

A genetic variant in *HK1* is associated with pro-anemic state and HbA1c but not other glycemic control related traits

Running head: *HK1* variant, glycated hemoglobin and anemia

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Objective: HbA1c is widely considered as a gold standard for monitoring effective blood glucose levels. Recently, a genome-wide association study reported an association between HbA1c and rs7072268 within *HK1* (encoding Hexokinase 1) which catalyzes the first step of glycolysis. HK1 deficiency in red blood cells (RBC) causes severe nonspherocytic hemolytic anemia in both humans and mice.

Research Design and Methods: The contribution of rs7072268 to HbA1c and the RBC-related traits was assessed in 6,953 non-diabetic (ND) European participants. We additionally analyzed the association with hematologic traits in 5,229 ND European individuals (where HbA1c was not measured) and 1,924 diabetic patients. Other glucose control related markers than HbA1c were analyzed in 18,694 ND European individuals. A type 2 diabetes case-control study included 7,447 French diabetic patients.

Results: Our study confirms a strong association between rs7072268-T allele and increased HbA1c ($\beta=0.029\%_{\text{HbA1c}}, P=2.22\times 10^{-7}$). Surprisingly, despite an adequate study power, rs7072268 showed no association with any other marker of glucose control (fasting- and 2h-post-OGTT-related parameters; $N=18,694$). In contrast, rs7072268-T allele decreases hemoglobin levels ($N=13,416; \beta=-0.054\text{g/dl}, P=3.74\times 10^{-6}$) and hematocrit ($N=11,492; \beta=-0.13\%_{\text{Hematocrit}}, P=2.26\times 10^{-4}$), suggesting a pro-anemic effect. The T-allele also increases risk for anemia ($N_{\text{cases}}=846, \text{OR}=1.13, P=0.018$).

Conclusions: *HK1* variation although strongly associated with HbA1c does not seem to be involved in blood glucose control. Since *HK1* rs7072268 is associated with reduced hemoglobin levels and favors anemia, we propose that HK1 may influence HbA1c levels through its anemic effect and/or its effect on glucose metabolism in RBC. These findings may have implications for T2D diagnosis and clinical management, as anemia is a frequent complication of the diabetic state.

Type 2 diabetes (T2D) is a major source of early excess morbidity and mortality that results from the lack of adequate control of blood glucose in most diabetic patients (1). In the absence of widely available continuous glucose monitoring, the glycosylated hemoglobin (HbA1c) assay has become the most popular index to evaluate the efficiency of T2D treatments on long-term blood glucose control (2; 3). HbA1c which is formed through the non-enzymatic attachment of glucose to the N-terminal of the β chain of hemoglobin is indeed commonly considered as a surrogate marker of mean blood glucose concentration over the previous 8–12 weeks (*i.e.* 120 days lifespan of erythrocytes) (4). Furthermore, the HbA1c assay is often used for confirming T2D diagnosis when fasting plasma glucose (FPG) is in the pre-diabetic range ($6.1 \text{ mM} \leq \text{FPG} < 7.0 \text{ mM}$ that define normal glycemia and overt diabetes (2)), as post prandial or post glucose load measurements of blood glucose are difficult to widely apply in clinical practice. However, the HbA1c measurement displays well known caveats such as genetically inherited hemoglobin defects or red blood cell (RBC) life span heterogeneity in hematologically normal people that would oblige the use of more complex measurement of glycosylated serum proteins or fructosamine as a surrogate of blood glucose levels (5; 6).

So far, several genome-wide association studies (GWAS) have identified 22 genes or loci increasing the risk for T2D and/or modulating FPG levels (7-19). Recently, Paré et al. reported a single nucleotide polymorphism (SNP) rs7072268 at the *Hexokinase 1* (*HK1*) locus (chr10q22) that strongly associates with increased HbA1c in a non-diabetic population (20). The four isozymes of the hexokinase family (named HK1, HK2, HK3 and glucokinase) contribute to commit glucose to the glycolytic pathway. The predominant HK1 isozyme is expressed in the vast majority of cells and tissues,

including cells that are strictly dependent on glucose uptake for their metabolic needs (21). Importantly, while most tissues express more than one HK isozyme, RBC glucose metabolism only depends on HK1 activity (22). In humans, mutations including non-synonymous substitutions in the active site of HK1 and intragenic deletions have been shown to cause HK1 enzymatic deficiency associated with autosomal recessive severe nonspherocytic hemolytic anemia (21; 23-25). A similar phenotype has been described in the Downeast Anemia (*dea*) mice displaying HK1 deficiency (22).

Based on these observations, we postulated that *HK1* genetic variation may modulate the maintenance of the RBC pool and thus indirectly alter HbA1c measurements independently of the ambient blood glucose concentration. We evaluated this hypothesis by assessing the impact of *HK1* rs7072268 on HbA1c, other glucose control-related traits, T2D risk and RBC-related parameters in several prospective and case/control European cohorts. Our data suggest that *HK1* variation through its anemic effect impairs HbA1c assays, which may have important clinical implications for both T2D diagnosis and management, as anemia is commonly associated with diabetes.

RESEARCH DESIGN AND METHODS

Study Participants: Clinical characteristics and data available on the studied populations are reported in Table 1. The study protocol was approved by the local ethics committee and participants from all studies described here (and parents of children) signed an informed consent form.

Genotyping of rs7072268 was performed in several cohorts:

D.E.S.I.R. The Data from the Epidemiological Study on the Insulin Resistance Syndrome (D.E.S.I.R.) cohort is a longitudinal French general population, described elsewhere (10; 26). We analyzed

4,590 non-diabetic D.E.S.I.R. participants successfully genotyped for rs7072268; of whom 3,795 were examined during the entire 9-year study.

Swiss Obese Adults. The Swiss cohort study of obese adults was fully described elsewhere (27). All of them were recruited for obesity surgery. We analyzed 2,363 non-diabetic participants successfully genotyped for rs7072268.

NFBC1986. The Northern Finland 1986 Birth Cohort (NFBC1986) is a prospective 1-year birth cohort including all Finnish Caucasian mothers with children whose expected date of birth fell between July 1st 1985 and June 30th 1986 in the two northernmost provinces of Finland (28). Clinical examination at 15-16 years follow-up was conducted between August 2001 and June 2002. We analyzed 5,287 non-diabetic participants in the NFBC1986 cohort successfully genotyped for rs7072268.

