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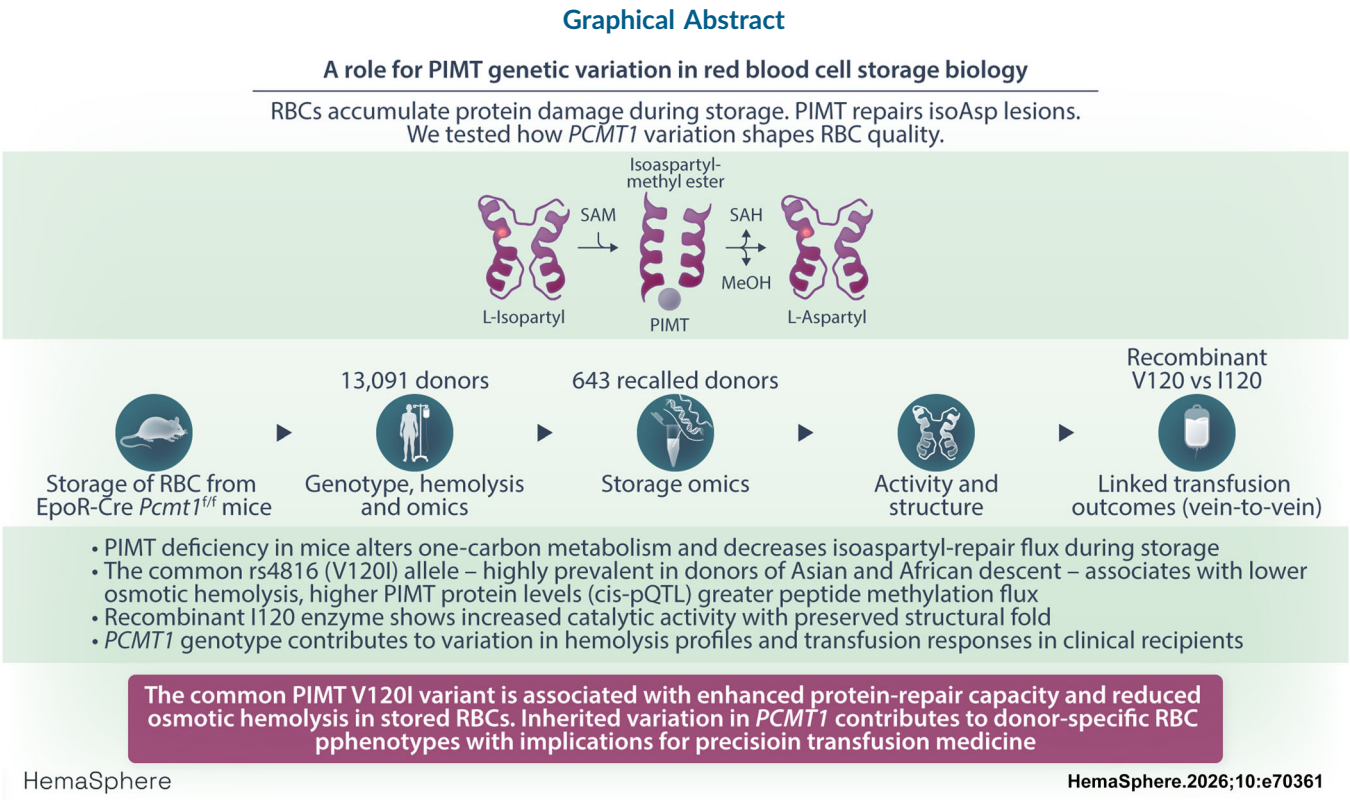
Graphical Abstract



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Improved red blood cell storage quality of blood from donors carrying the hypermorphic PIMT I120 variant

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Abstract

Protein-L-isoaspartate *O*-methyltransferase (PIMT), encoded by *PCMT1*, is a repair enzyme that corrects isoaspartyl lesions, preserving protein structure and function. While indispensable for neuronal integrity, its role in red blood cells (RBCs) and transfusion outcomes is incompletely understood. Here, we show that novel erythroid-specific *Pcmt1* knockout mice display profound remodeling of one-carbon metabolism, accumulation of repair intermediates, and destabilization of glycolytic and cytoskeletal proteins, yet maintain lower lipid peroxidation and normal post-transfusion recovery. Analysis of 13,091 blood donors from the Recipient Epidemiology and Donor Evaluation Study (REDS)-III Red Blood Cell Omics (RBC Omics) study revealed that common *PCMT1* variants associate with hemolysis phenotypes and regulate PIMT protein level, as gleaned by protein quantitative trait loci (pQTL) analyses. The nonsynonymous rs4816 (V120I) allele, enriched in donors of Asian or African ancestry, emerged as a beneficial variant: carriers exhibited lower osmotic hemolysis, altered peptide methylation flux, and higher PIMT protein levels. Recombinant expression confirmed that the I120 variant displays preserved global folding, but greater catalytic activity than the canonical V120 enzyme. Transfusion outcome data showed that *PCMT1* genotype influences hemoglobin increments and bilirubin responses in recipients. These findings identify PIMT as a novel determinant of RBC storage biology and establish rs4816 as a protective allele, with broader implications for donor diversity, transfusion efficacy, and proteome maintenance in aging.

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INTRODUCTION

Proteins are subject to spontaneous chemical modifications that accumulate with time and stress, progressively undermining their function. Among the most frequent and deleterious lesions is the formation of isoaspartyl (isoAsp) residues, generated through deamidation, isomerization, or racemization of asparaginyl and aspartyl residues.[1] IsoAsp incorporation distorts peptide backbones, alters enzymatic active sites, and disrupts protein–protein interactions. To cope with IsoAsp damage, cells rely on the repair enzyme protein-L-isoaspartate (D-aspartate) *O*-methyltransferase (PIMT, encoded by *PCMT1*).[2] By catalyzing the *S*-adenosyl-methionine (SAM)-dependent methyl esterification of isoAsp residues, PIMT initiates a rearrangement that can restore the canonical L-aspartyl form, thereby extending protein half-life and preserving cellular function. PIMT thus represents one of the few enzymatic systems devoted specifically to protein repair rather than degradation and replacement.

Global Pcmt1 knockout mice accumulate isoAsp-modified proteins, develop seizures,[3] and die prematurely, highlighting the enzyme's indispensability for neuronal integrity.[4] Heterozygous *PCMT1*$^{+/-}$ mice suffer from an accelerated brain aging phenotype.[5] Early work from Clarke and colleagues identified synaptic proteins, tau, and amyloid-β (Aβ) as prominent substrates, implicating PIMT deficiency in pathways relevant to Alzheimer's disease (AD).[6] Beyond direct protein repair, PIMT intersects with one-carbon metabolism, since both PIMT and DNA-methyltransferases consume SAM. Heightened demand for protein repair may therefore compete with DNA methylation, providing a plausible mechanism for the DNA hypomethylation characteristic of aging.[7,8]

Red blood cells (RBCs) provide another system in which PIMT function is crucial.[9–15] As enucleated cells, RBCs cannot synthesize new proteins and depend entirely on repair pathways to maintain their structure and metabolism across their ~120-day lifespan. We previously showed that methionine and SAM consumption increase during storage of human RBC units, correlating with oxidative stress and reflecting flux through PIMT-mediated repair.[16] Proteomic studies revealed widespread isoAsp and methyl-isoAsp modifications across cytoskeletal proteins (band 3, spectrin, and ankyrin) and glycolytic enzymes (GAPDH, BPGM, and LDH), often at catalytically or structurally critical sites.[16] Hyperoxic storage exacerbated isoAsp burden, whereas hypoxic storage alleviated it, demonstrating that redox environment modulates repair demand.[16] Thus, PIMT functions both as a repair enzyme and as a metabolic sensor of oxidative stress in stored RBCs. In vivo, global Pcmt1 knockout mice tolerate basal RBC circulation but exhibit heightened hemolysis and metabolic reprogramming when challenged with oxidants, underscoring PIMT's protective role under stress.[17] These findings built upon a body of classic literature from the 1990's identifying a deamidation of structural protein 4.1 as a hallmark of RBC aging in circulation.[18] However, interpretation has been limited by the neurological lethality of global deletion. To resolve this, here we generated novel erythroid-specific conditional knockout (cKO) mice (EpoR-Cre *Pcmt1*$^{flox/flox}$), providing the first tool to specifically interrogate PIMT function in RBCs without confounding systemic phenotypes.

Although PIMT's biochemical function is well characterized, naturally occurring genetic variation in *PCMT1* has received comparatively little attention. Several nonsynonymous variants have been described, with demonstrable effects on enzymatic function. Recombinant expression studies revealed that substitutions such as L191S, A150V, and P174H cause substantial activity loss (72%, 64%, and 61%, respectively), while A65V results in a milder reduction (~11%).[19] These variants are also associated with reduced thermal stability, increased aggregation propensity, or altered substrate affinity. In contrast, the V120I (rs4816) polymorphism is among the most prevalent coding variants and appears to confer increased activity relative to the canonical allele.[20] Such functional diversity suggests that *PCMT1* polymorphisms can span a spectrum from hypomorphic to hypermorphic, with potential consequences for proteome maintenance and cellular stress responses.

Population genetics analyses show that *PCMT1* alleles are unevenly distributed across human ancestries. The V120I variant, for example, is enriched in individuals of African and Asian descent compared to European populations.[20] Hypomorphic variants, while rarer, may also contribute to inter-individual differences in susceptibility to protein damage. Given that PIMT activity declines with age and that isoAsp accumulation is a recognized biomarker of proteome aging, genetic variation in *PCMT1* could help explain variability in aging trajectories, neurodegenerative disease risk, and RBC resilience across populations. In this sense, *PCMT1* provides an entry point into understanding how ancestry and genetic diversity intersect with the fundamental biology of proteome repair.

