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Distinction Between Homozygous and Heterozygous Subjects with Hereditary Haemochromatosis Using Iron Status Markers and Receiver Operating Characteristic (ROC) Analysis

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Summary: The purpose of the present study was:

1) to evaluate which of the four iron status markers (serum iron, serum transferrin, serum transferrin saturation, serum ferritin) displayed the highest discriminatory potential in the distinction between homozygous patients with hereditary haemochromatosis and heterozygous relatives,

2) to suggest optimum cut-off values for these iron status markers, and

3) to demonstrate how these cut-off values change if the expected utility from a correct diagnosis is incorporated into the analysis.

The patients and relatives were found by a nation-wide epidemiological survey. The study population consisted of 162 patients with clinically overt hereditary haemochromatosis and 84 asymptomatic heterozygous relatives. The statistical evaluation was performed using receiver operating characteristic (ROC) curve analysis. The diagnostic power of the iron markers is expressed as the area under the ROC curve. The optimum cut-off value is at the point where the slope of the ROC curve is equal to one. Changes in the optimum cut-off value at varying expected utility from a correct classification was estimated by changing the scaling of the ROC diagram. Serum iron and serum transferrin had the smallest area under the ROC curve, and were both unsuitable as discriminators. Near complete discrimination was obtained with serum transferrin saturation and serum ferritin concentrations, displaying the largest area under the ROC curve (0.991 and 0.998). The optimum threshold value for transferrin saturation was 61%, and for serum ferritin concentration 800 µg/l. The transferrin saturation level reflects the presence of the haemochromatosis allele, whereas the serum ferritin concentration indicates the degree of iron overload. Changes in the optimum cut-off value from varying expected utility implicated the importance of incorporating this discriminator.

Introduction

Hereditary haemochromatosis is a disorder of iron metabolism, characterized by intestinal hyperabsorption of iron, parenchymal iron overload and subsequent organ damage (1, 2). Much effort has been devoted to the search for a reliable non-invasive test of preclinical and early haemochromatosis, but so far the best discriminators are still biochemical iron status markers, serum transferrin saturation and serum ferritin concentration (1–6). There is virtually no overlapping of transferrin saturation in subjects being homozygous (h/h) for the haemochromatosis allele and normal (−/−) subjects, whereas discriminatory problems exist between homozygous and heterozygous (h/−) subjects (3–6). The present study uses receiver operating characteristic (ROC) curve analysis in the discrimination between subjects being homozygous and heterozygous for the haemochromatosis allele.

Subjects and Methods

The material consisted of samples from 162 Danish patients (126 men, 36 women, median age 55 years, range 29–81) with hereditary haemochromatosis, found by a nation-wide epidemiological survey (2, 6). All patients had clinically overt disease and were considered to be homozygous for the haemochromatosis allele. The diagnostic criteria and the selection of the patients have been described previously (2, 6). Pedigree studies were performed in 32 of the patients (probands) with haemochromatosis, HLA-typing being employed in 21 families (7). Altogether, 84 heterozygous subjects (39 men, 45 women) were identified and included in the study.

Blood samples were obtained in the fasting state in the morning. Serum iron was analyzed by spectrophotometry on a centrifugal analyser (Cobas Bio®, F. Hoffmann-La Roche AG, CH-4002 Basel) using guanidinium ferrozine as chromogen, following release from transferrin with HCl and subsequent reduction with ascorbic acid (Unimate 7 Iron®, Roche Diagnostic Systems, F. Hoffmann-La Roche AG, CH-4002 Basel). Samples were analyzed in duplicate. The overall analytical coefficient of variation was 3.1%.

Serum transferrin (IFCC relative molecular mass M_r 74 000) was measured by immunoturbidimetry on a centrifugal analyser (Cobas Bio®, F. Hoffmann-La Roche AG, CH-4002 Basel) using the Unimate 3 Transferrin® (Roche Diagnostic Systems, F. Hoffmann-La Roche AG, CH-4002 Basel). Calibration was performed with the human protein standard CRM 470. Samples were analysed in duplicate. The overall analytical coefficient of variation was 3.3%. The transferrin saturation in percent was calculated as: serum iron, µmol/l × 100/serum transferrin, µmol/l × 2.

Serum ferritin was measured with a two-site immunoradiometric assay, employing specially prepared paper discs as the solid phase (Phadebas Ferritin PRIST®, Pharmacia Diagnostics AB, S-75182 Uppsala) and described in detail elsewhere (8). Samples were run in duplicate. The overall analytical coefficient of variation was 8.8%. Calibration of the kit using the WHO Human Liver Ferritin International Standard 80/602, showed that a Phadebas value of 11 µg/l corresponded to a WHO value of 15 µg/l (9).

