

# ***HFE* S65C Variant Is Not Associated with Increased Transferrin Saturation in Voluntary Blood Donors**

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Naveen Arya,<sup>1</sup> Subrata Chakrabarti,<sup>2</sup> Robert A. Hegele,<sup>3</sup> Paul C. Adams<sup>1</sup>

**ABSTRACT:** Two amino acid variants in the *HFE* gene, C282Y and H63D, have been reported in most cases of hereditary hemochromatosis. A recently discovered novel amino acid variant of *HFE*, namely S65C, has been implicated to be responsible for a mild form of iron overload.

We determined genotypes of the *HFE* S65C variant in 230 voluntary blood donors with a transferrin saturation > 45%, who did not carry the *HFE* C282Y variant. The control group consisted of 248 first time blood donors who had a transferrin saturation < 45%. We also determined genotypes of the *HFE* H63D variant in the two groups.

For the *HFE* S65C variant, the frequency of the *HFE* C65 allele was 1.7% and 2.2% in the high and low transferrin saturation groups, respectively ( $p = 0.65$ ). In contrast, for the *HFE* H63D variant, the frequency of the *HFE* D63 allele was 24.8% and 14.7% in the high and low transferrin saturation groups, respectively ( $p = 0.0009$ ).

This study demonstrates no association of the *HFE* C65 allele with the phenotype of high transferrin saturation. The results do not support the use of DNA genotyping for the *HFE* S65C mutation in population screening studies for hemochromatosis.

## **INTRODUCTION**

Hemochromatosis is a common hereditary disease associated with progressive iron storage eventually leading to parenchymal damage of the liver, heart, pancreas and other organs (1). The hemochromatosis gene *HFE* was identified in 1996 (2), and genetic testing for hemochromatosis has become a diagnostic tool that alleviates the need for diagnostic liver biopsy in most cases (3). The *HFE* C282Y variant results from a single base substitution, 845G→A, and has two alleles, designated C282 and Y282. *HFE* Y282 is present in >90% of typical hemochromatosis patients (1). The proportion of subjects with hemochromatosis with the *HFE* Y282 allele varies between countries and is strongly affected by phenotypic assignment criteria. Another *HFE* mutation, H63D results from a single base substitution in exon 2, 187C→G, and has two alleles, designated H63 and D63. The *HFE* D63 allele has been associated with iron overload, specifically in

*HFE* D63/D63 homozygotes and in compound heterozygotes for both *HFE* Y282 and D63 alleles. However, these two *HFE* mutations do not account for all cases of iron overload. Approximately 5% to 10% of typical hemochromatosis patients do not carry either of the *HFE* C282Y or H63D mutations. Recently, a new *HFE* amino acid variant has been reported, namely S65C, which results from a single base substitution in exon 2, 193A→T and has two alleles, designated S65 and C65. *HFE* C65 has been implicated, through association, to be involved in the pathogenesis of hemochromatosis (4,5). This study set out to: (a) evaluate the association of *HFE* S65C with iron overload that is unexplained by C282Y, and (b) study the potential utility of genotyping *HFE* S65C as part of population screening for hemochromatosis.

## **MATERIALS AND METHODS**

A population screening study for hemochromatosis was conducted in 5,211 volun-

<sup>1</sup>Department of Medicine, University of Western Ontario, London, Ontario, Canada.

<sup>2</sup>Department of Pathology, University of Western Ontario, London, Ontario, Canada.

<sup>3</sup>Robarts Research Institute, University of Western Ontario, London, Ontario, Canada.

Reprint requests to: Dr. Paul C. Adams, Department of Medicine, London Health Sciences Centre, 339 Windermere Road, London, Ontario N6A 5A5. Fax: 519-663-3232. E-mail: padams@julian.uwo.ca.

**Table 1.** Genotype Frequencies in Voluntary Blood Donors of *HFE* S65C and H63D Mutations

<i>HFE</i> genotype	High TS group (N = 230) (%)	Low TS group (N = 248) (%)	p Value*
D63/D63	5.7	1.2	0.009
D63/H63	38.3	27.0	0.014
H63/H63	56.1	71.8	NS
C65/S65	3.5	4.4	NS
S65/S65	96.5	95.6	NS
C65/S65 plus D63/H63	2.2	3.6	NS

Note. \* - Chi square analysis, Fisher exact test, NS – not significant.

tary blood donors, which demonstrated that 1 in 327 blood donors was homozygous for *HFE* Y282/Y282 (6). The study had received approval from the Human Ethics Committee at the University of Western Ontario, and all participants provided informed consent for DNA testing. For the present study, we selected a subgroup of patients (cases) who were homozygous for *HFE* C282/C282, and yet had a transferrin saturation (TS) > 45%. This threshold has previously been recommended as a critical value for population screening in hemochromatosis (7). The control group consisted of first time blood donors who were homozygous for *HFE* C282/C282, but had TS < 45%. Donors with iron deficiency, defined as TS < 16%, were excluded from this study. All *HFE* Y282 homozygotes and heterozygotes were excluded, in order to eliminate the possible contribution of this allele to mild iron abnormalities. The high TS group had 230 subjects (cases) and the low TS group had 248 subjects (controls).

