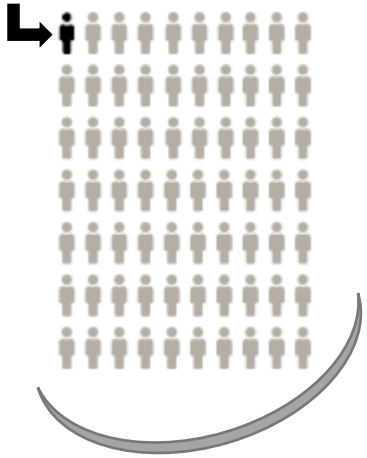
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High Serum Ferritin

1306 patients with
suspicion of Ferroportin disease

Ferroportin Disease



Differential diagnosis:
Metabolic syndrome,
Alcohol, Inflammation,
Hematological conditions…

Clinical Judgement





Subjective balance between patient's characteristics, confounders, and expertise

No genetic test



Genetic Test

Overlooked diagnosis ?

Negative predictive value ?

7% of patients tested positive

FERROPORTIN SCORE



Objective cut-off value :

- Age
- Sex
- Serum ferritin
- Liver Iron content
- High blood pressure or diabetes

<9.5 → **No genetic test**

Rules out Ferroportin disease

50% Unnecessary tests avoided

Sensitivity 93.6%
Negative predictive value 99%

≥ 9.5 →  **Genetic Test**

12.6% of patients tested positive

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A simple clinical score to promote and enhance ferroportin disease screening

Short Title: Screening score for Ferroportin disease

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Keywords: Ferroportin, iron overload, genetic testing, diagnostic score

Data availability statement: Data regarding patients included in the study are confidential. Some anonymous data can be made available upon request.

Electronic word count: 6408

Number of figures and tables: 1 figure; 5 tables

Conflict of interest statement:

Dr. LOUSTAUD-RATTI reports personal fees from Abbvie, personal fees from Gilead, personal fees from IPSEN, outside the submitted work.

Dr. Causse reports personal fees from AbbVie, personal fees from Gilead Sciences, personal fees from MSD, outside the submitted work.

Dr. SI AHMED reports grants and non-financial support from Abbvie, grants and non-financial support from Gilead, grants from MSD, outside the submitted work.

Dr. Ganne-Carrié reports personal fees and non-financial support from GILEAD, personal fees and non-financial support from BAYER, personal fees from IPSEN, outside the submitted work.

Dr. Loréal reports personal fees and other from Diafir, outside the submitted work.

Other authors have no conflict of interest to disclose.

Financial support: None

Authors contribution:

AL: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing

HH-R: Data curation, Investigation, Writing – review & editing

SC: Data curation, Validation, Writing – review & editing

VL-R: Data curation, Writing – review & editing

XC: Data curation, Writing – review & editing

SNSA: Data curation, Writing – review & editing

BD: Data curation, Writing – review & editing

CB: Data curation, Writing – review & editing

GP: Data curation, Writing – review & editing

CDK: Data curation, Writing – review & editing

NG-C: Data curation, Writing – review & editing

SD: Data curation, Writing – review & editing

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FP: Data curation, Writing – review & editing

OL: Data curation, Writing – review & editing

MR: Data curation, Writing – review & editing

LD: Data curation, Writing – review & editing

JM: Formal Analysis, Writing – review & editing

PA-M: Data curation, Validation, Writing – review & editing

YD: Writing – review & editing

EB-J: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Validation Writing – review & editing

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Abstract

Background and aims

Ferroportin disease (FD) is a rare genetic iron overload disorder with usually normal transferrin saturation (TSat). Similarities with secondary iron overload in the setting of metabolic syndrome favors overlooked diagnosis. Recent data suggest higher prevalence than suspected. The lack of definite criteria prompting genetic testing and costs precludes large scale molecular screening. Our aim was to coin a readily available scoring system to promote and enhance FD screening.

Methods

Derivation cohort included probands tested for FD from 2008 to 2016 in our rare disease network. Data were prospectively recorded. Univariate and multivariate logistic regression were used to determine significant criteria, and odd ratios were used to build a weighted score. Cut-off was defined through ROC curve with a predefined aim of 90% sensitivity. An independent cohort was used for cross validation.

Results

Derivation cohort included 1306 patients. Mean age was 55±14 years, ferritin 1351±1357 µg/L, and liver iron content (LIC) 166±77 µmol/g. Pathogenic variants (N=32) were identified in 71 patients. In multivariate analysis female gender, younger age, higher ferritin, higher LIC and the absence of hypertension or diabetes were significantly associated with the diagnosis of FD (AUROC in whole derivation cohort 0.83[0.78-0.88]). The weighted score was based on gender, age, the presence of hypertension or diabetes, ferritin level and LIC. AUROC in derivation cohort without missing value was 0.83[0.77-0.88]. Using 9.5 as cut-off sensitivity was 93.6[91.7-98.3] %, specificity 49.5[45.5-53.6] %, positive likelihood ratio 1.8[1.6-2.0] and negative likelihood ratio 0.17[0.04 – 0.37].

Conclusion

We describe a readily available score with definite criteria and good diagnosis performance that could help screening patients for Ferroportin disease in routine clinical practice.

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Lay summary

Increased iron burden associated to metabolic syndrome is a very common condition. Ferroportin disease is a dominant genetic iron overload disorder which prevalence is higher than initially though. They can be difficult to distinguish from each other, but the limited availability of genetic testing and the lack of definite guidelines restrain proficient screening. We herein describe a simple and definite clinical score to help the clinicians in the decision for genetic testing.

