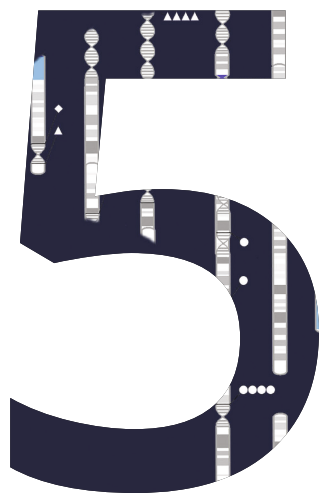
Genome-wide assocation study on the early phase insulin response to a liquid mixed meal: results from the NEO study



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ABSTRACT

Early phase insulin secretion is a determinant of postprandial glucose homeostasis. In this study, we aimed to identify novel genetic variants associated with the early phase insulin response to a liquid mixed meal by a genome-wide association study using a discovery and replication design embedded in the Netherlands Epidemiology of Obesity (NEO) study. The early phase insulin response was defined as the difference between the natural logarithm transformed insulin concentrations of the postprandial state at 30 minutes after a meal challenge and the fasting state (Δinsulin). After Bonferroni correction, rs505922 (Beta (MAF, P-value): -6.5% (0.32, $3.3x10^{-8}$)) located in the *ABO* gene reached genome-wide significant level (p-value<$5 x10^{-8}$) and was also replicated successfully (Beta (MAF, P-value): -7.8% (0.32, $7.2x10^{-5}$)). The function of the ABO gene was assessed using *in vitro* shRNA mediated knock-down of gene expression in the murine pancreatic β-cell line MIN6. Knocking down the *ABO* gene led to decreased insulin secretion in the murine pancreatic β-cell line. These data indicate that the previously identified elevated risk of type 2 diabetes for carriers of the ABO rs505922:C allele may be caused by decreased early phase insulin secretion.

INTRODUCTION

Impaired β-cell function is considered a key factor in the pathogenesis of type 2 diabetes (T2D) driven by insulin resistance (1). Insulin secretion in response to an intravenous glucose stimulus is a two-phase process: the first peak of insulin secretion occurs rapidly within 5 to 10 minutes after the glucose infusion, followed by a second peak depending on the degree and duration of glucose stimulus (1). Although the insulin response to ingested glucose (e.g. from a meal) does not exhibit a clear biphasic shape under physiological conditions, an early insulin response with rapid elevations of portal and peripheral insulin concentrations has been observed (2; 3). A previous study has found that the plasma insulin response at 30 minutes after an oral glucose load was inversely associated with the 2-hour plasma glucose concentrations in patients with impaired glucose tolerance (4). This implies that the early phase insulin secretion is a marker for postprandial glucose homeostasis and plays a role in the development of type 2 diabetes.

In the past decade, the genetics of glycaemic traits including fasting glucose, fasting insulin, 2-hour glucose after an oral glucose tolerance test (OGTT) and HbA1c have been extensively investigated by genome-wide association studies (GWAS) (5-7). Many of the genes associated with glycaemic traits from these GWAS are thought to be related to β-cell function. Among over 120 loci that were identified to associate with type 2 diabetes (T2D), a few loci resided in the *ABO* gene repeatedly popped up in the GWAS. *ABO* gene encodes proteins that determine ABO blood group system, and non-O blood group carriers showed an increased risk of T2D, myocardial infarction, peripheral vascular disease as well as venous thromboembolism from the previous epidemiological studies (8; 9). From all the GWAS on glycaemic traits, few studies have focused on the dynamic measures of insulin secretion and action to identify genetic variation that is associated with the insulin response. To date, there was only one large-scale GWAS focused on the dynamic measures of insulin secretion, reflected by the insulin levels at 30 minutes after an OGTT (10). From this study, in addition to previously identified glycaemic-trait associated loci, the imprinted *GRB10* gene was identified as an islet function regulator in men only. However, OGTT only assesses one component of metabolism, namely glucose metabolism. It has been suggested that the response of gastrointestinal hormones to dietary proteins and fat has considerable effects on β-cell insulin secretion after ingestion of a mixed meal (11; 12), which might be underestimated by studying OGTT alone. Thus far, the genetic basis of the early phase insulin secretion response to a mixed meal has not been studied.

In the current study, we conducted a GWAS on early phase insulin response to a liquid mixed meal. Discovery and replication were both performed in a large population-based cohort study, the Netherlands Epidemiology of Obesity (NEO) Study. To further

shed light on the function of the top genetic signal from the GWAS, *in vitro* shRNA knock-down gene expression experiment was performed of the top genetic signal from the GWAS by measuring glucose-stimulated insulin secretion in the murine pancreatic β-cell line MIN6.

MATERIALS AND METHODS

Study design and study population

The study was performed in a population-based prospective cohort study, the Netherlands Epidemiology of Obesity (NEO) study (13). All participants gave written informed consent and the Medical Ethical Committee of the Leiden University Medical Center (LUMC) approved the study design. Initiated from 2008, NEO study was designed to study pathways that lead to obesity-related diseases. Detailed information about the study design and data collection has been described elsewhere (13). Men and women aged between 45 and 65 years with a self-reported body mass index (BMI) of 27 kg/m^2 or higher living in the greater area of Leiden (in the west of the Netherlands) were eligible to participate in the NEO study. In addition, all inhabitants aged between 45 and 65 years from one municipality (Leiderdorp) were invited irrespective of their BMI. Participants were invited for a baseline visit at the NEO study center in the LUMC after an overnight fast. Prior to their visits, participants completed a questionnaire at home with demographic, lifestyle and clinical data. At the baseline visit, fasting blood samples were drawn. Within the next five minutes after the fasting blood draw, a liquid mixed meal (400mL with 600 kcal, with 16 percent of energy (En%) derived from protein, 50 En% carbohydrates, and 34 En% fat) was consumed and subsequent blood samples were drawn 30 and 150 minutes after the liquid mixed meal. Individuals were excluded from the analyses (Supplemental Figure 1) if 1) with self-reported type 1 or type 2 diabetes or taking any glucose-lowering medication, 2) with fasting glucose measures above 7 mmol/L at baseline, 3) with HbA1c above 6.5% at baseline, 4) violating overnight fasting, 5) violating liquid meal challenge protocol.

Genotyping and imputation

DNA was extracted from venous blood samples obtained from the antecubital vein. Genotyping was performed in the Centre National de Génotypage (Evry Cedex, France), using Illumina HumanCoreExome-24 BeadChip (Illumina Inc., San Diego, California, United States of America). The detailed quality control process has been described previously (14). Genotypes were further imputed to the 1000 Genome Project reference panel (v3 2011) (15) using IMPUTE (v2.2) software (16). All genetic variants

with an imputation quality below 0.4 or a minor allele frequency below 0.01 were not considered for the analyses in the present study.

Laboratory insulin measurements and early phase insulin response definitions

Participants fasted for at least 10 hours prior to the NEO study baseline visits. Blood samples were drawn after this overnight fast as well as 30 and 150 minutes after the consumption of a liquid mixed meal. Blood was drawn in tubes that were immersed in ice after collection and frozen. The tube contained frozen serum with a volume of 350ul. Insulin was measured by Insulin kit 200T-L2KIN2, an immunometric sandwich assay method (Siemens Dpc Immulite 2000 analyser, Siemens Healthcare Diagnostics Inc., USA). Glucose concentrations were determined by using standard enzymatic methods (Roche Modular Analytics P800, Almere, The Netherlands).

Early phase insulin response measures were estimated by four different methods: 1) insulin concentration at 30 minutes ($insulin_{30}$), 2) insulin concentration ratio between 30 minutes after the liquid meal and fasting state ($insulin_{30}/insulin_{0}$), 3) insulinogenic index (IGI) defined as a ratio of the area under the curve (AUC) of insulin to glucose, i.e., $AUC_{insulin(0\text{-}30\text{-}150\ minutes)}/AUC_{glucose(0\text{-}30\text{-}150\ minutes)}$, calculated by the trapezium rule, 4) insulin response to glucose during the first 30 minutes adjusted for BMI ($IR_{bmiadj} = insulin_{30}/(glucose_{30} \times BMI)$) (10). The four derived measures were natural logarithm transformed to better approximate normally distributed variables, and the second measure by insulin concentration ratio between 30 minutes after the liquid meal and fasting state was therefore equivalent to the difference of the natural logarithm transformed insulin concentrations between 30 minutes after the liquid meal and fasting state ($\Delta insulin = ln(insulin_{30})-(insulin_{0})$). Extreme measures, i.e., when the natural logarithm transformed values were more than four standard deviations (SD) from the mean were excluded, with one individual being removed in both the discovery and replication population for Δinsulin as well IR_{bmiadj} separately and four individuals removed in the replication population for Δinsulin (Supplemental Figure 1).

