Hemochromatosis classification: update and recommendations by the BIOIRON Society

Tracking no: BLD-2021-011338R2

Domenico Girelli (University of Verona, Italy) Fabiana Busti (University of Verona, Italy) Pierre Brissot (INSERM, Univ-Rennes 1, INRA, Institut Numecan, France) Ioav Cabantchik (Hebrew University, Jerusalem, Israel) Martina Muckenthaler (University of Heidelberg, Germany) Graca Porto (i3S/IBMC, Portugal)

Abstract:
Hemochromatosis (HC) is a genetically heterogeneous disorder in which uncontrolled intestinal iron absorption may lead to progressive iron overload responsible for disabling and life-threatening complications such as arthritis, diabetes, heart failure, hepatic cirrhosis, and hepatocellular carcinoma.

The recent advances in the knowledge of pathophysiology and molecular basis of iron metabolism have highlighted that HC is caused by mutations in at least five genes, resulting in insufficient hepcidin production or, rarely, resistance to hepcidin action. This has led to an HC classification based on different molecular subtypes, mainly reflecting successive gene discovery. This scheme was difficult to adopt in clinical practice and therefore needs revision. Here we present recommendations for unambiguous HC classification developed by a working group of the International Society for the Study of Iron in Biology and Medicine (BIOIRON Society) including both clinicians and basic scientists during a meeting in Heidelberg, Germany. We propose to deemphasize the use of the molecular subtype criteria in favor of a classification addressing both clinical issues and molecular complexity. Ferroportin Disease (former type 4a) has been excluded because of its distinct phenotype. The novel classification aims to be of practical help whenever a detailed molecular characterization of HC is not readily available.

Conflict of interest: COI declared - see note

COI notes: TG and EN are co-founder of Intrinsic LifeScience, and consultant for Protagonist, Vifor Pharma, Ionis Pharmaceuticals, and Disc Medicine. JR is consultant for Bond Biosciences and Gilead. The other authors declare no competing financial interests.

Preprint server: No;

Author contributions and disclosures: DG, PBr, GP, MM and IC led the panel and conceived the manuscript. DG and FB co-wrote the manuscript. PA, EB-J, PBi, BB, CC, RE, RF, TG, OL, GM, EN, APie, APip, DP, JR, MS, PS, DS, HZ critically revised and edited the manuscript

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement:

Clinical trial registration information (if any):

1 **Special Report**

2

3 **Article Title:** Hemochromatosis classification: update and recommendations by the

4 BIOIRON Society

5 **Short Title:** New Hemochromatosis classification by BIOIRON Society

6

7 **Authors:** Domenico Girelli[1]*, Fabiana Busti[1]*, Pierre Brissot[2]*, Ioav Cabantchik[3], Martina U.

8 Muckenthaler[4], Graça Porto[5], on behalf of the Nomenclature Committee of the International

9 Society for the Study of Iron in Biology and Medicine (BIOIRON Society)**

10

11 [1]Department of Medicine, Section of Internal Medicine, EuroBloodNet Center, University of Verona and
12 Azienda Ospedaliera Universitaria Integrata Verona, Italy
13 [2]INSERM, Univ-Rennes, INRA, UMR 1241, Institut NuMeCan, Rennes, France
14 [3]Della Pergola Chair, Alexander Silberman Institute of Life Sciences, Hebrew University, Jerusalem, Israel
15 [4]Department of Pediatric Oncology, Hematology, and Immunology and Molecular Medicine Partnership Unit,
16 University of Heidelberg, Heidelberg, Germany; Molecular Medicine Partnership Unit, European Molecular
17 Biology Laboratory, Heidelberg, Germany; Translational Lung Research Center, German Center for Lung
18 Research, Heidelberg, Germany; German Centre for Cardiovascular Research, Partner Site Heidelberg,
19 Mannheim, Germany
20 [17]Institute for Molecular and Cell Biology (IBMC), i3S (Instituto de Investigação e Inovação em Saúde),
21 Universidade do Porto, Porto, Portugal; Clinical Hematology, Santo António Hospital - Porto University

22

23

24 *equal contributors

25 **Full list of Authors and affiliations is reported at the end of the manuscript

26

27

28

29 **Corresponding author:** Domenico Girelli, Department of Medicine, Section of Internal

30 Medicine, Policlinico Giambattista Rossi, 37134 Verona, Italy. E-mail:

31 domenico.girelli@univr.it. Tel. +39 045 812 4262. Fax +39 045 802 7496.

32

33

34

35

36

37 **Text word count**: 4,031

38 **Abstract word count**: 200

39 **Number of figures**: 3

40 **Number of tables**: 4

41   **Number of references**: 110

42

43

44

45

46

47

Abstract

Hemochromatosis (HC) is a genetically heterogeneous disorder in which uncontrolled intestinal iron absorption may lead to progressive iron overload responsible for disabling and life-threatening complications such as arthritis, diabetes, heart failure, hepatic cirrhosis, and hepatocellular carcinoma.

The recent advances in the knowledge of pathophysiology and molecular basis of iron metabolism have highlighted that HC is caused by mutations in at least five genes, resulting in insufficient hepcidin production or, rarely, resistance to hepcidin action. This has led to an HC classification based on different molecular subtypes, mainly reflecting successive gene discovery. This scheme was difficult to adopt in clinical practice and therefore needs revision. Here we present recommendations for unambiguous HC classification developed by a working group of the International Society for the Study of Iron in Biology and Medicine (BIOIRON Society) including both clinicians and basic scientists during a meeting in Heidelberg, Germany. We propose to deemphasize the use of the molecular subtype criteria in favor of a classification addressing both clinical issues and molecular complexity. Ferroportin Disease (former type 4a) has been excluded because of its distinct phenotype. The novel classification aims to be of practical help whenever a detailed molecular characterization of HC is not readily available.

3

Historical Perspective

It is commonly accepted that the term "hemochromatosis" was coined by the German pathologist von Recklinghausen in 1889. Of note, this was during the *Versammlung Deutscher Naturforscher* (meeting of German scientists) held in Heidelberg, like the BIOIRON Society (formerly IBIS) meeting in 2019, which led to the current report. Following the description of patients with "bronze diabetes and cirrhosis" by French physicians led by Armand Trousseau in the mid-1800s, von Recklinghausen hypothesized that something circulating in the blood ("Hemo-") was responsible for skin and organ damage and pigmentation ("-chromatosis") (HC). Recognizing excess iron as the etiology of organ toxicity took several decades, and was attributable to Joseph Sheldon in 1935, who was also the first to suggest the genetic origin of the metabolic defect (for a comprehensive historical review, see[1]). Overall, these pioneers' works clearly defined a clinical-pathological entity caused by progressive iron accumulation and characterized by multi-organ damage (mainly in liver, pancreas, joints, heart, and endocrine glands), without signs of anemia (to the contrary, some patients show mildly increased Hb levels)[2-5]. In the 1950s, ferrokinetic studies revealed abnormally increased intestinal iron absorption as the key pathophysiological feature of HC,[6] and repeated/frequent phlebotomies were established as the mainstay of treatment[7]. In 1977, the seminal work by Marcel Simon and colleagues reported the tight linkage between the major histocompatibility complex (MHC) and the putative hemochromatosis gene on chromosome 6p, definitively demonstrating the genetic origin of the disease[8]. This paved the way to the discovery, in 1996, of the "hemochromatosis gene" *HFE* (alias "high Fe", official full name "homeostatic iron regulator")[9], which provided additional information about HC. Initially, it appeared that up to 95% of HC cases could be attributed to homozygosity for a single nucleotide change (845 G→A) causing the substitution of cysteine by tyrosine at amino acid 282 (*p.Cys282Tyr* or C282Y variant)[10-13]. A second *HFE* polymorphism, *p.His63Asp*, was detected, whose minor role became clearer later[14]. The meaning of the *p.Cys282Tyr*/*p.His63Asp* compound heterozygosity is discussed in detail below. The high frequency of *p.Cys282Tyr* homozygosity in the original studies resulted from the inclusion of patients mostly of Northern European ancestry, a region where the variant had originated around 4,000 BC.[15,16] Indeed, the *p.Cys282Tyr* variant, frequent in certain geographical regions,[17] is rare or even absent in large areas of the world, including Asian and African countries, as well as in native Americans.[18,19] Subsequent studies in Southern Europe, in the Mediterranean

4

115   area, and in Brazil found that at least one-third of subjects with a defined HC phenotype

116   were negative for *p.Cys282Tyr* at *HFE* genetic testing.[20,21]

117   Over time, it became evident that the genetic basis of HC was more heterogeneous

118   than initially assumed, and several variants in other iron-controlling genes (collectively

119   referred to as "non-*HFE* genes") were progressively associated with the disorder. These

120   include variants on genes coding for a second receptor for transferrin (*TFR2*),[22-24]

121   ferroportin (*SLC40A1*),[25] hepcidin (*HAMP*),[26,27] and hemojuvelin (*HJV*).[28,29] In particular,

122   the discovery of variants in the *HAMP* and *HJV* genes made it possible to define a severe

123   early-onset (*juvenile*) form of HC, with early cardiac and endocrine impairments, as a

124   molecularly distinct entity.

