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The p.H63D allele of the HFE gene protects against low iron stores in Sri Lanka

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ABSTRACT

In hereditary hemochromatosis, iron overload is associated with homozygosity for the p.C282Y mutation. A second mutation, p.H63D, occurs at significant frequencies in Europe, North Africa, the Middle East and Asia. Early studies in Sri Lanka indicated that the variant had arisen independently, suggesting that it had been the subject of selective pressure. However, its role in iron absorption is unclear.

In a survey of 7526 Sri Lankan secondary school students, we determined hemoglobin genotype and measured red cell indices, serum ferritin, transferrin receptor, iron zinc protoporphyrin and hepcidin. These variables were compared according to the presence or absence of the p.H63D variant in a subset of 1313 students for whom DNA samples were available. Students were classified as having low red cell indices if they had an MCV < 80 fl and/or MCH < 27 pg.

Hetero and/or homozygosity for the p.H63D variant was more common in students with normal than low red cell indices (16.4% and 11.9% respectively; $p = 0.019$). Iron biomarkers and red cell indices were greater in children with the p.H63D variant than in normal and this was statistically significant for MCV ($p = 0.046$).

Our findings suggest that selective pressure by mild iron deficiency contributes to the high frequencies of the p.H63D variant.

1. Introduction

Hereditary hemochromatosis is caused by inappropriate iron absorption and is among the commonest autosomal recessive diseases. After the hemochromatosis gene, *HFE*, was cloned, two presumed causative mutations were identified [1]. The first, a G-A transition at nucleotide 845 which changes amino acid 282 from cysteine to tyrosine (p.Cys282Tyr), is commonly known as C282Y. The C282Y mutation, thought to have arisen approximately 4000 years ago, occurs at a frequency of 1 in 12 to 1 in 15 in populations of northern European ancestry [2,3]. The second mutation, a C to G transversion at nucleotide 187 which changes amino acid 63 from histidine to aspartic acid (p.His63Asp), is commonly known as H63D and thought to be the older of the two mutations. It occurs across Europe and is also distributed widely in north Africa, the Middle East and most of Asia [3]. Due to the low penetrance of the H63D mutation, many regard it as a common variant rather than a mutation. Several other HFE mutations have been identified subsequently. That they are mutations and not variants has been confirmed in many cases by functional studies [4].

A previous study in Sri Lanka reported that the H63D variant was present on three new haplotypes suggesting that these mutations had arisen independently in this region [5]. These findings suggest that the *HFE* gene has been the subject of selective pressure. The present study was established to try to determine whether the H63D variant protects against iron deficiency.

Recently, a national survey of Sri Lankan secondary school students was carried out to (i) determine the frequency and distribution of hemoglobinopathy traits and (ii) assess iron status. Hemoglobin genotype

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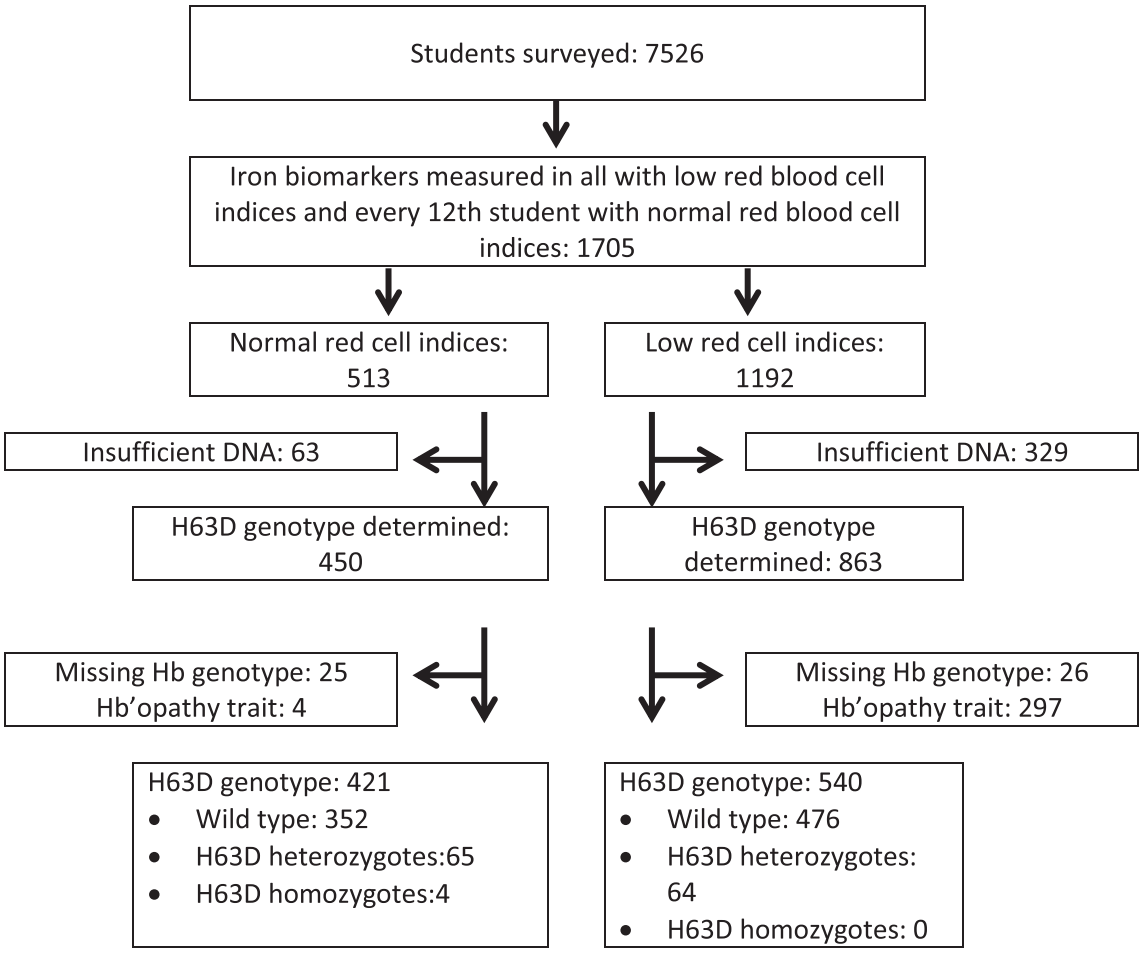

