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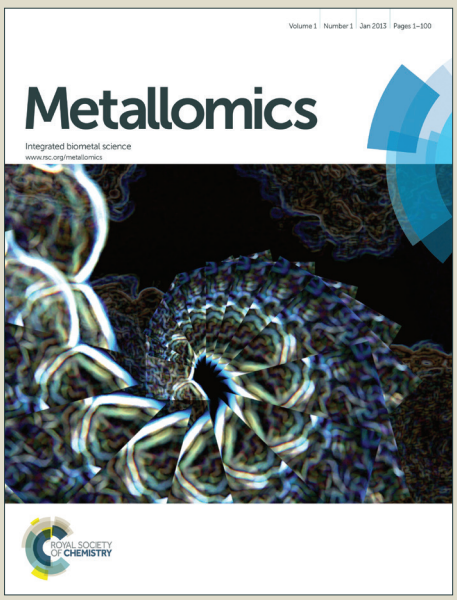

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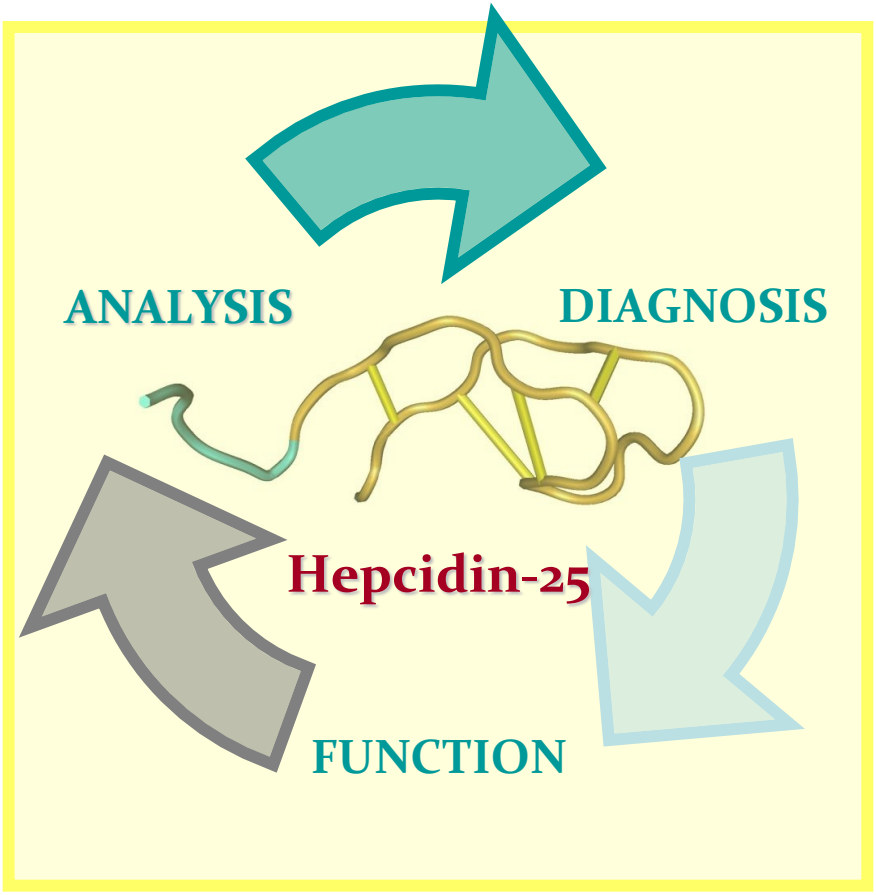

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HEPCIDIN QUANTIFICATION: METHODS AND UTILITY IN DIAGNOSIS

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ABSTRACT

Hepcidin is a 25-amino acid peptide hormone that is produced and secreted predominantly by hepatocytes, circulates in the bloodstream, and is excreted by the kidneys. Since the discovery of hepcidin and the elucidation of its important role in iron homeostasis, hepcidin has been suggested as a promising diagnostic marker for iron-related disorders. In this regard, a number of analytical methods have been developed in order to assess hepcidin concentration in different biological fluids, particularly serum and urine. In this critical review we have tried to address the issues still pending in the accurate determination of this peptide by evaluating the available analytical methodologies. Among them, the use of ELISA strategies (in competitive or sandwich formats) and molecular mass spectrometry (MS) including MALDI and/or LC-MS have been critically compared. The use of elemental mass spectrometry (ICP-MS) has been also included as a possible complementary tool to the previous ones. In addition, the manuscript has revised the existing and potentially emerging clinical applications of hepcidin testing for diagnosis. These include the iron disorders such as iron deficiency anemia (IDA, low hepcidin), anemia of chronic disease (ACD, high hepcidin) and the combined state of ACD and IDA or hemochromatosis. Other applications such as using hepcidin in assessing the response to existing therapies in cancer have been also revised in the manuscript.

