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RESEARCH PAPER

Association of genetic variants with response to iron supplements in pregnancy

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Abstract The incidence of iron deficiency anemia in pregnancy is high in India where iron supplementation is a regular practice. The response to oral iron is influenced by several factors such as age, body mass index, gravida, socioeconomic status, food, vitamin deficiency and compliance to supplements. The major challenge is to understand the various modulators of iron status in this high-risk group so that we can improve the diagnosis and the management of these patients. The current study was designed to evaluate the iron status during pregnancy and to identify factors which might be influencing their response to oral iron. We investigated a total of 181 pregnant women with anemia (Hb < 11 g/dl) and evaluated the impact of probable factors on anemia and their iron status. Assessment of the response was based on hemoglobin and serum ferritin or transferrin saturation level after 8 and 20 weeks of iron supplementation. Socioeconomic, clinical, hematological, biochemical and genetic factors were all evaluated. Molecular analysis revealed that *HFE* variant allele (G) (rs1799945) was significantly associated with an adequate response to iron supplementation. We identified five subjects with a sustained poor response, and targeted re-sequencing of eleven iron-related genes was performed in them. We have identified seven novel variants in them, and in silico analysis suggested that these variants may have an iron regulatory effect. Taken together, our findings underscore the association of genetic variants with response to supplements in pregnancy, and they can be extended to other diseases where anemia and iron deficiency coexist.

Keywords Iron deficiency anemia · Iron supplements · Pregnancy · Genetic variants

Introduction

Iron deficiency anemia is common in pregnancy, since the requirement of iron is extraordinarily high during this period. The total iron requirement during pregnancy is estimated to be about 1000 mg (fetus and placenta—300 mg; increase in red cell mass—500 mg and basal loss—200 mg), and this escalates up to 7–8 mg/day in the last trimester (Harju 1989; Bothwell 2000). The amount of iron that can be absorbed even from an optimal diet is lower than the actual requirement during this period (Bothwell 2000). According to the World Health Organization (WHO) report (2001), the percentage of women with iron deficiency anemia (IDA) can be as high as 80 % in developing countries. Anemia is considered to be a risk factor for spontaneous abortion, preterm delivery and low birth weight where severe anemia results in increased maternal morbidity and mortality (Allen 2000; Uche-Nwachi et al. 2010; Koura et al. 2012; Haider et al. 2013). Anemia is found to be responsible for 40 % of maternal deaths in India (Kalaivani 2009).

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Iron supplementation during pregnancy is universally accepted in order to meet the high iron demand during pregnancy. Despite the routine iron supplementation and preventive measures, iron deficiency anemia (IDA) continues to be a major public health concern. Rama Krishnan et al. reported a higher prevalence of iron deficiency at 32 weeks of gestation and 1 month postpartum despite the supplementation, which was not attributable to low rates of compliance (Ramakrishnan et al. 2004). In another study, the response to supplementation was only found to be 65 % despite a rate of compliance of 85 % (Christian et al. 2009). Several other studies have also reported that the response to iron supplements is not as expected (Milman et al. 1999, 2005a). In one of the earlier WHO sponsored trial in India, 25 % of the women remained anemic at the end of the study even after supplementing them with iron, vitamin B12 and protein. Even when hookworm infestation was excluded, 25 % continued to be anemic, indicating that it was not a major factor in the etiology of the anemia (Mathan et al. 1979). Another trial conducted by Indian Council of Medical Research (ICMR) revealed that the prevalence of anemia was similar in those who received supplements (88.1 %) and those who did not (87.5 %) (Yusufji et al. 1973; Mathan et al. 1979). These data indicate the possibility of other factors that might affect iron absorption and thus response to treatment. The reasons for the high prevalence of IDA and poor response were not clear, and it has not been extensively studied till date.

Recently, researchers have demonstrated the association of SNPs (single nucleotide polymorphisms) with iron status and hemoglobin. *TMPRSS6* genetic variants were found to be significantly associated with serum iron, hemoglobin (Hb), mean corpuscular volume (MCV) and mean cell hemoglobin (MCH) in genome-wide association studies (GWAS) (Benyamin et al. 2009; Chambers et al. 2009a). The serum transferrin level was found to be influenced by *HFE* variants, and serum iron was associated with transferrin gene polymorphisms and the RBC count with *TFR2* variants in these different studies (Tanaka et al. 2010). Haptoglobin 2-2 genotype and *TNF-α* genotype-308 AA have also been shown to be associated with low hemoglobin (Cox et al. 2008; Atkinson et al. 2008).