Fig. 1. Flow diagram for study of H63D variant in Sri Lankan adolescents. Hb = hemoglobin, Hb'opathy = hemoglobinopathy.

was determined and red cell indices, serum ferritin transferrin receptor, iron, zinc protoporphyrin and hepcidin levels were measured. The results of these investigations have already been published [6–9].

Subsequently, we determined the frequency of the H63D variant in a subset of students from this survey for whom DNA samples were available. Red cell indices and biomarkers of iron status were compared according to the presence or absence of the H63D variant. We report the findings in students without a hemoglobinopathy trait.

2. Methods

2.1. Study design

Details of the study population and recruitment methods in the cross-sectional survey have been published previously [6,7]. Briefly, as part of a national survey of the frequency and distribution of hemoglobinopathy traits, we recruited 7526 children aged 11–19 years attending schools across the 25 districts of Sri Lanka. Signed, informed consent was obtained from the parents/guardians of all participants prior to enrolment into the study. Iron biomarkers were measured in 1705 students; all 1192 students with low red cell indices and, as a comparison group, every 12th student with normal red cell indices (513 students).

Based on recommended guidelines students were classified as having low red cell indices if they had a mean cell volume (MCV) < 80 fl and/or a mean cell hemoglobin (MCH) < 27 pg, [10–12]. For the present study, sufficient DNA was available for the determination of H63D genotype in 1313/1705 (77.0%) students in whom iron biomarkers had been measured; 863 from the low red cell indices group, (including those with a hemoglobinopathy trait) and 450 from the normal red cell indices groups.

The study and the consent procedures were approved by the Ethics Committee, University of Kelaniya, Sri Lanka and Oxford University Tropical Research Ethics Committee, Oxford, UK.

2.2. Laboratory methods

Five milliliters venous blood was collected and transferred into an EDTA and a plain tube for each participant. The EDTA sample was used for the measurement of red cell indices, hemoglobin variants and zinc protoporphyrin (ZPP). DNA was extracted from the buffy coat.

The blood sample in the plain tube was allowed to clot, centrifuged and the serum removed and used for the measurement of iron biomarkers.

Details of the laboratory methods used for the measurement of hematological indices, hemoglobin variant detection, DNA extraction, serum iron, ferritin, transferrin receptor and hepcidin have been published previously [6–9].

ZPP was measured in whole blood using the Protofluor reagent system and a front-faced hematofluorimeter (Helena BioSciences, South Shields, UK). Serum iron was measured using a manual colorimetric assay (SI 257, Randox laboratories, County Antrim, UK). H63D genotype was determined by polymerase chain reaction (PCR) followed by restriction digest of the PCR product with Mbo1 (New England BioLabs, UK), [13].

