

The *TMPRSS6* variant (SNP rs855791) affects iron metabolism and oral iron absorption – a stable iron isotope study in Taiwanese women



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ABSTRACT

Genome wide studies have associated *TMPRSS6* rs855791 (2321 C>T) with iron status and hepcidin. It is unclear whether this polymorphism affects iron absorption. We administered standardized rice-based test meals containing 4 mg of labeled ⁵⁷Fe or ⁵⁸Fe as FeSO₄ on alternate days in non-anemic Taiwanese women (n=79, 44 TT variant, 35 CC variant). Fractional iron absorption was measured by erythrocyte incorporation of the tracers 14 days after administration. Compared to the CC variant, iron and transferrin saturation were lower ($P=0.001$; $P<0.001$, respectively) and serum hepcidin/transferrin saturation and serum hepcidin/serum iron ratios were higher ($P=0.042$; $P=0.088$, respectively) in the TT variant. Serum hepcidin did not differ between the groups ($P=0.862$). Geometric mean (95% Confidence Interval [CI]) fractional iron absorption, corrected to a serum ferritin of 15 µg/L, was 26.6% (95% CI: 24.0-29.5) in the CC variant and 18.5% (95% CI: 16.2-21.1) in the TT variant ($P=0.002$). Overall, predictors of iron absorption were: serum ferritin ($P<0.001$); genetic variant ($P=0.032$); and hepcidin ($P<0.001$). In the models by variant, in the CC variant the model explained 67-71% of variability in absorption and serum ferritin was the only significant predictor ($P<0.001$); while in the TT variant, the model explained only 35-43% of variability, and hemoglobin ($P=0.032$), soluble transferrin receptor ($P=0.004$) and hepcidin ($P<0.001$) were significant predictors. Women with the *TMPRSS6* rs855791 (2321 C>T) polymorphism show altered iron homeostasis which affects oral iron absorption and may increase their risk for iron deficiency. The trial was registered as clinicaltrials.gov. Identifier: NCT03317873, and funded by the Kaohsiung Chang-Gung Memorial Hospital, Kaohsiung, Taiwan, (grant CMRPG8F0721) and ETH Zurich, Switzerland.

Introduction

In the absence of a physiological iron excretion mechanism, long-term iron balance in humans is determined by dietary iron absorption. Systemically, iron absorption is controlled by hepcidin (Hep), a peptide hormone synthesized in hepatocytes¹ that regulates iron export from cells via its interaction with ferroportin.² Hep is synthesized in response to increasing body iron in a homeostatic feedback loop, involving iron sensing of iron-saturated transferrin by transferrin receptors (Tfr1 and Tfr2) and associated proteins (HFE, hemojuvelin) initiating a cascade involving bone morphogenic protein (BMP) receptor activation.^{3,4}

The *TMPRSS6* gene encodes the transmembrane serine protease matriptase-2, which interacts with hemojuvelin, modulating the Hep activation pathway.⁵ Consistently with this regulatory model, nonsense mutations in *TMPRSS6* cause

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iron refractory iron deficiency anemia (IRIDA), due to inappropriately elevated Hep levels.⁶ The ratio of serum Hep/transferrin saturation (Hep/TS) may be useful to differentiate subjects with IRIDA from subjects with chronic iron deficiency (ID),⁷ consistent with a disrupted feedback loop between TS and Hep.

Common genetic variants of *TMPRSS6*, are associated with erythrocyte parameters in human genome wide association studies.⁸⁻¹¹ The single nucleotide polymorphism (SNP) rs855791 (2321 C>T) of *TMPRSS6* has a population frequency of ≈ 0.5 in Caucasians,^{10,12} ≈ 0.6 in Japanese¹³ and $\approx 0.2-0.1$ in African Americans.^{10,12} It causes a non-synonymous substitution near the catalytic and active site of the protease,¹⁰ with a strong association with iron status, erythrocyte parameters,^{8-10,12,14-17} Hep¹⁸ and ratios of Hep to iron indices.^{19,20} T-allele variants in the rs855791 are associated with an increased risk for ID and iron deficiency anemia (IDA).^{16,17} In a case control study in Taiwan, homozygotes for the SNP rs855791 CC had a lower prevalence of IDA, compared to subjects with the CT or TT variant.²¹ In European populations, variants (TT) in rs855791 are associated with lower TS and serum ferritin (SF), higher Hep, and higher ratios of Hep to iron indices.¹⁸⁻²⁰ In first time blood donors, the TT variant was associated with larger decreases in SF and hemoglobin (Hb) after multiple donations, suggesting an impaired capacity to replenish stores following donation.²²

ID is considered the most prevalent nutritional deficiency worldwide and one of the leading causes of anemia among non-pregnant and pregnant women.²³ While iron status and dietary composition are the main determinants of iron absorption, individual factors other than iron status have been estimated to account for $\approx 50\%$ of the variance in iron absorption.²⁴ Furthermore, a strong familial tendency in iron absorption has been reported in mother child pairs using stable iron isotopes;^{25,26} this could be due to genetic, epigenetic or shared environmental mechanisms.

The genetic determinants of iron status and Hep metabolism in humans, including the effect of mutations in *TMPRSS6*, are poorly understood. The study aim was to compare iron absorption, Hep and other indices of iron metabolism in iron-sufficient Taiwanese women carrying the TT or the CC variant of the rs855791 SNP in the *TMPRSS6* gene. We hypothesized that the TT variant would be associated with higher serum Hep concentrations, higher ratios of Hep to iron indices, and lower iron absorption at comparable iron status.

