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A large-scale transcriptome-wide association study (TWAS) of 10 blood cell phenotypes reveals complexities of TWAS fine-mapping

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Abstract

Hematological measures are important intermediate clinical phenotypes for many acute and chronic diseases and are highly heritable. Although genomewide association studies (GWAS) have identified thousands of loci containing trait-associated variants, the causal genes underlying these associations are often uncertain. To better understand the underlying genetic regulatory mechanisms, we performed a transcriptome-wide association study (TWAS) to systematically

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

SUPPORTING INFORMATION

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investigate the association between genetically predicted gene expression and hematological measures in 54,542 Europeans from the Genetic Epidemiology Research on Aging cohort. We found 239 significant gene-trait associations with hematological measures; we replicated 71 associations at $p < 0.05$ in a TWAS meta-analysis consisting of up to 35,900 Europeans from the Women's Health Initiative, Atherosclerosis Risk in Communities Study, and BioMe Biobank. Additionally, we attempted to refine this list of candidate genes by performing conditional analyses, adjusting for individual variants previously associated with hematological measures, and performed further fine-mapping of TWAS loci. To facilitate interpretation of our findings, we designed an R Shiny application to interactively visualize our TWAS results by integrating them with additional genetic data sources (GWAS, TWAS from multiple reference panels, conditional analyses, known GWAS variants, etc.). Our results and application highlight frequently overlooked TWAS challenges and illustrate the complexity of TWAS fine-mapping.

Keywords

fine-mapping; hematological traits; R Shiny; TWAS

1 | INTRODUCTION

Genome-wide association studies (GWAS) have identified thousands of loci containing hematological traitassociated variants (i.e., variants associated with red cell, white cell, and platelet traits), and previous Mendelian randomization and phenome-wide association study analyses have highlighted the likely causal role of hematological trait-associated genetic variants in a variety of disorders, including autoimmune conditions and coronary heart disease (Astle et al., 2016; Chen et al., 2020; Vuckovic et al., 2020). Unfortunately, these individual single nucleotide polymorphism (SNP)-based GWAS make it difficult to identify regulatory variants with small effect sizes which in aggregate impact the same gene, even in very large sample sizes, and they identify regions of associated variants whose biological function is often not clear (Gamazon et al., 2015).

A transcriptome-wide association study (TWAS) is one gene-based method that systematically investigates the association between genetically predicted gene expression and phenotypes of interest, which can increase the power to identify novel trait-associated loci and may elucidate mechanisms of biological function (Gamazon et al., 2015; Gusev et al., 2018; Hu et al., 2019; Zhou et al., 2020). However, many challenges associated with TWAS, such as loci containing multiple associated genes, correlated gene expression, and bias with expression panels (Wainberg et al., 2019) can complicate TWAS results interpretation, particularly for well-studied traits with many identified genetic loci. Here we performed a large-scale TWAS of hematological measures using the PrediXcan method (Gamazon et al., 2015) to analyze data from 54,542 individuals of European ancestry from the Genetic Epidemiology Research on Adult Health and Aging (GERA) cohort (our discovery data set) (Banda et al., 2015; Kvale et al., 2015). Following the initial TWAS analysis, we explored several complimentary strategies (including conditional analysis, TWAS meta-analysis, TWAS using secondary gene expression reference panels, and fine-mapping tools) to improve TWAS results interpretation. Hematological phenotypes

are particularly good candidates for exploring TWAS analysis interpretation due to the availability of large RNA-sequencing datasets in a relevant tissue type, high heritability across traits, and a large number of known genetic associations, most with poorly understood mechanisms and target genes.

In addition to TWAS, we performed conditional analyses to evaluate if TWAS-identified genes represented novel statistical signals or were primarily driven by variants known from GWAS (Vuckovic et al., 2020); we replicated our significant set of gene-trait associations in a meta-analyzed sample of TWAS results containing 18,100 individuals from the Women's Health Initiative (WHI), 9345 individuals from the Atherosclerosis Risk in Communities Study (ARIC), and 8455 individuals from Mount Sinai Bio*Me* Biobank (BioMe), all of European ancestry (Table S1); and we compared the TWAS results between primary and secondary gene expression reference panels to determine if relevant tissues with smaller sample sizes support our primary TWAS findings.

We further employed several strategies to improve our understanding and interpretation of complex genomic regions containing multiple TWAS-identified genes. First, we used FOCUS (fine-mapping of causal gene sets (Mancuso et al., 2019)) to seek to identify a set of causal genes within genomic loci containing multiple significant TWAS gene-trait associations. FOCUS is a software used to fine-map TWAS statistics at genomic risk regions, while accounting for linkage dis-equilibrium (LD) among variants and predicted expression correlation among genes at those risk regions. Second, we developed a novel web-based tool (called *LocusXcanR*) for integrating and visualizing TWAS and GWAS results, as well as results from multiple expression reference datasets. Taking the results of each analysis into consideration, we highlight frequently overlooked challenges of TWAS interpretation, such as failure to consider the number of proximal genes which cannot be accurately imputed with a given reference panel, but which may still be influenced by variants identified in GWAS studies. Our results illustrate the complexity of TWAS interpretation and fine-mapping efforts and provide one resource for clarifying likely gene targets for hematological trait-related genetic loci. Consideration of additional annotation resources and TWAS limitations is necessary for confident identification of gene targets.

2 | METHODS

2.1 | Initial TWAS analysis

We applied the PrediXcan method (detailed in Online Supporting Information) to identify expression-trait associations using individual-level genotype and phenotype data from the GERA non-Hispanic White ethnic group. The GERA cohort includes over 100,000 adults who are members of the Kaiser Permanente Medical Care Plan, Northern California Region. Genotyping was completed as previously described (Kvale et al., 2015) and genotype data were phased without external reference and imputed to 1000 Genomes Phase 3 v5. Principal components analysis was used to characterize genetic structure in this European ancestry sample (Banda et al., 2015). Hematological measures were extracted from medical records, and the first visit was used for each participant in most cases. In total, 54,542 non-Hispanic White individuals with hematological measures were included in the analysis (see Online Supporting Information for further details).

Variants were filtered for imputation quality ($R^2 > 0.3$). Ten hematological measures were analyzed including platelet count (PLT), red blood cell counts (red blood cell count [RBC], hematocrit [HCT], hemoglobin [HGB], mean corpuscular volume [MCV], and red cell distribution width [RDW] indices), and white blood cell counts (white blood cell count [WBC], monocyte count [MONO], neutrophil count [NEUTRO], and lymphocyte count [LYMPH]) indices). Because the Depression Genes and Networks (DGN) cohort has the largest single whole blood RNA-seq data set (Battle et al., 2014; genes = 11,538, $n = 922$), we used DGN gene expression panel weights from PredictDB (see URLs) to predict gene expression levels in GERA.