Genome-wide association analyses

We conducted the GWAS on all the four pre-defined early phase insulin response measures across 22 autosomal chromosomes. The previous GWAS on type 2 diabetes has been shown no nominal evidence of heterogeneity of estimated odds ratios between obese and non-obese strata (17), so we divided the total NEO study population based on the graphical area of recruitment into a discovery cohort with oversampling on overweight and obese population and a replication cohort with a reference population of no BMI restriction (13). Additive linear regression analyses were performed separately for the discovery and replication cohort by SNPTEST v2, adjusted

for age, sex, BMI and the first four principal components to identify BMI-independent genetic signals. A P-value $<5\times10^{-8}$ was considered to be genome-wide significant, and a P-value $<1\times10^{-6}$ was considered a suggestive signal. Independent SNPs with a P-value $<1\times10^{-6}$ in the discovery stage were validated in the replication cohort. SNPs with a P-value <0.05 in the replication cohort were considered to be a successful replication. To further explore the BMI-dependent signals, association analyses were performed in the entire NEO cohort for the four pre-defined phenotypes, adjusted for age, sex, and the first four principal components.

To identify independent genetic loci that were associated with each early phase insulin response measure, the linkage disequilibrium-based clumping procedure was applied using PLINK (18). As a start, all the SNPs with P-values below a certain threshold ('-clump-p1') were set as "index" SNPs, and the other SNPs were clustered into different clumps based on their linkage disequilibrium (LD) and physical proximity to the "index" SNPs controlled by 'clump-r2' and 'clump-kb' separately in the command (19). In the current analyses, the following parameters were adopted: '--clump-p1', 1.0×10^{-6}, '--clump-r2', 0.1, '--clump-kb', 1000. The linkage disequilibrium patterns were based on the 1000 Genome v3 20101123 reference set of Utah Residents (CEPH) with Northern and Western European Ancestry (CEU) population (15). To further verify the independency of the selected loci from clumping, additional conditional analyses were performed using GCTA tool with the command of "--cojo-cond" (version 1.24.4) (20) in the entire chromosome region of the tag SNP, by conditioning on the tag SNP itself. The explained variance of the independent SNPs in each insulin response measure was estimated in the replication cohort. For each individual SNP the explained variance was estimated as the partial R^2 from the linear regression model, with the SNP as independent variable and insulin response (natural logarithm transformed measures) as dependent variable. In order to increase the statistical power, both discovery and replication cohorts were pooled to run the GWAS as a sensitivity analysis, followed by clumping and conditional analysis (similar to the split cohort analysis). As type 2 diabetes is a heterogeneous disease with more than 150 genetic variants that have been identified from the previous GWAS (21), the associations between T2D-assocaited SNPs and insulin response were further highlighted, to disentangle the potential insulin secretion-related T2D-associated loci.

Functional annotation of top SNPs

The independent top variants were searched in the human pancreatic islet cis-eQTL summary data from A. Varshney et al. (22), to identify the human pancreatic islet-specific expression patterns (expression quantitative trait loci (eQTLs)) of mRNAs associated with these variants. To further verify whether eQTL signals act as the putative

effector transcripts of the GWAS findings, we performed colocalization analysis using (Approximate) Bayes Factor colocalisation analyses (coloc.abf) in the R package "coloc", by p-values extracted from both our GWAS summary data and the eQTL summary data published by Varshney et al. (23; 24). Bayesian colocalization analysis evaluated the posterior probabilities of five different configurations based on a prior probability on the SNP level, and the configuration with the highest posterior probability will be the most likely situation. Subsequently, Bayesian fine mapping analysis was performed by the Bayesian fine mapping analysis (finemap.abf) implemented in the R package "coloc" (24), to determine the probability of the top SNPs to be causal for early phase insulin response. All the SNPs with p-value<5×10^{-8} in the same LD block as the top signal, which was tagged as lead SNP by plink clumping, were tested in the coloc.abf and finemap.abf. The phenome wide association studies (Phewas) of independent top variants was performed by GeneAtlas based on 118 non-binary and 599 binary traits of 408,455 related and unrelated UK Biobank participants (25). The regulatory function of the top variants were screened in HaploReg database (26) to explore their potential chromatin state and regulatory motif alterations.

ABO blood group determined by genotyping in the NEO study

Since the GWAS results indicated that ABO blood type and insulin response to a mixed liquid meal were associated, four *ABO* SNPs (rs8176719:insC, rs7853989:G>C, rs8176749:G>A, rs8176750:delC) were used to discriminate among the common *ABO* alleles: O^1, O^2, A^1, A^2 and B (27; 28) (Supplemental information). Six phenotypic ABO blood types (A_1, A_2, A_1B, A_2B, B, and O) were further derived from the 14 *ABO* allele combinations. Among them, A_1, A_2, A_1B, A_2B, and B were clustered as non-O group in the analysis (29). The comparisons of insulin response difference (Δinsulin) between O and non-O group was performed by Student's t-test, and analysis of variance (ANOVA) was used to compare the significance of the mean differences among the six phenotypic ABO blood type groups on insulin response (Δinsulin). To estimate the effect sizes and with 95% confidence interval (CI) of ABO blood type groups on Δinsulin, linear regression models were used with age and sex being adjusted in the model. For the classification of O and non-O group, non-O group was considered as the reference group; and for the six phenotypic ABO blood group types, blood group A_1 was used as the reference.

Modelling the effects of ABO gene expression changes on insulin secretion in vitro

We assessed the effects of lentivirus-mediated shRNA knock-down of *ABO* gene expression on glucose-stimulated insulin secretion (GSIS) in the murine pancreatic β-cell line MIN6. Lentivirus vectors were obtained from the Sigma MISSION library (murine ABO (shRNA ABO) clone TRCN0000110442; non-target control (shRNA ctrl)

clone SHC-002) and produced in the facility of Prof. RC Hoeben (LUMC, Department of Cell & Chemical Biology) as previously described (30). Cells were used four days post-transduction and all experiments were repeated three times (Supplemental information).

RESULTS

BMI was higher in the discovery cohort than the replication (30.1 kg/m^2 *versus* 25.5 kg/m^2) in all the four derived insulin response measures (Table 1). In addition, fasting glucose and insulin were also slightly higher in the discovery cohort compared with the replication (glucose: 5.5 mmol/L *versus* 5.3 mmol/L, insulin: 10.8-10.9 mU/L versus 7.7-7.8 mU/L). However, there was no difference in HbA1c between the two subpopulation (5.3% versus 5.3%).

Three out of the four early phase insulin response measures were highly correlated (Supplemental Figure 2), whereas Δinsulin (ln(insulin$_{30}$)-ln(insulin$_0$)) showed the most dissimilarity to the other three measures (Pearson correlations to insulin$_{30}$, IGI and IR$_{bmiadj}$: 0.38, 0.21, 0.46 separately). The Manhattan plot for the GWAS in the discovery population on Δinsulin is shown in Figure 1, and the other three Manhattan plots for insulin$_{30}$, IGI and IR$_{bmiadj}$ are shown in Supplemental Figure 4-6. After clumping and conditional analysis on the lead SNPs, six independent genome-wide significant SNPs (P-value<5×10^{-8}) by four different insulin response measures were identified in the discovery phase (Table 2). However, only rs505922:T>C from the GWAS on Δinsulin (Beta (MAF, P-value): -6.5% (0.32, 3.3x10^{-8})) was successfully replicated (Beta (MAF, P-value): -7.8% (0.32, 7.2x10^{-5})). Another ten SNPs across four different measures reached suggestive significance (P-value<1×10^{-6}) levels, which are shown in Supplemental Table 1. However, none of these SNPs were replicated.

