# Disordini del ferro.
# Nuovi approcci diagnostici

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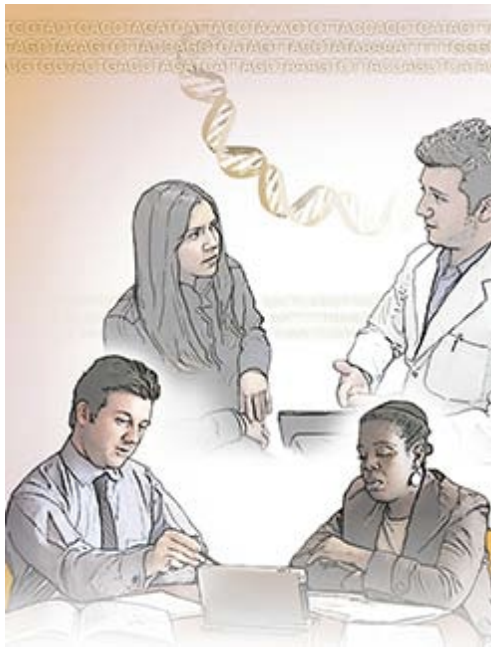
# 20 anni “d’oro” per il ferro…

**ASH 50th anniversary review**

Forging a field: the golden age of iron biology

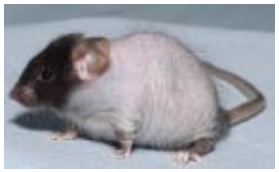
Andrews NC, Blood 2008



**impatto nella pratica clinica?**

**HFE discovery (1996)**

**Hepcidin discovery (2000-2001)**



**TMPRSS6/IRIDA discovery (2008-200)**

**ERFE discovery (2014)**

# Sommario

**1) Approccio diagnostico alla CARENZA marziale.**

***Il dosaggio dell'epcidina (possibile ruolo in casi selezionati).***

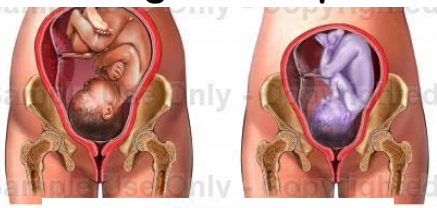
**2) Approccio diagnostico al SOVRACCARICO marziale (primitivo).**

***Ruolo della RM e dei test genetici di II livello basati sul Next Generation Sequencing (NGS).***

# Absolute iron deficiency

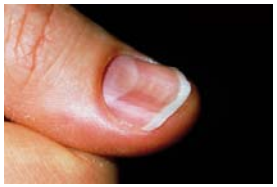
| | Normal | Negative iron balance | Iron-deficient erythropoiesis | Iron-deficiency anemia |
|---|---|---|---|---|
| Iron stores / Erythron iron | | | | |
| Marrow iron stores | 1-3+ | 0-1+ | 0 | 0 |
| Serum ferritin (µg/L) | 50-200 | <20 | <15 | <15 |

growth retardation
neurocognitive impairment



hair loss

restless leg syndrome



koilonychia

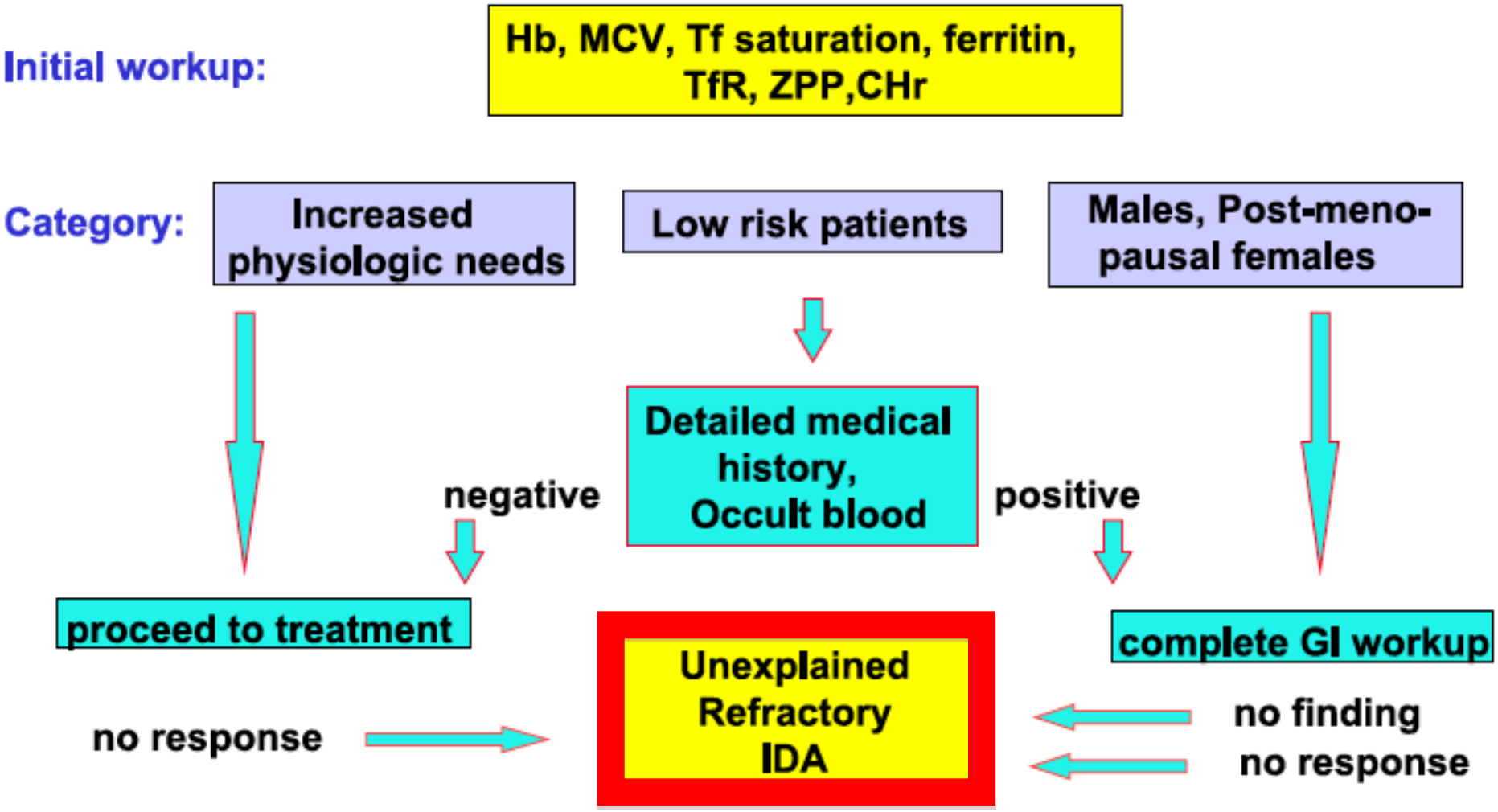
**Diagnosi facile**
**No "falsi positivi" per ferritina ↓↓**
**(differenza con iperferritinemia!)**

**Ricercare la causa**

"neuroasthenia"

irritability

# UNEXPLAINED/REFRACTORY iron deficiency anemia (IDA)




Hershko & Camaschella, Blood 2014

# IRON REFRACTORY IRON DEFICIENCY ANEMIA (IRIDA)

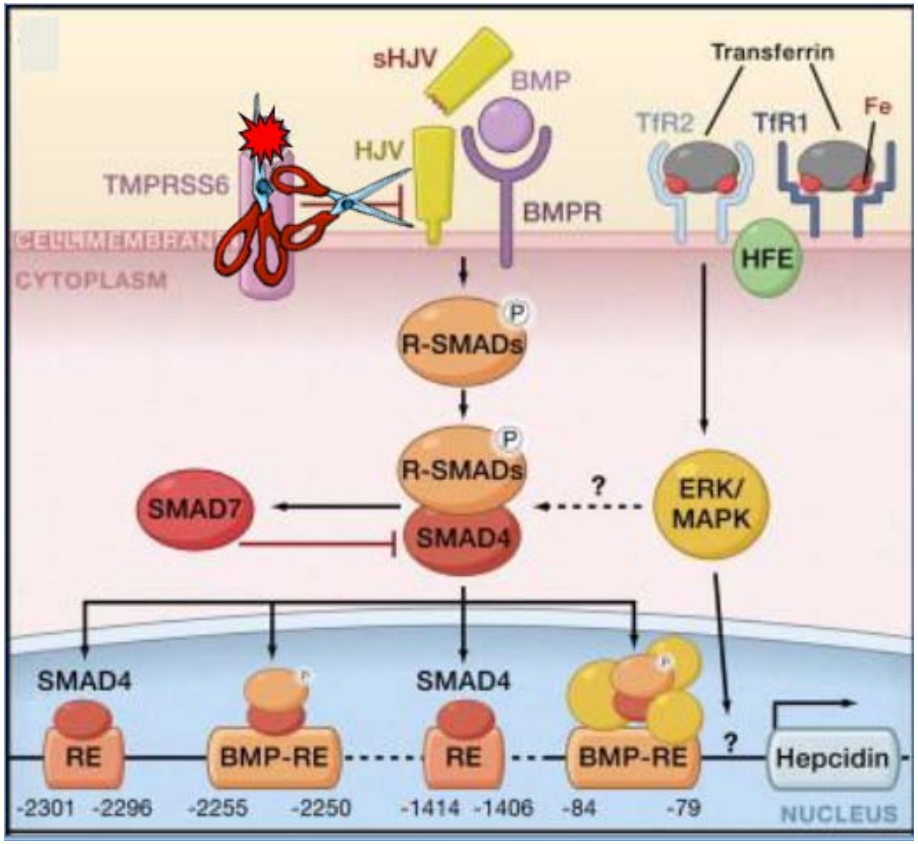

**Table 4. Proposed diagnostic workup for unexplained or refractory IDA**

| | *H pylori* | Autoimmune gastritis | Celiac disease | IRIDA |
|---|---|---|---|---|
| Screening | *H pylori* IgG antibodies or fecal antigen | Serum gastrin anti-parietal Abs anti-intrinsic factor Abs | TTG IgA Abs | Suggestive history and clinical assessment |
| Advanced | Urease breath test gastroscopy and biopsies (optional) | Gastroscopy and biopsies (recommended) | Duodenal biopsy, HLA screening for DQ2 or DQ8 genotypes | Sequencing of the TMPRSS6 gene |
| Response to specific treatment | *H pylori* eradication | NA | Gluten-free diet | NA |

NA, not applicable.

