BLOOD DONATION AND DONOR RECRUITMENT

Original article

Next generation sequencing to identify iron status and individualise blood donors' experience

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Background - Young adults form the majority of first-time blood donors to Australian Red Cross Lifeblood. However, these donors pose unique challenges for donor safety. Young blood donors, who are still undergoing neurological and physical development, have been found to have lower iron stores, and have higher risks of iron deficiency anaemia when compared to older adults and non-donors. Identifying young donors with higher iron stores may improve donor health and experience, increase donor retention, and reduce the burden on product donation. In addition, these measures could be used to individualise donation frequency.

Materials and methods - Stored DNA samples from young male donors (18-25 years; No.=47) were sequenced using a custom panel of genes identified in the literature to be associated with iron homeostasis. The custom sequencing panel used in this study identified and reported variants to human genome version 19 (Hg19).

Results - 82 gene variants were analysed. Only one of which, rs8177181, was found to have a statistically significant ($p<0.05$) association with plasma ferritin level. Heterozygous alleles of this *Transferrin* gene variant, rs8177181T>A, significantly predicted a positive effect on ferritin levels ($p=0.03$).

Discussion - This study identified gene variants involved in iron homeostasis using a custom sequencing panel and analysed their association with ferritin levels in a young male blood donor population. Additional studies of factors associated with iron deficiency in blood donors are required if a goal of personalised blood donation protocols is to be achieved.

Keywords: *iron deficiency, anaemia, blood donors, genome.*

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INTRODUCTION

Blood product transfusion is commonly used during surgery, trauma, and treatment for cancer and hematological disorders[1]. At least 1 in 3 Australians will require blood or blood products at some point in their lifetime[1]. Annually, Australian Red Cross Lifeblood (Lifeblood) collects approximately 1.7 million blood donations[2]. Young adults represent the largest group of first-time donors at Lifeblood. Donors aged <25 years made up 44.2% and 44.8% of first-time whole blood donors in 2007 and 2013, respectively[3]. Recruiting

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and involving young donors is thought to have the most long-term benefit with early priming of donors possibly leading to greater long-term donor retention[3]. However, concerns for the health of young adult donors have recently emerged due to findings of lower iron stores and higher risks of iron deficiency anaemia when compared to older donors and non-donors[4,5].

Blood donation can result in significant iron loss of approximately 200 to 250 mg per whole blood donation, risking iron deficiency (ID), or anaemia, as adequate iron levels in the body are essential for healthy red blood cell production[6,7]. Furthermore, donation frequency has been associated with ID[8]. Clinical presentations of ID and/or anaemia include fatigue, restless leg syndrome, pica and reduced work productivity[9]. Adolescents and young adults, who are still undergoing neurological and physical development, can experience additional unique and significant complications including a range of cognitive and behavioural deficits[10,11]. Although adolescents and young adults are considered a vulnerable group for ID post donation, they are also a crucial subgroup for maintaining transfusion supplies in Australia. Furthermore, repeat donors also contribute to the stability of the blood supply in Australia and a positive experience is central to retaining donors and encouraging first-time donors to return[12,13]. Negative experiences post-donation, including ID induced deferrals, could lead to permanent loss of donors early in their donation journey. This risk is particularly increased among younger donors who are less likely to return for various other reasons[3,12]. Concerns around young donor wellbeing have already led to an increase in the minimum age for donation in Australia (from 16 to 18 in 2018)[14]. Additionally, Lifeblood continues to conduct research addressing young donors' safety and behaviour[15].

To mitigate the risk of ID and anaemia among Australian donors, a standardised iron management strategy includes minimum hemoglobin (Hb) levels (males: >130 g/L; females: >120 g/L) and set donation frequencies (12 weeks for whole blood and 2 weeks for plasmapheresis and plateletpheresis)[16]. Additionally, a short course of iron supplementation post-donation for female blood donors aged 18-45 years is recommended[16]. The latter two strategies allow for red blood cell recovery post-donation. Donors with low venous Hb levels (males: <125 g/L; females: <115 g/L) are temporarily deferred from donation, and referred to their general practitioner for investigation and management[16]. Similar selection criteria are also used in several other countries such as USA and Canada[17]. However, Hb is not an accurate representation of total iron stores as anaemia only occurs in the late stages of the iron depletion process[8]. In addition, donor Hb recovery time post-donation may vary[18]. Alternative techniques have been used in an attempt to screen and monitor blood donors' iron levels. However, alternate methods, such as reticulocyte Hb[19] and zinc protoporphyrin levels[20], increase costs for donation services, are not available as point of care screening, and are often required in conjunction with Hb to predict ID, anaemia and risk of donor deferral[6,8,20].

