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PROTEOMICS IN TRANSLATIONAL AND CLINICAL RESEARCH

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I hereby declare that this thesis has been written solely by myself and that all the sources used in this thesis are cited and included in the references part. The research was carried out in the space of the Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacký University Olomouc.

In Olomouc ……

April 2016 Dušan Holub, M.Sc.

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Abstrakt

Předkládaná disertační práce si klade za úkol využít pokročilé proteomické metody v klinické praxi a diagnostice. V jejím úvodu jsou popsány jednotlivé fáze vývoje proteomických biomarkerů a to od jejich hledání, následné verifikace, validace až k měření klinických vzorků. Dále popisuje odlišné operační módy hmotnostního spektrometru, které jsou pro vývoj biomarkerů v daných fázích adekvátně vhodné. V teoretické části je také popsána regulace metabolismu železa a v této souvislosti význam hepcidinu, který byl analyzován, stejně tak i stručný teoretický úvod k analýze amyloidóz, což je druhá klinická jednotka, kterou práce zpracovává.

Následující, experimentální část práce je zaměřená na stanovení hladiny hepcidinu v lidském séru pomocí cílené proteomiky. Prvním cílem, který byl naplněn je vývoj a validace SPE-LC-MS/MS metody pro stanovení hladiny hepcidinu v lidském séru. Na základě dosažených výsledků vyplývá, že stanovení hladiny hepcidinu v séru, v kombinaci s existujícími diagnostickými metodami je přínosné pro porozumění patogeneze různých, zejména hematologických onemocnění spojených s narušenou homeostázou železa. Druhé experimentální téma je zaměřeno na přesnou typizaci depozit amyloidu pomocí proteomické a imunohistochemické analýzy. Vzorky tkání byly vyšetřeny pomocí obou zmíněných metod, přičemž v práci jsou diskutovány jejich výhody a nevýhody. Obě studované metody byly vyhodnoceny jako vhodné pro typizaci amyloidu, přičemž proteomický přístup vykázal mnohem slibnější výsledky v porovnání s imunohistochemickou typizací.

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Abstract

Aim of the present thesis is to use advanced proteomic methods in clinical practice and diagnostics. In introduction are described the different phases of development of proteomic biomarkers and from their searches, subsequent verification, validation and measurements of clinical samples. It also describes different operational modes of the mass spectrometer, which are suitable for particular development stages. The theoretical part also describes the regulation of iron metabolism and in this context the importance of hepcidin, which has been analysed, as well as a brief introduction to the theoretical analysis of amyloid, which is the second clinical entity studied in the thesis.

The experimental part of the work is focused on quantification of hepcidin in human serum using targeted proteomics. The first goal, to develop and validate SPE-LC-MS/MS method for measurement of hepcidin level in human was acomplished. Based on the obtained results, the determination of hepcidin levels in serum is useful for understanding the pathogenesis of various, especially hematologic diseases associated with impaired iron homeostasis. The second experimental subject is typing amyloid deposits using proteomic and immunohistochemical analyses. Tissue samples were examined using both of these methods, while at work are discussed their advantages and disadvantages. Boths studied methods were classified as suitable for amyloid typing, the proteomic approach has shown more promising results compared to immunohistochemical analysis.

TABLE OF CONTENTS

1 Introduction – Proteomics in translational and clinical research

1.1 Introduction to proteomics biomarker discovery

1.1.1 Work-flow of biomarker development

The goal of clinical proteomics is an identification of proteins showing different expression levels between disease and control subjects and a development of quantitative assays for their measurement (Schiess et al., 2009; Hüttenhain et al., 2009). These clinical assays would contribute to solutions of various clinical needs (e.g. prediction, diagnosis, prognosis, or treatment monitoring) (Domon & Aebersold, 2010). A complete proteomic biomarker discovery study includes the generation of candidate protein markers using a limited number of well-defined samples. It is followed by multiplexed verification of the differential proteins expression using a targeted platform on a larger number of sample sets, and unbiased validation of a marker panel using clinical assays in large retrospective and prospective collections of samples from target population (Surinova et al., 2011). The process of biomarker development is a long, difficult and uncertain path. All phases of proteomics biomarker development study are showed in Figure 1 and are described below in more detail.

Numerous proteomic studies demonstrated the impact of proteomic analysis on answering key biological questions (Cravatt et al., 2007) and discovery of more appropriate protein biomarkers (Schiess et al., 2009; Faca et al., 2007). Biomarkers are indicators of normal biological and pathogenic processes as well as pharmacologic responses to a therapeutic intervention. The clinically useful biomarkers enable clinicians to detect and identify a disease at an early stage, to stratify patients for a suitable treatment, and to follow disease progression and regression (Surinova et al., 2011). Biomarkers should be objectively measured and evaluated in clinical subjects. The clinically useful biomarkers, which have excellent performance (high specificity and sensitivity) could by measured in relatively non-invasive manner using readily obtained samples. For this reason, much interest has focused on the use of biofluids, such as plasma, cerebrospinal fluid, breath condensate, urine, and tissues, as the samples of choice for the measurement of biomarkers.

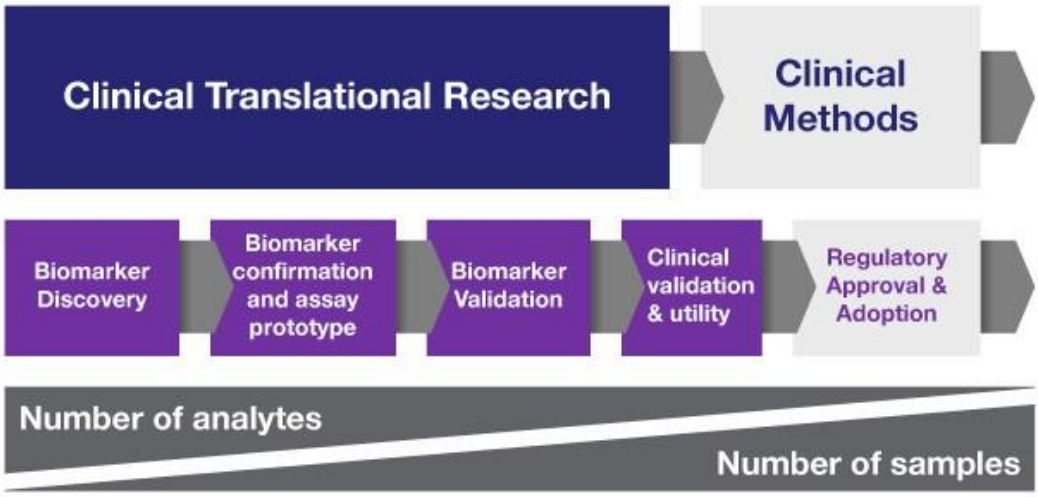

Figure 1. A general scheme of protein biomarker development workflow (https://www.thermofisher.com/cz/en/home/clinical/clinical-translational-research/protein-analysis/protein-biomarker-discovery-validation.html).

Phase I – Preclinical discovery

Discovery phase aims an identification of protein candidates for the defined clinical objective. The common proteomics strategies employ a discovery-driven unbiased approach by analyzing of small number of samples (10), which represent the case and control state in comparative manner, which generate a list of protein candidates with fold changes and their corresponding significance (Domon & Aebersold, 2010). Final list of protein biomarker candidates include protein from discovery phase, together with candidates gained from analyzing the literature and of the publicly available databases (Hüttenhain et al., 2009).

Phase II – Preclinical verification

In this phase, suitable multiplexed analytical platform need to be considered and evaluated in terms of reproducibility, dynamic range and limit of detection (Schiess et al., 2009). The sample size is represented by small cohort (10 – 50), where patients classification into case and control subjects is known (Surinova et al., 2011). Preclinical verification phase aims a development of reliable biomarker candidate assay that find a mean concentration difference between case and control subject.

Phase III – Preclinical validation

In this phase, the analytical (accuracy and precision) and clinical (sensitivity and specificity and indication of clinical utility) performance of a protein signature is determined (Domon & Aebersold, 2010). At this phase, retrospective study, based on known clinical data (disease cases and controls) is performed. Sample size ranges between 100 - 500 biological samples (Surinova et al., 2011). At this phase, an algorithm to weigh and intergrade the most promising protein assays into a protein panel is developed. Biomarker panel makes a promise to warrant clinical evaluation, only if the performance characteristics meet the defined clinical requirement (Surinova et al., 2011). This biomarker panel will be further applied for prospective study.

Phase IV – Clinical evaluation

The goal of the phase IV is a development of the final biomarker assay (e.g. ELISA) platform that fulfills the clinical requirements, such as an in vitro diagnostics (IVD) test, and a determination the true and false positive rates of subject classification into respective groups with the biomarker test (Surinova et al., 2011). Usually, sample size ranges between 500 - 1000 biological samples. Phase IV is a prospective study and involves newly recruited subjects (disease cases and controls) from the target population (Manolio et al., 2006).

Phase V - Disease control

In this phase, it is define the impact of the biomarker test, which is accepted for clinical use, in the disease burden on the target population. Outcome measures include survival time and potential effects of the biomarker test on costs and quality of life (Surinova et al., 2011).

1.1.2 Proteome and strategies for reduction of sample complexity

The proteomes of biological samples are extremely complex (Aebersold & Mann, 2003). The Human Genome Project has revealed that there are probably about 20 300 human genes and has given the word a resource of detailed information about the structure, organization and function of the complete set of the human genes. The proteome is far more complex than the genome owing alternative RNA splicing and post-translational modifications that lead to approximately 1 million proteins.

Additionally, the dynamic range in protein concentration present in the biological samples is wide (e.g. in the case of human plasma is more than ten orders of magnitude) (Figure 2).

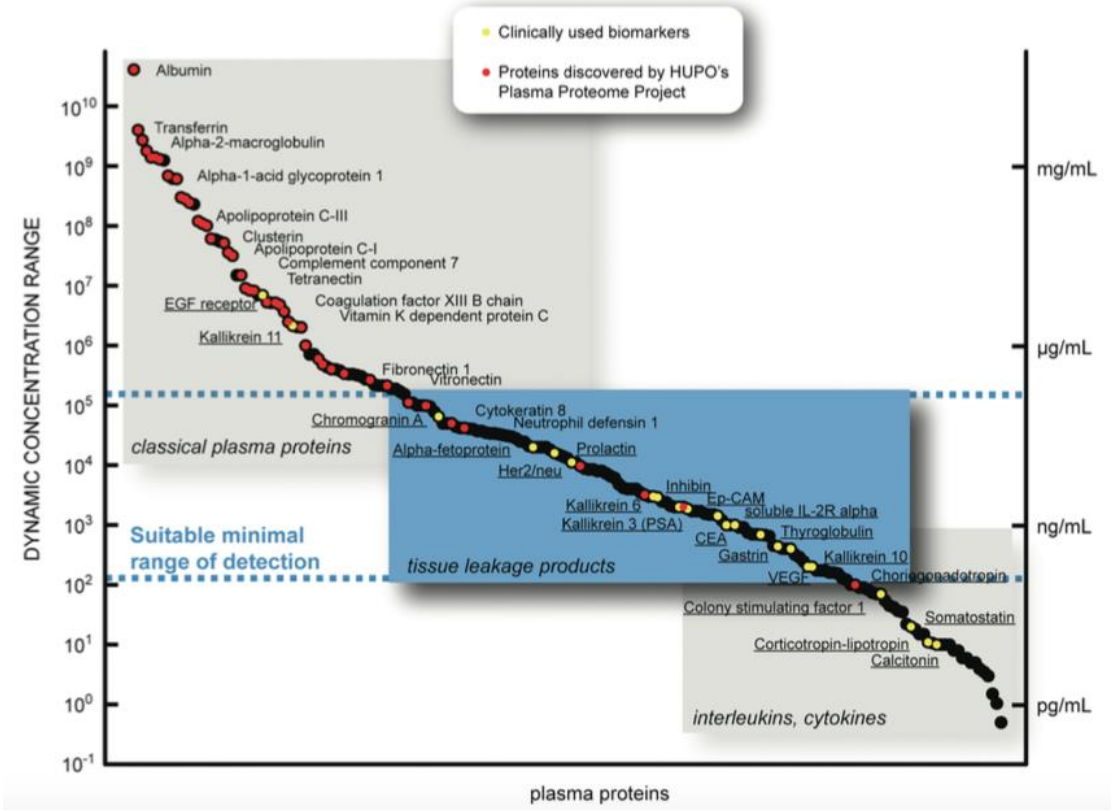

Figure 2. Dynamic plasma protein concentration range (Surinova et al., 2011).

For these reasons, the interrelated issues of high complexity and wide dynamic range of the analytes need to be addressed in comprehensive profiling of proteins for biomarker discovery studies. The most common strategies for reduction of sample complexity applied before LC-MS analysis are: 1) extensive fractionation, such as peptides fractionation based on pI, SCX, hydrophobicity and molecular size; 2) selective enrichment, such as cys-peptide enrichment (Liu et al., 2005), glycopeptide enrichment (Abbott & Pierce, 2010), phosphopeptide enrichment (Mann et al., 2002); 3) selective depletion, such as immunoaffinity based depletion of most abundant proteins in human plasma (Echan et al., 2005). Reduction of the sample complexity depends on the purpose of study and target proteins or post-translational modifications, which need to be quantified (Hüttenhain et al., 2009; Angel et al., 2012) (Figure 3).

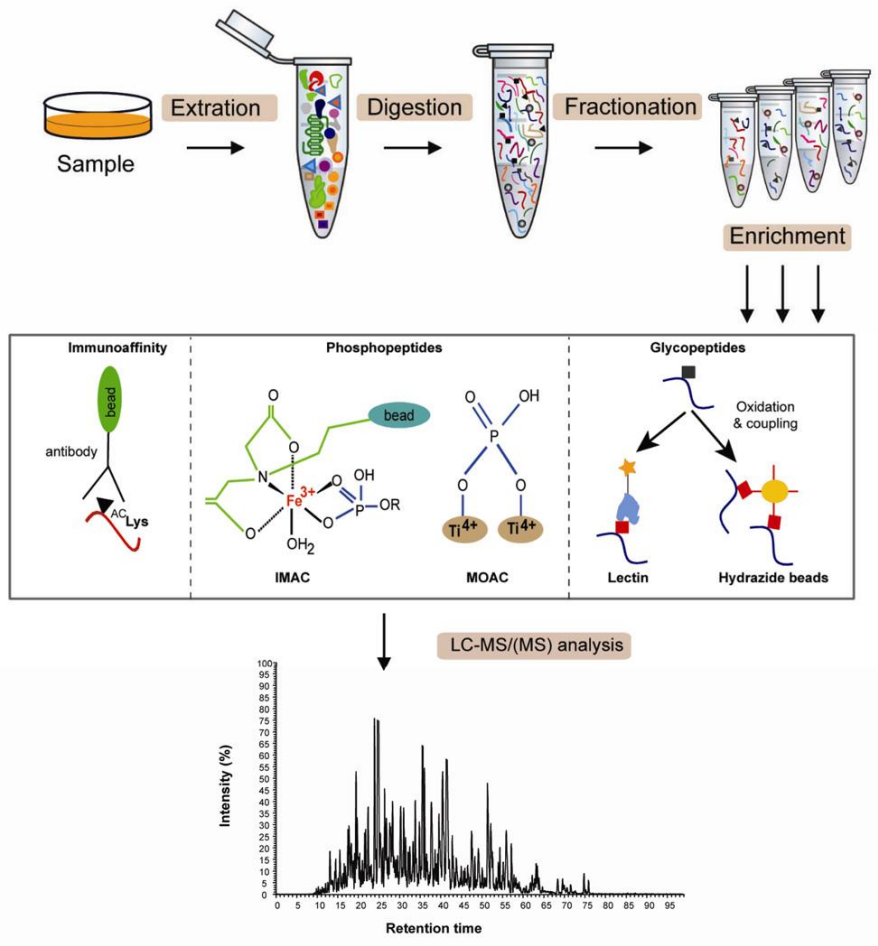

Figure 3. Reduction of sample complexity based on the targeted peptide capturing (Angel et al., 2012).

1.1.3 Bottom-up and top-down proteomics

Two proteomic approaches, bottom-up and top-down, were developed for peptide or protein profiling (Figure 4). The bottom-up approach, widely used in MS-based proteomics research, is applied for peptide profiling (Aebersold & Mann, 2003). It is also known as shotgun proteomics, involving digestion of a protein sample using a proteolytic enzyme, such as trypsin, Lys-C, for creation a complex peptide mixture. The digested samples are analyzed by liquid chromatography and tandem mass spectrometry (LC-MS/MS). The top-down approach is applied for protein profiling, involving separation of intact proteins from complex samples followed by mass spectrometry analysis. It enables a detection of native protein mass ions, sequence variants and combinations of post-translational modifications of biological proteins species (Kelleher et al., 2014).

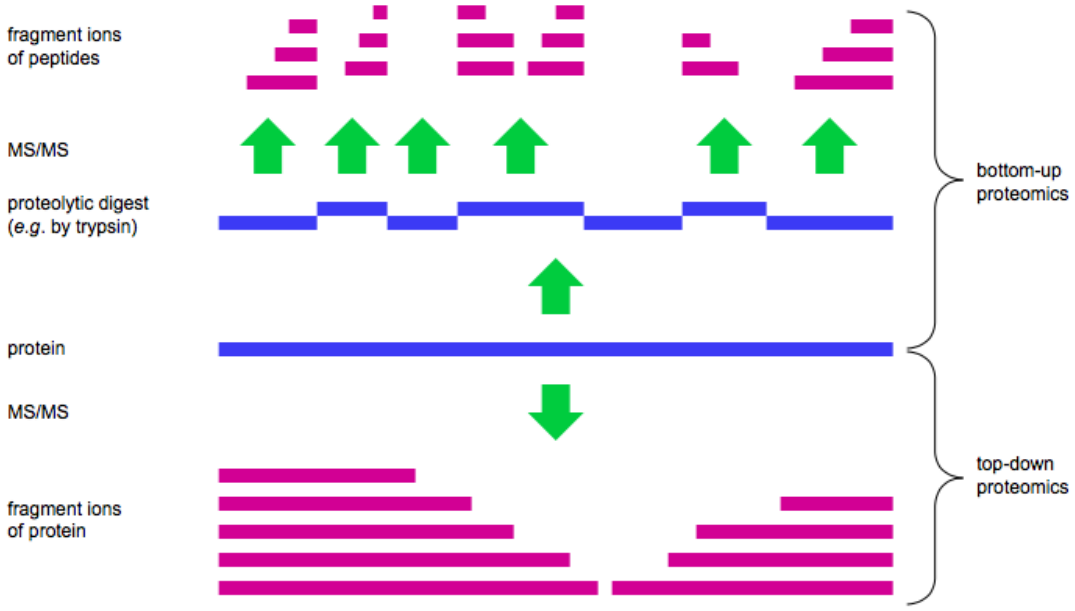

Figure 4. Bottom-up and top-down proteomics. Bottom-up proteomics is a method to identify proteins and characterize their sequences by proteolytic digestion of proteins prior to analysis by mass spectrometry (Aebersold R & Mann M, 2003). Top-down proteomics is a method of protein identification that uses an ion trapping mass spectrometer to store an isolated protein ion for mass measurement and tandem mass spectrometry analysis (Kelleher NL, 2004) (https://upload.wikimedia.org/wikipedia/commons/f/f0/Bottom-up_vs_top_down.svg).

1.1.4 Mass spectrometry

Mass spectrometry (MS)-based proteomics has become an important technology platform for qualitative and quantitative study of protein expression, modification, integration and degradation (Aebersold & Mann, 2003). Over the last two decades, mass spectrometry-based technologies overcame a rapid advances and a high degree of innovation for fulfilling the expectations of the proteomics and life science communities (Surinova et al., 2011). Common components of all mass spectrometers are an ion source, a mass analyzer and an ion detector. Liquid or dried protein samples are loaded into mass spectrometer and then vaporized and ionized by the ion source. Two most common ionization methods are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) (Mann et al., 2001). Commonly used mass analyzers include ion traps, orbitraps, quadrupoles and time of flight (TOF). Mass analyzers are used for separation of all analytes in a sample for global analysis or for filtration of specific ions towards the detector (Domon & Aebersold, 2010).

Mass spectrometer operation mode

The mass spectrometer operates in specific scanning mode. Various operating modes are available for unsupervised and supervised peptide profiling. Unsupervised profiling includes untargeted data acquisition, such as data dependent acquisition (DDA) and data independent acquisition (DIA) (Domon & Aebersold, 2010; Gillet et al., 2012). Supervised profiling includes targeted data acquisition, such as selected reaction monitoring (SRM) and parallel reaction monitoring (PRM) (Hüttenhain et al., 2009; Domon & Aebersold, 2010; Peterson et al., 2012).

Data dependent acquisition (DDA)

DDA is the most widely used mass spectrometry strategy for in-depth peptide profiling (Mann et al., 2001). A series of precursors for MS/MS analysis are selected based on preset parameters, such as ion intensity and charge state. In this mode, MS/MS spectrum of the peptide provides sufficient signals for peptide search process. The analyzed ions are excluded from the selection of precursors within a certain time windows. This allows analysis of peaks with lower ranking of the intensity. The logical selection of precursors in DDA mode enables a systemic acquisition and efficient data analysis in shotgun profiling. This MS strategy is ideally suitable for discovery phase and can achieve the identification of thousands of proteins from a biological sample (de Godoy et al., 2008). DDA was used for amyloid subtyping, subscribed in the third part of this thesis.

Data independent acquisition (DIA)

DIA is recently developed method based on parallel MS/MS analysis that selects several precursor ions concomitantly. The workflow skips the specific precursor selection step by fragmenting the entire mass range or using wide (10 - 25 Da) precursor windows stepwise, providing convoluted MS/MS spectra containing ion fragments derived from multiple precursors (Gillet et al., 2012). Fragments and precursor ions are identified after acquisition, by decoding their coelution profiles in MS/MS spectra over the chromatographic separation. Due to the unbiased nature and highly multiplexing capacity, DIA renders a universal approach for verification and validation phases in biomarker investigation. A variety of software's have been developed to DIA-date processing, such as DIA-Umpire, OpenSWATH, Skyline and Spectronaut^{TM}.

Selected reaction monitoring (SRM) and parallel reaction monitoring (PRM)

To date, SRM represents the most widely used and targeted proteomics method for accurate and reproducible quantification of proteins in complex biological samples (Hüttenhain et al., 2009) (Figure 5). It uses LC system for peptides separation on analytical column, which is connected in-line to the ESI source of triple quadrupole (QQQ) mass spectrometer. The first and third quadrupole (Q1, Q3) are used as mass filters with selection windows (0.7 - 1 Da) and the second quadrupole (Q2) serves as the collision cell. Based on the SRM setting, the Q1 selectively transmits precursor ions of a given m/z value to the collision cell at a given time. After fragmentation using collision-induced dissociation (CID) in Q2, several specific product ions are selectively transmitted through the Q3 to the detector (Lange et al., 2008). Targeted proteomics is focused on analysis of a subset of proteins, such as candidate biomarkers. SRM strategy offers multiplexing for protein measurements and can monitor several hundreds of peptides in a one-hour analysis (Stahl-Zeng et al. 2007). It shows the potential for fast verification of biomarker candidates in clinical samples and thereby closes the gap between discovery and validation of the biomarker development pipeline. This targeted method was used for quantification of hepcidin level in human serum, subscribed in the second part of this thesis.

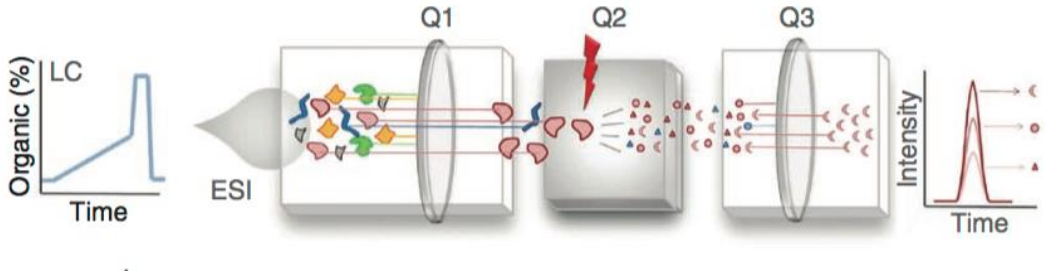

Figure 5. Principle of SRM using a triple quadrupole mass spectrometry (QQQ). In SRM experiment, a complex peptide sample is chromatographically separated on an analytical column that is connected in-line to an ESI source. Molecular ions of specific analyte are selected in Q1 and fragmented in Q2. A specific fragment ion from the target analyte is selected in Q3 and guided to the detector. Commonly, several transitions are monitored per peptide and several peptides per protein. On right, cycles through three transitions, corresponding to three different fragments of the target peptide, and the corresponding three SRM traces are shown (Picotti & Aebersold, 2012).

Targeted proteomic method, similar to SRM, is PRM, where all the detectable fragmentation ions from preselected precursor are recorded and used for quantification (Peterson et al., 2012). In this method, the Q3 is replaced with a high resolution

Orbitrap analyzer, which improves the identification and quantification of the targeted peptides.

In general, proteomics technology offers a robust, quantitatively accurate, and high throughput analytical platform for biomarker discovery, verification and validation. The integration of the discovery, verification and validation platforms with standardized protocols will streamline the general implementation of the LC-MS assays in clinical settings as well as in the translational research for personalized medicine. In the proposed thesis we will demonstrate usefulness of different proteomic approaches in identification of our molecules of interest (hepcidin-25 and amyloidogenic proteins) in different biological materials like serum and formalin fixed paraffin embedded tissue samples.

1.2 Gene and metabolic regulation of iron metabolism - Introduction to hepcidin proteomic analysis

1.2.1 Role of iron and its distribution in the human body

Iron is an essential trace element for nearly every living organism. The average adult human contains 3 – 4 g of iron, most of which is localized in erythrocyte hemoglobin (2 – 3 g of iron). Other major reserved organs of iron are liver and spleen, where it is stored in macrophages and hepatocytes in a specialized cytoplasmic iron storage protein, ferritin. Muscle contains iron predominantly stored in myoglobin that is an oxygen storage protein. All cells contain smaller concentrations of iron-containing proteins essential for energy production, synthetic metabolism, and other important functions. Iron is distributed to tissues through blood plasma, which contains only 2 – 4 mg of iron, bound to the iron-transport protein transferrin. Plasma iron turns over 20 – 25 mg iron per day (Ganz & Nemeth, 2012). Although smaller amounts of iron are also recovered by macrophages from other cell types, most plasma iron is derived from aged erythrocytes that are recycled by macrophages in the spleen and other organs. As the lifespan of human erythrocytes is 120 days, 0.8% (or 15 – 25 mg) of all erythrocyte iron must be recycled every day. In turn, iron is extracted from the plasma compartment mostly for hemoglobin synthesis by erythrocyte precursors, regulated separately by erythropoietin in response to tissue oxygenation. Despite rapid turnover and changes in iron utilization, plasma iron concentrations are generally stable, indicating that the delivery of iron from recycling macrophages into plasma is homeostatically controlled (Figure 6) (Ganz, 2013).

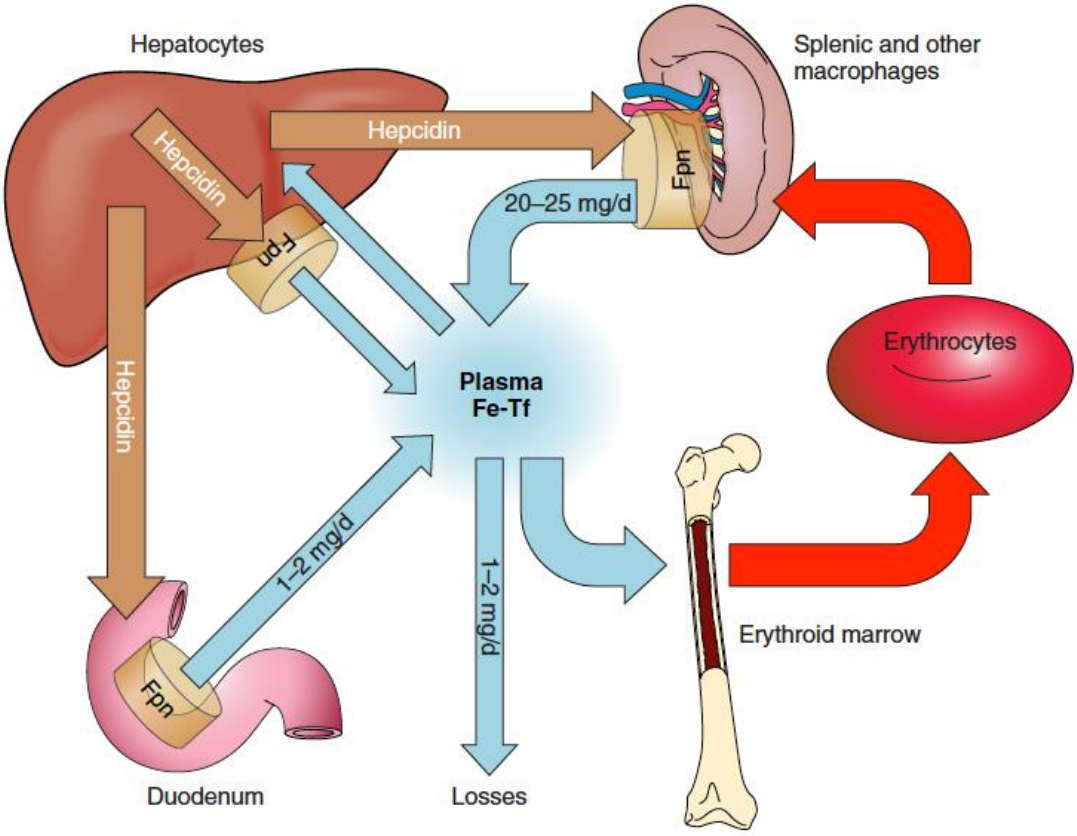

Figure 6. Major iron flows and their regulation by hepcidin and ferroportin. Transferrin bound iron is indicated by blue, and iron of erythrocytes by red colour. Hepcidin controls the iron flow into plasma by inducing of the ferroportin (brown) endocytosis and proteolysis (Ganz, 2013).

1.2.2 Hepcidin, history, sequence, structure, synthesis and its isoforms

Hepcidin is a key regulatory protein that controls intestinal absorption of iron and its distribution throughout the body (Nicolas et al., 2001; Nemeth & Ganz, 2006; Kartikasari et al., 2008). It was initially extracted from plasma ultrafiltrate (Krause et al., 2000) and urine (Park et al., 2001) samples as a small bactericidal peptide and named liver-expressed antimicrobial peptide (LEAP-1). Hepcidin has antimicrobial (*Escherichia coli*, *Neisseria cinerea*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and group B streptococcus bacteria) and antifungal activity (*Candida albicans*, *Aspergillus niger* and *Aspergillus fumigatus*) (Krause et al., 2000; Park et al., 2001). The name ‘hepcidin’ originates from the place of its synthesis in hepatocytes (hep-) and its antimicrobial activity (-cidin).

Produced by the liver, high level of hepcidin reduces iron absorption in response to oral or intravenous iron. On the other hand, limited iron decrease hepcidin levels, which allows enhanced intestinal iron absorption and release of storage iron. Hepcidin circulates in blood plasma mostly as a free molecule except for weak binding to

albumin and α_2-macroglobulin (Itkonen et al., 2012), is filtered and excreted by the kidneys (Park et al., 2001).

Hepcidin is a 25 amino acid protein (DTHFPICIFCCGCCHRSKCGMCCKT) with four disulphide bridges at Cys_7-Cys_{23}, Cys_{10}-Cys_{22}, Cys_{11}-Cys_{19} and Cys_{13}-Cys_{14} (Krause et al., 2000; Park et al., 2001, Figure 7). The NH_2-terminal hepcidin segment of six amino acids is highly conserved, unstructured, and it is crucial for interaction with its receptor ferroportin (Nemeth et al., 2006). The rest of the molecule is a bent β-hairpin cross-linked with highly conserved disulphide bonds (Jordan et al., 2009).

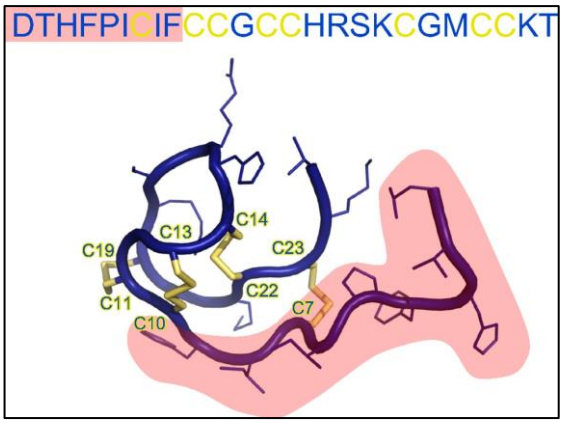

Figure 7. The amino acid sequence and structure of hepcidin. The NH2-terminal segment known to interact with ferroportin is shaded by light red. The characteristic cysteines and their disulfide bonds are shown by yellow (Ganz, 2013).

