

The risk allele load accelerates the age-dependent decline in beta cell function

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Abstract

Aims/hypothesis Among the novel type 2 diabetes risk loci identified by genome-wide association studies, *TCF7L2*, *HHEX*, *SLC30A8* and *CDKAL1* appear to affect beta cell function. In the present study we examined the effect of these genes' risk alleles on the age-dependent decline in insulin secretion.

Methods The SNPs rs7903146 (*TCF7L2*), rs7754840 (*CDKAL1*), rs7923837 (*HHEX*) and rs13266634 (*SLC30A8*) were genotyped in 1,412 non-diabetic patients, who were subsequently grouped according to their number of risk alleles. All participants underwent an OGTT. Insulin secretion was assessed by validated indices and proinsulin conversion by calculating $AUC_{\text{proinsulin}}/AUC_{\text{insulin}}$.

Results The number of risk alleles revealed a Gaussian distribution, with most participants carrying four risk alleles. Stratification into groups with low (LAL, up to three alleles), median (MAL, four alleles) and high (HAL, five to eight alleles) allele load resulted in MAL and HAL participants displaying significantly lower insulin secretion and proinsulin conversion than LAL participants ($p \leq 0.0014$ and $p = 0.0185$, respectively). In the overall cohort, age was negatively

associated with insulin secretion and proinsulin conversion (both $p < 0.0001$). MAL and HAL participants showed a significantly more pronounced decline in insulin secretion with increasing age than LAL participants ($p \leq 0.0325$; analysis of covariance), and after stratification for BMI this relationship was maintained in obese, but not non-obese, participants. Proinsulin conversion decreased with increasing age in MAL and HAL, but not LAL, participants ($p \leq 0.0003$ vs $p = 0.2$).

Conclusions/interpretation The risk allele load significantly accelerates the age-dependent decline in beta cell function, and this might be of particular importance in obese people.

Keywords Age · Beta cells · Insulin secretion · Proinsulin conversion

Abbreviations

ANCOVA	analysis of covariance
HAL	high allele load
LAL	low allele load
MAL	median allele load
SNP	single-nucleotide polymorphism
TÜF	Tübingen Family Study for type 2 diabetes

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Introduction

Gene–environment interactions are held responsible for the development of type 2 diabetes mellitus. More explicitly, common variation within several genes is thought to confer increased susceptibility to the adverse environmental challenges of the modern civilised world (e.g. high-calorie diets and reduced physical activity) [1]. Using large-scale single-nucleotide polymorphism (SNP) arrays, recent genome-wide

association studies identified a series of novel type 2 diabetes risk loci [2–6], and the maximum number risk alleles of these genes was subsequently shown to increase the risk of type 2 diabetes approximately 2.2-fold [7]. Among the novel risk loci, *TCF7L2*, *HHEX*, *SLC30A8* and *CDKAL1* have been reported to affect diverse beta cell functions, such as overall insulin release, proinsulin-to-insulin conversion, and glucose and incretin responsiveness of the beta cell [8–16].

We have shown earlier that ageing is associated with deteriorating insulin secretion and proinsulin conversion [17]. In order to better understand the role and importance of the risk loci mentioned above in beta cell dysfunction, we asked in this study whether the load of risk alleles of these genes influences the degree of age-dependent decline in beta cell function. To this end, we genotyped 1,412 non-diabetic German participants at increased risk of type 2 diabetes for the genome-wide association-derived diabetes risk SNPs rs7903146 in *TCF7L2*, rs7754840 in *CDKAL1*, rs7923837 in *HHEX* and rs13266634 in *SLC30A8*, divided them according to the number of risk alleles into groups with a low allele load (LAL, zero to three alleles), a median allele load (MAL, four alleles) and a high allele load (HAL, five to eight alleles), and investigated in these groups the associations between age and insulin secretion/proinsulin conversion.

