Philippine Journal of Science
147 (1): 99-112, March 2018
ISSN 0031 - 7683
Date Received: 29 May 2017

Blood Iron Concentration and Status in Pregnant Filipino Women with Single Nucleotide Polymorphisms in *HFE*, *TMPRSS6*, and *TF*

Vanessa Joy A. Timoteo[1*], Leslie Michelle M. Dalmacio[2], Jacus S. Nacis[1], Juanita M. Marcos[1], Marietta P. Rodriguez[1], and Mario V. Capanzana[1]

[1]Food and Nutrition Research Institute, Department of Science and Technology, DOST Complex, Bicutan, Taguig City 1631 Philippines
[2]Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines, 547 Pedro Gil St., Ermita, Manila 1000 Philippines

Anemia is a significant health problem in the Philippines, especially in pregnant women. Investigation of single nucleotide polymorphisms (SNPs) that are associated with blood iron concentration and status may identify the underlying genetic factors contributing to incidences of anemia, iron deficiency, and iron deficiency anemia (IDA) in pregnant Filipino women. This study determined the genotype distribution of SNPs in the hemochromatosis gene (*HFE*), transmembrane protease, serine 6 gene (*TMPRSS6*), and transferrin gene (*TF*) in pregnant Filipino women and their effects on levels of hemoglobin (Hb), hematocrit (Hct), serum ferritin (SF), serum iron (SI), total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC), and percent transferrin saturation (TS%). Non-parametric Mann Whitney U test and Analysis of Covariance were performed to evaluate the effect of SNPs on blood iron levels, where maternal age, age of gestation, iron supplementation, and area of residence were considered as covariates. The minor allele frequencies of SNPs in *TMPRSS6* and *TF* among the 109 pregnant women living in Quezon, Palawan are higher than previously reported values. Comparison across SNP genotypes show that: (1) carriers of the heterozygous CG of *HFE* rs1799945 have significantly lower Hct levels than carriers of the wild-type CC, (2) carriers of the homozygous risk genotypes of *TMPRSS6* rs855791 (TT) and rs4820268 (GG) have significantly higher UIBC levels than carriers of the wild-type CC and AA genotypes, (3) carriers of CT and risk TT genotypes of *TMPRSS6* rs855791 and AG and risk GG genotypes of rs4820268 have lower TS% than carriers of the wild-type genotypes, and (4) carriers of AG and risk AA genotypes of *TF* rs3811647 have significantly higher TIBC and UIBC levels than carriers of the wild-type GG genotype. These findings imply that SNPs in *TMPRSS6* and *TF* are potential genetic risk factors for anemia, iron deficiency, and IDA in Filipinos.

Key words: blood iron levels, *HFE*, pregnant Filipino women, SNPs, *TF*, *TMPRSS6*

INTRODUCTION

Anemia is a prevailing form of undernutrition in the Philippines. It has been a moderate public health significance based on the overall anemia prevalences recorded during the 1993 and 1998 National Nutrition Surveys (NNSs), which were at 28.9% and 30.6%, respectively (WHO 2001; FNRI-DOST 2015). It then became a mild public health significance in 2008 and 2013 with overall anemia prevalences of 19.5% and 11.1%, respectively. The current prevalence rate among pregnant Filipino women is at 25.2% (FNRI-DOST 2015). Pregnant

*Corresponding author: vjtimoteo@gmail.com

women are at high risk of developing anemia because of additional iron requirements for increased blood volume and fetal growth (NHLBI 2014).

Certain biochemical and hematological tests are utilized in the assessment of iron levels and concentration in human blood, as well as in the determination of anemia, iron deficiency, and IDA. Hemoglobin (Hb) and hematocrit (Hct) tests measure the levels of the iron-rich hemoglobin in red blood cells and the volume percentage of red blood cells in whole blood, respectively. These tests, which are commonly used to screen individuals for anemia, are recommended in determining the prevalence of anemia within a population in a field setting (WHO 2011a) . In practice, however, additional tests that are more specific and sensitive need to be carried out to detect iron deficiency and diagnose IDA. The serum ferritin (SF) test measures an individual's iron stores. Depleted iron stores normally indicate iron deficiency while high levels of iron stores suggest hemochromatosis or iron overload in the body (WHO 2011b) . The serum iron (SI) test measures the amount of iron in the blood, while the total iron binding capacity (TIBC) test measures the amount of transferrin protein in the blood that is void of iron. Reduced SI levels in combination with elevated TIBC levels confirm iron deficiency (WHO 2001). Levels of unsaturated iron binding capacity (UIBC) and percent transferrin saturation (TS%) can be calculated from SI and TIBC values. UIBC level is increased with iron deficiency while low TS% indicate iron deficiency (WHO 2001). Overall, the Hb, Hct, SF, SI, and TS% levels are decreased in the presence of IDA, while TIBC and UIBC levels are elevated (IDI 2009).

The advances in the field of biochemistry and molecular biology paved the way for the elucidation of the relationship between DNA sequence variations (the most common type of which is single nucleotide polymorphism or SNP) and susceptibility to diseases. There were reports that investigated the association of SNPs with indicators of blood iron concentration and status of anemia and iron deficiency across different populations. For instance, in a meta-analyses by Gichohi-Wainaina and co-authors (2015), a SNP in the *TMPRSS6* gene was found associated with lower Hb and SF concentrations among Caucasians, Asians, and African-Americans. Similarly, another SNP in the *TF* gene was associated with lower SF and body iron concentrations. However, no data exist to date on the association of SNPs with blood iron levels among Filipinos despite the relatively high anemia prevalences that were observed in this population.

Thus, this study was conducted to determine the genotype distribution of selected candidate SNPs in a group of pregnant women residing in Quezon, Palawan. Target SNPs were identified from scientific literature, where SNPs were associated with different indices of blood iron concentration and status across different populations. Current data on SNP frequencies present the potential susceptibility genes of anemia, iron deficiency, and IDA in pregnant Filipino women. This study is also the first to investigate the potential association of SNPs in the hemochromatosis gene (*HFE*), transmembrane protease, serine 6 gene (*TMPRSS6*), and transferrin gene (*TF*) with levels of Hb, Hct, SF, SI, TIBC, UIBC and TS% in Filipinos.

HFE protein is involved in the regulation of iron homeostasis, and SNPs in *HFE* (rs1800562 and rs1799945) are responsible for familial hemochromatosis (Barton et al. 2015). The SNP rs1800562 results in a cys282-to-tyr (C282Y) missense substitution, with a minor allele frequency (MAF) range of 0.030-0.090 in European populations (Rajeevan et al. 2012). On the other hand, rs1799945 results in a his63-to-asp (H63D) substitution that is observed to be less occurring among European populations. The compound heterozygosity for both H63D and C232Y mutations was observed in three cases (out of 115 with familial hemochromatosis) and four controls (out of 101) in the UK Haemochromatosis Consortium (1997) . In addition to the strong association of *HFE* SNPs with iron overload in the body, Datz and colleagues (1998) were able to show that the heterozygosity for these SNPs is protective against IDA.

TMPRSS6 encodes matriptase-2 enzyme, which is required to sense iron deficiency and promote iron absorption. The SNP rs855791 in *TMPRSS6* leads to a nonsynonymous val736-to-ala (V736A) change, while rs4820268 results in a synonymous asp521-to-asp (D521D) change. Both SNPs result in decreased levels of blood iron parameters and red blood cell indices across different populations (Gichohi-Wainaina et al. 2015). Several candidate gene and SNP studies and genome-wide association studies identified *TMPRSS6* to be strongly associated with red blood cell indices or iron parameters and, therefore, the susceptibility to anemia, iron deficiency, and IDA in general population.

Lastly, *TF* encodes transferrin, which is a serum protein that delivers iron to the cells. The SNP rs3811647 was found along intron 11 of the *TF* gene, while rs1799852 is a non-synonymous coding SNP located in exon 17. It is suggested that these SNPs function during transcription or post-transcription modification at the molecular level. Both SNPs were shown to also cause variations in blood iron indices (Benyamin et al. 2009b; Blanco-Rojo et al. 2011; McLaren et al. 2011, 2012).