All serum samples were tested in duplicate for each of the biomarker

Table 1
Frequency of H63D according to sex, ethnicity and red cell indices in students without a hemoglobinopathy trait.[a]

Variable	H63D genotype				
	Wild type N (%)	Heterozygote N (%)	Homozygote N (%)	X^2	*p* value
Demography					
Sex					
• Male	351 (86.9)	52 (12.9)	1 (0.2)	0.644	0.725
• Female	475 (85.7)	76 (13.7)	3 (0.5)		
Ethnicity					
• Sinhalese	522 (87.8)	71 (11.9)	2 (0.3)	4.37	0.358
• Muslim	56 (86.2)	9 (13.8)	0 (0.0)		
• Tamil	249 (83.0)	49 (16.3)	2 (0.7)		
Red cell indices					
• Low red cell indices	476 (88.1)	64 (11.9)	0	7.96	**0.019**
• Normal red cell indices	352 (83.6)	65 (15.4)	4 (1.0)		

[a] Sex or ethnic group was not recorded for 4 students.

assays. The following cut offs were used as determinants of iron deficiency: ferritin of < 15 ng/ml [14], transferrin receptor > 28.1 nmol/l; serum iron < 10.6 μmol/l in males and < 6.6 μmol/l in females; ZPP > 70 μmol/mol heme (in accordance with each manufacturer's kit insert); hepcidin < 3.2 ng/ml [9].

2.3. Statistical methods

Categorical variables were summarized using counts and percentages and compared using the Chi-squared test. Continuous variables tended to have skewed distributions; they were described using median and interquartile range and compared using the Mann-Whitney *U* test. Statistical analysis was 2-sided and significant at the 0.05 level. All data analysis was performed using SPSS statistical software version 25.

3. Results

The flow diagram for the study is shown in Fig. 1. Overall, the frequency of the H63D variant was 185/1313 (14.1%) and its frequency was similar according to sex and ethnic group (Table 1). The H63D variant was present in 42/301 (14.0%) students with a hemoglobinopathy trait and 133/961 (13.8%) without a hemoglobinopathy trait ($p = 1.0$). Hemoglobin genotype was not available for 51 students.

Since red cell indices and hepcidin levels may be lower, and transferrin receptor and ZPP levels may be higher in individuals who have hemoglobinopathy traits irrespective of iron status [15–17], we confined our analysis to 961 students with a normal hemoglobin genotype (Fig. 1), Hetero- and/or homozygosity for the H63D variant were significantly more common in students with normal than in those with low red cell indices: 69/421 (16.4%) and 64/540 (11.9%) respectively; ($p = 0.019$; Table 1). Red cell indices and biomarkers of iron status according to H63D genotype are shown in Table 2. MCV was statistically significantly greater in students with the H63D variant than in those with a normal HFE genotype ($p = 0.046$). Serum iron concentrations were also higher, and ZPP and hepcidin concentrations were lower in students with the H63D variant than those without, but these differences did not reach statistical significance (Fig. 2). Although there was no statistically significant difference in serum ferritin concentration according to H63D genotype, the frequency of the H63D variant was significantly greater in iron replete students (18.7%) than in those with low iron stores (ferritin < 15 ng/ml; 12.2%; $p = 0.041$, Table 3).

These findings were similar when males and females were analyzed separately.

Table 2
Red cell indices and iron biomarkers according to H63D genotype in students without a hemoglobinopathy trait.

Red cell indices/iron biomarker	H63D genotype			P value
	Wild type N Median (IQR)	Heterozygote N Median (IQR)	Homozygote N Median (IQR)	
Mean cell volume (fl)	814 81.7 (78.1–84.8)	129 83.0 (77.9–85.5)	4 88.8 (84.0–93.3)	**0.046**
Mean cell hemoglobin (pg)	828 27.9 (26.2–29.7)	129 28.1 (26.0–30.6)	4 30.9 (29.1–31.2)	0.126
Hemoglobin (g/dl)	802 13.5 (12.4–14.8)	124 13.7 (12.3–15.1)	4 13.9 (13.2–14.4)	0.738
Ferritin (ng/ml)	813 25.1 (11.9–42.5)	128 28.9 (12.4–46.9)	4 21.1 (16.9–34.2)	0.588
Transferrin Receptor (nmol/l)	821 27.3 (21.3–37.0)	127 28.5 (22.4–37.1)	4 28.1 (23.8–34.92)	0.536
Iron (nmol/l)	797 13.5 (9.1–18.3)	124 14.5 (8.4–19.1)	4 20.3 (16.2–23.7)	0.107
ZPP (umol/mol heme)	267 58.0 (42.0–86.0)	39 57.0 (45.0–110.0)	0 – –	0.586
Hepcidin (ng/ml)	728 3.73 (2.15–5.89)	104 3.07 (1.8–5.84)	4 3.04 (2.23–3.75)	0.461

4. Discussion

The H63D variant is unusual in that it occurs at reasonably high frequencies in many countries throughout the world including non-malarious regions. This is in contrast to the inherited disorders of hemoglobin, the commonest monogenic diseases of man, which only occur at high frequencies in countries within the tropical belt with past or present malaria. Early studies in Sri Lanka suggested that the H63D variant had arisen independently from the European haplotype suggesting that this variant had been the subject of selective pressure.

