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Exome Genotyping Identifies Pleiotropic Variants Associated with Red Blood Cell Traits

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Red blood cell (RBC) traits are important heritable clinical biomarkers and modifiers of disease severity. To identify coding genetic variants associated with these traits, we conducted meta-analyses of seven RBC phenotypes in 130,273 multi-ethnic individuals from studies genotyped on an exome array. After conditional analyses and replication in 27,480 independent individuals, we identified 16 new RBC variants. We found low-frequency missense variants in *MAP1A* (rs55707100, minor allele frequency [MAF] = 3.3%, $p = 2 \times 10^{-10}$ for hemoglobin [HGB]) and *HNF4A* (rs1800961, MAF = 2.4%, $p < 3 \times 10^{-8}$ for hematocrit [HCT] and HGB). In African Americans, we identified a nonsense variant in *CD36* associated with higher RBC distribution width (rs3211938, MAF = 8.7%, $p = 7 \times 10^{-11}$) and showed that it is associated with lower *CD36* expression and strong allelic imbalance in ex vivo differentiated human erythroblasts. We also identified a rare missense variant in *ALAS2* (rs201062903, MAF = 0.2%) associated with lower mean corpuscular volume and mean corpuscular hemoglobin ($p < 8 \times 10^{-9}$). Mendelian mutations in *ALAS2* are a cause of sideroblastic anemia and erythropoietic protoporphyria. Gene-based testing highlighted three rare missense variants in *PKLR*, a gene mutated in Mendelian non-spherocytic hemolytic anemia, associated with HGB and HCT (SKAT $p < 8 \times 10^{-7}$). These rare, low-frequency, and common RBC variants showed pleiotropy, being also associated with platelet, white blood cell, and lipid traits. Our association results and functional annotation suggest the involvement of new genes in human erythropoiesis. We also confirm that rare and low-frequency variants play a role in the architecture of complex human traits, although their phenotypic effect is generally smaller than originally anticipated.

Introduction

One in four cells in the human body is a mature enucleated red blood cell (RBC), also called an erythrocyte. RBC mean lifespan in adults is 100–120 days, requiring constant renewal. To that end, we produce on average 2.4 million RBCs per second in the bone marrow. This massive yet well-orchestrated cell proliferation process is necessary to

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accommodate RBCs' main function: to transport oxygen from the lungs to the peripheral organs, and carbon dioxide from the organs to the lungs. Hemoglobin (HGB), the metalloprotein that constitutes by far the most abundant biomolecule found in mature RBCs, is responsible for oxygen transport. In addition to their critical role in the circulatory system, RBCs also have secondary, often less-appreciated, functions. Within blood vessels, they respond

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to shear stress and produce the vasodilator nitric oxide to regulate vascular tonus.[1] RBCs participate in antimicrobial strategies to fight hemolytic pathogens[2] and in the inflammatory response, acting as a reservoir for multiple chemokines.[3] Furthermore, the direct involvement of RBCs in adhering to the vascular endothelium or supporting thrombin generation may help to promote blood coagulation or thrombosis.[4,5]

Given the paramount importance of RBCs in physiology, it is not surprising that monitoring their features is common practice in medicine to assess the overall health of patients. An excessive number of circulating RBCs (erythrocytosis [MIM: 133100]) can suggest a primary bone marrow disease, a myeloproliferative neoplasm such as polycythemia vera (MIM: 263300), or chronic hypoxemia due to congenital heart defects. Low HGB concentration and hematocrit (HCT) levels (anemia) can indicate inherited HGB or RBC structural gene mutations, malnutrition, or kidney diseases. By considering the volume (mean corpuscular volume [MCV]), hemoglobin content (mean corpuscular hemoglobin [MCH] and mean corpuscular hemoglobin concentration [MCHC]) or the distribution width (RDW) of RBCs, a physician can distinguish between the different causes of anemia (e.g., microcytic/hypochromic due to iron deficiency[6]). In addition, epidemiological studies have correlated high RDW values with a worse prognosis in heart failure patients.[7] RDW is also an independent predictor of overall mortality in healthy individuals, as well as a predictor of mortality in patients with various conditions such as cardiovascular diseases, obesity, malignancies, and chronic kidney disease.[8–12]

RBC count and indices vary among individuals, and 40%–90% of this phenotypic variation is heritable.[13–16] Identifying the genes and biological pathways that contribute to this inter-individual variation in RBC traits could highlight modifiers of severity and/or therapeutic options for several hematological diseases. Already, large-scale genome-wide association studies (GWASs) have found dozens of SNPs associated with one or more of these RBC traits.[17,18] However, owing to their design, GWASs are largely insensitive to rare (minor allele frequency [MAF] $< 1\%$) and low-frequency ($1\% \leq$ MAF $< 5\%$) genetic variants. Using an exome array, we previously performed an association study for HGB and HCT in 31,340 European-ancestry individuals and identified rare coding or splice site variants in the erythropoietin and β-globin genes.[19] Within the framework of the Blood-Cell Consortium (BCX),[20,21] we now report a larger genotyping-based exome survey of seven RBC traits conducted in up to 130,273 individuals, including 23,896 participants of non-European ancestry. With this experiment, our initial goals were to expand the list of rare and low-frequency coding or splice site variants associated with RBC traits and to explore whether the exome array can complement the GWAS approach to fine map RBC causal genes.

Subjects and Methods

Study Participants

The Blood-Cell Consortium (BCX) aims to identify novel common and rare variants associated with blood-cell traits using an exome array. BCX is comprised of more than 134,021 participants from 24 discovery cohorts and five ancestries: European, African American, Hispanic, East Asian, and South Asian. Detailed description of the participating cohorts is provided in Table S1. BCX is interested in the genetics of all main hematological measures and is divided into three main working groups: RBC, white blood cell (WBC),[21] and platelet (PLT).[20] For the RBC working group, we analyzed seven traits available in up to 130,273 individuals: RBC count ($\times 10^{12}$/L), HGB (g/dL), HCT (%), MCV (fL), MCH (pg), MCHC (g/dL), and RDW (%) (Table S2). The BCX procedures were in accordance with the institutional and national ethical standards of the responsible committees and proper informed consent was obtained.

