Research Paper

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

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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.

1. Introduction

Type 2 diabetes has become one of the main threats to human health in the 21st century (Zimmet et al., 2001). 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 (Nolan et al., 2011, Ashcroft and Rorsman, 2012). 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 (Grant et al., 2006, Helgason et al., 2007, Lin et al., 2016).

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 (Florez et al., 2006, Wang et al., 2007, Alibegovic et al., 2010, Giannini et al., 2014, Heni et al., 2010). 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 (Faerch et al., 2013, Schafer et al., 2007, Shu et al., 2009) and with increased hepatic glucose production (Boj et al., 2012, Cropano et al., 2017). 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 (Zhou et al., 2014). 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 (Le Bacquer et al., 2012). 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 (da Silva Xavier et al., 2009). 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

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Abbreviations: DI, disposition index; eGFR, estimated glomerular filtration rate; FSR, fractional synthesis rate; GC-MS, gas chromatography–mass spectrometry; IAC, immuno-affinity chromatography; OGTT, oral glucose tolerance test; OMM, oral minimal model; SI, insulin sensitivity index; SPE, solid phase extraction; t/T, tracer/tracee ratio; *TCF7L2*, transcription factor 7-like 2.

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measurements during OGTT, we are able to detect newly synthesized insulin (Jainandunsing et al., 2016a). 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.

2. Materials and Methods

2.1. 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 (Jainandunsing et al., 2015). 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 (Purcell et al., 2003). We performed our novel insulin synthesis test in 100 of these first-degree relatives: 48 (M18 F30) Caucasians and 52 (M26 F26) 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.

2.2. 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.

2.3. ^{13}C Leucine Bolus as Add-on to OGTT

We performed our protocol, immunoassay and enrichment measurements as described previously and evaluated extensively (Jainandunsing et al., 2016a). 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 (Jainandunsing et al., 2016b). The reasons for using

urine voids for our enrichment measurements have been published previously (Jainandunsing et al., 2016a). 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 (Jainandunsing et al., 2016a). 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 25 m, I.D. 0·22 mm, film 0·25 μm (SGE Analytical Science). The intra and inter-variability coefficient of variability of C-peptide enrichment measurements were 1.11% and 2.34%, respectively.

2.4. 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 (Breda et al., 2001). 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 (Barrett et al., 1998). eGFR was estimated with the modification of diet in renal disease formula (Levey et al., 1999).

2.5. 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 (%/h) = ($E_{collected}$ − E_{basal})/A × 60 min × 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 (Jainandunsing et al., 2016a). 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 Figs. 1 and 2 was calculated as FSR × 2 h (period of *de novo* synthesis between 90 and 120 min, as mentioned previously (Jainandunsing et al., 2016a)).

2.6. 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 CCAA GCTTCTGAGTCACACAGGCC). Sequence analysis of the PCR product was performed on a 310 Genetic Analyzer (ABI Prism), programmed

for POP-6 polymer, 1 mL syringe, with a 47 cm, 50 i.d. capillary. The retrieved sequence products were used for *TCF7L2* rs7903146 genotyping.

