RESEARCH ARTICLE

Interferon-gamma polymorphisms and risk of iron deficiency and anaemia in Gambian children [version 1; peer review: 1 approved]

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Abstract

Background: Anaemia is a major public health concern especially in African children living in malaria-endemic regions. Interferon-gamma (IFN-γ) is elevated during malaria infection and is thought to influence erythropoiesis and iron status. Genetic variants in the IFN-γ gene (IFNG) are associated with increased IFN-γ production. We investigated putative functional single nucleotide polymorphisms (SNPs) and haplotypes of IFNG in relation to nutritional iron status and anaemia in Gambian children over a malaria season.

Methods: We used previously available data from Gambian family trios to determine informative SNPs and then used the Agena Bioscience MassArray platform to type five SNPs from the IFNG gene in a cohort of 780 Gambian children. We also measured haemoglobin and biomarkers of iron status and inflammation at the start and end of a malaria season.

Results: We identified five IFNG haplotype-tagging SNPs (IFNG-1616 [rs2069705], IFNG+874 [rs2430561], IFNG+2200 [rs1861493], IFNG+3234 [rs2069718] and IFNG+5612 [rs2069728]). The IFNG+2200C [rs1861493] allele was associated with reduced haemoglobin concentrations (adjusted β -0.44 [95% CI -0.75, -0.12]; Bonferroni adjusted P = 0.03) and a trend
towards iron deficiency compared to wild-type at the end of the malaria season in multivariable models adjusted for potential confounders. A haplotype uniquely identified by IFNG+2200C was similarly associated with reduced haemoglobin levels and trends towards iron deficiency, anaemia and iron deficiency anaemia at the end of the malaria season in models adjusted for age, sex, village, inflammation and malaria parasitaemia.

**Conclusion:** We found limited statistical evidence linking IFNG polymorphisms with a risk of developing iron deficiency and anaemia in Gambian children. More definitive studies are needed to investigate the effects of genetically influenced IFN-γ levels on the risk of iron deficiency and anaemia in children living in malaria-endemic areas.

**Keywords**
Interferon-gamma, malaria, iron deficiency, anaemia, ferritin, hepcidin, zinc protoporphyrin, transferrin saturation, iron, IFNG, genetic polymorphisms, Africa, children

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Introduction
Malaria and iron deficiency are major public health problems for children living in sub-Saharan Africa. The majority (94%) of the 405,000 global deaths due to malaria in 2018 occurred in sub-Saharan Africa, where up to 24% of the population have malaria parasitaemia at any given time. In this region, iron deficiency (ID) and anaemia are highly prevalent, and may lead to impaired brain development, while iron deficiency anaemia (IDA) is a leading cause of years lived with disability in African children. Increasing evidence suggests that malaria may be contributing to ID and IDA. Previous studies reported that the prevalence of ID and IDA increased over the malaria season in Gambian and Kenyan children, and decreased with the interruption of malaria transmission in the Kenyan highlands.

Multiple factors may contribute towards the development of ID and IDA following a malaria infection. One such factor is interferon-gamma (IFN-γ), which is induced during acute and persistent malaria infection. Among other type 1 responses, IFN-γ is involved in regulating erythropoiesis and iron-regulatory proteins. IFN-γ has also been reported to increase the expression of hepcidin, and divalent metal transporter 1 (DMT1), while suppressing ferroportin, ferritin, and transferrin receptors. This regulation of iron proteins may be aimed at starving invading pathogens of iron, a critical nutrient for pathogen growth, but could also play an important role in the pathogenesis of ID and IDA. Indeed, higher IFN-γ levels have been reported in Kenyan children with severe malarial anaemia.

Single nucleotide polymorphisms (SNPs) in the IFN-γ gene have been associated with increased production of IFN-γ and susceptibility to severe malaria. Despite evidence that malaria induces the production of IFN-γ and that this cytokine influences iron regulation, it is not known whether variation in the IFNG gene influences the risk of ID and IDA among children in malaria-endemic areas. We investigated SNPs and haplotypes in the IFNG gene locus in relation to nutritional iron status and anaemia in a cohort of 780 Gambian children prior to and at the end of a malaria season, using an approach based on informative SNPs and Agena Bioscience MassArray platform typing.

Methods
Study area
The study was conducted in ten rural villages in the West Kiang region of The Gambia at the start (July 2001) and end (December 2001/January 2002) of a malaria season, as previously described. Malaria incidence is highly seasonal in The Gambia, with the majority of cases occurring between September and December. The study participants were from the Mandinka and Fulani ethnic groups. All of the Fulani children were located in a single village and ethnic group was accounted for in all analyses by adjusting for village.