Human hepcidin is encoded by *HAMP* gene, localized on chromosome 19 (Krause et al., 2000; Park et al., 2001). The mature hepcidin-25 is generated from an 84-amino acid prepropeptide containing a characteristic NH_2-terminal 24-amino acid signal sequence that is cleaved to yield the cellular intermediate prohepcidin. The generation of the mature 25-amino acid form requires furin-like prohormone convertases that cleave prohepcidin at the COOH-terminal peptide bond after a characteristic polybasic sequence (Valore & Ganz, 2008).

The NH_2-terminally truncated shorter isoforms of hepcidin (hepcidin-20 and hepcidin-22) are found in human urine and (hepcidin-20) in human plasma (Campostrini et al., 2012; Park et al., 2001). They are most likely the result of the degradation of the bioactive form of hepcidin (hepcidin-25), but the relevance of these isoforms still needs to be determined.

1.2.3 Regulation of iron homeostasis by hepcidin

The major regulatory action of hepcidin is binding the sole known cellular iron exporter ferropotin and controlling its membrane concentration (Nemeth et al., 2004). As ferroportin is the transporter that delivers dietary, stored, or recycled iron to plasma, the hepcidin-ferroportin interaction effectively controls the flux of iron into plasma and the iron supply to all the iron-consuming tissues. The molecular mechanisms responsible for ferroportin degradation rely on hepcidin-induced ubiquitination of lysines in a cytoplasmic loop of the exporter, its subsequent endocytosis and degradation in the lysosomes (Nemeth et al., 2004; Qiao et al., 2012; Ross et al., 2012). Hepcidin induced loss of ferroportin decreases iron transfer to plasma and causes hypoferremia. On the contrary, hepcidin deficiency increases iron transfer to plasma and causes systemic iron overload. This suggests that organisms can control plasma iron level and maintain iron metabolism homeostasis by modulating hepcidin expression (Kemma et al., 2008).

1.2.4 Factors responsible for the regulation of hepcidin synthesis

Hepcidin synthesis is regulated at the transcriptional level by multiple stimuli. The major regulators of hepcidin expression are iron status, inflammation and anaemia (Figure 8).

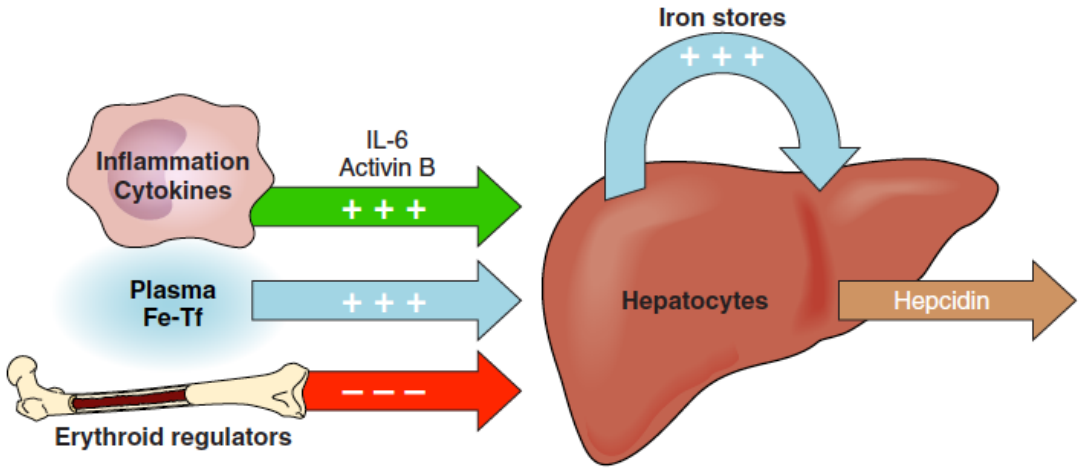

Figure 8. Regulation of hepcidin synthesis or inhibition in hepatocytes. The two major positive regulators are iron stores (blue), inflammation (green), and major negative regulator is erythroid activity (red) (Ganz, 2013).

In the iron-regulated pathway, extracellular iron in the form of holotransferrin (HoloTf) binds to its two receptors, TfR1 and TfR2, which communicate with each other via iron specific adaptor hereditary hemochromatosis protein (HFE) (Gao et al.,

2009) and sensitize the bone morphogenetic protein (BMP) receptor to its ligands such as BMP6 (Andriopoulos et al., 2009). Membrane linked coreceptor hemojuvelin also potentiate the BMP receptor activation, which then controls hepcidin transcription via the SMAD pathway (Wang et al., 2005). BMP signalling is also modulated by MT-2 protease, which cleaves hemojuvelin, and by neogenin, which may augment or stabilize membrane hemojuvelin (Lee et al., 2010; Silvestri et al., 2008). In hepatocytes, intracellular iron is also sensed, possible through a mechanism that enhances the expression of BMP6 mRNA and protein, eventually leading to activation of the BMP receptor (Feng et al., 2012). The BMP signalling pathway is initiated upon BMP binding to a BMP receptro complex on the external part of the cellulat membrane, which activates the receptor kinase activity to phosphorylate the cytoplasmic proteins SMAD1, SMAD5, and SMAD8. Then the transcription factor complex with SMAD4 is formed and translocated into the nucleus to induce the transcription of hepcidin gene (Zhao et al., 2013).

Hepcidin transcription is prominently increased by inflammation, predominantly through the activity of IL-6 cytokine, its receptor and its JAK-STAT3 pathway (Nemeth et al., 2004). Activated JAK2 is phoshorylating STAT3 which translocates into the nucleus and induces hepcidin expression through binding to the STAT3 response element in the hepcidin protomoter (Schmidt, 2015). Other cytokines, including IL-22 and activin B, also contribute to hepcidin transcription (Armitage et al., 2011; Besson-Fournier et al., 2012). The iron-regulated pathway and inflammatory pathway are the two well-understood regulators of hepcidin. Current understanding of molecular mechanisms of hepcidin regulation is shown in Figure 9.

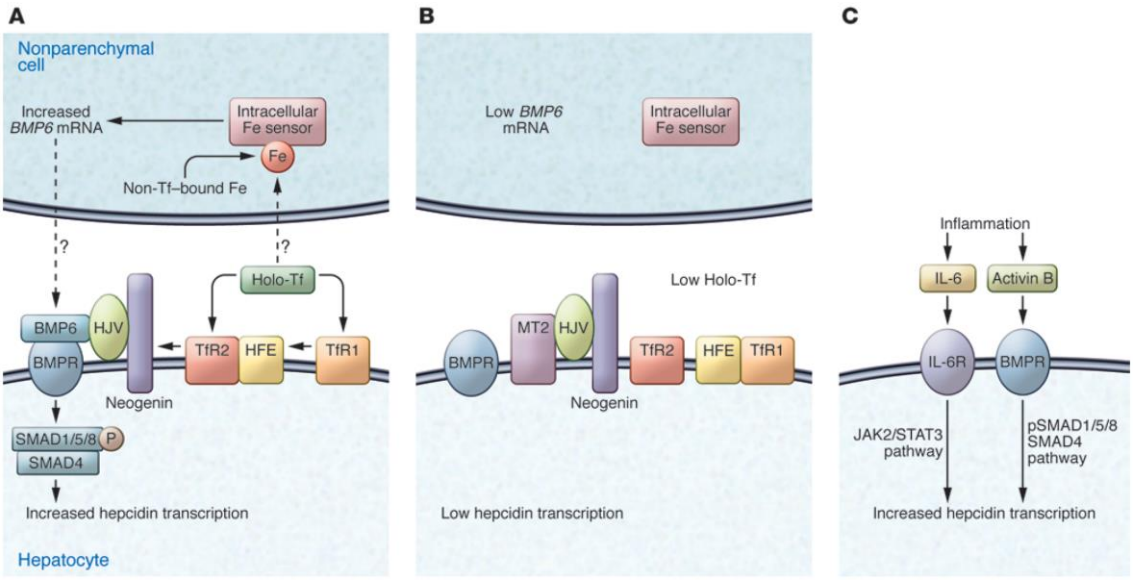

Figure 9. A current model of hepcidin transcription regulation by iron and inflammation. A) Under high iron conditions, increased loading of Tf with iron stabilizes TfR2, disrupts the HFE-TfR1 interaction, and induces BMP6 secretion, which facilitates the formation of a complex to induce hepcidin expression. B) Low iron conditions increase MT-2 protease, which induces the cleavage of hepatic HJV. The BMP6 secretion is reduced which is lowering the hepcidin expression. C) Inflammation induces the expression of IL-6 and actvitin B in the liver, which activates the transcription of hepcidin via the STAT3/JAK2 and BMP signalling pathway (Zhao et al., 2013).

It was confirmed that anaemia belongs to regulators of hepcidin expression (Ganz & Nemeth, 2012). Current model suggests that the bone marrow produces a hepcidin suppressor in response to erythropoietin (Liu et al., 2012; Masaratana et al., 2013). A similar effect has been postulated in anaemia with ineffective erythropoiesis where hepcidin is decreased despite iron overload and even in the absence of transfusions (Gardenghi et al., 2010). GDF-15, a BMP family member whose serum concentrations are increased in iron loading anemias, has been proposed as a hepcidin suppressor in β-thalassemia, but its contribution to hepcidin suppression and iron load in these conditions remains uncertain (Tanno et al., 2010). Further studies are needed to define the pathophysiological erythroid regulators of hepcidin.

1.2.5 Hepcidin and iron disorders

Hepcidin and its role in iron homeostasis helped to simplify and rationalized our understanding of pathogenesis of most common iron disorders. Some disorders were described as a result of mutations in genes that encode ferroportin, hepcidin or their physiological regulators (Table 1.).

Table 1. Hepcidin and iron disorders

Disorder	Genes mutated	Hepcidin levels
Hereditary hemochromatosis (hepdicin deficiency)	*HFE, TFR2, HFE2, HAMP*	Low
Hereditary hemochromatosis (hepdicin resistance)	*SLC40A1*	High
Ferroportin disease	*SLC40A1*	-
Iron-refractory iron deficiency anemia	*TMPRSS6*	High
Hypotransferrinemia	*TF*	Low
β-Thalassemia intermedia	*HBB*	Low
Chronic hepatitis C, alcoholic liver disease		Low
Anemia of inflammation		High
Anemia of chronic kidney diseases		High

HAMP gene encodes hepcidin; *HBB* gene encodes hemoglobin subunit beta (β-globin); *HFE* gene encodes hereditary hemochromatosis protein; *HFE2* gene encodes hemojuvelin; *TF* gene encodes transferrin; *TFR2* gene encodes transferrin receptor protein 2; *TMPRSS6* gene encodes transmembrane protease serine 6 (Matriptase-2); *SLC40A1* gene encodes solute carrier family 40 member 1 (Ferroportin-1).

Hereditary hemochromatosis (HH) is a genetic disorders characterized by the excessive absorption of dietary iron and its deposition into the liver and other organs, such as pancreas and heart. Liver injuries leading to cirrhosis and hepatocellular carcinoma are common clinical manifestations of HH, but the toxicity can also damage other organs. The various forms of HH are caused by mutations in *HFE, HFE2, TFR2*, or *HAMP* genes, and the common feature of HH is hepcidin deficiency (Ganz, 2011).

In rare cases, HH due to mutation in ferroportin encoding *SLC40A1* gene result in resistance to hepcidin and high hepcidin levels with parenchymal iron overload. The ferroportin mutation interferes with hepcidin binding and causes impairment of hepcidin-induced internalization of ferroportin (Sham et al., 2009).

In other cases of iron disorders with excess levels of hepcidin, iron-refractory iron deficiency anemia (IRIDA) is caused by mutation of *TMPRSS6* gene encoding transmembrane protease serine 6. Mutation in this enzyme leads to high levels of serum hepcidin despite severe iron deficiency (Finberg et al., 2008). This increase in hepcidin levels presumably caused due to increased BMP signaling, resulting from the inability of mutated enzyme to cleave membrane hemojuvelin (Silvestri et al., 2008).

Hypotransferrinemia is another rare genetic disorder characterized by a severe deficiency in serum transferrin, which leads to hepcidin deficiency. The defect causes iron-deficient erythropoiesis, a condition with iron deficiency anemia and severe iron overload in all non-hematopoietic tissues. The hepcidin deficiency can be caused by two ways: deceased stimulation of iron sensor (TfR1, TfR2 and HFE) that regulates hepcidin production and/or by the suppressive effects of erythropoietin-stimulated bone marrow on hepcidin production (Trombini et al., 2007).

The secondary iron disorders are caused by diseases, which originate outside of iron-homeostatic system. The β-thalassemia belongs to iron-loading anemias with ineffective erythropoiesis and high erythropoietin levels. Hepcidin concentration is low in patients who do not undergo transfusion (Papanikolaou et al., 2005), despite high serum ferritin and severe iron overload (Origa et al., 2007). Transfusion decreases erythropoietin and erythroid activity in the bone marrow and iron loading subsequently increasing hepcidin levels (Origa et al., 2007).

Chronic liver diseases, such as chronic hepatitis C, alcoholic liver disease, are accompanied by hepcidin deficiency and hepatic iron overload (Fargion et al., 2011). However, the mechanisms that impair hepcidin synthesis remain yet to be elucidated.

In the chronic kidney diseases, impaired renal functions cause increase in blood hepcidin concentration, which can lead to the characteristic anemia and erythropoietin resistance (Busbridge et al., 2009).

Anemia of inflammation, also called "anemia of chronic disease (ACD)", is accompanied by mild to moderate anemia with hypoferremia. In ACD, iron is stored in macrophages and intestinal iron absorption is decreased. Hemoglobin synthesis is impaired due to limited iron delivery to the maturing erythrocytes. Hepcidin synthesis is stimulated by cytokine (IL-6), which leads to trapping of iron into macrophages and enterocytes. Increased serum hepcidin concentrations have been found also in various diseases, such as acute infection, Castleman disease (Song et al., 2010), inflammatory diseases (Ganz et al., 2008), Hodgkin disease (Hohaus et al., 2010), malaria (de Mast et al., 2010), multiple myeloma (Maes et al., 2010) and in a subset of patients with solid tumors (Butterfield et al., 2010).

1.2.6 Methods used for hepcidin measurements

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 (Konz et al., 2014). Therefore, a number of analytical methods have been developed in order to assess hepcidin concentration in human or animal plasma, serum and urine.

Two types of strategies have been applied for hepcidin quantification – immunochemical (IC) and mass spectrometry (MS) based methods. The main difference between these two strategies is that while MS based methodologies permit us to distinguish between hepcidin forms (hepcidin-20, -22 and -25), methods based on immunochemical principles measure just the total hepcidin (all forms) concentration. Different methods, extraction strategies and applied standards for determination of the hepcidin level are summarized in Table 2.

Table 2. Characteristics of methods used for serum, plasma or urine hepcidin measurements.

Type of strategy	Methods	Hepcidin extraction		Standard	Purchased from	Reference
IC	Competitive ELISA	None	Ext.	Hepcidin-25[a]	Bachem	(Ganz et al., 2008)
IC	Competitive ELISA	None	Ext.	Recombinant Hepcidin-25	In house made	(Koliaraki et al., 2009)
IC	Competitive ELISA	None	Ext.	Hepcidin-25[a]	Peptide Int.	(Kroot et al., 2010)
IC	Competitive ELISA	None	Ext.	Modified Hepcidin-25 fragment	DRG	(Schwarz et al., 2011)
IC	Sandwich ELISA	None	Ext.	Hepcidin-25[a]	Peptide Int.	(Butterfield et al., 2010)
IC	Competitive RIA	None	Ext.	Hepcidin-25[a]	Bachem	(Busbridge et al., 2009)
IC	Competitive RIA	None	Ext.	Hepcidin-25[a]	Peptide Int.	(Grebenchtchikov et al., 2009)
MS	SELDI-TOF MS	IMAC	Ext.	Hepcidin-25[a]	Peptide Int.	(Altamura et al., 2009)
MS	SELDI-TOF MS	IMAC	Ext.	Hepcidin-25[a]	AltaBioscience	(Ward et al., 2008)
MS	MALDI-TOF MS	WCX	Ext.	Hepcidin-24[a]	Peptide Int.	(Swinkels et al., 2008)
MS	MALDI-TOF MS	SD ppt buffer	Int.	[$^{13}C_6$, $^{15}N_4$-Arg_{16}]-Hepcidin-25[a]	Sigma Aldrich	(Anderson et al., 2011)
MS	MALDI-TOF MS	WCX	Int.	Hepcidin-25$^{(+40\ Da)\ a}$	Peptide Inc.	(Laarakkers et al., 2013)
MS	LC-MS/MS (SRM)	Ultrafiltration	Int.	[$^{13}C_6$, $^{15}N_1$ - $Ile_{6,\ 8}$]-Hepcidin-25[a]	Bachem	(Kobold et al., 2008)
MS	LC-MS/MS (SRM)	ACN ppt, SPE (HLB)	Int.	Calcitonin gene-related peptide	Peptide Int.	(Murphy et al., 2007)
MS	LC-MS/MS (SRM)	TCA ppt	Int.	[$^{13}C_{18}$, $^{15}N_3$]-Hepcidin-25[a]	Peptide Int.	(Murao et al., 2007)
MS	LC-MS/MS (SRM)	SPE (HLB)	Int.	[$^{13}C_6$, $^{15}N_1$-Phe_4]-Hepcidin-25[a]	In house made	(Li et al., 2009)
MS	LC-MS/MS (SRM)	WCX	Int.	[$^{13}C_9$-Phe_9, $^{13}C_2$, $^{15}N_1$-Gly_{20}]-Hepcidin-25[a]	Peptide Int.	(Bansal et al., 2010)
MS	LC-HRMS	ACN ppt	Int.	[$^{13}C_{18}$, $^{15}N_3$]-Hepcidin-25[a]	Peptide Int.	(Rochat et al., 2013)
MS	LC-MS/MS (PRM)	SPE (Zorbax C-18)	Int.	[$^{13}C_9$, $^{15}N_1$-Phe_9]-Hepcidin-25[a]	Peptide Int.	(Li et al., 2015)

ACN, acetonitrile; ELISA, enzyme linked immunosorbent assay; Ext., external standard; HLB, hydrophilic lipophilic balanced reversed phase sorbent; HRMS, high resolution mass spectrometry IC, immunochemical based; IMAC, immobilized metal affinity chromatography; Int., internal standard; LC, liquid chromatography; MALDI, matrix assisted laser desorption/ionization; MS, mass spectrometry based; MS/MS, tandem MS; ppt, protein precipitation; PRM, parallel reaction monitoring; RIA, radioimmunoassay; SD ppt buffer, solvent-detergent precipitation buffer; SELDI, surface enhanced laser desorption/ionization; SPE, solid phase extraction; SRM, selected reaction monitoring; TCA, trichloroacetic acid; TOF, time of flight; WCX, weak cation exchange

[a] Synthetic standard

1.2.6.1 Immunochemical methods

Two main immunochemical strategies for hepcidin quantification are enzyme-linked immunosorbent assay (ELISA), that could be competitive (Kroot et al., 2010; Ganz et al., 2008; Koliaraki et al., 2009; Schwarz et al., 2011) or sandwich (Butterfield et al., 2010) and competitive radioimmunoassay (RIA) (Busbridge et al., 2009; Grebenchtchikov et al., 2009). The main benefits of both methods are high sample throughput, cost effective analysis and easily incorporated assay into routine labs. The advantage of RIA over ELISA is its high sensitivity for quantification of hepcidin level in human serum, where detection limits can reach 0.02 ng/mL (Grebenchtchikov et al., 2009). Nevertheless, the RIA is usually less accepted than ELISA due to the safety concerns regarding the use of radioisotopes. The main drawback of both methods is their inability to distinguish between hepcidin isoforms (hepcidin-20, -22 and -25). As hepcidin is small molecule, which contains four disulfide bonds, low diversity of hepcidin isoforms are not suitable candidates for traditional immunochemical methods.

1.2.6.2 Mass spectrometry based methods

Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) and surface enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF MS) are widely applied techniques for the qualitative analysis of molecules. However, this type of instrumentation is only seldom applied for quantitative analysis.

In SELDI-TOF MS analysis, proteins of interest are captured by specific interactions with different surfaces that are used as a laser desorption ionization target. Proteins, which do not bind to the surface, are removed by washing, while bound proteins are analysed by TOF mass spectrometry. In MALDI-TOF MS analysis, the weak cation exchange (WCX) magnetic beads (Swinkels et al., 2008; Kroot et al., 2010), immobilized metal affinity chromatography (IMAC; Ward et al., 2008; Altamura et al., 2009) or solvent detergent precipitation buffer (Anderson et al., 2011) are used for hepcidin enrichment for subsequent MALDI-TOF MS analysis.

Altamura at al. described IMAC-SELDI-TOF MS method, where quantification of the hepcidin levels was based on peak heights in mass spectra (Altamura et al., 2009), which do not always reflects the correct concentration of hepcidin in samples. The main benefits of these methods are simple to perform and higher sample throughput in

comparison to LC-MS. Ward et al. described IMAC-SELDI-TOF MS, which already used stable isotope labelled analogue of the hepcidin as internal standard and quantification was based on ratio of endogenous hepcidin and internal standard (Ward et al., 2008). SELDI-TOF MS assay was optimized also for quantification of urinary hepcidin level in sepsis (Altamura et al., 2009). This semi-quantitative assay, where quantification was based on peak height, was able to detect elevated level of hepcidin from patients with sepsis (Altamura et al., 2009).

Laarakkers et al. in 2013 described WCX-MALDI-TOF MS assay, with the stable isotope labelled hepcidin-25 that has higher molecular weight (+40 Da) compared to hepcidin-25. The implementation of this internal standard for correction of inter/intra-assay variation proved to be very useful. The assay enabled the quantification of levels of circulating hepcidin-25 and its isoforms (Laarakkers et al., 2013).

LC-MS/MS is commonly used for qualitative and quantitative analysis of biological samples. This instrumentation utilizes a combination of online separation technique and selective mass detection, such as SRM and PRM. Up to date, the different extraction procedures for hepcidin enrichment from the biological sample involved: ultrafiltration (Kobold et al., 2008), protein precipitation with trichloroacetic acid (Murao et al., 2007) or acetonitrile (Murphy et al., 2007), solid phase extraction (Li et al., 2009) and weak cation-exchange magnetic beads (Bansal et al., 2010). For the compensation of hepcidin losses during sample preparation, addition of a stable isotopic-labelled hepcidin, as internal standard, is recommended for all samples (standards, control quality and clinical samples). Subsequently, the quantification is based on hepcidin/IS ratio. Murphy et al. in 2007 first described a LC-MS/MS (SRM) assay for the quantification of human or mouse hepcidin in serum, using calcitonin gene-related peptide (CGRP) as internal standard (Murphy at al., 2007). However, the CGRP cannot be considered as an ideal internal standard because of its differences from the endogenous hepcidin in terms of mass, hydrophobicity, pI and net charge. Li et al. in 2009 developed a LC-MS/MS method based on the selected reaction monitoring (SRM) for quantification of hepcidin in human sera. This method was also validated according to Food Drug Administration (FDA) guidelines for bioanalytical methods validation (Li et al., 2009). This method with minimal changes was introduced into our proteomics lab in 2009.

Rochat et al. in 2013 developed an alternative method to the LC-MS/MS (SRM), LC-HRMS, for the absolute quantification of hepcidin and its isoforms in human plasma. After sample processing by protein precipitation and a drying reconstitution

step, the extracts were subsequently analysed onto a LC-MS (Exactive Plus™ Orbitrap mass spectrometer) that operated in high-resolution full-scan acquisition mode (Rochat et al., 2013). Later, Li et al. in 2015 developed two LC-MS/MS methods (SRM and PRM) for quantification of hepcidin level in monkey and mouse sera using the following treatment with small interfering RNA (siRNA) that targets hepcidin. As was shown in their study, at least 10-fold improvement in specificity and sensitivity of PRM analysis compared to SRM analysis was received due to significant reduction of background noise. Moreover, siRNA could be used as promising therapeutic agent for reducing hepcidin levels in serum of patients with ACD (Li et al., 2015).

1.3 Amyloidosis as an example of misfolded protein mediated disease - Introduction to proteomic typing of amyloid

Amyloidosis is a rare disorder characterized by the abnormal extracellular and/or intracellular deposition of misfolded amyloid proteins into various organs. These proteins polymerize into insoluble fibrils with a characteristic β–pleated sheet structure, and with other components, like apolipoproteins, glycosaminoglycans and serum amyloid P protein, which stabilize the fibrils, form amyloid. Amyloid accumulates in various tissues, and results in to disorganisation, damage and loss off function of the organs (Merlin & Bellotti, 2003). Amyloid deposition can be systemic (more frequent) or localized in the specific sites (less frequent). Additionally, amyloidosis can be acquired or hereditary disease (Hawkins, 2003; Desport et al., 2012).

The most frequent subtype of amyloidosis is (AL amyloidosis) characterized by the deposition of the amyloid fibrils of immunoglobulin light chain (AL κ or AL λ). AL amyloidosis is a systemic disease that belongs to plasmacellular dyscrasias, and in rare cases is associated with lymphoproliferative diseases (Telio et al., 2010; Schönland et al., 2012). Amyloidosis derived from transthyretin (ATTR) is the other common subtype, which results due to the misfolded wild type or mutated transthyretin (TTR) protein (Merlin & Bellotti, 2003). Chronic infections and autoimmune inflammations with increased levels of serum amyloid A (SAA) protein may results in AA amyloidosis. Mutations in the proteins, such as fibrinogen α, apolipoprotein A-I, apolipoprotein A-II, apolipoprotein A-IV, lysozyme, can lead to the hereditary systemic form of amyloidosis (Sipe et al., 2012; Sipe et al., 2014).

To date, there are 31 known extracellular and/or intracellular fibril proteins in humans that can cause amyloidosis, with individual fibril proteins specifying the subtype of this disease (Sipe et al., 2014). Available treatment modalities are dependent on the subtype of amyloidosis, and therefore, accurate diagnostic is essential. Clinically, the presence of amyloid deposits is first verified by histological examination of tissue samples obtained from an affected organ using specific staining methods, such as Congo red (CR), Sirius red (SR), metachromatic staining, etc. CR staining is the standard and generally applied technique for amyloid diagnosis, described by Puchtler et al. (Puchtler et al., 1965), and later modified by Linke (Linke, 2012). Amyloid fibrils with β–pleated sheet structures bind to CR dye, result in green, yellow or orange

birefringence under the polarized light (Sipe et al., 2014) (Table 3). Once the amyloid has been identified, more detailed characterization and typing are performed.

Table 3. Amyloid fibril protein and their precursors in human (Sipe et al., 2014)

Fibril protein	Precursor protein	Systemic and/or localized	Acquired or hereditary	Target organs
AL	Immunoglobulin Light Chain	S, L	A, H	All organs except CNS
AH	Immunoglobulin Heavy Chain	S, L	A	All organs except CNS
Aβ2M	β2-Microglobulin, wild type	L	A	Musculoskeletal system
	β2-Microglobulin, variant	S	H	ANS
ATTR	Transthyretin,wild type	S, L	A	Heart mainly in males, Tenosynovium
	Transthyretin, variants	S	H	PNS, ANS, heart, eye, leptomen
AA	(Apo) Serum Amyloid A	S	A	All organs except CNS
AApoAI	Apolipoprotein A I, variants	S	H	Heart, liver, kidney, PNS, testis, larynx (C terminal variants), skin (C terminal variants)
AApoAII	Apolipoprotein A II, variants	S	H	Kidney
AApoAIV	Apolipoprotein A IV, wild type	S	A	Kidney medulla and systemic
AGel	Gelsolin, variants	S	H	PNS, cornea
ALys	Lysozyme, variants	S	H	Kidney
ALect2	Leukocyte Chemotactic Factor-2	S	A	Kidney, primarily
AFib	Fibrinogen α, variants	S	H	Kidney, primarily
ACys	Cystatin C, variants	S	H	PNS, skin
ABri	ABriPP, variants	S	H	CNS
ADan	ADanPP, variants	L	H	CNS
Aβ	Aβ protein precursor, wild type	L	A	CNS
	Aβ protein precursor, variant	L	H	CNS
APrP	Prion protein, wild type	L	A	CJD, fatal insomnia
	Prion protein variants	L	H	CJD, GSS syndrome, fatal insomnia
ACal	(Pro)calcitonin	L	A	C-cell thyroid tumors
AIAPP	Islet Amyloid Polypeptide	L	A	Islets of langerhans, insulinomas
AANF	Atrial Nutriuretic Factor	L	A	Cardiac atria
APro	Prolactin	L	A	Pituitary prolactinomas, aging pituitary
AIns	Insulin	L	A	Iatrogenic, local injection
ASPC	Lung Surfactant Protein	L	A	Lung
AGal7	Galectin 7	L	A	Skin
ACor	Corneodesmin	L	A	Cornified epithelia, Hair folicles
AMed	Lactadherin	L	A	Senile aortic, media
AKer	Kerate-epithelin	L	A	Cornea, hereditary
ALac	Lactoferrin	L	A	Cornea
AOaap	Odontogenic Ameloblast-Associated Protein	L	A	Odontogenic tumors
ASem1	Semenogelin 1	L	A	Vasicula seminalis
AEnf	Enfurvitide	L	A	Iatrogenic

The most commonly used methods for amyloid subtyping are performed by immunohistochemistry (IHC) and immunofluorescence (IF) analysis of formalin-fixed paraffin-embedded (FFPE) and/or the native frozen fixed tissue samples (Linke, 2012).

IHC often produces ambivalent results because the antigenic epitope could be lost during the FFPE tissue preparations and contamination of samples by serum proteins can result in high background staining. Additionally, several antibodies are required for precise determination of the most frequent amyloid protein. Differences in sensitivity and specificity of the individual antibodies may further lead to misinterpretation of data (Satoskar et al., 2011).

Laser microdissection (LMD) followed by the combination of liquid chromatography (LC) with mass spectrometry (LMD-LC-MS/MS) is current advanced proteomic approach for the correct diagnosis and typing of amyloidosis (Vrana et al., 2010; Klein et al., 2011; Lavatelli et al., 2012; Theis et al., 2013). LMD-LC-MS/MS enables determination of complete protein composition and identification of the most abundant amyloid proteins using a minimal amount of tissue samples (Dasari et al., 2014) (Figure 10).

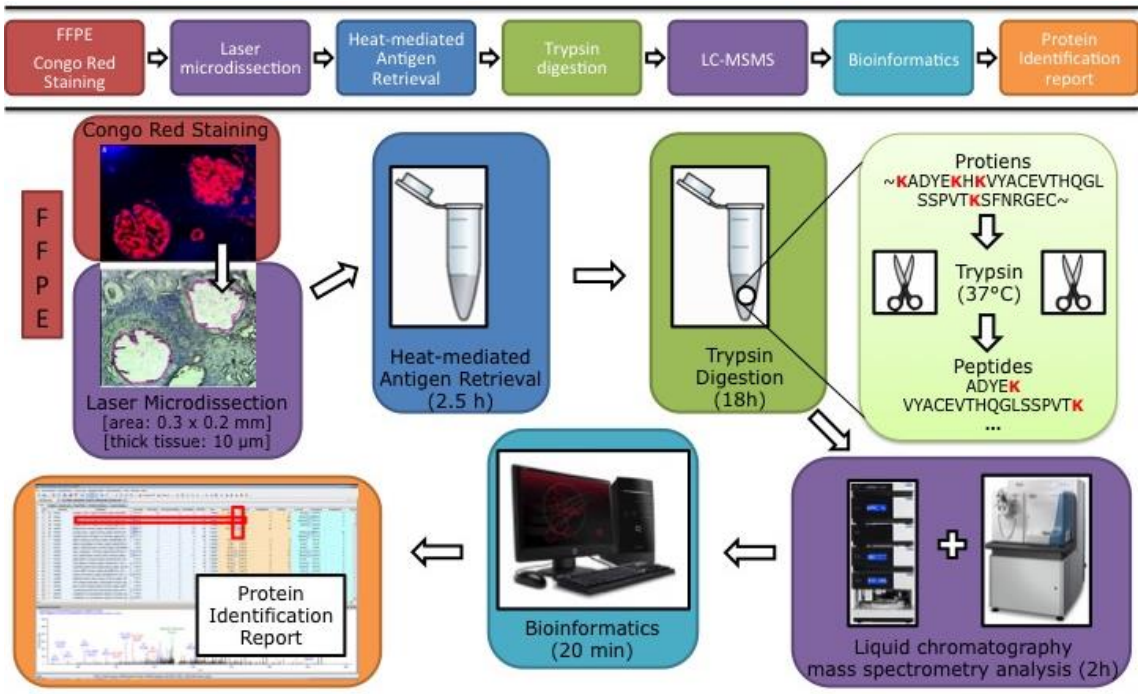

Figure 10. Proteomics workflow for characterizing the protein content of amyloid deposits.