Haguenau. The Haguenau community-based cohort of young adults investigates long-term consequences of being born small for gestational age and was fully described elsewhere (29). Briefly, subjects born between 1971 and 1985 were identified from a population-based registry of Haguenau (France). Non European ancestry subjects are estimated to be less than 0.1% of the general population (29). At a mean age of 22 years, participants under overnight fasting conditions underwent a medical examination to assess anthropometric and clinical parameters. We analyzed 1,455 non-diabetic participants successfully genotyped for rs7072268.

Obesity French Pedigrees. French children and adults with European ancestry from obesity families were recruited at the CNRS-UMR8090 unit (Lille, France) through an ongoing national media campaign (30). We analyzed 5,261 non-diabetic participants successfully genotyped for rs7072268.

French type 2 diabetes case-control study. We analysed 7,447 unrelated French individuals with T2D ascertained from the French T2D family and Obesity family studies, collected by the CNRS-UMR8090 unit; from the Endocrinology-Diabetology Department of the Corbeil-Essonnes Hospital (7) and from the Diabhycar/Diab2-Néphrogène/Surdiagène study (31). We used 5,380 unrelated normoglycemic participants (age at exam > 40 years) as controls ascertained from the D.E.S.I.R. cohort, the SU.VI.MAX study fully described elsewhere (32), and the French T2D family and Obesity family studies recruited by the CNRS-UMR8090 unit.

For each population, glycemic status was defined according to 1997 American Diabetes Association criteria (2): normal glucose, defined as fasting glucose < 6.1 mmol/l without hypoglycemic treatment; and T2D, defined as FPG \geq 7.0 mmol/l and/or treatment with anti-diabetic agents. For the Corbeil study, ‘overt nephropathy’ was defined as: microalbuminuria levels \geq 30 mg/24h or microalbuminuria levels \geq 20 mg/l in two out of three urinary takings.

Genotyping: Genotyping of SNP rs7072268 was performed using a TaqMan™ assay according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA, USA; AB assay ID C-30005592-10). Allelic discrimination was performed by capillary electrophoresis analysis using an Applied Biosystems 3730xl DNA Analyser and GeneMapper 3.7 software. Genotype success rate was at least 98% and no deviation ($p > 0.05$) from Hardy-Weinberg equilibrium was observed in any of the examined populations.

Genotyping of *MTNR1B*-rs10830963, *GCK*-rs1799884, *G6PC2*-rs560887 and *SLC30A8*-rs13266634 in the D.E.S.I.R. study had previously been reported (10; 19; 33; 34).

Statistical analyses: We analyzed the effect of SNP rs7072268 on quantitative traits

using linear regression models under an additive model adjusted for age, gender and BMI. To take into account familial relationships within the French obesity pedigrees, we tested the association between rs7072268 and glucose homeostasis-related traits using Gaussian models of generalized estimated equations (GEE) performed with STATA software. The estimates of the effect of rs7072268 on quantitative traits and their standard errors for each separate population were combined in the meta-analyses using the weighted inverse normal method. The overall effect and its confidence interval were estimated using the inverse variance method implemented in ‘meta.summaries’ function of R RMETA package. The effect of rs7072268 on diabetic status was assessed using a logistic regression model adjusted for age, gender and BMI. In the D.E.S.I.R. participants, the effect of the rs7072268 genotype on quantitative traits was assessed in non-diabetic individuals at baseline and using repeated measures at 3-, 6-, and 9-year follow-up visits. We used mixed models for analyses of repeated measures adjusted for age, gender and BMI. Using the QUANTO software, we estimated what significant effects of rs7072268 on glucose homeostasis related parameters we could expect in the related meta-analyses, with a detection power of 80%. Given the analyzed sample sizes, small effects of *HK1* rs7072268 (estimated at $\beta \leq |0.1|$) on glucose homeostasis related parameters can be detected with a power of 80%.

All statistical analyses were performed with R (version 2.6.1), SPSS (version 14.0 for Windows), QUANTO (version 1.2) and STATA software (version 5.0).

Indices calculation: The homeostasis model of pancreatic beta cell function (HOMA-B) was calculated as $HOMA-B = (20 \times \text{‘fasting serum insulin’}) / (\text{‘fasting plasma glucose’} - 3.5)$ where ‘fasting serum insulin’

is in mU/l and ‘fasting plasma glucose’ is in mmol/l (35).

The homeostasis model of insulin resistance (HOMA-IR) was calculated as $HOMA-IR = (\text{‘fasting plasma glucose’} \times \text{‘fasting serum insulin’}) / 22.5$ where ‘fasting serum insulin’ is in pmol/l and ‘fasting plasma glucose’ is in mmol/l (35).

The insulinogenic index, the insulin sensitivity index (ISI) and the disposition index (DI) were calculated from the oral glucose tolerance test (OGTT) according to the formulas:

Insulinogenic index = $(\text{‘serum insulin at 30 minutes’} - \text{‘fasting serum insulin’}) / \text{‘plasma glucose at 30 minutes’}$ where serum insulin is in pmol/l and plasma glucose is in mmol/l (36).

$ISI = 10,000 / \sqrt{(\text{‘fasting plasma glucose’} \times \text{‘fasting serum insulin’} \times \text{mean OGTT}_{\text{glucose}} \times \text{mean OGTT}_{\text{insulin}})}$ where serum insulin is in mU/l and plasma glucose is in mmol/l (37).

$DI = ISI \times 100 \times \text{‘serum insulin at 30 minutes’} / [(\text{‘plasma glucose at 30 minutes’} \times (\text{‘plasma glucose at 30 minutes’} - 3.89))]$ where serum insulin is in mU/l and plasma glucose is in mmol/l (38).

ISI and DI were only calculated in the French obese pedigrees as measurements of serum insulin and plasma glucose were available at 0, 30, 60, 90 and 120 minutes after glucose load. In the Haguenau study, measurements of serum insulin and plasma glucose were only available at 0, 30, and 120 minutes after glucose load.

RESULTS

SNP rs7072268 strongly associates with increased HbA1c level in non-diabetic individuals. We first genotyped SNP rs7072268 in 4,590 middle-aged non-diabetic individuals from the French D.E.S.I.R. population (mean age 47 years) and in 2,363 Swiss non-diabetic obese adults (mean age 41 years) (see clinical characteristics in Table 1).

