RESEARCH ARTICLE

# Identification of novel mutations in hemochromatosis genes by targeted next generation sequencing in Italian patients with unexplained iron overload

Sadaf Badar,[1] Fabiana Busti,[1] Alberto Ferrarini,[2] Luciano Xumerle,[2] Paolo Bozzini,[1] Paola Capelli,[3] Roberto Pozzi-Mucelli,[4] Natascia Campostrini,[1] Giovanna De Matteis,[5] Sergio Marin Vargas,[2] Alejandro Giorgetti,[2] Massimo Delledonne,[2] Oliviero Olivieri,[1] and Domenico Girelli[1,6]*

Hereditary hemochromatosis, one of the commonest genetic disorder in Caucasians, is mainly associated to homozygosity for the C282Y mutation in the *HFE* gene, which is highly prevalent (allele frequency up to near 10% in Northern Europe) and easily detectable through a widely available "first level" molecular test. However, in certain geographical regions like the Mediterranean area, up to 30% of patients with a HH phenotype has a negative or non-diagnostic (*i.e.* simple heterozygosity) test, because of a known heterogeneity involving at least four other genes (*HAMP*, *HJV*, *TFR2*, and *SLC40A1*). Mutations in such genes are generally rare/private, making the diagnosis of atypical HH essentially a matter of exclusion in clinical practice (from here the term of "non-HFE" HH), unless cumbersome traditional sequencing is applied. We developed a Next Generation Sequencing (NGS)-based test targeting the five HH genes, and applied it to patients with clinically relevant iron overload (IO) and a non-diagnostic first level genetic test. We identified several mutations, some of which were novel (*i.e. HFE* W163X, *HAMP* R59X, and *TFR2* D555N) and allowed molecular reclassification of "non-HFE" HH clinical diagnosis, particularly in some highly selected IO patients without concurring acquired risk factors. This NGS-based "second level" genetic test may represent a useful tool for molecular diagnosis of HH in patients in whom HH phenotype remains unexplained after the search of common *HFE* mutations.

Am. J. Hematol. 91:420–425, 2016. 

## Introduction

Hereditary hemochromatosis (HH) is a potentially lethal group of genetic disorders due to insufficient production or response to the iron regulatory hormone hepcidin, leading to progressive accumulation of toxic amounts of iron in vital organs like liver, heart, pancreas, and endocrine glands [1]. Early diagnosis of HH is mandatory, since a prompt treatment before the development of end-stage organ damage can partially or totally reverse the clinical phenotype, and can be associated to a near normal life-expectancy [2]. HH is among the commonest genetic diseases in Caucasian populations, being typically associated with homozygosity for the C282Y mutation in the *HFE* gene, which takes part in complex hepcidin regulation (for comprehensive reviews see [3,4]). This mutation, indeed, has an allele frequency up to near 10% in certain Northern European countries, like in Ireland [5]. On the other hand, only a minor fraction of C282Y homozygotes develop the disease, whose clinical penetrance is relevantly influenced by acquired and genetic risk factors. The main acquired cofactors, that acts by further suppressing hepcidin, are represented by alcohol abuse [6] and HCV infection [7]. The copresence of common features of the so-called metabolic syndrome [8] is also relevant in clinical practice, since they can contribute to further increase of serum ferritin levels, and are sometimes associated to a true iron overload [9,10]. The genetic modifiers remain still largely unknown, although recent studies have suggested a role for *GNPAT* [11], not confirmed by others [12,13]. Beyond the C282Y, another common *HFE* polymorphism, the H63D, can contribute to the development of biochemical and, sometimes, clinically relevant signs of iron overload (IO) when in compound heterozygosity with the C282Y mutation, particularly in subjects with one or more of the above mentioned cofactors [5]. A simple and widely available "first level" genetic test evaluating the presence of the C282Y and H63D variants is therefore a useful tool for clinicians facing with patients with suspected HH, although it should always be prescribed according to diagnostic

*Additional Supporting Information may be found in the online version of this article.*

[1]Department of Medicine, Section of Internal Medicine, University of Verona, Verona, Italy; [2]Department of Biotechnology, University of Verona, Verona, Italy; [3]Unit of Pathology, Azienda Ospedaliera Universitaria Integrata Verona, Verona, Italy; [4]Department of Diagnostics and Public Health, Section of Radiology, University of Verona, Verona, Italy; [5]Unit of Clinical Chemistry, Azienda Ospedaliera Universitaria Integrata Verona, Verona, Italy; [6]Veneto Regional Referral Center for Iron Metabolism Disorders, GIMFer (Gruppo Interdisciplinare Sulle Malattie Del Ferro), Azienda Ospedaliera Uiversitaria Integrata Verona, Verona, Italy

**Conflict of interest:** DG, OO, and MD, are stock-holders and scientific advisors of Personal Genomics s.l.r., a spin-off company of the University of Verona. The remaining Authors have nothing to disclose.

