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REVIEW

Appropriate Clinical Genetic Testing of Hemochromatosis Type 2–4, Including Ferroportin Disease

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Abstract: Hereditary hemochromatosis (HH) is an inherited iron overload disorder due to a deficiency of hepcidin, or a failure of hepcidin to degrade ferroportin. The most common form of HH, Type 1 HH, is most commonly due to a homozygous C282Y mutation in HFE and is relatively well understood in significance and action; however, other rare forms of HH (Types 2–4) exist and are more difficult to identify and diagnose in clinical practice. In this review, we describe the clinical characteristics of HH Type 2–4 and the mutation patterns that have been described in these conditions. We also review the different methods for genetic testing available in clinical practice and a pragmatic approach to the patient with suspected non-HFE HH.

Keywords: non-HFE, hemochromatosis, juvenile hemochromatosis, ferroportin disease, transferrin-receptor 2, HAMP, hepcidin

Introduction

Iron is an essential metal for its biological role in hemoglobin and myoglobin to deliver oxygen to tissues, and as a cofactor for a host of enzymes.[1,2] Body iron stores are regulated at the level of intestinal absorption, and there is no physiologic mechanism other than blood loss via menses for elimination of excess iron.[3] Therefore, prevention of iron overload must occur at the level of absorption in the duodenum.

Hepcidin

Hepcidin is a 25-amino acid peptide that regulates systemic iron homeostasis by inhibition of iron absorption.[4] Hepcidin originates in the liver in response to elevated iron levels and binds to ferroportin (FPN) on intestinal cells, resulting in a reduction of cellular iron export.[5] Hepcidin has major effects on control of iron absorption in enterocytes and macrophages, and is crucial to the regulation of overall serum iron levels.[6]

Iron Overload Disorders

Iron overload disorders may be classified as primary or secondary iron overload disorders. Primary iron overload disorders have a genetic basis, and can be attributed to either low hepcidin production or decreased binding interactions between hepcidin and FPN, the transmembrane cellular iron exporter.[7,8] These primary iron

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overload disorders are classified as different types of hereditary hemochromatosis. These disorders have variable phenotypic expression but all share the central defect of decreased hepcidin activity due to different mutations. The most frequent form of hereditary hemochromatosis is one of the most common genetic disorders among Caucasians, with a homozygote frequency of approximately 1 in 250 individuals of Northern European descent.[9,10] Type 1 or classical hereditary hemochromatosis, is due to mutations in *HFE*, the gene encoding the HFE protein.[11] Patients with *HFE*-associated HH may be asymptomatic; chronic fatigue and arthropathy may be early signs.[3] The most common *HFE* mutation is a guanine to alanine substitution at position 845 of the *HFE* gene, resulting in a cysteine to tyrosine change (p.C282Y).[11] This mutation is inherited in an autosomal recessive pattern, and p.C282Y heterozygotes generally do not express the hemochromatosis phenotype.[12] Clinical penetrance of p.C282Y hereditary hemochromatosis may be as low as 2%, and is influenced by other factors leading to iron overload such as excess alcohol intake or chronic hepatitis C[13]. The mutation responsible for p.C282Y was originally identified in 1996 by a full physical mapping of the 3-Mb genomic region around the *HLA-A3* allele on chromosome 6 in diagnosed HH patients.[11] Along with p.C282Y, a second missense mutation in *HFE* was also identified in the same physical mapping, with a cytosine to guanine change resulting in a substitution from histidine to aspartic acid, p.H63D.[11] p.H63D heterozygotes may have an increased likelihood of iron overload if they are compound heterozygotes with the p.C282Y mutation; this form of HH is known as type 1B hereditary hemochromatosis. However, heterozygosity or homozygosity for H63D alone is unlikely to result in expression of the hemochromatosis phenotype.[13]

The p.C282Y mutation is present in a large proportion of patients who present with the phenotype of hereditary hemochromatosis (80.6%).[14] Testing for *HFE*-associated hereditary hemochromatosis is readily available, as a multiplex-PCR can be performed to identify p.H63D and p.C282Y concurrently.[15]