Methods

Subjects

The study was performed at the Kaohsiung Chang Gung Memorial Hospital (K-CGMH) in Taiwan, between February 2018 and February 2019. The flow of study participants is shown Figure 1. We invited apparently healthy females, with no known history of thalassemia or anemia, aged between 20 to 45 years for screening, assessed their medical history and measured body weight and height, complete blood count, SF and the rs855791 genotype. Inclusion criteria are described in the *Online Supplementary Appendix*. All participants that were homozygous in the rs855791 (TT or CC), and fulfilled all inclusion criteria were recalled 1 week before the first test meal administration, where we assessed Hb, SF, C-reactive protein (CRP), and menstrual blood losses. Study inclusion criteria were: i) Hb > 120 g/L;

ii) SF 30–120 $\mu\text{g/L}$ and iii) CRP <5 mg/L. The ethical committees of ETH Zurich in Switzerland and the Chang Gung Memorial Foundation Institutional Review Board in Taiwan approved the study. All participants provided written informed consent, and the study was registered as clinicaltrials.gov. Identifier: NCT03317873.

On study days 1 and 3 (D1, D3), we administered two standardized rice test meals to fasting participants, labeled with 4 mg iron (⁵⁷Fe, and ⁵⁸Fe) as labeled ferrous sulfate (FeSO₄). A detailed description of the test meal administration and the preparation of stable iron isotopes, can be found in the *Online Supplementary Appendix*.

Laboratory analyses

We determined fractional iron absorption (FIA) based on the shift in the enrichment ratio of stable iron isotopes into the erythrocytes on D17. We performed the analyses by inductively coupled plasma mass spectrometry (MC-ICP-MS, Neptune; Thermo Finnigan) as previously described.²⁷ We calculated the amounts of ⁵⁷Fe, and ⁵⁸Fe isotopic labels in blood on D17 on the basis of the shift in iron isotope ratios and on the estimated amount of iron circulating in the body.²⁸ We corrected the FIA for SF to the cutoff for ID (15 $\mu\text{g/L}$),²⁹ and to 50 $\mu\text{g/L}$ as a level representing sufficient iron stores with a modification of the Cook *et al.* formula,³⁰ as described in the *Online Supplementary Appendix*. Procedures such as the assessment of menstrual blood loss, and laboratory measurements such as genotyping, measurement of erythrocyte parameters, CRP, acute phase protein α -1-acid glycoprotein (AGP), SF, serum iron (SFe), total iron-binding capacity (TIBC), Hep, and soluble transferrin receptor (sTfR) are also described in the *Online Supplementary Appendix*.

Sample size calculation

We based the sample size calculation on a design with two repeated measurements with a compound symmetry covariance structure. Based on previous studies from the Human Nutrition Laboratory, using log-transformed data, we assumed an intra-individual correlation of 0.7, and a standard deviation of 0.235. A difference of 30% in iron absorption was considered relevant. Therefore, we planned to recruit 40 subjects per variant, with 80% power and $\alpha = 0.05$, it allows two dropouts per group. Due to the imbalanced distribution of the minor allele in the Taiwanese population, and difficulties enrolling the planned number of CC subjects, we made a protocol amendment to include 35 CC and 45 TT subjects. This unbalanced distribution results in an estimated power of 75%.

Data and statistical analysis

We used IBM SPSS statistics (Version 24) for statistical analysis. After testing for normality, we used log-transformed data further analysis if not normally distributed. Normally distributed data is presented as means \pm standard deviation (SD), transformed normal data as geometric mean with the 95% Confidence Interval (95% CI), non-normal data as median and the interquartile range (IQR). Means or medians of red cell parameters, are based on the concentrations measured on D1. Means, medians, or geometric means of CRP, AGP, SF, SFe, TIBC, TS, sTfR, BIS, Hep, Hep/TS, Hep/SF, and FIA are based on concentrations measured on D1 and D3. We tested between-group differences for i) normally distributed variables using independent samples t-test and for ii) not normally distributed variables using Mann-Whitney U test, as well as iii) differences in CRP, AGP, SF, SFe, TIBC, TS, sTfR, BIS, Hep, Hep/TS, Hep/SF, and FIA by linear mixed models (LMM), with subjects' code as a random intercept, the corresponding variable as a dependent variable and the genotype as a fixed effect. We

