SUPPLEMENTARY APPENDIX

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

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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.[1] 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 $FeSO_4$ 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): $\log(FIA_C) = \log(FIA_O) + a * \log(SF_C/SF_O)$. 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)[8], 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[9] and assuming an 80% incorporation of absorbed iron into erythrocytes.[6]

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 $P < .05$.

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