Study design
We used previously collected data from a cohort of 780 children aged two to six years, recruited at the start of a malaria season as previously described. All children had a clinical examination and a blood sample collected for full blood count, malaria film, and biomarkers of iron status and inflammation at the start and end of the malaria season. Children with pyrexia (temperature ≥37.5°C) had appropriate clinical investigations, clinical treatment and a blood sample taken 2 weeks later after recovery from illness. All children received a 3-day course of mebendazole for possible hookworm infection at recruitment.

Laboratory procedures
Haemoglobin (Medonic CA 530 Haemoglobinimeter) and zinc protoporphyrin (ZnP) levels (Aviv Biomedical Hematofluorometer) were measured within 24 hours of sample collection. Hepcidin (Hepcidin-25 [human] ELISA kit, Bachem), ferritin (IMx ferritin assay, Microparticle Enzyme Immunoassay; Abbot Laboratories), soluble transferrin receptor (sTfR, Human sTfR ELISA; R&D Systems), serum iron, unsaturated iron binding capacity (UIBC, Ferrozine-based photometry and colorimetry; Hitachi 911 automated analyzer), and α1-antichymotrypsin (ACT, immunoturbidimetry, Cobas Mira Plus Bioanalyzer, Roche) were assayed according to the manufacturers’ instructions from plasma samples stored at -80°C. Transferrin saturation (TSAT) was calculated from plasma iron and UIBC (TSAT = [plasma iron / (UIBC + plasma iron)] X 100). Giemsa-stained thick and thin blood films were examined for Plasmodium falciparum and other Plasmodium species.

SNPs and haplotype construction
Genotypes were determined on whole-genome amplified DNA (primer extension pre-amplification) by the Agena Bioscience MassArray platform (formerly SEQUENOM) using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry as previously described. Details of the primer sequences and assays are given in Extended datafiles 1 and 2. The most informative haplotype-tagging SNPs (htSNPs) to type in Gambian subjects were identified by analysing the pattern of linkage disequilibrium (LD) in the IFNG gene loci using previously available data from 32 Gambian family trios. The PHASE program (http://stephenslab.uchicago.edu/software.html) version 2.1 was used to infer haplotypes from the genotypes of the study population and estimate the frequency of each inferred haplotype. The entropy maximization method was used to identify htSNPs that described >90% of the observed haplotypic diversity in this gene region. The HaploXT program (http://www.sph.umich.edu/csg/abecasis/GOLD/docs/haplotxt.html) was used to estimate pairwise LD statistics. Sickle cell (HbS, rs334) and glucose-6-phosphate deficiency (G6PD) deficiency (rs1050828 and rs1050829) were also genotyped using the Agena Bioscience MassArray platform.

Definition of terms
Inflammation was defined as ACT ≥0.6 g/L. ID was defined as ferritin <12 µg/L or <30 µg/L in the presence of inflammation or <15 µg/L in children ≥5 years, anaemia as Hb<11.0 g/dL (or Hb <11.5 g/dL in children ≥5 years) and IDA as ID plus anaemia.

Statistical analyses
Statistical analyses were conducted using STATA 15.1 (StataCorp. College Station, Texas, USA). Categorical data were expressed as proportions with corresponding percentages. Pearson chi-squared
test was used to compare the prevalence of malaria and iron status (ID, IDA and anaemia) at the start and end of the malaria season. Changes in haemoglobin levels and markers of iron status over the malaria season were assessed using the paired t-test. Biological data that were not normally distributed were log-transformed, and geometric means were calculated from original untransformed values.

Log-transformed markers of iron status and risk of ID, IDA and anaemia were analysed using univariable and multivariable linear and logistic regression models, as appropriate. Multivariable regression models were adjusted for age (grouped by year), sex, village (which also acted as a proxy for ethnic group), malaria parasitaemia and ACT. The Bonferroni correction for multiple testing was applied when the five SNPs and six haplotypes were considered individually as independent factors. For multivariable analyses, P values are noted as adj. P for non-Bonferroni corrected analyses and as Bonferroni adj. P for multivariable analyses that are Bonferroni corrected, and for univariable models P values are similarly presented as P or Bonferroni P if Bonferroni corrected. All analyses were considered statistically significant at P<0.05.