125   Hepcidin is the master regulator of iron homeostasis[30-33] and its identification has

126   represented a considerable advance in the comprehension of the pathophysiological

127   mechanisms underlying HC. For detailed reviews on hepcidin discovery, functions and

128   regulation, readers are referred elsewhere[34-37]. Briefly, hepcidin is a small peptide

129   hormone produced by the liver, that negatively controls circulating iron levels. Through

130   interaction with ferroportin[38-40] (its receptor and the only cellular iron exporter so far

131   identified in humans), hepcidin inhibits the absorption of dietary iron in the duodenum and

132   its release by spleen macrophages involved in recycling iron from senescent erythrocytes.

133   Molecular defects causing hepcidin deficiency result in uncontrolled intestinal iron

134   absorption, with progressive iron accumulation in tissues, ultimately leading to HC.[1] In

135   most cases gene defects cause insufficient production of hepcidin, while rarely ferroportin

136   resistance to hepcidin is observed (see below). As illustrated in **Figure 1**, hepcidin

137   regulation by iron is quite complex and involves numerous proteins[41,42] whose alterations

138   can compromise hormone synthesis or function. For this reason, identifying the molecular

139   causes of HC is far from simple and requires a deep knowledge of its pathogenetic basis,

140   which is still not completely clarified. In our view, the term "hemochromatosis" should be

141   reserved for this unique clinical entity caused by genetic lesions that primarily affect the

142   hepcidin-ferroportin system and not used to describe clinically distinct iron overload

143   conditions with other causes (see discussion below).

144

145   **The current clinical scenario**

146   Unlike in the past, fully expressed and potentially lethal HC (with liver cirrhosis,

147   diabetes, endocrine dysfunction, and heart failure) is seen rarely in current clinical

148   practice.[43] This can be ascribed to increased awareness of the disease, and, mostly, to the

5

routine assessment of iron biomarkers, particularly serum ferritin. Unfortunately, this is counterbalanced by an increased diagnostic challenge for non-experts in the iron field, primarily due to the lack of specificity of ferritin.

Ferritin is an essentially intracellular protein that serves to store iron safely. It is also present at very low concentration (µg/L) in serum, likely through secretion by macrophages[44]. Normal values usually range from 30 to 200 or 300 µg/L in females and males, respectively. The function of secreted extracellular ferritin remains largely unknown.[45,46] Several common conditions lead to increased serum ferritin levels, including virtually all inflammatory disorders, hepatic cytolysis (e.g., during acute or chronic liver disease), or the metabolic syndrome.[47,48] This translates into a huge number of consultations, overuse of the "first-level" genetic test looking for the presence of the common variants in the *HFE* gene, and even misdiagnosis due to incorrect interpretation of the results.[49]

The glycoprotein transferrin is the extracellular carrier of iron that is detectable at high concentration in blood (g/L; the third most abundant protein after albumin). Transferrin saturation (TSAT) is calculated as the ratio between serum iron and transferrin (multiplied by the correction factor 1.42) or, less reliably, between serum iron and total iron binding capacity, and expressed as a percentage. TSAT is much less requested in clinical practice but is much more informative about a possible diagnosis of HC. Normal TSAT varies between 20 and 45%. It has been estimated that hyperferritinemia with normal TSAT is associated with increased iron stores in less than 10% of cases.[50] Importantly, TSAT elevation is the hallmark of HC. In HC patients, high TSAT reflects the increased pool of circulating iron due to insufficient hepcidin production and typically precedes the rise of serum ferritin by several years.[51] TSAT tends to remain elevated even in subjects effectively iron-depleted by phlebotomies. Occasional reports of normal TSAT in *p.Cys282Tyr* homozygotes with hyperferritinemia should always prompt the search for additional cofactors that raise ferritin, such as metabolic syndrome or alcohol intake.[52,53]

Liver biopsy was once regarded as the gold standard for the diagnosis of HC, because it can reveal iron deposition in hepatocytes with the typical decreasing gradient from the periportal zone (most exposed to iron coming from the gut) to the central-lobular zone in the hepatic acinus. However, in current practice the demonstration of *p.Cys282Tyr* homozygosity along with elevated serum ferritin and TSAT is considered sufficient to make the diagnosis of HC. Liver biopsy remains useful for prognostic purposes in HC patients with serum ferritin levels repeatedly >1,000 µg/L, allowing the early identification of

6

183 advanced fibrosis or even subclinical cirrhosis. These conditions require close surveillance

184 for hepatocellular carcinoma even after iron depletion.[51] Nowadays, liver biopsy is seldom

185 performed due to its invasiveness, costs, and the increasing availability of non-invasive

186 tools. Indeed, magnetic resonance imaging (MRI) techniques have largely replaced it for

187 the determination of liver iron concentration (LIC). This is obtained indirectly by using

188 various MRI protocols, for which there is still no consensus on the best one. The choice of

189 the protocol mainly depends on local expertise, as well as on the available equipment and

190 software (for detailed reviews, see [54-56]). Moreover, hepatic transient elastography

191 (Fibroscan) is a reliable non-invasive method for detecting liver fibrosis in HC patients,

192 limiting the need for liver biopsy to those with indeterminate results.[57]

193 Currently, HC is typically suspected in subjects with no or minimal symptoms, increased

194 serum ferritin levels without alternative explanation, high TSAT, and evidence of increased

195 liver iron stores by MRI. Making the diagnosis at this preclinical early stage[51] has the

196 undoubted advantage of preventing organ damage by a relatively simple and cost-

197 effective treatment (phlebotomy), as well as of allowing normal life expectancy.[58,59]

198 The HFE genetic test, available in most laboratories, identifies the commonest inherited

199 defect in Caucasians predisposing to *HFE*-related HC, i.e. *p.Cys282Tyr* homozygosity.

200 Therefore, in Caucasians, an HFE genetic test is indicated when high TSAT is confirmed

201 irrespective of parenchymal IO demonstration. *p.Cys282Tyr* homozygosity has a variable

202 and difficult to predict clinical penetrance,[60-64] with several inherited and acquired modifiers

203 potentially contributing to the final phenotype. For this reason, *HFE*-related HC should not

204 be viewed as a simple monogenic disorder, but rather as the complex result of the

205 interplay of environmental, lifestyle, and still unidentified genetic cofactors.[1]

206   Regarding the compound *p.Cys282Tyr* and *p.His63Asp* heterozygosity, compelling

207 evidence exists that this genotype *per se* is characterized by minimal or no clinical

208 penetrance [65,66]. Thus, it cannot be considered diagnostic for HC (for a detailed discussion

209 see[14]), but at most as a susceptibility factor that can be associated with mild-to-moderate

210 IO only in case of digenic inheritance[67] (see below) or when other predominant causes of

211 liver disease are present, namely non-alcoholic fatty liver disease (NAFLD), alcohol, or

212 HCV. Of note, the latter two are known to cause acquired hepcidin suppression[41,68,69].

213 According to existing guidelines, whenever a subject with *p.Cys282Tyr/p.His63Asp*

214 compound heterozygosity has evidence of IO, a secondary cause of liver disease should

215 be sought and treated[70,71], with phlebotomies possibly considered as an adjunctive

216 treatment. On the other hand, the negative effects of an automatic HC (mis)diagnosis in

7

217 *p.Cys282Tyr/p.His63Asp* compound heterozygotes are commonly seen at referral centers.

218 They include patients' and family members' unnecessary anxiety, incomplete prior

219 investigations (e.g., serum ferritin but neither TSAT nor MRI), overlooking of other causes,

220 and/or unnecessary treatment by phlebotomies.

221   Clinical elements that should raise a definite suspicion of HC are reported in **Table 1**. In

222 Caucasians with a negative first-level *HFE* test (i.e., *p.Cys282Tyr* homozygosity is not

223 detected) and in non-Caucasians, a second-level genetic test should be considered in

224 order to identify rarer variants in the *HFE* or in other genes known to be linked to hepcidin

225 control. In general, these types of HC are less influenced by cofactors, and characterized

226 by a more severe and homogeneous clinical picture appearing at a younger age.[72] Their

227 molecular diagnosis is often complex, since variants in HC genes other than *HFE* are

228 typically private, i.e. restricted to members of only one or a few families. To this end,

229 modern approaches based on Next-Generation-Sequencing (NGS) have greatly expanded

230 the diagnostic possibilities in rarer HC, while at the same time opening enormous

231 challenges of interpretation of the results. NGS is generally available only at referral

232 centers and requires specific expertise to avoid misdiagnosis, with long wait time for

233 results (see below). Nonetheless, treatment of patients with a defined HC phenotype

234 should not be delayed pending the result of the genetic test. Recently, NGS methods have

235 also made it possible to estimate the global prevalence of HFE and non-HFE HC in

236 different populations,[19] as summarized in **Table 2**.

237   **Figure 2** illustrates a possible algorithm for diagnosis of HC, starting from clinical,

238 biochemical and imaging studies to molecular confirmation.

239

240   **The nomenclature of genetic disorders**

241   HC nomenclature suffers from a common problem of classifying genetic diseases. In

242 contrast to genes, diseases lack a standardized way to review official names and symbols

243 by formal committees (https://ghr.nlm.nih.gov/primer/mutationsanddisorders/naming).

244 Disease nomenclature is often derived from the name(s) of the physician(s) who first

245 described the condition, one major sign or symptom, or the biochemical/genetic underlying

246 defect. However, the growing comprehension of the pathophysiological or molecular

247 mechanisms that regulate diseases, as well as the identification of new phenotypes, may

248 require revision of the initial name by experts, in order to improve its usefulness in clinical

249 practice.