Rs505922:T>C, located in the first intron of *ABO* gene, explained 1.2% of the total variation in the natural logarithm transformed insulin response (Δinsulin) and had a per-allele decrease in Δinsulin of 6.5%. This SNP is in high linkage disequilibrium (LD) with the ABO blood type determining SNP rs657152:C>A (r2 = 0.90 in the NEO study). However, the LDs to the previously reported glycaemic-traits associated SNPs located in *ABO* gene are relatively moderate (LDs to rs651007:T>C: 0.40; LDs to rs579459:C>T: 0.40; LDs to rs635634:T>C: 0.48; LDs to rs507666:G>A: 0.49). Rs505922 has been reported as a human pancreatic islet eQTL for *ABO* gene expression in two independent studies with significant p-values (31; 32). In total 34 SNPs located in the same LD-block as rs505922:T>C were tested for the colocalization to the pancreatic islet eQTL reported by A. Varshney et al. (22) and their probability of being causal for the early phase insulin response. By doing the Bayesian colocalization analysis

TABLE 1 Characteristics of the discovery and replication cohort from the Netherlands Epidemiology of Obesity (NEO) study.

Characteristics	$Insulin_{30}$		ΔInsulin		Insulinogenic index (IGI)		Insulin response adjusted by BMI (IR_{bmiadj})	
	Discovery	**Replication**	**Discovery**	**Replication**	**Discovery**	**Replication**	**Discovery**	**Replication**
N	3,526	1,239	3,518	1,233	3,441	1,202	3,503	1,228
Age (year)	56 (51, 61)	56 (51, 61)	56 (51, 61)	56 (51, 61)	56 (51, 61)	56 (51, 61)	56 (51, 61)	56 (51, 61)
Women (N (%))	1,757 (50.0%)	678 (54.7%)	1,753 (49.8%)	674 (54.7%)	1,701 (49.4%)	654 (54.4%)	1,743 (49.8%)	669 (54.5%)
BMI (kg/m^2)	30.1 (28.3, 32.6)	25.5 (23.2, 27.9)	30.1 (28.3, 32.6)	25.5 (23.3, 27.9)	30.1 (28.3, 32.6)	25.5 (23.2, 27.9)	30.1 (28.3, 32.6)	25.5 (23.2, 27.9)
Fasting serum measures								
Glucose (mmol/L)	5.5 (5.2, 5.9)	5.3 (5.0, 5.6)	5.5 (5.2, 5.9)	5.3 (5.0, 5.6)	5.5 (5.2, 5.9)	5.3 (5.0, 5.6)	5.5 (5.2, 5.9)	5.3 (5.0, 5.6)
Insulin (mU/L)	10.9 (7.5, 15.7)	7.8 (5.2, 11.7)	10.9 (7.5, 15.7)	7.8 (5.2, 11.7)	10.8 (7.4, 15.6)	7.7 (5.2, 11.7)	10.9 (7.5, 15.7)	7.8 (5.2, 11.7)
HbA1c (%)	5.3 (5.2, 5.5)	5.3 (5.1, 5.4)	5.3 (5.2, 5.5)	5.3 (5.1, 5.4)	5.3 (5.2, 5.5)	5.3 (5.1, 5.4)	5.3 (5.2, 5.5)	5.3 (5.1, 5.4)
Serum measures at 30 minutes								
Glucose (mmol/L)	7.1 (6.2, 8.1)	6.2 (5.3, 7.2)	7.1 (6.2, 8.1)	6.2 (5.3, 7.2)	7.1 (6.2, 8.1)	6.2 (5.3, 7.2)	7.1 (6.2, 8.1)	6.2 (5.3, 7.2)
Insulin (mU/L)	61.4 (42.8, 89.6)	49.1 (34.9, 69.1)	61.3 (42.7, 89.6)	49.1 (34.9, 69.0)	61.3 (42.8, 89.4)	48.8 (34.6, 68.9)	61.4 (42.8, 89.6)	49.1 (34.8, 69.1)

Results are presented as median (inter quartile range) or number (percentage).
BMI, body mass index; HbA1c, hemoglobin A1c.

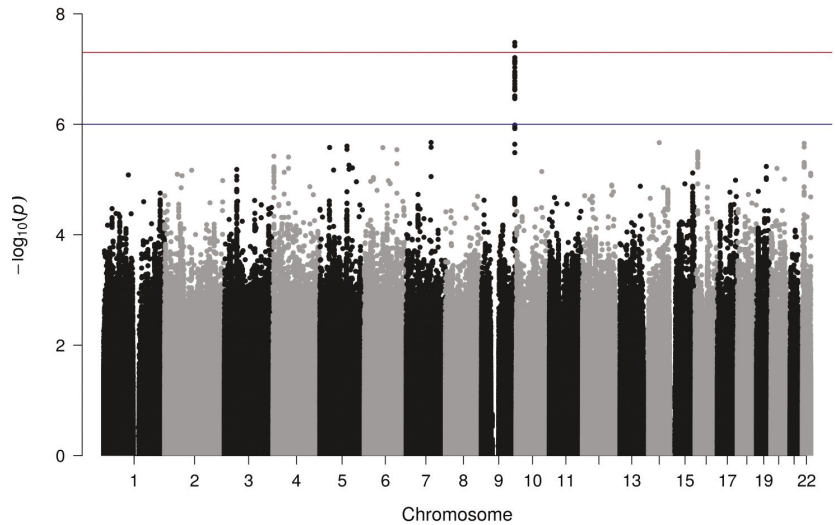

FIGURE 1 Manhattan plot for the genome-wide association study on Δinsulin (ln(insulin$_{30}$)-ln(insulin$_{0}$)) in the discovery cohort (n=3,518).
The red line represents the threshold for genome-wide significance (5×10^{-8}). The blue line represents the threshold for suggestive signals (1×10^{-6}).

on the 34 SNPs in the same LD-block as rs505922:T>C, the hypothesis H4, indicating the posterior probability of both traits being associated and sharing a single causal variant, was 96.7%, indicating a shared causal variant associated with both early-phase insulin response to a meal, and pancreatic islet ABO gene expression. The median probability of these tested 34 SNPs was 3%, with the highest probability of 8% being assigned to rs505922:T>C. From the regulatory annotations in HaploReg database, rs505922:T>C resides in a DNase I hypersensitive site in the pancreas and has enhancer marks (e.g., H3K4me1 and H3K4me3) recorded in the ENCODE Consortium data. By the Phewas of rs505922:T>C in the UK Biobank, 35 traits reached the genome-wide significant threshold (P-value<5×10^{-8}), and the top associated phenotype is the venous thromboembolic disease (P-value= 7.0×10^{-86}) (Supplemental Table 2).

Among 151 previously identified T2D-associated SNPs , 143 were found in the current NEO imputed genotype data (5 SNPs were removed due to MAF<1% and 3 SNPs were not present in the imputed data). Figure 2 shows the associations of these T2D-associated SNPs to Δinsulin in the entire NEO study population. None of the signals reached genome-wide significance (P-value<5×10^{-8}). However, when the significance level was set to the candidate SNP-wide significance (P-value<5.2×10^{-4}, 0.05/96, where 96 is the number of independent loci), rs2877716:T>C located in the intron of *ADCY5* was significant. Another six T2D-assocaited SNP reached candidate SNP-wide suggestive significance (P-value<1×10^{-2}).

TABLE 2 Independent lead SNPs that reached genome-wide significance under four different measures of early phase insulin response.

									Discovery cohort			Replication cohort		
Chr	**SNP**	**Phenotype**	**Position**	**Location**	**Gene**	**Effect/ Non-effect allele**	**Effect allele frequency[a]**	**Imputation quality[a]**	**Effect size per allele[b] (%)**	**SE**	**P-value**	**Effect size per allele[b] (%)**	**SE**	**P-value**
Lead SNPs														
3	rs115404340	$insulin_{30}$	67839179	Intron	*SUCLG2-AS1*	G/A	0.01	0.44	78.5	0.098	3.8E-9	-15.7	0.13	0.19
9	rs505922	ΔInsulin	136149229	Intron	*ABO*	C/T	0.32	1	-6.5	0.012	3.3E-8	-7.8	0.021	7.2E-5*
9	rs657152	IGI	136139265	Intron	*ABO*	A/C	0.34	0.98	8.1	0.011	3.0E-12	2.7	0.017	0.11
19	rs74889068	IGI	46199363	Intron	*QPCTL*	A/G	0.15	0.93	-8.8	0.016	3.8E-9	-2.1	0.024	0.36
3	rs115404340	IR_{bmiadj}	67839179	Intron	*SUCLG2-AS1*	G/A	0.01	0.42	72.5	0.092	3.4E-9	-9.8	0.12	0.41
19	rs74889068	IR_{bmiadj}	46199363	Intron	*QPCTL*	A/G	0.15	0.93	-10.9	0.018	4.4E-11	-4.0	0.028	0.14

Threshold for genome-wide significance is 5×10^{-8}. *A successful replication with p-value< 0.05.
[a] In the discovery cohort.
[b] Beta coefficient expressed as the percentage difference in the outcome by one copy of the effect allele.
Chr, chromosome; SNP, single nucleotide polymorphism; SE, standard error.