Genotyping and Quality-Control Steps

Participants from the different studies were genotyped on one of the following exome chip genotyping arrays: Illumina ExomeChip v.1.0, Illumina ExomeChip v.1.1_A, Illumina ExomeChip-12 v.1.1, Affymetrix Axiom Biobank Plus GSKBB1, or Illumina HumanOmniExpressExome Chip. Genotypes were then called either (1) with the Illumina GenomeStudio GENCALL and subsequently recalled using zCALL or (2) by the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium Exome Chip effort[22] (Table S3). The same quality-control steps were followed by each participating study. We excluded variants with low genotyping success rate (<95%, except for WHI that used a cutoff <90%) (Table S3). Samples with call rate < 95% (except for SOLID-TIMI 52 and STABILITY that used 94.5% and 93.5% cutoffs, respectively) after joint or zCALL calling and with outlying heterozygosity rate were also excluded. Other exclusions were deviation from Hardy-Weinberg equilibrium ($p < 1 \times 10^{-6}$) and gender mismatch. We performed principal-component analysis (PCA) or multidimensional scaling (MDS) and excluded sample outliers from the resulting plots through visual inspection, using populations from the 1000 Genomes Project to anchor these analyses. Keeping only autosomal and X chromosome variants for the analysis, we aligned all variants on the forward strand and created a uniform list of reference alleles using the --force alleles command in PLINK.[23] Finally, an indexed variant call format file (VCF) was created by each study and checked for allele alignment and any allele or strand flips using the checkVCF package.[24] Prior to performing meta-analyses of the association results provided by each participating study, we ran the EasyQC protocol[25] to check for population allele frequency deviations and proper trait transformation in each cohort.

Phenotype Modeling and Association Analyses

When possible, we excluded individuals with blood cancer, leukemia, lymphoma, bone marrow transplant, congenital or hereditary anemia, HIV, end-stage kidney disease, dialysis, splenectomy, or cirrhosis and those with extreme measurements of RBC traits (Table S1). We also excluded individuals on erythropoietin treatment or chemotherapy. Additionally, we excluded pregnant women and individuals with acute medical illness at the time the complete blood count (CBC) was done. For the seven RBC traits, within each study, we adjusted for age, age-squared, gender,

the first ten principal components, and, where applicable, other study-specific covariates such as study center via a linear regression model. Within each study, we then applied inverse normal transformation on the residuals and tested the phenotypes for association with the ExomeChip variants using either RVtests (v.20140416)[26] or RAREMETALWORKER.0.4.9.[27]

Discovery Meta-analyses

Score files generated by RVtests or RAREMETALWORKER from each participating study were used to carry out meta-analyses of the single variant association results using RareMETALS v.5.9.[28] All analyses were performed separately in each of European American (EA) and African American (AA) ancestries. In the multi-ancestry meta-analyses, we combined individuals of European, African American, Hispanic, East-Asian, and South-Asian ancestries (All). We included variants with allele frequency difference between the highest and lowest MAF < 0.3 for EA and AA ancestries and < 0.6 for the combined ancestry meta-analyses. For the gene-based analyses, we used score files and variance-covariance matrices from the study-specific association results and applied the sequence kernel association test (SKAT)[29] and variable threshold (VT) algorithms[30] in RareMETALS considering only missense, nonsense, and splice site variants with a MAF $< 1\%$. Gene-based analyses were also stratified by ancestry. Significance thresholds were determined using Bonferroni correction assuming ~250,000 independent variants ($p < 2 \times 10^{-7}$ for the single-variant analyses) and ~17,000 genes tested on the ExomeChip ($p < 3 \times 10^{-6}$ for the gene-based tests).

Conditional Analysis and Replication

In order to identify independent signals, we performed conditional analyses. In each round of conditional analysis, we conditioned on the most significant single variant in a 1 Mb window. These conditional analyses were performed at the meta-analysis level using RareMETALS. We repeated this step until there were no new signals identified in each region, defined as $p < 2 \times 10^{-7}$. We then checked for linkage disequilibrium (LD) within the list of variants that was retained from the conditional analyses. For variants that were in moderate-to-strong LD ($r^2 \geq 0.3$), we kept the most significant. We attempted replication of the final list of independent variants in eight additional studies that contributed a total of 27,480 individuals ($n = 21,473$ for EA and $n = 6,007$ for AA) (Table S4). The division of discovery and replication samples was dictated by timing because we collected all groups we were aware of for initial discovery and then found others who could participate only much later and hence were used for replication. These studies followed similar analytical procedures and steps as those followed by the discovery analysis (see above). A joint meta-analysis of the discovery and the replication results was carried out using a fixed-effects model and inverse-variance weighting as implemented in METAL.[31] We considered as replicated markers those with a nominal $p_{replication} < 0.05$ and an effect on phenotype in the same direction as in the discovery results.

Allelic Imbalance and Expression of *CD36*

We checked for allelic imbalance (AI) of the rs3211938 variant in *CD36* (MIM: 173510) as well as the expression of the gene in 12 samples of fetal liver erythroblasts obtained from anonymous donors. Details on the protocol including RNA extraction and sequencing can be found elsewhere.[32] We calculated the difference in the ratio of reads of the reference allele (T) and the alternate allele (G) of rs3211938. In brief, reads overlapping rs3211938 were counted with samtools (v.1.1) mpileup software using genome build hg19. We kept uniquely mapping reads using -q 50 argument (mapping quality > 50) and sites with base quality > 10. Statistical significance of the difference in the ratio of reads between the reference allele and the alternate allele was assessed with a binomial test. For each sample, we summed all reads overlapping all heterozygous SNPs and calculated the expected ratio within each SNP allele combination. Reads that fall in the top 25th coverage percentile were down-sampled so that the highest covered sites do not bias the expected ratio.[33] For rs3211938, the expected T:G ratio was 0.507.

Expression Quantitative Trait Loci Analysis

We cross-referenced our list of RBC novel variants with more than 100 separate expression quantitative trait loci (eQTL) published datasets. Datasets were collected through publications, publically available sources, and private collaborations. A general overview of a subset of >50 eQTL studies has been published,[34] with specific citations for >100 datasets included in the current query followed here. A complete list of tissues and studies used can be found in the Supplemental Data. We considered SNPs that are themselves expression SNPs (eSNP) when they meet a $p < 0.0001$ threshold or when they are in LD ($r^2 > 0.3$) with the best eSNP ($p < 0.0001$).

Results

Single-Variant Meta-analyses

We meta-analyzed ExomeChip results for seven RBC-related phenotypes (RBC count, HCT, HGB, MCH, MCHC, MCV, and RDW) available in up to 130,273 individuals from 24 studies and 5 ancestries (Tables S1–S3 and Figure S1). Across these different phenotypes, a total of 226 variants reached exome-wide significance ($p < 2 \times 10^{-7}$) in the combined ancestry analyses (Figures 1 and S2). Given that some of these RBC traits are correlated (Figure S3), these associated variants highlight 71 different loci (defined using a 1 Mb interval). Overall, we observed only modest inflation of the test statistics ($\lambda_{GC} = 1.03$–1.05), consistent with little confounding due to technical artifacts, population stratification, or cryptic relatedness.