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1. Background/Introduction

The past decade of research in the field of iron homeostasis has allowed the discovery of many new actors (enzymes, sensors, regulators, transporters, scavengers) involved in maintaining iron balance, both at the cellular and systemic levels. At the cellular level, iron homeostasis is orchestrated by the Iron Regulatory Proteins (IRP1 and 2) binding to RNA stem-loop structures called iron-responsive elements (IREs) in transcripts encoding proteins involved in iron uptake, storage and export. IRPs are activated by iron deficiency and function to increase cellular iron levels to ensure adequate iron content. Another iron-regulatory pathway aimed at iron conservation and optimization to ensure essential survival functions has been described more recently and concerns the TTP (tristetraprolin) pathway. TTP is a zinc-finger containing protein that binds to AU-rich elements of target mRNAs and reduces cellular iron utilization through selective suppression of non-essential iron-consuming pathways. These pathways have been recently reviewed.[1, 2]

At the level of the whole body organism, a major advance in our understanding of the molecular circuits that achieve iron balance has been the discovery of the iron regulatory hormone hepcidin and its receptor, the iron exporter ferroportin. Hepcidin is made primarily by the liver and controls plasma and tissue iron levels by inhibiting cellular iron efflux through degradation of ferroportin.[3] The molecular mechanisms responsible for ferroportin degradation rely on hepcidin-induced ferroportin ubiquitination of lysines in a cytoplasmic loop of the exporter, its subsequent endocytosis and its degradation in the lysosomes.

According to this iron-regulatory function and its ancestral one as a defensin-like peptide, hepcidin plays also a critical role in innate immunity both by restricting systemic iron necessary for pathogen growth and by harbouring direct antimicrobial activity. As befits an iron regulatory hormone, hepcidin gene expression is regulated by many iron cues (holotransferrin, cellular iron and ferritin) and in all situations requiring elevated input of iron,

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such as anaemia, hypoxia and increased erythropoiesis conditions, to face increased erythropoiesis. Strikingly, while the list of hepcidin regulators is rapidly increasing (including sugar, growth factors, hormones and vitamins), it seems that the signalling pathways connected to the external signals converge in most cases to the unique BMP/HJV/SMAD1, 5, 8 pathway.[4,5] In conditions of anaemia observed in infections/inflammatory disorders, iron abnormalities are partly explained by inappropriately high hepcidin expression mediated by IL-6 (and IL-6 family members)-dependent activation of the STAT3 pathway. Other hepcidin activators were demonstrated to be involved in inflammation such as Activin B, *via* the SMAD1, 5, 8, pathway, highlighting the crosstalk between the BMP-SMAD and the IL-6-STAT3 pathways (see Figure 1) in the setting of inflammation.[5,6]

Hepcidin is encoded by an 84 amino acid prepropeptide containing a typical N-terminal signal sequence and a pro-region with a consensus furin cleavage site immediately followed by the C-terminal 25 amino acid bioactive mature peptide, the so called iron-regulatory hormone.[6] Apart from the 25- mature peptide (amino acid sequence DTHFPI**C**IF**CC**G**CC**HRSK**C**GM**CC**KT), N-terminal truncated hepcidin 20, 22-, and more recently 24- peptides, have been observed in the urine and at low concentrations in the plasma. So far, the origin and the role of these smaller isoforms are still unknown.

The identification of critical regulators of liver hepcidin synthesis during various physiopathological conditions and the deciphering of the signalling transducing pathways have been extensively reviewed elsewhere.[7-10] Also, a considerable number of reviews have discussed the molecular mechanisms of hepcidin deregulation in iron related disorders and hepcidin-ferroportin-biased therapeutics and will be out of the scope of this manuscript.[11,12] Apart from these key exciting considerations concerning the development of new promising therapeutic strategies for the management of iron disorders, considerable hope is also attracted by the use of hepcidin in providing useful diagnostic and prognostic information to limit iron-

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mediated pathology. For that, reliable and robust methods for measuring hepcidin in human serum, plasma and urine are required to ensure the diagnostic value of hepcidin. This review describes the current methods, either based on mass spectrometry or immunochemical principles that have been developed since the discovery of hepcidin and discuss the clinical applications of hepcidin testing.

2. Hepcidin-25 quantitative analysis methods

Since the discovery of hepcidin in the year 2001, the development of analytical strategies that permit its accurate quantification in body fluids has been growing exponentially. In general terms, two types of strategies have been applied for hepcidin quantification: MS based methods (including LC/MS/MS, SELDI-TOF or MALDI-TOF) and those based on immunochemical principles (competitive or sandwich ELISA and radioimmunoassay, RIA). The main difference between these two general strategies is that while MS-based methodologies permit to discriminate between hepcidin forms (20, 22 and 25-hepcidin), most of the existing immunochemical based-assays measure total hepcidin concentration. On the other hand, ELISA methods provide high sample throughput and can be easily incorporated in routine labs, once they are optimized.