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Introduction

Ferroportin disease (FD) is an autosomal dominant iron overload disease caused by pathogenic variants in the *SLC40A1* gene located on chromosome 2.[1, 2] Ferroportin is the only known cellular iron exporter. [3] Mainly expressed in enterocytes and macrophages, it is the main regulator of iron egress in bloodstream and thus of iron absorption and recycling under the control of hepcidin.[4]

Although FD is considered a rare disease, a recent study using exome data suggests much higher prevalence than initially suspected, with an estimated *SLC40A1* pathogenic allele prevalence of 0.0364%, giving a predicted pathogenic genotype carrier rate of 1 in 1373 (95% CI: 920–1598).[5, 6] This suggests that FD is largely underdiagnosed.

The main pitfall in FD diagnosis is the heterogeneous and unspecific phenotypic expression that forestalls the use of large scale genetic screening. Unlike increased transferrin saturation (TSat) for HFE hemochromatosis, there are no definite clinical or biochemical criteria available in literature to prompt genetic test for FD.

FD is characterized by iron overload with limited if any clinical manifestation and normal TSat. [7] There are however two subtypes defined by the molecular consequences of the variants.[8] The common form (type A) is due to loss of the iron export function, blocking iron egress into the bloodstream and leading to iron build-up in the mesenchymal cell. From a clinical point of view this leads to iron overload with normal or low TSat, increased serum ferritin and macrophage iron deposition.[9] The rarer form (type B) is due to the resistance of ferroportin to hepcidin inhibition, leading to uncontrolled egress of iron into the bloodstream. The phenotype mimics HFE hemochromatosis, with iron overload, increased TSat, increased serum ferritin and hepatocellular iron deposition.[10, 11] Few cases of significant liver damage have been reported and were frequently associated with environmental and acquired cofactors.[12-15]. Likely because of increased TSat, liver damage is more frequent in type B form. [12, 16]

Until recently, FD was mostly reported in case series describing patients with massive iron overload, but family screening and wider awareness of the disease yielded diagnosis of milder forms. Overall FD seems to have a variable clinical presentation and penetrance. [7, 16]

Familial hyperferritinemia with iron overload and autosomal dominant inheritance can be very suggestive of FD and may be sufficient to prompt testing, but this finding is not always present. Difficulty in FD diagnosis is due to its phenotype, identical to the most common cause of

increased ferritin: Dysmetabolic Iron Overload Syndrome (DIOS).[17, 18] DIOS associates features of the metabolic syndrome, and a mildly increased iron burden with increased serum ferritin and normal or moderately increased TSat. Although the links between iron metabolism and insulin resistance remain largely unknown, there are close relationships between DIOS, nonalcoholic fatty liver disease and diabetes.[18] Increased serum ferritin or liver iron concentration are frequently observed in these settings.[19-21] Given the high prevalence of these conditions, there is a large number of patients with DIOS, and similarly a significant number of patients with FD have metabolic syndrome.

Because genetic tests are not routinely performed and the differential diagnosis is difficult, FD may be underdiagnosed. However large scale genetic testing would not be cost effective, therefore well-defined criteria are required to help clinicians screening patients for FD and assess the relevance of a genetic test.

The aim of our study was to determine criteria based on routinely available clinical data, to efficiently prompt genetic testing for FD.

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Patients and Methods

Patients

All patients referred to our National Reference Center for Hemochromatosis and iron metabolism disorder in Rennes between February 2008 and September 2016, for whom a genetic test for Ferroportin disease has been performed, were included in the derivation cohort.

The National Reference Center is a nationwide network that provides clinical diagnosis and therapeutic guidance to requiring practitioners. Patients' records are discussed in multidisciplinary weekly meetings, with at least one clinical practitioner, one biomolecular geneticist, one biochemist, and with scientific and administrative staff. If deemed appropriate, blood sample are sent and genetic testing is performed.

FD testing was performed in case of increased serum ferritin and increased liver iron content according to the algorithm established in our reference network, which is very similar to the one suggested by the EASL Guidelines. [22, 23] Briefly, confounding factors of increased ferritin must be assessed. If they are deemed not significant, HFE C282Y homozygosity must be ruled out if Tsat is increased. In patients with normal Tsat or if HFE hemochromatosis is ruled out, LIC must be assessed and if iron overload is confirmed FD testing is performed.

All cases were discussed in the multidisciplinary meeting and the cut-off value for iron overload was balanced according to family history and the presence of confounding factors (alcohol consumption, metabolic syndrome, hematological conditions, etc.).

A validation cohort was obtained from the CHU of Montpellier, a Competence Center, and member of the French network on rare disorders of iron metabolism. Patients referred to the centre between 1996 and 2017 for evaluation of hyperferritinemia underwent FD testing according to the algorithm recommended by the national network, including the exclusion of acquired factors.

Informed consent for molecular testing and data recording in the local database was obtained from all the subjects. The study was approved by the CHU Rennes ethic Committee.

Patient's relatives referred for family screening after a confirmed diagnose of FD and patients homozygous for the C282Y *HFE* mutation were excluded from both cohorts.

Data collected

The clinical characteristics of patients were prospectively recorded. When multiple values were available, we selected the closest one to the genetic testing prior to any iron depletion.

The following data were recorded: date of diagnosis, age, gender, family history of iron overload disorder, alcohol consumption, tobacco smoking, body-mass index, blood pressure, presence of diabetes, clinical symptoms (fatigue, cardiomyopathy, liver disease, hypogonadism, arthropathy), iron status (serum ferritin, serum transferrin saturation), blood count, lipid profile, liver enzymes, and C-reactive protein. High blood pressure and dyslipidemia were defined using the same criteria than criteria for the diagnosis of metabolic syndrome.