In the present study, we used IHC and LMD-LC-MS/MS for amyloid subtyping of twenty-two FFPE tissue samples, and summarized the advantages and failures of both methods for the diagnosis of amyloidosis.

2 Experimental part, survey of the results

2.1 Aims

Aim of the thesis was to introduce new mass spectrometry methods in clinical diagnostic as a potentially valuable tool, which is directly identifying proteins or peptides of the interests. Therefore, we have selected hepcidin as a prototype biomarker and amyloidosis as a disease with various pathogenic proteins, which have to be carefully distinguished and analyzed. Developed analytical methods were validated on the numerous patients' samples and diagnoses.

1. Hepcidin analysis

   a) Develop SPE-LC-MS/MS method for hepcidin quantification in serum.

   b) Use the SPE-LC-MS/MS method for hepcidin quantification in different heamatologic diseases (three studies).

2. Amyloid analysis

   a) Develop proteomic method for amyloid subtyping from formalin fixed paraffin embedded tissue samples.

   b) Compare proteomic and immunohistochemical analyses for amyloid subtyping from formalin fixed paraffin embedded tissue samples.

2.2 Development of the analytical method and clinical impact of hepcidin evaluation

2.2.1 Development of the SPE-LC-MS/MS method for hepcidin analysis

In the last decade it was shown that hepcidin is an important regulator of iron metabolism in the body and therefore as is described in the introduction in part **1.2.6 "Methods used for hepcidin measurements"** there was a huge effort to develop robust and reliable method for hepcidin analysis. The antibody-based methods (ELISA, IHC, RIA) are limited, because the tests are affected by the specificity of the antibody epitope. Therefore, we have decided to develop SPE-LC-MS/MS method, which is easy and cheap to perform with good sensitivity and reproducibility.

2.2.1.1 Materials and reagents

Human hepcidin and stable isotope-labelled [$^{13}C_9$, $^{15}N_1$-Phe_4]-hepcidin used as internal standard (IS) were purchased from Thermo Fischer Scientific (Thermo Fischer Scientific, Berlin, Germany). Formic acid, methanol and rabbit serum were purchased from Sigma Aldrich (Sigma Aldrich, Prague, Czech Republic). Solid phase extraction (SPE) Oasis HLB 96-well μElution plate was purchased from Waters (Waters, Prague, Czech Republic). Analytical column Polaris C18 A, 5 μm, 75 x 2.1 mm was purchased from Varian (Labicom, Olomouc, Czech Republic).

Amino acid sequences of the hepcidin are as follows (DTHFPICIFCCGCCHRSKCGMCCKT): the human hepcidin and stable isotope-labelled analogue of the hepcidin (IS), $C_{113}H_{170}N_{34}O_{21}S_9$, and $C_{104}{}^{13}C_9H_{170}N_{33}{}^{15}N_1O_{21}S_9$ with Mw = 2789.4 Da and 2799.4 Da, respectively.

2.2.1.2 Sample collection and storage

The blood collection and analysis of serum samples were approved by the Ethics Committee of Palacký University Hospital in Olomouc. Samples from patients and healthy subjects used for quantify hepcidin level in serum were obtained from the Paediatrics Department of the Faculty of Medicine and Dentistry, Palacký University Olomouc. Following collection, blood was allowed to clot at room temperature for

approx. 30 min. Cloted blood was than centrifuged at 1500x g for 10 min and the supernatant (serum) was separated and aliquoted into Eppendorf tubes, and stored at -80 °C. The study was designed according to the Declaration of Helsinki and informed consent was obtained from the adult patients and parents of children participants.

2.2.1.3 Preparation of standard solutions

Stock solutions of hepcidin and IS at 100 µg/mL concentration were prepared in 10% methanol. Both stock solutions were stored at -80 °C. Working solution of hepcidin (500 ng/mL) was prepared freshly by diluting stock solution with rabbit serum. Working solution of IS (100 ng/mL) was prepared freshly by diluting stock solution of IS in 50%/49.9%/0.1% (v/v/v) of methanol/water/formic acid. Calibration standards were prepared by spiking rabbit serum with hepcidin working solution, resulting in concentration of 2.5, 5, 10, 25, 50, 100, 250 and 500 ng/mL.

2.2.1.4 Solid-phase extraction

Solid phase extraction was performed in Oasis HLB 96-well µElution plate. 30% MeOH (pH 10) and 90% MeOH (pH 5) was used as washing and eluting solvents, respectively. After activating and conditioning of SPE plate with methanol and water, 200 µL of serum sample containing 50 µL of IS (100 ng/mL) and 100 µL of formic acid (0.1%) was loaded per wells. The SPE plate was subsequently washed with 200 µL of water, 200 µl of washing solvent and 200 µL of water. Elution was done using 50 µL of elution solvent. The eluate was then diluted with 150 µL of formic acid (0.1%). All these steps were done on Tomtec Quadra 4^{TM} robotic liquid handling workstation. The diluted elute was ready for LC-MS analysis.

2.2.1.5 Liquid chromatography and mass spectrometry

MRM optimization

All experiments were carried out on a QTRAP 5500 mass spectrometer equipped with Turbo V ion source. The system was controlled by Analyst software.

Precursor and product ions of human hepcidin were determined by direct infusion of 10 μg/mL of hepcidin in 50% methanol with 0.1% formic acid in positive ionization mode. Hepcidin with charge state +4 (698.0 m/z) was most predominant. The collision assisted dissociation of the hepcidin precursor ion (698.0 m/z, +4) produce major product ions at 354.1 m/z corresponding to the b_3^+ ion and at 501.4 m/z corresponding to the b_4^+ ion.

MS and MS/MS spectrum's of human hepcidin-25 and stable isotopic-labelled hepcidin (IS) are shown in Figure 11.

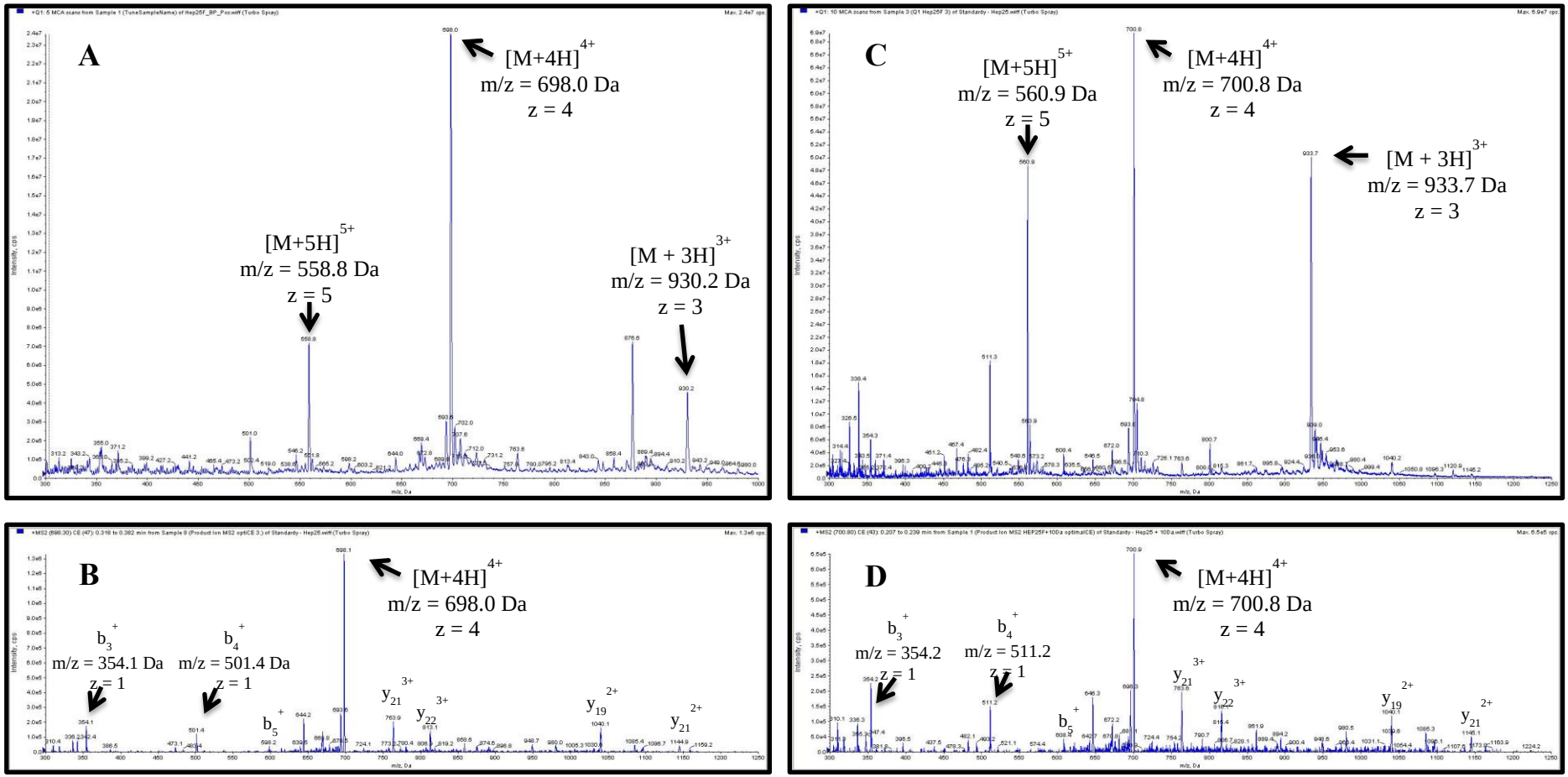

Figure 11. MS and MS/MS spectrum's of hepcidin-25 and IS collected on the QTRAP 5500 mass spectrometry. (A) MS spectrum of hepcidin-25. Various charge states (+3, +4, +5) were observed. (B) MS/MS spectrum of quadruply charged hepcidin ion $[M+4H]^{4+}$ at 698.0 m/z. The b_3^+ and b_4^+ product ions were used for hepcidin detection. (C) MS spectrum of IS (isotopic-labelled hepcidin). Similarly, various charge states were observed. (D) MS/MS spectrum of quadruply charged IS ion $[M+4H]^{4+}$ at 700.8 m/z. The b_3^+ and b_4^+ product ions were used for IS detection.

LC-MS/MS (SRM) analysis

The chromatographic system UltiMate® 3000 RSLCnano includes a ternary loading pump, column compartment and autosampler. The column compartment and autosampler were set to 40 °C and 8 °C, respectively. 20 µL of serum extract was injected. Separation was performed on a Polaris C18, 5 µm 2.1 x 75 mm column. The flow rate was set to 200 µL/min. The mobile phase was composed of (A) 5% methanol (v/v) and (B) 95% methanol (v/v): both with content of 0.1% formic acid. The gradient conditions were set as follows: isocratic 10% B at 0 - 1.5 min; 10% B to 90% B at 1.5 - 4.0 min; 90% B at 4.0 - 5.5 min; 90% B to 10% B at 5.5 - 5.6 min and isocratic 10% B at 5.6 - 8.6 min. Total run time was 8.6 min.

The LC system was coupled to a QTRAP 5500 mass spectrometer with Turbo V ion source. The ESI ion source voltage was +5000 V. The curtain gas was set to 20 psi, nebulized gas was set to 42 psi and the auxiliary gas was set to 55 psi. The source temperature was set to 600 °C. The optimized ion transitions of SRM were set 698.0→354.1 and 700.8→354.2 for hepcidin-25 and IS, respectively. Optimized SRM parameters for hepcidin-25 and IS are shown in Table 4.

Table 4. Optimized SRM parameters for hepcidin-25 and IS.

Compound name	Precursor ion [m/z]	Product ion [m/z]	Product ion name	Dwell time [ms]	DP [V]	EP [V]	CE [V]	CXP [V]
Hepcidin-25	698.0	354.1	b_3^+	100	80	10	41	11
Hepcidin-25	698.0	501.4	b_4^+	100	80	10	41	11
IS	700.8	354.2	b_3^+	100	80	10	41	11
IS	700.8	511.2	b_4^+	100	80	10	41	11

CE, collision energy; CXP, collision cell exit potential; DP, declustering potential; EP, entrance potential.

2.2.2 Method validation

Eight calibration standards were prepared at concentration of 2.5, 5, 10, 25, 50, 100, 250 and 500 ng/mL by serial dilutions in rabbit serum, which were run in duplicate at the beginning and at the end of each batch. The evaluation of the calibration curve was performed in three separately prepared batches. The calibration curve was derived from the peak area ratios (hepcidin/IS) using $1/x^2$ weighted linear least squared regression of the ratio and the concentration of the corresponding standards. The regression equation

from the calibration standards was used for back calculation of standard and validation samples concentrations.

Three analytical runs during separate days were used for evaluation of accuracy and precision. In each run, QC samples at concentrations of 5.0 (Low QC), 100 (Middle QC) and 500 ng/mL (High QC) were analysed (n = 6). The intra- and interday accuracy RE [%] and precision CV of assay were calculated from the measured QC concentrations.

Matrix effect was evaluated using six different lots of rabbit serum with the hepcidin concentration of 5 ng/mL (n = 3 for each lot). The mean RE [%] and CV were calculated for evaluate of matrix effect. Assay selectivity was evaluated through the control rabbit serum analysis of six different lots with or without IS.

To evaluate the freeze-thaw stability, three QC samples with concentrations of 5.0, 15.0 and 400 ng/mL were subjected to three freeze-thaw cycles including defrosting at room temperature and freezing at -60 °C to -80 °C for at least 12 h. To evaluate bench top stability, QC samples were left in the top of the bench at room temperature for more than 12 h prior to extraction. To evaluate the stability of extracted QC samples stored in the autosampler, calibration standards and QC samples were extracted and analysed. They were reinjected after storage into the autosampler at 8 °C for 2 days. Long-term stability was evaluated using QC samples stored in the freezer at -60 °C to -80 °C for 90 days prior to analysis.

Recovery was evaluated using QC samples analysed with concentration of 5.0, 25.0, 100 and 500 ng/mL. Recovery control solutions were prepared using SPE (Oasis HLB) extracts of rabbit control serum. The absolute peak areas of human hepcidin in the extracted QC samples were compared to the peak area obtained from the control samples at the same concentrations.

2.2.3 Results

2.2.3.1 The lower limit of quantitation and standard calibration curve

The standard calibration curves were validated with the concentration ranging from 2.50 to 500 ng/mL of hepcidin-25, from three separately prepared batches during different days. Weighted least squares regression analysis was used to construct calibration curves. The peak area of hepcidin-25/IS ratios and hepcidin-25 standard concentrations were plotted into graph. The representative calibration curve is shown in Figure 12. The average correlation coefficient (r^2) was 0.99. The interday accuracy as relative error (RE [%]) and precision as coefficient of variation (CV) for hepcidin calibration standards are presented in Table 5. The RE [%] of the mean back calculated concentrations of the standards from the theoretical concentration were between -1.6 to 3.6%. The lower limit of quantification (LLOQ), defined as the lowest concentration that could be measured with a precision better than 20% CV and accuracy within ±20% of the theoretical concentration was 2.50 ng/mL. The upper limit of quantification (ULOQ), defined as the highest concentration that could be measured with a precision better than 15% CV and accuracy within ±15% of the theoretical concentration was 500 ng/mL. Hepcidin-25 carryover was evaluated by analysing blank samples following the ULOQ standard (500 ng/mL). No significant carryover was observed.

Figure 12. Calibration curve for human hepcidin-25 extracted from rabbit serum.

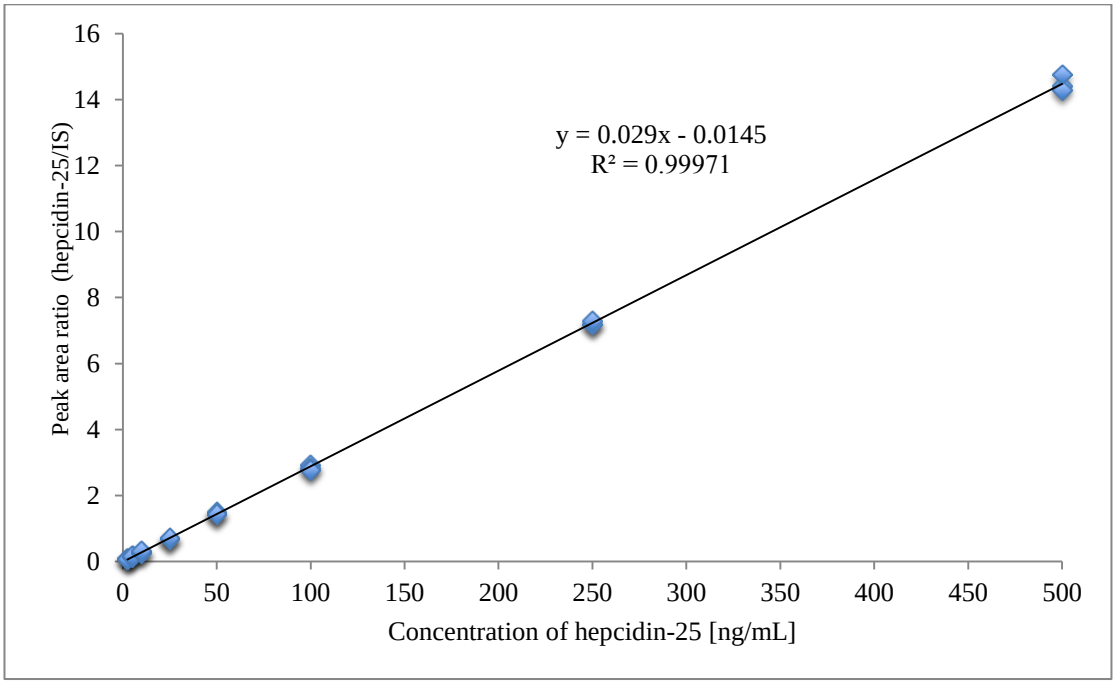

Table 5. Interday precision and accuracy of hepcidin-25 calibration standards.

Day	Statistic	Hepcidin-25 calibration standards [ng/mL]							
		Std. 1	Std. 2	Std. 3	Std. 4	Std. 5	Std. 6	Std. 7	Std. 8
		2.5	5.0	10.0	25.0	50.0	100	250	500
1		2.43	4.97	9.58	24.5	50.7	100.5	255.9	496.5
		2.70	5.06	10.1	25.1	49.4	98.4	248.1	520.2
2		2.67	5.00	10.05	26.0	53.0	98.6	237.2	470.2
		2.44	4.96	10.16	25.8	52.2	101.4	238.4	464.6
3		2.68	4.98	10.06	23.6	51.2	102.5	246.3	496.0
		2.62	5.24	9.74	24.2	50.1	108.5	272.6	504.6
	Mean	2.59	5.04	9.95	24.9	51.1	101.7	249.8	492.0
1-3	CV ([%], n=6)	4.3	1.9	2.1	3.3	2.4	3.3	4.8	3.9
	RE ([%], n=6)	3.6	0.7	-0.5	-0.5	2.2	1.7	-0.1	-1.6

CV, coefficient of variation; Std., standard; RE, relative error.

2.2.3.2 Intraday and interday accuracy and precision in QC samples

Intraday and interday accuracy and precision were obtained from three analytical runs during separate days. All runs contained six replicates of each QC concentration. Intraday and interday accuracy and precision results are shown in Table 6. The intraday and the interday CV were between 1.8 - 9.1% and 2.2 - 9.2%, respectively. The intraday and the interday RE were between -8.5 - 19.2% and -6.0 - 5.5%, respectively.

Table 6. Intraday and interday accuracy and precision of hepcidin-25 QC samples.

Day	Statistic	Concentration of QC samples [ng/mL]			
		LLOQ	Low QC	Middle QC	High QC
		2.5	5	100	500
1	Intraday mean (n=6)	2.54	4.77	99.6	482.5
	CV [%]	11.7	9.1	3.0	4.6
	RE [%]	1.8	-4.5	-0.4	-3.5
2	Intraday mean (n=6)	2.40	5.21	97.7	457.4
	CV [%]	6.1	7.0	2.2	1.8
	RE [%]	-4.1	4.2	-2.3	-8.5
3	Intraday mean (n=6)	2.45	5.30	119.2	469.8
	CV [%]	5.9	5.9	8.9	6.2
	RE [%]	-2.1	5.9	19.2	-6.1
1-3	Interday mean (n=18)	2.46	5.09	105.5	469.9
	CV [%]	2.4	4.6	9.2	2.2
	RE [%]	-1.5	1.9	5.5	-6.0

CV, coefficient of variation; LLOQ, lower limit of quantitation; QC, quality control sample; RE, relative error.

2.2.3.3 Matrix effect and selectivity

Matrix effect was not verified in our laboratory. It was found that it is possible to use the procedure with various types of matrices for quantification of hepcidin-25 level (e.g. rabbit, pig plasma or serum). The precision and accuracy were ±15% and 85 - 115%, respectively (Table 7) (Li at al., 2009; Rochat et al., 2013).

Table 7. Matrix effect (Li at al., 2008).

Matrix	n	Theoretical concentration of hepcidin-25 [ng/mL]	Determined concentration of hepcidin-25 [ng/mL]			Mean concentration of hepcidin-25 [ng/mL]	Mean RE [%]
Rabbit serum	1	5.0	5.51	4.81	5.60	5.31	6.1
	2	5.0	5.54	4.34	4.89	4.59	-8.2
	3	5.0	4.77	5.43	5.21	5.14	2.7
	4	5.0	4.45	6.01	4.05	4.84	-3.3
	5	5.0	4.27	5.14	4.43	4.61	-7.7
	6	5.0	4.47	4.70	5.24	4.80	-3.9

RE, relative error.

2.2.3.4 Stability

Freeze-thaw, bench-top, reinjection and long-term stabilities were not performed in our validation. These stability tests were published before and the representative results

are showed in Table 8 (Li at al., 2008; Rochat at al., 2013; Laarakkers et al., 2013; Li et al., 2015). Hepcidin-25 appeared to be stable in human serum after three freeze-thaw cycles (mean RE was between -7.3 and 4.3%) and at room temperature for 16 h before extraction (mean RE was between -4.0 and 2.8%). Good stability after 48 h storage in the autosampler at 8 °C (mean RE was between -3.3 and 0.8%) demonstrated the stability of hepcidin-25 in extracted solution. Long-term stability of hepcidin-25 in biomatrix was evaluated from the hepcidin-25 stability QC samples, which were stored in the freezer at -60 °C to -80 °C for 90 days and analysed against freshly prepared calibration curve (mean RE was between -2.0 and 6.5%).

Table 8. Hepcidin-25 stability tests (Li at al., 2008).

Theoretical concentration of hepcidin-25 [ng/mL]	Stability after 3 freeze-thaw cycles		Stability at room temperature for 16 h	
	Hepcidin-25 [ng/mL]	Mean RE [%]	Hepcidin-25 [ng/mL]	Mean RE [%]
5.00	4.91	-1.8	4.87	-2.6
15.0	13.9	-7.3	14.4	-4.0
400	417	4.3	411	2.8
Theoretical concentration of hepcidin-25 [ng/mL]	Stability in the autosampler at 8 °C for 48 h		Stability after storage at -80 °C for 90 days	
	Hepcidin-25 [ng/mL]	Mean RE [%]	Hepcidin-25 [ng/mL]	Mean RE [%]
5.00	5.04	0.8	4.90	-2.0
15.0	14.5	-3.3	15.7	4.7
400	388	-3.0	426	6.5

RE, relative error.

2.2.3.5 Extraction recovery

Recovery of the SPE (Oasis HLB) was determined by comparison of the absolute peak areas of human hepcidin in QC samples after SPE extraction and the peak areas of human hepcidin in recovery control solution with the same theoretical concentration (% RE = 100 x (Peak area $_{\text{Extracted sample with hepcicin}}$/Peak area$_{\text{Post-extracted SPIKED sample}}$). The mean SPE extraction recovery for hepcidin-25 was ≥ 60% for all tested concentrations.

The article describing the method is under review in Chemagazín (**see APENDIX 8.4**).

2.2.4 Impact of hepcidin evaluation in hematological diseases

In the first study, we focused on level of hepcidin, iron metabolism and erythropoietic activity in DBA patients, where knowledge of systemic iron regulation is limited (POSPÍŠILOVÁ, D., **D. HOLUB**, Z. ŽIDOVÁ, L. SULOVSKÁ, J. HOUDA, V. MIHÁL, I. HADAČOVÁ, L. RADOVÁ, P. DŽUBÁK, M. HAJDÚCH, V. DIVOKÝ a M. HORVÁTHOVÁ. Hepcidin levels in Diamond-Blackfan anemia reflect erythropoietic activity and transfusion dependency. Haematologica. 2014, **99**(7), e118-121. ISSN 0390-6078. IF: 5.868. PMID: 24727814) (**see APENDIX 8.1**).

Diamond-Blackfan anemia (DBA) is a rare congenital red cell aplasia associated with mutation in ribosomal proteins (RP) (Boria et al., 2010). The prevalence ranges are from 5 to 7 cases per million live births in DBA. Diagnostic criteria according to the international consensus include: (a) age less than 1 year, (b) macrocytic anemia with no other significant cytopenia, (c) reticulocytopenia and (d) normal bone marrow cellularity with a paucity of erythroid precursor (Vlachos et al., 2008). Other associated anomalies include: craniofacial dysmorphism, thumb anomalies, heart defects, skeletal and genito-urinary abnormalities.

The DBA cohort consisted of 25 patients, which was divided into four subgroups based on the therapy: 12 patients receiving regular transfusions with or without chelation therapy, 7 patients in remission without treatment, 4 patients on steroids and 2 patients treated with corticosteroids and occasional transfusions. A control group included 5 young healthy adults. The causative RP mutation (RPS7, RPS17, RPS19, RPS26, RPL5 and RPL11) was found in 20 patients in this study.

In the first group, (transfusion-dependent patients) we found reduced number of erythroblasts in the bone marrow (median 0.8%) and decreased sTfR levels (often under the lower limit of detection). These findings confirmed suppressed erythropoiesis that corresponded to elevated EPO level (2452 IU/L). Analysis of iron parameters showed increased serum iron (41.5 μmol/L), TSAT (91 %) and high ferritin levels (1290 ng/mL) (Table 9).

Table 9. Parameters of iron status and erythripoietic activity in subgroups of Diamond-Blackfan anemia patients.

	TDP (n=12)	RP (n=7)	SP (n=4)	S,T* (n=2)	Reference range
Age (years)	7.6 (1.0-27.9)	26.9 (13.3-35.8)	32.6 (27.0-42.9)	25.8 25.9	–
Hepcidin (ng/mL)	341.5 (165.1-572.6)	72.1 (29.5-153.3)	24.7 (8.5-100.1)	173.9 144.9	27.6 (13.1-104.8)[5]
Ferritin (ng/mL)	1290 (343-3747)	177 (42-1079)	188 (44-637)	3150 868	22-275
Hepcidin/ferritin ratio	0.240 (0.09-1.35)	0.222 (0.09-1.04)	0.151 (0.10-0.19)	0.06 0.17	0.35 (0.2-2.2)[5]
Fe (µmol/L)	41.5 (35.8-59.0)	20.0 (10.0-30.6)	21 (14.7-33.4)	39.6 43.2	7.2-29
TSAT (%)	91 (61-100)	38 (25-55)	64 (63-65)	93 94	21-48
LIC (mg/g d.w.)	7.3# (4.6-16.3)	*ND*	*ND*	*ND*	0.3-1.4
GDF15 (pg/mL)	676.2 (352.1-1694.8)	927.0 (556.3-2824.8)	1127.6 (408.8-2190.8)	4221.5 3497.1	223 (166-344)[5]
EB (%)	0.8 (0.4-8.2)	21.4 (8.4-34.0)	20.4 (16.8-33.4)	10.6 20.8	15.0-25.0
EPO (IU/L)	2452 (837-6476)	137 (20-1913)	441 (287-500)	615 4000	4.3-29.0
sTfR (mg/L)	*ND*† <0.5-1.2	2.3 (1.3-3.2)	2.4 (1.8-2.8)	1.4 1.4	1.9-4.4[6]

Values are shown as medians and the full range of variation TDP: transfusion-dependent patients; RP: patients in disease remission; SP: patients treated with steroids; S,T: patient on steroids and occasional transfusion; Fe: serum iron; TSAT: transferrin saturation; LIC: liver iron concentration, d.w.: dry weight; GDF15: growth differentiation factor 15; EB: erythroblasts in the bone marrow; EPO: serum erythropoietin; sTfR: soluble transferrin receptor; ND: not determined. *Individual values are shown; #values are available for 5 patients; †median could not be calculated as 9 of 12 patients had sTfR below the limit of detection (less than 0.5 mg/L) (Pospisilova et al., 2014).

This suggests that erythropoiesis is unable to utilize transferrin-bound iron effectively. Moreover, liver biopsy showed elevated liver iron concentration (7.3 mg/g) and massive iron stores in hepacytes and Kupffer cells (Figure 13). The macrophage iron loading can be attributed to non-effective erythrocyte-derived iron recycling (Hentze et al., 2010; Ganz & Nemeth, 2012). Patients on regular transfusion showed significantly elevated levels of hepcidin (341.5 ng/mL) (Figure 14).

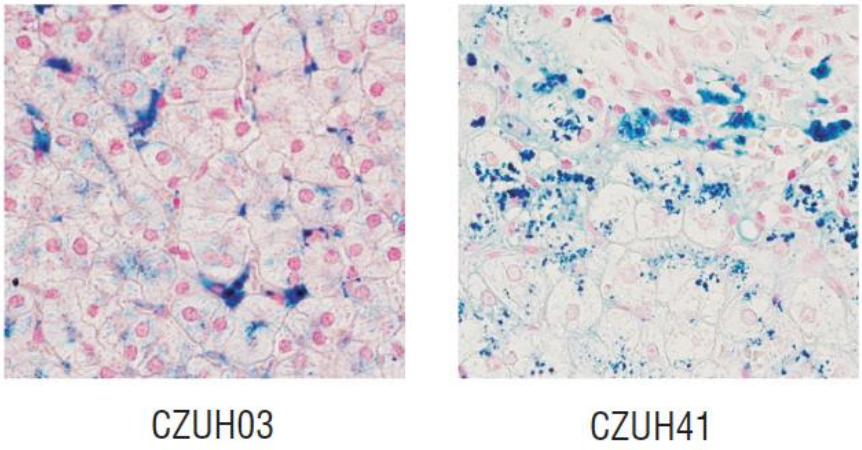

Figure 13. Perl's staining showing iron deposits in the liver of two transfusion-dependent DBA pacients (CZUH03 – LIC 4.6 mg/g d.w. and CZUH41 – LIC 10.3 mg/g d.w.) Massive iron sores (blue stain) can be found in bouth hepatocytes and Kupffer cells (Pospisilova et al., 2014).

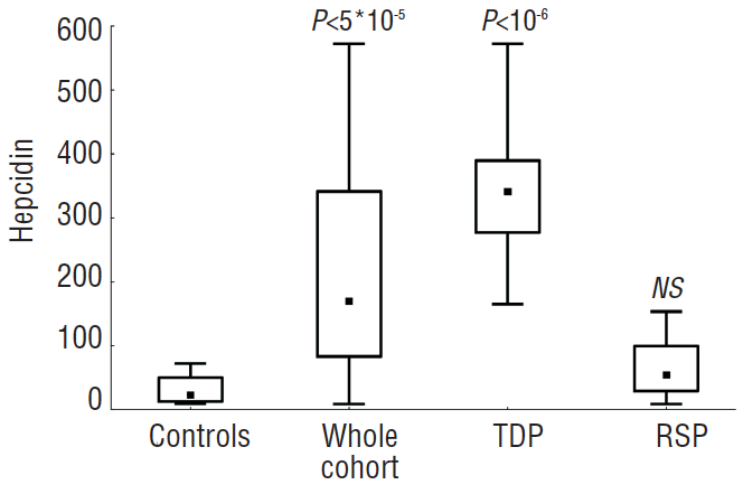

Figure 14. Significantly increased hepcidin levels in comparison with healthy controls were detected for the whole DBA cohort (n=25) and transfusion-dependent DBA patients (TDP, n=12); DBA patients in remission or treated with steroids (RSP, n=11) had hepcidin levels comparable with the controls (Pospisilova et al., 2014).