Large-scale donor-omics initiatives provide a powerful framework to explore these questions. The Recipient Epidemiology and Donor Evaluation Study (REDS)-III Red Blood Cell Omics (RBC Omics) study, which integrates genomic, metabolomic, lipidomic, and proteomic data from more than 13,000 blood donors, has revealed that omics-defined markers of the storage lesion are shaped not only by storage duration but also by donor characteristics such as sex,[21] age,[22] and body mass index,[23] inflammation[24] or energy status[25] at the time of donation, as well as by dietary, professional, and recreational exposures.[26] Importantly, genetic diversity emerged as a major driver of inter-donor differences in RBC hemolytic propensity[27] and transfusion outcomes.[28] Variants in genes involved in antioxidant defense, iron handling, and membrane remodeling—including G6PD,[29] STEAP3,[30] p53,[30] GPX4,[31] SLC22A16, and LPCAT3[32]—were shown to predispose RBCs to oxidative stress, lipid peroxidation, and storage hemolysis, offering an intervention window through supplementation of L-carnitine, vitamin E, or L-methionine to boost storage quality and transfusion efficacy.[31–34] Most of these alleles are deleterious and are disproportionately represented in donors of African ancestry[30]—or in wild rather than domesticated murine strains in diversity outbred population studies[30]—highlighting ancestry-linked genetic contributions to transfusion-relevant phenotypes. Together, these findings establish that the molecular heterogeneity of stored RBCs is encoded at the intersection of demographic, environmental, and genetic factors, thereby fostering the implementation of Precision Transfusion Medicine approaches.[35]

In light of the above, here we hypothesized that genetic and functional diversity in PIMT contributes to inter-individual differences in RBC storage quality and transfusion efficacy. We test this hypothesis by leveraging novel erythroid-specific cKO mice and multi-omics datasets from the REDS RBC Omics study.

METHODS

Extended details are provided in Supporting Information S1: Supplementary Materials—extended.

Mouse models, storage, and post-transfusion recovery studies in conditional EpoR-Cre PIMT$^{f/f}$ mice

Novel erythroid-specific conditional EpoR-Cre PIMT$^{f/f}$ mice were generated on a C57BL6/J background. Mouse post-transfusion recovery (PTR) studies were performed as previously described.[36] RBCs

from conditional erythroid-specific EpoR-Cre PIMT$^{f/f}$ mice and congenic controls (PIMT$^{f/f}$) (three recipients per group) were stored for 7 days at 4°C, followed by transfusion into Ubi-GFP mice, along with fresh mCherry tracer RBCs.

REDS RBC Omics cohort

The REDS RBC Omics program has been extensively detailed in prior publications.[37,38] Stored (Day 42) RBCs from 13,091 donors were tested for hemolysis, metabolomics, and donors were genotyped at 879,000 single-nucleotide polymorphisms (SNPs), including 717 *PCMT1* SNPs.[21,39] Donors ranking ≤5th or ≥95th percentile for hemolysis parameters ($n = 643$) were invited to return for a second donation (recalled cohort), for testing at storage Days 10, 23, and 42, including high-throughput mass spectrometry–based metabolomics,[34] proteomics,[40] lipidomics,[41] and elemental analysis[42] (1929 total samples each).

Protein quantitative trait loci studies

Protein quantitative trait loci (pQTL) analyses for PIMT protein levels and peptides covering the V120 region of PIMT sequence were performed in REDS Index and Recalled donors, consistently with published methods (e.g., GPX4,[31] G6PD[43]).

Determination of hemoglobin and bilirubin increment via the vein-to-vein database

Association of PIMT SNPs with hemoglobin increments was performed by interrogating the vein-to-vein database.[28]

Nuclear magnetic resonance and structural models

Nuclear magnetic resonance (NMR) studies were performed[44,45] on recombinant human PIMT proteins containing the reference (V120) or variant (I120) allele.

PIMT methyltransferase activity assay

Enzymatic activity of recombinant human PIMT variants was quantified using a synthetic isoAsp peptide substrate (WAGG(iso-D)ASGE—GenScript), in the presence of *S*-adenosyl-L-methionine (SAM) as methyl donor. Readouts included targeted UHPLC–MS measurements of SAM, *S*-adenosyl-homocysteine (SAH), the unmodified substrate peptide, its methyl-ester product WAGG(D-*O*-methyl)ASGE, and the succinimide intermediate.

RESULTS

Erythroid-specific PIMT deficiency disrupts one-carbon metabolism and storage remodeling of RBCs

To investigate PIMT function in RBCs, we generated erythroid-specific Pcmt1 cKO mice using EpoR-Cre excision of exon 3 (Figure 1A; Supporting Information S1: Figure 1A). Proteomic PCA confirmed separation of cKO and controls with near-complete loss of Pcmt1 protein (Supporting Information S1: Figure 1B,C). Metabolomics revealed marked perturbations in one-carbon and redox metabolism, including reduced SAM/SAH ratios, altered methyl-donor utilization, accumulation of SAM and 5-methyl-thioadenosine, and depletion of SAH, dimethylglycine, and serine (Figure 1B–E; Supporting Information S1: Figure 1E). Causal inference analysis confirmed rewired one-carbon flux in PIMT-deficient RBCs (Supporting Information S1: Figure 2).

PIMT deficiency perturbs proteome stability and isoAsp repair after storage

We next asked whether these metabolic defects impaired storage quality (Figure 2A). PTR of stored cKO RBCs was not significantly reduced (Figure 2B), despite clear biochemical alteration, notably SAM accumulation without corresponding SAH increases (Figure 2C). These results parallel our earlier studies on constitutive PIMT knockouts exsanguinated prior to seizure onset.[17]

RBC remodeling in cKO mice was exacerbated as a function of genotype and storage (Figure 2D–F), with significant changes in polyamine, folate, and redox cycles (Figure 1G; Supporting Information S1: Figure 3A,B). Of note, cKO mouse RBCs—at baseline and even more so after storage—were characterized by a significant and exponential accumulation of glyceraldehyde 3-phosphate (triose phosphate isomers—Supporting Information S1: Figure 3C), compatible with a metabolic blockade in glycolysis at the levels of redox-sensitive GAPDH, whose oxidation at active site residues C152, C156, and H179[46] or deamidation of N residues proximal to them[16] has been previously shown to exacerbate the storage lesion. Consistently, peptidomics demonstrated reduced accumulation of isoAsp-repair–linked methylated peptides in PIMT-deficient RBCs after storage (Figure 2H,I). Proteomic and peptidomic data further identified altered abundance of cytoskeletal and redox-related proteins (Figure 2E,F; Supporting Information S1: Figures 4 and 5). Pathway analyses pointed to enrichment of complement and coagulation components (markers of RBC hemolytic fragility[24]), ferroptosis,[30] one-carbon, and immune-related proteins in cKO RBCs, whereas glycolysis and proteasome-associated proteins were depleted (Supporting Information S1: Figures 4D,E and 5A–E), phenotypes reported in sub-populations of stored RBCs that are prone to post-transfusion splenic sequestration (small microcytic erythrocytes[47]).

Thus, although PIMT deficiency does not reduce short-term recovery, it destabilizes protein-repair processes, especially methylation of deamidated or dehydrated residues (Figure 2G–I) in hemoglobin, membrane proteins (SPTA1), and glycolytic enzymes (ALDOC, ENOA). Altogether, these results indicate that substantial biochemical and proteomic disruption of isoAsp repair does not necessarily translate into impaired short-term PTR when RBCs are transfused into healthy, autologous recipients.

PIMT deficiency is not associated with lipid oxidation during RBC storage

Unexpectedly, lipidomic and oxylipin analyses showed that cKO RBCs had reduced levels of oxidized fatty acid derivatives, including prostaglandins, lipid hydroperoxides, and lipid alcohols (e.g., HODEs, HETEs) at baseline and after storage (Supporting Information S1: Figure 6E). Complex lipids, including phosphatidylcholines, sphingolipids, and ceramides, were also depleted in cKO RBCs after storage (Supporting Information S1: Figure 6B). Together, these findings suggest that lipid and protein oxidation do not progress in parallel under all conditions. Rather, lipid oxidation can occur under relatively mild oxidative stress, whereas protein damage requiring PIMT-mediated repair becomes functionally relevant only at higher levels of oxidant burden.