We also recorded liver iron concentrations (LIC) as assessed by Magnetic Resonance Imaging (MRI) as previously described. [24, 25]

Genetic study

Sequencing of the entire coding region, intronic flanking sequences and promoter of the *SLC40A1* gene (NM_014585.5) was carried out. Sanger sequencing was performed until 2013 using the ABI Prism terminator cycle sequencing ready reaction kit and ABI prism 377 DNA sequencer (Applied Biosystems), or the BigDye terminator cycle sequencing kit and 3130 XL Genetic Analyser (Applied Biosystems). Since 2014, next-generation sequencing (NGS) has been performed using the Ion AmpliSeq Library Kit 2.0 and Ion PGM 200 Sequencing Kit (Life Technologies) following the manufacturer's protocol. Samples were sequenced on the Ion Torrent PGM and confirmed by Sanger analysis if a variant was found. To exclude association with variant in other genes involved in iron metabolism, sequencing of the *HFE, HAMP, HJV* and *TFR2* coding and intronic flanking regions was performed if no variant in SLC40A1 were identified in patients with increased Tsat before 2014, and for all patients since 2014. Moreover, microcytogenetic rearrangements were searched by multiplex ligation probe amplification (MLPA - MRC-Holland) on the five genes.

Sequencing the *SLC40A1* gene of the validation cohort was carried out using the SCA method based on Sanger sequencing until 2016,[26] and by next generation sequencing (Agilent SureSelectQXT®, Miseq® Illumina) since 2016. The same set of genes involved in iron disorders was also studied concomitantly to exclude any associations. Each identified pathogenic variant was subsequently confirmed by Sanger sequencing.

Variant clinical significance was classified according to ACMG (American College of Medical Genetics and Genomics) and AMP (Association for Molecular Pathology) international guidelines. [27] In our study, the diagnosis for FD was confirmed if the variant was classified as pathogenic or likely pathogenic. Variants of uncertain significance were considered as significant for the diagnosis of FD if phenotype was highly suggestive and/or if the variant was found in several unrelated patients with similar phenotype. Indeed pathogenicity is arduous to determine because FD is rare and molecular functional studies are time consuming and difficult to set up. Functional studies were performed when available to help us classify some of the variants. [8]

Statistical analysis

We aimed to build a model able to assess the likelihood of FD, according to genetic testing result.

Variables are summarized as mean and standard deviation or as number and percentage as appropriate. Candidate predictor variables for the diagnosis of FD were first selected by univariate analysis using logistic regression and a p value <0.2 for further analysis. Then these variables were included in a multivariate logistic regression model with stepwise backward selection according to likelihood ratio. To this end, continuous variables were categorized to account for missing data and thus divided in tertiles, or according to relevant values if available in literature. Collinearity was checked using correlation matrix and Variance Inflation Factor.

Then to assess the model accuracy, we ran the same model using continuous variables. The predicted value obtained by the model was used to plot the Receiver Operating Characteristics (ROC) curve.

The model was first determined in patients without missing data, then to assess the impact of missing values, multiple imputation was performed. The datasets were generated (n=100) with fully conditional specification imputation methods for missing values for the following variable: age, serum ferritin, BMI, LIC, transferrin saturation, ALAT, GGT, hemoglobin, c reactive protein, high blood pressure, diabetes, alcohol consumption, dyslipidemia. Logistic regression was then performed with the previously defined model for each dataset and then pooled.

We created a clinical and biochemical score using the rounded odd ratios of the logistic regression as the weights for each of the predictors. Performance of this new predictive model

was estimated by area under the ROC curve analysis. By then we determined the best cutoff value of the score to respect a predefined sensitivity of at least 90% in order to avoid underdiagnoses. Point estimate sensitivity, specificity and likelihood ratio with confidence intervals were determined for the selected for cut-off.

The relevance of the final model was assessed in a replication cohort to ascertain its generalization.

A p value <0.05 was considered significant. Statistical analysis was performed using SPSS 23.0 (IBM Corp, Armonk, NY).

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Results

Patients

During the study period, in the derivation cohort, 1306 index cases had a genetic test for FD. The population study consisted of 1046 men (80.1%) and 260 women (19.9%), with a mean age of 55.8±13.7 years(Table 1 and Supplementary Table 1).

Features of the metabolic syndrome were frequently found. The mean BMI was 25.4±3.8, with 11.7% patients having a BMI higher than 30. High Blood Pressure (HBP) was reported in 270/912 (29.6%) patients, dyslipidemia in 402/918 (43.7%) patients and diabetes in 80/715 (11.1%) patients.

The mean serum ferritin was 1351±1357 µg/L, and as expected in FD, most of them had normal TSat (793/1306 patients; 60.7%), with a mean value of 47.5±20.9%. LIC as determined by MRI was available for 927 (70.8%) patients, with a mean value of 166±77 µmol/g dry weight. Iron burden was assessed through amount of iron removed or liver biopsy in 204 (15.6%) patients and 177 (13.5%) patients had no information regarding body iron load.

The validation cohort included 205 patients, with similar characteristics to that of the initial cohort: 79.5% of patients were male, mean age was 55.0±14.2 years, mean serum ferritin was 1187±1012 µg/L, mean TSat was 49.6±21.5 %. LIC was available in 166/205(81%) patients, mean LIC was 155±84 µmol/g. HBP was reported in 35/166 (21.0%) of patients, and diabetes in 26/180 (14.4%) of patients.

