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The $\textit{TMPRSS6}$ variant (SNP rs855791) affects iron metabolism and oral iron absorption – a stable iron isotope study in Taiwanese women

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SB and SNP contributed equally to this publication.

Running head: A $\textit{TMPRSS6}$ polymorphism affects iron absorption

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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. In nonanemic Taiwanese women (n=79, 44 TT variant, 35 CC variant), we administered standardized rice-based test meals containing 4 mg of labeled $^{57}$Fe or $^{58}$Fe as FeSO$_4$ on alternate days. Fractional iron absorption was measured by erythrocyte incorporation of the tracers 14 days after administration. Compared to the CC variant, in the TT variant serum 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). Serum hepcidin did not differ between groups ($P=0.862$). Geometric mean (95% CI) fractional iron absorption, corrected to a serum ferritin of 15 μg/L, was 26.6% (24.0, 29.5) in the CC variant and 18.5% (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 ($P<0.001$); 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 at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) as NCT03317873, and funded by the Kaohsiung Chang-Gung Memorial Hospital, Kaohsiung, Taiwan, (grant CMRPG8F0721) and ETH Zurich, Switzerland.
Introduction

In 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, a peptide hormone synthesized in hepatocytes\(^1\) that regulates iron export from cells via its interaction with ferroportin.\(^2\) Hepcidin (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 hepcidin activation pathway.\(^5\) Consistently with this regulatory model, nonsense mutations in *TMPRSS6* cause iron refractory iron deficiency anemia (IRIDA), due to inappropriately elevated hepcidin levels.\(^6\) The ratio of serum hepcidin/transferrin saturation (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 hepcidin.

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 Japanese\(^13\) and ≈0.2-0.1 in African Americans.\(^10,12\) It causes a nonsynonymous substitution near the catalytic and active site of the protease,\(^10\) with a strong association with iron status, erythrocyte parameters\(^8,10,12,14-17\), Hepcidin\(^18\) 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 hepcidin 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. 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 ≈50% of the variance in iron absorption. Furthermore, a strong familial tendency in iron absorption has been reported in mother child pairs using stable iron isotopes; this could be due to genetic, epigenetic or shared environmental mechanisms.

The genetic determinants of iron status and hepcidin metabolism in humans, including the effect of mutations in TMPRSS6, are poorly understood. The study aim was to compare iron absorption, hepcidin 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 hepcidin concentrations, higher ratios of hepcidin 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 for screening apparently healthy females, with no known history of thalassemia or anemia, aged between 20 to 45 years, assessed medical history and measured body weight and height, complete blood count, SF and the rs855791 genotype. Inclusion criteria are described in the Online Supplementary Methods. All participants that were homozygous in the rs855791 (TT or CC), and fulfilled all inclusion criteria were recalled one week before the first test meal administration, where we assessed Hb, SF, C-reactive protein (CRP), and menstrual blood losses. Study inclusion criteria were: 1) Hb > 120 g/L; 2) SF 30 – 120 µg/L and 3) 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 at clinicaltrials.gov (NCT03317873).
On study days one and three (D1, D3), we administered two standardized rice test meals to fasting participants, labelled with 4 mg iron ($^{57}$Fe, and $^{58}$Fe) as labelled ferrous sulfate (FeSO$_4$). Detailed description of the test meal administration and preparation of stable iron isotopes, can be found in the Online Supplementary Methods.

**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 $^{57}$Fe, and $^{58}$Fe 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 µg/L)$^{29}$, and to 50 µg/L as a level representing sufficient iron stores with a modification of the Cook et al. formula,$^{30}$ as described in the Online Supplementary Methods. Procedures such as the assessment of menstrual blood loss, and laboratory measurements such as genotyping, measurement of erythrocyte parameters, CRP, acute phase protein alpha-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 Methods.

**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 2 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 ± 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 normally distributed variables with independent samples T-Test and for not normally distributed variables using Mann-Whitney U Test; 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 random intercept, the corresponding variable as dependent variable and genotype as fixed effect. We assessed Pearson’s correlations and differences between the coefficients with the Fishers r to z transformation. We assessed predictors of iron absorption with LMM using subjects’ code as random intercept, FIA as dependent variable, and 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 $P < .05$.