Other more rare forms of hereditary hemochromatosis relevant to the clinician may be caused by mutations in hemojuvelin (Type 2A HH), hepcidin antimicrobial peptide (Type 2B HH), transferrin receptor 2 (Type 3A HH), or ferroportin (Type 4 HH) (Table 1).[11,16–18] A recent publication by Sandhu et al is a comprehensive collection of cases of identified non-HFE hereditary hemochromatosis[19]. It is important to note that iron overload may also be caused by inherited mutations in the *H-ferritin gene* (Type 5 HH); however, Type 5 HH has only been identified in one Japanese family.[20] Additionally, aceruloplasminemia can be the source of iron overload and present with increased serum ferritin, but is not generally considered a form of hereditary hemochromatosis.[21]

Table 1 Hereditary Hemochromatosis Subtypes, Genes Affected and Most Common Mutations

Hereditary Hemochromatosis Type	Most Common Mutation
Type 1A (HFE)	p.C282Y[11]
Type 1B (HFE)	p.C282Y/p.H63D[11]
Type 2A (HJV)	p.G320V[16]
Type 2B (HAMP)	Various
Type 3 (TFR2)	Various
Type 4A (FPN)	p.V162del,[22] p.A77D[8]
Type 4B (FPN)	Various

Molecular Testing (General)

Molecular diagnosis of monogenic inherited diseases is based on identifying variants that may explain phenotypic patterns.[23] It is estimated that approximately 8% of all live births will be diagnosed with a genetic abnormality, with the majority due to monogenic autosomal variants.[24] Linkage mapping of recessive traits using restriction fragment length polymorphisms (RFLP) was first described as a method of diagnosis for recessive traits within consanguineous relatives in 1987,[25] and is particularly useful for autosomal recessive monogenic diseases such as cystic fibrosis.[26] However, linkage analysis limits resolution and may be unable to identify candidate genes.[23] In the late 20th century, identification efforts for pathogenic genes were typically carried out to identify monogenic diseases, and typically within single pedigrees.[27]

The Human Genome Project (HGP) represented a watershed moment in sequencing and identification of genetic disease.[28] The HGP identified 19,000 protein-coding (exome) genes, representing approximately 1% of the genome; however, a majority of phenotypes and pathogenic variants occur in exomic loci.[29,30] Massively parallel sequencing (MPS) or next-generation sequencing (NGS) drastically increased the data-to-price ratio of sequencing exomes, reducing genes of interest from 3 million base pairs of the genome to 20,000 single-nucleotide

polymorphisms (SNPs) of exomes.[31] There are two approaches to exome sequencing: whole-exome sequencing and array-based sequencing.[32] NGS can be used as a diagnostic tool for rare diseases and can reveal novel mutations within known clinical genes of interest.[33] Whole-exome sequencing (WES) focuses screening on exomes of genes of interest, as interpretation of introns is limited.[34] This method has proved effective in postmortem diagnosis in early sudden death due to monogenic disease variants.[35] Exome sequencing is valuable for diagnosis of prenatal disease, and WES of parent and child can be used to identify de novo mutations of interest.[31] For example, WES has expanded genetic diagnosis of terminal prenatal conditions after ultrasound abnormalities, even when karyotyping appears normal.[34] Definitive diagnosis after WES has been estimated to be approximately 25 to 35% and is especially useful in determining autosomal dominant mutations.[36] Chromosomal microarray analysis (CMA) examines changes in genes within a known chromosomal region.[37] CMA has higher sensitivity for nucleotide additions and deletions and is typically used to determine copy number variants (CNVs) or SNPs of clinical significance.[38] Genetic testing for diagnosis of disease is a rapidly evolving field, with genotyping not only being used for diagnosis of diseases but may also to guide individualized treatment regimens.[39]

Hereditary hemochromatosis may also be identified through molecular testing but is not as straightforward as simply testing for p.C282Y. In a genotype analysis of African Americans, primary iron overload was found to be due to varying causes and not as straightforward as the relative frequency of p.C282Y in Caucasian patients with primary iron overload.[40] Since the clinical consequences and diagnosis of HH have been described in detail, in this review we discuss and elucidate features and diagnosis of non-*HFE* hemochromatosis. A variety of options exist for clinicians to test for non-*HFE* hemochromatosis (Table 2). Genetic testing for HH is usually ordered in patients with elevated serum transferrin-iron saturation and/or ferritin; the diagnosis of non-*HFE* hemochromatosis is usually only suspected in patients who test negative for the p.C282Y mutation.[41]