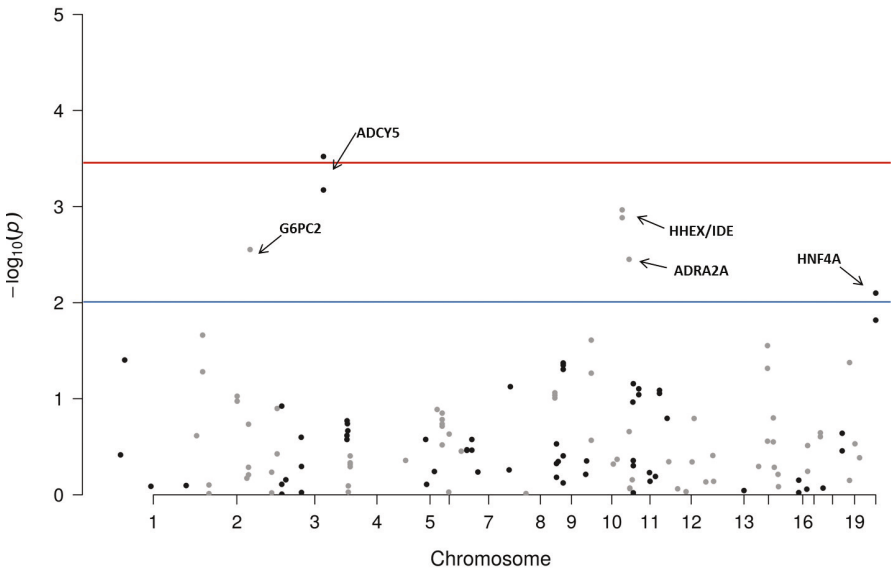

FIGURE 2 The associations between T2D-assocaited SNPs (N=143) and Δinsulin (ln(insulin$_{30}$)-ln(-insulin$_0$)) in the entire NEO cohort (N=4,751)
The red line represents the SNPs with p-value <5.2×10^{-4} (0.05/96, 96 being the number of independent loci). The blue line represents the SNPs with p-value <1×10^{-2}.

After pooling the discovery and replication cohort, a GWAS was performed on the Δinsulin measure in all the NEO samples (Supplemental Figure 7). After clumping and conditional analysis, two independent signals were identified (Supplemental Table 3). The top signal, rs676996:T>G, is in the same locus as rs505922:T>C (LD=0.97, Beta (MAF, P-value): -6.9% (0.32, 1.3x10^{-11})) located within the first intron of *ABO* gene. A new signal was identified in the second intron of *QPCTL* (rs2287019:C>T, Beta (MAF, P-value): -6.9% (0.19, 3.3x10^{-8})). However rs2287019 had a much weaker signal in discovery cohort alone (Beta (MAF, P-value): -5.5% (0.19, 1.4x10^{-4})). Compared with the top signals after BMI adjustment, BMI-dependent GWASs (without BMI adjustment in the model) for all the four pre-defined early phase insulin response measures identified nearly the same loci (Supplemental Figure 8-11).

The Δinsulin was further compared across six ABO blood types as well as the combined O and non-O blood type groups in the entire NEO population (Figure 3a-c). On average, insulin concentrations were lower among O blood type group than non-O both at fasting baseline and 30 minutes after the mixed meal challenge (Figure 3a), with a slope difference test p-value of 5.3×10^{-4}. Similarly, a stronger insulin response (Δinsulin) was observed in the O blood group individuals than the non-O group (Figure 3c, t-test p-value=1.8×10^{-4}), indicating a higher potential of postprandial insulin concentrations to increase among O blood group versus non-O blood group carriers.

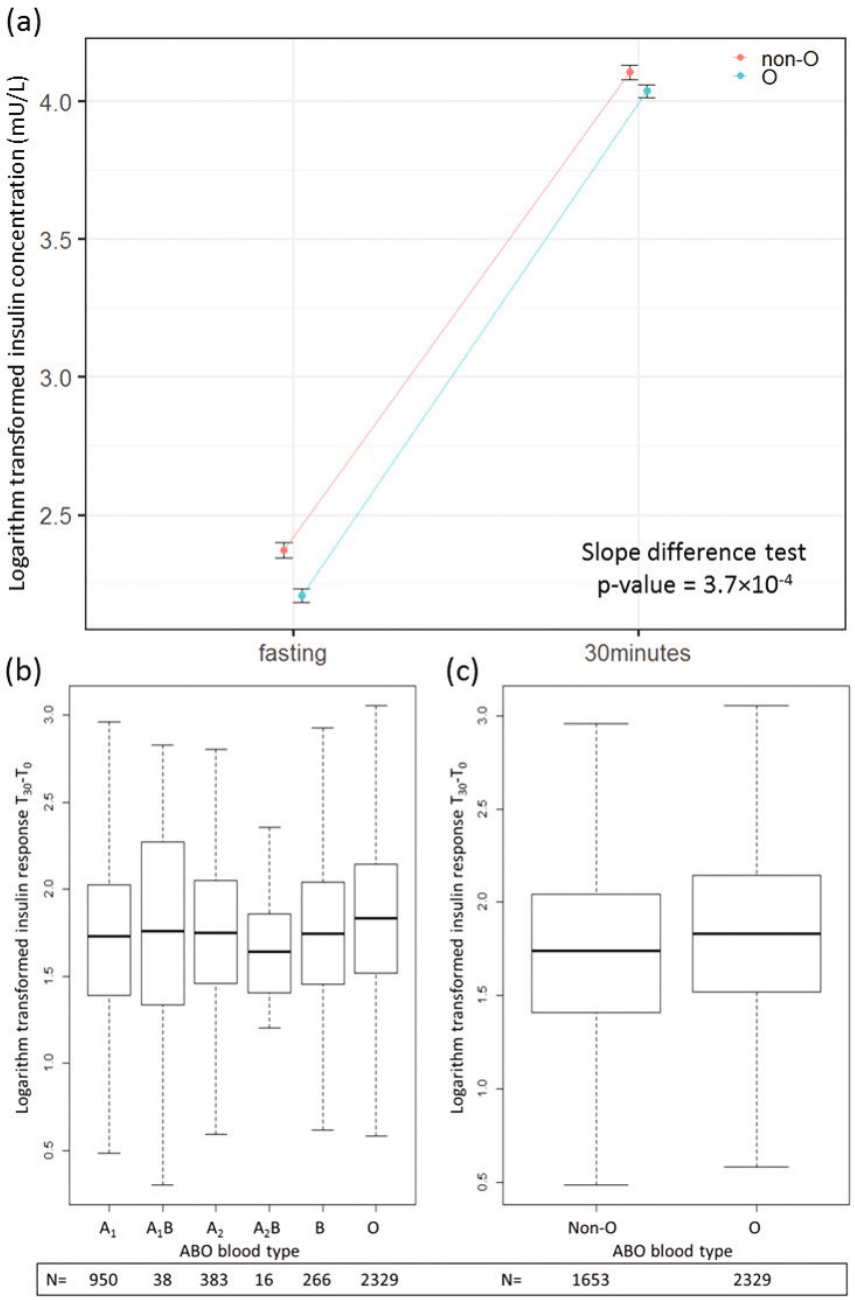

FIGURE 3 The distributions of natural logarithm transformed insulin response across different ABO blood groups in the NEO study. (a). A comparison of natural logarithm transformed insulin concentrations between O and non-O blood groups at fasting and 30 minutes after the liquid meal. (b). Comparisons between six phenotypic ABO blood type groups. (c). A comparison between O and non-O blood groups.

Compared with non-O group, individuals with O blood type group had a larger Δinsulin of 0.090 (95%CI: 0.060-0.12).

Next, *in vitro* experiments were performed in the murine pancreatic β-cell line MIN6. *ABO* gene expression was reduced by 60% in MIN6 cells transduced with the shRNA ABO lentivirus compared with the non-target shRNA controls (Supplemental Figure 12a). After glucose stimulation, the increase in insulin secretion was lower after transduction with the shRNA ABO lentivirus than the controls, indicating that down-regulation of the *ABO* gene led to a reduced glucose-stimulated insulin secretion (Supplemental Figure 12b).