In order to identify independent variants, we performed conditional analyses at the meta-analysis level adjusting for the effect of the most significant variant in a 1 Mb region in a stepwise manner (Subjects and Methods). After this analysis, we obtained a list of 126 independent variants associated with at least one RBC trait at $p < 2 \times 10^{-7}$ (Table S5). Selecting only variants that lie more than 1 Mb away from a known GWAS locus resulted in 23 independent variants located within 20 novel RBC loci, where novel is used to define loci not found in the existing literature (Table 1). We attempted to replicate these 126 variants in 8 independent cohorts totaling 27,480 participants (Table S5). Overall, we observed a strong replication, with 94 of the 126 variants showing consistent direction of effect between the discovery and replication analyses (binomial $p = 3 \times 10^{-8}$; Table S5). Of the 23 novel RBC variants, we replicated 16 at nominal $p < 0.05$ for at

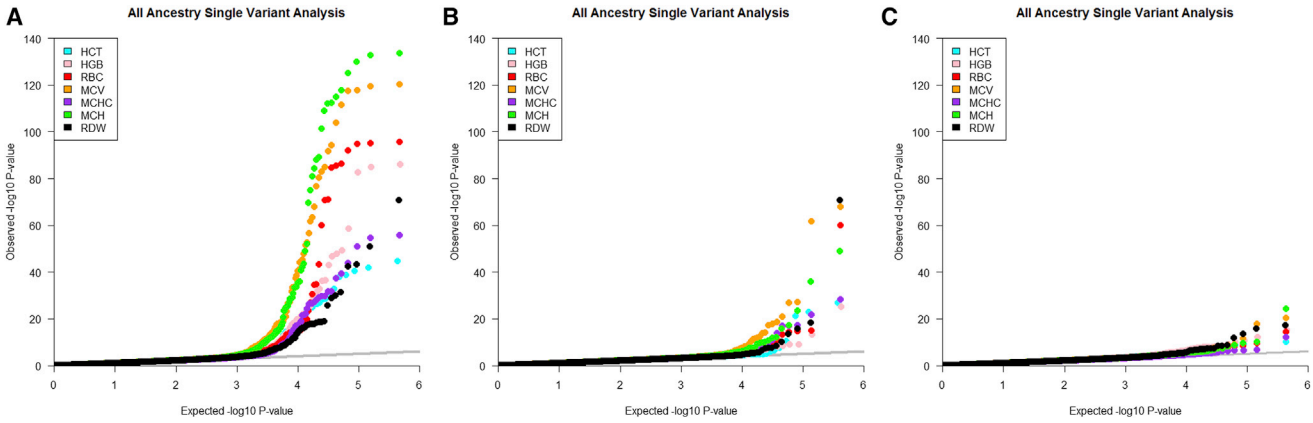

Figure 1. Quantile-Quantile Plots of Single-Variant Association Results in the All Ancestry Meta-analyses for the Seven Red Blood Cell Traits Analyzed

(A) Distribution of the single variant results for all variants tested on the exome array.
(B) Only markers with a minor allele frequency < 5% are shown here.
(C) Variants outside of known RBC GWAS regions. Variants that are within 1 Mb from a previously published RBC GWAS locus were excluded for this QQ plot.
Abbreviations are as follows: HCT, hematocrit; HGB, hemoglobin; RBC, red blood cell count; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; RDW, red blood cell distribution width.

least one RBC trait (binomial $p = 3 \times 10^{-16}$; Table 1). Out of these 16 novel and replicated RBC variants, there are five missense variants, including two variants with MAF < 5% in *MAP1A* (MIM: 600178) and *HNF4A* (MIM: 600281) and one nonsense variant in *CD36* (Table 1). Among the remaining nine novel and replicated RBC variants, there are five intronic, one synonymous, one 5′ UTR, and one intergenic marker (Table 1).

Prioritization of Candidate Genes and Genetic Variants

Our single-variant analyses in EA samples identified one rare missense variant in *ALAS2* (MIM: 301300) associated with MCV and MCH (rs201062903, p.Pro507Leu [c.1559C>T], MAF = 0.2%) (Table 1). The association with this variant did not replicate, potentially because of limited statistical power (the replication sample size for this rare marker was 5,044; see also Discussion). *ALAS2* encodes 5-aminolevulinate synthase 2, the rate-controlling enzyme of erythroid heme synthesis. Additionally, rare mutations in *ALAS2* cause X-linked sideroblastic anemia (MIM: 300751) and erythropoietic protoporphyria (MIM: 300752). Thus, despite the lack of replication, *ALAS2* remains an excellent candidate gene to modulate RBC traits. The *ALAS2* p.Pro507Leu variant, which is not reported in the ClinVar database, maps between two amino acids (Tyr506 and Thr508) that are important for catalytic activity and known to be mutated in cases of sideroblastic anemia.[35]

Two low-frequency missense variants identified in our analyses implicate *MAP1A* and *HNF4A* in RBC biology (Table 1). *MAP1A* encodes microtubule-associated protein 1A, a gene highly expressed in the nervous system and mostly studied in the context of neuronal diseases, although it is expressed in many additional tissues, including hematopoietic cells.[36] Deletion of *MAP1A* in the mouse causes defects in synaptic plasticity.[37] This observation is interesting given that inactivation of *ANK1* (MIM: 612641), another gene that encodes a cytoskeleton protein and is expressed in neurons and RBCs, is associated with neurological dysfunction in the mouse and spherocytosis and hemolytic anemia in humans (MIM: 182900). Our meta-analyses confirmed two known independent *ANK1* variants associated with MCHC: an intronic SNP (rs4737009, MAF = 19.8%, $p = 1.3 \times 10^{-8}$) and a low-frequency missense variant (rs34664882, p.Ala1462Val, MAF = 2.9%, $p = 1.7 \times 10^{-16}$) (Table S5; N.P., U.M.S., J.B.-J., and M.-H.C., unpublished data).[17]

In the accompanying BCX PLT article,[20] we report that the same *MAP1A* rs55707100 allele (p.Pro2349Leu [c.7046C>T]) associated here with decreased HGB concentration is also associated with increased PLT count. Furthermore, recent studies have identified associations between rs55707100 and HDL-cholesterol and triglyceride levels (S. Mukherjee, 2015, ASHG, conference). Adding to the complexity, the GTEx dataset indicates that rs55707100 is an expression quantitative trait locus (eQTL) for *ADAL* ($p_{eQTL} = 9 \times 10^{-11}$) but not for *MAP1A*.[38] *ADAL* is a poorly characterized adenosine deaminase-like protein that is highly expressed in human erythroblasts. However, the eQTL association between rs55707100 and *ADAL* could simply reflect "LD shadowing" from nearby markers that are much stronger eQTL variants for *ADAL*. Indeed, rs3742971 (a common variant located in *ADAL*'s 5′ UTR) is in partial LD with rs55707100 ($r^2 = 0.18$ in European populations from the 1000 Genomes Project) and strongly associated with *ADAL* expression levels ($p_{eQTL} = 6 \times 10^{-49}$).

