

**Supporting online information for Soranzo et al.
Supplementary Methods and Appendix**

Common variants at ten genomic loci influence hemoglobin A_{1c} levels via glycemc and non-glycemc pathways

Corresponding author(s):

Jose C. Florez MD, PhD

Diabetes Research Center (Diabetes Unit) and Center for Human Genetic Research

Simches Research Center – CPZN 5.250

Massachusetts General Hospital

185 Cambridge Street

Boston, MA 02114

Office: 617-643-3308

email: jcflorez@partners.org

Manuela Uda

Istituto di Neurogenetica e Neurofarmacologia

Consiglio Nazionale delle Ricerche

Cittadella Universitaria di Monserrato

Monserrato, Cagliari, Italy 09042

Office: +39 070675 4591

email: manuela.uda@inn.cnr.it

Nicholas J. Wareham

MRC Epidemiology Unit

Institute of Metabolic Science

Addenbrooke's Hospital

Hills Road

Cambridge, CB2 0QQ

Office: +44 (0)1223 330315

email: nick.wareham@mrc-epid.cam.ac.uk

Inês Barroso

Wellcome Trust Sanger Institute

Genome Campus

Hinxton, CB10 1SA

Office: +44 (0)1223 495341

email: ib1@sanger.ac.uk

James B. Meigs MD, MPH

General Medicine Division

Massachusetts General Hospital

50 Stanford St 9th Floor

Boston, MA 02114

Office: 617-724-3203

email: jmeigs@partners.org

Supplementary Methods

Genotyping, imputation and quality control

Study samples are described in **Table 1**. Study specific parameters and pre-imputation filters are specified in **Table S1**. Each study applied similar criteria for data calling. Prior to imputation, the criteria applied for exclusion of SNPs were: (i) minor allele frequency (MAF) <0.01 , (ii) Hardy-Weinberg equilibrium $P < 10^{-4}$ or 10^{-6} and (iii) call-rate <0.90 or 0.95 . Criteria applied for exclusion of samples were: (i) call-rate <0.95 or <0.97 , (ii) sex mismatch between genotypes and reported sex, and (iii) outliers as assessed by population structure analysis. Imputation of additional autosomal SNPs from the HapMap CEU (1) reference panel was performed using the software MACH (2) or IMPUTE (3).

As standard for imputation, we excluded sex chromosome-linked SNPs from analyses given the difficulty of accurately imputing non-autosomal SNPs and the poor overlap of X-chromosome SNPs across different platforms. SNPs were also excluded if the cohort-specific imputation quality was particularly poor (observed-over-expected variance ratio (r^2_{hat}) <0.3 if MACH was used for imputation, or proper-info <0.4 if IMPUTE was used) or if MAF < 0.01 . In total, up to 2.5 million genotyped or imputed autosomal SNPs were considered for meta-analysis. We only report on individual SNPs imputed or genotyped in $\geq 6,000$ participants.

Statistical methods for primary analyses

In each cohort we fitted a linear regression model using measured HbA_{1C} (%) as the dependent variable to evaluate the additive effect of genotyped and imputed SNPs. The model was adjusted for age, sex and/or study site and family structure (**Table S1**). The association was tested taking genotype and imputation uncertainty into account, using a missing data likelihood test as implemented in SNPTTEST (3) or by using allele dosages in the linear regression model as implemented in ProbABEL (4) or MACH2QTL (2) for unrelated samples or in Merlin (5) or using a linear mixed effects model implemented in the lmeKin function of the R kinship package for family-based studies. Regression estimates for each SNP were combined across studies in a meta-analysis using a fixed effect inverse-variance approach, as implemented in METAL (<http://www.sph.umich.edu/csg/abecasis/Metal/index.html>). The individual cohort analysis results were corrected prior to performing the meta-analysis for residual inflation of the test statistic using the genomic control method if the lambda coefficient was > 1.0 . Heterogeneity was assessed using the standard chi-square test implemented in METAL, Cochran's Q statistic and the I^2 statistics (6).