***Correspondence to:** Domenico Girelli, MD PhD, Department of Medicine, Section of Internal Medicine, University of Verona, and Veneto Region Referral Center for Iron Metabolism Disorders, Azienda Ospedaliera Universitaria Integrata Verona, Policlinico G.B. Rossi, 37134 Verona, Italy. E-mail: domenico.girelli@univr.it

**Contract grant sponsor:** Italian Ministry of Health; Contract grant number: RF2010-2312048.
**Contract grant sponsor:** University of Verona; Contract grant number: NGS-HH.
**Received for publication:** 9 December 2015; **Revised:** 8 January 2016; **Accepted:** 13 January 2016
Am. J. Hematol. 91:420–425, 2016.
**Published online:** 22 January 2016 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ajh.24304

algorithms included in guidelines [14,15]. On the other hand, soon after the discovery of the *HFE* gene in 1996 [16], it has become clear that HH is a genetically heterogeneous disorder, particularly in the Mediterranean area where up to near 30% of patients have a negative or "non-diagnostic" (*i.e.*, C282Y and H63D simple heterozygosity) first level genetic test [17]. Rare mutations in the *HFE* gene itself, and/or in additional four genes encoding hepcidin (*HAMP*) [18], its receptor ferroportin (*SLC40A1*) [19,20], or critical modulators of the iron-sensing machinery controlling hepcidin like type 2 transferrin receptor (*TFR2*) [21,22], and hemojuvelin (*HJV*) [23,24], have also been associated to HH. Such conditions are often collectively designated as "non-HFE" HH [25,26]. Of note, mutations in the above-mentioned genes are typically "private," *i.e.*, limited to just one family and/or few individuals, requiring DNA sequencing for establishing a possible molecular diagnosis. In clinical practice, "non-HFE" HH is still essentially a diagnosis of exclusion, which is made after a negative first level genetic test in subjects with clinical, biochemical, instrumental, and pathological findings suggesting *primary* iron overload. Only relatively few cases have been molecularly characterized at referral centers through traditional sequencing, which was hampered by time-consuming and costs, until recently. Recent breakthrough in the development of novel genomic technologies for high-throughput DNA sequencing, collectively known as "next generation sequencing" (NGS), have now the potential of revolutionizing the molecular diagnosis of inherited diseases [27]. This holds the promise of a significant impact on clinical care [28], with unprecedented accuracy, rapidity, and at constantly declining costs [29].

Here we describe the development of a NGS-based "second level" genetic test that simultaneously analyzes all the five hemochromatosis genes, whose performance was evaluated in patients attending our referral clinic because of the presence of relevant biochemical signs of IO and nondiagnostic first level genetic test, suggesting a possible atypical HH.

## Methods

*Patients.* The newly developed second level, NGS-based, genetic test for HH was applied to a total of 106 subjects. For this study we selected DNA samples from patients attending our Regional Referral Centre for Iron Disorders at the Azienda Ospedaliera Universitaria Integrata of Verona (http://www.gimferverona.org). All patients ($n = 34$; mean age 51.3 years, CIs: 47.5–55.2 years; 82.3% males) had relevant biochemical signs of iron overload (*i.e.*, mean serum ferritin 1,810 μg $L^{-1}$, CIs: 1,369–2,259 μg $L^{-1}$; mean ransferring saturation 62.3%, CIs: 54.3–70.2%), confirmed by liver iron content (LIC) measured through magnetic resonance (MR), and/or liver biopsy showing the typical periportal iron deposition. Patients were categorized into three groups. Group 1 ($n = 8$) included patients having IO without apparently relevant cofactors (*i.e.*, metabolic syndrome, HCV infection, alcohol abuse) and with non-diagnostic first level genetic test, defined as above. From a clinical point of view, these patients were provisionally considered affected by "non-HFE" HH with high probability. Group 2 ($n = 16$) included patients with a non-diagnostic first level genetic test and some acquired cofactors, which nevertheless were deemed disproportionate (*i.e.*, possibly unable to fully explain the IO phenotype) by clinical judgment. In such patients, the presence of other HH genetic variants could be theoretically anticipated as potential contributors to better explain the clinical/biochemical phenotype. Group 3 ($n = 10$) included historical patients attending our referral center with known rare mutations previously detected by traditional sequencing [19,22,30]. Such patients were included in the study as "methodological controls", *i.e.* to confirm the ability of the new test to catch the presence of the known genetic variants in the HH genes. Finally, a nearly double number ($n = 72$) of age- and sex-matched subjects with documented normal iron biochemical parameters (*i.e.*, serum ferritin levels between 30 and 200 or 300 μg $L^{-1}$ in females and males, respectively) were included in this study as "true controls." All patients and controls gave written informed consent to DNA analyses, according to study protocols approved by the Ethical Committee of the Azienda Ospedaliera Universitaria Integrata di Verona.

*Sample preparation.* Genomic DNA was extracted using kit (Promega, Madison, WI). DNA quality was determined on a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) while concentration was measured using Qubit dsDNA assay kit with Qubit fluorometer (Life Technologies, Carlsbad, CA). DNA integrity was assessed by gel electrophoresis on agarose gel (1%).