Ethics
Individual written informed consent was obtained from children’s parents or guardians and the study was approved by The Gambian Government and the Medical Research Council Ethics Review Committee (874/830).

Results
Characteristics of participants
A total of 756 children, including 403 males (53%) and 353 females (47%), were followed up to the end of the malaria season. Most of the children were from the Mandinka ethnic group (n = 681; 90%, compared to Fulani n = 75; 10%). A total of 99/751 (13.2%) children carried sickle cell trait (HbAS) and 136/683 (19.9%) children G6PD deficiency. The prevalence of ID and IDA increased over the malaria season (from 20.6% to 31.6% and from 11.9% to 21.7%, respectively), as previously reported. Individual markers of iron status also reflected an increase in ID over the malaria season. Table 1 summarises the characteristics of the study population and their iron status at the beginning and end of the malaria season.

IFNG single nucleotide polymorphisms and associations
We identified five IFNG haplotype tagging SNPs at positions IFNG-1616 (rs2069705), IFNG+874 (rs2430561), IFNG+2200 (rs1861493), IFNG+3234 (rs2069718) and IFNG+5612 (rs2069728) relative to the ATG start codon (Figure 1, Extended datafile 1). All of the IFNG SNPs were in Hardy Weinberg equilibrium. The IFNG+2200C (rs1861493, n=97) allele was associated with a reduced risk of malaria parasitaemia at the start of the malaria season.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Start</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age months, median (IQR)</td>
<td>46.11 (34.83, 59.17)</td>
<td>51.37 (40.47, 64.20)</td>
</tr>
<tr>
<td>Sex, Female (%)</td>
<td>353/756 (46.7%)</td>
<td>322/700 (46.0%)</td>
</tr>
<tr>
<td>Malaria parasitaemia (%)</td>
<td>89/754 (11.8%)</td>
<td>179/698 (25.6%)</td>
</tr>
<tr>
<td>Iron status*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron deficiency</td>
<td>151/734 (20.6%)</td>
<td>216/684 (31.6%)</td>
</tr>
<tr>
<td>Iron deficiency anaemia</td>
<td>86/720 (11.9%)</td>
<td>146/673 (21.7%)</td>
</tr>
<tr>
<td>Anaemia</td>
<td>475/736 (64.5%)</td>
<td>504/691 (72.9%)</td>
</tr>
<tr>
<td>Iron biomarkers**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepcidin (ng/ml)</td>
<td>11.1 (9.9, 12.4)</td>
<td>4.2 (3.7, 4.8)</td>
</tr>
<tr>
<td>Ferritin (µg/L)</td>
<td>25.2 (23.6, 26.8)</td>
<td>20.6 (19.1, 22.2)</td>
</tr>
<tr>
<td>ZnPp (µmol/mol Hb)</td>
<td>86.4 (82.9, 90.0)</td>
<td>113.4 (108.7, 118.3)</td>
</tr>
<tr>
<td>sTfR (mg/L)</td>
<td>3.45 (3.36, 3.54)</td>
<td>4.16 (4.05, 4.28)</td>
</tr>
<tr>
<td>UIBC (µmol/L)</td>
<td>56.8 (55.7, 57.8)</td>
<td>62.7 (61.3, 64.2)</td>
</tr>
<tr>
<td>Serum iron (µmol/L)</td>
<td>8.6 (8.3, 9.0)</td>
<td>8.4 (8.2, 8.7)</td>
</tr>
<tr>
<td>TSAT (%)</td>
<td>12.9 (12.4, 13.4)</td>
<td>11.6 (11.1, 12.1)</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>10.6 (10.5, 10.7)</td>
<td>10.0 (9.8, 10.1)</td>
</tr>
</tbody>
</table>

* Frequency and percentages are shown. ** Geometric means and 95% CIs are shown. †Iron deficiency was defined as ferritin <12µg/l (or ferritin <30µg/l in the presence of inflammation or <15 µg/L in children ≥5years); ‡iron deficiency anaemia as iron deficiency and anaemia; ‡‡anaemia as haemoglobin<11.0 g/dL or haemoglobin<11.5 g/dL in children ≥5years. ZnPp, zinc protoporphyrin; TSAT, transferrin saturation; sTfR, soluble transferrin receptor; UIBC, unsaturated iron binding capacity; and Hb, haemoglobin.
end of the malaria season (adj. OR 0.40 [95% CI 0.21, 0.77]; Bonferroni adj. P=0.03) but not at the start. The other IFNG SNPs were not associated with malaria parasitaemia at either time point (Extended datafile 3). The IFNG SNPs were not associated with HbAS or G6PD genotypes following Bonferroni correction in adjusted models. The IFNG+874T and IFNG+3234C alleles were associated with the Fulani ethnic group (OR 2.22 [95% CI 1.49, 3.31] Bonferroni P = 0.0005 and OR 1.56 [95% CI 1.09, 2.24]; Bonferroni P = 0.045, respectively). The minor allele frequencies by ethnic group are presented in Extended datafile 4.