Ferritin, an intracellular protein providing storage of iron for Hb production, has also been used as a marker of iron stores[8,21]. Low ferritin levels, particularly serum levels below 30 ng/mL, are suggestive of ID, irrespective of co-existing conditions[22]. However, the sensitivity of ferritin testing is reduced in the presence of inflammation which can falsely elevate results[8,21]. Blood donors represent a unique population, required to be feeling well on the day of donation, and have been found to have low levels of inflammation at the time of donation[8]. Hence, ferritin is likely to appropriately represent iron stores in blood donors and has a lower risk of falsely elevated results secondary to other inflammatory processes. Yet, ferritin results remain a marker of iron stores at a specific point in time, require repeated screening to determine ongoing suitability for blood donation and are not completed routinely for Lifeblood donations. Hence, targeted genomic testing to establish risk profiles for those most susceptible to ID has been considered[23]. This could provide individualised donation schedules and post-donation management strategies for donors following a single genomic screening test[23].

Various studies have identified associations between numerous genetic variants and iron metabolism[6,24-30]. Iron levels are controlled by the liver which secretes a peptide, hepcidin, that regulates the absorption of iron from the gut and recycling of iron from the breakdown of senescent red blood cells[31-34]. Iron overload results from insufficient hepcidin production and conversely excess hepcidin production is associated with ID[21]. The expression of hepcidin is itself dependent on a number of regulatory proteins[31,34]. Mutations in genes encoding these

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regulatory proteins and hepcidin are associated with iron disorders, leading to either too much iron (e.g., hereditary hemochromatosis) or insufficient iron (e.g., ID, anaemia)[32]. Single nucleotide variants (SNVs) associated with iron status and red cell parameters have been identified in a number of genes required for iron homeostasis[24]. Most commonly, variants in transmembrane protease, serine 6 (*TMPRSS6*), Transferrin (*TF*) and homeostatic iron regulator (*HFE*) genes have been associated with changes to iron, ferritin, transferrin, total iron binding capacity and Hb levels[24-26,30,35]. However, other reports have identified a broader range of genes. Ji *et al.*, identified a SNV in the BTB Domain containing 9 (*BTBD9*) gene to be associated with higher ferritin levels in male blood donors[24] and Torti *et al.*, reported a polymorphism in the hypoxia inducible factor 1 (*HIF1α*) gene to be associated with higher ferritin levels in males[36].

This study aimed to explore genetic variants affecting iron homeostasis among younger male blood donors. Identifying markers associated with ID or anaemia post-donation, or robust ferritin levels despite frequent donations could be incorporated into blood donor screening and personalised donor experiences in the future.

MATERIALS AND METHODS

Ethics and sample collection

Ethics approval was obtained from the Lifeblood Human Research Ethics Committee (Perros27052021).

Younger adults are likely to be early in their donation careers, with less cumulative iron loss from donation. Yet, young female donors are at risk of greater fluctuations in their iron stores due to menstrual blood loss and Lifeblood's recommendation for iron supplementation post-donation[8,16]. Hence, archived samples from young male donors exclusively were selected to assess the role genetic variants may play in iron metabolism for blood donors. This study used 48 archived genomic DNA (gDNA) samples from repeat blood donors who were recruited using convenience sampling in a previous study by Ji *et al.*[24], and stored by Lifeblood. Hb and plasma ferritin levels were available from data records. These results were obtained from patient samples which were tested within a week of their collection using the commercially available human ferritin ELISA kit (Sigma-Aldrich, Darmstadt, Germany)[24]. All samples were deidentified.

Donor demographic data and donation history were also available, including number and type of previous donations, duration since last donation, Hb, and ferritin levels. Donation-induced iron losses in the preceding 12 months were also determined by multiplying the number of donations by the approximate iron loss for the type of donation provided (220mg per whole blood and 18mg per plasmapheresis donation)[16]. gDNA was previously extracted using the EZ1 DNA Blood Kit (Qiagen, Hilden, Germany) on the EZ1 advance automated instrument (Qiagen) available at Lifeblood[24].

Library preparation and next generation sequencing

A custom next generation sequencing panel was developed consisting of genes associated with clinical cases of genetic iron overload, anaemia, or which have been functionally associated with the regulation of iron homeostasis in either animal models or *in vitro* experiments[24,29]. In addition to whole transcripts of these genes, the promoter regions of 11 genes described in McDonald *et al.*,[29] were also included in the custom sequencing panel. DNA libraries were prepared from gDNA using the Illumina DNA Prep with Enrichment kit according to manufacturer instructions (Illumina, San Diego, USA)[37]. Purified sample libraries were pooled at 12-plex and sequenced in two separate runs consisting of 24 samples in each. Libraries were sequenced on the MiSeq (Illumina) using the standard MiSeq 300 cycle V2 reagent kit (2x150bp). The MiSeq reporter software suite[37] was used to perform on-board quality filtering, read mapping of FASTQ reads to the human reference genome (GRCh37/hg19) using BWA-MEM[38] and variant calling using GATK[39].