Methods

Subjects Non-diabetic participants ($n=1412$) from southern Germany were recruited from the ongoing cross-sectional Tübingen Family Study for type 2 diabetes (TÜF), which currently includes ~2,000 individuals at an increased risk of diabetes (family history of type 2 diabetes, diagnosis of impaired fasting glycaemia). Type 2 diabetes was an exclusion criterion for the TÜF. More than 99.5% of TÜF participants are of European ancestry. More information about the TÜF study is given in [18]. Recruitment of the participants was based on the availability of DNA samples and complete data sets (glucose, insulin, C-peptide, and proinsulin measurements). All participants underwent physical examination, medical history assessment, routine blood tests, and an OGTT. The OGTT revealed that 72.9% of the non-diabetic participants had normal glucose tolerance, 9.9% had impaired fasting glycaemia, 9.4% had impaired glucose tolerance and 7.8% had impaired fasting glycaemia and impaired glucose tolerance. To ensure that we did not miss effects of the risk allele load on glucose tolerance, we left these subgroups in the analysis. The participants were not taking any medication known to affect glucose tolerance or insulin secretion. Characteristics of the overall study population were as follows: age, 39 ± 13 years; BMI, 28.5 ± 7.8 kg/m²; fasting glucose, 5.12 ± 0.58 mmol/l; 120 min glucose, 6.28 ± 1.68 mmol/l; fasting insulin, 62.6 ± 51.7 pmol/l; 120 min

insulin, 427 ± 450 pmol/l (mean \pm SD). Informed written consent was obtained from all participants, and the local ethics committee (Ethikkommission der Medizinischen Fakultät der Universität Tübingen) approved the protocol.

Genotyping DNA was isolated from whole blood using a commercial DNA isolation kit (NucleoSpin; Macherey & Nagel, Düren, Germany). The SNPs rs7903146 in *TCF7L2*, rs7754840 in *CDKAL1*, rs7923837 in *HHEX* and rs13266634 in *SLC30A8* were genotyped using the TaqMan assay (Applied Biosystems, Foster City, CA, USA), as previously described [9]. The overall genotyping success rate was 99.98%. All SNPs were distributed according to Hardy–Weinberg equilibrium. The genotype distribution and risk allele frequencies of the SNPs are given in Table 1.

OGTT After a 12 h overnight fast, the participants ingested a solution containing 75 g glucose. Venous blood samples were drawn at 0, 30, 60, 90 and 120 min, and plasma glucose, insulin, C-peptide and proinsulin concentrations were determined.

Blood analyses Venous plasma glucose was measured using a bedside glucose analyser (glucose oxidase method; Yellow Springs Instruments, Yellow Springs, OH, USA). Plasma insulin and C-peptide levels were determined with a commercial chemiluminescence system (ADVIA Centaur; Siemens Medical Solutions, Fernwald, Germany). Serum proinsulin concentrations were measured using a microparticle enzyme immunoassay (IBL, Hamburg, Germany). The proinsulin assay showed 0% cross-reactivity with human insulin and C-peptide and the insulin assay showed 0% cross-reactivity with human proinsulin.

Calculations The AUC of plasma glucose levels during the OGTT was calculated according to the trapezoid method as: $0.5 \times [0.5 \times c(\text{glucose})_0 + c(\text{glucose})_{30} + c(\text{glucose})_{60} + c(\text{glucose})_{90} + 0.5 \times c(\text{glucose})_{120}]$. The AUCs of plasma C-peptide, insulin and proinsulin were calculated in a similar way. Insulin secretion was assessed by calculating the ratio

Table 1 Genotype distribution and risk allele frequencies of the analysed SNPs

SNP (gene)	Genotype			Risk allele frequency
rs7903146 (<i>TCF7L2</i>)	CC	CT	TT	
<i>n</i>	704	591	117	0.29
rs7754840 (<i>CDKAL1</i>)	GG	GC	CC	
<i>n</i>	631	629	152	0.33
rs7923837 (<i>HHEX</i>)	GG	GA	AA	
<i>n</i>	555	680	177	0.63
rs13266634 (<i>SLC30A8</i>)	CC	CT	TT	
<i>n</i>	736	552	124	0.72