DISCUSSION

Rs505922 located in the first intron of the *ABO* gene was associated with Δinsulin and was also successfully replicated in another subsample of the NEO population. Further analyses revealed that ABO blood type and Δinsulin were associated. Blood group O carriers had a lower fasting insulin level, but showed a larger increase upon the mixed meal than the non-O blood group carriers. By *in vitro ABO* gene down regulation, a decreased insulin secretion was observed.

A recent study reported the association between rs505922:C in the *ABO* gene and an increased risk of T2D (OR=1.06, 95%CI: 1.04-1.09) (33). Rs505922 is in strong LD (r2=0.90) with the ABO blood type determining SNP rs8176719, and in a previous observational study (8), it was shown that the non-O blood group carriers have an increased risk of developing T2D. It can therefore be hypothesized that beta-cell function and insulin secretion capacity are different between the different ABO blood groups, which subsequently leads to different risks of T2D. The ABO gene encodes a glycosyltransferase that determines the glycosylation status of the glycoprotein H antigen that is expressed on all normal blood red cells. The A and B alleles encode enzymes with different activity, whereas the O allele is a null-variant. The ABO gene is expressed in a variety of tissues and thus in theory could affect the glycosylation status of numerous proteins. Our results indicate the activity of one or more of the proteins that are directly or indirectly involved in insulin secretion and/or clearance after a meal are affected by ABO mediated glycosylation status. Since the substrate specificity of the ABO protein beyond the glycoprotein H antigen is poorly defined, the identity of these proteins requires additional work. However, given the pleiotropic nature of the disease associations observed with the ABO gene locus, it is likely that ABO affects a variety of substrates. Although there are extensive differences between *in vitro* and *in vivo* conditions, the *in vitro* experiment described in the current study showed a clear effect on glucose stimulated insulin secretion after *ABO* gene down regulation in a

mouse cell line. Taken together, the previously reported high risk of T2D in those with non-O blood groups may be mediated through an increased baseline insulin level and decreased capacity to increase insulin levels in the early phase insulin response.

In addition to rs505922, alternative loci near the *ABO* gene have been associated with either fasting glucose levels (rs651007, rs579459, rs507666) or the risk of T2D (rs635634) (7; 34). All of these four SNPs are located in the intergenic region near the *ABO* gene. However, these SNPs show modest LDs to rs505922, which resides in the first intron of *ABO* gene. This observation may be very well explained by the omnigenic model for complex traits in the GWAS (35). In this hypothetical model, the disease risk is affected by a few strong effect "core" functional genetic variants as well as numerous small effect "peripheral" genes. Together they form a highly connected network. Some perturbation on the peripheral genes will transmit to the "core" gene and affect its function. It has also been shown that disease-associated genetic signals are significantly enriched in regions that are transcribed actively, which partially explained the observation of a large amount of genetic variants identified from GWAS located in the gene regulatory regions. Therefore, rs505922 may be another "peripheral" genetic signal that is involved in type 2 diabetes pathophysiological pathways, similar to other genetic markers identified previously in the intron and intergenic regions of *ABO* gene and other type 2 diabetes associated genes.

Several methodological aspects should be considered. The main strength of this study is the liquid meal that was provided to all the NEO participants. Moreover, epidemiological data were further investigated with functional analyses to verify the role of the *ABO* gene in the early phase insulin response. Nonetheless, the sample size of the genome-wide association study was too small to identify genetic variants with low-frequency and rare variants. Finally, another strength is that we followed up the top signal of the GWAS with an *in vitro* experiment.

CONCLUSION

A genetic variant, rs505922 in an intron of the *ABO* gene showed an association with the early phase insulin response to a liquid meal measured by Δinsulin (log ($insulin_{30}$/$insulin_0$)), and a subsequent *in vitro* analysis showed that knocking down the *ABO* gene affected glucose stimulated insulin secretion. A phenotypic difference in Δinsulin between O and non-O blood type groups was found, which may explain the role of ABO blood type in the risk of developing T2D.

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DUALITY OF INTEREST

Dennis Mook-Kanamori is a part-time clinical research consultant for Metabolon, Inc. All other authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

R. Li-Gao performed the analysis and wrote and edited the manuscript. F. Carlotti conducted the in vitro experiment and drafted and reviewed the manuscript. R. de Mutsert and F. R. Rosendaal contributed to study design and manuscript review. A. van Hylckama Vlieg, E. J. P. de Koning and J.W. Jukema contributed to results interpretation and manuscript review. K. Willems van Dijk and D. O. Mook-Kanamori contributed to study design, conceive the idea of the current study, interpret the results and review the manuscript.

GUARANTORS

R.Li-Gao is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

PRIOR PRESENTATION

Parts of this study were presented in abstract form at Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium meeting on 18-19 April 2018.

DATA AVAILABILITY STATEMENTS

The datasets generated (all the GWAS summary statistics results) during and analyzed during the current study are available from the corresponding author on request.

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SUPPLEMENTAL INFORMATION

ABO blood group determined by genotyping in the NEO study

In the NEO genotype data, only rs8176749:G>A was directly genotyped on the array. Rs7853989:G>C was derived by imputation, with an imputation quality of 0.99. Rs8176719:insC and rs8176750:delC were not available and replaced by rs8176645:A>T (R2=0.98 with rs8176719:insC, imputation quality of 0.80) and rs8176704:G>A (R2=0.99 with rs8176750:delC, imputation quality of 0.99) separately. The best guess genotypes for the imputed SNPs were determined by the highest probability of the genotype with a minimum threshold of 0.7. If the highest probability of the genotype was below the minimum threshold, the genotype of that SNP was set to missing. Theoretically, 15 combinations of the four ABO alleles were possible, and 14 out of the 15 combinations were observed in the current NEO study population.

Modelling the effects of ABO gene expression changes on insulin secretion in vitro

The knock-down efficiency was assessed by Q-PCR (Light Cycler 480-II Real-time PCR system - Roche) on total RNA extracted using RNeasy kit (Qiagen), and reversed transcribed using M-MLV reverse transcriptase (Invitrogen). Fold induction was calculated using the DeltaCT method with GAPDH as housekeeping gene. The primers used were the following: ABO-Fw: ACAGACACTGAACCATCCTGGGTT; ABO-Rv: AGACAAACACTGCGAAGGGAAGGA; GAPDH-Fw: ATCATCCCTGCATCCACTG; GAPDH-Rv: ATCATACTTGGCAGGTTTCTCC.

Transduced MIN6 cells were subjected to a GSIS assay. Briefly, cells were pre-incubated in a modified Krebs-Ringer bicarbonate buffer with HEPES (KRBH) containing 2 mmol/l glucose for 1.5 h at 37°C. They were then incubated in 2 mmol/l glucose KRBH buffer for 1 h at 37°C, then switched to 25 mmol/l glucose KRBH buffer for 1 h at 37°C, and again switched to 2 mmol/l glucose KRBH buffer for 1 h at 37°C. Insulin concentrations were determined in supernatant fractions by ELISA (Mercodia, Uppsala, Sweden). At the end of the assay, MIN6 cells were lysed by sonication in distilled water and total DNA content was determined by Quant-iT PicoGreen dsDNA kit (Invitrogen) for normalization.

SUPPLEMENTAL TABLE 1 Independent suggestive SNPs by the four different measures of early phase insulin response.

									Discovery cohort			Replication cohort		
Chr	**SNP**	**Phenotype**	**Position**	**Location**	**Gene**	**Effect/ Non-effect allele**	**Effect allele frequency[a]**	**Imputation quality**	**Effect size per allele[b] (%)**	**SE**	**P-value**	**Effect size per allele[b] (%)**	**SE**	**P-value**
Suggestive SNPs														
19	rs74889068	$Insulin_{30}$	46199363	Intron	*QPCTL*	A/G	0.15	0.94	-9.7	0.019	6.1E-8	-3.0	0.029	0.28
9	rs657152	$Insulin_{30}$	136139265	Intron	*ABO*	A/C	0.34	1	7.2	0.014	3.2E-7	1.0	0.021	0.64
3	rs62254949	$Insulin_{30}$	67933644	Intron	*SUCLG2-AS1*	G/A	0.12	0.95	10.5	0.020	7.7E-7	0.9	0.032	0.78
3	rs115404340	IGI	67839179	intron	*SUCLG2-AS1*	G/A	0.010	0.44	49.5	0.081	6.0E-7	-5.0	0.10	0.62
12	rs117236472	IGI	28121063	intron	*PTHLH*	T/C	0.014	0.65	32.5	0.057	7.6E-7	-7.3	0.093	0.41
17	rs34958340	IGI	48709115	nc transcript	*LOC101927253*	T/C	0.013	0.53	37.2	0.064	9.6E-7	-5.1	0.075	0.49
5	rs147351017	IR_{bmiadj}	132373452	intergenic	*ZCCHC10/HSPA4*	G/A	0.015	0.56	-28.2	0.066	5.1E-7	4.3	0.12	0.68
9	rs657152	IR_{bmiadj}	136139265	intron	*ABO*	A/C	0.34	1	6.5	0.013	6.1E-7	1.3	0.020	0.45
18	rs112752032	IR_{bmiadj}	24428770	intron	*AQP4-AS1*	G/A	0.011	0.41	54.4	0.088	7.8E-7	14.3	0.15	0.41
17	rs141652399	IR_{bmiadj}	78397039	intron	*ENDOV*	T/C	0.019	0.55	34.8	0.061	8.0E-7	-4.0	0.088	0.69

Threshold for the suggestive signals is 1×10^{-6}.
*A successful replication with p-value< 0.05.
[a] In the discovery cohort.
[b] Beta coefficient expressed as the percentage difference in the outcome by one copy of the effect allele.
Chr, chromosome; SNP, single nucleotide polymorphism; SE, standard error; nc transcript, transcript variant of a non-coding RNA gene.

