Regulation of hepcidin and its role as a diagnostic tool

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Disclosures

I am an employee of the Radboud University Medical Center that serves the medical, scientific and commercial community with high quality MS and ELISA hepcidin measurements at a fee-for service basis (www.hepcidinanalysis.com)

My team at Radboudumc participates in EUROCALIN, **a hepcidin-antagonist drug development collaboration** funded by the European Commission under its FP7 HEALTH program. We are responsible for the development of analytical assays for free and drug bound hepcidin.

Iron is essential and toxic

Iron is crucible for life

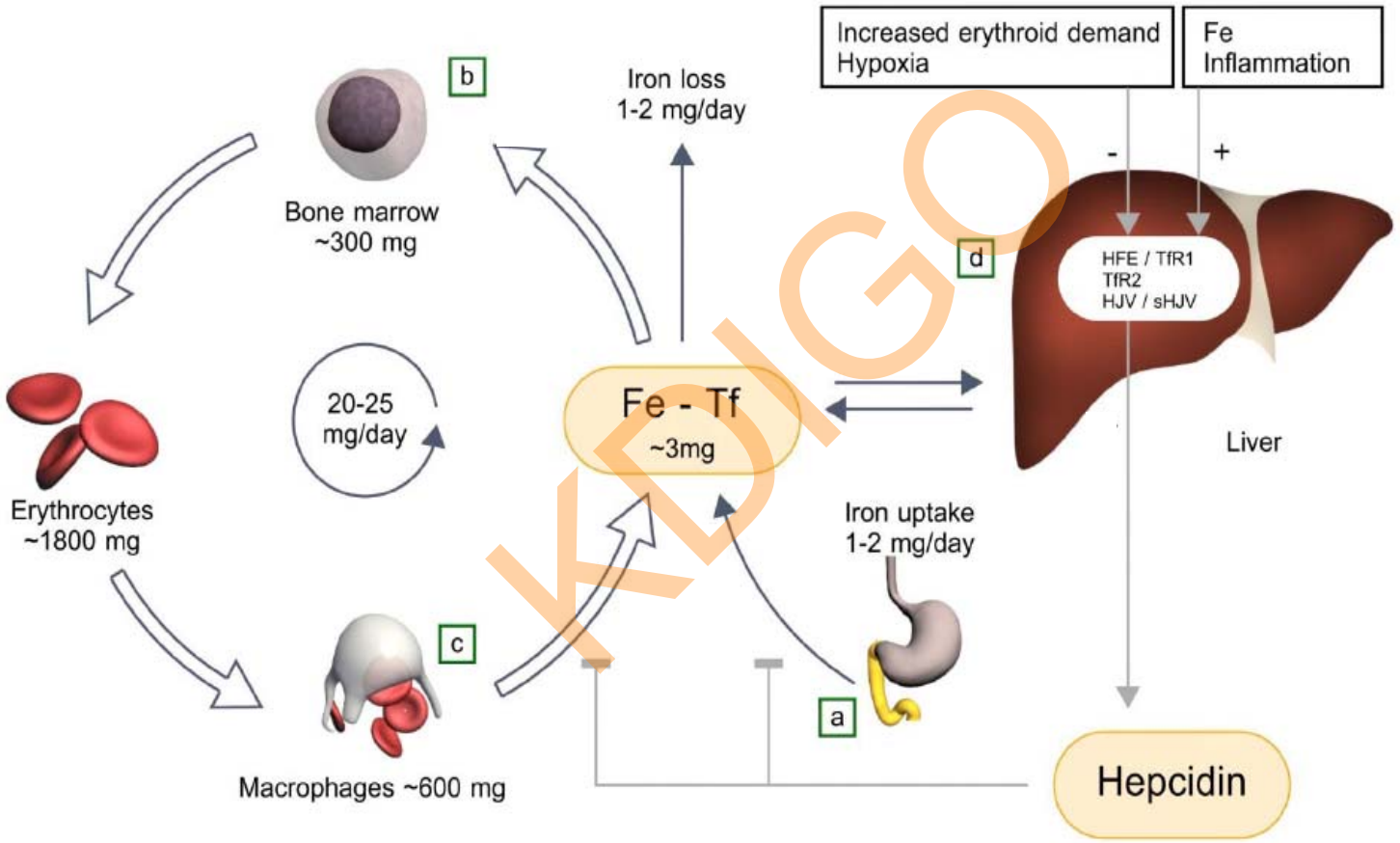
DNA synthesis, respiration and metabolic processes

(heme, Fe-S proteins and enzymes)

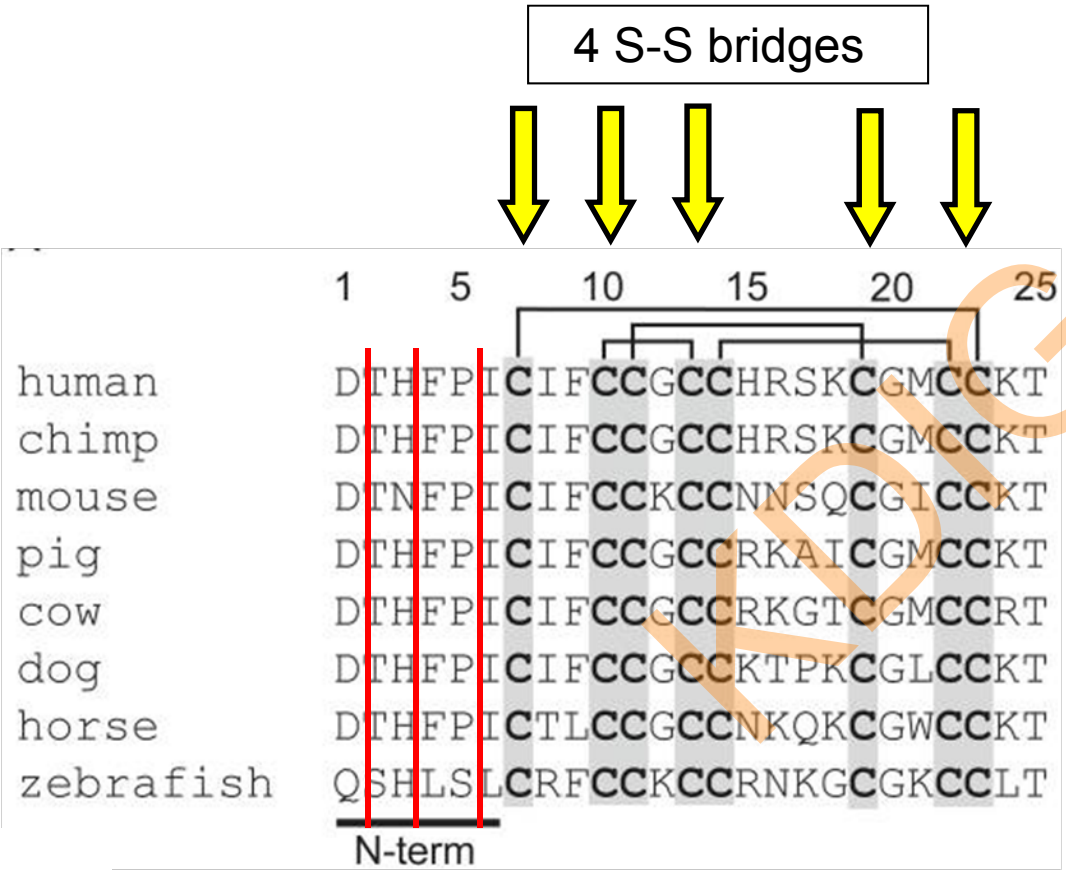
Iron loading is toxic, as it leads to free radical damage

Thus , iron levels must be tightly regulated both at the cellular and **the systemic level**