**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**
Serum ferritin 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 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 systemic 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% (6.87, 9.22), and in the CC variant was 6.50% (5.54, 7.62) ($P = 0.160$; Figure 2). FIA corrected to a SF concentration of 15 µg/L was significantly lower in the TT 18.5% (16.2, 21.1), compared to the CC variant 26.6% (24.0, 29.5) ($P = 0.002$; Table 2; Figure 2). When corrected to a SF of 50 µg/L, the TT variant had significantly higher FIA than the CC variant: 7.59% (6.66, 8.66), compared to 5.70% (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 ($\beta = -0.312$, $P < 0.001$), ($R^2_{\text{adjusted}} = 0.468$), Table 3. Stepwise deletion removed TS, PBAC and CRP from the model ($R^2_{\text{adjusted}} = 0.469$). 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 ($R^2_{\text{adjusted}} = 0.669$) (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 ($R^2_{\text{adjusted}} = 0.375$), 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 ($\beta = -0.217$, $P = 0.002$) ($R^2_{\text{adjusted}} = 0.688$). For the TT
variant, significant predictors are Hep, \( \beta = -0.411, P < 0.001 \) sTfR, \( \beta = 0.320, P = 0.003 \) and Hb \( \beta = -0.226, P = 0.038 \) \( R^2_{\text{adjusted}} = 0.356, \text{Table 5} \).

**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 standardized serum ferritin concentration of 15 µg/L, iron absorption was significantly lower in the TT variant. The TT variant also had lower transferrin saturation and serum iron and higher Hep/TS ratios, suggesting an altered interplay of serum iron, 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 labelled 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 \( \approx 50\% \) 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 \( (n = 17) \), nonheme-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, nonanemic 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 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 serum ferritin 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: hepcidin, soluble transferrin receptors, hemoglobin, and transferrin saturation, 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 hepcidin activation pathway, and we hypothesized the largest effects would be seen in an iron replete population, where hepcidin 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, hepcidin, 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. 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 hepcidin and transferrin saturation in the TT variant by inducing the translation of iron transporters (e.g., DMT1) and transcription factor HIF-2α. 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.

Our findings suggest that, at low serum ferritin 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 serum ferritin uses a similar approach as the original formula of Cook et al. 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 nonanemic controls, the CC variant was less frequent in the IDA group compared to the control group (12% versus 25%); this suggests the CC variant may reduce risk of IDA.
This effect is also suggested in our screening data: among the screened subjects, 60% of women with the TT variant had either an hemoglobin below 12 g/dl and/or a serum ferritin below the study inclusion criteria, compared to 42% of women with the CC variant. Also, among women who received iron supplementation because of 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 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 serum iron and transferrin saturation are lower, and total iron binding capacity higher in the TT variant.\textsuperscript{18-20} The higher Hep/TS ratio reported in women with the TT variant in our study has been previously described in Italian\textsuperscript{18} and Dutch populations.\textsuperscript{20} In our study, the lack of association between variant and hepcidin suggests a different modulation of iron regulatory signals (transferrin bound iron and iron stores) in the regulation of hepcidin 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.\textsuperscript{7}

A strength of this study is that iron absorption was assessed from an isotopically labelled 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.\textsuperscript{36,37} We performed iron absorption 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 serum ferritin 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 SNPs known to affect iron homeostasis.
However, the *HFE* rs1800562 (C282Y) mutation is known to be rare in Taiwanese women.\textsuperscript{38} In contrast, the *GNPAT* rs11558492 has been associated with a high-iron phenotype,\textsuperscript{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\textsuperscript{40}. We did not study heterozygotes, despite the fact that effects on iron absorption are conceivable in this group. Furthermore, unknown SNPs 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 hepcidin 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.
Contributors

Contribution: MBZ, DM, SB, and SNP designed the study; SNP, SCH and CTL conducted the study and collected the samples; SB 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.

Declaration of interests

Conflict-of-interest disclosure: None of the authors declare a conflict of interest.