Hershko & Camaschella, Blood 2014

# Hepcidin regulation by TMPRSS-6 and the IRIDA model (↑ hepcidin → malabsorption)



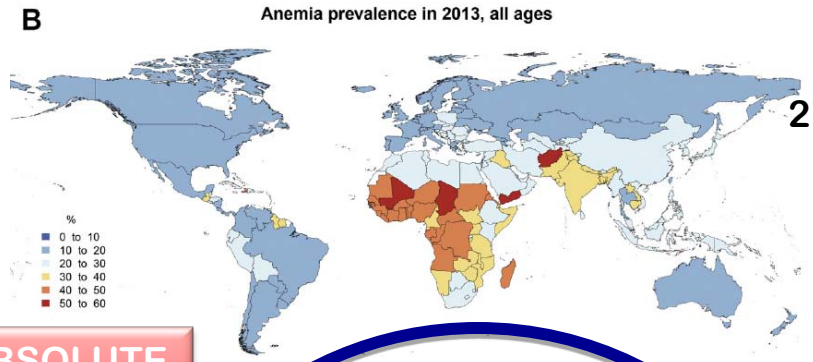

Courtesy of Clara Camaschella

TMPRSS6 mutations

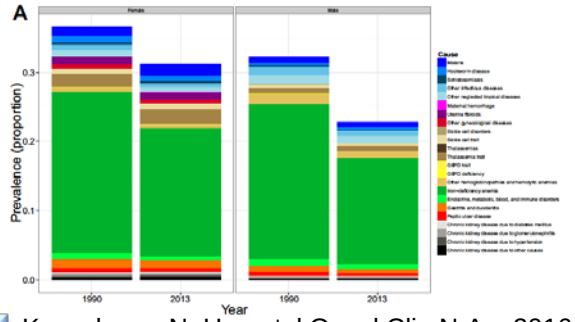
**Iron Refractory Iron Deficiency Anemia (IRIDA)**

- Post-natal microcytic hypochromic anemia with low TS%
- Refractoriness to oral iron
- Slow response to i.v. iron
- **Sometimes diagnosed in adulthood**
- Normal/high hepcidin levels (diagnosis)

# Absolute and "Functional" ID can coexist



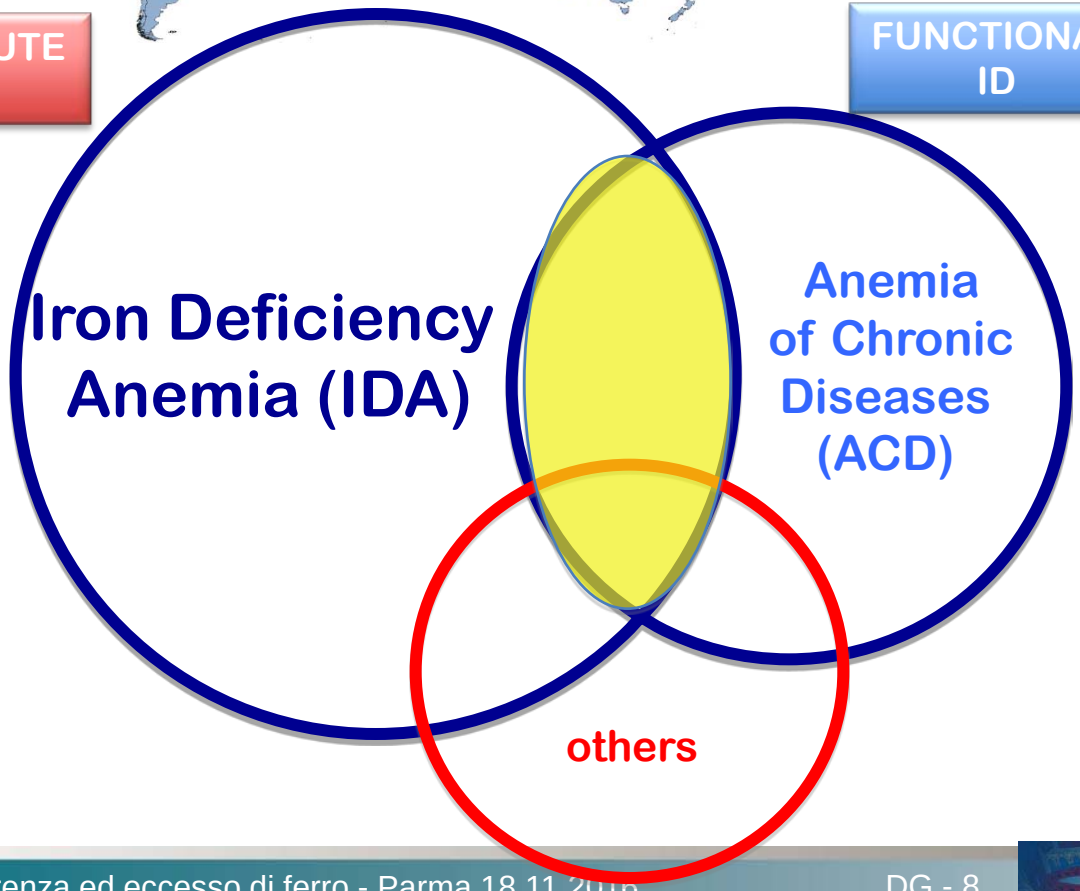

**ANEMIA:**
**27% of world population**
**≥60% by ID**




Kassebaum N, Hematol Oncol Clin N Am 2016

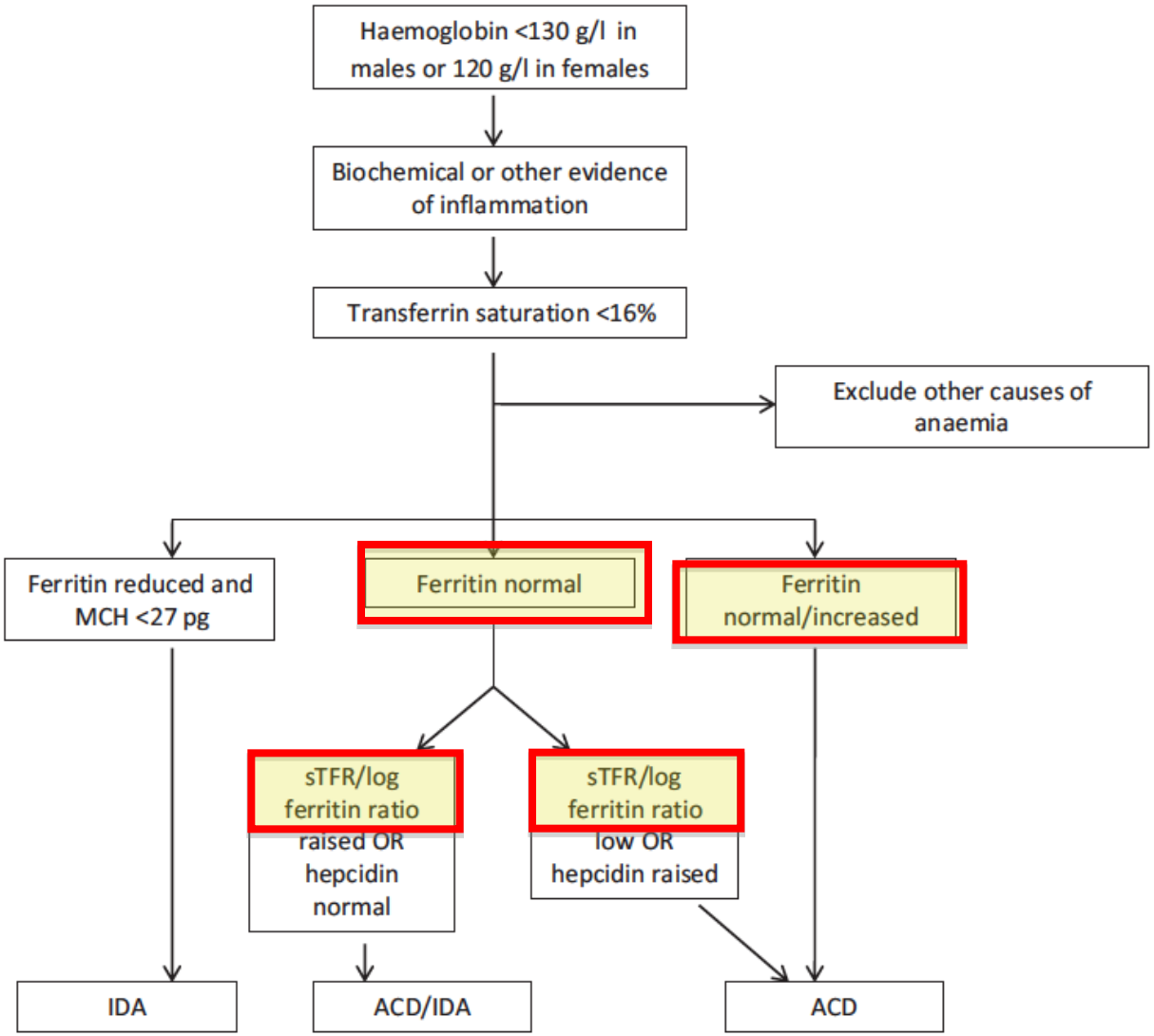
**ABSOLUTE ID**

**FUNCTIONAL ID**




- ✓ **IDA = most frequent cause of anemia worldwide**
- ✓ **ACD = 2° cause**
- ✓ **Mixed IDA/ACD not uncommon (i.e. RA or IBD): difficult to diagnose and manage**

# (Complex) algorithm for distinguishing ACD/IDA from "pure" ACD




Cullis JO, Brit J Haematol 2011

Ferritin, classical IDA biomarker = (acute phase reactant)

Controversy on the **cut-off** value defining **"increased" ferritin** (i.e. iron not beneficial)

- **100 μg/L** ? (Weiss G, NEJM 2005)
- **Pts. with HF respond to i.v. iron also with ferritin up to 300 μg/L** (Anker SD, NEJM 2009)

Algorithm requires serum Transferrin receptor (sTFR, not universally available, **lack of standardization**)

# Hepcidin assays: ready for the clinic?

***Blood* Spotlight**

## Hepcidin in the diagnosis of iron disorders

Domenico Girelli,[1] Elizabeta Nemeth,[2] and Dorine W. Swinkels[3]