Systemic iron homeostasis



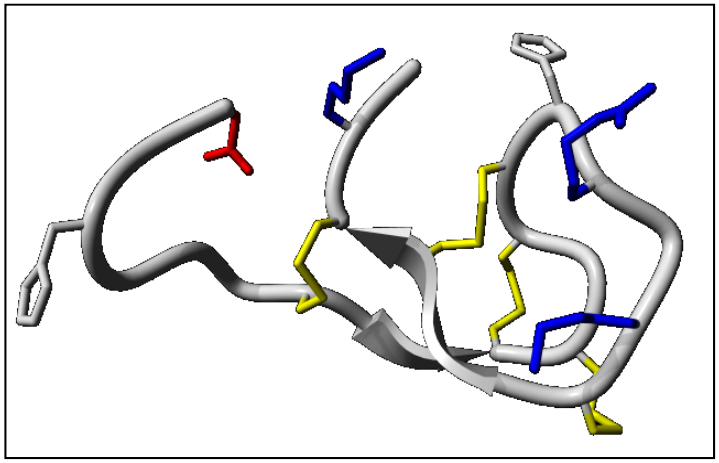
Hepcidin: characteristics



3 small hepcidin isoforms

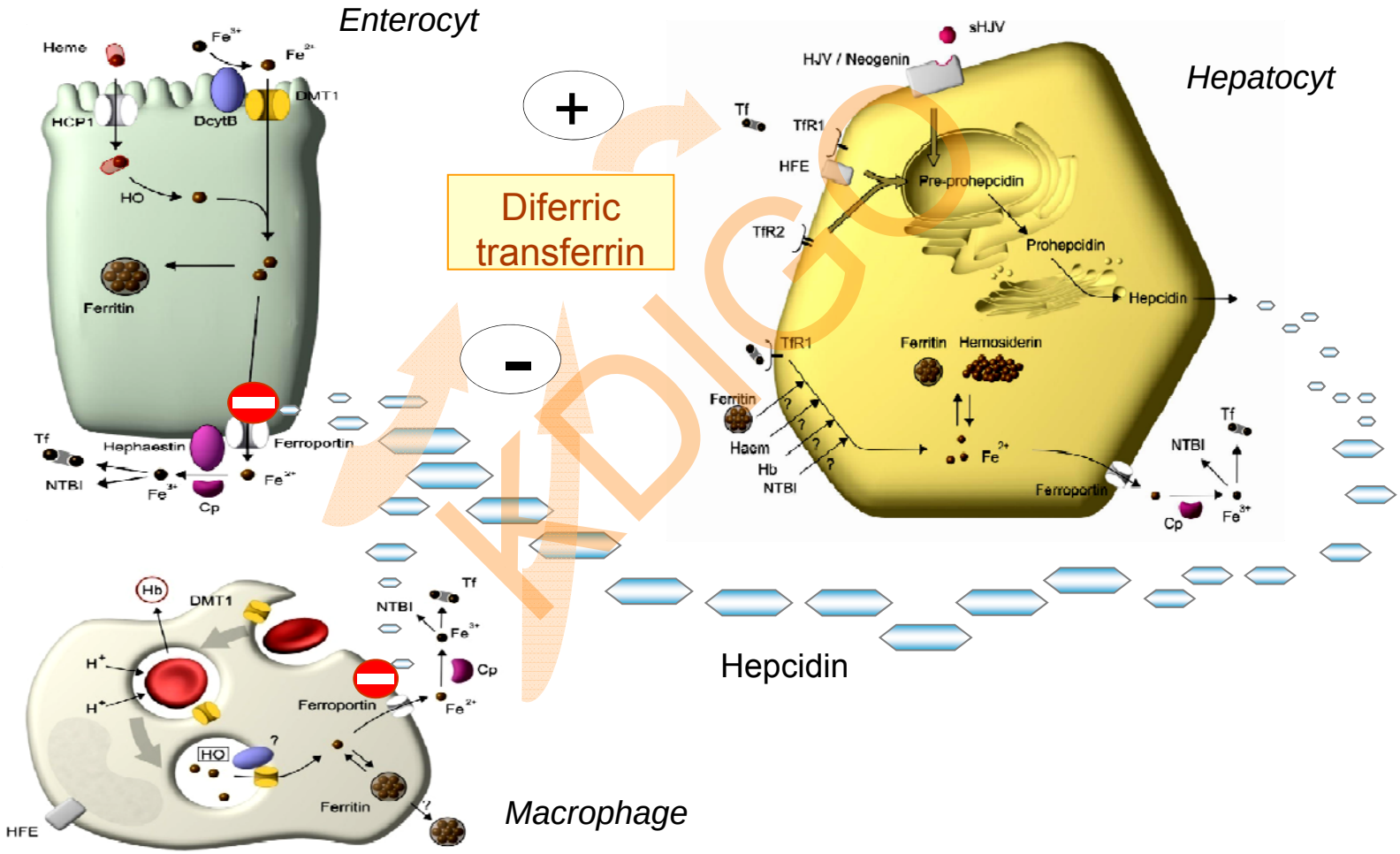
Properties

- Peptide hormone of 25 aa
- Production foremost in hepatocytes
- Highly conserved between species
- 4 disulphide bridges
- 4 isoforms: 25-, 24-, 22- en 20-aa
- Amphipathic (aggregation and sticking to lab plastics)