Conditional analyses

We used conditional analyses to infer whether the ten HbA_{1C} loci (**Table 2**) have associations with HbA_{1C} through glycaemic or non-glycaemic pathways by implementing a two-stage regression approach. First, we selected a subset of up to 23,654 non-diabetic participants from 15 cohorts having HbA_{1C} and fasting glucose (FG) levels measured, or up to 6,394 non-diabetic participants from 6 cohorts having HbA_{1C} and 2-hr post-challenge levels measured. In these participants we calculated separate regressions of HbA_{1C} and FG on each of the ten genome-wide significant SNPs; these estimates reflect the unadjusted effect of the genetic variants on HbA_{1C} and glucose. To identify the glucose-dependent and glucose-independent effects on HbA_{1C}, we adjusted the HbA_{1C} regressions on the genetic variants additionally for FG or 2-hr post challenge in models adjusted for sex, age and other study-specific covariates. We further meta-analyzed summary statistics using inverse-variance meta-analysis as in the primary analysis.

For the *ANK1* locus, an additional conditional analysis was carried out to test whether an independent association signal was present for SNP rs6474359. This signal appeared to be statistically independent from the lead SNP rs4737009, as shown by low linkage disequilibrium (pairwise r^2 with rs4737009 = 0.0001). In each study, the association at chromosome 8 was evaluated including SNP rs4737009 as an additional covariate to

the basic model, and then results were meta-analyzed as in the primary analysis.

Similarly, we used conditional analyses to assess whether the ten loci (**Table 2**) affect HbA_{1c} through hematological mechanisms. First, we selected a subset of up to 7,500 samples from four cohorts (KORA F3, KORA F4, SardiNIA and NHANES III) with available data for hemoglobin levels (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), iron levels and transferrin. In these participants we calculated separate regressions of HbA_{1c} and HbA_{1c} adjusted for each of the hematological parameters on each of the ten genome-wide significant SNPs. All models were also adjusted for sex, age and other study-specific covariates. We further meta-analyzed summary statistics using inverse-variance meta-analysis as in the primary analysis.

Calculation of explained variance

To estimate the total variance in HbA_{1c} explained by the ten lead SNPs (rs2779116, rs552976, rs1800562, rs1799884, rs4737009, rs16926246, rs1387153, rs7998202, rs1046896, rs855791), we fitted a regression model for each GWAS cohort including the ten SNPs, and calculated an estimate of the variance explained by the SNPs as sample size weighted average in the following samples: ARIC, B58C-T1DGC, B58C-WTCCC, BLSA, DESIR, EPIC cases, EPIC cohort, Fenland, FHS, GenomeEUtwin, KORA F3, KORA F4, Lollipop, NTR and SHIP. The cohort-specific total variance explained by the ten SNPs was calculated as the difference between the variance explained by the full model and the variance of a basic model including only sex, age and the study-specific covariates.

Calculation of HbA_{1c} genotype score

We defined a risk score for the ten leading SNPs as a weighted sum of the number of expected risk alleles, where the sum of the weights was set to the number of SNPs and the weights were proportional to the estimate of the effect size for each SNP (beta coefficients from the association model). The same approach was taken for the seven non-glycemic loci. Mean HbA_{1c} (%) levels according to the number of weighted risk alleles were computed in some of the largest population cohorts (FHS, ARIC, SardiNIA and KORA F4) with all seven or ten SNPs available (genotyped or imputed). For FHS and SardiNIA, a mixed effect model with a single variable with two groups (lower 10% versus upper 10%) was used to account for relatedness among participants, for the other studies, fixed-effects models were used. We carried out the same calculation using the seven non-glycemic loci (rs2779116, rs1800562, rs4737009, rs16926246, rs7998202, rs1046896, rs855791). For both ten and seven loci we calculated a weighted average difference in the HbA_{1c} level between the 10% tails of the genotype score distribution (N=200 in FHS, N=335 in SardiNIA, N=149 in KORA).