Applying an additive genetic model adjusted for age, gender and BMI, the rs7072268-T allele showed a consistent association with increased HbA1c in the D.E.S.I.R. study at baseline and over the 9-year follow-up ($\beta = 0.023$ %_{HbA1c} [0.016;0.031]_{95%CI}, $P = 1.76 \times 10^{-3}$ and $\beta = 0.022$ %_{HbA1c} [0.016;0.029]_{95%CI}, $P = 3.93 \times 10^{-4}$, respectively; Table 2) and in the Swiss obese adults sample set ($\beta = 0.046$ %_{HbA1c} [0.032;0.060]_{95%CI}, $P = 9.46 \times 10^{-4}$; Table 2). These results were unchanged when the additive genetic model was adjusted for age and gender only (data not shown). When we also included FPG level in the linear regression model, the significance of the effect on HbA1c was stronger in both studies as well as in a meta-analysis of the D.E.S.I.R. baseline data and the Swiss obese samples ($N = 6,953$; $\beta = 0.029$ %_{HbA1c} [0.018;0.040]_{95%CI}, combined $P = 2.22 \times 10^{-7}$; Table 2).

SNP rs7072268 does not associate with any other markers of glucose control in non-diabetic individuals. We then assessed the impact of the rs7072268-T allele on glucose homeostasis-related traits in D.E.S.I.R. and the Swiss samples. Applying an additive genetic model adjusted for age, gender and BMI, we did not find significant associations between rs7072268 and any glucose-related traits including fasting glucose, fasting insulin and the homeostasis models of pancreatic beta cell function (HOMA-B) and insulin resistance (HOMA-IR) (Table 3).

In order to support further these paradoxical findings, we tested the effect of rs7072268 on the same fasting traits in 12,003 additional non-diabetic individuals ascertained from the NFBC1986 study (age at the examination 16 years), from the French Haguenau cohort (mean age 22 years) and from French obesity pedigrees including both children and adults (mean age of 11 and 46 years, respectively) (see clinical characteristics in Table 1). HbA1c levels were not measured in these sample sets. Applying

an identically adjusted additive genetic model, we did not find significant associations with any of these traits analyzed in each cohort nor in the overall combined meta-analysis (Table 3). Furthermore, analyses of glucose and insulin levels after oral glucose load in 1,440 individuals from Haguenau; in 1,055 children and in 2,294 adults from the French obesity pedigrees did not show any significant associations (Table 4).

SNP rs7072268 associates with RBC-related parameters and anemia in non-diabetic individuals. Since our results so far suggested that the effect of rs7072268 on HbA1c was not due to differences in glycemic status, we assessed the impact of rs7072268 on RBC-related parameters available in D.E.S.I.R. and the Swiss obese adults sample set; and also in 5,229 participants from the NFBC1986 study (where RBC-related traits but not HbA1c were measured). Applying an additive genetic model adjusted for age, gender and BMI, our combined analysis demonstrated an association between the rs7072268-T allele and decreased hematocrit ($N = 11,492$; $\beta = -0.13$ %_{hematocrit} [-0.20;-0.06]_{95%CI}, combined $P = 2.26 \times 10^{-4}$; Table 5) and decreased hemoglobin levels ($\beta = -0.044$ g/dl [-0.071;-0.017]_{95%CI}, combined $P = 1.43 \times 10^{-3}$; Table 5). Combined case-control studies for anemia (stringently defined by hemoglobin ≤ 12 g/dl for females and ≤ 13 g/dl for men; $N_{cases} = 669$) from the same cohorts supported further the anemic effect of the rs7072268-T allele (OR = 1.13 [1.01;1.27]_{95%CI}; combined $P = 0.032$). We next studied the effects of variation at rs7072268 on mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) indices: since the P values for heterogeneity in effects on both traits were lower than 0.05, our analysis was performed in each cohort in isolation. In Swiss obese adults, the rs7072268-T allele associates with both decreased MCH and MCV parameters (β

= -0.21 pg/cell [-0.28 ; -0.14]_{95%CI}, $P = 2.16 \times 10^{-3}$ and $\beta = -0.56 \times 10^{-15}$ /cell [-0.72 ; -0.38]_{95%CI}, $P = 1.29 \times 10^{-3}$ respectively; Table 5), suggesting a microspherocytocytic anemic state. In the D.E.S.I.R. participants, the RBC count also showed a negative association with the rs7072268-T allele both at baseline ($\beta = -0.018 \times 10^{12}/l$ [-0.025 ; -0.011]_{95%CI}, $P = 8.01 \times 10^{-3}$; Table 5) and over the 9-year follow-up ($\beta = -0.020 \times 10^{12}/l$ [-0.027 ; -0.014]_{95%CI}, $P = 9.63 \times 10^{-4}$ respectively; Table 5).

Effect of SNP rs7072268 on RBC-related parameters in T2D individuals. The rs7072268-T allele is also associated with decreased hemoglobin level in 1,924 French T2D subjects from the Corbeil Hospital cohort where this parameter was measured ($\beta = -0.13$ g/dl [-0.16 ; -0.09]_{95%CI}, $P = 7.66 \times 10^{-4}$; Table 5). When the presence of overt nephropathy, the microalbuminuria level or the albumin-to-creatinine ratio were introduced in the linear regression model, this association remained significant ($P < 1.5 \times 10^{-3}$) suggesting that the effect of *HK1* on RBC is independent of diabetes-linked kidney disease. We also identified in T2D subjects a trend for association between the rs7072268-T allele and decreased MCV (Table 5).

Combined meta-analysis of SNP rs7072268 on RBC-related parameters. In a combined meta-analysis including non-diabetic and T2D participants, the rs7072268-T allele strongly associates with decreased hemoglobin levels ($N = 13,416$; $\beta = -0.054$ g/dl [-0.076 ; -0.031]_{95%CI}, combined $P = 3.74 \times 10^{-6}$; Table 5) and trend for an increased risk for clinical anemia ($N_{cases} = 836$; OR = 1.13 [1.02; 1.25]_{95%CI}, combined $P = 0.018$) is supported further.

Impact of SNP rs7072268 on T2D risk. We then assessed the contribution of rs7072268 to T2D risk in 7,447 T2D French individuals and 5,380 unrelated normoglycemic French controls (age at exam ≥ 40 years old). The T2D case-control analysis only displayed a nominal association

between the rs7072268-T allele and increased risk of T2D (OR = 1.07 [1.00; 1.14]_{95%CI}, $P = 0.045$; Table 6). These findings were not supported by GWAS meta-analyses carried out by the DIAGRAM+ consortium including 8,130 T2D and 38,987 controls European participants (OR = 0.98 [0.94; 1.02]_{95%CI}, $P = 0.40$) (unpublished data). Therefore, weak *HK1* rs7072268 effect found in our samples on increased T2D risk is not supported further in other European populations.