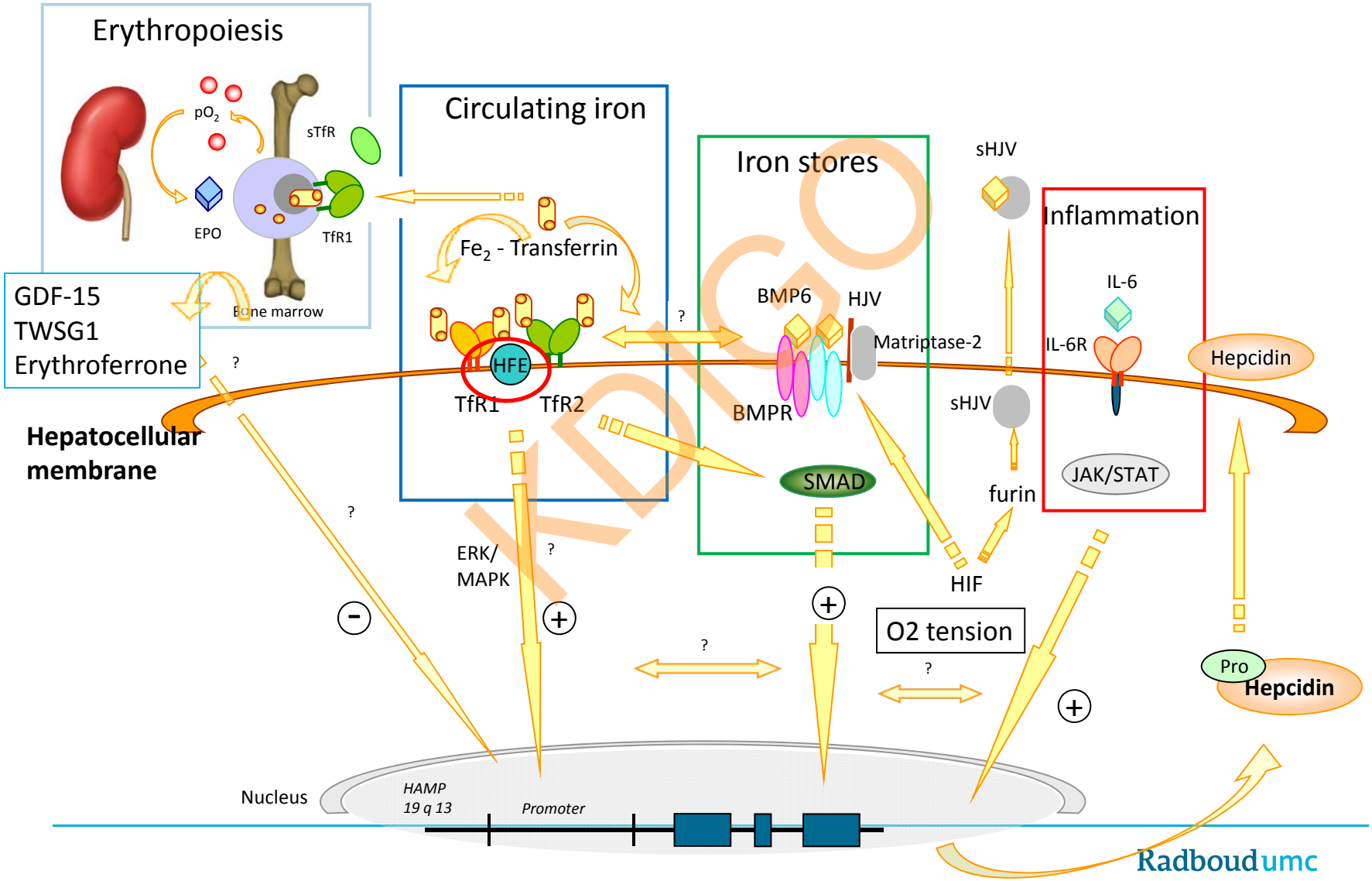


Park J Biol Chem 2001; Hunter J Biol Chem 2002; Ganz Blood 2003; Jordan J Biol Chem 2009; Peslova Blood 2009; Itkonen CCA 2011; Kroot Clin Chem 2011; Laarakkers PLoS ONE 2013

Hepcidin and ferroportin: mode of action

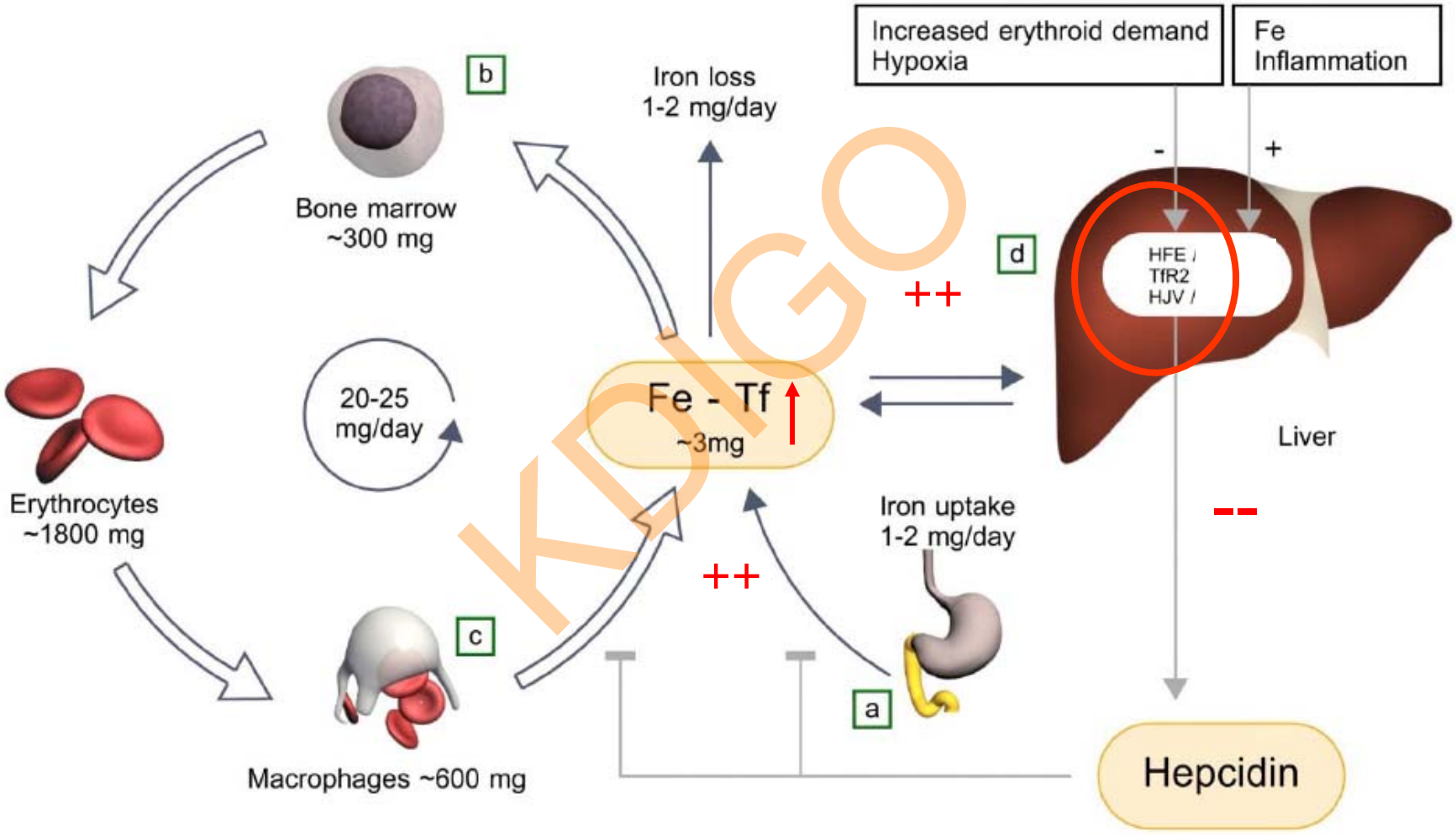


Regulatory pathways of hepcidin



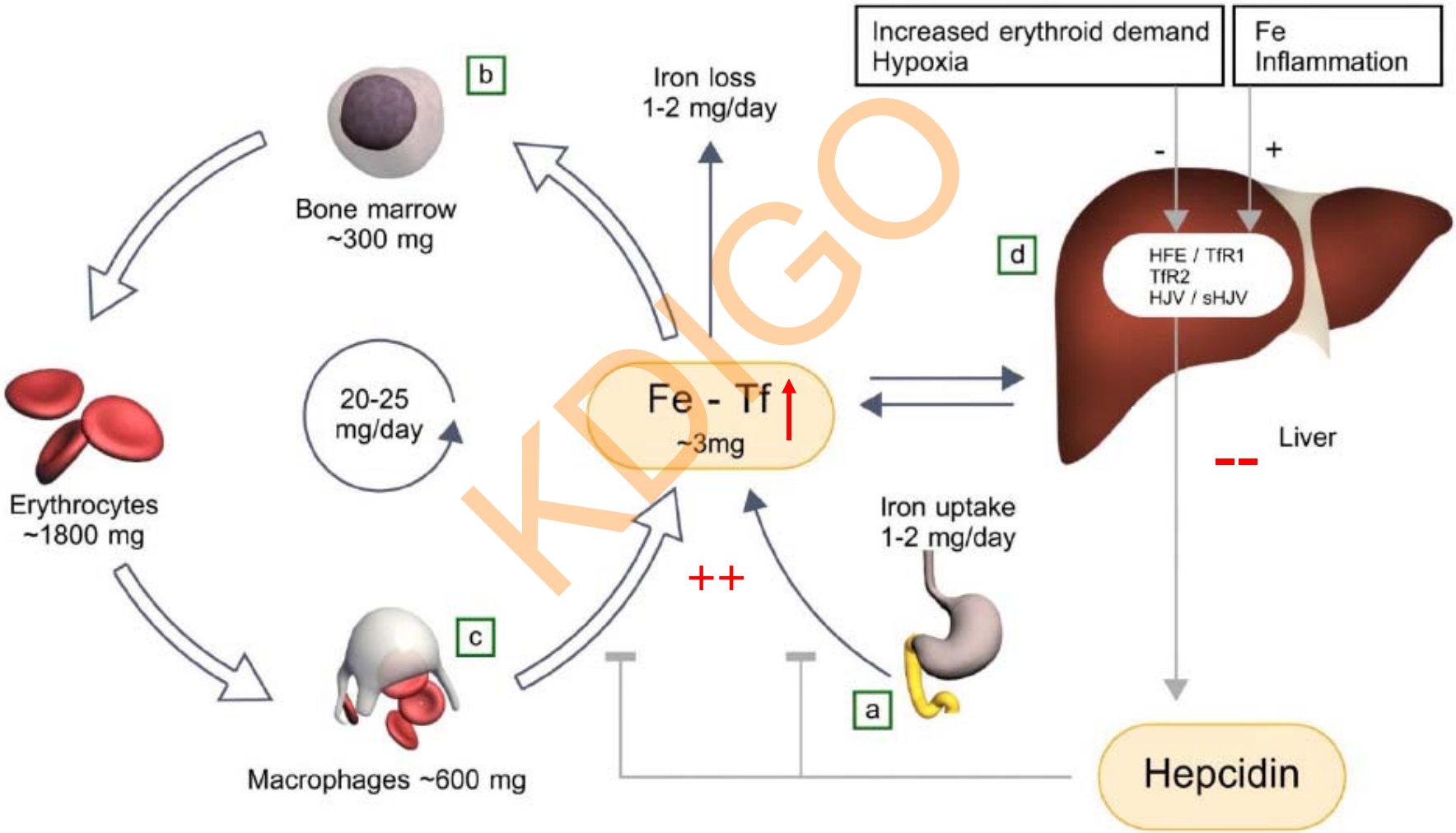
Kroot et al, Clin Chem 2011; Fleming et al. NEJM 2012