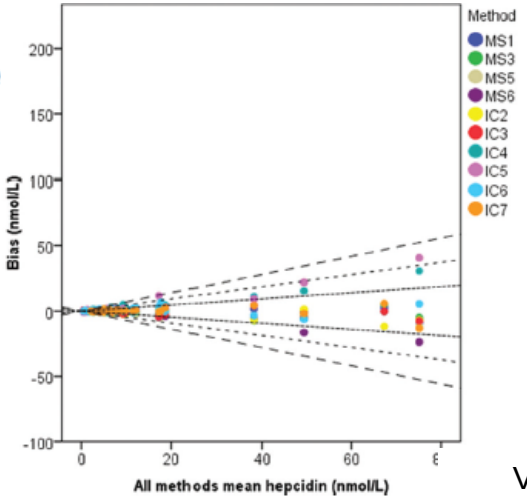
**Table S1. List of 10 analytically well performing* hepcidin assays available for clinical use.**

| | Method | Institution / Company | Ref. | Contact/Info | Clinical credentials |
|---|---|---|---|---|---|
| **A) Mass Spectrometry (MS)-based assays** | | | | | |
| 1. | WCX-MALDI-TOF | Radboudumc, www.hepcidinanalysis.com, Nijmegen (Netherlands) | 1, 2 | hepcidinanalysis@radboudumc.nl<br>www.hepcidinanalysis.com | Patient care: included in scope of accreditation of clinical laboratory (Dutch CCKL/RvA Code of Practice, in transation to EN ISO 15189)[6].<br>Clinical trials: Hepcidinanalysis.com is CRO and has validated test at GC(L)P like manner. |
| 2. | LC MS/MS with reversed phase extraction[1] | University Hospital of Verona (Italy) | 3 | malattie.ferro@ospedaleuniverona.it<br>www.gimferverona.org | Embedded in laboratory accredited by Regional Health authority (Veneto Region law no. 838, April 8, 2008). |
| 3. | LC MS/MS with hydrophilic-lipophilic-balanced (HLB) extraction | HUSLAB at Helsinki University Central Hospital (Finland) | 4 | huslab@hus.fi | Embedded in accredited clinical laboratory (Finnish Accreditation Service, T055, EN ISO/IEC 17025, EN ISO 15189)[6] |
| 4. | LC MS/MS with reversed phase extraction | Kanazawa Medical University / Medical Care Proteomics Biotechnology Co., Ltd. (Japan) | 5 | tomosugi@kanazawa-med.ac.jp / proteome@mcprot.co.jp | Research Use Only |
| **B) Immunochemical assays (IA)** | | | | | |
| 5. | In house developed c-ELISA | Radboudumc, www.hepcidinanalysis.com, Nijmegen (Netherlands) | 6 | hepcidinanalysis@radboudumc.nl<br>www.hepcidinanalysis.com | Research Use Only; conducted in compliance with Dutch CCKL/RvA Code of Practice, additional for research. |