Association with intermediate and disease endpoints

The top SNPs were additionally tested for association with other metabolic traits using available meta-analysis data from MAGIC (7). Associations with FG (n=40,934-46,184), fasting insulin (n=33,182-38,236), β -cell function by homeostasis model assessment (HOMA-B; n=31,434-36,464) and insulin resistance by homeostasis model assessment (HOMA-IR; n=31,884-37,035) were calculated as described previously (7). Associations with oral glucose tolerance tests (2-hr glucose, n=10,075-15,234 and 2-hr insulin, n=3,690-7,062) were calculated as described previously (8). Analyses of HbA_{1c} conditional on FG were calculated in a subset of the samples as described above. Associations with hematologic traits were obtained from a meta-analysis carried out on the four populations (KORA F3, KORA F4, SardiNIA and NHANES III) with available data for Hb, MCH, MCV iron levels and transferrin. Associations of *MTNR1B*, *GCK* and *G6PC2/ABCB11* with type 2 diabetes (T2D) were obtained from a total of 8,130 cases and 38,987 controls (or 6,206 cases and 36,049 controls for SNP rs1800562 (*HFE*)) from the DIAGRAM+ consortium (9).

For associations with coronary artery disease, we obtained summary statistics for 13,925 cases and 14,590 controls (in aggregate) from nine case-control collections. Sample characteristics and case/control definitions are given in **Table S5**, and study specific association results are given in **Table S6**. Pooled summary statistics (odds ratios, 95% confidence intervals and *P* values) were calculated under a fixed-effects model as there was no evidence for inter-cohort heterogeneity, using custom scripts implemented in the R environment (available from the authors on request).

Author Contributions:

PI: Ma. S. San., Mu. P. Rei., Da. Alt., Do. I. Boo., St. R. Bor., Jo. Cha., Fr. S. Col., Ec. J. C. Geu., Pa. Del., Ni. For., Ma. Fra., An. Ham., Ja. Koo., Jo. Kuu., Ma. Laa., Ma. Lat., Ru. J. F. Loo., Pa. K. E. Mag., Wi. Mär., Ru. McP., Ka. L. Moh., Da. M. Nat., Na. L. Ped., Da. J. Rad., Ig. Rud., Da. Sch., Pe. Sch., Ud. See., Ma. Ser., Ti. D. Spe., Ka. Ste., Da. P. Str., Mi. Stu., Un. Tho., An. Tön., He. Völ., Hu. Wat., HE. Wic., Go. H. Wil., Ja. F. Wil., Al. F. Wri., St. E. Eps., Ka. Kha., Ro. Rob., Ro. Sla., Go. Abe., Mi. Boe., Ph. Fro., Le. Gro., Ma. I. McC., Ma. Uda., Ni. J. War., In. Bar., Ja. B. Mei.

Data analysis: Ni. Sor., Se. San., El. Whe., Ch. Gie., Dö. Rad., Jo. Dup., Na. Bou., Cl. Lan., In. Pro., Sa. L. Ric., Al. F. R. Ste., Da. Bar., Ch. Fra., So. Gal., An. Goe., Da. Had., Jo. Hot., Ra. Hun., An. U. Jac., Ch. W. Kno., Au. Kon., Br. Küh., Cé. Lec., Mi. Li., Ru. J. F. Loo., Ji. Lua., Va. Lys., Re. Mäg., An. Mäl., Vi. E. Moo., Jo. F. Ped., Ma. Per., El. Por., Sa. Rip., Gh. Roc., Ve. Sal., Ri. Sax., Pe. Shr., Kj. Son., Da. P. Str., Ro. Str., Id. Sur., Al. Teu., Gu. Tho., Da. M. Wat., Ca. Zab., Ji. Zha., Go. Abe.

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Manuscript preparation: Ni. Sor., Se. San., El. Whe., Ch. Gie., Dö. Rad., Jo. Dup., Na. Bou., Cl. Lan., In. Pro., El. Sto., Ma. S. San., Ma. M. Hee., Mu. P. Rei., Al. F. R. Ste., An. Cao., Ro. Cla., Ma. Fra., An. Goe., An. Köt., Ru. McP., Jo. F. Ped., Le. Pel., Ma. Per., Da. J. Rad., Ud. See., Da. P. Str., St. E. Eps., Ro. Rob., Ma. D. Fle., Jo. C. Flo., Ma. Uda., Ni. J. War., In. Bar., Ja. B. Mei.