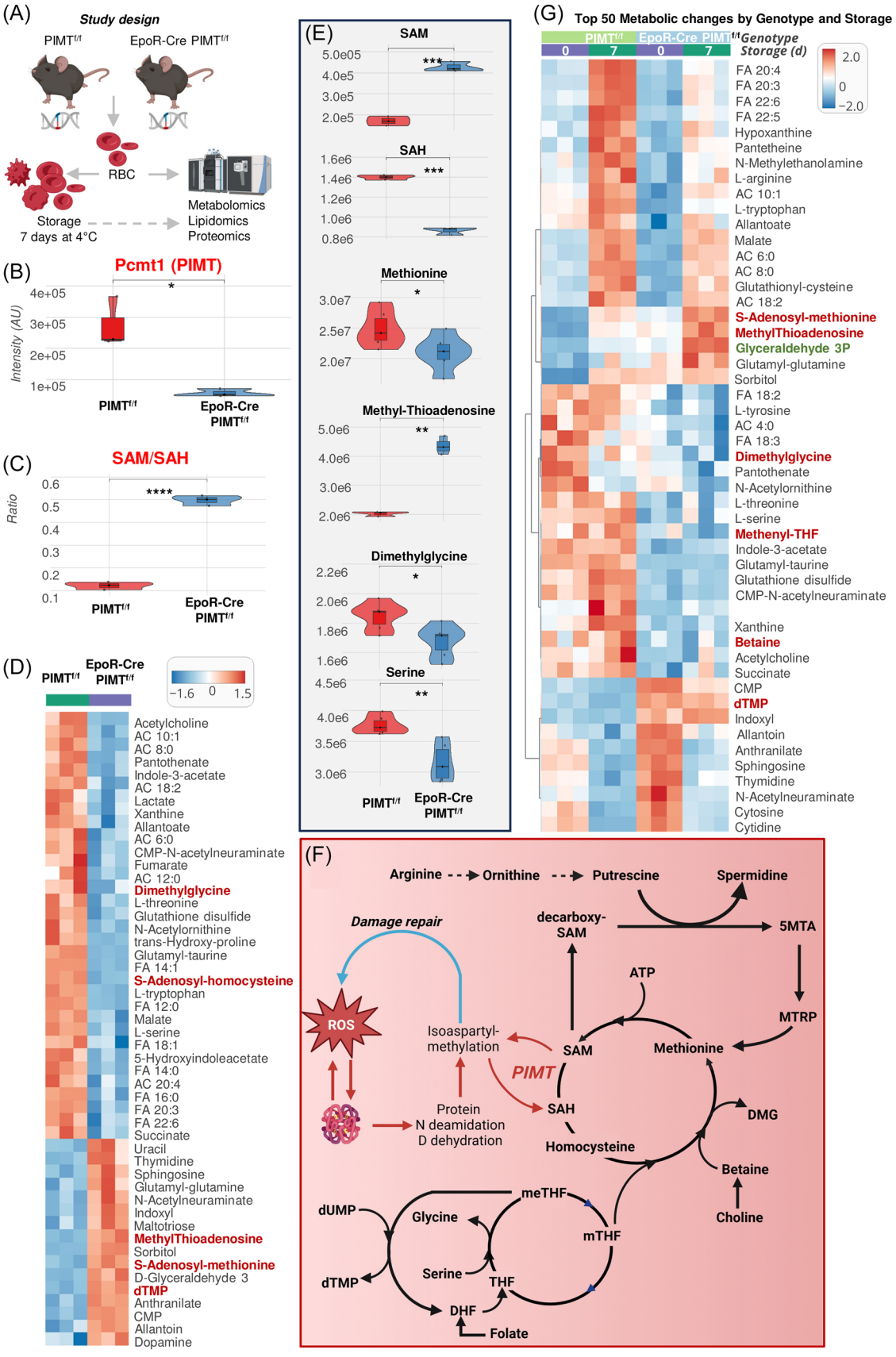


FIGURE 1 (See caption on next page).

FIGURE 1 **Erythroid-specific protein-L-isoaspartate *O*-methyltransferase (PIMT) deficiency alters one-carbon metabolism and storage-associated remodeling of red blood cells.** Experimental design **(A)**: red blood cells (RBCs) from *Pcmt1*$^{flox/flox}$ (control) and erythroid-specific *EpoR-Cre Pcmt1*$^{flox/flox}$ (PIMT conditional knockout [KO]) mice were stored for 7 days at 4°C and analyzed by metabolomics, lipidomics, and proteomics. Expression of *Pcmt1* (PIMT) measured in erythroid cells confirms loss of protein **(B)**. Consistently, the *S*-adenosyl-methionine (SAM)/*S*-adenosyl-homocysteine (SAH) ratio showed a significant reduction in methyl-donor consumption in PIMT-deficient RBCs **(C)**. Heatmap of top 50 metabolites (*t*-test) altered in conditional KO versus control RBCs at baseline **(D)**, highlighting in red font changes in methylation-related intermediates and redox-sensitive metabolites. Violin plots of representative metabolites in one-carbon metabolism (SAM, SAH, methionine, methyl-thioadenosine, dimethylglycine, and serine), demonstrating depletion of methyl donors and altered transmethylation flux in conditional knockout (cKO) RBCs **(E)**. Schematic illustrating the intersection of PIMT-dependent protein repair with one-carbon metabolism, polyamine synthesis, and folate cycling **(F)**. Heatmap of the top 50 metabolites significantly altered by genotype and storage duration (by linear discriminant analysis), highlighting accumulation of isoaspartyl (isoAsp) repair intermediates and remodeling of redox and one-carbon pathways **(G)**.

To validate these findings in humans, we next turned to the REDS-III donor cohort to test whether natural PCMT1 variation similarly influences hemolysis and omics phenotypes.

Common *PCMT1* variants modulate hemolysis in human donors

As part of the REDS RBC Omics study, 13,091 blood donors were genotyped for 717 *PCMT1* SNPs (Figure 3A). The nonsynonymous rs4816 (V120I) variant was most prevalent (minor allele frequency [MAF] ~ 0.3) and associated with ~15% reduced osmotic hemolysis ($P < e{-}50$), slight increases in oxidative hemolysis ($P < e{-}4$), and no change in storage hemolysis (Figure 3B–E). Given the large cohort size, statistically significant differences may reflect modest but consistent effect sizes with overlapping distributions. For example, we observed an allele-dose effect consistent with stepwise reduction in osmotic hemolysis from PIMT 120 VV to VI to II genotypes in 12,950 donors (means ± SD: 29.6% ± 13.4%, 28.2% ± 13.5%, and 25.3% ± 13.7%, respectively). An overall Kruskal–Wallis test yielded $P = 5.2 \times 10^{-40}$, with all pairwise comparisons remaining highly significant (VV vs. VI: $P = 3.1 \times 10^{-7}$; VV vs. II: $P = 3.0 \times 10^{-40}$; VI vs. II: $P = 1.7 \times 10^{-21}$). Other missense variants (R5H; V178I; K183R; M263I) were only found in heterozygosity in 11, 6 and 18 donors, respectively. The intronic variant rs9322219 also emerged as strongly associated with hemolysis traits, while both rs4816 (V120I) and rs9397805 variants were enriched in donors of Asian and African ancestry (MAF > 0.70—Figure 3F,G), consistent with prior reports.[20] To validate these associations, we next examined donors recalled as high-frequency donors (>3/year, $n = 150$) or with extreme hemolysis (<5th or >95th percentile, $n = 493$, Figure 4A).

PIMT protein and peptide levels stratify by genotype in recalled donors

In 643 recalled donors, PIMT protein abundance varied by sex, storage duration, and genotype, with I120 homozygotes exhibiting the highest levels (Figure 4B–D). PIMT was a top discriminant protein in proteomic LDA (Figure 4E), while metabolomics identified genotype-linked differences in metabolites related to osmotic fragility, like kynurenine,[24] sphingosine 1-phosphate,[48] bilirubin, and several phosphatidylserines—markers of eryptosis[49] (Figure 4F). The V120/I120 polymorphic peptide was lower in carriers of the variant allele, despite higher overall protein abundance, and methylated D or N-deamidated peptides increased with storage in all genotypes but were highest in I120 homozygotes at Days 10 and 21 (Figure 4H–J). These findings show that rs4816 (V120I) genotype influences both hemolysis phenotypes and PIMT protein/peptide abundance, consistent with higher repair flux in I120 carriers.

rs4816 (V120I) defines a strong cis-pQTL for PIMT protein and V120-containing peptide

To further test the genetic underpinnings of PIMT protein abundance, we performed pQTL mapping in the index donor cohort on mass spectrometry–derived measurements of PIMT protein levels in 13,091 donors. The analysis highlighted a strong ($P < e{-}18$) cis-pQTL hit on chromosome 6, on the region coding for *PCMT1*, with rs62441335 as the lead SNP (Figure 5A,B). In our cohort ($n = 12{,}755$ had available genotypes), rs62441335 and rs4816 (V120I) are in near-complete linkage disequilibrium ($r^2 = 0.999$, $D' \approx 1.00$; $\chi^2(1) = 12{,}746.6$, $P < 1 \times 10^{-300}$), indicating they tag the same haplotype with essentially no detectable recombination, and both SNPs are prevalent in donors of Asian or African descent (Supporting Information S1: Figure 7A–C). Also in the larger index cohort, PIMT abundance was proportionately higher in donors heterozygous or homozygous for the rs4816 I120 allele compared with donors homozygous for the V120 reference allele (Figure 5C). A second trans-QTL was observed on chromosome 7 on the region coding for IKZF1.

We then focused on peptide-level data for the V120-containing peptide from the recalled donor cohort. From this analysis, rs4816 (V120I) achieved even stronger genome-wide significance as a determinant of PIMT peptide levels (P-value = e−85—Figure 5D,E). Comparison of PIMT reference (WT – V120) and alternative allele (I120) through proteome-constrained modeling predicts that flux through PIMT V120I variant was approximately 40% greater than that of the WT PIMT variant across varying abundance levels of active enzyme (Figure 5F). The maximum predicted fluxes for the PIMT variants are 609 nmol/h/gDW for the V120 variant and 841 nmol/h/gDW for I120, when all available enzyme (~3.736 nmol/gDW) is actively utilized. Consequently, RBCs with the V120I variant require ~27% less PIMT to achieve the maximal flux of the WT variant, highlighting the increased catalytic efficiency of V120I PIMT variant at repairing L-isoAsp protein residues compared to the WT protein. The linear relationship between PIMT flux and abundance for both WT and V120I variants also indicates that no flux bottlenecks are imposed by other enzymes (e.g., methionine adenosyltransferase and adenosylhomocysteinase), making PIMT enzyme abundance and kinetic properties the primary determinants of PIMT flux when sufficient substrate is available.