2.2 | Conditional analysis of significant TWAS genes on known GWAS variants

To determine if TWAS results from GERA were driven by any previously reported genome-wide significant variant, we performed conditional analysis. For each statistically significant TWAS gene-trait association, the effect of predicted gene expression was conditioned on a set of previously reported GWAS sentinel variants (Vuckovic et al., 2020) meeting the following criteria: (1) the sentinel variant fell within a 1 Mb region of the TWAS gene, (2) the trait with which the GWAS variant was associated matched the TWAS analytical trait or was within the same trait category as the analytical trait (PLTs, red blood cell indices [HCT, HGB, MCV, RBC, and RDW], white blood cell indices [WBC, NEUTRO, MONO, and LYMPH]), and (3) the GWAS variant met an imputation quality threshold of $R^2 > 0.3$. We used a modified version of the cpgen R package (see Online Supporting Information) to perform the conditional analysis, accounting for a PLINK KING-robust kinship matrix (Manichaikul et al., 2010), which used only genotyped variants and excluded variants with minor allele frequency less than 5% and individuals missing more than 1% of SNPs.

2.3 | Replication of conditional TWAS results in meta-analyzed cohorts

To replicate the conditionally significant gene-trait associations in GERA, we compared each gene-trait association to a TWAS meta-analysis including ARIC, WHI, and BioMe cohorts (brief cohort summaries follow and are detailed in Online Supporting Information). All TWAS analyses were limited to self-reported white or European ancestry participants for easy comparability of the DGN European ancestry eQTL panel and with the largest single-ancestry blood cell trait GWAS (also conducted in European ancestry participants). We analyzed 10 hematological phenotypes (as noted above) across all cohorts; inverse normalized phenotypes were analyzed with appropriate covariate adjustments for demographic characteristics and principal components.

URLS
cpgen: https://github.com/cheuerde/cpgen
FOCUS: https://github.com/bogdanlab/focus
Human Protein Atlas: https://www.proteinatlas.org/
LocusXcanR R package for R Shiny application: https://github.com/amanda-tapia/LocusXcanR
LocusXcanR R Shiny application for GERA results: http://shiny.bios.unc.edu/gera-twas/
METAL: https://genome.sph.umich.edu/wiki/METAL_Documentation
Online Mendelian Inheritance in Man (OMIM): https://www.omim.org/
PLINK KING kinship matrix: https://www.cog-genomics.org/plink/2.0/distance#make_king
PredictDB: http://predictdb.org/
PrediXcan: https://github.com/hakyimlab/PrediXcan
R Shiny: https://shiny.rstudio.com/

Each cohort anlayzed in this study included only participants of European ancestry with hematological measures. Atherosclerosis Risk in Communities Study (ARIC) included 9345 participants ("The Atherosclerosis Risk in Communities [ARIC] Study: design and objectives," 1989; Women's Health Initiative (WHI) included 18,100 women (The Women's Health Initiative Study Group, 1998); and BioMe (the Mount Sinai Bio*Me* Biobank) included 8455 participants.

Replication of the conditionally significant GERA gene-trait associations was performed using meta-analyzed TWAS results from ARIC, WHI, and BioMe. As described above, PrediXcan was used to facilitate gene expression imputation and association in each cohort separately, and the meta-analysis association test was conducted using METAL (Willer et al., 2010). Seventeen gene-trait associations remained statistically significant after conditional analysis; thus, for this set of genes, we defined a Bonferroni-corrected statistically significant replication threshold at p-value $< 2.94 \times 10^{-3}$.

2.4 | Characterization of TWAS-identified fine-mapping loci

We next performed fine-mapping of TWAS-identified loci. Fine-mapping loci refers to fine-mapping analysis of trait-specific genomic locations that contain, and are centered at sentinel TWAS genes. That is, we took the set of trait-specific statistically significant GERA TWAS genes, selected the most significant gene in the set (the sentinel gene), and assigned it to a locus along with any other statistically significant TWAS genes within a 1 Mb window of the sentinel gene. We then selected the next most significant TWAS gene which had not yet been assigned to a locus and continued in this fashion until all statistically significant TWAS genes had been assigned to a locus.

We then defined locus categories based on whether the locus contained a single gene or multiple genes and whether the locus replicated in TWAS meta-analysis at either a lenient or strict threshold. For this fine-mapping analysis, the statistical significance of replicated genes was qualified based on two different thresholds – a stringent threshold Bonferroni-corrected for all 239 statistically significant TWAS gene-trait associations at p-value $< 2.09 \times 10^{-4}$, and a more lenient threshold at p-value < 0.05. Thus, locus categories were defined as follows: 1 = single gene locus, strict replication ($p < 2.09E{-}04$); 2 = single gene locus, replication ($p < 0.05$); 3 = single gene locus, no replication; 4 = multi gene locus, strict replication ($p < 2.09E{-}04$); 5 = multi gene locus, replication ($p < 0.05$); 6 = multi gene locus, no replication.

2.5 | TWAS fine-mapping strategies

To facilitate TWAS fine-mapping and allow for better interpretation of TWAS results, we employed several different strategies including the use of R Shiny, FOCUS, and secondary TWAS reference panels. Details of each method follow.

2.5.1 | R Shiny application—We used R's convenient Shiny package (version 1.5.0, implemented in R 4.0.3) to produce a web application (*LocusXcanR*) that displays our GERA TWAS results. All GERA TWAS results were produced using PrediXcan as described above. We also included GERA GWAS results in the R Shiny app; analysis

was performed using Bolt LMM as implemented in rvtests (Zhan et al., 2016). GERA conditional analysis results were produced using cpgen. Known GWAS sentinel variants were obtained from (Vuckovic et al., 2020). Model weights and model variants were taken from our primary DGN reference panel from PredictDB or secondary reference panels from PredictDB (Genotype-Tissue Expression [GTEx] Project; The Genotype-Tissue Expression GTEx project, 2013; whole blood [GWB] and Epstein-Barr virus [EBV] transformed lymphocytes [GTL]; and Multi-Ethnic Study of Atherosclerosis [MESA] [Liu et al., 2013] monocytes [MSA]. These are considered secondary reference panels due to their smaller sample sizes compared with DGN). Supplementary Methods contains further details relevant to R Shiny data and visualizations.