Given its occurrence in the HFE gene, selection for H63D is likely to be a result of protection against iron deficiency. Iron deficiency is common in young children, pregnant women and females of childbearing age in most countries of the world and is a complex condition that occurs in 3 progressive stages [18,19]. In iron depletion, characterized by low serum ferritin, iron stores are low but physiological functions not impaired. Cellular iron deficiency, characterized by low serum ferritin and raised transferrin receptor, reflects more marked iron insufficiency, as iron stores are exhausted and normal cellular physiological functions are impaired. Finally, in iron deficiency anemia, persistence of iron deficiency reduces red cell mass, reflected in reduced hemoglobin (Hb) concentration, low serum ferritin and raised transferrin receptor.

We have reported previously that low iron stores occurred in 19.2% of the student population in Sri Lanka [7]. We now show that, although hemoglobin and serum ferritin did not differ significantly according to H63D genotype, students with the H63D variant were less likely to have low iron stores (ferritin < 15 ng/ml), indicative of the early stages of iron deficiency, than those students with wild type genotype. Furthermore, heterozygosity for the H63D variant was significantly more common in students with normal red cell indices than in those with low red cell indices, and MCV was significantly greater in those students with the H63D variant, than those without. Our findings are consistent with studies in Polish children, where iron status and Hb concentrations were greater in those with H63D variant than those without [20,21].