In India, where IDA is prevalent, it is important to determine and understand whether genetic variants contribute to the iron status and anemia. These data will give not only insights into the genes which primarily regulate iron metabolism but also about the differential mechanism that may form the basis of IDA. This would also help us to explain the contrasting responses to supplementation. The lack of molecular studies to understand the effect of genetic variants in IDA and response to treatment demands additional research; hence, we undertook the present study to evaluate the influence of genetic variants on iron status in pregnant women with IDA. We screened two relevant missense variants in the *TMPRSS6* and *HFE* genes (rs855791 and rs1799945) which have previously been reported to have an association with iron status and to be considered as risk factor in IDA (Chambers et al. 2009b; Delbini et al. 2010; Blanco-Rojo et al. 2011). A set of eleven relevant iron-related genes were re-sequenced in poor responders to identify the molecular basis of poor response.

Subjects and methods

Study cohort

The Institutional Review Board (Ethics committee) of Christian Medical College, Vellore, approved this study (IRB No 7123 dated 21.04.2010), and informed written consent was obtained from all the study participants. Pregnant women at 10–18 weeks of gestation were included in the study. The inclusion criteria and the evaluation strategy are given in the flowchart (Figure S1). A detailed proforma was used to collect the patient information. Compliance to the treatment with iron supplements was assessed by counting and documenting the number of empty tablet strips at each visit.

Definitions

Anemia in pregnancy Hb < 11 g/dl (first trimester) and <10.5 g/dl in second and third trimester.

Iron deficiency anemia Anemia with ferritin level <12 ng/ml or TS < 16 %

Grading of Anemia severity Hb; 10–10.9 g/dl (mild); 7–9.9 g/dl (moderate); <7 g/dl (severe)

Good response to iron supplementation Hb > 10.5 g/dl with ferritin >12 ng/ml or transferrin saturation >16 %.

Good compliance ≥ 60 % of prescribed tablets consumed.

Poor compliance <60 % of prescribed tablets consumed.

Hematological and biochemical analysis

DNA was extracted from peripheral blood mononuclear cells (PBMNCs), and serum samples were stored for biochemical assays. Complete blood counts (CBC) were done using an automated hematology analyzer (SysmexKX21). Serum iron and unsaturated iron-binding capacity (UIBC) were measured in a Roche modular P800 according to standard protocols. Serum ferritin was analyzed by chemiluminescence immunoassay using the Advia

Centaur, Siemens XPI. Serum vitamin B12 and folate were measured in selected cases by an electro-chemiluminescence method (Roche E170). High-sensitive cellular reactive protein (CRP) was measured in selected cases to rule out anemia of inflammation. Serum hepcidin was quantified by using an enzyme immunoassay method from DRG, GmbH according to the manufacturer's protocol.

Genotyping of two significant genetic variants (rs855791 and rs1799945)

Two known polymorphisms *TMPRSS6* c.2207A>G (rs855791) and *HFE* c.187C>G (rs1799945) were screened in all subjects ($n = 181$) by RFLP (restriction fragment length polymorphism) using *Hae*III and *Bcl*I enzymes, respectively.

Targeted re-sequencing

Targeted re-sequencing of eleven genes (*TMPRSS6*, *SLC11A2*, *SLC40A1*, *STEAP3*, *HFE*, *HFE2*, *FTL*, *FHL*, *HAMP*, *TFR2* and *TF*) was performed using the Ion Torrent™ PGM instrument (Life Technologies, NY, USA) in five subjects with a persistent poor response. Next-generation sequencing (NGS) workflow involved first target enrichment and then library and template preparation followed by ion semiconductor-based sequencing as described previously (Chapla et al. 2015). The primer sequences for all the eleven genes are given in Table S1.

In silico analysis

To investigate the probable effect of novel mutations/variants, bio-informatics tools such as PolyPhen (polymorphism phenotyping-2) (http://genetics.bwh.harvard.edu/pph2/) and SIFT (sorting intolerant from tolerant) (http://sift.jcvi.org/www/SIFT_enst_submit.html) were used. PolyPhen-2 scores are assessed as 0.000 (probably benign) to 0.999 (probably damaging). SIFT scores % <0.05 indicate substitutions as intolerant. RegRNA 2.0 was used to predict regulatory RNA motifs and elements in premRNA sequence. The change in splice site was predicted using BDGP neural network (http://www.fruitfly.org/seq_tools/splice.html).