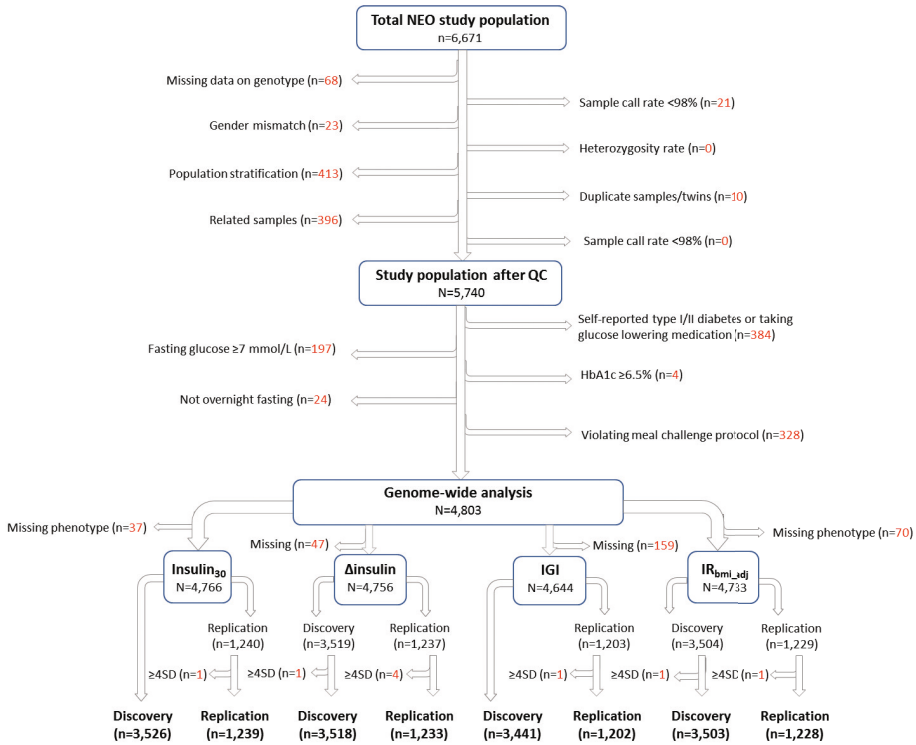
SUPPLEMENTAL TABLE 2 Phewas on rs505922 in the UK Biobank, with all the genome-wide significant phenotypes reported.

Phenotype	Beta	P-values
venous thromboembolic disease	-0.007	6.95E-86
deep venous thrombosis (dvt)	-0.006	6.27E-72
Haematocrit percentage	0.108	3.59E-68
Haemoglobin concentration	0.036	2.88E-66
I80 Phlebitis and thrombophlebitis	-0.004	4.54E-55
Red blood cell (erythrocyte) count	0.011	2.25E-48
Monocyte count	0.006	2.62E-40
pulmonary embolism +/- dvt	-0.003	9.56E-39
I26 Pulmonary embolism	-0.003	1.33E-37
I26-I28 Pulmonary heart disease and diseases of pulmonary circulation	-0.003	9.96E-33
White blood cell (leukocyte) count	0.043	2.75E-21
Impedance of arm (left)	-0.774	6.80E-21
Neutrophill count	0.028	5.79E-20
Impedance of arm (right)	-0.726	2.59E-19
Monocyte percentage	0.050	5.28E-18
Mean reticulocyte volume	-0.130	5.60E-16
high cholesterol	-0.006	2.58E-15
I84 Haemorrhoids	0.004	4.72E-14
Arm fat percentage (left)	-0.118	1.25E-13
Platelet distribution width	0.008	2.17E-13
Arm fat percentage (right)	-0.116	2.77E-13
Mean sphered cell volume	-0.076	8.28E-13
K57 Diverticular disease of intestine	0.004	1.76E-11
Trunk fat percentage	-0.102	2.66E-11
Trunk fat-free mass	0.039	2.80E-09
Trunk predicted mass	0.037	5.47E-09
Arm fat-free mass (left)	0.006	1.32E-08
Arm predicted mass (left)	0.005	1.76E-08
Eosinophill count	0.002	1.94E-08
K55-K64 Other diseases of intestines	0.005	3.39E-08
M79 Other soft tissue disorders, not elsewhere classified	-0.002	3.60E-08
Arm predicted mass (right)	0.005	3.71E-08
Arm fat-free mass (right)	0.005	4.05E-08
Body fat percentage	-0.073	4.07E-08
Lymphocyte percentage	-0.087	4.38E-08

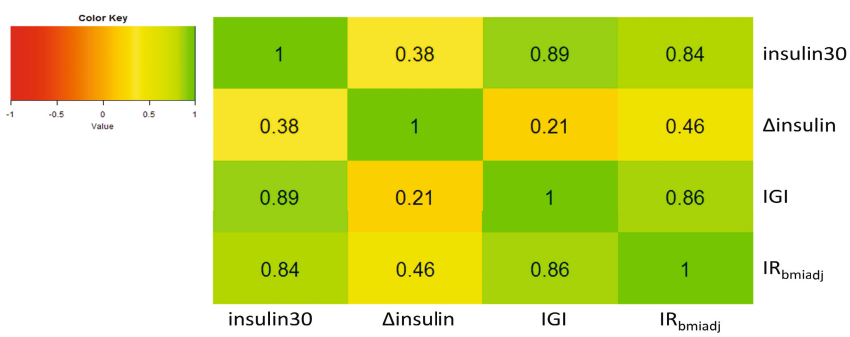
SUPPLEMENTAL TABLE 3 Genome-wide significant SNPs on Δinsulin in the entire NEO cohort (N=4,751).

								Discovery cohort (N=3,518)			Entire NEO cohort (N=4,751)		
Chr	**SNP**	**Position**	**Location**	**Gene**	**Effect/ Non-effect allele**	**Effect allele frequency[a]**	**Imputation quality**	**Effect size per allele[b] (%)**	**SE**	**P-value**	**Effect size per allele[b] (%)**	**SE**	**P-value**
9	rs676996*	136146077	intron	*ABO*	G/T	0.32	0.98	-6.4	0.012	6.2E-8	-6.9	0.011	1.3E-11
19	rs2287019	46202172	intron	*QPCTL*	T/C	0.19	1	-5.6	0.015	1.4E-4	-6.9	0.013	3.3E-8

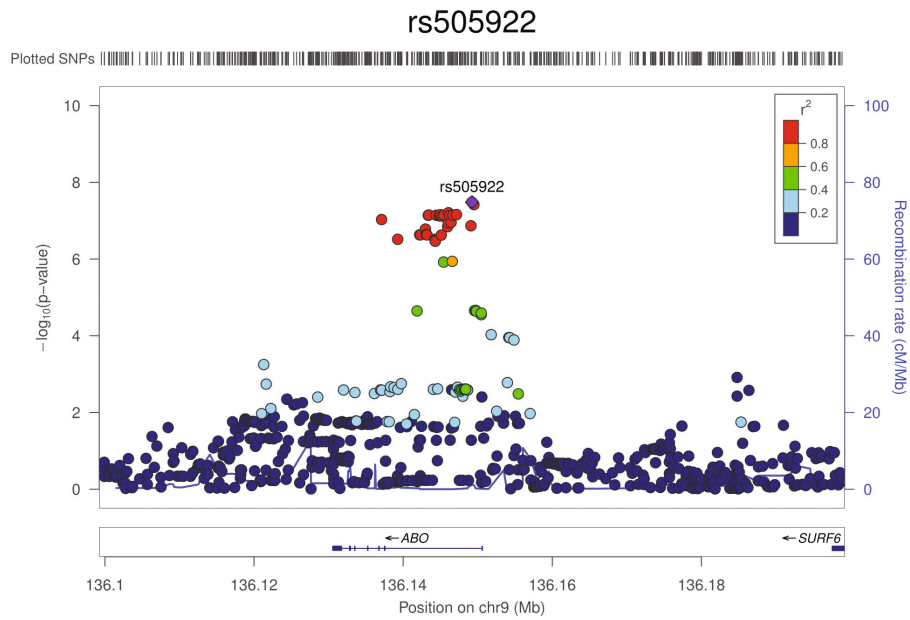
Threshold for genome-wide significance is 5×10^{-8}.
* The LD between rs676996 and rs505922 is 0.97 in the entire NEO population.
[a] Coding allele frequency in the entire NEO cohort.
[b] Beta coefficient expressed as the percentage difference in the outcome by one copy of the effect allele.
Chr, chromosome; SNP, single nucleotide polymorphism; SE, standard error.