8

250 Proper nomenclature, in fact, is an essential prerequisite for clear and effective

251 communication about a particular condition. Ideally, it should unequivocally evoke

252 disorders sharing the same pathogenesis and treatment, eventually helping clinicians to

253 provide an accurate diagnosis and management.

254

255 **What hemochromatosis is (and what it is not)**

256 Nomenclature and case definition of HC have long been recognized as a potential source

257 of confusion[73], especially when dealing with the report of genetic tests [14]. Among experts,

258 there was a common feeling that the HC classification needs to be revised in view of the

259 increasing awareness and knowledge of IO disorders. To this end, the BIOIRON Society

260 promoted a two-step process. The starting point was the preparation of a survey that was

261 sent to working group participants, including both expert clinicians and basic scientists

262 actively involved in the iron metabolism field, and consisting of nearly all who discovered

263 the hemochromatosis genes and hepcidin. The survey questions and the summary of

264 responses are available in the Supplementary Materials (S1). This was followed by a

265 critical collegial discussion during a specific session of the most recent Biennial Meeting of

266 the BIOIRON Society in Heidelberg. The recommendations reported here are the result of

267 such discussion, where the panelists eventually agreed on the novel classification. As an

268 integral part of the process, the panelists agreed on a robust definition of HC, based on

269 clinical presentation and widely available tools, as a prerequisite for genetic testing. The

270 main clinical, biochemical and imaging studies for the suspicion of HC are reported in

271 **Table 1**. Rigorously speaking, the term "hemochromatosis" should be reserved for a

272 unique genetic clinical-pathological condition characterized by increased TSAT, IO in the

273 liver (but not in the spleen), prevalent involvement of peri-portal hepatocytes with iron-

274 spared Kupffer cells, and signs and/or symptoms associated with IO. The panelists also

275 emphasized that the term "hemochromatosis" itself implies an IO of genetic origin, which is

276 why they would recommend avoiding the unnecessary use of qualifiers such as

277 "hereditary", "genetic" or "primary". Indeed, genetic defects in the hepcidin/ferroportin

278 regulatory axis (caused by variants in hepcidin regulators, the hepcidin gene itself, or in

279 ferroportin) are responsible for inadequate production or activity of hepcidin, or lack of

280 hepcidin responsiveness of ferroportin. Finally, the panelists agreed that the definition of

281 HC should also include the absence of hematological signs of a primary/predominant red

282 blood cell disorder, such as anemia or reticulocytosis (see **Table 1** for some exceptions to

283 this rule). This is needed to distinguish HC from other iron overload conditions, often

9

referred to as "iron-loading anemias",[74,75] which are similarly characterized by increased

TSAT and are nearly always genetically determined. In these conditions, the hepcidin

suppression is caused by factors released by erythropoietin-stimulated erythroblasts (e.g.

erythroferrone or ERFE),[76] as a consequence of ineffective erythropoiesis or compensated

chronic hemolysis, and not by variants in genes affecting the hepcidin-ferroportin axis. The

prototype of this group is non-transfusion dependent thalassemia (NTDT).[77] IO also occurs

in transfusion dependent inherited anemias, but in this case, it is mainly due to

transfusions *per se*, and hepcidin levels tend to be increased,[78] especially immediately

after the transfusion because of suppression of erythropoiesis.[79] **Figure 3** illustrates the

main mechanisms underlying the development of IO in hemochromatosis and iron loading

anemias. As mentioned above, the majority of iron loading anemias are inherited (for

recent comprehensive reviews, see[36,80-82]), including forms caused by variants in the

hemoglobin genes, in genes coding for red blood cell enzymes or membrane structures,

as well as congenital sideroblastic[81] or dyserythropoietic[82] anemias. Sometimes, variants

in genes directly regulating iron transport and utilization (such as *DMT1*, transferrin,

ceruloplasmin, and others) may be implicated as well.[80] Finally, IO due to hepcidin

inhibition can also occur independently of transfusions in some forms of myelodysplastic

syndromes[83], especially those characterized by ringed sideroblasts and increased

ineffective erythropoiesis associated with acquired somatic mutations in *SF3B1*[84]. In any

case, all these conditions should never be regarded as hemochromatosis because of the

distinct pathogenesis and treatment (e.g., phlebotomies are often not feasible).

The former classifications: strengths and shortcomings

The HC classifications reported by authoritative textbooks and recent reviews and

guidelines are based on a schema (**Table 3**), in which numbers and letters reflect the

chronology of first descriptions of genotype-phenotype correlations.[51,71,85,86] Four types are

included, with type 2 and type 4 further subdivided into subtypes A and B. They have the

advantage of being very informative from a molecular point of view, and officially endorsed

by OMIM (the Online Mendelian Inheritance of Man database), but also present several

caveats and inconsistencies. The main limitations of the current classifications are listed

below.

a. Poor applicability in clinical practice (Limitations due to costs and lack of

widespread expertise).

317   Apart from the genetic test looking for the common *HFE* variants, the identification of

318 the molecular defect causing rarer forms of HC is currently offered by few laboratories,

319 heterogeneously distributed and scattered worldwide. This requires that patients should

320 travel, or DNA should be sent to referral centers, with inevitable discomforts, delays and

321 costs. Moreover, although the second-level genetic test (mainly based on NGS approach)

322 has recently improved, with gradually decreasing costs, it demands advanced experience

323 for a rigorous interpretation, which can take several weeks.[14,87-89] Moreover, some cases

324 of HC still remain molecularly undiagnosed even after NGS, suggesting the possibility of

325 unknown gene(s) yet to be discovered.[89-91] For this reason, the cooperation between

326 geneticists, bioinformaticians, and clinicians is necessary to resolve the most difficult

327 cases. EuroBloodNet, the network connecting experts on rare hematological diseases, is

328 making great efforts in this direction (for details, see www.eurobloodnet.eu).

329   ***b.   Numerical subtypes do not capture the complex molecular pathogenesis of***

330 ***HC.***

331   Recent applications of NGS have highlighted that some patients with a provisional

332 diagnosis of *non-HFE* HC cannot be ascribed to any of the numerical subtypes listed in the

333 previous classification (**Table 3**). Essentially there are two reasons:

334   1) Some show a "digenic" inheritance, deriving from the combination of pathogenic

335 variants in two different genes involved in iron metabolism (e.g., single *p.Cys282Tyr* +

336 heterozygous variants in *HJV*, *HAMP* or *TFR2*).[90,92-95] Although there are still only few

337 cases reported, digenic inheritance must also be considered in cases whose *HFE*

338 genotype *per se* does not fully explain the clinical picture, for example in patients with

339 p.Cys282Tyr homozygosity and very early/severe IO.

340   2) Others do not display variants in any of the five classical hemochromatosis genes

341 (i.e., *HFE, HAMP, HJV, TFR2,* and *SLC40A1*). Recently, some small case-series[96-98] have

342 reported moderate late-onset IO in patients carrying variants in the *BMP6* gene, encoding

343 one of the major activators of hepcidin expression in response to iron[99]. The role of such

344 variants is still controversial, as they have been detected mostly in patients with a

345 substantial burden of acquired cofactors.[100] Nonetheless, they broaden the spectrum of

346 genetic defects potentially responsible for HC.

347   ***c.   Former Type 4A HC***

348   Former Type 4A HC actually represents an IO syndrome characterized by distinctive

349 clinical, biochemical, and pathological features, which do not fit the definition of HC.[101,102]

350 They include normal to low TSAT, iron retention in spleen and hepatic macrophages, and,

11

sometimes, poor tolerance to standard phlebotomies. The underlying molecular defect is the presence of loss-of-function (LOF) variants in the *SLC40A1* gene, that reduce expression or iron export capability of ferroportin at the cell surface. Therefore, iron is trapped inside iron-recycling macrophages (primarily in the spleen), resulting in a reduction of circulating iron, and a tendency to iron-restricted erythropoiesis. The corresponding clinical features are normal-to-low TSAT and, sometimes, the development of mild anemia after phlebotomies. Both these elements are clearly at variance with the case definition of HC according to **Table 1**. Another peculiarity is represented by autosomal dominant inheritance. Taking into consideration all these aspects, many authors have suggested adopting a specific terminology for this condition, such as Ferroportin disease (FD).[14,102] In spite of very high ferritin levels which may be evident even in young-adult subjects, FD phenotype is generally milder than in *HFE*-related HC, possibly because of the lower toxicity of iron accumulation in macrophages as compared to hepatocytes.[103].[102,103]

On the other hand, very rare gain-of-function (GOF) variants in the *SLC40A1* gene lead to ferroportin resistance to hepcidin, and cause IO conditions phenotypically and biochemically indistinguishable from hepcidin-deficient HC (former Type 4B HC).[104] Variants that interfere with hepcidin binding to ferroportin and also impair ferroportin stability or ability to export iron are also possible, potentially leading to a mixed or intermediate phenotype variably influenced by age or environmental factors.

d. Former Type 2 molecular subtypes are not always Juvenile forms and vice versa

As mentioned before, the term *juvenile* hemochromatosis classically designates an early-onset (within the second or third decades of life), fully-expressed HC phenotype showing similar penetrance in both genders and a tendency to present with cardiac and endocrine dysfunctions.[27,105] This phenotype is generally due to variants in *HJV* and *HAMP* genes causing much more severe iron hyperabsorption than the *HFE* mutations. However, recent studies have highlighted some age-overlap at diagnosis between the various molecular subtypes of HC.[72] Therefore, the term *juvenile* HC can be ambiguous if invariably attributed to variants in the *HAMP* and *HJV* genes, because in some of these patients the disease is diagnosed in adulthood. Similarly, the term can be misleading in HC patients with defects in genes other than *HJV* or *HAMP,* but with early-onset severe phenotypes.