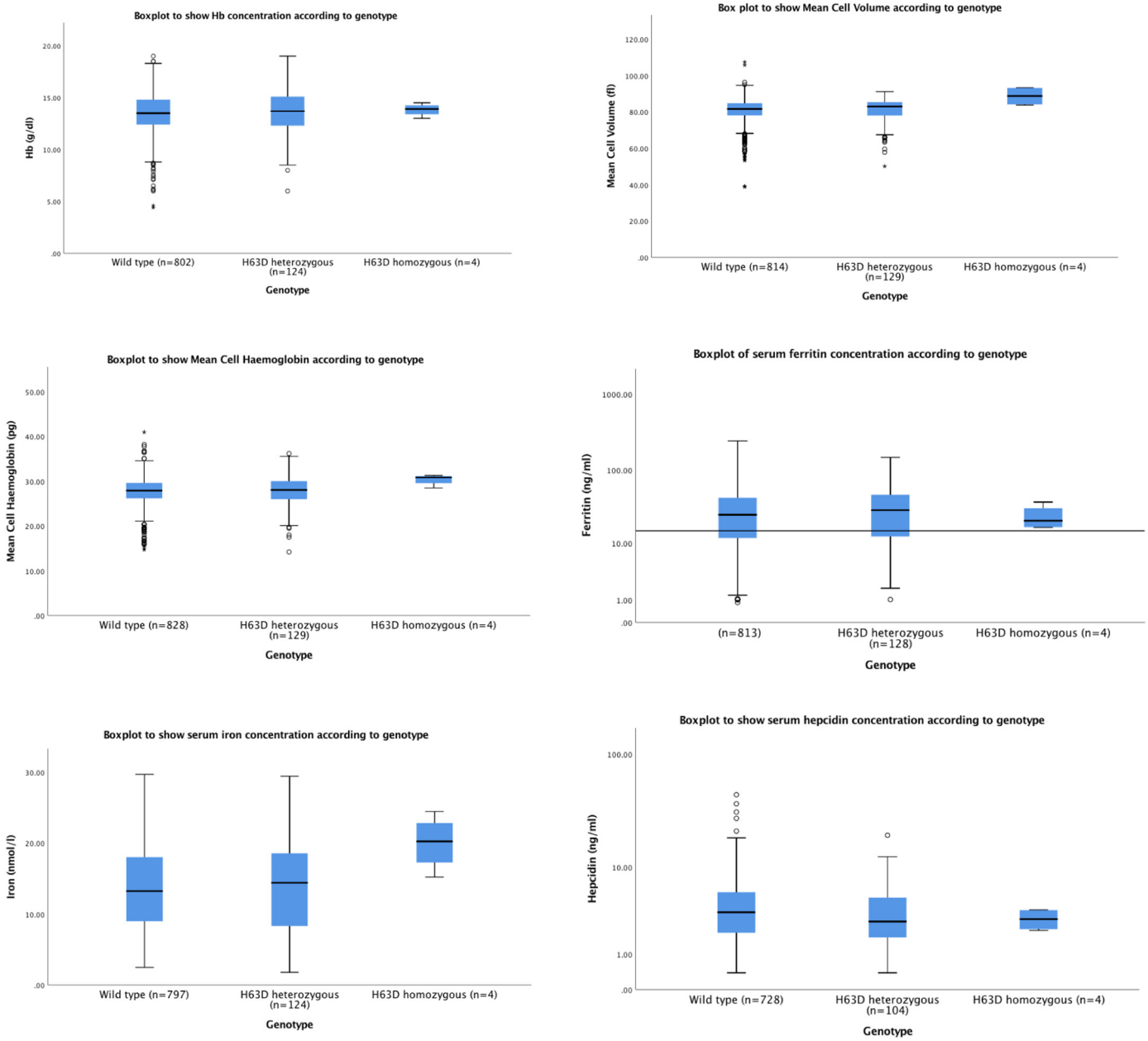

Fig. 2. Hb, red cell indices and iron biomarkers according to H63D genotype.
Box plots to show hemoglobin, red cell indices and iron biomarkers according to HFE genotype in secondary school students from Sri Lanka: (A) hemoglobin; (B) mean cell volume; (C) mean cell hemoglobin; (D) serum ferritin; (E) serum iron.; (F) serum hepcidin; Horizontal lines inside the box show the median value, box length is the interquartile range and whiskers show the range, excluding outliers. Outlying values 1.5 to 3, or > 3 box lengths from the upper and lower edge of the box are shown as open circles and stars respectively. The horizontal line in D shows the lower limit of the normal range for serum ferritin concentration.

Table 3
Iron status according to H63D genotype in students without a hemoglobinopathy trait.

Iron status	H63D genotype			X^2	p value
	Wild type N (%)	Heterozygote N (%)	Homozygote N (%)		
Iron replete[a]	201 (81.4)	44 (17.8)	2 (0.9)	3.87	**0.041**
Low iron stores (serum ferritin < 15 ng/ml)	260 (87.8)	36 (12.2%)	0 (0.0)		

[a] Red cell indices and iron biomarkers all within the normal range.

Our findings are consistent with protection against low iron stores providing the selective pressure for H63D in Sri Lanka, and this may also be relevant to the multiple countries in which this variant has been detected. However, the relatively small differences in iron stores according to H63D genotype and the lack of a difference according to more severe stages of iron deficiency or anemia calls this into question. It is possible that the reason we did not find differences in cellular iron deficiency or iron deficiency anemia according to H63D genotype in our study group was because we studied adolescents and, given that HFE gene variants result in progressive iron accumulation throughout life, we may have seen greater differences if we had studied older people. In a study of older people (aged 55 and over) H63D heterozygosity was associated with increased concentrations of all iron biomarkers [22]. However, in a longitudinal study of the natural history of

iron indices in Australian adults aged 40–69 years followed up over a 12 year period, iron biomarker concentrations in H63D heterozygotes remained within the normal range and were similar over time [23].

Also, the current study does not reflect the historical period over which selection for this variant has occurred. Iron deficiency became more frequent and severe, following the agricultural revolution that occurred about 10,000 years ago, when the human diet changed from predominantly meat eating to more cereal and plant based [24,25] and this may have resulted in greater selective pressure, than occurs today. As well as improvements in diet, available treatments for iron deficiency may have reduced the importance of the heterozygous advantage afforded by the H63D variant [25,26].

A further consideration regarding selective advantage is the relationship between iron status and susceptibility to infections. Although inheritance of an HFE variant resulting in increased iron stores might be considered to predispose to infections, conversely, iron deficiency within macrophages in hereditary hemochromatosis might offer protection against intracellular pathogens [27].

5. Conclusion

We have demonstrated that inheritance of the H63D variant in the HFE gene is associated with significantly but marginally improved iron status in adolescents in Sri Lanka. It is possible that improved iron status confers a selection advantage to carriers of this variant and accounts for the cosmopolitan distribution and high frequencies of the H63D variant throughout the world.

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Statement of authors' contributions

AA, DW, AP designed the study, AA, KR, SA and DW wrote the paper, AA, RR, AM, LP, KW, AA, CF, performed the laboratory analyses, RR, AM, LP, AP performed the field work, AA, SA, KR, HD, DW analyzed the data and interpreted the findings. All authors reviewed and approved the final manuscript.

Statement of interest

None of the authors have any declaration of interest to declare.

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