Statistical analysis

Statistical analysis of the data was carried out using the software SPSS, version 20. For categorical data, the Chi-square test was used. Appropriate statistical tests including *t* test for continuous variables, analysis of variance (ANOVA) for comparison of groups and Mann–Whitney and Kruskal–Wallis for nonparametric data were used. Regression analysis was used to estimate the relationships between the selected variables.

Results

Transferrin saturation and ferritin level predicts hemoglobin

A total of two hundred and twelve ($n = 212$) consecutive anemic (Hb < 11 g/dl) pregnant women (14.1 ± 2.4 weeks of gestation) who visited the antenatal clinic at our community health center were included in this study. One hundred and eighty-one subjects (81.5 %) were classified as iron deficient (Fig. 1) according to the defined criteria. Sixty-one non-anemic pregnant women served as healthy controls. The mean age in IDAP was 22.9 ± 3.1 and 22.6 ± 3.5 years in the controls.

The basal hemoglobin concentration in the IDAP group as mean ± SD was 9.34 ± 1.19 g/dl, and for the MCV, it was 70.7 ± 6.7 fl. The majority of the subjects were classed as having moderate anemia. The hematological and biochemical characteristics of these subjects at baseline and at follow-up are given in Table 1. IDAP subjects had significantly lower body mass index (BMI), Hb, MCV, iron, transferrin saturation and ferritin values than the control group. Transferrin saturation (TS %) and serum ferritin values positively correlated with the hemoglobin concentration at diagnosis and follow-up (TS % vs Hb; $r = 0.522$, $p < 0.0001$); (ferritin vs Hb; $r = 0.375$, $p < 0.0001$).

Response to iron supplements is not completely dependent on compliance

Iron supplementation with Autrin capsules (ferrous fumarate; 98.6 mg elemental iron) was started once a day from the time of diagnosis after administration of anti-helminthics. Compliance and response were assessed after 8 and 20 weeks of iron therapy. Response was assessed in 161 subjects after 8 weeks of supplements (first follow-up—F1) and in 139 subjects after 20 weeks of supplements (second follow-up—F2) (Fig. 1). The dropout rate was 11 % at F1 and 14.3 % at F2. The reasons for dropping out were: abortion ($n = 5$), preterm delivery ($n = 6$), refusal of tablet intake ($n = 3$), diabetes and cardiac complications ($n = 2$) and opting to undergo delivery at other centers ($n = 27$). The mean compliance at 8 weeks was 87.6 ± 18.1 % and at 20 weeks was 89.1 ± 14.3 %.

A good response was shown by 76.4 % (123/161) and 79.8 % (111/139) of the subjects at F1 and F2, respectively. Thirty-three (20.5 %) of the subjects had a poor response at F1 and seventeen (12.2 %) at F2. Sixteen

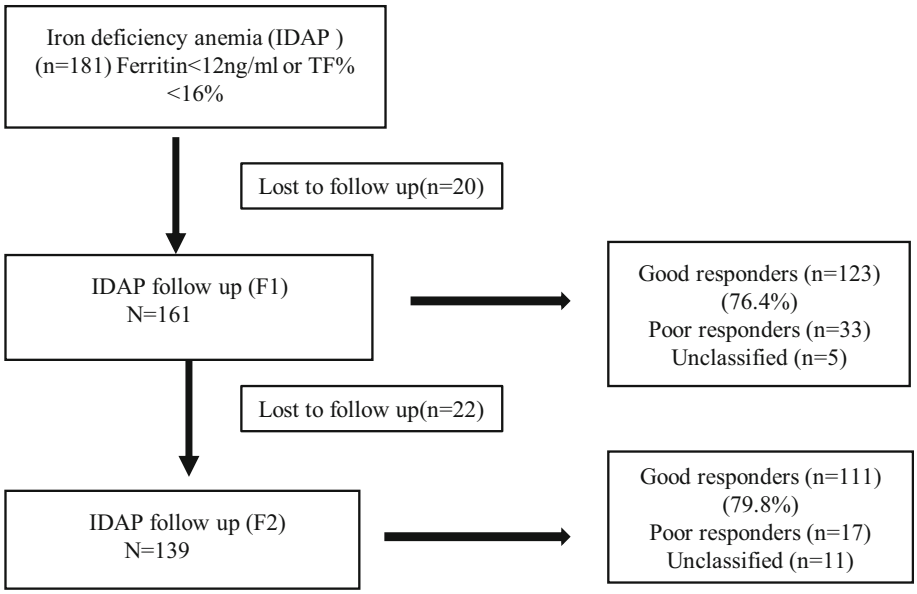

Fig. 1 Enrolment of subjects and follow-up in IDAP group

Table 1 Hematological and biochemical parameters in IDAP and controls at baseline and follow-up (F1 = first follow-up; F2 = second follow-up)