New classification of HC proposed by the working group

12

385   As a result of the two-step process described previously, the panelists propose a new

386   classification of HC (shown in **Table 4**), addressing both clinical issues (thereby

387   addressing the needs of general clinicians and subspecialists) and molecular precision.

388   The emphasis on clinical features obviates the current challenges represented by second-

389   level genetic testing for detecting rare variants in the *HFE* and non-*HFE* genes, which

390   could lead to delayed diagnosis and treatment. When criteria listed on **Table 1** are fulfilled,

391   the diagnosis of *HFE*-related HC can be made in the presence of *p.Cys282Tyr*

392   homozygosity. If an appropriately investigated patient has an unequivocal HC phenotype

393   without cofactors but is not a *p.Cys282Tyr* homozygote (and this includes compound

394   *p.Cys282Tyr* and *p.His63Asp* heterozygosity or *p.His63Asp* homozygosity), a provisional

395   diagnosis of "molecularly undefined" HC can be made, and phlebotomies started. In this

396   case, quantification of the total amount of iron removed by phlebotomies will serve as an

397   additional marker of IO. The panelists agree that, whenever possible, an accurate

398   molecular characterization remains important in these patients, especially for cascade

399   screening of asymptomatic siblings, or other first-degree relatives. To this end, patients

400   should be referred (or DNA should be sent) to a specialized center. Indeed, second-level

401   genetic tests have limitations that include costs, time-delay, and poor availability in certain

402   regions, and require a high level of expertise for interpretation. Based on NGS results,

403   some cases could be reclassified into *HFE*-related, digenic or non-*HFE* HC (as shown in

404   **Figure 2**).

405   Based on all the above considerations, we suggest adopting a new, more workable

406   classification of HC (shown in **Table 4**) capable of capturing the growing genetic

407   complexity of HC highlighted by NGS. In fact, the type-numerical classification does not

408   allow assignation of any subtypes to patients with complex genotypes deriving from

409   variants in two genes ("digenic" HC), nor to those who remain undefined after sequencing

410   of known HC genes.

411   Finally, the panelists agreed to abandon the current terminology of Type 4A and 4B HC,

412   related to LOF and GOF variants in the ferroportin gene, respectively. While Type B is in

413   all respects a form of HC (and it should be referred to as ferroportin-related HC), Type 4A

414   has the unique characteristics we described previously. Thus, it should be definitively

415   renamed as "Ferroportin Disease" and included in inherited rare disorders of iron

416   metabolism other than HC. It is important to recall that ferroportin mutations are

417   characterized by an autosomal dominant inheritance pattern, with important implications

418   for genetic testing of family members.

13

419   In summary, the novel classification proposed here is based on a pathophysiological

420   cornerstone (hepcidin deficiency) and a distinct clinical/biochemical phenotype. It

421   recognizes the difficulties of a complete molecular characterization and has the potential of

422   being easily shareable between practicing physicians and referral centers. Avoiding any

423   ambiguity is essential for clear and effective communication, that will facilitate proper

424   diagnosis and treatment of HC.

425

426

427

428

429

430

431

432

433

434

435

436

437

438

439

440

441

442

443

444

445

446

447

448

449

450

451

452

453 The Authors (Members of the Nomenclature Committee of the International Society for the
454 Study of Iron in Biology and Medicine - BIOIRON Society) and affiliations (alphabetical
455 order) are as follows:

456 **Paul C Adams[1], Edouard Bardou-Jacquet[2], Patricia Bignell[3], Pierre Brissot[4], Fabiana**
457 **Busti[5], Barbara Butzeck[6], Ioav Cabantchik[7], Clara Camaschella[8], Robert Evans[9],**
458 **Robert Fleming[10], Tomas Ganz[11], Domenico Girelli[5], Olivier Loréal[12], Giacomo**
459 **Marchi[5], Martina U. Muckenthaler[13], Elizabeta Nemeth[14], Antonello Pietrangelo[15],**
460 **Alberto Piperno[16], Graça Porto[17], Dianne Prince[9], John D. Ryan[18], Mayka Sanchez[19],**
461 **Paulo Santos[20], Dorine W. Swinkels[21,22], Heinz Zoller[22].**

462
463 [1]Department of Medicine, University of Western Ontario, London, Ontario, Canada.
464 [2]Univ Rennes, CHU Rennes, National Reference Center for Hemochromatosis and iron metabolism
465 disorder, F-35000, Rennes, France.
466 [3]Oxford University Hospitals NHS Foundation Trust, The Churchill Hospital, Oxford, UK
467 [4]INSERM, Univ-Rennes, INRA, UMR 1241, Institut NuMeCan, Rennes, France
468 [5]Department of Medicine, Section of Internal Medicine, EuroBloodNet Center, University of Verona and
469 Azienda Ospedaliera Universitaria Integrata Verona, Italy
470 [6]European Federation of Associations of Patients with Haemochromatosis EFAPH, Croissy-sur-Seine,
471 France.
472 [7]Della Pergola Chair, Alexander Silberman Institute of Life Sciences, Hebrew University, Jerusalem, Israel
473 [8]Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Milan, Italy
474 [9]Haemochromatosis International*, Bratton Fleming, Devon, UK
475 [10]Saint Louis University, St. Louis, Missouri, USA
476 [11]David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, California, USA
477 [12]INSERM, University of Rennes, INRAE, UMR 1241, AEM2 Platform, Nutrition Metabolisms and Cancer
478 (NuMeCan) Institute, Rennes, France.
479 [13]Department of Pediatric Oncology, Hematology, and Immunology and Molecular Medicine Partnership Unit,
480 University of Heidelberg, Heidelberg, Germany; Molecular Medicine Partnership Unit, European Molecular
481 Biology Laboratory, Heidelberg, Germany; Translational Lung Research Center, German Center for Lung
482 Research, Heidelberg, Germany; German Centre for Cardiovascular Research, Partner Site Heidelberg,
483 Mannheim, Germany
484 [14]Center for Iron Disorders, David Geffen School of Medicine, University of California, Los Angeles, CA, USA
485 [15]Department of Medical and Surgical Sciences, University of Modena and Reggio Emilia; Internal Medicine
486 and Centre for Hemochromatosis and Heredometabolic Liver Diseases, ERN -EuroBloodNet Center,
487 Azienda Ospedaliero-Universitaria di Modena, Policlinico, Modena, Italy
488 [16]University of Milano-Bicocca - Department of Medicine and Surgery, Monza, Italy; Medical Genetics -
489 ASST-Monza, S. Gerardo Hospital, Monza, Italy; Centre for Rare Diseases - Disorders of Iron Metabolism -
490 ASST-Monza, EuroBloodNet Referral Centre, S.Gerardo Hospital, Monza, Italy
491 [17]Institute for Molecular and Cell Biology (IBMC), i3S (Instituto de Investigação e Inovação em Saúde),
492 Universidade do Porto, Porto, Portugal; Clinical Hematology, Santo António Hospital - Porto University
493 Hospital Center (CHUP), Porto, Portugal; Molecular Pathology and Immunology, Abel Salazar Institute for
494 Biomedical Sciences (ICBAS), University of Porto, Porto, Portugal
495 [18]Hepatology Unit, Beaumont Hospital/Royal College of Surgeons in Ireland, Dublin, Ireland
496 [19]Department of Basic Sciences, Faculty of Medicine and Health Sciences, Universitat Internacional de
497 Catalunya (UIC); Sant Cugat del Valles, Barcelona, Spain
498 [20]Department of Pharmacology, Universidade Federal de Sao Paulo, São Paulo, Brazil
499 [21]Translational Metabolic Laboratory, Department of Laboratory Medicine, Radboud University Medical
500 Center, Nijmegen, Netherlands.
501 [22]Department of Blood Cell Research and Unit of Transfusion Medicine, Sanquin Research and Blood Bank,
502 Amsterdam, The Netherlands.
503 [23]Medical University of Innsbruck, Department of Medicine I, Christian Doppler Laboratory on Iron and
504 Phosphate Biology, Innsbruck, Austria.

505
506 *Hemochromatosis International: Global Alliance linking associations of patients with Hemochromatosis
507 worldwide (http://haemochromatosis-international.org/)

508
509

15

Authorship and conflict-of-interest statements

References

1. Pietrangelo A. Hereditary hemochromatosis: pathogenesis, diagnosis, and treatment. *Gastroenterology*. 2010;139(2):393-408, 408 e391-392.

2. Barton JC, Bertoli LF, Rothenberg BE. Peripheral blood erythrocyte parameters in hemochromatosis: evidence for increased erythrocyte hemoglobin content. *J Lab Clin Med*. 2000;135(1):96-104.