Statistical methods

In order to obtain an estimate of the diagnostic power of the four variables, ROC curves were constructed. A ROC curve is a plot of the true positive fraction, TPF, (n (true positive)/n (all diseased subjects)), as a function of the false positive fraction, FPF, (n (false positive)/n (all non-diseased subjects)), when the cut-off value runs from $-\infty$ to $+\infty$ (10). The ROC curves were fitted by the equation (11)

$$TPF = R \times FPF^{1/E} + (1 - R) \times (1 - (1 - FPF)^{E})$$

where E, which is > 1 for any test of practical value, determines the height of the vault of the curve, and R the skewness of the curve ($0 \leq R \leq 1$). The E and R parameters were estimated by non-linear regression. A least square lost function was used. The minimization algorithm was initiated by a *Rosenbrock* algorithm (to avoid local minima), followed by a quasi-*Newton* minimization algorithm (12). The area under the ROC curve was subsequently calculated as

$$Area = E/(E + 1)$$

and was taken as an estimate of accuracy.

The optimum cut-off value is situated at the point where the slope of the ROC curve is equal to one. To include the expected utility from a correct classification, the axes were scaled by the frequencies of the two conditions (homozygosity and heterozygosity) and the expected utility. The ordinate axis was multiplied by the frequency of homozygous subjects and by the benefit a homozygous patient would miss if he was misclassified, i.e., false negative. The abscissa axis was multiplied by the frequency of heterozygous subjects and by the cost of examining a non-diseased person (13). The cut-off values were calculated for various benefit/cost ratios.

Results

Iron status markers in homozygous patients and heterozygous subjects are shown in table 1. Homozygotes had significantly higher serum iron concentration, lower serum transferrin concentration, higher transferrin saturation, and higher serum ferritin concentration than heterozygotes.

The ROC curves for the four iron status markers are depicted in figure 1, and the area under the ROC curve, and optimum discriminatory values for each variable are displayed in table 2.

It is evident from the curve that serum iron concentration and serum transferrin concentration had the lowest discriminatory potential between homozygous and het-

Tab. 1 Iron status markers (median and range) in homozygous patients with clinical hereditary haemochromatosis, and in healthy heterozygous subjects.

	Serum iron (µmol/l)	Serum transferrin (µmol/l)	Serum transferrin saturation (%)	Serum ferritin (µg/l)
Homozygotes	39*	22*	87*	3400*
n = 162	17–61	9–36	52–100	800–12700
Heterozygotes	19	31	30	55
n = 84	6–38	17–49	12–70	8– 293

*) *Mann-Whitney* test: $p < 0.0001$

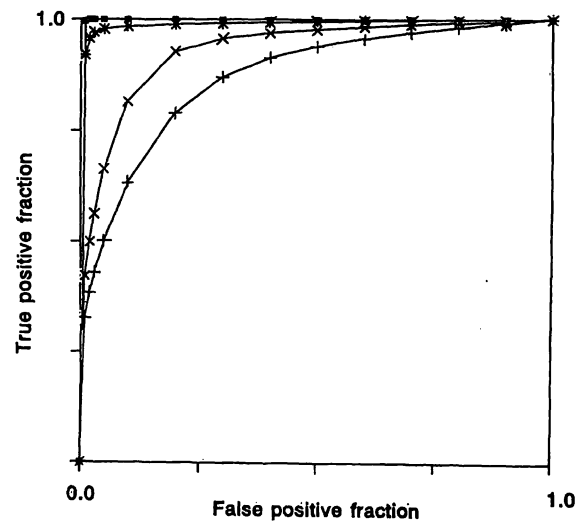

Fig. 1 Exponentially fitted receiver operating characteristic (ROC) curves for iron status markers in hereditary haemochromatosis.
× S-iron
+ S-transferrin
✳ Transferrin saturation
□ S-ferritin

Tab. 2 Optimum cut-off values for iron status markers in hereditary haemochromatosis at various levels of expected utility. The diagnostic power of the four markers are expressed as the area under the ROC curve. B denotes the benefit a homozygous patient would miss if he was misclassified, i. e., false negative. C denotes the cost of examining a non-diseased person. According to different levels of expected utility, corresponding cut-off values for various B/C ratios are shown (see text).

Iron status marker	Area under the ROC curve	Cut-off value B/C =		
		1/1	1/10	1/100
Serum iron (μmol/l)	0.935	30	18	16
Serum transferrin (μmol/l)	0.874	34	36	40
Serum transferrin saturation (%)	0.991	61	44	44
Serum ferritin (μg/l)	0.998	800		

erozygous subjects. The optimum cut-off value was 30 μmol/l for serum iron and 34 μmol/l for serum transferrin (tab. 2).

