Measurement of iron-regulatory hepcidin and investigation of its role in perinatal iron homeostasis

*Thesis book*

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Introduction

Iron is essential for every live organism, however it can also present a risk as it can contribute to the generation of free radicals. Therefore human organism keeps iron homeostasis under strict control. The role of some of these molecules (ferritin, transferrin, transferrin-receptor 1) in the iron homeostasis is well known for decades, while recently several new molecules were identified having significant role in the iron homeostasis. These molecules include hepcidin.

Hepcidin is a defense like peptide discovered in 2000. From that time it was demonstrated that hepcidin decreases the expression of iron transporting molecules, first of all that of ferroportin, the only iron exporting molecule. Hepcidin reduces gastrointestinal iron absorption, iron release from the macrophages, and hence it decreases serum iron levels. The primary source of hepcidin is the liver where it is synthesized as prohepcidin. Clinical investigations suggested that inflammation, and iron overloads increase, while hypoxia and anemia decrease hepcidin production.

Clarification of hepcidin role in iron homeostasis could provide an explanation to anemia of inflammation and chronic diseases, in addition it could imply to new diagnostic and therapy possibilities in the iron metabolism disorders, like hemochromatosis and anemia of inflammation.

During our work I developed an accessible reproducible method for hepcidin measurements and obtained some data about its possible impact on neonatal iron homeostasis.
AIMS

1. To synthesize simplified, characterized hepcidin derivatives, which might be suitable as standard molecules for the development of hepcidin measurements?

2. To develop a method for the measurement of urinary hepcidin levels.

3. To measure serum prohepcidin and urinary hepcidin levels at birth and 48 hour after birth in healthy human newborns.
1. Synthesis and chemical characterization of hepcidin and hepcidin derivatives

We have synthesized the linear 25-mer hepcidin as along with 7 and 13-mer truncated versions of 25-mer hepcidin: hepcidin-25 (1-25), C-terminal segment (13-25), N-terminal segment (1-7), N-terminal segment with pentaglycin spacer ((Gly)_5-1-7). We have also synthesized their N-terminal modified derivatives: one derivative with acetyl group (Ac1-25), one derivative with biotinyl group (biotin-(Gly)_5-1-7) and the further derivatives with biotinil-Acp group: biotin-Acp-25, biotin-Acp-13-25, biotin-Acp-1-7.

The peptides were synthesized by Fmoc/tBu solid phase methodology. Cleaved peptides and modified derivatives were purified and characterized with reversed phase high performance liquid chromatography (RP-HPLC) using semi-preparative column. The primary structure and the chemical characterization were performed with mass spectrometry (ESI-MS, MALDI-TOF), and with amino acid analysis. The table below shows the code and amino acid sequence of synthesized peptide derivatives

<table>
<thead>
<tr>
<th>Peptide name and/or code</th>
<th>Peptide sequence</th>
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<tbody>
<tr>
<td>hepcidin-25 (1-25)</td>
<td>1DTHFPICIFCCGCCHRSKCGMCCKT^{25}</td>
</tr>
<tr>
<td>N-terminal segment (1-7)</td>
<td>1DTHFPIC^{7}</td>
</tr>
<tr>
<td>C-terminal segment (13-25)</td>
<td>13CCHRSKCGMCCKT^{25}</td>
</tr>
<tr>
<td>(Gly)_5-1-7</td>
<td>(Gly)_5-1DTHFPIC^{7}</td>
</tr>
<tr>
<td>biotin-(Gly)_5-1-7</td>
<td>biotin-(Gly)_5-1DTHFPIC^{7}</td>
</tr>
<tr>
<td>Ac-1-25</td>
<td>Ac^-1DTHFPICIFCCGCCHRSKCGMCCKT^{25}</td>
</tr>
<tr>
<td>biotin-Acp-25</td>
<td>biotin-Acp^-1DTHFPICIFCCGCCHRSKCGMCCKT^{25}</td>
</tr>
<tr>
<td>biotin-Acp-13-25</td>
<td>biotin-Acp^-13CCHRSKCGMCCKT^{25}</td>
</tr>
<tr>
<td>biotin-Acp-1-7</td>
<td>biotin-Acp^-1DTHFPIC^{7}</td>
</tr>
</tbody>
</table>

The sequence of the synthesized peptide derivatives (Characters are the code of aminoacids [IUPAC nomenclature, www.iupac.org], numbers shows the aminoacid positions according to the sequence of native hepcidin).
2. Functional characterization of hepcidin derivatives by immunadsorption methods

We performed the dot blot just as the direct and competitive ELISA measurements using commercially available polyclonal antibodies and oxidized standard hepcidin molecule, and the self-synthesized peptide derivatives. At the beginning of our work there were two commercially available rabbit anti-human IgG polyclonal antibodies (α-7, α-13). The α-7 antibody recognizes the sequence pattern of DTHFPIC, while the α-13 antibody recognizes the sequence pattern of CCHRSGMCCKT (Alpha Diagnostic Inc.).