MATERIALS AND METHODS

Ethical Considerations

The study protocol was reviewed and approved by the Food and Nutrition Research Institute (FNRI) Institutional Ethics Review Committee (FIERC Registry No. 2012-09-28-0010-2). Accomplished and duly signed written informed consents were collected prior to the conduct of the study and all information obtained from the study participants were treated with utmost confidentiality. Upon completion of the study, biological samples and derivatives were disposed of properly.

Study Population and Research Design

The study followed a cross-sectional research design and employed a non-probability, purposive homogeneous sampling. All identified pregnant women living in selected *barangays* (districts) in Quezon, Palawan were oriented, recruited, and screened for the study. Quezon, Palawan was chosen as the study site because of previously reported high prevalences of anemia within the region based on NNSs results.

A total of 109 pregnant women satisfied the following inclusion-exclusion criteria of the study: (1) Filipino, (2) may or may not be taking up iron supplements and/or multivitamins, (3) assessed as healthy at the time of data collection, and (4) without disease or complications that may alter blood iron concentration and status (e.g., recent or current infection, kidney disease, chronic inflammatory disease). Data on anthropometric, biochemical, and clinical indices were obtained. The socio-demographic information and obstetrical history of the respondents were also taken through an interview using a pre-tested questionnaire.

Measurement of Anthropometric, Biochemical, and Hematological Indices

The height and weight of study participants were measured twice during screening using a microtoise (Seca™, USA) and a calibrated platform beam balance weighing scale (Detecto™, USA), respectively. Using the recommended weight-for-height table by month of pregnancy for Filipinos (Magbitang et al. 1988), study participants were classified as within, below, or above the prescribed weight-for-height values for pregnant women.

Blood samples were collected in health centers in the morning to minimize the effect of diurnal variations on levels of biochemical and hematological indices. Hemoglobin was measured on-site using a portable spectrophotometer (Odyssey DR/2800, Hach Company USA) following the cyanmethemoglobin method (ICSH 1978). Hb concentration of an in-house quality control blood sample was determined with the other samples collected in each assay day to monitor precision within $\pm$ 2 S.D., while accuracy of the method was determined using a tri-level control for blood samples (Liquichek Hematology-16 Control, Bio-Rad Laboratories, Irvine, CA 92618). Pregnant women with Hb < 11 g/dL were considered anemic based on the World Health Organization cut-off (WHO 2001). Hct was determined by filling a microhematocrit tube containing sodium heparin (Vitrex Medical A/S, Denmark) with well-mixed K2EDTA blood. Tubes were sealed with Sigillum wax plate (Vitrex Medical A/S, Denmark) and were balanced and centrifuged at 3,000 rpm for 15 min. A hematocrit reader was used to obtain the Hct reading in percentage. Pregnant women with Hct < 33% were considered anemic based on the WHO cut-off (WHO 2001). Hb and Hct levels were measured in duplicate.

The quantitative measurement of SF levels was done at the Philippine Nuclear Research Institute - Department of Science and Technology using solid-phase immunoradiometric assay (Coat-a-Count®, Siemens USA). The concentration of the bound antigen in the assay supernatant was measured using Genesys Gamma-1 single well gamma counter (Laboratory Technologies, Inc. USA) following the recommended manufacturer's protocol. Controls with at least two ferritin levels (low and high) were assayed together with the unknown samples. Pregnant women with SF < 15 ng/mL were considered with depleted iron stores (iron-deficient), while pregnant women with SF > 150 ng/mL were considered with severe risk of iron overload (WHO 2011b).

SI and TIBC levels were determined by the Philippine General Hospital - Medical Research Laboratory (PGH-MRL) using Ferrimat-Kit (Bio-Merieux, France). Reference ranges for normal values of SI and TIBC were at 0.6-1.6 mg/L and 2.51-3.63 mg/L, respectively. SI levels < 0.60 mg/L and TIBC levels > 3.63 mg/L may indicate IDA (CLSI 1998). Using the measured values of SI and TIBC, UIBC level was calculated as the difference between TIBC and SI values and TS% was calculated as: TS% = SI / TIBC x 100%.

Extraction of Genomic DNAs

Genomic DNAs (gDNAs) were extracted from EDTA-treated whole blood and buffy coat samples using QIAamp™ DNA Mini Kit (QIAGEN, Germany), following the recommended spin purification protocol from blood or body fluids. No additional RNase treatment was performed. The concentration and purity of gDNAs were determined by measuring absorbance at 260 nm and 280 nm (A_{260} and A_{280}, respectively) in triplicates using EPOCH Micro-Volume Spectrophotometer System (BioTek Instruments, Inc. USA), followed by the calculation of A_{260}/A_{280} ratio. Electrophoresis in 1% agarose gel stained with GelRed™ (Biotium, USA) was

performed to visualize the integrity of extracted gDNAs, wherein a TrackIt™ 1 Kb Plus DNA ladder (Invitrogen™, USA) was used as a molecular weight marker. gDNAs were diluted to make a standard working concentration of 5 ng/uL prior to real-time amplifications.

Selection of Candidate SNPs, Primer Design, and Optimization of Amplification Reactions

The target SNPs and genes were identified from published scientific works that presented the association of SNPs with the biochemical and hematological indices of blood iron concentration and status across different populations.

Sets of forward and reverse primers were synthesized by AITbiotech Singapore Pte Ltd and designed using Primer3Plus (accessible at http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) according to the following parameters: length between 20-25 bases, optimal at 23 bases; GC content of 40-60% (optimal at 50%), melting temperature (Tm) ranging 55-65° C, optimal Tm at 60°C (with a maximum Tm difference of five units), and length of amplification product within 100-250 base pairs (bp). The primers used in SNP genotyping were as follows: (1) *HFE* rs1800562 forward: AATGGGGATGGGACCTACCAG, reverse: CTCTCATCAGTCACATACCCCAG; (2) *HFE* rs1799945 forward: ACATGGTTAAGGCCTGTTGC, reverse: ATGTGATCCCACCCTTTCAG; (3) *TMPRSS6* rs855791 forward: ATCAGCAACGCTCTGCAGAAA, reverse: ACCTGACAGGCATCCTTCTTG; (4) *TMPRSS6* rs4820268 forward: GAAGCATGTAGCAGGCCTAGA, reverse: CTGATTGTCTCAACGGCAGC; (5) *TF* rs3811647 forward: ACACAAGTGGACCCTAAGCTG, reverse: AATGTGCAGGCCTCATGTCA; (6) *TF* rs1799852 forward: GCTTTCCCTCCCCAGAGAACTT, reverse: CACGACGGTATGAGAAGGGAC.

Real-time amplifications were carried out in the 96-well CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) of the FNRI Nutritional Genomics Laboratory, Philippines. The optimal annealing temperature (Ta) of each primer set was determined using gradient real-time PCR technique, where optimization reaction mixture consisted of 20 ng (4 µL) template gDNA, 2 µM forward and reverse primers and 5 µL of 2X SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc. Singapore Pte Ltd) in a total reaction volume of 10 µL. In each temperature in the gradient, reactions were in triplicate and control reactions without template (NTC) were included in each optimization run. The specificity of each primer set and PCR yield was visualized in 2% agarose gels stained with GelRed™ and loaded with an EZ Load™ 100 bp Molecular Ruler (Bio-Rad Laboratories, Inc. USA).

Genotyping via Capillary Sequencing and High Resolution Melt Assays

SNP genotyping was performed via capillary electrophoresis sequencing and high resolution melt (HRM) assays. Selected amplicons ($n \geq 50$) from each HRM assay were purified using GF-1 AmbiClean Kit (Gel & PCR) (Vivantis, Malaysia) following the recommended protocol for PCR clean-up of DNA bands ranging from 100 bp to 20 kilo base pairs (kb) and were submitted to AITbiotech for sequencing. Resulting sequences were validated through Basic Local Alignment Search Tool (accessible at https://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned against the FASTA sequence spanning the SNPs-of-interest using MultAlin (accessible at http://multalin.toulouse.inra.fr/multalin/). SNP genotypes were identified from resulting sequence electropherograms.