To facilitate analysis, variant call format files (VCF) were merged into a single multiVCF dataset using BCFtools[40] and the merged dataset was annotated for functional changes and dbSNP catalog numbers using wANNOVAR[41].

Statistical analysis

Prior to analysis, results were excluded if sequencing quality did not meet threshold or genotype variation was not consistent with Hardy-Weinberg equilibrium (HWE)[42]. Gene variants were then analysed for their association with donors' ferritin status. Where possible, following log transformation due to right-skewed distribution of results, plasma ferritin results were analysed as a continuous dependent variable. A multiple linear regression model was conducted for relationship of

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ferritin results with categorical gene variant (wild-type, heterozygous, homozygous), number of days since last donation, and iron loss (mg) in the previous 12 months as a result of blood donation as independent variables using the statistical software SPSS (IBM SPSS Statistics 23; IMB Australia Ltd., St. Leonards, NSW, Australia).

For gene variants with binomial results (wild-type and heterozygous results only), linear regression was not possible because of variant results (homozygous) with observations of zero. Therefore, a Fisher's exact test was calculated for these polymorphism's 3x3 contingency table using the statistical software R[43]. For this analysis, untransformed ferritin results were categorised as follows: low (ferritin ≤15 µg/L), medium (ferritin >15 and <95 µg/L), and high (ferritin ≥95 µg/L). Days since last donation and donation related iron loss (mg) in the previous 12 months were not able to be included as independent variables in this analysis.

To minimise the risk of Type I error because of multiple testing (82 tests), we used a False Discovery Rate (FDR) correction using the Benjamini-Hochberg procedure and report the adjusted p-values[44]. This method is designed to control the expected proportion of false discoveries. In doing so, it retains more power compared to, for example, Bonferroni correction, which controls the probability of at least one Type I error.

RESULTS

Cohort demographics

On review of results, 1 donor sample was excluded due to a therapeutic donation history and the identification of homozygous C282Y mutation of the *HFE* gene which is associated with hereditary hemochromatosis, and therefore not representative of the broader donor population. Of the remaining 47 samples, plasma ferritin levels ranged from 8.9 to 157.3 µg/L (median: 28.3 µg/L). Categorical ferritin results included 10 low samples, 32 medium samples and 5 high samples. The median ferritin results for the low, medium, and high categories were 11.7 µg/L, 30.7 µg/L and 103.8 µg/L, respectively (**Table I**). The average age of the participants was 22 years old (**Table I**). The median Hb level was 148 g/L, and was similar between ferritin groups. Duration since previous donation spanned from 13 days to 18 months with a median of 84 days. This median aligns with Lifeblood's whole blood donation frequency policy of every 12 weeks, whilst shorter durations represented those who completed apheresis donations. Iron loss in the previous 12 months as a result of blood donation showed considerable variation ranging from no losses due to a lack of donations in this time to 880mg (estimated loss as a result of 4 whole blood donations) the maximum permitted by Lifeblood's donation frequency policy.

Variants identified with involvement in iron homeostasis

285 genotype variants were identified across all targets and samples. VCF are filtered for various quality metrics (depth, variant frequency, strand bias) and are only defined as suitable if all metrics meet required thresholds. Results were excluded if sequencing was of poor quality (No.=28), minor allele frequencies (MAF) at the extremes (<0.10 and >0.90) (No.=170) or results were not consistent with HWE (No.=5). As a result, 82 variants involved in iron homeostasis were identified (**Table II**), with a mean MAF of 0.37 (range 0.1-0.9, SD 0.20).

Following FDR correction, rs8177181, an intron variant in the *TF* gene, was found to statistically significantly predict plasma ferritin result (p=0.03, R^2=0.357)

Table I - *Participant demographics, including by ferritin category*

	All (No.=47)	Low (No.=10)	Medium (No.=32)	High ferritin (No.=5)
	Median (IQR)			
Plasma ferritin (µg/L)	28.3 (16.7-45.7)	11.7 (10.5-12.2)	30.7 (23.3-43.9)	103.8 (98.5-146.2)
Hemoglobin (g/L)	148 (142-153)	141 (137-148)	148 (144-154)	144 (137-157)
Age (years)	22 (21-24)	23 (22-24)	22 (20-24)	24 (23-25)
Donations in prior 12 months[a] (No.)	3 (2-6)	3 (2-4)	4 (2-8)	2 (1-2)
Iron loss in prior 12 months[a] (mg)	418 (220-458)	467 (289-614)	281 (180-440)	346 (220-440)
Time since previous donation[a] (days)	84 (19-111)	94 (20-109)	80 (19-110)	109 (28-196)