Table 2 Associations of risk allele load with anthropometric and metabolic variables

Group	LAL	MAL	HAL	p_1 value	p_2 value
Number of risk alleles	0–3	4	5–8	–	–
n (female/male)	509 (332/177)	429 (283/146)	474(314/160)	–	–
Age (years)	39±1	39±1	39±1	0.5	0.5
BMI (kg/m ²)	29.0±0.4	27.9±0.4	28.4±0.3	0.06	0.08
Fasting glucose (mmol/l)	5.11±0.02	5.11±0.03	5.14±0.03	0.6	0.4
AUC glucose (mmol/l)	14.4±0.1	14.7±0.2	14.7±0.2	0.3	0.0109
AUC insulin (pmol/l)	1,010±37	872±32	834±31	0.0009	0.0017
AUC C-peptide (pmol/l)	4,855±86	4,417±81	4,389±73	<0.0001	0.0004
AUC proinsulin (pmol/l)	38.3±2.0	36.9±1.9	37.6±1.8	0.7	0.6
First-phase insulin (pmol/l)	1,375±40	1,185±35	1,156±34	<0.0001	<0.0001
AUC C-peptide/AUC glucose ($\times 10^{-9}$)	340±5	306±5	303±5	<0.0001	<0.0001
Insulinogenic index ($\times 10^{-9}$)	163±7	108±25	148±13	0.0002	0.0014
Disposition index (AU)	16,365±377	16,149±425	16,051±427	0.4	0.0340
AUC proinsulin/AUC insulin	0.050±0.003	0.053±0.002	0.054±0.002	0.0027	0.0185

All data are mean±SE (unadjusted)

p_1 , p values for comparison of unadjusted data (ANOVA)

p_2 , p values for comparison of adjusted data; age was adjusted for sex; BMI was adjusted for sex and age; plasma glucose, insulin, C-peptide and proinsulin concentrations and disposition index were adjusted for sex, age and BMI; other insulin secretion variables were adjusted for sex, age, BMI and insulin sensitivity (multivariate linear regression analysis)