In order to harmonize the results provided by the different analytical methods used for hepcidin analysis, two round robin exercises[13,14] have been conducted and the results of the second one have been just published.[14] The first one[13] revealed also big discrepancies between the results obtained with the different methodologies but could be used to establish the first recommendations for the harmonization of the various hepcidin assays. The results obtained in the second exercise[14] reveal that there is still a long way to obtain a reference method (and a reference material) for the determination of hepcidin in biological fluids. So far, the authors constructed algorithms (using linear regression model) which allow computing hepcidin consensus values (HEPCON) for the results obtained by the various methods. However, from an analytical point of view, there are still pending questions in hepcidin-25 quantification

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methods that could be improved. In the next sections, we will try to critically address those characteristics and remaining challenges.

2.1. Immunochemical methods

Together with the diversity of hepcidin forms (20-, 22-, 24- and 25-) the development of suitable immunochemical methods for hepcidin-25 has been always hampered by the small size of the molecule. This reduces dramatically the number of antigenic epitopes for antibody recognition and therefore, the selectivity of most designed antibodies. Additionally, hepcidin contains 4 disulfide bonds and due to such compact structure is not ideal for Western blotting analysis. Up to now, three main immunochemical strategies have been applied to hepcidin-25 quantification in body fluids: competitive or sandwich enzyme-linked immunosorbent assay (ELISA) and a radio-immunoassay (RIA). Figure 2 shows the main drawbacks/benefits, of each one of these methods. They all have been applied to the analysis of hepcidin-25, however, the use ELISA strategies has become more widely accepted than RIA strategies, particularly due to the safety concerns regarding the use of radioisotopes.

RIA is a very sensitive technique by which the analyte of interest (hepcidin-25 in this case) is mixed with a known amount of radioactive-labelled analyte and both species compete for the same antibody binding site. The radio-labelling is normally done by adding ^{125}I in the form of iodine that is attached either to tyrosine or histidine residues of proteins and peptides. The radiation intensity is inversely correlated with the concentration of un-labelled hepcidin present in the sample. For hepcidin-25 determination in serum samples, Grebenchtchikov et al.[15] have described a RIA that provided excellent detection limits (0.02 ng/mL) with good linearity and precision using a coated plate with an anti-rabbit IgG antibody before treatment with the anti-hepcidin antibody.

One of the first successful approaches for competitive ELISA (cELISA) of hepcidin-25 was designed by the group of T. Ganz, first reported in human urine[16] and further validated for serum in 2008.[17] This assay used biotinylated hepcidin as competitor and synthetic

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hepcidin for calibration. The competitive format of the assay implied the inverse correlation between hepcidin concentration and the measured absorbance, which is often less sensitive than the direct correlation. In any case, the method was sensitive enough to proof gender differences in serum hepcidin content resulting from the lower iron stores in women and also diurnal changes on the concentration of hepcidin similar to serum iron concentrations but it could not discriminate between hepcidin forms (20-, 22- and 25-) . Further comparison of such competitive assay with MS methods (based either on immunocapture, IC or on the enrichment of the peptide with weak cation exchange beads WCX, both followed by MALDI-TOF) revealed positive correlation among data ($p<0.001$). However, comparative concentration results obtained by WCX-TOF-MS and cELISA were different, particularly at the low concentration levels (< 5 nmol/L). The authors ascribe the differences, among others, to differences in the aggregation of the synthetic and native hepcidin during sample handling and to the serum matrix effects that could differentially influence the recognition of the antibody for biotynilated synthetic hepcidin-25 or native hepcidin. Similarly, the group of Koliaraki and co-workers reported a cELISA assay for hepcidin using a recombinant peptide and polyclonal antibodies raised against this peptide.[18] Once again, the recombinant peptide showed different behaviour to the native one.

In order to increase the assay selectivity by minimizing the problems of the peptide recognition by the use of a single anti-hepcidin antibody, subsequent publications documented the development of sandwich ELISA (sELISA) for more selective quantification of hepcidin-25.[19] For this purpose, two independent monoclonal antibodies, specific for hepcidin-25, hepcidin-20 or hepcidin-22 respectively were synthesized. The cross reactivity among forms of the peptide seemed to be really minimized in this case. In addition, the correlation between the ELISA and the LC-MS results was good for hepcidin-25 ($r^2=0.98$, $P<0.001$) proving the specificity of the assay. Together with the advantages of using double antibody recognition, the sandwich format provides signals that can be positively correlated with the hepcidin-25

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concentration. This yields in an overall limit of quantification of 0.01 μg/L, improved in respect to the previous assays. In addition, the obtained results by sELISA could be directly correlated (r=0.98, P<0.001) with the concentration obtained by LC-MS.

Unfortunately, although the obtained results by sELISA seemed to be highly promising, the second round robin exercise[14] revealed important differences still existing among methods (and even between methods based in the same measurement principle) in the results of hepcidin-25, particularly in real samples. These differences between assays might be attributed to differences in the values that laboratories and companies assign to the internal and external standards used by the different methods, to impurities in these standards or to losses of the standard during storage.