*Target capture and sequencing.* Capture of exonic regions of the 5 HH genes (*HFE*, *HFE2*, *TFR2*, *HAMP*, and *SLC40A1*) was performed starting from 225 ng of genomic DNA using a custom design HaloPlex$^{TM}$ Target Enrichment 1–500 kb Kit (Agilent Technologies, Santa Clara, CA) according to manufacturer instructions. Of note, this approach has recently been recommended for high-coverage capturing of regions spanning 100–500 Kb, particularly when relatively large batches have to be examined [31]. Library quality was determined using the Agilent High Sensitivity DNA kit on the Agilent 2100 bioanalyzer, and the quantity was measured using Qubit dsDNA assay kit with Qubit fluorometer (Life Technologies, Carlsbad, CA) and by quantitative PCR using the KAPA Library Quantification kit (Kapa Biosystems). Libraries were pooled at equimolar concentrations and sequenced with TruSeq PE Cluster Kit v3 and TruSeq SBS Kit v3 (Illumina, San Diego, CA) on Illumina HiSeq 1000 sequencer (Illumina, San Diego, CA) generating 100-bp paired-end reads at average target coverage of 1000X for captured regions.

*Data analysis.* Illumina adapter sequences were removed from sequence reads using Scythe software using default parameters (v. 0.994; https://github.com/vsbuffalo/scythe). Low quality read ends were trimmed using Sickle software (https://github.com/vsbuffalo/scythe). Reads were mapped against reference human genome version 19 (HG19) using BWA (Burrows Wheeler Aligner, version 0.7.10). Mappings and portion of mappings outside expected amplicons were removed using custom scripts. Duplicate PCR reads were removed using Picard, base quality scores were recalibrated using Genome Analysis Toolkit (GATK, version 3), and alignments near putative insertion/deletions were refined with GATK. Variants were called using Unified Genotyper module from GATK. Variant calls that failed to pass the following filters were eliminated from the call set: (i) $MQ0 \geq 4$ && $((MQ0/(1.0 * DP)) > 0.1)$, (ii) $DP < 5$, (iii) $QUAL < 30.0$, (iv) $QD < 5.0$. Coding and splice variants were annotated using the SNP & Variation Suite software (Version 8; Golden Helix, Bozeman, MT). The potential functional impact of identified variants was assessed using three different predictive algorithms, *i.e.* Sorting Intolerant From Tolerant (SIFT), Polymorphism Phenotyping version 2 (PolyPhen2 HumaVar), and MutationTaster (MT). The variants of interest were confirmed by traditional Sanger sequencing using a CEQ 8000 capillary sequencer and GenomeLab DTCS Quick Start kit (Beckman Coulter, Indianapolis, IN). When possible, some of the genetic variants that appeared most interesting were further studied by *in silico* modeling, as reported [32]. Methodological details and *ad hoc* references are reported in Supporting Information [53–62].

## Results

### Genetic variants and literature review

Supporting Information Table SI summarizes the variants found through NGS that survived rigorous filtering, in addition to the known HH associated *HFE* mutations (C282Y, H63D, S65C), and the known mutations previously found in methodological controls. For sake of completeness, we report also the variants found only in controls, which could be useful for comparison with future reports and for databases compilation [33,34]. Among the listed variants, five were apparently new, *i.e.* not reported in the following databases:

dbSNPs (http://www.ncbi.nlm.nih.gov/SNP/),
1,000 genome (http://www.1000genomes.org/category/sequence),
OMIM (http://www.ncbi.nlm.nih.gov/omim),

and ESP Databases (http://evs.gs.washington.edu/EVS/). We also checked a very recent paper reporting a catalogue of all hemochromatosis associated variants [35]. Two out of the new mutations, *i.e.*, the **H274fs** in *SLC40A1* and **M45fs** in *TFR2* were found in control subjects. The *SLC40A1* **H274fs** was found in a 43-year-old man with serum ferritin of 199 μg $L^{-1}$. Although no prediction could be inferred by the three predictive algorithms (Supporting Information Table SI), given that ferroportin disease is the only *dominant* non-HFE HH subtype, this variant is likely non pathogenic. The same applies to the *TFR2* **M54fs**, *i.e.*, no useful information from the predictive algorithms, but since type 3 HH is autosomal recessive we cannot draw any conclusion about its possible pathogenic role.

The three other novel private variants found only in patients from group 1, which could be considered very likely pathogenic, are described in detail below along with individuals' clinical data (Table I).

A sixth patient from group 1 with history of consanguinity was found homozygous for the *TFR2* **N241I** mutation. This variant has