DNA was extracted from peripheral blood leukocytes and was amplified using the polymerase chain reaction (PCR). Since the DNA changes underlying H63D and S65C are both located in *HFE* exon 2, primers and PCR conditions previously used to amplify *HFE* exon 2 for H63D analysis were used (8). Genotyping for *HFE* S65C was performed using the same conditions as for *HFE* H63D, except that the diagnostic restriction endonuclease was *Hinf*I (5 units in each reaction). PCR products were resolved on 3% agarose gels. The amplified *HFE*

C65 allele product yielded a single 208 bp fragment after digestion with *Hinf*I, whereas the amplified *HFE* S65 allele product yielded two smaller fragments, with sizes 147 bp and 61 bp (4). Patients with the *HFE* C65 allele by restriction analysis had the presence of the variant confirmed by direct DNA sequencing using the dideoxynucleotide chain termination method, with the QIAquick sequencing kit (QIAGEN Inc, Mississauga, Ontario, Canada). Putative samples with the *HFE* C65 allele were compared with a known positive control, kindly provided by Dr. Jim Barton, Birmingham, Alabama.

### Statistical Analysis

Chi-square analysis with the Fisher's exact test was used to compare differences in the allele frequencies of *HFE* H63D and S65C between cases and controls. Linkage disequilibrium coefficients were estimated using the method of Hill and Robertson (9). Power analysis demonstrated that this sample size could detect a statistical difference of 5% between the two groups, with a power of 0.9 and an alpha error of 0.05.

### RESULTS

The mean TS  $\pm$  standard deviation was 49%  $\pm$  4% and 30%  $\pm$  6% in the 230 cases and 248 controls, respectively.

The genotype frequencies of *HFE* H63D and S65C in the cases and controls are shown in Table 1. The genotype frequencies did not deviate significantly from Hardy-Weinberg expectations.

The frequency of the *HFE* C65 allele was 1.7% and 2.2% in cases and controls, respectively (p = 0.65). In contrast, the frequency of the *HFE* D63 allele was 24.8% and 14.7% in the cases and controls, respectively (p = 0.0009). Furthermore, the frequency of the *HFE* D63/D63 homozygotes was significantly higher in the cases compared to the controls (Table 1).

Clinical features of subjects who were heterozygous for *HFE* C65 are shown in Table 2.

**Table 2.** Clinical Attributes of *HFE* C65/S65 Heterozygotes

Age	Sex	Codon 63 genotype	Transferrin saturation (%)	Serum ferritin (µg/L)	Number of blood donations
Study group					
28	M	H63/H63	49	36	8
33	M	H63/H63	45	41	12
31	F	H63/H63	52	50	25
29	M	H63/H63	46	58	0
31	M	H63/H63	48	120	6
28	M	D63/H63	45	111	10
34	M	D63/H63	47	159	3
26	M	D63/H63	46	276	3
Control group					
38	M	H63/H63	33	32.7	0
39	M	H63/H63	31	15.2	0
43	M	H63/H63	27	112	0
29	F	H63/H63	43	16.5	0
38	F	H63/H63	30	30.8	0
43	M	H63/H63	23	37.6	0
31	M	H63/H63	40	93.1	0
34	F	H63/H63	26	53	0
44	F	H63/H63	18	19.4	0
36	M	D63/H63	38	24.8	0
38	M	D63/H63	39	81.6	0

There was no apparent difference in TS between simple heterozygotes for *HFE* C65/S65 and compound heterozygotes for *HFE* C65/S65 plus D63/H63.

Hill and Robertson's maximal likelihood estimate of linkage disequilibrium (D) between *HFE* D63 and C65 was 0.005 ( $r = 0.09$ ,  $P = 0.56$ , NS). However, no haplotype was found to unequivocally contain both *HFE* D65 and C65, strongly suggesting linkage disequilibrium, although the numbers were very small.

## DISCUSSION

In this study we have shown no association between *HFE* C65 and a high TS phenotype. Previous population screening studies have demonstrated an increased frequency of deleterious *HFE* alleles in subject with TS > 45% (10,11). The rare *HFE* C65 allele had a frequency of 1.7% and 2.2% in the high TS and low TS groups, respectively. There was sufficient power in this study to detect a clinically relevant

difference in *HFE* C65 allele frequency in the groups defined by high and low TS. Furthermore, if the *HFE* C65 allele had been associated with an increased risk of iron overload, then a difference in TS might have been expected in the compound heterozygotes (D63/H63 plus C65/S65) compared to the simple heterozygotes (D63/H63 plus S65/S65). Such a difference was not apparent, although the numbers of subjects in these subgroups were small.

In contrast, the *HFE* D63 allele and *HFE* D63/D63 homozygote frequencies were significantly higher in the high TS group compared to the low TS group. This is consistent with the results of previous screening studies, although it remains rare to have significant iron overload in *HFE* D63/D63 homozygotes (12).

This study differs from the previous studies by Mura and Barton, in which atypical proband cases with iron overload were found to have the *HFE* C65 allele (4,5). The extreme phenotypes used in those studies could have resulted in ascertainment bias, which we took care to minimize in our study by selecting a more subtle quantitative phenotype. Also, there could have been linkage disequilibrium with another, unmeasured functional variant within or flanking *HFE* in the patients studied by Mura and Barton, which was absent from the population from which our subjects were drawn. The population selected for this study was chosen from a screening population from voluntary blood donors. TS was selected to determine whether there was an increased risk for iron overload, since ferritin does not rise as early as TS in patients with iron overload. The control group had been selected on the basis that they were first time blood donors, and therefore their TS would not have been altered by repeated blood donations.

In conclusion, the evidence from this study does not support the hypothesis that the *HFE* C65 allele is associated with a genetic predisposition to iron overload.

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