2.7. 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 <.05. For differences between proportions, a Chi-squared test was used; differences were considered significant if the *P*-value was <.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 (Almasy and Blangero,
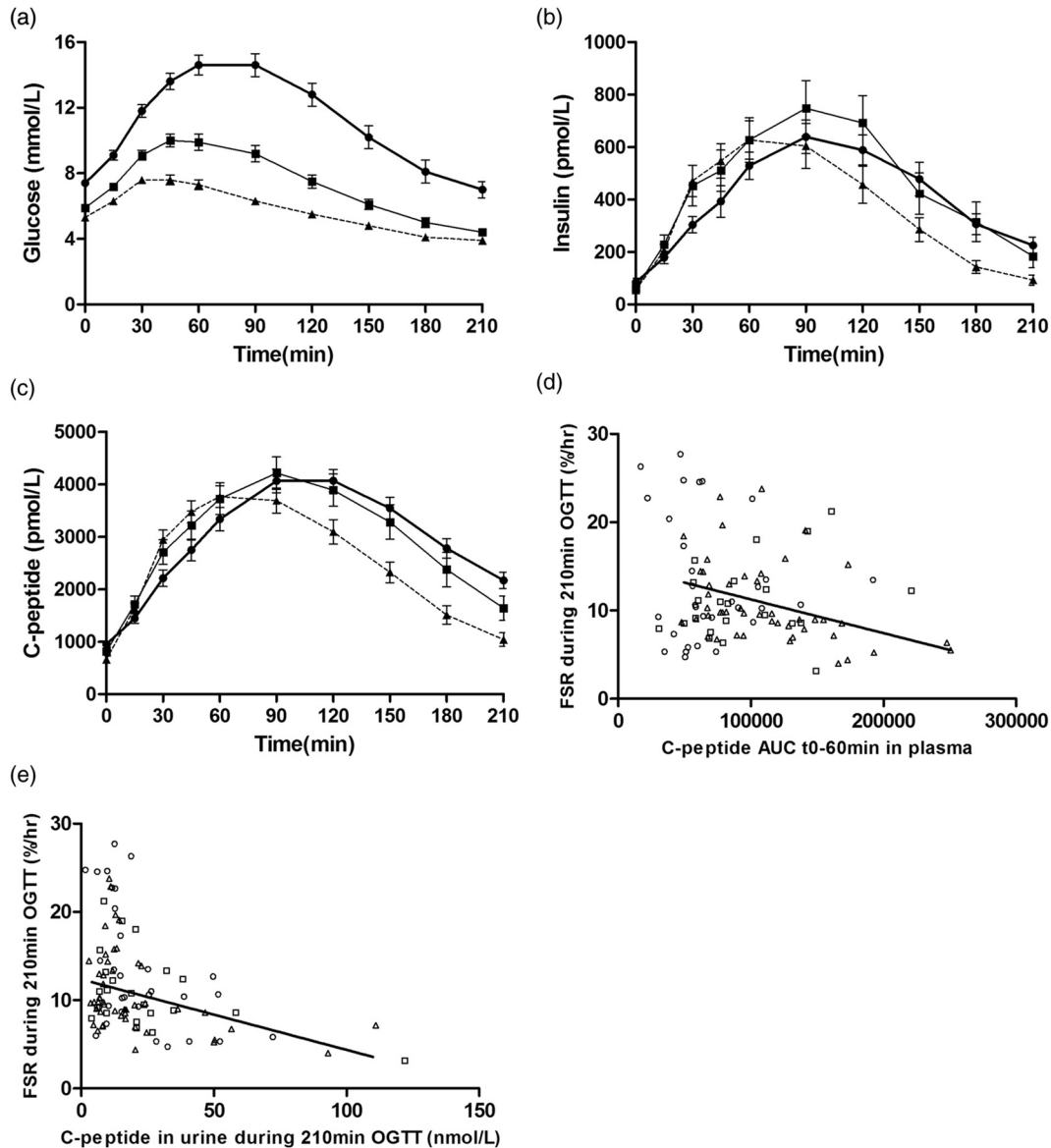

