroups towards decreasing insulin secretion by the beta-cells in the T2D group. The South Asian noMetS/noT2D and MetS/noT2D subgroups both had high beta-cell responsivity indices and the MetS/T2D subgroup had low values. The DIs are the product of ISI and beta-cell responsivity indices and independent of ethnicity followed a decreasing trend over the subgroups in the direction of T2D. Notably, a number of separate MetS traits had different associations in the two ethnicities: dyslipidemia had a strong relationship with ISI and DI in the South Asians, whereas blood pressure was associated with ISI and DI in the Europeans.

Over the last years several studies have examined the effect of lifestyle, dietary habits, and genetic polymorphisms on the development of MetS in different ethnicities, in order to identify high-risk populations such as the South Asians, who appear to have an increased predisposition for T2D [30-32]. Most of these reports focused on epidemiological analyses and evaluations of the impact of biochemical and physical characteristics (e.g. total abdominal fat, intra-abdominal adipose tissue) on the development of MetS. In a number of studies, ISI and beta-cell function were assessed in different ethnicities using fasting blood glucose and plasma insulin concentrations (for instance homeostatic model assessment - HOMA) [33-35]. These methods do not assess the dynamic response of beta-cells to glucose stimuli [36, 37]. Our study is the first to employ OMM in order to examine the relationships between MetS traits and ISI and beta-cell response. The estimations of ISI and insulin secretion by OMM are reasonably well correlated with those assessed by the hyperinsulinemic euglycemic clamp method [38].

We demonstrated for the first time, to our knowledge, that in South Asians and Europeans both ISI and beta-cell function are lower in subjects with MetS and in subjects with MetS and T2D compared to noMetS/noT2D subjects. In addition, we confirmed an increased insulin resistance in noMetS/noT2D South Asians compared to noMetS/noT2D Europeans [39, 40]. Moreover in South Asians with noMetS/noT2D the disposition indices were lower comparing to the disposition indices in Europeans and not statistically different from the disposition indices in South Asians with MetS/

noT2D. These findings indicate that South Asians are a vulnerable population and have an increased risk to develop chronic diseases such as MetS and T2D, in which insulin resistance and beta-cell function are key pathogenetic factors.

Our study showed that OMM could be used to evaluate glucose homeostasis in different populations and to examine associations between insulin resistance, abnormal beta-cell function and MetS traits. Waist-to-hip ratio reflects the association between visceral obesity and the causal path from insulin resistance to T2D [41-43]. Hypertriglyceridemia and increased enzymes involved in triglyceride transport and metabolism may have a toxic effect on beta-cells [44], but hypertriglyceridemia can be a consequence of insulin resistance and T2D as well. Remarkably, triglycerides and HDL were associated with ISI and DIs in South Asians. Unfortunately, we cannot infer from our data whether dyslipidemia was the cause or the consequence of insulin resistance and reduced DIs. In the European families, insulin resistance and reduced DI were associated with raised blood pressure. This suggests that disorders of carbohydrate metabolism were complicated by higher levels of blood pressure. A possible explanation is an impaired insulin signaling through PI3K signaling pathway and the increased vascular inflammation noted in insulin resistance which can decrease nitric oxide (NO) production and increase Endothelin-1 (ET-1) secretion leading to raised blood pressure [45,46]. The differences in the associations between metabolic syndrome traits, beta cell function and insulin sensitivity in different ethnicities have not been fully explained yet. A possible etiology is the different genetic profile of Europeans and South Asians; however further research is required towards this direction in order to understand the metabolic pathways and the effect of the genome on these associations.

In the present analysis, we investigated associations between metabolic syndrome traits and insulin sensitivity and beta cell function after adjusting for age and sex. Of note the results did not change significantly when we adjusted also for BMI. The only differences that we noticed were in Europeans where there was no longer an association between ISI, waist-to-hip ratio and diastolic blood pressure as well as a correlation between fasting glucose and ISI and DI_{static} (Supplementary Table 1).

Strengths and Limitations

We have recruited subjects through families with prevalent T2D. Family studies avoid a number of serious selection biases, but the findings cannot easily be generalized to the general population. Moreover, we prefer to analyze family data using a family matrix for instance SOLAR, but unfortunately there were too many small cells. Adjustment for family ties in regression analyses suffered from the same problem; therefore, we restricted the adjustment of all primary analyses to age, which correlates with

generation in kindreds. We did not analyze genetic variation, and transmission was not a topic of our research. Moreover, separate analyses of small and large families did not change the results (data not shown).

We chose the comparison between the MetS/T2D status groups and not between the two ethnic groups because South Asians and Europeans differ in many aspects and therefore we tried to avoid bias due to the ethnicity differences.

We have used the IDF criteria for MetS, because they take ethnic specific cut-off points for central obesity, in effect waist circumference, into account. South Asians residing in the Western world differ in lifestyle and phenotype from the South Asians living in South Asia, and therefore we tested the necessity of such ethnicity specific cut-off points: if the European cut-off points were used for all subjects, only three male South Asians would have not been classified as MetS. Still a number of arguments could be made to include analyses using a single cut-off point, but the effects on our results were negligible (data not shown) and therefore we decided to follow the IDF recommendations.

A limitation of the study was the relatively small sample size and for this reason these findings cannot be generalized to the broader community. Furthermore, we excluded the noMetS/T2D subgroup, because meaningful analysis was not possible, as there were only 4 South Asians and no Europeans counterparts.

CONCLUSIONS

In our study, we observed low ISIs in the MetS/T2D group relative to the noMetS/noT2D group in both ethnicities; a finding that indicates an association between insulin resistance and a pathologic metabolic state (T2D). The pathogenic mechanisms that lead to insulin resistance appear to differ among ethnicities, as ISI and DIs were associated with different metabolic traits in each ethnicity. The simultaneous assessment of insulin sensitivity and beta-cell function allowed us to study metabolic profiles that promote T2D and glucose intolerance. We used this primarily to explore the underlying mechanisms of disturbances in glucose metabolism, but also to explore the potential of designing ethnicity-specific risk models for early identification of subjects that are at risk for developing diabetes mellitus. In this study, South Asians, in contrast to Europeans, appear to be predisposed to insulin resistance and exhibit impaired beta-cell function at an earlier age. Dyslipidemia related to insulin resistance and pancreatic dysfunction may underlie or enhance this susceptibility of South Asians.

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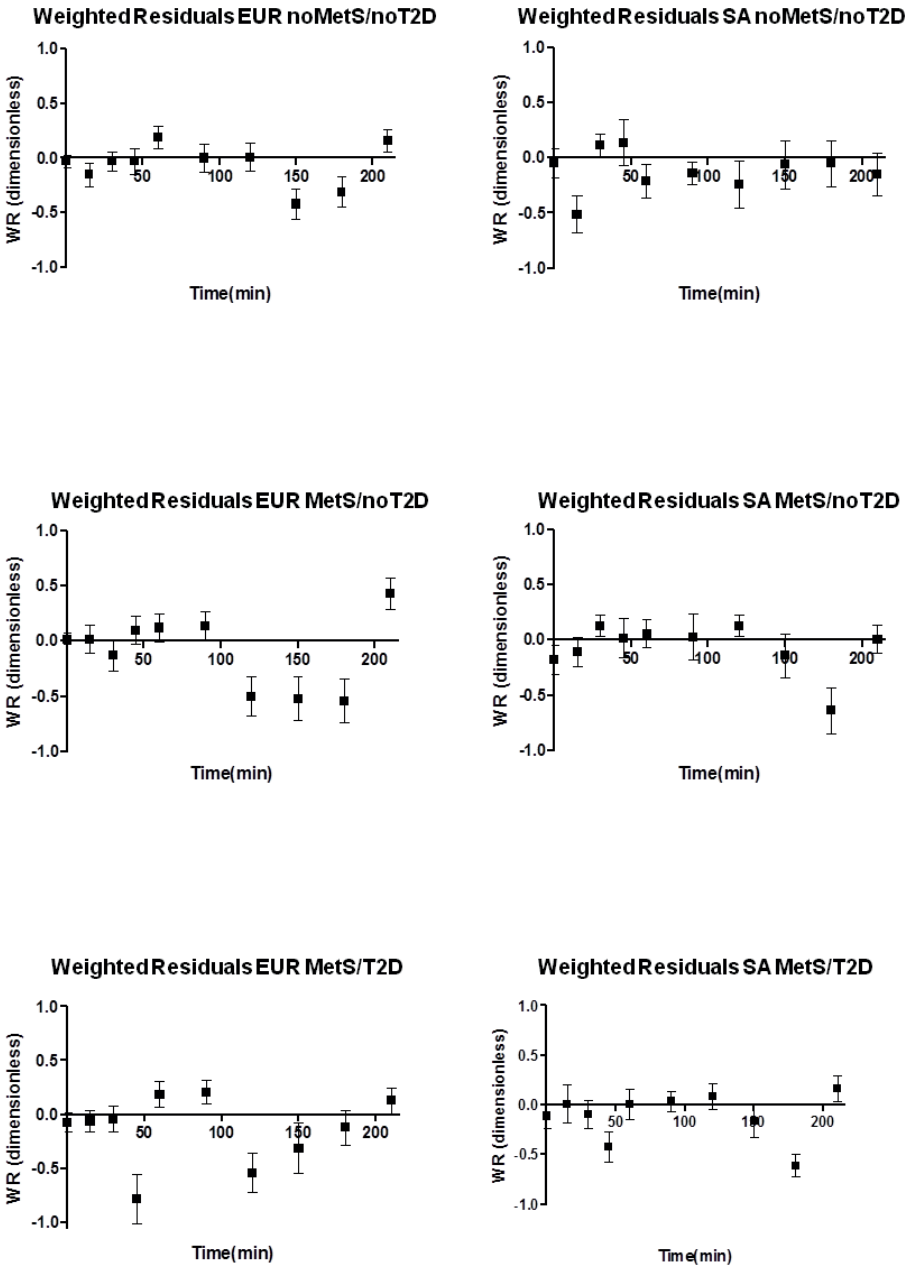
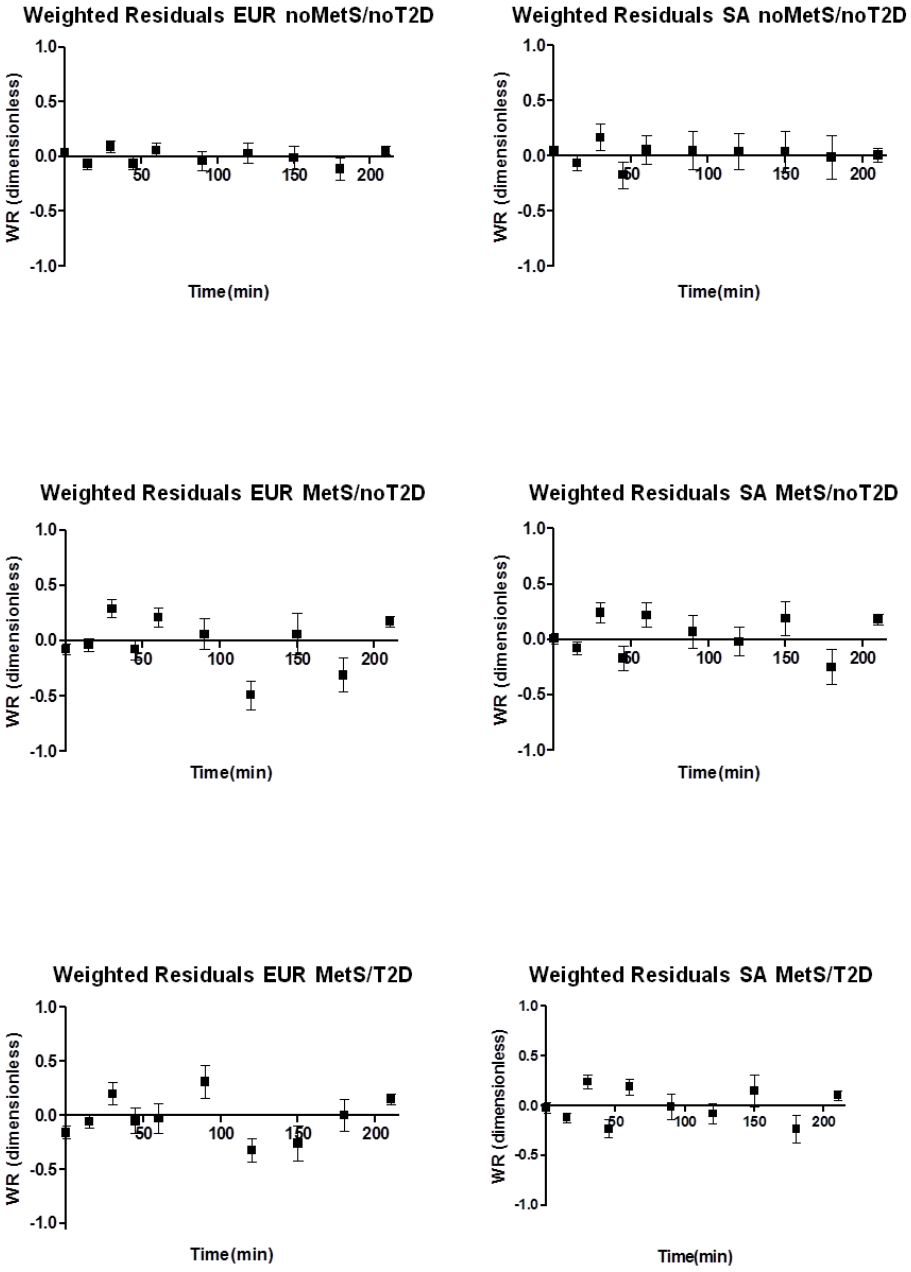
SUPPLEMENTARY MATERIAL



Supplementary Figure 1 | Average weighted residuals for the C-peptide oral minimal model.



Supplementary Figure 2 | Average weighted residuals for the glucose oral minimal model.

Supplementary Table 1 | Relationship between metabolic syndrome traits and insulin sensitivity index (ISI), dynamic disposition index ($DI_{dynamic}$) and static disposition index (DI_{static}) adjusted for sex, age and BMI according to ethnicity

	ISI	95% CI	*P*	$DI_{dynamic}$	95% CI	*P*	DI_{static}	95% CI	*P*
Metabolic syndrome trait	**x10^{-4} dl/kg/min per µU/ml**			**x10^{-10} dl/kg/min per µU/ml**			**x10^{-10} dl/kg/min per µU/ml**		
South Asians									
Waist-to-hip ratio	-60.75	-109 to -12.1	0.016	-9.03	-26.56 to 8.5	0.303	-0.694	-1.74 to 0.36	0.189
Triglycerides (mmol/L)	-6.72	-11.53 to -1.91	0.007	-1.84	-3.52 to -0.16	0.032	-0.130	-0.23 to -0.031	0.012
HDL (mmol/L)	24.6	14.12 to 35.08	<0.001	5.2	1.17 to 9.38	0.013	0.435	0.21 to 0.664	<0.001
Systolic blood pressure (mmHg)	-0.21	-0.44 to 0.023	0.077	-0.04	-0.12 to 0.04	0.322	-0.004	-0.009 to 0.000	0.074
Diastolic blood pressure (mmHg)	-0.22	-0.53 to 0.087	0.154	-0.04	-0.15 to 0.07	0.457	-0.004	-0.01 to 0.003	0.259
Fasting plasma glucose (mmol/L)	-2.73	-5.06 to -0.4	0.023	-1.11	-1.87 to -0.35	0.005	-0.069	-0.11 to -0.023	0.004
Europeans									
Waist-to-hip ratio	-47.1	-117.14 to 22.94	0.183	-16	-43.48 to 11.45	0.248	-1.162	-4.054 to 1.73	0.423
Triglycerides (mmol/L)	-6.9	-15.98 to 2.2	0.134	-0.039	-3.675 to 3.6	0.983	-0.104	-0.48 to 0.275	0.583
HDL (mmol/L)	0.87	-14.94 to 16.68	0.912	-1.072	-7.24 to 5.098	0.728	0.130	-0.514 to 0.774	0.687
Systolic blood pressure (mmHg)	-0.43	-0.76 to -0.100	0.012	-0.099	-0.23 to 0.036	0.146	-0.015	-0.029 to -0.001	0.036
Diastolic blood pressure (mmHg)	-0.4	-0.93 to 0.137	0.141	-0.05	-0.26 to 0.163	0.637	-0.007	-0.029 to 0.015	0.529
Fasting plasma glucose (mmol/L)	-3.27	-7.98.3 to 1.44	0.169	-1.95	-3.74 to -0.158	0.034	-0.144	-0.336 to 0.047	0.137

Sjaam Jainandunsing, J.L. Darcos Wattimena, Adrie J.M. Verhoeven, Janneke G. Langendonk, Trinet Rietveld, Aaron J. Isaacs, Eric J.G. Sijbrands, Felix W.M. de Rooij

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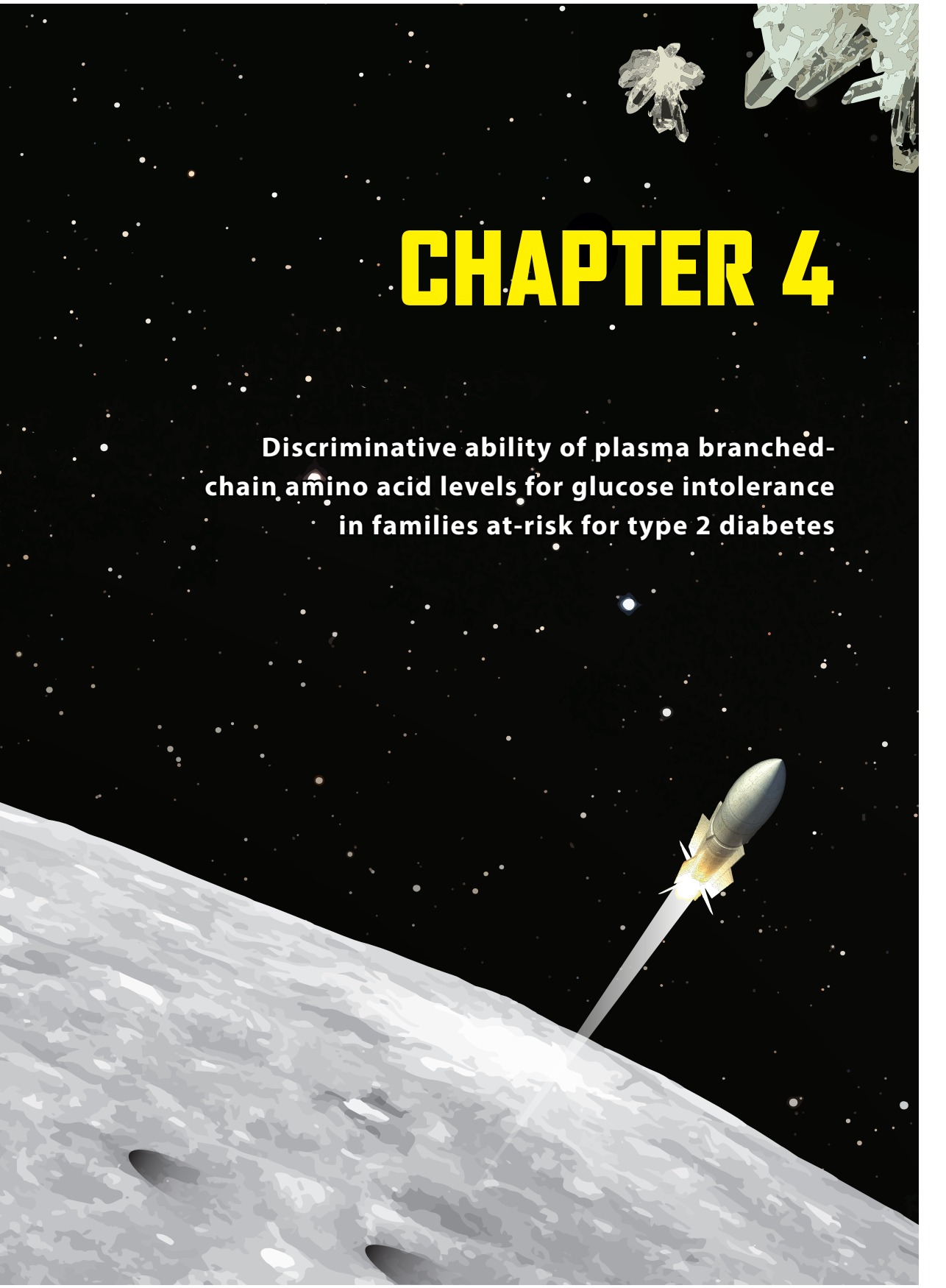


CHAPTER 4

Discriminative ability of plasma branched-chain amino acid levels for glucose intolerance in families at-risk for type 2 diabetes

ABSTRACT

Background– Insulin resistance and glucose intolerance have been associated with increased plasma levels of branched-chain amino acids (BCAA). BCAA levels do not predict T2DM in the population.

We determined the discriminative ability of fasting BCAA levels for glucose intolerance in nondiabetic relatives of patients with T2DM of two different ethnicities.

Methods– Based on oral glucose tolerance test (OGTT), first-degree relatives of patients with T2DM were categorized as normal glucose tolerance, prediabetes, or T2DM. Included were 34, 12, and 18 Caucasian and 22, 12, and 23 Asian Indian participants, respectively. BCAA levels were measured in fasting plasma together with alanine, phenylalanine and tyrosine. Insulin sensitivity and beta-cell function were assessed by indices derived from an extended OGTT and their relationship with plasma BCAA levels was assessed in multivariate regression analysis. The value of the amino acids for discriminating prediabetes among nondiabetic family members was determined with the area under the curve of receiver-operated characteristics (c-index).

Results–BCAA levels were higher in diabetic than in normoglycemic family members in the Caucasians (P = 0.001) but not in the Asian Indians. In both groups, BCAA levels were associated with waist-hip ratio (β = 0.31; P = 0.03 and β = 0.42; P = 0.001, respectively) but not with indices of insulin sensitivity or beta-cell function. The c-index of BCAA for discriminating prediabetes among non-diabetic participants was 0.83 and 0.74 in Caucasians and Asian Indians, respectively, which increased to 0.84 and 0.79 by also including the other amino acids. The c-index of fasting glucose for discriminating prediabetes increased from 0.91 to 0.92 in Caucasians and 0.85 to 0.97 (P = 0.04) in Asian Indians by inclusion of BCAA + alanine, phenylalanine and tyrosine.

Conclusions. Adding fasting plasma BCAA levels, combined with phenylalanine, tyrosine and alanine to fasting glucose improved discriminative ability for the prediabetic state within Asian Indian families at risk for T2DM. BCAA levels may serve as biomarkers for early development of glucose intolerance in these families.

INTRODUCTION

The incidence of obesity and type 2 diabetes (T2DM) is increasing worldwide, and the onset of the disease occurs at steadily earlier age. Biomarkers for early development of glucose intolerance and T2DM are highly needed to identify individuals at risk and initiate early preventive strategies. Raised plasma levels of branched-chain amino acids (BCAA) have long been linked to obesity, insulin resistance and T2DM [1-3]. In addition to BCAA, phenylalanine, tyrosine, and alanine are also associated with insulin resistance and glucose intolerance. In predominantly lean Chinese and Asian Indian men, this combined amino acid profile correlated with insulin resistance[4]. In healthy young adults, fasting plasma levels of BCAA, phenylalanine, and tyrosine were associated with an increase of the HOMA-insulin resistance index at 6 years follow-up [5]. Similarly, in obese children, elevated BCAA levels were positively associated with an increase in HOMA-insulin resistance index during the following 18 months [6]. In two independent prospective cohorts with normoglycemic adults followed for 12 years, elevated levels of these amino acids at baseline were associated with future development of T2DM [7]. Although fasting plasma amino acid levels have limited predictive value for future glucose intolerance and T2DM in the general population [5, 7], it may have a strong value for at-risk individuals [7]. Patients with the early onset of T2DM often descend from families with high risk of T2DM. This holds particularly for the Asian Indians living in the Netherlands, who develop T2DM at a young age and relatively low BMI, resulting in a fivefold increased risk of T2DM compared to Dutch Caucasians [8, 9]. We approached first-degree family members of these patients and identified individuals with normal and impaired glucose tolerance. We hypothesized that the fasting amino acid profile with raised BCAA, phenylalanine, tyrosine, and alanine levels is a potential biomarker for the development of early glucose intolerance or insulin resistance in family analyses.

In the present study, we determined the value of fasting BCAA and other amino acid levels in discriminating glucose-intolerant from glucose-tolerant subjects in families with a high risk of T2DM.

MATERIALS AND METHODS

Subjects

Index cases (individuals with T2DM) had been referred to the Outpatient clinic of Internal Medicine at the Erasmus University Medical Center in Rotterdam for complications associated with their T2DM. First-degree relatives were invited for the

study; this resulted in participation of individuals from 24 Caucasian families and 36 Asian Indian families living in the Netherlands, with two generations taken into account and an average of approximately two members of each family. Power calculation was performed with Quanto version 1.0[10], and was based on differences in early phase insulin secretion response (described further on in Materials and Methods section) between healthy South Asian and Caucasian individuals , during a pilot phase of the study, with alpha 0.05 and power 80%. At least 17 individuals with normal glucose tolerance, based on oral glucose tolerance test (OGTT) were required. Caucasian subjects had to be born in the Netherlands and both their parents had to be of ethnic Dutch Caucasian origin. Asian Indian subjects were eligible for the study if they were born in Surinam or the Netherlands and when both their parents were of Asian Indian origin. Subjects had to be at least 18 years old. The exclusion criteria were as follows: insulin-dependent diabetes mellitus, use of antidiabetic medication other than metformin, a history of pancreatitis, insulinoma, or other reasons, which made participation impossible. All participants underwent an OGTT and were diagnosed to have normal glucose tolerance, impaired glucose tolerance, or T2DM (normoglycemic, prediabetic, and diabetic) according to the WHO OGTT criteria[11]. Details about the study setup have been previously reported[12]. Written informed consent was obtained from all participants. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. The study protocol was approved by the Erasmus University Medical Center Medical Ethics Review Board (MEC-2009-242).

In vivo measurements

After an overnight fast, each participant underwent an extended OGTT and venous blood samples were collected at 15 min before and 15, 30, 45, 60, 90, 120, 150, 180 and 210 minutes after the oral administration of 75 grams glucose. Fasting blood was collected at 60 min before administration of glucose and used to reflect the t=0 value. Body length and weight were measured to the nearest 0.1 cm and 0.1 kg, respectively. Waist and hip circumferences were measured to the nearest cm in the standing position, halfway between the lowest rib and the iliac crest, and the maximum hip circumference, respectively.

Assays

Plasma glucose was measured by a hexokinase-based method (Gluco-Quant, Roche Diagnostics). Plasma insulin and C-peptide were measured by a competitive chemiluminescent immunoassay, supplied by Euro/DPC. The assay was performed

on a DPC Immulite 2000 analyzer (Euro/DPC) according to the manufacturer's recommendations. Serum creatinine was measured in fasting blood with the Creatinine Plus assay on a Roche/Hitachi analyzer.

Amino acid analysis

The amino acid concentrations in fasting plasma samples were determined by HPLC after precolumn derivatization with ortho-phthaldialdehyde/3-mercaptopropionic acid (OPA/MPA) and detection by fluorescence [13]. In short, 25 µl of plasma was deproteinized with 25 µl 6 % sulfosalicylic acid and, thereafter, derivatized with OPA/MPA in the borate buffer (pH 10). After 2 min, the reaction was neutralized with citric acid (1 M) and 5 µl was applied to a 4.6 x 50 mm 1.8 µm Eclipse C18 column (Agilent). The HPLC system consisted of two LC-10ADvp pumps, a SIL-10ADvp autosampler and a RF-10Axl fluorescence detector (Shimadzu). The mobile phase A was 40 mM NaPi buffer (pH 7.8) and mobile phase B consisted of acetonitrile/methanol/water (45/45/10, v/v/v). A linear gradient with a flow rate of 0.5 ml/min was started at 2% B in A, ending at 45.7% B in A at 16 min. Thereafter, the column was re-equilibrated. Concentrations of BCAA, alanine, phenylalanine, and tyrosine in plasma were calculated as the average of duplicate work-up.

Calculations

Indices for whole body insulin sensitivity and beta-cell function were derived from the extended OGTT, which included 11 blood samplings in 3.5 hr for glucose, insulin, and C-peptide measurements, as described in detail previously[12] .The clamp-validated insulin sensitivity index (ISI) was calculated according to Matsuda.[14] As a marker of beta-cell function, the overall disposition index (DI) was calculated as the product of ISI and the insulin secretion response [15]. As a measure of renal function, the estimated Glomerular Filtration Rate (eGFR) was calculated with the Modification of Diet in Renal Disease (MDRD) formula [16, 17].

Statistical analyses

Data are expressed as mean ± SD, unless indicated otherwise. Differences between subgroups were analyzed with ANOVA, followed by Bonferroni, and considered significant when P value < 0.0125. Inverse or log transformations were used when normality or equal variance assumptions were not met. Pearson correlation coefficients were determined by multivariate regression analysis and considered significant when P value < 0.05. As glucose intolerance and T2DM progresses with age in affected individuals, age was not included as an independent parameter in the multivariate regression analysis. The value of BCAA and other parameters in discriminating the

individual's state of glucose intolerance was determined by binary logistic regression analysis adjusted for family ties [18]. Differences between the area under the curve of receiver-operated characteristics (ROC AUC, concordance index or c-index) were calculated according to Hanley and McNeil[19] and considered significant when $P < 0.05$. All statistical tests were conducted using SPSS software, version 15.0, for Windows (SPSS Inc.).

RESULTS

Subject characteristics

Family members of 24 Caucasian and 36 Asian Indian index patients were included in the study. From the Caucasian families 34, 12, and 18 participants were normoglycemic, prediabetic and diabetic, respectively. These numbers were 22, 12, and 23 for the Asian Indians. The characteristics of these groups are shown in Table 1; OGTT AUC of plasma glucose, insulin, and C-peptide concentrations for all groups is depicted in Fig 1. Among the Caucasians, there were significant differences between the diabetic and the normoglycemic subgroups with respect to age, waist circumference, waist-hip ratio (WHR), ISI and DI, but not for body weight, BMI, or eGFR MDRD. For the DI, the prediabetic subgroup was intermediate between the normoglycemic and diabetic subgroup. The diabetic subgroup was significantly older than the prediabetic subgroup. For the other parameters, the prediabetic subgroup did neither differ significantly from the normoglycemic nor the diabetic subgroup. The Asian Indian family members were more homogeneous, as only differences were observed among subgroups for age and for the DI. Compared to the Caucasian T2DM patients, the Asian Indian patients with T2DM were younger ($P = 0.004$), shorter ($P < 0.001$) and weighed less ($P = 0.005$). No other remarkable differences between similar subgroups of both ethnicities were evident.

Fasting plasma amino acid levels

In the Caucasians, plasma levels of leucine, isoleucine, valine, alanine, phenylalanine, and tyrosine were all significantly higher in the T2DM than in the normoglycemic subgroup (Table 1). Although a similar trend is observed among the Asian Indians for most amino acids, none of the differences was significant. The Caucasian T2DM group had higher BCAA levels than the Caucasian normoglycemic group ($P = 0.001$). In contrast, the Asian Indian T2DM group did not have significantly higher total BCAA than the Asian Indian normoglycemic group ($P = 0.21$). There were no significant differences between fasting plasma amino acid levels between similar subgroups of the two ethnicities,

except that most amino acids, including total BCAA, were higher in the normoglycemic Asian Indians than in the normoglycemic Caucasians. Notably, levels of total BCAA, phenylalanine, and tyrosine in the normoglycemic Asian Indians were not statistically different from the diabetic Caucasians. To replicate known relationships between BCAA and a number of traits, illustrated in Fig. 2, we have performed multivariate analyses that are shown in Table 2. In Caucasians, WHR significantly explained 15.4% (P = 0.03) of the variance in BCAA concentrations. In Asian Indians, WHR explained 32.5% (P = 0.001) and eGFR MDRD 9.5% (P = 0.009) of the variance in BCAA concentrations.

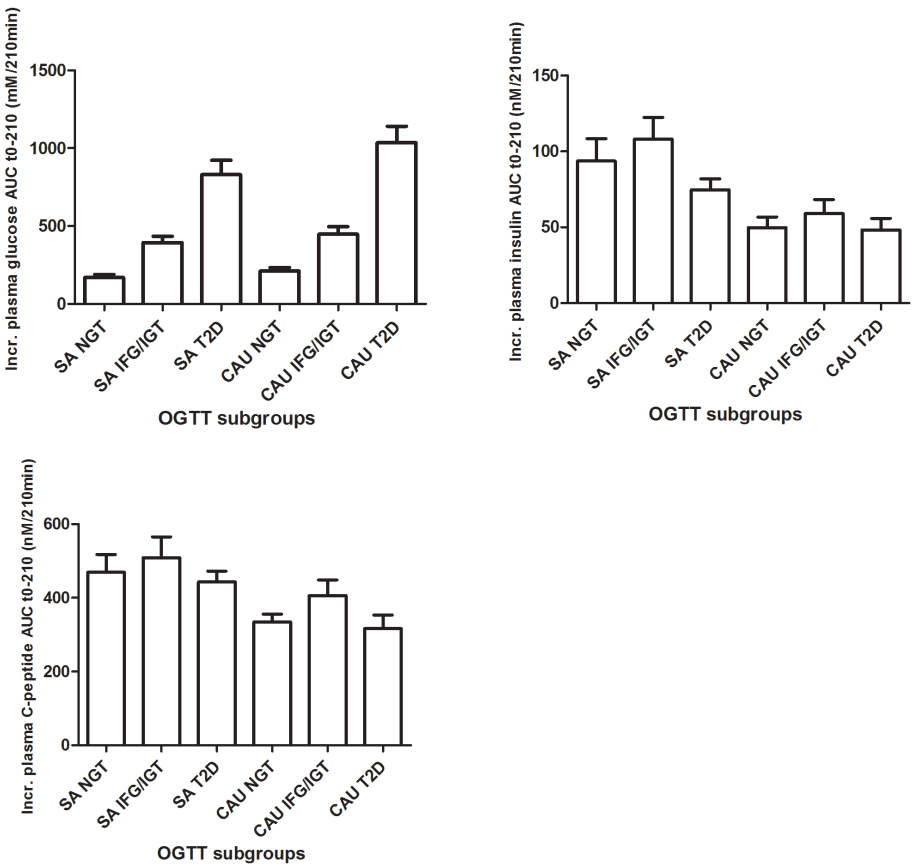


Figure 1 | Incremental (Incr.) plasma AUC of glucose, insulin, and C-peptide during the 210 min oral glucose tolerance test (OGTT) (mean±SEM) among WHO OGTT subgroups with normal glucose tolerance (NGT), impaired fasting glucose, and/or impaired glucose tolerance (IFG/IGT) and Type 2 Diabetes (T2D) from South Asian (SA) or Caucasian (Cau) origin. Incr. plasma glucose AUC; SA NGT versus T2DM SA P < 0.001, SA IFG/IGT versus T2DM P < 0.001, Cau NGT versus Cau T2DM P < 0.001, and Cau IFG/IGT versus Cau T2DM P < 0.001. Incr. plasma insulin AUC; SA NGT versus Cau NGT P < 0.01.