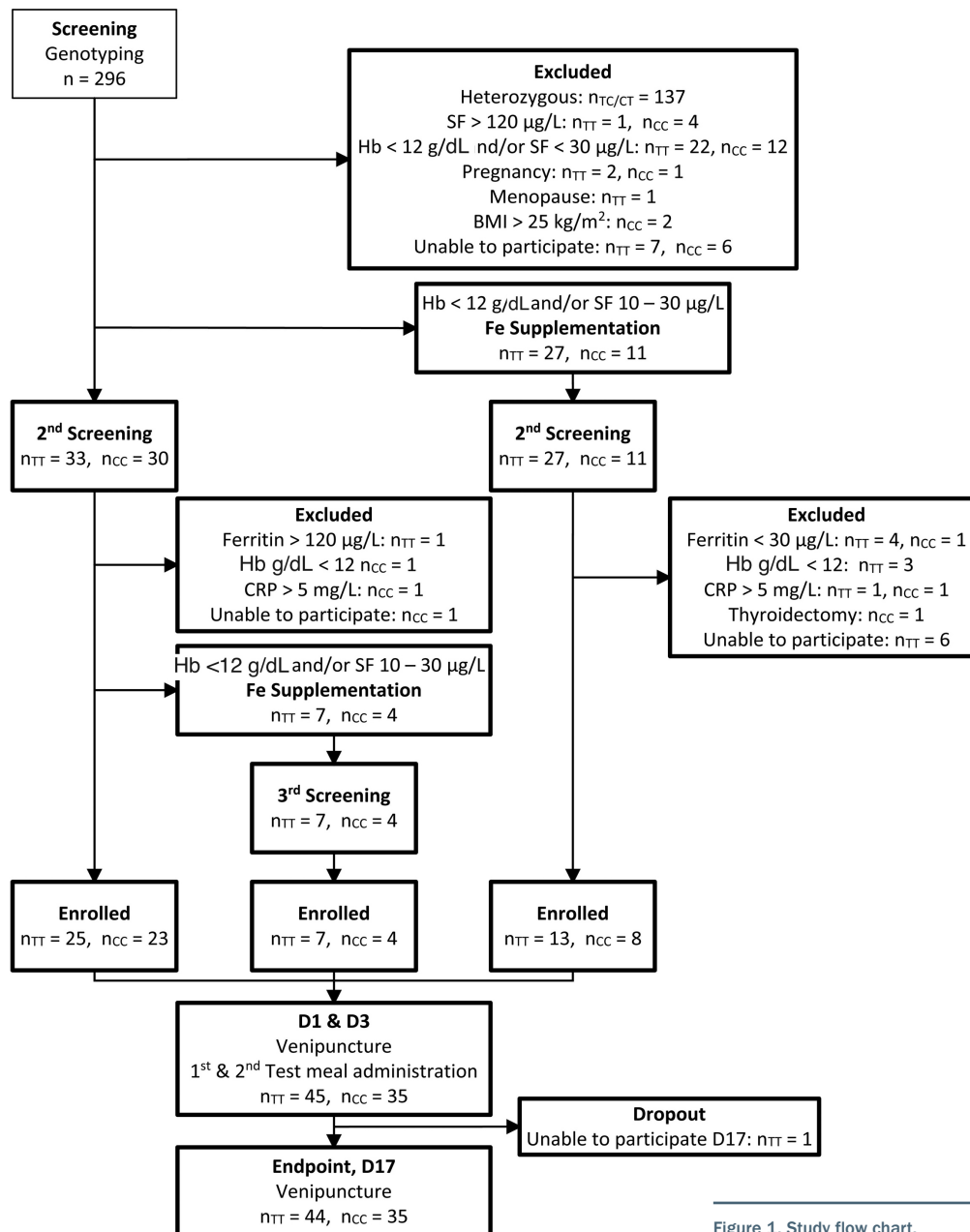


Figure 1. Study flow chart.

assessed Pearson's correlations and differences between the coefficients with with the Fisher Z-transformation. We assessed predictors of iron absorption with LMM using subjects' code as a random intercept, FLA as a dependent variable, and the genotype, Hb, SF, TS, sTfR, Hep and PBAC as fixed factors. We performed a backward linear regression to assess a minimal adequate model, and we fitted the variables in a LMM. Statistical significance was defined as $P < 0.05$.

Results

Subjects

We screened 296 women and identified 93 women carrying the TT variant and 66 with the CC variant, while 137 were excluded as heterozygotes (Figure 1). Of the identified subjects, 33 women with the TT variant and 30 with CC variant met all inclusion criteria. Thirty-four

women with TT variants and 15 with CC variants received iron supplements. After the iron supplementation period, 20 subjects with the TT and 12 subjects with the CC variant were included into the study. Finally, 35 subjects with CC and 45 with the TT variant fulfilled all study inclusion criteria and were enrolled (Figure 1). One woman with the TT variant left the study after study D3, thus, 79 women completed the study.

Iron indices

SF concentrations were balanced between the two variants, while SFe was lower in the TT compared to the CC variant ($P = 0.001$; Table 1). Similarly, TS was lower ($P < 0.001$), and TIBC higher ($P = 0.086$) in the TT variant (Table 1). While Hep did not differ between the groups ($P = 0.862$), the Hep/TS ratio ($P = 0.042$) and the Hep/SFe ratio ($P = 0.088$) were 28% and 25% higher in the TT variant, respectively (Table 1). None of the subjects had sys-

Table 1. Subject characteristics of Taiwanese women with the homozygous CC and TT variants of the rs855791 in *TMPRSS6*.

	CC	TT	P
n	35	45	-
Age, y*	34 ± 6	36 ± 7	0.436 [§]
Weight, kg*	54.6 ± 4.8	54.2 ± 5.2	0.717 [§]
Height, cm*	160 ± 4	160 ± 5	0.780 [§]
CRP, mg/L [†]	0.271 (0.213, 0.346)	0.388 (0.306, 0.491)	0.125
AGP, g/L*	0.426 ± 0.0962	0.455 ± 0.113	0.206
RBC, million/ μ L [‡]	4.51 (4.19-4.59)	4.58 (4.37-4.74)	0.032 [¶]
Hb, g/dL	13.3 ± 0.6	13.3 ± 0.7	0.762 [§]
HCT, % [‡]	40.0 (38.4-40.9)	40.3 (38.7-41.5)	0.349 [¶]
MCV, fL/cell [‡]	89.8 (88.3-92.0)	88.4 (86.4-90.6)	0.008 [¶]
MCH, pg/cell [‡]	30.0 (29.2-31.0)	29.6 (28.8-30.0)	0.018 [¶]
SF, μ g/L [†]	45.1 (41.0, 49.7)	47.0 (43.5, 50.9)	0.626
SFe, μ g/dL [†]	114.5 (105.2, 124.7)	90.3 (82.6, 98.7)	0.001
TIBC, μ g/dL	315.6 ± 29.7	327.3 ± 33.5	0.086
TS, % [†]	36.5 (33.3, 39.9)	27.7 (25.5, 30.2)	<0.001
sTfR, mg/L [†]	4.14 (3.97, 4.32)	4.33 (4.12, 4.55)	0.351
BIS, mg/kg BW	7.13 ± 1.62	7.12 ± 1.80	0.978
Hep, nM [†]	2.10 (1.80, 2.46)	2.06 (1.79, 2.37)	0.862
Hep/SFe, pmol/ μ g [†]	183.4 (160.3, 209.8)	227.8 (193.3, 268.4)	0.088
Hep/TS, pM/% [†]	57.6 (50.8, 65.4)	74.2 (63.0, 87.4)	0.042
Hep/SF, pmol/ μ g [†]	46.5 (40.7, 53.2)	43.7 (38.4, 49.8)	0.537
PBAC*	126 ± 63	171 ± 99	0.015 [§]