AU, arbitrary units

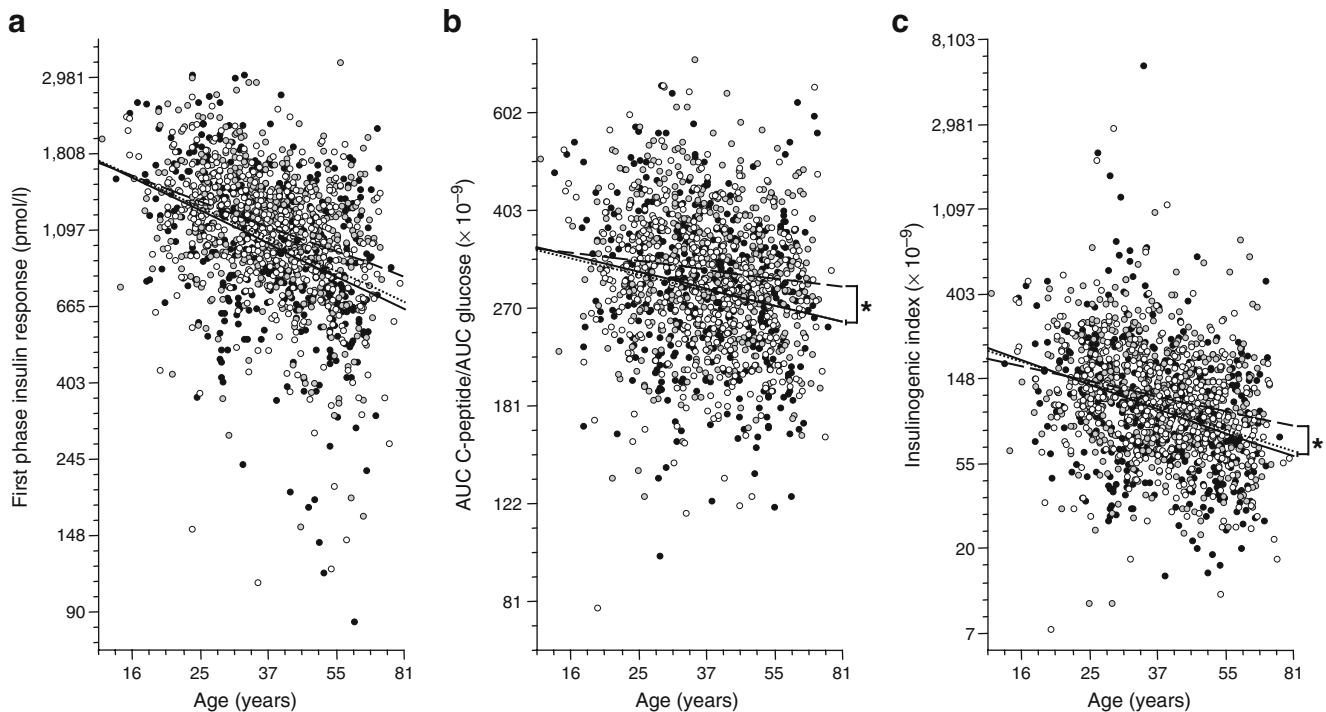


Fig. 1 Effect of risk allele load on the age-dependent decline in insulin secretion. All data were ln-transformed before statistical analysis and plotted using ln scales. First-phase insulin secretion (a), AUC_{C-peptide}/AUC_{glucose} (AUC C-peptide/AUC glucose) (b) and the insulinogenic index (c) were adjusted for sex, BMI and insulin sensitivity by multivariate linear regression modelling (leverage plots shown). Regression lines were compared by ANCOVA with

separate slopes (* $p < 0.05$). Grey circles (dashed regression line), LAL (a, $r = -0.33$, $p < 0.0001$; b, $r = -0.10$, $p = 0.0243$; c, $r = -0.22$, $p < 0.0001$); white circles (dotted regression line), MAL (a, $r = -0.40$, $p < 0.0001$; b, $r = -0.19$, $p < 0.0001$; c, $r = -0.34$, $p < 0.0001$); black circles (continuous regression line), HAL (a, $r = -0.38$, $p < 0.0001$; b, $r = -0.20$, $p < 0.0001$; c, $r = -0.33$, $p < 0.0001$)

of the AUC of C-peptide divided by the AUC of glucose ($AUC_{C\text{-peptide}}/AUC_{\text{glucose}}$). First-phase insulin secretion was estimated from plasma insulin and glucose concentrations as described in [19]: $1,283 + 1.829 \times c(\text{insulin})_{30} - 138.7 \times c(\text{glucose})_{30} + 3.772 \times c(\text{insulin})_0$, where c is concentration. The insulinogenic index was calculated as: $[c(\text{insulin})_{30} - c(\text{insulin})_0] / [c(\text{glucose})_{30} - c(\text{glucose})_0]$. Proinsulin conversion was assessed by calculating the ratio of the AUC of proinsulin divided by the AUC of insulin ($AUC_{\text{proinsulin}}/AUC_{\text{insulin}}$). Insulin sensitivity was estimated as proposed by Matsuda and DeFronzo [20]: $10,000 / [c(\text{glucose})_0 \times c(\text{insulin})_0 \times c(\text{glucose})_{\text{mean}} \times c(\text{insulin})_{\text{mean}}]^{1/2}$. The disposition index was calculated as first-phase insulin secretion multiplied by insulin sensitivity.