3. Urine hepcidin quantification

Solid phase extraction of urine

We performed the solid phase extraction of urinary sample using the methods published before (Macroprep gyanta, NP20 Proteinchip), than several SUPELCO solid phase extraction tubes. We tested the suitability of the Macroprep CM resin and NP20 protein chip during mass spectrometry (primarily SELDI-TOF) for purification of urine and concentrating the hepcidin. We tested the suitability of 7 SUPELCO solid phase extraction tubes (DSC-8, DSC-Ph, DSC-CN, DSC-18Lt, DSC-SAX, DSC-WCX and NH2).

Urine hepcidin quantification using MALDI-TOF mass spectrometry

The MALDI-TOF MS measurements were performed with a Bruker BIFLEX III mass spectrometer (Bruker, Bremen, Germany) equipped with a TOF analyzer.

4. Healthy newborns

Twenty healthy, full-term neonates were enrolled into the study. We collected from cord-blood samples at birth and then postnatal peripheral blood samples from infants suspected to be ill during the first 72 postnatal hours. (However, laboratory tests and a later medical history indicated the absence of disease) We also obtained urinary samples at the same time.

We performed the Mann-Whitney U test to analyze the difference between values obtained in samples at birth and postnatal samples. We used linear regression to test the association between changes in parameters of erythropoiesis, iron homeostasis and serum prohepcidin along with urinary hepcidin levels.
RESULTS AND DISCUSSION

1. Synthesis and chemical characterization of hepcidin and hepcidin derivatives

We have synthesized the N-terminally biotinylated derivatives for an immunadsorption method. The N-terminally acetylated derivative was used for a mass spectrometry method for urinary hepcidin measurement. The homogeneity of synthesized peptide derivatives was verified with RP-HPLC; the purity was up to 95%. Amino acid sequence was justified by amino acid sequence analysis. The calculated and measured mass weight of peptide derivatives did not differ significantly. Therefore we demonstrated that synthesized peptide derivatives are identical to the desired ones. Peptide derivatives are water soluble and hence are suitable for making solutions with appropriate concentrations for biological studies (1-5 mg/ml). Since our peptide derivatives contain cystein we also tested their stability in liophilised and liquid state after storage at 4ºC. We observed a dimerization rate of ~5-6% and ~15% in liophilised and liquid samples, respectively.

2. Functional characterization of hepcidin derivatives by immunadsorption methods

We performed the dot blot experiments in order to develop a method suitable for human urinary hepcidin measurements. We used our own peptide derivatives 1-25, 13-25 and 1-7, a commercially available, 25-mer, oxidized standard hepcidin molecule, and a human urinary sample during the dot-blot analysis. Results indicate that commercially available antibodies recognize the synthetic peptide derivatives 1-25, 13-25 and 1-7. Both antibodies recognize the 1-25 peptid derivatives. Affinity of 1-25 peptide derivatives to the polyclonal rabbit hepcidin antibodies was comparable with activity of commercially available peptide standard. According to our data we suppose that this dot blot method does not allow the measurement of the whole concentration range of hepcidin in human urinary samples.

We performed the ELISA experiments in order to develop a method which is suitable for human urine hepcidin quantification. For direct ELISA experiments we used the N-terminally biotinylated peptid derivatives (biotin-Acp-1-25, biotin-Acp-13-25, biotin-Acp-1-7 and biotin-(Gly)₅-1-7). Results support that commercially available antibodies recognize these peptide derivatives. Both antibodies recognized biotin-Acp-1-25 peptid derivative. According to the competitive ELISA experiments in case of 1-7 and biotin-Acp-1-7 (see figure below), just as in case (Gly)₅-1-7 and biotin-(Gly)₅-1-7 peptide pair the antigen-antibody reaction and the competition also functioned with α7 antibody.
Standard curves of the competitive ELISA using peptide 1-7 and biotin-Acp-1-7.

Concentration of biotin-Acp-1-7 peptide: 12.5 ng/μl.