Parameters	Baseline ($n = 181$) (10–18 weeks)	F1 ($n = 161$) (18–24 weeks)	F2 ($n = 139$) (30–36 weeks)	Controls ($n = 61$)
Age (years)	22.99 ± 3.12	23.1 ± 3.17	22.9 ± 3.16	22.61 ± 3.58
BMI	20.97 ± 3.76	NA	NA	22.21 ± 3.07
Hemoglobin (g/dl)	9.34 ± 1.19	10.8 ± 1.27	11.4 ± 1.4	12.62 ± 1.16
MCV (fl)	70.7 ± 6.7	81.3 ± 6.1	85.9 ± 6.9	86.77 ± 5.37
RBC ($\times 10^6$)/μl	4.2 ± 0.36	4.08 ± 0.4	3.9 ± 0.41	4.2 ± 0.37
IRON (μg/dl)	23 (8–152)	75 (10–291)	85 (17–282)	76 (38–168)
TIBC (μg/dl)	412.54 ± 74.2	389 (204–651)	404 (274–581)	394 ± 59.87
TF saturation (%)	6.69 ± 4.1	21.8 ± 13.9	22.9 ± 12.9	20.9 ± 8.9
Ferritin (ng/ml)	7.2 (0.2–57.4)	22.4 (1–127)	26.7 (2.4–218)	25 (8–79.8)

Controls—non-anemic pregnant women with gestational age 10–36 weeks

patients could not be classified (five in F1 and 11 in F2) because all the serum measurements were not obtained. Sixteen of the 33 poor responders (48.5 %) at F1 showed improvement in their hemoglobin iron and ferritin levels at F2, while nine still remained as poor responders. Causes of poor response were identified in four subjects as: poor compliance (<50 % of tablets taken, $n = 2$), vitamin B12 deficiency ($n = 2$). The remaining five subjects (3.6 %) were identified as having persistent anemia without any cause. The hematological, biochemical and other parameters observed in these five subjects are shown in Table 2.

Gravida predicts response to iron supplements

We analyzed factors that might influence iron status in pregnancy such as age, BMI, gravida, food pattern and compliance. Of these, only gravida had any significant influence. Interestingly, subjects with primigravida were predominantly good responders, 83 % (106/128) in F1 and 91.2 % (99/109) in F2. This was significantly better than subjects in second or third gravida: 64 % good responders (17/28) in F1 ($p = 0.019$) and 69 % (12/19) in F2 ($p = 0.004$). A poor response was significantly associated with multigravida in regression analysis both by univariate analysis—relative risk ratio (RR) 3.1, 95 % confidence interval (CI) 1.28–7.56; $p = 0.012$—and by multivariate analysis (RR 2.4, 95 % CI 0.97–5.90; $p = 0.05$).

HFE (rs1799945) variant allele "G" is associated with good response

Hemoglobin, serum iron, transferrin saturation and ferritin concentrations were all significantly higher at follow-up in the group with the *HFE* variant allele (CG or GG) as compared to those wild type (CC) (Table S2). The wild-type allele "C" was significantly associated with poor response compared to the mutant allele "G"(CC vs

Table 2 Details of pregnant women with sustained poor response

UPN	Age	Gravida	BMI	Hb_D	Hb_F1	Hb_F2	FT_D	FT_F1	FT_F2	TS %_D	TS %_F1	TS %_F2	C _F1	C_F2	Hep _D (ng/ml)	Hep_F2 (ng/ml)
IP183	22	1	23.6	9.7	8	8	5	3.8	3.7	5.18	6.28	3.47	100	91.46	4.08	3.50
IP138	32	2	24.4	9.2	8.9	7.8	8.1	8.7	7.1	6.58	7.19	3.41	85.5	89.89	5.20	5.60
IP196	22	2	22.2	9.6	9.8	9.1	6.4	14.6	6.8	3.55	5.97	5.77	98.2	98.73	3.51	3.67
IP157	22	2	21.1	8.3	9.2	10.3	4.5	4.5	8.1	5.20	4.98	9.17	85.7	100	5.02	5.22
IP220	26	1	16.5	8.5	8.6	9.4	5.3	12.6	11.4	4.54	4.80	5.45	100	100	5.50	6.50

UPN, unique patient number; X_D, at diagnosis; F1, follow-up at 8 weeks; F2, follow-up at 20 weeks

Hb, hemoglobin; FT, ferritin; TS %, transferrin saturation; C, compliance; Hep, hepcidin

CG + GG., RR 10; 95 % CI 0.01–0.81; $p = 0.031$). A similar association was also observed by multivariate analysis (RR 10; 95 % CI 0.02–1.03; $p = 0.054$). Although subjects with wild genotype (AA) for SNP rs855791 had significantly higher levels of serum iron compared to GG genotype at F2 ($p = 0.014$) (Table S3), this was not associated with a better response to the iron supplementation.