Pathogenic variants

In the derivation cohort 32 pathogenic variants were identified in 71 patients (Table 2). According to the ACMG classification, 10 variants were of uncertain significance, 21 were likely pathogenic and 1 was pathogenic. A large majority of variants were found in only one patient, and 2 were found in more than 10 patients (NM_014585.5(*SLC40A1*):c.610G>A p.(Gly204Ser), rs387907377, and NM_014585.5(*SLC40A1*):c.1469G>A p.(Gly490Asp), rs1060501102)

In the validation cohort, 8 pathogenic variants were found in 10 patients. Two of them were not found in the derivation cohort: NM_014585.5(*SLC40A1*):c.1328C>T p.(Pro443Leu) and c.-59_-45del15, rs770737502.

Clinical characteristics associated with Ferroportin Disease

In univariate logistic regression (Table 1), sex, age, HBP, diabetes, dyslipidemia, LIC, ferritin, AST, ALT, hemoglobin, CRP were significantly associated with FD ($p < 0.2$).

As three parameters related to metabolic syndrome had a p value <0.2 in univariate analysis, binary composite variables were also submitted to the univariate analysis (presence of HBP or diabetes (p<0.2), presence of HBP or dyslipidemia (p>0.2), presence of diabetes or dyslipidemia (p<0.2), and presence of HBP or diabetes or dyslipidemia (p<0.2)), then to the multivariate analysis if p<0.2 in order to assess if the final score could be simplified.

Multivariate analysis using categorical variables, showed that female sex, younger age, higher ferritin, higher LIC and the absence of HBP or diabetes were significantly associated with the diagnosis of FD (Table 3). Multivariate analysis using these five parameters as continuous variables with multiple imputation to account for missing value showed similar results (Table 4). No significant collinearity was found between the variables in the multivariate model.

Diagnosis accuracy of models was assessed through Receiver Operating Characteristic (ROC) curve analysis and showed an area under the curve of 0.83[0.78-0.88] and 0.83[0.78-0.84], for the raw and categorical variable model respectively (Fig.1).

Setting up a practical clinical score and defining cut-off value for genetic testing

We used the derivation cohort to set up a simple and convenient score for clinical practice. The five variables determined by the multivariate analysis were weighted according to the resulting β-coefficients of the logistic regression. Because cut-off values based on division into quantiles were not convenient, we used rounded value or cut-off based on the literature when available. The simplified clinical score is shown in Table 5. ROC curve analysis of the prediction as determined by this simplified score showed an area under the curve of 0.83[0.78-0.88] (Fig1).

As aforementioned, for the purpose of a rare genetic disease, a sensitivity of at least 90% was required. The best cut-off was ≥9.5, yielding a sensitivity of 93.6[91.7-98.3] %, specificity 49.5[45.5-53.6] %, positive predictive value 12.6[9.4-16.6] %, negative predictive value 99.0[96.9-99.7] %, positive likelihood ratio 1.8[1.6-2.0] and negative likelihood ratio 0.17[0.04-0.37]. We had 3 false-negative cases. An obese 55 year-old man with the p.Met173Val variant which was also found in another patient who had a very clear phenotype without confounding factor. His obesity might be an explanation, as obesity induces higher

hepcidin levels and lower iron burden in patients with *HFE* hemochromatosis. [28] A 56 year-old man with high blood pressure, overweight (BMI=26) and the likely pathogenic p.Val97Met variant, this patient had discrepancy between high ferritin (1881µg/L) and low LIC (65µmol/g). A 68 year-old man with a pathogenic variant (p.Asn185Thr), who also had β-thalassemia minor.

Overall, 46.5% of patients had a score <9.5, therefore using this score we could have ruled out FD without performing genetic test in half of the patients without noticeable risk of underdiagnoses. Furthermore, this would enhance the positivity rate of genetic testing from 7.2% to 12.6%.

Validation cohort

In the validation cohort 9 of 174 (5.17%) patients had FD and 87 (50.0%) patients had a score ≥9.5, yielding a sensitivity of 77.7[40.1-96.0] %, specificity 51.5[43.6-59.3] %, a positive likelihood ratio 1.6[1.0-2.3] and negative likelihood ratio 0.43[0.12-1.4]. ROC curve analysis of the prediction as determined by this simplified score showed an area under the curve of 0.77[0.60-0.95].

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Discussion

Rare diseases like Ferroportin disease suffer from underdiagnoses and poor guidance for screening in routine clinical practice. Our study is the first based on large derivation and validation cohorts to establish definite clinical and biochemical criteria for the screening of Ferroportin disease and guidance to perform genetic test. We conceived a simple clinical Ferroportin score based on sex, age, the presence of high blood pressure or diabetes, serum ferritin and liver iron content. The diagnosis performance is good with an AUC of 0.83 as assessed by ROC curve analysis, and using a score ≥9.5 as cut-off, the sensitivity was 93.6%, specificity 49.5% and a negative likelihood ratio of 0.17 emphasizing its relevance. Using this score would have allowed to conveniently rule out FD in half of both cohorts without requiring expert advice or genetic test, and with minimal risk of underdiagnose. An online calculator for the score is available: http://centre-reference-fer-rennes.org/score-ferroportine/.