Table 1 | Characteristics of Normoglycemic, Prediabetic and Diabetic Subgroups in T2DM High-Risk Caucasian and Asian Indian families.

	Caucasians (24 families)			Asian Indians (36 families)		
	normoglycemic	**prediabetic**	**diabetic**	**normoglycemic**	**prediabetic**	**diabetic**
n	34	12	18	22	12	23
Sex: male/female,(% male)	11/23(32.4)	4/8(33.3)	9/9(50.0)	10/12(45.5)	8/4(66.7)	11/12(47.8)
Age, years	38.9±9.4‡	44.5±11.4‡	63.2±7.6*†¶	39.6±11.6¶	46.3±8.8	52.3±8.8‡§
Weight, kg	81.1±15.7	94.1±30.4	90.5±15.0¶	78.7±13.8	78.7±14.5	74.4±12.4‡
Length, cm	1.75±0.10	1.75±0.07	1.76±0.07¶	1.69±0.11	1.67±0.11	1.61±0.08‡
BMI, kg/m2	26.3±4.1	30.4±8.5	29.3±4.9	27.6±4.1	27.9±2.9	28.6±4.1
Waist, cm	91±15‡	105±20	105±14*	94±10	98±13	97±11
Hip, cm	108±8	116±20	112±10	105±5	103±6	104±9
WHR	0.84±0.09‡	0.90±0.07	0.94±0.08*	0.90±0.07	0.95±0.10	0.93±0.08
ISI	8.2±0.9‡	5.0±1.0	4.2±0.8*	5.0±0.9	3.2±0.5	2.9±0.3
DI (Log transformed)	-1.00±0.14†‡	-1.77±0.17*‡	-2.86±0.14*† ¶	-0.54±0.12 || ¶	-1.36±0.15§ ¶	-2.09±0.14‡ § ||
eGFR MDRD (ml/min/(1.73m2)	101.8±3.2	104.1±7.6	94.2±3.9	103.2±5.0	112.9±6.8	102.5±5.7
Leucine, μM	123.7±4.0‡	124.0±7.3	149.8±7.3*	142.2±6.1	149.6±9.6	165.6±8.3
Isoleucine, μM	63.4±2.6‡§	66.3±5.0	81.9±5.2*	79.6±4.0*	81.4±7.3	92.9±4.9
Valine, μM	214.1±6.3‡§	231.8±16.9	272.8±10.9*	260.1±11.0*	248.7±20.1	284.2±10.3
BCAA, μM	401.2±12.1‡§	422.1±28.8	504.7±22.6*	481.9±20.4*	479.4±35.8	542.6±22.0
Alanine, μM	350.9±10.6‡	361.8±17.2	437.3±24.8*	396.0±17.8	401.4±19.7	435.8±15.4
Phenylalanine, μM	58.4±1.5§	57.9±2.9	64.6±1.7	67.4±2.0*	63.1±3.5	69.7±2.0
Tyrosine, μM	61.7±2.3‡§	64.1±3.6	77.3±3.5*	82.7±2.9*	79.5±5.4	92.1±4.2

Data are means ± SD, except for ISI, DI and eGFR MDRD, which are in mean ± SEM. Symbols represent significance with $P < 0.0125$ in the corresponding subgroup versus other subgroups as stated in the Materials and Methods section; *=versus Cau normoglycemics, † =versus Cau prediabetics, ‡ = versus Cau diabetics, § = versus AI normoglycemics, || = versus AI prediabetics, ¶ = versus AI diabetics. AI, Asian Indians; BCAA, branched-chain amino acids; Cau, Caucasians; DI, disposition index; eGFR MDRD, estimated Glomerular Filtration Rate by Modification of Diet in Renal Disease; ISI, insulin sensitivity index; WHR, waist-hip ratio.

Discriminative ability of amino acid profile for glucose intolerance

Before we tested the discriminative ability of BCAA between normoglycemia and prediabetes, we repeated our univariate analyses of BCAA with the earlier mentioned traits, but now restricted to the normoglycemic and prediabetic subgroups for both ethnicities. Even when we excluded their respective T2DM subgroups, our regression analyses remained significant (data not shown). The ability of the amino acid profile to discriminate between glucose-tolerant and glucose-intolerant family members was determined by the ROC curves (Table 3). The c-index of total BCAA was 0.83 in the Caucasians and 0.74 in the Asian Indians, which increased when combining BCAA levels with those of alanine, phenylalanine, and tyrosine to 0.84 and 0.79, respectively. The corresponding c-indices for fasting glucose were 0.91 and 0.85. When amino acid levels were combined with fasting glucose, the c-index increased to 0.92 and 0.97 (P = 0.04 vs. fasting glucose alone) for the Caucasian and Asian Indian family members, respectively (Fig. 3).

Table 2 | Stepwise Regression Analysis Explaining Variance in % of BCAA Concentrations in Both Ethnicities, with WHR, eGFR MDRD, ISI, and DI as Determinants

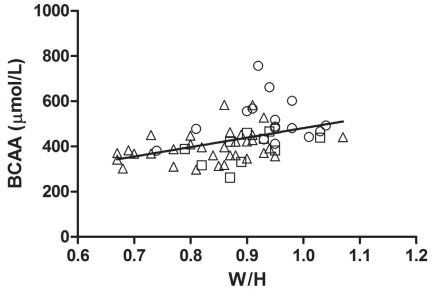
	Caucasians			Asian Indians		
Determinant	***b* (95% CI)**	**β**	**%**	***b***	**β**	**%**
WHR	260.71 (25.69 to 495.72)	0.31	15.4(P = 0.03)	543.61 (242.38 to 844.85)	0.42	32.5(P = 0.001)
eGFR MDRD	Excluded from model	-	-	-1.12 (-1.94 to -0.30)	-0.30	9.5 (P = 0.009)
ISI	Excluded from model	-	-	-7.69 (-16.12 to 0.74)	-0.21	3.8 (P = 0.073)
DI	-20.52 (-43.19 to 2.15)	-0.25	5.5(P = 0.075)	Excluded from model	-	-

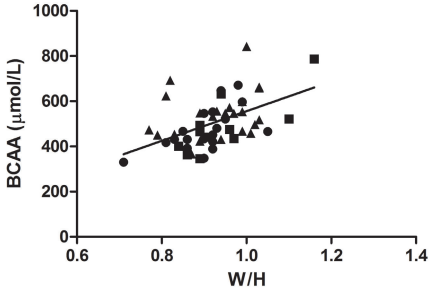
In South Asians, WHR explained 32.5% and eGFR MDRD 9.5% of the variance in BCAA concentrations. ISI explained 3.8% in this model, although statistical significance was not reached (P = 0.073). DI did not contribute to the variance observed. In indigenous Dutch, WHR explained 15.4% of the variance in BCAA concentrations. DI may explain 5.5% in this model, however, this was not significant (P = 0.075). eGFR MDRD and ISI did not contribute to the differences observed. DI, disposition index; eGFR MDRD, estimated Glomerular Filtration Rate by Modification of Diet in Renal Disease; ISI, insulin sensitivity index; WHR, waist–hip ratio

Table 3 | Ability to Discriminate Between Normoglycemic and Prediabetic Relatives Within Families with T2D

	BCAA	Amino acids	Fasting glucose	Fasting glucose+ BCAA	Fasting glucose+ Amino Acids
Caucasians	0.83	0.84	0.91	0.92	0.92
Asian Indians	0.74*	0.79*	0.85*	0.89	0.97*

The c-index (ROC AUC) of BCAA, amino acids (BCAA, phenylalanine, tyrosine, and alanine), fasting glucose and BCAA or amino acids as add-on to fasting glucose, for prediabetes. *c-Index of fasting glucose+ amino acids versus BCAA (P = 0.01), amino acids (P = 0.02) or fasting glucose (P = 0.04)


BCAA (µmol/L)
1000
800
600
400
200
0
0.6
0.7
0.8
0.9
1.0
1.1
1.2
W/H


BCAA (µmol/L)
1000
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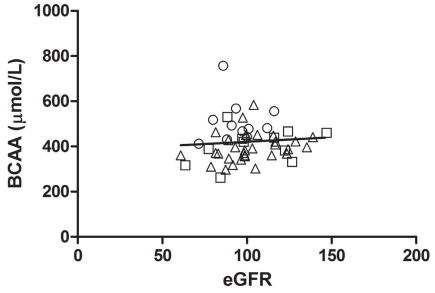
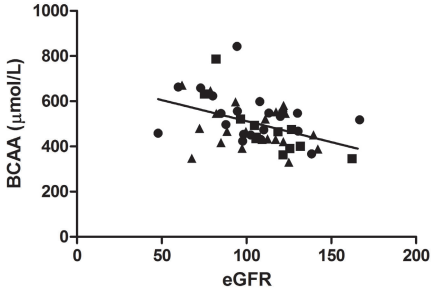
1.4
W/H


BCAA (µmol/L)
1000
800
600
400
200
0
0
50
100
150
200
eGFR


BCAA (µmol/L)
1000
800
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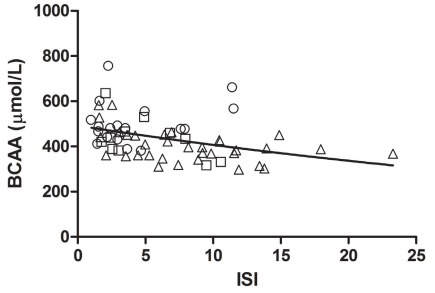
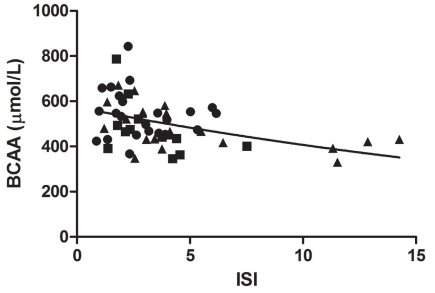
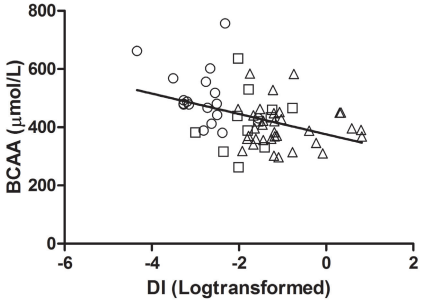
200
0
0
50
100
150
200
eGFR


BCAA (µmol/L)
1000
800
600
400
200
0
0
5
10
15
20
25
ISI


BCAA (µmol/L)
1000
800
600
400
200
0
0
5
10
15
ISI


BCAA (µmol/L)
800
600
400
200
0
-6
-4
-2
0
2
DI (Logtransformed)

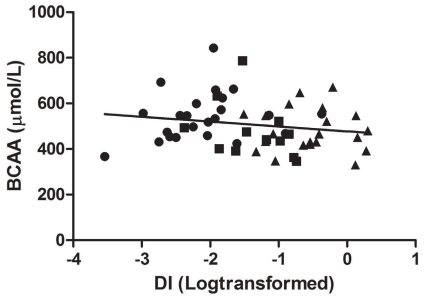

BCAA (µmol/L)
1000
800
600
400
200
0
-4
-3
-2
-1
0
1
DI (Logtransformed)

Figure 2 | relationships of branched-chain amino acids (BCAA) with waist/hip ratio (W/H), glomerular filtration rate (eGFR), insulin sensitivity index (ISI) and insulin disposition index (DI) in Caucasian (*left, open circles*) and Asian Indian (*right, closed circles*) families (*triangles* are normal individuals, *squares* are individuals with prediabetes and *circles* individuals with T2DM, respectively). In Asian Indians, the relationship between BCAA and W/H (R^2= 0.25; $P < 0.001$), eGFR (R^2= 0.19; $P = 0.001$), and ISI (R^2=0.14; $P < 0.001$) was significant; the relationship between BCAA and DI (R^2=0.03; $P = 0.18$) was not significant. In Caucasians, the relationship between BCAA and W/H (R^2=0.18; $P < 0.001$), BCAA and ISI (R^2=0.14; $P < 0.001$) and BCAA and DI (R^2=0.16; $P < 0.001$) was significant; the relationship between BCAA and eGFR (R^2=0.007; $P = 0.54$) was not significant

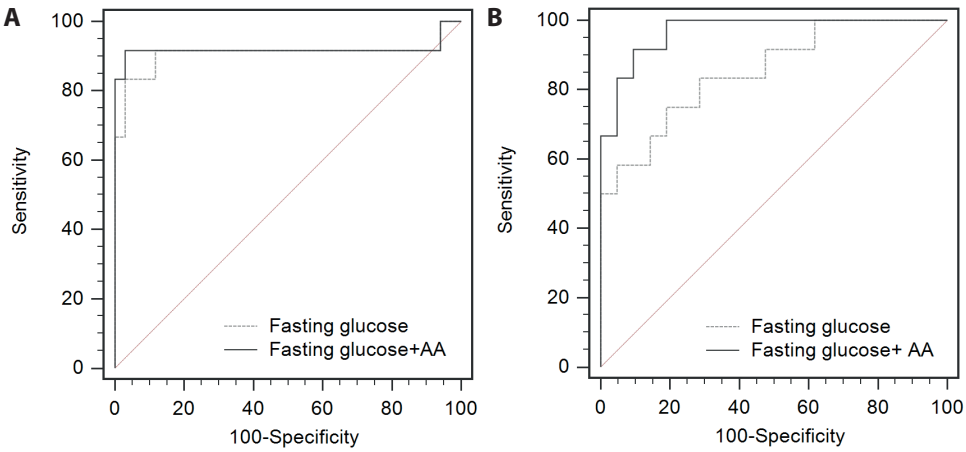


Figure 3 | ROC curves for prediabetes among nondiabetic members of Caucasian **(A)** and Asian Indian **(B)** families with high T2DM risk. For both ethnicities, the ROC curve for fasting glucose alone (*grey short dashed line*) and fasting glucose+amino acids (BCAA, phenylalanine, tyrosine, and alanine, *black continuous line*) to discriminate between OGTT-defined prediabetic from normoglycemic condition, are depicted.

DISCUSSION

We found that fasting BCAA, aromatic amino acids and alanine levels were correlated with glucose intolerance and well discriminated between the prediabetic and the normoglycemic members of the Caucasian and fairly well for the Asian Indian families. However, in the Caucasian families, fasting glucose had high discriminative ability that was not improved by adding amino acid levels, whereas in the Asian Indian families, adding of amino acid levels to fasting glucose substantially improved the ability to discriminate normoglycemic from prediabetic family members in the Asian Indian families.

Prediabetes is defined by impaired fasting glucose and/or impaired glucose tolerance. We restricted the criteria for subgroups within the families to the OGTT measurements. Compared to prediabetic Caucasians, more Asian Indians had impaired glucose tolerance without impaired fasting glucose. This is the reason that an elevated level of plasma amino acids was a predictor of impaired glucose tolerance on top of the fasting glucose levels in Asian Indians but not in Caucasians. We also tested the discriminative ability of branched-chain alpha-keto acids, but we did not observe an improvement over BCAA (data not shown).

The Asian Indians living in the Netherlands have a high propensity to develop obesity and T2DM. They develop T2DM at an earlier age and at lower BMI than Caucasians [8, 9]. In both ethnicities, ISI and adequate beta-cell function became less going from the normoglycemic through the prediabetic to the diabetic state, but ISI was not significantly different between the Asian Indian subgroups. In multivariate regression analysis, BCAA levels marginally associated with the ISI among Asian Indians and with the beta-cell function index among Caucasians. High BCAA levels are the result of impaired BCAA catabolism occurring in parallel with resistance to insulin, as has been observed during hyperinsulinemic euglycemic clamping of nonobese and T2DM subjects[20]. In line, metabolites related to BCAA catabolism associate with impaired fasting glucose and T2DM[21]. Moreover, insulin resistance decreases the hepatic expression of branched-chain alpha-keto acid dehydrogenase, the rate-limiting enzyme of BCAA catabolism[22]. Nonetheless, the causes for high BCAA levels may differ between ethnicities and an effect of beta-cell function has not been excluded.

We found that WHR was a stronger determinant of fasting plasma BCAA levels in our Asian Indians than in Caucasian subjects. A number of studies support a role of visceral fat in the BCAA catabolism: insulin resistant, obese sisters and brothers of monozygotic twins discordant for obesity had downregulated mitochondrial pathways for BCAA degradation in adipose tissue [23]; expression of enzymes involved in BCAA catabolism in visceral fat was lower in obese women with than those without T2DM [24]; and BCAA levels are reduced by bariatric surgery[25, 26]. Visceral fat is clearly involved in the development of the metabolic syndrome among normoglycemic Asian Indians[27]. Moreover, large differences have been observed in distributions of fat tissue among Asian Indian neonates compared to other ethnic groups[28]. Intriguingly, our normoglycemic Asian Indians had fasting plasma BCAA and other amino acid levels that were already in the range of the Caucasian patients with T2DM. This may result from these differences in fat tissue distribution early in life.

Our work has a number of potential weaknesses: inherent to the used methodology of family screening we studied relatively small groups. We did not assess prior food intake. However, fasting plasma BCAA originate predominantly from endogenous sources [29, 30] and diet does not influence fasting BCAA levels.[4, 7] Screening of families with prevalent diabetes at our outpatient clinic resulted in selection on a high a priori chance of diabetes. In line, family ties increased the c-indices. However, we need to emphasize that our findings cannot be generalized to population-based screening approaches that are not based on family screening. Our findings are of interest to clinicians, who screen relatives of patients in high-risk families.

In conclusion, we found that BCAA levels increase with the degree of glucose intolerance in families at-risk for T2DM of Caucasian descent, but not in Asian Indian families living in the Netherlands. However, when combined with fasting glucose, BCAA levels together with the other amino acids reliably discriminated normoglycemic from prediabetic family members in both ethnicities, and significantly improved the discriminative ability of fasting glucose alone among the Asian Indian families. Amino acid profiles in fasting blood, particularly of BCAA, may be used as biomarkers to improve detection of the glucose-intolerant subject at risk for development of T2DM.

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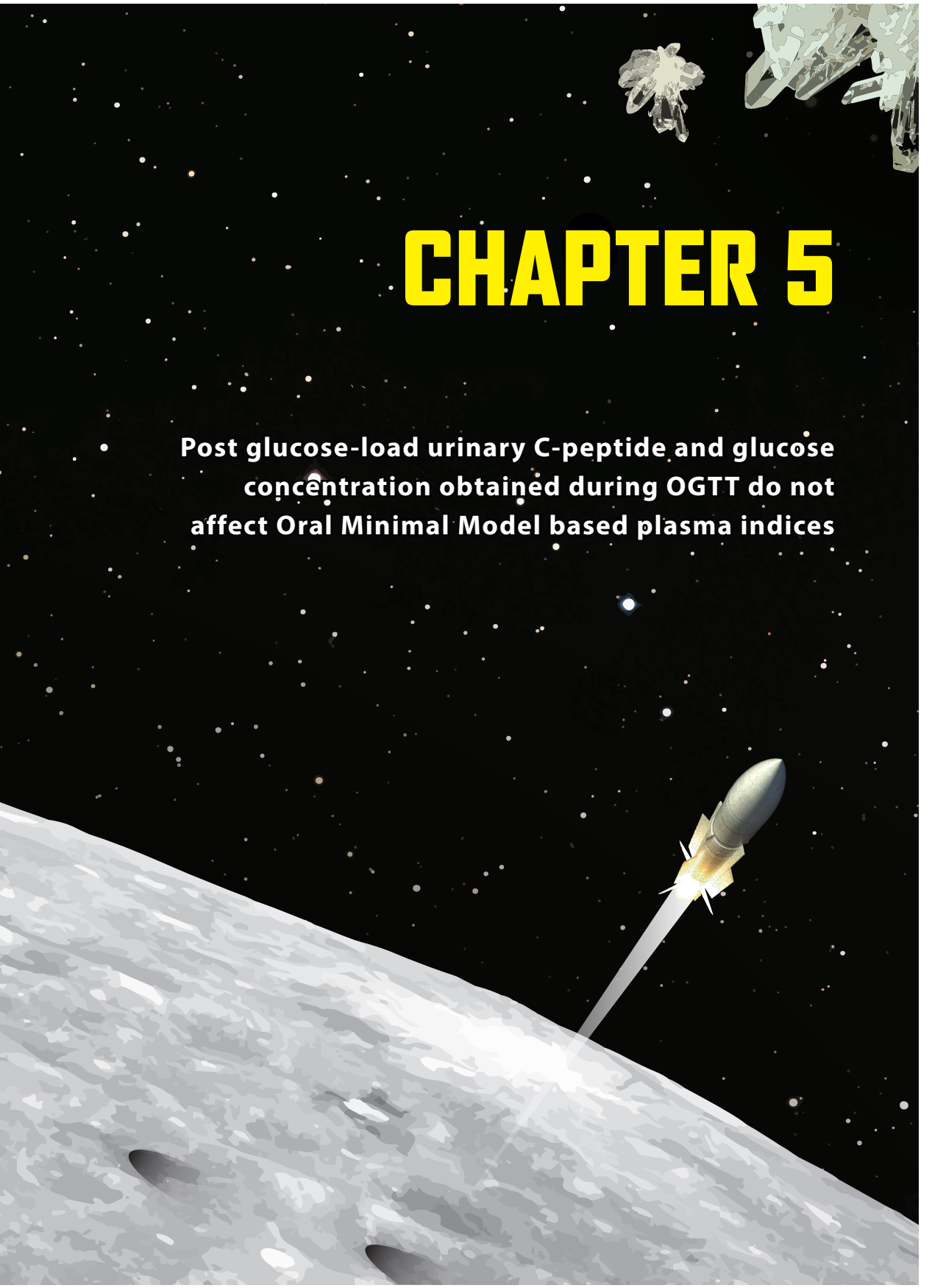

CHAPTER 5

Post glucose-load urinary C-peptide and glucose concentration obtained during OGTT do not affect Oral Minimal Model based plasma indices

ABSTRACT

The purpose of this study was to investigate how renal loss of both C-peptide and glucose during oral glucose tolerance test (OGTT) relate to and affect plasma derived oral minimal model (OMM) indices. All individuals were recruited during family screening between August 2007 and January 2011 and underwent a 3.5 h OGTT, collecting nine plasma samples and urine during OGTT. We obtained the following three subgroups: normoglycemic, at risk and T2D. We recruited South Asian and Caucasian families and we report separate analyses if differences occurred. Plasma glucose, insulin and C-peptide concentrations were analyzed as AUCs during OGTT, OMM estimate of renal C-peptide secretion, and OMM beta-cell and insulin sensitivity indices were calculated to obtain disposition indices. Post-glucose load glucose and C-peptide in urine were measured and related to plasma-based indices. Urinary glucose corresponded well with plasma glucose AUC (Cau $r = 0.64$, $P < 0.01$, SA $r = 0.69$, $P < 0.01$), S_I (Cau $r = -0.51$, $P < 0.01$, SA $r = -0.41$, $P < 0.01$), $\Phi_{dynamic}$ (Cau $r = -0.41$, $P < 0.01$, SA $r = -0.57$, $P < 0.01$), and Φ_{oral} (Cau $r = -0.6$, $P < 0.01$, SA $r = -0.73$, $P < 0.01$). Urinary C-peptide corresponded well to plasma C-peptide AUC (Cau $r = 0.45$, $P < 0.01$, SA $r = 0.33$, $P < 0.05$) and OMM estimate of renal C-peptide secretion ($r = 0.42$, $P < 0.01$). In general, glucose excretion plasma threshold for the presence of glucose in urine was ~10-10.5mmol/L in non-T2D individuals, but not measurable in T2D individuals. Renal glucose secretion during OGTT did not influence OMM indices in general nor in T2D patients (renal clearance range 0-2.1%, with median 0.2% of plasma glucose AUC). C-indices of urinary glucose to detect various stages of glucose intolerance were excellent (Cau 0.83-0.98; SA 0.75-0.89).

The limited role of renal glucose secretion validates the neglecting of urinary glucose secretion in kinetic models of glucose homeostasis using plasma glucose concentrations. Both C-peptide and glucose in urine collected during OGTT might be used as non-invasive measures for endogenous insulin secretion and glucose tolerance state.

INTRODUCTION

Mathematical approaches based on compartmental pharmacokinetic/pharmacodynamic (PK/PD) principles are used to describe the biphasic glucose-insulin system in oral function tests[1, 2], with the oral minimal model (OMM) as one of the most widely accepted approaches [3]. However, the contribution of renal clearance of endogenous glucose, insulin and C-peptide during oral glucose tolerance test (OGTT) in various stages of glucose tolerance remains largely unclear. As renal extraction of insulin is negligible[4], we focused on the relationship between plasma and urine concentrations of both C-peptide and glucose, collected during OGTT in the post-glucose load phase. This was performed in families to obtain groups with different risk for T2D and we recruited families of South Asian and Caucasian origin to enable generalization of our findings. Especially, South Asians with T2D may be at high risk for chronic kidney disease[5]. We questioned to which degree C-peptide and glucose excretion in urine influence OGTT based plasma indices. Moreover, we compared the OMM derived estimates of renal C-peptide excretion with actual urinary C-peptide concentration. In addition, renal loss of glucose is not taken into consideration in OMM, and the extent to which renal clearance might require correction of plasma-derived OMM calculations is unknown.

METHODS

Subjects and anthropometric data

Patients were recruited from South Asian and Caucasian families with high risk of T2D after family screening from the Outpatient Clinic of the Erasmus Medical University Centre as described previously[6]. The first-degree relatives of patients with T2D attending our Clinic (index cases), who did not have T2D were recruited from 36 South Asian families and 24 Caucasian families, with 2 generations taken into account. Data were obtained from 57 (M29 F28) South Asians and 64 (M24 F40) Caucasians who all underwent an OGTT. Index cases were on metformin use only, and had at least one sibling with T2D. Informed written consent to the study was obtained from all participants. The study protocol was approved by the Erasmus University Medical Center Medical Ethics Review Board. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

OGTT

Venous blood was drawn via an intravenous canula, at time-points 60 and 15 min before 75g glucose load and 15, 30, 45, 60, 90, 120, 150, 180 and 210 minutes after glucose load to measure glucose, insulin and C-peptide concentrations. The WHO criteria for the OGTT were used to define normal glucose tolerance (NGT), impaired fasting glucose and/or impaired glucose tolerance (IFG/IGT) or Type 2 Diabetes (T2D) status among subjects. After emptying their bladder prior to glucose load, urine was collected until 210 minutes after glucose-load. As there were no significant differences between baseline values of plasma glucose, insulin or C-peptide obtained at -60 min or -15 min, and as -60 min was sampled before our study subjects had emptied their bladder prior to glucose load, we chose -60 min as the representative baseline value. In 7 of 18 Caucasians with T2D, urine was not collected, because it was not included in our protocol at that time. As this group did not significantly differ from the remaining group, we used data of n=18 for all plasma indices, but data of n=11 for all analyses with urinary glucose and C-peptide measurements in Caucasians.

Immunoassay

Plasma and urine glucose was measured by a hexokinase-based method (Gluco-quant; Roche Diagnostics, Mannheim, Germany). Plasma and urine C-peptide, and plasma insulin were measured separately by a competitive chemiluminescent immunoassay, supplied by Euro/DPC. The assay was performed on a DPC Immulite 2000 analyzer (Euro/DPC) according to the manufacturer's recommended protocol. Serum creatinine was measured with an enzymatic procedure based on creatinine conversion with the Creatinine Plus assay on a Roche/Hitachi analyzer. Urine creatinine was measured based on the Jaffe alkaline picrate method.

Calculations for OMM

The OMM was used to describe the plasma glucose, insulin and C-peptide concentrations after oral glucose stimulus[7]. With C-peptide minimal model we assessed parameters for beta-cell function; basal responsivity of beta-cells due to basal glucose potentiation $\Phi_{basal}(min^{-1})$, static responsivity of beta-cells due to glucose potentiation Φ_{static} (10^{-9} min^{-1}) , dynamic responsivity of beta-cells due to glucose potentiation $\Phi_{dynamic}$ (10^{-9}), total responsivity of beta-cells due to glucose potentiation Φ_{oral} (10^{-9} min^{-1}) and delay in response to glucose potentiation T (min). With glucose minimal model we assessed parameters for insulin sensitivity; insulin sensitivity S_I (10^{-5} $dl*kg^{-1}*min^{-1}$ per pM). Parameters from both models were multiplied with each other for calculation of disposition indices (DI), which are beta-cell function measures corrected for insulin sensitivity; $DI_{basal} = \Phi_{basal} * S_I$, $DI_{static} = \Phi_{static} * S_I$, $DI_{dynamic} = \Phi_{dynamic} * S_I$

and $DI_{oral} = \Phi_{oral} * S_I$. Parameters of OMM were estimated with SAAM2 software[8]. Incremental plasma AUC of C-peptide and glucose within a given time period was calculated according to trapezoidal rule, with subtraction of basal concentration. For urinary glucose, we estimated plasma glucose threshold (when exceeded glucose in urine is present) separately among both our T2D and non T2D (NGT+IFG/IGT) groups; we calculated plasma glucose AUCs from 8.5 to 11.5 mmol/L with an interval of 0.5mmol/L to detect the most suitable plasma glucose threshold. Stepwise exclusion was performed of T2D and non T2D individuals, based on whether their plasma glucose AUC was above a given threshold or not. Also, to determine the relative renal loss of glucose from total plasma glucose AUC, renal clearance of glucose was calculated with absolute amount of urinary glucose/ total plasma glucose AUC. For comparison with urinary C-peptide, we used AUC from flux k01 (Fig. 1) from OMM, representing irreversibly metabolized C-peptide from central compartment. Estimated glomerular filtration rate (eGFR) was estimated with the modification of diet in renal disease (MDRD) formula [9, 10].

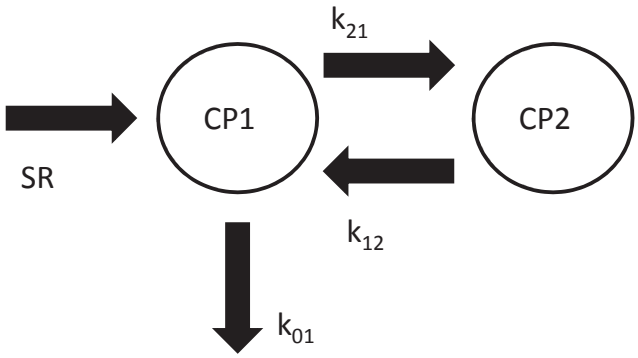

Figure 1a | Two-compartment model of C-peptide kinetics that is integrated in oral minimal model (OMM). SR is prehepatic insulin secretion rate, based on C-peptide curve. CP1 represents amount of C-peptide in central compartment, and CP2 amount of C-peptide in peripheral compartment. k_{21} and k_{12} are C-peptide transfer rate between CP1 and CP2; k_{01} describes metabolization of C-peptide from CP1. In this study, plasma AUC of CP1*k_{01} , or OMM flux $k_{01,}$ is related to actual measured C-peptide concentration in urine during OGTT. *Adapted from van Cauter et al[1].*

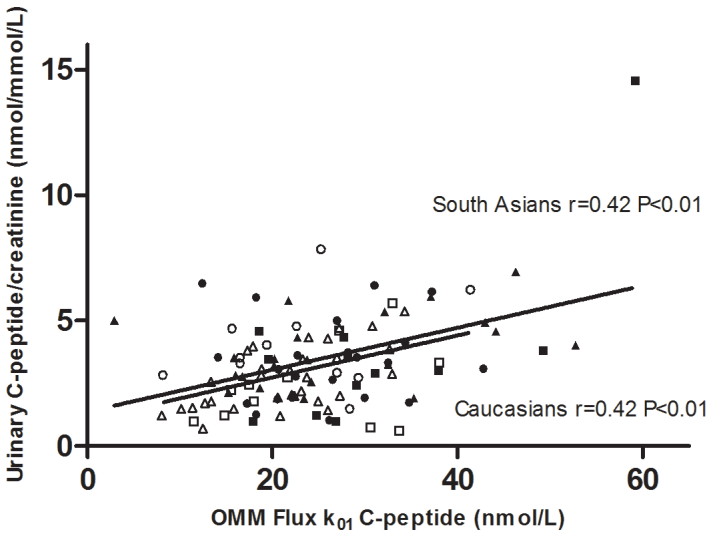

Figure 1b | Correlation between C-peptide in urine and OMM flux k01. NGT (*triangle*), IFG/IGT (*square*) and T2D (*circle*) subgroups for South Asian (*closed*) and Caucasian (*open*) families.

Statistical analyses

Data analysis: Data are expressed as mean ± SEM, or indicated otherwise. Comparisons within the subgroups of the ethnicities were done with ANOVA, with the other two subgroups of same ethnicity and with the corresponding other ethnic subgroup. Differences were considered statistically significant when the two-sided *P* value was <0.0125. Urinary glucose and C-peptide were correlated with plasma indices, with Spearman's correlation within each ethnicity, with significance at *P* value < 0.05. For urinary glucose and C-peptide concentrations, AUC of receiver-operated characteristics (ROC) curves (concordance indices or c-indices), adjusted for family ties by binary logistic regression analysis [11], were calculated to detect IFG/IGT and/or T2D status. All statistical tests were conducted with the use of SPSS, version 15.0, for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Baseline characteristics

Baseline characteristics are shown in Table 1, and incremental plasma AUC of primary data glucose, insulin and C-peptide during OGTT in Fig. 2. South Asian T2D individuals were younger ($P = 0.004$), shorter ($P < 0.001$) and weighed less ($P = 0.005$) than Caucasian

T2D individuals. W/H increased from NGT to T2D in Caucasians ($P = 0.003$), but not in South Asians. In both ethnicities, incremental plasma glucose AUCs from NGT or IFG/IGT were significantly lower than T2D ($P < 0.001$). In both ethnicities, no significant differences were found in incremental plasma insulin AUC between NGT, IFG/IGT and T2D; however, a difference was found between South Asian NGT and Caucasian NGT ($P < 0.01$). In both ethnicities, no significant differences were found in incremental plasma C-peptide AUC between NGT, IFG/IGT, and T2D. In both ethnicities, no significant differences were found in Φ_{basal} between NGT, IFG/IGT, and T2D; whereas, $\Phi_{dynamic}$, Φ_{static} and Φ_{total} decreased with increasing glucose intolerance. No significant differences were found in delay T. With OMM, we observed a decrease in insulin sensitivity in both ethnicities with increasing glucose intolerance ($P < 0.001$), however, differences were lower among the South Asian subgroups. All disposition indices decreased with increasing glucose intolerance. Overall, South Asian NGT and IFG/IGT demonstrated lower DI indices when compared to Caucasian NGT, whereas those of South Asian T2D were higher than their Caucasian counterparts. In both ethnicities glucose in urine increased with increasing glucose intolerance; both creatinine unadjusted and adjusted urinary glucose in T2D subgroup were significantly higher when compared to NGT or IFG/IGT subgroups ($P < 0.001$). In Caucasians, creatinine adjusted C-peptide was higher in T2D when compared to NGT and IFG/IGT combined (t test $P = 0.005$); no differences were found among South Asians.