[a] Inclusive of whole blood or plasma donations, IQR: interquartile range

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Table II - *Custom sequencing panel and variant results*

Function in iron homeostasis	Gene	dbSNP reference	FDR adjusted p value
Master regulator	*HAMP*	No gene variants identified	
Hepcidin regulation	*GDF15 (MIC1)*	rs1058587	0.166
		rs1059022	0.167
		rs1059369*	0.690
		rs1059519	0.204
		rs1804826*	0.690
		rs6413435	0.164
	TWSG1	No gene variants identified	
	BMP2	rs1049007	0.203
		rs235768	0.196
BMP/SMAD hepcidin regulation	*HFE2 (HJV)*	No gene variants identified	
	TMPRSS6 (MT2)	rs11704654	0.197
		rs200434923	0.168
		rs2111833	0.263
		rs2235321	0.185
		rs2235324	0.270
		rs5756515	0.193
		rs4820268	0.160
		rs881144*	0.690
	BMP6	rs17557	0.177
	ACVR1 (ALK2)	rs2227861	0.163
	ACVR2A	rs1128919	0.166
	BMPR1A (ALK3)	rs11528010	0.165
	SMAD1	rs13144151	0.168
	SMAD4	No gene variants identified	
	SMAD5	No gene variants identified	
	SMAD6	rs34643453	0.164
		rs4776822	0.198
		rs62005619	0.192
	SMAD7	rs3764482	0.239
	SMAD9	No gene variants identified	
Iron sensing	*ACO1 (IRP1)*	rs3780473	0.163
	IREB2 (IRP2)	rs13180	0.166
		rs954144	0.165
Iron transport	*FTL*	rs2230267	0.250
	TF	rs4481157	0.157
		rs8177179	0.186
		rs8177181	0.030
		rs8177184	0.060
		rs1049296	0.167
		rs1130459	0.176
		rs12769	0.165
		rs8649	0.168
	SLC40A1 (FPN))	rs11568351	0.244
		rs13008848	0.225
		rs2304704	0.175
	SLC11A2 (DMT-1)	rs1048230	0.182
		rs224575	0.166
	TFRC (TFR1)	rs1805051	0.202
		rs3817672	0.165
Metal ion transporter	*SLC11A1 (NRAMP)*	rs2276631	0.202

*binomial gene variant results, Fisher's exact used to calculate p value. FDR: false discovery rate.

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Table II - *Custom sequencing panel and variant results (continued from previous page)*

Zinc/iron transporter	*SLC39A14 (ZIP14)*	rs2293144	0.238
		rs3052256*	0.690
		rs33999442*	0.690
		rs896378	0.178
Transcription factor	*NFE2L2*	No gene variants identified	
Ferroxidase	*CP*	No gene variants identified	
	APP	No gene variants identified	
	HEPHL1	rs2511403	0.167
Ferric reductase	*CYBRD1 (DCYTB)*	rs10455	0.196
		rs2356782	0.167
		rs3731976	0.186
Trafficking	*NDFIP1*	rs3761758*	0.758
		rs3761759	0.208
	NDFIP2	rs11549502	0.165
	ZFYVE16	rs2544600	0.165
Hypoxia/oxidative stress	*HIF1α*	rs11549465*	0.690
	EPAS1 (HIF2α)	No gene variants identified	
	HMOX1	No gene variants identified	
Mutated in hereditary Hemochromatosis/ Iron sensing	*HFE*	rs1800562	0.169
	TFR2	rs2075674	0.198
Chaperone/HFE interaction	*B2M*	No gene variants identified	
Hematopoiesis	*BTBD9*	No gene variants identified	
Erythropoiesis	*EPO*	No gene variants identified	
Encodes small protein which is antigen to Vel blood group	*SMIM1*	No gene variants identified	
Transcription regulator protein	*BACH1*	rs388707	0.162
Promotor regions	Promoter region of HFE	rs1061482	0.216
		rs1800702	0.203
		rs2794720	0.192
	Promoter region of HAMP	rs10414846	0.170
		rs10421768	0.178
		rs1882694	0.166
		rs1882695	0.174
		rs66868858	0.188
		rs7256866	0.183
	Promoter region of HFE2 (HJV)	rs10910811	0.167
	Promoter region of SLC40A1	rs12693542	0.225
		rs13015236	0.225
		rs3811621	0.202
	Promoter region of FTL	No gene variants identified	
	Promoter region of FTH1	No gene variants identified	
	Promoter region of BMP6	rs1107495	0.162
	Promoter region of TMPRSS6	rs228909	0.189
		rs5756516	0.186
	Promoter region of TFRC	rs11915082	0.161
		rs3827554	0.171
		rs3827555	0.166
		rs41300395	0.168
		rs6583288	0.165
	Promoter region of TFRC2	No gene variants identified	
	Promoter region of TF	No gene variants identified	

*binomial gene variant results, Fisher's exact used to calculate p value. FDR: false discovery rate.