3. Beutler E, Felitti V, Gelbart T, Waalen J. Haematological effects of the C282Y HFE mutation in homozygous and heterozygous states among subjects of northern and southern European ancestry. *Br J Haematol*. 2003;120(5):887-893.

4. Datz C, Haas T, Rinner H, Sandhofer F, Patsch W, Paulweber B. Heterozygosity for the C282Y mutation in the hemochromatosis gene is associated with increased serum iron, transferrin saturation, and hemoglobin in young women: a protective role against iron deficiency? *Clin Chem*. 1998;44(12):2429-2432.

5. McLaren CE, Barton JC, Gordeuk VR, et al. Determinants and characteristics of mean corpuscular volume and hemoglobin concentration in white HFE C282Y homozygotes in the hemochromatosis and iron overload screening study. *Am J Hematol*. 2007;82(10):898-905.

6. Alper T, Savage DV, Bothwell TH. Radioiron studies in a case of hemochromatosis. *J Lab Clin Med*. 1951;37(5):665-675.

7. Crosby WH. A history of phlebotomy therapy for hemochromatosis. *Am J Med Sci*. 1991;301(1):28-31.

8. Simon M, Bourel M, Genetet B, Fauchet R. Idiopathic hemochromatosis. Demonstration of recessive transmission and early detection by family HLA typing. *N Engl J Med*. 1977;297(19):1017-1021.

9. Feder JN, Gnirke A, Thomas W, et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat Genet*. 1996;13(4):399-408.

10. Jazwinska EC. Current status in identifying the haemochromatosis gene. *J Gastroenterol Hepatol*. 1996;11(11):1041-1043.

11. Jazwinska EC, Cullen LM, Busfield F, et al. Haemochromatosis and HLA-H. *Nat Genet*. 1996;14(3):249-251.

12. Jazwinska EC, Powell LW. Hemochromatosis and "HLA-H": definite! *Hepatology*. 1997;25(2):495-496.

13. Cullen LM, Gao X, Easteal S, Jazwinska EC. The hemochromatosis 845 G-->A and 187 C-->G mutations: prevalence in non-Caucasian populations. *Am J Hum Genet*. 1998;62(6):1403-1407.

14. Porto G, Brissot P, Swinkels DW, et al. EMQN best practice guidelines for the molecular genetic diagnosis of hereditary hemochromatosis (HH). *Eur J Hum Genet*. 2016;24(4):479-495.

15. Distante S, Robson KJ, Graham-Campbell J, Arnaiz-Villena A, Brissot P, Worwood M. The origin and spread of the HFE-C282Y haemochromatosis mutation. *Hum Genet*. 2004;115(4):269-279.

16. Cassidy LM, Martiniano R, Murphy EM, et al. Neolithic and Bronze Age migration to Ireland and establishment of the insular Atlantic genome. *Proc Natl Acad Sci U S A*. 2016;113(2):368-373.

17. McCullough JM, Heath KM, Smith AM. Hemochromatosis: Niche Construction and the Genetic Domino Effect in the European Neolithic. *Hum Biol*. 2015;87(1):39-58.

18. Lok CY, Merryweather-Clarke AT, Viprakasit V, et al. Iron overload in the Asian community. *Blood*. 2009;114(1):20-25.

19. Wallace DF, Subramaniam VN. The global prevalence of HFE and non-HFE hemochromatosis estimated from analysis of next-generation sequencing data. *Genet Med*. 2016;18(6):618-626.

20. Piperno A, Sampietro M, Pietrangelo A, et al. Heterogeneity of hemochromatosis in Italy. *Gastroenterology*. 1998;114(5):996-1002.

21. Santos PC, Cancado RD, Pereira AC, et al. Hereditary hemochromatosis: mutations in genes involved in iron homeostasis in Brazilian patients. *Blood Cells Mol Dis*. 2011;46(4):302-307.

22. Camaschella C, Roetto A, Cali A, et al. The gene TFR2 is mutated in a new type of haemochromatosis mapping to 7q22. *Nat Genet*. 2000;25(1):14-15.

23. Roetto A, Daraio F, Alberti F, et al. Hemochromatosis due to mutations in transferrin receptor 2. *Blood Cells Mol Dis*. 2002;29(3):465-470.

24. Girelli D, Bozzini C, Roetto A, et al. Clinical and pathologic findings in hemochromatosis type 3 due to a novel mutation in transferrin receptor 2 gene. *Gastroenterology*. 2002;122(5):1295-1302.

25. Montosi G, Donovan A, Totaro A, et al. Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene. *J Clin Invest*. 2001;108(4):619-623.

26. Roetto A, Papanikolaou G, Politou M, et al. Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. *Nat Genet*. 2003;33(1):21-22.

27. Camaschella C, Roetto A, De Gobbi M. Juvenile hemochromatosis. *Semin Hematol*. 2002;39(4):242-248.

28. Papanikolaou G, Samuels ME, Ludwig EH, et al. Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. *Nat Genet*. 2004;36(1):77-82.

29. Lanzara C, Roetto A, Daraio F, et al. Spectrum of hemojuvelin gene mutations in 1q-linked juvenile hemochromatosis. *Blood*. 2004;103(11):4317-4321.

30. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem*. 2001;276(11):7806-7810.

31. Pigeon C, Ilyin G, Courselaud B, et al. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem*. 2001;276(11):7811-7819.

32. Andrews NC. Forging a field: the golden age of iron biology. *Blood*. 2008;112(2):219-230.

33. Ganz T, Nemeth E. Hepcidin and iron homeostasis. *Biochim Biophys Acta*. 2012;1823(9):1434-1443.

34. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of Mammalian iron metabolism. *Cell*. 2010;142(1):24-38.

35. Ganz T. Hepcidin and iron regulation, 10 years later. *Blood*. 2011;117(17):4425-4433.

36. Camaschella C, Nai A, Silvestri L. Iron metabolism and iron disorders revisited in the hepcidin era. *Haematologica*. 2020.

37. Muckenthaler MU, Rivella S, Hentze MW, Galy B. A Red Carpet for Iron Metabolism. *Cell*. 2017;168(3):344-361.

38. Aschemeyer S, Qiao B, Stefanova D, et al. Structure-function analysis of ferroportin defines the binding site and an alternative mechanism of action of hepcidin. *Blood*. 2018;131(8):899-910.

39. Pan Y, Ren Z, Gao S, et al. Structural basis of ion transport and inhibition in ferroportin. *Nat Commun*. 2020;11(1):5686.

40. Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin Regulates Cellular Iron Efflux by Binding to Ferroportin and Inducing Its Internalization. *Science*. 2004;306(5704):2090-2093.

41. Girelli D, Nemeth E, Swinkels DW. Hepcidin in the diagnosis of iron disorders. *Blood*. 2016;127(23):2809-2813.

42. Wang CY, Babitt JL. Liver iron sensing and body iron homeostasis. *Blood*. 2019;133(1):18-29.

43. Deugnier Y, Morcet J, Laine F, et al. Reduced phenotypic expression in genetic hemochromatosis with time: Role of exposure to non-genetic modifiers. *J Hepatol*. 2019;70(1):118-125.

44. Cohen LA, Gutierrez L, Weiss A, et al. Serum ferritin is derived primarily from macrophages through a nonclassical secretory pathway. *Blood*. 2010;116(9):1574-1584.

45. Arosio P, Elia L, Poli M. Ferritin, cellular iron storage and regulation. *IUBMB Life*. 2017;69(6):414-422.

46. Arosio P, Carmona F, Gozzelino R, Maccarinelli F, Poli M. The importance of eukaryotic ferritins in iron handling and cytoprotection. *Biochem J*. 2015;472(1):1-15.

47. Knovich MA, Storey JA, Coffman LG, Torti SV, Torti FM. Ferritin for the clinician. *Blood Rev*. 2009;23(3):95-104.

48. Sandnes M, Ulvik RJ, Vorland M, Reikvam H. Hyperferritinemia-A Clinical Overview. *J Clin Med*. 2021;10(9).

49. Cullis JO, Fitzsimons EJ, Griffiths WJ, Tsochatzis E, Thomas DW, British Society for H. Investigation and management of a raised serum ferritin. *Br J Haematol*. 2018;181(3):331-340.

50. Adams PC, Barton JC. A diagnostic approach to hyperferritinemia with a non-elevated transferrin saturation. *J Hepatol*. 2011;55(2):453-458.