References

### Tables

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

<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>TT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>35</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Age, y*</td>
<td>34 ± 6</td>
<td>36 ± 7</td>
<td>0.436§</td>
</tr>
<tr>
<td>Weight, kg*</td>
<td>54.6 ± 4.8</td>
<td>54.2 ± 5.2</td>
<td>0.717§</td>
</tr>
<tr>
<td>Height, cm*</td>
<td>160 ± 4</td>
<td>160 ± 5</td>
<td>0.780§</td>
</tr>
<tr>
<td>CRP, mg/L†</td>
<td>0.271 (0.213, 0.346)</td>
<td>0.388 (0.306, 0.491)</td>
<td>0.125§</td>
</tr>
<tr>
<td>AGP, g/L*</td>
<td>0.426 ± 0.0962</td>
<td>0.455 ± 0.113</td>
<td>0.206§</td>
</tr>
<tr>
<td>RBC, million/µl‡</td>
<td>4.51 (4.19-4.59)</td>
<td>4.58 (4.37-4.74)</td>
<td>0.032¶</td>
</tr>
<tr>
<td>Hb, g/dl*</td>
<td>13.3 ± 0.6</td>
<td>13.3 ± 0.7</td>
<td>0.762§</td>
</tr>
<tr>
<td>HCT, %‡</td>
<td>40.0 (38.4-40.9)</td>
<td>40.3 (38.7-41.5)</td>
<td>0.349¶</td>
</tr>
<tr>
<td>MCV, fl/cell‡</td>
<td>30.0 (29.2-31.0)</td>
<td>29.6 (28.8-30.0)</td>
<td>0.018§</td>
</tr>
<tr>
<td>MCH, pg/cell‡</td>
<td>45.1 (41.0, 49.7)</td>
<td>47.0 (43.5, 50.9)</td>
<td>0.626¶</td>
</tr>
<tr>
<td>SF, µg/L†</td>
<td>114.5 (105.2, 124.7)</td>
<td>90.3 (82.6, 98.7)</td>
<td>0.001¶</td>
</tr>
<tr>
<td>TIBC, µg/dL*</td>
<td>316.6 ± 29.7</td>
<td>327.3 ± 33.5</td>
<td>0.086¶</td>
</tr>
</tbody>
</table>

Anthropometrics, RBC indices and Hb were assessed on D1, inflammation, iron parameters, plasma hepcidin concentration were assessed on D1 and D3. AGP, acute phase protein alpha-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.

*Means ± SD.
†Geometric means (95% CI).
‡Medians (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.

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

<table>
<thead>
<tr>
<th></th>
<th>CC variant</th>
<th>TT variant</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIA D1, %</td>
<td>6.50 (5.14, 8.22)*</td>
<td>7.99 (6.39, 9.98)†</td>
<td>0.206‡</td>
</tr>
<tr>
<td>FIA D3, %</td>
<td>6.49 (5.17, 8.15)*</td>
<td>7.93 (6.50, 9.68)†</td>
<td>0.183‡</td>
</tr>
<tr>
<td>FIA D1 &amp; D3, %</td>
<td>6.50 (5.54, 7.62)</td>
<td>7.96 (6.87, 9.22)</td>
<td>0.160§</td>
</tr>
<tr>
<td>FIA D1 &amp; D3, SF15corr, %</td>
<td></td>
<td>26.6 (24.0, 29.5)</td>
<td>43.7 (38.4, 49.8)</td>
</tr>
</tbody>
</table>

Values are the geometric means and the 95% 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 \( P = 0.984 \).
† 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: \( \log(FIAC) = \log(FIA0) + a \cdot \log(SFC/SFO) \), with
\[ a_{CC} = -1.28, a_{TT} = -0.74. \]

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

<table>
<thead>
<tr>
<th>Variables</th>
<th>Overall Model*</th>
<th>Minimal adequate model†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>SE</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.14</td>
<td>0.10</td>
</tr>
<tr>
<td>Variant (CC vs. TT)‡</td>
<td>-0.35</td>
<td>0.16</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>-0.13</td>
<td>0.07</td>
</tr>
<tr>
<td>Serum Ferritin</td>
<td>-0.39</td>
<td>0.08</td>
</tr>
<tr>
<td>Transferrin Saturation</td>
<td>0.11</td>
<td>0.06</td>
</tr>
<tr>
<td>Soluble Transferrin Receptor</td>
<td>0.12</td>
<td>0.07</td>
</tr>
<tr>
<td>Plasma Hepcidin</td>
<td>-0.31</td>
<td>0.06</td>
</tr>
<tr>
<td>PBAC</td>
<td>0.07</td>
<td>0.08</td>
</tr>
<tr>
<td>C-reactive Protein</td>
<td>-0.05</td>
<td>0.07</td>
</tr>
</tbody>
</table>

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).

Hb, PBAC were assessed on D1, inflammation, iron parameters, plasma hepcidin concentration, and FIA are based on data measured on D1 and D3.

*Regression model fit: \( R^2 = 0.498; R^2_{\text{adjusted}} = 0.468. \)

†Assessed by backward linear regression; regression model fit: \( R^2 = 0.486; R^2_{\text{adjusted}} = 0.469. \)

‡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 (n_{CC} = 35, n_{TT} = 44).