Table 2 Available Testing for Non-HFE Related HH Subtypes, as of 6/22/21

Hereditary Hemochromatosis Type	Companies Available
Type 2A (HJV)	Blueprint Genetics,[42] Fulgent,[43] Invitae,[44] Prevention Genetics,[45] Ivami,[46]
Type 2B (HAMP)	Blueprint Genetics,[42] Fulgent,[43] Invitae,[44] Prevention Genetics,[45] Ivami,[46]
Type 3 (TFR2)	Blueprint Genetics,[42] Invitae,[44] Ivami,[46] Prevention Genetics,[45] Baylor Genetics Laboratory[47]
Type 4A (FPN)	Blueprint Genetics,[42] Invitae,[44] Ivami,[46] Prevention Genetics[45]
Type 4B (FPN)	Blueprint Genetics,[42] Invitae, Ivami,[46] Prevention Genetics[45]

Type 2A- HJV

Type 2 hereditary hemochromatosis, also known as juvenile hemochromatosis (JH), is an early-onset form of HH. Two subtypes of juvenile hemochromatosis have been identified: type 2A and type 2B. Type 2A hereditary hemochromatosis is caused by mutations in *HJV*, located on chromosome 1q which encodes hemojuvelin (HJV), which is expressed in the same tissues as hepcidin.[48] The median age of presentation for Type 2A is 25 years, and while Type 1 hemochromatosis shows a male predominance, HH Type 2A affects sexes equally.[2] Type 2A HH has a distinct set of clinical symptoms compared to Type 1 HH, and characteristically includes iron overload, hypogonadotropic hypogonadism, diabetes and most significantly, cardiomyopathy.[49] Type 2A HH may also result in amenorrhea in women, further increasing body iron stores.[50] If untreated, cardiomyopathy is a frequent cause of death among JH probands.[51,52] Based on family members of one lineage, it was suggested that clinical penetrance of *HJV* mutations is higher than mutations in *HFE*.[50] The earliest age at which patients present with symptoms of iron overload associated with *HJV* mutations was age 4 in an African American patient with the homozygous nonsense mutation p.R54X.[53]

Originally known as *HFE2, HJV* was first mapped by Papanikolaou et al, from a Greek family.[16] Six candidate mutations were identified based on polymorphisms compared to controls in ten Greek families, one Canadian family and one French family in juvenile hemochromatosis patients without p.C282Y mutations.[16] The most common mutation in *HJV* is a guanine to thymine transition originally discovered by Sanger sequencing, resulting in a glycine to valine substitution in position 320 of HJV (p.G320V).[16] WES also revealed a homozygous substitution

in *HJV* from guanine to thymine in position 959, confirming WES validity in diagnosis of p.G320V hemochromatosis.[54] The second mutation in *HJV* identified by Papanikolaou et al was a substitution of thymine to adenine at position 665, known as p.I222N.[16] The p.I281T mutation has been detected both as a homozygous mutation in a Greek patient and as compound heterozygous with the nonsense mutation p.C321X in Chinese patients.[16,55] Mutations in *HJV* are not always identified in patients with juvenile hemochromatosis; *HJV* mutations in Japan (p.D249H, p.Q312X) have resulted in HH phenotypes with onset in middle age.[56]

In a PCR-RFLP study in Alabama of Caucasians and African Americans there were no p.G320V homozygotes and only one heterozygote for p.I222N was found, indicating the relative rarity of these mutations in the population.[57] It has been proposed that WES should be considered for a possible diagnosis of Type 2A HH only after demonstration of elevated ferritin and transferrin saturation, and negative *HFE* and p.G320V testing.[54] Classical symptoms of Type 2A HH accompanied by secondary hypothyroidism and iron overload in the pituitary may be due to a frameshift mutation from a cytosine deletion at position 697 of the HJV gene, resulting in a stop codon at amino acid 245 of *HJV*.[58] Commercially available testing for Type 2A HH includes single gene panels for p.G320V available from Fulgent[43] and Invitae;[44] exome sequencing available from Blueprint Genetics,[42] Prevention Genetics,[45] the Valencian Institute of Microbiology (Ivami)[46] and Invitae.[44] Companies identified for testing were identified by the use of search engine, using keywords "non-HFE hemochromatosis testing, HJV testing".