In each HRM assay, PCRs were performed in a total reaction volume of 20 µL with 22 ng (4.4 µL) template DNA, 2 µM forward and reverse primers, 11 µL of 2X SsoFast™ EvaGreen® Supermix, and 3.3 µL nuclease-free water. All PCRs were run in duplicate, which included two PCR reactions per gDNA sample, positive controls for all genotypes, and NTCs. Table 1 presents

Table 1. PCR cycling conditions used in each HRM assay[a].

HRM Assay	Enzyme Activation	Denaturation	Annealing/ Extension (with plate read)	Number of Cycles of Denaturation and Annealing	Melt Curve
HFE rs1800562	98°C/3 min	98°C/30 s	59.0°C/30 s	40	65-95°C at 0.2°C/10 s increments
TMPRSS6 rs855791			58.8°C/30 s		
TF rs1799852			59.4°C/30 s		
HFE rs1799945[b]	98°C/2 min	98°C/5 s	56.3°C/30 s		
TF rs3811647			57.5°C/30 s		
TMPRSS6 rs4820268	98°C/2 min	98°C/5 s	56.8°C/30 s	50	

[a]Before melt curve generation, denaturation was set again at 95°C/30 s followed by cooling to 60°C/1 min.
[b]A 3-step run protocol, where an extension step at 72°C for 30 s was included during the annealing/extension step (with plate read), has been adapted to improve PCR performance.

the cycling conditions specific to each assay. Analyses of characteristic melt curve profiles and genotype discrimination were performed using Bio-Rad Precision Melt Analysis ™ software.

Data Processing and Statistical Analyses

Data processing and statistical analyses were performed using IBM® SPSS® Statistics 20.0 (USA). Continuous variables such as anthropometric (height, weight, and BMI), hematological (Hb and Hct), and biochemical indices (SF, SI, TIBC, UIBC, and TS%) were expressed as mean $\pm$ S.D., mean ranks, or geometric means with confidence interval (CI). A normal distribution of the parameters was determined by the Kolmogorov-Smirnoff test. SF and TS% values were log-transformed to account for the non-normal distribution of SF and TS% data.

Genotype and allele frequencies were expressed as number and percentage values. For each SNP, MAF was computed as: MAF = minor allele count/total allele count. Deviation from the Hardy-Weinberg principle was determined by Chi-Square Goodness-of-Fit test.

The codominant (additive) model was used in comparing differences across the SNP genotypes. Analysis of variance (ANOVA) with Tukey post-hoc test was used to initially compare variables among groups. Analysis of covariance (ANCOVA) with a Sidak correction was used to assess differences in levels of blood iron concentration and status across SNP genotypes in *TMPRSS6* and *TF*, where maternal age, gestational age, daily iron supplementation, and area of residence were treated as covariates. On the other hand, the non-parametric Mann Whitney U test was used in determining differences in blood iron levels based on *HFE* rs1799945 genotypes, since there were only two genotype groups observed among the participants. All computations used 95% CI and a p value of < 0.05 was considered statistically significant.

RESULTS

Characteristics of Study Participants

Table 2 presents the general characteristics of the study population (N=109) as well as the mean levels of the biochemical and hematological parameters based on maternal age grouping. There was no significant difference observed in the anthropometric, biochemical, and hematological profiles among age groups.

Based on the weight-for-weight table by month of pregnancy, only one-third of the participants had the desired weight based on gestational age. Majority were observed to be underweight (i.e., weight below the recommended values), while a few were overweight (i.e., weight above the recommended values).

Based on the cut-offs used in the study to define iron concentration and status, the mean levels of the biochemical and hematological parameters of the study population were found normal except for the TIBC. However, stratification based on maternal age showed that participants in early (i.e., adolescent) and late (i.e., old-age) pregnancies had lower mean Hb, Hct, SF, SI, and TS% levels and higher mean TIBC and UIBC levels. Additionally, the mean Hb and Hct levels were lowest among women at late pregnancies while mean SF, SI, and TS% levels were lowest at early pregnancies. The mean TIBC and UIBC levels were found to be highest at early pregnancies. It was also observed that the mean Hb and Hct levels of study participants at late pregnancies were below the cut-offs set by the WHO (2001), while the mean TIBC levels for all maternal age groups exceeded the normal reference range defined by the PGH-MRL. In terms of anthropometric measurements, the study participants at early pregnancies have the lowest mean weight and BMI values.

Among the 109 study participants, only 59 (accounting for 54% of the study population) had their daily source of exogenous iron from supplements and/or multivitamins for pregnant women (i.e., ferrous sulfate, Usanatal, Martham, etc.). Despite iron supplementation, 22 pregnant women were found anemic while 28 pregnant women were iron-deficient based on Hb and SF cut-offs set by the WHO, respectively. The overall incidences of anemia and iron deficiency in the study population was 37.6% (n=41) and 47.7% (n=52), respectively.

Table 3 shows the biochemical and hematological profiles of the study participants after grouping based on trimester of pregnancy. The mean levels of Hb, Hct, SF, log SF, TS%, and log TS% of pregnant women who were in their first trimester of pregnancy were significantly higher than the pregnant women who were in their second and third trimesters of pregnancy. The mean UIBC and TIBC levels of the pregnant women who were in their first trimester of pregnancy were significantly lower than those in their second and their trimesters of pregnancy. There was no significant difference in SI levels in terms of trimester of pregnancy, but it was shown to decrease towards the end of pregnancy. These trends were similarly observed even after further grouping the participants based on maternal age groups. The differences observed were, however, not statistically significant.

SNP Genotypes and Association with Levels of Blood Iron Concentration and Status

The genotype and allele frequencies of SNPs among the study participants are presented in Table 4. More than 30% of the study participants carry the homozygous minor (risk) genotypes of *TMPRSS*6 rs855791 and rs4820268,

Table 2. Characteristics of the study population[a].

Parameters	Maternal age (years)			Total (*N*=109)
	≤ 19 (*n*=10)	20-34 (*n*=72)	≥ 35 (*n*=27)	
Age (yr)*	17.7 ± 1.42	25.8 ± 4.09	37.48 ± 2.26	28.0 ± 6.94
	(15-19)[b]	(20-34)[c]	(35-42)[d]	
Height (cm)	150.20 ± 6.57	150.76 ± 5.13	148.86 ± 4.63	150.24 ± 5.17
	(142.20-161.50)	(141.00-164.00)	(142.25-161.55)	
Weight (kg)	49.62 ± 11.14	52.09 ± 8.36	51.97 ± 9.84	51.83 ± 8.95
	(35.55-70.00)	(36.30-75.10)	(37.10-70.30)	
Within prescribed weight-for-height	3 (2.8)	28 (25.7)	5 (4.6)	36 (33.0)
Below prescribed weight-for-height	5 (4.6)	25 (22.9)	11 (10.1)	41 (37.6)
Above prescribed weight-for-height	2 (1.8)	19 (17.4)	11 (10.1)	32 (29.4)
Hb (g/dL)	11.22 ± 0.54	11.30 ± 1.08	10.86 ± 1.19	11.18 ± 1.08
	(10.4-12.2)	(8.7-14.6)	(8.6-12.9)	
Hct (%)	33.40 ± 2.22	34.22 ± 3.35	32.85 ± 3.52	33.81 ± 3.33
	(30-38)	(27-45)	(28-40)	
SF (ng/mL)	26.50 ± 27.09	31.92 ± 31.90	27.81 ± 36.07	30.40 ± 32.37
	(5-86)	(3-177)	(3-142)	
SI (mg/L)	0.88 ± 0.40	1.05 ± 0.44	1.02 ± 0.32	1.02 ± 0.41
	(0.54-1.92)	(0.26-2.69)	(0.40-1.79)	
TIBC (mg/L)	4.60 ± 1.16	4.33 ± 1.02	4.42 ± 1.08	4.38 ± 1.04
	(2.70-6.46)	(2.60-6.38)	(2.69-6.25)	
UIBC (mg/L)	3.72 ± 1.38	3.29 ± 1.08	3.40 ± 1.16	3.35 ± 1.12
	(1.93-5.65)	(1.37-5.38)	(1.76-5.39)	
TS% (%)	21.27 ± 12.65	25.35 ± 11.23	24.65 ± 10.26	24.80 ± 11.09
	(9.87-49.87)	(5.84-47.89)	(9.28-43.21)	
Without iron supplementation	9 (8.3)	30 (27.5)	11 (10.1)	50 (45.9)
With daily iron supplementation	1 (0.9)	42 (38.5)	16 (14.7)	59 (54.1)

[a]Data are presented as mean ± S.D., where the minimum and maximum values were also presented per parameter in each maternal age group. Data on weigh-for-height by month of pregnancy and iron supplementation of study participants were presented as *n* (% based on *N*).
*Different alphabets for the superscript indicate significant differences observed between age groups using ANOVA with Tukey post-hoc test, $p<0.001$.