Statistical analyses Before statistical analysis, all non-normally distributed variables were ln-transformed in order to approximate normal distribution. Comparisons of unadjusted data between the risk allele groups were performed using one-way ANOVA. Adjustment of indices of beta cell function was performed using multivariate linear regression analysis (least squares method). Leverage plots are shown. Regression lines were compared using analysis of covariance (ANCOVA) with separate slopes. Upon combination

of the MAL and HAL groups, the study was sufficiently powered ($1 - \beta > 0.8$) to detect differences as small as 2.5% ($AUC_{C\text{-peptide}}/AUC_{\text{glucose}}$) and 6.5% ($AUC_{\text{proinsulin}}/AUC_{\text{insulin}}$) between the slopes of the regression lines. Results with p values < 0.05 were considered statistically significant. The JMP 7.0 statistical software package (SAS Institute, Cary, NC, USA) was used.

Results

After genotyping of the overall population ($n=1412$) for the four diabetes risk SNPs in *TCF7L2*, *CDKAL1*, *HHEX* and *SLC30A8* and grouping of the individuals according to their number of risk alleles, the risk alleles were found to follow a Gaussian distribution, most participants carrying four risk alleles (Electronic supplementary material [ESM] Table 1). The anthropometric and metabolic variables of these groups are presented in ESM Table 1. We then stratified the cohort according to their allele load and defined carriers of up to three alleles as LAL participants, carriers of four alleles as MAL participants, and those with five to eight alleles as HAL participants. For summation of the risk alleles, we weighted each risk allele equally. As shown in Table 2, this

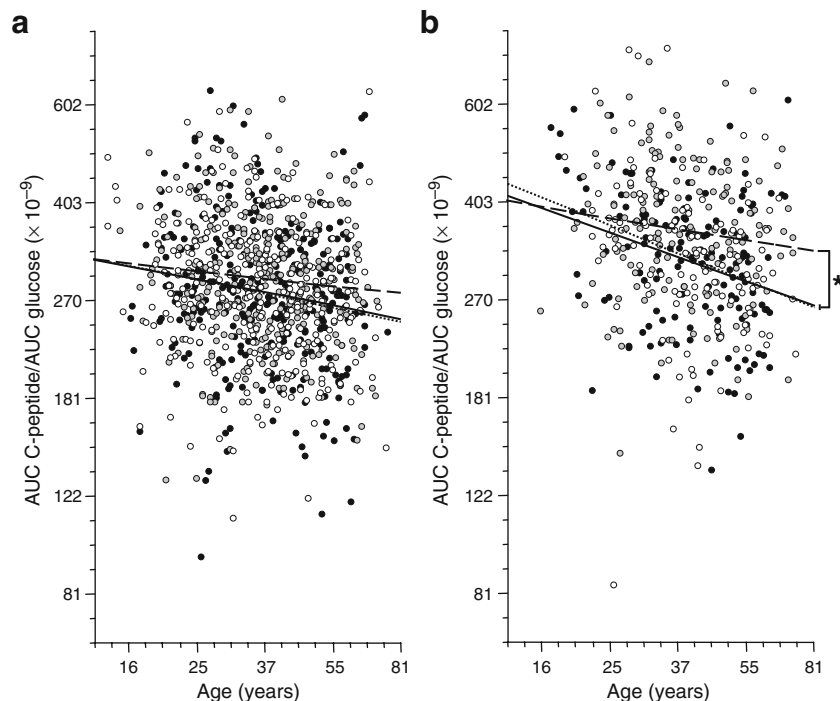


Fig. 2 Effect of risk allele load on the age-dependent decline in insulin secretion in non-obese and obese participants. All data were ln-transformed before statistical analysis and plotted using ln scales. $AUC_{C\text{-peptide}}/AUC_{\text{glucose}}$ ($AUC_{C\text{-peptide}}/AUC_{\text{glucose}}$) was adjusted for sex and insulin sensitivity using multivariate linear regression modelling (leverage plots shown). Participants were stratified according to their BMI: non-obese, $BMI < 30 \text{ kg/m}^2$ ($n=966$; **a**); obese,