Type 2B- HAMP

The *HAMP* gene located on chromosome 19 directly encodes HAMP, a propeptide that is cleaved into the 25 amino acid hepcidin. Clinical features of *HAMP* hemochromatosis are similar to those of Type 2A HH.[13] *HAMP* mutations are relatively rare and heterozygosity in *HAMP* can be observed as a modifier of the p.C282Y *HFE* phenotype.[59] Microsatellite analysis of chromosome 19q13 in two individuals of Italian and Greek descent with juvenile hemochromatosis (JH) but with no *HJV* mutations revealed two *HAMP* mutations: a frameshift mutation resulting from a guanine deletion at position 3 of *HAMP* (93delG) and a cytosine to guanine transversion resulting in a nonsense mutation (p.R56X).[60] This led *HAMP*-associated HH to be classified as a subtype of JH. PCR screening of the *HAMP* gene in 21 unrelated JH patients identified a missense from cysteine to arginine (p.C70R), removing a stabilizing disulfide in the structure of hepcidin.[61] Mutations in *HAMP* have also been observed in both Russia and Portugal, and the overall geographic range is not completely defined.[61,62]

A mutation in the 5′UTR of *HAMP* mRNA has been identified in Portuguese patients, creating a mutant initiation codon resulting in iron overload phenotypes from mistranslation of hepcidin.[63]

As in Type 2A HH, testing for Type 2B HH includes exome sequencing available from Blueprint Genetics[42] Prevention Genetics,[45] Valencian Institute of Microbiology (Ivami)[46] and Invitae,[44] and next-generation sequencing from Fulgent.[43] Companies identified for testing were identified by the use of search engine, using keywords "non-HFE hemochromatosis testing, HAMP testing".

Type 3- TFR2

Type 3 hereditary hemochromatosis is caused by mutations in the transferrin receptor 2 (*TFR2*) gene on chromosome 7; *TFR2* is relatively long with a length of 20 kB and many mutations and polymorphisms have been identified.[64] It was initially thought that HFE formed dimers in the duodenum with transferrin receptor 2 (TFR2), but TFR2 acts as an independent regulator of hepcidin in hepatocytes.[65] TFR2-associated hemochromatosis is described as intermediate in clinical presentation between HFE-HH and HJV-HH, and mutations in TFR2 can produce a form of HH resembling juvenile hemochromatosis.[66,67]

TFR2-associated hereditary hemochromatosis is rare, with approximately 65 cases in 44 family lineages across a wide geographic area.[68] Although TFR2-HH was initially discovered in Italy, subsequent cases were found in Scotland, Spain, Japan and Taiwan.[68–71] However, *TFR2*-associated HH is still most commonly found in Italy and Japan.

Mutations in *TFR2* were first discovered upon analysis of two unrelated families of Sicilian origin (one heavily inbred) presenting with hemochromatosis but with no mutations in p.C282Y.[17] Linkage mapping excluding *HFE* and *HJV* revealed homozygosity in the region of *TFR2* on chromosome 7; microsatellite analysis confirmed a cytosine to guanine substitution in exon 6 of *TFR2*, creating a nonsense mutation (p.Y250X) in *TFR2*.[17]

The second mutation in *TFR2* to be discovered was within a consanguineous family, and was an insertion of a cytosine into exon 2, resulting in a nonsense mutation at amino acid 60 (p.E60X).[72]

A homozygous deletion in exon 16 of *TFR2* resulting in deletion of amino acids 594–597 was found in a proband of Northern Italian descent with known iron overload, and intrafamilial haplotyping of affected siblings detected early-onset HH phenotype.[69] Three potential mutations of *TFR2* were proposed in a Scottish man in 2006, including p.R396X, and p.G792R, but could not be directly attributed to the observed HH phenotype.[70] Despite the relative rarity of HH in Asian populations, WES of *TFR2* in a Taiwanese woman found a mutation of guanosine to adenosine in exon 11 (p.R481H) resulting in extreme iron overload and diabetes mellitus.[71] The first described change in TFR2 mRNA levels was a splice site mutation resulting in skipping of exon 4 in *TFR2* reported in a woman of Southern Italian descent.[73] In a study of four unrelated Spanish patients with HH symptoms and no mutations in *HFE, HJV, HAMP* and *SLC40A1*, homozygous mutations in *TFR2* were discovered, including a missense mutation p.G792R, and nonsense mutations p.Q306X and p.Q672X.[68]

TFR2 sequencing as a single gene is available from Invitae[44] and Fulgent,[43] and whole exome sequencing is available from Blueprint Genetics,[42] Ivami[46] and Prevention Genetics.[45] Additionally, Baylor Genetics Laboratories has created a sequence-specific test designed to detect private mutations.[47] Companies identified for testing were identified by the use of search engine, using keywords "non-HFE hemochromatosis testing, TFR2 testing".