2.5.2 | Fine-mapping Of CaUsal gene Sets (FOCUS)—We used the Fine-mapping Of CaUsal gene Sets (FOCUS) (Mancuso et al., 2019) software to fine-map TWAS statistics at genomic risk regions. As input, we used GERA GWAS summary data along with eQTL weights from PredictDB Depression Genes and Networks whole blood data, and the European LD reference panel from 1000 Genomes Phase 3. The software outputs a credible set of genes at each locus which can be used to explain observed genomic risk.

2.5.3 | TWAS secondary reference panels—We used a set of secondary gene expression reference panels relevant to whole blood to assist with fine-mapping by assessing consistency between our primary TWAS results from DGN and TWAS results from secondary reference panels. Thus, in addition to DGN weights, weights for gene expression using RNA sequencing data were obtained from the Genotype-Tissue Expression project (version 7) (Zhang & Lin, 2013) (whole blood, genes = 6208; and EBV transformed lymphocytes, genes = 3000), and Multi-Ethnic Study of Atherosclerosis (Europeans only, monocytes, genes = 4647) (Mogil et al., 2018).

2.6 | Ethics statement

We performed secondary data analysis on deidentified data only (exempt research). All individual studies included were approved by relevant local institutional review boards, and participants provided written informed consent.

3 | RESULTS

3.1 | Initial TWAS analysis

We applied the PrediXcan method to identify expression-trait associations using individual-level genotype and phenotype data from the GERA non-Hispanic White ethnic group. Analyzed blood cell traits included PLT, RBC, HCT, HGB, MCV, RDW, WBC, MONO, NEUTRO, and LYMPH indices. We used DGN whole blood expression panel weights from PredictDB (a database of weights provided by PrediXcan; see URLs) to predict gene expression levels in GERA. Among the 11,538 genes in the DGN expression panel, 11,438 genes were predicted in GERA and 51% of those genes achieved DGN model $R^2 > 0.05$ (see Table S2 for model R^2 values for significant genes and Table S3 for genes included in DGN but not predicted in GERA). The number of GERA variants used for prediction was equal to the number of variants included in the prediction model (i.e., complete variant

matching) for 74% of the predicted genes; the remaining genes used fewer variants from GERA than were included in the prediction models. We tested each of the 11,438 predicted genes individually for association with each of the 10 hematological measures, resulting in a Bonferroni-corrected *p*-value threshold of $p < 4.37 \times 10^{-7}$. Through the subsequent study analyses, we will refer to these results as "marginal TWAS."

Overall, we identified 295 statistically significant marginal TWAS associations ($p < 4.37 \times 10^{-7}$), with each hematological measure having at least one significant association (Table S2). Among these, 47 marginal TWAS associations fell into the major histocompatibility complex (MHC) or HLA region (GRCh37; chr 6: 28,477,797–33,448,354) and were not considered in subsequent analyses (Table S4); disentangling a set of causal genes within the MHC region is exceptionally difficult due to the highly polymorphic genetic loci and complex LD in the region. Another nine significant associations included genes which contained only a single variant in the prediction model. These associations were also not included in subsequent analyses (Table S4). The remaining 239 significant associations included genes predicted from 2 to 112 variants, with a median of 21 variants used in predictive models. Among this set of 239 associations, we replicated 71 at $p < 0.05$ with same direction of effect for the blood cell trait in TWAS meta-analysis.

3.2 | Conditional analysis of significant TWAS genes on known GWAS variants

To determine whether any of the 239 remaining significant gene-trait associations were novel signals, not driven by any previously reported genome-wide significant variant, we performed conditional analysis. Since we performed TWAS with individual-level data, we conditioned the predicted gene expression value of each statistically significant marginal TWAS gene on the set of nearby (within ± 1 Mb of the gene) sentinel GWAS variants within the hematological category (RBC, WBC, and PLT) from the largest current European ancestry focused GWAS for hematological measures (Vuckovic et al., 2020). We found that 222 (93%) of all marginal TWAS significant associations were attenuated by known GWAS variants and became nonsignificant ($p > 4.37 \times 10^{-7}$) upon conditional analysis. Another 15 of the associations remained significant after conditional analysis, and the remaining two associations did not have GWAS-reported variants within a 1 Mb window of the gene (Table S5).

3.3 | Replication of conditional TWAS results in meta-analyzed cohorts

For confirmation of robust and/or novel signal, we attempted to replicate these 17 conditionally significant findings in a TWAS meta-analysis which included up to 32,036 European ancestry individuals from three cohorts: ARIC, WHI, and BioMe. Two of the 17 conditionally significant gene-trait associations (*HIST1H2BO*-HGB and *HIST1H2BO*-RDW) included in the replication set met the stringent significance threshold ($p < 2.94 \times 10^{-3}$). However, *HIST1H2BO* is situated within 1 Mb of the MHC region already excluded above (GRCh37; chr 6: 28,477,797–33,448,354), with this signal potentially reflecting long-range LD with the MHC region, and has poor model $R^2 = 0.016$. Additionally, *OR2B6* associated with HGB, MCV, and RWD; *ZNF192* associated with HGB, MCV, and RDW; and *ZSCAN12* associated with MCV meet a more lenient significance threshold ($p < 0.05$). Yet, *OR2B6*, *ZNF192*, and *ZSCAN12* are also located on chromosome 6 within

approximately 500 kb of the MHC region and all have poor model $R^2 < 0.015$. The remaining seven gene-trait associations did not meet any replication criteria.

3.4 | Characterization of TWAS-Identified fine-mapping loci

Based on the conditional analysis, previously reported GWAS sentinel variants were at least partly responsible for and attenuated 93% of the significant marginal TWAS signals. Thus, we next examined if TWAS aided fine-mapping and identification of regulatory mechanisms at these loci. To better contextualize if fine-mapping in GERA was consistent in additional cohorts, we also examined replication of TWAS significant genes in these known loci. The 239 marginal TWAS associations resided in 120 trait-specific, physically nonoverlapping *cis* loci (i.e., the *cis* region of each locus is ± 1 Mb of the locus's TWAS sentinel gene start and end positions). Over half (57%) of these loci contained only a single significant gene, while another 19% contained two significant genes. The remaining 24% of nonoverlapping loci contained three or more significant genes, with up to 11 significant genes at a single locus. These 120 loci contained 87 unique index genes (defined as the most significant TWAS gene within the locus). Most loci did not contain a TWAS gene that replicated in meta-analysis (67% total; i.e., 47% of all loci were single-gene loci that did not replicate plus 20% of all loci were multi-gene loci that did not contain any gene that replicated, even at a marginal level). Ten percent of all loci were single-gene loci that met a marginal replication threshold ($p < 0.05$), and 20% of all loci were multi-gene loci that met this marginal threshold for at least one gene at the locus. The remaining four loci (3%) contained multiple TWAS genes with at least one gene meeting a strict replication threshold ($p < 2.09 \times 10^{-4}$) (Table S2). These included the following index gene-phenotype associations: *TRIM68*-MCV, *USP49*-MCV, *PSMD3*-NEUTRO, and *PSMD3*-WBC.