Fig. 1. a–c: Glucose, insulin and C-peptide curves during Oral Glucose Tolerance Test (OGTT) according to glucose tolerance state. (a) Plasma glucose (above), (b) insulin (middle) and (c) C-peptide (below) curves during 210 min OGTT of individuals with normal glucose tolerance (triangle, dashed line), 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; $P = .03$, according to Student's unpaired t test), respectively. d–e: Correlation plot of fractional synthesis rate (FSR, %/h) 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 ($r = -0.243$, $P = .015$; above) and (e) between C-peptide in urine during 210 min OGTT ($r = -0.39$, $P < .001$; below), respectively among individuals with normal glucose tolerance (triangle), impaired fasting glucose/impaired glucose tolerance (square) or type 2 diabetes (circle).
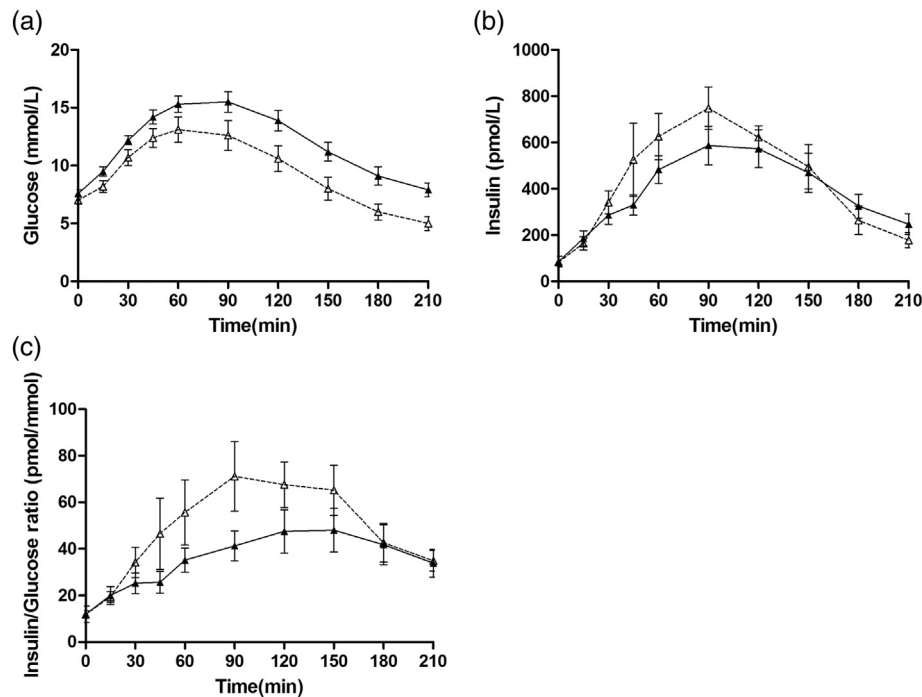
Fig. 2. a–c: Glucose, insulin and insulin/glucose ratio curves during Oral Glucose Tolerance Test (OGTT) in type 2 diabetes subgroup according to RS7903146 genotype (f) Plasma glucose (above), (g) insulin (middle) and (h) insulin/glucose ratio (below) curves during 210 min OGTT of individuals with type 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; $P = 041$, according to Student's unpaired t test.

1998). Effects of independent variables in multiple regression analyses were considered significant if the P-value was <.05. The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

3. Results

3.1. 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, $P = .48$) and urine volume during OGTT (443 ± 60 *versus* 461 ± 36 mL, respectively, $P = .79$), 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 (Jainandunsing et al., 2016a). 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 ($P < .001$) and W/H ratio ($P = .002$) 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 ($P < .001$) and lower β cell DI parameters ($P < .001$ for $DI_{dynamic}$; $P = .005$ for DI_{static}; and $P = .004$ for DI_{oral}) when compared with the non-type 2 diabetes subgroup. The type 2 diabetes subgroup had a higher FSR ($P = .030$) 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-

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	.004[†]
Sex (male/female)	17/30	11/11	16/15	.330[†]
Age (years)	40 ± 2	44 ± 2	56 ± 2	<.001
BMI (kg/m^2)	27·1 ± 0·6	29·3 ± 1·4	29·4 ± 0·8	.148
W/H ratio	0·86 ± 0·01	0·92 ± 0·2	0·94 ± 0·01	.002
SI (10^{-5} dL kg^{-1} min^{-1} per pM)	23 ± 3	11 ± 2	6 ± 1	<.001
$DI_{dynamic}$ (10^{-14} dL kg^{-1} min^{-1} per pM)	6263 ± 1034	2174 ± 576	545 ± 140	<.001
DI_{static} (10^{-14} dL kg^{-1} min^{-2} per pM)	496 ± 109	131 ± 29	47 ± 9	.005
DI_{oral} (10^{-14} dL kg^{-1} min^{-2} per pM)	569 ± 122	152 ± 33	51 ± 10	.004
FSR (%/h)	10·8 ± 0·7	11·1 ± 0·9	13·5 ± 1·3	.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.