The second low-frequency missense variant associated with HGB and HCT maps within the coding sequence of

Table 1. Association Results of Variants in Novel Loci Associated with Red Blood Cell Traits

Marker Info						Discovery				Replication				Combined	
Trait	Position	A1/A2	SNP	Annotation	Gene	n	AF (A2)	Beta (SE)	p Value	n	AF (A2)	Beta (SE)	p Value	Beta (SE)	p Value
RDW-EA	1: 25,768,937	A/G	rs10903129*	intron	*TMEM57-RHD*	45,573	0.544	0.037 (0.007)	1.19×10^{-7}	18,475	0.560	0.023 (0.011)	0.0373	0.033 (0.006)	2.41×10^{-8}
RDW-All	1: 25,768,937	A/G	rs10903129*	intron	*TMEM57-RHD*	56,194	0.568	0.034 (0.006)	9.58×10^{-8}	24,474	0.600	0.021 (0.01)	0.0252	0.03 (0.005)	1.32×10^{-8}
HCT-All	1: 155,162,067	C/T	rs4072037*	synonymous	*MUC1*	109,875	0.554	0.025 (0.005)	5.82×10^{-8}	25,006	0.563	0.038 (0.009)	5.96×10^{-5}	0.027 (0.004)	3.47×10^{-11}
HGB-All	2: 27,741,237	T/C	rs780094	intron	*GCKR*	130,273	0.626	0.024 (0.004)	7.14×10^{-8}	3,162	0.626	−0.012 (0.026)	0.6410	0.023 (0.044)	1.68×10^{-7}
RBC-All	2: 219,509,618	C/A	rs2230115*	missense	*ZNF142*	74,488	0.509	0.033 (0.006)	9.74×10^{-9}	27,442	0.477	0.024 (0.01)	0.0167	0.031 (0.005)	7.11×10^{-10}
HCT-All	3: 56,771,251	A/C	rs3772219*	missense	*ARHGEF3*	109,875	0.338	−0.028 (0.005)	2.38×10^{-9}	25,006	0.366	−0.021 (0.01)	0.0292	−0.027 (0.004)	2.56×10^{-10}
HGB-All	3: 56,771,251	A/C	rs3772219*	missense	*ARHGEF3*	130,273	0.336	−0.026 (0.004)	3.76×10^{-9}	27,749	0.367	−0.02 (0.009)	0.0331	−0.025 (0.004)	4.33×10^{-10}
HCT-EA	4: 88,008,782	G/A	rs236985	intron	*AFF1*	87,444	0.394	0.032 (0.005)	3.89×10^{-10}	19,968	0.405	0.02 (0.011)	0.0626	0.03 (0.005)	1.14×10^{-10}
RBC-EA	4: 88,008,782	G/A	rs236985*	intron	*AFF1*	60,231	0.393	0.034 (0.006)	3.50×10^{-8}	21,435	0.405	0.023 (0.011)	0.0273	0.031 (0.005)	4.22×10^{-9}
HGB-EA	4: 88,030,261	G/T	rs442177*	intron	*AFF1*	106,377	0.595	−0.034 (0.005)	3.97×10^{-13}	21,743	0.586	−0.029 (0.01)	0.0052	−0.033 (0.004)	8.23×10^{-15}
RDW-EA	5: 127,371,588	A/G	rs10063647*	intron	*LINC01184-SLC12A2*	45,573	0.463	−0.05 (0.007)	1.72×10^{-13}	18,475	0.480	−0.033 (0.011)	0.0018	−0.045 (0.006)	2.88×10^{-15}
RDW-All	5: 127,371,588	A/G	rs10063647*	intron	*LINC01184-SLC12A2*	56,194	0.506	−0.044 (0.006)	2.11×10^{-12}	24,474	0.545	−0.03 (0.01)	0.0014	−0.04 (0.005)	2.37×10^{-14}
RDW-EA	5: 127,522,543	C/T	rs10089*	utr_5p	*LINC01184-SLC12A2*	45,573	0.21	0.051 (0.008)	8.45×10^{-10}	16,692	0.215	0.058 (0.014)	2.71×10^{-5}	0.053 (0.007)	1.15×10^{-13}
RDW-All	5: 127,522,543	C/T	rs10089*	utr_5p	*LINC01184-SLC12A2*	56,194	0.207	0.044 (0.008)	4.08×10^{-9}	22,691	0.208	0.045 (0.012)	0.0001	0.044 (0.006)	2.73×10^{-12}
HGB-All	6: 7,247,344	C/A	rs35742417*	missense	*RREB1*	130,273	0.174	0.030 (0.005)	1.17×10^{-8}	4,074	0.207	0.065 (0.028)	0.0190	0.032 (0.005)	1.50×10^{-9}
RDW-AA	7: 80,300,449	T/G	rs3211938*	nonsense	*CD36*	6,666	0.087	0.174 (0.031)	2.36×10^{-8}	5,999	0.086	0.139 (0.032)	1.83×10^{-5}	0.161 (0.025)	7.09×10^{-11}
RDW-All	7: 80,300,449	T/G	rs3211938*	nonsense	*CD36*	55,510	0.012	0.171 (0.029)	5.29×10^{-9}	22,691	0.023	0.139 (0.032)	1.61×10^{-5}	0.157 (0.022)	5.12×10^{-13}
RDW-EA	8: 126,490,972	A/T	rs2954029*	intergenic	*TRIB1*	45,573	0.46	0.036 (0.007)	1.53×10^{-7}	16,692	0.466	0.026 (0.011)	0.0210	0.034 (0.006)	1.29×10^{-8}
RDW-All	8: 126,490,972	A/T	rs2954029*	intergenic	*TRIB1*	56,194	0.439	0.032 (0.006)	1.83×10^{-7}	22,691	0.432	0.021 (0.01)	0.0298	0.029 (0.005)	2.54×10^{-8}
MCH-All	10: 105,659,826	T/C	rs2487999	missense	*OBFC1*	66,318	0.869	0.047 (0.009)	4.12×10^{-8}	26,749	0.861	0.025 (0.013)	0.0601	0.041 (0.007)	1.75×10^{-8}
MCH-AA	11: 92,722,761	G/A	rs1447352	intergenic	*MTNR1B*	8,273	0.557	0.089 (0.016)	1.85×10^{-8}	5,038	0.562	−0.022 (0.02)	0.2713	0.07 (0.014)	1.08×10^{-6}
HGB-EA	15: 43,820,717	C/T	rs55707100*	missense	*MAP1A*	106,377	0.033	−0.071 (0.013)	1.65×10^{-8}	21,743	0.0223	−0.099 (0.033)	0.0028	−0.075 (0.012)	2.31×10^{-10}
MCV-AA	16: 1,551,082	A/G	rs2667662*	intron	*TELO2*	10,849	0.725	−0.099 (0.015)	1.79×10^{-10}	5,034	0.724	−0.093 (0.022)	3.02×10^{-5}	−0.098 (0.014)	7.32×10^{-12}
MCV-AA	16: 2,812,939	C/A	rs2240140*	missense	*SRRM2*	8,525	0.118	0.134 (0.025)	7.08×10^{-8}	6,002	0.124	0.106 (0.027)	0.0001	0.128 (0.022)	5.24×10^{-9}