Associations with iron and anaemia
We found that the IFNG SNPs were not associated with ID, IDA or anaemia at the start of the malaria season in multivariable logistic regression analyses adjusted for age, sex, village, ACT and malaria parasitaemia following Bonferroni adjustment (Extended datafile 4). The IFNG SNPs were similarly not significantly associated with ID, IDA or anaemia at the end of the malaria season after Bonferroni correction. The IFNG+2200C allele (rs1861493) was associated with trends towards increased risk of anaemia (adj. OR 1.86 [95% CI 1.02, 3.38]; adj. P = 0.04), IDA (adj. OR 1.81 [95% CI 1.01, 3.25]; adj. P = 0.05), and ID (adj. OR 1.63 [95% CI 0.95, 2.80]; adj. P = 0.08) in multivariable logistic regression models, but not after Bonferroni correction (Table 2). Children carrying the IFNG+2200C allele had lower haemoglobin levels (9.7 g/dl [95% CI 9.4, 10.1]) compared to those with the IFNG+2200 TT genotype (10.0 g/dl [95% CI 9.8, 10.1]; adj. P = 0.006 and Bonferroni adj. P = 0.03), as well as a trend towards ID in other markers of iron status at the end of the malaria season in adjusted linear regression models (Table 3).

IFNG haplotypes
Haplotype analysis identified ten haplotypes (four with less than 1% population frequency) resolved by SNPs at nucleotide positions -1616, +874, +2200, +3234 and +5612 in the IFNG gene locus (Figure 1). Six haplotypes accounted for most of the variation. The wild-type haplotype (1, IFNG-CATTCT) was present at a frequency of 35% in the Gambian children, while haplotype 6 (IFNG-CACTCT), uniquely identified by the IFNG+2200 SNP, was present at a frequency of 7%. Using haplotype 1 as the reference, haplotype 6 was associated with a trend towards increased risk of ID (adj. OR 1.58 [95% CI 0.93, 2.69]), IDA (adj. OR 1.71 [95% CI 0.94, 3.10]) and anaemia (adj. OR 1.67 [95% CI 0.93, 3.01]) at the end of the malaria season (Table 4). Haplotype 6 was also associated with reduced haemoglobin concentrations (adj. β -0.48 [95% CI -0.79, -0.18]; P = 0.002) and TSAT (adj. β -0.15 [95% CI -0.27, -0.03]; P = 0.02), and higher ZnPP levels (adj. β 0.06 [95% CI 0.01, 0.12]; P = 0.02) and a trend towards reduced ferritin levels compared to the wild-type haplotype (Table 5).

Discussion
In this study we observed an increase in the prevalence of ID, IDA and anaemia across the malaria season in Gambian children. Dietary iron insufficiency may be an important cause since the malaria season also coincides with the ‘hungry season’ in The Gambia when there is a scarcity of staple foods. We hypothesized that IFN-γ, a pro-inflammatory cytokine induced during malaria infection19, might play a role in influencing the risk of ID and anaemia in children exposed to malaria. In addition to directly reducing erythrocyte half-life19, evidence suggests that IFN-γ induces hepcidin and inhibits ferroportin, hence reducing iron absorption and promoting sequestration of iron in macrophages17,18. Consequently, high levels of IFN-γ induced during malaria infections may concomitantly lead to ID and anaemia.

We found that the IFNG+2200C (rs1861493) allele, located at intron 3 of the IFNG gene, was associated with reduced haemoglobin levels and a trend towards ID, IDA and anaemia at the end of a malaria season in multivariable analyses adjusting for potential confounders. We then constructed haplotypes to increase the probability of capturing functional mutations which might reside within a given haplotype. Haplotype 6 (uniquely identified by the IFNG+2200C allele), was associated with reduced haemoglobin levels and TSAT and increased ZnPP levels in keeping with iron deficiency compared to the wild-type haplotype. Haplotype 6 was similarly associated with trends towards increased risk of ID, IDA and anaemia at the end of the malaria season.