Recombinant PIMT I120 variant shows enhanced enzymatic activity

To directly test the biochemical consequences of rs4816 (V120I), and expand previous reports of an increased protein stability and potential higher activity of the I120 variant,[20] we expressed and purified recombinant V120 and I120 PIMT proteins (Figure 6A). Activity

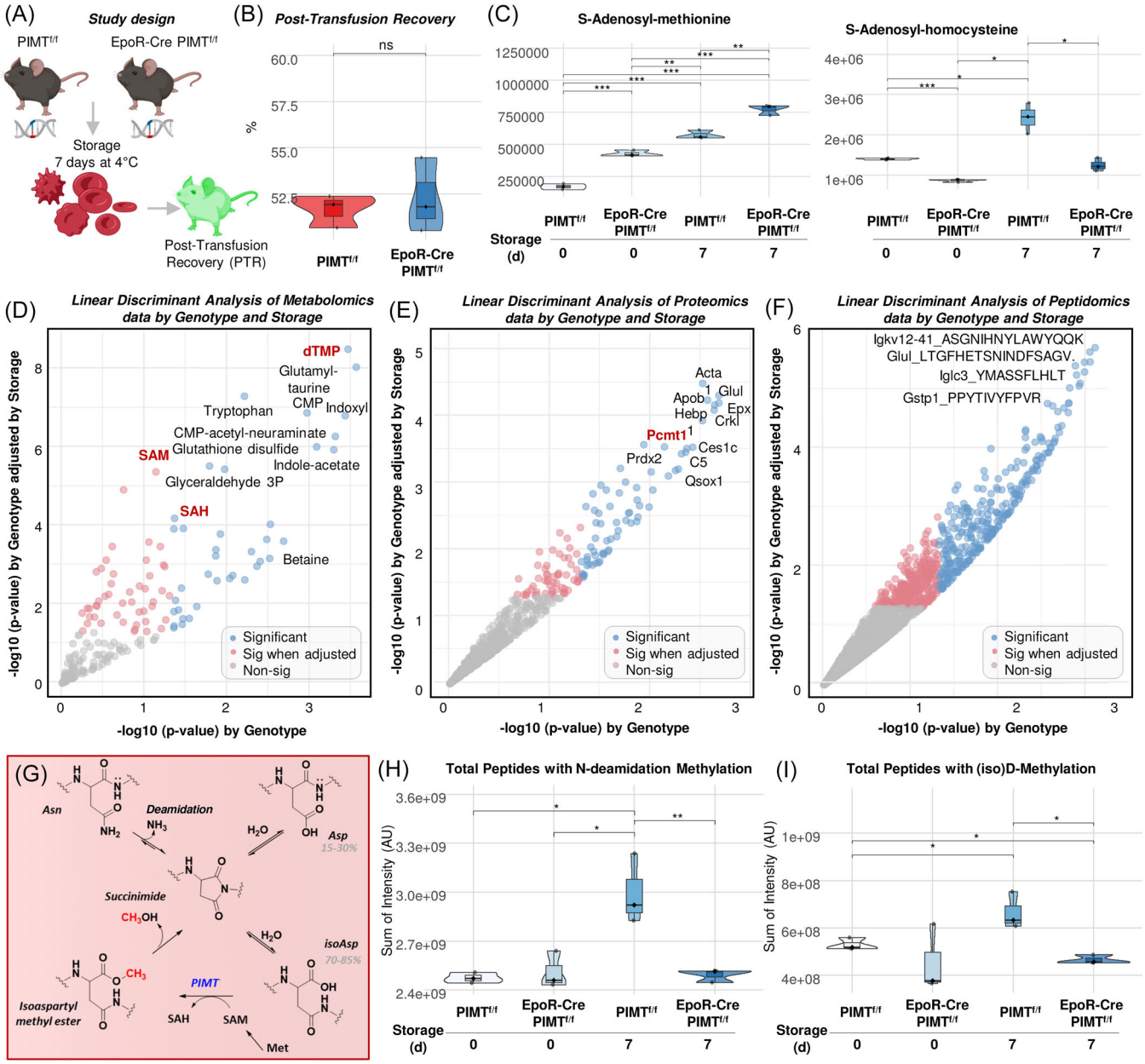


FIGURE 2 **Protein-L-isoaspartate *O*-methyltransferase (PIMT) deficiency impacts proteome stability after red blood cell (RBC) storage, with no significant effects on post-transfusion recovery.** Experimental design **(A)**: RBCs from Pcmt1$^{flox/flox}$ (control) and erythroid-specific EpoR-Cre Pcmt1$^{flox/flox}$ (PIMT conditional knockout [KO]) mice were stored for 7 days at 4°C and transfused to assess post-transfusion recovery (PTR). PTR did not differ significantly at 24 h between groups **(B)**. Metabolomics showed depletion of *S*-adenosyl-homocysteine (SAH) and accumulation of *S*-adenosyl-methionine (SAM) in PIMT-deficient RBCs at baseline and after storage **(C)**. Linear discriminant analysis (−log10 of P-values) of metabolomics **(D)**, proteomics **(E)**, and peptidomics **(F)** data by genotype (unadjusted or adjusted by storage duration) revealed significant differences in one-carbon and redox metabolism, cytoskeletal proteins, and peptides undergoing isoAsp-related modifications, with PIMT highlighted among the top discriminants. Schematic **(G)** of isoaspartyl (isoAsp) formation and repair by PIMT illustrates the biochemical cycle disrupted by enzyme deficiency. Quantification of peptides with N-deamidation followed by methylation **(H)** or (isoAsp)-methylation **(I)** confirmed increased repair flux in RBCs after storage, a response that is ablated in conditional KO mice.

assays using a synthetic isoAsp-containing peptide (WAGG(iso-D) ASGE) in the presence of SAM demonstrated that the I120 (rs4816) PIMT variant exhibits higher catalytic activity than the reference V120 proteoform (Figure 6B,C). Nonlinear Michaelis–Menten fitting yielded an apparent K_m of 11.34 μM for V120 and 12.54 μM for I120, corresponding to a modest increase in K_m (~12%) for the variant enzyme. In contrast, the I120 variant displayed an approximately 30%–35% higher apparent V_{max} under identical assay conditions, indicating enhanced maximal catalytic capacity. Double-reciprocal (Lineweaver–Burk) plots are provided in Supporting Information S1: Figure 8 to facilitate visualization of these kinetic differences, but kinetic parameters reported here are derived from nonlinear fitting to avoid bias introduced by reciprocal transformation. Together, these data indicate that the V120I substitution enhances catalytic turnover at saturating substrate concentrations, with a modest trade-off in substrate affinity. These findings are consistent with prior reports

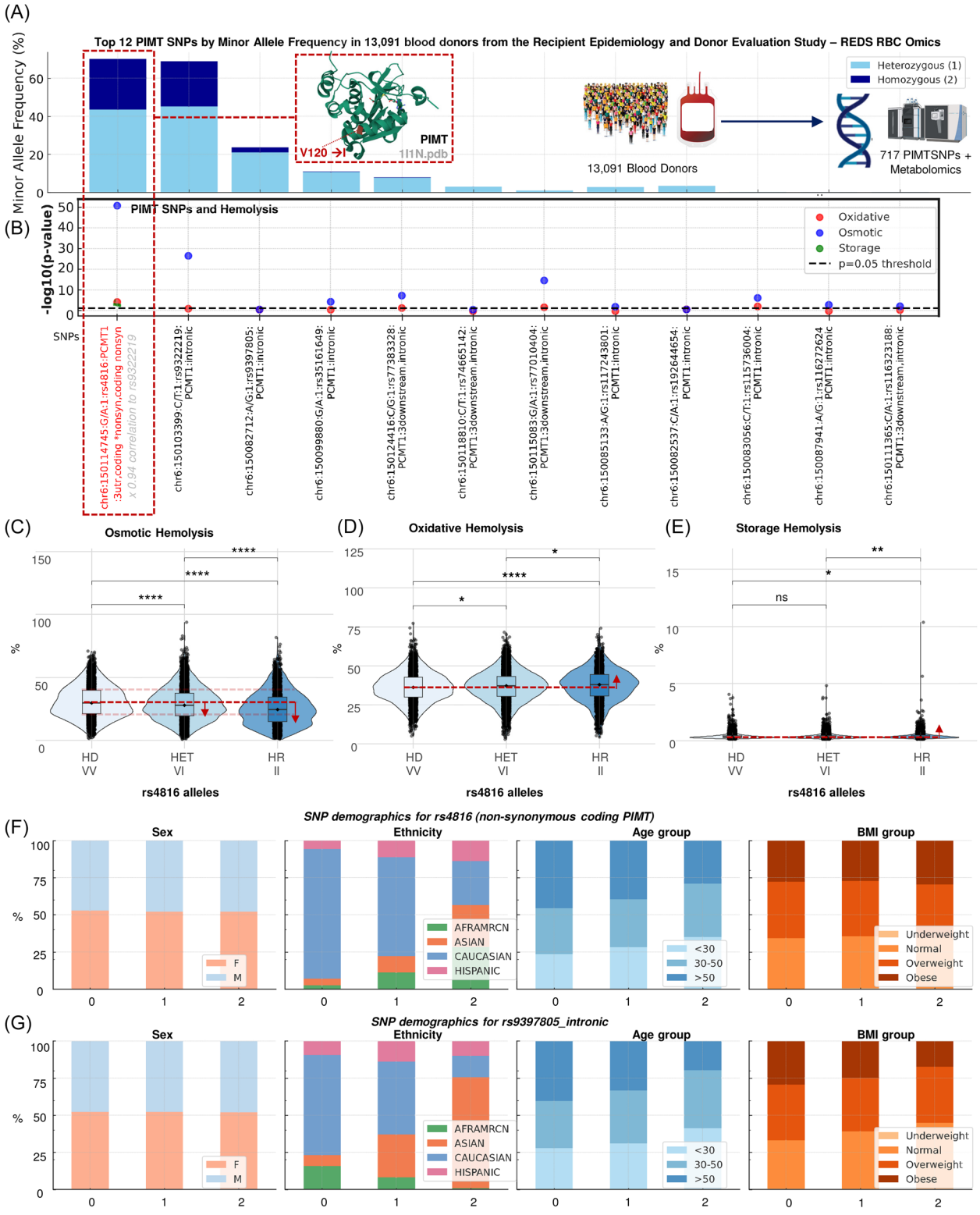


FIGURE 3 **Common *PCMT1* variants associate with hemolysis phenotypes in the Recipient Epidemiology Donor Evaluation Study (REDS)-III Red Blood Cell Omics (RBC Omics) cohort.** Study overview and top 12 *PCMT1* single-nucleotide polymorphisms (SNPs) by minor allele frequency (MAF) across donors (n = 13,091), highlighting the most frequent nonsynonymous coding variant *rs4816* (V120I) **(A)**. Association analysis (−log10 P-values) of *PCMT1* SNPs with osmotic, oxidative, and storage hemolysis; rs4816 surpassed the significance threshold for oxidative and osmotic hemolysis **(B)**. Violin plots of hemolysis measures stratified by rs4816 genotype: homozygous reference (HD, VV), heterozygous (HET, VI), and homozygous rare allele (HR, II): rs4816 carriers demonstrated significantly reduced osmotic hemolysis—up to 15% decrease **(C)**—minimally increased oxidative hemolysis—up to 3% increase **(D)**, while storage hemolysis showed no significant difference **(E)**. Demographic distribution of rs4816 **(F)** and the top associated intronic SNP rs9397805 **(G)** by sex, ethnicity, age group, and body mass index (BMI), illustrating enrichment of both SNPs in donors of Asian or African ancestry. The top associated intronic SNP (rs9397805), which is not in strong linkage disequilibrium with rs4816 (V120I), identifies a regulatory signal distinct from the protein-coding haplotype.