$BMI \geq 30 \text{ kg/m}^2$ ($n=446$; **b**). Regression lines were compared by ANCOVA with separate slopes ($*p < 0.05$). Grey circles (dashed regression line), LAL (**a**, $r = -0.10$, $p = 0.089$ [NS]; **b**, $r = -0.12$, $p = 0.1$ [NS]); white circles (dotted regression line), MAL (**a**, $r = -0.10$, $p = 0.0018$; **b**, $r = -0.27$, $p = 0.0027$); black circles (continuous regression line), HAL (**a**, $r = -0.315$, $p = 0.0070$; **b**, $r = -0.29$, $p = 0.0004$)

procedure resulted in MAL and HAL groups that displayed significantly lower insulin secretion and lower proinsulin conversion ($AUC_{\text{proinsulin}}/AUC_{\text{insulin}}$ is an inverse measure of proinsulin conversion) than the LAL group ($p \leq 0.0014$ and $p = 0.0185$, respectively; adjusted for sex, age, BMI and insulin sensitivity). Furthermore, MAL and HAL participants had a significantly lower disposition index than LAL participants ($p = 0.0340$; adjusted for sex, age, and BMI).

In the overall population, age was negatively associated with both insulin secretion and proinsulin conversion (both $p < 0.0001$; adjusted for sex, BMI and insulin sensitivity). As shown in Fig. 1a–c, MAL and HAL participants revealed a more pronounced decline in insulin secretion with increasing age than LAL participants, and this effect reached statistical significance using $AUC_{\text{C-peptide}}/AUC_{\text{glucose}}$ and the insulinogenic index as insulin secretion indices (MAL+HAL vs LAL, $p = 0.0325$ and $p = 0.0262$, respectively; ANCOVA; Fig. 1b, c). Weaker differences in the slopes of the regression lines were seen with the disposition index (ESM Fig. 1). To examine whether the effect of the risk allele load on the age-dependent decline in insulin secretion was due to a skewed distribution between the groups of a single SNP with a predominant effect, we included in addition to the allele load each individual SNP, separately, in the ANCOVA and asked whether this procedure abolished the significant effect of the allele load. By this kind of analysis, we could not detect a predominant effect of any of the individual SNPs (in the dominant or the additive inheritance model) over the effect of the allele load. To assess the influence of obesity status on this allele load effect, we stratified the cohort into non-obese ($BMI < 30 \text{ kg/m}^2$) and obese ($BMI \geq 30 \text{ kg/m}^2$) groups instead of adjusting for BMI. This showed that the effect of the allele load on $AUC_{\text{C-peptide}}/AUC_{\text{glucose}}$ was significant in the obese but not in the non-obese participants ($p = 0.0299$ vs $p = 0.1$; ANCOVA; Fig. 2). Furthermore, MAL and HAL participants revealed a decline in proinsulin conversion with increasing age ($p \leq 0.0003$; adjusted for sex, BMI and insulin sensitivity; Fig. 3). However, we did not detect a correlation between age and proinsulin conversion in the LAL group ($p = 0.2$; Fig. 3).

Discussion

In the present study, we examined the effects of the diabetes risk alleles of the *TCF7L2*, *HHEX*, *SLC30A8* and *CDKAL1* genes on the age-dependent decline in insulin secretion. In accordance with earlier studies that examined each SNP separately [8–16] and a more recent study that reported a cumulative effect of the number of alleles on the risk of type 2 diabetes [7], we found that a high load of risk alleles negatively affected insulin secretion and proinsulin conversion. Furthermore, in the present study we confirmed our