In case of 13-25 and biotin-Acp-13-25 peptide-pair with α13 antibody, just as in case of 1-25 and biotin-Acp-1-25 peptide-pair with α7 and α13 antibodies the antigen-antibody reaction were evolved but the competition was not detectable. The suspected cause of this phenomenon is the presence of dimers and oligomers formed under circumstances of ELISA.

Our synthesized peptide derivatives contain one or more cysteins. Between cysteins intra- and intermolecular disulfide bonds may develop that could affect the result of antibody based methods. Therefore we tested the formation of disulfide bonds in our peptide derivatives under experimental conditions used for ELISA. With two different methods we demonstrated that truncated peptide derivatives (1-7, biotin-Acp-1-7, Gly)s-1-7 and biotin-(Gly)s-1-7) do not form dimmers, while other peptide derivatives (13-25, biotin-Acp-13-25, 1-25 and biotin-Acp-1-25) do.

**Summary of hepcidin peptide derivatives synthesis and characterization:**

The synthesis or isolation of native hepcidin is a complex process. The lack of a general available hepcidin standard presents a great barrier to the development of a method for urinary hepcidin measurements. The truncated hepcidin peptide derivatives (1-7 peptid, Gly)s-1-7 derivatives) produced in our lab are recognized by the commercially available antibodies. Another advantage of these derivatives is that they are easy, quick and cheap to be
produced in large quantities compared with the native form. These simplified peptide derivatives might be suitable representatives of the 25 amino acid form of hepcidin in immune adsorption method using commercially available polyclonal antibodies.

3. Urine hepcidin quantification using MALDI-TOF mass spectrometry

Solid phase extraction of urine

According to previous data urinary hepcidin concentration is low. Furthermore, there are several urinary substances may interfere with quantitative measurements. The direct measurement of human urinary samples with MALDI-TOF without any pretreatment is not convenient and reliable.

During method development we tested the suitability of methods used previously for urine purification during hepcidin measurement (Macroprep, NP20 Proteinchip). Macroprep resin was used previously for urine purification in HPLC and immunodot methods. Our preliminary experiments showed that even a small volume of urinary sample is suitable for purification with cationic exchange resin; however, it is an effective but time-consuming procedure with a low reproducibility. In SELDI-TOF based methods for urinary hepcidin quantification NP20 Protein chip was used for urine purification. According to our data, however, the purification with NP20 Protein chip is not effective enough and is also not reproducible for MALDI-TOF MS measurements.

To find an effective, reproducible, quick method for urine purification and hepcidin concentration we tested seven different SPE tubes (Supelco), which were selected especially to hepcidin by reversed-phase chemistry attribution (DSC-8, DSC-Ph, DSC-CN, DSC-18Lt, DSC-SAX, DSC-WCX and NH2) and measured with MALDI-TOF mass spectrometry. Our results showed that these solid phase tubes are suitable for hepcidin extraction from urine with good recovery. Considering the MALDI-TOF MS signal intensity and relative selectivity we have chosen the “DSC-8” tube for additional measurements.

Measurement of urine hepcidin levels using MALDI-TOF mass spectrometry

We used the N-terminal acetilated peptide derivative (ac-1-25) in MALDI-TOF measurements as a hepcidin-related internal standard. The peptide derivative 1-25 and Ac-1-25 crystallized well with matrix and possessed good ionization efficiency under MALDI conditions. The measured molecular weights were 2797.0 and 2839.9 Da at 1-25 and ac-1-25 peptide derivative, respectively, therefore we were able to avoid peak-overlapping in MALDI-TOF MS spectra. In order to compare the ionization efficiency of standards, i.e., ac-1-25
peptide (AcHep) to that of 1-25 peptides (Hep), these two peptides were mixed in different concentration ratios and the MALDI-TOF MS spectra of these mixtures were recorded. MALDI-TOF MS intensity ratios (I_{Hep}/I_{AcHep}) were plotted on figure below as a function of the corresponding concentration ratio (c_{Hep}/c_{AcHep}). (where I_{Hep} and I_{AcHep}, c_{Hep} and c_{AcHep} stand for the MALDI-TOF MS intensity and concentration of the 1-25 and ac-1-25 hepcidin, respectively).