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(**Table III**). Heterozygous genotype, rs8177181T>A, significantly predicted a positive effect on ferritin levels (p=0.001) in our study cohort.

Table III - *Detailed log-linear regression values for rs8177181T>A*

	Estimate	95% CI	SE	Coefficient p value
Constant	1.489	1.289 to 1.689	0.099	
Days since previous donation	0.0004	−0.0003 to 0.001	0.0004	0.255
Iron loss (mg) in previous 12 months	−0.0005	−0.0009 to -0.00007	0.0002	0.023
Gene variant - heterozygous	0.290	0.121 to 0.459	0.084	0.001
Gene variant - homozygous	0.487	−0.061 to 1.035	0.271	0.080

DISCUSSION

ID and associated anaemia remain complex issues affecting the Australian blood donor population. Optimization of blood donation frequency through identification of factors affecting iron homeostasis may improve blood donor safety and retention. Genetic sequencing may be a novel screening strategy for categorising blood donors based on their ability to replenish iron stores. A panel of genetic variants developed like in this study could be used to improve donor iron health, increase donor retention, and reduce the burden on blood product supply in Australia. This study has provided further data regarding variants in genes involved in iron metabolism in a young male blood donor population. Furthermore, the development of our custom iron gene panel can be further explored now that its feasibility has been established.

The intron substitution T>A at the triallelic site within *TF* gene rs8177181 was identified to be statistically associated to ferritin levels for males with a heterozygous genotype. McClain *et al.*, when investigating adipose tissue transferrin and insulin resistance, reported rs8177181T>A variant to be independently associated with lower expressions of *TF* transcript in adipose tissue[45]. However, an association between this genotype and plasma transferrin or ferritin levels was not observed in their cohort. Therefore, McClain *et al.*, concluded this *TF* polymorphism was specific only to adipocyte *TF* expression[45]. Nevertheless, the association of the rs8177181A variant with low expressions of *TF* transcript may warrant further investigation given transferrin is negatively regulated by iron. Further investigation of other variants of the *TF* gene may also be warranted to evaluate if *TF* expression in blood donors is associated with higher systemic iron.

A recent genome-wide association study of blood donors also found a number of genes not known to be directly involved with iron homeostasis were associated with ferritin levels. These included WD Repeat, Sterile Alpha Motif And U-Box Domain Containing 1 (*WDSUB1*), Keratinocyte Differentiation Factor 1 (*KDF1*), Contactin Associated Protein 2 (*CNTNAP2*), Bromodomain Adjacent To Zinc Finger Domain 2B (*BAZ2B*), SET Domain Containing 7 (*SETD7*), Teneurin Transmembrane Protein 3 (*TENM3*), Dishevelled Binding Antagonist Of Beta Catenin 2 (*DACT2*), CAMP Responsive Element Binding Protein 5 (*CREB5*), LHFPL Tetraspan Subfamily Member 3 (*LHFPL3*), Myosin XVI (*MYO16*), ADP Ribosylation Factor 6 (*ARF6*), Gliomedin (*GLDN*), Mitochondrial Ribosomal Protein S23 (*MRPS23*), Zinc Finger And BTB Domain Containing 7C (*ZBTB7C*)[46]. These findings suggest further genetic mechanisms may be responsible for iron homeostasis and a broader range of genes should be included in further panels assessing factors influencing iron status. This is supported by Moksnes *et al.*, who utilised a genome-wide association meta-analyses and identified variants in genes associated with Parkinson's disease (DnaJ Heat Shock Protein Family (Hsp40) Member C13, *DNAJC13*), Hb concentration (SprT-Like N-Terminal Domain, *SPRTN*, Fc Gamma Receptor IIa, *FCGR2A*, Carbamoyl-Phosphate Synthase 1, *CPS1*, Patatin Like Phospholipase Domain Containing 3, PNPLA3, and ATP Binding Cassette Subfamily A Member 6, *ABCA6*), and fibrinolysis and bleeding (Serpin Family F Member 2, *SERPINF2*) to be related to iron status biomarkers[47]. However, this meta-analysis addressed disease and mortality, therefore applicability of these gene variants in blood donors may be limited. Further genome studies of blood donor's iron status would be benefited by including a wide array of genes in order to better understand genetic mechanisms of iron regulation.

