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DETECTION OF *HFE* HAEMOCHROMATOSIS IN THE CLINIC AND COMMUNITY USING STANDARD ERYTHROCYTE TESTS

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Abstract

Detection of *HFE* Haemochromatosis (HH) is challenging in the absence of clinical features. HH subjects have elevated erythrocyte parameters compared to those without HH, but it remains unclear how this could be applied in clinical practice. Thus, we determined the sensitivity, specificity and clinical utility of erythrocyte parameters in 144 HH subjects with (n=122) or without (n=22) clinical and/or biochemical expression of iron overload, 1844 general population controls, and 700 chronic disease subjects. For both expressing and non-expressing HH subjects, the mean pre- and post-phlebotomy values of mean cell volume (MCV) and mean cell haemoglobin (MCH) were always significantly higher when compared to all other groups and demonstrated excellent diagnostic utility for detection of HH in men and women (AUROC 0.83-0.9; maximal sensitivity and specificity 82% and 78%) using cut-off values for MCV >91 fL or MCH >31 pg, respectively. Between 34 and 62% of all HH subjects would be detected, and less than 4% of all non-HH subjects would undergo unnecessary testing, if those with MCV or MCH values greater than 94 fL or 32.2 pg, respectively, were evaluated.

Keywords

HFE Haemochromatosis; screening; diagnosis; mean cell volume; mean cell haemoglobin

Abbreviations

AUROC curve – area under receiver operator characteristic curve

HH – *HFE* Haemochromatosis

MCH – mean cell haemoglobin

MCV – mean cell volume

Introduction

HFE hemochromatosis (HH) is a genetic disorder most commonly attributed in populations of northern European descent to the presence of a homozygous C282Y mutation in the HFE-gene product[1-5]. Most HH subjects are now ascertained with relatively few or no symptoms on the basis of family screening or incidental documentation of elevated serum transferrin saturation and ferritin levels[2, 3, 5]. Biochemical or genetic screening is generally reserved for subjects with a family history of the disorder or clinical presentation with symptoms characteristic of the disorder[2, 3, 5]. Whilst there has been much debate regarding general population screening, it is not currently recommended[2, 3, 6, 7].

There is much evidence supporting the clinical benefits of early detection, including improvement of symptoms and signs of disease[2, 3, 5, 8,9]. Treatment of HH individuals with elevated ferritin levels prior to the onset of cirrhosis is associated not only with long-term survival identical to that of age- and gender-matched controls in the general population[8] but also to marked improvement in symptoms and quality of life[9]. Many individuals with HH derive satisfaction from knowing that the treatment of their disorder results in the provision of blood products for transfusion purposes[10].

Previous studies have reported that HH subjects exhibited significant differences in peripheral blood erythrocyte parameters compared with controls[11-12]. The mean values of haemoglobin (Hb), haematocrit, mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) were all elevated compared with controls. After therapeutic phlebotomy, the MCV, MCH and MCHC values of HH subjects all decreased but remained elevated compared with controls. However, in these studies up to 16% of subjects had cirrhosis, raising the possibility that clinical presentations were more advanced than is currently observed[11-12]. The aim of our study was to evaluate the sensitivity and specificity of erythrocyte parameters as screening tests for detection of HH compared with general population controls and

randomly selected subjects with a range of chronic diseases including liver disease, rheumatological disease, diabetes mellitus and chronic pulmonary disease.