describing increased enzymatic activity (>20% in RBCs), greater thermal stability, and reduced substrate affinity of the I120 variant relative to V120.[50]

To characterize the difference between the V120 and the I120 variants on the atomic level, we used a suite of 2D and 3D NMR experiments, which allowed us to assign 60% of the backbone resonances of the I120 construct. By overlaying the 2D ^{15}N-HSQC fingerprint spectra of both I120 and V120 (Figure 6D), we could observe that both proteins are well-folded, and they exhibit minor chemical shift perturbations (CSPs) (Figure 6E–G). This indicates that

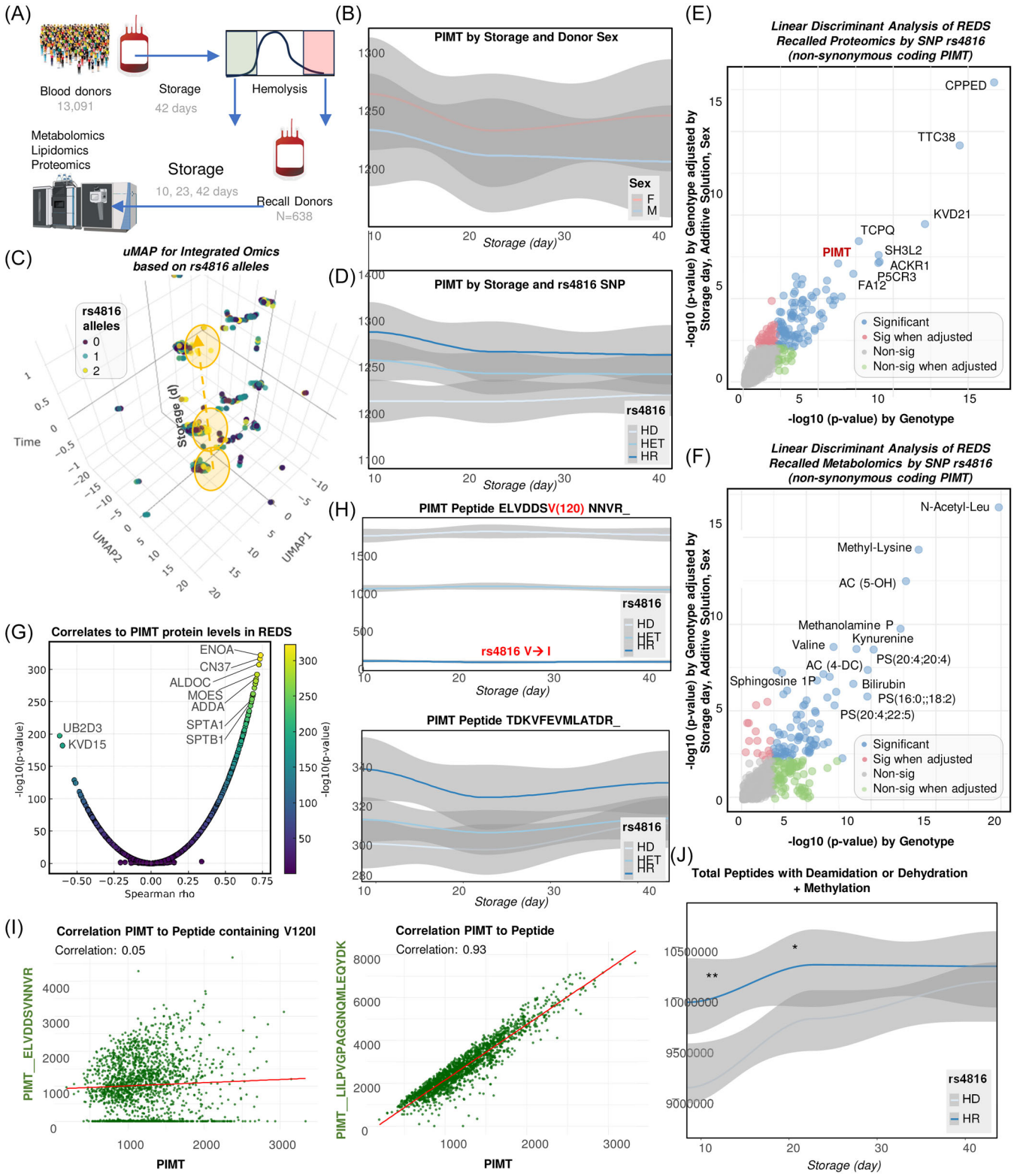


FIGURE 4 (See caption on next page).

FIGURE 4 **Protein-L-isoaspartate *O*-methyltransferase (PIMT) protein and peptide levels in recalled donors stratified by rs4816 (V120I) alleles.** Study overview: 643 donors from the red blood cell (RBC) Omics index cohort, who ranked above the 95th or below the 5th percentile for osmotic, oxidative, or storage hemolysis, were recalled and donated a second independent unit for metabolomics, proteomics, and peptidomics analyses **(A)**. PIMT protein abundance across storage was influenced by donor sex, with higher levels in female donors and slow decrease by storage duration **(B)**. Uniform Manifold Approximation and Projection (uMAP) of omics data of the recalled donors showed stratification by rs4816 alleles **(C)**. Specifically, stratification by rs4816 genotype showed increased PIMT levels in homozygous carriers of the I120 allele (HR, II) compared to heterozygotes (HET, VI) and homozygous reference donors (HD, VV) **(D)**. Linear discriminant analysis (−log10 P-values) of proteomics data by genotype, adjusted for storage, age, sex, and ancestry, identified PIMT among the top discriminant proteins **(E)**, while metabolomics analysis revealed significant differences in metabolic markers of osmotic fragility—kynurenine, sphingosine 1-phosphate and methyl-lysine, bilirubin, and several phosphatidylserines **(F)**. Correlation of PIMT protein levels to the RBC proteome showed a strong positive association with several membrane proteins (SPTA1, SPTB1) and glycolytic enzymes (ALDOC, ENOA) **(G)**. PIMT peptides containing the rs4816 V120/I120 site and additional isoAsp-prone motifs (ELVDD(V120)NNVR, TDKFVEVWLATDR) showed genotype-dependent abundance during storage **(H)**. Correlation analysis demonstrated that PIMT protein levels were tightly linked to the abundance of its associated peptides, except for the peptide containing the V120I polymorphism, which showed weak correlation **(I)**. Quantification of total peptides with deamidation or dehydration followed by methylation showed increased accumulation during storage in reference allele carriers, a trend blunted in rs4816 I120 homozygotes **(J)**.

the mutation does not cause major structural or conformational changes in the apo form of the enzyme. Therefore, we hypothesize that the difference in enzymatic activity reflects altered local conformational dynamics, particularly in regions involved in substrate or cofactor engagement, rather than global changes in protein fold. This aligns with the computationally modeled increase in active-site flexibility or accessibility of PIMT I120, without compromising overall structural stability, consistent with the dynamics simulations reported by Rutherford and Dagget, who modeled the I120 variant within <10 Å from the SAM-binding site[50] based on solved human V120 PIMT crystal structures.[51,52] These data establish that the I120 variant represents a hypermorphic allele at both proteomic and biochemical levels, confirming omics observations in REDS donors.

PIMT protein levels and genotype associate with transfusion outcomes

Having linked *PCMT1* variation to hemolysis and biochemical remodeling in both index and recalled cohorts, we next asked whether donor PIMT levels and genotypes influence transfusion efficacy. In the index cohort, PIMT protein abundance was identified as the top proteome-wide positive correlate of both oxidative and storage hemolysis (Figure 7A–C). Since transcriptional regulatory mechanisms for PIMT remain incompletely understood, it could be speculated that donors with elevated hemolytic fragility upregulate PIMT expression during erythropoiesis as a compensatory mechanism. In recalled donors, osmotic fragility confirmed lower hemolysis in I120 carriers (Figure 7D), validating index cohort results (Figure 3C).

Integration with the vein-to-vein database revealed that genetic variation also affected clinical outcomes. In 4511 single-unit transfusion events, hemoglobin increments were not significantly different in recipients of I120 donor RBCs, but were significantly reduced (40% decrease, P-value < 0.0001) in rs9397805 carriers (Figure 7F). In 1165 events with bilirubin measurements, rs9397805 carriers showed reduced increments, consistent with lower in vivo hemolysis following transfusion, whereas rs4816 (V120I) showed no significant effect (Figure 7G). Supplementary hive plot analyses further illustrated that I120 donors clustered with higher SAM/SAH balance, greater peptide methylation, and reduced hemolysis (Supporting Information S1: Figures 9 and 10).