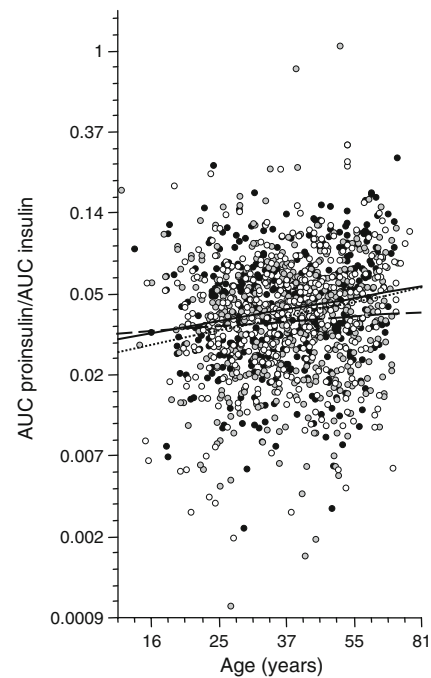


Fig. 3 Effect of risk allele load on the age-dependent decline in proinsulin conversion. All data were ln-transformed before statistical analysis and plotted using ln scales. $AUC_{\text{proinsulin}}/AUC_{\text{insulin}}$ ($AUC_{\text{proinsulin}}/AUC_{\text{insulin}}$) was used as an inverse measure of proinsulin conversion and was adjusted for sex, BMI and insulin sensitivity using multivariate linear regression modelling (leverage plot shown). Grey circles (dashed regression line), LAL ($r = 0.06$, $p = 0.2$ [NS]); white circles (dotted regression line), MAL ($r = 0.20$, $p < 0.0001$); black circles (continuous regression line), HAL ($r = 0.18$, $p = 0.0003$)

earlier results showing deterioration of insulin secretion and proinsulin conversion with increasing age [17].

Most interestingly however, we demonstrated in this study that a high load of risk alleles significantly accelerates the age-dependent decline in insulin secretion, with 50-year-old carriers of five to eight alleles (of the risk loci tested) displaying the same insulin secretion as 70-year-old carriers of only three or fewer alleles. Furthermore, 70-year-old participants with five to eight risk alleles revealed a 13–18% reduction in insulin secretion compared with carriers of up to three alleles of the same age (depending on the insulin secretion index used). Even though our findings are limited to a non-diabetic cohort, these findings, when extrapolated, suggest that the individual number of risk alleles determines the onset of beta cell failure and type 2 diabetes.

Genetic susceptibility to type 2 diabetes has been reported to be modulated by obesity: SNPs known to affect insulin action were associated with type 2 diabetes in obese participants only, whereas SNPs known to modulate beta cell function (i.e. SNPs in *GCK*, *HNF1A*, *SLC30A8* and *TCF7L2*) were shown to be associated with type 2 diabetes in non-obese participants [21]. We therefore asked whether the effect of the allele load on the age-dependent decline in insulin secretion was influenced by obesity status. Interest-

ingly, the effect of the allele load was significant only in the obese participants. Thus, our data appear inconsistent with the results of the previous study. However, our study differed from the previous study in several points: (1) we did not assess the effects of the individual gene variants separately, but performed stratification according to the allele load; (2) we included *HHEX* and *CDKAL1*, two genes not investigated in the previous study; and (3) we did not investigate the effect of the allele load on the endpoint of diabetes. Even though the studies are not really comparable, both stress the importance of gene–environment interactions in the development of beta cell dysfunction and type 2 diabetes.

Finally, we found that participants with few risk alleles did not display impaired proinsulin conversion with increasing age, whereas participants with a high allele load showed an age-dependent decline in proinsulin conversion (i.e. an increase in the $AUC_{\text{proinsulin}}/AUC_{\text{insulin}}$ ratio) with age. Thus, defects in insulin processing, probably caused by reductions in proprotein convertase activities [22], are most probably due to genetics.

Even though we obtained significant results with different, i.e. insulin- and C-peptide-derived, measures of insulin secretion and demonstrated that our study was sufficiently powered to detect even small effect sizes, the moderate sample sizes of our risk allele groups may represent a limitation. Therefore, replication of our findings in larger cohorts is needed.

In conclusion, we show here that the risk allele load significantly accelerates the age-dependent decline in beta cell function and hence might promote beta cell failure and type 2 diabetes. This detrimental effect of the risk alleles might be of particular importance in obese participants.

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Duality of interest statement The authors declare that there is no duality of interest associated with this manuscript.

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