Methods

Subjects

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Human Research Ethics Committees of The University of Western Australia and The Queensland Institute of Medical Research. Consecutive HH subjects (n=122) aged between 20 and 70 years who were referred for initial diagnosis and treatment from primary care to metropolitan community-based Hepatology services between 2005 and 2015 were included in the study. Only subjects with C282Y homozygosity were included. Our exclusion criteria were; pregnant or lactating women, venesection in the previous 12 months, malignancy, and acute inflammatory conditions. Laboratory investigations available for HH subjects at initial assessment included haematology, serum iron biochemistry, HH genotyping, liver biochemistry, chronic viral hepatitis serology, screens for autoimmune liver disease, Wilson disease, alpha-1 antitrypsin genotype, and whether subjects were thought to have cirrhosis. All HH subjects with serum ferritin levels elevated above 300 μg/L underwent therapeutic phlebotomy at weekly intervals until the ferritin level was between 50 and 100 μg/L. Haematology parameters and serum iron biochemistry were monitored throughout therapy. A second group of 22 men and women with HH who did not have elevated ferritin levels or clinical symptoms were included for comparison purposes. General population control subjects comprised 900 men and 944 women aged between 20 and 70 years who did not have a C282Y homozygous nor C282Y/H63D compound heterozygous mutation and who were enrolled in the Busselton Population Study, Western Australia[4]. Baseline haematology parameters were available for all subjects. All blood tests were performed in accredited laboratories. For comparison with other chronic inflammatory diseases, we randomly selected a total of 700 subjects, with equal numbers of men and women, attending outpatient clinics with chronic liver disease (including cirrhosis, chronic

hepatitis C, and chronic hepatitis B), rheumatological disease (rheumatoid arthritis and non-rheumatoid arthritis), diabetes mellitus, or chronic pulmonary disease.

Statistical Analysis

All data are presented as the mean ± SEM unless otherwise specified. Analysis of variance was used to analyse differences between groups and area under the receiver operator characteristic (AUROC) curve analysis was performed for evaluation of sensitivity and specificity of erythrocyte parameters in the diagnosis of HH. All statistical tests were conducted using GraphPad Prism 7 (GraphPad Software, San Diego, CA). Statistical significance was assigned for $p<0.05$.

Results

Patient Characteristics

Clinical characteristics of HH subjects at initial diagnosis are shown in Table 1. 70 men and 52 women with clinical and/or biochemical expression of HH were enrolled in this study. Approximately 44% of men and 48% of women in this cohort experienced symptoms related to HH, with the most common symptom being lethargy and joint pain. 14 of 70 men (20%) and 6 of 52 women (12%) had a history of excessive alcohol consumption (greater than 3 standard drinks per day for men or 2 standard drinks per day for women). More men than women had abnormal liver biochemistry ($p=0.004$). 4 of 70 men whilst none of the women had clinically or histologically confirmed cirrhosis.

Subjects with HH were significantly younger compared to their gender-matched controls and those with cirrhosis, diabetes and pulmonary disease (Table 2). Men with HH were significantly younger compared to gender-matched controls with rheumatoid and non-rheumatoid arthritis but women were significantly younger compared to gender-matched controls with rheumatoid arthritis only. As expected, clinically detected HH subjects had significantly higher serum ferritin levels compared to the control and chronic disease subjects ($p<0.001$). Phlebotomy treatment resulted in significant reductions in ferritin levels in both men and women with HH compared with pre-

treatment values ($p<0.0001$ for both men and women, Table 2). The maximal combinations of sensitivity and specificity for ferritin using cut-off values determined in our study for the detection of HH in pre-treatment men (ferritin cut-off 380 μg/L) was 92% and 89%, respectively whilst in women (ferritin cut-off 227 μg/L) both these values were 92%.

For pre-treatment, post-treatment and non-expressing HH subjects, the mean pre-treatment values of MCV and MCH in men and women were significantly higher when compared to the general population control and chronic disease other groups (Table 2, $p<0.001$). The same was observed for MCV and MCH following treatment of iron-loaded HH subjects. The MCV and MCH also demonstrated excellent diagnostic utility for detection of HH, especially for pre-treatment HH, as shown in the AUROC analysis (Tables 3 and 4, Figures 1 and 2). For pre-treatment men and women, the AUROC ranged between 0.83 and 0.9, providing maximal sensitivity and specificity of 82% and 78% using cut-off values for MCV >91 fL or MCH >31 pg, respectively.

The 90% sensitivity and 90% specificity cut-off thresholds for MCV and MCH are reported in Table 5. Less than 10% of subjects with HH would be left undiagnosed if subjects with values below the 90% sensitivity threshold values for MCV or MCH were not further assessed. Up to 9% of non-HH subjects in the general population also have MCV or MCH values below these thresholds.