Effect of renal glucose loss on plasma-derived OMM indices

The absolute amount of glucose concentration collected in urine voids of T2D patients did not influence glucose minimal model measurements, as a result of the small variation of renal clearance (range 0-2.1%, with median 0.2% of total plasma glucose AUC).

Relation urine markers with plasma indices of C-peptide and glucose, OMM and eGFR

Relationships between urinary glucose and C-peptide with plasma indices in both ethnicities can be found in Table 2. In both ethnicities, urinary glucose was positively associated with plasma glucose AUC and negatively associated with S_I and DI values. In both ethnicities, urinary C-peptide was positively associated with plasma C-peptide AUC. Plasma AUC of OMM flux k01 reflecting C-peptide from central compartment that is irreversibly metabolized, correlated with creatinine-adjusted urinary C-peptide (Fig. 1b; $r = 0.42$; $P < 0.01$). Creatinine unadjusted and adjusted urinary glucose as well as C-peptide had no significant correlation with eGFR.

Table 1 | Clinical characteristics in persons with NGT, IGT and/or IFG, and type 2 diabetes.

	South Asian			Caucasian		
	NGT	IFG/IGT	T2D	NGT	IFG/IGT	T2D
n	22	12	23	34	12	18
Sex(male/female), n%(male)	10/12(45.5)	8/4(66.7)	11/12(47.8)	11/23(32.4)	4/8(33.3)	9/9(50.0)
Age (years)	39.6±11.6¶	46.3±8.8	52.3±8.8‡§	38.9±9.4‡	44.5±11.4‡	63.2±7.6*†¶
Weight (kg)	78.7±13.8	78.7±14.5	74.4±12.4‡	81.1±15.7	94.1±30.4	90.5±15.0¶
Length (m)	1.69±0.1	1.67±0.1	1.61±0.1‡	1.75±0.1	1.75±0.1	1.76±0.1¶
BMI (kg/m2)	27.6±4.1	27.9±2.9	28.6±4.1	26.3±4.1	30.4±8.5	29.3±4.9
Waist (cm)	94±10	98±13	97±11	91±15‡	105±20	105±14*
Hip (cm)	105±5	103±6	104±9	108±8	116±20	112±10
W/H ratio	0.90±0.07	0.95±0.10	0.93±0.08	0.84±0.09‡	0.90±0.07	0.94±0.08*
eGFR MDRD(ml/min)	103.2±5.0	112.9±6.8	102.5±5.7	101.8±3.2	104.1±7.6	94.2±3.9
Φ_{basal} (min^{-1})	0.50±0.05	0.54±0.07	0.42±0.02	0.36±0.02	0.36±0.03	0.38±0.05
$\Phi_{dynamic}$ (10^{-9})	365.36±41.89¶	228.19±62.60	114.52±36.50§	252.55±28.55‡	140.48±40.55	94.46±15.10*
Φ_{static} (10^{-9} min^{-1})	21.28±1.17‖¶	12.64±1.35§	8.33 ±1.24§	18.34±1.71‡	11.97±1.46	4.19±0.52*
Φ_{oral} (10^{-9} min^{-1})	24.96±1.38‖¶	14.66±1.49§	9.43±1.58§	21.37±1.89‡	13.88±1.86	4.92±0.61*
T (min)	13.15±2.61	8.93±1.05	14.84±2.06	10.35±1.06	11.69±1.74	13.86±2.50
S_I (10^{-5} $dl*kg^{-1}*min^{-1}$ per pM)	15.60±2.60¶	8.30±1.80	7.60±1.40§	26.70±3.70‡	12.90±3.60	5.80±1.50*
DI_{basal} (10^{-5} $dl*kg^{-1}*min^{-2}$ per pM)	6.40±0.70¶	3.90±0.60	3.10±0.60§	8.70±1.20‡	4.30±1.20	1.60±0.30*
$DI_{dynamic}$ (10^{-14} $dl*kg^{-1}*min^{-1}$ per pM)	5263.30±902.50¶	2116.00±888.30	739.20±205.20§	6389.70±1373.20‡	1999.90±631.50	390.70±115.10*
DI_{static} (10^{-14} $dl*kg^{-1}*min^{-2}$ per pM)	323.40±52.80‖¶	111.60±32.90§	60.50±11.60§	558.90±147.60‡	144.30±43.40	23.40±7.10*
DI_{oral} (10^{-14} $dl*kg^{-1}*min^{-2}$ per pM)	379.90±62.40‖¶	130.30±38.10§	67.70±13.00§	637.10±164.60‡	165.10±49.10	26.80±8.00*
Glucose urine (mmol/l)	0.31±0.24¶	2.13±1.29¶	24.99±7.02§‖	0.19±0.06‡	4.55±3.37‡	32.68±6.57*†[A]
Glucose/creatinine ratio urine	0.04±0.02¶	0.33±0.20¶	5.60±1.67§‖	0.03±0.01‡	0.24±0.14‡	5.01±1.08*†[A]
C-peptide urine (nmol/l)	19.0±3.2	21.2±4.3	19.1±3.0	20.2±4.5	26.6±10.1	26.9±5.9[A]
C-peptide/creatinine ratio urine	3.8±0.3	3.8±1.0	3.4±0.4	2.7±0.2	2.4±0.5	4.0±0.5[A]

Anthropometric data are means±SD, with *n* or *n*(%); data of OMM and urinary glucose and C-peptide concentrations are means±SEM. Symbols represent significance with $P < 0.0125$ in the corresponding subgroup versus other subgroups as stated in methods section; *=versus Cau NGT, † =versus Cau IFG/IGT, ‡ = versus Cau T2D, § = versus SA NGT, ‖ = versus SA IFG/IGT, ¶ = versus SA T2D. [A]Caucasian T2D n=11, for reasons mentioned in methods section

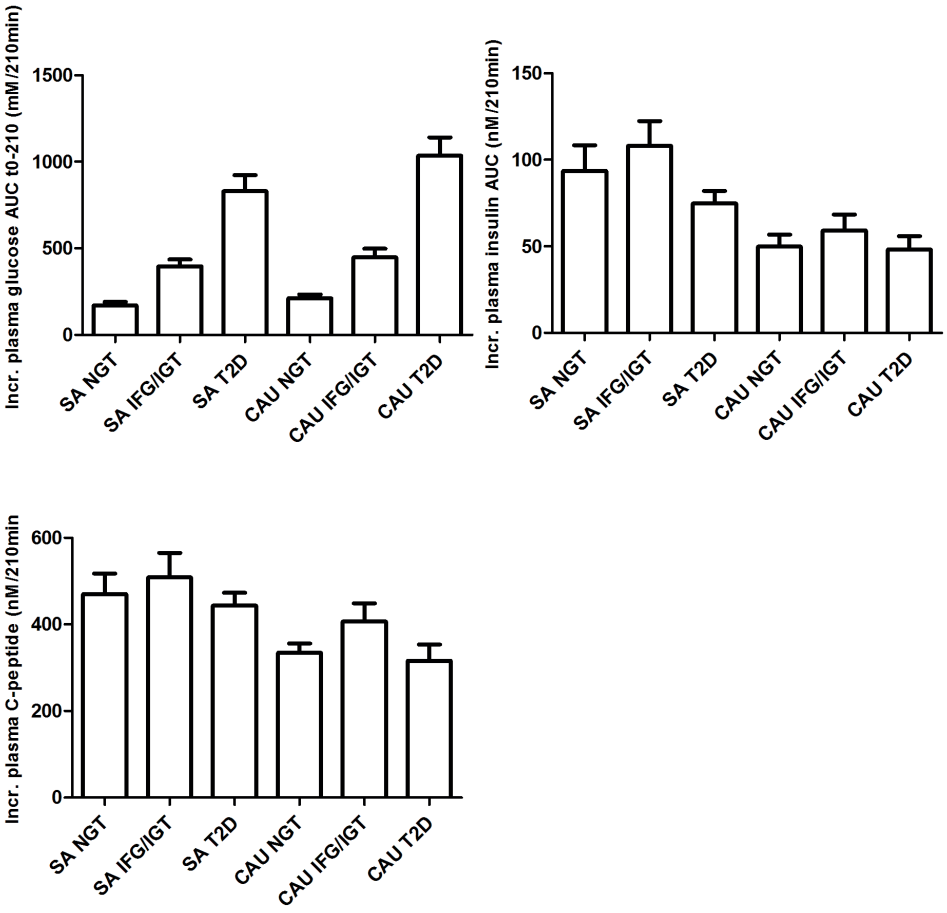

Figure 2 | Incremental (Incr.) plasma AUC of glucose, insulin and C-peptide during 210 min OGTT (mean±SEM) among WHO OGTT subgroups with normal glucose tolerance (NGT), impaired fasting glucose and/or impaired glucose tolerance (IFG/IGT) and Type 2 Diabetes (T2D) from South Asian (SA) or Caucasian (Cau) origin. Incr. plasma glucose AUC; SA NGT versus T2D SA $P < 0.001$, SA IFG/IGT versus T2D $P < 0.001$, Cau NGT versus Cau T2D $P < 0.001$,Cau IFG/IGT versus Cau T2D $P < 0.001$. Incr. plasma insulin AUC; SA NGT versus Cau NGT $P < 0.01$.

Table 2 | Spearman's correlation between urine markers and plasma indices

	Incr C-peptide AUC	Incr. Glucose AUC	S_I	$\Phi_{dynamic}$	Φ_{static}	$DI_{dynamic}$	DI_{static}
South Asians							
Glucose urine	-0.023	0.757**	-0.459**	-0.578**	-0.774**	-0.687**	-0.730**
Glucose/creatinine ratio urine	-0.059	0.690**	-0.414**	-0.565**	-0.727**	-0.653**	-0.674**
C-peptide urine	0.331*	0.170	-0.151	0.153	0.036	0.010	-0.060
C-peptide/creatinine ratio urine	0.301*	0.069	-0.148	0.100	0.068	-0.057	-0.002
Caucasians							
Glucose urine	0.175	0.669**	-0.639**	-0.208	-0.571**	-0.663**	-0.763**
Glucose/creatinine ratio urine	0.036	0.644**	-0.513**	-0.412**	-0.610**	-0.687**	-0.656**
C-peptide urine	0.485**	0.346*	-0.458**	0.178	-0.039	-0.269	-0.398**
C-peptide/creatinine ratio urine	0.453**	0.254	-0.202	-0.055	-0.053	-0.198	-0.168

*P <0.05 **P <0.01

Table 3 | Two groups, one with and one without glucose in urine. With increasing glucose threshold, stepwise exclusion was performed separately among non T2D (NGT+IFG/IGT) and T2D subgroups, based on their absence of having above threshold glucose AUC (mean+/-SEM).

			Caucasians[A]		South Asians	
			N (stepwise)	Glucose AUC	N (stepwise)	Glucose AUC
Treshold 8.5 mmol/L	Urine glucose-	Non T2D	3	28.8±14.7	2	71.25±57.0
		T2D	0	0	1	33.8
	Urine glucose+	Non T2D	19	116.6±29.3	14	116.2±28.9
		T2D	11	1012.9±143.4	20	684.5±107.0
Treshold 9 mmol/L	Urine glucose-	Non T2D	2	17.25±6.0	2	41.3±37.5
		T2D	0	0	1	12.0
	Urine glucose+	Non T2D	16	103.6±28.0	12	99.7±26.0
		T2D	11	922.7±139.1	20	614.0±102.2
Treshold 9.5 mmol/L	Urine glucose-	Non T2D	2	2.25±1.50	1	45.8
		T2D	0	0	0	0
	Urine glucose+	Non T2D	11	112.1±28.5	11	75.8±23.0
		T2D	11	837.8±133.8	20	545.1±97.3
Treshold 10 mmol/L	Urine glucose-	Non T2D	***0***	***0***	1	16.5
		T2D	0	0	0	0
	Urine glucose+	Non T2D	9	99.0±25.6	8	67.5±22.4
		T2D	11	759.3±128.5	20	480.3±91.8
Treshold 10.5 mmol/L	Urine glucose-	Non T2D	0	0	***0***	***0***
		T2D	0	0	0	0
	Urine glucose+	Non T2D	8	77.6±20.6	7	46.9±18.6
		T2D	11	683.1±123.2	20	420.5±85.8
Treshold 11.0 mmol/L	Urine glucose-	Non T2D	0	0	0	0
		T2D	0	0	0	0
	Urine glucose+	Non T2D	8	53.3±16.5	7	25.9±13.9
		T2D	11	608.8±117.8	19	383.8±81.4
Treshold 11.5 mmol/L	Urine glucose-	Non T2D	0	0	0	0
		T2D	0	0	0	0
	Urine glucose+	Non T2D	7	38.6±13.4	4	21.2±13.0
		T2D	11	538.9±111.8	17	367.0±78.7

[A]Caucasian T2D n=11, for reasons mentioned in methods section

Estimation of glucose threshold among non-T2D and T2D subgroups

Our stepwise exclusion approach to estimate glucose threshold during OGTT can be found in Table 3. It led to glucose threshold of ~10 mmol/L among Caucasian NGT and IFG/IGT individuals. In South Asians, glucose threshold for NGT and IFG/IGT individuals did not differ much, being ~10.5 mmol/L. Glucose threshold during OGTT varied considerably among T2D patients from both ethnicities and was therefore not assessable.

ROC values of urine markers for detection of glucose tolerance state

We examined the areas under ROC curves of glucose and C-peptide in urine unadjusted as well as adjusted for creatinine (adjusted are the values between brackets), respectively. We have found clear differences between the two ethnicities and therefore performed separate analyses. Urinary glucose concentration demonstrated high capability to discriminate between T2D and the combination of the other two subgroups (NGT and IFG/IGT); 0.976 (0.996) in Caucasians and 0.893 (0.898) in South Asians, respectively. We also calculated c-indices of urinary glucose for NGT versus the combination of IFG/IGT and T2D; 0.904 (0.908) in Caucasians and 0.894 (0.877) in South Asians, and c-indices for NGT versus the IFG/IGT subgroup; 0.826 (0.827) in Caucasians and 0.748 (0.736) in South Asians, respectively (Fig. 3).

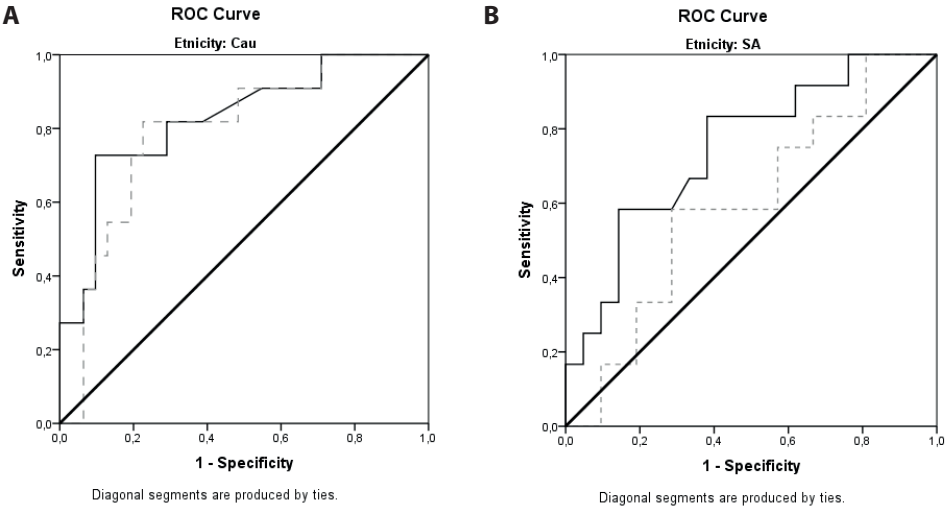

Figure 3 | Receiver-operated characteristics (ROC) curve for discriminatory ability between individuals with normal glucose tolerance (NGT) versus impaired fasting glucose/ impaired glucose tolerance (IFG/IGT) with urinary glucose (*closed line*) or C-peptide (*dashed line*) concentration obtained from urine collected during OGTT (both unadjusted for urine creatinine), in Caucasians **(A)** and South Asians **(B)**.

The c-indices of urinary C-peptide concentration for the detection of glucose tolerance status were for discrimination between T2D and the combination of two other subgroups; 0.658 (0.742) in Caucasians and 0.503 (0.565) in South Asians, respectively. The c-indices for NGT versus the combination of IFG/IGT and T2D were; 0.692 (0.733) in Caucasians and 0.556 (0.584) in South Asians. The c-indices for NGT versus IFG/IGT were; 0.792 (0.792)in Caucasians and 0.595 (0.599) in South Asians, respectively (Fig. 3).

DISCUSSION

We found that urinary C-peptide collected during OGTT correlated with plasma C-peptide indices and OMM-derived estimates of renal C-peptide excretion. Urinary glucose collected during OGTT also correlated well with plasma indices, especially with plasma glucose AUC. Urinary glucose was mainly present in urine of patients with T2D, however, the loss of glucose in the urine was too small to influence general OMM calculations. Among the patients with T2D the urinary glucose concentration showed large variation but discriminated well between normal and abnormal glucose tolerance.

Both glucose minimal model as C-peptide minimal model assess plasma glucose concentrations during OGTT with the first model also applying an area under the curve constraint; the amount of circulating glucose is expected to be a fixed parameter based on the amount of glucose load used as stimulus. The knowledge about the effects of dynamic glucose loss on OMM based parameters due to renal handling after stimulus is limited[7]. We hypothesized that the variance of the urinary glucose excretion could be a serious confounder, but we found that renal loss of glucose does not influence OMM based parameters of insulin sensitivity and beta-cell function. This finding is highly relevant for glucose homeostasis based kinetic models in general.

Overnight, 24 h fasting and after a mixed meal urine collection studies demonstrated the value of urinary C-peptide as non-invasive measure for endogenous insulin secretion in people with and without diabetes[12-17]. As stricter metabolic control affects urinary C-peptide, it might be of use to follow-up the insulin secretory function[18-20]. In line, we found that during OGTT, urinary C-peptide correlated well with plasma values reflecting the endogenous pancreatic secretion. For urinary C-peptide, we did not observe a relationship between eGFR MDRD. This is in agreement with previous studies, where the presence of micro albuminuria, or renal impairment with reduced filtration rate did not alter the relationship between urinary and plasma C-peptide[21, 22].

Glucose is cleared by the kidney and predominantly reabsorbed by the sodium-glucose co-transporter 2 (SGLT2) in the proximal tubules. The urinary glucose excretion threshold is believed to be around 10 mmol/L in individuals without T2D[23, 24], which is in accordance with our estimations, with the exception of our individuals with T2D. Our patients with T2D did not use SGLT2 inhibitors. With clamp steady state studies it was demonstrated that a glucose threshold is not applicable to individuals with T2D due to a large variation in their glucose excretion; and glucosuria is present even when treated patients with T2D return to euglycemic conditions [25, 26]. Hence, using the OGTT the post glucose load urinary glucose concentration may be useful as non-invasive marker to detect abnormal glucose tolerance, but it is not suited to monitor treatment.

The strength of the present study lies in the fact that we used OMM and assessed urine parameters in two different ethnicities and in all stages of glucose tolerance. Among the weaknesses of our study are the limited sample size, the limited possibilities to translate our findings of the *extended* OGTT into clinical applications and using estimate eGFR MDRD instead of measuring GFR directly as a measure for renal function. Although our groups were relatively small, differences between subgroups and ethnicities became apparent with this relatively simple and low-cost test procedure. In contrast to the customary 24 h urine collections obtained at home, we collected urine in the hospital setting, during an extended version of OGTT. Validity of reduced amount of sampling and sampling time after stimulus has been demonstrated previously in healthy individuals, resulting in a more practical application of OMM[27]. We were also able to reduce amount of sampling, as we found no significant differences between plasma indices obtained from our above-described final 210min-post-glucose load nine samples OGTT versus an earlier performed pilot with 210min-post-glucose load 13 samples OGTT, which also included sampling at t=5, t=10, t=20 and t=25min (data not shown). The participants in our study had no severe kidney failure and no history of renal disease.

In conclusion, urinary C-peptide corresponded well to OMM-derived estimates of renal C-peptide clearance and the renal glucose secretion during OGTT did not influence OMM indices.

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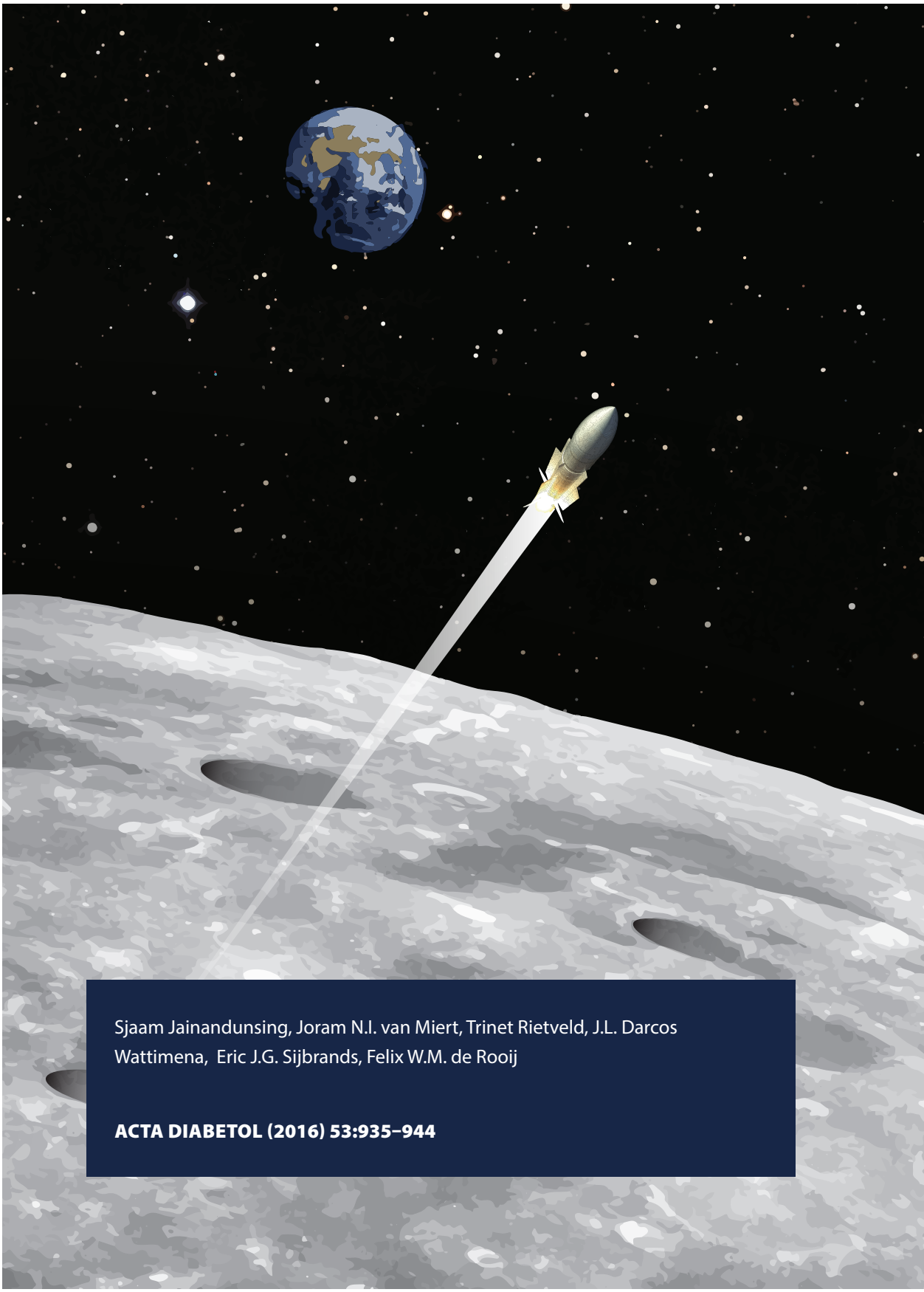

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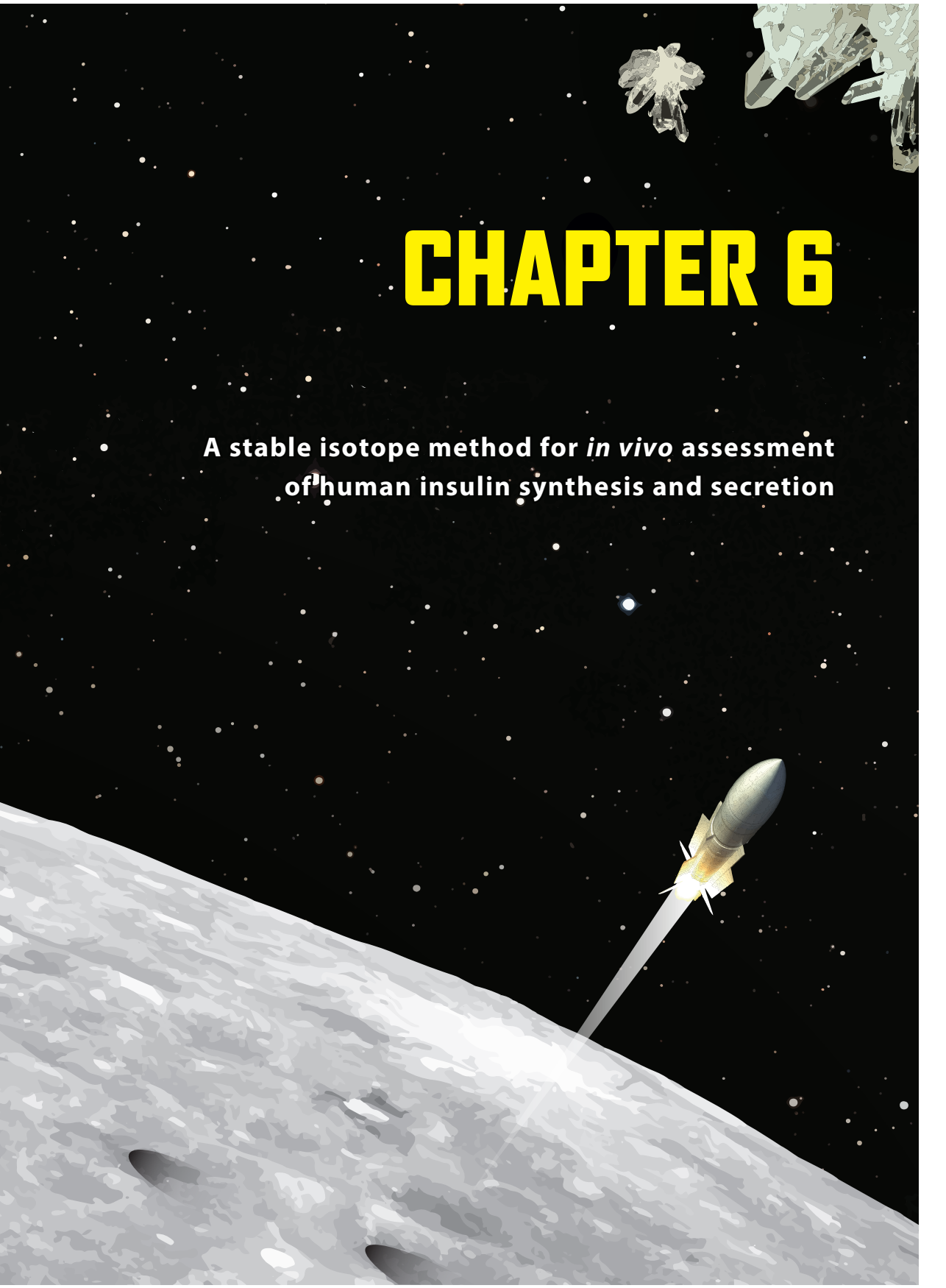

CHAPTER 6

A stable isotope method for *in vivo* assessment of human insulin synthesis and secretion

ABSTRACT

Aims. *In vitro*, beta cells immediately secrete stored but readily releasable insulin in response to a rise of glucose. During a prolonged insulin response this is followed by newly synthesized insulin. Our aim was to develop an *in vivo* test to determine the ratio between readily available and newly synthesized insulin after a stimulus in humans by labelling newly synthesized insulin.

Methods. A stable isotope tracer of 1.0 g ^{13}C leucine with C-peptide as target peptide was administered 45 min prior to 75 g glucose load of a frequently blood sampled 210 minutes oral glucose tolerance test (OGTT). Our OGTT also encompassed collection of urine, which has a high content of C-peptide. Prior, the optimal conditions under which the tracer ^{13}C leucine was administered for enrichment of (pre) proinsulin were established. Also, techniques to obtain urinary C-peptide under highly purified circumstances were set up. Our main outcome measure was the stable isotope enrichment of *de novo* C-peptide, which we related to early plasma insulin and glucose AUC. Twelve healthy Caucasian individuals (M4F8, age 41.8±2.3, BMI 28.3±1.7) with normal glucose tolerance underwent our OGTT.

Results. We found that during a 75 g OGTT, newly synthesized insulin contributed approximately 20% of total insulin secretion. The pattern of isotope enrichment obtained by collecting multiple urine voids was suggestive that the newly synthesized insulin contributes to the late phase of insulin secretion. *De novo* C-peptide correlated negatively with both early plasma insulin AUC ($r = -0.629$, $P = 0.028$) and early plasma glucose AUC ($r = -0.605$, $P = 0.037$).

Conclusions. With stable isotope technique added to OGTT, we were able to measure newly synthesized insulin in healthy individuals. This new technique holds the promise that it is feasible to develop a direct *in vivo* beta cell function test.

BACKGROUND

Abnormal function of the pancreatic beta cells is crucial to the development of type 2 diabetes (T2D)[1]. An *in vivo* test of the dynamics of insulin excretion could be used in pathogenetic studies and to examine drug effects in patients with T2D. In the present study, we explored whether it is feasible to develop a test with a stable isotope tracer to quantify the newly synthesized insulin.

Insulin synthesis and secretion by beta cells is regulated predominantly by changes in plasma glucose concentrations and in particular by the rate of these changes (supplemental figure 1)[2, 3]. Following an acute rise of glucose concentrations, a biphasic insulin secretion response occurs[4-6]. This results from the glucose transport into beta cells through the glucose transporter 2 (GLUT2) [7] , which activates calcium-dependent triggering as well as calcium-independent amplifying pathways[8] . In the so-called storage-limited model[9-13], insulin is secreted by exocytosis of two distinct pools of granules, which are the storage and trafficking units for insulin within beta cells as well as the site of conversion from predecessor (pre)proinsulin to insulin and co-secreted C-peptide [14] . A 'readily releasable pool' (RRP) of granules near the plasma membrane is responsible for the rapid first-phase release (via the triggering pathway) and the translocation of a more distal 'storage granule pool' (SGP) serves as replenishment of the RRP and results in the more sustained second phase [15, 16] . After an *in vitro* glucose stimulus, rat pancreatic islets have a biphasic insulin response and synthesize *de novo* proinsulin, which is stored in newly synthesized granules and subsequently secreted after 1 h [17, 18] . However, the dynamics of newly synthesized insulin and granular secretion of (*de novo)* insulin have not yet been investigated in humans *in vivo*.

In the present study, we determined insulin secretory function with a novel method by following insulin kinetics during an oral glucose tolerance test (OGTT) preceded by administration of a bolus of the stable isotope tracer ^{13}C leucine. We hypothesized that the *in vitro* findings would be reflected in the time course of changes in labelled and unlabelled insulin and C-peptide, providing an *in vivo* test to characterize beta cell dynamics in humans.

METHODS

Study design

Firstly, we optimized our method using ^{13}C leucine during an OGTT according to an earlier described bolus dose technique[19-21], by examining, dosage and distribution. The equilibrium phase between the isotope enrichment in the extracellular fluid was assessed by measuring ^{13}C leucine in plasma and the isotope enrichment in the intracellular fluid assessed by measuring the transamination product of intracellular leucine α-ketoisocaproic acid (KIC) in both plasma and saliva. We also assessed the potential stimulatory effects of ^{13}C leucine on insulin or C-peptide, as doses with essential amino acids may affect various metabolic processes in tissues [22, 23]. C-peptide *de novo* synthesis was calculated by its fractional synthesis rate (FSR). We tested whether ^{13}C enrichment was derived from purified C-peptide accurately by comparing several procedures. After standardization of our protocol, we used the ^{13}C leucine OGTT among subjects with normal glucose tolerance and compared the enrichment results with standard OGTT parameters. On top of basal enrichment of C-peptide, an increase in this ratio during OGTT represents *de novo* synthesized insulin(illustrated schematically in Fig 1a).

Subjects

We had two study groups of healthy volunteers, one for the pilot phase (n = 12) in which we explored, developed, and tested the method and thereafter a group (n = 12) to perform the newly developed analyses. In the pilot phase, with subgroups obtained from the 12 healthy individuals, we tested a number of components of the method, regarding dosage, equilibrium stage and final precursor enrichment (KIC) curves, based on the availability of samples on a given time point. These individuals were not on medication known to influence glucose metabolism and did not have endocrine, hepatic and renal disease. The WHO criteria for fasting and 120-min plasma glucose values were used to categorize the study subjects as being in a normal glucose tolerance state. The ^{13}C leucine OGTT was performed at the clinical research unit of the department of Internal Medicine of Erasmus MC. Informed written consent for the study was obtained from all participants, and the Erasmus Medical Centre Medical Ethics Review Board approved the study protocol.

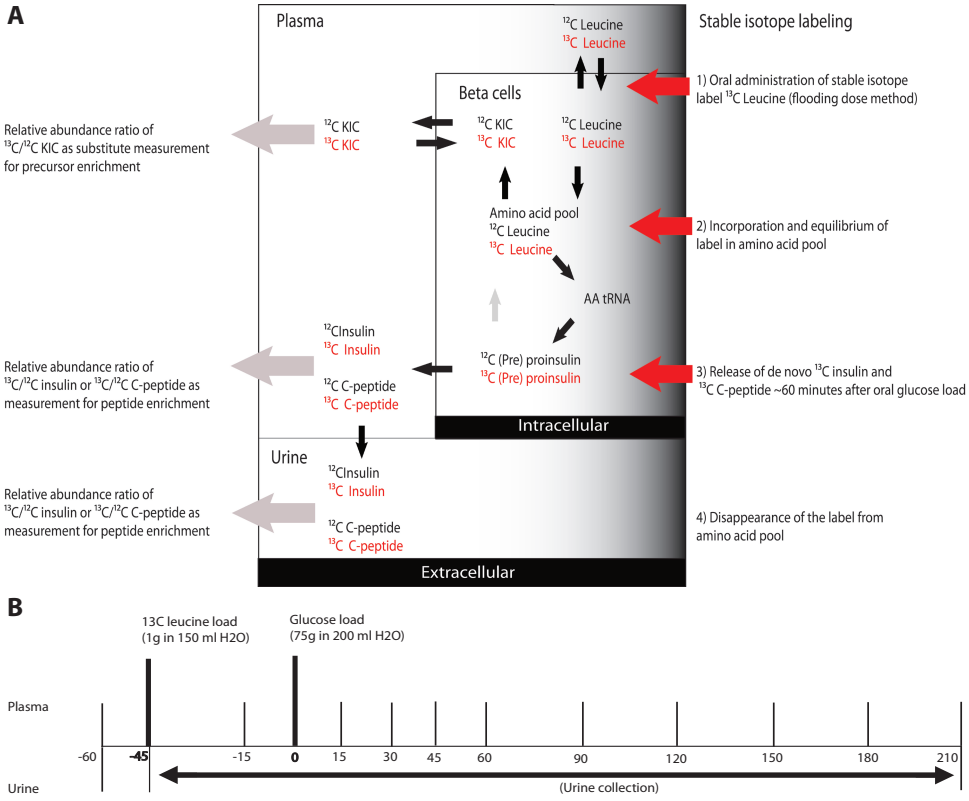

Figure 1 | (A) Model of ^{13}C leucine tracer incorporation in precursor amino acid pool for enrichment of *de novo* insulin and C-peptide; and **(B)** Schematic overview ^{13}C leucine OGTT sampling.