Our results show that 5 parameters were significantly associated with the likelihood of FD diagnosis in multivariate analysis: age, sex, HBP-diabetes, ferritin level, LIC. All of them are definite and readily available in clinical practice and coherent as they are already used in the routine workup of iron overload.[22] It is not surprising that higher iron overload increases the likelihood of positive genetic test for FD, in particular LIC by confirming liver iron store [24, 25] as confounding factors and secondary iron overload are usually associated with milder increases. It is important to note that quantification of liver iron store have a considerable weight in our score and must thus be performed adequately and according to acknowledged methods and sequences. These are well described and freely available software (like http://mrquantif.org) allowing to perform quantification with any MRI devices regardless of magnetic field or manufacturers. [29]

Although most patients had normal TSat and were thus investigated for type A FD, one third had increased TSat suggesting type B FD. This may explain why TSat was eventually not significantly associated with a positive genetic test. Performing subgroup analysis limited to patients with low to normal versus increased TSat, showed the score had similar performance using the same cut-off (data not shown).

Our finding that the presence of high blood pressure or diabetes reduces the likelihood of positive genetic test is consistent with the expected causes of acquired iron overload or

increased serum ferritin. These are two parameters of the metabolic syndrome, which is one of the most common causes of hyperferritinemia and mild iron overload. [17, 21] Of note, our score shows similar performance (with the same cut-off value) in a subgroup analysis restricted to patients presenting with at least one features of the metabolic syndrome (data not shown). The fact that higher age and male gender are also negative criteria of our score could be similarly interpreted. Indeed metabolic syndrome is more frequent in male population in Europe and its prevalence increases with age.[30] Furthermore this may be explained by the increasing frequency of secondary causes of iron overload with age.

Overall this score allows translating what would be an intuitive interpretation of iron overload workup results by an expert, into a definite decision-making process accessible to any clinician. Because clinicians can be reluctant to seek expert advice for common condition as secondary iron overload, and as experts are not always readily available, the diagnosis might be overlooked.[5] Although our score does not encompass some clinically relevant characteristics like family history, it will help significantly reduce the need for expert advice and complex genetic testing.

The diagnosis performance of the resulting score is satisfactory with an AUROC of 0.83[0.78-0.88]. Using 9.5 as cut-off, sensitivity is 93.6[91.7-98.3]%, and specificity 49.5[45.5-59.6]% which are satisfactory for screening rare diseases. The negative likelihood ratio of 0.17[0.04-0.37] suggest the score has a significant added value in ruling out FD, whereas the positive likelihood ratio 1.8[1.6-2.0] suggests it only slightly increases the likelihood of FD diagnosis. Thus this score fulfill its role as a convenient screening tool, mainly to rule out FD without requiring expert advice or genetic testing.

The main strength of our study is the very large cohort recruited through a nationwide network dedicated to rare disease and with prospective data recording. The large sample size in the derivation cohorts yield enough power to confidently assess sensitivity.[31] Moreover the performance of the score was cross validated in a validation cohort from another independent center strengthening our results and suggesting that this simplified clinical score can be generalized in a population of European descent.

Because the parameters included in the score are not specific to FD, it might be considered for screening of other types of genetic iron overload. However the population of our study and its design does not allow to tests this hypothesis. First HFE C282Y homozygotes were excluded from our cohorts because there is a wide consensus to perform this test as a first line screening in the Caucasian population as soon as serum transferrin saturation is increased. [23, 32] Moreover

the HFE C282Y test is inexpensive and widely available therefore using our score in this setting is unlikely to provide benefits. Regarding iron overload related to the other genes involved in the hepcidin metabolism, although the idea is appealing, our population is inadequate to proper assessment. Besides the fact that the phenotype is characterized by increased serum transferrin saturation, the prevalence is extremely lower than that of FD [5], consequently the number expected in our study are too low to tests the score.

One weakness of our study is the lack of information regarding family history in probands. Although dominant inheritance is an important feature of FD, it is not taken into account in our clinical score. This is due to the lack of accuracy in the pedigree of most of probands as when history of iron overload or increased serum ferritin was reported, we rarely had definite information allowing assertion of iron overload in relatives. Another bias induced by the large spectrum of referral pathway is missing value for some variables. We assessed the impact of missing value using multiple imputation method, which confirmed it did not alter our multivariate model.

A major challenge of our results is the potential referral bias that could limit our score generalizability, and therefore emphasize the question of its adequate positioning in clinical practice. Because patients were addressed for genetic testing only if physicians had suspicion of FD, our population study is not a general population of patients with increased ferritin. However this is in line with practice guidelines advocating to rule out other causes of increased ferritin before considering genetic disease. Survey conducted in our center revealed that referring physicians were of diverse specialty (20% general practitioners, 56% liver or GI specialist, 11% internal medicine and 14% others) and diverse center profile (25% tertiary care, 31% secondary care and 42% primary care), therefore reflecting a wide spectrum of clinical practice. Moreover the independent validation cohort has characteristics very similar to our population. This strongly suggests that our population is generalizable to the population of patients in which first line evaluation has ruled out other obvious causes of increased serum ferritin. Therefore we think our score should not be considered as a first line screening tool of increased serum ferritin but as a second line evaluation once others confounding causes of increased ferritin have been ruled out.