In the second group, patients in disease remission or in steroids showed improved erytropoiesis (normal/higher number of bone marrow erythroblasts and level of sTfR in normal range). This group had slightly increased or normal level of ferritin and near normal serum iron and TSAT. This indicates an improved erythropoietic activity increases iron utilization and consequently leads to amelioration of hyperferritinemia. Restoring erythropoietic activity also attenuated hepcidin expression. We conclude that hepcidin correlated with ferritin, reflecting hepcidin stimulation by the patients iron

overload. The relationship between EPO and number of erythroblasts in the bone marrow showed that EPO is stimulated in response to suppressed erythropoiesis and hypoxia. A positive correlation between hepcidin and EPO demonstrates that hepcidin suppression by EPO requires active erythropoiesis in the bone marrow (Figure 15).

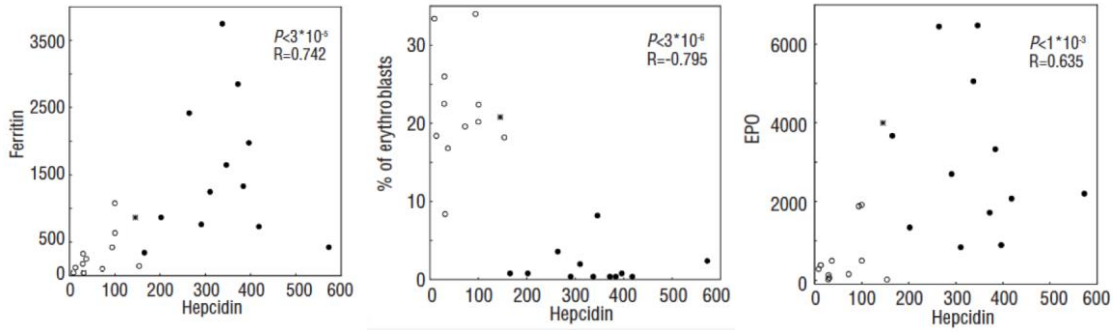

Figure 15. Hepcidin levels showed positive correlation with ferritin, negative correlation with the number of erythroblasts in the bone marrow and positive correlation with serum EPO (Pospisilova et al., 2014).

We found significantly increased levels of GDF15 in the whole DBA cohort presumably due to increased apoptosis of bone marrow erythroblast (Figure 16). No correlation between hepcidin and GDF15 indicates that GDF15 is not playing hepcidin-regulatory role in DBA.

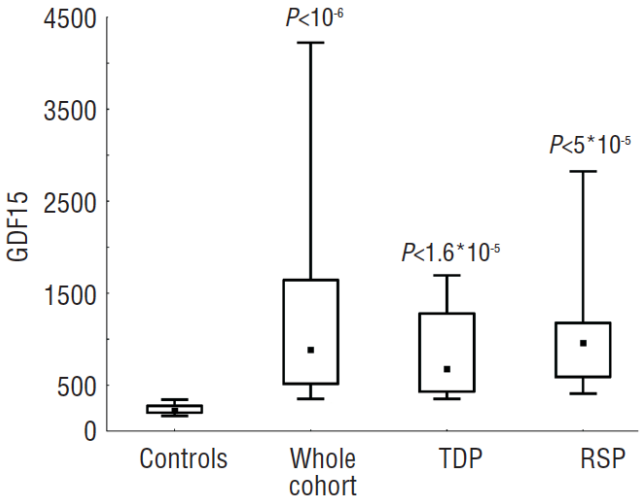

Figure 16. GDF15 levels were significantly elevated in the whole DBA cohort when compared to healthy controls (Pospisilova et al., 2014).

DBA patients with different severities of anemia and different treatment have diverse levels of hepcidin and iron overload. Hepcidin production in DBA reflects variable erythropoietic activity in the bone marrow and futher contributes to the heterogenity of the disease.

In the second study, we focused on iron metabolism and erythropoiesis in patients with erythrocyte membrane defects and thalassemia carriers (Sulovska, L., Holub, D., Zidova, Z., Divoka, M., Hajduch, M., Mihal, V., Vrbkova, J., Horvathova, M., & Pospisilova, D. (2015). Characterization of iron metabolism and erythropoiesis in erythrocyte membrane defects and thalassemia traits. *Biomedical Papers,* 1–7. http://doi.org/10.5507/bp.2015.054) (**see APENDIX 8.2**).

The cohort consisted of 20 patients with erythrocyte membrane defects (18 with HS and 2 with HE), 13 with thalassemia minor and 1 patient with thalassemia major. A control group included 47 children examined prior to planned minor surgery and none of the subjects had signs of an inflammatory process at the time of examination as documented by normal C-reactive protein levels. The hematological data in patients with erythrocyte membrane defects showed significantly reduced RBC count and significantly increased MCHC in comparison with healthy controls (Table 10).

Table 10. Hematological parameters (Sulovska et al., 2015).

	Controls	Thalassemia carriers	Erythrocyte membrane defects	Thalassemia major
RBC (10^{12}/L)	4.78 ± 0.60	5.55** ± 0.315	4.23* ± 0.71	2.97
Hb (g/dL)	13.0 ± 1.3	10.6*** ± 1.1	12.0 ± 1.9	8.2
MCV (fL)	82.56 ± 6.87	59.1*** ± 5.68	79.1 ± 5.73	79.8
MCH (pg)	28.29 ± 2.62	19.23*** ± 2.27	28.45 ± 1.86	27.6
MCHC (g/dL)	34.32 ± 1.22	32.46* ± 1.21	36.09** ± 0.87	34.6
Ret (ratio)	0.010 ± 0.003	0.012 ± 0.004	0.066*** ± 0.045	0.012

Values are shown as means ± SD; single values are given for the patient with thalassemia major. RBC, red blood cells count; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration, Ret, reticulocytes count. Statistical significance relative to control group: *P value<0.05; **P value<0.01; ***P value<0.001.

These findings reflect compensatory reticulocytosis in response to chronic hemolysis. The markers of erythropoietic activity showed elevated EPO (34 IU/L) and sTfR (9.58 mg/L) levels in the group of erythrocyte membrane defect than in healthy controls. This indicates increased erythropoietic activity in the bone marrow patients with erythrocyte membrane defect. As a marker of ineffective erythropoiesis, serum GDF15 levels were found elevated in the group of erythrocyte membrane defect (387 pg/mL) and thalassemia carriers (653 pg/mL) in compare to healthy controls (205 pg/mL). The parameters of iron metabolism showed increased level of serum ferritin and reduced (not significant) hepcidin levels (Table 11).

Table 11: Iron status parameters (Sulovska et al., 2015).

	Controls	Thalassemia carriers	Erythrocyte membrane defects	Thalassemia major
%TSAT	33.07 ± 14.09	30.9 ± 10.86	29.05 ± 10.22	59.9
Serum Fe (μM/L)	20.87 ± 8.37	16.75 ± 5.57	15.04 ± 4.57	22
Ferritin (μg/L)	34.62 ± 20.1	40.23 ± 39.15	99.54** ± 45.52	2872
Hepcidin (ng/mL)	25.86 ± 26.46	14.73 ± 14.27	15.34 ± 16.56	56.2
Hepcidin/ferritin	0.658 ± 0.564	0.341 ± 0.149	0.177** ± 0.173	0.02
(hepcidin/ferritin)/sTfR	0.184 ± 0.189	0.072 ± 0.050	0.023*** ± 0.021	0.001

The hepcidin/ferritin ratio was significantly lower in erythrocyte membrane defects group compared to healthy controls and indicated inappropriate suppression of hepcidin synthesis. The hematological data in thalassemia carriers showed raised RBC count and reduced Hb, MCV, MCH and MCHC compared to healthy controls. Elevated sTfR and GDF15 were found, which indicate increased but ineffective erythropoiesis in the bone marrow. Our data are similar to recently publish data for schoolchildren with β-thalassemia trait (Guimarães et al., 2015). The parameters of iron metabolism showed reduced (not significant) hepcidin levels. The reduction (not significant) of hepcidin/ferritin ratio was found in thalassemia carriers compared to healthy controls and indicated inappropriate suppression of hepcidin synthesis. Our patient with β-thalassemia major with the most severe form of anemia showed reduced RBC count and Hb level. The very low hepcidin/ferritine ratio showed that hepcidin was disproportionally low with respect to iron loading. The low hepcidin/ferritin ratio and elevated sTfR and GDF15 reflect increased ineffective erythropoiesis with higher iron demand leading to increased iron absorption and iron recycling (Figure 17).

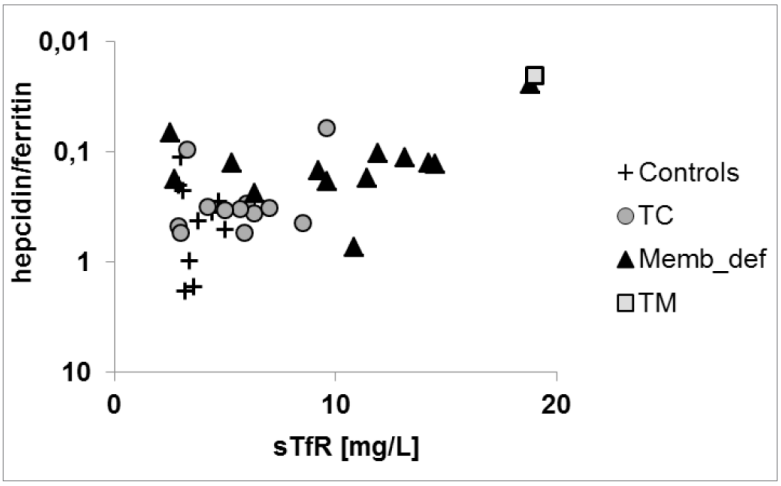

Figure 17. Differentiation of patients based on the plot of hepcidin/ferritin ratio against sTfR. Patients with erythrocyte membrane defect (Memb_def. n=13), thalassemia carriers (TC, n=12) and thalassemia major (TM, n=1) dislocate from healthy controls (n=9) (Sulovska et al., 2015).

The iron overload in β-thalassemia major is a result of regular transfusion therapy and disrupted hepcidin synthesis and this finding has previously been described (Pasricha et al., 2013). Finally, we calculated (hepcidin/ferritin)/sTfR ratio which combines the parameters of iron status and erythropoietic activity (Figure 17). This ratio was reduced in β-thalassemia (0.001), erythrocyte membrane defects group (0.023) and thalassemia carrier group (0.072) in comparison to healthy controls (0.184). The findings suggest that pediatric patients with erythrocyte membrane defects and thalassemia traits are more susceptible to iron overload than the general population. The hepcidin/ferritin ratio also reflects the severity of the anemia and can be used to follow up patients with erythrocyte membrane defects or thalassemia carriers for evalution of the disease with focus on alterations in erythropoietic activity and potential risk of iron overload.

In the third study, the iron status and hepcidin level was studied, which was targeting iron metabolism in patients with pyruvate kinase deficiency (Mojzikova, R., Koralkova, P., Holub, D., Zidova, Z., Pospisilova, D., Cermak, J., Striezencova-Laluhova, Z., Indrak, K., Sukova, M., Partschova, M., Kucerova, J., Horvathova, M., Divoky,V. (2014). Iron status in patients with pyruvate kinase deficiency: neonatal hyperferritinaemia associated with a novel frameshift deletion in the PKLR gene (p.Arg518fs), and low hepcidin to ferritin ratios. *British Journal of Haematology*, 165(4), 556–563. http://doi.org/10.1111/bjh.12779) (**see APENDIX 8.3**).

The research was done in collaboration with Department of Biology, Faculty of Medicine and Dentistry, Palacky University.

Pyruvate kinase deficiency is an inherited lack of the enzyme pyruvate kinase, which is used by red blood cells. Without this enzyme, red blood cells break down, resulting in low levels of these cells. Disease is therefore in general characterized by chronic haemolysis, ineffective erythropoiesis and a requirement for blood transfusions. In the pyruvate kinase deficiency study, we investigated 11 patients and found 9 different disease-causing mutations in the PKLR gene. Two of these mutations were novel (the point mutation c.878A>T and the frameshift deletion c.1553delG) and were associated with atypical severe phenotype (involving neonatal hyperferritinanemia). All patients had secondary iron overload due to chronic heamolysis, ineffective erythropoiesis and transfusion therapy. Clinical and heamatological data of the patients with pyruvate kinase deficiency are showed in Table 12.

Table 12. Clinical and haematological data of the patients with pyruvate kinase deficiency (Mojzikova et al., 2014).

Patient	Sex	Age	Neonatal jaundice	ExTx	Spleen	Hb, g/l	Ret,% [0·5–3·0]	Bilirubin, µmol/l [0–23]	Ferritin, µg/l [6–200]*	TfS [0·21–0·48]	Hepcidin, µg/l [13·1–104·8]	GDF15, ng/l [166–344]	EPO, iu/l [4·3–29]
1	M	9 years	Yes	Yes	Out	79	32	38	2000§	0·79	n.d.	3052	n.d
2	M	6 months	Yes	Yes	In	77	7·2	33	4852	0·97	90·7	935	46·2
		33 months				98	4·8	49	145§	n.d.	16	1399	78·1
3	F	7 years	Yes	Yes†	In	95	10·6	35	16	0·26	5·7	851	454
4	M	10 months	Yes	Yes	In	75	7·5	59	303	n.d.	15·5	876	123
5	M	7 years	Yes	Yes†	In	97	8·1	39	205	0·50	24·5	386	n.d
6	F	42 years	No	Yes‡	In	103	5·9	50	645	0·37	11·4	2581	46·8
7	F	22 years	Yes	Yes	Out	65	80·5	139	372§	0·88	9·1	514	122
8	F	31 years	Yes	Yes	In	95	6·6	34	948	0·54	29·1	805	126
9	M	3 months	Yes	Yes	In	67	2·1	172	802	n.d.	n.d.	2163	n.d
10	F	25 years	Yes	Yes	In	73	25·3	124	29·7	0·20	5·7	1259	n.d
11	M	50 years	No	No	In	121	5·2	49	906	0·34	68·8	854	51·5

Analysis of factors regulating iron homeostasis revealed that the levels of hepcidin in patients (median, 15.8 μg/l) compared to healthy age-matched controls (median, 27.6 μg/l) were low, however, the difference was not statistically significant. Therefore, we calculated the hepcidin/ferritin ratio, which represents a more accurate estimation of proper hepcidin production with respect to iron loading, and found it significantly low in pyruvate kinase deficient patients (median, 0.06) compared with healthy controls (median, 0.35) (Figure 18). The effect of chelation therapy on hepcidin levels and hepcidin/ferritin ration is difficult to address in our cohort.

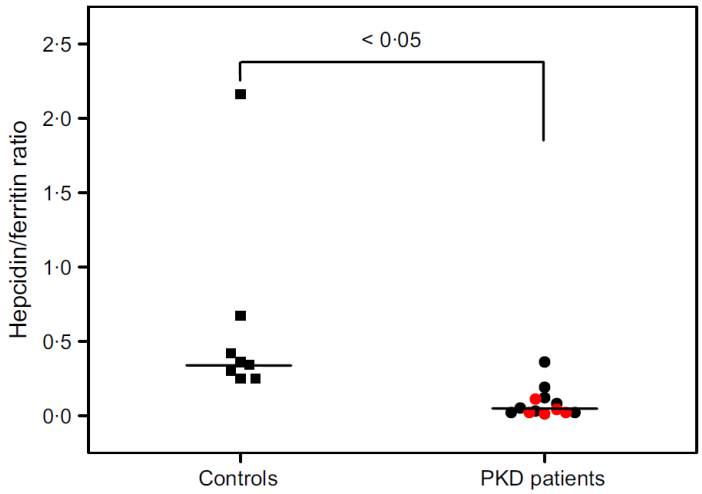

Figure 18. Hepcidin/ferritin ratio in PK-deficient patients. The hepcidin/ferritin ratio was significantly lower in PK-deficient patients compared to healthy controls ($P < 0.05$). The thick horizontal lines represent the medians for each group. PKD, pyruvate kinase-deficient (Mojzikova et al., 2014).

Additionally, pyruvate kinase deficient patients showed elevated levels of GDF15 and EPO compared to healthy controls. No correlation between GDF15 and hepcidin (or hepcidin/ferritin ratio) as well as EPO and hepcidin (or hepcidin/ferritin ratio) were observed. This suggests that GDF15 and EPO are indirect suppressors of hepcidin. However, the main erythroid-derived regulator of hepcidin synthesis in pyruvate kinase deficiency remains yet to be identified.

Based on our results and published studies of another research groups, it is clear that hepcidin analysis is providing new and deep insight into the iron metabolism regulation. Sometimes the results are surprising and leading to the new hypothesis and conclusions. Interpretation of such results is complex and difficult and frequently touching the barrier of current knowledge in the field. Even based on our research we are suggesting that there are existing not discovered regulators of hepcidin synthesis which have to be identified.

2.3 Development of mass spectrometry based method for amyloid typing – impact on clinical diagnostics

2.3.1 Introduction to amyloid typing

Objective: The subtype of amyloid classes from twenty-two formalin-fixed, paraffin-embedded tissues samples by immunohistochemical and proteomic analyses.

Methods: Amyloid subtyping was performed on human heart, liver, kidney, tongue and small intestine tissues samples by IHC and LMD-LC-MS/MS, and the results were compared to the clinical data.

Results: LMD-LC-MS/MS analysis clearly subtyped AL amyloidosis in all twenty-two FFPE tissue samples. In contrast, IHC analysis correctly subtyped AL amyloidosis only in eight FFPE tissue samples. In majority of the sample, where AL amyloidosis was subtyped by LMD-LC-MS/MS, IHC staining intensity was on the same level for transthyretin and serum amyloid A as for Ig κ and Ig λ antibodies, suggesting low Ig κ or Ig λ reactivity and additional antibodies are necessary.

Conclusion: Both methods used in the study were found suitable for amyloid subtyping, where LMD-LC-MS/MS showing more promising results than IHC.

2.3.2 Methods

2.3.2.1 Collection of the samples

Tissue samples were obtained by routinely done autopsy examination at the Department of Clinical and Molecular Pathology between years 2008 and 2013. There were eight men and three women in the selected patient group with the age range from 49 to 84. The median age of these patients was 69. Twenty-two FFPE samples with previously diagnosed amyloid deposits were chosen for our pilot study. Samples were prepared from two different organs per case, which included myocardial, liver, kidney, tongue and small intestine tissues.

2.3.2.2 Histology and immunohistochemistry

Three-µm and 5-µm thick sections of FFPE tissue were prepared for histological and IHC examination, respectively. Histological examination was performed using CR and SR staining to visualise the amyloid deposits. IHC examination was done after deparaffinization, followed by endogenous peroxidase blocking and heat-mediated antigen retrieval using two antibody panels. First panel "Routine panel" included four antibodies against the most common systemic (AL κ, AL λ, AA, ATTR) amyloidosis: anti-human lambda light chains (Ig λ, FLEX polyclonal rabbit ready-to-use, dilution 1:10), anti-human kappa light chains (Ig κ, FLEX polyclonal rabbit ready-to-use, dilution 1:20), anti-human amyloid A (SAA, monoclonal mouse, clone mc1, dilution 1:100) and anti-human prealbumin (TTR, polyclonal rabbit, dilution 1:4000). All antibodies were purchased from DAKO (Glostrup, Denmark). For the second panel "Extra panel" we used two antibodies to help to subtype the correct amyloid class for AL amyloidosis, and included: anti-human kappa light chains (Ig κ (KRA/KUN), polyclonal rabbit, dilution 1:2000), anti-human lambda light chains (Ig λ (ULI/LAT), polyclonal rabbit, dilution 1:500). These antibodies were purchased from amYmed (Martinsried, Germany). In accordance with the semi-quantitative evaluation of IHC staining, the intensity was classified as negative (-), weak (+), moderate (++) and strong (+++).

2.3.2.3 Sample preparation and LMD-LC-MS/MS analysis

Tissue samples were prepared according to previously described methods methods (Klein et al., 2012; Theis et al., 2013; Sethi et al., 2012). Five-µm thick sections of FFPE tissues were placed on membrane slides (Molecular Machines & Industries GmbH, Eching, Germany) and stained with CR or SR. Positive stained amyloid deposits were dissected using Laser Microdissection System MMI CellCut (Molecular Machines & Industries, Eching, Germany). Three separate areas were handled in all tissue samples. Each dissected specimen contained a tissue volume of at least 0.6 nl. The excised materials were collected into three individual 0.5 mL microcentrifuge tube cap (Molecular Machines & Industries, Eching, Germany) containing 35 µL of 10 mM Tris/1 mM EDTA/0.002% Zwittergent 3-16 buffer. Collected materials were then heated at 98 °C for 90 min. Subsequently, the samples were sonicated in a water bath for 60 min and digested using 0.5 µg trypsin overnight at 37 °C. The peptides obtained

following digestion were reduced with 3 μL of 0.1 M dithiotreitol (Sigma Aldrich, Munich, Germany) at 95 °C for 5 min.

The peptide mixtures were loaded onto C18 Acclaim PepMap Nano Trap Column (Thermo Fisher Scientific, Bremen, Germany). Peptides were separated on EASY-Spray column C18, 75 μm × 15 cm (Thermo Fisher Scientific, Bremen, Germany) using a 60-min gradient of 5–35% acetonitrile in 0.1% formic acid. Eluted peptides were analysed with Orbitrap Elite Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) operated in data-dependent mode. Full MS scans were collected in the Orbitrap at a resolution of 60,000. The top ten most intense precursor ions were sequentially isolated for the collision-induced dissociation, and the resulting tandem mass spectra (MS/MS) were collected in the linear ion trap. The raw data were processed by MaxQuant software (Cox & Mann, 2008). The tandem mass spectra were matched against a composite protein sequence database using search engine Andromeda (Cox et al., 2011). The composite database contains protein sequences obtained from the SwissProt database selected for the human subspecies, known human immunoglobulin variant domains, known amyloid fibril protein mutations collected from literature and common contaminants (Dasari et al., 2014). The search engine was configured to detect semitryptic peptides from the composite database while looking for the following variable modifications: oxidation of methionine (+15.996 Da) and n-terminal pyroglutamic acid (−17.023 Da). The cutoff of the global false discovery rate (FDR) for the peptide and protein identification was set to 0.01 (Theis et al., 2013).

For every case, a personalized proteomic profile was created that listed all protein identifications in each of the dissections along with their respective MS/MS spectral counts. The number of the MS/MS spectra matching to a protein is considered as a semi-quantitative measure of its abundance. The amyloidosis subtype was considered to be the most abundant amyloid protein (Table 15, labelled dark-grey) detected across all dissected areas.

2.3.3 Results

Clinical features

The clinical diagnosis and characteristics of the patients are listed in Table 13. Multiple myeloma (MM) and monoclonal gammopathy of undetermined significance

(MGUS) was diagnosed in three and seven patients, respectively. Clinical diagnosis was unknown in the case 10. Serum paraproteinemia of IgA λ light chain type (cases 7 and 8), IgG κ light chain type (case 1), and IgD κ light chain type (case 2) was detected by the serum protein electrophoresis and immunofixation. Increased levels of serum free κ light chain (FLC κ), and λ light chain (FLC λ) were found in the cases 1, 2, 3, 4, 5 and 6, 7, 8, 9, 11, respectively. All patients except cases 8 and 9 had abnormal free light chain ratio (FLC κ/λ ratio). Cardiomyopathy (stage Mayo 3) was diagnosed in all patients. Proteinuria was detected in ten patients (for the case 10 no data were available) and eight patients met the criteria for renal insufficiency (proteinuria with increased creatinine and urea; cases 1, 2, 3, 4, 6, 7, 8 and 9).

Table 13. Clinical diagnosis and characteristics of patients

Case	Sex	Age	Clinical Diagnosis	Serum Protein	FLC κ [mg/L]	FLC λ [mg/L]	FLC κ/λ ratio	Cardiomyopathy	Nephropathy
1	M	68	MGUS	IgG κ	449.5	91.0	4.939	Mayo 3	NS + RI
2	M	58	MM	IgD κ	241. 5	1	241.4	Mayo 3	NS + RI
3	M	49	MGUS	κ	2114.3	36.7	57.61	Mayo 3	NS + RI
4	M	70	MM	κ	906.3	16.5	54.90	Mayo 3	NS + RI
5	F	78	MM	κ	1588.2	24.4	65.09	Mayo 3	NS
6	M	63	MGUS	λ	18.2	593.4	0.031	Mayo 3	NS + RI
7	F	75	MGUS	IgA λ	18.7	284.7	0.066	Mayo 3	NS + RI
8	M	84	MGUS	IgA λ	47.2	84.1	0.561	Mayo 3	NS + RI
9	F	67	MGUS	λ	21.1	70.1	0.302	Mayo 3	NS + RI
10	M	77	-	ND	ND	ND	ND	Mayo 3	-
11	M	49	MGUS	λ	22.6	495.1	0.046	Mayo 3	NS

FLC, free light chains; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; ND, not determined; NS, nephrotic syndrome; RI, renal insufficiency (creatinine level ≥ 130 μmol/L); Mayo, Mayo Clinic staging system (1-3) based on troponin T and NT-proBNP levels
Reference range: FLC κ: 3.3–19.4 mg/L; FLC λ: 5.7–26.3 mg/L; FLC κ/λ ratio 0.26–1.65

LMD-LC-MS/MS and IHC analysis

Results from IHC and proteomics analysis are summarized in Table 14. LMD-LC-MS/MS analysis identified Ig kappa chain C region and Ig lambda-2 chain C region as the most abundant amyloid fibril proteins for the examined tissues in five (1, 2, 3, 4, 5) and six (6, 7, 8, 9, 10, 11) cases, respectively. AL amyloidosis was clearly subtyped in all eleven cases, where in five cases it was AL κ type, and in six cases, it was AL λ type. The results from LMD-LC-MS/MS analysis completely correlated with the clinical symptoms in all patients. Detailed results from LMD-LC-MS/MS analysis are summarized in Table 15.

IHC analysis that used "Routine panel" of antibodies subtyped amyloid fibril protein just in five specimens from three patients (both tissues in cases 6, 8, 9, and heart tissue sample in cases 5 and 11). Heart tissue samples of case 1 failed in IHC staining with all four antibodies, therefore the typing could not be done. Surprisingly, both tissues of case 3 had a positive reaction with TTR antibody, and negative reaction with Ig κ and Ig λ antibodies. However, clinical diagnosis and proteomic analysis subtype AL κ amyloidosis in this case. In six other cases (2, 4, 5, 7, 10, 11), one or both examined tissues had positive reaction for more than one antibody. The vast majority of tissues had a false positive reaction with TTR (14 of 22) and SAA antibody (6 of 22) (Figure 19).

IHC analysis that used "Extra panel" of antibodies (in Table 14 marked as +IHC), subtyped correctly amyloid fibril protein in twelve specimens (both tissues in cases 4, 6, 8, 9, and heart tissue samples in cases 1, 5, 7, 11). IHC analysis was not conclusive in the remaining ten specimens. These examined tissues (both tissues in cases 2, 3, 10, and liver tissue samples in the cases 1, 5, 7, 11) had positive reaction for more than one antibody.

Table 14. Amyloid subtyping based on IHC and proteomics analysis

Case	Tissue	IHC: Routine panel[a]				IHC subtyping	+IHC: Extra panel[a]		+IHC subtyping	Proteomics subtyping	Classified amyloid
		Ig κ[b]	Ig λ[b]	SAA[b]	TTR[b]		Ig κ[c]	Ig λ[c]			
1	Heart	-	-	-	-	-	++	-	AL κ	AL κ	AL κ
	Liver	-	+ / -	-	-	AL λ	+	-	NS	AL κ	
2	Heart	+	-	+ / ++	+	AA	++	+	NS	AL κ	AL κ
	Kidney	+	-	++	++	NS	++	+	NS	AL κ	
3	Heart	-	-	-	+ / ++	ATTR	++	-	NS	AL κ	AL κ
	Liver	-	-	-	+	ATTR	+	-	NS	AL κ	
4	Heart	+	-	-	+	NS	++	-	AL κ	AL κ	AL κ
	Tongue	+	-	-	+	NS	++	-	AL κ	AL κ	
5	Heart	++	-	-	-	AL κ	++	+ / -	AL κ	AL κ	AL κ
	Liver	+	-	+	-	NS	+	-	NS	AL κ	
6	Heart	-	+	-	-	AL λ	-	++	AL λ	AL λ	AL λ
	Liver	-	+	-	-	AL λ	+	++	AL λ	AL λ	
7	Heart	-	++	-	++	NS	++	+++	AL λ	AL λ	AL λ
	Liver	-	++	-	++	NS	++	++	NS	AL λ	
8	Heart	-	++	-	+	AL λ	+	+++	AL λ	AL λ	AL λ
	Kidney	-	++	-	+ / -	AL λ	+	+++	AL λ	AL λ	
9	Heart	-	+	-	-	AL λ	-	++	AL λ	AL λ	AL λ
	Liver	-	++	-	-	AL λ	-	+	AL λ	AL λ	
10	Heart	-	++ / +++	+ / ++	++ / +++	NS	+	++	NS	AL λ	AL λ
	Small intestine	-	+ / ++	+ / ++	++	NS	++	++	NS	AL λ	
11	Heart	-	++	+	+	AL λ	+ / -	++	AL λ	AL λ	AL λ
	Liver	-	++	-	+ / ++	NS	-	+	NS	AL λ	

Ig κ, immunoglobulin light chain kappa; Ig λ, immunoglobulin light chain lambda; NS, no immunospecific staining; SAA, serum amyloid A; TTR, transthyretin; AL κ, amyloidosis derived from immunoglobulin light chain kappa; AL λ, amyloidosis derived from immunoglobulin light chain lambda; AA, amyloidosis derived from serum amyloid A; ATTR, amyloidosis derived from transthyretin.

[a] The intensity of IHC staining was classified as negative (-), weak (+), moderate (++) and strong (+++); [b]Antibodies from DAKO; [c]Antibodies from amYmed.

Table 15. Amyloid subtyping based on the proteomics analysis

			The MS/MS counts of the most abundant amyloid proteins																	
Case	Tissue	Biological replicates	Ig kappa chain C region	Apolipoprotein E	Ig gamma-1 chain C region	Serum amyloid P-component	Ig gamma-3 chain C region	Apolipoprotein A-IV	Ig kappa chain V-III region SIE	Ig gamma-2 chain C region	Ig kappa chain V-III region VG	Ig alpha-1 chain C region	Apolipoprotein A-I	Ig lambda-2 chain C regions	Gelsolin	Transthyretin	Ig kappa chain V-III region POM	Fibrinogen alpha chain	⋮	Proteomics subtyping
1	Heart	3	51	36	20	19	9	9	8	7	7	5	5	4	4	3	3	2	...	**AL κ**
	Liver	3	64	61	15	33	5	11	9	3	9	6	7	6	2	4	3	4	...	**AL κ**
2	Heart	3	56	25	4	10	1	37	2	1	0	1	29	2	7	11	0	11	...	**AL κ**
	Kidney	2	44	17	4	5	1	24	4	0	0	1	31	2	13	14	0	2	...	**AL κ**
3	Heart	3	37	35	2	9	1	53	1	0	0	1	15	2	2	10	0	3	...	**AL κ**
	Liver	3	34	62	6	9	3	26	1	0	0	1	4	2	1	9	0	10	...	**AL κ**
4	Heart	3	107	32	13	16	7	45	2	6	0	3	12	4	6	14	1	4	...	**AL κ**
	Tongue	3	40	33	12	12	5	7	2	5	0	3	8	4	0	6	0	10	...	**AL κ**
5	Heart	3	12	12	1	5	1	12	1	0	0	1	4	1	0	2	4	0	...	**AL κ**
	Liver	2	20	11	2	13	2	38	5	0	1	4	4	2	2	2	5	2	...	**AL κ**
6	Heart	3	7	11	6	5	2	29	1	1	0	4	8	14	2	7	1	4	...	**AL λ**
	Liver	3	3	16	6	9	2	32	2	1	1	3	6	12	1	8	0	3	...	**AL λ**
7	Heart	3	6	20	7	20	3	42	2	1	0	4	17	31	6	8	0	10	...	**AL λ**
	Liver	1	3	15	6	27	2	31	1	0	0	3	13	17	1	6	0	3	...	**AL λ**
8	Heart	3	5	17	3	15	2	40	1	1	0	5	9	25	1	4	0	6	...	**AL λ**
	Kidney	3	11	23	9	31	4	53	2	5	1	8	8	29	4	10	0	4	...	**AL λ**
9	Heart	3	5	25	5	12	2	10	1	1	0	2	10	30	5	3	0	5	...	**AL λ**
	Liver	3	3	44	6	28	2	13	1	1	0	3	13	30	0	4	0	8	...	**AL λ**
10	Heart	3	10	22	6	8	3	31	0	4	0	3	15	26	4	3	0	4	...	**AL λ**
	Small intestine	3	13	43	15	13	6	14	2	10	1	7	5	32	5	5	1	4	...	**AL λ**
11	Heart	3	1	14	1	7	0	31	0	1	0	1	1	30	2	1	0	1	...	**AL λ**
	Liver	3	1	44	0	10	0	24	0	0	0	2	0	34	0	2	0	9	...	**AL λ**

..., indicates the most abundant amyloid protein in the sample; ..., indicates proteins that are associated with the amyloid formation.