Transferrin saturation and serum ferritin concentration had far better discriminatory abilities. Almost complete discrimination was obtained with these two variables, displaying the largest area under the ROC curve. The optimum cut-off value was 61% for transferrin saturation and 800 μg/l for serum ferritin concentration (tab. 2).

Discussion

Until specific DNA-probes which can detect the haemochromatosis allele have been developed, the available discriminators consist of the biochemical tests serum transferrin saturation and serum ferritin concentration. Due to the biological variation, it is often difficult to define the threshold value, by which the clinician will be able to discriminate between normal and abnormal. The ROC analysis has been developed to cope specifically with this problem.

It appeared that both serum iron concentration and serum transferrin concentration were unsuitable as discriminators in haemochromatosis, both displaying a high degree of misclassification. Similar results have been reported in other studies (3, 4, 6).

Serum ferritin concentration and transferrin saturation displayed the highest discriminatory ability, with an optimum calculated value for transferrin saturation of 61%. The diagnostic potential of transferrin saturation has been evaluated by other statistical methods (3–6). *Dadone* et al. (3) examined laboratory data in pedigree studies of haemochromatosis patients, using discriminant analysis, and found that transferrin saturation was superior to serum ferritin concentration in predicting the genotype. A threshold transferrin saturation value of 62% correctly predicted homozygosity in 92% of the cases (3).

Borwein et al. (5) evaluated the diagnostic potential of transferrin saturation alone and in combination with serum ferritin concentration in pedigree studies using a likelihood analysis model. A transferrin saturation of 55% provided the best statistical combination of true-positive and true-negative results. A cut-off point of 55% for transferrin saturation and a cut-off point at the 90th percentile for serum ferritin concentration were sufficient for detection of haemochromatosis, when the results of one or both tests were positive (5).

Bassett et al. (4) assessed the value of transferrin saturation and serum ferritin concentration using sensitivity, specificity, predictive value of a positive test (PV_{pos}) and predictive value of a negative test (PV_{neg}). Transferrin saturation and serum ferritin concentration demonstrated high levels of sensitivity and specificity. The authors suggested a cut-off point of 55% for transferrin saturation. The diagnostic efficiency was not improved if transferrin saturation and serum ferritin concentration were combined. However, serum ferritin concentration appeared to be an accurate indicator of body iron overload (4).

In a previous report (6), the present series was analyzed using predictive values, PV_{pos}, PV_{neg}, and efficiency according to *Vecchio* (14). The maximum efficiency for the four iron status markers was obtained with the following discriminatory values: serum iron, 29 μmol/l; serum transferrin, 30 μmol/l; transferrin saturation, 60%; serum ferritin, 800 μg/l (6). Clearly, the cut-off values calculated by ROC analysis were similar or close to those calculated by the *Vecchio* principles.

Three different statistical methods, discriminant analysis (3), predictive analysis according to *Vecchio* (6) and ROC analysis have indicated a transferrin saturation threshold value of 60–62% as being most efficient in the discrimination between subjects being homozygous and heterozygous for the haemochromatosis allele.

ROC analysis gives the opportunity to adjust for the lost benefit of false negative and cost of false positive misclassifications. Untreated haemochromatosis may lead to premature death, most often due to cirrhosis of the liver and/or hepatoma (1, 2). If the genetic abnormality is recognized in the preclinical phase, overt disease can be prevented by depletion of the iron overload through therapeutic phlebotomy.

The disease prevalence of hereditary haemochromatosis among Danish male blood donors is estimated to 0.37–0.46% (15). The ultimative cost of a false negative misclassification may be permanent disability and premature death, whereas the cost of a false positive misclassification includes the expense of an additional serum ferritin measurement and a liver biopsy.

The transferrin saturation level reflects the presence of the haemochromatosis allele, while the serum ferritin concentration reflects the level of iron overload. Serum ferritin concentration per se cannot therefore be used in the general screening procedure, as subjects with preclinical haemochromatosis may have normal or only slightly elevated concentrations. When a transferrin saturation above the critical level has been identified, serum ferritin concentration should be measured in order to assess the magnitude of the iron overload, i. e., the severity of the disease. The lowest ferritin concentration found in homozygous subjects with clinically overt disease in this series was 800 µg/l, while homozygous subjects with preclinical disease display values ranging from 50 to 600 µg/l (2). According to the present results, a critical serum ferritin value of 800 µg/l seems appropriate to discriminate between patients with preclinical and clinical disease.

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