The aim of our simplified clinical Ferroportin score is to promote appropriate genetic testing and improve awareness for the disease. First description of the disease was based on patients with massive iron overload. [2, 33] but later studies showed that similarly to other type of hemochromatosis penetrance is variable.[34, 35] This heterogeneous expression can lead to

overlook or misdiagnosis, especially with metabolic syndrome.[17] The lack of definite criteria prompting testing available in literature precludes efficient screening as it requires most clinicians to refer to tertiary center which in most cases would prove to be unnecessary. This likely makes non-expert physicians reluctant to screen for FD. Having a simple score with readily available parameter allowing withholding genetic testing with good reliability might thus enhance the willingness to screen for FD and improves the awareness for the disease. From the expert center point of view, using our score might significantly increase the positivity rate of genetic testing, meaning many tests could be avoided if they had been performed according to score values and thus significantly improve cost effectiveness of FD screening. This will eventually lead to a better availability of genetic testing and allows screening of a larger number of patients.

Having an accurate diagnosis of FD will provide several benefits. From a patient perspective it provides relief from uncertainty that has deleterious psychological and social consequences, [36] allows to avoid unnecessary exploration, helps in providing adequate treatment, improves adherence to follow-up and helps the patient finding support. Moreover having a definite diagnosis would allow to perform family screening and prevent relatives to undergo unnecessary tests. Although there is still no longitudinal study regarding the natural history and prognosis of FD, bloodletting treatment is usually recommended. Tolerance is frequently worse than in HFE hemochromatosis because of anemia, therefore it must be monitored cautiously. From a more general point of view and by analogy with what happened for *HFE* hemochromatosis after wide availability of DNA testing [37], wider use of FD screening and diagnosis will allow to better understand its epidemiology, physiopathology and phenotypic expression.

In conclusion, our results provide a simple clinical score to increases the proficiency of genetic testing for Ferroportin disease, based on 5 definite and readily available criteria with a good diagnosis performance. Even though this score was set up in patients of European descent in which HFE hemochromatosis has been ruled out, therefore its use in other populations should be validated first, it could help screening patients for Ferroportin disease in routine clinical practice.

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Table 1. Characteristics of the derivation cohort and univariate comparison between patients with and without *SLC40A1* pathogenic variant

Results are shown as Mean (±SD) or N (%) as appropriate. HBP, high blood pressure; BMI, body mass index; LIC, liver iron concentration; TSat, transferrin saturation; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; CRP, C reactive protein; WHO rec, World Health Organization recommendations regarding alcohol consumption. [a] *p* value of univariate logistic regression.

		Total n=1306	***No pathogenic variant present*** n=1235	***Pathogenic variant present*** n=71	***p-value***
Sex	**Male**	1046	1003 (81.2%)	43 (60.6%)	p<0.001
	Female	260 (19.9%)	232 (18.8%)	28(39.4%)	
Alcohol consumption	**None**	527 (40.4%)	495 (40.1%)	32 (45.1%)	p=0.42
	< WHO rec.	368 (28.2%)	347 (28.1%)	21 (29.6%)	
	> WHO rec.	67 (5.1%)	62 (5.0%)	5 (7.0%)	
	Missing	344 (26.3%)	331 (26.8%)	13 (18.3%)	
Dyslipidemia	**No**	516 (39.5%)	479 (38.8%)	37 (52.1%)	p=0.07
	Yes	402 (30.8%)	383 (31.0%)	19 (26.8%)	
	Missing	388 (29.7%)	373 (30.2%)	15 (21.1%)	
HBP or Diabetes	**No**	506 (38.7%)	464 (37.6%)	42 (59.2%)	p=0.002
	Yes	313 (24.0%)	301 (24.4%)	12 (16.9%)	
	Missing	487 (37.3%)	470 (38.1%)	17 (23.9%)	
Age (y) (N=1305)		55.8 (±13.7)	56.1 (±13.6)	50.5 (±14.1)	p=0.001
BMI (kg/m²) (N=965)		25.4 (±3.8)	25.4 (±3.8)	25.2 (±4.1)	p=0.7
LIC (µmol/g) (N=927)		166 (±77)	163 (±75)	222 (±84)	p<0.001
TSat (%) (N=1152)		47.5 (±20.9)	47.3 (±20.7)	50.0 (±24.7)	p=0.31
Ferritin level (µg/L) (N=1206)		1351	1268 (±1097)	2725 (±3242)	p<0.001
ALT level (IU/L) (N=974)		39.3 (±28.6)	38.8 (±28.2)	46.7 (±33.8)	p=0.05
GGT level (IU/L) (N=884)		70.4	70.2 (±143.7)	74.1 (±164.6)	p=0.8
Hemoglobin (g/dL) (N=944)		14.5 (±1.6)	14.5 (±1.6)	14.2 (±1.3)	p=0.18
CRP (mg/L) (N=712)		3.9 (±9.1)	3.8 (±9.3)	4.7 (±5.9)	p=0.56

Table 1. Pathogenic variants found in the derivation cohort.

DNA and protein position are shown according to the NM_014585.5 reference sequence. Pathogenicity level is determined according to the American College of Medical Genetics and Genomics guidelines.[25] All variants were found in heterozygous state. (N, number of cases). Accession number (rs) of variants are provided as referenced in the Single Nucleotide Polymorphism Database (dbSNP).