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Our study was limited by a small cohort, underpowering the statistical analysis of the results. However, of the variants analysed, greater than 80% had a minor allele frequency within 10% of the population frequency seen on the Genome Aggregation Database[48]. This suggests the variants included were a reasonable representation of variants within the general population. Yet, only a limited number of variants were able to be analysed as a result of reduced numbers of heterozygous or homozygous variants within the cohort, as evidenced by the need to exclude a large proportion of results due to MAFs less than 10% or greater than 90%. Additionally, the coefficient of determination (R^2) remained low, suggesting that the gene variants in this linear regression model explain only a modest amount of variability in our ferritin levels. Further research addressing genomic factors influencing iron homeostasis should include larger participant numbers, from a wide variety of ethnic backgrounds, to improve the ability of identifying SNVs associated with plasma ferritin levels. Furthermore, expansion of participant recruitment to include females and older individuals may also be warranted to ensure increased sample size, address effects of confounders, and address applicability to the wider donor population. Additionally, information regarding dietary iron, iron supplementation, and blood loss should be collected to ensure their effect can be included in analyses. Iron supplementation use may be of increased importance given mutations in the *TMPRSS6* gene have been associated with iron deficiency anaemia refractory to oral iron supplementation, a strategy which is currently implemented for young female donors[49]. Hence, cohorts with mutations in this gene may benefit significantly from further research and personalised donation plans. Cohort expansion may be achieved through the development of a blood donor databank, while confounding variables could also be observed over time through longitudinal donor follow-up. The sequencing panel developed in this study could also be expanded as reported genes, as listed above, are linked to iron homeostasis.

CONCLUSIONS

This study identified statistically significant associations of one gene variant, rs8177181, from a panel of genes known to be involved with iron homeostasis and ferritin in young male blood donors. However, the use of a wider panel of genes and increased participant numbers should be considered as more evidence relating to iron homeostasis emerges and larger databanks are amassed. A custom iron gene panel for blood donors could provide further benefit and understanding about donors' personal iron replenishment potential, and reduce risks of deferrals. We have demonstrated that whilst iron homeostasis remains complex with multiple influencing mechanisms, as further associated genes are identified, gene variant identification remains a possibility for customising blood donors' experiences including donation frequency and follow up protocols.

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AUTHORSHIP CONTRIBUTIONS

RF, AP, AS, and ER assisted with project development. ER developed panel. ER and MO completed laboratory work. AS, ER, EV, GJ, MO, MW and JP assisted with data analysis and interpretation. GJ prepared the manuscript. All Authors reviewed and contributed to manuscript.

The Authors declare no conflicts of interest.

REFERENCES

1. Australian Government: Department of Health and Aged Care; c2020 About blood and blood products. Available at: https://www.health.gov.au/health-topics/blood-and-blood-products/about-blood-and-blood-products. Accessed on 18/08/2022.
2. Australian Red Cross Lifeblood; c2021-2022. Why donate blood? Available at: https://www.lifeblood.com.au/blood/learn-about-blood/why-donate-blood. Accessed on 01/08/2022.
3. Priyono A, Masser BM, Dyda A, Davison TE, Irving DO, Karki S. Long-term return and donation pattern of those who begin donating at different ages: A retrospective cohort analysis of blood donors in Australia. Transfusion 2021; 61: 799-810. doi: 10.1111/trf.16187.
4. Patel EU, White JL, Bloch EM, Grabowski MK, Gehrie EA, Lokhandwala PM, et al. Association of blood donation with iron deficiency among adolescent and adult females in the United States: a nationally representative study. Transfusion 2019; 59: 1723-1733. doi: 10.1111/trf.15179.
5. Spencer BR, Bialkowski W, Creel DV, Cable RG, Kiss JE, Stone M, et al. Elevated risk for iron depletion in high-school age blood donors. Transfusion 2019; 59: 1706-1716. doi: 10.1111/trf.15133.
6. Mast AE, Langer JC, Guo Y, Bialkowski W, Spencer BR, Lee TH, et al. Genetic and behavioral modification of hemoglobin and iron status among first-time and high-intensity blood donors. Transfusion 2020; 60: 747-758. doi: 10.1111/trf.15743.