Anthropometrics, red blood cell (RBC) indices and hemoglobin (Hb) were assessed on day (D) 1, inflammation, iron parameters, plasma hepcidin concentration were assessed on D1 and D3. AGP: acute phase protein α -1-acid glycoprotein; BIS: body iron stores; CRP: C-reactive protein; Hb: hemoglobin; HCT: hematocrit; Hep: plasma hepcidin; MCH: mean corpuscular hemoglobin; MCV: mean corpuscular volume; PBAC: pictorial blood-loss assessment chart; SF: serum ferritin; SFe: serum iron; sTfR: soluble transferrin receptor; TS: transferrin saturation; y: years. *Means \pm standard deviation (SD); [†]geometric means (95% Confidence Interval [CI]); [‡]medians (interquartile range [IQR]); [§]differences were assessed by two-sided independent *t*-test; ^{||}differences were assessed by fitting linear mixed models with genotype as fixed effect, participants as the random effects, and the corresponding variable as dependent variable; [¶]differences were assessed by Mann-Whitney U test.

temic inflammation, during the study period (Table 1). The menstrual blood loss scores (PBAC) was higher in the TT variant ($P=0.015$, Table 1).

Fractional iron absorption

The uncorrected FIA on D1 and D3 within variant did not differ (Table 2; Figure 2) but the Pearson's correlation between days (D1 and D3) FIA was stronger in the CC ($r=0.86$) than in the TT variant ($r=0.67$; for both, $P<0.001$; Figure 2). The mean uncorrected FIA of D1 and D3 of the TT variant, was 7.96% (95% CI: 6.87-9.22), and in the CC variant was 6.50% (95% CI: 5.54-7.62) ($P=0.160$; Figure 2). FIA corrected to a SF concentration of 15 μ g/L was significantly lower in the TT 18.5% (95% CI: 16.2-21.1), compared to the CC variant 26.6% (95% CI: 24.0-29.5) ($P=0.002$; Table 2; Figure 2). When corrected to a SF of 50 μ g/L, the TT variant had significantly higher FIA than the CC variant: 7.59% (95% CI: 6.66-8.66), compared to 5.70% (95% CI: 5.15-6.31) ($P=0.012$).

Correlation of fractional iron absorption, iron indices and hepcidin

The Pearson's correlation between FIA and SF was more pronounced in the CC variant ($r=-0.79$, $P<0.001$) than in the TT variant ($r=-0.45$, $P=0.002$) and there was a difference in the strength of the correlation between groups ($P<0.001$, Figure 3). Fractional absorption was correlated with TS only in the CC variant (CC: $r=-0.45$, $P=0.006$; TT $r=-0.14$, $P=0.360$) and the correlation coefficients tended to differ ($P=0.070$). The correlation of FIA with Hep was

more pronounced ($P=0.004$) in the CC variant (CC: $r=-0.81$, $P<0.001$, TT: $r=-0.45$, $P=0.002$).

Predictors of fractional iron absorption

Genetic variant ($\beta=-0.346$, $P=0.032$) was a significant predictor of overall FIA along with SF ($\beta=-0.393$, $P<0.001$), and Hep ($\beta=-0.312$, $P<0.001$), ($R^2_{adjusted}=0.468$), Table 3. Stepwise deletion removed TS, PBAC and CRP from the model ($R^2_{adjusted}=0.469$). In the prediction model by variant, in the CC variant only SF was significantly associated with FIA ($\beta=-0.696$, $P<0.001$), explaining 67% of the variability in iron absorption ($R^2_{adjusted}=0.669$) (Table 4). In contrast, in the TT variant, Hep ($\beta=-0.353$, $P<0.001$), sTfR ($\beta=0.317$, $P=0.004$), Hb ($\beta=-0.252$, $P=0.023$), and TS ($\beta=0.199$, $P=0.011$) were associated with FIA (Table 4), but with a substantially lower coefficient of determination ($R^2_{adjusted}=0.375$), explaining 38% of the variability. In the minimal adequate model (Table 5), for the CC variant, significant predictors are SF ($\beta=-0.667$, $P<0.001$) and Hep ($\beta=-0.217$, $P=0.002$) ($R^2_{adjusted}=0.688$). For the TT variant, significant predictors are Hep, ($\beta=-0.411$, $P<0.001$) sTfR, ($\beta=0.320$, $P=0.003$) and Hb ($\beta=-0.226$, $P=0.038$) ($R^2_{adjusted}=0.356$, Table 5).

Discussion

Our study shows that the *TMPRSS6* rs855791 TT variant is associated with lower iron absorption in an overall model controlling for other iron status indicators. At a

Table 2. Fractional iron absorption from rice meals in CC and TT variants of the rs855791.