rs (dbSNP)	Gene variant	Protein variant	Probands (N)	Pathogenicity level
Not referenced	c.140C>T	p.Ser47Phe	4	
Not referenced	c.134C>A	p.Ala45Glu	1	
rs201485374	c.306A>G	p.Ser102Ser	1	
rs1278518794	c.517A>G	p.Met173Val	2	Uncertain
rs1436123615	c.1035G>C	p.Leu345Phe	1	
Not referenced	c.1048G>A	p.Ala350Thr	1	
Not referenced	c.1049C>T	p.Ala350Val	1	
Not referenced	c.1151T>G	p.Leu384Trp	1	
rs572245704	c.1384G>A	p.Val462Ile	1	
rs863224768	c.1520A>G	p.His507Arg	1	
Not referenced	c.188T>C	p.Val63Ala	1	
Not referenced	c.212C>T	p.Ser71Phe	3	
rs978427853	c.238G>A	p.Gly80Ser	1	
rs387907374	c.262A>G	p.Arg88Gly	1	
rs886055361	c.289G>A	p.Val97Met	1	
Not referenced	c.442A>G	p.Thr148Ala	1	
rs1172102948	c.485_487del	p.Val162del	3	
rs104893663	c.470A>G	p.Asp157Gly	1	
rs1449300685	c.533G>A	p.Arg178Gln	6	Likely pathogenic
rs765023388	c.536G>C	p.Arg179Thr	1	
Not referenced	c.554A>C	p.Asn185Thr	2	
rs387907377	c.610G>A	p.Gly204Ser	15	
rs1205429003	c.611G>T	p.Gly204Val	1	
Not referenced	c.616G>C	p.Gly206Arg	2	
Not referenced	c.679T>G	p.Tyr227Asp	1	
rs753603407	c.689C>A	p.Thr230Asn	1	
rs1278475785	c.698T>C	p.Leu233Pro	1	

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rs104893664	c.800G>A	p.Gly267Asp	1	
Not referenced	c.968G>A	p.Gly323Asp	1	
rs1313335539	c.1468G>A	p.Gly490Ser	1	
rs1409883266	c.1502A>G	p.Tyr501Cys	1	
rs1060501102	c.1469G>A	p.Gly490Asp	11	Pathogenic

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Table 2. Multivariate analysis of the clinical characteristics associated with the diagnosis of Ferroportin disease using categorical variables in the derivation cohort.

Variables significantly associated with the diagnosis of Ferroportin disease (*p* value <0.2) in univariate analysis were included in a multivariate binary logistic regression model with stepwise backward selection according to likelihood ratio. Model was first determined in patients without missing data then after multiple imputation. OR, Odd ratio; CI95%, 95% confidence interval; HBP, high blood pressure; LIC, liver iron concentration.

	Without Missing Data (N=663)			**Multiple Imputation**	
	OR	**CI95%**	***p* value**	**OR**	**CI95%**
Sex (Male)					
Male (N=547)	1			1	
Female (N=116)	1.78	0.87-3.64	0.12	2.62	1.51-4.53
Age (Years)			0.08		
≤40 (N=74)	5.20	1.22-22.14	0.026	5.08	1.57-16.40
41-≤70 (N=486)	3.30	0.96-11.39	0.059	2.83	0.99-8.11
>70 (N=103)	1			1	
HBP or Diabetes			0.001		
No (N=402)	3.72	1.45-6.1	0.003	2.6	1.36-4.98
Yes (N=261)	1			1	
LIC (µmol/g)			0.007		
≤96 (N=111)	1			1	
97-≤160 (N=216)	2.41	0.50-11.78	0.27	1.65	0.47-5.84
161-≤200 (N=152)	4.49	0.94-21.50	0.06	2.74	0.81-9.28
>200 (N=184)	7.88	1.76-35.19	0.007	3.86	1.21-12.35
Ferritin level (µg/L)			0.0003		
≤700 (N=128)	1			1	
701-≤1000 (N=167)	1.22	0.35-4.21	0.75	1.43	0.50-4.12
1001-≤1500 (N=207)	1.62	0.52-5.00	0.41	2.34	0.92-5.96
>1500 (N=161)	6.01	2.08-17.36	0.0009	5.70	2.35-13.82

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Table 3. Multivariate analysis of the clinical characteristics associated with the diagnosis of Ferroportin disease using continuous variables in the derivation cohort. Variable significantly associated with the diagnosis of Ferroportin disease in multivariate analysis were included in a logistic regression model as continuous variable. To account for missing value, multiple imputation was performed to generate 100 dataset, the model was run in each dataset and then combined. OR, Odds ratio; CI95%, confidence interval of 95%; HBP, high blood pressure ; LIC, liver iron concentration;

N=1305	**OR**	**CI95%**
Sex (Female)	2.57	1.50-4.39
Age (per 10 Years)	0.76	0.64-0.91
HBP or Diabetes (Absence)	2.38	1.23-4.59
LIC (per 10µmol/g)	1.04	1.01-1.08
Ferritin (per 100µg/L)	1.03	1.02-1.05

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Table 4. Clinical simplified Ferroportin score.

LIC: liver iron concentration.

Sex	Male	0
	Female	2.5
Age (years)	≤40	5
	41-≤70	3
	>70	0
High blood pressure or diabetes	Yes	0
	No	3
LIC (µmol/g)	≤96	0
	97-≤160	1.5
	161-≤200	3
	>200	4
Ferritin level (µg/L)	≤700	0
	701-≤1000	1.5
	1001-≤1500	2.5
	>1500	6

Score range: 0 to 20.5 points

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Figure 1. Accuracy of the diagnostic model for Ferroportin disease.

Receiver operating characteristic curve of the multivariate models using continuous variable, categorical variables, and the clinical simplified score in patients without missing data in the derivation cohort.