(*Continued on next page*)

Table 1. ***Continued***

Marker Info						Discovery				Replication				Combined	
Trait	**Position**	**A1/A2**	**SNP**	**Annotation**	**Gene**	**n**	**AF (A2)**	**Beta (SE)**	**p Value**	**n**	**AF (A2)**	**Beta (SE)**	**p Value**	**Beta (SE)**	**p Value**
HCT-EA	17: 59,017,025	T/C	rs8080784	intron	*BCAS3-TBX2*	79,344	0.158	−0.039 (0.007)	2.62×10^{-8}	19,968	0.148	0.011 (0.014)	0.4349	−0.029 (0.006)	3.39×10^{-6}
HGB-EA	17: 59,483,766	C/T	rs8068318	intron	*BCAS3-TBX2*	106,377	0.722	−0.026 (0.005)	1.53×10^{-7}	21,743	0.730	−0.021 (0.011)	0.0565	−0.025 (0.005)	2.55×10^{-8}
MCV-EA	20: 31,140,165	C/T	rs4911241*	intron	*NOL4L*	61,462	0.241	−0.04 (0.007)	1.25×10^{-8}	21,714	0.252	−0.025 (0.012)	0.0302	−0.036 (0.006)	2.01×10^{-9}
RDW-EA	20: 31,140,165	C/T	rs4911241*	intron	*NOL4L*	45,573	0.242	0.043 (0.008)	5.79×10^{-8}	18,475	0.240	0.049 (0.012)	7.44×10^{-5}	0.045 (0.007)	2.01×10^{-11}
RDW-All	20: 31,140,165	C/T	rs4911241*	intron	*NOL4L*	56,194	0.235	0.038 (0.007)	1.56×10^{-7}	24,474	0.222	0.044 (0.011)	6.10×10^{-5}	0.04 (0.006)	4.60×10^{-11}
HCT-EA	20: 43,042,364	C/T	rs1800961*	missense	*HNF4A*	79,344	0.024	0.083 (0.015)	1.44×10^{-8}	19,968	0.033	0.082 (0.028)	0.0037	0.083 (0.013)	1.91×10^{-10}
HGB-EA	20: 43,042,364	C/T	rs1800961*	missense	*HNF4A*	98,277	0.032	0.073 (0.013)	2.53×10^{-8}	21,743	0.032	0.062 (0.027)	0.0232	0.071 (0.012)	1.93×10^{-9}
HCT-All	20: 43,042,364	C/T	rs1800961*	missense	*HNF4A*	100,313	0.022	0.077 (0.014)	2.31×10^{-8}	25,006	0.027	0.091 (0.028)	0.0010	0.08 (0.012)	9.88×10^{-11}
HGB-All	22: 44,324,727	C/G	rs738409	missense	*PNPLA3*	130,273	0.223	0.028 (0.005)	2.24×10^{-8}	4,074	0.218	0.053 (0.027)	0.0504	0.029 (0.005)	4.81×10^{-9}
MCH-EA	X: 55,039,960	G/A	rs201062903	missense	*ALAS2*	52,758	0.002	−0.324 (0.053)	7.32×10^{-10}	5,855	0.001	−0.291 (0.235)	0.215	−0.323 (0.052)	5.81×10^{-10}
MCH-All	X: 55,039,960	G/A	rs201062903	missense	*ALAS2*	65,067	0.002	−0.322 (0.051)	3.36×10^{-10}	10,893	0.001	−0.276 (0.224)	0.218	−0.321 (0.051)	2.68×10^{-10}
MCV-EA	X: 55,039,960	G/A	rs201062903	missense	*ALAS2*	60,211	0.002	−0.285 (0.049)	7.11×10^{-9}	5,044	0.001	−0.178 (0.248)	0.472	−0.282 (0.049)	6.11×10^{-9}

Variants in novel loci with $p < 2 \times 10^{-7}$ and that were retained after conditional analyses are presented here. All variants are >1 Mb apart from a known GWAS signal for RBC traits. Chromosome positions are given on human genome build hg19. Allele frequency and effect size are given for the alternate (A2) allele. Replication was carried out in six cohorts for EA and two cohorts for AA and was performed in RareMetals; meta-analyses of the discovery and replication cohorts are presented under "Combined" and were carried out in METAL. Asterisks (*) indicate variants that replicated with a nominal $p < 0.05$. Abbreviations are as follows: EA, European American; AA, African American; All, combined ancestry (EA + AA + Asians + Hispanics); A1, reference allele; A2, alternate allele; N, sample size; AF, allele frequency; SE, standard error; HCT, hematocrit; HGB, hemoglobin; RBC, red blood cell count; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; RDW, red blood cell distribution width.