Impact of the five established genetic determinants of HbA1c on HbA1c levels, fasting plasma glucose and RBC-related parameters in D.E.S.I.R. We then analysed the contribution of four previously reported genetic determinants of HbA1c (*i.e.* *MTNR1B*-rs10830963 (9; 34), *GCK*-rs1799884 (20), *G6PC2*-rs560887 (20) and *SLC30A8*-rs13266634 (20)) on HbA1c levels in the D.E.S.I.R. cohort. We confirmed the contribution of these SNPs to HbA1c levels in ~4,500 non-diabetic individuals from the D.E.S.I.R. study at baseline, except for the *SLC30A8*-rs13266634 that displayed only a trend for association with HbA1c levels ($P_{MTNR1B} = 2.25 \times 10^{-4}$, $P_{GCK} = 1.32 \times 10^{-4}$, $P_{G6PC2} = 2.31 \times 10^{-6}$ and $P_{SLC30A8} = 0.063$; Table 7). Analysis of *HK1*-rs7072263 combined with the four other SNPs demonstrated a significant additive effect on HbA1c levels ($\beta_{per-allele} = 0.032$ %HbA1c, $P = 1.49 \times 10^{-15}$; Figure 1). Individuals carrying seven or more ‘high HbA1c’ alleles ($N = 415$; ~11% of the European population) show a mean of 0.17 % increase in HbA1c as compared with individuals carrying fewer than two ‘high HbA1c’ alleles ($N = 219$) (Figure 1).

We then assessed the effect of *MTNR1B*-rs10830963, *GCK*-rs1799884, *G6PC2*-rs560887 and *SLC30A8*-rs13266634 on FPG levels and RBC-related parameters including RBC count, hemoglobin and hematocrit levels. As previously reported (9; 10; 19; 33), the four SNPs are strongly

associated with FPG levels (Table 7). SNPs *GCK*-rs1799884, *G6PC2*-rs560887 and *SLC30A8*-rs13266634 are not associated with RBC-related parameters (Table 7). In contrast, the *MTNR1B*-rs10830963-T allele associates with decreased RBC count, hemoglobin and hematocrit levels ($\beta = -0.017 \times 10^{12}/l$ [-0.025;-0.001]_{95%CI}, $P = 0.022$; $\beta = -0.055$ g/dl [-0.076;-0.033]_{95%CI}, $P = 0.011$ and $\beta = -0.19$ %hematocrit [-0.25;-0.12]_{95%CI}, $P = 4.13 \times 10^{-3}$, respectively; Table 7).

DISCUSSION

Our data unambiguously demonstrate that *HK1* rs7072268 strongly associates with increased HbA1c levels in European general populations, as reported by Paré et al. (20). In contrast, we failed to find any further association with other quantitative metabolic traits commonly used to monitor glucose control. In addition, it is unlikely that *HK1* rs7072268 significantly increases risk for T2D. Our data suggest that the effect of *HK1* variation on HbA1c levels may be due to a molecular mechanism involving RBC function, rather than related to impaired blood glucose homeostasis. In this regard, we found that the *HK1* rs7072268-T allele increasing HbA1c is strongly associated with both reduced hemoglobin and hematocrit levels (the Spearman correlation between hematocrit and hemoglobin levels in non-diabetic people from D.E.S.I.R. is: $r^2 = 0.94$, $P < 0.0001$). In addition, the rs7072268-T allele contributes to increase the risk of clinical anemia. However, this result has to be confirmed in large scale and more powered case/control studies. To support our findings, *dea* mice having HK1 deficiency also display lower RBC count, hemoglobin and hematocrit levels (22). Indeed, these mice show a severe anemia with extensive tissue iron deposition and marked reticulocytosis that results from significant intravascular hemolysis (22). About 20 patients with nonspherocytic hemolytic anemia due to HK1 deficiency have been

described so far (21), but there is no information available about their HbA1c levels. SNP rs7072268 is located in the first intron of the *HK1* isoform *HK1-R* specifically expressed in RBC and is in intermediate linkage disequilibrium with a common non-synonymous coding SNP rs1133189 (according to the HapMap CEU population: $r^2 = 0.58$). Although we have no obvious information about the truly causative common SNPs in the *HK1* locus associated with anemia (that might be obtained from fine mapping studies), we speculate they may impair *HK1* expression and/or the maturation of this hexokinase enzymatic isoform in reticulocytes and in mature RBC, as known in monogenic HK1 deficiency (21; 23).

In RBC, the oxygen affinity of hemoglobin is strongly regulated by 2,3-biphosphoglycerate (2,3-DPG) produced by a bypass in glycolysis (21). Increasing 2,3-DPG levels cause a decreased oxygen affinity and thus improve the transfer of oxygen to tissues and ameliorate the anemic state. HK1 deficiency contributes to decrease 2,3-DPG levels and thus annuls its beneficial effect (21). HK1 is also known to bind in mitochondria to the voltage-dependent anion channels (VDACs), known as mitochondrial porins (39). Mitochondrial associated hexokinase activity has been shown to protect cells from entering apoptosis via the blockade of the interaction of the pro-apoptotic BAX with the VDAC (40-42). We speculate that *HK1* variation may impair the HK1 anti-apoptotic effect in reticulocytes (*i.e.* the precursors of RBC), as well as in kidney and brain where *HK1* is expressed (21; 43). It may have deleterious effects on maturation of RBC as well as on erythropoiesis via decreased synthesis of kidney and brain erythropoietin (Epo).

The mechanism by which *HK1* related anemia increases HbA1c levels is unknown. Using a conditional regression model, we failed to clearly show that the *HK1* effect on

HbA1c was affected by adjustment for the hemoglobin or hematocrit levels (see Table A1 in the online appendix at <http://diabetes.diabetesjournals.org>). This may suggest that the hemoglobin or hematocrit levels would explain a small variance of HbA1c. But, larger studies will be needful to confirm these findings. A higher turnover of the RBC pool should diminish protein glycation due to the reduced hemoglobin half life (5). Alternatively, we speculate that the enhanced accumulation of unprocessed glucose resulting from the *HK1* deficiency may favor hemoglobin glycation within RBC, which in turn may increase the RBC death rate via their impaired deformability (44). Importantly, anemia due to iron deficiency often seen in late pregnancy, also causes increased HbA1c levels (45) and HbA1c levels significantly decreases after iron or vitamin B12 treatment in patients with iron or vitamin B12 deficiency anemia, respectively (46; 47). Therefore, different anemia inducing mechanisms increase HbA1c levels.