Variation of I_{Hep}/I_{AcHep} ratio with the c_{Hep}/c_{AcHep} ratio (I_{Hep} and I_{AcHep}, c_{Hep} and c_{AcHep} stand for the MALDI-TOF MS intensity and concentration of 1-25 and ac-1-25 peptide, respectively)

As it is evident from figure above the I_{Hep}/I_{AcHep} ratio varies linearly with the c_{Hep}/c_{AcHep} ratio yielding a slope of 1.31 and an intercept of 0.25 (mean: 1.412; SD: 0.123). This result indicates that the synthetic 1-25 peptide has slightly higher ionization efficiency than synthetic acetyl-1-25 peptide, and linearity in the range of concentration ratio 0.2 – 2.5 can be attained. (The purity and peptide content did not differ significantly).

We also tested whether the relative intensity of 1-25 peptides to the ac-1-25 peptide changes with absolute concentration. Therefore, the two peptides (hepcidin 1-25 and acetyl-1-25) were mixed in 1:1 ratio but at different concentrations ranging from 0.6 to 30 mg/L and the MALDI-TOF MS spectra of these mixtures were recorded. The ratio of the measured intensity of peptides (I_{Hep}/I_{AcHep}) did not differ significantly from the value (y=1.3132) calculated from the curve (mean: 1.412; SD: 0.123).
MALDI-TOF spectra of mixture of purified urine from a healthy volunteer and ac-1-25 peptide (ac-1-25 peptide concentrations in the different blocks: (a: 0.6; b: 3; c 6; d: 18 mg/L).

Furthermore, in order to assess the effect of added internal standard concentration on the calculated hepcidin concentration in urine sample, the MALDI-TOF MS peak intensity of hepcidin from a healthy human volunteer’s urinary sample were compared to that of the internal standard (ac-1-25) added at six different concentrations (see figure above) added to the samples and the adherent urinary hepcidin concentrations were calculated based on the calibration curve.

We have observed that the concentration of added internal standard has minimal effect on calculated hepcidin concentration, i.e., minimal charge competition or signal suppression occurs for hepcidin-25 in this concentration range. However, the intensities of hepcidin isoforms (i.e., hepcidin-20 and hepcidin-22) decrease with increasing the concentration of internal standard, i.e., the presence of internal standard in higher concentration hinder the detection of these hepcidin isoforms. Therefore we compared the MALDI-TOF MS peak intensity of hepcidin from healthy newborns urine sample to two concentrations of internal standard.
Summary of measurement of urine hepcidin levels:

We developed a novel MALDI-TOF MS based semi-quantitative, reproducible method for measuring hepcidin concentration in human urine using a synthesized hepcidin related internal standard. We used an easy and quick solid phase extraction for purification of urine and demonstrated that this method is comparable to the previously described method (Macroprep and NP20 proteinchip). This new semi quantitative assay for human urine hepcidin will extend the possibilities for developing an available reproducible method, and the synthetic, acetylated hepcidin could be a uniform internal standard in hepcidin assays.

4. Healthy newborns

The results of red blood cell and iron parameters in healthy newborns were in the same range as those reported previously. Red blood cell count, hemoglobin, hematocrit, mean corpuscular hemoglobin concentration (MCHC) and ferritin levels in postnatal samples were higher than in corresponding cord blood samples, while iron levels and transferrin saturation were lower.

Prohepcidin

Prohepcidin was detected in each cord blood and postnatal sample. Compared with cord blood samples, serum prohepcidin levels in postnatal samples were higher in 13 neonates, while they were lower in 7 neonates (see figure below).

Changes in serum prohepcidin levels in the first postnatal days. Time of postnatal blood collecting (median; range): 39; 18-114.
This indicates that newborns may be able to synthesize prohepcidin (data from animal model shows that fetus able to synthesize prohepcidin). We found no significant variation between cord blood and postnatal samples (median, range: 93.92, 51.81-150.93 in cord blood; 107.15, 0.32-245.44 in postnatal samples ng/ml respectively). However, it is still uncertain whether prohepcidin measured in cord blood is exclusively of fetal origin, or is in part maternal prohepcidin which has crossed the placenta.

Serum prohepcidin levels showed no association with any of investigated iron homeostasis or blood cell parameters, but related to MCHC in cord blood ($r=0.558$, $p=0.013$). The association with MCHC was not present in postnatal samples (see figure below).

![Graph showing Association between serum prohepcidin and MCHC in cord blood.](image)

Association between serum prohepcidin and MCHC in cord blood.