3.5 | TWAS fine-mapping strategies

To facilitate TWAS fine-mapping and allow for better interpretation of whether a given TWAS-identified gene was truly likely to associate with hematological variation, or whether it was likely to be a spurious association due to correlation of expression with nearby genes or other factors, we created an R Shiny application (*LocusXcanR*) to interactively visualize TWAS sentinel genes in context, one locus at a time. *LocusXcanR* allowed us to integrate multiple sources of information from our primary TWAS analysis, including gene expression prediction models, TWAS meta-analysis, TWAS using secondary reference panels (whole blood and EBV transformed lymphocytes from GTEx, and monocytes from MESA), GWAS analysis of all hematological measures, and correlation among genetic variants (i.e., LD) and among predicted gene expression levels. We highlight several loci to demonstrate the utility of the application, showcase some of the challenges that arise when TWAS identifies multiple significant genes at a single locus, and illustrate some of the complexities that are inherent in TWAS fine-mapping. In the sections that follow, we feature TWAS genes that fall into loci with a low, intermediate, or high level of complexity. All the figures in the following sections originate from *LocusXcanR* (http://shiny.bios.unc.edu/gera-twas/), which could be readily adapted to future TWAS analyses for other complex traits.

3.5.1 | *HK1* locus—The *HK1* gene is known to be associated with several red blood cell traits including HGB, MCV, HCT, mean corpuscular hemoglobin, RBC, and RDW in GWAS

analyses (Vuckovic et al., 2020) and is a Mendelian gene for hemolytic anemia [MIM 142600]. Our TWAS results confirmed previously reported *HK1* GWAS associations with HCT and MCV (assigned based on nearest gene for lead GWAS variants). The marginal TWAS tests for association between *HK1* and HCT ($p = 3.84 \times 10^{-8}$) and MCV ($p = 1.05 \times 10^{-7}$) were statistically significant (Figure 1); associations were all but eliminated by conditional analysis on known GWAS sentinel variants (HCT $p = 2.58 \times 10^{-1}$; MCV p $= 4.36 \times 10^{-2}$); *HK1* with HCT replicated in meta-analysis ($p = 4.63 \times 10^{-2}$); and *HK1* was the most significant TWAS gene among only two other genes (*HKDC1* and *TSPAN15*) implicated by GWAS at these loci, with the other two genes showing no TWAS signal. Thus, results point simply to *HK1* as the most likely causal gene at this locus.

3.5.2 | *CREB5* locus—The marginal TWAS tests for association between *CREB5* and NEUTRO ($p = 1.41 \times 10^{-12}$) and WBC ($p = 4.01 \times 10^{-10}$) were the only TWAS significant associations at this locus (Figure 2a), and associations were essentially eliminated by conditional analysis on known GWAS sentinel variants (NEUTRO $p = 9.04 \times 10^{-1}$; WBC $p = 3.97 \times 10^{-1}$). However, at this locus, both *CREB5* and *JAZF1* (TWAS NEUTRO $p = 5.26 \times 10^{-3}$, WBC $p = 2.42 \times 10^{-3}$) had previously been annotated as being the nearest and/or assigned gene for one or more GWAS sentinel variants. Predicted gene expression for *CREB5* and *JAZF1* was not highly correlated (r^2 between 0.0–0.2), and the genes appeared to share only a single, nonsignificant predictive model variant (Figure 2b). *CREB5* and *JAZF1* both replicated at a lenient significance threshold for NEUTRO ($p = 1.25 \times 10^{-2}$, $p = 8.98 \times 10^{-3}$, respectively), and *CREB5* also replicated at a lenient threshold for WBC ($p = 2.61 \times 10^{-2}$) but *JAZF1*-WBC did not replicate ($p = 0.11$). Both genes appeared in the GTEx whole blood (GWB) and MESA monocyte (MSA) secondary reference panels, but neither gene met the significance threshold for either reference panel. Importantly, Human Protein Atlas (Uhlén et al., 2015) reported that *CREB5* was enhanced in blood and brain tissues and was specifically cell type enriched for NEUTRO (Human Protein Atlas., 2020b). *JAZF1* on the other hand had low tissue specificity (Human Protein Atlas., 2020d). Together the TWAS, GWAS, and Human Protein Atlas results point to *CREB5* as the most likely, and most biologically plausible, gene over *JAZF1* at this locus.

3.5.3 | *CD164* locus—The *CD164* gene is known to play a role in hematopoiesis (Watt et al., 1998; Zannettino et al., 1998) and has been associated with several blood cell indices in GWAS analyses (Vuckovic et al., 2020). Our TWAS results prioritized *CD164* over other genes at the locus as being significantly associated with MCV ($p = 2.54 \times 10^{-12}$) and categorized it into a multi-gene locus along with *MICAL1* ($p = 4.20 \times 10^{-7}$) (Figure 3a). Conditional analysis on sentinel GWAS variants all but eliminated the TWAS signal for both *CD164* ($p = 8.61 \times 10^{-2}$) and *MICAL1* ($p = 1.26 \times 10^{-1}$). Interestingly, Figure 3b shows that *CD164* and *MICAL1* were not highly correlated in their predicted gene expression (r^2: 0.2–0.4) and did not share any predictive model variants. We also note that both genes replicated in meta-analysis at a lenient threshold (*CD164* $p = 1.56 \times 10^{-3}$; *MICAL1* $p = 7.06 \times 10^{-3}$; Figure 3c). Additionally, while *MICAL1* is not available in secondary reference panels, *CD164* met the TWAS significance threshold for its association with MCV in GTEx whole blood and MESA monocytes (GTEx $p = 2.61 \times 10^{-9}$ and MESA $p = 4.09 \times 10^{-8}$). Thus, the evidence at this locus suggests that expression of *CD164* and *MICAL1* are both

reasonable candidates for being regulated by red cell-associated genetic variants although we note that Human Protein Atlas reports low tissue specificity for MICAL1 (Human Protein Atlas., 2020f).