Other genes associated with RBC-related parameters may also interfere with the glycation of hemoglobin. In this regard, our present data suggest that genetic variation in *MTNR1B* (encoding melatonin receptor 2), which strongly influences both HbA1c and fasting glucose (9), also associates with decreased RBC-count, hemoglobin and hematocrit levels. Melatonin is a neurohormone mainly involved in the regulation of circadian rhythms. Recently, Bozek et al. provided evidence of a circadian oscillation of *Epo* gene expression in the kidney (48), a tissue that strongly expresses *MTNR1B* in rat (49). In contrast, three other genetic determinants of HbA1c (*i.e.* *GCK*, *G6PC2* and *SLC30A8*) modulate fasting glucose but do not influence hematologic parameters measured in our cohorts. Altogether, HbA1c levels seem to be largely genetically determined (Figure 1) possibly via

the modulation of blood glucose and/or hematologic parameters.

As both the American Diabetes Association and the European Association for the Study of Diabetes have proposed to use HbA1c as a criterion for T2D diagnosis (an individual with HbA1c lower than 6 % is considered as non-diabetic), both genetic and environmental factors (including iron and vitamin B12) interacting with RBC function and survival, have to be taken into consideration to better interpret HbA1c levels in the general population. Furthermore, diabetes by itself is a known cause for anemia through a range of deleterious mechanisms (44), and it would be important to better determine the impact of anemia on HbA1c assays.

In conclusion, our study suggests mechanisms that may underlie the consistent association between *HK1* genetic variation and HbA1c, but also identifies for the first time a gene contributing to common pro-anemic state. At a time where the utility of GWAS is debated for disease prediction (50), our study highlights GWAS power to identify physiological determinants of complex conditions such as anemia having serious implications for health.

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Figure 1. Cumulative effect of *HK1*-rs7072268, *MTNR1B*-rs10830963, *GCK*-rs1799884, *G6PC2*-rs560887 and *SLC30A8*-rs13266634 on HbA1c in non-diabetic individuals from the D.E.S.I.R. study

A linear regression model was carried out applying an additive model adjusted for age, gender and BMI. Data are presented as mean [95% CI]. β coefficient corresponds to the increase in HbA1c levels (%) by additional ‘high HbA1c’ alleles. The numbers of individuals per category of ‘high HbA1c’ alleles and corresponding percentages are shown below the graph.

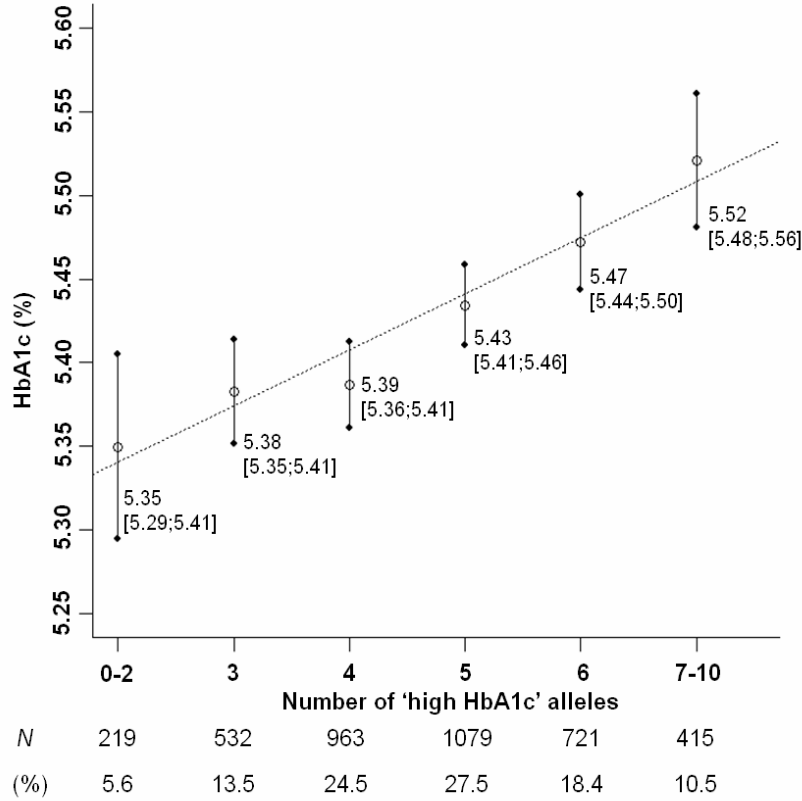


Table 1. Clinical characteristics and available data on the study populations with successful genotyping for rs7072268

Study Populations	D.E.S.I.R. at baseline	Swiss obese adults	NFBC1986	Haguenau	French children from obesity pedigrees	French adults from obesity pedigrees	T2D case-control study	
							French T2D case	French controls
<i>N</i> (M/F)	4,590(2,259 / 2,331)	2,363 (511 / 1852)	5,287 (2,628 / 2,659)	1,455 (690 / 765)	1,411 (678 / 733)	3,850 (1,454 / 2,396)	7,447 (4,752 / 2,695)	5,380 (2,293 / 3,087)
Age (years)	47.1 ± 10.0	40.8 ± 11.1	16.0	22.1 ± 3.9	11.4 ± 3.3	46.3 ± 15.2	62.7 ± 10.3	53.0 ± 8.3
BMI (kg/m ²)	24.6 ± 3.7	43.1 ± 7.2	21.3 ± 3.7	22.6 ± 4.1	26.2 ± 7.4	32.5 ± 9.4	30.7 ± 6.2	25.2 ± 5.0
Fasting glucose (mmol/l)	5.3 ± 0.5	5.1 ± 0.6	5.2 ± 0.4	4.8 ± 0.4	4.9 ± 0.5	5.3 ± 0.7	NA	NA
Fasting insulin (pmol/l)	39.2 (28.6;55.8)	110.4 (75.9;165.6)	66.2 (51.2;85.6)	32.3 (22.2;43.8)	69.0 (42.8;109.7)	55.9(33.3;89.7)	NA	NA
HbA1c (%)	5.43 ± 0.40	5.59 ± 0.48	NA	NA	NA	NA	NA	NA
Association study with rs7072268:								
HbA1c	■	■						
Fasting metabolic traits	■	■	■	■	■	■		
Metabolic traits during an OGTT				■	■	■		
RBC-related parameters	■	■	■				■*	

T2D, type 2 diabetes; OGTT, oral glucose tolerance test; RBC, red blood cell; NA, not applicable or not available

Data are presented as mean ± standard deviation or median (interquartile range).

Available data are presented as '■'.

*RBC-related parameters were only available in T2D patients from the Corbeil-Hospital.