	CC variant	TT variant	P
FIA _{D1} , %	6.50 (5.14, 8.22)*	7.99 (6.39, 9.98) [†]	0.206 [‡]
FIA _{D3} , %	6.49 (5.17, 8.15)*	7.93 (6.50, 9.68) [†]	0.183 [‡]
FIA _{D1} & D ₃ , %	6.50 (5.54, 7.62)	7.96 (6.87, 9.22)	0.160 [§]
FIA _{D1} & D ₃ , SF15corr, %	26.6 (24.0, 29.5)	18.5 (16.2, 21.1)	0.002 [§]

Values are the geometric means and the 95% Confidence Interval (CI). D1: study day 1; D3: study day 3; FIA: fractional iron absorption; SF15corr: serum ferritin correction to a concentration of 15 µg/L. *differences between study day one and three were assessed by paired *t* test $P=0.935$. [†]differences between the two variants were assessed by two-sided independent *t*-test. [‡]differences between the two variants were assessed by fitting linear mixed models with genotype as fixed effect, participants as the random effects, and the corresponding variable as dependent variable. [§]correction was done using the formula: $\log(\text{FIAC}) = \log(\text{FIAO}) + a \cdot \log(\text{SFC/SFO})$, with $a_{\text{CC}} = -1.28$, $a_{\text{TT}} = -0.74$.

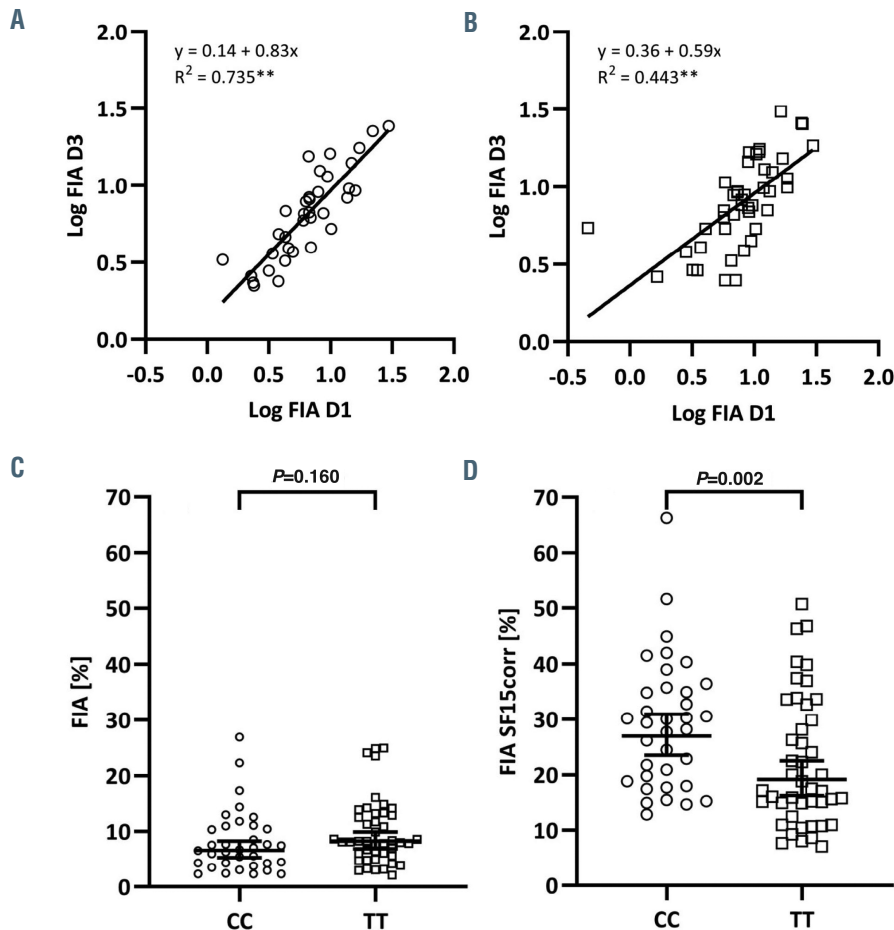


Figure 2. Correlation of the inter-individual fractional iron absorption (A and B) and fractional iron absorption (C-D) in rs855791 variants. (A and B) Fractional iron absorption (FIA) measured from identical rice test meals on study day one and three, separated by variant in the TMPRSS6 rs855791, in (A) the CC variant (○ n=35), and in (B) the TT (□ n=44). The Pearson's correlation factors are: 0.86, and 0.67 for the CC and TT, respectively (both, ** $P<0.001$). (C and D) Each point represents the mean of the FIA on day 1 (D1) and D3 from two identical rice meals, the line represents the geometric mean and the bars the 95% Confidence interval (CI). (C) the measured FIA versus (D) the FIA corrected to a serum ferritin (SF) concentration of 15 µg/L are shown. Differences between the two variants were assessed by fitting linear mixed models with genotype as fixed effect, participants as the random effects, and FIA or FIA corrected for SF as the dependent variable CC (○, n=35) versus TT (□, n=44).

standardized SF concentration of 15 µg/L, iron absorption was significantly lower in the TT variant. The TT variant also had lower TS and SFe and higher Hep/TS ratios, suggesting an altered interplay of SFe, hepcidin, iron stores and the regulation of dietary absorption compared to the CC variant. Similarly, known predictors of iron absorption explained much less of the variability in iron absorption in the TT variant. To our knowledge, this is the first study comparing dietary iron absorption using stable iron isotopes among the common SNP rs855791 of the *TMPRSS6*, which has been associated with iron status and red blood cell parameters in various genome wide association studies and large cross-sectional studies.^{10,12,15-17}