3.5.4 | *PSMD3* locus—The *PSMD3* locus contained a much higher level of complexity because it fell into a region containing many marginal TWAS genes, had a complex gene-gene correlation and LD pattern, and included a combination of genes previously reported by GWAS as well as genes that had not been reported by GWAS. Thus, TWAS results did not clearly pinpoint the most likely causal gene. While *PSMD3* appeared as the index TWAS gene associated with WBC (Figure 4a), eight other genes were also TWAS significant at this locus. Five of those genes (*IKZF3, GSDMB, ORMDL3, MED24*, and *CCR7*) replicated at a lenient significance threshold ($p < 0.05$), and *PSMD3* replicated at a more stringent threshold ($p < 2.09 \times 10^{-4}$) (Figure 4c). We saw a complex network of shared model variants and correlation/LD patterns in Figure 4b, notably with *MED24* and *CCR7* (the next most significant genes at this locus) being only slightly correlated (r^2 between 0.2 and 0.4) with *PSMD3*. The FOCUS fine-mapping results (Figure 4d) aligned to the TWAS results and indicated *PSMD3* and *MED24* as the most likely causal genes at the locus, each having posterior inclusion probabilities (PIPs) equal to 1.0. PIPs for all other genes at this locus, including *CCR7*, were less than 0.021 (Figure 4d).

4 | DISCUSSION

Our follow-up fine-mapping, replication, and conditional analyses for a large-scale TWAS of 10 hematological measures demonstrates that results from marginal TWAS analyses alone cannot illuminate causal genes at loci for these traits.

4.1 | TWAS discovery analyses

While 17 of our 239 marginal gene-trait associations did remain significant after conditional analysis or contained no known GWAS sentinel variants within a 1 Mb region of the gene, we found no substantive evidence from meta-analysis nor secondary reference panels to support these associations as novel discoveries for hematological traits. Conditional analyses suggested that nearly all our TWAS findings were driven at least in part by GWAS sentinel variants from the largest recent European-focused GWAS analysis for hematological measures (Vuckovic et al., 2020). This is perhaps not surprising given the greater statistical power for this GWAS analysis, which was conducted in 563,085 participants (vs. 54,542 participants in our analysis). However, for 61 gene-trait associations (26%), some residual signal ($p < 0.05$) remained after conditioning on GWAS. For example, although *JAK2* is a well-known blood cell-associated signal from GWAS (Vuckovic et al., 2020) and the Mendelian disease literature for platelet disorders [MIM 147796], its association with platelet count remained statistically significant after conditional analysis. Thus, our TWAS results also suggested that there are likely additional regulatory variants at the *JAK2* locus which are not tagged by current GWAS single variants. Similarly, for other gene-trait associations retaining some residual significance after conditional analysis, our results suggest that additional small-effect regulatory variants remain to be discovered for

these genes which associate with blood cell indices, illustrating the power advantages from aggregate tests like TWAS.

4.2 | TWAS Fine-Mapping

For TWAS fine-mapping, we grouped the 239 TWAS-wide significant gene-trait associations into 120 loci. To effectively interpret these results, we introduced *LocusXcanR*, an R Shiny application that integrates TWAS and GWAS information into locus-specific, interactive visualizations which we use to assist with TWAS fine-mapping and TWAS results interpretation. We showed the utility of *LocusXcanR* by highlighting the varying levels of complexity at several TWAS loci and demonstrating where TWAS aligns with or provides advantages over GWAS. For example, the *HK1*-MCV locus showed a very simple genomic locus in which we found that TWAS confirmed what we already knew from GWAS. Coding variants in *HK1* are known to be associated with hemolytic anemia due to hexokinase deficiency [MIM 142600], providing a clear link to red blood cell-related traits.

The *CREB5* locus further demonstrated one of the advantages of TWAS over GWAS in that the TWAS results provided clarity regarding the likely causal gene at the locus. At this locus, *CREB5* and *JAZF1* had both been implicated by GWAS, likely assigned based on their physical proximity to the GWAS sentinel variant. However, *CREB5* showed a stronger TWAS signal, replicated in the much smaller meta-analysis sample, and Human Protein Atlas provided clear evidence of enrichment in blood (specifically neutrophils) (Human Protein Atlas., 2020b, 2020d; Uhlén et al., 2015). These results in aggregate supported *CREB5* as the likely causal gene at this locus, even though *CREB5* may not be the closest gene in proximity to all sentinel GWAS variants within the region.

4.3 | TWAS challenges

We further highlight the challenges, particularly at multi-gene loci, which should be considered when interpreting TWAS findings, including total and/or predicted expression correlation, shared predictive model variants, the relevance of reference tissue panel, biological plausibility, and so forth and demonstrated the importance of interpreting TWAS results in context. Although TWAS is useful for prioritizing candidate causal genes, researchers should guard against the hasty conclusion that the most significant gene is the only causal gene or even the most likely causal gene. For example, the conclusion at the *CD164* locus is not evident from TWAS results. While TWAS points to *CD164* as the causal gene, as does existing knowledge of the gene's biological function, taking the full context of this locus into consideration, it is not out of the realm of possibilities that both *CD164* and *MICAL1* are causal at this locus.

Furthermore, at the *PSMD3* locus we see potentially misleading TWAS results when marginal TWAS statistics are interpreted alone. The *PSMD3*-WBC association appears as the sentinel gene at this locus. However, several pieces of evidence support other genes, including *CCR7*, as the most likely biologically plausible causal gene at the locus. First, *PSMD3* and *MED24* have no immune cell specificity (Human Protein Atlas., 2020e, 2020g; Uhlén et al., 2015). Second, *CCR7* was also TWAS significant, it replicated at a lenient threshold in meta-analysis, and is enriched for expression in blood and lymphoid tissues,

especially T-cells (Human Protein Atlas., 2020a). However, *CCR7* was not highly correlated with nor does it appear to share model variants with lead gene *PSMD3*, and the FOCUS results show a posterior inclusion probability of only 0.001. Finally, *CCR7* is known to be involved in the migration of neutrophils to lymph nodes (Beauvillain et al., 2011). While it is certainly possible at multi-gene TWAS loci for multiple genes to be contributing to trait regulation, it is also possible for spurious or nonrelevant genes to be identified based on shared eQTLs across tissues that are not relevant to a given trait or correlation of gene expression.

Moreover, proximal genes which cannot be accurately imputed with a given reference panel, but which may still be influenced by variants identified by GWAS studies, must also be considered. For example, the gene colony-stimulating factor 3 (*CSF3*), which has a known key role in the production, differentiation, and function of granulocytes [MIM 138970], is also situated within the *PSMD3* locus. However, this gene has very low constitutive expression in whole blood (Human Protein Atlas., 2020c), and it is not depicted in Figure 4 (or *LocusXcanR*) because a predictive model could not be fit for this gene in the DGN reference panel (likely due to very low expression); therefore, *CFS3* cannot be detected as a possible target gene at this locus (Supplementary Table 7 contains *CSF3*, along with other genes that have been assigned by one or more GWAS variants but are not included in DGN). This genomic region is extremely complex and highly pleiotropic, and any interpretation of this locus using TWAS results alone is likely to be overly simplistic. This complex locus shows the importance of considering statistical evidence from TWAS, GWAS, and FOCUS fine-mapping as well as trait biology in the interpretation of TWAS findings.

