

Genetic factors associated with iron storage in Australian blood donors

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Background. Blood donors are at risk of developing iron deficiency and/or iron deficiency anaemia. This may affect their health and affect their eligibility to give subsequent donations. Investigating genetic factors that may predispose donors to high or low iron stores is of interest; this may assist with providing optimal management strategies for maintaining donor health. This study aimed to investigate whether the presence of selected single nucleotide polymorphisms (SNPs) affecting parameters of iron status were associated with ferritin levels in Australian donors.

Materials and methods. Samples (n=800) were collected from non-first-time blood donors in Queensland. Plasma ferritin levels were quantified and the genotypes for ten SNPs, identified by a review of relevant literature, were determined for each sample. Associations between SNPs and ferritin levels were investigated.

Results. Three SNPs were associated with ferritin levels. In male donors, high ferritin levels were associated with the variant allele (G) of the SNP rs3923809 in the *BTBD9* gene. An association with ferritin levels was also identified with the SNP rs235756 in the *BMP2* gene in males. The SNP rs4820268 in the *TMPRSS6* gene was associated with ferritin levels in females, with donors with the AG genotype being three times more likely to have low ferritin levels.

Discussion. Variants in the genes *TMPRSS*, *BTBD9* and *BMP2* were associated with ferritin levels in Australian blood donors. These findings provide support that genetic testing may be useful for the generation of predictive algorithms that may allow for management strategies to be tailor-made for individual donors.

Keywords: ferritin, single nucleotide polymorphism, iron deficiency, health, safety.

Introduction

Blood donation leads to iron loss in blood donors. This increases the risk of developing iron deficiency (ID) or iron-deficiency anaemia (IDA) in regular donors, especially young females, and those with low initial ferritin levels^{1,2}. Clinically significant ID can be associated with a number of health issues, including fatigue, pica, restless leg syndrome, and impaired cognitive performance³. In addition, ID-induced donor deferral imposes a negative experience on enthusiastic donors, and may result in permanent donor loss and a long-term adverse impact on blood collections⁴.

The current donor selection procedure in Australia, as well as in other countries including the USA and Canada, uses capillary haemoglobin (Hb) level as the only criterion to assess whether the iron status of a donor is acceptable for blood donation⁵⁻⁷. However, Hb does not reflect body iron stores and discrepancies exist between Hb measuring devices⁸. Various approaches have been explored to predict the suitability of a donor for blood donation, including adding pathology tests or developing suitability models in donor screening^{9,10}.

However, these predictors/predictive models increase cost and lack large-scale validation^{11,12}.

Despite the fact that the same management strategies, such as minimum Hb requirements and pre-determined donation intervals (12 weeks for whole blood and 2 weeks for plasmapheresis in Australia)⁵, are applied, some blood donors become iron deficient more easily than others after donation¹³. In addition, the ability of individuals to recover normal iron levels varies, which indicates that there are contributing factors in addition to dietary iron intake or supplementation¹⁴. In recent decades, various studies have reported the associations between genetic variants and iron metabolism. Single nucleotide polymorphisms (SNPs) associated with parameters of iron status and red cell indices have been identified in genes involved in iron regulation in various population studies. For example, variants in the *TMPRSS6* gene are associated with decreased serum iron¹⁵, serum ferritin¹⁶ and Hb^{15,17}. SNPs in the *TF* gene are associated with increased serum transferrin levels^{17,18}, total iron binding capacity¹⁷ and serum ferritin levels¹⁸. The SNP rs7385804 in the