Less than 10% of subjects who underwent further work-up for HH for values above the 90% specificity thresholds for MCV or MCH would not have HH. Overall, 1-4% of non-HH subjects in the general population control group have MCV or MCH values above the 90% specificity threshold, whilst 34% to 62% of pre-treatment HH subjects fit in this category. This group is substantially enriched for finding HH (likelihood ratio up to 30 times more likely than non-HH subjects).

Discussion

HH in populations of northern European descent is generally suspected in the clinic following elucidation of a family history, clinical features compatible with a diagnosis or detection of elevated serum iron studies. Definitive diagnosis requires documentation of elevated body iron stores and confirmatory testing for causative *HFE* gene mutations[5,13]. Screening for HH outside these settings or in asymptomatic general populations of appropriate genetic background are not currently recommended and is not cost-effective using combinations of *HFE* genotyping, transferrin saturation, and serum ferritin[2, 3, 6, 7].

Ascertaining those at risk of HH would be made easier if other routine blood tests could provide indications for the likely presence of C282Y homozygosity. In this study we evaluated the potential utility of standard erythrocyte parameters as tests for the detection of C282Y homozygosity. We have found that MCV and MCH were the most appropriate erythrocyte parameters and these values were significantly higher in both men and women with C282Y homozygosity, independent of their treatment status.

The observations of the current study combined with the earlier study of Barton et al.[11-12] suggests that MCV and MCH could add to the range of parameters that inform the clinician on a likelihood of HH. Erythrocyte parameters are commonly ordered for routine clinical assessment and when within the reference range they are often paid limited attention. For example, Australia has a population of 24 million[14] and there are almost 12 million full blood examinations performed per annum which assess Hb, MCH, MCV and other erythrocyte parameters[15]. There are over twice as many full blood examinations performed each year compared with the combined totals of serum iron studies, ferritin or *HFE* genotyping[15]. Targeted further assessment of subjects with MCV >94 fL would identify 34% of untreated men and 62% of untreated women with HH in our general population. This would result in unnecessary further evaluation of less than 4% of the general population who do not have HH. Utilisation of a MCH threshold of >32.2 pg would identify 34% of untreated men and 47% of untreated women with HH, incurring unnecessary testing in 1% of the

general population who do not have HH. The yield of identification of unsuspected HH if evaluation is limited to those above the 90% specificity cut-offs in Table 5 is up to 30 times that of general population screening. Further cost modelling analysis of the utility of such an approach is warranted.

We extended our analysis to include populations with chronic disease such as chronic hepatitis B, chronic hepatitis C, cirrhosis, rheumatologic diseases, diabetes mellitus and chronic pulmonary diseases. Overall, the utility of MCH can be extended to these chronic disease groups, with less discriminatory value for MCV especially in those with chronic hepatitis C, cirrhosis or rheumatoid arthritis. The 90% sensitivity thresholds remained constant within the general and chronic diseases populations. Thus, subjects with values below these values have an extremely low likelihood of having HH and can be excluded from further evaluation at the cost of missing less than 10% of unsuspected HH in our community. The 90% specificity cut-off values were higher in the combined chronic diseases group compared with the general population. Less than 8% of the combined chronic diseases group who do not have HH have values greater than the 90% specificity cut-offs for MCV or MCH.

The mechanisms underlying the erythrocyte manifestations of C282Y homozygosity are unclear. The persistence of elevated MCV and MCH values in C282Y homozygous subjects following adequate phlebotomy therapy indicates that the abnormality is not purely a reflection of iron status. Indeed, when we compared the erythrocyte parameters of a small group of HH subjects who had not yet developed elevated ferritin levels or clinical features with our study cohorts, we observed the same findings of elevated MCV and MCH. Thus, the erythrocyte anomalies occur before elevation of total body iron stores develops. Interestingly, MCV and MCH values increase significantly and incrementally from wild-type HFE to C282Y simple heterozygotes and then further again in C282Y/H63D compound heterozygotes[16]. The levels of MCV and MCH which we observed in our C282Y homozygotes are markedly greater in comparison with our previously published observations in C282Y/H63D compound heterozygotes[16]. Taken together, these observations