Table 3. Biochemical and hematological profiles of study participants based on trimester of pregnancy per maternal age group[a].

	Hb, g/dL*	Hct, %*	SF, ng/mL*	SI, mg/L	TIBC, mg/L*	UIBC, mg/L*	TS%, %*
Age ≤ 19 yr							
1st Trimester, *n*=2	11.85 ± 0.50	36.00 ± 2.83	68.50 ± 24.75	1.35 ± 0.81	3.28 ± 0.81	1.93 ± 0.00	39.20 ± 15.10
2nd Trimester, *n*=5	10.96 ± 0.42	32.00 ± 1.58	21.60 ± 17.02	0.82 ± 0.22	4.52 ± 0.96	3.71 ± 1.16	19.53 ± 8.78
3rd Trimester, *n*=3	11.23 ± 0.50	34.00 ± 1.00	6.67 ± 1.53	0.69 ± 0.11	5.62 ± 0.77	4.93 ± 0.67	12.22 ± 0.55
Aged 20-34 yr							
1st Trimester, *n*=14	12.35 ± 1.07	37.57 ± 3.88	54.43 ± 44.35	1.12 ± 0.36	3.70 ± 0.88	2.59 ± 0.87	31.02 ± 9.73
2nd Trimester, *n*=37	11.09 ± 0.88	33.38 ± 2.70	27.92 ± 26.85	1.03 ± 0.40	4.23 ± 1.00	3.20 ± 1.10	25.85 ± 11.73
3rd Trimester, *n*=21	10.95 ± 1.03	33.48 ± 2.66	23.30 ± 24.63	1.03 ± 0.57	4.94 ± 0.86	3.91 ± 0.83	20.69 ± 9.66
Age ≥ 35 yr							
1st Trimester, *n*=5	10.90 ± 1.92	33.60 ± 4.16	50.80 ± 53.49	0.98 ± 0.28	3.24 ± 0.38	2.27 ± 0.28	29.87 ± 6.95

Table 3 continued next page . . .

	Hb, g/dL*	Hct, %*	SF, ng/mL*	SI, mg/L	TIBC, mg/L*	UIBC, mg/L*	TS%, %*
2nd Trimester, *n*=11	10.96 ± 0.61	32.27 ± 1.90	32.36 ± 40.22	1.02 ± 0.38	4.40 ± 1.03	3.39 ± 1.19	24.94 ± 12.40
3rd Trimester, *n*=11	10.75 ± 1.35	33.09 ± 4.57	12.82 ± 9.27	1.04 ± 0.29	4.96 ± 0.94	3.92 ± 1.06	21.99 ± 8.89
***N* = 109**							
1st Trimester, *n*=21	11.96 ± 1.37	36.48 ± 4.07	54.90 ± 43.64	1.10 ± 0.38	3.55 ± 0.78	2.45 ± 0.75	31.53 ± 9.45
2nd Trimester, *n*=53	11.05 ± 0.79	33.02 ± 2.50	28.25 ± 28.99	1.01 ± 0.38	4.29 ± 0.99	3.29 ± 1.11	25.07 ± 11.58
3rd Trimester, *n*=35	10.91 ± 1.09	33.40 ± 3.23	18.97 ± 20.27	1.00 ± 0.47	5.00 ± 0.87	4.00 ± 0.92	20.38 ± 9.22
p-value, *n*=109	0.001	< 0.001	< 0.001	0.605	< 0.001	< 0.001	0.001

[a]Data are presented as mean ± S.D.
*Significant differences observed between trimesters using ANOVA with Tukey post-hoc test

Table 4. Distribution of SNPs associated with blood iron concentration and status among study participants[a].

Genes and SNPs Investigated	Genotype Frequencies			Allele Frequencies		
	Homozygote Wild-type	Heterozygote	Homozygote Minor	Major Allele	Minor Allele	χ^2
HFE rs1800562	GG	AG	AA	G	A	
	109 (100)	-	-	218 (100)	-	-
HFE rs1799945	CC	CG	GG	C	G	
	107 (98.2)	2 (1.8)	-	216 (99.1)	2 (0.9)	-
TMPRSS6 rs855791	CC	CT	TT	C	T	
	28 (25.7)	45 (41.3)	36 (33.0)	101 (46.3)	117 (53.7)	3.14
TMPRSS6 rs4820268[b]	AA	AG	GG	A	G	
	27 (24.8)	43 (39.4)	39 (35.8)	97 (44.5)	121 (55.5)	4.39
TF rs3811647	GG	AG	AA	G	A	
	38 (34.9)	46 (42.2)	25 (22.9)	122 (56.0)	96 (44.0)	2.25
TF rs1799852	CC	CT	TT	C	T	
	40 (36.7)	55 (50.5)	14 (12.8)	135 (61.9)	83 (38.1)	0.54

[a]Data are presented as *n* (% based on *N*).
[b]SNP not in HW equilibrium

~23% carry the homozygous minor (risk) genotype of *TF* rs3811647, and only ~13% carry the homozygous minor genotype of *TF* rs1799852. There were ~2% of the study participants carrying the heterozygous genotype of *HFE* rs1799945, while only the major (wild-type) genotype of *HFE* rs1800562 was 100% present among the study participants. The calculated MAFs of *TMPRSS6* rs855791 and rs4820268 are greater than 0.5; hence, the T and G alleles of these SNPs are the major alleles in the study population. *TMPRSS6* rs4820268 was found to be deviating from Hardy-Weinberg proportions.

Table 5 presents the mean levels of the biochemical and hematological indicators of the study participants as they were grouped according to SNP genotypes. In terms of *HFE* rs1799945, carriers of the CG genotype have higher mean levels of SF, SI, and TS% and lower mean levels of TIBC and UIBC than the wild-type CC genotype carriers. On the other hand, the CC carriers have higher mean Hb and Hct levels. With *TMPRSS6* rs855791, the presence of two copies of the risk T allele (i.e., TT genotype) resulted in more reduced mean Hb, Hct, SF, SI, and TS% levels and higher mean TIBC and UIBC levels. No statistically significant difference was observed, however, in the levels of blood iron concentration and status of study participants based on *TMPRSS6* rs855791 genotypes. There were significant differences in the mean SI and TS% levels based on *TMPRSS6* rs4820268 genotypes. Carriers of the homozygous risk GG genotype have significantly lower mean SI level (p=0.033) than the wild-type AA carriers. Carriers of the risk GG and AG genotypes have also significantly lower mean TS% levels than the wild-type AA carriers (p=0.022, p=0.031). The study participants carrying the homozygous risk GG of *TMPRSS6* rs4820268 have the lowest mean Hb, Hct, and TS% levels and highest mean TIBC and UIBC levels. Significant differences in the mean TIBC and UIBC levels based on *TF* rs3811647 and rs1799852 genotypes were also observed. Carriers of the homozygous risk AA and AG genotypes of rs3811647 have

Table 5. Biochemical and hematological profiles based on SNP genotypes of study participants[a].