TFR2 gene is associated with iron status and red cell indices^{17,19}. In addition, SNPs have been discovered in other genes involved in iron metabolism or storage, including the genes *HFE*²⁰, *BMP2*²¹, *CYBRD1*²², *HIF1A*²³, and *EPO*¹⁹. Some SNPs that are located on genes not directly involved in iron regulation may also be associated with parameters of iron status. For example, the SNPs rs3923809, and rs9296249 in the *BTBD9* gene are associated with decreased serum ferritin, and restless leg syndrome, respectively^{24,25}. The SNP rs1175550 in the *SMIM1* gene influences the expression of the red blood cell antigen Vel, and is also associated with lower mean corpuscular Hb concentration in Europeans^{26,27}. These polymorphisms may directly affect iron metabolism through yet unknown mechanisms, or be associated with proteins that participate in iron regulation signalling pathways. Evidence as such raises the question of whether generic factors contribute to differences in responses to iron loss between individuals. This study aims to investigate whether the presence of genetic variants in parameters of iron status were associated with ferritin levels in Australian blood donors. With a focus on genetic factors associated with changes in iron metabolism and storage that would be of relevance in blood donors in Australia, we focused on ten SNPs in six different genes identified through a review of large population-wide published studies: rs9296249 and rs3923809 in the *BTBD9* gene^{24,25}; rs855791 and rs4820268 in the *TMPRSS6* gene^{15-17,28}; rs7385804 in the *TFR2* gene^{17,28}; rs3811647, rs1799852 and rs2280673 in the *TF* gene¹⁸; rs235756 in the *BMP2* gene²¹, and, rs1175550 in the *SMIM1* gene²⁷.

Materials and methods

Samples

Samples (n=800) were collected from non-first-time blood donors in Queensland, Australia. Samples were collected in tubes containing ethylenediamine tetraacetic acid (EDTA) anticoagulant (BD Vacutainer® Whole Blood Collection tube with spray-coated dipotassium EDTA 6 mL; Becton Dickinson, Franklin Lakes, NJ, USA) before centrifugation at 2,500 rpm for 5 minutes, as per routine procedures. These samples were collected for routine infectious disease screening as part of the normal blood donation procedure. They were recovered for use in this study after such testing was complete, and prior to their usual discard. All were investigated within 1 week of collection and were stored at 4 °C before use in this study. The following data were collected for each donor/sample: age, sex, Hb level, number and type of prior donations (ever and in the previous 12 months) and time since previous donation. Donation-induced iron loss (in mg, in the

preceding 12 months) for each donor was calculated using the following formula: number of whole blood donations × 220 mg (average iron loss in one whole blood donation) + number of plasma donations × 18 mg (average iron loss in one plasmapheresis donation), and was recorded as one of the parameters⁵. Samples were anonymised prior to any laboratory testing. Ethical approval was provided by the Blood Service Human Research Ethics Committee (2014#12) and the University of Queensland School of Medicine Ethics Committee (2014-SOMILRE-0107).

Ferritin testing

Plasma ferritin levels were quantified with the Human Ferritin ELISA kit (Sigma-Aldrich, Castle Hill, NSW, Australia), in accordance with the manufacturer's instructions. Colorimetric changes were read on a plate reader (Synergy H1 Multi-Mode Reader; BioTek, Winooski, VT, USA) at 450 nm immediately, and the ferritin result of each sample was determined automatically by the in-built Gen5™ software (BioTek).

DNA extraction

Genomic DNA was extracted from whole blood (separated plasma was resuspended with the cell pellet to recreate a whole blood sample) using the EZ1 DNA Blood kit (QIAGEN, Hilden, Germany) on an EZ1 Advance machine (QIAGEN), according to the manufacturer's directions. The concentration of DNA in each sample was quantified with a NanoDrop™ 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Single nucleotide polymorphism analysis

The genotypes of the candidate SNPs were determined using TaqMan SNP genotyping assays (Thermo Fisher Scientific, Mulgrave, Australia) according to the manufacturer's instructions (assay details are provided in the Online Supplementary Content, Table SI) using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Scoresby, VIC, Australia). Each reaction well had a total volume of 15 µL, which included DNA template (10 ng), TaqMan SNP reagent (0.375 µL) and genotyping Master Mix (7.5 µL). Standard cycling conditions were used: 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Due to weak expression of alleles for one of the SNPs (rs1175550), modified conditions were used and included 50 ng of DNA template followed by 50 cycles of 15 seconds at 95 °C and 1 minute at 60 °C for the polymerase chain reaction amplification. An automated genotype call from the StepOnePlus™ instrument (StepOne™ Software v2.2.2, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used.