<table>
<thead>
<tr>
<th>Variables</th>
<th>CC variant*</th>
<th>TT Variant†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>SE</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.0003</td>
<td>0.08</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>Serum Ferritin</td>
<td>-0.70</td>
<td>0.010</td>
</tr>
<tr>
<td>Transferrin Saturation</td>
<td>-0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>Soluble Transferrin Receptor</td>
<td>-0.12</td>
<td>0.08</td>
</tr>
<tr>
<td>Plasma Hepcidin</td>
<td>-0.16</td>
<td>0.08</td>
</tr>
<tr>
<td>PBAC</td>
<td>-0.12</td>
<td>0.09</td>
</tr>
<tr>
<td>CRP</td>
<td>0.04</td>
<td>0.08</td>
</tr>
</tbody>
</table>

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.

Hb, PBAC were assessed on D1, inflammation, iron parameters, plasma hepcidin concentration, and FIA are based on data measured on D1 and D3.

*Regression model fit of CC variant: \( R^2 = 0.707; R^2_{\text{adjusted}} = 0.669. \)

†Regression model fit of TT variant: \( R^2 = 0.432; R^2_{\text{adjusted}} = 0.375. \)
Table 5: The minimal adequate model and predictors of iron absorption in variants of the TMPRSS6 rs855791 (n_CC = 35, n_TT = 44).

<table>
<thead>
<tr>
<th>Variables</th>
<th>CC variant*</th>
<th>TT Variant†</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>SE</td>
<td>P</td>
<td>β</td>
<td>SE</td>
<td>P</td>
<td>β</td>
<td>SE</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.0004</td>
<td>0.08</td>
<td>0.996</td>
<td>-0.01</td>
<td>0.11</td>
<td>0.911</td>
<td>-0.23</td>
<td>0.11</td>
<td>0.038</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>removed from the model‡</td>
<td>-0.23</td>
<td>0.11</td>
<td>0.038</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Ferritin</td>
<td>-0.67</td>
<td>0.09</td>
<td>&lt;0.001</td>
<td>removed from the model‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin Saturation</td>
<td>removed from the model‡</td>
<td>0.32</td>
<td>0.10</td>
<td>0.003</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble Transferrin Receptor</td>
<td>removed from the model‡</td>
<td>-0.22</td>
<td>0.07</td>
<td>0.002</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Hepcidin</td>
<td>-0.11</td>
<td>0.09</td>
<td>0.226</td>
<td>removed from the model‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>removed from the model‡</td>
<td>removed from the model‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.

Hb, PBAC were assessed on D1, inflammation, iron parameters, plasma hepcidin concentration, and FIA are based on data measured on D1 and D3.

*Regression model fit of CC variant: $R^2 = 0.702$; $R^2_{adj.} = 0.688$.
†Regression model fit of TT variant: $R^2 = 0.378$; $R^2_{adj.} = 0.356$.
‡Removed variable by the backward regression to assess the minimal adequate model.

**Figures**

**Figure 1:** Study flow chart.

**Figure 2:** Fractional iron absorption (FIA) in rs855791 variants (A-B) and correlation of the inter-individual FIA (C-D). A-B: Each point represents the mean of the FIA on day one and day three from two identical rice meals, the line represents the geometric mean and the bars the 95% CI. (A) the measured FIA, versus (B) the FIA corrected to a serum ferritin concentration of 15 µg/L is 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). C-D: FIA measured from identical rice test meals on study day one and three, separated by variant in the TMPRSS6 rs855791, in (C) the CC variant (○, n = 35), and in (D) 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).

**Figure 3:** Correlations of FIA. Between FIA and (A, B) serum ferritin; (C, D) transferrin saturation; and (E, F) hepcidin, 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 one and three. 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. ** = $P < 0.001$, * = $P < 0.05$.**
Figure 1: Study flow chart.
Online Supplementary Methods

Subjects
Inclusion criteria for the study were: 1) homozygous in the SNP rs855791 (wild type (CC) or mutation (TT)); 2) 20-45 years of age; 3) body mass index (BMI) 18.5 – 25 kg/m2; 4) body weight < 65 kg; 5) nonanemic, defined as Hb > 120 g/L; 6) iron sufficient, defined as SF > 30 µg/L; 7) no high body iron stores, defined as SF < 120 µg/L; 8) no oligomenorrhea or amenorrhea; 9) not pregnant or lactating; 10) no chronic disease; 11) no blood donation, transfusion or significant blood loss over the previous six months; 12) no use of long-term medication; 13) no use of vitamin/mineral supplements during the study and two weeks prior the first test meal and 14) no night shift work one month prior the study. We invited women who fulfilled all criteria to participate into the study. If the participant met all inclusion criteria, except a SF < 30 µg/L and/or a Hb < 120 g/L, they were assigned to an iron supplement group and consumed daily 27 mg Fe as ferrous fumarate (Multivitamins + Iron Stresstabs®, Pfitzer Inc.) for three months and were then invited for rescreening.