[*] $P < .05$ Student's unpaired t-test of T2D *versus* non-T2D (NGT and IFG/IGT subgroups combined) unless otherwise stated.

[†] $P < .05$ Chi-squared test among given subgroups.

peptide area under the curve t0–60 min and urinary C-peptide (Fig. 1d-e; ($r = -0.243$, $P = .015$ and $r = -0.39$, $P < .001$, respectively).

3.2. 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 ($P = .004$, Table 1). Ordinal regression analysis revealed a significant association between the *TCF7L2* rs7903146 genotypes (CC, CT or TT) and the three WHO OGTT categories ($B = 1.06$, $P = .009$). As we found a significant interaction of WHO OGTT subgroup with CC, CT or TT carriership on the variance of FSR ($\beta_{interaction} = 0.11$, $P = .002$), 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 $DI_{dynamic}$ ($P = .043$ 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 ($P = .041$). 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, $P = .93$) 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 ($\beta = -1.986$, $P = .002$), whereas in the type 2 diabetes subgroup, the *TCF7L2* rs7903146 variant was associated with increased insulin synthesis ($\beta = 29.893$, $P = .01$). 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 ($P = .018$),

while no differences were found within the Caucasian non-type 2 diabetes subgroup.

4. 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 (Poitout and Robertson, 2008, Ottosson-Laakso et al., 2017), 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 (Avrahami et al., 2017) 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 (Dorrell et al., 2016). 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 (Aguayo-Mazzucato et al., 2017). 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 (Talchai et al., 2012, Wang et al., 2014, Brereton et al., 2014, Szabat et al., 2016) 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 (Cinti et al., 2016). 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

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/m²)	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 (%/h)	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.
  * $P < .05$ 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]	**.002**	29.893	[52.768;7.018]	**.010**
Ethnicity	−2·431	[−4·024;-0·838]	**.003**	10.083	[−8.913;29.079]	.298
Gender	1·325	[−0·500; 3·150]	.155	−5.415	[−22.502;11.672]	.535
Age	0·136	[0·065;0·207]	**<.001**	0.686	[−0.208;1.580]	.132
W/H ratio	−0·04	[−0·124;0·044]	.352	0.00212	[−0.076;0.081]	.958
SI	0·059	[−0·037;0·155]	.229	0.233	[3.336;−2.870]	.883
DI$_{dynamic}$	0·0004	[−0·003;0·003]	.790	−0.034	[−0.085;0.017]	.191
OGTT category	−0·02	[−0·044;0·004]	.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 <.05 significance.

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 (Westermark et al., 2011), as well as ATP, which might increase islet inflammation through activation of islet macrophages (Weitz et al., 2018), 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 (Loos et al., 2007, Alibegovic et al., 2010), reduced exocytosis (da Silva Xavier et al., 2009), impaired proinsulin-to-insulin conversion (Loos et al., 2007, Kirchhoff et al., 2008, Stolerman et al., 2009), and decreased β cell mass (Le Bacquer et al., 2012). 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 (Zhou et al., 2014, Mitchell et al., 2015) 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 (Alibegovic et al., 2010). 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

(Cropano et al., 2017). 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 (Bindraban et al., 2008, Chandie Shaw et al., 2006). Previously, we have reported homogenous insulin-resistant conditions in South Asian families regardless of their glucose tolerance (Jainandunsing et al., 2015), 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 (Constan et al., 1975). 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 (Jainandunsing

et al., 2016a). 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 (Kim et al., 2017), 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 (Templeman et al., 2017). 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 (Gregg et al., 2012). 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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Conflicts of Interest

The authors declare that they have no competing interests.

Author Contributions

S.J. and F.W.M. de R. designed the study and experiments. S.J. performed the data collection and analysis. S.J. and E.J.G.S. wrote the first version of the manuscript. H. K-L, J.L.D.W., T.R. and J.N.I. van M. participated in data analysis and manuscript preparation. F.W.M. de R. and E.J.G.S. are the guarantors of this work and had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors critically revised the manuscript and approved the final version.

Conflict of Interest Statement

The authors have declared that no conflict of interest exists.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.03.026.

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