**TABLE I.** Clinical and Biochemical Features of Group 1 Patients With Iron Overload, Minimal or No Acquired Cofactors, and Non-diagnostic 1st Level Genetic Test

| Pt. ID | Age/sex | 1stlevel genetic test | TS, Ferritin | LIC[a] (MR) | Liver biopsy | NGS | Reference | Clinical-molecular interpretation |
|---|---|---|---|---|---|---|---|---|
| #01 | 70/M | Wild-type[b] | 80%, 1,450 μg $l^{-1}$ | – | no (iron removed >10 g) | – | – | Remains unexplained |
| #02 | 39/M | H63D +/− | 61%, >1,000 μg $l^{-1}$ | – | yes (HH "pre-cirrhotic") | HFE W163X +/− | Novel | Atypical Type 1 HH (HFE-related) |
| #03 | 48/M | H63D +/− | 73%, >1,000 μg $l^{-1}$ | 300 | No | HAMP R59X +/− | Novel | Digenic HH (HFE/HAMP)? |
| #04 | 62/F | Wild-type[b] | 60%, 1,786 μg $l^{-1}$ | 270 | yes (siderosis 3+, periportal gradient) | – | – | Remains unexplained |
| #05 | 58/M | H63D +/+ | n.a., 1,089 μg $l^{-1}$ | 160 | No | TFR2 D555N +/− | Novel | Digenic HH (HFE/TFR2)? |
| #06 | 35/F | Wild-type[b] | 100%, n.a. | – | yes (HH "pre-cirrhotic") | TFR2 N241I +/+ | Bardou-Jacquet E et al.[36] | Type 3 HH |
| #07 | 66/M | Wild-type[b] | 48%, 2,352 μg $l^{-1}$ | 250 | yes (siderosis 2–3+, periportal gradient) | – | – | Remains unexplained |
| #08[c] | 47/M | H63D +/− | 95%, 6,242 μg $l^{-1}$ | – | no (iron removed >10 g) | SLC40A1 A69T +/− | – | Type 4 HH |

[a] μM $g^{-1}$.
[b] = for both C282Y and H63D HFE variants.
[c] This peculiar patient has been described in detail elsewhere (Ferbo et al., submitted).

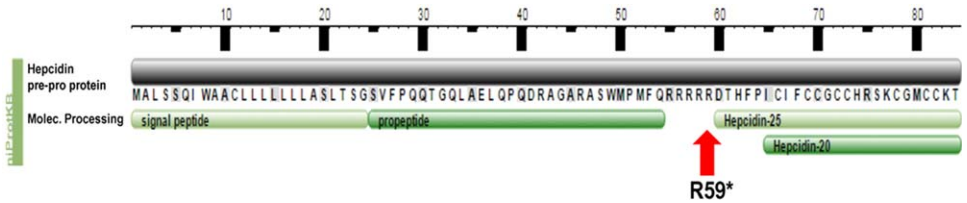


**Figure 1.** Functional consequences of the *HAMP* R59X mutation. The sequence of hepcidin pre-pro-protein is shown (UniProtKB entry P81172). Arginine 59 is located upstream of the region coding the mature hepcidin-25 peptide, which derives from subsequent cleavage of a precursor (pre-pro-hepcidin). This stop codon mutation prevent the possibility of producing the mature peptide. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

been previously reported as pathogenic by others [36], since it was found in a young male whose HH was clinically diagnosed at the age of 17 years. Our data are also consistent with a highly penetrant mutation, since our patient was a female whose HH was clinically diagnosed during the fertile period (at the age of 35 years) because of sustained increase of transaminases, 100 percent transferrin saturation, and a liver biopsy showing iron overload and advanced fibrosis (Table I, patient ID = #06). In this patient we also found a *SLC40A1* variant (rs11568344, Supporting Information Table SI), which was already reported as a benign polymorphism by others [37].

The *TFR2* rs41295942 variant (resulting in mutation **R581H** was previously described by early reports using traditional sequencing, with contrasting or biased results [38–40]. It was also specifically investigated as a potential modifier of the C282Y genotype in a recent report [41], but no effect was found. In our study, this variant was found in homozygosity in a control subject (a 47-year-old male with serum ferritin of 167 μg $L^{-1}$). This finding appears to definitively rule out a possible pathogenic role of this *TFR2* variant. The *HJV* **A197G** variant (rs7540883) that we found in heterozygosity in a control subject was also previously reported by others [37], but without a definite conclusion on its possible pathogenetic role. It was only "possibly damaging" according PolyPhen2, and benign according to SIFT and MT, which seems more likely. Other variants already annotated/reported [42,43] and classified as benign/polymorphisms are reported in Supporting Information Table SI.

## Novel private mutations with high probability of being pathogenic

Three interesting novel variants were found in patients from group 1 (Table I). Patient #03 carried a stop-codon mutation (**R59X**) in the *HAMP* gene. Of note, the Arginine at position 59 is located immediately upstream the sequence containing the hepcidin-25 mature peptide (Fig. 1). Thus, this mutation results in the synthesis of a pre-pro-hepcidin that cannot be processed into the functional peptide. This patient was diagnosed at the age of 48 years because of altered iron parameters (serum ferritin >1,000 μg $L^{-1}$, transferrin saturation >70%) and substantial iron accumulation at MRI, but he was clinically asymptomatic and refused liver biopsy (Table I). At the first level genetic test he was only heterozygous for the *HFE* H63D variant. Thus, after NGS the patient could be reclassified as an example of HH due to digenic inheritance, *i.e.* the presence of two or more variants in distinct genes involved in the same pathway (hepcidin sensing/production) sufficient to give rise to the clinical disease [44].