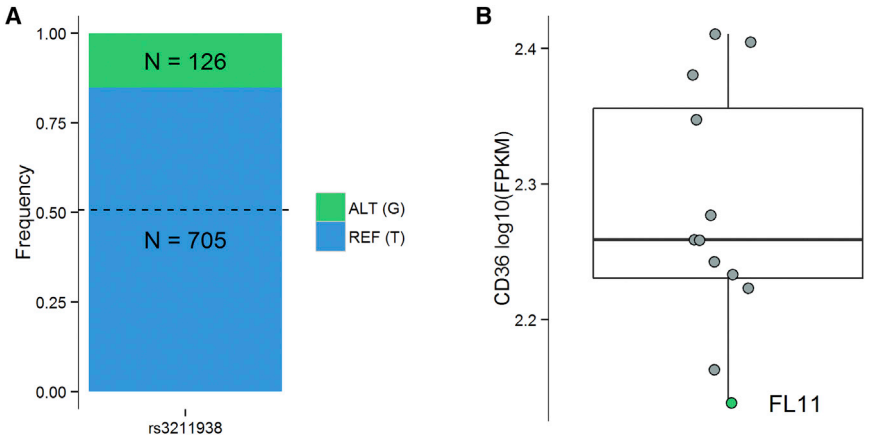

Figure 2. *CD36* Expression in Human Erythroblasts

(A) In a dataset of 12 human fetal liver erythroblasts, all samples were homozygous at rs3211938 for the reference T-allele with the exception of one heterozygous sample (FL11). FL11 demonstrated strong allelic imbalance: we observed 705 reads for the reference allele (T) and 126 reads for the alternate allele (G) (binomial $p = 4.9 \times 10^{-95}$).

(B) FL11 (in green) shows the lowest *CD36* expression level when compared to the other 11 samples. Abbreviation is as follows: FPKM, fragments per kilobase of transcript per million mapped reads.

the transcription factor HNF4A (Table 1). This marker, rs1800961 (p.Thr117Ile [c.350C>T]), has previously been associated with HDL and total cholesterol, C-reactive protein, fibrinogen, and coagulation factor VII levels.[39–42] Mutations in *HNF4A* cause maturity-onset diabetes of the young (MODY [MIM: 125851]) and a common intronic SNP in *HNF4A* (rs4812829) has been associated with type 2 diabetes (MIM: 125853) risk.[43] The missense rs1800961 associated with HGB and HCT is only in weak LD with rs4812829 ($r^2 = 0.021$ in EA populations from the 1000 Genomes Project). Querying recently released ExomeChip data from Type 2 Diabetes Genetics (Web Resources), we found that rs1800961 is also associated with T2D risk in ~82,000 participants ($p = 9.5 \times 10^{-7}$, odds ratio = 1.16). *HNF4A* is expressed in the kidney and could influence HGB and HCT through the regulation of erythropoietin production.[44] It is also abundantly expressed in the liver, where it could indirectly affect HGB and HCT levels through an effect on blood lipid levels (see Discussion). *HNF4A* is detectable at low levels in erythroblasts, and the BLUEPRINT Project has found that some *HNF4A* isoforms may be more highly expressed in this cell type (Figure S4).[45]

In AA, we identified a nonsense variant (rs3211938, p.Tyr325Ter [c.975T>G], MAF = 8.7%, $p = 7.1 \times 10^{-11}$) in *CD36* associated with RDW. This variant displays a wide variation in allele frequency between AA and EA ($MAF_{EA} = 0.01\%$). The association is slightly improved in the ancestry-combined meta-analysis ($p = 5.1 \times 10^{-13}$) because there is also evidence of association in Hispanics (MAF = 1.9%, p = 0.022) (Table 1). We examined a dataset of ex vivo differentiated human erythroblasts to determine whether this *CD36* nonsense variant shows allelic imbalance (AI).[32] All samples were homozygous at rs3211938 for the reference allele with the exception of one heterozygous sample (FL11). FL11 had the lowest level of *CD36* expression among the 12 samples tested and demonstrated strong AI where we observe 705 sequence reads for the reference allele (T) versus 126 for the alternate allele (G) ($p = 4.9 \times 10^{-95}$; Figure 2). To confirm this finding in independent samples, we queried the GTEx dataset, which has compiled RNA-sequencing and genotype information from multiple human tissues.[38] GTEx does not include data for human erythroblasts. However, it detected a strong eQTL effect of rs3211938 on *CD36* expression in whole blood ($p_{eQTL} = 1.1 \times 10^{-15}$), with carriers of the G-allele expressing less *CD36* (Figure S5). Furthermore, GTEx reported evidence for moderate AI in multiple tissues for *CD36*-rs3211938, with the G-allele being under-represented among sequence reads (Figure S5). These results strongly support our observations in human erythroblasts.

eQTL Analysis

To prioritize additional causal genes at RBC loci that contain non-coding variants, we cross-referenced our list of novel variants with more than 100 published eQTL datasets (Subjects and Methods). Overall, 12 variants were significant eQTLs in a wide variety of tissues (Table S6). The most interesting eQTL finding is the association between rs10903129, a common marker associated with RDW in our analyses and located within an intron of *TMEM57* (MIM: 610301), and the expression of *RHD* (MIM: 111680) in whole blood. *RHD* is located 112 kb downstream of *TMEM57* and encodes the D antigen of the clinically significant Rhesus (Rh) blood group. rs10903129 has also been associated with total cholesterol levels and erythrocyte sedimentation rate (ESR).[46,47] The association with ESR is particularly intriguing given that it is considered a non-specific indicator of inflammation. As described above, RDW is also abnormal in chronic diseases, such as atherosclerosis and diabetes, which have an important inflammation component.

Gene-Based Association Testing

Despite our large sample size, statistical power remains limited for rare variants of weak-to-moderate phenotypic effect. To try to capture these genetic factors, we performed gene-based testing by aggregating coding and splice site variants with MAF < 1% within each gene (Subjects and Methods). The SKAT analyses identified two genes: *ALAS2* associated with MCH and *PKLR* (MIM: 609712) associated with HGB and HCT (Table 2). The *ALAS2* signal was driven

Table 2. Gene-Based Association Results

Trait	Gene	n	Number of Variants Analyzed	VT p Value	SKAT p Value	Top Variant	Top-Variant MAF	Top-Variant p Value
HGB-EA	*PKLR*	106,377	15	1.92×10^{-5}	7.02×10^{-7}	rs116100695	0.003	1.17×10^{-5}
HGB-All	*PKLR*	130,273	15	0.00016	6.57×10^{-7}	rs116100695	0.003	1.94×10^{-5}
HCT-All	*PKLR*	109,875	15	3.96×10^{-5}	7.95×10^{-7}	rs116100695	0.003	2.49×10^{-5}
MCH-EA	*ALAS2*	54,009	11	4.78×10^{-6}	5.79×10^{-7}	rs201062903	0.002	7.32×10^{-10}
MCHC-All	*ALPK3*	84,841	28	1.95×10^{-6}	0.793	rs202037221	3.0×10^{-5}	0.0005

Gene-based results of the VT and SKAT algorithms for genes associated with RBC traits at $p < 3 \times 10^{-6}$. We analyzed non-synonymous coding (nonsense, missense) and splice site variants with a minor allele frequency (MAF) < 1%. Abbreviations are as follows: EA, European American; All, combined ancestry (EA + AA + Asians + Hispanics); n, sample size; HCT, hematocrit; HGB, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin.