Anthropomorphic measurements

Body height and weight were measured to the nearest 0.1 cm and 0.1 kg, respectively. Waist was measured in cm halfway between the lowest rib and the iliac crest, hip was measured as the maximum circumference of the hips in the standing position in cm and from these measurements the waist-to-hip-ratio was calculated. Systolic and diastolic blood pressures were measured with an electronic blood pressure monitor (Datascope Accutorr Plus Inc., Montvale, NJ) after 5-min rest in the sitting position.

Standardized ^{13}C leucine OGTT

The timing of sampling is shown in Fig. 1b. A total of 75 g glucose was dissolved in 200 ml H2O and administered orally after a 10-h overnight fast. A bolus dose of 1 g ^{13}C leucine was dissolved in 150 ml H2O and administered orally 45 min (-45 min) prior

to this oral glucose load. Venous blood samples were drawn before the oral intake of the ^{13}C leucine solution (-60 min) and thereafter (-15 min). After glucose load, venous blood samples were drawn at time-points 15, 30, 45, 60, 90, 120, 150, 180 and 210 min for measurement of glucose, insulin and C-peptide concentrations (11 sampling time-points in total). Urine voids were collected in the fasting state (before oral ^{13}C leucine solution intake) and during OGTT (total urine collected in period after ^{13}C leucine solution intake until 210 min post-glucose load). In these two collections, C-peptide concentrations were measured. For a subset of individuals, urine collection during OGTT was performed in multiple portions, enabling us to observe possible trends over time. In supplementary material we explain how enrichment was measured in purified urinary C-peptide. For all subjects, we performed these analyses in triplicate from the start of solid phase extraction.

Measurements

Insulin and C-peptide concentrations are given in pmol/l and glucose concentrations given in mmol/l. Area under Curve (AUC) of C-peptide and glucose was calculated according to the trapezoid method[24] .

Calculations of beta cell function enrichment parameters

1) Dependency on de novo insulin

Enrichment expressed in tracer/tracee ratio (t/T) in purified C-peptide in urine at baseline and in urine collected during the ^{13}C leucine OGTT was used as initial measurement. These numbers were used for correlation analyses with routine OGTT parameters. Leucine and precursor KIC enrichment are expressed in mole per cent excess (MPE).

2) FSR of de novo insulin

The FSR of C-peptide *de novo* synthesis was calculated; FSR was expressed as percentage (%) during OGTT and calculated with the following formula[19, 22] ;

$$FSR(\%/hr) = (E_{collected} - E_{basal})/A \times 60min \times 100\%$$

where is the enrichment of leucine in purified C-peptide from urine collected during the total duration of the ^{13}C leucine OGTT, is the natural enrichment in baseline urine, and Area (A) is the AUC in enrichment of KIC from 90 to 210 min during OGTT, and used as substitute for enrichment of precursor pool. The factor 100 is used to convert FSR into %/h. Tracer-based synthesis measurement is based on a series of events: firstly the secretion time, which in this is case the period between oral administration of ^{13}C

leucine and first appearance of enriched C-peptide; secondly the period of *de novo* synthesis of C-peptide. This period is used for calculation of A; and thirdly the period of disappearance of stable isotope ^{13}C leucine and decrease in precursor enrichment. Regarding these events, FSR calculation was based on a fixed model: this model is based on earlier *in vitro* literature regarding biphasic responses, with a period of secretion time for *de novo* synthesis within OGTT estimated as 0-90 min post-glucose load [19]; period of *de novo* synthesis was estimated as 90-210 min, and we assumed this time period based on 1) previous literature where in vitro isolated rodent islet cells exposed to high glucose concentrations produced *de novo* insulin after 60 min [17, 18] 2) and taking into account both leucine and glucose absorption in our gut; period of disappearance of stable isotope is not taken into consideration in this model.

3) Estimated absolute de novo C-peptide concentration in both urine and plasma

Total urinary C-peptide concentration was multiplied with the overall fractional synthesis (FS) during the 2 h of OGTT (t90-210min) to obtain absolute *de novo* C-peptide estimated in urine. Total plasma C-peptide AUC was multiplied with FS during the 2 h of OGTT (t90-210min) to obtain absolute *de novo* C-peptide estimated in plasma.

Statistical analyses

Data are expressed as mean ± SD, or indicated otherwise. Comparisons within persons were made with paired t test. For correlation analyses, Spearman's rho was used. Differences were considered statistically significant when P value was < 0.05. All statistical tests were conducted with the use of SPSS, version 15.0, for Windows (SPSS Inc., Chicago, IL, USA).

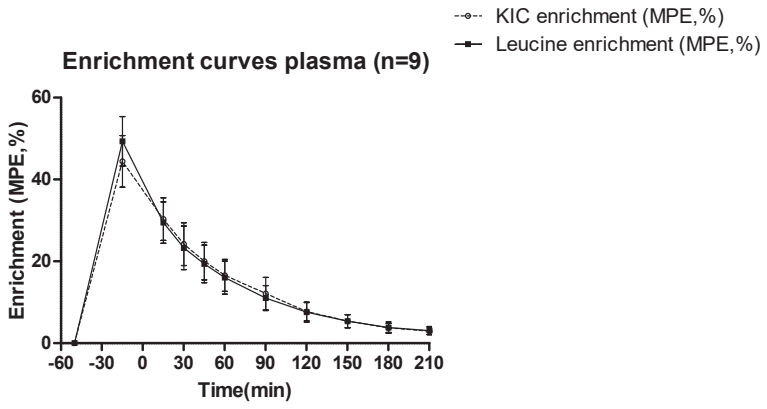

Figure 2 | Final leucine and KIC enrichment curves (MPE, mean± SD) in healthy individuals (n = 9)

RESULTS

Dosage, distribution, single pool kinetics and final conditions of the bolus dose method

First, the optimal conditions for stable isotope administration during an OGTT were determined. We studied the effect of 1.0 g ^{13}C leucine administration on plasma insulin concentrations among non-diabetics (n = 7, M4 F3, age 31.2±16.1, BMI 23.2 ±2.2), with paired t test of insulin concentration in both groups before and 15 min after administration. Since no significant increase in insulin or C-peptide concentration was observed, this dosage was maintained (supplemental figure 2a). To test whether 1.0 g ^{13}C leucine and timing of administration would result in enough precursor enrichment, we examined isotope enrichment in the extracellular fluid (leucine MPE) and isotope enrichment in the intracellular fluid (KIC MPE) in both plasma and saliva in non-diabetics during OGTT (n = 6, M4 F2, age 35.5 ±17.4 BMI 23.9 ± 3.3). No significant difference in the amount of average leucine MPE and KIC MPE/min between plasma and saliva was observed after ^{13}C leucine administration: equilibrium with KIC MPE as valid surrogate marker was assumed with high precursor enrichment (supplemental figure 2b). With this final protocol, we combined the data of total nine non-diabetic individuals (M4F5, age 31.9±10.5, BMI 24.8±3.0) into a final KIC MPE and leucine MPE curve (Fig 2). From this curve, the AUC from t=90 to t=210 min was used to estimate A. As we observed a small variance in A, with a value of 0.109 ± SEM 0.016 (t120-210 min), we used this as a fixed parameter in our final model. Single-pool kinetics of both ^{13}C leucine and ^{13}C KIC in both plasma and saliva are mentioned in supplemental table 1.

^{13}C leucine enrichment and OGTT based plasma/urine parameters

We used our final model in 12 subjects with normal glucose tolerance. Their general characteristics as well as their enrichment measurements are described in Table 1, detailed individual characteristics in supplemental table 2, and their OGTT curves in Fig. 3ac. In general, $^{13}C/^{12}C$ enrichment in C-peptide from basal urine (the naturally occurring enrichment) did not differ much between subjects. It was estimated that on average, *de novo* synthesis represented ~20% of total C-peptide released during a 210 min OGTT. In correlation analyses with OGTT parameters, $^{13}C/^{12}C$ leucine enrichment was negatively correlated with early C-peptide release (Fig. 3d), and also negatively correlated with excesses of glucose concentrations (Fig. 3e). Finally, in order to demonstrate the trend of enrichment post-glucose load, we collected multiple urine voids during OGTT. Supplemental Fig. 3 illustrates that $^{13}C/^{12}C$ leucine enrichment had its maximum more towards the late phase of the OGTT.

Table 1 | Clinical characteristics of individuals with normal glucose tolerance

	NGT
n	12
Sex(male/female)	4/8
Age (years)	41.8±2.3
Weight (kg)	88.2±6.21
Height (m)	1.76±0.03
BMI (kg/m2)	28.3±1.7
Waist (cm)	101.8±4.7
Hip (cm)	113.0±2.85
W/H ratio	0.90±0.02
RR systolic (mmHg)	121±4
RR diastolic (mmHg)	76±2
Basal C-peptide enrichment (t/T)	0.273±0.0004
Collected C-peptide enrichment (t/T)	0.295±0.002
FSR(%/hr), total FS (%) during 210min OGTT between brackets	9.9±1.0 (19.8±1.9)
Total urinary C-peptide (pmol/L*210min)	8746±1585
De novo urinary cpep (pmol/L*210min)	1633±305
Total plasma C-peptide AUC (pmol/L*210min)	388129±35252
De novo plasma C-peptide AUC (pmol/L*210min)	74367±8727

Data are means± SEM. BMI is Body Mass Index. W/H ratio is waist/hip ratio. RR is Riva-Rocci (blood pressure). FSR is Fractional Synthesis Rate. FS is fractional synthesis. OGTT is oral glucose tolerance test. AUC is area under curve.

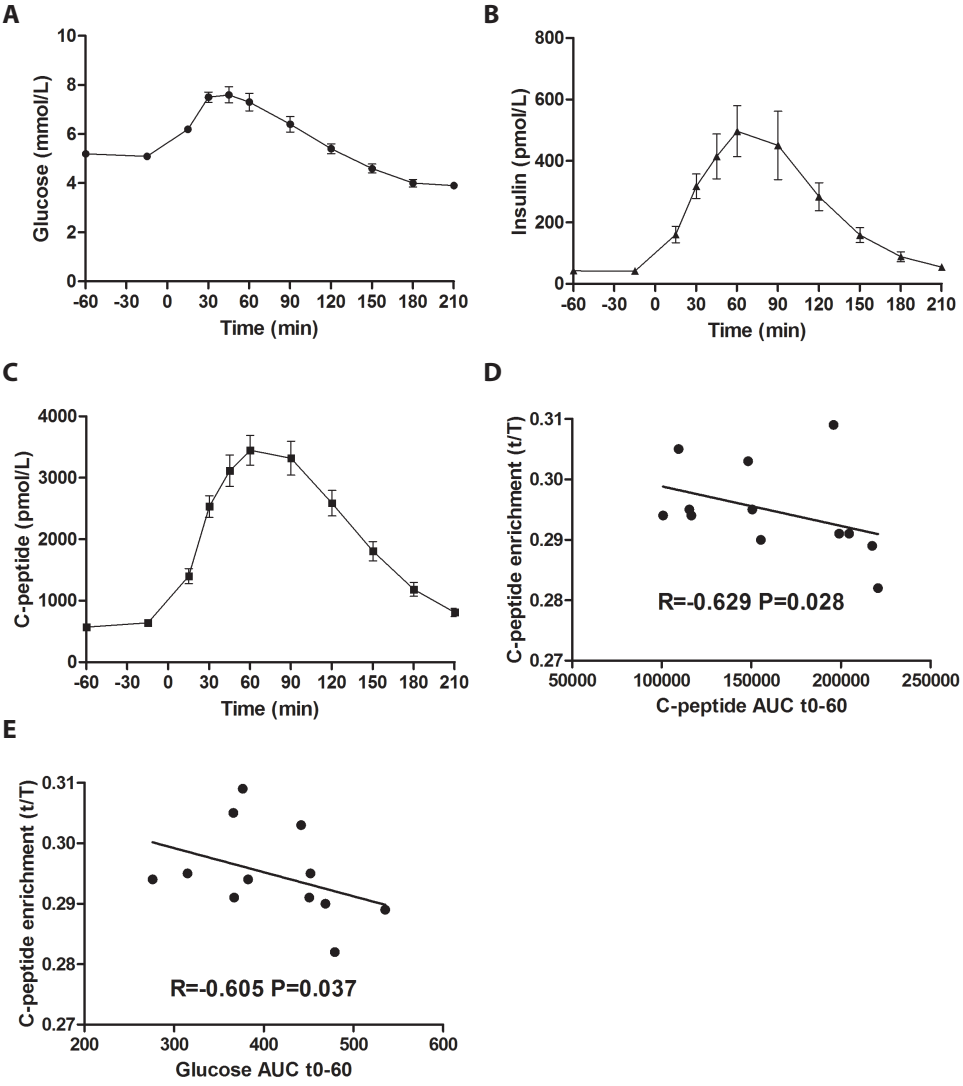

Figure 3 | OGTT curves (mean±SEM) for **(A)** glucose, **(B)** insuline and **(C)** C-peptide. Spearman's correlation of C-peptide enrichment (t/T) obtained from urine collected during OGTT with OGTT parameters d C-peptide 0-60 min AUC; and e glucose disposal 0-60 min AUC

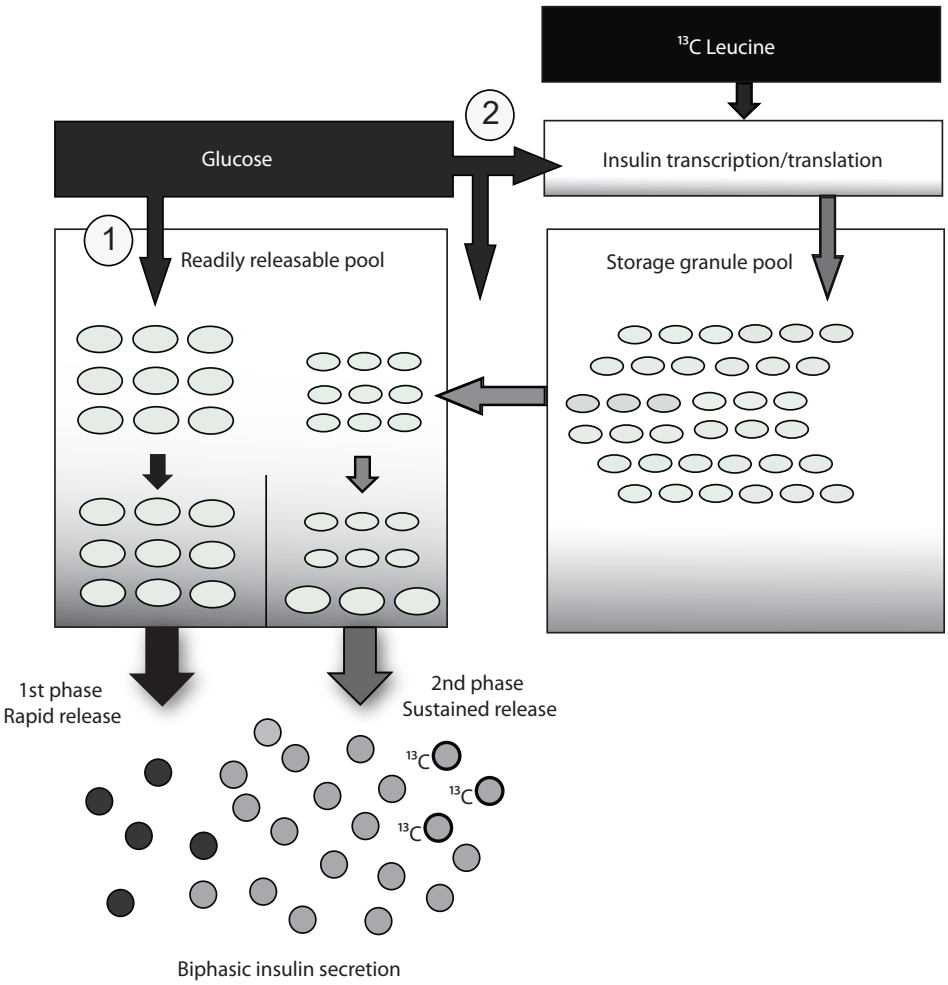

Figure 4 | Schematic overview of assessment of beta cell function with stable isotope method during OGTT. Oral glucose load initiates beta cell response in a biphasic secretion modus. Two pools of granules (*oval shaped*) are responsible for insulin secretion (*sphere shaped*): 1st phase insulin release (*dark spheres*) is delivered by a readily releasable pool located at the cell periphery (1), and second more sustainable phase (*light spheres*) is delivered by a storage granule pool located more distantly (2). The storage granule pool contains pre-existing insulin and insulin that is newly synthesized during OGTT. This newly synthesized insulin (*light spheres with ^{13}C labelling*) can be measured with stable isotope techniques, adding a novel beta cell function test to investigate T2D development within classical OGTT.

DISCUSSION

Using ^{13}C-leucine to label peptides during a 75 g OGTT in healthy volunteers, we found that newly synthesized insulin contributed a substantial portion (approximately 20%) to the secreted insulin during 210 min. The pattern of isotope enrichment suggested that the newly synthesized insulin contributed mainly to the second phase of insulin secretion: the negative association of post glucose load C-peptide enrichment with early plasma C-peptide AUC might imply that a low first phase of insulin secretion is followed by a relatively high dependency on *de novo* synthesis. This is the first *in vivo* study where stable isotope labelling has been used to explore synthesis and release of insulin in humans.

Early in vitro studies following radioactive labelled insulin in pancreatic islets in response to high glucose concentration demonstrated an increase in radioactive insulin release after more than 1 h delay [17, 18] . Moreover, we had to consider both leucine and glucose absorption in the gut . The observed enrichment during our OGTT from 90 to 210 min suggests an increase in *de novo* insulin production, while it has been assumed that roughly only 15% of stored insulin is being secreted by the pancreas when exposed to high glucose levels. The enrichment of C-peptide in the presence of a large insulin storage capacity of the pancreas supports the idea of a preferential secretion of *de novo* insulin under high glucose load conditions [25, 26] . In line, late phase *in vivo* insulin release in our healthy volunteers was not fully explained by *de novo* synthesis, as had been observed *in vitro* [27] . In Fig. 4, we propose a schematic overview of insulin synthesis including labelling with ^{13}C-leucine, SGP, RRP and secretion of granules.

C-peptide was preferred to insulin as a measure of insulin biosynthesis and enrichment measurement. Both are secreted in equimolar rate, but C-peptide is more stable than insulin, is cleared predominantly by the kidneys, and has a higher availability due to its longer half time in plasma as well as being secreted in higher amounts into urine. In addition and in contrast to insulin, C-peptide does not have a significant first-pass liver clearance or other peripheral tissue degradation pathways, which vary largely between individuals with different metabolic conditions influencing insulin synthesis and turnover. Urine was sampled during OGTT, as urine is easily available and contains C-peptide in higher abundance than plasma, which is an advantage for isolation of the small C-peptide. Urinary C-peptide excretion reflects endogenous insulin secretion[28] , and provides a potential for a non-invasive method to follow beta cell dynamics with stable isotopes.

^{13}C leucine was used as a tracer, as insulin and C-peptide contain six potential sites for enrichment. We assumed that there would be negligible isotopic effects or recycling of the stable isotope in the current setting[29-31]. We cannot exclude a contribution of recycling of the tracer present in basal proteins, but the isotope bolus method during a restricted period reduces such effects to negligible proportions. We also assumed that our measured general distribution of precursor surrogate ^{13}C KIC in plasma also reflects enrichment in amino acid pools of pancreatic beta cells[22, 32] , and that the ^{13}C leucine enriched C-peptide has the same properties during our purification procedures as normal C-peptide[33] . Stimulation of protein synthesis in peripheral tissue has been observed on administration of amino acids [19, 23, 34] . Therefore, leucine as well as its derivative KIC could have had effects on the beta cells, albeit to a substantially lesser extent than glucose[35, 36], but we did not find such a metabolic effect of administration of ^{13}C leucine on insulin and C-peptide levels. We believe this metabolic effect is minimized by using a bolus dose technique instead of a flooding dose method or infusion labelling techniques, resulting in a substantial lower required amount of ^{13}C leucine administration. The use of a relatively low amount, together with the timing of administration of ^{13}C leucine (45 min before glucose load), differs from previous studies in which a metabolic effect was observed using substantially higher amounts of leucine simultaneously with glucose[37]. Although a continuous infusion labelling technique would result in a preferred constant precursor enrichment, the bolus dose method is less time consuming with seemingly similar results [20], and it has a proven capability to achieve equilibrium of intra- and extracellular pancreatic amino acid pools [19-21] .

There are some technical considerations regarding the use of tracer/tracee ratio in urinary C-peptide as marker for *de novo* synthesis. C-peptide ^{13}C enrichment could be underestimated when there is still a demand for insulin and presumably also for *de novo* insulin synthesis in the late phase of the OGTT based on disappearance of label into the extravascular pool. This would result in diminished precursor enrichment. We have purified C-peptide from human urine. This method is not only of benefit for further C-peptide oriented studies, but also provides an overview of how to manage the purification of other low abundant peptides from human bodily fluids. SPE-IAC demonstrated highly purified C-peptide on 1D HPLC analysis, preventing of loss of C-peptide by reduction in the number of steps required for purification. Loss of C-peptide during work-up procedure was also reduced by optimization of the surface materials[38]. It remains the question whether or not this procedure will suffice in individuals with T2D, with possible fewer quantities of urinary C-peptide, and excessive urinary protein and peptide contamination due to diabetic nephropathy.

Although our method used for enrichment measurements was reproducible, urine C-peptide (ELISA measured) concentration was independent, and increased enrichment could be observed when using 1 or 4g of ^{13}C leucine (supplemental Fig. 3) in the same individual, it is not a purely quantitative method, as it is based on the ratio of labelled to unlabelled C-peptide, rather than the absolute amount of tracer. Considerations for qualitative or quantitative measurements have been discussed previously[39]. Taken together, the increase over time of enrichment after a leucine bolus, the increase over time of the production of C-peptide after a glucose load and the use of urine voidance instead of blood make our results an overall approximation of *de novo* synthesis of insulin during OGTT. With only two measurements to determine the enrichment, the current model simplifies the non-steady state nature of insulin secretion after an acute oral glucose stimulus for beta cell secretion. Of course, the tracer ^{13}C leucine enrichment could be measured in frequently sampled plasma for more detail. Further technical improvements to increase the recovery of purified C-peptide (or insulin) from plasma and enhanced mass spectrometry efficiency for measuring *de novo* synthesis measurement are required if intravenous glucose stimulus techniques are considered to test beta cell function. Such an approach may make a clearer distinction between the first phase and the second phase insulin response and facilitates more detailed modelling[5].

The stable isotope labelling techniques used for this study purpose provide a base for improved phenotyping of individuals with metabolic syndrome and predisposition for T2D, which could open the opportunity for the earlier initiation of preventive beta cell focused strategies to inhibit the progression to T2D. Moreover, our method could also be applied for monitoring of beta cell capacity during beta cell potentiating medication.

In conclusion, we have developed an *in vivo* stable isotope tracer method to investigate beta cell dynamics in humans that is able to distinguish between already available and *de novo* synthesized insulin. Future research is required to test the value of the method to screen for impaired insulin secretion as part of beta cell dysfunction.

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SUPPLEMENTARY MATERIAL

Methods for measuring $^{13}C/^{12}C$ leucine ratio in purified urinary C-peptide

Materials and chemicals

All chemicals used were of analytical grade and all solvents of chromatographic grade and were purchased from VWR International (West Chester, Pennsylvania, USA). Buffers and solutions were prepared with deionized water (Milli-Q grade). OASIS HLB cartridge columns were purchased from Waters (Milford, MA). ZIPTIP pipette tips were purchased from Merck Millipore (Darmstadt, Germany). The human C-peptide mouse antibodies were purchased from HyTest Ltd. (Turku, Finland). Cyanogen-bromide-activated Sepharose 4B required for immunoaffinity columns was purchased from GE Healthcare (Diegem, Belgium).

The phosphate buffered saline (PBS) was prepared according to Thevis, et al. [1]« , consisting out of 0.12 M Na3PO4 and 0.5 M NaCl in deionized LC-MS H2O, pH adjusted to 8.0 with 3 M HCL. ^{13}C leucine (99% purity) was purchased from Cambridge Isotope Laboratories. Possible presence of C-peptide mouse IgG antibodies due to harsh elution conditions or leakage of columns in our post- immunoaffinity chromatography (IAC) sample elute was measured with Mouse IgG total Ready-SET-Go! ELISA from Bioscience (San Diego, CA) according to manufacturer's protocol. Urinary C-peptide was measured after each purification step with C/PEP/EASIA ELISA from DIAsource ImmunoAssays S.A. (Cat. no. KAP0401, Nivelles, Belgium). Plasma glucose was measured by a hexokinase-based method (Gluco-quant; Roche Diagnostics, Mannheim, Germany). Plasma insulin and C-peptide, and urinary C-peptide were measured separately by a competitive chemiluminescent immunoassay, supplied by Euro/DPC. The assay was performed on a DPC Immulite 2000 analyzer (Euro/DPC), according to the manufacturer's recommended protocol. Serum creatinine was measured with an enzymatic procedure based on creatinine conversion, with the Creatinine Plus assay on a Roche/Hitachi analyzer. Urine creatinine was measured based on the Jaffe alkaline picrate method, with commercially available creatinine from Merck, Darmstadt, Germany used for making reference values.

Purification of urinary C-peptide

Solid phase extraction (SPE), followed by IAC was used for purification of C-peptide from urine. C-peptide recovery and possible contamination were evaluated at each step of the purification.

Solid Phase Extraction

For our work-up procedure 100 pmol of absolute C-peptide was required, and based on ELISA measurements of urinary C-peptide concentration, a variable volume of urine was used in order to obtain the absolute amount of 100pmol . These samples were first loaded on a 3cc 60mg OASIS HLB cartridge columns from Waters (Milford, MA) for solid-phase extraction (SPE); Urine was diluted with 0.5% TFA in a ratio of 5:3.5 and centrifuged during 10 minutes at 700 g at room temperature. The supernatant was loaded on SPE column, which had been preconditioned with 1 ml methanol acetonitril, followed by 1 ml 0.1% TFA. The cartridge was washed with 3X0.50ml 0.1% TFA and dried. Elution of the cartridge followed, using 2X0.25ml 50% acetonitril containing 0.1% TFA into an Eppendorf tube and this eluate was dried using a SpeedVac concentrator (Thermo Scientific Savant SPD131DDA SpeedVac concentrator, Thermo Scientific RVT4104 Refrigerated Vapor Trap). The dried residue was resuspended in 1ml PBS buffer, incubated during 15 minutes in an ultrasonic bath and transferred to an IAC column.

Extraction of C-peptide from urine by IAC.

IAC columns were made with anti-human C-peptide monoclonal mouse antibodies (HyTest). These antibodies were coupled to cyanogen-bromide-activated Sepharose 4B (GE Healthcare, Diegem) with a capacity of 1 mg IgG/mL (0.5 mL per column) according to enclosed protocol. The IAC columns were stored in PBS and were washed 3X with 1ml PBS before use. The resuspended eluate from SPE procedure was loaded on the IAC column and after mixed incubation during 60 minutes, the effluent was collected and the column washed with 3X 0.50ml PBS and 3X 0.50ml H2O. Bound C-peptide was eluted with total 1.8ml 0.1% TFA. Hereafter, the IAC columns were washed with 3X 0.50ml 0.1% TFA en 2X0.50ml LC-MS H20 and stored again in PBS.

1Dimensional (1D) High-performance Liquid Chromatography (HPLC) of the IAC elute

IAC eluate was captured in prehydrolysed vials for gas chromatography-mass spectrometry (GC-MS) analysis for all final enrichment analyses. In parallel, we also worked up random samples with SPE and IAC for further work-up on 1D HPLC; for this procedure IAC eluate was captured in vials coated with different albumin concentrations of 1% 0.5.%,0.1%, 0.05%, 0.01%, 0.001% and 0% H20 solution Separation of C-peptide in IAC hydrolysate from possible nonspecific IAC-bound contaminants including BSA from the work-up procedure was performed on HPLC interfaced to an UV detector (Spectrasystem), after establishing optimal conditions with recombinant human C-peptide. The HPLC was equipped with a Polaris C18, Varian column (50X2mm, particle

size 3µm, pore size 180 A). The mobile phase consisted of (A) H2O containing 0.1% TFA and (B) acetonitril. A sample volume of 100 µl was injected into the HPLC system, and a gradient of 15%B to 90%B in 13 min at a flow rate of 0.5 ml/min was used.

GC-MS analysis of precursor surrogate $^{13}C/^{12}C$ KIC from plasma and $^{13}C/^{12}C$ leucine from target peptide purified urinary C-peptide

We measured $^{13}C/^{12}C$ in KIC in plasma on all time points during OGTT. The amino acids of from plasma were derivatized with N-Methyl-N-tert-butyldimethyl-silyltrifluoroacetamide in pyridine during 60 minutes at 60°C to their t-butyldimethylsylil derivatives. The ^{13}C enrichment was determined by gas chromatography–mass spectrometry by measuring the fragments of natural ^{12}C and ^{13}C KIC, respectively. Gas chromatography–mass spectrometry analyses were carried out on a Carlo Erba GC8000 gas chromatograph coupled to a Fisons MD800 mass spectrometer (Interscience BV) by on column injection of 1 µL on a 25-m 0.22-mm fused silica capillary column, coated with 0.11 µm of HT5 (SGE, Victoria, Australia).With regard to $^{13}C/^{12}C$ leucine in purified urinary C-peptide, 2 ml 6 M HCl was added to the remaining dried eluate after IAC, the tube was flushed with nitrogen and capped. After incubation during 24 hours at 110° C the hydrolysate was dried using a SpeedVac concentrator. The ^{13}C enrichment was determined by gas chromatography–mass spectrometry by measuring the fragments 302 and 303 of natural and ^{13}C leucine, GC-MS analyses of all urine samples were performed with DSQ II Mass Spectrometer Detector (Thermo Electron Corporation) and GC column BPX5 column 25m, I.D. 0.22 mm, film 0.25µm (SGE Analytical Science)

Results

Purification of C-peptide, validation, inter- and intra-variability of ^{13}C leucine enrichment

The recovery of C-peptide after SPE was ~100% and after SPE-IAC ~50-60% (supplemental figure 4a). An albumin coating with at least 0.001% BSA solution of the collection tube for IAC eluate was required to maintain an adequate ~40% recovery of C-peptide for the workup towards 1D HPLC to confirm purity (supplemental figure 4b). Leakage of IAC columns was present, but the amounts were negligible; sometimes even below detection threshold levels (supplemental figure 4c). With these procedures, at least 100 pmol of C-peptide was required to perform enrichment measurements. In order to test the purity of C-peptide we did the following:

1) OASIS-IAC method is our standard work-up procedure, we also performed 4 more intensive methods to isolate C-peptide ;a) OASIS-IAC followed by an additional OASIS method b) OASIS-IAC followed by an additional ZIPTIP method c) OASIS-

IAC followed by an additional1D HPLC method d) same as method c, but using 250 pmol C-peptide. In procedures c and d we added the 1D HPLC method as an additional separation step for C-peptide. We loaded IAC eluate onto 1D HPLC (supplemental figure 5a) When using this procedure as an additional purification step, we isolated the C-peptide peak by capturing the fractions between 5.8 and 8.8 minutes In each fraction, we measured C-peptide concentration with ELISA (supplemental figure 5b). The concentrations correlated well with their chromatographic peak (supplemental figure 5c). When related to our original urine concentration, recovery rate in all HPLC fraction combined was around 30%. However, with these additional purification step no increase in enrichment was found (supplemental figure 5d), so we maintained our OASIS-IAC procedure.

2) In order to be sure that enrichment came from C-peptide, we also excluded the possibility of interference by free ^{13}C leucine in collected urine, by adding 1000mg ^{13}C leucine to urine prior to steps for C-peptide purification; this did not affect enrichment measurements (supplemental figure 6a). Addition of 500 pmol of recombinant C-peptide to collected urine prior to steps for C-peptide purification resulted in lowering op C-peptide enrichment; as recombinant C-peptide had an enrichment of ~ 0.273, the measured decrease in enrichment corresponded well with the theoretical estimated decrease (supplemental figure 6b). Oral intake of 4 gr ^{13}C leucine instead of 1 gr resulted in increase of C-peptide enrichment (supplemental figure 6c).
3) During GC-MS analysis, we used isoleucine, an amino acid not present in C-peptide as a marker for the amount of contamination and compared its amount to that of leucine. The ratio of leucine-isoleucine peaks was in general 10-1, this would imply that the range of contamination does not result in substantial underestimation in the the range of enrichment that we measured

With our final procedure, both intra and inter-variability coefficient of variability of C-peptide enrichment measurements were 1.11% and 2.34%, respectively.