17

623 51.   Brissot P, Pietrangelo A, Adams PC, de Graaff B, McLaren CE, Loreal O. Haemochromatosis.
624 *Nat Rev Dis Primers*. 2018;4:18016.
625 52.   Adams PC, Reboussin DM, Press RD, et al. Biological variability of transferrin saturation and
626 unsaturated iron-binding capacity. *Am J Med*. 2007;120(11):999 e991-997.
627 53.   Adams PCaBJC. Iron overload and cirrhosis in referred HFE p.C282Y homozygotes with normal
628 transferrin saturation and elevated serum ferritin. *Can Liver Journal*. 2020;3(2):188-193.
629 54.   Golfeyz S, Lewis S, Weisberg IS. Hemochromatosis: pathophysiology, evaluation, and
630 management of hepatic iron overload with a focus on MRI. *Expert Rev Gastroenterol Hepatol*.
631 2018;12(8):767-778.
632 55.   Henninger B, Alustiza J, Garbowski M, Gandon Y. Practical guide to quantification of hepatic
633 iron with MRI. *Eur Radiol*. 2020;30(1):383-393.
634 56.   Franca M, Carvalho JG. MR imaging assessment and quantification of liver iron. *Abdom Radiol
635 (NY)*. 2020;45(11):3400-3412.
636 57.   Legros L, Bardou-Jacquet E, Latournerie M, et al. Non-invasive assessment of liver fibrosis in
637 C282Y homozygous HFE hemochromatosis. *Liver Int*. 2015;35(6):1731-1738.
638 58.   Niederau C, Fischer R, Sonnenberg A, Stremmel W, Trampisch HJ, Strohmeyer G. Survival and
639 causes of death in cirrhotic and in noncirrhotic patients with primary hemochromatosis. *N Engl J Med*.
640 1985;313(20):1256-1262.
641 59.   Prabhu A, Cargill T, Roberts N, Ryan JD. Systematic Review of the Clinical Outcomes of Iron
642 Reduction in Hereditary Hemochromatosis. *Hepatology*. 2020;72(4):1469-1482.
643 60.   Beutler E, Felitti VJ, Koziol JA, Ho NJ, Gelbart T. Penetrance of 845G--> A (C282Y) HFE
644 hereditary haemochromatosis mutation in the USA. *Lancet*. 2002;359(9302):211-218.
645 61.   Waalen J, Nordestgaard BG, Beutler E. The penetrance of hereditary hemochromatosis. *Best
646 Pract Res Clin Haematol*. 2005;18(2):203-220.
647 62.   Adams PC, Reboussin DM, Barton JC, et al. Hemochromatosis and iron-overload screening in
648 a racially diverse population. *N Engl J Med*. 2005;352(17):1769-1778.
649 63.   Pilling LC, Tamosauskaite J, Jones G, et al. Common conditions associated with hereditary
650 haemochromatosis genetic variants: cohort study in UK Biobank. *BMJ*. 2019;364:k5222.
651 64.   Allen KJ, Gurrin LC, Constantine CC, et al. Iron-overload-related disease in HFE hereditary
652 hemochromatosis. *N Engl J Med*. 2008;358(3):221-230.
653 65.   Gurrin LC, Bertalli NA, Dalton GW, et al. HFE C282Y/H63D compound heterozygotes are at low
654 risk of hemochromatosis-related morbidity. *Hepatology*. 2009;50(1):94-101.
655 66.   Walsh A, Dixon JL, Ramm GA, et al. The clinical relevance of compound heterozygosity for the
656 C282Y and H63D substitutions in hemochromatosis. *Clin Gastroenterol Hepatol*. 2006;4(11):1403-
657 1410.
658 67.   Biasiotto G, Roetto A, Daraio F, et al. Identification of new mutations of hepcidin and
659 hemojuvelin in patients with HFE C282Y allele. *Blood Cells Mol Dis*. 2004;33(3):338-343.
660 68.   Dostalikova-Cimburova M, Balusikova K, Kratka K, et al. Role of duodenal iron transporters and
661 hepcidin in patients with alcoholic liver disease. *J Cell Mol Med*. 2014;18(9):1840-1850.
662 69.   Girelli D, Pasino M, Goodnough JB, et al. Reduced serum hepcidin levels in patients with
663 chronic hepatitis C. *J Hepatol*. 2009;51(5):845-852.
664 70.   European Association For The Study Of The L. EASL clinical practice guidelines for HFE
665 hemochromatosis. *J Hepatol*. 2010;53(1):3-22.
666 71.   Kowdley KV, Brown KE, Ahn J, Sundaram V. ACG Clinical Guideline: Hereditary
667 Hemochromatosis. *Am J Gastroenterol*. 2019;114(8):1202-1218.
668 72.   Sandhu K, Flintoff K, Chatfield MD, et al. Phenotypic analysis of hemochromatosis subtypes
669 reveals variations in severity of iron overload and clinical disease. *Blood*. 2018;132(1):101-110.
670 73.   Adams PC. Hemochromatosis case definition: out of focus? *Nat Clin Pract Gastroenterol
671 Hepatol*. 2006;3(4):178-179.
672 74.   Camaschella C, Nai A. Ineffective erythropoiesis and regulation of iron status in iron loading
673 anaemias. *Br J Haematol*. 2016;172(4):512-523.
674 75.   Finch C. Regulators of iron balance in humans. *Blood*. 1994;84(6):1697-1702.
675 76.   Coffey R, Ganz T. Erythroferrone: An Erythroid Regulator of Hepcidin and Iron Metabolism.
676 *Hemasphere*. 2018;2(2):e35.
677 77.   Musallam KM, Cappellini MD, Wood JC, Taher AT. Iron overload in non-transfusion-dependent
678 thalassemia: a clinical perspective. *Blood Rev*. 2012;26 Suppl 1:S16-19.
679 78.   Origa R, Galanello R, Ganz T, et al. Liver iron concentrations and urinary hepcidin in beta-
680 thalassemia. *Haematologica*. 2007;92(5):583-588.

681   79.   Pasricha SR, Frazer DM, Bowden DK, Anderson GJ. Transfusion suppresses erythropoiesis
682   and increases hepcidin in adult patients with beta-thalassemia major: a longitudinal study. *Blood*.
683   2013;122(1):124-133.
684   80.   Brissot P, Bernard DG, Brissot E, Loréal O, Troadec MB. Rare anemias due to genetic iron
685   metabolism defects. *Mutat Res*. 2018;777:52-63.
686   81.   Ducamp S, Fleming MD. The molecular genetics of sideroblastic anemia. *Blood*.
687   2019;133(1):59-69.
688   82.   Iolascon A, Andolfo I, Russo R. Congenital dyserythropoietic anemias. *Blood*.
689   2020;136(11):1274-1283.
690   83.   Santini V, Girelli D, Sanna A, et al. Hepcidin levels and their determinants in different types of
691   myelodysplastic syndromes. *PLoS One*. 2011;6(8):e23109.
692   84.   Bondu S, Alary AS, Lefevre C, et al. A variant erythroferrone disrupts iron homeostasis in
693   SF3B1-mutated myelodysplastic syndrome. *Sci Transl Med*. 2019;11(500).
694   85.   Fleming RE, Ponka P. Iron overload in human disease. *N Engl J Med*. 2012;366(4):348-359.
695   86.   Powell LW, Seckington RC, Deugnier Y. Haemochromatosis. *Lancet*. 2016;388(10045):706-
696   716.
697   87.   Manolio TA, Fowler DM, Starita LM, et al. Bedside Back to Bench: Building Bridges between
698   Basic and Clinical Genomic Research. *Cell*. 2017;169(1):6-12.
699   88.   Fernandez-Marmiesse A, Gouveia S, Couce ML. NGS Technologies as a Turning Point in Rare
700   Disease Research , Diagnosis and Treatment. *Curr Med Chem*. 2018;25(3):404-432.
701   89.   Wright CF, FitzPatrick DR, Firth HV. Paediatric genomics: diagnosing rare disease in children.
702   *Nat Rev Genet*. 2018;19(5):325.
703   90.   Badar S, Busti F, Ferrarini A, et al. Identification of novel mutations in hemochromatosis genes
704   by targeted next generation sequencing in Italian patients with unexplained iron overload. *Am J
705   Hematol*. 2016;91(4):420-425.
706   91.   McDonald CJ, Ostini L, Wallace DF, Lyons A, Crawford DH, Subramaniam VN. Next-generation
707   sequencing: Application of a novel platform to analyze atypical iron disorders. *J Hepatol*.
708   2015;63(5):1288-1293.
709   92.   Merryweather-Clarke AT, Cadet E, Bomford A, et al. Digenic inheritance of mutations in HAMP
710   and HFE results in different types of haemochromatosis. *Hum Mol Genet*. 2003;12(17):2241-2247.
711   93.   Gan EK, Trinder D, Ayonrinde OT, Olynyk JK. Genetics of hereditary hemochromatosis: a
712   clinical perspective. *Expert Rev Endocrinol Metab*. 2009;4(3):225-239.
713   94.   Rametta R, Dongiovanni P, Baselli GA, et al. Impact of natural neuromedin-B receptor variants
714   on iron metabolism. *Am J Hematol*. 2020;95(2):167-177.
715   95.   Lanktree MB, Sadikovic B, Waye JS, et al. Clinical evaluation of a hemochromatosis next-
716   generation sequencing gene panel. *Eur J Haematol*. 2017;98(3):228-234.
717   96.   Piubelli C, Castagna A, Marchi G, et al. Identification of new BMP6 pro-peptide mutations in
718   patients with iron overload. *Am J Hematol*. 2017;92(6):562-568.
719   97.   Daher R, Kannengiesser C, Houamel D, et al. Heterozygous Mutations in BMP6 Pro-peptide
720   Lead to Inappropriate Hepcidin Synthesis and Moderate Iron Overload in Humans. *Gastroenterology*.
721   2016;150(3):672-683 e674.
722   98.   Alvarenga AM, da Silva NK, Fonseca PFS, et al. Novel mutations in the bone morphogenetic
723   protein 6 gene in patients with iron overload and non-homozygous genotype for the HFE p.Cys282Tyr
724   mutation. *Blood Cells Mol Dis*. 2020;84:102444.
725   99.   Xiao X, Alfaro-Magallanes VM, Babitt JL. Bone morphogenic proteins in iron homeostasis.
726   *Bone*. 2020;138:115495.
727   100.   McDonald CJ, Rishi G, Secondes ES, et al. Evaluation of a bone morphogenetic protein 6
728   variant as a cause of iron loading. *Hum Genomics*. 2018;12(1):23.
729   101.   Pietrangelo A. The ferroportin disease. *Blood Cells Mol Dis*. 2004;32(1):131-138.
730   102.   Pietrangelo A. Ferroportin disease: pathogenesis, diagnosis and treatment. *Haematologica*.
731   2017;102(12):1972-1984.
732   103.   Vlasveld LT, Janssen R, Bardou-Jacquet E, et al. Twenty Years of Ferroportin Disease: A
733   Review or An Update of Published Clinical, Biochemical, Molecular, and Functional Features.
734   *Pharmaceuticals (Basel)*. 2019;12(3).
735   104.   Bardou-Jacquet E, Ben Ali Z, Beaumont-Epinette MP, Loreal O, Jouanolle AM, Brissot P. Non-
736   HFE hemochromatosis: pathophysiological and diagnostic aspects. *Clin Res Hepatol Gastroenterol*.
737   2014;38(2):143-154.
738   105.   De Gobbi M, Roetto A, Piperno A, et al. Natural history of juvenile haemochromatosis. *Br J
739   Haematol*. 2002;117(4):973-979.