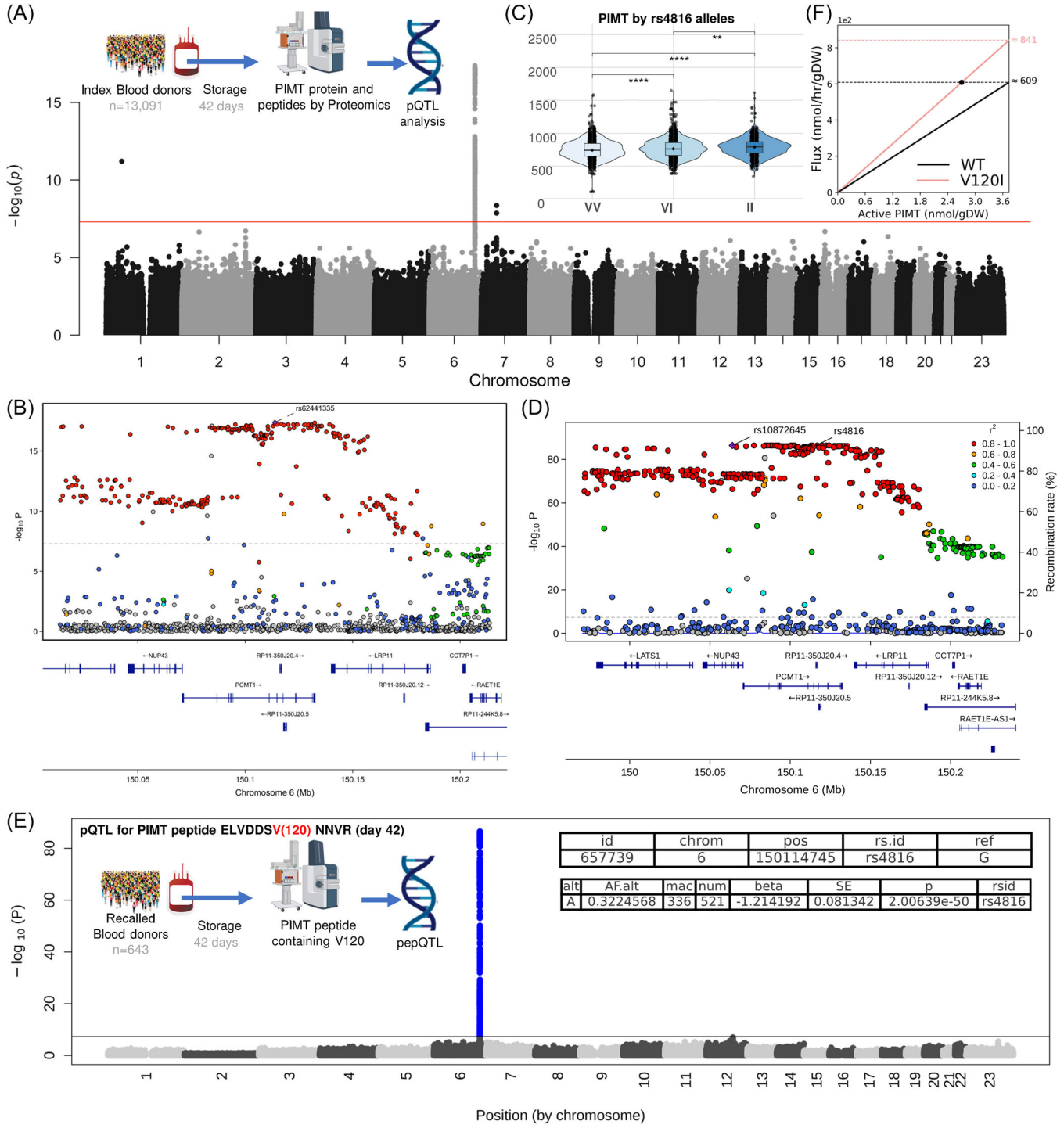
Of note, protective effects of rs4816 (V120I) on osmotic hemolysis and deleterious effects on oxidative fragility synergized with *G6PD* V68M mutant alleles—coding for hypomorphic A− variant (Supporting Information S1: Figure 10E,F). Notably, "protective" alleles at these loci reflect distinct molecular mechanisms: preserved enzymatic function for G6PD (V68), versus enhanced catalytic activity for PIMT (I120). However, while PIMT I120 is prevalent in donors of Asian or African descent, G6PD M68 is prevalent only in donors of African ancestry (~13%),[29] with negligible co-inheritance of both traits (only 61 donors were homozygous/hemizygous for both traits—*G6PD* is X-linked).

Taken together, these results indicate that the consequences of PIMT deficiency or variation are readily detectable at the omics level, yet may not translate into impaired PTR when tested in an autologous recipient setting, like in the mouse experiment described above. Instead, their impact becomes unmasked under additional oxidative or clinical stress. This is consistent with our previous *Pcmt1* knockout mouse studies involving bone marrow transplant into ablated wild-type recipients, where in vivo hemolysis was revealed only after phenylhydrazine treatment.[17] In humans, *PCMT1* variants influenced transfusion outcomes in heterologous recipients requiring transfusion (i.e., with clinical comorbidities).

DISCUSSION

In this study, we identify PCMT1 genetic variation and PIMT protein levels as key determinants of RBC storage quality, with translational implications extending beyond transfusion medicine. To clarify the relationships among PCMT1-associated variants identified in this study, it is important to distinguish two genetically and functionally distinct signals. The exonic rs4816 (V120I) variant, which encodes the V120I substitution in PIMT, is in near-complete linkage disequilibrium with the intronic variant rs62441335; together, these variants define a single haplotype associated with increased PIMT protein abundance, enhanced enzymatic activity, and increased peptide methylation flux. In contrast, the intronic variant rs9397805 shows only modest linkage disequilibrium with rs4816 ($r^2 \approx 0.17$) and represents an independent regulatory signal. This variant associates with hemolysis metrics and transfusion outcomes without altering PIMT protein sequence or catalytic activity. Thus, rs4816/rs62441335 defines a protein abundance and activity haplotype, whereas rs9397805 defines a distinct regulatory haplotype influencing RBC fragility and transfusion performance through mechanisms independent of the V120I substitution.

The nonsynonymous rs4816 (V120I) variant emerged as a positive allele, associated with reduced osmotic hemolysis, attenuated protein damage, and greater peptide methylation flux, effectively functioning as a "good storage" polymorphism. This contrasts with most genetic variants uncovered in RBC Omics analyses to date, such as *G6PD*,[27] *STEAP3*,[30] *p53*,[30] *GPX4*,[31] and *LPCAT3*,[30] which predominantly confer increased susceptibility to oxidative stress and



id	chrom	pos	rs.id	ref
657739	6	150114745	rs4816	G

alt	AF.alt	mac	num	beta	SE	p	rsid
A	0.3224568	336	521	-1.214192	0.081342	2.00639e-50	rs4816

FIGURE 5 **rs4816 (V120I) is a strong protein quantitative trait loci (pQTL) for protein-l-isoaspartate *O*-methyltransferase (PIMT) peptides in recalled donors.** Study overview: mass spectrometry–based measurements of PIMT in 13,091 index units were strongly associated with a cis-QTL hit on chromosome 6 **(A)** mapping on the coding region for *Pcmt1* **(B)**—lead single-nucleotide polymorphism (SNP) rs62441335. Violin plot of PIMT protein abundance in the 13,091 REDS index donors stratified by rs4816 genotype (V/V, V/I, I/I), demonstrating a stepwise increase in PIMT abundance with increasing dosage of the I120 allele **(C)**. In the RBC Omics recall cohort ($n = 643$), proteomics and peptidomics were performed on stored RBCs to test for protein quantitative trait loci (pQTL) **(D, E)**. A Manhattan plot of pQTL analysis for the PIMT peptide ELVDDS(V120)NNVR at Day 42 storage showed a genome-wide significant association with the *PCMT1* locus on chromosome 6, specifically rs4816 (V120I) **(E)**. Locus zoom analysis identified rs4816 as the lead SNP, with strong linkage disequilibrium across the *PCMT1* locus (D). Proteomics-informed genome-scale reconstruction of RBC metabolism in donors with 0 or 2 alleles of rs4816 predicts significant increases in fluxes through PIMT in donors carrying the I120 alleles **(F)**.