Type 4A- FPN Disease

Type 4A hereditary hemochromatosis is also known as classic ferroportin disease (FD), and can be categorized as its own disease associated with iron overload. Outside of HFE-HH, FD is the most prevalent form of genetic iron overload regardless of race.[18,74] FD is caused by pathogenic mutations in *Fpn* or *SLC40A1*, which encodes ferroportin (FPN), also known as Solute Carrier Family 40 Member 1 protein. FD is distinct from hereditary hemochromatosis because it is not associated with high transferrin-iron saturation or low hepcidin concentrations, and unlike other forms of HH, FD is typically inherited in an autosomal dominant pattern.[74,75] In FD, the hepcidin-FPN interaction marking FPN for degradation is disturbed, resulting in a heterogeneity of clinical symptoms.[22]

The most common FD genotypes are an alanine to aspartic acid at residue 77 (p.A77D) substitution in exon 3 of the *FPN* gene and a deletion of valine at residue 162 (p.V162del).[8,22] p.A77D was detected by PCR amplification of exons in the FPN gene comparing probands and control groups.[8] Two missense mutations causing FD (p.I152F and p.L233P) were identified in probands by denaturing high-performance liquid chromatography (DHPLC), where *Fpn* mutations were transfected in vitro into kidney cell lines and then injected into zebrafish embryos, using immunofluorescence to detect FPN localization and degradation in the cell.[76]

Molecular testing for *SLC40A1* includes whole exome sequencing is available from Blueprint Genetics,[42] Ivami[46] and Prevention Genetics,[45] and next-generation sequencing from Fulgent.[43] Companies identified for testing were identified by the use of search engine, using keywords "non-HFE hemochromatosis testing, FPN testing".

Type 4B

Type 4B hereditary hemochromatosis also results from a mutation in *SLC40A1*; however, Type 4B HH is not caused by a change in expression of *SLC40A1*, but rather is a gain-of-function mutation in *FPN*. This "resistant" form of FPN is no longer susceptible to degradation by hepcidin binding and remains hyperactive on the cell membrane.[77] Types 4A and 4B HH can be distinguished clinically by the finding of a low to normal transferrin saturation (TS) in type 4A and high TS in type 4B.[22] MRI has been suggested as a non-invasive tool to distinguish between the gain-of-function and loss-of-function forms of Type 4 HH.[78]

The first form of Type 4B HH was discovered by RFLP analysis of Thai and Vietnamese patients by an autosomal dominant mutation p.C326Y creating a resistant FPN with no effect on protein localization.[79] The cysteine residue at position 326 is involved in a disulfide interaction between hepcidin and FPN, and any change in the cysteine at position 326 of *FPN* results in minimal hepcidin internalization.[80,81] An Australian proband with cirrhosis and parenchymal iron overload onset was identified with relatively early onset of disease at 32 years old, with a change in the asparagine residue to aspartic acid at position 144.[82] A splice mutation in *SLC40A1* was detected by PCR analysis of *HFE, HJV, HAMP, TFR2*

compared against a cDNA library in a middle-aged Chinese woman with unexplained iron overload.[83]

Conclusion

Primary iron overload not due to mutations in *HFE* can be due to a variety of mutations in *HJV, HAMP, TFR2* and/or *FPN*. These rare forms of non-*HFE* HH and difficult to diagnose with certainty, given the variety of mutations, many of which are private. Moreover, compound heterozygosity can add challenges to the diagnosis when *HFE* mutations accompany *HJV* or *HAMP* mutations (Figure 1). It is also important to note that the majority of suspected HH cases are not attributable to any genetic cause, so adequate clinical suspicion is necessary for diagnosis of genetic HH and it is important to eliminate secondary causes of iron overload. Clinicians should always seek alternative explanations for iron overload in patients with iron overload in the absence of *HFE* mutations before considering a diagnosis of non-HFE hereditary hemochromatosis given the rarity of these disorders. Patient genetic