Cont'd

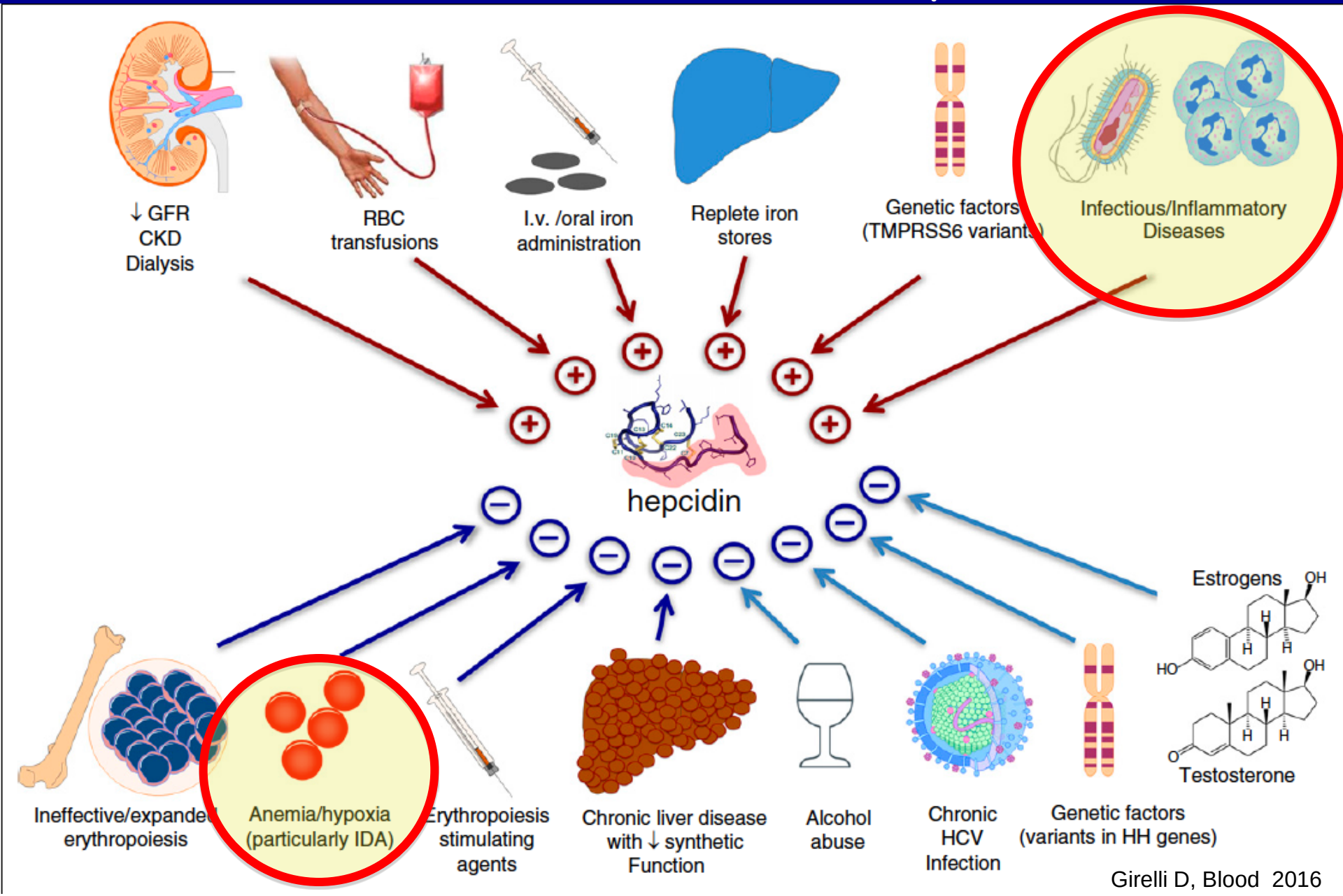
Girelli D, Blood 2016

## Toward Worldwide Hepcidin Assay Harmonization: Identification of a Commutable Secondary Reference Material



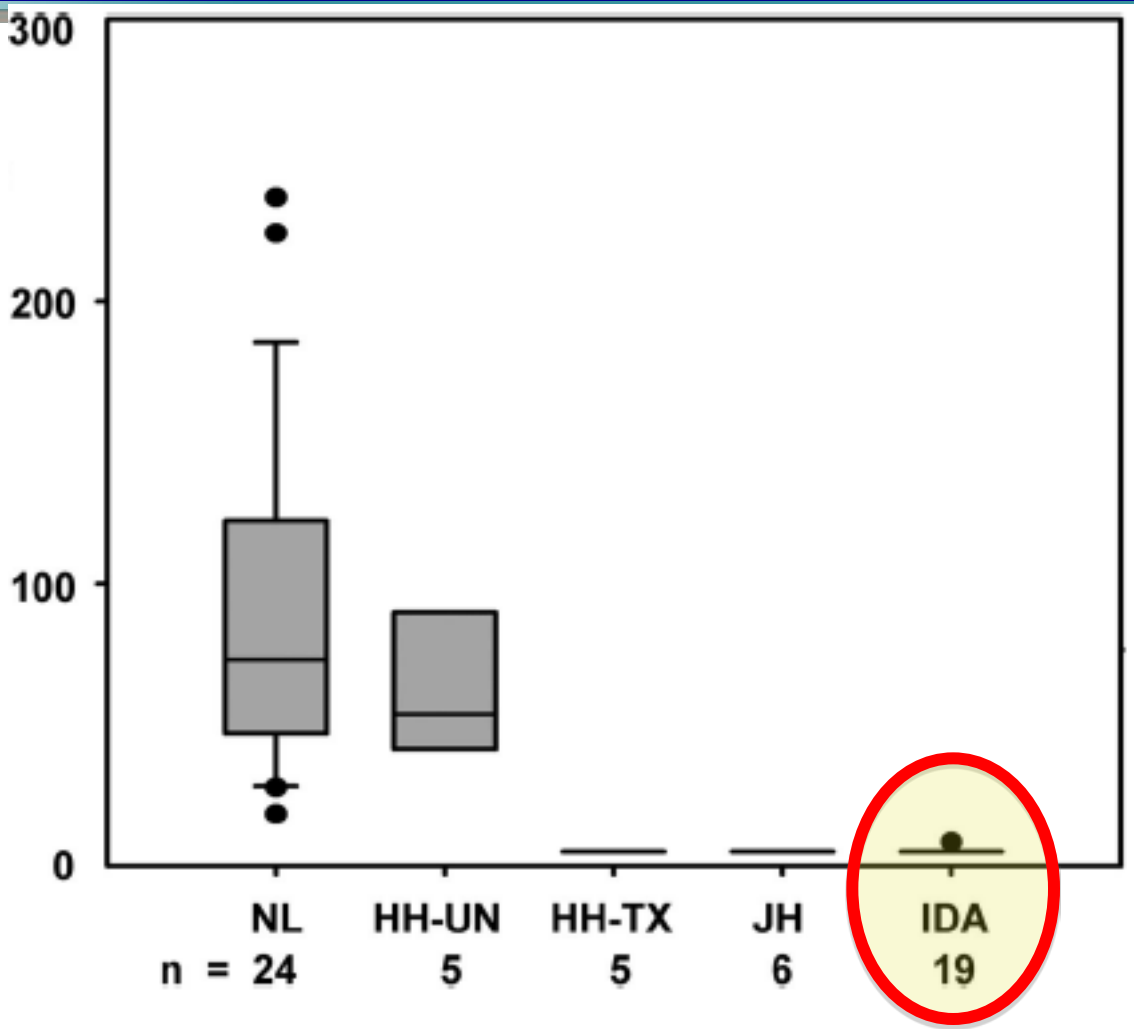

Van der Vorm L, Clin Chem 2016

# Clinical conditions known to influence hepcidin levels




Girelli D, Blood 2016

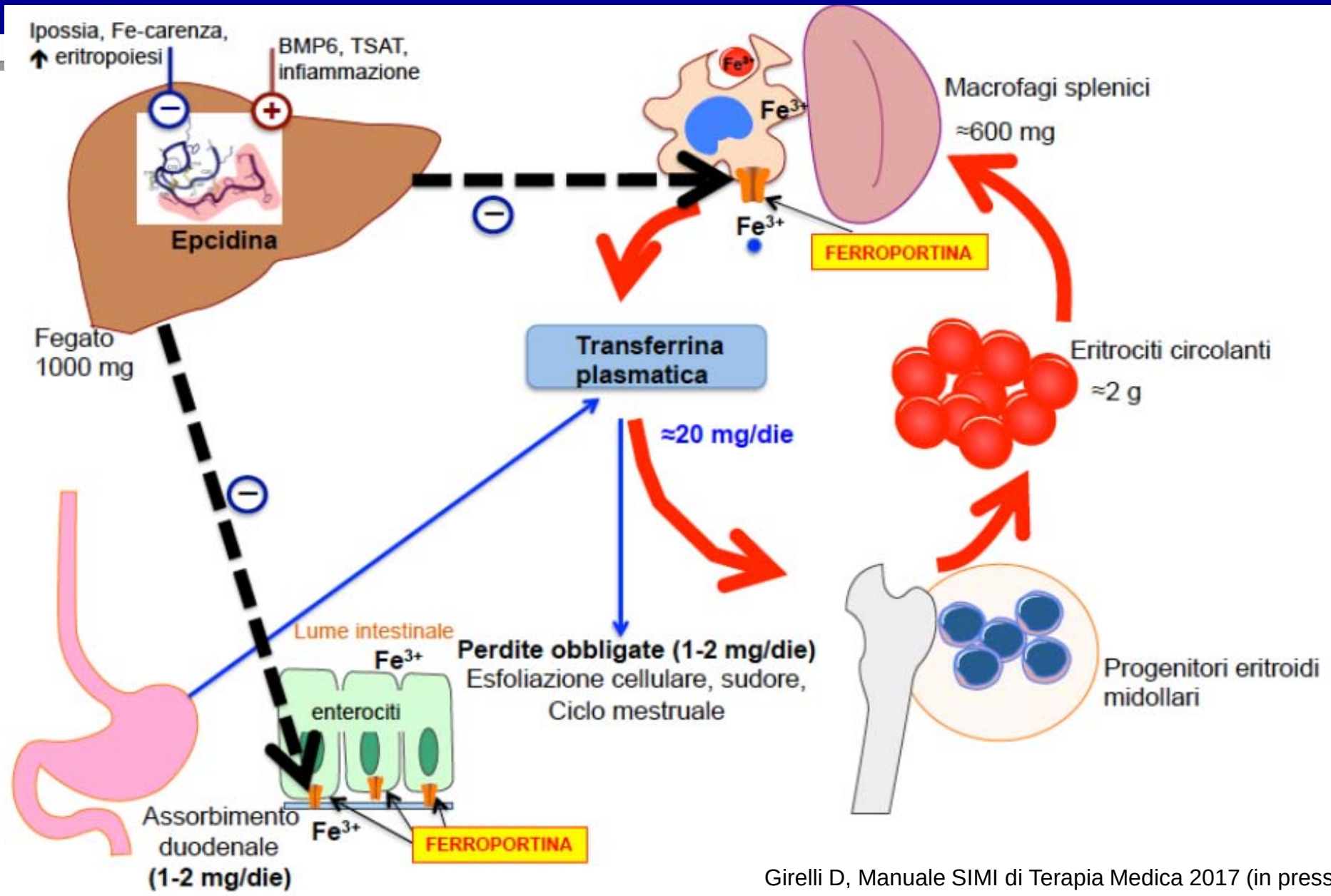
# Serum Hepcidin levels are suppressed in IDA




**Less influenced by concurrent inflammation as compared to ferritin (suppression tends to prevail)**

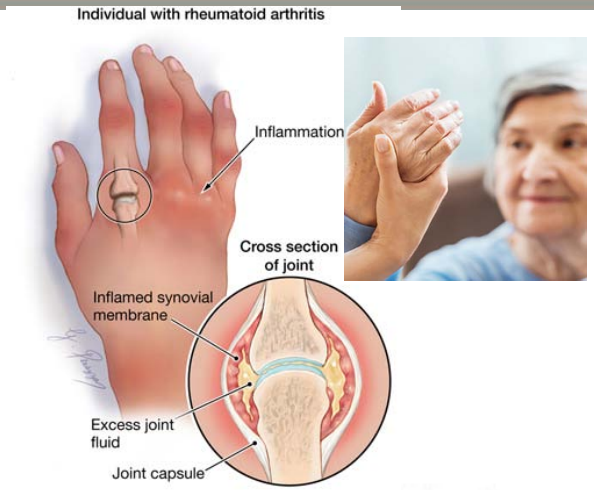
Ganz T, Blood 2008

# Metabolismo del ferro



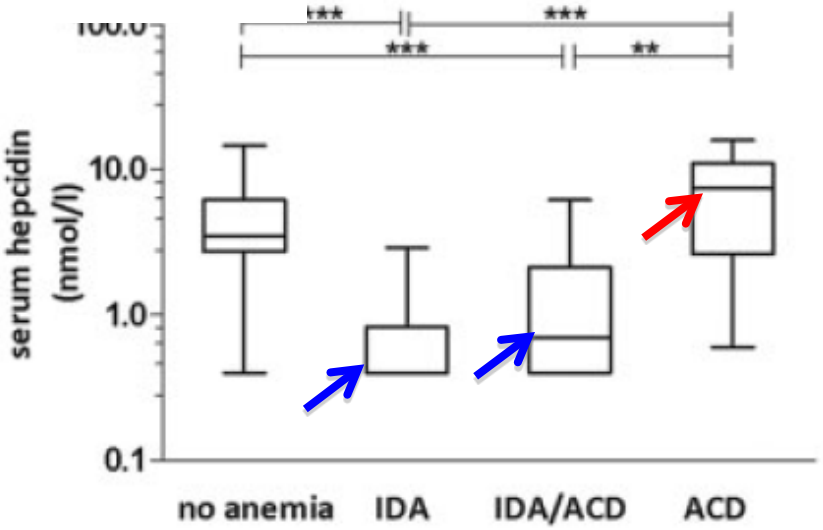

Girelli D, Manuale SIMI di Terapia Medica 2017 (in press)

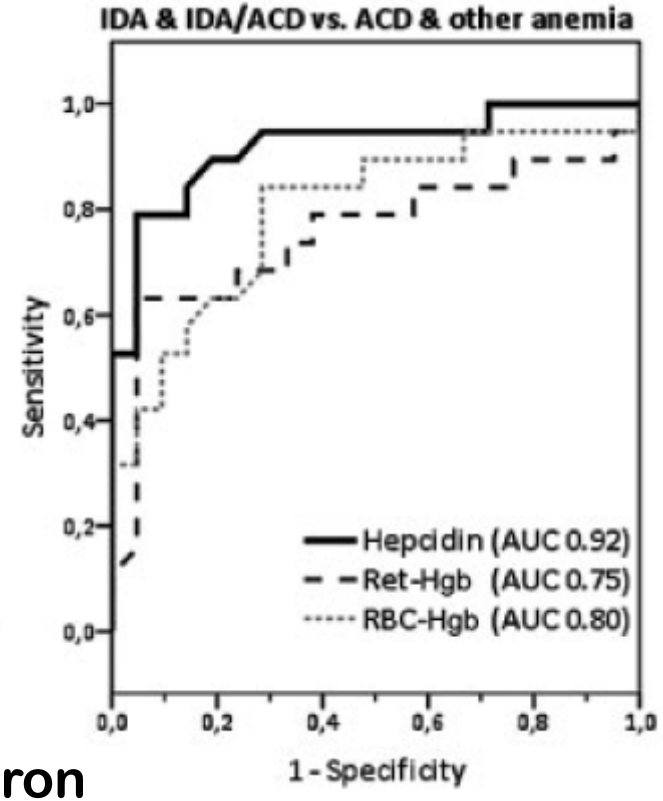
# Low hepcidin levels allow detection of concurrent iron deficiency in anemic patients with Rheumatoid Arthritis






**F, 67 years, fatigue, other Tx (PPI, DOA for concurrent AF), vegetarian, Hb 9.3 g/dl, MCV 79 fl, PCR 30 mg/l, ferritin 187 μg/l, TSAT 12%...**




**pts. with ↓hepcidin may benefit from iron**

van Santen S, Arthritis & Rheumatism 2011

# Low hepcidin levels allow detection of concurrent iron deficiency in anemic patients with IBD

## Serum Hepcidin in Inflammatory Bowel Diseases: Biological and Clinical Significance

*Gaetano Bergamaschi, MD,* Antonio Di Sabatino, MD,* Riccardo Albertini, MD,† Filippo Costanzo, MD,* Marco Guerci, MD,* Michela Masotti, MD,* Alessandra Pasini, PhD,* Alessandro Massari, MD,* Natascia Campostrini, MD,‡ Michela Corbella, MD,‡ Domenico Girelli, MD,‡ and Gino Roberto Corazza, MD**

**TABLE 2.** Serum Hepcidin-25 Concentrations in Different Groups of IBD Patients and Controls

| Study Population (N) | Serum Hepcidin, nM | *P* |
|---|---|---|
| CD (22) | 2.43 (1.38–4.29) | NS |
| UC (32) | 1.33 (0.85–2.06) | — |
| IBD, quiescent disease (28) | 1.58 (1.05–2.40) | NS |
| IBD, active disease (26) | 2.35 (1.28–4.29) | — |
| Nonanemic IBD (28) | 1.70 (1.10–2.63) | NS |
| IBD with anemia (26) | 1.86 (1.05–3.31) | 0.006[a] |
| IDA (9) | 0.21 (0.19–0.23) | |
| IDA + AI (4) | 1.06 (0.01–4.61) | |
| AI (7) | 7.61 (2.15–23.94) | |