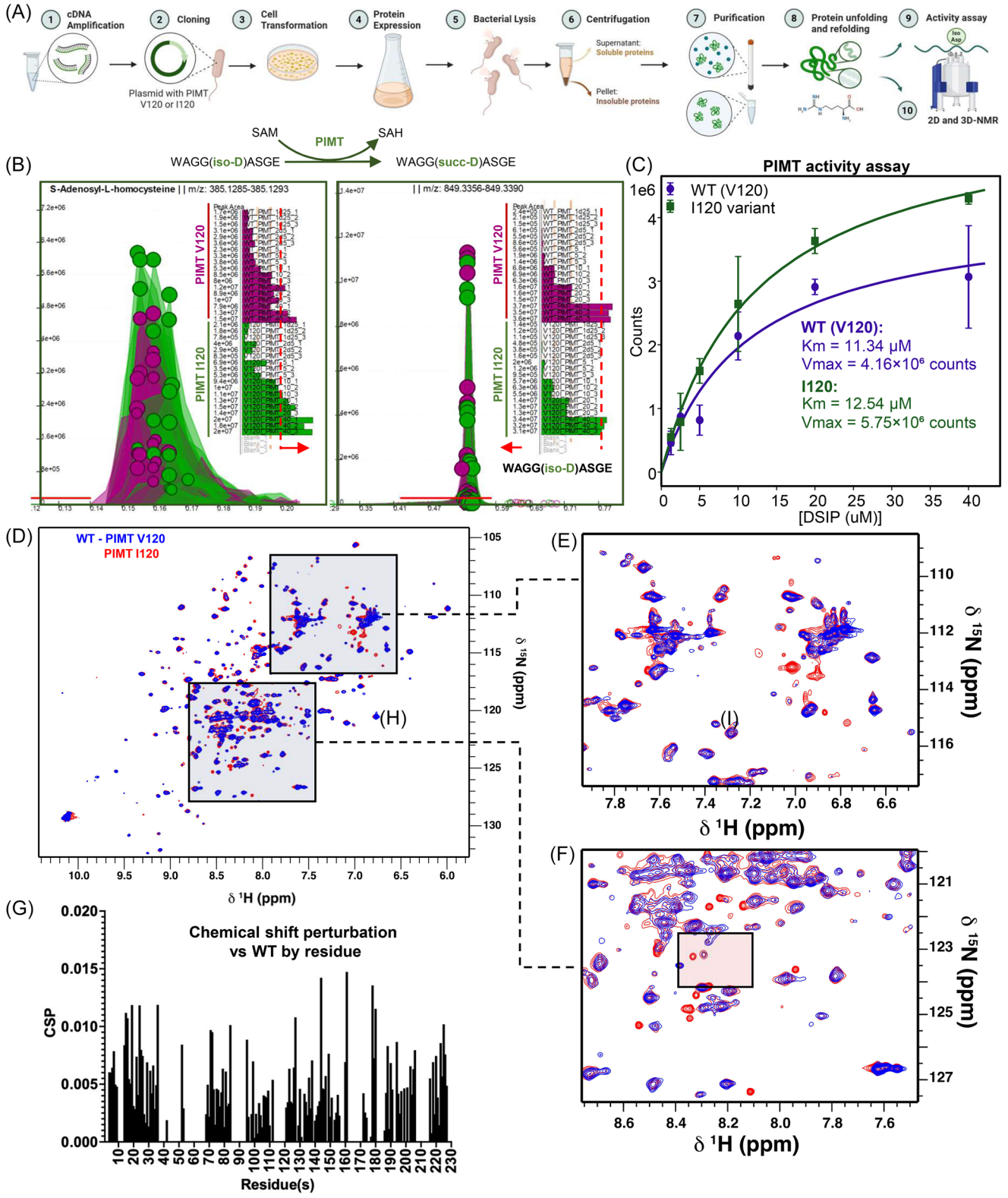


FIGURE 6 **Recombinant expression, activity profiling, and nuclear magnetic resonance (NMR) characterization of the protein-L-isoaspartate *O*-methyltransferase (PIMT) V120I variant.** Workflow for cloning, bacterial expression, purification, and enzymatic characterization of recombinant wild-type (V120) and variant (I120) PIMT proteins **(A)**. In vitro methylation assays using *S*-adenosyl-methionine (SAM) and a custom isoaspartyl-containing peptide substrate WAGG(iso-D)ASGE, with targeted mass spectrometry used to quantify SAM, *S*-adenosyl-homocysteine (SAH), substrate, methylated product, and the succinimide intermediate **(B)**. Michaelis–Menten analysis demonstrating a higher apparent V_{max} for the I120 (green) variant relative to WT (V120—purple), with a modest increase in K_m **(C)**. Two-dimensional ^{1}H–^{15}N-HSQC spectra of uniformly ^{15}N-labeled WT (blue) and I120 (red) PIMT reveal nearly identical peak dispersion, indicating that both proteins adopt the same global fold **(D)**. Enlarged regions **(E, F)** highlighting localized chemical-shift and intensity perturbations between WT and I120. Residue-wise chemical shift perturbation (CSP) plot for assigned amide resonances **(G)**.

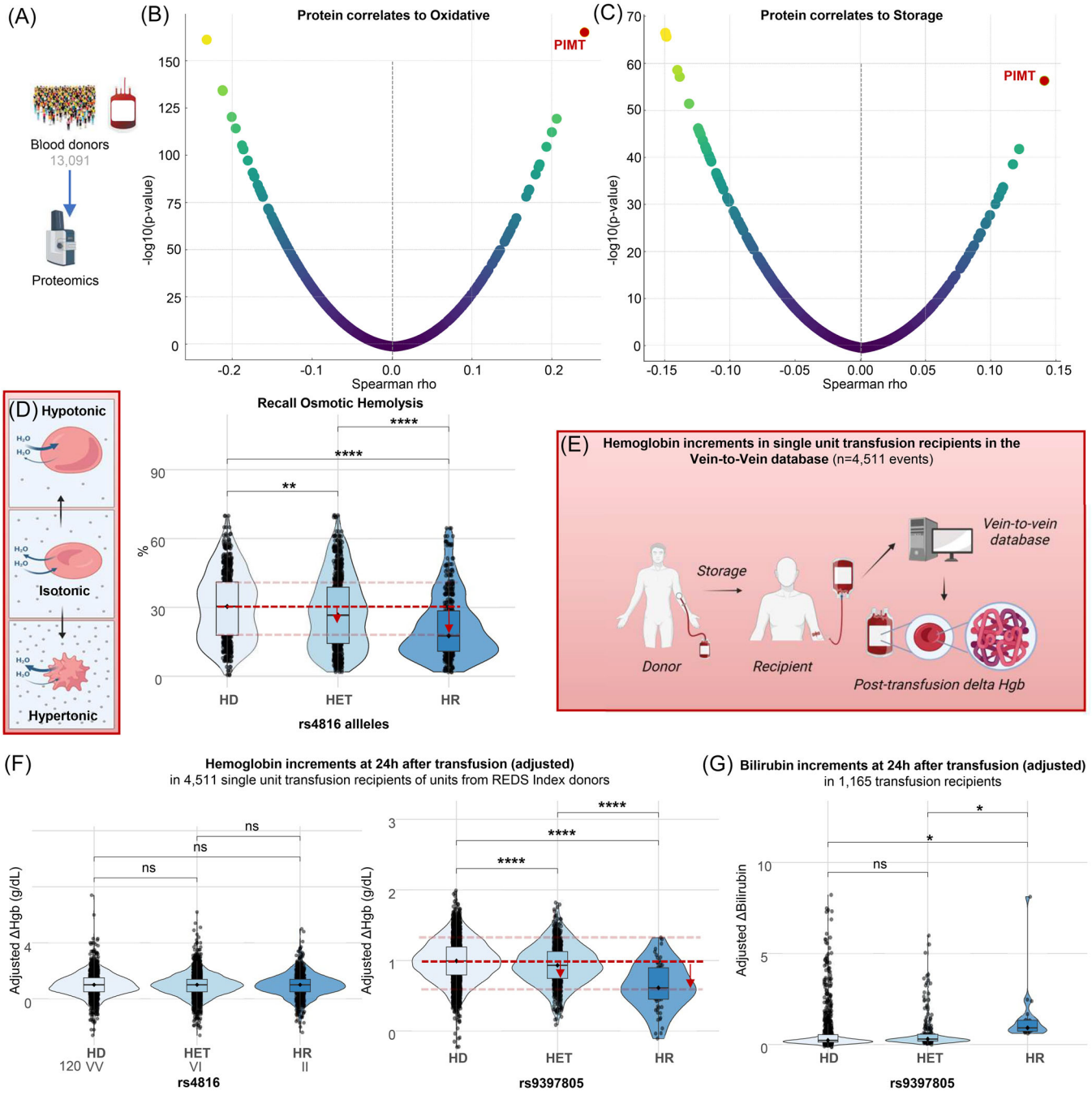


FIGURE 7 **Protein-L-isoaspartate *O*-methyltransferase (PIMT) protein levels and genotype associate with hemolysis and transfusion outcomes.** PIMT protein levels were quantified by high-throughput proteomics in the Recipient Epidemiology Donor Evaluation Study (REDS)-III index cohort (n = 13,091) **(A)**. Correlation analyses showed that PIMT protein levels were the strongest proteomic predictors of oxidative hemolysis **(B)** and storage hemolysis in this cohort **(C)**. In the recall cohort, osmotic fragility assays demonstrated significantly lower hemolysis in carriers of the I120 allele (rs4816), with homozygous I120 donors showing the greatest resistance **(D)**, validating observations in the index cohort (Figure 3C). Integration with the vein-to-vein database linked donor genotype to transfusion outcomes: schematic of workflow illustrating donor–recipient matching and outcome tracking **(E)**. In 4511 single-unit transfusion events, hemoglobin increments at 24 h were not significantly different in recipients of red blood cell (RBC) units from donors carrying the I120 allele of rs4816 (homozygous reference [HD, VV], heterozygous [HET, VI], and homozygous rare allele [HR, II]), but significantly lower (40% decrease) when donors carried the intronic variant rs9397805 **(F)**. In 1165 events with bilirubin measurements, rs9397805 carriers showed reduced post-transfusion bilirubin increments, while rs4816 showed no significant association **(G)**. Reduced bilirubin increments in rs9397805 carriers are consistent with decreased post-transfusion RBC breakdown. Transfusion outcomes stratified by PCMT1 genotype demonstrate that rs9397805 (intronic, regulatory) but not rs4816 (V120I, coding) associates with altered hemoglobin and bilirubin increments.

hemolysis, with deleterious SNPs especially prevalent in donors of African descent. Thus, in donors of African ancestry, interpretation of storage and hemolysis phenotypes is further shaped by the high prevalence of strong, high-impact modifiers of red cell redox biology—including G6PD deficiency, altered iron handling, and susceptibility to lipid peroxidation and ferroptosis—which can dominate or mask additive effects of PCMT1 variation. On the other hand, the enrichment of V120I among donors of Asian descent is particularly

striking and aligns with epidemiological observations that AD and related dementias appear to occur at somewhat lower incidence in Asian and Asian-American populations,[53] though these data are heterogeneous and confounded by healthcare access, diagnosis rates, and other genetic traits (e.g., APOE variants[54]). Conversely, African American populations—despite similarly high frequencies of the PIMT I120 allele—experience a higher incidence of AD compared to white populations,[55] a disparity driven by a convergence of cardiovascular risk factors, systemic health inequities, and ancestry-specific genetic variants (e.g., APOE R145C or structural variants enriched in African ancestry[56]), providing important context for interpreting population-specific effects of protein-repair pathways. However, PIMT has long been recognized for its role in repairing isoAsp lesions in neuronal proteins such as tau and Aβ, and isoAsp accumulation accelerates their aggregation.[6] The demonstration here that a common coding variant enhances PIMT activity in RBCs raises the possibility that the same allele might confer more efficient protein repair in long-lived brain proteins, with consequences for neurodegeneration, echoing lifespan extension by 32%–39% in *Drosophila* overexpressing PIMT.[57]

Our conditional erythroid-specific PIMT knockout mice provide a mechanistic context. Ablation of PIMT in RBCs and erythroid precursors did not result in seizures in the absence of perturbation, separating erythroid from neurological phenotypes, at least in young, unstressed mice. Storage of cKO RBCs on a C57BL6/J background, which exhibits relatively limited storage-induced oxidant stress, resulted in exacerbated protein oxidation but modest lipidomic alterations, disentangling the relative contribution of protein versus lipid damage to storage-associated lesions. These mice, now available to the community, offer a platform to test whether additional phenotypes, including neurocognitive decline during aging, will emerge under specific stressors.