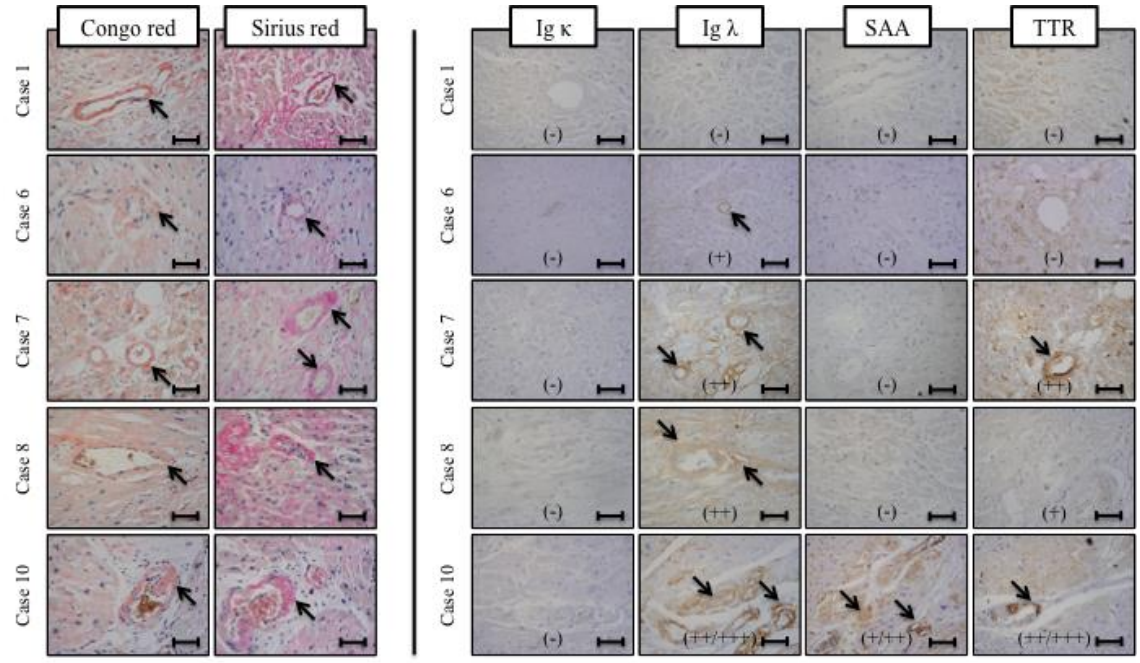

Figure 19. Confirmation of the presence of the amyloid deposition using CR and SR staining is on the left side. Amyloid subtyping using IHC analysis (IHC: Routine panel) is on the right side (only selected cases are demonstrated). Myocardial tissue sample of the case 1 failed in IHC staining with all four antibodies. In cases 6 and 8, IHC analysis certainly determined amyloid fibril protein (AL λ). In cases 7 and 10 examined tissues showed positive reaction for more then one antibody. IHC staining intensity was classified as negative (-), weak (+), moderate (++) and strong (+++). Scale bar = 10 μm.

2.3.4 Discussion

Precise typing of amyloidosis in tissues is crucial for the treatment and prognosis (Leung et al., 2012). Amyloidosis has an incidence of approximately 8 - 10 cases per million per year (Merlini & Bellotti, 2003). The population in Czech Republic was last recorded at 10.5 million people in 2014, thus incidence of amyloidosis corresponds 84 - 105 cases. In the present study we used multidisciplinary diagnostic approaches including clinical evaluation and biochemical tests combined with the special staining (CR, SR), IHC and proteomics analysis for amyloid diagnosis and typing of approximately 10% cases in the Czech Republic. Clinical features of the patients with amyloidosis in this study corresponded with typical organ involvement, such as cardiomyopathy and proteinuria/RI that are most common organ failures in AL amyloidosis patients (Desport et al., 2012). The data from LMD-LC-MS/MS analysis correlated with the clinical data, where AL κ and AL λ were determined as the most abundant amyloid fibril proteins in patients with high concentration of serum FLC κ and FLC λ, respectively (Table 14 and 15).

IHC and LMD-LC-MS/MS are predominant methods, which have been recently applied to the amyloid subtyping (Linke, 2012; Theis et al., 2013). Fundamentally, results from these two methods differed in the current study. IHC subtyped correctly only eight (36%) of the twenty-two FFPE samples; whereas LMD-LC-MS/MS precisely identified AL amyloid fibril proteins in all 22 FFPE samples. In addition to the most abundant amyloid protein, LMD-LC-MS/MS also identified serum amyloid P, apolipoprotein E and apolipoprotein A-IV in the amyloid deposits (Table 15, labelled light-grey). It was previously shown that these proteins are associated with amyloid formation (Rodriguez et al., 2008).

According to IHC, the fourteen of twenty-two examined samples had false positive reaction with TTR and/or SAA antibodies, where the intensity of IHC staining in some case was even higher than with Ig κ or Ig λ antibody. Much effort has been developed for the detailed study of IHC insufficiency for final subtyping of AL amyloidosis (Schönland et al., 2012; Picken, 2007). The most critical factors that affect IHC were found to be: heterogeneity of variable domains in the amino-terminal end of the light chains, preanalytical effect of the formalin mediated tissue fixation, antigen masking due to protein folding, fragmentation of light chain molecules, and variable quality of the commercially available antibodies (Schönland et al., 2012). All these factors could

decrease the reactivity of Ig κ or Ig λ antibodies. In such cases, the intensity of IHC staining could be the same for more than one amyloid protein involved in amyloid formation, and the most abundant amyloid protein may not be conclusively identified (Satoskar et al., 2011; Lachmann et al., 2002 ; Novak et al., 2004). This was case 2 in the present study, where LMD-LC-MS/MS identified AL κ as the most abundant amyloid protein. However, the intensity of IHC staining for Ig κ antibody was classified as weak (+), while that of TTR and SAA antibody was moderate (++). In addition, the presence of transthyretin in the samples of case 2 was confirmed by LMD-LC-MS/MS analysis, however, their abundance was lower than Ig κ protein (Table 15). These results support previous findings and confirm that IHC is insufficient for amyloid subtyping in this case. The increased specificity of IHC can be achieved by the application of several different antibodies targeted against the same Ig κ or Ig λ, which was previously shown in several studies (Schönland et al., 2012; Linke, 2012). The application of two to four antibodies against Ig κ or Ig λ led to the precise diagnosis of up to 94% of the examined cases (Schönland et al., 2012; Linke, 2012; Chee et al., 2010). Based on our IHC results with "Routine panel" of antibodies, we decided to apply additional two antibodies ("Extra panel") targeted against the Ig κ or Ig λ. This step helped to increase specificity of the IHC staining from 36 to 54.5%. This specificity is not definitive due to the small sample size, and need further examination.

Despite the failures, IHC method is still the most preferred method in about 83% of laboratories and does not need any high-tech equipment (Linke, 2012). In contrast, the cost of LMD and LC-MS are high and therefore, are available only in the specialized institutions. Contrastively, LMD-LC-MS/MS assay was able to determine the correct typing of amyloidosis in all cases without the previous knowledge of the clinical data. Taken together, both methods are essential for the amyloid subtyping, with IHC being the first-choice. However and according to our finding, LMD-LC-MS/MS will be of valuable when clinical data are inconsistent of IHC, and IHC staining results in dual positive or negative outcomes.

3 Summary

In the proposed thesis we are developing and using new proteomic mass spectrometry based methods in the evaluation of the clinical and patients' samples. Our work is focused on hematological diseases where we are analyzing key regulatory molecule hepcidin and on amyloidosis where we can directly analyze presence of amyloidogenic proteins and make amyloidosis diagnosis more accurate.

Hepcidin is the key regulatory molecule responsible for the maintenance of iron homeostasis in the body. In the frame of the first aim of proposed thesis and as a necessary prerequisite to the clinical studies targeting the iron metabolism in the different hematological diagnoses, we have developed SPE-LC-MS/MS method for quantification of human hepcidin-25 level in serum (**section 2.2.1**). We tried to improve the technical reproducibility of the metod by elimination manual steps like pipetting by the use of the Tomtec Quadra pipetting station and parallelization of solid phase extraction (SPE) for sample preparation prior to LC-MS/MS (SRM) measurement by use of Oasis HLB μElution 96 well extraction plates. Hepcidin was quantified using a chemically synthesized hepcidin standard and corresponding IS in rabbit serum as the surrogate calibration matrix. This method has been adapted and validated according to Food and Drug Administration (FDA) agency guidelines for bioanalytical methods validation (Li et al., 2009). The method is capable of measuring hepcidin concentration in over the range of 2.5 to 500 ng/mL and is robust and reliable in terms of accuracy and precision for monitoring hepcidin level in serum. The hepcidin was analyzed and evaluated in various diseases and in the healthy subjects as well as. Over 1300 serum samples from patients or healthy subjects were analyzed to date (data not shown) (**Holub et al., 2016; under review**).

In the first Diamond-Blackfan anemia study, we evaluated the selected markers of erythropoietic activity and iron metabolism including hepcidin level with different severities of anemia and treatment strategies (**section 2.2.4**). In the first group, (transfusion-dependent patients) we found reduced number of erythroblasts in the bone marrow and decreased sTfR levels. These findings confirmed suppressed erythropoiesis that corresponded to elevated EPO level. Analysis of iron parameters showed increased serum iron, TSAT and high ferritin levels with significantly elevated levels of hepcidin. This suggests that erythropoiesis is unable to utilize transferrin-bound iron effectively.

Moreover, liver biopsy showed elevated liver iron concentration and massive iron stores in hepatocytes and Kupffer cells. The macrophage iron loading can be attributed to non-effective erythrocyte-derived iron recycling (Hentze et al., 2010; Ganz & Nemeth, 2012). In the second group, patients in disease remission or steroids treated showed improved erytropoiesis (normal/higher number of bone marrow erythroblasts and level of sTfR in normal range). This group had slightly increased or normal level of ferritin and near normal serum iron and TSAT. This indicates an improved erythropoietic activity increases iron utilization and consequently leads to amelioration of hyperferritinemia. Restoring erythropoietic activity also attenuated hepcidin expression. We conclude that hepcidin correlates with ferritin, reflecting transcription stimulation of hepcidin expression by the patients iron overload. The relationship between EPO and number of erythroblasts in the bone marrow showed that EPO is stimulated in response to suppressed erythropoiesis and hypoxia. A positive correlation between hepcidin and EPO demonstrates that hepcidin suppression by EPO requires active erythropoiesis in the bone marrow. We found significantly increased levels of GDF15 in the whole DBA cohort presumably due to increased apoptosis of bone marrow erythroblast. No correlation between hepcidin and GDF15 indicates that GDF15 is not playing hepcidin-regulatory role in DBA (**Pospisilova et al., 2014**).

In the second study, we investigated patients with erythrocyte membrane defects and thalassemia carriers. The hematological data in patients with erythrocyte membrane defects showed significantly reduced RBC count and significantly increased MCHC in compare with healthy controls. These finding reflect compensatory reticulocytosis in response to chronic hemolysis. The markers of erythropoietic activity showed elevated EPO and sTfR levels in the group of erythrocyte membrane defect. This indicates increased erythropoietic activity in the bone marrow patients with erythrocyte membrane defect. As a marker of ineffective erythropoiesis, serum GDF15 levels were found elevated in the group of erythrocyte membrane defect in compare with healthy control. The parameters of iron metabolism showed increased level of serum ferritin and reduced (not significant) hepcidin levels. The hepcidin/ferritin ratio was significantly lower in erythrocyte membrane defects group compared to healthy controls and indicated inappropriate suppression of hepcidin synthesis. The hematological data in thalassemia carriers showed raised RBC count and reduced Hb, MCV, MCH and MCHC compared to healthy controls. Elevated sTfR and GDF15 were found, which indicate increased but ineffective erythropoiesis in the bone marrow. The parameters of

iron metabolism showed reduced (not significant) hepcidin levels. The reduction (not significant) of hepcidin/ferritin ratio was found in thalassemia carriers compared to healthy controls and indicated inappropriate suppression of hepcidin synthesis. Our patient with β-thalassemia major with the most severe form of anemia showed reduced RBC count and Hb level. The very low hepcidin/ferritine ratio showed that hepcidin was disproportionally low with respect to iron loading. The low hepcidin/ferritin ratio and elevated sTfR and GDF15 reflect increased ineffective erythropoiesis with higher iron demand leading to increased iron absorption and iron recycling. The iron overload in β-thalassemia major is a result of regular transfusion therapy and disrupted hepcidin synthesis and this finding has previously been described (Pasricha et al., 2013). Finally, we calculated (hepcidin/ferritin)/sTfR ratio which combines the parameters of iron status and erythropoietic activity. This ratio was reduced in β-thalassemia (0.001), erythrocyte membrane defects group (0.023) and thalassemia carrier group (0.072) in comparison to healthy controls (0.184). This ratio also reflects the severity of the anemia and can be used to follow up patients with erythrocyte membrane defects or thalassemia carriers for disease development with focus on alterations in erythropoietic activity and potential risk of iron overload (**Sulovska et al., 2015**).

In the third pyruvate kinase deficiency study, we investigated 11 patients and found 9 different mutations in the PKLR gene. Two of these mutations were novel and were associated with atypical severe phenotype (involving neonatal hyperferritinanemia). All patients had secondary iron overload due to chronic heamolysis, ineffective erythropoiesis and transfusion therapy. We have observed low levels of hepcidin in PKD patients compared to healthy controls, however the difference was not statistical significant. Therefore, we calculated the hepcidin/ferritin ratio, and found it significantly low in pyruvate kinase deficient patients compared with healthy controls. Additionally, pyruvate kinase deficient patients showed elevated levels of GDF15 and EPO compared to healthy controls. No correlation between GDF15 or EPO and hepcidin (or hepcidin/ferritin ratio) were observed. This suggests that GDF15 and EPO are indirect suppressors of hepcidin expression. The main erythroid-derived regulator of hepcidin expression in pyruvate kinase deficiency remains yet to be identified (**Mojzikova et al., 2014**).

In conclusion, hepcidin assay is particularly attractive for diagnosis and risk stratification of iron overloading at various hematological disorders. Quantification of

hepcidin levels in serum with combination with existing diagnostic methods helps to simplify and rationalize understanding of the pathogenesis of the various iron disorders.

In the second part of experimental section of my thesis, I present amyloid subtyping results, which were analysed by IHC and mass spectrometry based proteomics analysis on twenty-two FFPE patient tissue samples from 11 patients with diagnosed amylodosis (**Section 2.3**). Our aim was to perform and to compare amyloid subtyping by available and routinely used immunohistochemistry methods, which are highly dependent on the used antibodies with proteomic method, which is based on the direct identification of the amyloidogenic proteins. Determination of the amyloidosis subtype was possible in all eleven examined cases. Considering the clinical diagnosis, we found that proteomics analysis is very accurate and suitable method for the precise diagnosis of amyloidosis with 100% sensitivity and 100% specificity in the present study. On the other hand, sensitivity and specificity of IHC analysis was 96% and 36% respectively, suggesting that in many individual cases the IHC method is insufficient and additional analysis, including usage of multiple antibodies against the same amyloidogenic protein, is required for the improvement of proper amyloid subtyping.

We can conclude that methods, which are based on the direct proteomic analysis, are providing more accurate results than method, which are dependent on the specificity of the antibodies (ELISA, IHC). Moreover, such methods can't frequently distinguished precursor and active fragments like in the case of studied peptide hepcidin, where only hepcidin-25 is showing activity and precursors protein and shorter fragments hepcidin-21 and hepcidin-21 are inactive. Similar situation is in the case of amyloidosis, where we can detect amyloidogenic proteins directly and in some cases even with the causative amyloidogenic mutations at protein level. However, the complexity of the mass spectrometry based proteomic methods, expensive instrumentation and personnel qualification is high and will be at least in the near future used only in the research and diagnostic centres providing the servis to the clinical facilities.

4 Souhrn

V rámci předkládané dizertační práce jsme vyvinuli a využili nové na hmotnostní spektrometrii založené metody použitelné v analýze klinických a pacientských vzorků.

Naše práce byla zaměřená na hematologická onemocnění, kde jsme analyzovali klíčovou regulační molekulu hepcidin a na amyloidózu, kde můžeme přímo analyzovat přítomnost amyloidogenního proteinu a tím významně zpřesnit diagnostiku amyloidózy.

Hepcidin je klíčová regulační molekula odpovědná za udržování homeostázy železa v těle. V rámci prvního cíle předkládané dizertační práce a také jako nezbytný předpoklad pro další klinické studie cílící metabolismus železa u rozdílných hematologických diagnóz, jsme vyvinuli SPE-LC-MS/MS metodu pro kvantifikaci lidského hepcidinu-25 v séru (**část 2.2.1**). Pokusili jsme se vylepšit technickou reprodukovatelnost metody pomocí nahrazení manuálních kroků jako je například pipetování použitím automatické Tomtec Quadra pipetovací stanice a také paralelizací extrakce na pevné fázi pro přípravu vzorků před vlastím LC-MS/MS (SRM) měřením pomocí použití Oasis HLB µElution 96 jamkových extrakčních destiček. Hepcidin byl kvantifikován pomocí chemicky syntetizovaného standardu hepcidinu a korespondujícího interního standardu v králičím séru jako alternativní komplexní matrici. Tato metoda byla adaptovaná a validovaná podle "Food and Drug Administration agency (FDA)" pravidel pro validaci bioanalytických metod (Li et al., 2009). Metoda umožňuje spolehlivě měřit koncentraci hepcidinu v rozsahu 2,5 – 500 ng/mL a je robustní a spolehlivá ve smyslu přesnosti a preciznosti pro monitorování koncentrace hepcidinu v séru. Hepcidin byl analyzován a hodnocen u různých hematologických onemocnění stejně jako u zdravých subjektů. Doposud jsme změřili vice než 1300 pacientských a kontrolních vzorků (nepublikovaná data) (**Holub et al., 2016**, v recenzním řízení).

V první studii, zabývající se Blakfanovou-Daimondovou anemií (DBA) jsme hodnotili vybrané parametry aktivity krvetvorby a metabolismu železa včetně hladiny hepcidinu s rozdílnou tíží anémie a léčebné strategie (**část 2.2.4**). V první skupině (pacienti závislí na transfuzi) jsme nalezli v kostní dřeni redukovaný počet erytroblastů a snížené hladiny sTfR. Tento nález potvrzuje potlačenou erytropoézu, která

koresponduje se zvýšenou hladinou EPO. Analýza parametrů metabolismu železa objevila zvýšenou hladinu železa v séru, TSAT a vysoké hodnoty feritinu se signifikantně zvýšenou hladinou hepcidinu. Na základě toho můžeme usuzovat, že krvetvorba nedokáže efektivně využívat železo navázané na transferin. Navíc jaterní biopsie ukázala zvýšenou koncentraci železa v játrech a masivní koncentraci železa v hepatocytech a Kupfferových buňkách. Železo akumulované v makrofázích může být připsáno na vrub neefektivnímu recyklování železa v rámci krvetvorby (Hentze et al., 2010; Ganz & Nemeth, 2012). Ve druhé studované skupině (pacienti s DBA v remisi onemocnění nebo na léčbě steroidy) jsme pozorovali zlepšenou krvetvorbu (normální/vyšší počet erytroblastů v kostní dřeni a normální hladinu sTfR). Tato skupina měla lehce zvýšenou nebo normální hodnotu ferritinu a téměř normální koncentraci železa v séru a TSAT. Tento nález ukazuje na zlepšenou aktivitu krvetvorby, lepší využití železa a v důsledku toho i úpravu hodnot hyperferritinémie. Obnovená erytropoetická aktivita také vede ke snížení hladiny hepcidinu. Na základě těchto dat jsme došli k závěru, že hladina hepcidinu koreluje s hladinou ferritinu a tak je výrazem transkripční stimulace exprese při přetížení železem. Pozorovaný vztah mezi EPO a počtem erytroblastů v kostní dřeni ukázal, že tvorba EPO je stimulovaná jako odpověď na nízkou krvetvorbu a hypoxii. Kladná korelace mezi koncentrací hepcidinu a EPO ukazuje, že potlačení exprese hepcidinu pomocí EPO vyžaduje aktivní krvetvorbu v kostní dřeni. Pozorovali jsme také signifikantně zvýšené hodnoty GDF15 u celé skupiny DBA pacientů a to pravděpodobně díky výšené apoptóze u erytroblastů v kostní dřeni. Nenalezená korelace mezi hepcidinem a GDF15 ukazuje, že GDF 15 nehraje žádnou roli v regulaci hepcidinu u pacientů s DBA (**Pospisilova et al., 2014**).

V druhé studii jsme se zaměřili na pacienty s defekty na membráně erytrocytů a na přenašeče talasémie. Hematologické parametry u pacientů s defekty na membráně erytrocytů ukázaly signifikantně redukovaný počet erytrocytů a významně zvýšený MCHC ve srovnání se zdravými kontrolami. Tyto nálezy odpovídají kompenzátorní retikulocytóze jako odpovědi na chronickou hemolýzu. Pozorovali jsme zvýšenou hladinu EPO a sTfR ve skupině pacientů s membránovými defekty na erytrocytech, což signalizuje zvýšenou erytropoetickou aktivitu v kostní dřeni. Přitom marker neefektivní erytropoézy, sérová hladina GDF15, byla zvýšená. Dále jsme pozorovali zvýšenou hladinu ferritinu a nesignifikantně sníženou hladinu hepcidinu. Poměr hepcidin/ferritin byl signifikantně nižší u pacientů s membránovými defekty, než ve zdravé kontrole a ukazoval na nevhodné potlačení syntézy hepcidinu. U přenašečů talasémie jsme

pozorovali zvýšený počet erytrocytů a snížené hodnoty Hb, MCV, MCH a MCHC ve srovnání se zdravými kontrolami. Zvýšené koncentrace sTfR a GDF15 ukazují na zvýšenou, ale neúčinnou krvetvorbu v kostní dřeni. V rámci parametrů metabolismu železa jsme pozorovali sníženou (nesignifikantně) hladinu hepcidinu. Redukovaný (nesignifikantně) poměr hepcidin/ferritin byl pozorován u přenašečů talasémie ve srovnání se zdravými kontrolami a je důsledkem nevhodného potlačení syntézy hepcidinu. U jednoho pacienta s β-talasémií major s nejtěžší formou anémie jsme pozorovali redukovaný počet RBC a hladinu hemoglobinu. Naproti tomu nízký poměr hepcidin/ferritin ukázal, že nízká hladina hepcidinu neodpovídá vysoké saturaci železem. Takto snížený poměr a zvýšené hodnoty sTfR a GDF15 jsou pozorovány zřejmě v důsledku neúčinné krvetvorby s vysokými požadavky na železo, což vede k jeho zvýšené absorpci a recyklaci. Přetížení železem u β-talasémie major je výsledkem pravidelné transfúzní terapie a narušené regulace syntézy hepcidinu, což již bylo v minulosti již popsáno (Pasricha et al., 2013). Závěrem jsme hodnotili (hepcidin/ferritin)/sTfR poměr, který kombinuje parametry zásob železa a erytropoetické aktivity. Pozorovali jsme redukovaný poměr u β-talasémie (0,001), skupiny s membránovými defekty na erytrocytech (0,023) a skupině přenašečů talasémie (0,072) ve srovnání se zdravými kontrolami (0,184). Tento poměr také odpovídá tíži anémie a může být použitý pro sledování pacientů s membránovými defekty nebo u přenašečů talasémie a hodnocení vývoje onemocnění se zaměřením na změny v erytropoetické aktivitě a rizika spojená s přetížením železem (**Sulovska et al., 2015**).

Ve třetí studii zaměřené na deficit pyruvát kinázy (PKD) bylo vyšetřeno 11 pacientů a nalezli jsme 9 rozdílných mutací v PKLR genu. Dvě z těchto mutací jsou nové a byly asociovány s atypicky těžkým fenotypem (včetně novorozenecké hyperferritinémie). Všichni pacienti měli sekundární přetížení železem v důsledku chronické hemolýzy, neefektivní krvetvorby a transfúzní terapie. Pozorovali jsme nízké hladiny hepcidinu, nicméně pokles nebyl statisticky signifikantní. Proto jsme hodnotili poměr hepcidin/ferritin a zjistili jsme, že je významně snížený ve skupině s deficitem pyruvát kinázy. Kromě toho pacienti s PKD vykazovali zvýšené hodnoty GDF15 a EPO ve srovnání se zdravými kontrolami. Nenalezli jsme žádnou korelaci mezi GDF15 nebo EPO a hepcidinem (nebo poměrem hepcidin/ferritin). To ukazuje, že GDF15 a EPO jsou nepřímými supresory tvorby hepcidinu. Je pravděpodobné, že hlavní regulátor exprese hepcidinu v souvislosti tvorbou červených krvinek čeká ještě na své objevení

(**Mojzikova et al., 2014**).

Závěrem můžeme konstatovat, že vyšetření hepcidinu je zajímavé pro diagnostiku a vyhodnocení rizika přetížení železem u řady hematologických onemocnění. Stanovení hladiny hepcidinu v séru s kombinací existujících diagnostickým metod pomáhá zjednodušit a racionalizovat porozumění patogeneze řady onemocnění spojených s metabolismem železa.

Ve druhé části experimentální sekce mé dizertační práce prezentuji výsledky typizace amyloidu, kdy byla použita imunohistochemická metoda (IHC) a hmotnostně spektrometrická proteomická analýza u dvaceti dvou formaldehydem fixovaných, parafinovaných tkáňových vzorků od celkem 11 pacientů s diagnózou amyloidózy (část 2.3). Naším cílem bylo provést a porovnat v současnosti dostupné a k typizaci amyloidu používané na protilátkách závislé imunohistochemické metody s novou proteomickou metodou, která je založená na přímé identifikaci amyloidogenních proteinů. Stanovení podtypu amyloidózy bylo možné u všech 11 vyšetřovaných pacientů. Zjistili jsme, že pro účely klinické diagnostiky amyloidózy je proteomická analýza velmi přesnou a vhodnou metodou se 100% sensitivitou a 100% specificitou v naši studii. Naproti tomu sensitivita a specificita IHC metody byla pouze 96 a 36%, což ukazuje, že v řadě případů je IHC metoda nedostačující a dodatečné analýzy za použití dalších protilátek vůči amyloidogenním proteinům je nezbytné pro zpřesnění typizace amyloidu.

Závěrem můžeme zhodnotit, že metody založené na proteomické analýze mohou v některých případech poskytovat přesnější výsledky, než metody, které jsou založené na specificitě protilátek (ELISA, IHC). Kromě toho tyto metody často nedokáží rozlišit prekurzorové formy od bioaktivní formy, jak to je například v případě hepcidinu, kdy pouze hepcidin-25 vykazuje bioaktivitu, zatímco prekurzorová forma a zkrácené formy hepcidinu (hepcidin-20 a hepcidin-22) bioaktivitu nemají. Podobná situace je i v případě amyloidózy, kdy detekujeme přímo peptidy amyloidogenních proteinů a zároveň v některých případech můžeme identifikovat i jejich mutace na úrovni aminokyselinové sekvence. Nicméně v případě proteomické analýzy narážíme v současnosti na komplexitu hmotnostně spektrometrických a proteomických metod, nákladné přístrojové vybavení a požadavek na kvalitně vyškolený personál. Proto je

velmi pravděpodobné, že v blízké budoucnosti bude hmotnostně spektrometrická, proteomická analýza dostupná pouze ve výzkumných a diagnostických centrech poskytujících servis klinickým pracovištím.

5 Abbreviations

AA	amyloid fibrils derived from serum amyloid A
AA amyloidosis	amyloidosis derived from serum amyloid A
AApoAI	amyloid fibril derived apolipoprotein A-I
AApoAI amyloidosis	amyloidosis derived from apolipoprotein A-I
AApoAIV	amyloid fibrils derived from apolipoprotein A-IV
AApoAIV amyloidosis	amyloidosis derived from apolipoprotein A-IV
ACD	anaemia of chronic disease
ACN	acetonitrile
AFib	amyloid fibrils derived from fibrinogen α
AFib amyloidosis	amyloidosis derived from fibrinogen α
AL	amyloid fibrils derived from immunoglobulin light chain
AL amyloidosis	amyloidosis derived from immunoglobulin light chain
AL κ	amyloid fibrils derived from immunoglobulin light chain kappa
AL κ amyloidosis	amyloidosis derived from immunoglobulin light chain kappa
AL λ	amyloid fibrils derived from immunoglobulin light chain lambda
AL λ amyloidosis	amyloidosis derived from immunoglobulin light chain lambda
ATP	adenosine triphosphate
ATTR	amyloid fibrils derived from transthyretin
ATTR amyloidosis	amyloidosis derived from transthyretin
BMP6	bone morphogenetic protein 6
CE	collision energy
CR	congo red
CGRP	calcitonin gene-related paptide
CV	coefficient of variation
CXP	collision cell exit potential
Da	Dalton unit
DBA	Diamond-Blackfan anemia
DP	declustering potential
ELISA	enzyme linked immunosorbent assay
EP	entrance potential
EPO	erythropoietin

FDA	U.S. Food and Drug Administration
FDR	false discovery rate
FFPE	formalin-fixed, paraffin embedded tissue
FLC	free light chains
FLC κ	free κ light chain
FLC λ	free λ light chain
FLC κ/λ	κ/λ free light chain ratio
Fpn	ferroportin
GDF-15	growth/differentiation factor 15
HAMP	hepcidin
Hb	hemoglobin
HBB	hemoglobin subunit beta
HFE	hereditary hemochromatosis
HFE2	hemojuvelin
HH	hereditary hemochromatosis
HJV	hemojuvelin
HLB	hydrophilic lipophilic balanced reversed phase sorbent
HoloTf	holotransferrin
HRMS	high resolution mass spectrometry
IC	Immunochemical method
IF	immunofluorescence
Ig κ	immunoglobulin light chain kappa or antibody against immunoglobulin light chain kappa
Ig λ	immunoglobulin light chain lambda or antibody immunoglobulin light chain lambda
IHC	immunohistochemistry
IL-6	interleukine-6
IL-6R	interleukine-6 receptor
IL-22	interleukine-22
IMAC	immobilized metal affinity chromatography
IS	internal standard
IRIDA	iron-refractory iron deficiency anemia
JAK	Janus kinase
LC	liquid chromatography

LC-MS	liquid chromatography - mass spectrometry
LEAP-1	liver-expressed antimicrobial peptide, hepcidin
LLOQ	lower limit of quantification
LMD	laser microdissection
LMD-LC-MS	laser microdissection followed by the combination of liquid chromatography with mass spectrometry
MALDI	matrix assisted laser desorption/ionization
Mayo	Mayo clinic staging system for cardiomyopathy
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MGUS	monoclonal gammopathy of undetermined significance
MM	multiple myeloma
MS	mass spectrometry, mass spectrum
MS/MS	tandem mass spectrometry
Mw	molecular weight
mRNA	messenger RNA
MT-2	matriptase-2
m/z	mass to charge ratio
ND	not determined
NS	nephrotic syndrome
pI	isoelectric point
PK	pyruvate kinase
PKLR	pyruvate kinase gene
ppt	protein precipitation
psi	pounds per square inch
PRM	parallel reaction monitoring
QC	quality control sample
SAA	serum amyloid A
SD ppt buffer	solvent-detergent precipitation buffer
SELDI	surface enhanced laser desorption/ionization
siRNA	small interfering RNA
SLC40A1	solute carrier family 40 member 1
SPE	solid phase extraction

SR	sirius red
SRM	serum reaction monitoring
STAT3	signal transducer and activator of transcription 3
Std.	standard
sTfR	soluble transferrin receptor
RBC	red blood cells count
RE	relative error
RI	renal insufficiency
RIA	radioimmunoassay
RNA	ribonucleic acid
RP	ribosomal protein
TCA	trichloroacetic acid
Tf	transferrin
TfR1	transferrin receptor protein 1
TfR2	transferrin receptor protein 2
TMPRSS6	transmembrane protease serine 6
TOF	time of flight
TSAT	transferrin saturation
TTR	transthyretin
ULOQ	upper limit of quantification
WCX	weak cation exchange

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8 Appendix – Full text publications related to the thesis

8.1 Hepcidin levels in Diamond-Blackfan anemia reflect erythropoietic activity and transfusion dependency

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Hepcidin levels in Diamond-Blackfan anemia reflect erythropoietic activity and transfusion dependency

Diamond-Blackfan anemia (DBA) is a rare congenital red cell aplasia associated with mutations in ribosomal proteins (RP) in 49-71% of cases.[1] DBA is a clinically heterogeneous disorder with one-third of patients developing transfusion-acquired iron overload.[2] The severity of anemia and transfusion dependency in DBA is comparable to transfusion-dependent beta-thalassemia major. However, moderate to severe suppression of erythropoiesis in DBA[2] is in contrast to accelerated ineffective erythropoiesis in β-thalassemia.[3,4] Knowledge of systemic iron regulation in DBA is limited.