740 106.   Beutler E, Griffin MJ, Gelbart T, West C. A previously undescribed nonsense mutation of the
741 HFE gene. *Clin Genet*. 2002;61(1):40-42.
742 107.   Mendes AI, Ferro A, Martins R, et al. Non-classical hereditary hemochromatosis in Portugal:
743 novel mutations identified in iron metabolism-related genes. *Ann Hematol*. 2009;88(3):229-234.
744 108.   Zhang W, Wang X, Duan W, et al. HFE-Related Hemochromatosis in a Chinese Patient: The
745 First Reported Case. *Front Genet*. 2020;11:77.
746 109.   Hamdi-Roze H, Beaumont-Epinette MP, Ben Ali Z, et al. Rare HFE variants are the most
747 frequent cause of hemochromatosis in non-c282y homozygous patients with hemochromatosis. *Am J
748 Hematol*. 2016;91(12):1202-1205.
749 110.   Le Gac G, Congiu R, Gourlaouen I, Cau M, Ferec C, Melis MA. Homozygous deletion of HFE is
750 the common cause of hemochromatosis in Sardinia. *Haematologica*. 2010;95(4):685-687.
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775

Table 1. Main clinical, biochemical and imaging elements for the suspicion of Hemochromatosis

Leading
+ TSAT >45% (mainstay)
+ S-Ferritin >200 µg/L (females) or >300 µg/L (males)
+ imaging evidence of liver IO (MRI* and/or biopsy**)
+ Iron deposits in hepatocytes (if biopsy is performed)
+ Absence of "predominant" acquired risk factors for hepcidin deficiency (e.g., alcohol abuse or end-stage liver disease) and iatrogenic iron overload (e.g. regular transfusions)
+ Absence of hematological signs of a primary red blood cell disorder, such as anemia*** (i.e. Hb>120 g/L in females, >130 g/L in males) and/or reticulocytosis
Not always present
± Signs and/or symptoms associated with IO: - skin pigmentation, asthenia - persistent increase of aminotransferases, hepatomegaly, cirrhosis, hepatocellular carcinoma - joint pain, arthritis, chondrocalcinosis, reduced bone mineral density - diabetes mellitus, hypopituitarism, hypoparathyroidism, hypogonadotropic hypogonadism - cardiomyopathy, heart failure, cardiac arrhythmias

TSAT = transferrin saturation; IO = iron overload; MRI = Magnetic Resonance Imaging.

*Liver Iron Content (LIC) quantification by MRI can be obtained using different protocols, which vary depending on local expertise and equipment. With these limitations, any LIC value higher than the upper normal limit (generally set at 36-40 µmol/g dry weight) should lead to consideration of phlebotomies in HC patients. Similarly, LIC >100-120 µmol/g and >240-300 µmol/g are generally considered as overt and severe IO, respectively (see text and ref.[55,56]).

**Liver biopsy should be considered in patients with ferritin >1,000 µg/L or signs of liver damage.

***Exceptions may occur, e.g. in HC patients with diagnosis delayed after the appearance of liver cirrhosis, in whom anemia may be observed because of hypersplenism or gastrointestinal bleeding, or subjects with beta-thalassemia trait, whose coexistence is not rare in Mediterranean countries.

Table 2. Combined pathogenic allele frequency for HC genes in the 1000 Genomes Project (1000G), Exome Sequencing Project (ESP), and Exome Aggregation Consortium (ExAc) datasets (modified by Wallace et al.)[19]

Gene	1000G	ESP6500	ExAc	Geographical distribution
HFE (p.Cys282Tyr)	0.013	0.048	0.0324	Highest prevalence in Northern Europe
HFE (non-p.Cys282Tyr)	0.001	0.0002	0.000307	
HJV		0.00074	0.000316	Highest prevalence in Southern Asia
TFR2	0.0004	0.0003	0.000102	Most frequent among non-Finnish European populations
HAMP	0.0002		0.0000165	Several populations
SLC40A1	0.0008	0.0009	0.00034	Several populations (highest prevalence among Africans)

825
826
827
828 **Table 3. Former classification of HC**
829

Classification	Gene involved and location	Inheritance	TSAT	Other clinical features
Type 1	*HFE;* chr.6	AR	Increased	Adult-onset; more severe in males; highly variable clinical expression, with predominant liver damage and arthritis
Type 2A	*HJV* (hemojuvelin); chr.1	AR	Increased	Earlier onset (e.g.<30 years old); similar severity in both sexes; prevalent cardiac and endocrine involvement
Type 2B	*HAMP* (hepcidin); chr.19	AR	Increased	Earlier onset (e.g.<30 years old); similar severity in both sexes; prevalent cardiac and endocrine involvement
Type 3	*TFR2* (transferrin receptor 2); chr.7	AR	Increased	Very rare (look for parental consanguinity); clinically similar to Type 1, with an earlier onset
Type 4A	*SLC40A1* (ferroportin); chr.2	AD	Low-normal	Adult-onset; IO in the spleen; mild anemia; possible low tolerance to venesection
Type 4B	*SLC40A1* (ferroportin); chr.2	AD	Increased	Very rare; in general, clinically similar to Type 1, but more severe/early onset forms are reported

830
831
832
833
834
835
836
837
838
839
840
841

Table 4. New classification of HC proposed by the working group

Novel classification	Molecular pattern	Note
HFE-related	*p.Cys282Tyr* homozygosity or compound heterozygosity of *p.Cys282Tyr* with other rare *HFE* pathogenic variants[106-109] or *HFE* deletion[110]	Low penetrance; consider presence of host-related or environmental cofactors for IO. In subjects with other HFE genotypes (e.g. *p.Cys282Tyr/His63Asp* compound heterozygosity or *p.His63Asp* homozygosity) consider second-line genetic testing for rarer variants.
Non HFE-related	Rare pathogenic variants in "*non-HFE*" genes: - *HJV*-related - *HAMP*-related - *TFR2*-related - *SLC40A1* (GOF)-related	Potentially, mutations in any hepcidin-regulatory gene may be causative (the effects of novel mutations should be confirmed through functional and epidemiological studies). Molecular subtypes characterization only at specialized centers, but the diagnosis of non-*HFE* related HC is sufficient to start phlebotomies at non-specialized centers*.
Digenic**	Double heterozygosity and/or double homozygosity/heterozygosity for mutations in two different genes involved in iron metabolism (*HFE* and/or *non-HFE*)	More commonly, *p.Cys282Tyr* mutation in *HFE* gene might coexist with mutation in other genes; rarely, both mutations involve *non-HFE* genes
Molecularly undefined	Molecular characterization (still) not available after sequencing of known genes (provisional diagnosis)	Patients should be referred (or DNA should be sent) to specialized centers

*Providing that iron overload is confirmed by MRI. If this is not accessible, close monitoring of Hb level is needed to avoid the occurrence of anemia.

** Caution is needed to interpret as digenic inheritance results from NGS outputs reporting several variants in gene panels. Whenever possible, strict criteria for defining pathogenic variants should be adopted, and corroborated by family segregation and/or functional studies.

Figure 1. Hepcidin regulation by iron. Increase in transferrin saturation induces hepcidin transcription via the BMP/SMAD signaling pathway. Diferric transferrin binds to TfR2, while BMP6 and BMP2 secreted by liver sinusoidal endothelial cells (LSECs) bind to BMP receptors on hepatocytes. These events trigger phosphorylation of regulatory SMAD1/5/8, recruitment of SMAD4, and translocation of the SMAD complex to the nucleus for activating hepcidin transcription upon binding to BMP/SMAD responsive element in the *HAMP* promoter. BMPs can be trapped by ERFE, leading to hepcidin inhibition in iron loading anemias. Efficient iron signaling requires the BMP co-receptor HJV and the protein HFE, and is negatively regulated by the transmembrane serine protease matriptase-2 (TMPRSS6). The complex molecular pathogenesis of HC reflects the numerous proteins involved in regulation of the hepcidin-ferroportin axis.