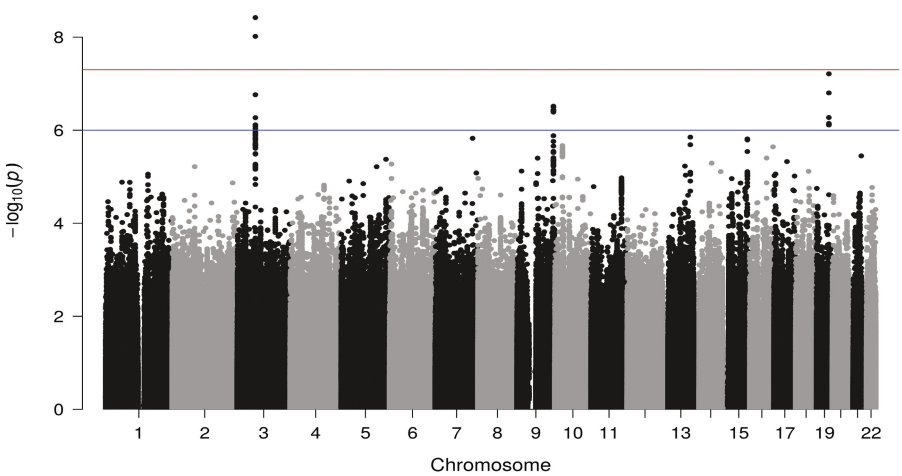
SUPPLEMENTAL FIGURE 1 Quality control steps for the genome-wide association analyses with four different measures of insulin response and exclusion criteria for the discovery and replication cohort.



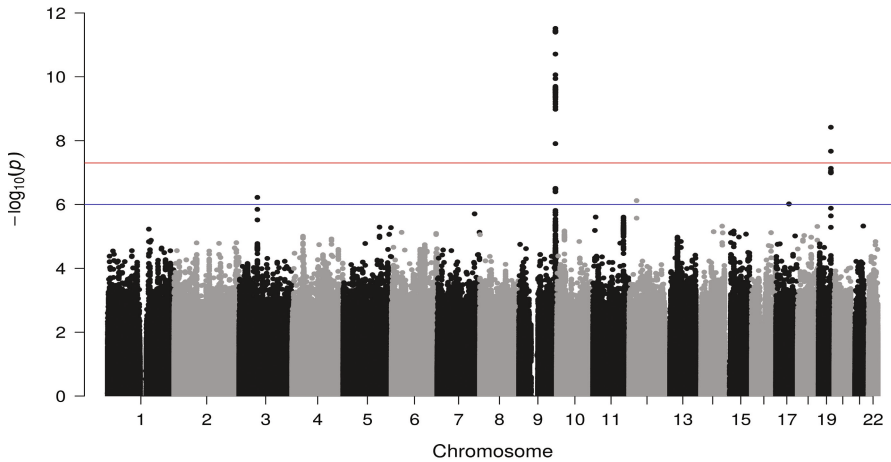
SUPPLEMENTAL FIGURE 2 Phenotypic correlation matrix of four different measures of the early phase insulin response.



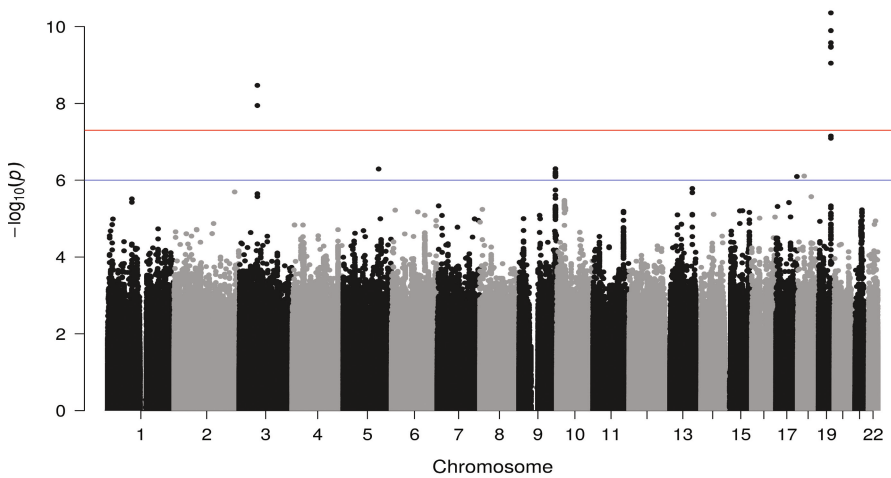
SUPPLEMENTARY FIGURE 3 Regional association plot for the independent genome-wide significant SNP identified from Δinsulin, i.e. rs505922. The purple diamond indicates the lead SNP for the locus.



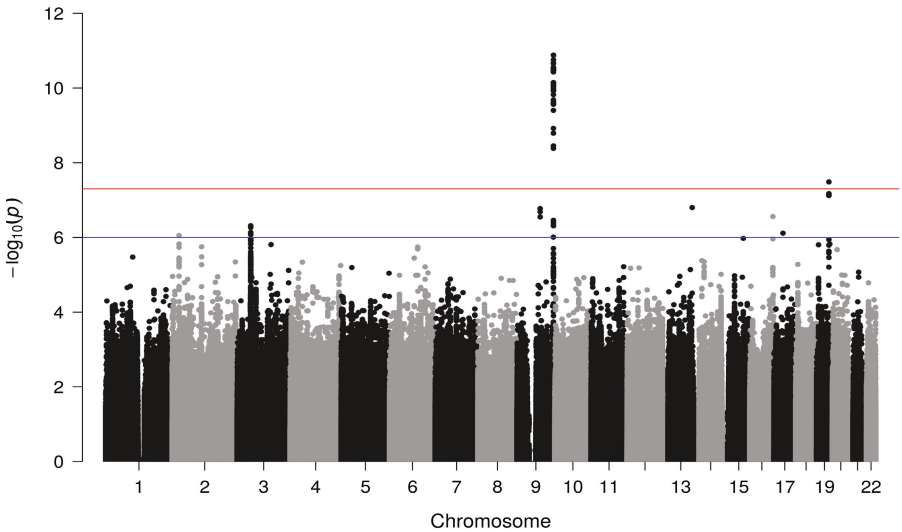
SUPPLEMENTAL FIGURE 4 Manhattan plot for the genome-wide association study on insulin measures at 30 minutes ($insulin_{30}$) in the discovery cohort (n=3,526).



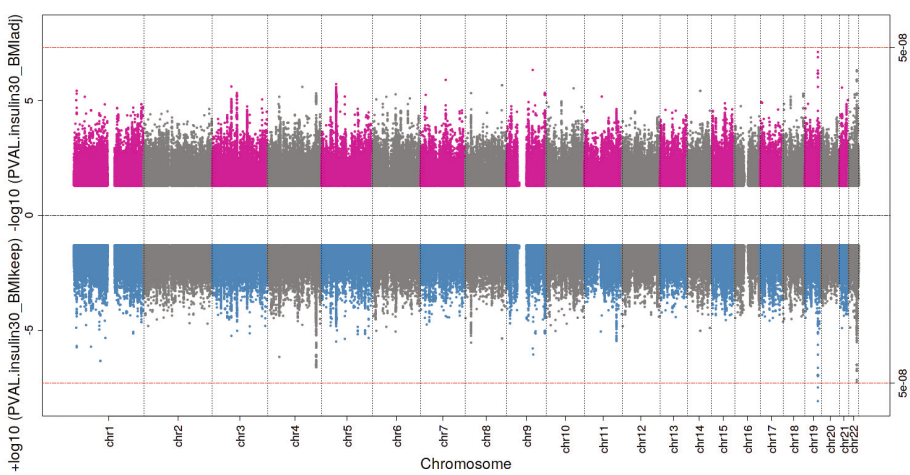
SUPPLEMENTAL FIGURE 5 Manhattan plot for the genome-wide association study on insulinogenic index (IGI) in the discovery cohort (n=3,441).