In this study, we assessed selected markers of erythropoietic activity and iron metabolism including the key molecule of this process, hepcidin, in DBA patients from the Czech National DBA Registry (Table 1 and *Online Supplementary Table S1*).[5] The cohort was made up of 12 patients receiving regular transfusions with or without chelation therapy, 4 patients on steroids, 7 patients in remission without treatment and 2 patients treated with corticosteroids and occasional transfusions. Nine patients, mostly from the transfusion-dependent group, were concomitantly treated with leucine (*Online Supplementary Table S1*).[5]

In transfusion-dependent patients, reduced number of erythroblasts in the bone marrow (median 0.8%), together with markedly decreased soluble transferrin receptor (sTfR, often under the lower limit of detection), confirmed severely suppressed erythropoiesis that corresponded to substantially elevated erythropoietin (EPO) levels (median 2452 IU/L) (Table 1). Analysis of iron parameters showed increased serum iron, transferrin saturation (TSAT) and high ferritin levels (median 1290 ng/mL) (Table 1). This suggests that erythropoiesis of these patients is not able to utilize transferrin-bound iron effectively. Liver biopsy in 5 selected transfusion-dependent patients consistently showed markedly elevated liver iron concentration (LIC) (Table 1). Moreover, massive iron stores were detected in both hepatocytes and Kupffer cells (Figure 1A), distinguishing DBA from β-thalassemia major with iron deposits predominantly in macrophages.[4] The macrophage iron loading can be attributed to non-effective erythrocyte-derived iron recycling.[8,9] Iron deposits in hepatocytes likely resulted from increased iron uptake by these cells. The involvement of non-transferrin-bound iron (NTBI) needs to be considered, as NTBI can be found in the plasma of patients with oversaturated transferrin and liver is the primary site for deposition of free iron from plasma.[10,11] Since 2009, all our transfusion-dependent patients have been monitored for potential cardiac, liver and pancreatic iron overload by magnetic resonance imaging (MRI).[12]

Contrary to transfusion-dependent DBA patients, patients who are currently in disease remission or on steroids showed improved erythropoiesis as documented by near normal/higher number of bone marrow erythroblasts and the levels of sTfR within the normal range (Table 1). These patients had slightly increased or even normal levels of ferritin and near normal serum iron and TSAT

Table 1. Parameters of iron status and erythropoietic activity in subgroups of Diamond-Blackfan anemia patients.

	TDP (n=12)	RP (n=7)	SP (n=4)	S,T* (n=2)	Reference range
Age (years)	7.6 (1.0-27.9)	26.9 (13.3-35.8)	32.6 (27.0-42.9)	25.8 25.9	–
Hepcidin (ng/mL)	341.5 (165.1-572.6)	72.1 (29.5-153.3)	24.7 (8.5-100.1)	173.9 144.9	27.6 (13.1-104.8)[5]
Ferritin (ng/mL)	1290 (343-3747)	177 (42-1079)	188 (44-637)	3150 868	22-275
Hepcidin/ferritin ratio	0.240 (0.09-1.35)	0.222 (0.09-1.04)	0.151 (0.10-0.19)	0.06 0.17	0.35 (0.2-2.2)[5]
Fe (μmol/L)	41.5 (35.8-59.0)	20.0 (10.0-30.6)	21 (14.7-33.4)	39.6 43.2	7.2-29
TSAT (%)	91 (61-100)	38 (25-55)	64 (63-65)	93 94	21-48
LIC (mg/g d.w.)	7.3# (4.6-16.3)	*ND*	*ND*	*ND*	0.3-1.4
GDF15 (pg/mL)	676.2 (352.1-1694.8)	927.0 (556.3-2824.8)	1127.6 (408.8-2190.8)	4221.5 3497.1	223 (166-344)[5]
EB (%)	0.8 (0.4-8.2)	21.4 (8.4-34.0)	20.4 (16.8-33.4)	10.6 20.8	15.0-25.0
EPO (IU/L)	2452 (837-6476)	137 (20-1913)	441 (287-500)	615 4000	4.3-29.0
sTfR (mg/L)	*ND*° <0.5-1.2	2.3 (1.3-3.2)	2.4 (1.8-2.8)	1.4 1.4	1.9-4.4[4]

*Values are shown as medians and the full range of variation. TDP: transfusion-dependent patients; RP: patients in disease remission; SP: patients treated with steroids; S,T: patient on steroids and occasional transfusion; Fe: serum iron; TSAT: transferrin saturation; LIC: liver iron concentration, d.w.: dry weight; GDF15: growth differentiation factor 15; EB: erythroblasts in the bone marrow; EPO: serum erythropoietin; sTfR: soluble transferrin receptor; ND: not determined. *individual values are shown; #values available for 5 patients; °median could not be calculated as 9 of 12 patients had sTfR below the limit of detection (less than 0.5 mg/L). Detailed description of individual methods can be found in the Online Supplementary Appendix. Pre-transfusion samples were collected for TDP.*

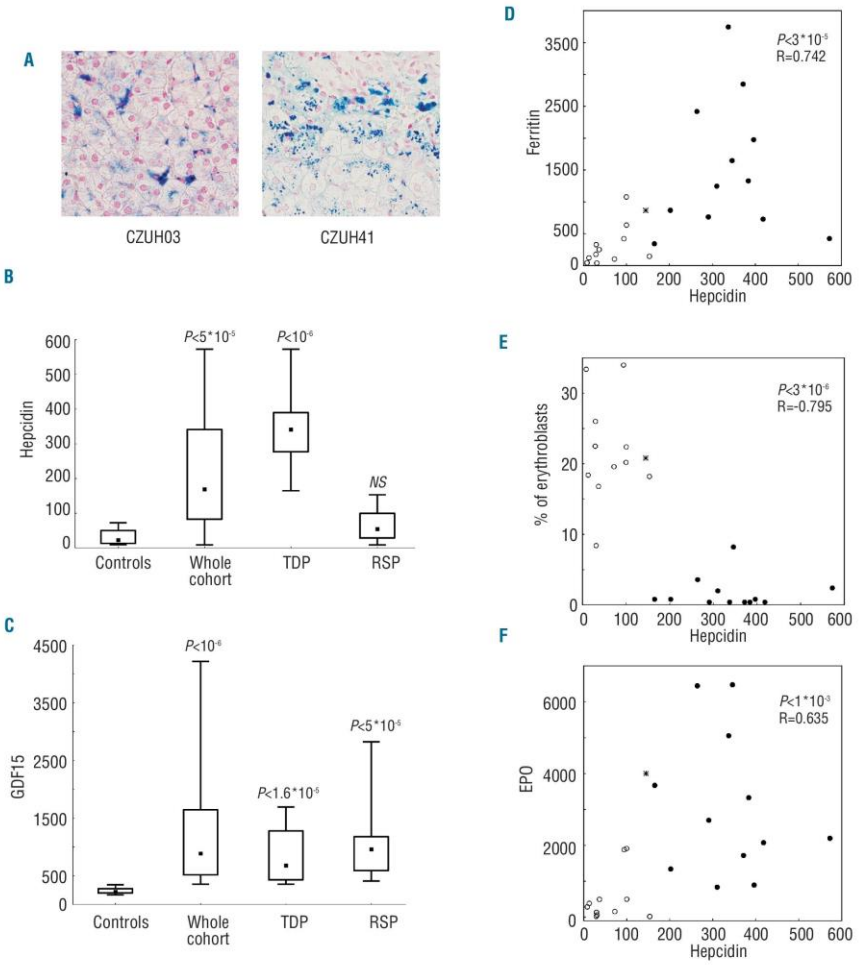

Figure 1. Hepatic iron distribution, hepcidin and GDF15 levels and correlation analyses. (A) Perl's staining showing iron deposits in the liver of 2 transfusion-dependent DBA patients; CZUH03 at the age of 10 (LIC, 4.6 mg/g d.w.) and CZUH41 at the age of 5 (10.3 mg/g d.w.). Massive iron stores (blue stain) can be found in both hepatocytes and Kupffer cells. Similar pattern of iron staining was obtained for the remaining 3 transfusion-dependent DBA patients analyzed (*data not shown*). (B) Significantly increased hepcidin levels in comparison with healthy controls were detected for the whole DBA cohort (n=25) and transfusion-dependent DBA patients (TDP, n=12); DBA patients in remission or treated with steroids (RSP, n=11) had hepcidin levels comparable with the controls. (C) GDF15 levels were significantly elevated in the whole DBA cohort (n=25), TDP (n=12) and RSP (n=11) when compared to healthy controls. (D-F) Hepcidin levels showed the following correlations: positive with ferritin (D), negative with the number of erythroblasts in the bone marrow (E) and positive with serum EPO (F). Closed and open circles depict TDP and RSP, respectively. Asterisk shows one patient on steroids and occasional transfusions; the other similarly treated patient (CZUH37) was excluded from statistical analyses due to concomitant occurrence of DBA and HFE C282Y homozygous mutation. The image in (A) was visualized with an Olympus IX 71 light microscope (Olympus, Hamburg, Germany), original magnification 400× and acquired with an Olympus DP 50 camera driven by DP controller software (provided by Olympus). Data in (B and C) are shown in a box plot depicting median (closed square), 25-75% range (box), and the highest and lowest value (the highest and the lowest whiskers, respectively). Image was assembled and labeled using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA). *NS*: not significant.

(Table 1). This indicates that, in patients who achieved a remission state or who are hematologically stable on steroids, the improved erythropoietic activity increases iron utilization and consequently leads to amelioration of hyperferritinemia. The effect of leucine treatment on iron metabolism seems to be indirect (via improved erythropoiesis).[7]

Using reverse-phase liquid chromatography,[5] we observed significantly higher hepcidin levels for the whole DBA cohort in comparison with healthy controls (median 165.1 *vs.* 27.6 pg/mL; $P<0.00005$) (Figure 1B). When hepcidin was assessed in individual subgroups of DBA patients, patients on regular transfusions showed significantly elevated hepcidin (median 341.5 pg/mL), as previously shown for transfusion-dependent β-thalassemia major patients.[4] Increased hepcidin above the median value of controls was detected even in the youngest patients aged 1.0 and 2.7 years (6-times and 20-times, respectively). This suggests that the absent or diminished erythropoiesis in the bone marrow, together with transfusions, is the driving factor for iron overload from early childhood. In contrast, DBA patients in remission or on steroids had hepcidin levels comparable to controls (medians 72.1 and 24.7 pg/mL, respectively) (Figure 1B). As erythropoiesis is known to produce a signal for hepcidin suppression,[8,9] DBA patients with restored erythropoietic activity are likely to be able to attenuate hepcidin expression and thus increase the iron pool available for improved erythropoiesis. In agreement with this concept, we detected a trend towards lower hepcidin-ferritin ratio in patients on steroids (median 0.151) or patients in remission (median 0.222) when compared to transfusion-dependent patients (median 0.240) (Table 1), although these individual values were not significantly different from the hepcidin-ferritin ratio of healthy controls (median 0.35).[5] Indeed, the hepcidin-ferritin ratio, which indicates suppression of hepcidin proportional to iron loading, is much higher in transfused DBA patients (range 0.09-1.35) than the hepcidin-ferritin ratio reported for transfusion-dependent β-thalasemia major patients (range 0.02-0.3),[4] suggesting that the erythroid drive suppressing hepcidin is much stronger in β-thalassemia and not completely attenuated by transfusions. On the other hand, the bone marrow of DBA patients receiving transfusions is probably not releasing the putative erythroid suppressor of hepcidin production.

In the 2 patients evaluated independently, hepcidin-ferritin ratio was more comparable with patients on steroids, reflecting their improved erythropoiesis (Table 1). Nevertheless, patient CZUH37 inherited a homozygous C282Y HFE mutation (*Online Supplementary Table S2*), which may contribute to inappropriately low levels of hepcidin (173.9 ng/mL) for the observed hyperferritinemia (3150 ng/mL).[10]

We next examined the levels of growth differentiation factor 15 (GDF15), a candidate negative regulator of hepcidin in β-thalassemia and a marker of ineffective erythropoiesis.[3] Significantly increased levels of GDF15 were detected for the whole DBA cohort as well as for the groups receiving different treatments when compared to normal controls (Figure 1C). We suppose that elevated levels of GDF15 in DBA patients may reflect the increased apoptosis of bone marrow erythroblasts that we observed in DBA patients selected for the TUNEL assay (*Online Supplementary Figure S1*).[3]

Lastly, we assessed which of the aforementioned signals/markers contribute to the regulation of hepcidin synthesis in DBA. Hepcidin positively correlated with ferritin ($P=0.00003$) (Figure 1D), reflecting hepcidin stimulation by the patients' iron overload. An inverse correlation between the percentage of bone marrow erythroblasts and hepcidin levels ($P=0.000003$) (Figure 1E) is consistent with negative regulation of hepcidin synthesis by erythropoietic activity. Although a negative correlation between EPO and the number of erythroblasts in the bone marrow (*data not shown*) confirms that EPO is stimulated in response to suppressed erythropoiesis and hypoxia, a positive correlation between hepcidin and EPO ($P=0.001$) (Figure 1F) demonstrates that hepcidin suppression by EPO requires active erythropoiesis in the bone marrow.[14,15] Similarly, no correlation between hepcidin or hepcidin-ferritin ratio and GDF15 indicates that GDF15 is not playing a hepcidin-regulatory role in DBA.

We conclude that DBA patients with different severities of anemia and different treatment strategies have diverse levels of hepcidin and iron overload. Hepcidin production in DBA reflects variable erythropoietic activity in the bone marrow and further contributes to the heterogeneity of this disease. It will be important to address whether some of these dissimilarities can be attributed to different types of disease-causing RP mutations.

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Hepcidin levels in Diamond-Blackfan anemia reflect erythropoietic activity and transfusion dependency

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Supplemental Material and Methods

Patients

The DBA cohort consists of twenty-five patients (eleven males and fourteen females) from the Czech National DBA Registry at the age of 1.0-42.9 years (median 23.7 years) with different clinical severity. Most of the patients have been previously described;[1] the group also includes five newly diagnosed patients. The causative RP mutation (RPS7, RPS17, RPS19, RPS26, RPL5 and RPL 11, respectively) was found in twenty patients; five patients are negative for all known RP mutations. Detailed characteristics of the patients are shown in Supplementary Table 1.

The study and the informed consent as per the Declaration of Helsinki were approved by the Ethics Committee of Palacky University Hospital, Olomouc, Czech Republic. A cohort of 12 children examined prior to planned minor surgery (inguinal or umbilical hernia, plastic surgery) and 5 young healthy adults served as controls for hepcidin levels interpretation; the normal range for GDF15 was determined in a group of four children and four adults.[2]

Hematological and biochemical analysis

Blood was taken during routinely performed venous puncture; for transfusion-dependent patients pre-transfusion samples were collected. Blood counts were examined on Sysmex XE-500 analyzer (Sysmex). Biochemical serum parameters of iron metabolism and inflammation: serum iron (Fe), total iron binding capacity (TIBC), transferrin saturation (TSAT), soluble transferrin receptor (sTfR) and CRP levels were determined with standard methods. The patients' serum erythropoietin (EPO) concentrations were measured by a solid-phase chemiluminescent immunochemical reaction.[1] Bone marrow samples were used for cytological and histopathological examination and immunohistochemistry. Liver biopsies were subjected to histopathological examination, determination of liver iron concentration (LIC, mg/g dry weight) and Perl's Prussian blue staining for non-heme iron.

Hepcidin analysis

The serum hepcidin levels were measured by reverse-phase liquid chromatography using the UltiMate 3000 Nano LC System (Thermo Fisher Scientific, Sunnyvale, CA, USA) coupled to the QTRAP 5500 mass spectrometer (AB SCIEX, Framingham, MA, USA) as we previously described.[2]

GDF15 measurements

The serum levels of growth differentiation factor 15 (GDF15) were quantified according to the manufacturer's instructions using the Human GDF-15 Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA).[2]

Immunohistochemistry

Bone marrow biopsy samples were fixed in neutral-buffered formalin for 24 hours and embedded into paraffin. Immunohistochemical staining was performed as previously described.[3] Glycophorin A staining (H-60, 1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was followed by analysis of apoptosis using the alkaline phosphatase (AP) In Situ Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The slides were analyzed with an Olympus BX 51 light microscope (Olympus, Hamburg, Germany).

HFE mutational analysis

The sequence analysis of the two most common HFE variants (p.C282Y and p.H63D) in exons 4 and 2 (accession: NM_000410.3) was performed as previously described.[2]

Statistical methods

All statistical analyses were done using the Statistica 10 software (StatSoft, Inc.). The Mann-Whitney test was used in comparisons between patients and healthy donors. The Spearman coefficient was used in correlation analyses. The significance level was set at $p < 0.05$ in all analyses.

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Supplementary Table S1

Clinical and laboratory parameters

Patient Number	Mutated RP	Sex	Age (years)	Age at Dg.of Anemia	SGA (BW (g))	Anomalies	Short Stature	Current Treatment	Leucine	eADA (nmol hour^{-1} mg Hb^{-1})
CZUH10	RPS19	F	**23.7**	New.	Yes (2500)	CD	No	T, DRX	Yes	90
CZUH14	RPS19	F	**27.9**	2 m	Yes (2450)	No	Yes	T, DRX	Yes	254
CZUH01	RPS26	M	**20.2**	2 m	No (3010)	No	Yes	T, DRX	Yes	96
CZUH03	RPS26	M	**15.5**	New.	Yes (2300)	Vesicoureteral reflux	No	T, DRX	Yes	68
CZUH41	RPL5	M	**9.6**	New.	Yes (1900)	Thenar hypoplasia, CD, HAP, PDA	No	T, DRX	Yes	*ND*
CZUH18	No	F	**17.3**	New.	No (2900)	No	Yes	T, DRX	No	17
CZUH43	RPS19	F	**5.5**	New.	No (3320)	ASD, CD	No	T, DRX	Yes	74
CZUH44	RPS26	F	**5.0**	New.	No (2900)	Ribs malformation, ASD	No	T, DRX	No	*ND*
CZUH46	RPS26	F	**3.0**	New.	No (2950)	Microcephaly	No	T, DRX	No	*ND*
CZUH45	RPS19	M	**4.7**	New.	No (3580)	No	No	T, DRX	Yes	*ND*
CZUH49	No	F	**2.7**	2 m	No	CD, kidney dystopia	No	T	No	*ND*
CZUH50	RPS19	M	**1.0**	4 w	No (2820)	Atypical thumb position	No	T	No	No
CZUH11	RPS19	F	**30.9**	6 w	No (2650)	No	No	R	No	204
CZUH15	RPS19	M	**21.7**	New.	Yes (1650, 35 w)*	CD, astenism	No	R	No	145
CZUH04	RPL5	M	**26.7**	3 m	Yes (2300)	Thenar hypoplasia, CD, HAP	Yes	R	No	487
CZUH25	No	F	**26.9**	6 m	No (3150)	Dermal syndactylia, low hair border, CD	Yes	R	No	183
CZUH51	RPS19	M	**34.0**	6 w	No (3300)	No	Yes	R	No	*ND*
CZUH20	No	F	**35.8**	New.	No (2550)	Kidney aplasia	Yes	R	No	191
CZUH33	No	F	**13.3**	New.	No (3150)	Vesicorenal reflux, CD	No	R	No$^{\#}$	78
CZUH19	No	F	**42.9**	3 m	No (3800)	No	Yes	S-LD	No	387
CZUH07	RPS7	F	**20.8**	5 m	No (3080)	No	No	S	No	429
CZUH12	No	M	**27.0**	10 m	No (3400)	ASD	No	S-LD	No	745
CZUH21	RPS17	M	**38.2**	1 m	No (2600)	Thenar hypoplasia	No	S	Yes	212
CZUH37	RPL11	F	**25.8**	7 m	Yes (2010)	Thenar hypoplasia, triphalangeal thumb, HAP	No	S, T‡, DRX	No	303
CZUH24	RPL5	M	**25.9**	1 m	Yes (2450)	Thenar hypoplasia, CD, HAP	No†	S, T†	Yes	78

Patients CZUH45, 46, 49, 50 and 51 are newly diagnosed. RPS, small subunit ribosomal protein; RPL, large subunit ribosomal protein; New., newborn age; SGA, small for gestational age (SGA babies are those whose birth weight lie below or are equal to –2 standard deviations for that gestational age. They have usually been the subject of intrauterine growth restriction (IUGR)); BW, birth weight; *, born before week 38 of gestation; CD, craniofacial dysmorphism; HAP, high arched palate; PDA, patent ductus arteriosus; ASD, atrial septal defect; †, after growth hormone treatment; T, transfusions (10-17 transfusions per patient per year); DRX, deferasirox; R, remission; S, steroids (the maintenance dose, 0.5 mg/kg on alternate days); S-LD, low doses of steroids (i.e. less than 0.3 mg/kg twice a week); ‡, occasional transfusions (CZUH37: 2-8 transfusions per year, CZUH24: 6-7 transfusions per year); **#**, published patient in remission after leucine treatment;[4] eADA, erythrocyte adenosine deaminase, normal values: 24 – 96 nmol hour^{-1} mg Hb^{-1}; *ND*, not determined.

Supplementary Table S2

HFE mutational screening

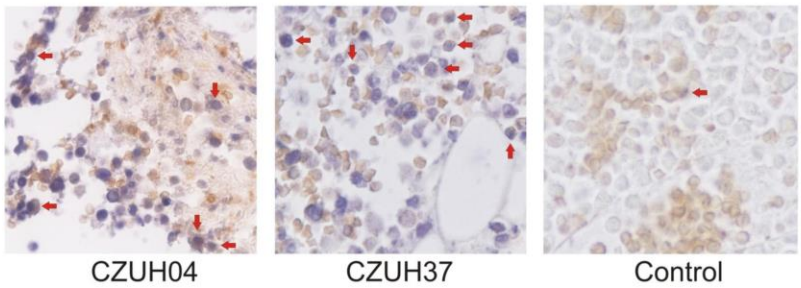
Patient Number	C282Y	H63D
CZUH10	**wt/wt**	**wt/wt**
CZUH14	**wt/wt**	**wt/wt**
CZUH01	**wt/wt**	**wt/wt**
CZUH03	**wt/wt**	**wt/H63D**
CZUH41	**wt/wt**	**wt/wt**
CZUH18	**wt/wt**	**wt/wt**
CZUH43	**wt/wt**	**wt/wt**
CZUH44	**wt/wt**	**wt/H63D**
CZUH46	**wt/wt**	**wt/wt**
CZUH45	**wt/wt**	*NA*
CZUH49	**wt/wt**	**wt/wt**
CZUH50	**wt/wt**	**wt/wt**
CZUH11	**wt/wt**	**wt/wt**
CZUH15	*NA*	*NA*
CZUH04	**wt/wt**	**wt/wt**
CZUH25	**wt/wt**	**wt/wt**
CZUH51	**wt/wt**	**wt/wt**
CZUH20	**wt/wt**	**wt/wt**
CZUH33	**wt/wt**	**wt/wt**
CZUH19	**wt/wt**	**wt/H63D**
CZUH07	**wt/wt**	**wt/wt**
CZUH12	**wt/wt**	**wt/wt**
CZUH21	**wt/wt**	**wt/wt**
CZUH37	**C282Y/C282Y**	*NA*
CZUH24	**wt/wt**	**wt/wt**

NA, not analyzed

Supplementary Figure legend

Supplementary Figure S1. Apoptosis of bone marrow erythroblasts. TUNEL assay shows significant elevation in the number of erythroid Glycophorin A^+ cells (brown color) undergoing apoptosis ($TUNEL^+$, dark purple color) in the bone marrow of two DBA patients compared to healthy control. The arrows indicate Glycophorin $A^+/TUNEL^+$ cells. Immunohistochemical slides were analyzed with an Olympus BX 51 light microscope (Olympus), original magnification 400×. Digital images were acquired with an Olympus DP 50 camera driven by DP controller software (provided by Olympus). Images were cropped, assembled and labeled using Adobe Photoshop software (Adobe Systems).

Supplementary Figure S1



8.2 Iron status in patients with pyruvate kinase deficiency: neonatal hyperferritinaemia associated with a novel frameshift deletion in the PKLR gene (p.Arg518fs), and low hepcidin to ferritin ratios

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bjh research paper

Iron status in patients with pyruvate kinase deficiency: neonatal hyperferritinaemia associated with a novel frameshift deletion in the *PKLR* gene (p.Arg518fs), and low hepcidin to ferritin ratios

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Summary

Pyruvate kinase (PK) deficiency is an iron-loading anaemia characterized by chronic haemolysis, ineffective erythropoiesis and a requirement for blood transfusion in most cases. We studied 11 patients from 10 unrelated families and found nine different disease-causing *PKLR* mutations. Two of these mutations - the point mutation c.878A>T (p.Asp293Val) and the frameshift deletion c.1553delG (p.(Arg518Leufs*12)) - have not been previously described in the literature. This frameshift deletion was associated with an unusually severe phenotype involving neonatal hyperferritinaemia that is not typical of PK deficiency. No disease-causing mutations in genes associated with haemochromatosis could be found. Inappropriately low levels of hepcidin with respect to iron loading were detected in all PK-deficient patients with increased ferritin, confirming the predominant effect of accelerated erythropoiesis on hepcidin production. Although the levels of a putative hepcidin suppressor, growth differentiation factor-15, were increased in PK-deficient patients, no negative correlation with hepcidin was found. This result indicates the existence of another as-yet unidentified erythroid regulator of hepcidin synthesis in PK deficiency.

Keywords: red blood cell, pyruvate kinase deficiency, iron overload, hepcidin, ferritin.

Erythrocyte pyruvate kinase (PK) deficiency, the most common cause of nonspherocytic haemolytic anaemia in central and northern Europe, is characterized by a variable degree of haemolysis and increased risk of iron overload. The mechanism of haemolysis is not clearly understood; however, the disruption of glycolysis leading to ATP depletion is one of the major contributing factors (Zanella *et al*, 2005). An appropriate diagnosis can be made only by measuring the erythrocyte PK enzyme activity and by direct mutation analysis of the gene encoding PK (*PKLR*). More than 220 mutations in the *PKLR* gene (1q22) have been identified worldwide since the first mutation was described in 1961 (Valentine *et al*, 1961). The disease is transmitted as a recessive trait, usually affecting only homozygotes or compound heterozygotes. The clinical manifestations vary from mild to severe anaemia requiring repeated transfusions. Rare cases of

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neonatal death and non-immune hydrops foetalis have also been reported (Ferreira *et al*, 2000). Similar to patients with β-thalassaemia, PK-deficient patients may develop secondary iron overload. Although repeated blood transfusions were considered to be the major cause, iron accumulation also affects non-transfused PK-deficient patients (Zanella *et al*, 1993). The pathogenesis of iron overload appears to be multifactorial, involving chronic haemolysis, ineffective erythropoiesis, splenectomy and, eventually, coinheritance of hereditary haemochromatosis (Zanella *et al*, 2001). Importantly, the levels of hepcidin, the negative regulator of iron absorption in the gut and iron recycling from macrophages (Ganz, 2004), are reduced in patients with PK deficiency (Finkenstedt *et al*, 2008). Based on recent analyses, growth differentiation factor-15 (GDF15) is one of the candidate molecules proposed to suppress hepcidin production and enhance iron loading in the setting of ineffective erythropoiesis (Tanno & Miller, 2010; Tanno *et al*, 2010). The negative correlation between hepcidin and GDF15 was first reported in patients with β-thalassaemia (Tanno *et al*, 2007). Although GDF15 levels are increased in PK deficiency, the magnitude of GDF15 elevation is markedly lower than that in β-thalassaemia (Tanno *et al*, 2010). Here, we analysed *PKLR* mutations and iron status parameters in a cohort of 11 patients with PK deficiency. Our genetic data, together with calculations of the hepcidin/ferritin ratio, indicate the involvement of an as-yet unknown erythroid-derived hepcidin suppressor in PK deficiency.

Design and methods

Patients, haematological and biochemical analysis

We investigated 11 patients with congenital nonspherocytic haemolytic anaemia from 10 unrelated Czech and Slovak families. The patient cohort consisted of five adults, three children and three infants. The Ethics Committee of Palacky University Hospital approved the collection and analysis of samples. Informed consent was obtained according to the Declaration of Helsinki. Red blood cell parameters were measured using a Sysmex XE-500 analyser (Sysmex Corp., Kobe, Japan). Serum parameters of iron metabolism, including iron, ferritin and transferrin saturation as well as bilirubin levels, were determined with standard biochemical methods. PK and hexokinase (HK) activities were measured in erythrocyte lysates that were free of leucocytes and platelets according to the methods recommended by the International Committee for Standardization in Haematology (Beutler *et al*, 1977). PK and HK activities were expressed as u/g haemoglobin (Hb). HK activity was included to estimate the contribution of young red blood cells to the total PK activity. The reference values for enzyme activities were established from 10 normal subjects. GDF15 serum concentrations were measured with an enzyme-linked immunosorbent assay (ELISA) kit for human GDF15 according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The normal range for GDF15 was determined from eight healthy controls (four children and four adults). The serum erythropoietin (EPO) concentrations were measured by radioimmunoassay (Zadrazil *et al*, 2007).

Hepcidin measurement

The method for hepcidin measurement was established based on the protocol published by Li *et al* (2009). Briefly, each sample was prepared from 200 μl of human serum. Calibration standards were prepared from unmodified synthetic human hepcidin (DTHFPICIFCCGCCHRSKCGMCCKT) dissolved in rabbit serum. Hepcidin labelled with a stable isotope [$^{13}C_9$, $^{15}N_1$-Phe$_4$] (50 ng) was added to each sample as an internal standard. Both hepcidin variants were synthesized by Thermo Fisher Scientific (Ulm, Germany). The samples were extracted on an Oasis HLB μElution plate and separated by reverse-phase liquid chromatography using an UltiMate 3000 Nano LC System (Thermo Fisher Scientific, Sunnyvale, CA, USA) coupled to a QTRAP 5500 mass spectrometer (AB SCIEX, Framingham, MA, USA). Subsequent detection of hepcidin was performed by selected reaction monitoring method. The acquired data were processed using Analyst® software (AB SCIEX, version 1.5.1). The assay was validated by Intrinsic LifeSciences LLC (La Jolla, CA, USA), and the normal control range was determined in 18 healthy individuals (10 children and eight adults).

Molecular analysis

Genomic DNA was isolated from peripheral blood drawn in EDTA using a QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA, USA). The exons of the following were amplified by polymerase chain reaction (PCR): *PKLR* (accession: NM_000298.5) (Lenzner *et al*, 1997); genes known to be responsible for hereditary haemochromatosis (Camaschella & Poggiali, 2011), including *HFE* (accession: NM_000410.3), *HFE2* (haemojuvelin) (accession: NM_213653.3), *HAMP* (hepcidin) (accession: NM_021175.2), *TFR2* (transferrin receptor 2) (accession: NM_003227.3) and *SLC40A1* (ferroportin 1) (accession: NM_014585.5); and a gene encoding the light chain of ferritin *(FTL)* (accession: NM_000146.3). The iron response element (IRE) regions of the 5′-UTR of *SLC40A1* and *FTL* were also amplified. PCR primers and conditions are available upon request. The PCR products were purified with a QIAquick kit (Qiagen) and sequenced using the BigDye terminator kit (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using the ABI Prism 310 Genetic Analyser (Applied Biosystems) with software provided by the manufacturer.

Statistical methods

Student's *t*-test was used to determine the statistical difference between the patients and controls using Origin 6.1

software (OriginLab Corporation, Northampton, MA, USA). The Spearman coefficient for correlation analyses was calculated using Statistica 10 software (StatSoft, Inc., Tulsa, OK, USA). *P* values <0·05 were considered statistically significant.

Results

Patients' characteristics and PK activity

The clinical findings of nine unrelated patients and two siblings obtained at the time of diagnosis are summarized in Table I. All patients suffered from mild to severe haemolytic anaemia (with Hb levels ranging from 65 to 121 g/l) and hyperbilirubinaemia (33 to 172 μmol/l). All but one of these patients had increased reticulocyte counts (4·8 to 80·5%). Neonatal jaundice was reported in most of the patients. Seven patients were transfusion-dependent; three were concomitantly treated with chelators and two underwent splenectomy. Two paediatric patients received transfusions during infancy, one adult patient was on intermittent transfusion therapy and only one patient had never received a transfusion in his lifetime. In 10 patients, erythrocyte PK activity was below the normal reference values (4·60–6·28 u/g Hb) (Table II). In one case (Patient 7), the activity was false normal (6·58 u/g Hb) due to substantial reticulocytosis (80·5%) and PK deficiency was therefore established based on low PK/HK activity ratio (1·6; normal range 3·6–6·3) (Table II). Reduced PK activity compared with the controls was also detected in the available healthy parents, confirming a congenital defect in the child. All parents tested were later shown to be heterozygous carriers of specific *PKLR* mutations (Table II).