Beyond genetic variation, our work highlights the systemic metabolic cost of maintaining isoAsp repair under stress. In both mouse and human RBCs, oxidant challenge increased uptake of methyl-group donors, particularly methionine and choline, to fuel PIMT-mediated repair. Given that the human circulation contains ~25 trillion RBCs, even modest increases in methyl-donor consumption per cell can represent a substantial systemic sink, potentially competing with other tissues for limited SAM-dependent reactions. Circulating methionine concentrations in plasma average ~30 μM and free choline ~10 μM, corresponding to total extracellular pools on the order of a few hundred micromoles. If oxidant stress were to increase RBC consumption of methyl donors by ~25%, this could deplete tens to hundreds of micromoles from the circulating pool within hours, a magnitude comparable to daily dietary intake in individuals with marginal nutrition. Because synthesis of phosphatidylcholine and sphingomyelin through the PEMT pathway requires three methyl groups per molecule, and because sphingomyelins are particularly demanding for methyl-donor availability and critical for myelin integrity in the brain, such a redistribution of substrates toward the RBC compartment could directly limit availability for other tissues. In this context, methyl-donor competition could affect epigenetic regulation, nucleotide biosynthesis, and myelin lipid homeostasis in the nervous system. These considerations suggest that RBC stress responses may have consequences extending beyond transfusion biology, especially in aged or nutritionally deficient individuals in whom methyl donor reserves are already constrained. Literature on choline metabolism and cognitive health supports this concern[58–61]: low choline intake is linked to memory impairment, whereas supplementation protects against neurodegeneration. Similarly, genetic defects impacting choline uptake (e.g., FLVCR1) are etiologically linked to neurocognitive deficits that in part overlap with mutant *Pcmt1* phenotypes.[62] The intersection of RBC metabolic stress, PIMT activity, and systemic methyl-donor availability therefore raises provocative questions about the crosstalk between transfusion medicine, systemic metabolism, and brain health.

Another central theme emerging from our work is that the phenotypic consequences of PIMT deficiency are threshold-dependent rather than constitutive. In mice, erythroid-specific Pcmt1 knockout profoundly destabilized one-carbon metabolism and proteome integrity, yet did not impair PTR when RBCs were transfused into healthy, autologous recipients. This apparent paradox reflects the physiological context in which PTR is measured: RBCs are transfused into recipients with minimal oxidative burden, where even substantial biochemical lesions may remain functionally silent over short time scales. In contrast, storage ex vivo, oxidant challenge, or transfusion into clinically stressed recipients represent conditions in which proteostatic and redox stress are markedly higher, unmasking the importance of isoAsp repair.

This concept is further supported by our prior studies in global Pcmt1 knockout mice,[17] in which overt hemolysis became evident only after phenylhydrazine challenge. Importantly, across commercially available laboratory mouse strains, C57BL6/J mice—the genetic background upon which our conditional mice were generated in this study—are recognized as relatively "good storers,"[30] with a low degree of storage-associated oxidant stress to proteins.[63] It is thus plausible that introducing the knockout onto a more oxidant-sensitive background could accentuate this phenotype. Rather than indicating a true disconnect between lipid and protein damage pathways, our data suggest that these processes operate on different oxidative thresholds: lipid peroxidation can occur under milder stress, whereas protein damage requiring PIMT-mediated repair becomes functionally relevant only at higher degrees of oxidant exposure.

Similarly, in human cohorts, associations of PCMT1 variants with hemolysis and transfusion phenotypes were most evident in recalled donors at the extremes of fragility and in recipients with comorbidities captured in the vein-to-vein database. These findings collectively indicate that PIMT functions as a molecular safeguard whose loss creates a hidden vulnerability, silent under physiological steady state but revealed under oxidative or clinical stress. While transfusion outcomes in human cohorts are inherently influenced by multiple donor and recipient factors, the consistency of these associations with stress-dependent phenotypes observed in murine *Pcmt1* deficiency supports a context-driven, rather than confounded, explanation for the observed differences.

Several limitations warrant consideration. First, although we demonstrate clear biochemical and omics signatures of PIMT deficiency in both mice and humans, functional consequences for transfusion efficacy were modest in healthy murine recipients and became apparent only under additional stress, underscoring the context dependency of PIMT phenotypes. Second, the vein-to-vein database, although uniquely powerful, reflects transfusion practice in a heterogeneous patient population where multiple donor and recipient factors converge, limiting causal attribution to single genetic variants. Third, we focused on common *PCMT1* variants; rarer hypomorphic alleles (L191S, A150V, P174H, and A65V) are unlikely to be represented in healthy donor cohorts and remain insufficiently studied, particularly in underrepresented populations where the "healthy blood donor effect"[64] may not apply. Fourth, NMR studies were limited to comparison of the apo forms of PIMT V120/I120, though further studies addressing dynamics in the presence of cofactors SAM/SAH and substrates are warranted. In addition, temperature-dependent kinetic analyses were not performed in this study. Such experiments would be required to deconvolve whether the increased catalytic efficiency of the V120I variant arises from reduced activation enthalpy, a more favorable activation entropy, or altered

coupling between chemical turnover and product release. Addressing these questions will require dedicated pre–steady-state and thermodynamic analyses beyond steady-state flux measurements and will be an important focus of future work. Future studies using humanized *Pcmt1* knock-in models carrying the V120I variant, or erythroid progenitors engineered to express this hypermorphic allele, would provide a powerful system to directly test its protective effects, particularly during aging or under conditions of oxidant stress. Based on the enhanced catalytic activity, preserved global fold, and stress-resistant phenotypes observed in human donors, we would predict that the V120I variant confers increased resilience of the RBC proteome rather than qualitatively distinct biology relative to the murine enzyme.

Despite these limitations, our integration of mouse genetics, recombinant biochemistry, human omics, and linked transfusion outcomes illustrates the bidirectional utility of animal and donor cohorts for discovery and validation, as well as the power of combining mechanistic and population-scale approaches. The convergence of findings across systems validates PIMT as a determinant of RBC storage quality and a candidate contributor to inter-individual and inter-ancestry variation in (RBC) proteome maintenance. These results further encourage the use of precision transfusion strategies based on donor genetics and offer a model of how molecular individuality in donor blood may inform broader aspects of human aging and health. Pragmatically, donor genetic information for *Pcmt1* SNPs could be incorporated into inventory management strategies, analogous to emerging uses of array-based genotyping for transfusion-relevant blood cell antigens[65] or G6PD status.[43] Within this framework, units from donors carrying the rs4816 (V120I) hypermorphic variant may be particularly advantageous for recipients in whom oxidative or inflammatory stress is expected to be high, including critically ill patients, individuals with sepsis or trauma, chronically transfused populations, and vulnerable recipients such as neonates or elderly individuals with limited physiological reserve. Conversely, units from donors carrying regulatory variants such as rs9397805, which associate with poorer transfusion indices, may warrant closer consideration in these settings.

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AUTHOR CONTRIBUTIONS

Shaun Bevers: Investigation; methodology; formal analysis; project administration; data curation; visualization. **Aaron V. Issaian:** Investigation; methodology. **Ariel Hay:** Investigation. **Gregory R. Keele:** Formal analysis; software. **Monika Dzieciatkowska:** Methodology; investigation; formal analysis. **Julie A. Reisz:** Methodology; supervision; resources. **Zachary B. Haiman:** Software; visualization. **Travis Nemkov:** Methodology; supervision. **Daniel Stephenson:** Methodology; investigation. **Amy L. Moore:** Methodology; software. **Xutao Deng:** Software; methodology. **Mars Stone:** Resources; supervision. **Kirk C. Hansen:** Resources; supervision; project administration. **Steve Kleinman:** Resources; supervision; funding acquisition. **Philip J. Norris:** Resources; supervision; funding acquisition. **Michael P. Busch:** Resources; supervision; funding acquisition. **Bernhard O. Palsson:** Resources; supervision. **Nareg H. Roubinian:** Formal analysis; supervision; data curation; software; methodology; writing—review and editing. **John Janetzko:** Supervision; writing—review and editing. **Beat Vögeli:** Supervision; resources; validation. **Grier P. Page:** Supervision; resources; software; methodology. **David N. Jones:** Methodology; investigation; formal analysis; visualization; supervision; data curation; resources; writing—review and editing. **Morkos A. Henen:** Data curation; supervision; resources; methodology; investigation; formal analysis; software; project administration; writing—review and editing. **Elan Z. Eisenmesser:** Supervision; data curation; resources; formal analysis; methodology; investigation; writing—review and editing; project administration. **James C. Zimring:** Supervision; resources; project administration. **Angelo D'Alessandro:** Conceptualization; investigation; funding acquisition; writing—original draft; visualization; validation; methodology; formal analysis; project administration; data curation; supervision; resources.

CLINICAL TRIAL

The NHLBI Recipient Epidemiology and Donor Evaluation Study (REDS)-III Red Blood Cell Omics (RBC Omics) and vein-to-vein databases are accessible at https://biolincc.nhlbi.nih.gov/studies/reds_iii/, and Genomics data are deposited at dbGaP Study Accession: phs001955.v1.p1.

CONFLICT OF INTEREST STATEMENT

The authors declare that A.D., K.C.H., and T.N. are founders of Omix Technologies Inc. A.D. and T.N. are Scientific Advisory Board (SAB) members for Hemanext Inc. A.D. is a SAB member for Macopharma Inc and SynthMed Biotechnologies. All the other authors have no conflicts to disclose in relation to this study.

DATA AVAILABILITY STATEMENT

All raw data and elaborations are included in Supporting Information S2: Table 1. Conditional EpoR-Cre PIMT$^{f/f}$ mice are available upon reasonable request, finalization of the material transfer agreement, and after institutional ACUC approval through Dr James Zimring (james.zimring@blood.ca). Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact (angelo.dalessandro@cuanschutz.edu).

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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