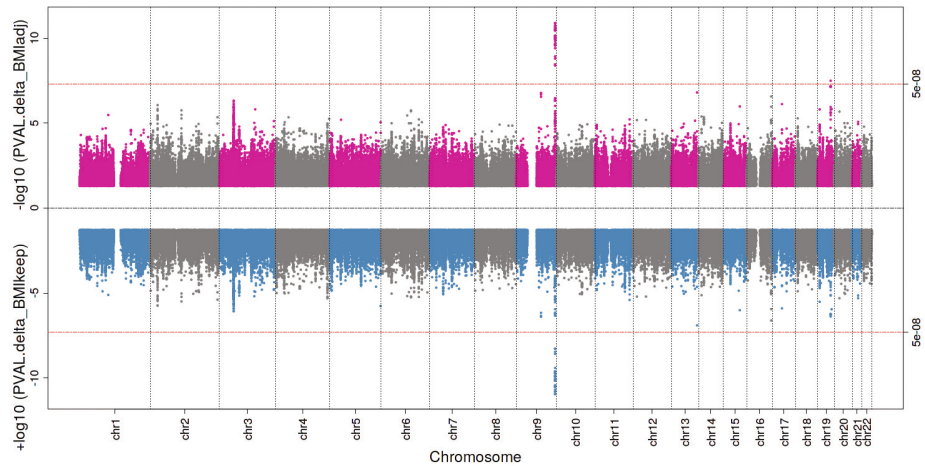
SUPPLEMENTAL FIGURE 6 Manhattan plot for the genome-wide association study on insulin response to glucose at 30 minutes, adjusted on BMI (IR_{bmiadj}) in the discovery cohort (n=3,503).



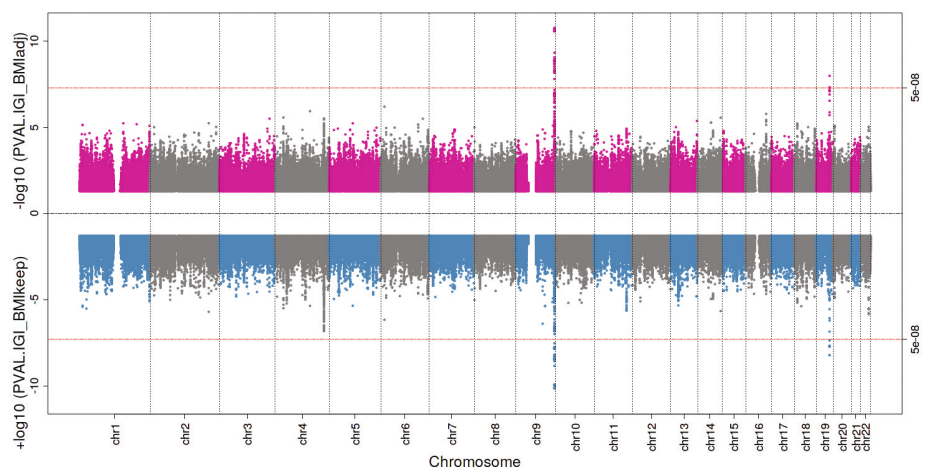
SUPPLEMENTAL FIGURE 7 Manhattan plot for the genome-wide association study on Δinsulin (Δinsulin=ln(insulin$_{30}$)- ln(insulin$_{0}$)) in the entire NEO cohort (n=4,751).



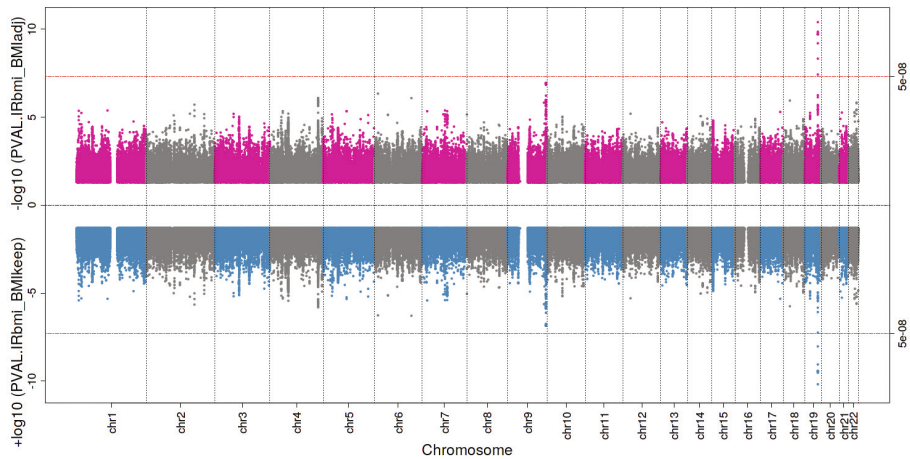
SUPPLEMENTAL FIGURE 8 Miami plot for the genome-wide association study on insulin measures at 30 minutes (insulin$_{30}$) in the entire NEO cohort (n=4,765), with (top panel) /without (bottom panel) adjustment on BMI.



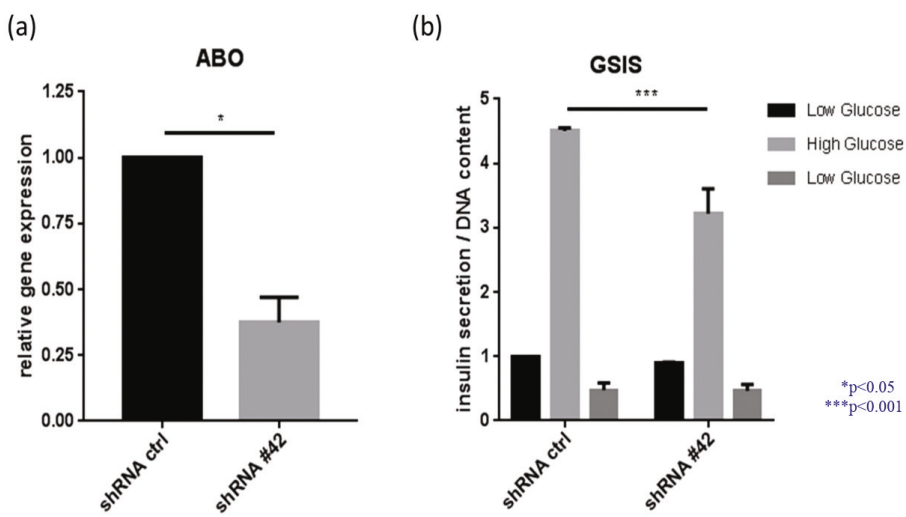
SUPPLEMENTAL FIGURE 9 Miami plot for the genome-wide association study on Δinsulin (Δinsulin=ln(insulin$_{30}$)- ln(insulin$_{0}$)) in the entire NEO cohort (n=4,751), with (top panel) /without (bottom panel) adjustment on BMI.



SUPPLEMENTAL FIGURE 10 Miami plot for the genome-wide association study on insulinogenic index (IGI) in the entire NEO cohort (n=4,642), with (top panel) /without (bottom panel) adjustment on BMI.



SUPPLEMENTAL FIGURE 11 Miami plot for the genome-wide association study on insulin response to glucose at 30 minutes, adjusted on BMI (IR_{bmiadj}) in the entire NEO cohort (n=4,731), with (top panel) /without (bottom panel) adjustment on BMI.



SUPPLEMENTAL FIGURE 12 Modelling the effects of ABO gene expression changes on insulin secretion in vitro: (a) *ABO* gene expression is reduced by 60% in MIN6 cells transduced with the shRNA ABO lentivirus as compared with non-target shRNA ctrl. (b) Glucose-stimulated insulin secretion (GSIS) tests performed on MIN6 cells transduced with shRNA ABO or shRNA ctrl lentivirus.