Novel and rare gene variants are associated with poor response

There were five subjects with a persistent poor response at F1 and F2. Their serum hepcidin was low at baseline as well as at follow-up. Celiac disease can result in poor iron absorption, but this was excluded in all the five subjects since the test for tissue transglutaminase antibody (serum anti-TTG) was negative. These subjects were selected for targeted re-sequencing of eleven genes important for iron metabolism and transport: *DMT1*, *DCYTB*, *SLC40A1*, *FTH*, *FTL*, *TF*, *TFR2*, *TMPRSS6*, *HFE*, *HFE-2* and *STEAP3* using the NGS Ion Torrent platform. The results showed that 99.2 % of the target genes were sequenced, at a minimum of 20× depth. These results were validated and confirmed by Sanger sequencing.

A total of 75 SNPs including eight novel variants were identified (refer to Table S4 and S5 note: intronic variants are not shown). Details of eight novel variants are summarized here: three in TF, two in SLC11A2, one in TFR2, one in SLC40A1 and one in HAMP (Table 3). These novel nucleotide changes have not been described in either the Ensembl or 1000 genome databases. We screened for these changes in randomly selected good responders ($n = 40$). Seven of the novel variants listed above were found to be absent, but the HAMP variant was present in one of the subjects tested. In addition, three rare variants (P555S in TMPRSS6, T495M in STEAP3 and c.34+6 T>G in SLC11A2) were also identified. These missense variants T495M (*STEAP3*) and P555S (*TMPRSS6*) are found to be rare (MAF; minor allele frequency <0.01). We screened for these variants in 40 non-anemic controls and 40 good responders. T495M was present in 12.5 % of good responders and 20 % of controls. P555S was present in 22.5 % good responders and 17.5 % of controls. However, the genotype frequency was not statistically different between the groups.

Discussion

The prevalence of anemia in developing countries varies from 35 to 75 %, and IDA is ranked ninth among 26 risk factors in the global burden of disease study (Allen 1997; Stoltzfus 2003). Iron deficiency anemia still remains a

Table 3 Details of rare and novel variants identified in poor responders by next-generation sequencing

Gene	Nucleotide change	Type of change	Amino acid change	Prediction
STEAP3[a]	c.1484 C>T	Substitution	T495M	Damaging (0.995)
TMPRSS6[a]	c.1663 G>A	Substitution	P555S	Benign (0.00)
SLC40A1[b]	IVS4+20C>T	Intronic	NA	None
TF[b]	IVS1+41 A>T	Intronic	NA	miR-644 binding site
TF[b]	IVS5+25 A>G	Intronic	NA	miR-1291 and 328 binding site Intronic enhancer
TF[b]	IVS9+299C>T	Intronic	NA	Intronic enhancer
SLC11A2[b]	3′UTR+776G>A	3′UTR	NA	miR-548m miR-555 binding site
SLC11A2[b]	IVS6+200 G>A	Intronic	NA	None
TFR2[b]	c.472–74 C>G	Intronic	NA	Affect splicing
HAMP[b]	IVS 2+12 A>C	Intronic	NA	Intronic enhancer

NA not applicable

[a] Rare variants

[b] Novel variants

major public health concern, especially affecting children and pregnant women. Oral iron supplementation is a preventive measure undertaken in all developing countries. However, previous studies and clinical trials have indicated the unpredictability of the response to iron prophylaxis in pregnancy (Mahomed 2000; Müngen 2003). The previous data showed that 27 mg of oral iron per day is effective dose to alleviate anemia (Eskeland et al. 1997; Milman et al. 2005b; Milman 2012). Based on the literature and study evidence, we used 100 mg of elemental iron as an anemia prophylaxis in our study. Our study demonstrates that oral iron is effective in correcting anemia with an average tablet intake for 57.6 ± 14.4 days.