Figure 1. Schematic representation of the IFNG gene locus. The IFNG single nucleotide polymorphisms (SNP) are designated according to the nucleotide position relative to the transcriptional starting site of IFNG.
Table 2. *IFNG* genotypes and risk of iron deficiency, iron deficiency anaemia and anaemia at the end of the malaria season.

<table>
<thead>
<tr>
<th>DBSNP number</th>
<th>Genotype</th>
<th>No (%)</th>
<th>Iron Deficiency&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Iron Deficiency Anaemia&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Anaemia&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>OR (95% CI)</td>
<td>Adj P</td>
<td>Bf adj P</td>
</tr>
<tr>
<td>rs2069705</td>
<td>IFN-1616 CC</td>
<td>206 (29.9)</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td></td>
<td>IFN-1616 CT</td>
<td>332 (48.2)</td>
<td>0.82 (0.53, 1.28)</td>
<td>0.40</td>
<td>1</td>
</tr>
<tr>
<td>rs2430561</td>
<td>IFN+874 AA</td>
<td>509 (71.4)</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td></td>
<td>IFN+874 AT</td>
<td>182 (25.5)</td>
<td>0.71 (0.44, 1.14)</td>
<td>0.22</td>
<td>0.76</td>
</tr>
<tr>
<td>rs1861493</td>
<td>IFN+2200 TT</td>
<td>598 (86.0)</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td></td>
<td>IFN+2200 CT/CC</td>
<td>97 (14.0)</td>
<td>1.63 (0.95, 2.80)</td>
<td>0.08</td>
<td>0.38</td>
</tr>
<tr>
<td>rs2069718</td>
<td>IFN+3234 TT</td>
<td>339 (54.7)</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td></td>
<td>IFN+3234 CT</td>
<td>268 (37.3)</td>
<td>0.87 (0.58, 1.32)</td>
<td>0.52</td>
<td>1</td>
</tr>
<tr>
<td>rs2069728</td>
<td>IFN+5612 TT</td>
<td>326 (47.0)</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td></td>
<td>IFN+5612 CT</td>
<td>302 (43.6)</td>
<td>0.85 (0.57, 1.28)</td>
<td>0.44</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>IFN+5612 TT</td>
<td>65 (9.4)</td>
<td>0.97 (0.48, 1.96)</td>
<td>0.93</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are number (%) in each group; <sup>b</sup>Ferritin <12 µg/L (or ferritin<30 µg/L in the presence of inflammation or <15 µg/L in children ≥5 years); <sup>c</sup>Iron deficiency<sup>1</sup> and anaemia<sup>2</sup>; <sup>d</sup>Haemoglobin<11.0 g/dL (or haemoglobin<11.5 g/dL in children ≥5 years); <sup>e</sup>The mutant alleles for *IFNG*+2200 were combined because of the low frequency of *IFNG*+2200CC (n=4) in the study population; <sup>f</sup>Odds ratios (OR) and P values were derived by multivariable logistic regression adjusted for age, sex, village (a proxy for location and ethnic group), α1-antichymotrypsin level and the presence of malaria parasites on blood film; <sup>g</sup>P values additionally adjusted for Bonferroni correction for multiple testing.

Table 3. *IFNG+2200* SNPs and markers of iron status at the end of the malaria season.

<table>
<thead>
<tr>
<th>Iron Marker</th>
<th>n</th>
<th>IFNG+2200 TT&lt;sup&gt;b&lt;/sup&gt;</th>
<th>n</th>
<th>IFNG+2200 CT/CC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Adj P&lt;sup&gt;†&lt;/sup&gt;</th>
<th>Bf adj P&lt;sup&gt;‡&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepcidin (ng/ml)</td>
<td>500</td>
<td>4.4 (3.8, 5.1)</td>
<td>84</td>
<td>3.2 (2.2, 4.6)</td>
<td>0.09</td>
<td>0.44</td>
</tr>
<tr>
<td>Ferritin (µg/L)</td>
<td>545</td>
<td>21.4 (19.7, 23.2)</td>
<td>91</td>
<td>16.9 (13.6, 21.1)</td>
<td>0.04</td>
<td>0.21</td>
</tr>
<tr>
<td>ZnPP (µmol/mol Hb)</td>
<td>550</td>
<td>112.4 (107.2, 117.8)</td>
<td>91</td>
<td>121.2 (107.0, 137.3)</td>
<td>0.06</td>
<td>0.32</td>
</tr>
<tr>
<td>sTIR (mg/L)</td>
<td>508</td>
<td>4.2 (4.0, 4.3)</td>
<td>86</td>
<td>4.2 (3.9, 4.6)</td>
<td>0.38</td>
<td>1</td>
</tr>
<tr>
<td>UIBC (µmol/L)</td>
<td>530</td>
<td>61.6 (60.1, 63.2)</td>
<td>87</td>
<td>67.9 (62.6, 73.8)</td>
<td>0.04</td>
<td>0.19</td>
</tr>
<tr>
<td>Serum iron (µmol/L)</td>
<td>532</td>
<td>8.6 (8.3, 8.9)</td>
<td>88</td>
<td>7.9 (7.1, 8.7)</td>
<td>0.19</td>
<td>0.95</td>
</tr>
<tr>
<td>TSAT (%)</td>
<td>528</td>
<td>12.0 (11.4, 12.5)</td>
<td>87</td>
<td>10.1 (8.8, 11.6)</td>
<td>0.04</td>
<td>0.19</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>546</td>
<td>10.0 (9.8, 10.1)</td>
<td>92</td>
<td>9.7 (9.4, 10.1)</td>
<td>0.006</td>
<td>0.03</td>
</tr>
</tbody>
</table>