Bergamaschi G, Inflamm Bowel Dis 2013

# Hepcidin in the diagnosis of iron disorders

**Table 1. Hepcidin measurement in clinical practice: a decalogue for the hematologist**

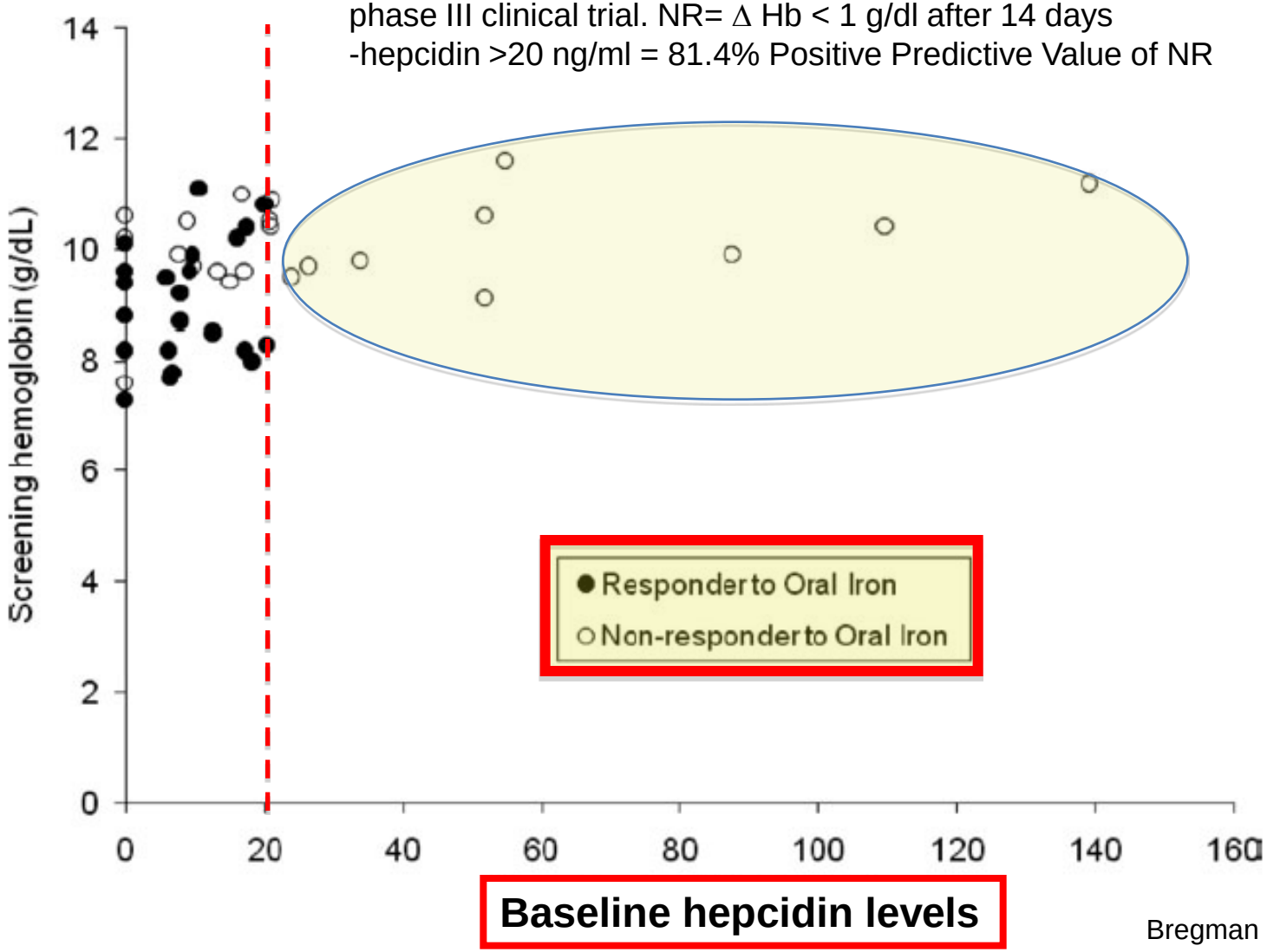
| | Comments | Reference |
|---|---|---|
| **Checklist before ordering the assay** | | |
| 1. Ensure local availability of a validated assay | See text and supplemental Table 1 | 19 |
| 2. Ensure control of preanalytical conditions (including diurnal rhythm) | See text | 8, 25, 26 |
| 3. Refer to age- and sex-specific ranges | Significant differences between males and females, particularly during fertile period | 22, 23 |
| 4. Interpret hepcidin value into a minimum laboratory context (CBC, ferritin, transferrin saturation, CRP, serum creatinine, and liver function tests) | See Figure 1 | — |
| 5. Be aware of any potential confounders/comorbidities in the individual patient | See Figure 1 | — |
| **Most promising applications** | | |
| 6. Evaluation of suspected IRIDA | Virtually diagnostic in an appropriate clinical context | 54, 55 |
| 7. Evaluation of IO disorders | For example, ferroportin disease due to hepcidin resistant mutations (see text) | 41, 42, 49, 51, 56, 57 |
| 8. Diagnosis of concomitant ID in patients with ACD | Promising reports in rheumatoid arthritis and inflammatory bowel disease patients, and in African children | 32, 58-60 |
| 9. Guide for iron therapy | For example, selection of patients for direct IV supplementation; oral administration in children from developing countries with high prevalence of infectious diseases (see text) | 6, 32, 58, 61-63 |
| 10. Monitoring of treatments targeting the hepcidin/ferroportin axis | To be confirmed by further studies | 64 |

ACD, anemia of chronic disease; CBC, complete blood count; CRP, C-reactive protein; ID, iron deficiency; IO, iron overload; IRIDA, iron-refractory iron deficiency anemia.

Girelli D, Blood 2016

# Hepcidin predicts nonresponsiveness to oral iron in IDA

phase III clinical trial. NR= Δ Hb < 1 g/dl after 14 days
-hepcidin >20 ng/ml = 81.4% Positive Predictive Value of NR




Bregman DB, Am J Hematol 2013

# Oral iron supplements increase hepcidin and decrease iron absorption from daily or twice-daily doses in iron-depleted young women

**Key Points**

- Iron supplements at doses of 60 mg Fe as $FeSO_4$ or higher increase hepcidin for up to 24 hours and are associated with lower iron absorption on the following day.

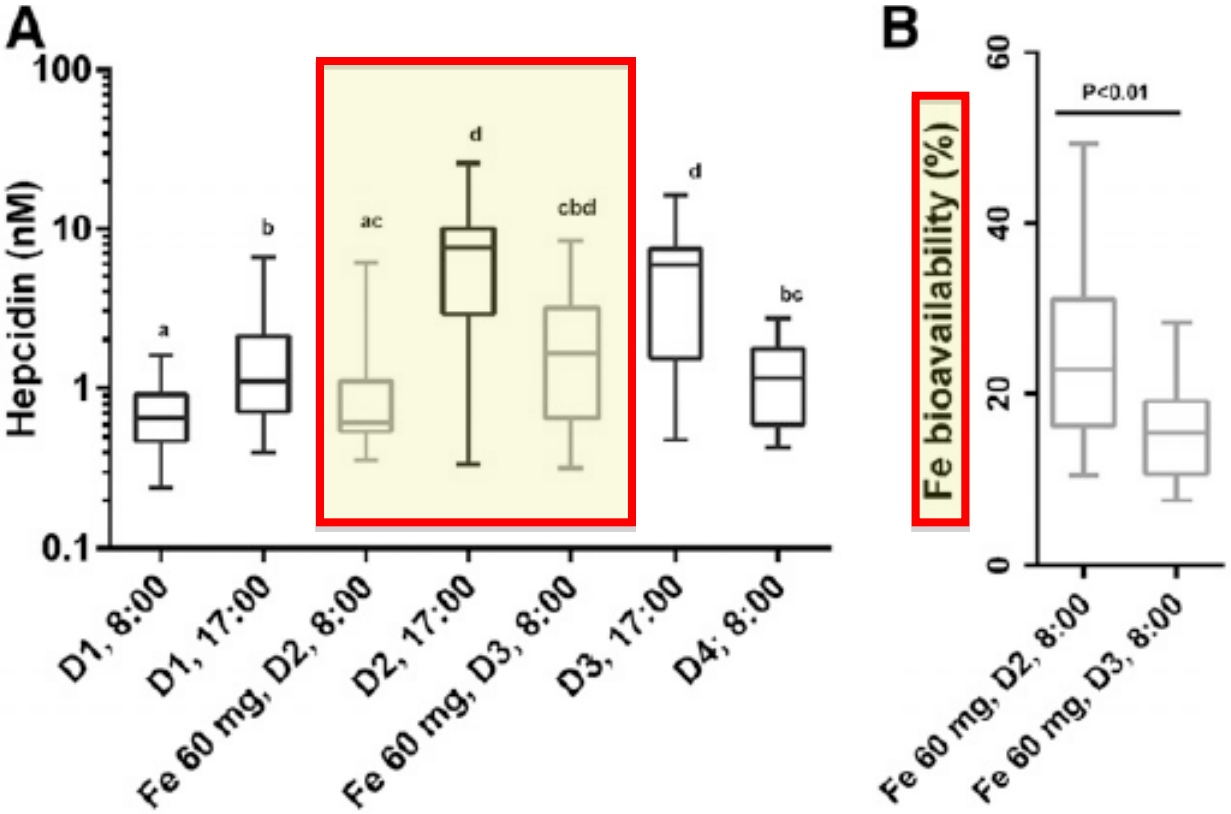
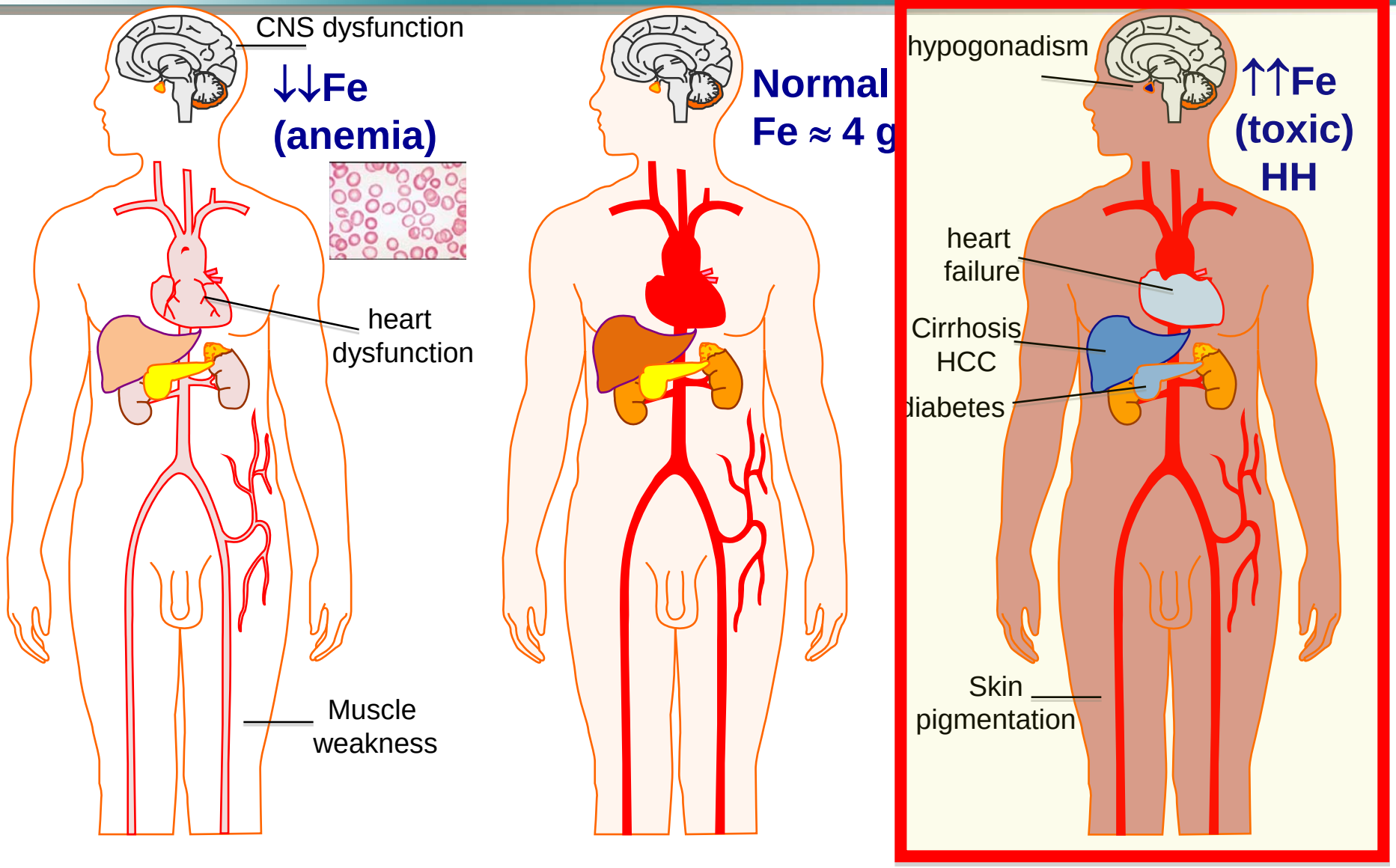