Patient #02 was also a simple H63D heterozygous at first level genetic test, which clearly did not explain a highly penetrant HH phenotype with advanced fibrosis at liver biopsy performed at the age of 39 years (Table I). NGS revealed a new stop-codon mutation in the *HFE* gene (**W163X**), whose modeling is reported in Fig. 2. According to *in silico* analysis, the mutation determines a dramatic change of protein structure, with the truncated protein being predicted to lack some amino acids critical for the surface interaction between HFE and transferrin receptor 1 (*TFR1*). It could affect also other interactions, *e.g.* BMP type I receptor ALK3). Such effects would eventually prevent the signaling that gives rise to hepcidin production. The final functional effect of this mutation can be considered similar to that of the C282Y mutation, which has been reported to prevent the correct membrane localization of HFE protein [45]. Thus, the molecular diagnosis in this patient previously classified as likely affected by "non-HFE" HH, was indeed "atypical" type 1 (or *HFE*-related) HH due to compound heterozygosity for a novel and a known mutation (W163X/H63D).

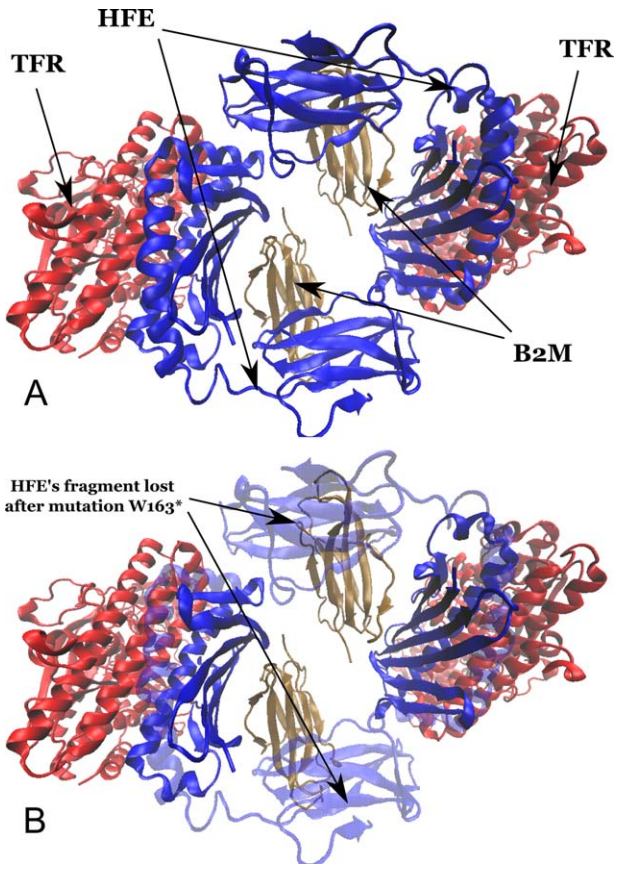


**Figure 2.** Functional consequences of the *HFE* W163X mutation. (A) Comparison between Hemochromatosis protein complex with Transferrin Receptor (TFR) and Beta-2-Microglobulin (B2M) crystal structure (PDB accession code: 1DE4) before and after the mutation *HFE* W163*. HFE (blue), TFR (red) and B2M (brownish) proteins before the mutation. (B) Putative HFE's fragment lost (light blue regions) after the mutation of TRP163 to a stop codon. These regions, when present, are actively involved in functional interactions with their molecular partners. Noteworthy, it is otherwise likely that, because of Nonsense-Mediated mRNA Decay (NMD), no HFE protein is produced when TRP163 change a stop codon. NMD is a translation-coupled mechanism that eliminates mRNAs containing premature translation-termination codons (PTC). In mammalian cells, NMD is also linked to pre-mRNA splicing, as in many instances strong mRNA reduction occurs only when the PTC is located upstream of an intron [61]. This picture is thus aimed at showing, in a structural context, the regions that are lost upon mutation.

Patient #05 was a 58-year-old male with marked hyperferritinemia and substantial liver iron deposition at MRI (Table I), which, in absence of cofactors, were not explained by the H63D homozygosity at first level genetic test. NGS revealed a new (**D555N**) *TFR2* mutation that was predicted as pathogenetic by all three algorithms (Supporting Information Table SI), and whose modeling is reported in Supporting Information Fig. S1. Briefly, the mutation is predicted to change substantially the electrostatic properties of a putative calcium binding cavity on protein crystal structure, through the substitution of a negative charge with a positive one (Supporting Information Fig. S1). This patient could be also reclassified as HH likely due to digenic HFE/TFR2 inheritance.

Overall, including the previously reported *TFR2* **N241I** mutation, the NGS-based second level genetic test allowed to find a plausible molecular diagnosis in four out of seven patients from group 1, *i.e.*, those previously classified provisionally as "non-HFE HH."

It is also noteworthy that no potentially pathogenic variant was found in patients from group 2, *i.e.*, IO patients who were *a priori* less likely to have pathogenic mutations because of the co-presence of acquired cofactors.

Finally, regarding the performance of our second level genetic test, all the mutations previously described in the "methodological" group, including *HJV* N196K [30], *TFR2* AVAQ 594-597 deletion [22], and *SLC40A1* L233P/I152F [19], were confirmed by NGS. Moreover, all the new potentially causative variants detected by NGS were confirmed by Sanger sequencing (Supporting Information Fig. S2).