<sup>b</sup>Values are geometric means (95% confidence interval); <sup>†</sup>The wild-type alleles represent children with *IFNG*+2200TT genotype (n=598). The mutant alleles represent children with the *IFNG*+2200CT (n=93) and *IFNG*+2200CC (n=4) genotypes combined; <sup‡</sup>Significance values were derived by multivariable linear regression with each log-transformed iron marker as a dependent variable and adjusted for age, sex, village (a proxy for location and ethnic group), α1-antichymotrypsin level and the presence of malaria parasites on blood film; <sup>‡</sup>P values adjusted for Bonferroni correction for the five tested SNPs. n, number of children; Hb, haemoglobin; ZnPP, zinc protoporphyrin; sTIR, soluble transferrin receptor; UIBC, unsaturated iron binding capacity; and TSAT, transferrin saturation.

So how might the *IFNG+2200C* genotype and a haplotype uniquely defined by this genotype potentially lead to reduced haemoglobin levels and ID at the end of the malaria season? A possible explanation may be through increasing *IFNG* gene expression and IFN-γ levels. The *IFNG+2200C* allele was associated with increased IFN-γ levels in Kawasaki disease patients<sup>5</sup>, although another study in patients with ankylosing spondylitis found no difference in IFN-γ levels by *IFNG+2200 genotypes<sup>6</sup>. Elevated IFN-γ levels promote dyserythropoiesis, anaemia and iron dysregulation. IFN-γ inhibits proliferation of
Table 4. IFNG haplotypes and risk of iron deficiency, iron deficiency anaemia and anaemia at the end of the malaria season.

<table>
<thead>
<tr>
<th>Hap ID</th>
<th>Haplotype</th>
<th>Freq (No)</th>
<th>Iron Deficiency</th>
<th>Iron Deficiency Anaemia</th>
<th>Anaemia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>OR (95% CI)</td>
<td>Adj P</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>Hap1</td>
<td>CATTTC</td>
<td>0.35 (536)</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Hap2</td>
<td>TATTT</td>
<td>0.19 (283)</td>
<td>0.82 (0.55, 1.21)</td>
<td>0.31</td>
<td>0.69 (0.44, 1.07)</td>
</tr>
<tr>
<td>Hap3</td>
<td>TTTCC</td>
<td>0.16 (240)</td>
<td>0.70 (0.46, 1.06)</td>
<td>0.09</td>
<td>0.68 (0.43, 1.08)</td>
</tr>
<tr>
<td>Hap4</td>
<td>CATTTC</td>
<td>0.11 (171)</td>
<td>1.14 (0.73, 1.78)</td>
<td>0.73</td>
<td>0.91 (0.53, 1.56)</td>
</tr>
<tr>
<td>Hap5</td>
<td>TATCC</td>
<td>0.11 (166)</td>
<td>1.19 (0.77, 1.85)</td>
<td>0.58</td>
<td>0.91 (0.53, 1.55)</td>
</tr>
<tr>
<td>Hap6</td>
<td>CACTCC</td>
<td>0.07 (100)</td>
<td>1.58 (0.93, 2.69)</td>
<td>0.09</td>
<td>1.71 (0.94, 3.10)</td>
</tr>
</tbody>
</table>

* The haplotype configuration is as follows: IFNG+1616, IFNG+874, IFNG+2200, IFNG+3234, IFNG+5612. Minor alleles are indicated by bold type; * ferritin <12µg/L or ferritin<30µg/L in the presence of inflammation or <15 µg/L in children <5 years); † iron deficiency and anaemia; ‡ haemoglobin<11.0g/dL (or haemoglobin<11.5g/dL in children <5 years); ¶ odds ratios (OR) and P values were derived by multivariable logistic regression adjusted for age, sex, village, α1-antichymotrypsin level and malaria parasitaemia. Hap, haplotype; Freq, frequency of the haplotypes in the study population; no, number of haplotype alleles in the study population.