**Figure 3. A supplemental iron dose of 60 mg Fe results in an increase in hepcidin after 24 hours and in a decreased iron absorption from the consecutive dose**

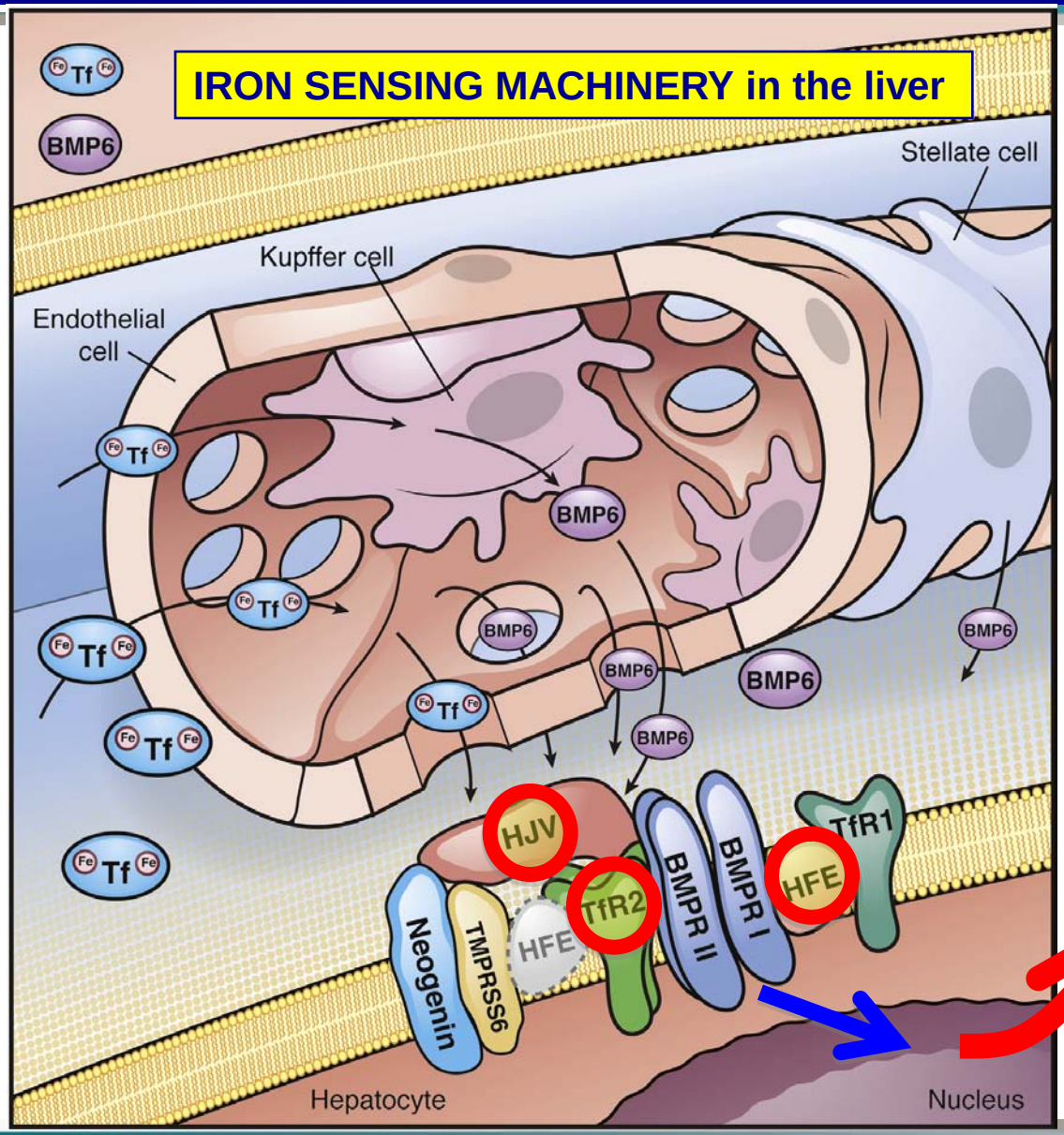
blood

Moretti D, Blood 2015

# Iron Deficiency vs Iron Overload



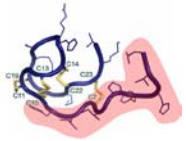
# Mechanisms of hepcidin deficiency (or resistance)




Mutations in any of the genes encoding proteins involved in iron sensing (***HFE***, ***TFR2***, ***HJV***)…

…but also in the genes encoding for hepcidin (***HAMP***) or its receptor ferroportin (***GoF*** mut. on ***SLC40A1**** → hepcidin-**resistance**).

**hepcidin transcription**



Pietrangelo A, Gastroenterology 2015

# HH: a genetically heterogeneous disorder

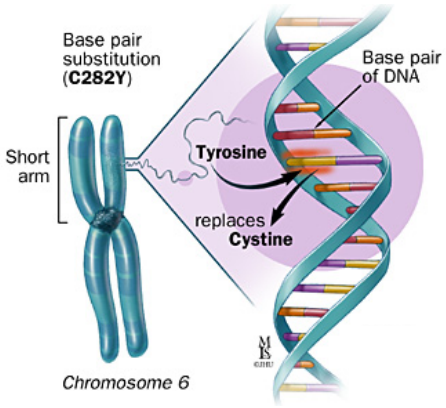
**Table 1. Heritable Forms of Systemic Iron Overload According to the Pathophysiological Defect.***

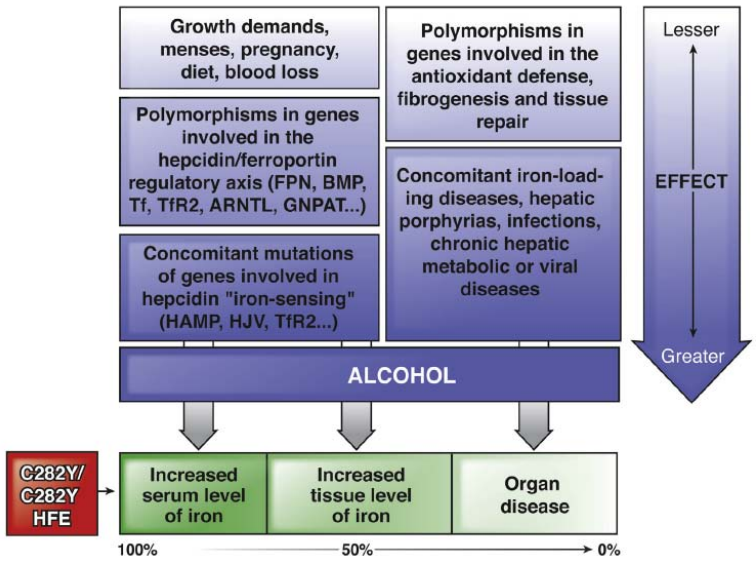
| Disorder | Gene and Inheritance | Age at Presentation | Neurologic Symptoms | Anemia | Transferrin Saturation |
|---|---|---|---|---|---|
| **Impaired hepcidin–ferroportin axis** | | | | | |
| HH type I | *HFE*, AR | Adult | No | No | High |
| HH type IIA | *HFE2*, AR | Child to young adult | No | No | High |
| HH type IIB | *HAMP*, AR | Child to young adult | No | No | High |
| HH type III | *TFR2*, AR | Young adult | No | No | High |
| HH type IVA (atypical HH) | *FP* (LOF), AD | Adult | No | Variable | Low initially |
| HH type IVB | *FP* (GOF), AD | Adult | No | No | High |
| **Impaired iron transport** | | | | | |
| Inadequate release to erythron: aceruloplasminemia | *CP*, AR | Adult | Yes | Yes | Low |
| Inadequate uptake by erythron | | | | | |
| DMT1 mutations | *DMT1*, AR | Child | No | Yes | High |
| Hypotransferrinemia | *TF*, AR | Variable | No | Yes | High |
| **Ineffective erythropoiesis** | | | | | |
| Thalassemia | *Globin*, AR | Child | No | Yes | High |
| Congenital sideroblastic anemia | *ALAS2*, XL; *SLC25A38*, AR; *GLRX5*, AR; *ABCB7*, XL | Variable | *ALAS2* and *SLC25A38*: no; *GLRX5* and *ABCB7*: yes | Yes | High |
| Congenital dyserythropoietic anemia | | | | | |
| Type I | *DAN1*, AR | Child | No | Yes | High |
| Type II | *SEC23B*, AR | Child | No | Yes | High |
| Type III | Unknown, AD | Child | No | Yes | High |