Hemochromatosis: low hepcidin

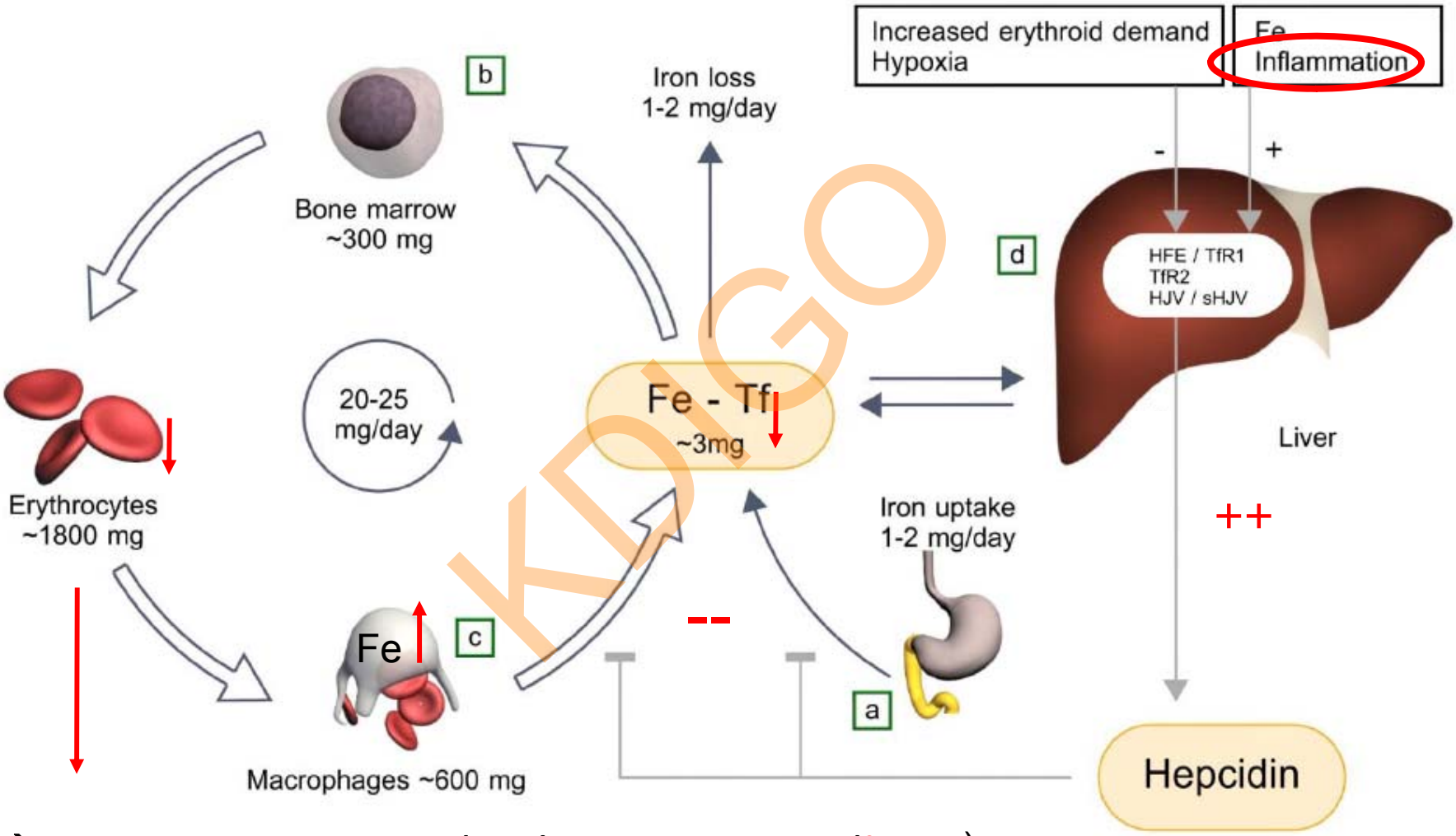


Nemeth *et al.* Blood 2003 and 2004; Papanikolaou *et al.,* Nat Genet. 2004; Kemna *et al.* Blood 2005; Kemna *et al.* Clin Chem 2007; van Dijk *et al.* British J. Haematol 2008; Girelli *et al.* Haematologica 2011