Supplemental Table 1 | Single pool model analysis of substrate kinetics

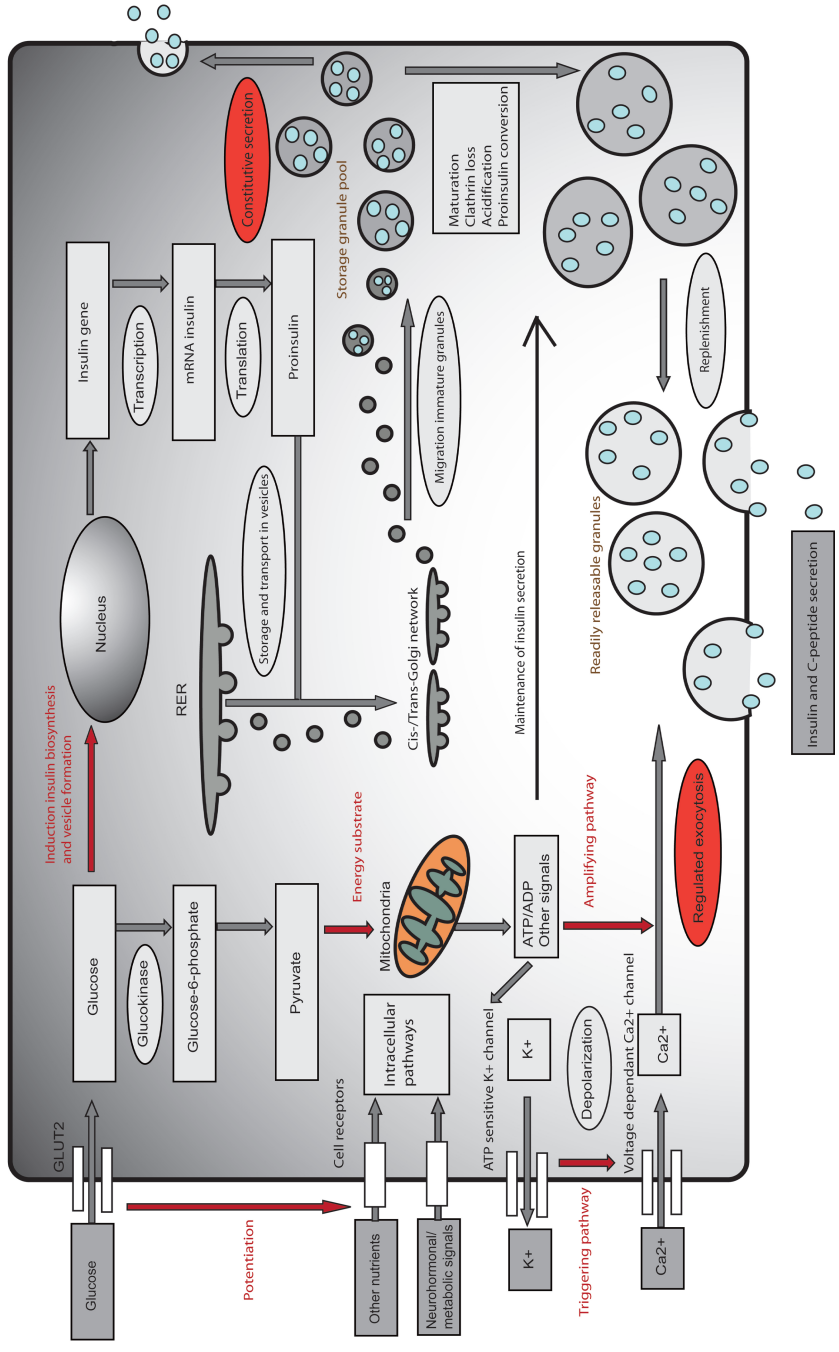
	Subjects		Subjects	
n	9		6	
Male/female	M4F5		4M2F	
Age (yrs)	31.9±10.5		35.5±17.4	
BMI (kg/l^2)	24.8±3.0		23.9±3.2	
	Plasma leucine kinetics	**Plasma KIC kinetics**	**Saliva leucine kinetics**	**Saliva KIC kinetics**
E(0)	1.18±0.20	0.99±0.18	0.59±0.20	0.70±0.14
k (min^{-1})	0.051±0.014	0.034±0.012	0.093±0.038	0.042±0.098
Q (µmol)	66277±12165	79658±14286	142044±44817	111692±19316
Ra (min^{-1})	51.8±13.6	34.2±12.1	93.3±37.6	42.2±9.9
T ½ (min)	14.4±4.6	23.4±11.2	8.5±3.3	17.3±4.7
TT (min)	20.8±6.6	33.8±16.1	12.3±4.8	25.0±6.8
MRT (min)	20.8±6.6	33.8±16.1	12.3±4.8	25.0±6.8

Data is in mean±SD. BMI is body mass index. Kinetic parameters mentioned in the table are isotopic enrichment of the first sample (E(0)) , rate constant for elimination (k), pool size (Q), rate of appearance (Ra), one half-life ($T^{1/2}$), turnover time (TT), mean residence time (MRT)

Supplemental Table 2 | Individual characteristics

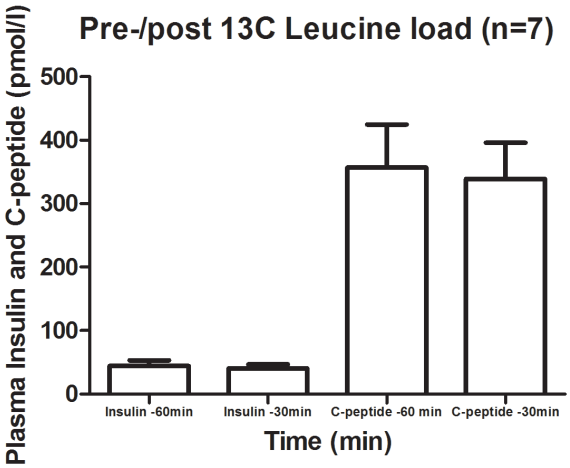
Person	Gender (M=male, F=female)	Age(years)	E basal (t/T)	E collected (t/T)	Delta E	A (t90-210min)	Total C-peptide urine (pmol/L)	Total C-peptide plasma AUC (pmol/L)	De novo (% of total) urinary cpep (pmol/L)		De novo (% of total) plasma AUC (pmol/L)	
1	M	37	0.273	0.295	0.021	0.109	5091	169583	1000	19.6	33313	19.6
2	M	61	0.273	0.282	0.010	0.109	21338	598463	1874	8.8	52568	8.8
3	M	38	0.274	0.289	0.016	0.109	9879	545370	1414	14.3	78097	14.3
4	M	41	0.271	0.290	0.019	0.109	2096	359963	368	17.6	63201	17.6
5	F	39	0.273	0.294	0.021	0.109	1515	216533	296	19.6	42360	19.6
6	F	44	0.275	0.309	0.035	0.109	7544	382425	2397	31.8	121507	31.8
7	F	45	0.273	0.305	0.031	0.109	5154	322785	1486	28.8	93079	28.8
8	F	43	0.273	0.291	0.019	0.109	9422	428355	1609	17.1	73142	17.1
9	F	45	0.276	0.295	0.019	0.109	8453	404565	1451	17.2	69443	17.2
10	F	41	0.273	0.294	0.021	0.109	10251	345765	1940	18.9	65449	18.9
11	F	35	0.273	0.291	0.017	0.109	8768	401175	1382	15.8	63214	15.8
12	F	27	0.272	0.303	0.031	0.109	15437	482565	4383	28.4	137028	28.4

Inidividual characteristics of our study subjects. M=male, F=female. E basal is natural enrichment of C-peptide in urine obtained at baseline, E collected is enrichment of C-peptide in urine obtained during 75gr 210min OGTT, Delta E is the difference between E basal and E collected, A is the area under curve (AUC) of the decay in enrichment of precursor KIC (t/T) calculated for t90-210min (based on our earlier performed pilot study as mentioned in our original article). Total C-peptide in urine and plasma were obtained during the OGTT, for both we calculated the contribution of de novo synthesized C-peptide, in absolute amounts as well as in percentage.



Supplemental Figure 1 | Schematic overview of key intracellular mechanisms induced by glucose in pancreatic beta cells. Insulin is secreted predominantly through regulated exocytosis. Under conditions of enduring high glucose concentration, a rapid insulin release from a ready releasable pool of granules is followed by a more sustained insulin release through release from a storage granule pool. De novo synthesis of (pro-)insulin replenishes the storage granule pool, and is eventually also secreted. More details about this process are mentioned in the text.

A



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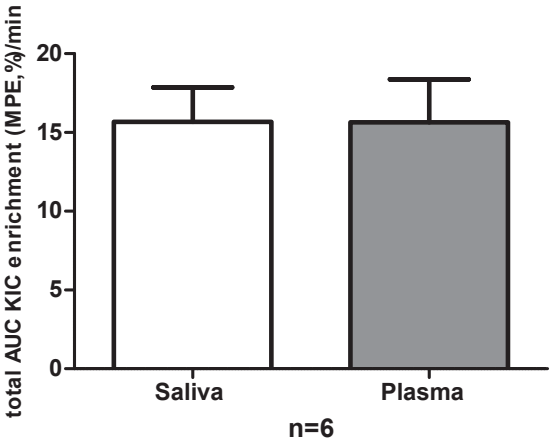
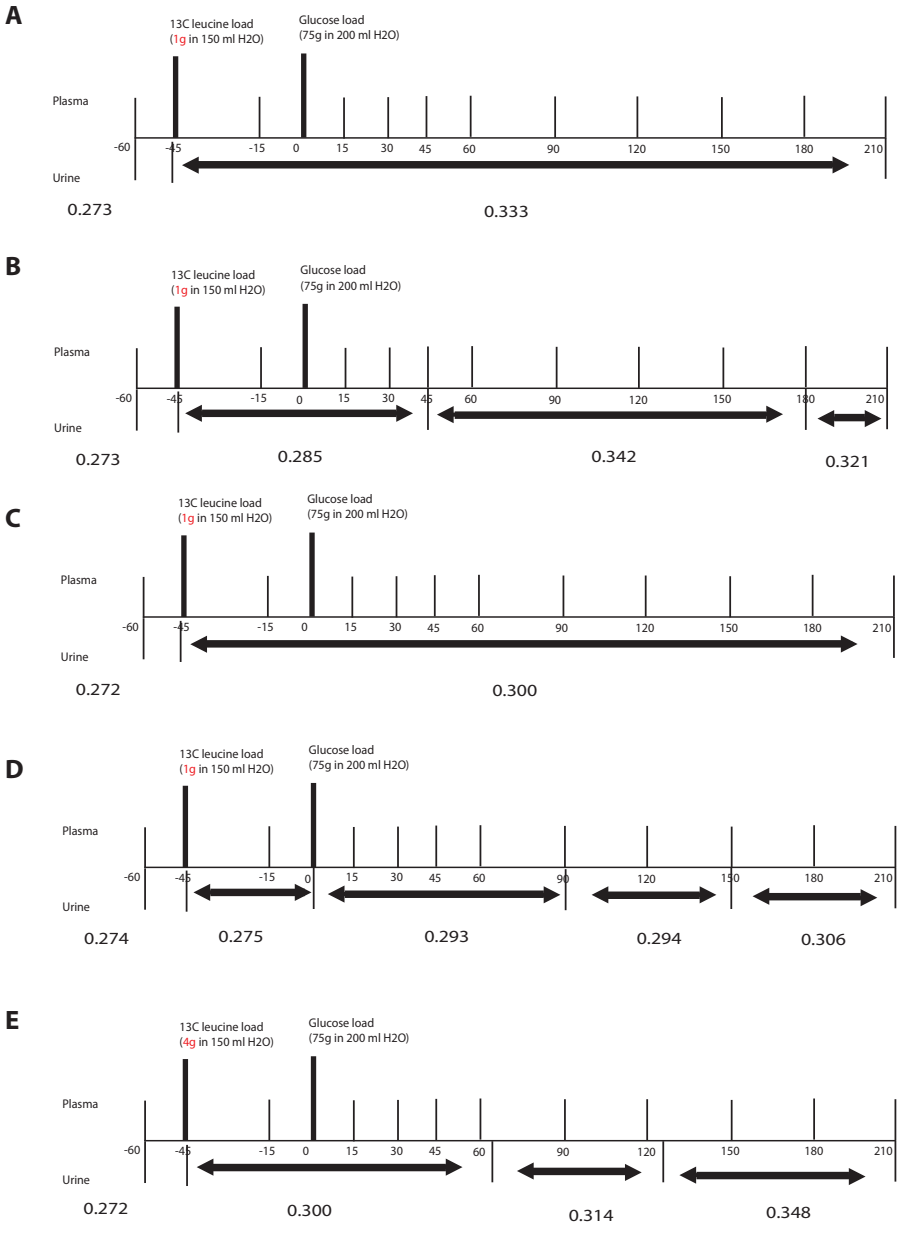
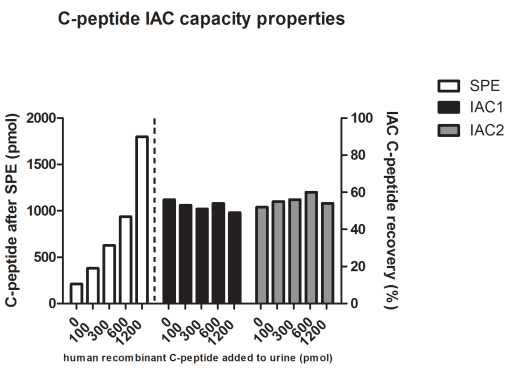


Supplemental figure 2 | (A) Determining dosage of tracer; effect on insulin concentration (mean+/- SEM). T=time before glucose load in minutes, 1gr of ^{13}C leucine was administered at T -45; **(B)** Distribution of tracer; average KIC enrichment (MPE) per minute after ^{13}c leucine administration in plasma and saliva (mean+/- SD)



Supplemental figure 3 | Illustration of urine collected over multiple time-points during OGTT. 5 schematic overviews of enrichment measurements (t/T) urine collected between given time-points are visible. The 2 first overviews are from individual 1; enrichment in basal and total collected urine **(A)** and enrichment in different portions of urine collected during the OGTT **(B)** are mentioned. The 3 overviews below are from individual 2; enrichment in basal and total collected urine **(C)** and enrichment in different portions of urine collected during the OGTT **(D)** are mentioned. Also this individual underwent the OGTT with 4gr ^{13}C leucine **(E)**.

A



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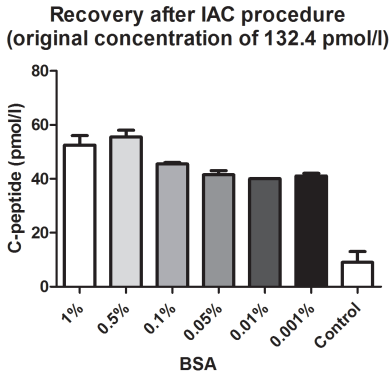

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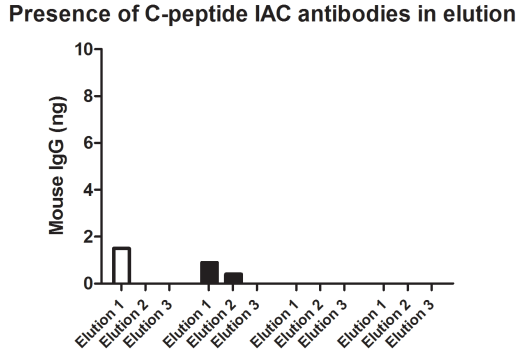
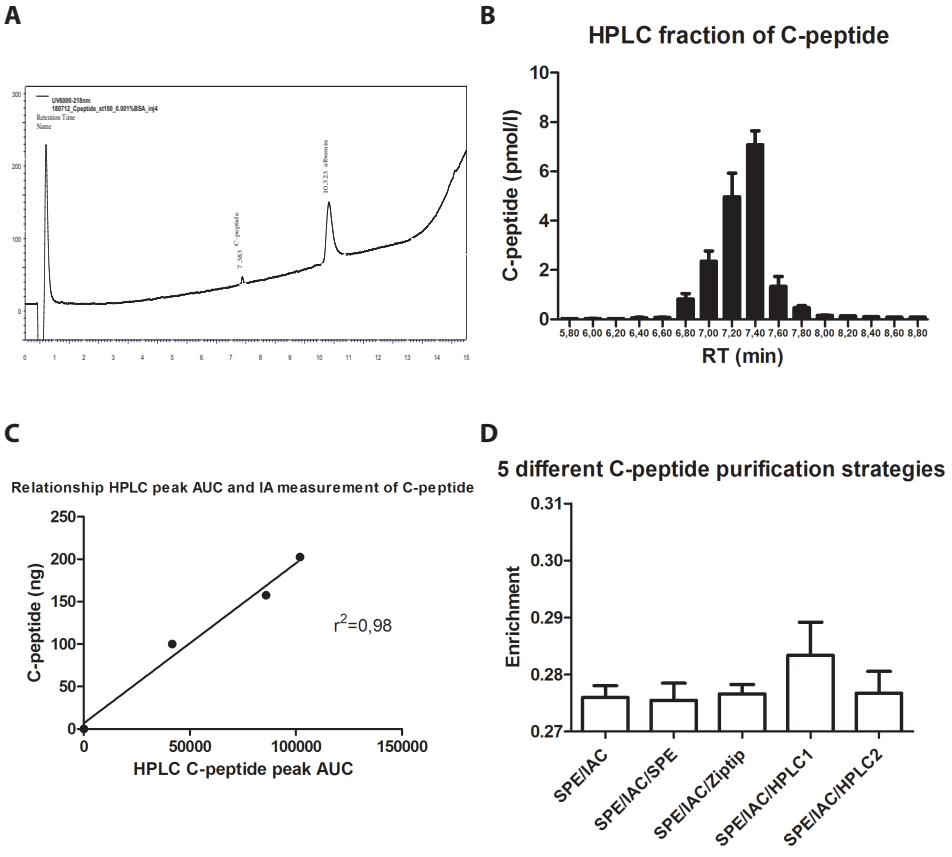
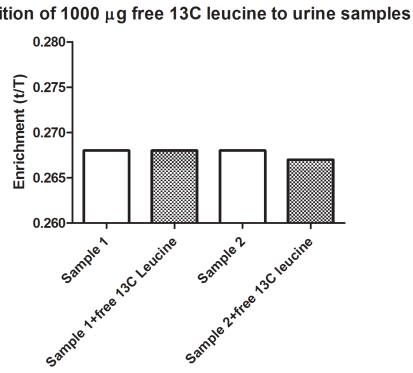


Supplemental Figure 4 | (A) recovery rate of C-peptide after (Solid Phase Extraction) SPE with increasing concentrations loaded on the OASIS column remained ~100%. In our procedure, SPE was followed by Immunoaffinity Chromatography (IAC), where recovery rate of C-peptide (tested with 2 different columns IAC1 and IAC2) was ~50-60%. **(B)** In order to prevent loss of C-peptide to surface absorption for possible further purification steps, at least 0.001% bovine serum albumin (BSA) was required, maintaining ~40% recovery of C-peptide, data in mean±SEM (addition of BSA was required when we tested an additional chromatographic separation step after SPE and IAC, however as we used SPE followed by IAC, this addition was not used in our final work-up procedure); and **(C)** Possible antibody leakage of IAC columns were tested, however the amounts measured were negligible, and occasionally the amounts were below the detection limit.



Supplemental Figure 5 | (A) After Solid Phase Extraction (SPE) and Immunoaffinity Chromatography (IAC), we performed a chromatogram of C-peptide by High-performance liquid chromatography (HPLC), with a retention time of 7.383. As albumin coating of tubes with 0.001% BSA capturing the IAC eluate was necessary for prevention of C-peptide loss, an albumin peak is visible as well. **(B)** Fractions around the 1D HPLC retention time for C-peptide were collected (concentrations in duplo in mean±SEM), and with ELISA measurements we confirmed presence of C-peptide. **(C)** There was a clear relationship between the 1D HPLC C-peptide peak AUC and the ELISA measured C-peptide concentration (r2 0.98, P<0.001). **(D)** We compared five different purification methods for their amount of C-peptide enrichment (t/T) in 7 different urine samples (mean±SEM): SPE followed by IAC (SPE-IAC), SPE-IAC followed by SPE (SPE-IAC-SPE), SPE-IAC followed by Ziptip (SPE-IAC-ZIPTIP), SPE-IAC followed by HPLC (SPE-IAC-HPLC1) with 100pmol C-peptide, and SPE-IAC-HPLC2 with 250pmol C-peptide, however no method was found to be superior, and thus the SPE-IAC strategy was maintained.

A



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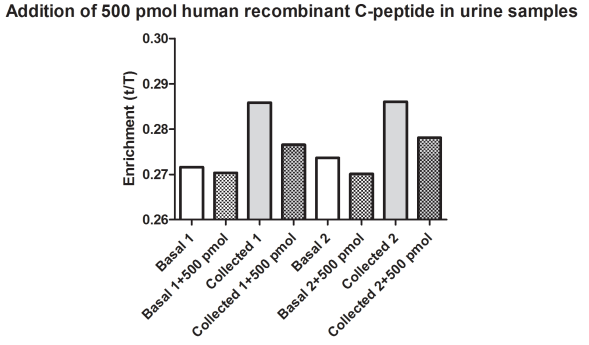

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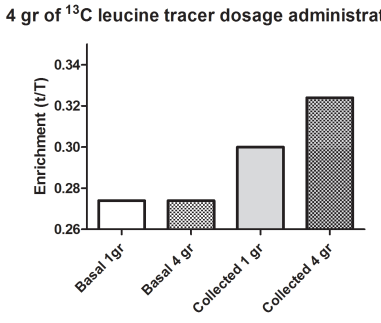

Supplemental Figure 6 | (A) As renal loss of free ^{13}C leucine after oral load might theoretically interfere in our C-peptide enrichment (t/T) measurement, we tested the addition of 1000mg free ^{13}C leucine to urine before our C-peptide purification work-up procedure; free ^{13}C leucine did not alter the our enrichment measurements; **(B)** Addition of 500pmol recombinant C-peptide resulted in lower enrichment measurements, when added to 100pmol C-peptide from urine collected after ^{13}C leucine oral intake; in our two collected urine examples after addition of 500 pmol recombinant C-peptide (with 0.273 enrichment) the enrichment went down from 0.286 to 0.277 and from 0.286 to 0.278, respectively. This corresponded with the theoretical estimated lowering effect, which was estimated to go towards ~0.277; and **(C)** Results of an individual who performed our OGTT test with 1gr ^{13}C leucine en 4gr ^{13}C leucine oral ingestion; enrichment of Cpeptide in basal urine was the same, but enrichment of C-peptide in collected urine increased from 0.300 to 0.324.

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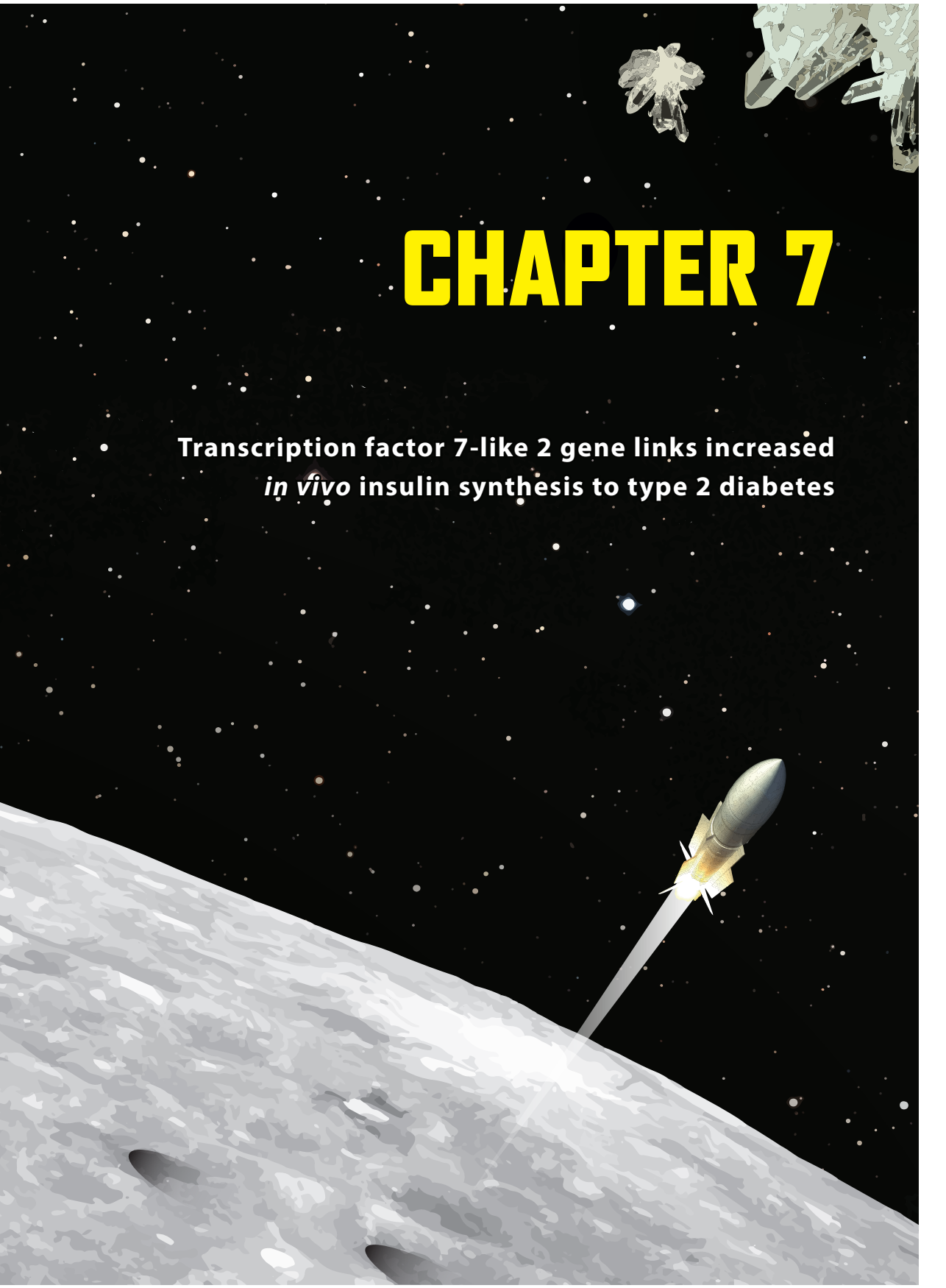
EBIOMEDICINE. 2018 APR;30:295-302



CHAPTER 7

- **Transcription factor 7-like 2 gene links increased *in vivo* insulin synthesis to type 2 diabetes**

ABSTRACT

Transcription factor 7-like 2 (*TCF7L2*) is the main susceptibility gene for type 2 diabetes, primarily through impairing the insulin secretion by pancreatic β cells. However, the exact *in vivo* mechanisms remain poorly understood. We performed a family study and determined if the T risk allele of the rs7903146 in the *TCF7L2* gene increases the risk of type 2 diabetes based on real-time stable isotope measurements of insulin synthesis during an oral glucose tolerance test. In addition, we performed oral minimal model (OMM) analyses to assess insulin sensitivity and β cell function indices. Compared to unaffected relatives, individuals with type 2 diabetes had lower OMM indices and a higher level of insulin synthesis. We found a T allele-dosage effect on insulin synthesis and on glucose tolerance status, therefore insulin synthesis was higher among T-allele carriers with type 2 diabetes than in wild-type individuals. These results suggest that hyperinsulinemia is not only an adaptation to insulin resistance, but also a direct cause of type 2 diabetes.

Highlights

- We developed a test to follow insulin synthesis in real-time *in vivo* and used it in type 2 diabetes high-risk families
- Insulin synthesis was increased in individuals with non-insulin treated type 2 diabetes
- A variant of the *TCF7L2* gene linked insulin synthesis with type 2 diabetes

Research in context. Transcription factor 7-like 2 (*TCF7L2)* is the main susceptibility gene for type 2 diabetes, predominantly by affecting insulin secretion of pancreatic β cells. However the exact *in vivo* mechanisms remain poorly understood. We investigated the relationship between the *TCF7L2* rs7903146 variant and real-time insulin synthesis measurements *in vivo*. We found that genetically increased insulin synthesis contributed to development of type 2 diabetes. Our data implies that hyperinsulinemia is a sign not only of resistance to insulin but also of intrinsic β cell dysfunction. Our findings can help in the understanding and treatment of type 2 diabetes. The glucose-sensitive *TFC7L2* pathway might be a target for intervention.

INTRODUCTION

Type 2 diabetes has become one of the main threats to human health in the 21st century[1]. This complex disease results from interactions between lifestyle and genes that are predominantly involved in the development or function of the insulin-secreting pancreatic β cells[2, 3]. The rs7903146 T allele of transcription factor 7-like 2 (*TCF7L2)*, a Wnt-signaling transcription factor gene, has consistently been linked to type 2 diabetes across different ethnicities[4-6].

The results of several studies that have looked at the effects of the *TCF7L2* variant suggest that it has a context-dependent influence on the availability of insulin. For example, obesity, insulin resistance, and hyperglycemia appear to enhance the effects of the *TCF7L2* variant.[7-11]. While the reasons underlying the context-dependent influence of the *TCF7L2* variant are largely unknown, several mechanisms have been proposed for how they might contribute to type 2 diabetes. *TCF7L2* variants have been associated with impaired incretin-stimulated insulin secretion[12-14] and with increased hepatic glucose production.[15, 16] Another mechanism might be that *TCF7L2* regulates insulin synthesis and processing in β cells, as suggested by the expression profiles of human pancreatic islets cells[17]. In human homozygotes for the *TCF7L2* rs7903146 T allele, pancreatic islet size is increased, β cell volume is relatively small, and glucose-stimulated insulin secretion *in vitro* is reduced[18]. These human data suggest a combination of morphological and functional β cell differences based on the T allele. Silencing of *TCF7L2* in rodent islets or clonal β cell lines also results in reduced glucose-stimulated insulin secretion, reduced preproinsulin gene expression, reduced incretin-stimulated insulin secretion, and defective exocytosis of the insulin containing granules[19]. Clearly, a number of different mechanisms related to regulating insulin synthesis and processing in beta cells underlie this type of genetically induced β cell dysfunction.

Numerous studies demonstrated a link between the *TCF7L2* rs7903146 T allele and insulin secretion, but it is unknown if altered *de novo* insulin synthesis contributes to this relationship *in vivo* and, consequently, if insulin synthesis is a target for preventive strategies for type 2 diabetes.

We recently developed a novel method that enables to follow real-time insulin synthesis *in vivo* during an oral glucose tolerance test (OGTT); with stable isotope ^{13}C leucine used as a tracer and insulin co-secretory product C-peptide as its target peptide for enrichment measurements during OGTT, we are able to detect newly synthesized insulin[20]. Here, we applied this technique in family analyses to determine whether

individuals with type 2 diabetes have defective insulin synthesis, and used Mendelian randomization with *TCF7L2* rs7903146 to determine if variation of *in vivo* insulin synthesis is causally related to type 2 diabetes.

MATERIALS AND METHODS

Subjects

We recruited families with a high risk of type 2 diabetes by systematic family screening at the outpatient clinic of the Erasmus University Medical Center as described previously[21]. Out of 83 patients with type 2 diabetes we identified 60 high-risk families of whom 19 Caucasian and 27 South Asian families decided to participate in the present study. Taking patients with type 2 diabetes attending our clinic as index cases, we recruited their first-degree relatives, taking two generations into account. Both parents of the South Asian probands and relatives were of South Asian origin with their roots in Surinam, and Caucasian probands and relatives were born in the Netherlands with both parents of Caucasian Dutch origin. All individuals with type 2 diabetes were only treated with metformin and received dietary advice. Based on the frequency of the genetic variant rs7903146 (CT/TT), alpha 0.05, power 80%, and 1:2 ratio of affected (type 2 diabetes) to unaffected (non-type 2 diabetes), we found that 32 individuals with type 2 diabetes and 64 without type 2 diabetes were required for allelic test of association[22]. We performed our novel insulin synthesis test in 100 of these first-degree relatives: 48 (M18 F30) Caucasians and 52 (M26F26) South Asians. For the OGTT, individuals were divided in subgroups with normal glucose tolerance (NGT), impaired fasting glucose/ impaired glucose tolerance (IFG/IGT), or type 2 diabetes, based on World Health Organization criteria. Written informed consent for the study was obtained from all participants prior to inclusion in the study. The study protocol was approved by the Erasmus University Medical Center Medical Ethics Review Board. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

Anthropomorphic data

To determine body mass index (BMI), body height and weight were measured to the nearest 0.1 cm and 0.1 kg. Waist circumference was measured in cm halfway between the lowest rib and the iliac crest; the maximum circumference of the hips was measured in cm in the standing position; and from these measurements, the waist-to-hip (W/H) ratio was calculated.

^{13}C Leucine bolus as add-on to OGTT

We performed our protocol, immunoassay and enrichment measurements as described previously and evaluated extensively[20]. In summary: 75 g of glucose was dissolved in 200 ml H_2O and administered orally after a ten-hour overnight fast. A bolus dose of 1 g of ^{13}C leucine was dissolved in 150 ml H_2O and administered orally 45 min (-45 min) prior to this oral glucose load. Venous blood samples were drawn before the oral intake of the ^{13}C leucine solution (-60 min) and thereafter (-15 min) and at several time points until 210 min after the glucose load. Urine voids were collected in the fasting state (before oral ^{13}C leucine solution intake) and during OGTT (total urine collected in period after ^{13}C leucine solution intake until 210 min after the glucose load). In these two collections, urine C-peptide concentrations were measured, which reflects endogenous C-peptide secretion[23]. The reasons for using urine voids for our enrichment measurements have been published previously[20]. For all subjects, we performed enrichment analyses of urinary C-peptide in triplicate from the start of solid phase extraction (SPE), which is the first step for purification of C-peptide from urine. On top of basal enrichment of urinary C-peptide, an increase in enrichment during OGTT represents *de novo* synthesized insulin.

Details regarding the enrichment measurements are mentioned in a technical addendum elsewhere [20]. In summary: All chemicals were of analytical grade and all solvents of chromatographic grade and were purchased from VWR International (West Chester, Pennsylvania, USA). Buffers and solutions were prepared with deionized water (Milli-Q grade). OASIS HLB cartridge columns for SPE were purchased from Waters (Milford, MA). The human C-peptide mouse antibodies were purchased from HyTest Ltd. (Turku, Finland). Cyanogen-bromide-activated Sepharose 4B required for immunoaffinity chromatography (IAC) was purchased from GE Healthcare (Diegem, Belgium). ^{13}C leucine (99% purity) was purchased from Cambridge Isotope Laboratories. SPE followed by IAC was used for purification of 100 pmol of absolute C-peptide from urine. Subsequently, ^{13}C enrichment in purified C-peptide was determined by gas chromatography–mass spectrometry (GC-MS) by measuring the fragments 302 and 303 of naturally occurring and ^{13}C-labeled leucine. GC-MS analyses of purified C-peptide from all urine samples were performed with DSQ II Mass Spectrometer Detector (Thermo Electron Corporation) and GC column BPX5 column 25m, I.D. 0.22 mm, film 0.25µm (SGE Analytical Science). The intra and inter-variability coefficient of variability of C-peptide enrichment measurements were 1.11% and 2.34%, respectively.

Calculation of OGTT indices and estimated Glomerular Filtration Rate (eGFR)

The Oral minimal model (OMM) was used to describe the plasma glucose, insulin and C-peptide concentrations after oral glucose stimulus.[24] We used the C-peptide minimal model to assess the following parameters for beta- cell function: the static responsivity of β cells due to glucose potentiation, Φ_{static} (10^{-9} min^{-1}); the dynamic responsivity of β cells due to glucose potentiation, $\Phi_{dynamic}$ (10^{-9}); and the total responsivity of β cells due to glucose potentiation, Φ_{oral} (10^{-9} min^{-1}). We used the glucose minimal model to assess the insulin sensitivity index, SI (10^{-5} dL kg^{-1} min^{-1} per pM). Parameters from both models were multiplied with each other to calculate the respective disposition indices (DI): DI_{static}, $DI_{dynamic}$ and DI_{oral}. OMM parameters were estimated using SAAM II software.[25] eGFR was estimated with the modification of diet in renal disease formula[26].