Mutational analysis of the PKLR gene

Direct sequence analysis of the *PKLR* gene in the patient cohort revealed nine different mutations in either the homozygous state (four patients) or the compound heterozygous state (seven patients) (Table II). Seven of the mutations are known; five of these (c.1594C>T, c.1529G>A, c.1493G>A, c.1456C>T, and c.994G>A) have previously been identified in Czech and Slovak populations (Lenzner *et al*, 1997). The c.823G>A substitution and the insertion-deletion (c.347_350delinsAACATTG) were identified in these populations for the first time. The clinical picture and the severity of the disease in our patients bearing the above-mentioned PK mutations were comparable to the phenotype previously reported for patients with identical mutations (Baronciani *et al*, 1995; Zanella *et al*, 1997, 2005, 2007). The c.878A>T and c.1553delG mutations are novel and have not been previously described in the literature.

The maternally inherited c.878A>T missense mutation was detected in a heterozygous state with the previously reported, paternally inherited c.1529G>A substitution (Fig 1A, Table II), which is the most common *PKLR* mutation in the northern and central European population (Zanella *et al*, 2005). The c.878A>T mutation involves the alteration of positively charged aspartic acid to hydrophobic valine on the

Table I. Clinical and haematological data of the patients.

Patient	Sex	Age	Neonatal jaundice	ExTx	Spleen	Hb, g/l	Ret,% [0·5–3·0]	Bilirubin, μmol/l [0–23]	Ferritin, μg/l [6–200]*	TfS [0·21–0·48]	Hepcidin, μg/l [13·1–104·8]	GDF15, ng/l [166–344]	EPO, iu/l [4·3–29]
1	M	9 years	Yes	Yes	Out	79	32	38	2000§	0·79	n.d.	3052	n.d
2	M	6 months	Yes	Yes	In	77	7·2	33	4852	0·97	90·7	935	46·2
		33 months				98	4·8	49	145§	n.d.	16	1399	78·1
3	F	7 years	Yes	Yes†	In	95	10·6	35	16	0·26	5·7	851	454
4	M	10 months	Yes	Yes	In	75	7·5	59	303	n.d.	15·5	876	123
5	M	7 years	Yes	Yes†	In	97	8·1	39	205	0·50	24·5	386	n.d
6	F	42 years	No	Yes‡	In	103	5·9	50	645	0·37	11·4	2581	46·8
7	F	22 years	Yes	Yes	Out	65	80·5	139	372§	0·88	9·1	514	122
8	F	31 years	Yes	Yes	In	95	6·6	34	948	0·54	29·1	805	126
9	M	3 months	Yes	Yes	In	67	2·1	172	802	n.d.	n.d.	2163	n.d
10	F	25 years	Yes	Yes	In	73	25·3	124	29·7	0·20	5·7	1259	n.d
11	M	50 years	No	No	In	121	5·2	49	906	0·34	68·8	854	51·5

Patients 3 and 4 are siblings; ExTx: exchange transfusion; TfS: transferrin saturation; GDF15: growth differentiation factor-15; EPO (serum erythropoietin); n.d.: not determined.

*normal range for ferritin levels, women: <200, men: <300, children: <70 μg/l (Brugnara, 2009). The control range for GDF15 was determined from eight healthy controls (four children and four adults).

†Dependent on blood transfusion during the first year of life due to severe anaemia.

‡Intermittent ExTx.

§Patients on chelation therapy. Two sets of data are shown for Patient 2; the first set represents values at the time of diagnosis, the second corresponds to values after 6 months of chelation therapy.

Table II. Biochemical and molecular data of the patients and their family members.

Patient	PK activity, u/g Hb	PK/HK	*PKLR* mutation	Amino acid alteration
1	2·64	0·78	c.[347_350delinsAACATTG]; [347_350delinsAACATTG]	p.[Arg116_Leu117delinsGlnHisCys]; [Arg116_Leu117delinsGlnHisCys]
1 (mother)	2·19	2·92	c.[347_350delinsAACATTG];[=]	p.[Arg116_Leu117delinsGlnHisCys];[=]
1 (father)	2·19	1·97	c.[347_350delinsAACATTG];[=]	p.[Arg116_Leu117delinsGlnHisCys];[=]
2	1·63	1·63	c.[1553delG];[1553delG]	p.[Arg518Leufs*12];[Arg518Leufs*12]
2 (mother)	2·57	2·3	c.[1553delG];[=]	p.[Arg518Leufs*12];[=]
3	1·43	0·51	c.[1529G>A];[1594C>T]	p.[Arg510Gln];[Arg532Trp]
4	1·14	0·46	c.[1529G>A];[1594C>T]	p.[Arg510Gln];[Arg532Trp]
4 (mother)	1·33	1·75	c.[1529G>A];[=]	p.[Arg510Gln];[=]
4 (father)	1·57	1·51	c.[1594C>T];[=]	p.[Arg532Trp];[=]
5	1·73	1·75	c.[1493G>A];[1529G>A]	p.[Arg498His];[Arg510Gln]
6	1·16	0·84	c.[1456C>T];[1594C>T]	p.[Arg486Trp];[Arg532Trp]
7	6·58	1·6	c.[1594C>T];[1594C>T]	p.[Arg532Trp];[(Arg532Trp)]
8	1·56	0·81	c.[878A>T];[1529G>A]	p.[Asp293Val];[Arg510Gln]
8 (father)	3·41	3·48	c.[1529G>A];[=]	p.[Arg510Gln];[=]
8 (mother)	4·04	3·98	c.[878A>T];[=]	p.[Asp293Val];[=]
9	1·72	2·07	c.[823G>A];[1594C>T]	p.[Gly275Arg];[Arg532Trp]
9 (father)	2·45	2·62	c.[823G>A];[=]	p.[Gly275Arg];[=]
9 (mother)	–	–	c.[1594C>T];[=]	p.[Arg532Trp];[=]
10	2·32	1·12	c.[1529G>A];[1529G>A]	p.[Arg510Gln];[(Arg510Gln)]
11	1·54	1·14	c.[994G>A];[1456C>T]	p.[Gly332Ser];[Arg532Trp]

PK: pyruvate kinase (normal activity range: 4·60–6·28 u/g Hb); PK/HK: pyruvate kinase and hexokinase activity ratio (normal range: 3·6–6·3). The normal activity range was determined in 10 healthy controls.

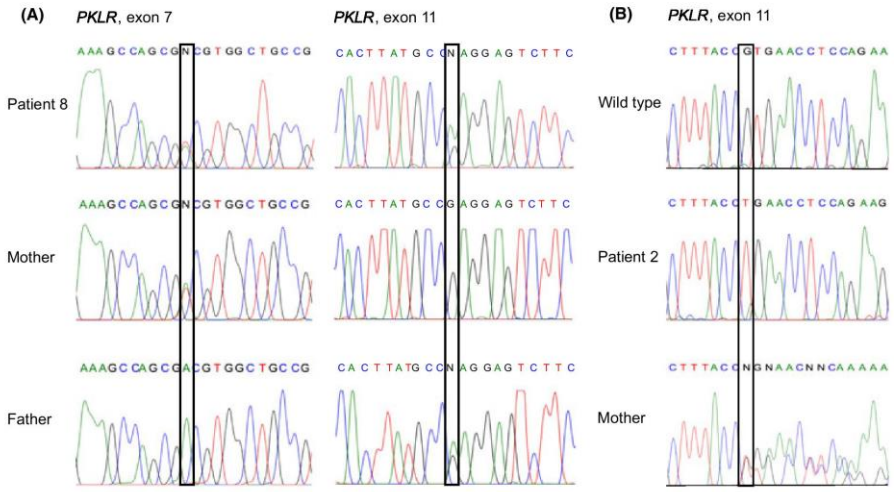

Fig 1. Detection of two novel *PKLR* mutations by sequencing amplified DNA. (A) In the family of Patient 8, the propositus carried the newly described c.878A>T mutation in exon 7 and the c.1529G>A mutation in exon 11; the mother was heterozygous for the c.878A>T mutation, and the father was heterozygous for the c.1529G>A mutation. (B) In the family of Patient 2, the propositus was homozygous for the second newly described mutation in the *PKLR* gene, a single nucleotide deletion (c.1553delG) in exon 11 leading to a frameshift (p.(Arg518Leufs*12)) and a premature stop codon. One allele was inherited from his heterozygous mother; his father was unavailable for the analyses.

surface of the A domain of PK. This alteration might increase the hydrophobicity of PK, which is thermodynamically less favourable for protein folding. PK activity was markedly reduced to 1·56 u/g Hb in an affected patient (Patient 8) who presented with a mild phenotype (Hb 95 g/l, reticulocytes 6·6%, and bilirubin 34 μmol/l; Table I).

The novel PKLR frameshift deletion associated with atypical severe phenotype involving neonatal hyperferritinaemia

Patient 2 carried the second previously unidentified frameshift deletion, c.1553delG (p.(Arg518Leufs*12), short p.Arg518fs), in a homozygous state; the mother is a heterozygous carrier of this deletion (Fig 1B, Table II). Because the father is unknown and thus unavailable for analyses, a possible hemizygous genomic deletion (loss of heterozygosity) was excluded by quantitative PCR-based copy number analysis (Supplementary methods; data not shown). The mutation causes premature termination of translation at codon 529, with predicted formation of a truncated protein lacking 46 C-terminal amino acids including the critical activator binding site Arg532 (Valentini *et al*, 2002). The activity of PK was reduced in both the patient (1·63 u/g Hb) and his mother (2·57 u/g Hb). The patient's clinical picture was very severe from birth, with a need for repeated blood transfusions. The patient exhibited profound anaemia (Hb, 77 g/l), with signs of hypoxia, neonatal jaundice (bilirubin, 90 μmol/l), thrombocytopenia, prominent hepatosplenomegaly and dramatic hyperferritinaemia (in the range of 3500–5000 μg/l), and transferrin saturation reaching almost 100%. Elevated levels of liver enzymes (not shown) suggested possible liver damage, which led us to perform liver biopsy. Massive iron deposits in both hepatocytes and Kupffer cells corresponding to grade IV haemosiderosis were detected. Taken together, these findings suggested the presence of another co-inherited defect in the child aside from PK deficiency. Sideroblastic anaemia was ruled out by the absence of sideroblasts in the bone marrow. The nucleotide sequence of the IRE region of the *FTL* gene, which encodes L-ferritin, was not mutated; thus, a possible association with hereditary hyperferritinaemia cataract syndrome (HHCS) was excluded (Brooks *et al*, 2002). Sequence analysis of all known genes that cause primary haemochromatosis (*HFE*, *HFE2*, *HAMP*, *SLC40A1* and *TFR2*) (Camaschella & Poggiali, 2011) did not reveal any causative mutations. Only five different previously described SNP variations were identified in *TFR2* (c.2255G>A (p.Arg752His) - rs41295942), *SLC40A1* (c.44-24G>C - rs1439816, c.-330CGG[8] - rs16836041, c.663T>C (p.Val221 =) - rs2304704) and *FTL* (c.163T>C (p.Leu55 =)) (Table SI). Of these polymorphisms, only rs1439816:G>C was previously shown to be associated with the clinical aggressiveness (liver damage) of hereditary haemochromatosis 1 caused by the p.Cys282Tyr *HFE* mutation; however, this SNP had no effect on ferritin levels in the control group, and it was therefore concluded that the negative effect of the rs1439816:C allele was restricted to pathological conditions (Altès *et al*, 2009). Because nine of our remaining PK-deficient patients were also homozygous for the rs1439816:C allele (Table SI), this polymorphism probably does not contribute to the severe phenotype of this patient and cannot explain his iron overload. Recently, at an age of two and a half years, the patient's ferritin levels dramatically decreased (to 145 μg/l), which may be a result of combined chelation therapy lasting 6 months and a prolonged interval between transfusions. Based on these new findings, we conclude that the severe neonatal phenotype of this patient was caused by the *PKLR* mutation itself.

Iron status in PK-deficient patients

One of the main health complications in PK deficiency is secondary iron overload with multifactorial pathogenesis (Zanella *et al*, 1993, 2001). In our cohort, nine patients had elevated serum ferritin (median, 724 μg/l), including the patient with no transfusion history (Patient 11). Of nine available cases, five showed a concomitant increase in transferrin saturation (from 0·50 to 0·97) (Table I).

Firstly, we assessed a possible effect of splenectomy on iron loading. One of our two splenectomized patients (Patient 7) showed a progressive increase in ferritin levels after spleen removal. Nevertheless, sustained haemolysis was observed in this patient after spleen surgery, which probably contributed to a further increase in her ferritin levels. On the other hand, no significant difference in ferritin was recorded for the second splenectomized patient (Patient 1); splenectomy improved this patient's anaemia, nevertheless his pre-splenectomy ferritin levels were already dramatically elevated (2213 μg/l). This patient is a child; thus, the contribution of spleen removal to iron loading may become more apparent over time.

We next searched for the presence of known *HFE* mutations (p.His63Asp, p.Cys282Tyr), which have been proposed as a condition that may predispose a patient to increased iron absorption (Arruda *et al*, 2000; Zanella *et al*, 2001). Abnormal *HFE* genotypes were identified in four of our patients (Table SI). One of these patients (Patient 3), who was a p.[His63Asp];[Cys282Tyr] *HFE* compound heterozygote, was the only paediatric patient from the group with normal ferritin levels.

Hepcidin levels in PK deficiency

To address the role of hepcidin in the pathogenesis of iron overload in our patient cohort, we established a proteomics-based method for hepcidin measurement. As expected, the levels of hepcidin were reduced in PK-deficient patients (median, 15·8 μg/l) (Table I) compared with healthy age-matched controls (median, 27·6 μg/l) (Table I), reflecting the impact of ineffective erythropoiesis in PK deficiency. The difference, however, was not statistically significant. We

therefore calculated the hepcidin/ferritin ratio which represents a more accurate estimation of proper hepcidin production with respect to iron loading, and found it to be very low in our PK-deficient patients (median, 0·06) compared with a healthy age-matched group of controls (median, 0·35) (Fig 2). Even the patient with the highest hepcidin level (Patient 2) showed a dramatically reduced ratio, which was detected in repeated measurements (ranging from 0·01 to 0·04) and eventually increased to 0·11 as a response to 6 months of chelation therapy and improved anaemia (Hb, 98 g/l) (Table I and Fig 2). The direct effect of chelation therapy on hepcidin levels and hepcidin/ferritin ratio is difficult to address in our cohort, given the complexity of positive and negative signals regulating hepcidin, the small number of patients receiving chelation therapy, and the lack of hepcidin pre-chelation values for remaining patients.

Candidate erythroid signals regulating hepcidin

To clarify the involvement of erythropoiesis in iron metabolism in PK deficiency, serum levels of GDF15 and EPO, two markers of erythropoietic activity known to be associated with hepcidin production, were evaluated. The levels of GDF15, a putative negative regulator of hepcidin production in the setting of ineffective erythropoiesis (Tanno & Miller, 2010), were determined using a commercial enzyme-linked immunosorbent assay. PK-deficient patients showed elevated GDF15 compared with healthy age-matched controls (median, 905·5 μg/l and 223 μg/l, respectively) (Table I), confirming previously reported results (Zanella *et al*, 2005). Importantly, no correlation between GDF15 and hepcidin or GDF15 and hepcidin/ferritin ratio was observed. In addition, all available patients from our cohort showed increased levels of EPO (median, 100·1 iu/l) (Table I), probably reflecting tissue hypoxia. Also, EPO levels did not correlate with hepcidin or hepcidin/ferritin ratio.

Lastly, as inflammatory processes are known to independently influence hepcidin levels (Ganz, 2004), it is important to note that none of our patients had signs of inflammation at the time of the analyses. In concordance, a substantial increase in hepcidin (from 90·7 μg/l to 386 μg/l) was observed in one of our patients (Patient 2) during bacterial infection.

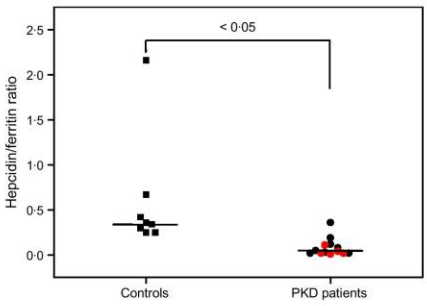

Fig 2. Hepcidin/ferritin ratio in PK-deficient patients. The hepcidin/ferritin ratio was significantly lower in PK-deficient patients compared to healthy controls ($P < 0·05$). The red dots in the graph indicate the hepcidin/ferritin ratio calculated from five repeated measurements (1·5 months, 9·5 months, 10 months, 20·5 months, and 33 months) for Patient 2 with a p.(Arg518Leufs*12) mutation in the *PKLR* gene, which is associated with hyperferritinaemia occurring from the neonatal period through to infancy (2 years of age). Only two hepcidin/ferritin ratios (calculated from those hepcidin and ferritin values shown in Table I) were included into the statistical analysis for this patient. The thick horizontal lines represent the medians for each group. PKD, pyruvate kinase-deficient.

Discussion

In this report we characterized *PKLR* mutations and systemic iron metabolism in eleven patients with PK deficiency from ten unrelated families. Nine different disease-causing *PKLR* mutations were identified; two of these mutations - the point mutation c.A878T (p.D293V) and the frameshift deletion c.1553delG (p.R518fs) - were novel. The patient affected by the frameshift deletion presented with an unusually severe phenotype involving neonatal hyperferritinaemia, which eventually ameliorated by two and a half years of age, probably in response to combined chelation therapy and a prolonged interval between transfusions.

PK-deficient patients usually develop secondary iron overload over time as a consequence of chronic haemolysis, ineffective erythropoiesis and transfusion therapy; splenectomy and inheritance of *HFE* mutations have been proposed as additional risk factors (Zanella *et al*, 2001). Consistently, all our transfusion-dependent patients presented iron overload; one patient has developed hyperferritinaemia independently of blood transfusions. Similar findings, iron overload independent of blood transfusions in PK deficiency, were previously reported by Zanella *et al* (1993). Although splenectomy reduces the symptoms of haemolysis, it is also known to be a risk factor for iron loading. Due to a low number of splenectomized patients in our cohort, however, the involvement of spleen surgery cannot be adequately addressed. The contribution of co-inherited *HFE* mutations to excessive iron accumulation is also difficult to assess, especially because three of the *HFE* mutant patients were children and HFE-related haemochromatosis is an adult-onset disease (Pietrangelo, 2010). Moreover, one paediatric patient with abnormal *HFE* genotype did not have any signs of iron overload. Based on these observations we can conclude that the *HFE* genotype does not play a major role in determining iron loading in PK-deficient paediatric patients; however, it may be important to follow up these patients due to a potential higher risk of iron overload in adulthood (Zanella *et al*, 2001).

Analysis of factors regulating iron homeostasis revealed that hepcidin levels were relatively low for the degree of iron loading in all PK-deficient patients with increased ferritin. Similar results were published for β-thalassaemia intermedia (Kearney *et al*, 2007; Origa *et al*, 2007), which clearly confirm the suppression of hepcidin by an erythroid signal that overrides iron loading-induced signalling. Although the levels of the putative hepcidin suppressor, GDF15, were increased in our patient cohort, the levels were considerably lower than those reported for β-thalassaemia (Tanno *et al*, 2010). No correlation between GDF15 and hepcidin or hepcidin/ferritin ratio indicates that GDF15 can only be used as a marker of accelerated ineffective erythropoiesis in PK deficiency (Tanno & Miller, 2010; Tanno *et al*, 2010).

Besides elevated GDF15 in the patients' serum, we also found increased levels of EPO (Ashby *et al*, 2010; Ganz, 2011) that again did not correlate with hepcidin. This evidence further suggests that EPO by itself is an indirect suppressor of hepcidin as mice with disrupted erythropoiesis are not able to attenuate hepcidin synthesis in response to EPO administration (Pak *et al*, 2006; Vokurka *et al*, 2006). In addition to GDF15 and EPO, a suppressive effect of twisted gastrulation protein homolog 1 (TWSG1) (Tanno *et al*, 2009) and hypoxia (Yoon *et al*, 2006; Ganz, 2011) should be considered with respect to the regulation of hepcidin production. Nevertheless, the role of TWSG1 has not been clearly confirmed so far (Tanno *et al*, 2010). Although hypoxia inducible factor (HIF) signalling was reported to directly and also indirectly (through EPO) downregulate hepcidin (Gordeuk *et al*, 2011), the exact signalling remains elusive. Altogether, our findings indicate that the main erythroid-derived regulator of hepcidin synthesis in PK deficiency remains to be identified.

In conclusion, we have shown that the hepcidin/ferritin ratio is consistently low in PK deficiency, which reflects relatively low hepcidin levels, even in PK deficiency associated with hyperferritinaemia.

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Author contributions

RM and PK designed the study, performed enzyme assays and some molecular analyses, collected and analysed data and contributed to manuscript writing. DH established the hepcidin assay. ZZ determined GDF15 levels. DP, JC, ZSL, KI and MS treated the patients, collected patient material and provided clinical information. MP and JK participated in molecular analyses. MH contributed to the design of the study, interpretation of the results and wrote the manuscript. VD participated in the design of the study, interpretation of the results and revision/editing of the manuscript.

Conflict of interest

The authors declare no competing financial interests.

Supporting Information

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

Methods S1. qPCR-based copy number analysis.
Table SI. *HFE*, *TFR2* and *SLC40A1* SNPs.

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Supplementary Method.

Copy number of *PKLR* exon 10 was assessed by SYBR Green I based absolute quantification, using standard curve method. Concentration values of *PKLR* were normalized to *RPPH1* housekeeping gene that is commonly used as a copy number variation (CNV) reference, as it's an autosomal gene with no reported CNV in population. The reaction was run in triplicates. The copy number value of Patient 2 was compared to six non-PK-deficient patient samples. The primer sequences are available upon request.

Table SI. *HFE*, *TFR2* and *SLC40A1* SNPs.

Patient	*HFE* mutation	*TFR2* p.Arg752His	*SLC40A1* CGG(7_8)	*SLC40A1* c.44-24G>C
1	wt	wt	CGG[7];[8]	[C];[C]
1 (mother)	wt	wt	CGG[8];[8]	[C];[C]
1 (father)	wt	wt	CGG[7];[7]	[C];[C]
2	wt	[Arg752His];[=]	CGG[8];[8]	[C];[C]
2 (mother)	wt	wt	CGG[8];[8]	[C];[C]
3	[His63Asp];[Cys282Tyr]	wt	CGG[8];[8]	[C];[C]
4	[His63Asp];[=]	wt	CGG[8];[8]	[C];[C]
3,4 (mother)	[Cys282Tyr];[=]	wt	CGG[8];[8]	[C];[C]
3,4 (father)	[His63Asp];[=]	wt	CGG[7];[8]	[C];[C]
5	[His63Asp];[=]	wt	CGG[8];[8]	[C];[C]
6	[His63Asp];[(His63Asp)]	wt	CGG[8];[8]	[C];[C]
7	wt	wt	CGG[7];[8]	[C];[C]
8	wt	wt	CGG[7];[8]	[C];[C]
8 (mother)	wt	wt	CGG[7];[7]	[C];[G]
8 (father)	wt	wt	CGG[7];[8]	[C];[C]
9	wt	wt	CGG[7];[8]	[C];[G]
9 (mother)	wt	wt	CGG[8];[8]	[C];[C]
9 (father)	wt	wt	CGG[7];[8]	[C];[G]
10	wt	wt	CGG[8];[8]	[C];[C]
11	wt	wt	CGG[8];[8]	[C];[C]

Rem.: *HFE* screened for p.Cys282Tyr, p.His63Asp, p.Ser65Cys, and p.Val256Ile; *TFR2* for p.Arg752His and *SLC40A1* for microsatellite polymorphism c.-330CGG(7_8) and c.44-24G>C.

CGG[7];[8] in *SLC40A1*column is short for c.-330CGG[7];[8]. [C];[C] and [C];[G] in *SLC40A1*column is short for c.[44-24G>C];[44-24G>C] and c.[44-24G>C];[=], respectively.

8.3 Characterization of iron metabolism and erythropoiesis in erythrocyte membrane defects and thalassemia traits

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Characterization of iron metabolism and erythropoiesis in erythrocyte membrane defects and thalassemia traits

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Background and Aims. Erythropoiesis is closely related to iron metabolism in a balanced homeostasis. Analyses of diverse erythroid and iron metabolism disorders have shown that disrupted erythropoiesis negatively affects iron homeostasis and vice versa. The aim of this study was to characterize the relationship between erythropoietic activity and iron homeostasis in pediatric patients with erythrocyte membrane defects and thalassemia traits.
Methods. Selected markers of erythropoietic activity (erythropoietin, soluble transferrin receptor - sTfR and growth differentiation factor 15) and iron status parameters (serum iron, ferritin and hepcidin) were evaluated in pediatric patients with erythrocyte membrane defects and thalassemia traits.
Results. The patients with erythrocyte membrane defects and thalassemia traits had altered iron homeostasis due to disturbed erythropoiesis. In comparison with healthy controls, they had a normal to low hepcidin/ferritin ratio and concomitantly elevated sTfR.
Conclusion. The findings suggest that pediatric patients with erythrocyte membrane defects and thalassemia traits are more susceptible to iron overload than the general population and that the (hepcidin/ferritin)/sTfR ratio can be used to monitor any worsening of the disease.

Key words: hepcidin, erythropoietic activity, erythrocyte membrane defects, thalassemia trait

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INTRODUCTION

Anemia, one of the most common pathological conditions of childhood, can adversely affect development owing to inappropriate oxygen supply to developing organs and tissues. The most frequent cause is nutritional iron deficiency known as iron deficiency anemia (IDA) which results in hypochromic and microcytic red blood cells. IDA however must be distinguished from inherited microcytic anemias, especially β-thalassemia, which is characterized by imbalanced production of globin chains. Anemia can also be associated with excessive destruction of red blood cells (hemolysis); the most common causes of inherited hemolytic anemia in the Czech population are erythrocyte membrane defects, predominantly hereditary spherocytosis.

Thalassemia

Thalassemias are a heterogeneous group of inherited disorders characterized by reduced or absent synthesis of one or several globin chains which are a part of the hemoglobin (Hb) molecule[1]. Under physiological conditions, the ratio of α- and non-α-globin chains (i.e. predominantly β-globins in adults) in red blood cells is balanced (1:1). A reduced amount of α- or β-globin chain produced by a mutant allele is referred as α^+ or β^+ thalassemia while a total absence of α- or β-globin chain production is called α^0 or β^0 thalassemia. The pathophysiology and clinical symptoms of thalassemia are related to the extent of imbalance between α- and β-globin chain synthesis. Heterozygous carriers of a β-thalassemia allele are usually asymptomatic, with hypochromic and microcytic erythrocytes, mild anemia accompanied by compensatory increase in the number of red blood cells (erytrocytosis) and slightly increased erythropoiesis in bone marrow. This type of thalassemia is known as β-thalassemia minor or β-thalassemia trait and represents the most common cause of hereditary microcytic anemia in the Czech Republic. More profound imbalance in α- and β-globin chain synthesis leads to β-thalassemia intermedia; patients exhibit moderate anemia with signs of hemolysis, ineffective erythropoiesis and splenomegaly. Some patients may require occasional transfusions. The incidence of β-thalassemia intermedia in the Czech Republic is very low. The most severe form of β-thalassemia is β-thalassemia major. Hb levels at diagnosis may be below 3.0 g/dL (normal range: 12.0 – 15.0 g/dL) and patients require regular transfusion therapy[1]. To date, no cases of β-thalassemia major have

been reported in the Czech population but sporadic cases have been diagnosed in immigrants. The clinical picture of α-thalassemia is dependent on the degree of reduction of α-globin chain synthesis; i.e. on total number of mutated α-globin genes. Four α-globin genes are present in the normal diploid human genome. Individuals with deletion/inactivation of one allele are asymptomatic and considered silent carriers; the incidence in the Czech Republic may be relatively high. Individuals who inherit two mutant alleles are usually clinically asymptomatic with mild hypochromic microcytic anemia and slightly increased red blood cell count. This condition, called α-thalassemia minor or α-thalassemia trait, is relatively rare in the Czech Republic (M. Divoka, unpublished data). Hemoglobin H disease results from mutation affecting three α-globin genes and presents with moderate to severe anemia. Sporadic cases may be found in the Czech population. The inactivation of all four α-globin genes is lethal and leads to hydrops fetalis and fetal death[2].

Erythrocyte membrane defects

Hereditary defects of erythrocyte membrane proteins manifest as clinically and genetically heterogeneous disorders. The result is large numbers of abnormally shaped erythrocytes, i.e. spherical (for hereditary spherocytosis - HS) or elliptical (for hereditary elliptocytosis – HE) rather than the normal biconcave disk shape[3]. These abnormal erythrocytes have a shorter than average life-span for normal red blood cells and die prematurely. This leads to a compensatory increase in reticulocyte count which is more pronounced in HS than HE. The patient may be asymptomatic or present with varying degree of hemolytic anemia, increased serum bilirubin and splenomegaly. Transfusion therapy for the most severe cases of HS and HE may lead to iron overload which can cause severe health problems[3].

Hepcidin and iron metabolism

Erythropoiesis and iron metabolism reciprocate in the coordinated supply of iron and globin synthesis, essential for normal red blood cell production. Analyses of diverse erythroid and iron metabolism disorders have shown that disrupted erythropoiesis negatively affects iron homeostasis and vice versa.

Iron (Fe) is vital for a number of metabolic processes, most importantly, oxygen transport as a part of the Hb molecule. However, because of its potential toxicity, iron levels must be strictly regulated. Hepcidin, the key molecule regulating plasma iron levels, is produced by hepatocytes[4]. Hepcidin binds to an iron exporter, ferroportin expressed in the cytoplasmic membrane. This induces ferroportin internalization and degradation, resulting in blocking iron release from sites of iron absorption (duodenal enterocytes), iron recycling (macrophages) and iron storage (hepatocytes). Hepcidin synthesis is stimulated in response to increased iron stores and inflammation. It is attenuated in response to iron deficiency and accelerated erythropoiesis due to anemia, ineffective erythropoiesis and hypoxia[5]. The molecules that induce hepcidin synthesis in response to increased iron levels and inflammation are relatively well- defined[4,5]. On the other hand, the identity of negative regulators of hepcidin is still elusive. Growth differentiation factor 15 (GDF15) was proposed as a candidate molecule in the suppression of hepcidin production in patients with β-thalassemia[6]. Later analyses showed that GDF15 was elevated in disease states associated with ineffective erythropoiesis[7]. More recent data indicate the existence of other negative erythroid regulators of hepcidin synthesis[8]. It is accepted that these signals are released by erythroid precursors in bone marrow[9] and override the signals of iron stores[5].

Imbalances in iron metabolism (including hepcidin levels) and their interconnection with defective erythropoiesis have been widely studied in β-thalassemia (intermedia and major) (ref.[10]), congenital dyserythropoietic anemia[11], pyruvate kinase deficiency[12] and Diamond-Blackfan anemia[13]. In this study, we investigated the relationship between iron metabolism and erythropoietic activity in pediatric patients with erythrocyte membrane defects and thalassemia traits.

MATERIALS AND METHODS

Characterization of the cohort

The cohort consisted of 20 patients with erythrocyte membrane defect (18 with HS and 2 with HE) (age range 2-18 years), 13 with thalassemia minor: 10 with the β-thalassemia trait and 3 with the α-thalassemia trait (age range 3-17 years) and 1 patient with thalassemia major (TM) (age 7 years), an immigrant from Moldova. Thalassemia carriers did not require any treatment. The patient with TM was on regular transfusion therapy (1 transfusion unit every three weeks). A control group included 47 children examined prior to planned minor surgery (inguinal or umbilical hernia, plastic surgery). The study and informed consent as per the Declaration of Helsinki were approved by the Ethics Committee of Palacky University Hospital, Olomouc, Czech Republic. None of the subjects had signs of an inflammatory process at the time of examination as documented by normal C-reactive protein (CRP) levels (not shown).

Hematological and biochemical analysis

Blood was taken during routinely performed venous puncture; for the TM patient a pre-transfusion sample was collected. Blood counts and erythrocyte characteristics were examined on Sysmex XE-500 analyzer (Sysmex) for all subjects included in the study. Biochemical serum parameters of iron metabolism and inflammation: serum iron (Fe), ferritin, transferrin saturation (TSAT), soluble transferrin receptor (sTfR) and CRP levels were determined using standard methods; for the control group, samples from 10 to 13 children were analyzed. The serum erythropoietin (EPO) concentration was measured by a solid-phase chemiluminescent immunochemical reaction[14]. Laboratory reference values are given for EPO instead of direct control group measurements.