Iron deficiency anemia: low hepcidin



Inflammation: elevated hepcidin → functional iron deficiency

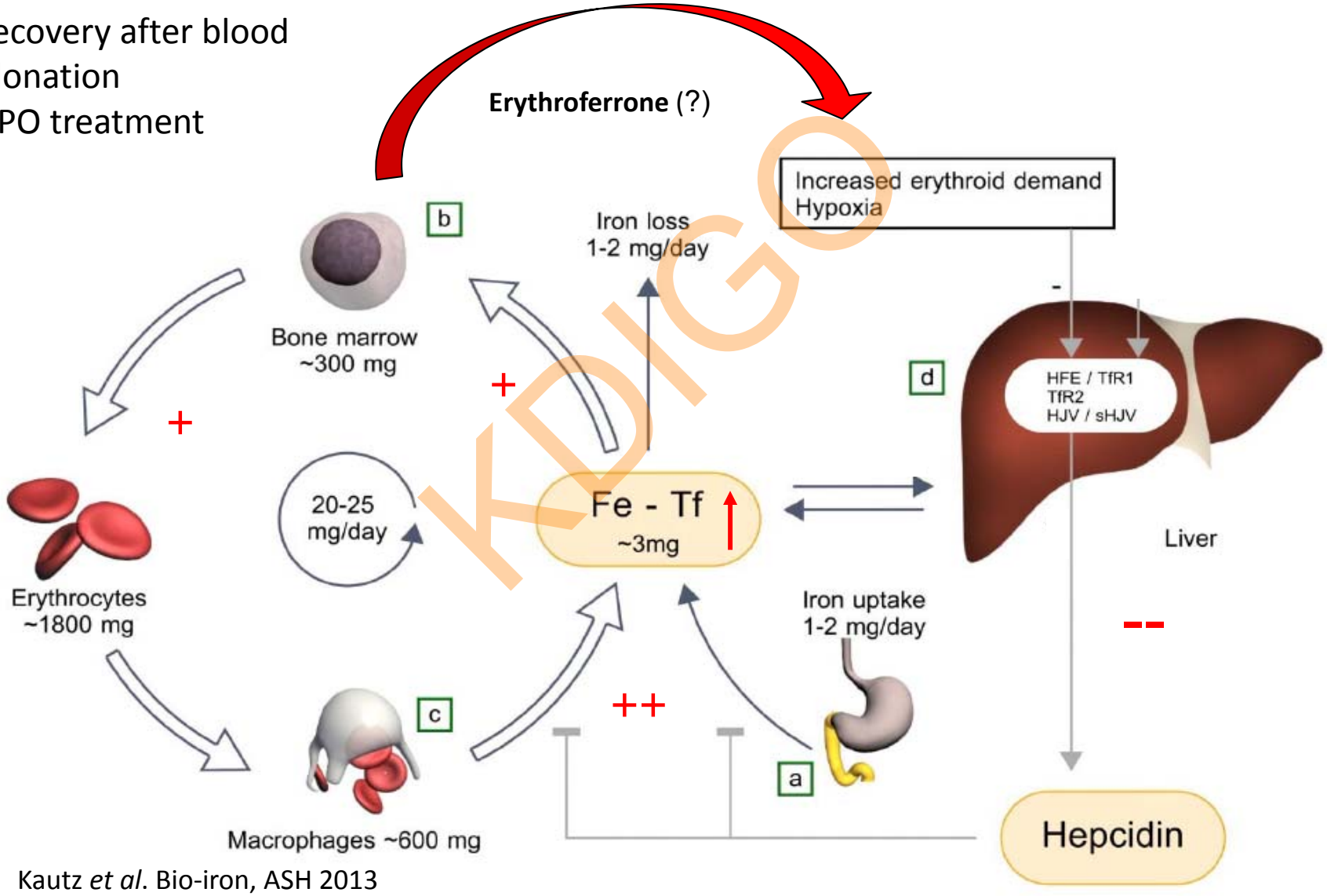


→ **Anemia, low circulating iron (TSAT) and elevated stores (ferritin)**

Pigeon, 2001; Fleming, 2001; Nemeth, 2003 and 2004; Kemna, 2005

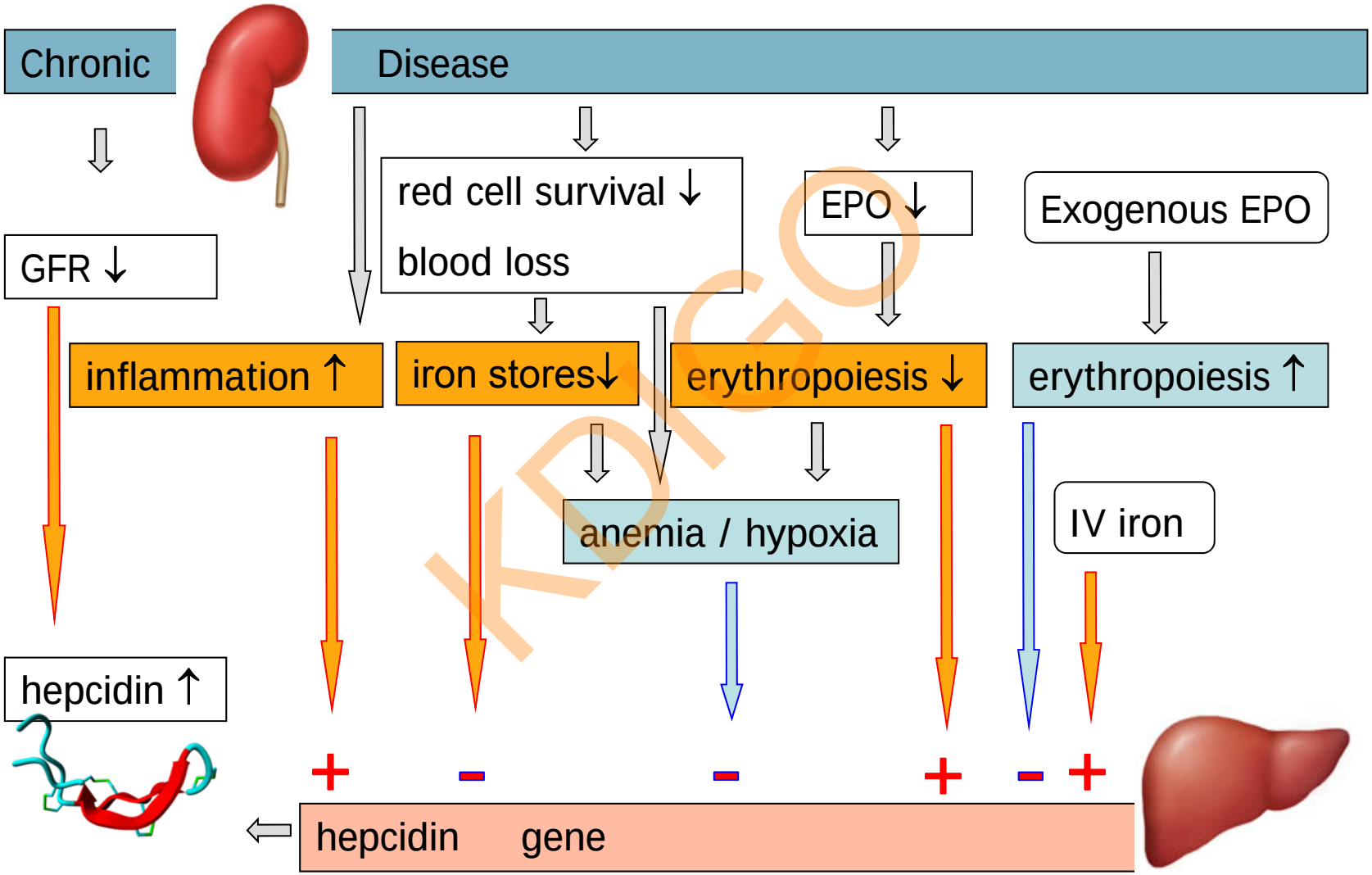
Increased erythropoiesis: low hepcidin

- recovery after blood donation
- EPO treatment



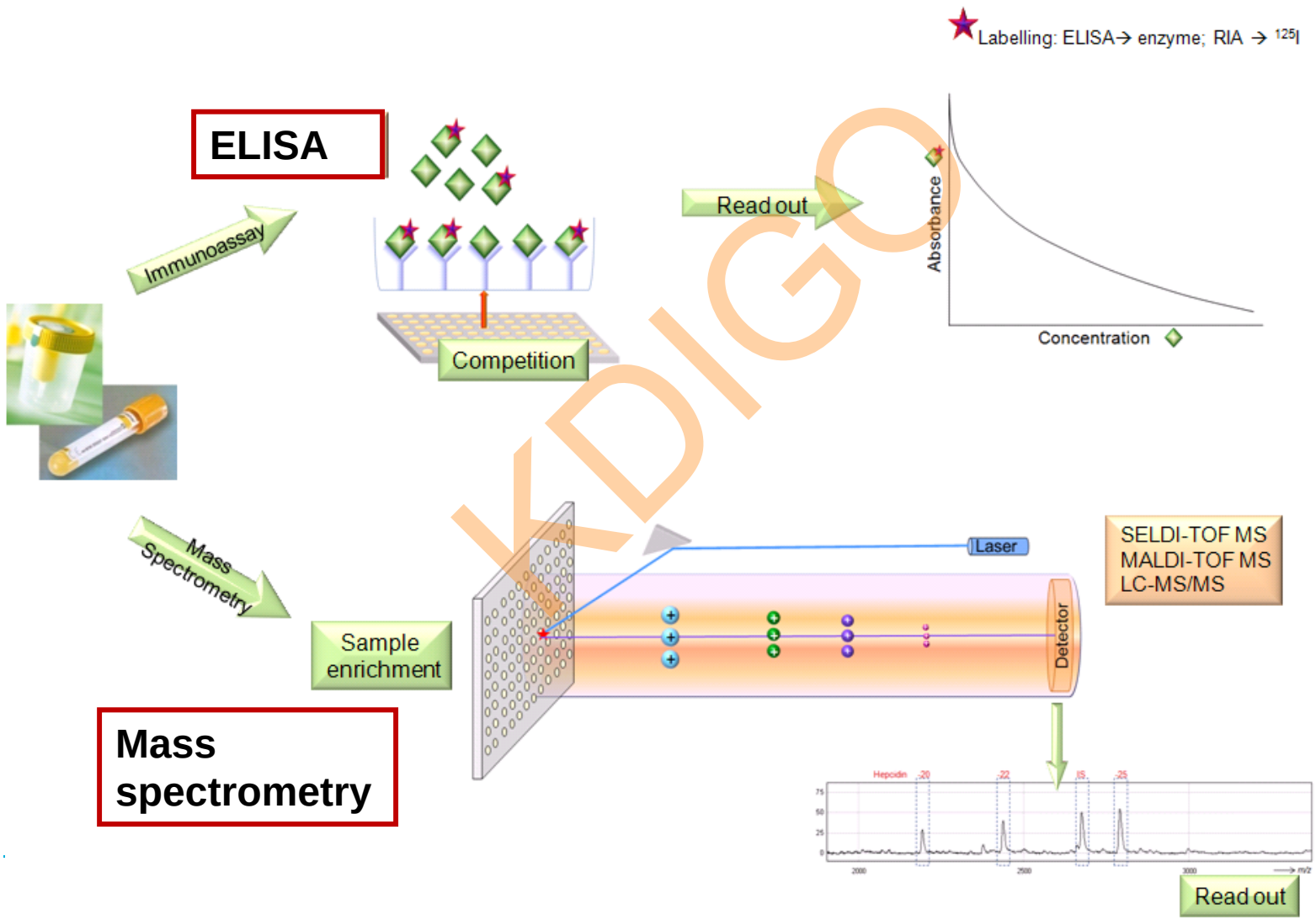
Kautz *et al*. Bio-iron, ASH 2013

Hepcidin in CKD results from the relative strengths of opposing stimuli

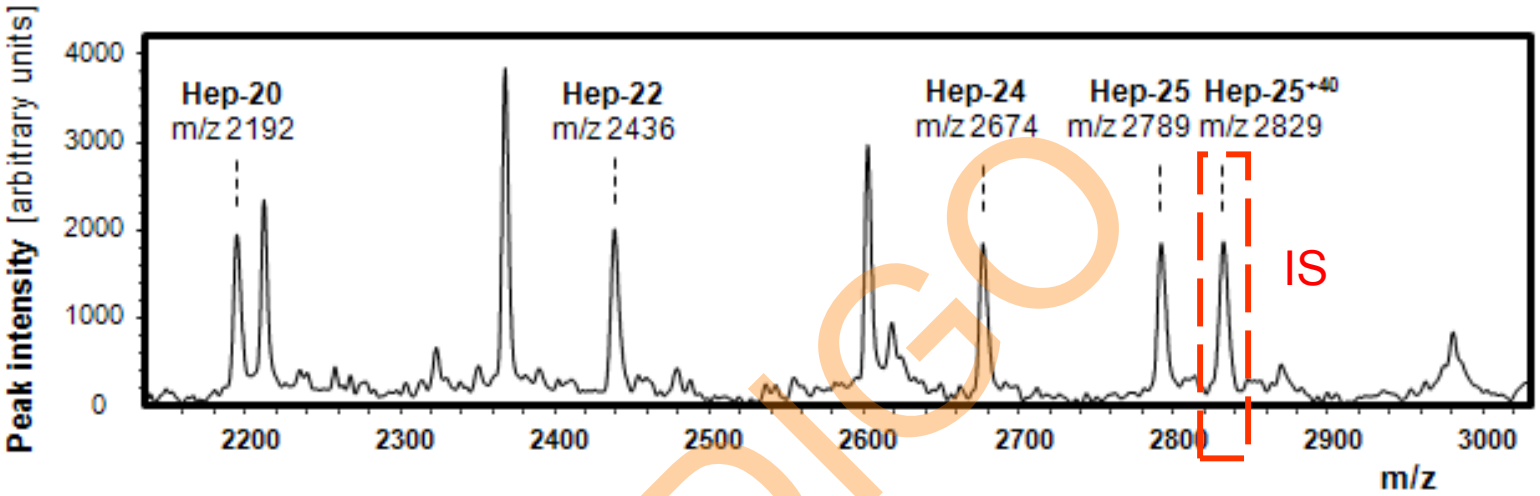


Swinkels & Wetzels, NDT 2008; Yamada, Kidney Int 2009

Principle of hepcidin assays



Mass spectrometry profile from patiënt with CKD



Laarakkers et al. PLOS ONE 2013; www.hepcidinanalysis.com

- Mass spectrometry can measure hepcidin isoforms separately
- ELISA overestimates hepcidin-25 levels in the presence of hepcidin isoforms*
- The clinical relevance of specific measurement of hepcidin-25 versus total is unknown

* Except for sandwhich ELISA, Butterfield, 2010

ELISA versus mass spectrometry

Characteristic	Remarks
Costs	Lower for ELISA
Lower limit of detection	Depending on antibody and MS methodology
Reproducibility	MS generally better
Specificity	c-ELISA measures total hepcidin; MS measures hepcidin-25

Kroot *et al.* Clin Chem 2010; Galesloot *et al.* Blood 2011; Kroot *et al*. Clin Chem 2011; Laarakkers *et al.* PLoS ONE 2013; www.hepcidinanalysis.com