Table 2. Association of rs7072268 with HbA1c level in non-diabetic individuals from the D.E.S.I.R. study (at baseline and over the 9-year follow-up study) and from the Swiss obese adults sample-set

			Mean HbA1c level (%) by genotype*			Additive model adjusted for age, gender and BMI		Additive model adjusted for age, gender, BMI and FPG level	
	<i>N</i>	T-allele frequency	CC	CT	TT	Per T allele effect [†] (% _{HbA1c}) [95% CI]	<i>P</i>	Per T allele effect [†] (% _{HbA1c}) [95% CI]	<i>P</i>
D.E.S.I.R. At baseline	4,590	0.49	5.40 ± 0.41	5.43 ± 0.39	5.45 ± 0.39	0.023 [0.016;0.031]	1.76 × 10 ⁻³	0.026 [0.018;0.033]	4.03 × 10 ⁻⁴
Swiss obese adults	2,363	0.54	5.56 ± 0.45	5.58 ± 0.48	5.64 ± 0.49	0.046 [0.032;0.060]	9.46 × 10 ⁻⁴	0.035 [0.026;0.044]	1.13 × 10 ⁻⁴
Meta-analysis	6,953	-	-	-	-	0.028 [0.016;0.041]	1.53 × 10 ⁻⁵	0.029 [0.018;0.040]	2.22 × 10 ⁻⁷
D.E.S.I.R. over the 9-year follow-up study [‡]	15,073	0.49	-	-	-	0.022 [0.016;0.029]	3.93 × 10 ⁻⁴	0.023 [0.017;0.029]	1.20 × 10 ⁻⁴

Association between rs7072268 and HbA1c was assessed applying an additive model adjusted for age, gender and BMI or adjusted for age, gender, BMI and fasting plasma glucose (FPG).

*Data are presented as mean ± standard deviation.

[†]Per T-allele effect size means the regression coefficient β .

[‡]*P* values and regression coefficients β are calculated from mixed models described in the statistical analysis section.

Table 3. Associations between rs7072268 and glucose homeostasis-related traits in non-diabetic individuals from several European cohorts

	T-allele frequency	N	Glucose homeostasis-related traits	Mean data level by genotype*			P
				CC	CT	TT	
D.E.S.I.R.	0.49	4,590	Fasting glucose(mmol/l)	5.29 ± 0.53	5.27 ± 0.52	5.28 ± 0.54	0.66
			Fasting insulin [†] (pmol/l)	39.22(28.63-56.82)	39.15(28.54-55.61)	39.58(28.82-55.78)	0.78
			HOMA-B [†]	67.65(48.06-94.05)	67.67(49.19-95.41)	69.51(49.52-93.61)	0.79
			HOMA-IR [†]	9.15(6.43-13.66)	9.17(6.48-13.23)	9.19(6.44-13.72)	0.74
Swiss Obese adults	0.54	2,101	Fasting glucose(mmol/l)	5.14 ± 0.63	5.11 ± 0.58	5.16 ± 0.57	0.44
			Fasting insulin [†] (pmol/l)	103.5(69-158.7)	110.4(75.9-165.6)	110.4(75.9-172.2)	0.08
			HOMA-B [†]	200.0(132.5-306.1)	216.0(137.7-329.2)	200.0(137.5-314.3)	0.24
			HOMA-IR [†]	24.2(15.5-36.2)	24.9(16.9-36.7)	25.5(16.3-38.3)	0.10
NFBC1986	0.40	5,287	Fasting glucose(mmol/l)	5.15 ± 0.44	5.15 ± 0.43	5.14 ± 0.41	0.81
			Fasting insulin [†] (pmol/l)	66.24(51.06-87.63)	66.24(51.06-84.67)	67.62(51.06-86.25)	0.53
			HOMA-B [†]	118.67(90.00-156.67)	117.89(92.00-156.21)	120.00(90.00-156.67)	0.75
			HOMA-IR [†]	15.03(11.50-20.16)	15.12(11.43-19.77)	15.35(11.57-19.73)	0.52
Hagenau	0.52	1,455	Fasting glucose(mmol/l)	4.76 ± 0.35	4.80 ± 0.38	4.79 ± 0.39	0.29
			Fasting insulin [†] (pmol/l)	33.01(22.96-44.49)	33.01(22.96-44.49)	30.49 (21.53-43.59)	0.82
			HOMA-B [†]	78.09(50.61-112.93)	75.09(51.23-107.05)	72.82(50.06-104.93)	0.74
			HOMA-IR [†]	7.08(4.79-9.48)	6.97(4.89-9.55)	6.57(4.48-9.34)	0.72
French children from obesity pedigrees	0.49	1,411	Fasting glucose(mmol/l)	4.89 ± 0.47	4.93 ± 0.48	4.86 ± 0.51	0.30
			Fasting insulin [†] (pmol/l)	68.31(42.78-107.30)	68.31(42.44-107.30)	70.38(44.85-112.47)	0.89
			HOMA-B [†]	151.76(99.44-225.38)	145.33(94.87-229.43)	152.73(94.44-253.33)	0.66
			HOMA-IR [†]	15.13(8.89-23.09)	14.95(8.98-24.04)	14.72(9.44-25.07)	0.98
French adults from obesity pedigrees	0.51	3,850	Fasting glucose(mmol/l)	5.33 ± 0.68	5.34 ± 0.69	5.36 ± 0.67	0.76
			Fasting insulin [†] (pmol/l)	54.17(33.12-84.70)	55.20(33.12-89.01)	58.65(34.50-93.84)	0.25
			HOMA-B [†]	87.55(56.32-141.14)	93.52(57.38-142.71)	96.36(59.64-151.54)	0.45
			HOMA-IR [†]	12.74(7.42-20.62)	13.26(7.43-21.42)	14.03(7.82-23.30)	0.23
Overall Meta-analysis	-	18,694	Fasting glucose(mmol/l)	-	-	-	0.93
			Fasting insulin [†] (pmol/l)	-	-	-	0.79
			HOMA-B [†]	-	-	-	0.90
			HOMA-IR [†]	-	-	-	0.81

Associations between rs7072268 and glucose homeostasis-related traits were assessed applying an additive model adjusted for age, gender and BMI, except for the NFBC1986 (an adjustment for gender and BMI was only performed as they were all 16 years old).

HOMA-B, homeostasis model of pancreatic beta cell function; *HOMA-IR*, homeostasis model of insulin resistance

*Data are presented as mean ± standard deviation and for logarithmically transformed data as median (interquartile range).

[†]Values of fasting serum insulin, HOMA-B and HOMA-IR were logarithmically transformed before statistical analysis.