Our results that serum prohepcidin shows association with MCHC, is important because there are no generally used parameter for iron stores in fetus. Some parameter are dependent on gestational age (such as mean corpuscular volume, mean corpuscular hemoglobin), while others are influenced by the physiological stress of birth, the time the unilical cord is cut (serum iron, ferritin, hemoglobin, hematocrit) and hemolysis due to oxidative stress during the first days of life (mean corpuscular volume). The ratio of Hb/Ht (i.e. MCHC) is independent of these factors and it is a potentially suitable indicator for the recent intrauterine iron supply. Therefore, MCHC may reflect the adequacy of iron supply for the fetus during the last trimester such as Nicolas et al suggested. Our finding that prohepcidin is closely and positively associated with MCHC in cord blood may indicate the possible implication of prohepcidin in the regulation of iron uptake of the fetus.
Interestingly, infants with detectable non protein bound iron (NPBI) values (n=6) presented with lower prohepcidin levels than those without NPBI (n=14) (serum prohepcidin (median, range in infants with detectable NPBI: 64.52, 51.81-121.81; and with undetectable NPBI: 101.14, 54.43-150.93 ng/ml; p=0.047). NPBI showed no association with serum iron levels, however prohepcidin may associate with antioxidant defense.

**Hepcidin**

Hepcidin level was determined in urinary samples obtained at birth and postnatal in 17 healthy newborn baby. During our measurements urinary hepcidin level was not detectable in some infants, while in others urinary hepcidin exceeded the upper limit of published healthy adult’s reference range.

Urinary hepcidin levels increased postnatal in 11/17 newborn (median, range; at birth: 0 (0-5016) postnatal: 3647 (0-14359) ng/mg creatinin; p=0,013; see figure below) and this change was significant even after the adjustment of sampling date. The difference was significant between the two groups, urine hepcidin level were increased in postnatal samples. This correlation was true after the sample date correction too.

![Changes in urinary hepcidin levels during postnatal](image)

Urinary hepcidin levels associated with iron serum level and total iron binding capacity but were independent from erythropoietic parameters, as well as CRP, NPBI or serum prohepcidin levels. (This finding is in contrast to that obtained in adults and suggesting an
association between urinary hepcidin levels and erythropoietic and serum iron homeostasis-parameters).

In newborns with higher urinary hepcidin levels, serum iron level and total iron binding capacity decreased. This finding corresponds to physiological effects of hepcidin, but it does not explain the decrease of total iron binding capacity. In our patients total iron binding capacity did not alter significantly during the early postnatal period. There was no association between hepcidin level and NPBI values. However according to the close association between hepcidin and total iron binding capacity one can assume that hepcidin is responsible for increased antioxidant defense.

Summary of healthy newborns results

During the first postnatal days in human healthy newborns urinary hepcidin levels significantly increased, while serum iron levels dropped. This finding suggests the role of hepcidin may in the early postnatal adaptation of iron homeostasis.
1. According to native 25-mer human hepcidin amino acid sequence we performed the solid phase synthesis of 9 hepcidin peptide derivatives (1-25, biotin-Acp-1-25, 13-25, biotin-Acp-13-25, 1-7, biotin-Acp-1-7, (Gly)$_5$-1-7, biotinil-(Gly)$_5$-1-7 and ac-1-25). After the chemical characterization of synthesized peptide derivatives (amino acid analysis, RP-HPLC and ESI-MS) we have demonstrated that the purity of desired peptides is 95% purity.

2. We functionally characterized the peptide derivatives and established structure-function relationship:
   a. We proved that commercially available α13 antibody recognizes the 1-25, biotin-Acp-1-25, 13-25 and biotin-Acp-13-25 peptide derivatives.
   b. We proved that commercially available α7 antibody recognizes the 1-25, biotin-Acp-1-25, 1-7, biotin-Acp-1-7 and (Gly)$_5$-1-7, biotinil-(Gly)$_5$-1-7 peptide derivatives.

3. We proved that the self-synthetic 1-25 and ac-1-25 peptide derivatives have good ionization efficiency under MALDI conditions.

4. We used a quick solid phase extraction method for purification of urine and demonstrated that this method is comparable to the previously described method. We presented a novel MALDI-TOF MS-based semi-quantitative, reproducible method for measurement of hepcidin concentration in human urine using a hepcidin-related internal standard produced in our lab.

5. We demonstrated that during early post adaptation of healthy human newborns serum prohepcidin does not fluctuate significantly in contrast to urinary hepcidin levels. During postnatal adaptation serum prohepcidin levels associated significantly with MCHC, while urinary hepcidin levels correlated with serum iron levels and total iron binding capacity values. Serum prohepcidin and urinary hepcidin levels showed no correlation with each other. Interestingly, healthy newborns with detectable non protein bound iron values presented with lower prohepcidin levels than those with undetectable non protein bound iron values.
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Publications