## Discussion

This study, along with a very similar one in Australian patients recently published by McDonald and colleagues [40], further illustrates the usefulness of an NGS-based approach test for the molecular diagnosis in patients with a provisional diagnosis of "non-HFE" HH. Indeed, in highly selected patients attending our referral center for Iron Disorders with a unexplained HH phenotype, because of either non-diagnostic 1st level genetic test or absence of acquired risk factors for nongenetic IO, we could obtain a plausible molecular explanation in five out of eight, finding three novel variants in *HFE*, *HAMP*, and *TFR2*. The same test also allowed us to detect a rare *SCL40A1* mutation in one patient with a distinct phenotype, who has been described separately (Ferbo L, manuscript under revision). A limitation lies on the fact that NGS was exonic, so there still could be causative mutations in the analyzed genes within non-exonic sequences. Anyway, similarly to our results, McDonald et al. [40], using a different NGS platform and an enlarged panel of genes possibly involved in a broad range of iron disorders ($n = 39$), were able to identify a likely causal mutation (in *HJV*, *SCL40A1*, *HFE*, or *TFR2*, but not in *HAMP*) in five out of eight patients with "atypical IO." Of note, notwithstanding the broad panel used, even in their study some patients with a HH phenotype remained molecularly "unexplained" after extensive NGS. While, of course, no NGS method, whatever the platform used, can ensure 100% sensitivity in detecting causal mutations, it is tempting to conclude that some still unknown genes could be involved in determining HH-like clinical phenotypes in humans. These genes could be eventually detected by using other NGS-based techniques like whole genome/exome sequencing [27], however their application is presently limited not only by costs, but also by difficulties in interpretation and ethical problem in reporting incidental findings [46].

Until now, the diagnosis of atypical or "non-HFE" HH is largely a diagnosis of exclusion, and our knowledge on these conditions is mainly based on individual studied by traditional Sanger sequencing. Current recommendations suggest a stepwise approach for the molecular diagnosis of atypical systemic iron overload disorders, in which sequencing should be made initially according to clinical features (*i.e.*, prioritizing *HJV* and *HAMP* in patients with early onset IO) [5,14]. While this is reasonable, it is time consuming and does not take appropriately into account the possible occurrence of digenic inheritance [47]. This new approach of simultaneous NGS-based sequencing of the five HH genes at a reasonable cost will clearly simplify the molecular diagnosis in a near future. Moreover, it will allow to expand/refine knowledge on the clinical features of the distinct HH subtypes. For example, based on the few cases reported until now, there is some controversy regarding whether or not type 3 (*TFR2*-related) HH has an earlier (or intermediate) onset as compared to type 1 [36]. Similarly, it is unclear whether or not type 4 HH (*SLC40A1*-related or ferroportin disease) is a relatively mild form of HH with low incidence of overt cirrhosis and HCC [20]. A widespread application of NGS-based molecular diagnosis is expected to substantially increase the accuracy of HH molecular diagnosis, allowing, preferably through the creation of *ad hoc* registries, a more precise phenotypic description of HH subtypes.

Noteworthy, we did not find any relevant mutation in patients from group 2, *i.e.*, those with IO possibly out of proportion with

respect to the burden of acquired risk factors. The role of the co-presence of variants in the iron-sensing proteins (*HJV*, *HAMP*, *TFR2*) has been previously investigated in C282Y homozygotes as possible genetic cofactors able to increase clinical expression, but they have been detected only rarely in sporadic patients [30,48–50]. Accordingly, our results suggest that variants in such genes are unlikely to act as modifiers also in patients with IO due to acquired risk factors. Of course, we cannot exclude in such patients the influence of other genetic modifiers not included in our restricted diagnostic panel [51,52]. Nonetheless, this underlines the critical importance of an accurate clinical selection before ordering any genetic test, which should be done only in subjects with the highest pretest probability. In our experience, while NGS-based methods clearly represent a major technical advance, interpretation of the clinical relevance of molecular data remains a difficult task, requiring close and repeated contacts among clinicians, molecular analysts, and bioinformatic experts. Nonetheless, our study provides further evidence of the usefulness of a NGS-based approach targeting a specific gene panel for the molecular diagnosis of patients clinically classified as "atypical" or non-HFE HH after first level genetic testing.

## Acknowledgments

This work was performed (in part) in the LURM (Laboratorio Universitario Ricerca Medica) Research Center, University of Verona.

## Author Contributions

SB performed sequencing, reviewed literature/databases, and co-wrote the manuscript. DG designed the research, reviewed the study results and genotype/phenotype correlations, and co-wrote/edited the manuscript. FB, PB, RPM, PC, OO participated in clinical enrolling/work-up of HH patients. AF, LX, GDM, NC, and MD performed the molecular/biochemical/bioinformatic analyses, and edited the manuscript. SMV and AG performed *in silico* modeling of mutations.