5 | LIMITATIONS

While we have used PrediXcan and pre-calculated PredictDB weights for our analysis, we note a limitation in doing so. The variants included in PredictDB were not always available in our analytical cohort (generally due to poor imputation quality), so some predictive models did not use all PredictDB weights. We note that 70% of our TWAS significant genes were predicted with complete variant matching (i.e. used all model variants) and 85% of TWAS significant genes used at least 90% of model variants; we have included this information in Table S2 for transparency, and these details should be taken into account when interpreting TWAS results.

The cohorts that we have included in our TWAS meta-analysis also pose some limitations on our ability to replicate GERA TWAS sentinel genes. The smaller sample sizes of the meta-analyzed cohorts are likely the primary reason why GERA TWAS sentinel genes fail to replicate. Additionally, it may be the case that major contributing variants exhibit differential allele frequencies across cohorts; although this is less likely than in multi-ethnic analyses because all cohorts are of European ancestry, it could still contribute to poorer power for replication. Furthermore, differences in imputation quality across cohorts could also explain the failure to replicate TWAS sentinel genes in meta-analysis.

Although FOCUS, in some cases, helps to identify a set of the most likely causal genes at a locus, we have shown that it does not always provide enough evidence above and beyond

TWAS to fully identify a putative causal gene set at a complex locus. Additionally, FOCUS performs a summary statistics-based TWAS method and then proceeds to fine-mapping the TWAS results from this method. However, we performed TWAS using PrediXcan, and thus, the fine-mapping results from FOCUS may not exactly match our PrediXcan TWAS results. In future, the FOCUS software could be extended to take pre-calculated TWAS results as input (using the TWAS method of the researcher's choosing), bypassing the need to use GWAS summary statistics or to recompute predicted gene expression.

Our analysis is primarily conducted using whole blood TWAS weights only, with supplemental TWAS results available in our app for a few other blood-related tissues (whole blood and EBV transformed lymphocytes from GTEx and monocytes from MESA); we felt this was the most prudent approach to limit false positives and reduce needed multiple testing correction, versus an approach using TWAS weights in, for example, all GTEx tissues. However, this choice could be inappropriate if the main relevant tissue at some blood cell-related loci is not in fact whole blood, and it limits our ability to use FOCUS fine-mapping to overcome the choice of tissue for TWAS training. Joint/multiple tissue TWAS approaches such as UTMOST (Hu et al., 2019) and MR-JTI (Zhou et al., 2020) could be employed in the future to assess the relevance of other tissues at blood-cell-related loci.

6 | SUMMARY

In summary, we found that TWAS results enrich our understanding of GWAS, can help to explain trait variation, and are superior to merely selecting the nearest gene. We have shown that the gene, or genes, implicated in TWAS, in some cases, clearly overlap with what is known in GWAS and from prior knowledge of important genes in hematopoietic processes. However, while we showed that TWAS may help in some cases to pinpoint likely causal genes, we emphasize the need for investigators not to interpret TWAS results alone and out of context. We introduced an R Shiny application and demonstrated its utility in assisting researchers in this endeavor by leveraging the TWAS and GWAS information available from the analytical cohort and interactively visualizing results one locus at a time. The results of this analysis are accessible online (http://shiny.bios.unc.edu/gera-twas/), and we also made the layout of this application available for others to import and analyze their own TWAS results in the *LocusXcanR* R package, available on GitHub (https://github.com/amanda-tapia/LocusXcanR). Together with a clearer understanding of the relationship between TWAS and GWAS results, biological insight, and subject matter expertise, TWAS results can help us formulate mechanistic hypotheses for functional experimental validation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

Data are available through dbGaP (GERA- phs000674, ARIC- phs000557, WHI- phs000386, and BioMe- phs000925), or by application to study coordinating centers with approved manuscript proposals (ARIC-https://sites.cscc.unc.edu/aric/, WHI https://www.whi.org/, and BioMe-https://icahn.mssm.edu/research/ipm/programs/biome-biobank/, GERA https://divisionofresearch.kaiserpermanente.org/genetics/rpgeh/biobank/).

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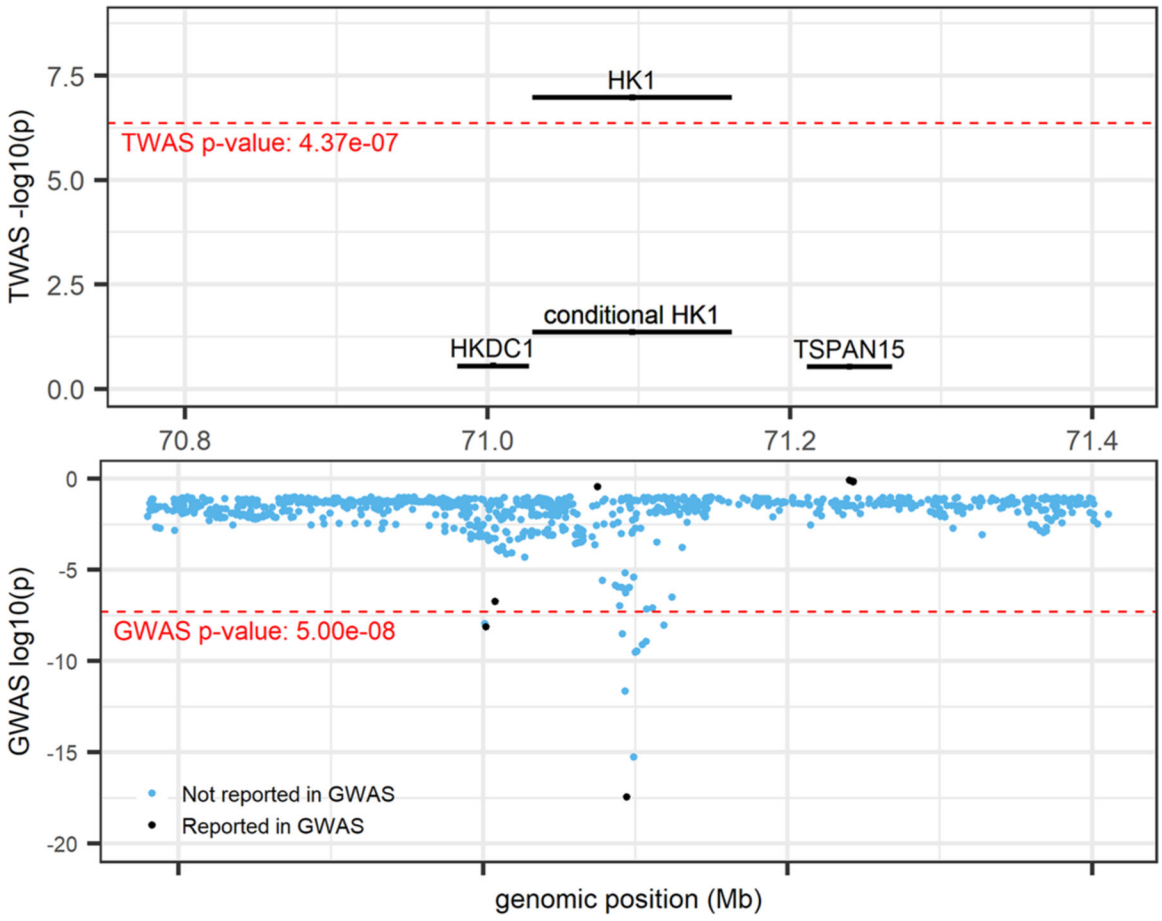