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7. Sørensen E, Rigas AS, Didriksen M, Burgdorf KS, Thorner LW, Pedersen OB, et al. Genetic factors influencing hemoglobin levels in 15,567 blood donors: results from the Danish Blood Donor Study. Transfusion 2019; 59: 226-231. doi: 10.1111/trf.15075.
8. Kiss JE. Laboratory and genetic assessment of iron deficiency in blood donors. Clin Lab Med 2015; 35: 73-91. doi: 10.1016/j.cll.2014.10.011.
9. Fillet AM, Martinaud C, Malard L, Le Cam S, Hejl C, Chenus F, et al. Iron deficiency among French whole-blood donors: first assessment and identification of predictive factors. Vox Sang 2021; 116: 42-52. doi: 10.1111/vox.12991.
10. McCann JC, Ames BN. An overview of evidence for a causal relation between iron deficiency during development and deficits in cognitive or behavioral function. Am J Clin Nutr 2007; 85: 931-945. doi: 10.1093/ajcn/85.4.931.
11. Khedr E, Hamed SA, Elbeih E, El-Shereef H, Ahmad Y, Ahmed S. Iron states and cognitive abilities in young adults: neuropsychological and neurophysiological assessment. Eur Arch Psychiatry Clin Neurosci 2008; 258: 489-496. doi: 10.1007/s00406-008-0822-y.
12. Germain M, Glynn SA, Schreiber GB, Gelinas S, King M, Jones M, et al. Determinants of return behavior: a comparison of current and lapsed donors. Transfusion 2007; 47: 1862-1870. doi: 10.1111/j.1537-2995.2007.01409.x.
13. Nguyen DD, Devita DA, Hirschler NV, Murphy EL. Blood donor satisfaction and intention of future donation. Transfusion 2008; 48: 742-748. doi: 10.1111/j.1537-2995.2007.01600.x.
14. Australian Red Cross Lifeblood; c2018. Minimium age for blood donation increases to 18. Available at: https://transfusion.com.au/bsib_february2018_1. Accessed on 06/09/2022.
15. Australian Red Cross Lifeblood; c2021-2022. Donor behaviour. Available at: https://www.lifeblood.com.au/our-research/program/donor-behaviour. Accessed on 06/09/2022.
16. Australian Red Cross Lifeblood; c2021-2022. Anaemia and iron deficiency in blood donors. Available at: https://transfusion.com.au/anaemia_management/iron_deficiency_anaemia/anaemia_and_iron_deficiency_blood_donors. Accessed on 08/04/2022.
17. Goldman M, Magnussen K, Gorlin J, Lozano M, Speedy J, Keller A, et al. International Forum regarding practices related to donor haemoglobin and iron. Vox Sang 2016; 111: 449-455. doi: 10.1111/vox.12431.
18. Nasserinejad K, van Rosmalen J, van den Hurk K, Baart M, Hoekstra T, Rizopoulos D, et al. Prevalence and determinants of declining versus stable hemoglobin levels in whole blood donors. Transfusion 2015; 55: 1955-1963. doi: 10.1111/trf.13066.
19. Semmelrock MJ, Raggam RB, Amrein K, Avian A, Schallmoser K, Lanzer G, et al. Reticulocyte hemoglobin content allows early and reliable detection of functional iron deficiency in blood donors. Clin Chim Acta 2012; 413: 678-682. doi: 10.1016/j.cca.2011.12.006.
20. Baart AM, de Kort WL, Moons KG, Atsma F, Vergouwe Y. Zinc protoporphyrin levels have added value in the prediction of low hemoglobin deferral in whole blood donors. Transfusion 2013; 53: 1661-1669. doi: 10.1111/j.1537-2995.2012.03957.x.
21. Camaschella C, Nai A, Silvestri L. Iron metabolism and iron disorders revisited in the hepcidin era. Haematologica 2020; 105: 260-272. doi: 10.3324/haematol.2019.232124.
22. Gastroenterological Society of Australia. *Iron deficiency: clinical update*. Report. 2nd ed. Victoria, Australia: Geda; 2015.
23. Sorensen E, Grau K, Berg T, Simonsen AC, Magnussen K, Erikstrup C, et al. A genetic risk factor for low serum ferritin levels in Danish blood donors. Transfusion 2012; 52: 2585-2589. doi: 10.1111/j.1537-2995.2012.03629.x.
24. Ji Y, Flower R, Hyland C, Saiepour N, Faddy H. Genetic factors associated with iron storage in Australian blood donors. Blood Transfus 2018; 16: 123-129. doi: 10.2450/2016.0138-16.
25. An P, Wu Q, Wang H, Guan Y, Mu M, Liao Y, et al. TMPRSS6, but not TF, TFR2 or BMP2 variants are associated with increased risk of iron-deficiency anemia. Human Molecular Genetics 2012; 21: 2124-2131. doi: 10.1093/hmg/dds028.
26. Benyamin B, McRae AF, Zhu G, Gordon S, Henders AK, Palotie A, et al. Variants in TF and HFE explain approximately 40% of genetic variation in serum-transferrin levels. Am J Hum Genet 2009; 84: 60-65. doi: 10.1016/j.ajhg.2008.11.011.