In humans, inter-subject absorption of nonheme iron shows a wide variation in healthy young women. Zimmermann *et al.* reported a variation from 1% to 58% in iron absorption from standardized test meals labeled with 4 mg Fe as stable isotopes.³¹ Some of this variation is

due to differences in iron status and meal matrix, however, taken together, it has been estimated that iron status and food factors predict only ≈50% of the variance in iron absorption in a population.²⁴ Cook *et al.*³² reported a striking positive correlation in body iron in iron-replete mothers and their young children and suggested this close correlation was due to a shared diet and/or possible genetic determinants of iron status such as shared iron-regulatory genes. In Mexican (n=18), and Senegalese mother-child pairs (n=17), non-heme-iron absorption measured with stable isotopes exhibited strong and intermediate correlations, respectively.^{25,26} A common polymorphism in the transferrin protein (G277S) has been associated with ID in American women,³³ but a stable isotope study comparing 25 iron deficient, non-anemic women who had either a heterozygous G277S/G277G or wild-type G277G/G277G genotype did not find a significant difference in iron absorption.³⁴ However, the G277S carriers did not show

Table 3. Predictors of iron absorption in healthy Taiwanese women (n=79).

Variables	Overall Model*			Minimal Adequate Model†		
	β	SE	P	β	SE	P
Intercept	0.14	0.10	0.159	0.13	0.01	0.197
Variant (CC vs. TT)‡	-0.35	0.16	0.032	-0.30	0.15	0.045
Hemoglobin	-0.13	0.07	0.085	-0.11	0.07	0.154
Serum Ferritin	-0.39	0.08	<0.001	-0.41	0.07	<0.001
Transferrin Saturation	0.11	0.06	0.063	removed from the model§		
Soluble Transferrin Receptor	0.12	0.07	0.083	0.12	0.07	0.105
Plasma Hepcidin	-0.31	0.06	<0.001	-0.29	0.06	<0.001
PBAC	0.07	0.08	0.389	removed from the model§		
C-reactive Protein	-0.05	0.07	0.434	removed from the model§		

Analyzed by LMM using standardized variables, dependent variable: fractional iron absorption; fixed factors: potential continuous or categorical predictors; random effects: Subjects' code. Shown are standardized β -coefficients standard errors (SE). Hemoglobin, menstrual blood loss scores (PBAC) were assessed on day (D) 1, inflammation, iron parameters, plasma hepcidin concentration, and fractional iron absorption (FIA) are based on data measured on D1 and D3. *Regression model fit: $R^2=0.498$; $R^2_{adjusted}=0.468$; †assessed by backward linear regression; regression model fit: $R^2=0.486$; $R^2_{adjusted}=0.469$; ‡nominal variable; 1=CC, 2=TT; §removed variable by the backward regression to assess the minimal adequate model.

Table 4. Potential predictors of iron absorption in variants of the *TMPRSS6* rs855791 (nCC=35, nTT=44).

Variables	CC Variant*			TT Variant†		
	β	SE	P	β	SE	P
Intercept	0.0003	0.08	0.997	-0.02	0.10	0.851
Hemoglobin	0.05	0.09	0.559	-0.25	0.11	0.023
Serum Ferritin	-0.70	0.010	<0.001	-0.20	0.11	0.060
Transferrin Saturation	-0.02	0.07	0.752	0.20	0.08	0.011
Soluble Transferrin Receptor	-0.12	0.08	0.161	0.32	0.10	0.004
Plasma Hepcidin	-0.16	0.08	0.053	-0.35	0.08	<0.001
PBAC	-0.12	0.09	0.229	0.14	0.11	0.211
C-reactive Protein	0.04	0.08	0.614	-0.05	0.10	0.610

Analyzed by LMM using standardized variables, dependent variable: fractional iron absorption; fixed factors: potential continuous or categorical predictors; random effects: Subjects' code. Shown are standardized β -coefficients with their standard errors. Hemoglobin, menstrual blood loss scores (PBAC) were assessed on day (D) 1, inflammation, iron parameters, plasma hepcidin concentration, and fractional iron absorption are based on data measured on D1 and D3. *Regression model fit of CC variant: $R^2=0.707$; $R^2_{adjusted}=0.669$; †regression model fit of TT variant: $R^2=0.432$; $R^2_{adjusted}=0.375$.

the typical inverse correlation between iron absorption and SF.³⁴ Similarly, in our study with the *TMPRSS6* rs855791 mutation, in the TT variant, the correlation between iron absorption and SF, and iron absorption and Hep, were only weak and moderate, respectively. In contrast to the CC variant, where both these correlations are strong. Also the models computed by variant show remarkable differences. In the CC variant SF alone is significantly associated with FIA, explaining 67% of the variability. In the TT variant, in contrast, several factors identify as being associated with FIA: Hep, sTfR, Hb, and TS, and their total contribution explain only 38% of the variability in iron absorption.