SNP Genotypes	Hb, g/dL	Hct, %	SF, ng/mL	SI, mg/L*	TIBC, mg/L*	UIBC, mg/L*	TS%*
***HFE* rs1799945**							
CC, *n*=107	11.19 ± 1.09	33.90 ± 3.29	30.37 ± 32.66	1.02 ± 0.41	4.39 ± 1.05	3.37 ± 1.13	24.61 ± 11.07
CG, *n*=2	10.65 ± 0.78	29.00 ± 1.41	32.00 ± 9.90	1.31 ± 0.10	3.82 ± 0.64	2.51 ± 0.74	35.06 ± 8.51
***TMPRSS6* rs855791**							
CC, *n*=28	11.31 ± 0.89	33.89 ± 2.86	35.36 ± 39.27	1.16 ± 0.48	4.22 ± 1.01	3.05 ± 1.08	29.03 ± 11.95
CT, *n*=45	11.24 ± 1.22	34.09 ± 3.75	28.82 ± 25.09	0.99 ± 0.39	4.38 ± 4.50	3.39 ± 1.07	23.50 ± 9.54
TT, *n*=36	11.01 ± 1.04	33.39 ± 3.17	28.53 ± 35.02	0.96 ± 0.36	4.50 ± 1.07	3.54 ± 1.21	23.15 ± 11.65
***TMPRSS6* rs4820268**							
AA, *n*=27	11.39 ± 0.90	34.19 ± 2.96	37.44 ± 39.15	1.20 ± 0.47[b]	4.18 ± 1.02	2.98 ± 1.05	30.08 ± 11.48[e]
AG, *n*=43	11.18 ± 1.21	33.81 ± 3.70	25.53 ± 23.43	0.98 ± 0.39[c]	4.37 ± 0.98	3.39 ± 1.02	23.31 ± 9.69[f]
GG, *n*=39	11.04 ± 1.05	33.54 ± 3.20	30.90 ± 35.51	0.95 ± 0.35[d]	4.52 ± 1.13	3.57 ± 1.24	22.80 ± 11.36[g]
***TF* rs3811647**							
GG, *n*=38	11.42 ± 1.06	34.42 ± 3.65	36.71 ± 33.07	1.05 ± 0.40	3.90 ± 0.86[h]	2.85 ± 0.91[k]	28.10 ± 11.20
AG, *n*=46	11.07 ± 1.18	33.65 ± 3.36	25.93 ± 29.84	0.96 ± 0.42	4.43 ± 1.01[i]	3.47 ± 1.08[l]	22.89 ± 10.60
AA, *n*=25	11.03 ± 0.89	33.16 ± 2.67	29.04 ± 35.46	1.09 ± 0.40	5.00 ± 1.04[j]	3.91 ± 1.21[m]	23.33 ± 11.10
***TF* rs1799852**							
CC, *n*=40	11.22 ± 0.96	33.83 ± 3.27	24.33 ± 28.30	1.07 ± 0.45	4.85 ± 0.91[n]	3.78 ± 1.05[q]	23.06 ± 10.56
CT, *n*=55	11.07 ± 1.22	33.64 ± 3.54	30.29 ± 27.22	0.98 ± 0.40	4.11 ± 1.00[o]	3.13 ± 1.06[r]	25.12 ± 10.77
TT, *n*=14	11.53 ± 0.74	34.43 ± 2.79	48.21 ± 52.65	1.06 ± 0.36	4.08 ± 1.16[p]	3.02 ± 1.27[s]	28.53 ± 13.46

[a]Data are presented as mean ± S.D. Analysis of SF and TS% were based also on log-transformed values.
*Different alphabets for the superscript indicate significant differences observed using ANOVA with Tukey post-hoc test in: (1) SI levels between *TMPRSS6* rs4820268 genotypes, p=0.028, (2) TS% levels between *TMPRSS6* rs4820268 genotypes, p=0.016, (3) TIBC levels between *TF* rs3811647 genotypes, p<0.001, (4) UIBC levels between *TF* rs3811647, p=0.001, (5) TIBC levels between *TF* rs1799852 genotypes, p=0.001, and (6) UIBC levels between *TF* rs1799852 genotypes, p=0.009.

significantly higher mean TIBC (p<0.001 and p=0.037) and UIBC (p<0.001 and p=0.022) levels than those with the wild-type GG genotype. AA and AG carriers have lower blood iron levels than wild-type GG carriers. With *TF* rs1799852, the study participants carrying the homozygous minor TT have, in general, the highest mean Hb, Hct, SF, SI, and TS% levels and lowest mean TIBC and UIBC levels. TT and CT carriers have significantly lower mean TIBC levels than the CC carriers (p=0.038 and p=0.002, respectively). Similarly, CT carriers have significantly lower mean UIBC level (p=0.013) than CC carriers.

Table 6 presents the differences in the mean ranks and adjusted means of blood iron levels between the two *HFE* rs1799945 genotypes and across the *TMPRSS6* and *TF* SNP genotypes, respectively. Several significant differences in levels of blood iron indices across SNP genotypes were identified. First, carriers of the homozygous wild-type CC genotype of *HFE* rs1799945 was found to have significantly higher Hct levels than the carriers of the CG genotype (p=0.022). Second, *TMPRSS6* rs855791 was significantly associated with UIBC and TS% levels. The mean UIBC level of the homozygous risk TT carriers was significantly higher than the carriers of homozygous wild-type CC genotype (p=0.019). The mean TS% levels of risk TT and CT genotypes were also significantly lower than the wild-type CC carriers (p=0.031 and p=0.029). Third, *TMPRSS6* rs4820268 was also significantly associated with UIBC and TS% levels. The mean UIBC level of the homozygous risk GG carriers was also significantly higher than the carriers of homozygous wild-type AA genotype (p=0.021). The mean TS% levels of risk GG and AG genotypes were also significantly higher than the wild-type AA carriers (p=0.020 and p=0.017). Lastly, *TF* rs3811647 was significantly associated with levels of TIBC and UIBC. The mean TIBC levels of the homozygous risk AA and AG carriers were significantly higher than the homozygous wild-type GG carriers (p=0.003, p=0.045). Likewise, the mean UIBC levels of AA and AG carriers were significantly higher than the GG carriers (p=0.009, p=0.019).

DISCUSSION

A total of 109 pregnant women aged 15-42 years old and living in Quezon, Palawan, Philippines participated in the study. More than half of the study participants were

Table 6. Differences in blood iron concentration and status across *HFE*, *TMPRSS6*, and *TF* SNP genotypes of study participants[a].

SNP Genotypes	Hb, g/dL	Hct, %*	SF, ng/mL	SI, mg/L	TIBC, mg/L*	UIBC, mg/L*	TS%, %*
***HFE* rs1799945**							
CC, *n*=107	55.39	55.87[b]	54.65	54.43	55.30	55.47	54.79
CG, *n*=2	34.00	8.50[c]	73.75	85.75	38.75	30.00	66.50
***TMPRSS6* rs855791**							
CC, *n*=28	11.32	33.93	36.59	1.16	4.10	2.94	29.48
	(10.9, 11.7)	(32.8, 35.1)	(25.3, 47.9)	(1.0, 1.3)	(3.8, 4.4)	(2.6, 3.3)[d]	(25.9, 33.0)[g]
CT, *n*=45	11.21	33.99	27.53	0.99	4.47	3.48	23.11
	(10.9, 11.5)	(33.1, 34.9)	(18.6, 36.5)	(0.9, 1.1)	(4.2, 4.7)	(3.2, 3.7)[e]	(20.3, 25.9)[h]
TT, *n*=36	11.04	33.49	29.18	0.96	4.48	3.52	23.28
	(10.7, 11.4)	(32.5, 34.5)	(19.3, 39.1)	(0.8, 1.1)	(4.2, 4.7)	(3.2, 3.8)[f]	(20.2, 26.4)[i]
***TMPRSS6* rs4820268**							
AA, *n*=27	11.35	34.09	36.89	1.19	4.13	2.94	29.89
	(11.0, 11.7)	(32.9, 35.3)	(25.4, 48.4)	(1.0, 1.3)	(3.8, 4.4)	(2.6, 3.3)[j]	(26.3, 33.5)[m]
AG, *n*=43	11.18	33.81	24.77	0.98	4.44	3.46	22.96
	(10.9, 11.5)	(32.9, 34.7)	(15.7, 33.8)	(0.9, 1.1)	(4.2, 4.7)	(3.2, 3.7)[k]	(20.1, 25.8)[n]
GG, *n*=39	11.06	33.62	32.13	0.96	4.48	3.52	23.31
	(10.7, 11.4)	(32.6, 34.6)	(22.6, 41.6)	(0.8, 1.1)	(4.2, 4.7)	(3.3, 3.8)[l]	(20.3, 26.3)[o]
***TF* rs3811647**							
GG, *n*=38	11.42	34.57	34.64	1.06	4.02	2.97	27.42
	(11.1, 11.8)	(33.6, 35.6)	(24.9, 44.4)	(0.9, 1.2)	(3.8, 4.3)[p]	(2.7, 3.2)[s]	(24.3, 30.5)
AG, *n*=46	11.07	33.65	25.28	0.97	4.48	3.52	22.73
	(10.8, 11.4)	(32.7, 34.5)	(16.5, 34.1)	(0.8, 1.1)	(4.3, 4.7)[q]	(3.3, 3.8)[t]	(19.9, 25.6)
AA, *n*=25	11.01	32.95	33.39	1.08	4.73	3.65	24.65
	(10.6, 11.4)	(31.7, 34.2)	(20.9, 45.9)	(0.9, 1.2)	(4.4, 5.1)[r]	(3.3, 4.0)[u]	(20.7, 28.7)
***TF* rs1799852**							
CC, *n*=40	11.26	33.75	27.95	1.06	4.60	3.54	24.34
	(10.9, 11.6)	(32.7, 34.8)	(18.1, 37.8)	(0.9, 1.2)	(4.3, 4.9)	(3.3, 3.8)	(21.1, 27.6)
CT, *n*=55	11.04	33.66	28.20	0.98	4.22	3.25	24.22
	(10.8, 11.3)	(32.8, 34.5)	(20.2, 36.2)	(0.9, 1.1)	(4.0, 4.4)	(3.0, 3.5)	(21.6, 26.9)
TT, *n*=14	11.54	34.55	46.11	1.11	4.36	3.25	28.41
	(11.0, 12.1)	(32.8, 36.3)	(29.7, 62.5)	(0.9, 1.3)	(3.9, 4.8)	(2.8, 3.7)	(23.0, 33.8)