Hepcidin is not standardized

Round Robin 1 (2008-2009)

- 8 laboratories, 8 methods
- urine samples (n=8)
- plasma pools (n=7)
- 4 days of triplicate measurements

Round Robin 2 (2010-2012)

- 16 laboratories, 21 methods
- plasma pools (n=10),
- blank plasma (n=1)
- blank plasma spiked with synthetic hepcidin of 2 commercial sources
- (blinded) duplicate measurements

- absolute levels differ
- methods *generally* correlate
  showed good/acceptable reproducibility
- synthetic hepcidin not useful as calibrator
- a "gold standard" is lacking
- no universal reference values or clinical decision limits

Kroot et al. Haematologica 2009; Kroot et al. Am J Hemat 2012

Reference ranges of hepcidin in our lab

nM	Men (N=1,066)				Women (N=882)			
			95% reference range				95% reference range	
Age, years	N	Median	P2.5	P97.5	N	Median	P2.5	P97.5
18-24	10	9.1	2.3	17.8	21	2.6	0.7	10.5
25-29	16	8.4	0.5	24.2	28	3.1	0.6	11.0
30-34	18	7.4	0.8	25.0	24	3.9	0.2	21.0
35-39	22	6.4	0.7	19.4	36	3.3		
40-44	19	10.2	1.6	17.8	65	4.8	0.3	24.2
45-49	76	8.2	1.3	21.0	110	3.5	0.3	14.6
50-54	106	7.0	0.3	22.0	140	5.4	0.4	22.8
55-59	173	7.7	0.4	24.8	129	8.5	0.8	21.7
60-64	179	7.9	0.3	22.7	137	8.2		
65-69	186	9.0	0.5	22.2	95	8.4		
70-74	133	8.4	1.0	26.9	62	8.7	1.0	37.8
75-79	99	6.8	0.8	25.5	16	9.2	2.1	29.0
80-84	22	6.8	3.5	20.1	10	11.9	1.6	19.2
≥85	7	11.3	3.4	20.5	9	6.7	1.2	24.5
All	1,066	7.8	0.6	23.3	882	6.5	0.5	23.2