Our hypothesis that the CC variant would have increased iron absorption was based on a regulatory model of *TMPRSS6* acting as a negative regulator of the Hep activation pathway, and we hypothesized the largest effects would be seen in an iron replete population, where Hep expression would be activated. However, our findings indicate the effects of the genetic variant are likely most relevant at low iron status (Figure 3); at lower SF, women with the TT variant were less able to upregulate iron absorption, which could increase the risk for ID. Further, the overall model (Table 3) shows that iron status indices, Hep, and genotype, but not inflammation and menstrual blood loss are associated with fractional iron absorption. A recent large study in blood donors suggests an impaired capacity in the TT

variant to replenish iron stores after repeated blood donations, even if the possibly protective CC variant was not enriched in high intensity donors.²² It is also possible that cellular mechanisms controlled by iron regulatory proteins are, especially at intermediate serum iron levels, able to compensate for the altered interplay of Hep and TS in the TT variant by inducing the translation of iron transporters (e.g., DMT1) and transcription factor HIF-2 α .³⁵ Such a compensatory mechanism was suggested in a recent study in women in whom an acute inflammatory stimulus increased Hep but did not affect iron absorption.³⁶

Our findings suggest that, at low SF concentrations, women with the TT variant have lower iron absorption, whereas when iron stores are replete, they may be less able to downregulate iron absorption compared to the CC variant. Our variant-specific FIA correction to SF uses a similar approach as the original formula of Cook *et al.*³⁰ used to correct dietary absorption measurements for the individual iron status; that formula employs a slope of -1 between log FIA and log SF. We propose adapted, regression formulas with slopes of -1.28 and -0.74 for CC and TT variants, respectively (Figure 3).

In a case control study in Taiwanese women comparing women with IDA to non-anemic controls, the CC variant was less frequent in the IDA group compared to the control group (12% vs. 25%); this suggests the CC variant

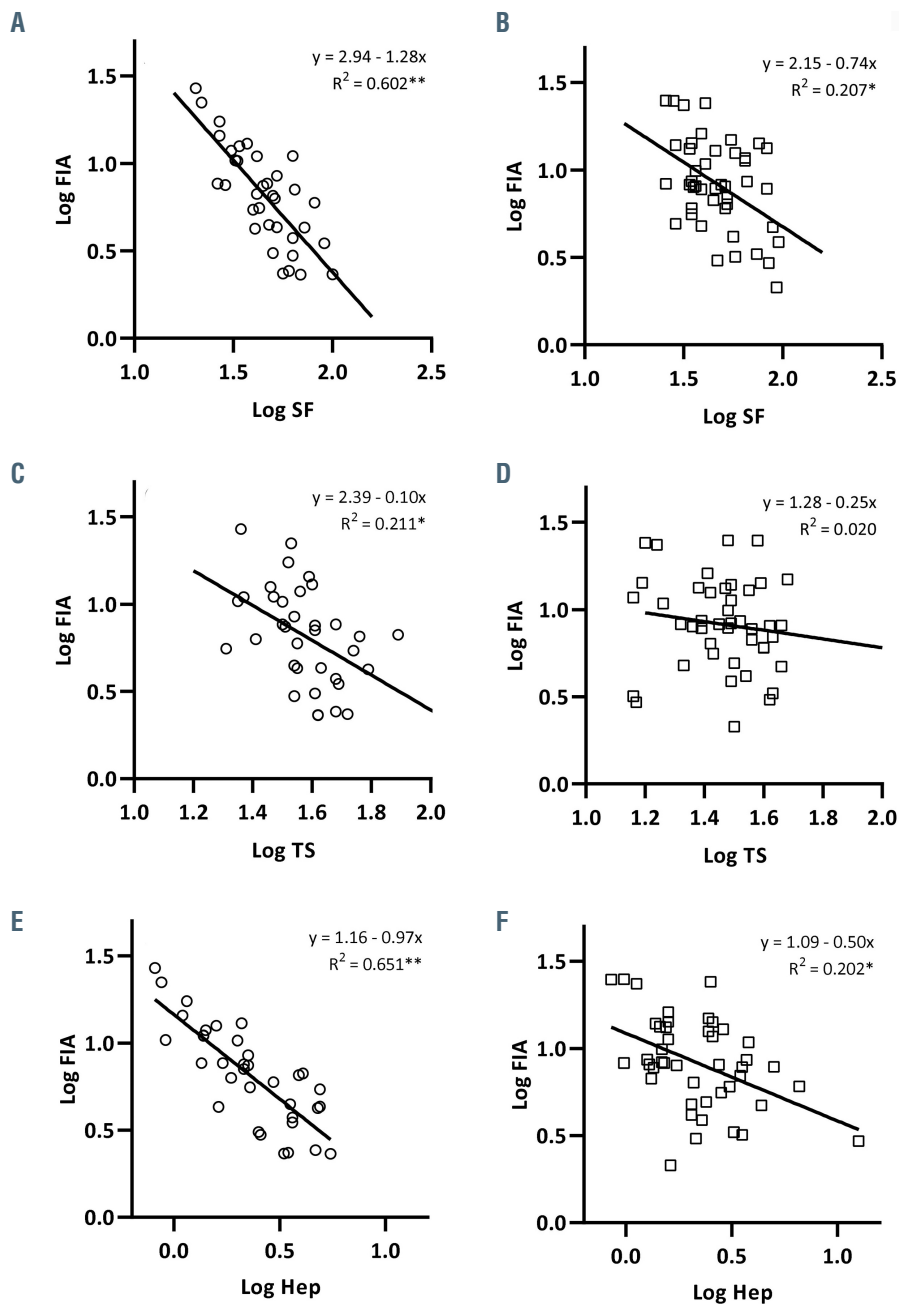


Figure 3. Correlations of fractional iron absorption. Correlations between fractional iron absorption (FIA) and serum ferritin (SF) (A and B), transferrin saturation (TS) (C and D) and hepcidin (Hep) (E and F) of the participants separated by the variants in the *TMPRSS6* rs855791, CC (○, n=35) and TT (□, n=44). Each point represents one participant and their mean of FIA, SF, TS, and Hep measured on study day 1 (D1) and D3. Pearson's correlation factors *r* for FIA to SF correlation are: -0.79 ($P < 0.001$) and -0.45 ($P = 0.002$); for FIA to TS correlation: -0.45 ($P = 0.006$) and -0.14 ($P = 0.360$); for FIA to Hep correlation: -0.81 ($P < 0.001$) and -0.45, ($P = 0.002$), for the CC variants and TT variants respectively. $^{**}P < 0.001$, $^*P < 0.05$.