There are numerous studies on the efficacy of treatment, dosage and treatment outcome in the IDA of pregnancy, but these have not evaluated the association of genetic variants with iron status, especially in pregnancy (Madhavan Nair et al. 2004; Singh et al. 2013; Haider et al. 2013). A study conducted by Prema et al. documented about 4.3 % of non-responders even after parenteral iron administration. Studies have also shown the different degrees of response to iron supplements in pregnancy, but the factors which can influence this response have never been addressed (Beard 2000; Agarwal et al. 2006; Christian et al. 2009). Hence, we undertook this study to address the possible genetic factors affecting iron status in pregnancy. Our study cohort served as an ideal group to reveal the association of genetic variants with iron status. This is because of the uniform iron supplementation with regular follow-up and the absence of confounding factors like menorrhagia and chronic disorders.

The majority of the subjects (41 %) presented with mild anemia, 35.2 % with moderate and 3.7 % with severe anemia. These data are similar to other studies (Baig-Ansari et al. 2008). IDA was observed in 181 of the subjects recruited constituting 81.5 % of the total number. In 1973, Baker et al. from our center found that 96 % of pregnant women had low serum iron and transferrin saturation, this is higher than in our study (Yusufji et al. 1973). In another study, Rajaratnam et al. (Rajaratnam et al. 2000) reported only 29.5 % of subjects with IDA in pregnancy (based on Hb < 11 g/dl and ferritin < 12 ng/ml). This is much lower than in our study. Earlier studies showed that, in pregnancy, the degree of anemia that is present is influenced by gravida and parity which were inversely related to education and socioeconomic status (Vijaynath et al. 2010; Al-Farsi et al. 2011). Both parity and gravida have also shown to be associated with a higher incidence of anemia (Al-Farsi et al. 2011). Our study suggests that multigravida not only increases the prevalence of anemia in pregnant women, but it is also associated with a poor response to iron supplements. There were more subjects with multigravida (36 %) who showed a poor response to treatment at F1 compared to primigravida (17 %). A similar pattern was also observed at F2 (31.65 vs 8.8 %). We propose that this might be due to the low iron stores in women who have already had one or more pregnancies. This observation may indicate that the multigravida subjects require a higher daily dose of iron for a longer time to ameliorate their anemia.

The degree of anemia positively correlated with the measured ferritin levels and transferrin saturation. Similar to the previous reports (Rehu et al. 2010; van Santen et al. 2013), serum hepcidin was low in anemic subjects in our study, excluding the possibility of IRIDA. The poor response to oral iron and the low serum ferritin in a subgroup

of patients suggest the possible defects in iron absorption. Therefore, we ruled out known causes of malabsorption and chronic illness (C-reactive protein negative) in our study subjects.

The *HFE* variant rs1799945 (H63D) had a protective effect against anemia with a significant improvement in hemoglobin, iron and ferritin values at follow-up. These data clearly establish the association of genetic variants in modulating iron absorption and thus response to treatment. The *TMPRSS6* rs855791 allele (A) was significantly associated with higher serum iron and hemoglobin values. The presence of novel and rare variants in *STEAP3*, *TMPRSS6*, *SLC11A2*, *SLC40A1*, *HAMP* and *TF* genes indicates that there is a probable genetic association with iron status as reported in other populations (Delbini et al. 2010; Kloss-Brandstätter et al. 2012).

In silico analysis of these novel variants revealed their importance as probable sites of miRNA binding and as intronic regulatory regions. Further validation and functional characterization of these variants should now be performed in a larger study. The presence of these rare variants (T495M and P555S) across our study population in non-anemic controls as well as good and poor responders suggests that these variants are common in our population and that probably their presence is a risk factor for iron deficiency. The T495M variant in the *STEAP3* gene was predicted to have a probable damaging effect on the protein. We could not identify any mutations which can lead to iron refractory anemia; however, the synergistic effect of these identified genetic variants on anemia should not be overlooked. The functional characterization of these identified rare and novel variants will help us to explore the differential mechanisms of iron regulation and thus patient susceptibility to IDA.

This is the first time that data have been produced which demonstrate the association between genetic variants and response to supplements in pregnancy. Our study suggests that genetic variants could be playing a significant role in modulating the response to oral iron along with other known modulators such as gravida and socioeconomic status. Our study demonstrates a significant impact of genetic variants in modulating iron status and response to oral iron in pregnancy. This association must be explored further in IDA to confirm and strengthen these initial findings.

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Conflict of interest The authors declare that they have no conflict of interest.

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