The influence of the IFNG+2200C allele on haemoglobin and iron status was only observed at the end of the malaria season. We hypothesized that the effects of this SNP may be most marked when expression of IFNG is upregulated, such as during malaria infections1,2,4. This also highlights the influence of gene-environment interactions in promoting disease, in this case ID, IDA and anaemia. It is unlikely that the decreased haemoglobin levels observed in individuals carrying the IFNG+2200C haplotype was due to increased malaria since these children had reduced prevalence of malaria parasitaemia at the end of the malaria season. It is possible that higher IFN-γ levels, putatively produced by IFNG+2200C carriers, may induce a protective proinflammatory response against malaria1,9, but at the expense of iron homeostasis. The IFNG+874TT (rs2430561) genotype, located at the first intron coinciding with the NFκB binding region, has also been associated with higher production of IFN-γ2,34,40. However, studies have found no association between IFNG+874TT and malaria25 or aplastic anaemia39, and in our study we observed a trend towards a decreased risk of ID in these individuals. Further investigations are required on a cellular level to explore putative functional effects of IFNG genotypes on IFN-γ levels and iron status.

To our knowledge, this is the first study examining the role of IFNG gene polymorphisms in relation to iron status. We found that the IFNG+2200C (rs1861493) allele, and a haplotype defined by this allele, were associated with reduced haemoglobin levels and a trend towards ID at the end of the malaria season, a finding that may be due to increased IFN-γ levels25. However, our study had a number of important limitations and our findings should be viewed with considerable caution. The study was conducted in a single site and had relatively small numbers (n = 756). Additionally, many of our findings were of marginal significance with wide confidence intervals and lost statistical significance after correction for multiple testing with Bonferroni adjustment. It is also unclear if our findings have clinical relevance at an individual level. The IFNG+2200C SNP may also be in linkage disequilibrium (LD) with another genetic variant within the haplotype that might influence IFN-γ levels and / or measures of iron status in Gambian populations. Finally, we did not measure IFN-γ levels to determine if they differed between genotypes at the end of the malaria season. Thus, our findings need to be examined in larger population-based studies, in other malaria-exposed populations, and functional assays are needed to identify whether genetic variation in the IFNG gene influences iron status. However, our study supports the hypothesis that preventing and treating malaria infection may improve haemoglobin levels and iron status in African children8.
Table 5. *IFNG* haplotypes and markers of iron status at the end of the malaria season.