27. Chambers JC, Zhang W, Li Y, Sehmi J, Wass MN, Zabaneh D, et al. Genome-wide association study identifies variants in TMPRSS6 associated with hemoglobin levels. Nat Genet 2009; 41: 1170-1172. doi: 10.1038/ng.462.
28. Ganesh SK, Zakai NA, van Rooij FJ, Soranzo N, Smith AV, Nalls MA, et al. Multiple loci influence erythrocyte phenotypes in the CHARGE Consortium. Nat Genet 2009; 41: 1191-1198. doi: 10.1038/ng.466.
29. McDonald CJ, Ostini L, Wallace DF, Lyons A, Crawford DH, Subramaniam VN. Next-generation sequencing: application of a novel platform to analyze atypical iron disorders. J Hepatol 2015; 63: 1288-1293. doi: 10.1016/j.jhep.2015.06.027.
30. Kloss-Brandstätter A, Erhart G, Lamina C, Meister B, Haun M, Coassin S, et al. Candidate gene sequencing of SLC11A2 and TMPRSS6 in a family with severe anaemia: common SNPs, rare haplotypes, no causative mutation. PLoS One 2012; 7: e35015. doi: 10.1371/journal.pone.0035015.
31. Muckenthaler MU, Rivella S, Hentze MW, Galy B. A Red carpet for iron metabolism. Cell 2017; 168: 344-361. doi: 10.1016/j.cell.2016.12.034.
32. Pagani A, Nai A, Silvestri L, Camaschella C. Hepcidin and anemia: a tight relationship. Front Physioll 2019; 10: 1294. doi: 10.3389/fphys.2019.01294.
33. Rishi G, Subramaniam VN. The relationship between systemic iron homeostasis and erythropoiesis. Bioscience Reports 2017; 37: BSR20170195. doi: 10.1042/bsr20170195.
34. Rishi G, Wallace DF, Subramaniam VN. Hepcidin: regulation of the master iron regulator. Biosci Rep 2015; 35: e00192. doi: 10.1042/BSR20150014.
35. Benyamin B, Ferreira MA, Willemsen G, Gordon S, Middelberg RP, McEvoy BP, et al. Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. Nat Genet 2009; 41: 1173-1175. doi: 10.1038/ng.456.
36. Torti L, Teofili L, Capodimonti S, Nuzzolo ER, Iachininoto MG, Massini G, et al. Hypoxia-inducible factor-1alpha(Pro-582-Ser) polymorphism prevents iron deprivation in healthy blood donors. Blood Transfus 2013; 11: 553-557. doi: 10.2450/2013.0076-13.
37. Illumina; c2022. MiSeq Reporter built-in data analysis software. Available at: https://sapac.illumina.com/systems/sequencing-platforms/miseq/products-services/miseq-reporter.html. Accessed on 25/08/2022.
38. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009; 25: 1754-1760. doi: 10.1093/bioinformatics/btp324.
39. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010; 20: 1297-1303. doi: 10.1101/gr.107524.110.
40. Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, et al. Twelve years of SAMtools and BCFtools. Gigascience 2021; 10: giab008. doi: 10.1093/gigascience/giab008.
41. Chang X, Wang K. wANNOVAR: annotating genetic variants for personal genomes via the web. J Med Genet 2012; 49: 433-436. doi: 10.1136/jmedgenet-2012-100918.
42. Court Lab Calculator. Court MH. Available at: https://www.researchgate.net/profile/Khalid_Hassan10/post/Does_anyone_know_how_to_calculate_Hardy_Weinberg_Equilibrium_for_14_different_alleles/attachment/59d64ad979197b80779a50fd/AS:477303651213312@1490809466020/download/Court+lab+-+HW+calculator-+important.xls. Accessed on 30/06/2022.
43. Team RC. R: A language and environment for statistical computing. Vienna, Austria; c2020. Available at: https://www.R-project.org/. Accessed on 07/06/2022.
44. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society 1995; 57: 289-300. doi: 10.1111/j.2517-6161.1995.tb02031.x.
45. McClain DA, Sharma NK, Jain S, Harrison A, Salaye LN, Comeau ME, et al. Adipose tissue transferrin and insulin resistance. J Clin Endocrinol Metab 2018; 103: 4197-4208. doi: 10.1210/jc.2018-00770.
46. Timmer T, Tanck M, Penkett C, Stirrups K, Gleadall N, de Kort W, et al. Genetic determinants of ferritin, haemoglobin levels and haemoglobin trajectories: results from Donor InSight. Vox Sang 2021; 116: 755-765. doi: 10.1111/vox.13066.
47. Moksnes MR, Graham SE, Wu KH, Hansen AF, Gagliano Taliun SA, Zhou W, et al. Genome-wide meta-analysis of iron status biomarkers and the effect of iron on all-cause mortality in HUNT. Commun Biol 2022; 5: 591. doi: 10.1038/s42003-022-03529-z.
48. gnomAD. [Internet]. Genome aggregation database. Available at: https://gnomad.broadinstitute.org/. Accessed on 04/08/2022.
49. McLaren CE, McLachlan S, Garner CP, Vulpe CD, Gordeuk VR, Eckfeldt JH, et al. Associations between single nucleotide polymorphisms in iron-related genes and iron status in multiethnic populations. PLoS One 2012; 7: e38339. doi: 10.1371/journal.pone.0038339.

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