[a]Data are presented as mean ranks for *HFE* rs1799945 genotypes using non-parametric Mann-Whitney U test. Data are presented as geometric means and confidence intervals for *TMPRSS6* rs855791, *TMPRSS6* rs4820268, *TF* rs3811647, and *TF* rs1799852 genotypes after adjusting and including maternal age, gestational age, iron supplementation, and area of residence as covariates for ANCOVA. Analysis of SF and TS% were based also on log-transformed values.

*Different alphabets for the superscript indicate significant differences observed in: (1) Hct levels between *HFE* rs1799945 genotypes, p=0.022; (2) UIBC and TS% levels across *TMPRSS6* rs855791 genotypes, p=0. 015, p=0.016; (3) UIBC and TS% levels across *TMPRSS6* rs4820268 genotypes, p=0.021, p=0.009, (4) TIBC and UIBC levels across *TF* rs3811647 genotypes, p=0.003, p=0.004.

taking up iron supplements daily. Based on maternal age grouping, lowest mean levels of blood iron were detected in groups at high-risk pregnancies. Hb and Hct as indicators of anemia were lowest in pregnant women aged 35 yr and over (i.e., old-age), while SF as an indicator of iron deficiency and body iron stores was lowest in pregnant women aged 19 yr and below (i.e., adolescents). These findings substantiate reports on the adverse effect of early or late pregnancies on iron status, which increases risk for maternal anemia and ultimately poor maternal and child health outcomes (Darnton-Hill & Mkparu 2015; WHO 2004; Mei et al. 2011). Additionally,

by grouping the study participants based on trimester of pregnancy, the continuous decrease in blood iron status (especially on hemoglobin and serum ferritin levels) throughout pregnancy was demonstrated (Scholl 2011). There is a continuous decrease in levels of blood iron concentration towards the end of pregnancy, reaching its lowest points of Hb, Hct, SF, SI, and TS% during the third trimester, while TIBC and UIBC are at its highest values. In this study, the mean levels of blood iron indices were found to be significantly different between trimesters, with each indicator decreasing in levels with an increase in trimester of pregnancy. Resulting TIBC levels in the current study also support the reports of the CDC (1998) and AACC (2015) in that pregnancy can raise TIBC readings, especially at late stages.

Proportions of the study population were found anemic (37.6%) and iron-deficient (47.7%) based on the WHO cut-offs for Hb and SF levels in pregnant women, respectively. Despite daily iron supplementation, there were participants who remained anemic and iron-deficient. Surprisingly, more than half of the anemic or iron-deficient pregnant women were already on iron supplementation. According to the WHO, not all pregnant women may fully respond to oral iron supplementation and that the iron supplement may only partially correct the hematological deficit because of the relatively large increase in iron requirement during pregnancy. Additionally, some pregnant women cannot sufficiently absorb additional iron when on treatment or supplementation (WHO 2001). Thus, there is really a need to identify the underlying genetic factors of anemia and iron deficiency in the Filipino population. Identification of the candidate genes and SNPs that are associated with blood iron levels among Filipinos shall lead to more innovative strategies and solutions (i.e., pharmacogenomics and personalized medicine or nutrition) towards the reduction of prevalences of anemia, iron deficiency, and IDA in the country (Laing 2011).

One of the most relevant findings of this study is the high minor allele frequencies of SNPs in *TMPRSS6* and *TF* in the study population. The calculated MAFs in the study population were higher than the resulting MAFs in the 1000 Genomes Project: 0.54 vs. 0.3954/1980 in rs855791, 0.56 vs. 0.4565/2286 in rs4820268, 0.44 vs. 0.340/1706 in rs3811647, and 0.38 vs. 0.1426/715 in rs1799852 (Sherry et al. 2001). Furthermore, a two-fold difference in the MAF of *TF* rs1799852 from the 1000 Genomes Project was observed. The current MAFs are close to the reported frequency data of nearby Asian populations (i.e., Han Chinese from the Coriell Cell Repository and International HapMap Project and Japanese from the HapMap phase 3) (dbSNP Short Genetic Variations 2017) and this may support why anemia is also prevalent in other Asian populations. It should also be noted that the supposedly minor T and G alleles of *TMPRSS6* rs855791 and rs4820268, respectively, are the major alleles in the study population. Deviation from the Hardy-Weinberg equilibrium of *TMPRSS6* rs4820268 was observed to be significant; hence, this should be further evaluated and established using a larger population size. On the other hand, the SNPs in *HFE* (rs1800562 or C282Y and rs1799945 or H63D) were reported to be common only among Americans and Caucasians (IDI 2009). In this study, the absence of the minor A of rs1800562 among the participants suggests that the minor A allele may not be truly present in Filipinos. Additionally, there is only a small percentage of the minor G of rs1799945 in the study population. These replicated previous findings on the rarity of *HFE* gene variants among Asians (Sherry et al. 2001).

This pilot study also provided relevant information on the potential association of SNPs in *HFE*, *TMPRSS6*, and *TF* with the levels of blood iron concentration and status among pregnant Filipino women. SNPs in *HFE* lead to hemochromatosis or the condition of having too much iron in the body. Hence, the presence of the minor A and G alleles (i.e., heterozygote) were reported to be protective against IDA (Datz et al. 1998). In this study, a significant association between *HFE* rs1799945 homozygous wild-type CC and higher Hct levels was observed; and the participants carrying the minor G allele of rs1799945 have lower Hb and Hct levels. If these hematological indicators are to be considered, CG of rs1799945 may not protect against anemia and iron deficiency in pregnant Filipino women. But looking into the other parameters, CG carriers were observed to have higher levels of SF, SI, and TS% but lower TIBC and UIBC levels. Blanco-Rojo and colleagues (2011) had previously shown that *HFE* rs1799945 is associated with higher iron status in women with the minor G allele than those carrying the homozygous wild-type CC genotype. HFE is known to regulate intestinal iron absorption by modulating the expression of hepcidin, a hormone that functions in systemic iron metabolism. Hence, efficient means of utilizing nutritional iron, in addition to enhanced absorption of dietary iron, may explain the current observations. It should be noted, however, that a very small percentage of the study participants carry the CG genotype. This stresses the need to validate and establish the role of *HFE* rs1799945 in the pathogenesis of anemia, iron deficiency, and IDA in Filipinos using a larger study population.