## References


1. Pietrangelo A. Hereditary hemochromatosis: Pathogenesis, diagnosis, and treatment. Gastroenterology 2010;139:393–408. 408 e391-392.
2. Brissot P, Troadec MB, Bardou-Jacquet E, et al. Current approach to hemochromatosis. Blood Rev 2008;22:195–210.
3. Ganz T. Systemic iron homeostasis. Physiol Rev 2013;93:1721–1741.
4. Hentze MW, Muckenthaler MU, Galy B, et al. Two to tango: Regulation of Mammalian iron metabolism. Cell 2010;142:24–38.
5. Pietrangelo A. Genetics, genetic testing, and management of hemochromatosis: 15 years since hepcidin. Gastroenterology 2015;149:1240–1251. e1244.
6. Harrison-Findik DD, Klein E, Crist C, et al. Iron-mediated regulation of liver hepcidin expression in rats and mice is abolished by alcohol. Hepatology 2007;46:1979–1985.
7. Girelli D, Pasino M, Goodnough JB, et al. Reduced serum hepcidin levels in patients with chronic hepatitis C. J Hepatol 2009;51:845–852.
8. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. Lancet 2005;365:1415–1428.
9. Fargion S. Dysmetabolic iron overload syndrome. Haematologica 1999;84:97–98.
10. Riva A, Trombini P, Mariani R, et al. Revaluation of clinical and histological criteria for diagnosis of dysmetabolic iron overload syndrome. World J Gastroenterol 2008;14:4745–4752.
11. McLaren CE, Emond MJ, Subramaniam VN, et al. Exome sequencing in HFE C282Y homozygous men with extreme phenotypes identifies a GNPAT variant associated with severe iron overload. Hepatology 2015;62:429–439.
12. Bardou-Jacquet E, de Tayrac M, Mosser J, et al. GNPAT Variant associated with severe iron overload in HFE hemochromatosis. Hepatology 2015;62:1917–1918.
13. Ryan E, Russell J, Ryan JD, et al. GNPAT variant is not associated with severe iron overload in Irish C282Y homozygotes. Hepatology 2015.
14. European Association for the Study of the L. EASL clinical practice guidelines for HFE hemochromatosis. J Hepatol 2010;53:3–22.
15. Bacon BR, Adams PC, Kowdley KV, et al. Diagnosis and management of hemochromatosis: 2011 practice guideline by the American Association for the Study of liver diseases. Hepatology 2011;54:328–343.
16. 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;13:399–408.
17. Piperno A, Sampietro M, Pietrangelo A, et al. Heterogeneity of hemochromatosis in Italy. Gastroenterology 1998;114:996–1002.
18. Roetto A, Papanikolaou G, Politou M, et al. Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. Nat Genet 2003;33:21–22.
19. Girelli D, De Domenico I, Bozzini C, et al. Clinical, pathological, and molecular correlates in ferroportin disease: A study of two novel mutations. J Hepatol 2008;49:664–671.
20. Pietrangelo A. The ferroportin disease. Blood Cells Mol Dis 2004;32:131–138.
21. Roetto A, Daraio F, Alberti F, et al. Hemochromatosis due to mutations in transferrin receptor 2. Blood Cells Mol Dis 2002;29:465–470.
22. 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;122:1295–1302.
23. Papanikolaou G, Samuels ME, Ludwig EH, et al. Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nat Genet 2004;36:77–82.
24. Lanzara C, Roetto A, Daraio F, et al. Spectrum of hemojuvelin gene mutations in 1q-linked juvenile hemochromatosis. Blood 2004;103:4317–4321.
25. Camaschella C. Iron and hepcidin: A story of recycling and balance. Hematol Am Soc Hematol Educ Prog 2013;2013:1–8.
26. Pietrangelo A. Non-HFE hemochromatosis. Semin Liver Dis 2005;25:450–460.
27. Katsanis SH, Katsanis N. Molecular genetic testing and the future of clinical genomics. Nat Rev Genet 2013;14:415–426.
28. Sankaran VG, Gallagher PG. Applications of high-throughput DNA sequencing to benign hematology. Blood 2013;122:3575–3582.
29. Lee H, Deignan JL, Dorrani N, et al. Clinical exome sequencing for genetic identification of rare Mendelian disorders. JAMA 2014;312:1880–1887.
30. Biasiotto G, Roetto A, Daraio F, et al. Identification of new mutations of hepcidin and hemojuvelin in patients with HFE C282Y allele. Blood Cells Mol Dis 2004;33:338–343.
31. Altmuller J, Budde BS, Nurnberg P. Enrichment of target sequences for next-generation sequencing applications in research and diagnostics. Biol Chem 2014;395:231–237.
32. Montioli R, Dindo M, Giorgetti A, et al. A comprehensive picture of the mutations associated with aromatic amino acid decarboxylase deficiency: From molecular mechanisms to therapy implications. Hum Mol Genet 2014;23:5429–5440.
33. Matthijs G, Souche E, Alders M, et al. Guidelines for diagnostic next-generation sequencing. Eur J Hum Genet 2016;24:2–5.
34. 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 2015.
35. Wallace DF, Subramaniam VN. The global prevalence of HFE and non-HFE hemochromatosis estimated from analysis of next-generation sequencing data. Genet Med 2015.
36. Bardou-Jacquet E, Cunat S, Beaumont-Epinette MP, et al. Variable age of onset and clinical severity in transferrin receptor 2 related haemochromatosis: Novel observations. Br J Haematol 2013;162:278–281.
37. 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;46:302–307.
38. Biasiotto G, Belloli S, Ruggeri G, et al. Identification of new mutations of the HFE, hepcidin, and transferrin receptor 2 genes by denaturing HPLC analysis of individuals with biochemical indications of iron overload. Clin Chem 2003;49:1981–1988.
39. An P, Wu Q, Wang H, et al. TMPRSS6, but not TF, TFR2 or BMP2 variants are associated with increased risk of iron-deficiency anemia. Hum Mol Genet 2012;21:2124–2131.
40. McDonald CJ, Ostini L, Wallace DF, et al. Next-generation sequencing: Application of a novel platform to analyze atypical iron disorders. J Hepatol 2015;63:1288–1293.
41. Radio FC, Majore S, Aurizi C, et al. Hereditary hemochromatosis type 1 phenotype modifiers in Italian patients. The controversial role of variants in HAMP, BMP2, FTL and SLC40A1 genes. Blood Cells Mol Dis 2015;55:71–75.
42. Lee PL, Halloran C, West C, et al. Mutation analysis of the transferrin receptor-2 gene in patients with iron overload. Blood Cells Mol Dis 2001;27:285–289.
43. Del-Castillo-Rueda A, Moreno-Carralero MI, Cuadrado-Grande N, et al. Mutations in the HFE, TFR2, and SLC40A1 genes in patients with hemochromatosis. Gene 2012;508:15–20.
44. Pietrangelo A. Hereditary hemochromatosis—A new look at an old disease. N Engl J Med 2004;350:2383–2397.
45. Waheed A, Parkkila S, Zhou XY, et al. Hereditary hemochromatosis: Effects of C282Y and H63D mutations on association with beta2-microglobulin, intracellular processing, and cell surface expression of the HFE protein in COS-7 cells. Proc Natl Acad Sci USA 1997;94:12384–12389.
46. Hehir-Kwa JY, Claustres M, Hastings RJ, et al. Towards a European consensus for reporting incidental findings during clinical NGS testing. Eur J Hum Genet 2015;23:1601–1606.
47. Pietrangelo A, Caleffi A, Henrion J, et al. Juvenile hemochromatosis associated with pathogenic mutations of adult hemochromatosis genes. Gastroenterology 2005;128:470–479.
48. Jacolot S, Le Gac G, Scotet V, et al. HAMP as a modifier gene that increases the phenotypic expression of the HFE pC282Y homozygous genotype. Blood 2004;103:2835–2840.