suggest that the MCV and MCH changes may be due to the direct effects of C282Y and H63D mutations in HFE *per se*. It is known that the HFE protein is not expressed in erythrocytes[17], and it has previously been proposed that the elevated MCV and MCH in subjects with HH may be due to increased iron supply to erythroblasts[18]. Although other studies have reported that European and United States subjects with HH exhibit higher Hb levels compared with controls[18-20], we have not observed this in our Australian subjects. It is possible that this difference may be due to geographic variation in other genetic or environmental factors that influence iron status[21,22].

Our observations confirm and extend those of previous investigators[11-12], indicating these findings are valid and relevant to HH. As we have not studied subjects with non-C282Y HH, we are unable to confirm whether similar erythrocyte manifestations may be present in these disorders. Furthermore, as we have not evaluated infants, children or adolescents, the proposed approach using erythrocyte parameters can only be recommended for adults. Another limitation is the relatively small number of subjects with chronic disease that were analysed and larger sample sizes would likely add strength to our observations of the utility of MCV and MCH screening in subjects with chronic inflammatory conditions. Ideally, the most appropriate means of validating the utility of the proposed screening strategy outlined in Figure 3 would be to conduct a prospective study.

Conclusion

We conclude that MCV and MCH are able to guide further assessment for the presence of otherwise unsuspected HH, with added advantages over ferritin including utility following successful therapy and in screening chronic disease subjects. Further cost analyses of such approaches to screening are warranted to guide further consideration of clinical implementation.

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Declaration of interests

The authors report no conflicts of interest.

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Figure Legends

FIGURE 1. AUROC curves for MCV and MCH in pre- and post-treatment HH men. Comparisons were made with the control group and the combined chronic diseases group. The AUROC for each analysis is shown within each graph.

FIGURE 2. AUROC curves for MCV and MCH in pre- and post-treatment HH women. Comparisons were made with the control group and the combined chronic diseases group. The AUROC for each analysis is shown within each graph.

FIGURE 3. Suggested approach to investigation for presence of HH in subjects based on provision of MCV or MCH results from full blood picture analysis.

TABLE 1. Characteristics of men and women with *HFE* hemochromatosis at diagnosis.

	Male	**Female**	**P value***
n	70	52	
Symptoms	31	25	0.72
Lethargy	30	17	0.27
Joint pain	12	12	0.49
Alcohol excess history[1]	14	6	0.32
Cirrhosis	4	0	0.13
Abnormal liver biochemistry[2]	22	5	0.004

* Fisher's exact test

[1]greater than 3 standard drinks per day for males or 2 standard drinks per day for females.

[2]Refers to levels above the cut-off for upper end of the reported reference range for liver biochemical tests.

TABLE 2. General and erythrocyte characteristics of clinically or biochemically diagnosed study subjects.

Male	**N**	**Age (yrs)**	**Hb (g/L)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/L)**	**Ferritin (µg/L)**
HH	70						
Pre-treat		43±2	153±2	94±0.5	32±0.2	345±1	1225±138
Post-treat		43±2	149±2	93±0.5	32±0.2	343±1	63±4***
Non-expressing	9	36±6	152±3	95±1	33±0.3	343±2	152±3***
Busselton	900	50±0.5**	150±0.3	89±0.1***	30±0.05***	343±0.2	217±7***
Noncirrhotic							
Hepatitis C	50	53±1	150±3	92±0.7	31±0.3**	339±1	283±29***
Hepatitis B	50	45±2	152±2	89±0.8**	30±0.3***	337±2*	528±154***
Cirrhosis	50	52±1*	146±4	92±0.9	31±0.5***	338±1	406±52***
Arthritis							
Rheumatoid	50	62±2***	145±2*	92±0.8	31±0.3***	334±2***	237±35***
Non-rheumatoid	50	56±2**	150±2	91±0.6***	30±0.3***	338±2*	212±34***
Diabetes	50	59±2***	139±3***	87±0.7***	29±0.3***	334±1**	264±43***
Chronic Lung	50	67±2***	142±3***	90±0.9***	30±0.3***	330±2***	300±83***