Second are the significant associations of *TMPRSS6* rs855791 and rs4820268 with higher UIBC and lower TS% after adjusting for covariates, as well as the decrease in blood iron levels with increasing copies of the risk alleles in both SNPs. *TMPRSS6* was discovered as the strongest

genetic link of IDA in 2009, and to date, *TMPRSS6* has the strongest reported association with indices of blood iron concentration and status (Pei et al. 2014). Variants in this gene have been studied across different populations since then in order to clearly define its role in the pathogenesis of anemia, iron deficiency, and IDA. Although the exact mechanism by how each SNP works is yet to be fully understood, it is suggested that *TMPRSS6* SNPs function by altering hepcidin transcription in response to systemic iron concentrations (Gichohi-Wainaina et al. 2015). Chambers co-workers (2009) reported through a genome-wide association study that Hb concentration is lowered by 0.13 g/dL per copy of the risk T allele in rs855791, while the variants of rs4820268 were also strongly associated with lower Hb levels in a population of European and Indian Asian ancestry. Benyamin and co-workers (2009a) then showed that the risk T allele is also associated with SI, TS%, and erythrocyte mean cell volume in Australian twin families. An and co-authors (2012) also showed that rs855791 is a genetic risk factor for both iron deficiency and IDA in a population of elderly Chinese women, where *TMPRSS6* SNPs were also associated with lower levels of Hb and SI in this population. A significant association of *TMPRSS6* rs4820268 with SI concentration was also reported by Tanaka and colleagues (2010). The minor G allele of rs4820268 was initially shown to be associated with reduced SI levels of the study participants. Hence, the present study replicates the association of *TMPRSS6* rs855791 and rs4820268 with lowered blood iron concentration and status in pregnant Filipino women. The identified associations between *TMPRSS6* SNPs and indicators of blood iron status together with the computed high MAFs among the participants may explain the high incidences of anemia and iron deficiency in the study population. These initial findings and the role of *TMPRSS6* as a susceptibility gene of anemia and iron deficiency in Filipinos, however, need further validation.

Lastly, this study shows the significant association of SNPs in *TF* with TIBC and UIBC levels in pregnant Filipino women. Transferrin plays an important role in iron metabolism by carrying iron in blood plasma. Severe mutations in the *TF* gene lead to atransferrinaemia or the absence of serum transferrin which, in turn, leads to severe microcytic (small RBCs) and hypochromic (pale RBCs) anemia (Gichohi-Wainaina et al. 2015). In the current study, the homozygous wild-type GG of *TF* rs3811647 was significantly associated with lower TIBC and UIBC levels of the study participants, while the homozygous wild-type CC of *TF* rs1799852 was found to have higher TIBC and UIBC levels (indicative of IDA) than heterozygous CT. Additionally, the study participants with the minor allele of *TF* rs3811647 (AG and AA) have lower mean levels of Hb, Hct, and SF, whereas the carriers of the homozygous minor TT genotype of *TF* rs1799852 have the highest mean Hb, Hct, and SF levels. McLaren and co-workers (2011) previously reported the association of rs3811647 with levels of TIBC among iron-deficient cases and iron-replete controls, and that increasing values of TIBC are associated with increasing copies of the minor A allele. Benyamin and co-authors (2009b) had also shown that *TF* rs3811647 and rs1799852 were significantly associated with serum transferrin or TIBC level. It was also reported that the minor A allele of rs3811647 confers risk to IDA while the minor T allele of rs1799852 protects against IDA (Blanco-Rojo et al. 2011). This study replicated the association of rs3811647 with higher TIBC levels; hence, supporting previous claims that rs3811647 minor A presents susceptibility to IDA. This study also showed the association of rs1799852 with decreased TIBC levels, which demonstrates the protective role of rs1799852 minor T against IDA. Nonetheless, these findings need to be validated using a larger population of pregnant Filipino women.

CONCLUSION

The present study provides relevant information on the genotype distribution of SNPs in *HFE*, *TMPRSS6*, and *TF*, and their potential association with levels of blood iron concentration and status among pregnant Filipino women. The SNPs investigated have relatively higher MAFs in the study population as compared to existing data, which qualified the T allele of *TMPRSS6* rs855791 and G allele of rs482068 as the major alleles in the study population. The SNPs in *TMPRSS6* and *TF* rs3811647 were shown to be potentially associated with reduced blood iron levels, while *HFE* rs1799945 and *TF* rs1799852 seem to be protective against anemia and iron deficiency. These SNPs may potentially function as markers or genetic risk factors for anemia, iron deficiency, and IDA in pregnant Filipino women.

It is highly recommended to replicate the SNP genotyping and perform association studies using a larger study population in order to fully establish the association of candidate SNPs with anemia and iron deficiency in Filipinos. Other genes and SNPs that were also reported to be associated with blood iron concentration and status warrant further exploration among Filipinos.

ACKNOWLEDGMENT

The authors are indebted to: Mr. Rod Erick L. Agarrado for his assistance from blood collection up to the conduct of downstream analyses in the Nutritional Genomics Laboratory of the DOST-FNRI; Ms. Ma. Lynell

Valdeabella-Maniego and Mr. Glen Melvin P. Gironella of the Nutritional Assessment and Monitoring Division, DOST-FNRI for their guidance and review of the statistical analyses; Ms. Maria-Julia Golloso-Gubat and Ms. Leah A. Perlas of the Nutrition Research and Development Group, DOST-FNRI for their technical review of the paper; the entire program team of "S&T Based Solutions towards Sustainable Strategy for Child Malnutrition: The First 1000 Days Window of Opportunity"; the local health and nutrition officers in municipalities of Quezon, Palawan, Philippines, and; the participants of the study.

Funding: This work was supported by the General Fund of the Department of Science and Technology-Food and Nutrition Research Institute and Grants-in-Aid Funds of the Department of Science and Technology-Philippine Council for Industry, Energy, and Emerging Technology Research and Development.

REFERENCES

[AACC] American Association for Clinical Chemistry. 2015. TIBC, UIBC, and Transferrin. Retrieved from https://labtestsonline.org/understanding/analytes/tibc/tab/all/ on Mar 2017.

AN P, WU Q, WANG H, GUAN Y, MU M, LIAO Y, ZHOU D, SONG P, WANG C, MENG L, MAN Q, LI L, ZHANG J, WANG F. 2012. *TMPRSS6*, but not *TF*, *TFR2* or *BMP2* variants are associated with increased risk of iron-deficiency anemia. Hum Mol Genet 21(9):2124-31. doi:10.1093/hmg/dds028.

BARTON JC, EDWARDS CQ, ACTON RT. 2015. *HFE* gene: Structure, function, mutations, and associated iron abnormalities. Gene 574(2):179-192. https://doi.org/10.1016/j.gene.2015.10.009.

BENYAMIN B, FERREIRA MAR, WILLEMSEN G, GORDON S, MIDDELBERG RPS, MCEVOY BP, HOTTENGA J-J, HENDERS AK, CAMPBELL MJ, WALLACE L, FRAZER IH, HEATH AC, DE GEUS EJC, NYHOLT DR, VISSCHER PM, PENNINX BW, BOOMSMA DI, MARTIN NG, MONTGOMERY GW, WHITFIELD JB. 2009a. Common variants in *TMPRSS6* are associated with iron status and erythrocyte volume. Nature Genetics 41(11):1173-75. doi:10.1038/ng.456.

BENYAMIN B, MCRAE AF, ZHU G, GORDON S, HENDERS AK, PALOTIE A, PELTONEN L, MARTIN NG, MONTGOMERY GW, WHITFIELD JB, VISSCHER PM. 2009b. Variants in *TF* and *HFE* Explain ~40% of Genetic Variation in Serum-Transferrin Levels. Am J Hum Genet 84(1):60-65. doi:10.1016/j.ajhg.2008.11.011.

BLANCO-ROJO R, BAEZA-RICHER C, LOPEZ-PARRA AM, PEREZ-GRANADOS AM, BRICHS A, BERTONCINI S, BUIL A, ARROYO-PARDO E, SORIA JM, VAQUERO MP. 2011. Four variants in transferrin and *HFE* genes as potential markers of iron deficiency anaemia risk: an association study in menstruating women. Nutr Metab 8:69. doi:10.1186/1743-7075-8-69.

[CDC] U.S. Centers for Disease Control and Prevention. 1998. Recommendations to Prevent and Control Iron Deficiency in the United States. Morb Mortal Wkly Rep 47(RR-3):1-36.