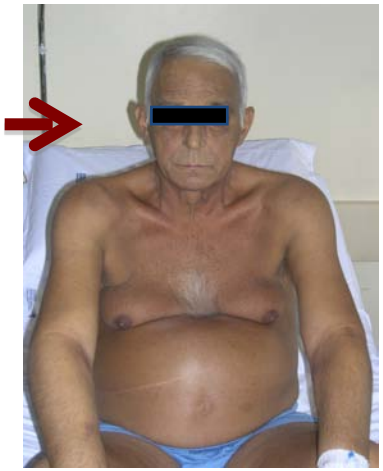
Fleming RE, N Engl J Med 2012

# Type 1 (*HFE*-related, "classic") HH

The commonest genetic disorder in European populations (carriers ≈ 1:200)



Recessive T
> Males
IV-V decades

**↓↓ clinical penetrance influenced by environmental/genetic (largely unknown) factors**

Pietrangelo A, Gastroenterology 2015

Simple molecular diagnosis through a widely available 1st level genetic test:
**C282Y homozygotes** or **C282Y/H63D compound heterozygotes (?)**

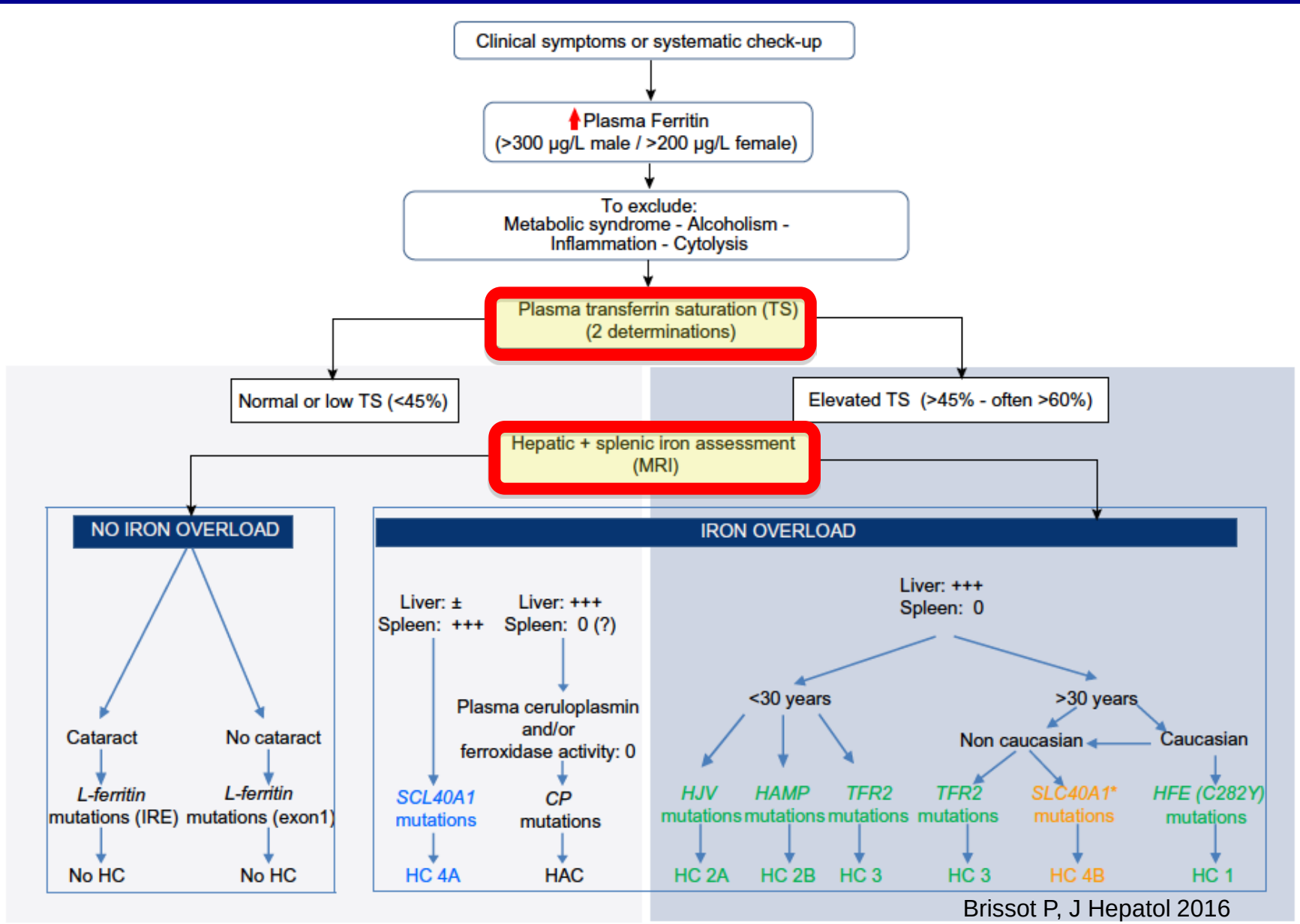
# "Non-*HFE* HH": common features

*Diagnosis of exclusion in pts. with a consistent IO phenotype and negative 1st level genetic test*

- ✓ Far more rare than type 1 (*HFE*-related) HH
- ✓ Worldwide distribution (not restricted to Northern EU descent)
- ✓ Mostly private mutations in at least 4 other genes (*HJV*, *HAMP*, *TFR2*, *SLC40A1**) → difficult molecular diagnosis

*Gain of Function

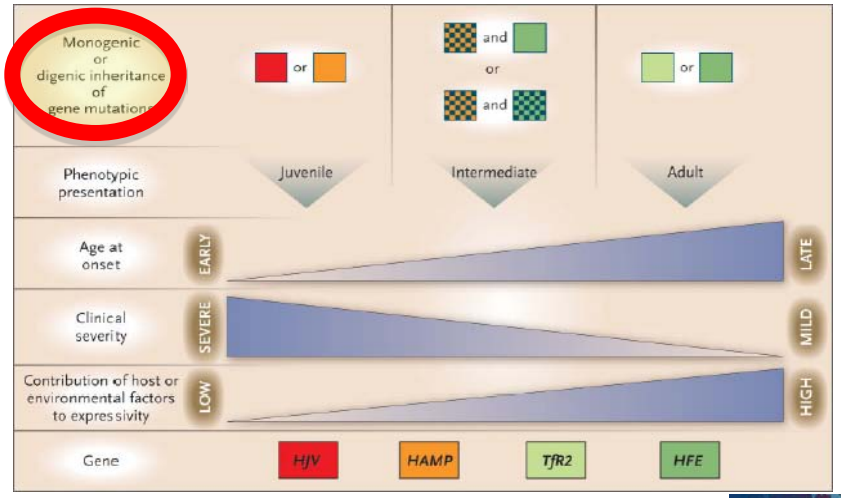
# Diagnostic approach to hyperferritinemia




Brissot P, J Hepatol 2016

# Molecular diagnosis of "non-*HFE*" IO disorders: classical approach

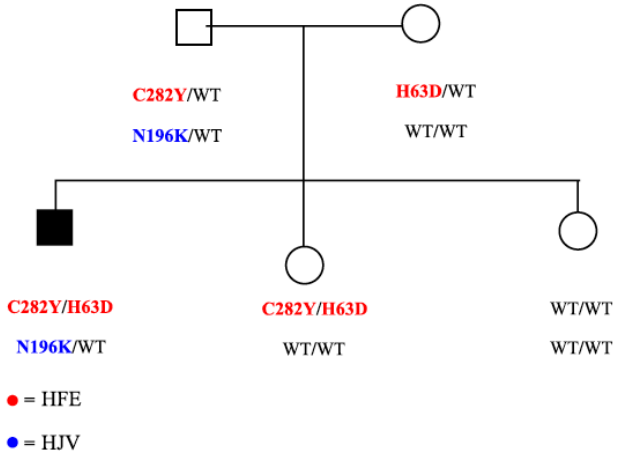
- ✓ traditional sequencing (Sanger) at referral centers
- ✓ Stepwise approach with selection/prioritization of candidate gene(s) according to clinical clues:
  - ☑ age of onset (e.g. *HJV* in early-onset)
  - ☑ ethnicity (e.g. *TFR2* in non-Caucasians)
- ✓ Relatively complex, time consuming
- ✓ Potentially overlooking digenic inheritance



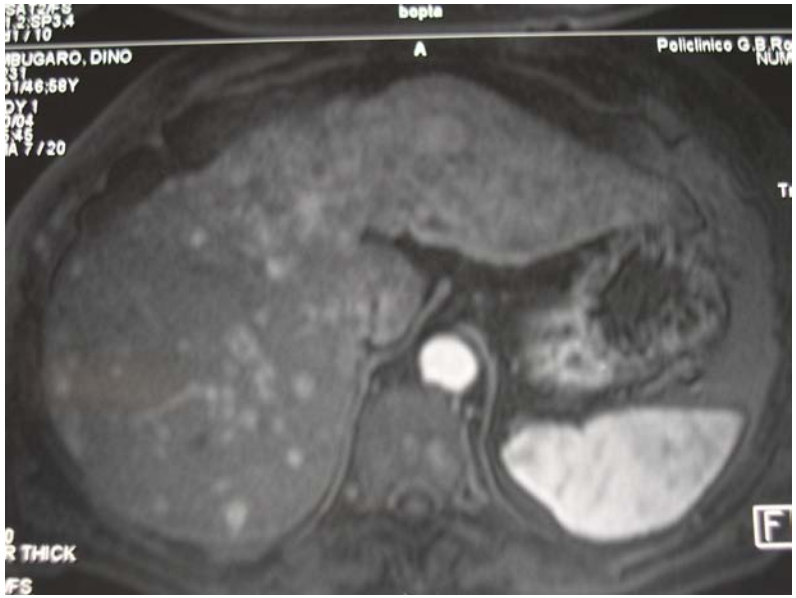

Pietrangelo A, N Engl J Med 2004

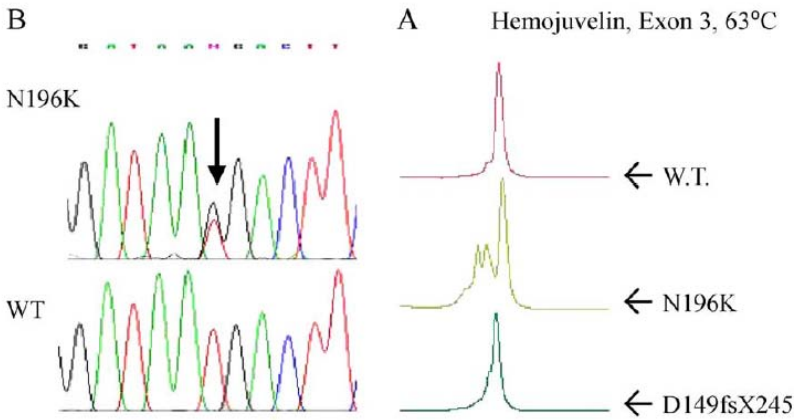
# Digenic inheritance in “highly penetrant” HH