Data analysis

All SNPs were tested for Hardy-Weinberg equilibrium (HWE) using the Court Lab Calculator²⁹. Only SNPs that did not differ from HWE ($p > 0.05$) were included in subsequent analyses. Samples were stratified into three groups based on plasma ferritin levels: high, medium and low. Males and females were analysed separately due to known differences in ferritin levels⁵: for male donors the three groups were ≤ 15 ng/mL (low), > 15 and ≤ 100 ng/mL (medium), and > 100 ng/mL (high), while for female donors they were ≤ 8 ng/mL (low), > 8 and ≤ 60 ng/mL (medium), and > 60 ng/mL (high). A multinomial regression analysis was performed using the Statistical Package for the Social Sciences (IBM SPSS Statistics 19; IMB Australia Ltd., St. Leonards, NSW, Australia) to analyse the association between ferritin levels and donor parameters (age, sex, Hb level, number and type of prior donations (ever and in the previous 12 months) and time since previous donation) as well as each individual SNP. Both high and low ferritin groups were compared with the medium group to identify SNPs that may be associated with high or low ferritin levels. The homozygous wild-type allele for each SNP was used as the reference genotype to compare the effect of having one or two variant alleles on donor ferritin levels. Donors with missing demographic or genotypic information were excluded from analyses. SNPs with an overall p -value less than 0.05 were adjusted for significant donor parameters.

Results

A total of 800 non-first-time donors were tested in this study. Of these, 54% were male (Online Supplementary Data, Table SII), and more than half of the donors (484 individuals, 61%) were greater than 45 years old. Almost half of all donors studied (377) had given a previous donation within the preceding 3 months, and the majority were men. A total of 31% of donors had an iron loss of less than 220 mg (equal to 1 whole blood donation) within the last 12 months, while 28% had an iron loss of between 440 and 880 mg (equal to 2 to 4 whole blood donations). Interestingly, ten donors, nine of whom were male, had iron loss equal to greater than four whole blood donations (880 mg) per year, but maintained normal Hb levels.

All 800 samples were successfully genotyped for the selected ten SNPs (Online Supplementary Content, Table SIII). Most SNPs were frequent in our donor cohort (mean allele frequency: 0.22–0.46), except for the SNP rs1799852 (mean allele frequency: 0.10). SNP frequencies obtained in Australian donors were similar to those that were reported in the National Centre for Biotechnology Information (NCBI) by previous studies. None of the SNPs differed from the HWE.

Not surprisingly, Hb levels were associated with ferritin levels in both male and female donors (overall $p = 0.024$, and $p < 0.001$, respectively) (Online Supplementary Content, Table SIV). For both sexes, increased amounts of iron loss had a negative effect on ferritin level ($p < 0.001$), while a longer donation interval was positively associated with iron stores (overall $p < 0.001$ for males and $p = 0.013$ for females). Age was significantly associated with ferritin levels in female donors only ($p = 0.018$).

Out of the ten SNPs analysed, rs3923809 ($p = 0.027$) and rs235756 ($p = 0.029$) were significantly associated with ferritin levels in male donors (Table I). Male donors with one G allele within the SNP rs3923809 were two times more likely to have high ferritin levels ($p = 0.011$), while having two G alleles increased this likelihood to three times ($p = 0.004$) (Table II). After adjusting for donation-induced iron loss and days since previous donation, the G allele of the SNP rs3923809 remained significantly associated with the high ferritin group, although this SNP was not significant overall ($p = 0.073$) (Table II). For the SNP rs235756, the impact of heterozygosity or homozygosity of the variant genotype on ferritin levels could not be determined (individual $p > 0.05$) (Table II). This SNP remained significantly associated with ferritin levels ($p = 0.038$) after adjusting for donor parameters, although the significance of heterozygosity and homozygosity of the variant allele remained to be determined ($p > 0.05$) (Table II). Low numbers of homozygous variant genotypes were present in high and low ferritin groups (Online Supplementary

Table I – Association between SNP and ferritin levels.