49. Le Gac G, Scotet V, Ka C, et al. The recently identified type 2A juvenile haemochromatosis gene (HJV), a second candidate modifier of the C282Y homozygous phenotype. Hum Mol Genet 2004;13:1913–1918.
50. Milet J, Dehais V, Bourgain C, et al. Common variants in the BMP2, BMP4, and HJV genes of the hepcidin regulation pathway modulate HFE hemochromatosis penetrance. Am J Hum Genet 2007;81:799–807.
51. Benyamin B, Esko T, Ried JS, et al. Novel loci affecting iron homeostasis and their effects in individuals at risk for hemochromatosis. Nat Commun 2014;5:4926.
52. de Tayrac M, Roth MP, Jouanolle AM, et al. Genome-wide association study identifies TF as a significant modifier gene of iron metabolism in HFE hemochromatosis. J Hepatol 2015;62: 664–672.
53. Humphrey W, Dalke A, Schulten K. VMD: Visual molecular dynamics. J Mol Graph 1996;14: 27–38.
54. Finn RD, Bateman A, Clements J, et al. Pfam: The protein families database. Nucleic Acids Res 2014;42:D222–D230.
55. Murzin AG, Brenner SE, Hubbard T, et al. SCOP: A structural classification of proteins database for the investigation of sequences and structures. J Mol Biol 1995;247:536–540.
56. Bernstein FC, Koetzle TF, Williams GJ, et al. The Protein Data Bank. A computer-based archival file for macromolecular structures. Eur J Biochem 1977;80:319–324.
57. Ashkenazy H, Erez E, Martz E, et al. ConSurf 2010: Calculating evolutionary conservation in sequence and structure of proteins and nucleic acids. Nucleic Acids Res 2010;38:W529–W533.
58. Arnold K, Bordoli L, Kopp J, et al. The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. Bioinformatics 2006;22:195–201.
59. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera—A visualization system for exploratory research and analysis. J Comput Chem 2004;25:1605–1612.
60. Baker NA, Sept D, Joseph S, et al. Electrostatics of nanosystems: Application to microtubules and the ribosome. Proc Natl Acad Sci USA 2001;98:10037–10041.
61. Brogna S, Wen J. Nonsense-mediated mRNA decay (NMD) mechanisms. Nat Struct Mol Biol 2009;16:107–113.
62. Biasini M, Bienert S, Waterhouse A, et al. SWISS-MODEL: Modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res 2014;42:W252–W258.