Median: 4.1 nM

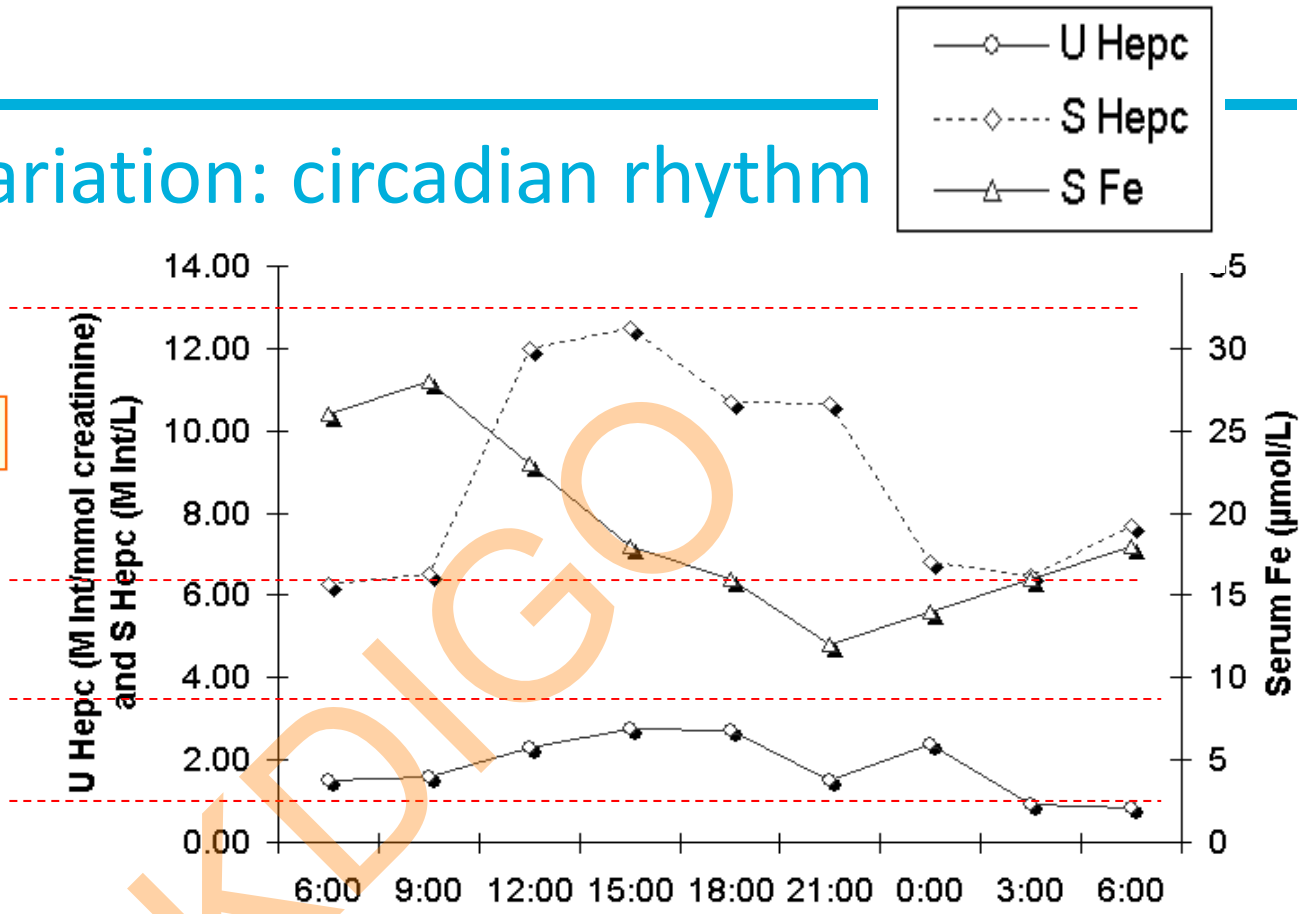
Median: 8.5 nM

Galesloot *et al.* Blood 2011; www.hepcidinanalysis.com

Biological variation: circadian rhythm

Serum Hepcidin

Urine Hepcidin



Circadian rhythm in healthy controls, but not in HD patients

Kemna, Clin Chem 2007; Ganz, Blood 2008; Ashby, Kidney Int. 2008; Kroot, Anal Biochem 2009, Troutt, Clin Chem 2012; Schaap, Clin Chem 2013

Biological hepcidin fluctuations in HD patients

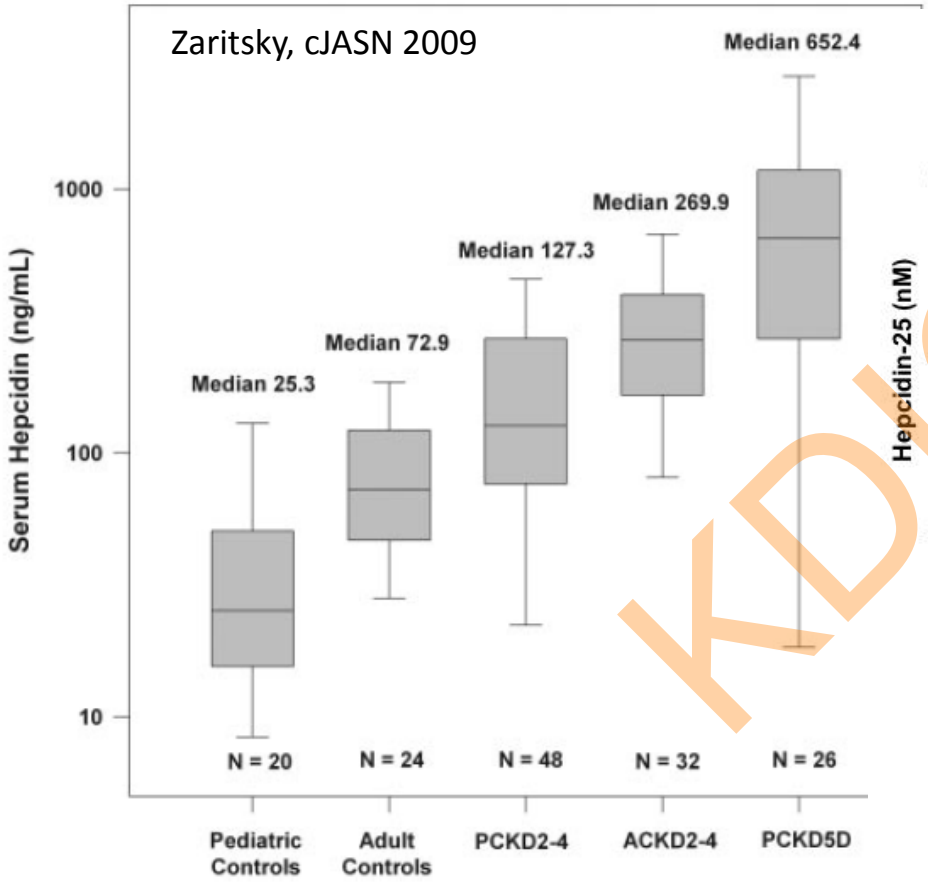
- median of intra-individual CV_i > 20 %
  - baseline to 3-6 weeks
  - both MS and ELISA
  - in 2 independent studies