FIGURE 1.

*HK1*locus (locus 60; chr 10: 70,029,740–72,161,638; trait = MCV) from R Shiny. TWAS results (top panel) and GWAS results (bottom panel). Marginal and conditional results for *HK1* are presented in the top panel. Black-colored genes and variants denote those previously reported by UK Biobank and BCX GWAS (Vuckovic et al., 2020), blue variants denote those not previously reported as UK Biobank and BCX GWAS sentinel variants. GWAS, genome-wide association studies; TWAS, transcriptome-wide association study

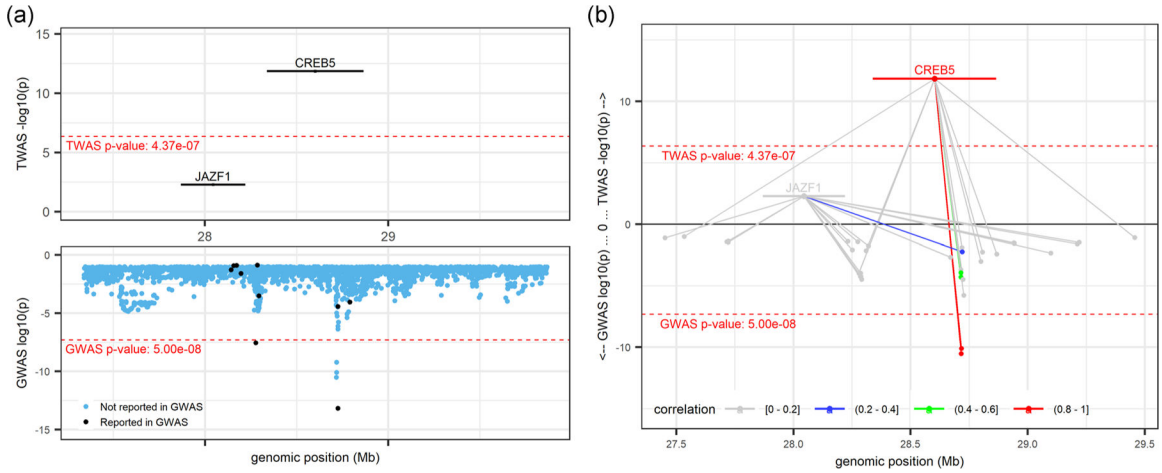


FIGURE 2.

CREB5 locus (locus 40; chr 7: 27,338,940–29,865,511; trait = NEUTRO) from R Shiny. TWAS results (top panels) and GWAS results (bottom panels). Marginal TWAS result displayed in (a), with Black-colored genes and variants denoting those previously reported by GWAS, blue variants denote those not previously reported as GWAS sentinel variants. (b) Mirrored-Manhattan locus-zoom plot displaying genes connected to their predictive model variants. Color scale, increasing from light gray to red, indicates the predicted expression correlation (r^2) between the index TWAS gene and all other genes in the locus and the LD between the index variant and all other variants in the locus. GWAS, genome-wide association studies; TWAS, transcriptome-wide association study