2.2 Mass spectrometry based methods

2.2.1. MALDI- and SELDI-TOFMS.- Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOFMS) is a widely applied technique and generally accepted by the scientific community for the qualitative analysis molecules. Although this technique has been seldom applied for quantitative analysis, there are MALDI-TOFMS based strategies dealing with quantitative analysis of hepcidin-25 in biological fluids that will be revised later. Somehow this is not surprising since MALDI-TOFMS, among the different MS-based techniques applied for hepcidin quantification, is simple to operate and can offer fast and relatively accurate analyses.[20] Furthermore, it achieves better mass resolution compared to SELDI-TOFMS (surface-enhanced laser desorption ionization) and generally shows higher sample throughput capabilities than LC-MS methods.[21] However, reliable quantification with this technique still remains challenging: MALDI-TOFMS is mainly limited by its poor reproducibility.[22] In this vein, the preparation of the sample target is particularly crucial. This is ascribed to the small volumes usually loaded on the MALDI target (typically a few μL per spot) which might affect the absolute amount of analyte per spot, limiting the spot-to-spot reproducibility. Furthermore, LASER fluctuations, matrix

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effects on the ionization and poor shot-to-shot reproducibility restrict the application of MALDI-TOFMS for quantitative biomolecule analysis.[23]

Some of these limitations can be corrected by the proper use of a suitable internal standard. For the quantification of serum hepcidin-25 by MALDI-TOFMS, synthetic hepcidin-24 has been applied as internal standard.[24] In this vein, human serum and internal standard were mixed and a polyclonal anti-hepcidin-25 antibody bound to protein-A sepharose beads was added for analyte extraction. Since mixing of internal standard and sample was performed before extraction, analyte losses should be compensated. For validation, the developed immunocapture (IC)-TOFMS strategy was compared to a competitive cELISA method which was presented by the same authors. The developed method was able to distinguish between hepcidin-25 concentrations in iron deficiency anaemia (IDA) and anaemia of chronic disease (ACD) patients. However, since hepcidin-24 could be also present in the samples, the use of an isotopically enriched molecule - corresponding to the analyte of interest - is more desirable as (ideal) internal standard.

Isotopically enriched hepcidin-25 ("heavy" hepcidin-25, $^{15}N_4$,$^{13}C_6$-Ar) as internal standard was recently applied by Anderson et al.[25] to determine the peptide hormone in human urine and plasma samples by MALDI-TOFMS. For cross validation, both methods were compared to an existing immunochemical assay. Although a good correlation between the distinct assays platforms was obtained, a slope of <1 indicates an offset between the MS methods and the antibody based assay. According to the authors, this may be due to spectral interferences of the MALDI-TOFMS method. However, the increased values determined by the immunochemical assay may also be related to cross reactivity caused by hepcidin-20 and hepcidin-22. Therefore, an orthogonal cross validation (e.g. to LC-MS) may be required to elucidate the origin of the discrepancies found in this work.

If no "heavy" hepcidin-25 can be applied as internal standard, the standard addition method in combination with an internal standard could be an alternative strategy to minimize

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matrix effects during analysis. Furthermore, signal fluctuations can be compensated by the internal standard. In this regard, one recent study describes the use of the standard addition method for the quantification of urinary hepcidin-25.[20] Here, quantitative recoveries (98%) were obtained in the case of aqueous hepcidin standards and 70% for hepcidin spiked in urine blanks. After validation of the developed method in terms of intra- and inter-day precisions and LOD, the authors could successfully determine endogenous hepcidin in urinary samples. However, the authors did not clarify why mixing of sample and synthetic hepcidin was realized after the sample treatment. This step could be carried out in advance in order to compensate sample losses during extraction individually for each sample.

Most of the reviewed MALDI-strategies show the potential of high sample throughput while delivering precise (semi)-quantitative results. However, fully quantitative analysis is still difficult to conduct using this type of instrumentation. Moreover, most MALDI-TOFMS methods do not offer MS/MS capabilities, resulting is a restriction in terms of selectivity, specially compared to LC-MS. Therefore, the separation of spectral interferences is of particular importance here.

2.2.2. LC-MS based strategies.- LC-MS, in contrast to MALDI-TOFMS, is commonly used for qualitative and quantitative analysis of biological samples. This is probably due to the very high selectivity and sensitivity provided by this technique: LC-MS combines the merits of chromatography and mass selective MS detection by hyphenation of an online separation technique. Since the ESI source is prone to generate multiple charged species, the formation of different molecule ions of hepcidin ($[Hep + nH]^{n+}$) is typically observed. For quantification, multiple reaction monitoring (MRM) can be a useful tool, particularly in the case of hepcidin since several transitions (pair of precursor ion and product ion) can be monitored. However, for hepcidin, as for any other analyte, the most intense transition needs to be determined. This principle of operation explains the high selectivity obtained, which is particular important if the analyte is embedded in a complex matrix like -

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in the case of hepcidin - serum or urine. Furthermore, an extremely low spectral background is obtained, resulting in very low detection limits.