Table 4. Associations between rs7072268 and quantitative metabolic traits during an OGTT in non-diabetic French individuals from the Haguenau study and obesity pedigrees

	N	T-allele frequency	Quantitative metabolic traits during an OGTT		Mean data level by genotype*			P
					CC	CT	TT	
French Children from obesity pedigrees	1,055	0.49	Plasma glucose (mmol/l)	30min post OGTT	7.24 ± 1.42	7.20 ± 1.52	7.29 ± 1.49	0.85
				120min post OGTT	5.47 ± 1.13	5.39 ± 1.18	5.39 ± 1.16	0.22
			Serum insulin [†] (pmol/l)	30min post OGTT	498(283-732)	448(275-698)	461(274-763)	0.57
				120min post OGTT	206(107-411)	193(99-401)	213(100-451)	0.72
			Insulinogenic Index [†]	58.7(34.5-84.7)	54.4(31.6-82.4)	54.3(33.4-89.9)	0.96	
			ISI [†]	32.5(21.3-55.4)	37.0(23.4-58.1)	33.8(21.3-57.2)	0.43	
			DI [†]	10,025(5,539-18,125)	10,827(6,013-18,391)	9,012(5,345-16,832)	0.83	
French Children from obesity pedigrees	2,294	0.51	Plasma glucose (mmol/l)	30min post OGTT	8.22 ± 1.67	8.40 ± 1.90	8.32 ± 1.85	0.70
				120min post OGTT	5.68 ± 1.95	5.72 ± 1.92	5.78 ± 1.97	0.43
			Serum insulin [†] (pmol/l)	30min post OGTT	293(167-490)	305(182-481)	295(165-485)	0.97
				120min post OGTT	168(79-366)	182(83-370)	190(91-364)	0.27
			Insulinogenic Index [†]	30.4(16.5-50.5)	30.4(16.5-50.0)	28.5(16.1-49.6)	0.56	
			ISI [†]	106.6(60.2-192.6)	102.4(62.3-170.0)	107.0(57.5-174.8)	0.46	
			DI [†]	13,046(6,496-26,909)	12,806(6,130-25,563)	13,005(5,841-24,931)	0.41	
Haguenau	1,440	0.52	Plasma glucose (mmol/l)	30min post OGTT	7.51 ± 1.42	7.61 ± 1.46	7.49 ± 1.40	0.60
				120min post OGTT	5.40 ± 1.22	5.30 ± 1.14	5.27 ± 1.18	0.17
			Serum insulin [†] (pmol/l)	30min post OGTT	294(185-445)	287 (187-420)	287(181-434)	0.82
				120min post OGTT	165(93-266)	172(108-273)	165(101-266)	0.99
			Insulinogenic Index [†]	34.9(20.6-53.6)	33.1(21.6-50.8)	34.8(21.6-50.9)	0.87	
Overall Meta-analysis	4789	-	Plasma glucose (mmol/l)	30min post OGTT	-	-	-	0.99
				120min post OGTT	-	-	-	-
			Serum insulin [†] (pmol/l)	30min post OGTT	-	-	-	0.99
				120min post OGTT	-	-	-	0.24
			Insulinogenic Index [†]	-	-	0.84	0.71	
			ISI [†]	-	-	0.92	0.83	
			DI [†]	-	-	-	0.42	

Associations between rs7072268 and quantitative metabolic traits during an OGTT were assessed applying an additive model adjusted for age, gender and BMI. *OGTT*, oral glucose tolerance test; *ISI*, Insulin Sensitivity Index; *DI*, disposition index

*Data are presented as mean ± standard deviation and for logarithmically transformed data as median (interquartile range).

[†]Values of serum insulin, ISI, DI and Insulinogenic Index were logarithmically transformed before statistical analysis.

‡Meta-analyses of both ISI and DI included association data of the participants from obesity French pedigrees only.

Table 5. Associations between rs7072268 and RBC-related parameters in non-diabetic individuals from D.E.S.I.R. (at baseline and over the 9-year follow-up), the Swiss obese adults and the NFBC1986; and in diabetic French participants from the Corbeil T2D study