Gene	SNP name	Overall p-value*			
		Male		Female	
		Before†	After‡	Before†	After‡
BTBD9	rs9296249	0.641		0.996	
	rs3923809	0.027	0.073	0.748	
TMPRSS6	rs855791	0.874		0.869	
	rs4820268	0.980		0.043	0.025
TFR2	rs7385804	0.512		0.480	
TF	rs3811647	0.824		0.686	
	rs1799852	0.260		0.736	
	rs2280673	0.473		0.377	
BMP2	rs235756	0.029	0.038	0.711	
SMIM1	rs1175550	0.961		0.425	

*Overall p -value < 0.05 indicates this particular SNP has a statistically significant impact on ferritin levels; †before adjusting for donor parameters; ‡after adjusting for significant donor parameters (donation-induced iron loss and time since previous donation for male donors; age, donation-induced iron loss and time since previous donation for female donors); Shaded cells show SNP significantly ($p < 0.05$) associated with ferritin levels. SNP: single nucleotide polymorphism.

Table II - Association between ferritin levels and *BTBD9* rs3923809 or *BMP2* rs235756 in male donors.

SNP	Before adjusting for donor parameters			
	<i>Ferritin</i> ≤15 ng/mL			
	<i>GG</i> vs <i>AA</i>		<i>AG</i> vs <i>AA</i>	
	<i>OR</i> (95% <i>CI</i>)	<i>p</i> -value	<i>OR</i> (95% <i>CI</i>)	<i>p</i> -value
rs3923809	1.491(0.395-5.632)	0.556	1.438 (0.651-3.174)	0.369
rs235756	0.297 (0.037-2.406)	0.255	1.967 (0.865-4.471)	0.106
	<i>Ferritin</i> >100 ng/mL			
	3.253 (1.443-7.333)	0.004	2.091 (1.186-3.688)	0.011
	0.434 (0.172-1.091)	0.076	0.742 (0.436-1.262)	0.271
	After adjusting for significant donor factors§			
	<i>Ferritin</i> ≤15 ng/mL			
	1.949 (0.493-7.709)	0.342	1.565 (0.682-3.592)	0.291
	0.327 (0.039-2.768)	0.305	2.077 (0.868-4.973)	0.101
	<i>Ferritin</i> >100 ng/mL			
	2.715 (1.164-6.330)	0.021	1.967 (1.097-3.525)	0.023
	0.448 (0.176-1.141)	0.092	0.724 (0.417-1.254)	0.249

Shaded cells indicate the genotype was significantly ($p < 0.05$) associated with this particular ferritin group compared to the wild-type (AA) genotype; §Significant donor factors include time since previous donation and donation-induced iron loss in the previous 12 months. SNP: single nucleotide polymorphism; OR: odds ratio; CI: confidence interval.

Data, Table SV), suggesting that this could be due to the small sample size.

Out of the ten SNPs analysed, only one SNP, rs4820268, was significantly associated with ferritin levels, specifically low ferritin levels, in female donors (Table I). The overall association remained significant ($p = 0.025$) after adjusting for significant donor parameters. Females with the AG genotype within this SNP were three times more likely to have low ferritin levels ($p = 0.010$) (Table III), with this observation remaining statistically significant ($p = 0.012$) after adjusting for donor parameters.

Among the seven other SNPs investigated, the SNP rs1799852 (*TF* gene) could not be analysed because of the very low frequency (8/800 homozygous variant [TT] genotype) (Online Supplementary Content, Table SVI). However, males with the TT genotype of this SNP had the highest mean ferritin values (145 ng/mL) among those with all genotypes, in both sexes. Given that this SNP is proposed to be associated with increased serum ferritin in Australians of European descent¹⁸, analysis of this SNP in a larger number of samples would allow determination of whether there is indeed an association between this SNP and donor ferritin levels. The SNP rs1175550 was not shown to be associated with ferritin levels (Table I); however, female donors with the variant allele had a smaller ferritin range of 4-68 ng/mL, compared to those in other genotype groups (Online

Supplementary Data, Table SVI). This is likely to be a result of the small numbers of this genotype identified in our study population, or could be due to the impact of the homozygous variant allele. Additional studies are required to assess the impact of this SNP on ferritin levels of female donors.