CHAMBERS JC, ZHANG W, LI Y, SEHMI J, WASS MN, ZABANEH D, HOGGART C, BAYELE H, MCCARTHY MI, PELTONEN L, FREIMER NB, SRAI SK, MAXWELL PH, STERNBERG MJE, RUOKONEN A, ABECASIS G, JARVELIN M-R, SCOTT J, ELLIOTT P, KOONER JS. 2009. Genome-wide association study identifies variants in *TMPRSS6* associated with hemoglobin levels. Nature Genetics 41(11):1170-72. doi:10.1038/ng.462.

[CLSI] Clinical and Laboratory Standards Institute. 1998. Determination of Serum Iron, Total Iron-Binding Capacity and Percent Transferrin Saturation; Approved Standard. Volume 18(19). ISBN 1-56238-362-0, ISSN 0273-3099.

CORPET F. 1988. Multiple sequence alignment with hierarchical clustering. Nucl Acids Res 16(22):10881-90.

DARNTON-HILL I, MKPARU UC. 2015. Micronutrients in Pregnancy in Low- and Middle-Income Countries. Nutrients 7(3):1744-68. doi: 10.3390/nu7031744.

Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. (dbSNP Build ID: rs1800562). Available from: http://www.ncbi.nlm.nih.gov/SNP/.

Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. (dbSNP Build ID: rs1799945). Available from: http://www.ncbi.nlm.nih.gov/SNP/.

Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. (dbSNP Build ID: rs855791). Available from: http://www.ncbi.nlm.nih.gov/SNP/.

Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. (dbSNP

Build ID: rs4820268). Available from: http://www.ncbi.nlm.nih.gov/SNP/.

Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. (dbSNP Build ID: rs3811647). Available from: http://www.ncbi.nlm.nih.gov/SNP/.

Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. (dbSNP Build ID: rs1799852). Available from: http://www.ncbi.nlm.nih.gov/SNP/.

DATZ C, HAAS T, RINNER H, SANDHOFER F, PATSCH W, PAULWEBER B. 1998. Heterozygosity for the C282Y mutation in the hemochromatosis gene is associated with increased serum iron, transferrin saturation, and hemoglobin in young women: a protective role against iron deficiency? Clin Chem 44:2429-32.

[FNRI-DOST] Food and Nutrition Research Institute - Department of Science and Technology. 2015. Philippine Nutrition Facts and Figures 2013. FNRI Bldg., DOST Compound, Bicutan, Taguig City, Metro Manila, Philippines.

GICHOHI-WAINAINA WN, SWINKELS DW, ZIMMERMANN MB, FESKENS EJ, Melse-Boonstra A. 2015. Inter-ethnic differences in genetic variants within the transmembrane protease, serine 6 (*TMPRSS6*) gene associated with iron status indicators: A systematic review with meta-analyses. Genes Nutr 10(1):442. doi: 10.1007/s12263-014-0442-2.

[ICSH] International Council for Standardization in Haematology. 1978. Recommendations for reference method for haemoglobinometry in human blood (ICSH Standard EP 6/2: 1977) and specifications for international haemiglobincyanide reference preparation (ICSH Standard EP 6/3: 1977). J Clin Pathol 31(2):139-143.

[IDI] Iron Disorders Institute. 2009. Classic Hemochromatosis. Retrieved from http://www.irondisorders.org/classic-hemochromatosis on Mar 2017.

LAING RE, HESS P, SHEN Y, WANG J, HU SX. 2011. The Role and Impact of SNPs in Pharmacogenomics and Personalized Medicine. Curr Drug Metab 12(5):460-86. DOI: 10.2174/138920011795495268.

MAGBITANG JA, TANGCO JBM, DE LA CRUZ EO, FLORES EG, GUANLAO FE. 1988. Weight-for-Height as a Measure of Nutritional Status in Filipino Pregnant Women. Asia Pac J Public Health 2(2):96-104.

MCLAREN CE, GARNER CP, CONSTANTINE CC, MCLACHLAN S, VULPE CD, SNIVELY BM, GORDEUK VR, NICKERSON DA, COOK JD, LEIENDECKER-FOSTER C, BECKMAN KB, ECKFELDT JH, BARCELLOS LF, MURRAY JA, ADAMS PC, ACTON RT, KILLEEN AA, MCLAREN GD. 2011. Genome-Wide Association Study Identifies Genetic Loci Associated with Iron Deficiency. PLoS One 6(3):e17390. doi:10.1371/journal.pone.0017390.

MCLAREN CE, MCLACHLAN S, GARNER CP, VULPE CD, GORDEUK VR, ECKFELDT JH, ADAMS PC, ACTON RT, MURRAY JA, LEIENDECKER-FOSTER C, SNIVELY BM, BARCELLOS LF, COOK JD, MCLAREN GD. 2012. Associations between Single Nucleotide Polymorphisms in Iron-Related Genes and Iron Status in Multiethnic Populations. PLoS One 7(6):e38339. https://doi.org/10.1371/journal.pone.0038339.

MEI Z, COGSWELL ME, LOOKER AC, PFEIFFER CM, CUSICK SE, LACHER DA, GRUMMER-STRAWN LM. 2011. Assessment of iron status in US pregnant women from the National Health and Nutrition Examination Survey (NHANES), 1999–2006. Am J Clin Nutr 93(6):1312-1320. doi: 10.3945/ajcn.110.007195.

[NHLBI] National Heart, Lung, and Blood Institute, US National Institutes of Health. 2014. Who Is at Risk for Iron-Deficiency Anemia? Retrieved from https://www.nhlbi.nih.gov/health/health-topics/topics/ida/atrisk on March 2017.

PEI SN, MA MC, YOU HL, FU HC, KUO CY, RAU KM, WANG MC, LEE CT. 2014. TMPRSS6 rs855791 polymorphism influences the susceptibility to iron deficiency anemia in women at reproductive age. Int J Med Sci 11(6):614-9. doi: 10.7150/ijms.8582.

RAJEEVAN H, SOUNDARARAJAN U, KIDD JR, PAKSTIS AJ, KIDD KK. 2012. ALFRED: an allele frequency resource for research and teaching. Nucleic Acids Res 40(D1): D1010-D1015. doi:10.1093/nar/gkr924.

SCHOLL TO. 2011. Maternal iron status: relation to fetal growth, length of gestation and the neonate's iron endowment. Nutr Rev 69(Suppl 1):S23-S29. doi:10.1111/j.1753-4887.2011.00429.x.

SHERRY ST, WARD MH, KHOLODOV M, BAKER J, PHAN L, SMIGIELSKI EM, SIROTKIN K. 2001. dbSNP: the NCBI database of genetic variation. Nucleic Acids Res 29(1):308-11.

TANAKA T, ROY CN, YAO W, MATTEINI A, SEMBA RD, ARKING D, WALSTON JD, FRIED LP, SINGLETON A, GURALNIK J, ABECASIS

GR, BANDINELLI S, LONGO DL, FERRUCCI L. 2010. A genome-wide association analysis of serum iron concentrations. Blood 115:94-96. doi:10.1182/blood-2009-07-232496.

The UK Haemochromatosis Consortium. 1997. A simple genetic test identifies 90% of UK patients with haemochromatosis. Gut 41(6):841-844.

UNTERGASSER A, CUTCUTACHE I, KORESSAAR T, YE J, FAIRCLOTH BC, REMM M, ROZEN SG. 2012. Primer3--new capabilities and interfaces. Nucleic Acids Res 40(15):e115.

[WHO] World Health Organization. 2004. Adolescent Pregnancy. Issues in Adolescent Health and Development. Geneva. ISBN 92-4-159145-5.

[WHO] World Health Organization. 2001. Iron Deficiency Anaemia: Assessment, Prevention and Control: A guide for programme managers. Geneva: World Health Organization (WHO/NHD/01.3).

[WHO] World Health Organization (WHO/NMH/NHD/MNM/11.1). 2011a. Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity. Vitamin and Mineral Nutrition Information System. Geneva.

[WHO] World Health Organization (WHO/NMH/NHD/MNM/11.2). 2011b. Serum ferritin concentrations for the assessment of iron status and iron deficiency in populations. Vitamin and Mineral Nutrition Information System. Geneva.