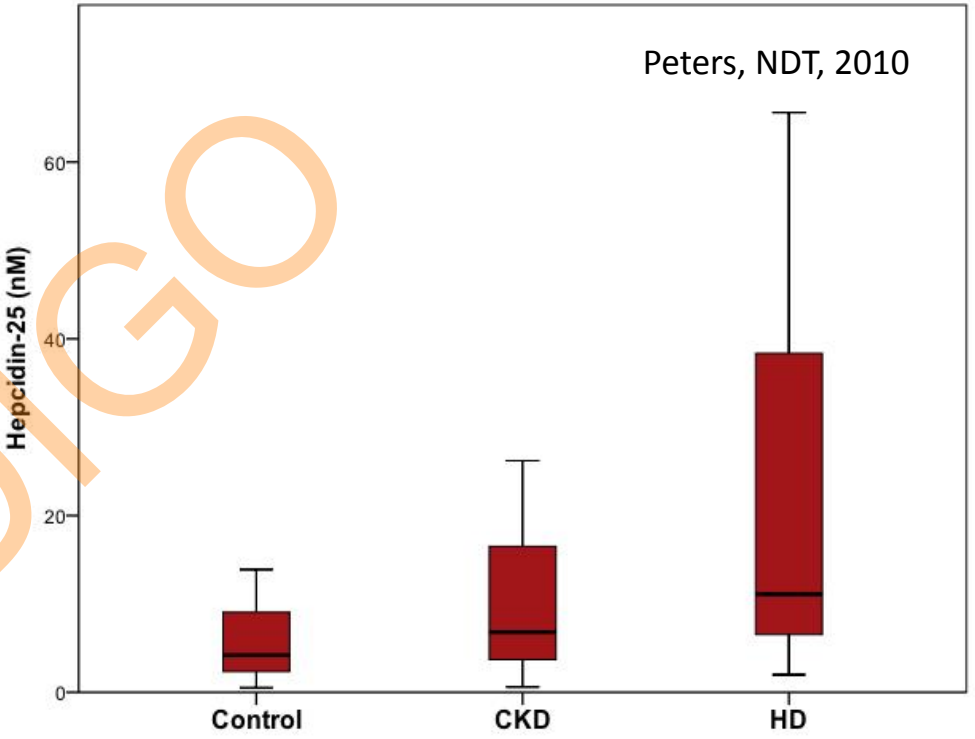
- wide variation in within-individual correlations with CRP and ferritin (significant in small % of patients)

→ Precludes clinical decision making on one single measurement

Ford et al, Kidney Int 2010; Peters et al. NDT 2012

Hepcidin is increased in CKD*





*Controls and patients are not matched for age and gender and ferritin levels in patients >(>>) controls

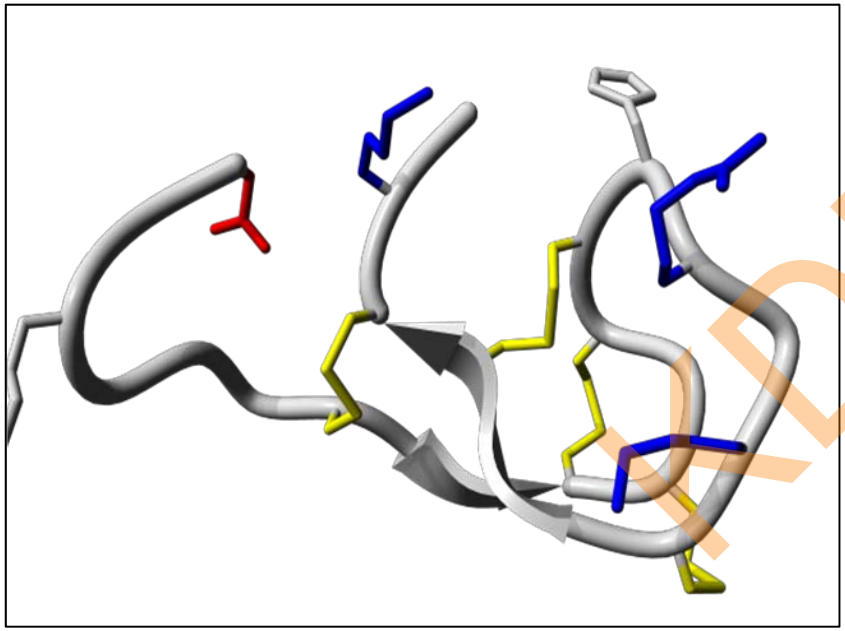
Tomosugi, 2006; Ashby, 2009; Camprostini, 2010; Kurugano, 2010; Troutt, 2013; Valenti, 2013

Associates of elevated hepcidin levels in CKD

Parameter/outcome		association	remarks
elevated ferritin	HD/non-HD	+++	major determinant
low GFR	non-HD	+	inconsistent
CRP and Il-6	HD/non-HD	++	
ESA resistance or response	HD	+/-	inconsistent
effect of iron supplementation on Hb	HD	-	small study, no control group
type of dialyzer	HD	+/-	inconsistent
renal anemia	Non-HD	+	prediction
atherosclerosis	HD	+	CV events/arterial stifness

Tomosugi 2006; Kato 2008; Ashby 2009; Weiss 2009; Costa 2009, Valenti 2009; Zaritsky 2009; Peters 2010; van der Putten 2010; Weiss, 2009; Kuragano 2010; Camprostini , 2010T Tessitore 2010; Ford, 2010; Kroot 2011, Uehata 2012; van der Weerd 2012; Nihata 2012, Troutt, 2013

Application of hepcidin in the clinic



Most promising applications of hepcidin

- Screen for Iron Refractory Iron defiency Anemia (IRIDA) in patients with unexplained microcytic anemia
- Differentiate anemia of chronic disease from iron deficiency anemia
- Guidance for oral iron supplementation
- Treatment with hepcidin antagonists: assess indication and monitor
- Chronic kidney disease: target for treatment and prognostic biomarker

Kroot et al. Clin Chem 2011, review

Hepcidin in CKD

1. Improved our insights on pathophysiology of anemia

2. **Disappointing** diagnostic **management tool**
   - *neither* a marker of ESA resistance
   - *nor* a marker of iron responsiveness
   - high intra-variability in HD patients

3. Promising **biomarker** for:
   - progression of renal anemia
   - CV events
   - **risk stratification** for IV iron therapy
     → high levels: interrupt iron suppl. and use hepcidin antagonists

4. **Target of treatment**

Van der Weerd, Gaillard, manuscript in preparation

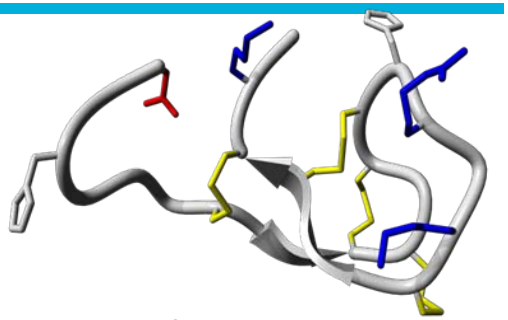
Summary

1. pre-analytical hepcidin handling is critical for reliable results
2. lack of validated assays, for which reference values are known
3. hepcidin assays are not standardized
4. c-ELISA measures total hepcidin, whereas MS measures hepcidin-25
5. Hepcidin is regulated by erythropoiesis, circulating iron, iron stores and inflammation

Summary; in CKD:

1. hepcidin levels are increased
2. hepcidin levels result from the relative strengths of opposing stimuli
3. ferritin is increased and major determinant of hepcidin
4. rel. high levels of the smaller hepcidin isoforms, of unknown significance
5. 25 aa form is the iron regulatory hormone
6. hepcidin is a
   a. poor diagnostic management tool
   b. promising prognostic biomarker for progression of renal anemia and CV-disease
   c. promising tool for risk stratification of IV iron treatment
   d. promising treatment target

Research agenda



- harmonise hepcidin assays
- assess clinical relevance of specific hepcidin-25 measurement
- increase number of reliable and accessible assays
- publish validation of commercial assays
- collect evidence for added value of hepcidin in CKD in well designed large diagnostic studies and controls
- define clinical decision limits of hepcidin
- clinical trials with hepcidin antagonists