<table>
<thead>
<tr>
<th>Hap</th>
<th>Hepcidin (ng/ml)</th>
<th>Adj P</th>
<th>Ferritin (µg/L)</th>
<th>Adj P</th>
<th>ZnPP (µmol/mol Hb)</th>
<th>Adj P</th>
<th>TSAT (%)</th>
<th>Adj P</th>
<th>sTfR (mg/L)</th>
<th>Adj P</th>
<th>Hb (g/dl)</th>
<th>Adj P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CATTC</td>
<td>4.3 (3.7, 5.0)</td>
<td>Ref</td>
<td>19.4 (17.8, 21.1)</td>
<td>Ref</td>
<td>115.3 (109.6, 121.3)</td>
<td>Ref</td>
<td>11.5 (11.0, 12.1)</td>
<td>Ref</td>
<td>4.2 (4.0, 4.3)</td>
<td>Ref</td>
<td>10.0 (9.8, 10.1)</td>
<td>Ref</td>
</tr>
<tr>
<td>TATT</td>
<td>4.5 (3.5, 5.6)</td>
<td>0.69</td>
<td>22.3 (19.7, 25.3)</td>
<td>0.06</td>
<td>113.0 (105.0, 121.6)</td>
<td>0.41</td>
<td>11.7 (11.0, 12.6)</td>
<td>0.93</td>
<td>4.2 (4.0, 4.4)</td>
<td>0.76</td>
<td>9.9 (9.7, 10.1)</td>
<td>0.22</td>
</tr>
<tr>
<td>TTCC</td>
<td>4.3 (3.3, 5.5)</td>
<td>0.95</td>
<td>24.4 (21.3, 27.9)</td>
<td>0.05</td>
<td>115.1 (107.3, 123.5)</td>
<td>0.54</td>
<td>11.3 (10.4, 12.4)</td>
<td>0.58</td>
<td>4.1 (3.9, 4.4)</td>
<td>0.50</td>
<td>10.0 (9.7, 10.2)</td>
<td>0.85</td>
</tr>
<tr>
<td>CATT</td>
<td>5.2 (4.0, 6.7)</td>
<td>0.28</td>
<td>20.9 (17.8, 20.5)</td>
<td>0.54</td>
<td>103.8 (95.9, 112.4)</td>
<td>0.26</td>
<td>12.9 (11.9, 14.0)</td>
<td>0.09</td>
<td>4.0 (3.8, 4.3)</td>
<td>0.30</td>
<td>10.2 (9.9, 10.4)</td>
<td>0.31</td>
</tr>
<tr>
<td>TATC</td>
<td>3.5 (2.6, 4.7)</td>
<td>0.16</td>
<td>18.8 (16.0, 22.1)</td>
<td>0.64</td>
<td>109.3 (99.9, 120.1)</td>
<td>0.87</td>
<td>11.5 (10.5, 12.6)</td>
<td>0.92</td>
<td>4.2 (4.0, 4.5)</td>
<td>0.80</td>
<td>10.0 (9.8, 10.3)</td>
<td>0.74</td>
</tr>
<tr>
<td>CACT</td>
<td>3.2 (2.3, 4.6)</td>
<td>0.11</td>
<td>16.6 (13.4, 20.5)</td>
<td>0.08</td>
<td>123.5 (109.1, 139.8)</td>
<td>0.02</td>
<td>9.8 (8.5, 11.3)</td>
<td>0.02</td>
<td>4.2 (3.9, 4.6)</td>
<td>0.28</td>
<td>9.7 (9.3, 10.0)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Values are geometric means (95% confidence interval). 1 Significance (P) values were derived by multivariable linear regression with each log-transformed iron marker as a dependent variable and adjusted for age, sex, village (a proxy for location and ethnic group), α1-antichymotrypsin level and the presence of malaria parasites on blood film. Hap, haplotype; Hb, haemoglobin; ZnPP, zinc protoporphyrin; sTfR, soluble transferrin receptor; and TSAT, transferrin saturation.
Data availability

Underlying data

Havard Dataverse: Replication Data for: Interferon-gamma polymorphisms and risk of iron deficiency and anaemia in Gambian children, https://doi.org/10.7910/DVN/2NKJID. This project contains the following underlying data:

- IFNG_final_data_v2 (dataset containing demographic information and results of laboratory assays for participants included in the study).
- IFNG_analysis_KM (contains the codes used for data analysis).
- KMokaya_IFNG_Codebook (contains variable description and labels).

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Extended Data


This project contains the following extended data:

- Extended datafile 1. Assayed IFNG single nucleotide polymorphisms
- Extended datafile 2. Agena Biosciences (formerly SEQUENOM) MassARRAY® primer-extension definitions data for the five IFNG polymorphisms
- Extended datafile 3. IFNG single nucleotide polymorphisms and risk of malaria parasitemia at the start and end of the malaria season
- Extended datafile 4. IFNG genotypes and risk of iron deficiency, iron deficiency anaemia and anaemia at the start of the malaria season

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

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References


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Abstract:
It would be good if the authors provided the age range of children enrolled in the study (2 – 6 years). This could be added in the Methods section at the end of the sentence ‘….780 Gambian children.’

The last sentence at the end of the Results section mentions a number of covariates that were adjusted for in the models. One such covariate is ‘malaria parasitaemia’.

Was malaria parasitaemia adjusted for only at the beginning and at the end of the malaria season or did it capture all the malaria cases during the study period? Does ‘malaria parasitaemia’ mean malaria episodes during the study period? If it only captures the two time points, what would the effect of ‘additional episodes’ during the study period have on the results? In malaria endemic areas, repeated infections are more likely to result in anaemia than just the patent infection. The authors need to clarify on this and if there were no malaria cases in-between the two time-points they need to indicate so.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes
Are the conclusions drawn adequately supported by the results?
Yes

*Competing Interests:* No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.