Calculations for C-peptide enrichment parameters

Enrichment parameters were expressed as tracer/tracee ratio (t/T) derived from the levels of purified C-peptide detected in urine at baseline and those in urine collected during the ^{13}C leucine OGTT. The fractional synthesis rate (FSR) of *de novo* C-peptide synthesis during OGTT was expressed as a percentage (%/hr) and calculated using the following formula: FSR (%/hr) = $(E_{collected} - E_{basal})/A \times 60min \times 100\%$, where $E_{collected}$ is the enrichment of leucine in purified C-peptide from urine collected during the total duration of the ^{13}C leucine OGTT; E_{basal} is the natural enrichment in baseline urine; and area (A) is the area under the curve in the enrichment of α-ketoisocaproic acid from 90 min to 210 min during OGTT, and used as substitute for enrichment of precursor pool, which was calculated as described previously.[20] The factor 100 is used to convert FSR into % per hour. In cases (n=47) where enrichment data for C-peptide from baseline urine were missing due to low C-peptide concentrations, we used a t/T ratio of 0.273, as this value reflects the natural enrichment which was virtually universal for all individuals in our subgroups and corresponds with the calculated theoretical natural isotope ratio in leucine. Total insulin synthesis during our 210 min OGTT mentioned in figure 3 was calculated as FSR x 2h (period of *de novo* synthesis between 90-210 min, as mentioned previously[20]).

Blood sampling for DNA isolation and gene analysis of *TCF7L2* rs7903146

Genomic DNA was isolated from venous whole blood sampled in ethylenediamine tetraacetic acid tubes using a QiAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany). Synthetic oligonucleotide primers were used for PCR to amplify a fragment of the *TCF7L2* gene (forward primer GCCGTCAGATGGTAATGCAGAT, reverse primer CCAAGCTTCTGAGTCACACAGGCC). Sequence analysis of the PCR product was

performed on a 310 Genetic Analyzer (ABI Prism), programmed for POP-6 polymer, 1mL syringe, with a 47 cm, 50 i.d. capillary. The retrieved sequence products were used for *TCF7L2* rs7903146 genotyping.

Statistical analyses

All numerical data were expressed as mean ± SEM. Comparisons between two given subgroups were performed with an unpaired t test. Differences were considered statistically significant if the *P* value was < 0.05. For differences between proportions, a Chi-squared test was used; differences were considered significant if the *P* value was < 0.05. Pearson's correlations were used to assess the associations between FSR and OGTT parameters. Statistical tests were conducted using SPSS version 20.0 for Windows (SPSS Inc., Chicago, IL, USA). Multiple regression analyses to explain variance of insulin synthesis with given independent variables were performed using the SOLAR software package, which takes into account family matrices.[27] Effects of independent variables in multiple regression analyses were considered significant if the *P* value was < 0.05. The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

RESULTS

Insulin synthesis across the stages of glucose tolerance

Prior to our main analysis, which is the investigation of the relationship between *TCF7L2* rs7903146 and insulin synthesis, we first explored how this novel β cell phenotype behaved across the different stages of glucose tolerance. Based on the results of the OGTT, we obtained three subgroups; NGT (n=47), IFG/IG (n=22) and non-insulin-treated type 2 diabetes (n=31). The clinical and biochemical features of the cohorts are described in Table 1. Individuals with type 2 diabetes underwent the same modified OGTT procedure as the non-type 2 diabetes group, as they were not known with renal disease, and as, in comparison to the non-type 2 diabetes group, there was no difference in eGFR (100 ± 4 versus 103± 3 mL/min/1.73 m2, respectively, *P* = 0.48) and urine volume during OGTT (443± 60 versus 461± 36 mL, respectively, *P* = 0.79), and as there was no difference in metabolization rate of our tracer ^{13}C leucine between both groups (Supplemental Fig. 1). Also, during our C-peptide purification work-up, no additional background contamination was observed in urine obtained from individuals with type 2 diabetes based on the ratio between amino acids not present in C-peptide versus amino acids that are present in C-peptide, as described previously [20]. This implies that possible protein loss in urine among individuals with type 2 diabetes did

not interfere with our enrichment measurements. Our individuals with type 2 diabetes had an average disease duration of 9.8±1.5 year, and they had a higher age ($P < 0.001$) and W/H ratio ($P = 0.002$) relative to individuals from the non-type 2 diabetes subgroup (NGT and IFG/IGT combined).

In addition, we also assessed indices for insulin sensitivity and β cell function based on the OMM, and for insulin synthesis. We found that patients in the type 2 diabetes subgroup had a lower SI ($P < 0.001$) and lower β cell DI parameters ($P < 0.001$ for $DI_{dynamic}$; $P = 0.005$ for DI_{static}; and $P = 0.004$ for DI_{oral}) when compared with the non-type 2 diabetes subgroup. The type 2 diabetes subgroup had a higher FSR ($P = 0.030$) when compared with the non-type 2 diabetes subgroup (table 1). For all subgroups, OGTT plasma glucose, insulin, C-peptide curves and contribution of their respective total insulin synthesis during OGTT are provided (Fig. 1a-c), as well as correlation plots between FSR and plasma C-peptide area under the curve t0-60min and urinary C-peptide (Fig. 1d-e; ($r = -0.243$, $P = 0.015$ and $r = -0.39$, $P < 0.001$, respectively).

Table 1 | General characteristics of families at high risk of type 2 diabetes.

	NGT	IFG/IGT	T2D	*P* value*
n	47	22	31	
rs7903146: CC/CT/TT	29/10/8	9/10/3	10/20/1	0.004 †
Sex (male/female)	17/30	11/11	16/15	0.330 †
Age (years)	40±2	44±2	56±2	<0.001
BMI (kg/m^2)	27.1±0.6	29.3±1.4	29.4±0.8	0.148
W/H ratio	0.86±0.01	0.92±0.2	0.94±0.01	0.002
SI (10^{-5} dL kg^{-1} min^{-1} per pM)	23±3	11±2	6±1	<0.001
$DI_{dynamic}$ (10^{-14} dL kg^{-1} min^{-1} per pM)	6263±1034	2174±576	545±140	<0.001
DI_{static} (10^{-14} dL kg^{-1} min^{-2} per pM)	496±109	131±29	47±9	0.005
DI_{oral} (10^{-14} dL kg^{-1} min^{-2} per pM)	569±122	152±33	51±10	0.004
FSR (%/hr)	10.8±0.7	11.1±0.9	13.5±1.3	0.030

Subjects are grouped according to normal glucose tolerance (NGT), impaired fasting glucose/impaired glucose tolerance (IFG/IGT) and type 2 diabetes (T2D), with numerical data presented as mean±SEM. BMI is body mass index; W/H ratio is waist-to-hip ratio; SI is insulin sensitivity index derived from oral minimal model (OMM); $DI_{dynamic}$, DI_{static} and DI_{oral} are OMM-derived β cell disposition indices as described in the material and methods section; FSR is fractional synthesis rate. * $P < 0.05$ Student's unpaired t test of T2D versus non-T2D (NGT and IFG/IGT subgroups combined) unless otherwise stated. † $P < 0.05$ Chi-squared test among given subgroups.

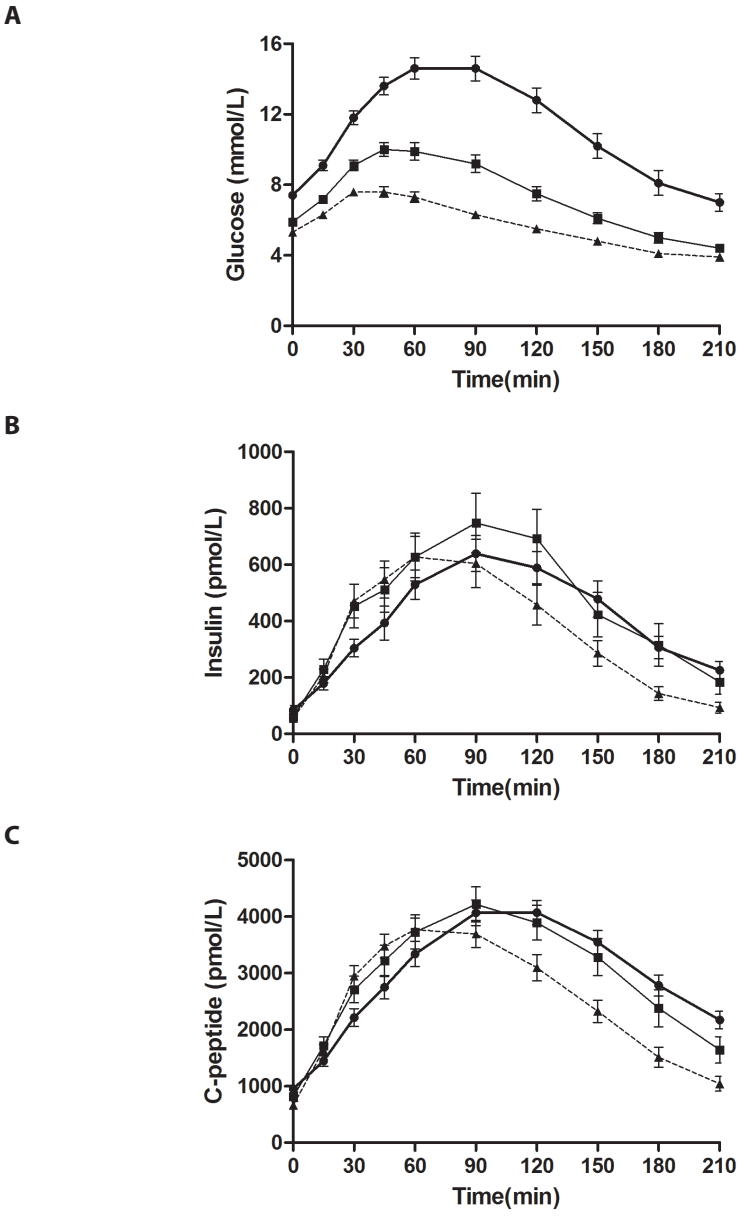
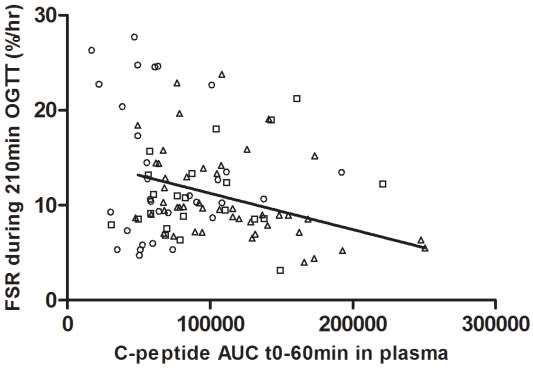


Figure 1a-c | Glucose, insulin and C-peptide curves during Oral Glucose Tolerance Test (OGTT) according to glucose tolerance state. **(A)** Plasma glucose, **(B)** insulin and **(C)** C-peptide curves during 210 min OGTT of individuals with normal glucose tolerance (*triangle, dashed line*), impaired fasting glucose/impaired glucose tolerance (*square, thin line*) and type 2 diabetes (*circle, thick line*), respectively (mean±SEM). Their corresponding total insulin synthesis measurements made during OGTT are approximately 21.6%, 22.1% and 27.0% (non-type 2 diabetes subgroups versus type 2 diabetes subgroup; $P = 0.03$, according to Student's unpaired t test), respectively.

D



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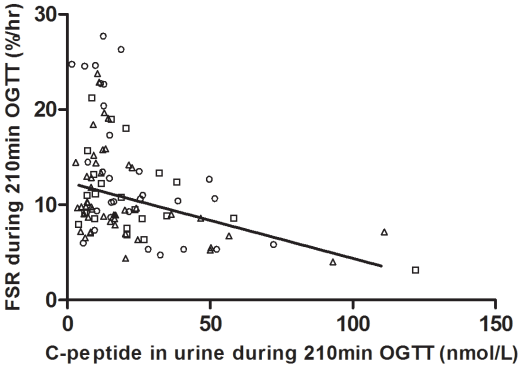

Figure 1d-e | Correlation plot of fractional synthesis rate (FSR, %/hr) during Oral Glucose Tolerance Test (OGTT) with OGTT parameters **(D)** Pearson's correlations between FSR and plasma C-peptide area under curve (AUC) t0-60min (r = -0.243, *P* = 0.015; *above*) and **(E)** between C-peptide in urine during 210 min OGTT (r = -0.39, *P* < 0.001; *below*), respectively among individuals with normal glucose tolerance (*triangle*), impaired fasting glucose/impaired glucose tolerance (*square*) or type 2 diabetes (*circle*).

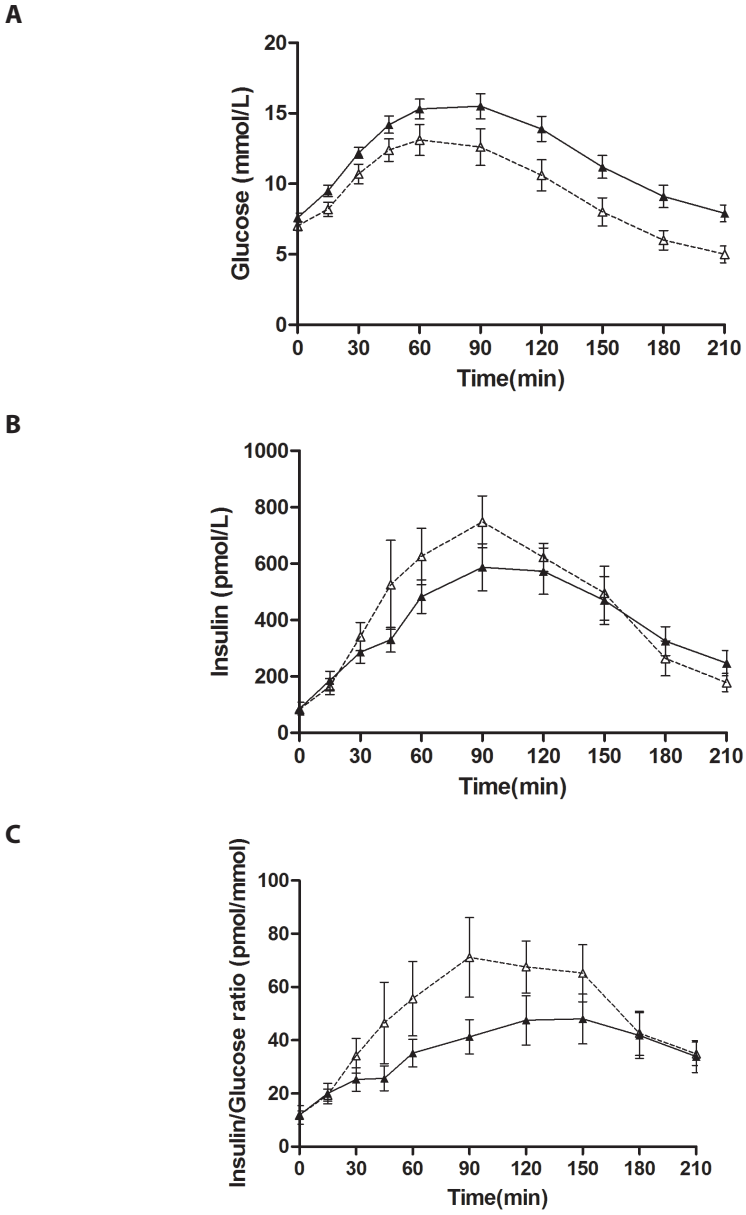

Figure 2 | Glucose, insulin and insulin/glucose ratio curves during Oral Glucose Tolerance Test (OGTT) in type 2 diabetes subgroup according to RS7903146 genotype Plasma glucose **(A)**, insulin **(B)** and insulin/glucose ratio **(C)** curves during 210 min OGTT of individuals with type 2 diabetes with either *TCF7L2* RS7903146 CC wild-type genotype (*dashed line, open triangles*) or CT/TT carriership (*continuous line, closed triangles*), respectively(mean±SEM). Their corresponding total insulin synthesis measurements during OGTT are approximately 20.8% versus 29.9%, respectively; $P = 0.041$, according to Student's unpaired t test.

Relationship between T allele of *TCF7L2* rs7903146 and insulin synthesis

The proportion of individuals with *TCF7L2* rs7903146 CT and TT genotypes increased with increasing glucose intolerance (P = 0.004, Table 1). Ordinal regression analysis revealed a significant association between the *TCF7L2* rs7903146 genotypes (CC, CT or TT) and the three WHO OGTT categories (B =1.06, P =0.009). As we found a significant interaction of WHO OGTT subgroup with CC, CT or TT carriership on the variance of FSR ($\beta_{interaction}$=0.11, P = 0.002), we performed additional subgroup analyses to compare the effect of the presence of the T allele on OMM indices and FSR (Table 2). In both non-type 2 diabetes and type 2 diabetes subgroups, there was a trend for the T-allele carriers to have a lower SI and lower $DI_{dynamic}$ (P = 0.043 within non-type 2 diabetes subgroup and P = 0.047 within type 2 diabetes subgroup), DI_{static} and DI_{oral} (P = 0.029 within type 2 diabetes subgroup) when compared with wild-type individuals. In the type 2 diabetes subgroup, FSR was increased in the T-allele carriers compared with wild-type (P = 0.041). Fig. 2a-c illustrates glucose, insulin and insulin/glucose ratio curves with contribution of their respective total insulin synthesis during OGTT for type 2 diabetes individuals with wild-type and T-allele carriership. Between these groups, there was no difference in their average disease duration (wild-type group 9.6±3.0 versus T-allele carriership group 9.9±1.8 years, P = 0.93) which could explain the difference in insulin synthesis.

For further in-depth analysis of the effects of the T allele on insulin synthesis within our family matrices, we performed multiple regression analyses in the non-type 2 diabetes subgroup and type 2 diabetes subgroup. Next to ethnicity, gender, and age (and in the non-type 2 diabetes subgroup also WHO OGTT category), these analyses also included testing for a possible influence of obesity (expressed by W/H ratio), insulin resistance (expressed as SI), and/or loss of first-phase insulin release (expressed as $DI_{dynamic}$), and we found opposing effects (Table 3). In the non-type 2 diabetes subgroup, the *TCF7L2* rs7903146 variant was associated with reduced insulin synthesis (β -1.986, P = 0.002), whereas in the type 2 diabetes subgroup, the *TCF7L2* rs7903146 variant was associated with increased insulin synthesis (β 29.893, P = 0.01). While in our multiple regression models W/H ratio, SI, and $DI_{dynamic}$ did not contribute to FSR, ethnicity did contribute to FSR in the non-type 2 diabetes subgroup analyses. Therefore, in our final analysis, we also analyzed FSR in the South Asian non-type 2 diabetes and the Caucasian non-type 2 diabetes subgroups (Supplemental Fig. 2). In the South Asian non-type 2 diabetes subgroup, the T-allele carriers had a lower FSR than that of wild-type individuals (P = 0.018), while no differences were found within the Caucasian non-type 2 diabetes subgroup.

Table 2 | Differences between transcription factor 7-like 2 CC wild-type genotype and CT/TT carriers in oral glucose tolerance test response.

	Non-T2D		T2D	
***TCF7L2* rs7903146**	**CC**	**CT/TT**	**CC**	**CT/TT**
n	38	31	10	21
NGT / (IFG/IGT)	29/9	18/13		
Sex (male/female)	16/22	12/19	7/3	9/12
Age (years)	42±2	40±2	55±3	56±2
BMI (kg/m^2)	27.9±0.9	27.6±0.9	30.9±0.9	28.7±1.0
W/H ratio	0.86±0.01	0.90±0.02*	0.96±0.03	0.93±0.02
SI (10^{-5} dL kg^{-1} min^{-1} per pM)	23±3	15±3	9±2	5±1
$DI_{dynamic}$ (10^{-14} dL kg^{-1} min^{-1} per pM)	6343±1175	3262±811*	1106±357	278±70*
DI_{static} (10^{-14} dL kg^{-1} min^{-2} per pM)	462±128	278±69	73±16	34±11
DI_{oral} (10^{-14} dL kg^{-1} min^{-2} per pM)	537±144	313±75	83±19	36±11*
FSR (%/hr)	11.6±0.8	10.0±0.6	10.4±1.2	14.9±1.7*

Non-type 2 diabetes (non-T2D) and T2D subgroups were compared, with numerical data presented as mean±SEM. NGT are individuals with normal glucose tolerance; IFG/IGT are individuals with impaired fasting glucose/impaired glucose tolerance; BMI is body mass index; W/H ratio is waist-to-hip ratio; SI is oral minimal model (OMM) based insulin sensitivity index; $DI_{dynamic}$, DI_{static} and DI_{oral} are OMM derived-disposition indices for β cell function as described in the main text; FSR is fractional synthesis rate of insulin synthesis. *$P < 0.05$ Student's unpaired t test based on CC wild-type genotype versus CT/TT carriers in each subgroup.

Table 3 | Multiple regression analyses on family matrices according to non-type 2 diabetes (non-T2D) and T2D subgroups.

	Non-T2D			T2D		
	β	**95% CI**	**P-value**	**β**	**95% CI**	***P* value**
TCF7L2 rs7903146	-1.986	[-3.229;-0.743]	**0.002**	29.893	[52.768;7.018]	**0.010**
Ethnicity	-2.431	[-4.024;-0.838]	**0.003**	10.083	[-8.913;29.079]	0.298
Gender	1.325	[-0.500; 3.150]	0.155	-5.415	[-22.502;11.672]	0.535
Age	0.136	[0.065;0.207]	**<0.001**	0.686	[-0.208;1.580]	0.132
W/H ratio	-0.04	[-0.124;0.044]	0.352	0.00212	[-0.076;0.081]	0.958
SI	0.059	[-0.037;0.155]	0.229	0.233	[3.336;-2.870]	0.883
$DI_{dynamic}$	0.0004	[-0.003;0.003]	0.790	-0.034	[-0.085;0.017]	0.191
OGTT category	-0.02	[-0.044;0.004]	0.096	-	-	-
R2	48.5%			52.2%		

Model trait: fractional synthesis rate (FSR) during 210 min oral glucose tolerance test (OGTT). Covariates: rs7903146 (CC or CT/TT), ethnicity, gender, age, waist/hip (W/H) ratio, oral minimal model (OMM) based insulin sensitivity index (SI) and OMM-based disposition index $DI_{dynamic}$. In the model for the non-T2D subgroup, we also included WHO OGTT category as covariate. Bold values indicate *P* values that reached < 0.05 significance.

DISCUSSION

The results of this study show that individuals with type 2 diabetes have defective insulin synthesis, and that this has a genetic background. We found that the *TCF7L2* rs7903146 T allele has a gene-dose effect on insulin synthesis and on glucose tolerance status. Compared to individuals without type 2 diabetes those with type 2 diabetes had higher insulin synthesis and lower OMM β cell indices during OGTT, and this increased synthesis was more prominent in individuals carrying a T allele.

These findings are based on a stable isotope based method that allows us to assess a previously undetectable parameter – newly synthesized insulin. The results suggest that individuals with type 2 diabetes, whose response to the OGTT is characterized by a reduction in both first-phase and overall insulin release, depend more on insulin synthesis during the second phase of insulin release. Although other factors like glucotoxicity might lead to β cell dysfunction in type 2 diabetes through reduced insulin gene expression, [28, 29] our data demonstrate that among our individuals with type 2 diabetes there is actually a shift from readily available insulin towards increased *de novo* insulin synthesis. There is increasing data about heterogeneity of β cells [30] and a gradual shift towards expression of specific β cell subpopulations that might contribute to the pathogenesis of type 2 diabetes. Out of four subtypes of human β cell populations one subtype with specific cell surface markers was related with increased impairment of glucose-stimulated insulin secretion in type 2 diabetes[31]. In mouse and human pancreas, the pattern of expression of aging markers in β cells suggests that β cell heterogeneity is based on the life cycle stage of β cells, and an increase of aging markers in β cells was observed during artificial insulin resistance[32]. These studies support the concept of β cell stress and apoptosis. Another mechanism for β cell deficiency in type 2 diabetes is beta cell dedifferentiation. In several animal models it has been demonstrated that hyperglycemic conditions can alter the differentiation status of β cells[33-36] with loss of β cell characteristic traits and/or conversion to other endocrine cells. The amount of dedifferentiated β cells was found to be more prominent in pancreatic islets of humans with type 2 diabetes compared with controls[37]. Further research is required whether a preferential secretion of de novo insulin synthesis in our individuals with type 2 diabetes is to some degree a marker for β cell heterogeneity under OGTT conditions *in vivo*. However, it is tempting to speculate that among individuals with type 2 diabetes, who already have reduced β cell mass secondary to apoptosis and/or dedifferentiation, the *in vivo* higher demand for insulin synthesis might further contribute to β cell heterogeneity, exhaustion and eventually apoptosis, and consequently also be one of the underlying causes of loss of β cell mass. In addition and accompanying increased insulin synthesis, an increased release of insulin-

co-release products like islet amyloid polypeptide, which has cytotoxic effects on β cells[38], as well as ATP, which might increase islet inflammation through activation of islet macrophages[39], could contribute to enhanced β cell deterioration. Our methodology is less suited for studies of insulin-treated type 2 diabetes, therefore our group of individuals with type 2 diabetes may have been in a relatively homogeneous early stage of the disease. Future prospective studies focused on how insulin synthesis is linked to the duration of the disease are required for additional insights in pathogenesis and progression of type 2 diabetes.

The findings among individuals with type 2 diabetes were even more pronounced in T-allele carriers. This increase in insulin synthesis in T-allele carriers who have non-insulin-treated type 2 diabetes might be explained by other factors that studies associated with *TCF7L2* SNPs; these include reduced early phase insulin secretion,[9, 40] reduced exocytosis,[19] impaired proinsulin-to-insulin conversion,[40-42] and decreased β cell mass[18]One may even argue that, because the *TCF7L2* rs7903146 variant is associated with type 2 diabetes, all other factors associated with type 2 diabetes will also be associated with the *TCF7L2* variant. However, although *TCF7L2* rs7903146 indeed has a consistent and relatively strong association with type 2 diabetes, it explains only a negligible fraction of the heritability of type 2 diabetes. One could also comment that defective insulin synthesis is not a cause of type 2 diabetes, but that it is rather just a secondary factor associated with the disease. However, we observed a more intricate relationship based on a significant genetic interaction that confirms the effect of the *TCF7L2* rs7903146 variant on insulin synthesis within type 2 diabetes. Also, in our multiple regression model, first-phase insulin release did not contribute to FSR. Nevertheless, *TCF7L2* is known to influence the expression of multiple genes in different pathways[17, 43] and our findings do not rule out this pleiotropy.

Our finding that South Asian *TCF7L2* T-allele carriers without type 2 diabetes had lower insulin synthesis than their wild-type counterparts was not unexpected. An inability to increase insulin secretion to compensate for insulin resistance has been previously demonstrated among healthy T-allele risk carriers in whom insulin resistance was artificially induced.[9] Also, in a recently published multi-ethnic cohort study comprising of obese adolescents, the *TCF7L2* rs7903146 was related to impaired β cell function and led to an increased risk of progression from prediabetes to type 2 diabetes[16]. As insulin resistance is a key characteristic of our South Asian population, this might explain the differences between T-allele risk carriers and wild-type that were apparent in South Asians without type 2 diabetes, while such differences were not seen in Caucasians without type 2 diabetes. This reduction in insulin synthesis might contribute to their risk of type 2 diabetes. Strikingly, the average age of onset of type

2 diabetes was 12 years earlier in our South Asian population than in the Caucasians. This does tie in with the fact that the prevalence of type 2 diabetes is known to be nearly fivefold higher among South Asians than among indigenous Dutch.[44, 45] Previously, we have reported homogenous insulin-resistant conditions in South Asian families regardless of their glucose tolerance,[21] and their insulin resistance might also augment the harmful effects of β cell-related gene variants other than those of *TCF7L2*. However, the increase in insulin synthesis that we observed in individuals with type 2 diabetes during the OGTT in this study indicates an opposite effect under hyperglycemic conditions, underlining the likelihood that glucose levels also influence the *TCF7L2* variant effect. This previously unrecognized interaction warrants future research into the influence of a glucose stimulus on multifunctional aspects of β cell pathways, as we cannot exclude the possibility that for other genotypes the β cell phenotype will also depend on whether or not the person has type 2 diabetes. Future studies with individuals with type 2 diabetes versus controls with artificially increased glucose levels are required to provide more insights in this interaction.

In terms of technical problems, a relatively large number of subjects had very low basal C-peptide concentrations in combination with relatively low basal volumes of urine and consequently we were unable to harvest enough amounts of absolute C-peptide for enrichment measurements. Although subjects were asked to empty their bladders at baseline, they might have already emptied their bladders at home prior to the start of the OGTT. Despite these technical problems, basal urine C-peptide enrichment measurements provided stable values for all subgroups with a small variance and corresponds with the calculated theoretical natural isotope ratio in leucine. Although we had expected difficulties with the urinary C-peptide enrichment measurements for individuals with type 2 diabetes due to protein contamination, such problems did not arise, probably because these patients had no history of diabetic nephropathy and they were not (yet) taking insulin. Our C-peptide antibodies used for the IAC procedure for C-peptide purification have cross-reactivity with insulin precursor proinsulin. However, the excretion of proinsulin in urine is negligible compared to C-peptide, with daily urinary excretion of 0.05% versus 5-10% of pancreatic secretion, respectively[46]. Subsequently, during the process towards actual enrichment measurements of urinary C-peptide, we did not find evidence that proinsulin or other insulin precursors played a significant role in affecting these measurements. These and other technical issues have been described previously[20]. Interestingly, because of the cross-reactivity trait of the antibodies used, future research focused on a similar purification methodology in plasma could provide us with in-depth real-time analyses of rs7903146 effects on proinsulin processing.

This is the first time that stable isotope-based tracer technique has been used to measure insulin synthesis and secretion in individuals with glucose intolerance, and the first time such an approach has been applied to analyze the pathogenesis of type 2 diabetes. Our test was able to find differences between individuals with and without type 2 diabetes as well as between *TCF7L2* T-allele carriers and C-allele homozygotes. Based on our enrichment data, late phase hyperinsulinemia observed in early stages of type 2 diabetes not only reflects a decrease of insulin sensitivity and insulin clearance[47], but also points at abnormal β cell function with a change in dynamics of insulin secretion in the context of a programmed increase of insulin synthesis. Moreover, this altered β cell function with increased insulin synthesis itself might be a significant contributor for sustaining insulin resistance[48]. In particular, it might partially explain why lifestyle intervention in individuals with type 2 diabetes for the overwhelming majority does not lead to partial or complete remission of their disease[49]. Future research focused on further assessment of the pathophysiology of this increased insulin synthesis state may provide opportunities for further development of specific therapies to decrease the demand for insulin synthesis.

In conclusion, using a novel stable isotope-based technique to follow *de novo* insulin synthesis *in vivo*, we have found that the *TCF7L2* rs7903146 gene variant provides a link between type 2 diabetes and variations in the levels of newly synthesized insulin. Our findings suggest that the glucose-sensitive *TCF7L2* pathway is a potential target for interventions that prevent type 2 diabetes.