Figure 2. Proposal of an algorithm for the diagnosis of HC, from clinical/biochemical and imaging studies to molecular confirmation. <u>Important note</u>: in Caucasians, *HFE* genotyping is indicated with the specific purpose of detecting *p.Cys282YTyr* homozygosity and, if confirmed, to recommend appropriate preventive treatment by phlebotomies. Asian, African, and native American subjects with defined HC phenotype could be directly referred to second-level genetic test. In populations with a frequent component of northern European ancestry, such as African-Americans and Hispanics, there may be still a role for *HFE* genetic testing.

Figure 3. Iron homeostasis in normal conditions (A) and mechanisms leading to iron accumulation in HC (B) and in iron-loading anemias (C: non-transfusion dependent; D: transfusion-dependent). In HC, iron hyperabsorption through the portal vein leads to iron accumulation in liver parenchymal cells, initially with a typical portal-central gradient (see histology) and sparing of macrophages (Kupffer cells). In non-transfusion dependent anemias with ineffective erythropoiesis hepcidin insufficiency is also central to the pathogenesis of IO, but it is due to suppression by soluble factors (e.g. ERFE) produced by ineffective/expanded erythroblasts, rather than to a genetic defect in pathways regulating hepcidin synthesis. In transfusion dependent anemias, regular red blood cells (RBCs) transfusions represent the major contributing factor to IO; in these conditions, hepcidin is relatively upregulated by iron, but fluctuates in response to intermittent erythropoiesis suppression by transfusions.
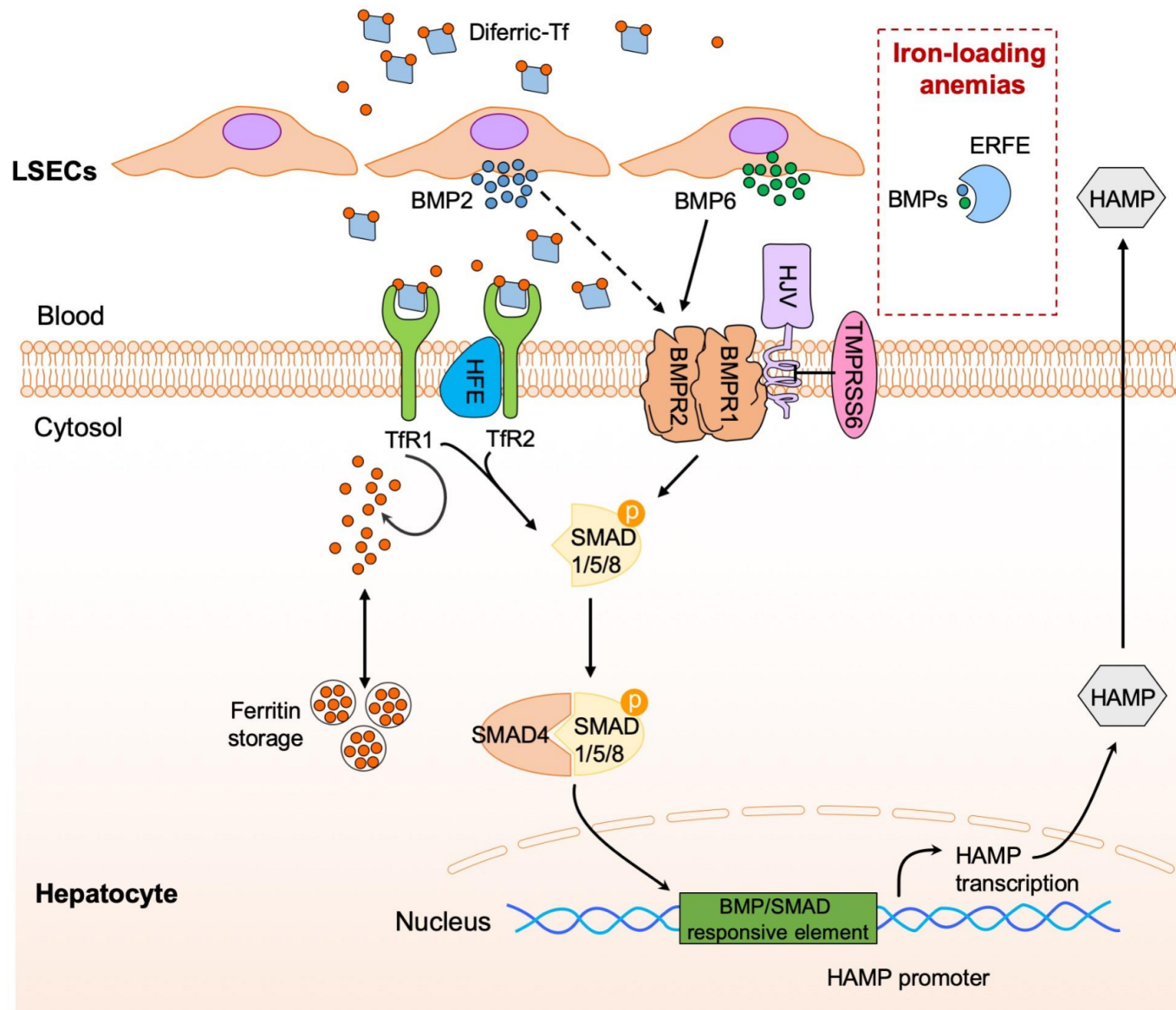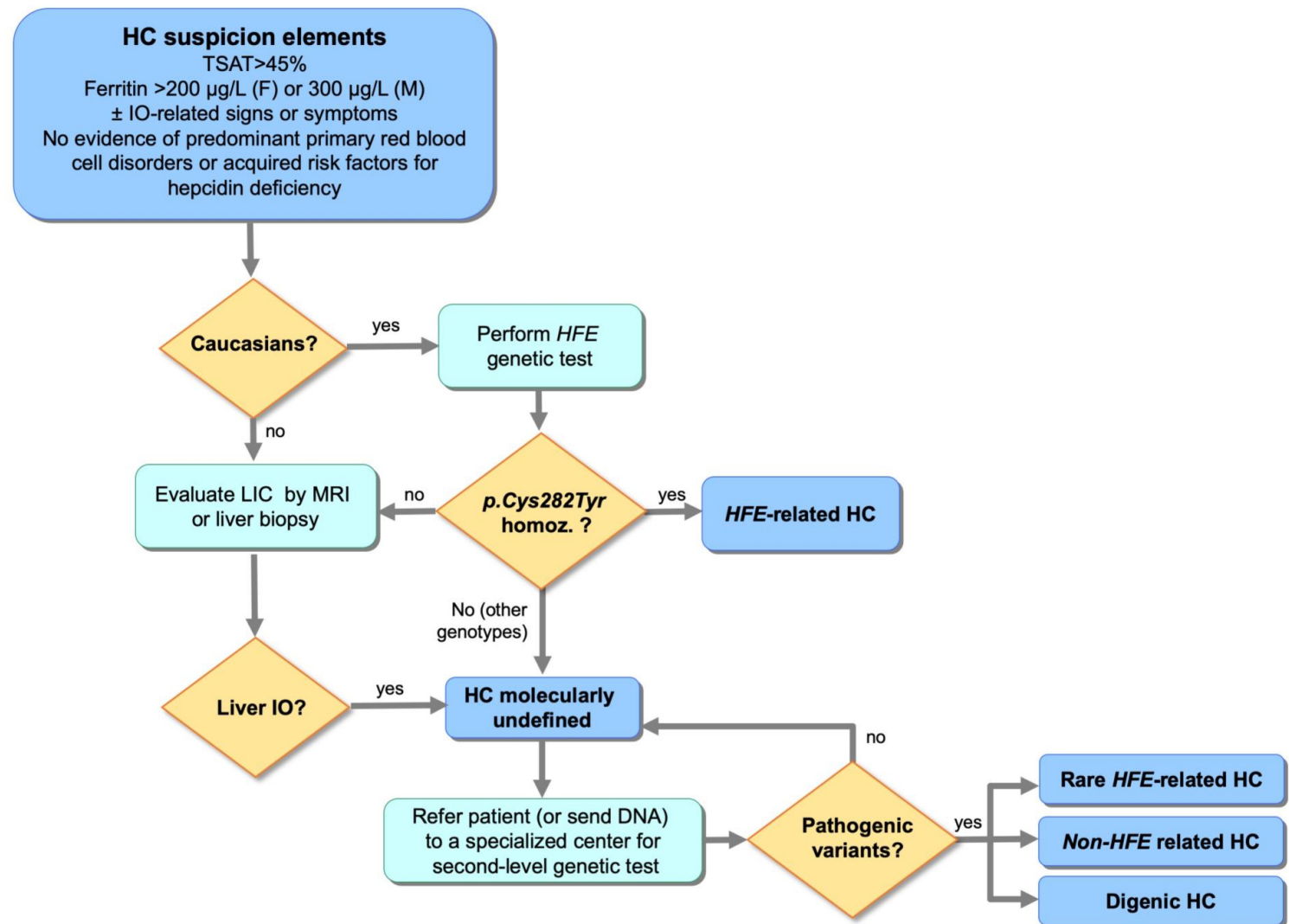
Figure 1

Figure 2

Figure 3

A. Normal conditions
B. Hemochromatosis
C. Iron-loading anemias — NON-TRANSFUSION-DEPENDENT
D. Iron-loading anemias — TRANSFUSION-DEPENDENT