by a single rare missense variant (rs201062903) and was described above. *PKLR* encodes the erythrocyte pyruvate kinase (PK) that catalyzes the last step of glycolysis. PK deficiency, usually caused by recessive mutations, is one of the main causes of non-spherocytic hemolytic anemia (MIM: 266200). In fact, one of the variants identified in our meta-analysis (rs116100695, p.Arg486Trp [c.1456T>G], MAF = 0.3%, $beta_{HGB} = -0.242$ g/dl, $p_{HGB} = 1.2 \times 10^{-5}$) is a frequent cause of PK deficiency in Italian and Spanish subjects.[48,49] This variant was confirmed in the replication cohorts ($p_{replication} = 0.039$; Table S7). Two additional *PKLR* rare missense variants contribute to the gene-based association statistic with HGB and HCT: rs61755431 (p.Arg569Gln [c.1706G>A], MAF = 0.2%, $beta_{HGB} = -0.179$ g/dl, $p_{HGB} = 0.006$) and rs8177988 (p.Val506Ile [c.1516G>A], MAF = 0.6%, $beta_{HGB} = +0.116$ g/dl, $p_{HGB} = 0.003$). It is noteworthy that the p.Val506Ile substitution is associated with increased HGB concentration given that this amino acid maps to a PKLR structural domain necessary for protein interaction.[50] This heterogeneity of effect among the *PKLR* missense variants might explain why SKAT's result is more significant than VT's for this gene (Table 2). A third gene, *ALPK3*, was identified only in the VT analysis for association with MCHC (Table 2). *ALPK3* encodes a kinase previously implicated in cardiomyocyte differentiation.[51] We could not test for replication because of the rarity of *ALPK3*'s coding alleles (Table S7).

RBC Variants and Pleiotropic Effects

Besides the overlap within the RBC traits themselves, we identified seven novel RBC variants associated with other blood-cell type traits or with lipid levels (Figure 3 and Table 3). To assess whether the genetic associations with RBC traits are independent of lipid levels, we performed additional analyses in a subset of BCX participants from three of our studies (FHS, MHIBB, and WHI) ranging from ~10,000 to 23,000 individuals. We repeated the association analyses for five RBC loci (*TMEM57-RHD* rs10903129, *AFF1* rs442177, *TRIB1* rs2954029, *MAP1A* rs55707100, and *HNF4A* rs1800961) additionally adjusting for the respective lipid trait and combined the results across the three studies using fixed-effect meta-analysis (Table S8). There was little or no change in the effect size or p values associated with the five RBC trait loci upon adjustment for the corresponding lipid trait, suggesting that the RBC and lipid associations are independent of one another and thus represent true "pleiotropic" genetic effects.

A correlated response to or role in inflammation might explain why some of the RBC variants are also associated with WBC, PLT, or lipid traits. Another plausible explanation for the concomitant association of several markers with RBC, WBC, and PLT phenotypes could be a more general effect of these genes on the proliferation or differentiation of hematopoietic progenitor cells. This is most likely the case for *JAK2* (MIM: 147796) and *SH2B3* (MIM: 605093), two key regulators of hematopoietic cells (Figure 3). In this category, we also observed two novel findings, *AFF1* (MIM: 159557) and *NOL4L*, which are associated with RBC and WBC phenotypes and have been previously implicated in leukemia.[53,54] Finally, we identified a novel missense variant in *ARHGEF3* (MIM: 612115) associated with HGB and HCT. In addition to its association with PLT traits, *ARHGEF3* plays a role in the regulation of iron uptake and erythroid cell maturation.[55]

Discussion

We present multi-ethnic meta-analyses of seven RBC traits using ExomeChip results of 130,273 individuals. Our statistical thresholds to declare significance at the discovery stage ($p < 2 \times 10^{-7}$ in the single-variant analyses) was adjusted for the approximate number of variants genotyped on the ExomeChip (Bonferroni correction for 250,000 variants), but we decided not to adjust it for the seven RBC phenotypes tested because of the high correlation between some of these traits (Figure S3). Instead, we relied on independent replication to distinguish true from probably false positive associations. Despite the limited size of our replication set (27,480 individuals), it was encouraging to detect a strong replication of direction of effect for known and novel RBC variants, suggesting a low false discovery rate. In total, we identified 23 novel

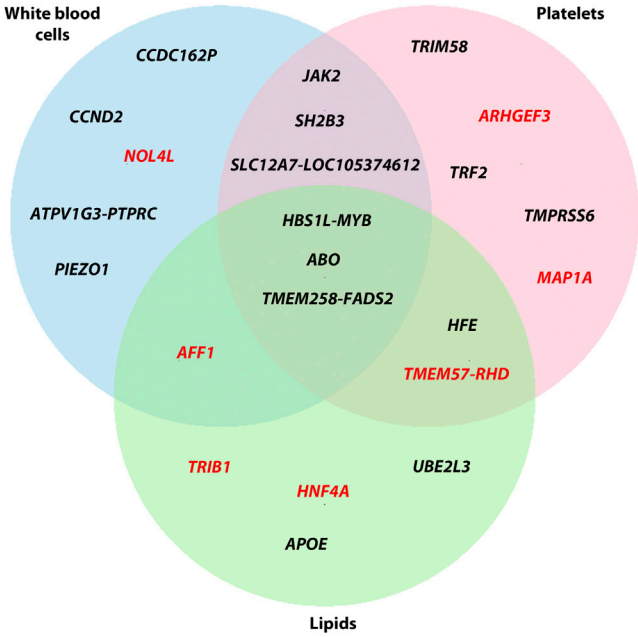

Figure 3. Venn Diagram Summarizing Pleiotropic Effects for Genetic Variants Associated with Red Blood Cell Traits

We considered variants only if their association p values with white blood cell (WBC) traits, platelet (PLT) traits, or with lipid levels was $p < 1 \times 10^{-4}$. Results for WBC and PLT are from the accompanying Blood-Cell Consortium articles.[20,21] Results for lipids have previously been published (Table 3). Genes highlighted in red are novel RBC trait findings.

variants associated with RBC traits in the single-variant analyses and a collection of three rare missense variants in *PKLR* associated with HGB and HCT in the gene-based analyses. Out of the 23 novel RBC variants, 16 were replicated at $p < 0.05$ in the independent samples (Table 1). To inform our replication criteria, we conducted a power analysis using a sample size of 20,000 and considering multiple combinations of allele frequencies and effect sizes. Based on allele frequency and effect size, one of our most difficult to replicate variants was rs1800961 (MAF = 0.022, Beta = 0.028). However, we still had approximately 56% power to detect this association in the replication stage.