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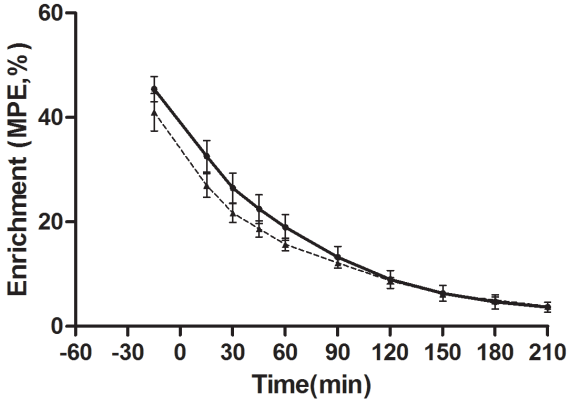
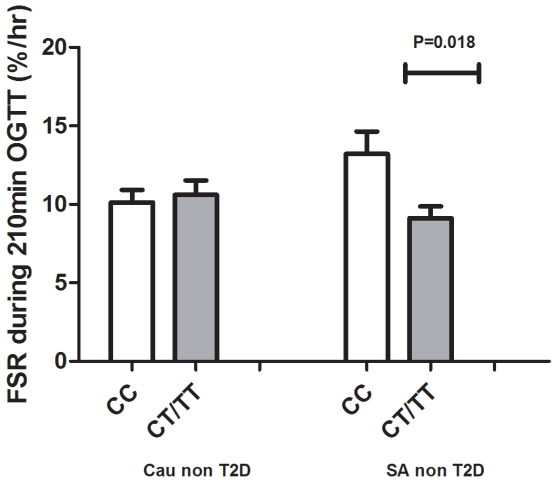
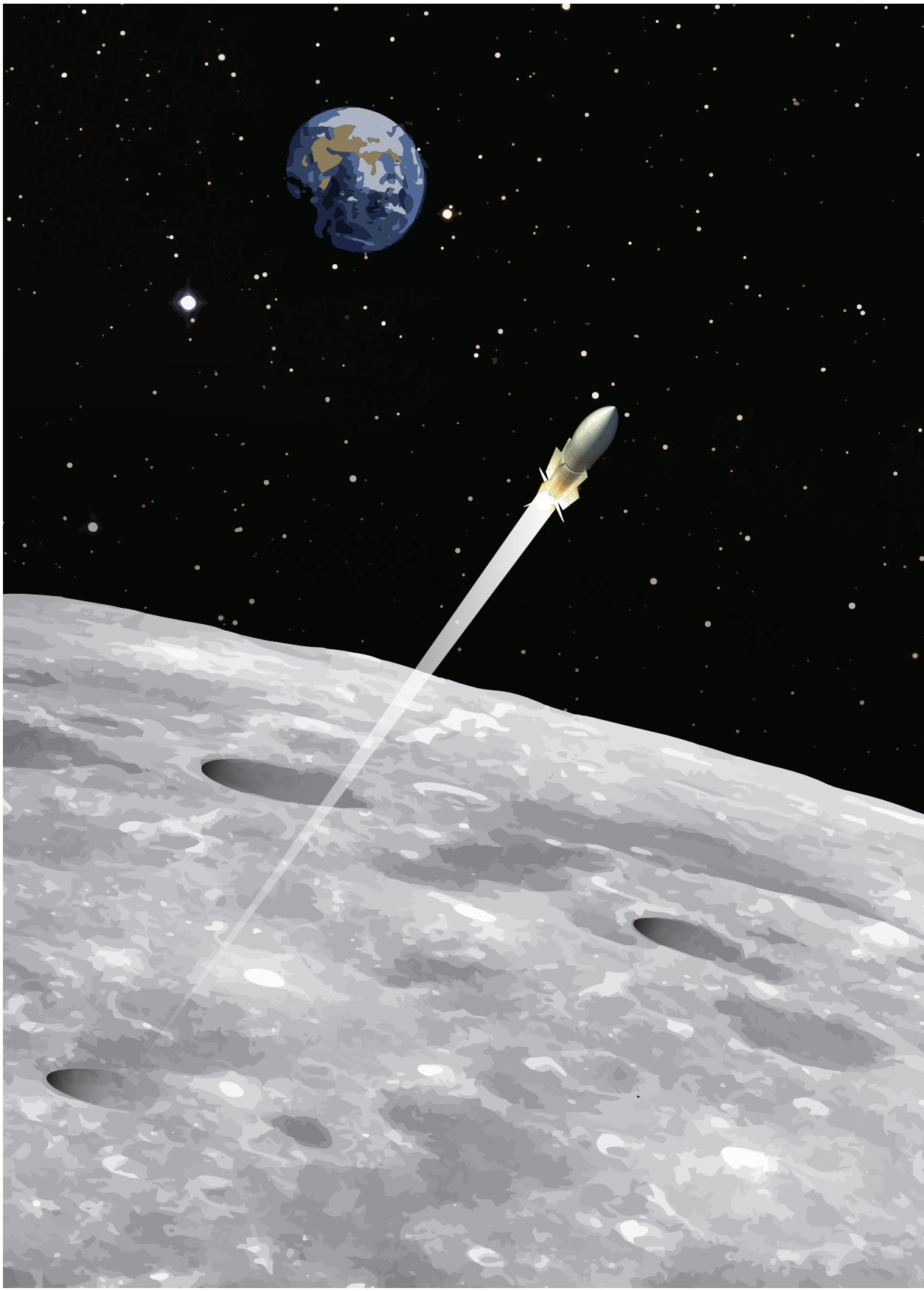
SUPPLEMENTARY MATERIAL

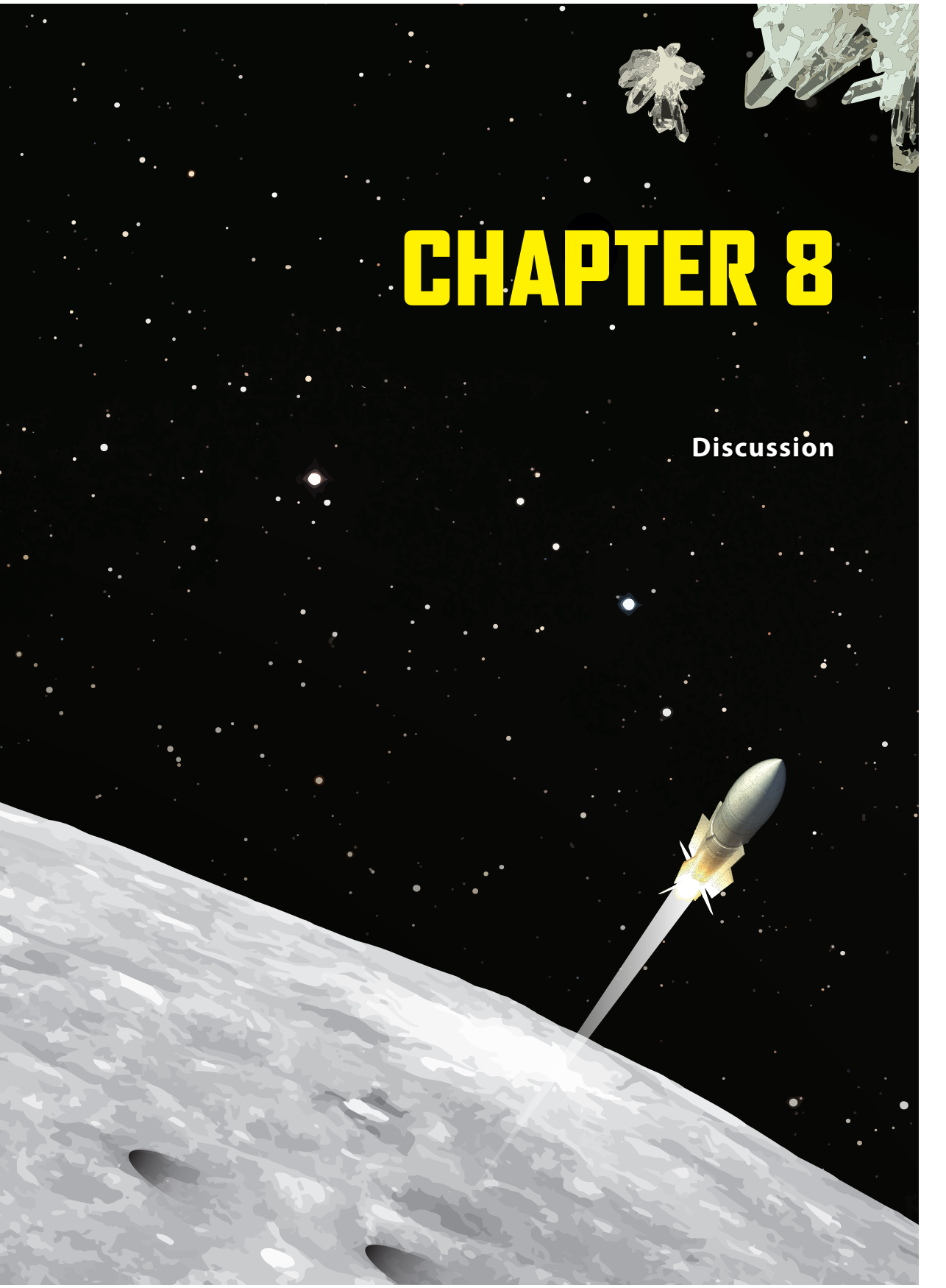


Supplemental Figure 1 | Curves of plasma ^{13}C alpha-ketoisocaproate indicating the metabolization rate of the ^{13}C leucine tracer. At t=-45 min the tracer ^{13}C leucine was applied and at t=0 min the oral glucose bolus was administered. The ^{13}C enrichment of alpha-ketoisocaproate (in mole percent excess, MPE, mean±SEM) was similar in individuals without type 2 diabetes (n=7; continuous line) and with type 2 diabetes (n=6; dashed line) during our Oral Glucose Tolerance Test. Hence, the metabolization rate of our tracer ^{13}C leucine was similar in both groups during our test.



Supplemental figure 2 | Insulin synthesis in the non-type 2 diabetes (non-T2D) subgroup according to ethnicity. Fractional synthesis rate (FSR, %/hr) during 210 min oral glucose tolerance test (OGTT) among Caucasian (Cau) or South Asian (SA) non-T2D subgroups, based on *TCF7L2* RS7903146 CC wild-type genotype or CT/TT carriership; data are in mean±SEM; P=0.018, according to Student's unpaired t test.





CHAPTER 8

Discussion

DISCUSSION

In the current chapter, I discuss the implications of our findings, and propose a pathophysiological model with a role for the increased insulin synthesis state in type 2 diabetes (T2D) pathogenesis. Hereafter, I present an overview of research opportunities that our method provides, with technical considerations.

Implications of our findings in general

Failure of the insulin secreting pancreatic beta cells is essential in T2D pathogenesis[1]. Multiple steps are involved in insulin biosynthesis and secretion, including glucose sensing, synthesis, storage and release of insulin, which all may contribute to the development of T2D. These individual steps are difficult to assess *in vivo*. Tests that can detect qualitative and quantitative changes in beta cell function could be used to further unravel the underlying mechanisms and perhaps identify distinct phenotypes of T2D. In addition, detailed testing of beta cell function is likely to be of prognostic significance, as the available indices for beta cell function have been shown to be superior to fasting and 2 hour glucose levels in predicting the onset of T2D during a ten year follow-up period[2]. Previously, the fate of *in vivo* insulin synthesis was unknown [3]. We found that improved beta cell phenotyping *in vivo* with a dynamic stable isotope labeling procedure provides crucial information about T2D pathogenesis. In the T2D high-risk families, genetically increased insulin synthesis contributes to T2D, implying that the delayed hyperinsulinemia is not only an adaptation to insulin resistance but also a direct cause of T2D as part of an intrinsic beta cell defect. This "final launch" of newly synthesized insulin provides an essential clue in the cascade of beta cell exhaustion and deterioration, with the implications of our most characteristic findings illustrated in figure 1.

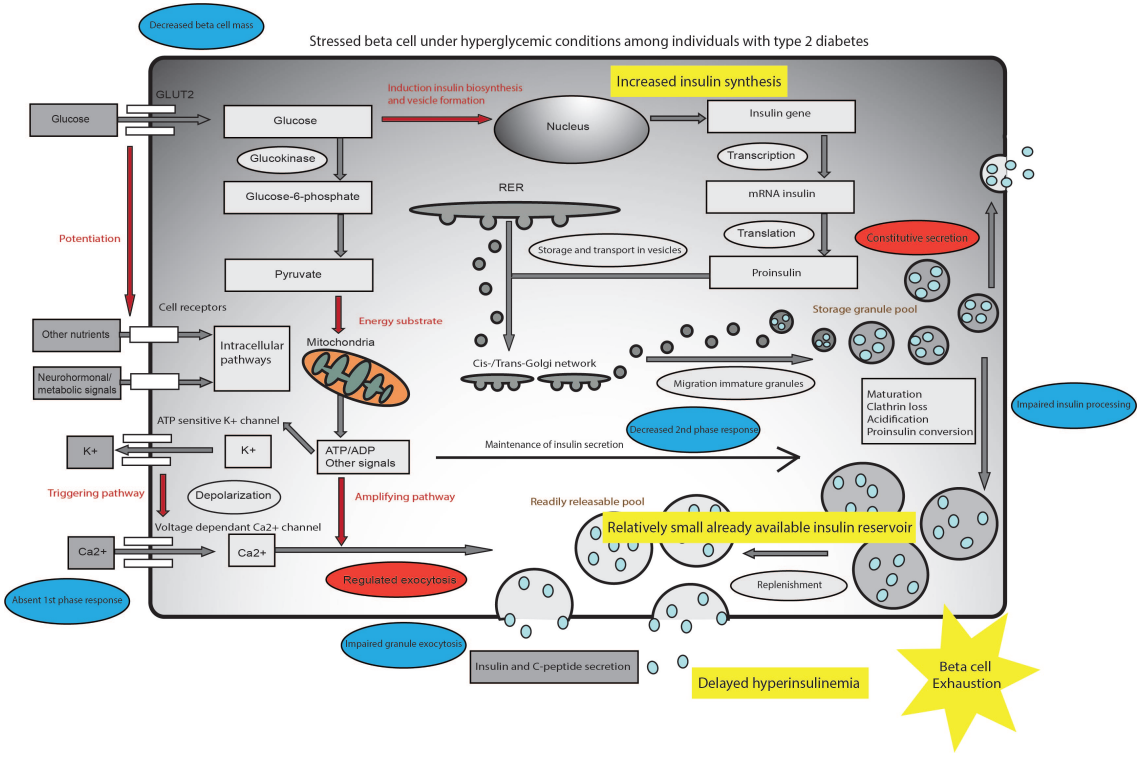


Figure 1 | Schematic overview of beta cell pathogenesis in T2D based on *in vivo* insulin synthesis measurements. Schematic overview of key intracellular mechanisms induced by glucose in pancreatic beta cells. Insulin is secreted predominantly through regulated exocytosis. Under conditions of enduring high glucose concentration, for instance during an oral glucose tolerance test, a rapid first phase insulin release from a ready releasable pool of granules is followed by a more sustained insulin release through release from a storage granule pool. De novo synthesis of (pro-) insulin replenishes the storage granule pool, and is eventually also secreted. Under hyperglycemic conditions among individuals with type 2 diabetes, there is an abnormal delayed insulin response due to a stressed beta cell with an overall decreased reservoir of already made and available insulin due to a reduced early and second phase insulin secretion, reduced exocytosis, impaired proinsulin-to-insulin conversion and decreased beta cell mass (blue spheres). The effects mentioned in the blue spheres are increased in carriers of the T risk allele of the rs7903146 in the *TCF7L2* gene with T2D. A decrease in the already available insulin pool and a higher dependency on insulin synthesis contribute to a delayed hyperinsulinemia which might accelerate beta cell exhaustion and finally apoptosis.

Our findings fundamentally changed the way how OGTT curves can be interpreted, as they add a novel distinction between "new" and "old" insulin. From a beta cell perspective, a more delayed insulin response observed in individuals with T2D compared to individuals without T2D as illustrated in figure 1b of chapter 8, or in carriers of the T risk allele of the rs7903146 in the *TCF7L2* gene with T2D versus wildtype as illustrated in figure 2b of chapter 8, respectively reflects more dependency on newly synthesized insulin. These observations further underline what was stated in chapter 8, that hyperinsulinemia might be a part of an intrinsic beta cell defect, and actually contribute to insulin resistance, as there is evidence that hyperinsulinemia itself impairs insulin signaling directly or leads to insulin resistance through other pathways in peripheral tissues[4].

Technical considerations and future perspective

Regarding the tracer ^{13}C leucine, the oral bolus dose method instead of the constant infusion method was chosen in our clinical setting, as the procedure has a shorter time requirement with minimal concern about precursor enrichment, gives comparable intra- and extracellular enrichments eliminating uncertainty about precursor enrichment, is non-invasive which means less manipulation of the subjects, and with relatively low dosage and early timing of administration of the tracer, we did not find a metabolic effect on plasma insulin and/or C-peptide concentrations (supplemental figure 2a of chapter 6). As the constant infusion method requires a steady-state condition, with the bolus dose method we obtained a relatively faster precursor enrichment, this is crucial as our target peptide is part of an immediate secretory response during a dynamic beta cell stimulus test. One of the main concerns that we had, based on the findings of previous literature and our results described in chapter 3, was whether or not a decreased catabolism of branched chain amino acids observed in individuals with T2D (figure 2), could lead to differences in the rate of the metabolization rate of ^{13}C leucine between individuals with and individuals without T2D. In chapter 7 we did not find differences in this rate (supplemental figure 1 of chapter 7).

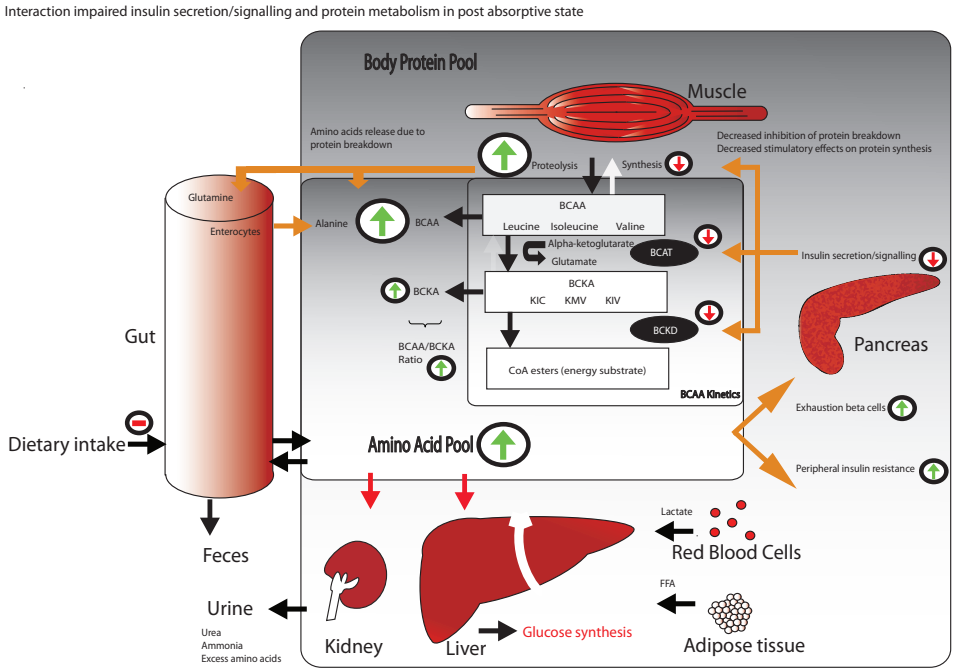


Figure 2 | Vicious circle in glucose homeostasis in type 2 diabetes (T2D) through amino acid turnover; alterations in several key factors involved in catabolism and/or anabolism may lead to rising branched-chain amino acid (BCAA) concentrations, and subsequently worsen glucose homeostasis. Although there are differences in BCAA catabolism between individuals with and without T2D, the metabolization rate of our tracer ^{13}C leucine was similar in both groups.

Regarding the target peptide, a more detailed exploration of insulin synthesis could be possible by altering our current method by measuring enrichment of urinary C-peptide over multiple collected urine voids with/without the use of an urine catheter, and/or by measuring enrichment levels of insulin/C-peptide over multiple (or pooled) time-points in plasma. For plasma however, we found that our current isolation procedure is not suitable as harvesting of the target peptide still requires improvement (data not shown) and further reduction of background noise to perform actual enrichment measurements is required. As future improvements, we could increase our target peptide enrichment by increasing the orally administered ^{13}C leucine dose, however an accompanying increase in metabolic effects due to the tracer itself is warranted. Alternatively, we could also maintain the current dose but use 6 ^{13}C atom labeled ^{13}C leucine instead of the present single ^{13}C. Also, cross reactivity of the antibodies used in our immunoaffinity chromatography (IAC) step might play a more significant role in plasma (figure 3).

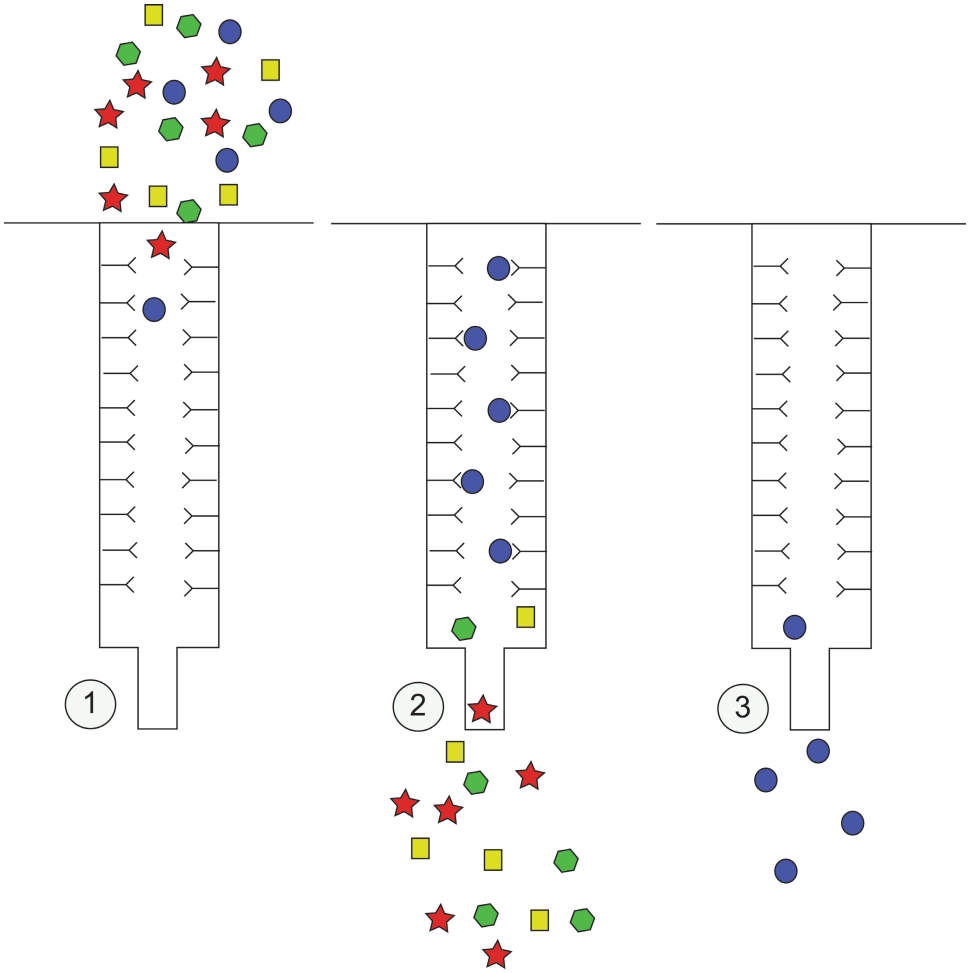


Figure 3 | Schematic overview of immunoaffinity chromatography (IAC): 1) sample goes through IAC column 2) Binding of specific peptides in column and loss of contamination 3) elution of isolated target peptide. Cross reactivity with other insulin related metabolites with the antibodies used might provide unique opportunities.

Genetic, epigenetic and posttranslational defects could lead to abnormal insulin and C-peptide variants with antagonistic properties, which could contribute to insulin resistance and T2D development. The antagonistic properties are believed to be more prominent when there are changes to the alpha chain of insulin, as it is the most bioactive part in *in vitro* studies[5].The variants include abnormal metabolites through amino acid alterations, impaired insulin precursor processing ((pre)proinsulin), truncation, glycation and protein complex formation of insulin[6-12]. These variants

might also contribute to discrepancies observed between quantification methods based on antibody assays. In contrast, mass spectrometry (MS) is considered to be a reference standard for quantification, and when compared to immunoassay measurements (IA) in urine and plasma, generally demonstrates lower insulin and C-peptide concentrations[13-19]. We also had a similar outcome in an exploratory study, as we compared plasma insulin concentrations between IA (Immunolite 2000 produced by EURO/DBC®) versus MS (Triple-Quad LC-MS from Agilent Technologies®; bovin insulin was used as internal standard), from 40 human plasma heparin samples that were obtained from healthy and diabetic males and females from our T2D high-risk families included in our study as mentioned in chapter 2 and consisted out of samples at fasting state and a random selection of non-fasting samples. There was a significant linear relationship between both types of measurements (fig 4a; r=0.90, p<0,0001). Although the difference between measurements using these two methods were within the limits of agreement (fig 4b), Bland-Altman analysis predominantly showed a non-concentration dependent positive residual between IA insulin versus MS insulin (fig 4b and 4c), and this was more prominent among individuals with T2D.

This difference suggests the presence of abnormal variants, and MS studies have indeed demonstrated several abnormal insulin and C-peptide variants, increasing with an abnormal glucose state[9, 10, 20]. It would be interesting to investigate how an increased insulin synthesis relates to insulin processing *in vivo*, as proinsulin misfolding and aggregation could contribute to beta cell failure due to increased endoplasmatic reticulum stress, a decreased conversion to insulin which in turn might lead to a vicious cycle with an even more increased demand for insulin synthesis[21]. We can follow this processing in more detail and real-time with our stable isotope method, as the C-peptide antibody used in our IAC procedure for urinary C-peptide isolation also has cross reactivity with insulin related metabolites, including proinsulin. In contrast to urine, insulin precursors in plasma are present in high abundance and the dynamics of proinsulin processing could be followed in more detail, with separate fractional synthesis rate (FSR) measurements for both C -peptide and proinsulin (figure 5). By comparing the enrichment we could confirm whether or not “old” proinsulin is secreted from an already available pool of granules, or that it is actually actively newly synthesized but wrongly processed and intact proinsulin released during OGTT.

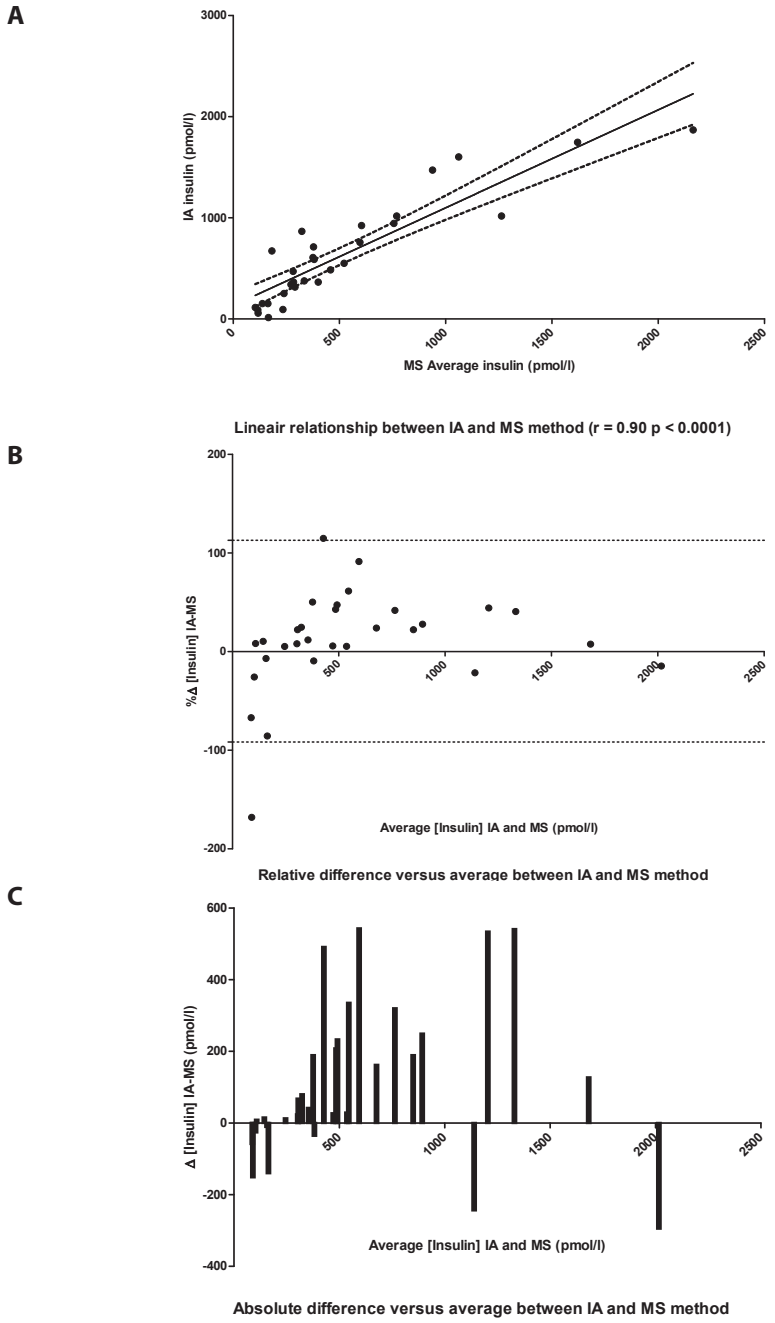


Figure 4 | (A) Relationship between immunoassay (IA) and mass spectrometry (MS) insulin measurements(r=0,90, p<0,0001). **(B)** and **(C)**: Relative and absolute Bland-Altman analysis between IA and MS measurements, respectively.

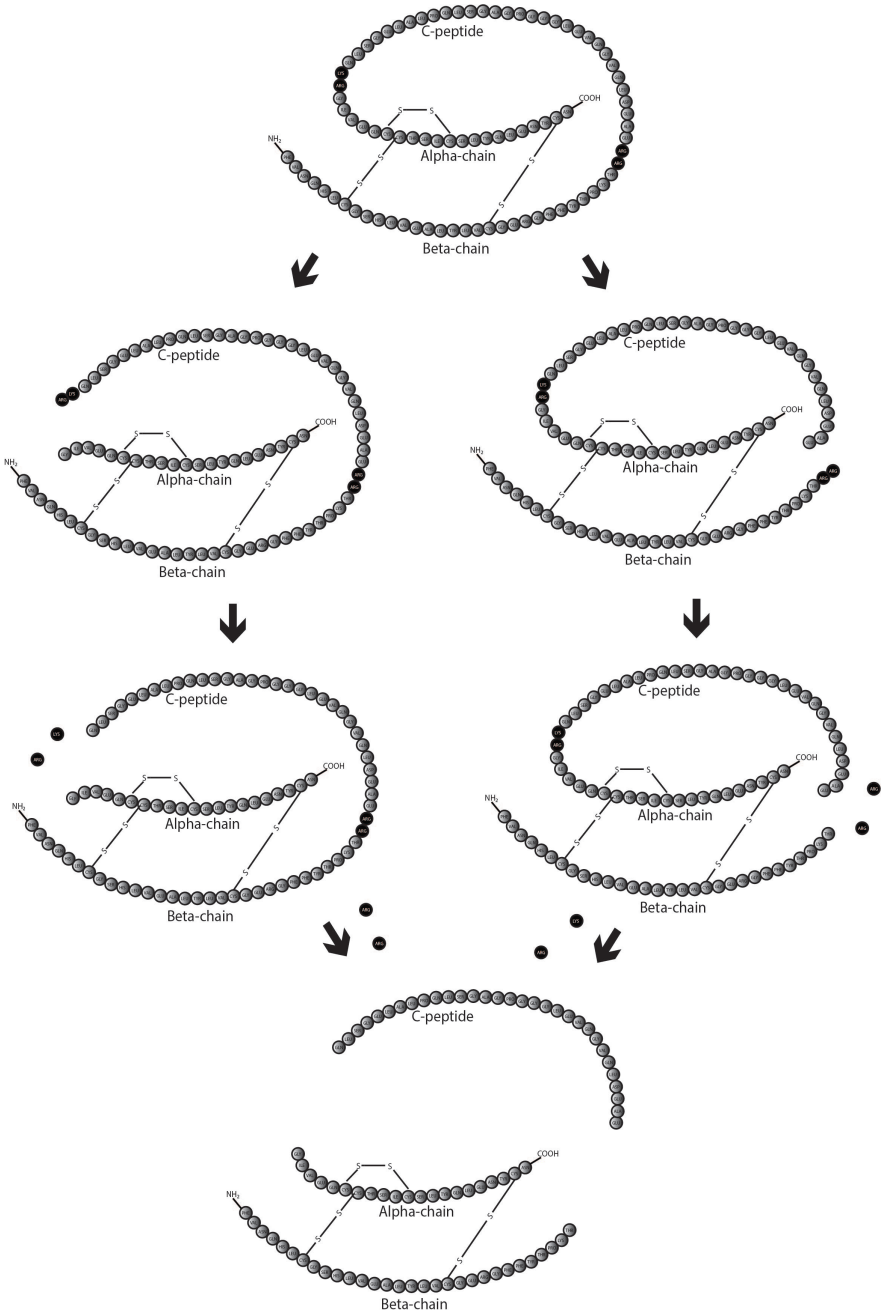


Figure 5 | Proinsulin processing towards insulin and C-peptide after cleavage by prohormone convertase enzymes. All precursors of insulin and C-peptide could be released into plasma. With our stable isotope method we used C-peptide as target peptide, however the enrichment in precursors could be measured as well.

In addition to a more accurate interpretation of insulin secretion after glucose load, our stable isotope method has potential for integration in other stress tests and/or studies of (simultaneously) measuring the synthesis of other peptide hormones. We used an oral glucose tolerance test (OGTT), one of the most standardized tests, which incorporates our physiological enteral response[22], other target peptides for enrichment measurements within OGTT could be the incretin hormones and/or other beta cell secretory proteins such as amylin.

As our stable isotope method provides real-time measurements of insulin synthesis, it could be integrated in existing models, most notably in the oral minimal model[23]: we used the C-peptide minimal model indices based on our OGTT in several studies, and could also add more detail to the model itself (figure 6a). The secretion rate is divided in a dynamic compartment (directly releasable insulin: SR_d) and a static compartment (replenishment insulin: SR_s). Based on our data, the SR_s could actually be divided in two secretion rates, one for already made replenishment insulin and one for newly synthesized replenishment insulin, with their own secretion time, for the latter based on when the novo insulin synthesis actually is secreted. This new SRs beta cell parameter could be multiplied with the insulin sensitivity index derived from the Glucose minimal model (figure 6b), to obtain a disposition index, to correct this beta cell function parameter for insulin resistance. Further research would be required with more detailed FSR analyses preferably in plasma before evaluating the additional value of these beta cell function parameters. Also, the concept of insulin resistance can be investigated further from a beta cell perspective. We primarily used IAC to isolate our target peptide in urine, but we could also use this procedure with insulin antibodies for plasma and specifically isolate and test the capability of our isolated products on insulin signaling *in vitro* by adding the isolates from human plasma from different moments in time during OGTT to a cell model (figure 6b).

One final aspect that has not been addressed is how the stable isotope measurements might relate to beta cell mass. Estimating beta cell mass separate from function *in vivo* is essential, as it gives insights if drugs or other interventions not only enhance beta cell function but also preserve mass, leading to measures for preventing apoptosis and/or reverse dedifferentiation. Similar to factually all pancreas stress tests, the insulin response during an OGTT is a result of both beta cell function and mass. *In vivo* imaging techniques are currently developed with an optimal imaging probe to specifically quantify beta cell mass, including positron emission tomography, single-photon emission computed tomography and/or magnetic resonance imaging[25]. Hopefully, these novel imaging methods will enable to visualize changes of the beta cells during life. As mentioned in chapter 7, insulin synthesis could reflect beta cell

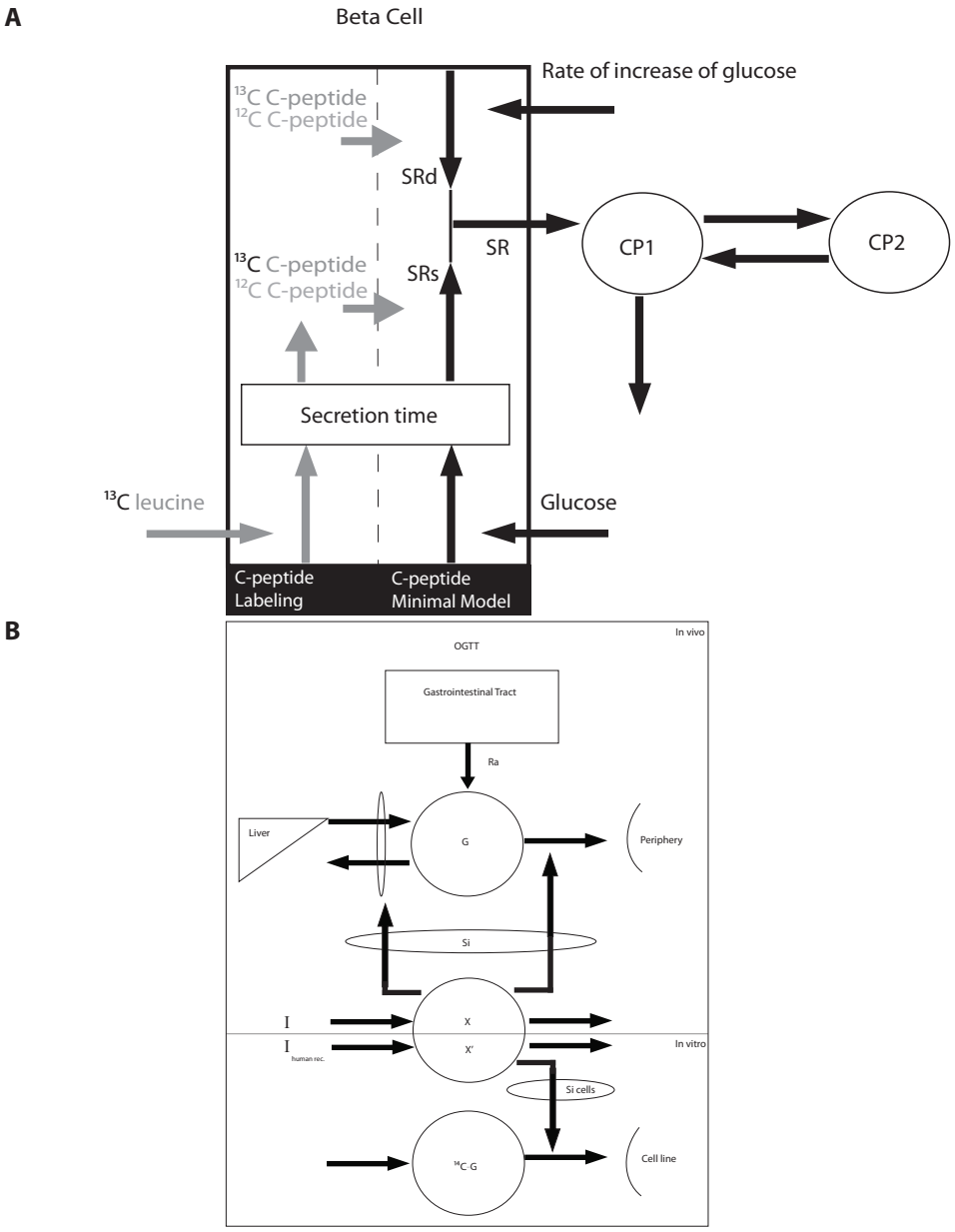


Figure 6 | (A) Modified version of C-peptide minimal model with integration of our novel beta cell phenotype. **(B)** The Glucose minimal model, with below addition of an in vitro variant with a human peripheral tissue cell line for assessment of insulin sensitivity with recombinant insulin. The latter could also be performed from the perspective of beta cell function by adding endogenous insulin and related precursors after isolation with immunoaffinity chromatography to a cell model to test the efficiency for insulin signaling in this cell model. Figure 6 a and b have been adapted and altered from figure 2 of ref [24] and modified with permission from the publisher.

heterogeneity with a specific population or populations of beta cells responsible for de novo synthesis, or a coordinated secretory response to glucose of the whole spectrum of beta cells in different degrees. Combining future imaging techniques with in-depth beta cell profiling could give crucial insights about the contributions to T2D of beta cell function and mass, and whether insulin synthesis also reflects beta cell mass or purely elasticity. As a surrogate measure, we could also relate our insulin synthesis measurements with the current best estimate of beta cell mass, by performing a test investigating the glucose potentiation of the first-phase insulin response induced by arginine in the same study group.