However, some LC-MS devices are less attractive for quantification of biomolecules. This is particularly true for Q-TOF mass analyzers, which offer higher mass resolution but are limited by a smaller linear dynamic range[26] and poorer sensitivity, compared to triple quadrupole devices. Nevertheless, Crockett et al.[27] applied a LC-Q-TOFMS for hepcidin-25 quantification. Separation of hepcidin-25 from remaining impurities is was conducted by reversed phase HPLC and quantification was achieved by using synthetic hepcidin-24 ($^{13}C_6{}^{15}N_2$)Lys-24 as external standard. Although neither the employed mass analyzer nor the use of the external standard (which is not suitable for the correction of matrix effects) are ideal for a reliable quantification of hepcidin-25, the method was characterized as "well performing" in the Second Round Robin for plasma hepcidin methods.[14]

In contrast, most recently presented LC-MS methods dealing with hepcidin quantification in biological fluids are based on MRM. In this vein, isotopically labelled hepcidin standards are applied as internal standards. For the compensation of analyte loses, mixing of endogenous hepcidin and internal standard are performed before hepcidin extraction: hepcidin-25 ($^{13}C_9{}^{15}N$)Phe-9,($^{13}C_2{}^{15}N$)Gly-20) was used for the quantification of hepcidin-25 in human plasma samples by LC-chip–nanoESI-MS/MS.[28] Bansal et al.[29] presented an assay for the determination of serum hepcidin based on UPLC-MS/MS and hepcidin-25 ($^{13}C_6$)Phe-9,($^{15}N_1{}^{13}C_2$)Gly-20) as internal standard. The quantification of hepcidin in serum and urine sample was achieved also by Hwang et al.[30] who presented an LC-MS/MS assay using hepcidin-25 ($^{13}C_6{}^{15}N_2$)Lys-24 while Wolff et al.[31] applied hepcidin-25 ($^{13}C_6{}^{15}N_1$)Ile-2 as internal standard for this purpose.

All these internal standards seem adequate for the robust quantification of hepcidin-25 since their chemical and physical behaviour should be almost identical compared to endogenous hepcidin-25. Since quantification relies on signal ratios and not on signal

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intensities like, mixing of standard and analyte can be conducted at an early stage of sample treatment in order to improve analytical accuracy. However, the final accuracy of the hepcidin determination strongly depends on the accuracy of the internal standard concentration itself. Many of the isotopically labelled standards are quantified by amino acid analysis. This quantification procedure includes two critical steps, the hydrolysis of the peptide bonds and the derivatization of the resulting amino acids. Under typical conditions which result in the almost complete hydrolysis of a peptide, some amino acids like Ser and Thr (both present in hepcidin-25) are decomposed while Cys and Met are derivatized to cysteic and methionic acids, respectively.[32]

In addition, since amino acids barely respond to UV- or fluorescence detection, a derivatization step is conducted after hydrolysis which can be achieved either pre- or post-column. Finally, the amino acids are separated by reversed phase chromatography and detected by absorbance or fluorescence. Therefore, precaution regarding the purity grade of the hepcidin internal standards should be taken, since the presence of other peptides or impurities could lead to an overestimation of the analyte's concentration, ultimately limiting the accuracy of the hepcidin measurement.

As demonstrated in recent studies, LC-MS can be a powerful tool for the quasi-absolute quantification of hepcidin if the methods are carefully validated.

2.2.3. ICP-MS based strategies.-To overcome some of the main limitations of molecular MS used as a tool for quantitative analysis, such as the matrix and species dependent ionization efficiency, inorganic MS, namely ICP-MS (inductively coupled plasma mass spectrometry) has proven to be a useful complementary technique.[33] In the plasma, the generation of ions is independent from the structure of the analyte. Therefore, the detector response is proportional to the number of atoms, more precisely isotopes, of all species ionized in the plasma at a given time. This means, in contrast to molecular MS, specific standards are not necessarily required, allowing the quantification of certain species by

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analyzing simple inorganic standards containing the same element in a known concentration.[34]

In this vein, sulphur can serve as a natural element tag, since hepcidin is composed of 25 amino acids out of which 9 contain sulphur (8 cysteines and 1 methionine). The presence of this element enables the quantitative analysis of hepcidin by ICP-MS without derivatization. Konz et al.[35] presented a strategy for hepcidin-25 quantification in urine samples based on sulphur monitoring by ICP-MS after chromatographic separation of concomitant species. Then, hepcidin quantification was carried out by sulphur post-column isotope dilution analysis (IDA), taking into account the well defined stoichiometry between sulphur and hepcidin-25 (9:1). Although the general idea of this analytical strategy is quite promising, the authors were not able to quantify hepcidin-25 in urine samples mainly due to lack of sensitivity.