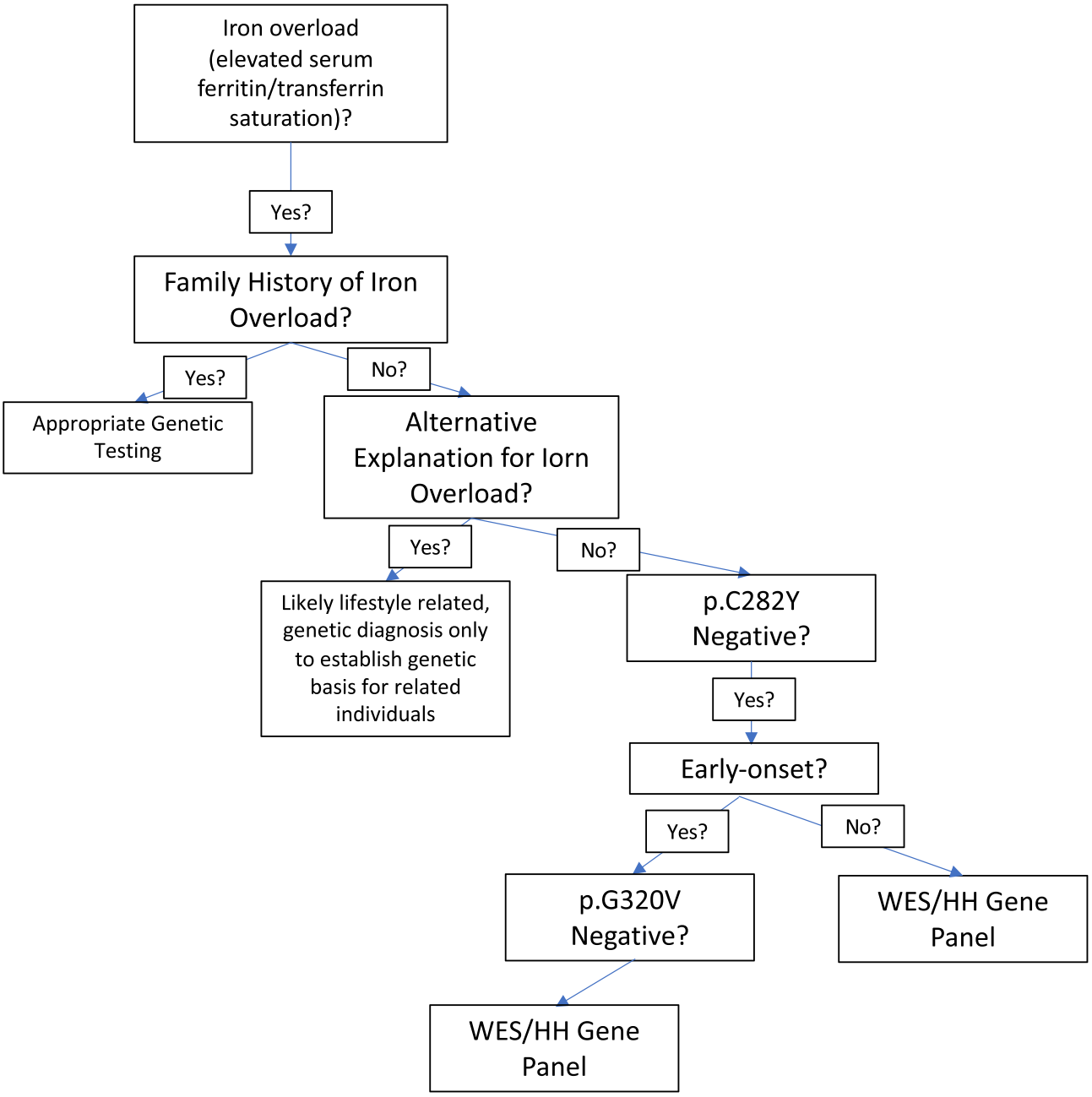

Figure 1 Flowchart for clinician approach to patients with unexplained iron overload.

history and ancestry should be taken into account before diagnosis and treatment with therapeutic phlebotomy.[13] Genetic testing for non-HFE HH should be considered in patients with documented iron overload after other causes if iron overload have been excluded, especially among patients with a family history of iron overload. Several clinical laboratories offer genetic testing for these forms of HH and appropriate use of genetic testing may provide confirmation of the diagnosis and prognosis.

Disclosure

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