Table III - Association between ferritin levels and *TMPRSS6* rs4820268 in female donors.

	Before adjusting for donor parameters			
	<i>Ferritin</i> ≤8 ng/mL			
	<i>GG</i> vs <i>AA</i>		<i>AG</i> vs <i>AA</i>	
	<i>OR</i> (95% <i>CI</i>)	<i>p</i> -value	<i>OR</i> (95% <i>CI</i>)	<i>p</i> -value
	1.808 (0.580-5.641)	0.307	3.348 (1.328-8.438)	0.010
	<i>Ferritin</i> >60 ng/mL			
	0.775 (0.339-1.769)	0.545	0.890 (0.379-1.462)	0.391
	After adjusting for significant donor factors*			
	<i>Ferritin</i> ≤8 ng/mL			
	2.073 (0.646-6.647)	0.220	3.443 (1.314-9.021)	0.012
	<i>Ferritin</i> >60 ng/mL			
	0.573 (0.238-1.378)	0.214	0.548 (0.265-1.133)	0.104

Shaded cells indicate the genotype was significantly ($p < 0.05$) associated with this particular ferritin group compared to the wild-type (AA) genotype; *Significant donor factors include age, time since previous donation and donation-induced iron loss in the previous 12 months. SNP: single nucleotide polymorphism; OR: odds ratio; CI: confidence interval.

Discussion

ID is a significant issue for frequent whole blood donors, with being female, particularly a younger female, time since previous donation and number of donations in the past 2 years identified as independent predictors for ID erythropoiesis². ID is implicated in a range of adverse effects, ranging from fatigue³⁰ to impaired cognitive performance, especially in young individuals who are undergoing neurological and physical development^{31,32}. Given that blood donation is associated with ID and IDA, donors may suffer from these adverse effects depending on the severity of iron depletion. In this study, we investigated whether SNP involved in parameters of iron status were associated with ferritin levels in a cohort of Australian blood donors. We show that the SNPs rs4820268, rs235756 and rs3923809 were potentially associated with changes in iron metabolism and lead to variations in ferritin levels.

The SNP rs4820268 has been shown to be associated with decreased serum iron, serum ferritin and red cell indices in Australians of European descent¹⁸, Chinese Han¹⁷, and Italian populations²⁸. In our study, a similar finding was obtained, whereby this SNP was significantly associated with low ferritin levels in female donors. This demonstrates the presence of a common variant that may potentially lower iron stores in healthy individuals. The SNP rs4820268 is a synonymous substitution found in exon 13 of the *TMPRSS6* gene³³. The *TMPRSS6* gene encodes a membrane serine protease, Matriptase-2, which can strongly suppress the expression of hepcidin - the principal negative iron regulator in human³⁴. A synonymous substitution does not result in amino acid changes, although it may cause inefficient translation of the variant codon, and lead to altered structure of clinically important proteins³⁵. The SNP rs4820268 may, therefore, lead to decreased production or function of Matriptase-2 and thus result in variations in iron stores. Further analyses including measuring the mRNA or total protein levels may assist in understanding the mechanism.

The SNP rs235756 was identified in a study of hemochromatosis patients, in which individuals with this SNP had increased serum ferritin levels²¹. However, this result was not replicated in a study that involved 2,139 Chinese women¹⁷. In our study, an impact of this SNP was observed to have an impact on ferritin levels in males, however, the significance of homozygosity and heterozygosity of the variant allele could not be determined. This was probably a result of low statistical power or not enough samples in the variant genotype group. The SNP rs235756 is located in the 3' flanking region of the *BMP2* gene, which is rapidly removed while pre-mature RNA is processed into mature mRNA³⁶. However, this region often has sequences that

affect the formation of the 3' end of the mRNA, and may also contain enhancer or protein-binding sites³⁷. The presence of the SNP rs235756 at this region may, therefore, lead to altered bone morphogenetic protein (BMP) 2 mRNA regulation and decreased function/production of encoded protein. The BMP2 signalling pathway is proposed to induce hepcidin expression and decrease systemic iron levels³⁸. SNPs in the *BMP2* gene may, therefore, compromise the stimulation of hepcidin expression, leading to increased iron absorption and accumulation. Additional studies with a larger sample size would be necessary to further understand the role of this SNP in Australian donors.