The same authors exploited the specific capabilities of hepcidin-25 for binding Cu(II) ions forming a stable complex[36,37] that could be used for its determination after chromatographic separation of the Cu excess.[38] The binding of Cu^{2+} with hepcidin-25 occurs, specifically, in the N-terminal region (in fact, the MS/MS spectrum of the complex shows the specific DTH-Cu intact fragment). Since none of the truncated forms possess the specific N-terminal sequence, they do not exhibit divalent metal binding properties. The hepcidin concentrations determined by this HPLC-ICP-MS method show a good agreement compared to published data as well as to the results of a commercially sandwich ELISA kit. Furthermore, the authors affirm that only hepcidin-25 is detected since hepcidin-22 and -20 show no complex-formation tendency with copper ions. Compared to molecular MS approaches such as MALDI/SELDI-MS or LC-MS, ICP-based ionization is compound independent. However, in contrast to molecular MS, no additional information regarding other isoforms (hepcidin-22 or 20) can be obtained by elemental MS.

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In the light of the current state of hepcidin analysis there seems to be no gold standard for hepcidin quantification available so far. This is mainly due to the high requirements such assay should fulfil, e.g. high sample throughput, high accuracy and specificity, no need for expensive equipment etc. Taking this into account, it seems to be worth considering if the aim to determine the gold analytical technique for its quantification should be revised. It might be a better option to establish an ideal strategy for different challenges, such as high sample throughput for clinical routine analysis and/or selective detection of all hepcidin isoforms for biomarker studies and/or absolute quantification of the peptide hormone for method validation. The high sample throughput provided by ELISA assays is limited, so far, by the selectivity. The MS methods are hampered by several other drawbacks although right now, they provide the adequate sensitivity and selectivity for conducting hepcidin-25 determination in biological samples. The development of more specific antibodies together with sensitive detection systems (e.g. electrochemoluminiscence) could be the best way to go once full validation is done using MS-based methods

3. Clinical applications of hepcidin testing

The first cELISA assay measuring urinary hepcidin in 2003[16] has allowed to demonstrate for the first time the linkage of hepcidin induction to inflammation and to support the key role of the hormone as a mediator of anemia of chronic disease, ACD (also known as anemia of inflammation).[39] This result was rapidly followed by the demonstration of the reverse situation, i.e. decreased urinary hepcidin levels, in patients with severe juvenile hemochromatosis.[40]

Since these pioneer studies demonstrating a causative role of the hormone in iron-related disease, a long way has been covered permitting today to classify iron-related disorders into two main classes according to abnormal hepcidin levels. In summary, hepcidin

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deficiency has been established to result in iron overload (both hereditary hemochromatosis and iron-loading anemias such as β-thalassemia). Conversely, hepcidin over expression has been proposed to contribute to the development of iron deficiency and anaemia's in infections, inflammatory diseases, some cancers, chronic kidney disease and in IRIDA, a genetic iron-refractory iron deficiency anaemia caused by loss-of function mutations in *TMPRSS6*, a negative regulator of hepcidin gene expression.[41] From these studies, therapeutic options have rapidly emerged and specific hepcidin-targeted strategies are already applied today in clinical trials.

Apart from using hepcidin value for therapeutic applications, the possible uses of hepcidin in diagnostic are diverse. An area of interest these last years has been the potential value of hepcidin to discriminate between iron deficiency anemia (IDA, low hepcidin), ACD (high hepcidin) and the combined state of ACD and IDA. The differentiation between ACD and ACD/IDA is of clinical importance to determine whether patients suffering from a concomitant true IDA during infection/inflammation could benefit or not from an oral iron therapy (similar to the one administrated in IDA alone).

In this line, van Santen et al., in a cross-sectional study on 155 patients with rheumatoid arthritis (RA), demonstrated that serum hepcidin was a potential useful indicator for detecting iron deficiency in RA patients with anaemia and active inflammation.[42] Similarly, Theurl et al[43] found in a study of 67 patients that ACD/IDA patients had significantly lower serum hepcidin levels than subjects with ACD alone, although the degree of inflammation was comparable, highlighting that hepcidin determination can aid in selecting the best iron therapy to be administrated to these patients. In the same vein, hepcidin level was measured in a cohort of 240 individuals with IDA to determine whether they may respond adequately or not to a course of oral iron.[44] The authors conclude that indeed, patient hepcidin baseline levels could be used to predict which patients will respond to oral iron supplementation.

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Challenging these results, Thomas et al. in a single centre study on 155 patients reported that hepcidin-25 was not a good marker for differentiation between ACD and ACD/IDA but the authors comment that they have used, in contrast to the other studies, the ferritin index to differentiate these two conditions.[45]

In chronic kidney disease (CKD) hepcidin determination has also been speculated as a diagnostic marker. Anaemia is highly prevalent in CKD patients primarily due to inadequate production of erythropoietin (EPO) and many patients are treated with ESAs. However, many patients are poorly responsive to this therapy due to abnormalities in iron balance that may be caused by increased levels of hepcidin. Indeed, in patients with CKD (as with patients with other chronic inflammatory diseases) a large number of studies have reported increased levels of hepcidin (most likely due to chronic inflammation and reduced renal clearance) that can contribute to reduce duodenal iron uptake and iron release from cellular iron stores. In the study by Van der Putten et al,[46] the authors suggest that hepcidin level, as a marker of iron overload, can predict EPO response at an early stage of treatment. Similarly, in cancer patients, where anaemia is highly prevalent and its inappropriate treatment may aggravate the outcome of the disease, systemic hepcidin has been proposed as a predictor of response to EPO therapy.[47]