In summary, we performed extensive in-depth profiling of beta cell function in T2D high-risk families, introducing a novel stable isotope method to follow insulin synthesis *in vivo*. We believe our findings will give rise to new concepts about T2D, its prevention and treatment. Of course, these routes will be long and in that respect the findings are preliminary, but this work will certainly change our thinking about hyperinsulinemia and T2D pathogenesis.

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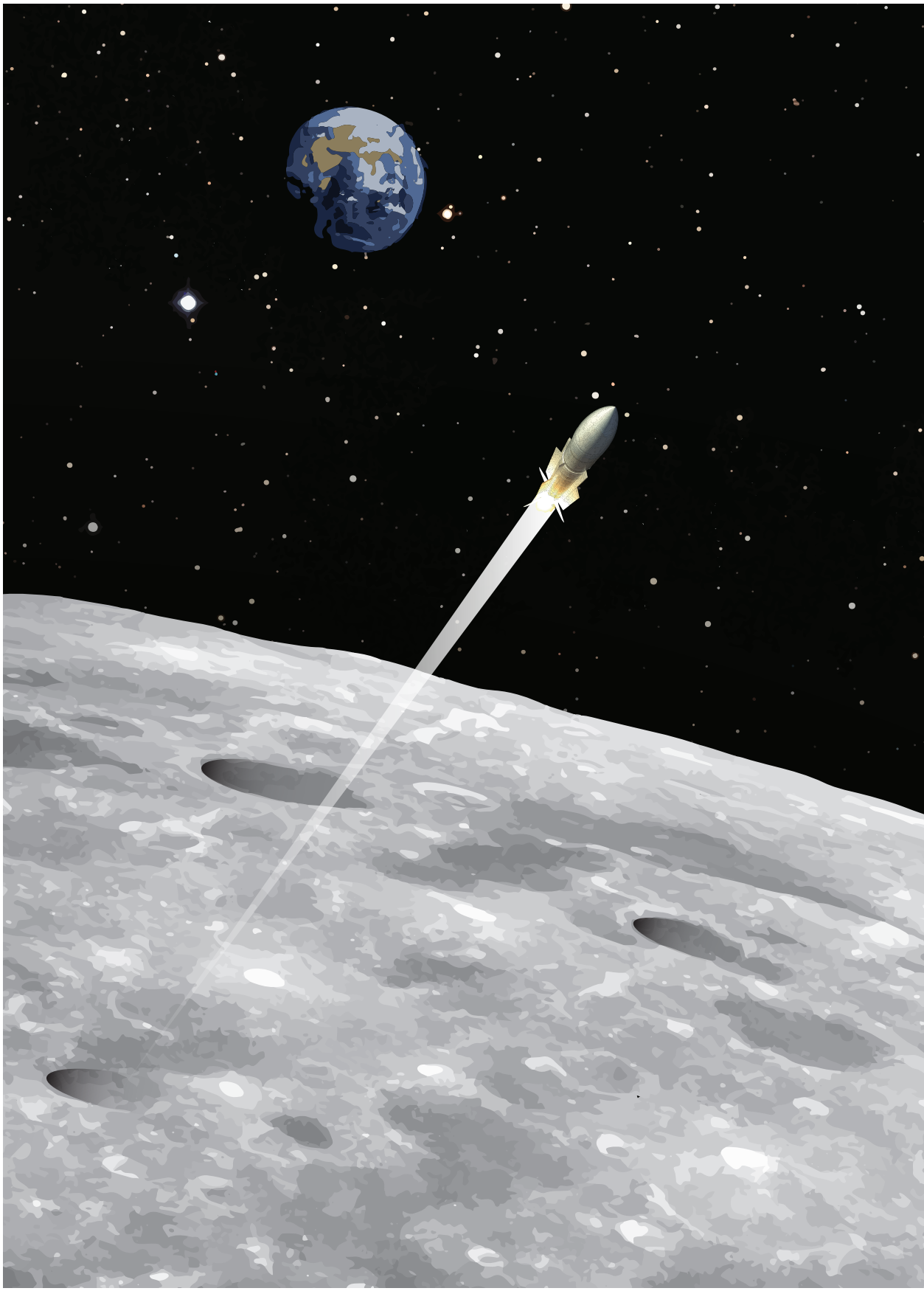

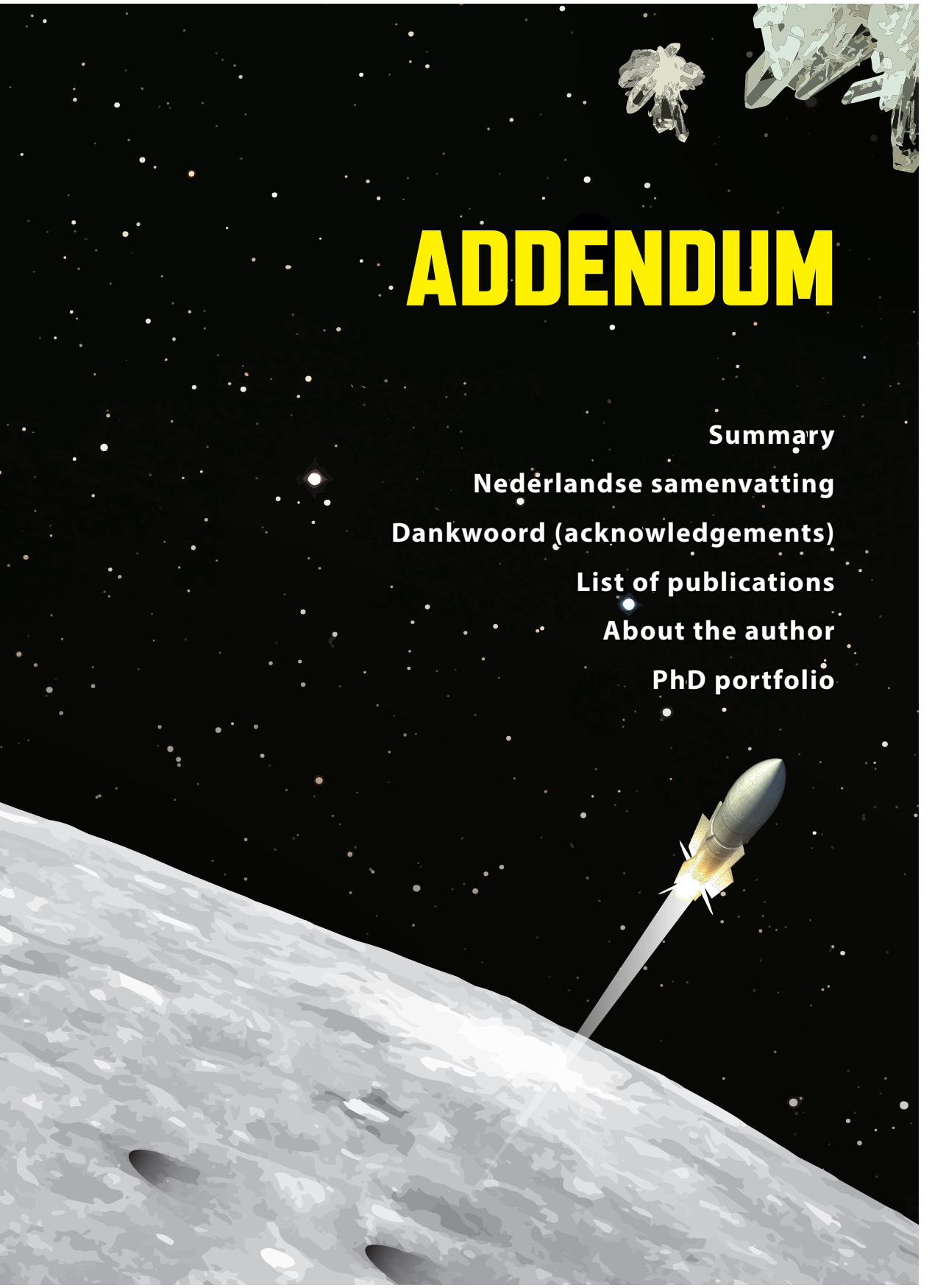

ADDENDUM

SUMMARY

In chapter 1 the normal physiology of insulin biosynthesis is described including the biphasic pattern of insulin secretion from the perspective of the storage-limited model and its regulatory mechanisms, beta cell pathogenesis and current available tests to characterize beta cell function and mass. In addition, I explained the value of stable isotope techniques to an existing standardized model for measurement of newly synthesized insulin as part of an in-depth analysis of beta cell function *in vivo*.

In chapter 2 the relationship between early and late beta cell response and type 2 diabetes (T2D) in South Asian and Caucasian individuals from T2D high-risk families was investigated. Based on classical oral glucose tolerance test (OGTT) indices, South Asians on average had lower insulin sensitivity (ISI) with rapid decline of their early and late beta cell response. Adjusted early insulin secretion rate (ISR) was significantly associated with both glucose disposal and with late ISR in South Asians, but not in Caucasians, with significant interaction between ethnicity and early ISR. Ordinal regression analyses confirmed that glucose tolerance state among South Asians was solely predicted by early ISR and not by ISI as insulin resistance was a homogenous feature, while in Caucasian families both ISI and early ISR were related to glucose tolerance state. In conclusion, in South Asian individuals, rapid beta cell deterioration might occur under insulin resistant conditions, and alterations in beta cell dynamics may give an explanation to their extreme early onset of T2D.

In chapter 3 metabolic syndrome traits were characterized among South Asian and Caucasian individuals from T2D high-risk families. Individuals from both ethnicities were classified in three groups according to the occurrence of metabolic syndrome (MetS) and T2D. ISI and disposition indices (dynamic and static DIs) for beta cell function were calculated with the use of oral minimal model (OMM). Similarly to my findings in chapter 2, South Asians without MetS were more insulin resistant than Caucasians without MetS. In the South Asians, ISI, dynamic DI, and static DI were associated significantly with plasma high-density lipoprotein cholesterol and triglycerides. In the Caucasians, ISI was associated with waist-to-hip ratio and systolic and diastolic blood pressure, while static DI was related to the systolic blood pressure. These findings suggest that there are ethnic differences with distinct metabolic syndrome phenotypes.

In chapter 4 these metabolic syndrome phenotypes were further addressed in the same families with amino acid profiling, as insulin resistance and glucose intolerance have been associated with increased plasma levels of branched-chain amino acids (BCAA). BCAA levels were measured in fasting plasma together with alanine, phenylalanine,

and tyrosine. Insulin sensitivity and beta cell function were assessed with classical OGTT indices. BCAA levels were higher in diabetic than in normoglycemic family members in the Caucasians but not in the South Asians. In both groups in stepwise regression analyses, BCAA levels were associated with waist-hip ratio but not with indices of insulin sensitivity or beta cell function. Adding fasting plasma BCAA levels, combined with phenylalanine, tyrosine and alanine to fasting glucose improved discriminative ability for the prediabetic state within Asian Indian families at risk for T2DM. BCAA levels may serve as biomarkers for early development of glucose intolerance in these families.

In chapter 5, I investigated how renal loss of both C-peptide and glucose during OGTT relate to and affect plasma-derived OMM indices. In South Asian and Caucasian individuals from T2D high-risk families, plasma glucose, insulin, and C-peptide concentrations were analyzed as area under curves (AUCs) during OGTT, OMM estimate of renal C-peptide secretion, and OMM beta cell and insulin sensitivity indices were calculated to obtain disposition indices. Urinary glucose corresponded well with plasma glucose AUC, beta cell and insulin sensitivity analyses. Urinary C-peptide corresponded well to plasma C-peptide AUC and OMM estimate of renal C-peptide secretion. Renal glucose secretion during OGTT did not influence OMM indices in any subgroup based on glucose tolerance state. C-indices of urinary glucose to detect various stages of glucose intolerance were excellent. The limited role of renal glucose secretion validates the neglecting of urinary glucose secretion in kinetic models of glucose homeostasis using plasma glucose concentrations. Both C-peptide and glucose in urine collected during OGTT might be used as non-invasive measures for endogenous insulin secretion and glucose tolerance state.

In chapter 6 I introduce my *in vivo* stable isotope based method to determine the ratio between readily available and newly synthesized insulin after a beta cell stimulus in humans by labelling newly synthesized insulin. Stable isotope tracer ^{13}C leucine with urinary C-peptide as target peptide was administered prior to an extended OGTT. I also collected urine, which has a high content of C-peptide, at basal level and during OGTT. I established optimal conditions under which the tracer was administered for enrichment purposes and describe the techniques to isolate C-peptide from urine under highly purified circumstances. Among healthy Caucasian individuals with normal glucose tolerance, I found that newly synthesized insulin is present *in vivo* and contributed approximately 20 % of total insulin secretion. The pattern of isotope enrichment obtained by collecting multiple urine voids was suggestive that *de novo* insulin contributes to the late phase of insulin secretion. In conclusion, it is possible to measure newly synthesized insulin in healthy individuals.

In chapter 7 my stable isotope based method was used as an in-depth beta cell assessment to understand the role of transcription factor 7-like 2 (*TCF7L2*), the main susceptibility gene for type 2 diabetes, in beta cell pathogenesis. I performed a study in T2D high-risk families and determined if the T risk allele of the rs7903146 in the *TCF7L2* gene increases the risk of type 2 diabetes based on real-time stable isotope measurements of insulin synthesis. In addition, OMM analyses were performed to assess insulin sensitivity and beta cell function indices. Compared to unaffected relatives, individuals with type 2 diabetes had lower OMM indices and a higher level of insulin synthesis. I found a T allele-dosage effect on insulin synthesis and on glucose tolerance status, therefore insulin synthesis was higher among T-allele carriers with type 2 diabetes than in wild-type individuals. These results suggest that hyperinsulinemia is not only an adaptation to insulin resistance, but also a direct cause of type 2 diabetes.

In chapter 8, I propose a pathophysiological model for T2D pathogenesis which includes my insulin synthesis findings. In addition, I described how my insulin synthesis findings have implications for insulin curve interpretation after stimulus in general, but also for the current concepts of hyperinsulinemia. Finally, suggestions for further research options are made.

NEDERLANDSE SAMENVATTING

In hoofdstuk 1 wordt de normale fysiologie van insuline biosynthese beschreven inclusief het bifasische patroon van insuline secretie vanuit het perspectief van het storage-limited model, de betrokken regulatoire mechanismen, bètacel pathogenese en beschikbare testen om bètacel functie en massa te karakteriseren. Voorts heb ik de toegevoegde waarde van stabiele isotoop technieken aan een bestaande gestandaardiseerde model besproken, om zo nieuw gesynthetiseerde insuline te meten als onderdeel van een diepgaande fenotypering van de bètacellen *in vivo*.

In hoofdstuk 2 wordt de relatie tussen vroege en late bètacel respons en diabetes mellitus type 2 (DM type 2) in Zuid-Aziatische en Kaukasische individuen afkomstig uit families met hoog risico voor DM type 2 onderzocht. Op basis van klassieke indices welke gebaseerd zijn op de orale glucose tolerantie test (OGTT), hadden Zuid-Aziaten gemiddeld een lagere insuline gevoeligheid (ISI) met snellere achteruitgang van de vroege en late bètacel respons. De gecorrigeerde vroege insulin secretion rate (ISR) was significant geassocieerd met zowel de glucose klaring als de late ISR bij de Zuid-Aziaten, maar niet bij de Kaukasiërs, met een significante interactie tussen etniciteit en vroege ISR. Ordinale regressie analyses bevestigden dat de glucose tolerantie status bij de Zuid-Aziatische families alleen werd voorspeld door de vroege ISR en niet door ISI, daar de ISI een homogene kenmerk was voor deze groep, terwijl in Kaukasische families zowel ISI als vroege ISR gerelateerd waren aan de glucose tolerantie status. Concluderend kan in insuline resistente Zuid-Aziatische individuen een snellere verval van bètacellen plaatsvinden, en veranderingen in de bètacel dynamiek kunnen een verklaring geven voor het voor hen kenmerkende extreem vroeg optreden van DM type 2.

In hoofdstuk 3 werden kenmerken van het metabool syndroom van Zuid-Aziatische en Kaukasische individuen gekarakteriseerd. Individuen van beide etniciteiten werden ingedeeld in drie groepen op basis van het voorkomen van metabool syndroom (MetS) en T2D. ISI en disposition indices (dynamische en statische DI's) voor bètacel functie werden berekend met behulp van de oral minimal model (OMM). Net als de bevindingen in hoofdstuk 2 waren Zuid-Aziaten zonder MetS meer insulineresistent dan Kaukasiërs zonder MetS. In de Zuid-Aziaten waren ISI, dynamische DI en statische DI significant geassocieerd met het high-density lipoproteïne cholesterol en triglyceriden in plasma. In de Kaukasiërs was ISI geassocieerd met de waist-to-hip ratio en de systolische en diastolische bloeddruk, terwijl statische DI gerelateerd was aan de systolische bloeddruk. Dit suggereert dat er etnische verschillen zijn met verschillende fenotypes van het metabool syndroom.

In hoofdstuk 4 werden deze fenotypes van het metabool syndroom verder onderzocht in dezelfde families middels aminozuur profilering, omdat insulineresistentie en glucose-intolerantie in verband zijn gebracht met verhoogde plasma waardes van vertakte keten aminozuren (BCAA). BCAA waardes werden gemeten in nuchter afgenomen plasma samen met alanine, fenylalanine en tyrosine. Insulinegevoeligheid en bètacel functie werden beoordeeld met klassieke indices verkregen uit een OGTT. De BCAA waarden waren hoger bij diabetische dan bij normoglycemische familieleden onder de Kaukasiërs maar dit was niet het geval in de Zuid-Aziatische families. In beide groepen werden BCAA waarden in stapsgewijze regressieanalyses geassocieerd met waist-to-hip ratio maar niet met indices voor insulinegevoeligheid of bètacel functie. Het toevoegen van de nuchtere BCAA waarden gecombineerd met fenylalanine, tyrosine en alanine aan het nuchtere glucose verbeterde het discriminerend vermogen in het aantonen van de prediabetische toestand binnen Zuid-Aziatische families met een hoog risico op DM type 2. BCAA waarden kunnen dienen als biomarkers voor de vroege ontwikkeling van glucose intolerantie in deze families.

In hoofdstuk 5 heb ik onderzocht hoe renaal verlies van zowel C-peptide als glucose tijdens een OGTT associeert met en invloed heeft op de OMM indices. In Zuid-Aziatische en Kaukasische individuen uit families met een hoog risico voor DM type 2 werden glucose, insuline en C-peptide concentraties in plasma tijdens de OGTT geanalyseerd als oppervlakte onder de curve (AUC's), voorts berekende ik middels de OMM een schatting van de te verwachten renale C-peptide-uitscheiding, en OMM bètacel functie indices en ISI indices werden berekend om DI's te verkrijgen. De glucose in urine kwam goed overeen met de glucose AUC in plasma, de bètacel functie indices en ISI. Urine C-peptide kwam goed overeen met de C-peptide AUC in plasma en met de vanuit de OMM verkregen schatting van renale C-peptide secretie. Renale glucose secretie tijdens de OGTT had geen invloed op de indices verkregen uit de OMM in alle subgroepen, verdeeld op basis van glucose tolerantie status. C-indices van glucose in de urine om verschillende stadia van glucose intolerantie te detecteren waren uitstekend. De beperkte rol van renale glucose secretie valideert het verwaarlozen van het verlies van glucose in urine tijdens de OGTT in kinetische modellen voor glucose homeostase welke zijn gebaseerd op glucose concentraties in plasma. Zowel C-peptide als glucose in urine verzameld tijdens de OGTT kunnen worden gebruikt als non-invasieve metingen voor endogene insuline secretie en de glucose tolerantie status.

In hoofdstuk 6 introduceer ik de *in vivo* stabiele isotoop methode om de ratio vast te stellen tussen reeds aanwezige en nieuw gesynthetiseerde insuline na een bètacel stimulus in mensen middels het labelen van nieuw gesynthetiseerde insuline. Stabiele isotoop ^{13}C leucine, met C-peptide in urine als target peptide, werd toegediend

vooraf aan een verlengde OGTT. Ik verzamelde ook urine, wat een grote hoeveelheid C-peptide bevat, basaal en tijdens de OGTT. De optimale condities waaronder de tracer toegediend kan worden voor verrijkingsdoeleinden werd gevonden en de technieken om zeer zuiver C-peptide uit urine te isoleren werden opgezet. In gezonde Kaukasische individuen met een normale glucose tolerantie detecteerde ik de aanwezigheid van nieuw gesynthetiseerde insuline *in vivo* welke voor ongeveer 20% bijdroeg aan de totale insuline secretie. Het patroon van de verrijking verkregen door het verzamelen van meerdere urine porties was suggestief dat de *de novo* insuline bijdraagt aan de late fase van insuline secretie. Concluderend is het mogelijk om nieuw gesynthetiseerde insuline te meten in gezonde individuen.

In hoofdstuk 7 werd de nieuwe stabiele isotoop methode gebruikt voor bètacel fenotypering om de rol van transcriptie factor 7-like 2 (*TCF7L2*), het voornaamste vatbaarheidsgen voor DM type 2, in de pathogenese van bètacellen te begrijpen. Ik voerde een studie uit in families met hoog risico voor DM type 2 en onderzochten of het T risico allel van de rs7903146 in het *TCF7L2* gen het risico op DM type 2 verhoogt op basis van real-time stabiele isotoop metingen van de insuline synthese. Aanvullend werden OMM analyses verricht om ISI en bètacel functie indices te verkrijgen. Vergeleken met familieleden zonder DM type 2, hadden individuen met DM type 2 lagere OMM waardes en was de insuline synthese verhoogd. Het T allel had een dosis-afhankelijk effect op insuline synthese en glucose tolerantie status: de insuline synthese was hoger bij T allel dragers met DM type 2 vergeleken met wild-type. Blijkbaar is hyperinsulinemie niet slechts een aanpassing aan insuline resistentie, maar ook een directe oorzaak voor DM type 2.

In hoofdstuk 8 stel ik een pathofysiologisch model voor de pathogenese van DM type 2 welke mijn insuline synthese bevindingen integreert. Aanvullend heb ik beschreven welke implicaties mijn bevindingen hebben voor de interpretatie van insuline curves na een stimulus in het algemeen, maar ook voor de huidige concepten van hyperinsulinemie. Tenslotte doe ik suggesties voor toekomstige onderzoeksmogelijkheden.

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LIST OF PUBLICATIONS

1. Transcription factor 7-like 2 gene links increased in vivo insulin synthesis to type 2 diabetes.- **Jainandunsing S**, Koole HR, van Miert JNI, Rietveld T, Wattimena JLD, Sijbrands EJG, de Rooij FWM.- EBioMedicine. 2018 Apr;30:295-302

2. A stable isotope method for in vivo assessment of human insulin synthesis and secretion.- **Jainandunsing S**, van Miert JNI, Rietveld T, Darcos Wattimena JL, Sijbrands EJG, de Rooij FWM.- Acta Diabetol. 2016 Dec;53(6):935-944.

3. Post-glucose-load urinary C-peptide and glucose concentration obtained during OGTT do not affect oral minimal model-based plasma indices.- **Jainandunsing S**, Wattimena JL, Rietveld T, van Miert JN, Sijbrands EJ, de Rooij FW.- Endocrine. 2016 May;52(2):253-62.

4. Failing beta-cell adaptation in South Asian families with a high risk of type 2 diabetes.- **Jainandunsing S**, Özcan B, Rietveld T, van Miert JN, Isaacs AJ, Langendonk JG, de Rooij FW, Sijbrands EJ.- Acta Diabetol. 2015 Feb;52(1):11-9.

5. Discriminative Ability of Plasma Branched-Chain Amino Acid Levels for Glucose Intolerance in Families At Risk for Type 2 Diabetes.- **Jainandunsing S**, Wattimena JL, Verhoeven AJ, Langendonk JG, Rietveld T, Isaacs AJ, Sijbrands EJ, de Rooij FW.- Metab Syndr Relat Disord. 2016 Apr;14(3):175-81.

6. The Relationship of Metabolic Syndrome Traits with Beta-Cell Function and Insulin Sensitivity by Oral Minimal Model Assessment in South Asian and European Families Residing in the Netherlands.- Geragotou T, **Jainandunsing S**, Özcan B, de Rooij FW, Kokkinos A, Tentolouris N, Sijbrands EJ.- J Diabetes Res. 2016;2016:9286303.

7. Urinary renin, but not angiotensinogen or aldosterone, reflects the renal renin-angiotensin-aldosterone system activity and the efficacy of renin-angiotensin-aldosterone system blockade in the kidney.- van den Heuvel M, Batenburg WW, **Jainandunsing S**, Garrelds IM, van Gool JM, Feelders RA, van den Meiracker AH, Danser AH.- J Hypertens. 2011 Nov;29(11):2147-55.

8. Monogenetic disorders of the cholesterol metabolism and premature cardiovascular disease.- van Schie MC, **Jainandunsing S**, van Lennep JER.- Eur J Pharmacol. 2017 Dec 5;816:146-153.

9. Cardio-abdominal echinococcosis: A man with a visible pulsating abdominal mass.- **Jainandunsing S**, Oei L, Oei EHG, Budde RPJ, Alsma J, Hellemond JJV, Maat APWM, Schurink CAM.- IDCases. 2017 Dec 27;11:46-47.

ABOUT THE AUTHOR

Sjaam Jainandunsing werd geboren op 4 augustus 1982 in Rotterdam. Vanaf september 2002 studeerde hij geneeskunde aan de Erasmus Universiteit in Rotterdam. In december 2008 behaalde hij het artsendiploma. In januari 2009 startte hij als arts-onderzoeker wat leidde tot een promotietraject vanaf augustus 2009 op de afdeling Interne Geneeskunde, sectie Farmacologie, Vasculaire en Metabole ziekten in het Erasmus MC onder begeleiding van prof. dr. E.J.G. Sijbrands en dr. F.W.M. de Rooij. Het betrof een onderzoek naar het verbeteren van het fenotyperen van de beta cel functie *in vivo* in mensen middels stabiele isotoop technieken. In januari 2013 begon hij met de opleiding tot internist in het Amphia Ziekenhuis in Breda (opleiders: dr. C. van Guldener en dr. J.W.J. van Esser). Sinds januari 2016 is hij werkzaam in het Erasmus MC (opleiders: dr. S.C.E. Klein Nagelvoort – Schuit en dr. A.A.M. Zandbergen), waar hij sinds januari 2017 bezig is met zijn fellowship Vasculaire Geneeskunde (opleider: Prof. dr. E.J.G. Sijbrands). Daarnaast zit hij sinds januari 2018 in het bestuur van het Nederlands Vasculair Forum (voorzitter prof. dr. H.A.H. Kaasjager). Voortbordurend op zijn thesis en interesse voor diabetes heeft hij in september en oktober 2018 in het kader van zowel research als kliniek in het Inselspital in Bern, Zwitserland gewerkt in het team van prof. dr. C. Stettler en dr. L. Bally. Hij zit momenteel in de laatste maanden van zijn specialisatie.

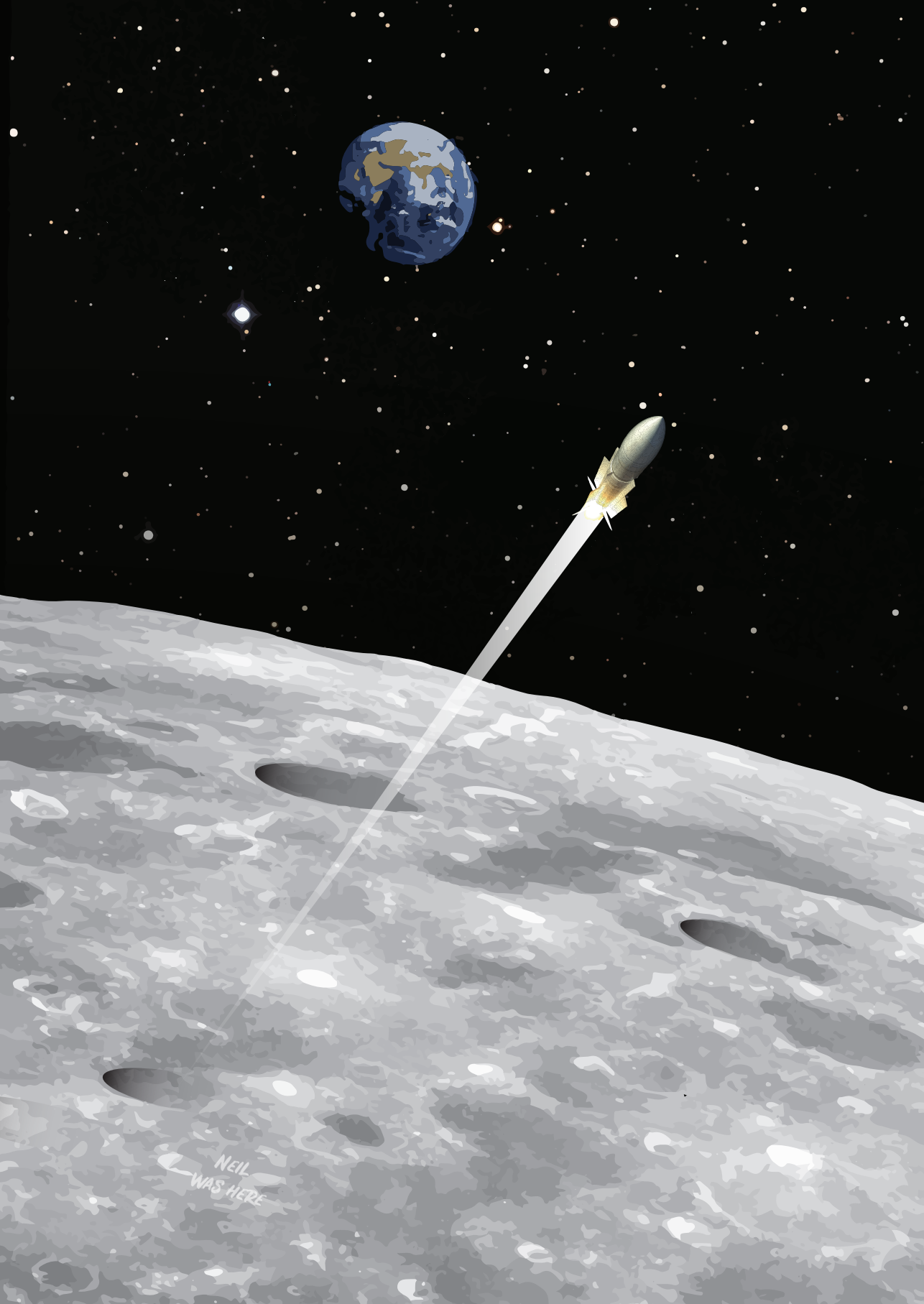
PHD PORTFOLIO

A summary of PhD training and teaching activities

PhD candidate:	Sjaam Jainandunsing
Erasmus MC Department:	Pharmacology, Vascular and Metabolic Diseases
Research school:	Cardiovascular Research School Erasmus University Rotterdam
Period:	Aug 2009-Dec 2012
Promotor:	Prof. Dr. E.J.G. Sijbrands
Co-promotor:	Dr. F.W.M. de Rooij

PhD training	Year	Workload (ECTS):
General academic skills		
Course Center for Patient Oriented Research	2009	0.7
Course biomedical English writing and communication	2011	3.0
Research skills		
Course NIHES introduction to data-analysis	2010	5.7
Course NIHES principles of research in medicine and epidemiology	2010	5.7
Course NIHES family based genetic analysis	2011	5.7
Course NIHES regression analysis	2011	1.0
Course NIHES repeated measurements	2011	5.7
In-depth courses		
Course MOLMED biomedical research techniques	2009	1.5
Course MOLMED molecular biology in cardiovascular disease	2011	5.7
Conferences and presentations		
Science Days Internal Medicine, Antwerp, Belgium: Analysis of phenotype and genotype with regard to beta cell function in T2D within South Asians living in the Netherlands (poster)	2009	0.6
Science Days Internal Medicine, Antwerp, Belgium: Diabetes and Alzheimer Congres, Paris, France	2010	0.8
Characterization of insulin biosynthesis and secretion in T2D high-risk families (oral presentation)	2011	0.6
Science Days Internal Medicine , Antwerp, Belgium: Early impairment of branched-chain amino acid kinetics among South Asians (poster)	2012	0.6
Resolve project TU Eindhoven, modeling the interplay of fat and carbohydrate metabolism with application in metabolic syndrome and type 2 diabetes, Eindhoven, The Netherlands: Oral minimal model applied in T2D high-risk families (oral presentation)	2013	0.6

PhD training	Year	Workload (ECTS):
Seminars		
Vascular Rounds	2009-2012	
Diabetes Platform	2009-2012	
Dutch Lipopprotein Club	2010-2011	
PhD Day	2011	
Vascular Lectures	2009-2012	2.1
Workshops		
Workshop Photoshop and Illustrator CS5	2011	0.3
Workshop Indesign CS5	2011	0.2
Workshop Graphing styles and plot versus tables	2012	0.2
Scientific meetings		
AIO/post-doc meetings at the department of Pharmacology, Vascular and Metabolic Diseases	2009-2012	
Journal club at the department of Pharmacology, Vascular and Metabolic Diseases	2009-2012	
Teaching activities		
Vascular Lecture: Defining the concept of insulin resistance (oral presentation)	2009	0.4
Research Seminar "diagnosis and risk of ischemic heart disease": Predicting type 2 diabetes (oral presentation)	2010	0.4
Scientific internship Junior Med School students	2009-2012	2.0
Supervising third-year medical students	2009-2012	1.0
Supervising doctorate of medical students	2009-2012	3.0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