The SNP rs3923809 is found in the intronic region of the *BTBD9* gene, which is proposed to exert diverse biological functions, although it has not so far been shown to be involved in iron metabolism³⁹⁻⁴¹. This SNP was associated with decreased serum ferritin levels, 13% per A allele in an Icelandic population, and increased susceptibility to restless leg syndrome²⁵. We found that although this SNP was initially associated with ferritin levels in male donors, the association was not significant overall after adjusting for donor parameters. However, the G allele of this SNP remained significantly associated with high ferritin levels after adjusting for significant donor parameters, which is in accordance with the Icelandic study²⁵. The insignificant results of the overall analysis could be a consequence of a small sample size not being able to achieve statistical significance. Thus, additional studies with a larger sample size are necessary to confirm the impact of this SNP. Although introns are removed before mRNA is translated into mature proteins, an intronic SNP present in the regulatory region of the gene may affect alternative splicing of the mRNA and lead to the formation of an alternative protein isoform⁴², or change the levels of gene expression of one or more genes⁴³. The SNP rs3923809 may, therefore, lead to altered iron metabolic pathways: (i) by contributing to altered expression of BTBD protein, which may indirectly affect iron storage through an unknown mechanism; (ii) by affecting the expression of genes involved in iron metabolism; or (iii) through association with an as yet unidentified SNP that is associated with ferritin levels. Future investigations involving DNA sequencing and cDNA analysis would be required to elucidate possible mechanisms, with the outcome having the potential to provide additional evidence in relation to novel pathways involved in iron regulation.

Our study has a number of limitations. As this study utilised convenience samples remaining after routine testing, plasma rather than serum was investigated. Our initial analysis suggested plasma and serum ferritin levels are not interchangeable, with plasma ferritin levels estimated to be half of those in serum⁴⁴. In the

absence of a plasma ferritin reference range, we therefore assigned low, medium and high ferritin levels based on this approximate conversion. This allowed us to stratify donors based on ferritin levels, but did not allow direct comparison with the serum ferritin ranges used for assessing ID and IDA or suitability to donate blood. Additional studies using serum samples are required to further validate our findings. In addition, information on the donors' diet, use of iron supplementation, menopausal status (female donors) and other factors that may affect ferritin levels (such as infection or inflammation) were not obtained. A large longitudinal study is required to understand the contribution of such factors. Furthermore, we analysed the association between various SNPs and donors' ferritin levels at their current donation; additional studies could involve testing donors' ferritin levels at two sequential donations or longitudinally over many donations, so as to identify SNPs associated with iron recovery after donation. Moreover, as serum ferritin levels are influenced by infection and inflammation⁴⁵, additional studies could look at other parameters involved in iron status, such as transferrin iron-binding capacity or percent transferrin saturation⁴⁶.

Conclusions

This study provides evidence of an association between genetic factors in blood donors and plasma ferritin levels. Although further validation is required, the three SNPs identified, rs3923809, rs235756 and rs4820268, have the potential to be useful in identifying the most suitable donation frequencies for eligible blood donors who pass the initial Hb screen. For example, male donors with a variant SNP rs3923809 allele were more likely to have high ferritin levels and may, therefore, be suitable for more frequent donation or double red blood cell donation, while female donors heterozygous for the SNP rs4820268 were more likely to have low ferritin levels and thus may be better managed with a longer donation interval. In addition, significant genotypes discovered in future analyses may assist in generating a SNP panel for accurately predicting ferritin variations between blood donations. Overall, this study provides support that genetic testing may be useful for the generation of predictive algorithms to assist in maintaining iron levels in regular blood donors with varied donation patterns, and thus, allow for improved, individualised donor management that better caters for donor health and wellbeing.

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Authorship contributions

YJ performed the experimental work, analysed the data and prepared the first draft of this manuscript. All Authors contributed to the study design, data interpretation, data analysis/consolidation and drafting/approval of the manuscript.

The Authors declare no conflicts of interest.

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