However, there is some controversies in the literature as for the use of hepcidin in chronic disease to guide clinical decisions[48] and for some authors the large intra-individual variability of the hormone preclude its use as a marker of iron status.[49, 50]

Using hepcidin in assessing the response to existing therapies in cancer also represents an attractive possibility. The link between iron and cancer has been largely described and iron is usually considered as a cofactor for tumour cell proliferation both by promoting ROS-induced DNA damage and cell transformation, and by activating enzymes involved in the proliferation process through DNA synthesis. Compared to normal cells, tumour cells have high iron demands and increased iron induces resistance to chemotherapeutic agents.[51]

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Although the hepcidin-ferroportin pathway has been largely investigated in cancer cells, and despite several clinical studies reporting hepcidin levels in cancer patients (renal carcinoma, colorectal carcinoma, prostate cancer, lung cancer…) a comprehensive view of hepcidin as a pro-oncogen is far from being understood. In addition, the use of measuring circulating hepcidin in cancer for diagnosis and prognosis is uncertain, in particular because it is still unknown whether localized hepcidin production by the tumour cell could influence systemic hepcidin.

Finally, in hereditary hemochromatosis, hepcidin has been proposed as a biomarker to optimize the frequencies of phlebotomies. Indeed phlebotomy has been reported to decrease hepcidin levels and therefore could have a negative effect by exacerbating intestinal iron absorption.[52]

In conclusion, whether hepcidin could constitute a clinically useful marker for the evaluation of iron disorder is not clearly established and especially whether its dosage will be superior to the conventional methods used so far for evaluating iron deficiency is still unclear. Indeed, traditional methods rely on ferritin and transferrin saturation,[53] two markers with well known limitations due to fluctuation in response to various non-iron related conditions, conditions that are also to be taken into consideration for hepcidin, in particular inflammation. Finally and above all, there is an urgent need for the standardization of hepcidin assays to determine clinically useful reference ranges.

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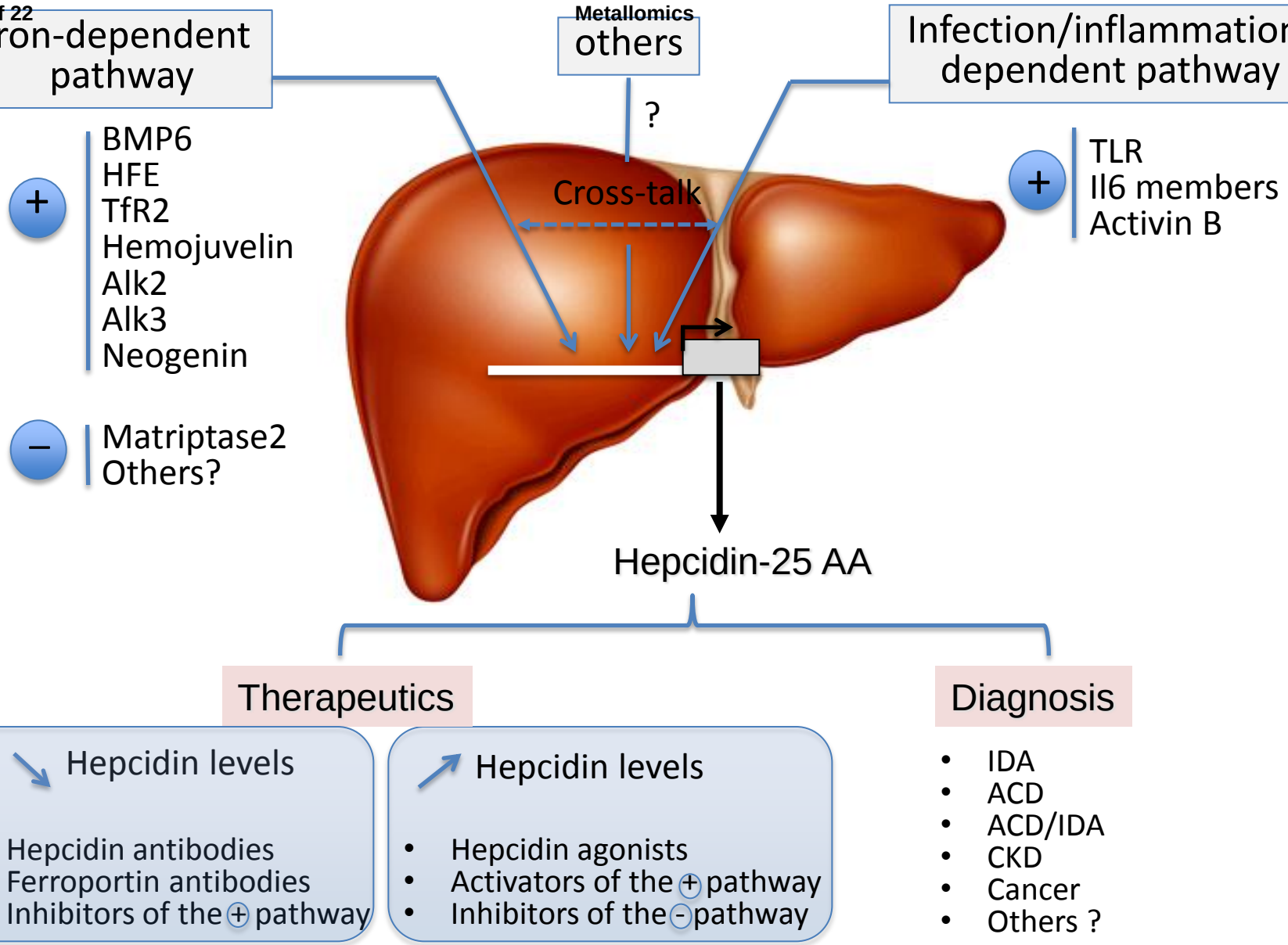