Test meal administration
We planned the test meal administrations on study days one and three (D1, D3) to be within the luteal phase of each subject menstrual cycle (MC); we administered the first test meal on the 14th MC day at the earliest, and the second test meal at the latest on the final day of the MC. On all study days (D1, D3, and D17), participants came to the K-CGMH in the morning after an overnight fast (no food intake after 8 pm and no drinks after midnight). Before each test meal administration, we collected a blood sample by venipuncture for determination of Hb, SF, serum iron (SFe), total iron binding capacity (TIBC), Hep, soluble transferrin receptor (sTfR), CRP, and the acute phase protein alpha-1-acid glycoprotein (AGP). On D1, the standardized test meals contained 4 mg iron ($^{57}$Fe) as labelled ferrous sulfate (FeSO$_4$), on D3 with 4 mg iron ($^{58}$Fe) as labelled FeSO$_4$. Participants consumed the entire test meal under supervision, and then remained fasting for three hours. On study D17, we collected a blood sample for determination of incorporation of stable iron isotopes into erythrocytes.

Preparation of stable iron isotopes, test meals and label administration
We labelled FeSO$_4$ with isotopically enriched elemental Fe, $^{57}$Fe, and $^{58}$Fe (Chemgas, Boulogne-Billancourt, France) as previously described. The test meals consisted of a rice
meal (273 g rice) (Rift Valley Nine, Taiwan Rice, Taiwan Costco) with 25 g seaweed sauce (Sea Tangle Seaweed Sauce, Gurume, Gurume Industrial Co., LTD). The labelled FeSO₄ solution was added to the meal just before consumption. The subjects consumed 350 ml bottled water with each meal.

**Assessment of menstrual blood loss**
We estimated menstrual blood losses using the semi-quantitative pictorial blood-loss assessment chart (PBAC), as previously described,² and defined menorrhagia as PBAC score > 100.

**Laboratory analyses**
We extracted DNA from peripheral leukocytes with a DNA extraction kit (QIAamp® DNA Mini kit, QIAGEN). We determined the TMRPSS6 rs855791 C>T polymorphism by sequencing allele specific PCR (TaqMan® SNP Genotyping Assay, ABI) using two forward allele-specific primers which differ by a single nucleotide complementary to the nucleotide of interest, and a common reverse primer in the PCR. We confirmed random samples (10%) by direct sequencing.

We collected venous blood samples using heparinized tubes (for immunoassays, isotopic analyses, hepcidin concentration), EDTA tubes (for erythrocytes and isotopic composition analysis), and non-anticoagulated tubes (for serum iron parameters). We assessed erythrocytes parameters by Sysmex XE-5000 or Sysmex XN-Series (Sysmex Co, Kobe, Japan). At screening, we determined CRP using a CRP kit (Fujifilm Wako Corporation, Osaka, Japan) and SF using by a two-site EIA (ADVIA Centaur, Siemens Healthcare Diagnostics, NY, USA). We measured SFe, and TIBC using a Fe/UIBC kit (Shino Corporation, Kanagawa, Japan). We shipped frozen samples to ETH Zurich for determination of CRP, AGP, and sTfR, Hep, and isotopic ratio. We measured CRP, AGP, and sTfR from D1, D3, and D17 by immunoassay,³ and calculated body iron stores (BIS, mg/kg)⁴ TS using the formula (SFe/TIBC) × 100. We measured Hep concentrations with the c-ELISA DRG Hepcidin 25 (bioactive) HS ELISA (DRG Instruments GmbH, Marburg, Germany).

**Fractional iron absorption correction to iron status.**
We corrected the FIA for SF with a modification of the Cook et al. formula,⁷ using the variants specific regression slope (a): \( \log(FIA_C) = \log(FIA_O) + a \times \log\left(\frac{SF_C}{SF_O}\right) \). Where FIA_C is the corrected, and FIA_O the observed FIA, SF_C is the corrected, and SF_O is the
observed SF. We corrected FIA to the cutoff for ID (15 µg/L), and to 50 µg/L as a level representing sufficient iron stores. We calculated circulating iron in the body based on hemoglobin and blood volume, derived from the participant’s height and weight and assuming an 80% incorporation of absorbed iron into erythrocytes.

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 2 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 ± 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 normally distributed variables with independent samples T-Test and for not normally distributed variables using Mann-Whitney U Test; 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 random intercept, the corresponding variable as dependent variable and genotype as fixed effect. We assessed Pearson’s correlations and differences between the coefficients with the Fishers r to z transformation. We assessed predictors of iron absorption with LMM using subjects’ code as random intercept, FIA as dependent variable, and 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 $P < .05$.

References