	T-allele frequency	N	RBC-related parameters	Mean data level by genotype*			Per T allele effect [†] [95% CI]	P
				CC	CT	TT		
D.E.S.I.R. At baseline	0.49	4,576	RBC count ($\times 10^{12}/l$)	4.82 \pm 0.41	4.79 \pm 0.41	4.78 \pm 0.41	-0.018 [-0.025;-0.011]	8.01 $\times 10^{-3}$
			Hematocrit (%)	43.66 \pm 3.61	43.50 \pm 3.61	43.28 \pm 3.67	-0.18 [-0.24;-0.12]	2.11 $\times 10^{-3}$
			Hemoglobin (g/dl)	14.41 \pm 1.26	14.36 \pm 1.24	14.30 \pm 1.28	-0.054 [-0.074;-0.035]	5.20 $\times 10^{-3}$
			MCH (pg/cell)	29.95 \pm 1.54	30.00 \pm 1.57	29.94 \pm 1.64	-	0.98
			MCV ($\times 10^{-15}$ liters/cell)	90.73 \pm 4.18	90.88 \pm 4.33	90.65 \pm 4.34	-	0.68
			MCHC (%)	33.01 \pm 0.96	33.01 \pm 1.06	33.03 \pm 0.97	-	0.57
Swiss Obese adults	0.54	1,687	RBC count ($\times 10^{12}/l$)	4.81 \pm 0.37	4.84 \pm 0.39	4.84 \pm 0.38	-	0.31
			Hematocrit (%)	43.19 \pm 3.32	43.19 \pm 3.36	42.93 \pm 3.10	-0.17 [-0.27;-0.070]	0.087
			Hemoglobin (g/dl)	14.35 \pm 1.19	14.28 \pm 1.26	14.22 \pm 1.24	-0.081 [-0.115;-0.046]	0.019
			MCH (pg/cell)	29.86 \pm 1.77	29.68 \pm 1.85	29.46 \pm 2.22	-0.21 [-0.28;-0.14]	2.16 $\times 10^{-3}$
			MCV ($\times 10^{-15}$ liters/cell)	90.05 \pm 4.77	89.55 \pm 4.56	88.92 \pm 5.39	-0.56 [-0.72;-0.38]	1.29 $\times 10^{-3}$
			MCHC (%)	33.16 \pm 1.18	33.15 \pm 1.12	33.11 \pm 1.18	-	0.29
NFBC1986	0.40	5,229	RBC count ($\times 10^{12}/l$)	4.71 \pm 0.40	4.70 \pm 0.42	4.70 \pm 0.42	-	0.66
			Hematocrit (%)	40.67 \pm 3.35	40.49 \pm 3.53	40.49 \pm 3.55	-0.086 [-0.137;-0.035]	0.094
			Hemoglobin (g/dl)	13.77 \pm 1.20	13.71 \pm 1.23	13.20 \pm 1.28	-0.030 [-0.047;-0.012]	0.087
			MCH (pg/cell)	29.40 \pm 1.77	29.31 \pm 1.87	29.30 \pm 1.85	-	0.12
			MCV ($\times 10^{-15}$ liters/cell)	86.42 \pm 4.05	86.28 \pm 4.21	86.32 \pm 4.45	-	0.45
			MCHC (%)	33.89 \pm 0.95	33.84 \pm 0.98	33.86 \pm 0.97	-	0.24
Meta-analysis		11,492	RBC count ($\times 10^{12}/l$)	-	-	-	-0.0068 [-0.015;0.0015]	0.11
			Hematocrit (%)	-	-	-	-0.13 [-0.20;-0.06]	2.26 $\times 10^{-4}$
			Hemoglobin (g/dl)	-	-	-	-0.044 [-0.071;-0.017]	1.43 $\times 10^{-3}$
			MCH (pg/cell)	-	-	-	NA [‡]	NA [‡]
			MCV ($\times 10^{-15}$ liters/cell)	-	-	-	NA [‡]	NA [‡]
			MCHC (%)	-	-	-	0.0005 [-0.036;0.037]	0.42
Corbeil T2D study	0.52	1,924	Hemoglobin (g/dl)	14.30 \pm 1.32	14.25 \pm 1.33	14.07 \pm 1.35	-0.13 [-0.16;-0.09]	7.66 $\times 10^{-4}$
			MCV ($\times 10^{-15}$ liters/cell)	90.26 \pm 6.20	90.07 \pm 5.49	89.63 \pm 6.10	-0.33 [-0.51;-0.15]	0.070
Overall Meta-analysis	-	13,416	Hemoglobin (g/dl)	-	-	-	-0.054 [-0.076;-0.031]	3.74 $\times 10^{-6}$
			MCV ($\times 10^{-15}$ liters/cell)	-	-	-	NA [‡]	NA [‡]
D.E.S.I.R. over the 9-year follow-up study [§]	0.49	15,119	RBC count ($\times 10^{12}/l$)	-	-	-	-0.020 [-0.027;-0.014]	9.63 $\times 10^{-4}$
			Hematocrit (%)	-	-	-	-0.17 [-0.22;-0.12]	3.73 $\times 10^{-4}$
			Hemoglobin (g/dl)	-	-	-	-0.055 [-0.071;-0.038]	1.04 $\times 10^{-3}$
			MCH (pg/cell)	-	-	-	-	0.43
			MCV ($\times 10^{-15}$ liters/cell)	-	-	-	-	0.72
			MCHC (%)	-	-	-	-	0.55

Associations between rs7072268 and RBC-related parameters were assessed applying an additive model adjusted for age, gender and BMI. RBC, red blood cell; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; NA, not applicable; T2D, type 2 diabetes.

*Data are presented as mean ± standard deviation.

†Per T-allele effect size means the regression coefficient β. We only displayed the T-allele effect when P was lower than 0.10.

‡The P values for heterogeneity in effects on both MCH and MCV indices were lower than 0.05. We thus considered these two traits as not applicable for overall meta-analyses.

§P values and regression coefficients β are calculated from mixed additive models.

Table 6. French type 2 diabetes case control analyses according to SNP rs7072268

	T-allele frequency	N	n [frequency] in each genotypic group			OR* [95% CI]	P
			CC	CT	TT		
T2D French participants	0.51	7,447	1,784 [0.24]	3,708 [0.50]	1,955 [0.26]	Ref.	-
French controls	0.50	5,380	1,327 [0.25]	2,715 [0.50]	1,338 [0.25]	1.069 [1.001;1.142]	0.045

Type 2 diabetes (T2D) was defined according to 1997 American Diabetes Association criteria (2).

*OR from additive logistic regression models adjusted for age, sex and BMI

Table 7. Association of HbA1c, fasting glucose, hemoglobin, hematocrit and RBC count with candidate SNPs in non-diabetic participants from the D.E.S.I.R. study at baseline

	HK1 rs7072268-T (frequency: 0.49; N=4,590)		MTNR1B rs10830963-G (frequency: 0.28; N=4,597)		GCK rs1799884-A (frequency: 0.27; N=4,406)		G6PC2 rs560887-A (frequency: 0.30; N=4,339)		SLC30A8 rs13266634-T (frequency: 0.30; N=4,488)	
	β [95% CI]	P	β [95% CI]	P	β [95% CI]	P	β [95% CI]	P	β [95% CI]	P
HbA1c (%)	0.023 [0.016;0.031]	1.76 × 10 ⁻³	0.031 [0.023;0.039]	2.25 × 10 ⁻⁴	0.038 [0.028;0.048]	1.32 × 10 ⁻⁴	-0.040 [-0.049;-0.032]	2.31 × 10 ⁻⁶	-0.016 [-0.024;-0.007]	0.063
Fasting glucose (mmol/l)	-0.004 [-0.014;0.006]	0.66	0.093 [0.082;0.104]	1.32 × 10 ⁻¹⁶	0.054 [0.041;0.067]	4.63 × 10 ⁻⁵	-0.077 [-0.089;-0.066]	4.72 × 10 ⁻¹²	-0.039 [-0.050;-0.028]	4.54 × 10 ⁻⁴
Hemoglobin (g/dl)	-0.054 [-0.074;-0.035]	5.20 × 10 ⁻³	-0.055 [-0.076;-0.033]	0.011	0.023 [-0.002;0.049]	0.35	0.010[-0.012;0.031]	0.65	0.004 [-0.017;0.026]	0.85
Hematocrit (%)	-0.18[-0.24;-0.12]	2.11 × 10 ⁻³	-0.19[-0.25;-0.12]	4.13 × 10 ⁻³	0.020[-0.058;0.097]	0.80	0.066 [0.0009;0.13]	0.31	0.008 [-0.057;0.073]	0.91
RBC count (×10 ¹² /l)	-0.018 [-0.025;-0.011]	8.01 × 10 ⁻³	-0.017 [-0.025;-0.0097]	0.022	0.001[-0.008;0.010]	0.88	0.0005 [-0.007;0.008]	0.94	0.002 [-0.005;0.010]	0.78

Associations between SNPs and quantitative traits were assessed applying an additive model adjusted for age, gender and BMI.

RBC, red blood cell