Figure 1. Schematic representation of the regulation of liver hepcidin gene expression and the potential use of the hormone in therapeutics and diagnosis

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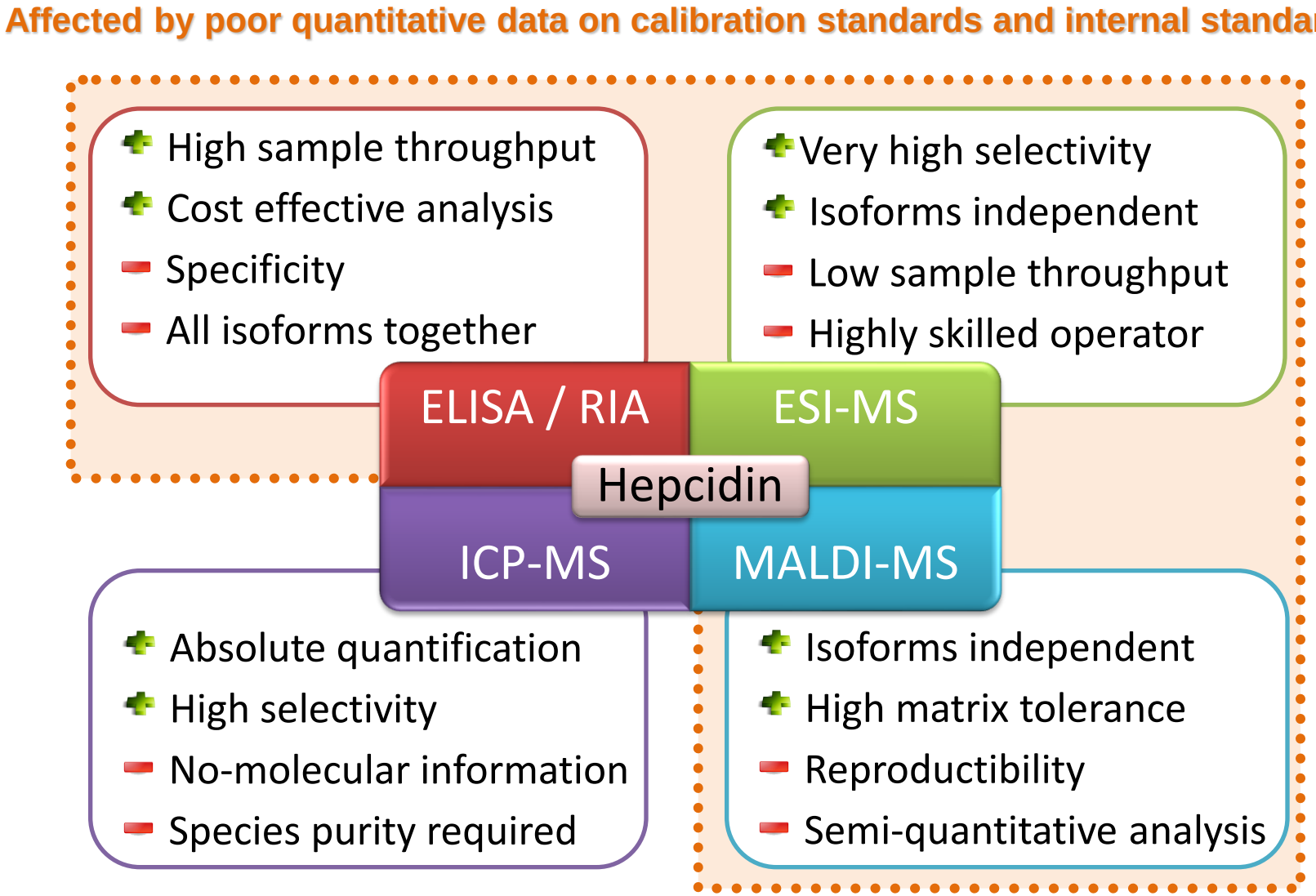

Figure 2. Advantages and limitations of the current methodologies used for hepcidin-25 quantification in biological fluids.

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