may reduce risk of IDA.²¹ This effect is also suggested in our screening data: among the screened subjects, 60% of women with the TT variant had either Hb below 12 g/dL and/or SF below the study inclusion criteria, compared to 42% of women with the CC variant. Also, among women who received iron supplementation due to ID, 80% of the women with the CC variant replenished their iron stores, in contrast to only 59% of women with the TT variant. While this is consistent with the view that women with the TT variant are at a higher risk for ID and may have a blunted response to iron supplements when body iron stores are low, this hypothesis needs confirmation in larger prospective trials. Further mechanistic studies in monozygotic twins would be particularly informative as they may distinguish potential genetic and epigenetic sources of variability in iron absorption.

Our findings are consistent with previous studies that have shown that Sfe and transferrin saturation are lower,

and TIBC is higher in the TT variant.¹⁸⁻²⁰ The higher Hep/TS ratio reported in women with the TT variant in our study has been previously described in Italian¹⁸ and Dutch populations.²⁰ In our study, the lack of association between variant and Hep suggests a different modulation of iron regulatory signals (transferrin bound iron and iron stores) in the regulation of Hep between the two different variants. Consistent with this interpretation, a recent study has suggested that the Hep/TS ratio may be a useful diagnostic marker to differentiate IRIDA patients from those with chronic ID.⁷

A strength of this study is that iron absorption was assessed from an isotopically labeled standardized labeled test meals in a relatively large number of subjects, using erythrocyte incorporation of stable iron isotope labels. Due to its precision, this approach allows, in combination with iron indices, to study regulatory aspects of iron metabolism in humans.^{36,37} We performed iron absorption

Table 5. The minimal adequate model and predictors of iron absorption in variants of the *TMPRSS6* rs855791 ($n_{CC}=35$, $n_{TT}=44$).

Variables	CC variant*			TT Variant†		
	β	SE	P	β	SE	P
Intercept	0.0004	0.08	0.996	-0.01	0.11	0.911
Hemoglobin		removed from the model‡	-0.23	0.11	0.038	
Serum Ferritin	-0.67	0.09	<0.001		removed from the model‡	
Transferrin Saturation		removed from the model‡			removed from the model‡	
Soluble Transferrin Receptor		removed from the model‡	0.32	0.10	0.003	
Plasma Hepcidin	-0.22	0.07	0.002	-0.41	0.08	<0.001
PBAC	-0.11	0.09	0.226		removed from the model‡	
C-reactive protein		removed from the model			removed from the model‡	

The minimal adequate model is assessed by backward linear regression using standardized variables. Parameters shown are analyzed by LMM, dependent variable: fractional iron absorption; fixed factors: potential continuous or categorical predictors; random effects: Subjects' code. Shown are standardized β -coefficients with their standard errors (SE). Hemoglobin, menstrual blood loss scores (PBAC) were assessed on day (D) 1, inflammation, iron parameters, plasma hepcidin concentration, and fractional iron absorption are based on data measured on D1 and D3. *Regression model fit of CC variant: $R^2=0.702$; $R^2_{adjusted}=0.688$; †regression model fit of TT variant: $R^2=0.378$; $R^2_{adjusted}=0.356$; ‡removed variable by the backward regression to assess the minimal adequate model.

measurements twice in each subject; this increased statistical power and allowed us to make intra-individual comparisons. Our study also has limitations: our assessment of menstrual loss using PBAC is semi-quantitative, and while we found no association with iron absorption after correcting for iron status in the overall model, we cannot fully exclude a potential effect of menstrual blood loss on iron absorption. Our proposed genotype-specific slopes of SF and iron absorption are based on a relatively narrow range of iron status and should be studied in populations with broader iron status distribution. We focused our hypothesis on a single SNP, and we did not study the interplay with other SNP known to affect iron homeostasis. However, the *HFE* rs1800562 (C282Y) mutation is known to be rare in Taiwanese women.³⁸ In contrast, the *GNPAT* rs11558492 has been associated with a high-iron phenotype,³⁹ and a recent study in Taiwanese women has shown a minor allele frequency of 12%, and a significant higher serum iron response after a supplement.⁴⁰ We did not study heterozygotes, despite the fact that effects on iron absorption are conceivable in this group. Furthermore, unknown SNP associated with rs855791 may explain the observed effects. However, we think this possibility is unlikely, as rs855791 has been repeatedly shown to be associated with iron status, as discussed above.

To summarize, we have shown that in a fully adjusted model of iron absorption, women with the TT variant have lower iron absorption compared to women with the CC variant. This may be associated with higher Hep/TS and Hep/SFe ratios, suggesting impaired negative feedback on Hep synthesis by circulating iron. Furthermore, in the TT variant, regulation of iron absorption is less well

predicted by iron stores. Thus, our findings suggest women with the TT variant are less able to upregulate iron absorption at low iron status, which may increase their risk of ID.

Disclosures

No conflicts of interest to disclose.

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Contributions

MBZ, DM, SB, and SNP designed the study; SNP, SCH and CTL conducted the study and collected the samples; SB and CZ analyzed the samples and performed the statistical analyses, SB, DM, MBZ, and SNP participated in the data interpretation; SB wrote the first draft of the manuscript; all authors edited the manuscript and approved the final version.

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