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APPENDIX - Biological function of candidate genes in associated regions

***G6PC2* (glucose-6-phosphatase, catalytic, 2) /*ABCB11* (ATP-binding cassette, sub-family B, member 11)**

***GCK* (glucokinase)**

Common genetic variants in *G6PC2* (10; 11), *GCK* (12), *GCKR* (glucokinase regulator) (13) and *MTNR1B* (melatonin receptor 1B) (14-16) have recently been identified as loci regulating FG in genome-wide association studies of diabetes-free adults. *GCK* and *G6PC2* are expressed in the pancreas and code for proteins that are key regulators of the provision of FG. Glucokinases/hexokinases phosphorylate glucose to produce glucose-6-phosphate, the first step in most glucose metabolism pathways. Alternative splicing of *GCK* results in three tissue-specific forms of glucokinase, one found in pancreatic islet β cells and two found in liver. The protein localizes to the outer membrane of mitochondria. In contrast to other hexokinases, this enzyme is not inhibited by its product glucose-6-phosphate but remains active while glucose is abundant. Rare inactivating mutations in *GCK* cause maturity-onset diabetes of the young, type 2, a disorder characterized by mild, stable fasting hyperglycemia (17). In contrast, activating mutations in *GCK* cause persistent hyperinsulinemic hypoglycemia of infancy, in which the threshold for glucose-stimulated insulin release is reduced. Thus, both disorders highlight the role of glucokinase

in regulating insulin secretion as the glucose sensor of β cells (18). *G6PC2* encodes an enzyme belonging to the glucose-6-phosphatase catalytic subunit family that is involved in the hydrolysis of glucose-6-phosphate, the terminal step in gluconeogenic and glycogenolytic pathways; in this manner it is directly linked to the availability of glucose for release into the bloodstream. The protein product encoded by this gene is found in pancreatic islets and does not exhibit phosphohydrolase activity, but it is a major target of cell-mediated autoimmunity in diabetes. *G6PC2* and *GCK* are likely candidates for altering an individual's physiologic glucostat set point in the absence of progressive hyperglycemia and symptomatic disease. Lead HbA_{1c} SNPs for the *G6PC2/ABCB11* (rs552976) and *GCK* loci (rs1799884) are in high linkage disequilibrium with the most significant SNPs previously identified for FG ($r^2=0.69$ between rs552976 and rs560887 and $r^2=1$ between rs1799884 and rs4607517).

Within the same recombination hot spot (Hapmap II data) of *GCK*: *CAMK2B* (calcium/calmodulin-dependent protein kinase II beta)

The product of this gene belongs to the serine/threonine protein kinase family and to the Ca²⁺/calmodulin-dependent protein kinase subfamily. It is possible that distinct isoforms of this chain have different cellular localizations and interact differently with calmodulin. Eight transcript variants encoding eight distinct isoforms have been identified for this gene, some of them are expressed in β cells.

***MTNR1B* (MELATONIN RECEPTOR 1B)**

MTNR1A and *MTNR1B* encode two of the known human melatonin receptors (19). *MTNR1B* is a G-protein coupled cell surface receptor that is highly expressed in the brain and retina. It is also transcribed in human pancreatic islets and rodent insulinoma cell lines (20). Insulin secretion demonstrates a circadian rhythm which is disrupted in T2D (21). Human pancreatic melatonin receptor expression is elevated in T2D based on elevated mRNA levels as well as more intense immunostaining (22). In previous work we and others have established that the common variants near *MTNR1B* modulate of FG and increase T2D risk, suggesting a link between circadian rhythm regulation and glucose homeostasis (14; 16). Lyssenko and colleagues demonstrated that the risk variant of SNP rs10830963 is associated with impaired early insulin secretion (15). Ronn et al. found that the SNP identified in Europeans was associated with an increased risk of T2D and increased FG in a Han Chinese population (23). Increased expression of *MTNR1B* in pancreatic β -cells and melatonin-mediated impaired insulin secretion in risk allele carriers (14-16; 23-25) suggest mechanisms for *MTNR1B* variants to alter glucose levels in healthy individuals (14-16). No other known gene is located in the same recombination hotspot as *MTNR1B*.