41 year-old male with liver cirrhosis and HHC, bronze hyperpigmentation and diabetes




First level genetic test:
*HFE* C282/H63D compound het…



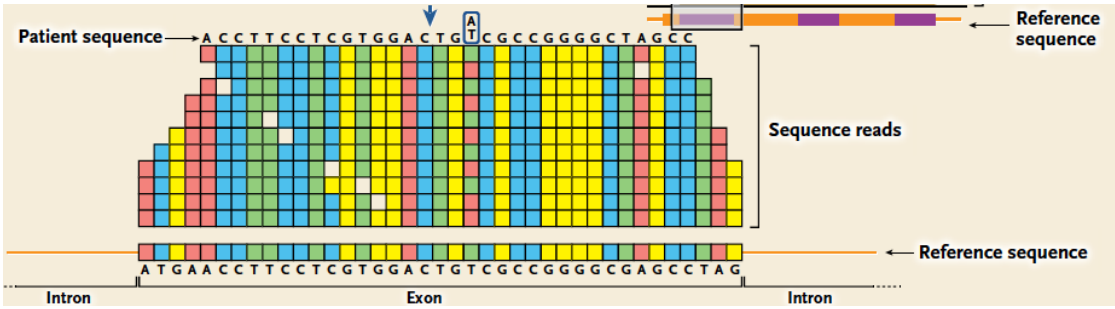



… plus
*HJV* N196K het.

Biasiotto G, Blood Cells Mol Dis 2004

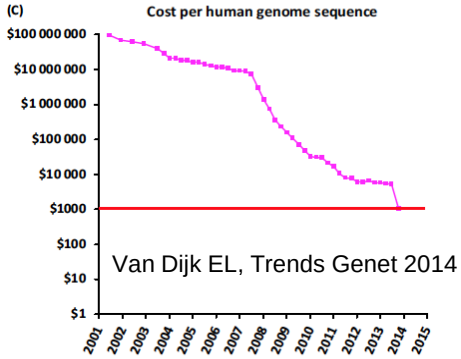
# The advent of NGS: a revolution in molecular diagnosis

PROS-1: rapid, **simultaneous** high-coverage sequencing of target genes




Biesecker LG, N Engl J Med 2014

CONS-1: **costs** (though **constantly** ↓)




Van Dijk EL, Trends Genet 2014

CONS-2: lot of variants of uncertain pathogenic significance found → **difficult interpretation**

Close/continuous interaction between geneticists, bioinformatics and clinicians needed.

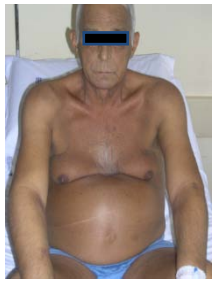
# Diagnosing non-*HFE* HH by NGS: recent experiences - 1

Identification of novel mutations in hemochromatosis genes* AJH

*HAMP, H1V, HFE, TFR2

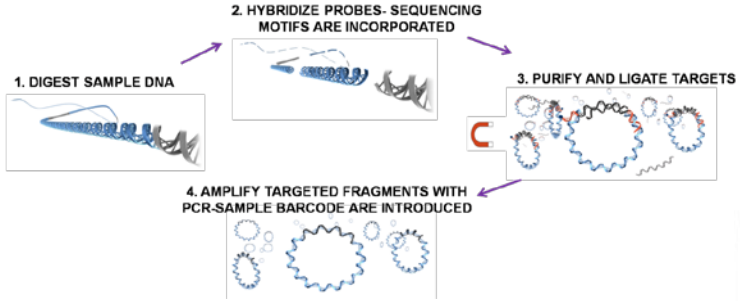
| Pt. ID | 1stlevel genetic test | TS, Ferritin | NGS | Reference | Clinical-molecular interpretation |
|---|---|---|---|---|---|
| #01 | Wild-type[b] | 80%, 1,450 µg $l^{-1}$ | - | - | Remains unexplained |
| #02 | H63D +/− | 61%, >1,000 µg $l^{-1}$ | HFE W163X +/− | Novel | Atypical Type 1 HH (HFE-related) |
| #03 | H63D +/− | 73%, >1,000 µg $l^{-1}$ | HAMP R59X +/− | Novel | Digenic HH (HFE/HAMP)? |
| #04 | Wild-type[b] | 60%, 1,786 µg $l^{-1}$ | - | - | Remains unexplained |
| #05 | H63D +/+ | n.a., 1,089 µg $l^{-1}$ | TFR2 D555N +/− | Novel | Digenic HH (HFE/TFR2)? |
| #06 | Wild-type[b] | 100%, n.a. | TFR2 N241I +/+ | Bardou-Jacquet E et al.[36] | Type 3 HH |
| #07 | Wild-type[b] | 48%, 2,352 µg $l^{-1}$ | - | - | Remains unexplained |
| #08[c] | H63D +/− | 95%, 6,242 µg $l^{-1}$ | SLC40A1 A69T +/− | - | Type 4 HH |

# Our current approach: broad panel of “iron genes” (n=50)



**Pts. selection**

**Halo-Plex™ PCR Technology For Target Capturing**

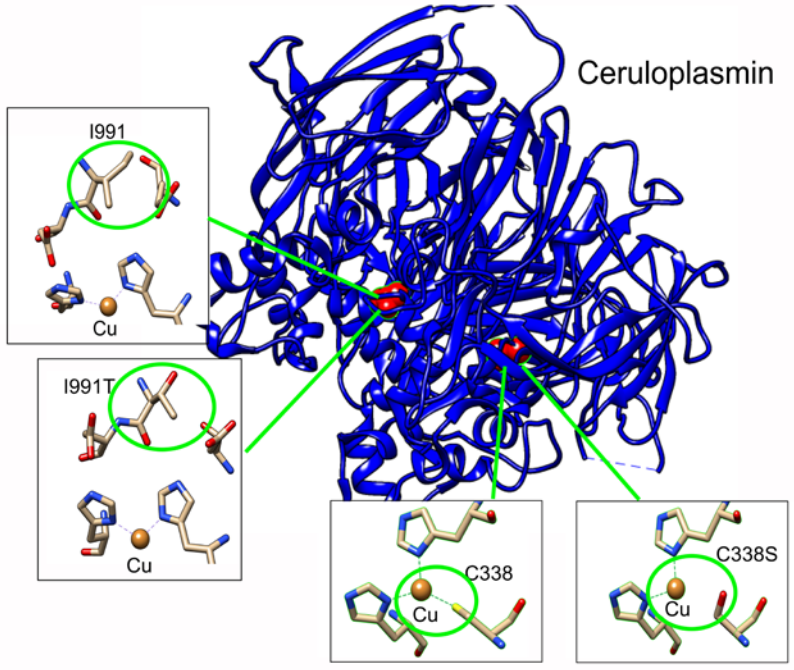



**targeted NGS by Illumina MiSeqDx™**

| | Iron metabolism Genetic Disorders | Genes Included in the panel | |
|---|---|---|---|
| | | Disease Causing genes | Modulators Genes |
| Iron Overload | *HFE HH* | HFE (and upstream region) | ACO1, ARNTL (intron), B2M, BMP4, BMP2, BMPR1A, BMPR1B, BMPR2, CBRD1 (and upstream region), CREB3,EGLN1, EGLN3, EPAS1, EPOR, EPO, EPOR, FADS2, FAM132B, FLVCR1, FURIN (and upstream region), GDF2, GDF15 (and upstream region), GNPAT,HEPH, HIF1A, HP, IL1A, IL6, IL6R, IREB2, LCN2, NCOA4, NEO1, PCSK7 (and upstream regions), RGMA, RGMB, SCARA5, SERPINA1, SMAD1, SMAD4, SMAD5, SMAD9, STEAP3, STAT3, TFRC, TWSG1, VHL |
| | *NON-HFE HH* | SLC40A1, TFR2, HFE2, HAMP (and upstream region), BMP6 | |
| | *Aceruloplasminemia* | CP | |
| | *Atransferrinemia* | TF | |
| | *DMT1-Related iron disorders* | SCL11A2 | |
| | *Autosomal Dominant Iron Overload* | FTH1 (and upstream region) | |
| | *Sideroblastic Anemia* | ALAS2 | |
| | *Congenital dyserythropoietic anemia type II* | SEC23B | |
| No Iron Overload | *Genetic Hyperferritinemia* | FTL (and upstream region) | |
| | *Genetic Hyperferritinemia cataract* | | |
| Iron Deficiency | *IRIDA* | TMPRSS6 | |

# Clinical applications

- 46 y-old male
- Ferritin 2,100-1,300 μg/l
- Low TSAT, mild anemia
- MRI: LIC ↑↑ 300 μM $g^{-1}$
- Asymptomatic (N neurological examination!)




- NGS-based “broad panel” test:
  compound heterozygous for p.Cys338Ser and p.Ile99Thr (both new) in *CP* gene
  → **ACERULOPLASMINEMIA**
  (serum ceruloplasmin undetectable)

# The Verona Interdisciplinary group on Iron Disorders

Participants Units

1. Internal Medicine
2. Clinical Chemistry & Molecular Biology
3. Blood Bank / Transfusional Service
4. Radiology
5. Pathology
6. Gastroenterology

Paolo Bozzini, Fabiana Busti, Paola Capelli, Annalisa Castagna, Michela Corbella, Massimo Delledonne, Giorgio Gandini, Alejandro Giorgetti, Giacomo Marchi, Oliviero Olivieri, Roberto Pozzi-Mucelli, Monica Rizzi, Alice Vianello, Luciano Xumerle.

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