We identified a nonsense variant in *CD36* associated with RDW in African Americans. CD36 is a type B scavenger receptor located on the surface of many cell types, including endothelial cells, platelets, monocytes, and erythrocytes. CD36 is a marker of erythroid progenitor differentiation[56] and might also be involved in macrophage-mediated clearance of red blood cells.[57] Furthermore, CD36 plays a role in many biological pathways such as lipid metabolism/transport and atherosclerosis, hemostasis, and inflammation.[58] The nonsense *CD36* variant identified in our RDW meta-analysis (rs3211938, Table 1) has previously been associated with platelet count, HDL cholesterol, and C-reactive protein levels in African Americans[59,60] and malaria resistance in Africans.[61,62] The *CD36* locus shows a signature of natural selection in AA populations[63] and the MAF of rs3211938 varies widely between continents: in the 1000 Genomes Project, the minor allele is absent from European populations but reaches frequency of 24%–29% in some African populations.[64] To characterize the molecular mechanism by which rs3211938 can impact RDW, we identified one heterozygous sample among a collection of ex vivo differentiated human erythroblasts.[32] In erythroblasts from this donor, we noted a strong allelic imbalance (Figure 2). Importantly, this result was confirmed in independent samples from the GTex dataset (Figure S5). At the molecular level, this *CD36* expression phenotype could be explained by nonsense-mediated mRNA decay or the regulatory effect of non-coding genetic variants in LD with rs3211938.

We observed that many new RBC variants are pleiotropic, being often associated with more than one RBC index as well as with WBC, PLT, and lipid traits (Figure 3). These shared effects could imply that the underlying causal genes at these RBC loci generally controlled blood cell proliferation or modulate inflammatory responses. An additional explanation for the link between RBC traits and lipid variants might be the cholesterol content of RBC membranes. As mentioned earlier, RBC corresponds to a large fraction (~25%) of the cells found in the human body. Genetic variation that modulates RBC count or volume could impact circulating lipid levels. In support of this hypothesis, it has been observed that a thalassemia allele is strongly associated with cholesterol levels in the Sardinian population.[65] In total, we found ten loci associated with lipid levels and RBC indices, including four novel RBC variants (*AFF1*, *TMEM57-RHD*, *TRIB1*, *HNF4A*) (Figure 3).

In summary, our multi-ethnic meta-analyses have expanded the genetic knowledge of erythrocyte biology and identified new common, low-frequency, and rare RBC variants. Many of the new RBC variants are pleiotropic, affecting other complex traits such as WBC, PLT, and blood lipid levels. Although our report demonstrates the utility of the ExomeChip for genetic discovery, it also highlights the challenge to attribute gene causality based only on association results. This is particularly evident for loci with common variants, for which coding and non-coding markers are often statistically equivalent. For instance, we found no examples of RBC coding variants that entirely explain RBC GWAS signals among the seven loci that had both a sentinel GWAS variant and ExomeChip coding markers. Although increasing sample sizes will continue to yield additional RBC loci, it has become incredibly clear that only a combination of well-powered genetic studies, transcriptomic and epigenomic surveys, and functional experiments (e.g., using genome editing) will ultimately pinpoint causal variants and genes that control RBC phenotypes.

Supplemental Data

Supplemental Data include a note on the eQTL analyses, information on supplementary funding, five figures, and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.05.007.

Table 3. Overlap of Red Blood Cell Markers with Other Blood Cell Traits and/or Lipid

SNP	Position	A1/A2	AF (A2)	Annotation	Gene	Trait	Beta	p Value
rs10903129	1: 25,768,937	A/G	0.568	intron	*TMEM57-RHD*	RDW	0.037	1.19×10^{-7}
						TC[46]	0.061	5.40×10^{-10}
						PLT	−0.021	7.06×10^{-6}
rs3772219	3: 56,771,251	A/C	0.338	missense	*ARHGEF3*	HCT*	−0.028	2.38×10^{-9}
						HGB*	−0.026	3.76×10^{-9}
						PLT	0.031	5.93×10^{-10}
rs442177	4: 88,030,261	G/T	0.595	intron	*AFF1*	HGB	−0.034	3.97×10^{-13}
						TG[40]	−0.031	1.00×10^{-18}
						BASO	−0.030	1.99×10^{-5}
rs2954029	8: 126,490,972	A/T	0.439	intergenic	*TRIB1*	RDW	0.036	1.53×10^{-7}
						TG[40]	−0.076	1.00×10^{-7}
rs55707100	15: 43,820,717	C/T	0.033	missense	*MAP1A*	HGB	−0.071	1.65×10^{-8}
						PLT	0.095	7.03×10^{-14}
						TG[52]	0.090	1.40×10^{-17}
rs4911241	20: 31,140,165	C/T	0.241	intron	*NOL4L*	MCV	−0.040	1.25×10^{-8}
						RDW	0.043	5.79×10^{-8}
						BASO	−0.051	1.35×10^{-10}
						MONO	−0.033	3.57×10^{-5}
rs1800961	20: 43,042,364	C/T	0.032	missense	*HNF4A*	HCT	0.083	1.44×10^{-8}
						HGB	0.073	2.53×10^{-8}
						HDL[40]	−0.127	2.00×10^{-34}

Shown here are significant novel variants from the RBC traits association analyses that overlap with other blood-cell traits or with lipids. Results for the white blood cell and platelet traits are from the Blood Cell Consortium, and results for lipids are from the published literature. Results are presented for European-ancestry individuals, except in the presence of an asterisk (*), which stands for result from "All" ancestry. The allele frequency and direction of the effect (beta) is given for the A2 allele. Abbreviations are as follows: A1, reference allele; A2, alternate allele; AF, allele frequency; HCT, hematocrit; HGB, hemoglobin; MCV, mean corpuscular volume; RDW, red blood cell distribution width; TC, total cholesterol; PLT, platelet; TG, triglycerides; WBC, white blood cells; BASO, basophils; MONO, monocytes; HDL, HDL cholesterol.

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Web Resources

BCX ExomeChip association results, http://www.mhi-humangenetics.org/en/resources
CheckVCF, https://github.com/zhanxw/checkVCF
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
OMIM, http://www.omim.org/
RareMETALS, http://genome.sph.umich.edu/wiki/RareMETALS
RareMetalWorker, http://genome.sph.umich.edu/wiki/RAREMETALWORKER
RvTests, http://genome.sph.umich.edu/wiki/RvTests
Type 2 Diabetes Genetics, http://www.type2diabetesgenetics.org/

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