***SPTA1* (spectrin, alpha, erythrocytic 1 (elliptocytosis 2))**

Spectrin is an actin crosslinking and molecular scaffold protein that links the plasma membrane to the actin cytoskeleton, and functions in the determination of cell shape, arrangement of transmembrane proteins, and organization of organelles. It is a tetramer made up of α - β dimers linked in a head-to-head arrangement. This gene is one member of a family of α -spectrin genes. The encoded protein is primarily composed of 22 spectrin repeats that are involved in dimer formation. It forms weaker tetramer interactions than non-erythrocytic α -spectrin, which may increase the plasma membrane elasticity and deformability of red blood cells. Rare mutations in *SPTA1* are responsible for elliptocytosis type 2 [MIM 130600], pyropoikilocytosis [MIM 266140], and spherocytic hemolytic anemia [MIM 270970] (26).

ANK1 (ankyrin 1, erythrocytic)

ANK1 encodes erythrocytic ankyrin 1, an integral membrane protein linked to the underlying spectrin-actin cytoskeleton, and plays a role in cell motility and maintenance of specialized membrane domains. Multiple isoforms of ankyrin with different affinities for various target proteins are expressed in a tissue-specific, developmentally regulated manner. Most ankyrins are typically composed of three structural domains: an amino-terminal domain containing multiple ankyrin repeats; a central region with a highly conserved spectrin binding domain; and a carboxy-terminal regulatory domain which is the least conserved and subject to variation. Ankyrin 1, the prototype of this family, was first discovered in the erythrocytes, but since has also been found in brain and muscle. Mutations in *ANK1* are found in approximately half of all patients with hereditary spherocytosis (26-28). Complex patterns of alternative splicing in the regulatory domain, giving rise to different isoforms of ankyrin 1 have been described. Truncated muscle-specific isoforms of ankyrin 1 resulting from usage of an alternate promoter have also been identified.

5' region of ANK1: NKX6-3 (NK6 homeobox 3)

The NKX family of homeodomain proteins controls numerous developmental processes. Members of the NKX6 subfamily, including *NKX6-3*, are involved in development of the central nervous system (CNS), gastrointestinal tract and pancreas (29).

HK1 (HEXOKINASE 1)

Mammalian hexokinase comprises four isozymes that vary in properties and tissue distribution (*HK1*, *HK2*, *HK3*, and *GCK*) (30). Hexokinase catalyzes the first step in glucose metabolism, converting glucose to glucose-6-phosphate via phosphorylation. Hexokinase is normally found in the cytoplasm of the cell and *HK1* is the predominant isoform found in erythrocytes (30). It is also expressed in other tissues such as muscle and brain (31). Rare mutations in *HK1* have been described to cause non-spherocytic hemolytic anemia (31-33). Pare et al. reported that two intronic SNPs (rs2305198 and rs7072268) are associated with HbA_{1c} (31).

3' region of HK1: TACR2 tachykinin receptor 2. This gene belongs to a family of genes that encode receptors for tachykinins, characterized by interactions with G proteins and 7 hydrophobic transmembrane regions. This gene encodes the receptor for the tachykinin neuropeptide substance K, also referred to as neurokinin A.

ATP11A (ATPase TYPE 11A)

Altered membrane integrity may cause a potassium leak in erythrocytes, leading to higher sodium/potassium ATPase activity, increased glycolytic activity, and lower intracellular glucose concentrations. In this regard, the association of ATPase TYPE 11A (*ATP11A*) with HbA_{1c} is particularly intriguing. *ATP11A* is a P-type ATPase that is involved in the transport of ions across membranes through phosphorylation and de-phosphorylation. Kikuno et al. isolated a partial cDNA encoding *ATP11A* and RT-PCR analysis detected widespread but moderate expression with lowest levels in spleen, pancreas, and testis (34). Resistance to farnesyltransferase inhibitors in Bcr/Abl positive lymphoblastic leukemia cells has been associated with overexpression of *ATP11A* (35). Altered membrane permeability and monovalent ion leak is also a cause of erythrocyte over- or under-hydration and several hereditary stomatocytoses (for instance, MIM %185000, MIM %194380).