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rs753603407	c.689C>A	p.Thr230Asn	1	
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rs104893664	c.800G>A	p.Gly267Asp	1	
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701-≤1000 (N=167)	1.22	0.35-4.21	0.75	1.43	0.50-4.12
1001-≤1500 (N=207)	1.62	0.52-5.00	0.41	2.34	0.92-5.96
>1500 (N=161)	6.01	2.08-17.36	0.0009	5.70	2.35-13.82

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Table 3. Multivariate analysis of the clinical characteristics associated with the diagnosis of Ferroportin disease using continuous variables in the derivation cohort. Variable significantly associated with the diagnosis of Ferroportin disease in multivariate analysis were included in a logistic regression model as continuous variable. To account for missing value, multiple imputation was performed to generate 100 dataset, the model was run in each dataset and then combined. OR, Odds ratio; CI95%, confidence interval of 95%; HBP, high blood pressure ; LIC, liver iron concentration;

N=1305	**OR**	**CI95%**
Sex (Female)	2.57	1.50-4.39
Age (per 10 Years)	0.76	0.64-0.91
HBP or Diabetes (Absence)	2.38	1.23-4.59
LIC (per 10µmol/g)	1.04	1.01-1.08
Ferritin (per 100µg/L)	1.03	1.02-1.05

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Table 4. Clinical simplified Ferroportin score.

LIC: liver iron concentration.

Sex	Male	0
	Female	2.5
Age (years)	≤40	5
	41-≤70	3
	>70	0
High blood pressure or diabetes	Yes	0
	No	3
LIC (µmol/g)	≤96	0
	97-≤160	1.5
	161-≤200	3
	>200	4
Ferritin level (µg/L)	≤700	0
	701-≤1000	1.5
	1001-≤1500	2.5
	>1500	6

Score range: 0 to 20.5 points

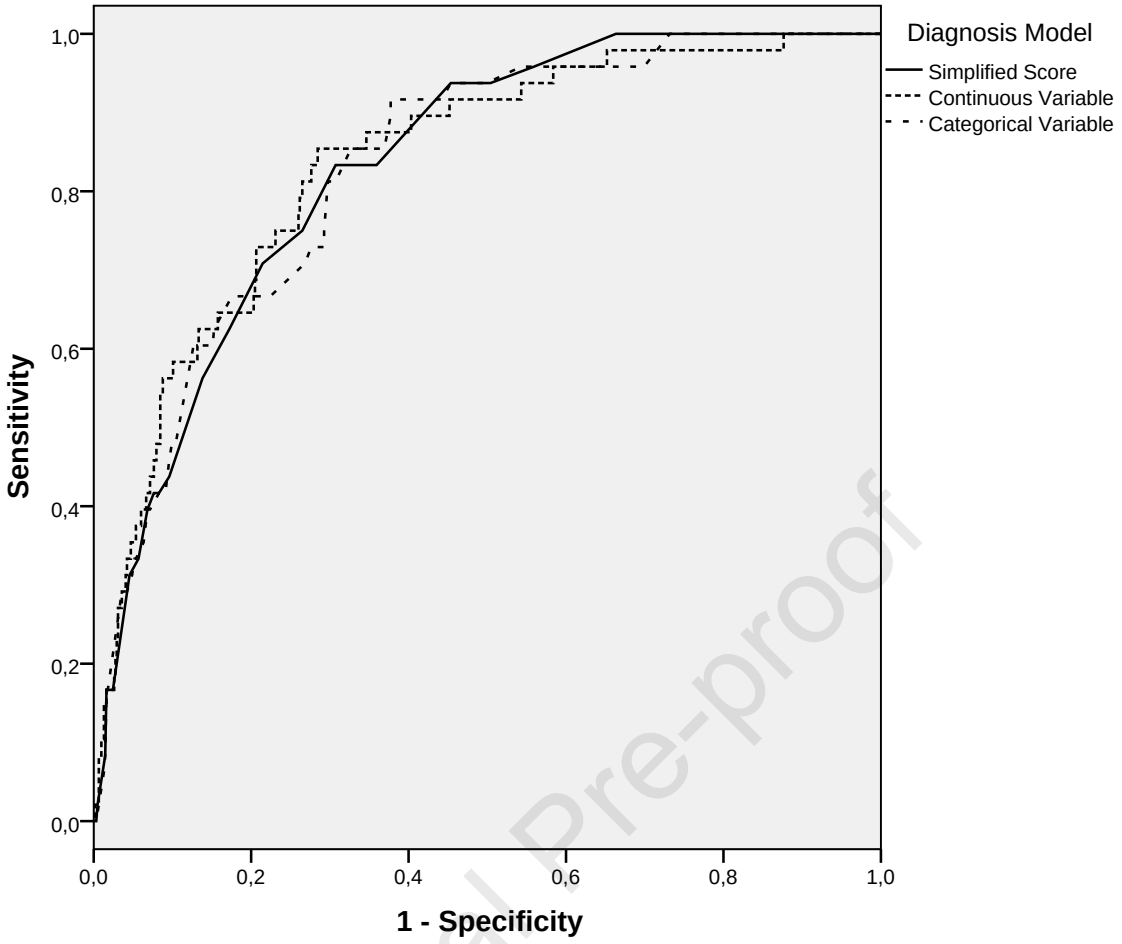
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Figure 1. Accuracy of the diagnostic model for Ferroportin disease.

Receiver operating characteristic curve of the multivariate models using continuous variable, categorical variables, and the clinical simplified score in patients without missing data in the derivation cohort.

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- Iron metabolism disorders related to metabolic syndrome are very common conditions that might be difficult to distinguish from Ferroportin disease
- Ferroportin disease prevalence is higher than initially thought
- The lack of definite criteria for genetic testing and its limited availability hinders proficient Ferroportin disease screening
- A simple score, based on clinical features with definite cut off, can efficiently rules out Ferroportine disease or prompt genetic testing

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