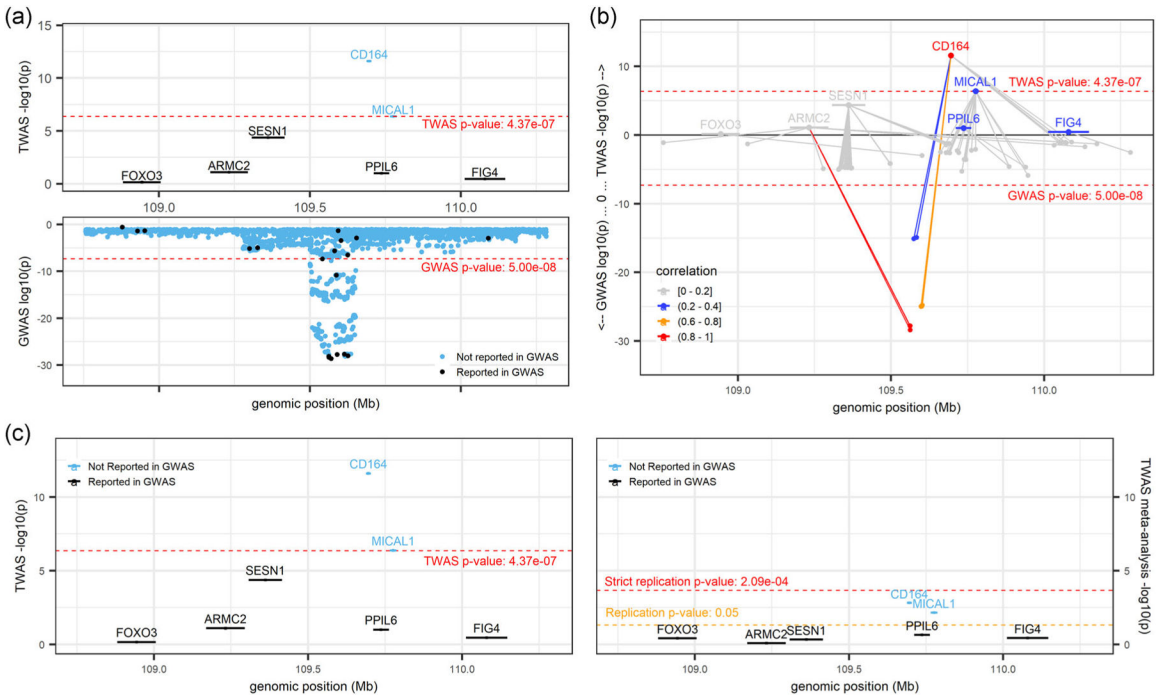
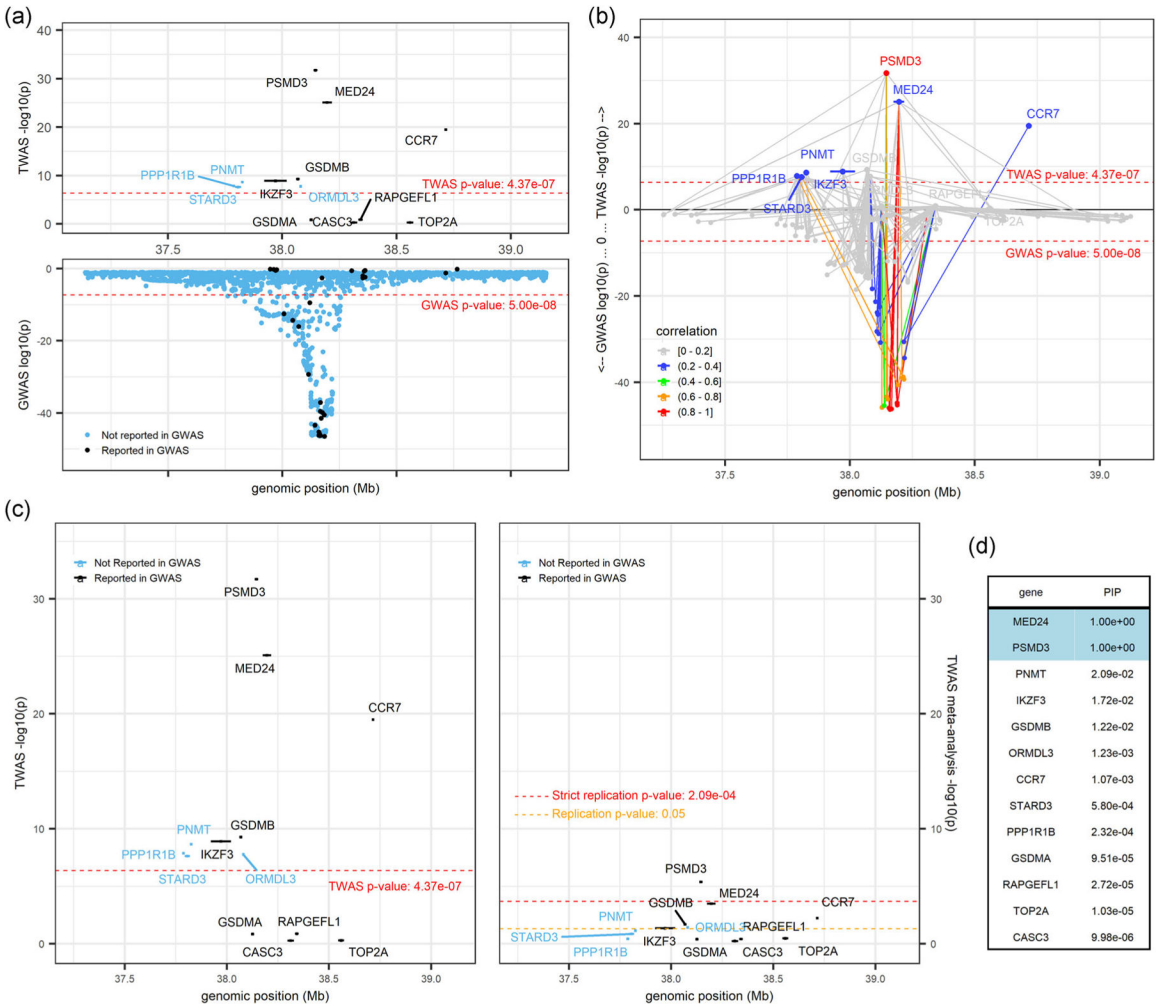


FIGURE 3.

CD164 locus (locus 36; chr 6: 108,687,717–110,703,762; trait = MCV) from R Shiny. (a) Marginal TWAS results in the top panel and GWAS results in the bottom panel. Black-colored genes and variants denote those previously implicated by GWAS, and blue-colored genes and variants denote those not previously implicated by GWAS. (b) Mirrored-Manhattan locus-zoom plot displaying genes connected to their predictive model variants. TWAS results in the top panel, GWAS results in the bottom panel. Color scale, increasing from light gray to red, indicates the predicted expression correlation (r^2) between the index TWAS gene and all other genes in the locus and the LD between the index variant and all other variants in the locus. (c) Comparison of marginal TWAS (left panel) and TWAS meta-analysis (right panel). Black-colored genes denote those previously implicated by GWAS sentinel variants, and blue genes denote those not previously implicated by GWAS sentinel variants. GWAS, genome-wide association studies; TWAS, transcriptome-wide association study



gene	PIP
MED24	1.00e+00
PSMD3	1.00e+00
PNMT	2.09e-02
IKZF3	1.72e-02
GSDMB	1.22e-02
ORMDL3	1.23e-03
CCR7	1.07e-03
STARD3	5.80e-04
PPP1R1B	2.32e-04
GSDMA	9.51e-05
RAPGEFL1	2.72e-05
TOP2A	1.03e-05
CASC3	9.98e-06

FIGURE 4.

PSMD3 locus (locus 101; chr 17: 37,137,050–39,154,213; trait = white blood cell count). (a) displays marginal TWAS results (top panel) and GWAS results (bottom panel), with genes and variants colored in blue and black to denote those not reported by GWAS and those reported by GWAS, respectively. (b) is a mirrored-Manhattan locus-zoom plot displaying genes connected to their predictive model variants with TWAS results (top panel) and GWAS results (bottom panel). Color scale, increasing from light gray to red, indicates the predicted expression correlation (r^2) between the index TWAS gene and all other genes in the locus and the LD between the index variant and all other variants in the locus. (c) presents marginal TWAS results (left panel) and meta-analysis TWAS results (right panel), with genes colored in blue and black to denote those not reported by GWAS and those reported by GWAS, respectively. (d) displays the FOCUS posterior inclusion probabilities (PIPs) for each gene at this locus. FOCUS, Fine-mapping Of CaUsal gene Sets; GWAS, genome-wide association studies; TWAS, transcriptome-wide association study