FN3K (FRUCTOSAMINE 3-KINASE)

Fructosamine 3-kinase is an intracellular enzyme that catalyzes the phosphorylation of

fructosamines formed by glycation. The fructosamine 3-phosphates that are formed are unstable and they undergo spontaneous decomposition (36). *FN3K* is therefore involved in de-glycation. Inhibition of *FN3K* in erythrocytes leads to an increase in HbA_{1C} (37). Delpierre et al. demonstrated that purified *FN3K* catalyzed ATP-dependent phosphorylation of a synthetic fructosamine (38). Fructosamine 3-kinase is active in erythrocytes and in the lens, which are characterized by slow protein turnover, and may be more susceptible to protein glycation (37). An RNA analysis from 11 different human tissues demonstrates that *FN3K* is widely expressed in a variety of cell types (39). There is wide inter-individual variability in *FN3K* activity but little correlation between *FN3K* activity and the levels of HbA_{1C} (36). HbA_{1C} variability has been associated with one SNP in the promoter region and one SNP in the exon 6 of the *FN3K* gene (P <0.0001) (36).

***HFE* (HEMOCHROMATOSIS)**

HFE encodes a membrane protein that appears involved in iron sensing through the interaction with the transferrin receptor (40). Defects in this gene can cause hereditary hemochromatosis (MIM 235200), a recessive iron storage disorder due to inappropriately low hepcidin levels. The A allele at rs1800562 codes for the pathological C→Y mutation at position 262, for the most common cause of hereditary hemochromatosis. The prevalence of the *HFE* mutation is higher in patients with T2D than those without diabetes, and iron overload associated with hemochromatosis is a risk factor for T2D (41; 42); however, our data show that the hemochromatosis risk A allele is associated with *lower* levels of HbA_{1C} as described in the main manuscript.

***TMPRSS6* (TRANSMEMBRANE PROTEASE, SERINE 6)**

TMPRSS6 (also referred to as matriptase-2) is a type II transmembrane serine protease enzyme that hydrolyzes a variety of synthetic substrates as well as endogenous proteins, such as fibronectin, fibrinogen, and type I collagen (43). *TMPRSS6* is involved in regulation of iron homeostasis through the control of hepcidin expression (44). The T allele at SNP rs855791 encodes a missense mutation [Val736Ala] that has been detected, together with other mutations, in families with iron-refractory iron deficiency anemia (IRIDA, MIM 206200). This mutation leads to the overproduction of hepcidin and, in turn, to defective iron absorption and utilization (45). Similar to *HFE* above, mutations in *TMPRSS6* 'uncouple' iron stores sensing from the regulation of iron absorption; however in IRIDA, *TMPRSS6* mutations result in inappropriately elevated hepcidin and an opposite phenotype from hemochromatosis. Northern blot analysis of multiple human tissues revealed expression of the *TMPRSS6* in the fetal and adult liver (43). In our data, the IRIDA risk T allele is associated with lower MCH and *higher* HbA_{1C} levels, as one would predict in a state of iron deficiency and disproportionately lower hemoglobin concentrations, thereby raising the measured percentage of glycated hemoglobin. Thus, our association results suggest the presence of two complementary and directionally consistent pathways that through deficiency or excess make iron metabolism a key determinant of measured levels of hemoglobin glycation in erythrocytes.

5' region of *TMPRSS6*: *KCTD17* (potassium channel tetramerisation domain containing 17), also known as *REN*. *KCTD17* has unknown function. Overexpression of Ren in mice induced neuronal differentiation, growth arrest, and p27(KIP1) expression in central and peripheral neural progenitor cell lines. Inhibition of Ren impaired retinoic acid induction of neurogenin-1 coded by *NEUROG1* and NeuroD coded by *NEUROD1* expression. *NEUROD1* harbors known rare mutations of maturity onset diabetes of the young (46) and regulates expression of the insulin gene, whereas p27 is encoded by *CDKN1B*, a T2D-associated locus (9).

3' region of *TMPRSS6*: *IL2RB* (interleukin 2 receptor B). The interleukin 2 receptor is involved in T cell-mediated immune responses. Both the intermediate and high affinity forms of the receptor are involved in receptor-mediated endocytosis and transduction of mitogenic signals from interleukin 2.

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