Female	**N**	**Age (yrs)**	**Hb (g/L)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/L)**	**Ferritin (µg/L)**
HH	52						
Pre-treat		45±2	138±1	96±0.7	32±0.2	339±1	510±39
Post-treat		46±2	139±2	94±0.8	32±0.3	342±2	68±6***
Non-expressing	13	48±3	143±3	97±1	33±0.3	341±2	117±22***
Busselton	944	54±0.5**	134±0.3	88±0.2***	31±0.06***	341±0.2	100±4***
Noncirrhotic							
Hepatitis C	50	51±1	137±2	91±0.7***	31±0.3***	337±1	164±34***
Hepatitis B	50	42±2	132±2	88±0.8***	29±0.4***	331±1	119±37***
Cirrhosis	50	58±1***	123±3***	93±0.1	31±0.4***	333±1	281±61***
Arthritis							
Rheumatoid	50	58±2*	130±2*	90±0.8***	30±0.3***	329±2	167±31***
Non-rheumatoid	50	52±2	138±21	90±0.7***	30±0.3***	330±2	107±12***
Diabetes	50	59±2***	128±3**	87±0.8***	29±0.3***	330±2	151±20***
Chronic Lung	50	68±2***	132±2	89±0.6***	29±0.2***	328±2	128±25***

All values ±SEM. All statistical comparisons are with the HH pre-treatment group. The Busselton and Chronic Diseases Groups are comprised of subjects who do not possess C282Y homozygosity or C282Y/C63D compound heterozygosity. *p<0.05; **p<0.01; ***p<0.001

TABLE 3. Sensitivity, specificity, and AUROC curve analysis for MCV and MCH.

MCV > 91fL	**Sensitivity**		**Specificity**		**ROC AUC**	
	Pre	Post	Pre	Post	Pre	Post
Control Population	77	69	69	69	0.83	0.74
Chronic Disease Groups Combined	77	68	56	56	0.71	0.64

MCH > 31pg	**Sensitivity**		**Specificity**		**ROC AUC**	
	Pre	Post	Pre	Post	Pre	Post
Control Population	77	65	75	75	0.83	0.75
Chronic Disease Groups Combined	77	65	69	69	0.78	0.72

Results are presented for male HH pre- and post-treatment subjects compared with the control population and the combined chronic disease groups. Cut-off values were selected to give the highest combination of sensitivity and specificity in the general population.

TABLE 4. Sensitivity, specificity, and AUROC analysis for MCV and MCH.

MCV > 91fL	**Sensitivity**		**Specificity**		**ROC AUC**	
	Pre	Post	Pre	Post	Pre	Post
Control Population	82	77	78	78	0.90	0.82
Chronic Disease Groups Combined	82	75	66	66	0.81	0.73

MCH > 31pg	**Sensitivity**		**Specificity**		**ROC AUC**	
	Pre	Post	Pre	Post	Pre	Post
Control Population	72	79	71	72	0.84	0.82
Chronic Disease Groups Combined	72	79	74	74	0.84	0.81

Results are presented for female HH pre- and post-treatment subjects compared with the control population and the combined chronic disease groups. Cut-off values were selected to give the highest combination of sensitivity and specificity in general population.

TABLE 5. Sensitivity and specificity cut-off thresholds for MCV and MCH in control and chronic disease populations.

MCV (fl)	**90% Sensitivity**	**90% Specificity**
Control Population	90.0	94.0
Chronic Disease Groups Combined	90.0	97.5

MCH (pg)	**90% Sensitivity**	**90% Specificity**
Control Population	31.0	32.2
Chronic Disease Groups Combined	31.0	33.2

Less than 10 percent of subjects with HH would be left undiagnosed if subjects with values below the 90 percent sensitivity threshold were not further assessed. Less than 10 percent of subjects who underwent further work-up for HH for values above the 90% specificity threshold would not have HH.