The diagnosis of HS and HE was made on the basis of elevated bilirubin levels (mean 29 μmol/L, patients range

8–70 μmol/L, normal range 5–21 μmol/L), positive results of cryohemolysis test and blood smear assessment. Thalassemias were diagnosed on the basis of hemoglobin electrophoresis and globin gene Sanger sequencing[15].

Hepcidin analysis

The serum hepcidin levels were measured by reverse-phase liquid chromatography using the UltiMate 3000 Nano LC System (Thermo Fisher Scientific, Sunnyvale, CA, USA) coupled to the QTRAP 5500 mass spectrometer (AB SCIEX, Framingham, MA, USA) as we previously described[12]. Hepcidin was determined for 47 healthy controls, 12 thalassemia carriers and 20 patients with erythrocyte membrane defect.

GDF15 measurements

The serum levels of growth differentiation factor 15 (GDF15) were quantified according to the manufacturer's instructions using the Human GDF15 Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA). GDF15 measurements were performed on 9 thalassemia carriers and 11 patients with erythrocyte membrane defect.

Statistical analysis

ANOVA and Dunnett's test for normally distributed variables or non-parametric Kruskal-Wallis test with multiple comparisons were used for the statistical evaluation. All statistical analyses were done using the STATISTICA, (StatSoft, Inc., software version 12). The significance level was set at 5% for all analyses.

RESULTS

Hematological data and erythropoietic activity

We first analyzed the hematological data. Carriers of α- thalassemia were evaluated together with β-thalassemia carriers due to similarities in clinical picture. They showed mild to severe anemia with a compensatory increase in RBC counts (Table 1). Hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were significantly lower compared to healthy controls. In the erythrocyte membrane defect group, the anemia was milder, with significantly reduced RBC count

Table 1. Hematological parameters.

	Controls	Thalassemia carriers	Erythrocyte membrane defects	Thalassemia major
RBC (10^{12}/L)	4.78 ± 0.60	5.55** ± 0.315	4.23* ± 0.71	2.97
Hb (g/dL)	13.0 ± 1.3	10.6*** ± 1.1	12.0 ± 1.9	8.2
MCV (fL)	82.56 ± 6.87	59.1*** ± 5.68	79.1 ± 5.73	79.8
MCH (pg)	28.29 ± 2.62	19.23*** ± 2.27	28.45 ± 1.86	27.6
MCHC (g/dL)	34.32 ± 1.22	32.46* ± 1.21	36.09** ± 0.87	34.6
Ret (ratio)	0.010 ± 0.003	0.012 ± 0.004	0.066*** ± 0.045	0.012

Values are shown as means ± SD; single values are given for the patient with thalassemia major. RBC, red blood cells count; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration, Ret, reticulocytes count. Statistical significance relative to control group: *P value<0.05; **P value<0.01; ***P value<0.001.

Table 2. Parameters of erythropoietic activity.

	Controls	Thalassemia carriers	Erythrocyte membrane defects	Thalassemia major
sTfR (mg/L)	3.7 ± 0.74	6.89 ± 5.02	9.58** ± 4.89	19
EPO (IU/L)	[4.3-29]+	18.0 ± 22.67	34.04 ± 30.45	> 4000
GDF 15 (pg/mL)	205 ± 27	653** ± 545	387 ± 131	8316.7

Values are shown as means ± SD; single values are given for the patient with thalassemia major. sTfR, soluble transferrin receptor; EPO, erythropoietin; GDF 15, growth differentiation factor 15; + reference range. Statistical significance relative to control group: **P value<0.01.

Table 3. Iron status parameters.

	Controls	Thalassemia carriers	Erythrocyte membrane defects	Thalassemia major
%TSAT	33.07 ± 14.09	30.9 ± 10.86	29.05 ± 10.22	59.9
Serum Fe (μM/L)	20.87 ± 8.37	16.75 ± 5.57	15.04 ± 4.57	22
Ferritin (μg/L)	34.62 ± 20.1	40.23 ± 39.15	99.54** ± 45.52	2872
Hepcidin (ng/mL)	25.86 ± 26.46	14.73 ± 14.27	15.34 ± 16.56	56.2
Hepcidin/ferritin	0.658 ± 0.564	0.341 ± 0.149	0.177** ± 0.173	0.02
(hepcidin/ferritin)/sTfR	0.184 ± 0.189	0.072 ± 0.050	0.023*** ± 0.021	0.001

Values are shown as means ± SD; single values are given for the patient with thalassemia major. TSAT, transferrin saturation; Fe, iron; sTfR, soluble transferrin receptor. Statistical significance relative to control group: **P value<0.01, ***P value<0.001.

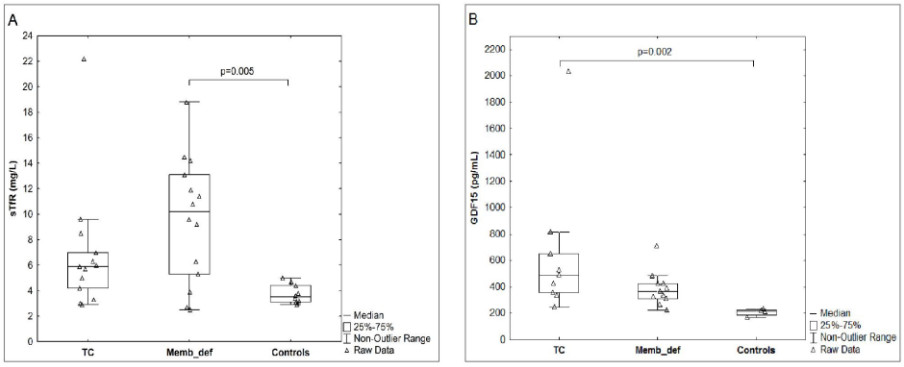

Fig. 1. Elevated markers of erythropoietic activity. (A) sTfR was increased in both patients' groups when compared to the controls (n=9), but only in patients with erythrocyte membrane defect (Memb_def; n=14) the difference reached statistical significance; thalassemia carriers (TC, n=13). (B) The elevation of GDF15 in comparison to the controls was significant for TC (n=9), but not for Memb_def (n=11). sTfR, soluble transferrin receptor; GDF15, growth differentiation factor 15. The graphs show individual values (triangles), 25-75% range with median (box and horizontal line, respectively) and the non-outlier range (the highest and the lowest whiskers, respectively).

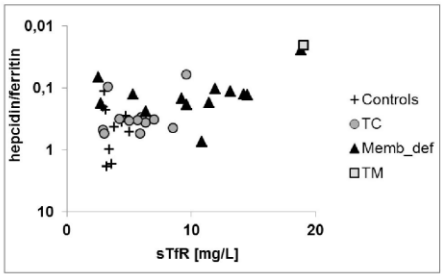

Fig. 2. Differentiation of patients based on the plot of hepcidin/ferritin ratio against sTfR[15]. Patients with erythrocyte membrane defect (Memb_def, n=13), thalassemia carriers (TC, n=12) and thalassemia major (TM, n=1) dislocate from healthy controls (n=9).

and moderate changes in RBC characteristics (Table 1) with the exception of MCHC, which was significantly increased. In addition, compensatory reticulocytosis in response to chronic hemolysis was observed in the majority of patients with erythrocyte membrane defect causing the number of reticulocytes to be significantly different from the control group (Table 1).

We next examined the markers of erythropoietic activity, i.e. serum erythropoietin (EPO) and soluble transferrin receptor (sTfR). EPO was elevated above the reference range (4.3 - 29 IU/L) only in the group of erythrocyte membrane defects (mean 34 IU/L, Table 2); in thalassemia carriers the mean value fell within the reference range (mean 18.0 IU/L, Table 2). sTfR was also significantly higher for patients with erythrocyte membrane defect (mean 9.58 mg/L) than in healthy controls (3.7 mg/L, Table 2 and Fig. 1A). In the thalassemia carriers' group mean sTfR (6.89 mg/L) was 1.9 times higher than mean sTfR of the controls (Table 2 and Fig. 1A). Although the difference was not statistically significant, 10 out of 13 thalassemia carriers had sTfR above the mean control value (Fig. 1A). This indicates increased erythropoietic activity in the bone marrow of thalassemia carriers and patients with erythrocyte membrane defect. To assess the degree of ineffective erythropoiesis[16,17], serum growth differentiation factor 15 (GDF15) was measured using ELISA. The mean GDF15 level was increased in both groups (thalassemia carriers, 653 pg/mL; erythrocyte membrane defects, 387 pg/mL) compared to the controls (205 pg/mL), but only in thalassemia carriers did the difference reach statistical significance (Table 2 and Fig. 1B).

Our only patient with thalassemia major (TM), who is a Moldovan immigrant living in the Czech Republic,

presented with the most severe anemia (Hb of 8.2 g/dL), reduced RBC number, elevated sTfR and EPO levels and the highest GDF15 (Table 1 and 2).

Iron status and hepcidin

To assess any disturbance in iron homeostasis, selected parameters of iron metabolism were evaluated. As shown in Table 3, serum iron (sFe) and transferrin saturation (TSAT) were comparable in all groups. Patients with erythrocyte membrane defect had significantly increased levels of serum ferritin (mean 99.54 μg/L; mean for controls, 34.62 μg/L). Ferritin levels of thalassemia carriers did not differ from healthy controls (Table 3). Hepcidin levels were reduced in thalassemia carriers (mean 14.73 ng/mL) and erythrocyte membrane defects (mean 15.34 ng/mL) compared to controls (mean 25.86 ng/mL, Table 3), but this was not statistically significant. We therefore calculated the hepcidin/ferritin ratio which represents a measure of appropriate response of hepcidin to iron stores. As shown in Table 3, this ratio was significantly lower in the erythrocyte membrane defects group (0.177) compared to healthy individuals (0.658). The reduction of hepcidin/ferritin ratio in thalassemia carriers (to 0.341) reached only borderline statistical significance; even though it was twice as low as in the controls. This indicates inappropriate suppression of hepcidin synthesis.

Along with the changes in hematological parameters, alterations in iron status in our TM patient was consistent with the data published on large cohorts of TM patients[10,18,19]. She showed normal sFe, but significant hyperferritinemia (ferritin level of 2872 μg/L, Table 3). Her serum hepcidin was twice as high (56.2 ng/mL) as in healthy controls (25.86 ng/mL). However, the hepcidin/ferritin ratio, which was 0.02 (control hepcidin/ferritin ratio is 0.658) indicated that hepcidin was disproportionally low with respect to iron loading (Table 3).

Finally, we calculated the ratio of (hepcidin/ferritin) to sTfR which combines the parameters of iron status and erythropoietic activity. Recently, it has been suggested that this formula distinguishes adult thalassemia carriers from healthy controls and reflects the severity of the anemia[20]. As shown in Table 3, this ratio is reduced in both thalassemia carrier group (0.072) and erythrocyte membrane defects (0.023) compared to healthy individuals (0.184) confirming disordered interaction between iron metabolism and erythropoiesis. When sTfR is plotted against the hepcidin/ferritin ratio, thalassemia carriers and patients with erythrocyte membrane defect, tend to diverge from controls (Fig. 2); showing slight to moderate reduction in hepcidin/ferritin ratio and concomitant increase in sTfR that is consistent with accelerated bone marrow erythropoiesis. The TM patient had the lowest (hepcidin/ferritin)/sTfR ratio (0.001) and can be found in the upper right corner on the graph plotting sTfR against hepcidin/ferritin with the lowest hepcidin/ferritin and highest sTfR (Fig. 2). This result confirms increased bone marrow erythropoiesis and inadequate suppression of hepcidin to the degree of iron loading.

DISCUSSION

A number of congenital anemias are coupled with disrupted iron balance and secondary iron overload. The molecular pathophysiology involves suppression of hepcidin; the key molecule inhibiting iron absorption and recycling[21]. An important finding is that erythroid signals dominate over iron store signals[5]. As a result, patients with ineffective erythropoiesis and concomitantly increased iron levels have inappropriately low hepcidin with respect to iron loading which leads to the accumulation of excessive iron in parenchymal tissues. These relatively suppressed hepcidin levels, explain iron overload in patients with β-thalassemia intermedia that are transfusion independent[18]. On the other hand, the primary cause of iron overload in β-thalassemia major is regular transfusion therapy; hepcidin is higher than in β-thalassemia intermedia due to the suppressive effect of transfusions on ineffective erythropoiesis[19]. Recently, it was shown that carriers of β- or α-thalassemia allele too, have altered iron metabolism parameters and erythropoiesis despite the absence of clinical symptoms[20,22].

In this study, we analyzed for the first time, whether and how disrupted erythropoiesis in erythrocyte membrane defects, namely HS and HE, influences iron metabolism. The anemia in these patients was hyperchromic and milder than for thalassemia carriers. Due to chronic hemolysis it was accompanied by increased reticulocyte count. However, serum EPO was higher than in thalassemia carriers and together with significantly elevated sTfR, reflected increased erytropoietic activity in the bone marrow. In addition, slightly increased levels of GDF15 indicated some degree of ineffective erythropoiesis[7].

The evaluation of parameters of iron metabolism revealed normal serum Fe, but significantly elevated ferritin levels. As in thalassemia carriers, hepcidin and hepcidin/ferritin ratio were reduced 1.7 and 3.7 times, respectively, compared to the controls. The patients with erythrocyte membrane defects also had a significantly lower (hepcidin/ferritin)/sTfR index than controls and dislocated from them on the graph when sTfR was plotted against hepcidin/ferritin. The erythrocyte membrane defects were shifted more to the top right on the graph than thalassemia carriers indicating more pronounced disruption in the erythropoiesis-hepcidin-iron stores axis. These results also suggest that hepcidin synthesis is inappropriately attenuated in patients with HS and HE. The direct signals need to be identified because hepcidin inhibition by EPO is indirect and requires active bone marrow erythropoiesis[13,23,24] and GDF15 is considered a marker of ineffective erythropoiesis rather than the main suppressor of hepcidin[17]. Very promising is the description of an erythroid regulator, named erythroferrone which stimulates hepcidin synthesis in response to chronic bleeding and EPO administration[8]. The human assay for erythroferrone measurements which would enable us to determine its levels under physiological and different pathological conditions, including erythrocyte membrane defects, is currently under development.

We also showed that pediatric thalassemia carriers have altered RBC parameters; raised RBC count and reduced Hb level, MCV, MCH and MCHC. Elevated sTfR and GDF15 indicate increased but ineffective erythropoiesis in the bone marrow. Similar data were recently published for schoolchildren with β-thalassemia trait from Sri Lanka[22] and also for adult thalassemia carriers[20]. However, the study on adult thalassemia carriers showed that the β-thalassemia trait is associated with more profound negative effects on erythropoiesis than the α-thalassemia trait[20]. We were unable to make such a comparison because of only three α-thalassemia carriers included in our cohort. Although their parameters were comparable with β-thalassemia carriers, it will be important to analyze larger numbers of children with α-thalassemia trait to draw final conclusions. Alternatively, the clinical and laboratory difference between α- and β-thalassemia carriers may become more obvious over time. It is also likely that the difference reflects the heterogeneity at the molecular level; mutant alleles causing more profound imbalance in globin production would be associated with more severe alteration in erythropoiesis.

In the thalassemia carrier group, all individuals were iron replete with sFe and ferritin indistinguishable from healthy controls. However, they showed reduced hepcidin levels and reduced hepcidin/ferritin ratio (both parameters approximately twice). These results are consistent with the data obtained on pediatric β-thalassemia carriers from Sri Lanka[22]. On the other hand, Guimaraes et al.[20] found that only adult α-thalassemia carriers had significantly decreased hepcidin, while hepcidin of adult β-thalassemia carriers was insignificantly higher than in controls. Again, we cannot draw any conclusions due to the minimal number of α-thalassemia carriers we analyzed. The differences between β-thalassemia carriers may again reflect differences between pediatric age and adulthood or may eventually be also influenced by variable severity of the different β-thalassemia alleles. Nevertheless, when the hepcidin/ferritin ratio was plotted against sTfR, our thalassemia carriers dislocate from the control group like the α- and β-thalassemia carriers in Guimaraes's study[20]. This result is consistent with the altered erythropoiesis and iron homeostasis in the thalassemia trait.

The clinical picture of anemia and the hematological and biochemical laboratory findings of our patient with TM were in agreement with the published data on TM subjects, including inappropriate suppression of hepcidin with respect to the degree of iron overload[18]. The low hepcidin/ferritin ratio and elevated sTfR and GDF15 reflect increased ineffective erythropoiesis which signals higher iron demand leading to increased iron absorption and recycling. This is consistent with the finding that iron overload in TM is a result of regular transfusion therapy and disturbed hepcidin synthesis[19].

CONCLUSIONS

Pediatric patients with erythrocyte membrane defect and thalassemia carriers showed alterations in erythropoiesis and iron metabolism. The regulation of iron homeostasis appeared to be more disbalanced in erythrocyte membrane defects than in thalassemia carriers, but to a lesser extent than in the TM subject. We propose that the degree of alteration may reflect the severity of disease-causing mutation. We confirmed the results of Guimarães et al.[20], that the (hepcidin/ferritin)/sTfR ratio distinguishes thalassemia carriers from healthy controls and showed that it also differentiates patients with erythrocyte membrane defect. We propose that these individuals may be more susceptible to iron overload than the general population. As suggested for thalassemia carriers[20], the (hepcidin/ferritin)/sTfR ratio can be used to follow up patients with erythrocyte membrane defects for evolution of the disease with a focus on alterations in erythropoietic activity and potential risk of iron overload. This ratio may also eventually help to make treatment choices including timing of splenectomy in patients with erythrocyte membrane defect and excessive hemolysis.

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Author contributions: LS: data collection and analysis, contributed to GDF15 measurements, manuscript writing; DH: measured the levels of hepcidin; ZZ: determined GDF-15 levels; MD performed Hb electrophoresis, Sanger sequencing and cryohemolysis tests; MHa: responsibility for the hepcidin assay; VM: collected the patients' material and provided clinical information; JV: performed statistical analyses; MHo, DP: study design, results interpretation; All authors contributed to the editing of the manuscript.

Conflict of interest statement: None declared.

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8.4 Stanovení hladiny hepcidinu-25 v séru pomocí SPE-LC-MS/MS metody

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Stanovení hladiny hepcidinu-25 v séru pomocí SPE-LC-MS/MS metody

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1. Úvod

Hepcidin je 25-aminokyselinový peptidový hormon, který je klíčovým regulátorem homeostázy železa v lidském těle [1]. Je syntetizován v játrech a je sekretován do plasmy. Hepcidin se váže na buněčný exportérový kanál pro železo – feroportin, což vede k internalizaci a degradaci tohoto komplexu a v důsledku ve snížení exportu železa z buněk do plasmy [1, 2].

Zvýšené zásoby železa a zánětlivé stavy indukují syntézu hepcidinu, zatímco k potlačení jeho syntézy dochází při hypoxii, anémii a zvýšené neefektivní erytropoéze v kostní dřeni. Deficit hepcidinu hraje ústřední roli při vstřebávání železa při hereditární hemochromatóze a talasemii. Syntézu hepcidinu indukují různé infekční stavy, což vede ke snížení hladiny železa, které je nezbytné pro přežití invazivních patogenů [1].

Od objevení hepcidinu v roce 2001 a osvětlení jeho důležité role v regulaci metabolismu železa, byl hepcidin navržen jako slibný diagnostický marker pro onemocnění z této skupiny. Desítky analytických metod byly vyvinuty pro stanovení hladiny hepcidinu v séru či plasmě. Metody lze rozdělit do dvou skupin: imunochemické metody [3] a metody založené na hmotnostní spektrometrii [4, 5]. Imunochemické metody v případě stanovení hepcidinu mají omezenou přesnost s ohledem na vysokou homologii mezi pro-hepcidinem (prekurzorový protein) a hepcidinem samotným.

V tomto článku se budeme věnovat preferované metodě pro stanovení hladiny hepcidinu v séru pomocí SPE-LC-MS/MS a její validaci, která zahrnovala hodnocení linearity a analytického rozsahu metody, jednodenní a mezidenní přesnosti a správnosti.

2. Materiál a metody

2.1. Materiál a chemikálie

Lidský hepcidin a izotopicky značený [$^{13}C_9$, $^{15}N_1$-F_4]-hepcidin (IS, interní standard) byly pořízeny od firmy Thermo Fisher Scientific (Thermo Fisher Scientific, Berlín, Německo); kyselina mravenčí (FA), metanol (MeOH) a králičí sérum od firmy Sigma Aldrich (Sigma Aldrich, Praha, Česko); 96-jamková destička pro extrakci na pevné fázi (SPE - Oasis HLB matrice) od firmy Waters (Waters, Praha, Česko); analytická kolona Polaris C18 A, 5 µm, 75 x 2,1 mm od firmy Varian (Labicom, Olomouc, Česko).

2.2. Příprava standardů

Zásobní roztoky peptidů o koncentraci 100 µg/ml byly připraveny rozpuštěním hepcidinu a IS v 10 % MeOH. Oba roztoky byly alikvotovány a skladovány při – 80°C. Pracovní roztok hepcidinu o koncentraci 500 ng/ml byl připraven vždy čerstvý a to naředěním jeho zásobního roztoku v králičím séru. Pracovní roztok IS o koncentraci 100 ng/ml byl připraven vždy čerstvý a to naředěním jeho zásobního roztoku ve směsi MeOH/H_2O/FA 50%/49,9%/0.1% (v/v/v). Kalibrační standardy hepcidinu byly připraveny naředěním pracovního roztoku hepcidinu v králičím séru na koncentraci 2,5; 5; 10; 25; 100; 250 a 500 ng/ml.

2.3. Extrakce na pevné fázi

Extrakce na pevné fázi byla provedena v 96-jamkové extrakční destičce (Oasis HLB). Byl připraven promývací (30 % MeOH, pH 10) a eluční roztok (90 % MeOH, pH 5). Po aktivaci MeOH a kondiciování HLB matrice pomocí H_2O, bylo do každé jamky SPE destičky naneseno 200 µl vzorku séra obsahující 50 µl 100 ng/ml IS a 100 µl 0,1 % FA. SPE destička

byla následně promyta 200 µl H_2O, 200 µl promývacího roztoku a opět 200 µl H_2O. Eluce proteinů byla provedena pomocí 50 µl elučního roztoku. Eluát byl sbírán do nové 96 jamkové destičky a byl naředěn 150 µl 0,1 % FA. Všechny popsané kroky byly provedeny na robotické stanici Tomtec Quadra 4^{TM} (Tomtec, USA). Naředěné eluáty byly připraveny pro LC-MS/MS analýzu.

2.4. SRM optimalizace

Všechny experimenty vedoucí k optimalizaci metody byly provedeny na hmotnostním spektrometru QTRAP 5500 (AB Sciex, Praha, Česko). Systém byl kontrolován přes software Analyst (verze 1.5.1, AB Sciex, Praha, Česko). Prekursorové a produktové ionty byly určeny na základě přímé infuze hepcidinu (10 µg/ml) v pozitivním ionizačním módu (Tab 1).

Tab. 1 – SRM přechody a optimalizované parametry pro hepcidin a IS

2.5. Kapalinová chromatografie a hmotnostní spektrometrie

Analýze vzorku předcházela separace pomocí kapalinové chromatografie (UltiMate® 3000 RSLCnano, Dionex). Peptidy byly separovány na koloně s reversní fází Polaris C18 A, 5 µm (75 x 2,1 mm), která byla vyhřívána na 40°C. Mobilní fáze se skládala z 5% MeOH s 0,1 % FA (A) a 95 % MeOH s 0,1 % FA (B). V čase 0-1,5 min bylo složení mobilní fáze 10 % B, mezi 1,5 až 4 min byl aplikován gradient 10-90 % B; mezi 4-5,5 min bylo složení mobilní fáze 90 % B; mezi 5,5-5,6 min byl aplikován gradient 90-10 % B a mezi 5,6-8,6 min bylo složení mobilní fáze 10 % B; průtoková rychlost mobilní fáze byla nastavena na 200 µl/min. Detekce a kvantifikace analytů byla provedena na hmotnostním spektrometru QTRAP 5500 (AB Sciex) s ionizací elektrosprejem. Podmínky pro sběr dat SRM metodou jsou uvedeny v tabulce 1. Napětí na elektrospreji bylo nastaveno na + 5000 V. Průtoky plynů byly nastaveny následovně: CUR na 20 psi, GS1 na 42 psi a GS2 na 55 psi. Teplota ionizačního zdroje byla nastavena na 600 °C.

2.6. Validace metody

Pro posouzení linearity (Obr. 1), jednodenní přesnosti a správnosti (Tab. 2) bylo připraveno osm standardních vzorků králičího séra s koncentraci hepcidinu 2,5, 5, 10, 25, 50, 100, 250 a 500 ng/ml. Celkem byly připraveny tři nezávislé série vzorků a každý vzorek byl proměřen dvakrát. Kalibrační křivka byla sestavena z poměru plochy píků (hepcidin/IS) a koncentrace odpovídajících standardů. Regresní rovnice byla použita k zpětnému výpočtu koncentrace hepcidinu ve vzorku (Obr 1). Pro posouzení mezidenní přesnosti a správnosti byly připraveny tři vzorky králičího séra s koncentrací hepcidinu 5, 100, 500 ng/ml. Každý vzorek byl proměřen šestkrát. Toto měření se opakovalo ve třech po sobě následujících dnech (Tab. 3).

Obr. 1 – Kalibrační křivka hepcidinu

3. Výsledky a diskuze

Výběr prekursorových a produktových iontů pro SRM přechody byl proveden v MS a MS/MS skenovacím módu. Nejvíce zastoupené prekursorové ionty byly čtyřnásobně nabité $[M+4H^+]^{4+}$ ionty hepcidinu a interního standardu. Jejich fragmentací byly získány produktové ionty, z nichž nejvíce intenzivní se jevily b_3^+ a b_4^+ ionty. U těchto produktových iontů byla stanovena jejich optimalizována kolizní energie (CE). V tabulce 1 jsou shrnuty SRM přechody a jejich optimalizované kolizní energie pro jednotlivé analyty. Li et al. v roce 2009 popsal metodu pro stanovení hladiny hepcidinu v séru, přičemž na hmotnostním spektrometru API 4000 použil pouze jeden SRM přechod pro kvantifikaci hepcidinu (698,1→354,1) [4]. Používat pouze jeden přechod nemusí být zcela spolehlivé pro výslednou kvantifikaci analytu, z tohoto důvodu jsme v naší metodě použili dva přechody, jeden SRM přechod slouží pro kvantifikaci a druhý SRM přechod pro verifikaci hepcidinu (Tab. 1). Hodnocení linearity a citlivosti metody bylo posouzeno na základě sestavené kalibrační křivky pomocí korelačního koeficientu a limitu kvantifikace (Obr. 1). Hepcidin vykazoval ve zvoleném

rozsahu 2,5 - 500 ng/ml vynikající linearitu s korelačním koeficientem 0,9997. Při hodnocení limitu kvantifikace LOQ jsme vycházeli z poměru signálu k šumu S/N > 10, přičemž tuto podmínku splňoval i kalibrační vzorek s nejnižší koncentraci hepcidinu (2,5 ng/ml). Jednodenní a mezidenní přesnost a správnost, jsou pro jednotlivé koncentrace shrnuty v tabulce 2 a 3, splňují kritéria pro validaci metody (přesnost ≤ 15 % a správnost mezi 85 – 115 %) [5].

Tab. 2 – Jednodenní přesnost a správnost metody

Tab. 3 – Vícedenní přesnost a správnost metody

4. Závěr

Stanovení hladiny hepcidinu má velmi důležitou roli v hodnocení homeostázy železa. Tato práce byla zaměřena na vývoj a částečnou validaci metody pro stanovení hladiny hepcidinu v séru, a to technikou kapalinové chromatografie ve spojení s hmotnostní spektrometrií. Metoda je v současné době dostupná odborné veřejnosti a pacientům v proteomické laboratoři Ústavu molekulární a translační medicíny v Olomouci. Metoda byla v minulosti použita pro stanovení hladiny hepcidinu v séru u pacientů trpících Blackfanovou-Diamondovou anémií [6], talasemií a sférocytózou [7]. Vyvinutá SPE-LC-MS/MS metoda pro stanovení hladiny hepcidinu v séru je vysoce spolehlivá a časově nenáročná.

Poděkování

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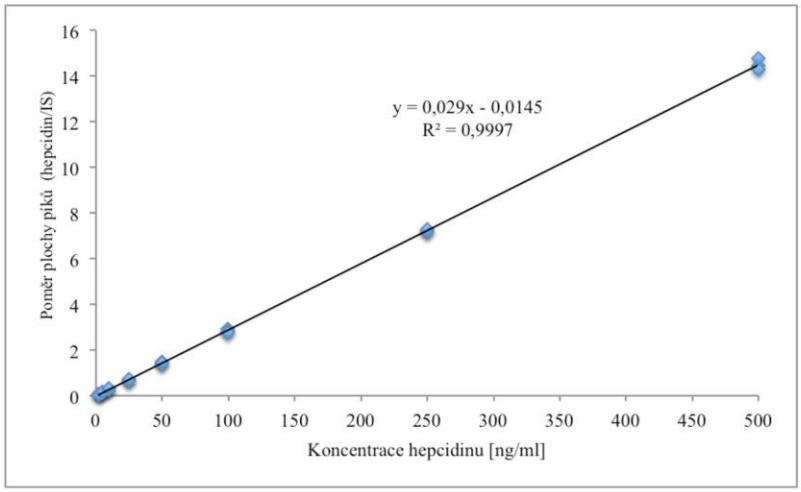
Abstract

Quantitative determination of hepcidin in serum using LC-MS/MS

Hepcidin is 25-amino acid peptide hormone that plays an important role in iron homeostasis. An accurate assessment of serum hepcidin is critical to understand its role in various iron disorders. In the present study, the method employing a liquid chromatography and tandem-mass spectrometry for quantification of hepcidin-25 level in serum is described. Method fulfilled the criteria for validation and was successfully used for determination of hepcidin level of patients with Diamond-Blackfan anemia, thalassemia and spherocytosis.

Keywords: proteomics, hepcidin, mass spectrometry, SRM

Obr. 1 – Kalibrační křivka hepcidinu v séru



Tab. 1 – SRM přechody a optimalizované parametry pro hepcidin a IS

Analyt	SRM přechod [m/z]	Popis produktového iontu	Doba měření [ms]	DP [V]	EP [V]	CE [V]	CXP [V]
Hepcidin	698,0→354,1	b_3^+	100	80	10	41	11
Hepcidin	698,0→501,4	b_4^+	100	80	10	41	11
IS	700,8→354,2	b_3^+	100	80	10	41	11
IS	700,8→511,2	b_4^+	100	80	10	41	11

IS, interní standard (izotopicky značený [$^{13}C_9$, $^{15}N_1$-F_4]-hepcidin)

Tab. 2 – Jednodenní přesnost a správnost metody

Série vzorků	Standardy hepcidinu [ng/ml]							
	Std. 1	Std. 2	Std. 3	Std. 4	Std. 5	Std. 6	Std. 7	Std. 8
	2,5	5	10	25	50	100	250	500
1	2,43	4,97	9,58	24,5	50,7	100,5	255,9	496,5
	2,70	5,06	10,1	25,1	49,4	98,4	248,1	520,2
2	2,67	5,00	10,05	26,0	53,0	98,6	237,2	470,2
	2,44	4,96	10,16	25,8	52,2	101,4	238,4	464,6
3	2,68	4,98	10,06	23,6	51,2	102,5	246,3	496,0
	2,62	5,24	9,74	24,2	50,1	108,5	272,6	504,6
Průměr (n = 6)	2,59	5,04	9,95	24,9	51,1	101,7	249,8	492,0
Přesnost [%]	4,7%	2,1%	2,3%	3,8%	2,6%	3,7%	5,3%	4,3%
Správnost [%]	103,6%	100,7%	99,5%	99,5%	102,2%	101,7%	99,9%	98,4%

Std., standard

Tab. 3 – Mezidenní přesnost a správnost metody

Den	Jednodenní přesnost a správnost	Koncentrace hepcidinu [ng/ml]		
		Vzorek 1	Vzorek 2	Vzorek 3
		5	100	500
Den 1	Průměr (n = 6)	4,77	99,6	482,5
	Přesnost [%]	9,1%	3,0%	4,6%
	Správnost [%]	95,4%	99,6%	96,5%
Den 2	Průměr (n = 6)	5,21	97,7	457,4
	Přesnost [%]	0,07	0,022	0,018
	Správnost [%]	7,0%	2,2%	1,8%
Den 3	Průměr (n = 6)	5,3	119,2	469,8
	Přesnost [%]	5,9%	8,9%	6,2%
	Správnost [%]	106,0%	119,2%	94,0%
Mezidenní přesnost a správnost	Průměr (n = 18)	5,09	105,5	469,9
	Přesnost [%]	4,6%	9,2%	2,2%
	Správnost [%]	101,8%	105,5%	94,0%