RESEARCH ARTICLE

Proteomics reveals reduced expression of transketolase in pyrimidine 5′-nucleotidase deficient patients

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Purpose: To date, it remains a challenge to correctly and timely diagnose red blood cell (RBC) enzymopathies that result in hereditary nonspherocytic hemolytic anemia (HNSHA), the third most common of which is pyrimidine 5′-nucleotidase (P5N) deficiency with just over 100 cases recognized and confirmed worldwide.
Experimental design: We have investigated the RBC proteome of a patient with P5N deficiency due to a homozygous frameshift mutation in the *NT5C3A* gene. Protein expression levels were analyzed against healthy controls and against patients with hemolytic anemia of different origin, to account for the patient's elevated reticulocyte versus RBC ratio.
Results: Stringent relative quantification of the patient's protein levels revealed reduced levels of P5N, and unexpectedly, also decreased levels of transketolase, an enzyme involved in the nonoxidative phase of the pentose phosphate pathway, one of the few key pathways active in RBCs. Immunoblotting of whole blood samples from this and other P5N-deficient patients with dissimilar mutations indicated that P5N deficiency was correlated with reduced transketolase levels.
Conclusions and clinical relevance: Consequently, insight into patient RBC proteomes illustrates potential benefit of coupling quantitative proteomics strategies with routine HNSHA diagnostic procedures. Proteomics facilitates finding novel biomarkers for HNSHA patients, for example, suffering from P5N deficiency, providing new prospects for future diagnosis and therapy.

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1 Introduction

Matured red blood cells (RBCs) have lost their nuclei and all other cytoplasmic organelles. This results in cells with an extremely flexible biconcave shape, which allows their circulation throughout the body, even across the smallest of

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Abbreviations: **ATP**, adenosine triphosphate; **GTP**, guanosine triphosphate; **HA**, hemolytic anemia; **HCs**, healthy controls; **HNSHA**, hereditary nonspherocytic hemolytic anemia; **NAD**, nicotinamide adenine dinucleotide; **NADP**, nicotinamide adenine dinucleotide phosphate (reduced form); **NADPH**, nicotinamide adenine dinucleotide phosphate (oxidized form); **P5N**, pyrimidine 5′-nucleotidase; **PPP**, pentose phosphate pathway; **RBC**, red blood cell; **SCX**, strong cation exchange; **TKT**, transketolase

Clinical Relevance

To date, accurate and timely diagnosis of red blood cell enzymopathies causing hereditary nonspherocytic hemolytic anemia remains a challenge, since these are rare diseases. Mutations in the gene encoding pyrimidine 5′-nucleotidase (P5N; the *NT5C3A* gene) cause an enzymatic disorder. At present, just over 100 patients are known worldwide, who show mostly unrelated *NT5C3A* gene mutations. One feature of this enzymopathy is marked basophilic stippling, which is detected in less than 5% of the cell population during peripheral blood smear tests. Moreover, basophilic stippling can also be observed in other congenital or acquired disorders such as lead poisoning, β-thalassemia trait, and sideroblastic anemia. Protein P5N itself cannot be used as protein biomarker, because of the existing wide diversity of mutations, therefore we used quantitative proteomics to find novel potential protein biomarkers, which could be utilized regardless of the origin of the *NT5C3A* gene mutation. We have identified transketolase as an RBC pentose phosphate pathway enzyme of which the reduced expression levels correlate well with P5N deficiency, although we could only verify that for four patients. This discovery opens new opportunities for improved insight into the pathophysiology of P5N deficiencies, and possibly into the pathogenesis of hemolysis.

microcapillaries. The biconcave shape further provides an optimal area for the RBCs' main task—uptake, transport, and delivery of oxygen to all tissues. While the intracellular oxidative power is high, RBCs need to keep the iron of the hemoglobin in the divalent state, and also keep cellular metabolic enzymes active by preventing their oxidation. As soon as one of these vital functions is lost, RBCs are quickly removed from circulation.

RBCs ultimately require metabolic energy for all these key tasks, which they solely obtain from anaerobic conversion of glucose in the Embden–Meyerhof pathway to generate ATP (Fig. 1) [1, 2]. For reducing capacity, the RBC needs NAD+, which is formed in the step catalyzed by glyceraldehyde-3-phosphate dehydrogenase [1]. These NAD+ molecules are converted into NADH, which is used to reduce methemoglobin to hemoglobin. Other reducing power is created by the reduction of NADP+ into NADPH in the pentose phosphate pathway (PPP) or hexose monophosphate shunt. The NADPH functions primarily as an input for the reduction of oxidized glutathione in the glutathione metabolism pathway [1]. An overview of this interconnectivity in RBC metabolic pathways is illustrated in Fig. 1.

RBCs cannot synthesize new proteins due to the absence of a nucleus and other cytosolic organelles. Therefore, enzyme deficiencies affect RBC metabolism, resulting in acute or chronic nonspherocytic hemolytic anemia (HA). A number of rare enzymopathies have been described for enzymes crucial for the RBC, such as deficiencies of glucose-6-phosphate dehydrogenase (PPP) and pyruvate kinase (Embden–Meyerhof pathway) [1, 3–5]. These RBC enzyme deficiencies generally have a clear cause-and-effect relationship, which is however not applicable to all RBC enzyme deficiencies.

A key pathway important for proper RBC function is the nucleotide metabolism pathway, part of which involves purine metabolism, important for the cellular ATP and GTP pools. ATP constitutes approximately 85% of the nucleotide pool within the RBC; GTP is present only in small amounts and the pyrimidine nucleotides in trace amounts [6]. Since pyrimidine nucleotides are not required for energy supply in the RBC, these are being catabolized by pyrimidine 5′-nucleotidase (P5N) in the final steps of RNA degradation. In these steps, these are dephosphorylated to membrane-diffusible nucleosides during final maturation of reticulocytes into RBCs [7]. Mutations in the gene encoding P5N (the *NT5C3A* gene) cause an enzymatic disorder [7]. Presently, only over 100—mostly unrelated cases—are known worldwide. A typical feature of this enzymopathy is marked basophilic stippling, as detected in less than 5% of the cell population during peripheral blood smear examination [8, 9]. Furthermore, a number of epiphenomena have been detected, such as a decrease in ribose pyrophosphokinase activity, an increase in pyrimidine nucleoside monophosphate kinase activity, and a marked increase in reduced glutathione levels, as reviewed by Corrons [6]. The pathophysiology of inherited HA as a result of P5N deficiency has been studied for many years. It has been suggested that RBC metabolism is substantially affected by the accumulation of the pyrimidine nucleotides and their derivatives [7, 10, 11].

To date, little is known about the effect of P5N deficiency on the entirety of proteins in the RBCs. Such knowledge would provide deeper insight into the overall effects of this enzymatic dysfunction. In this study, we therefore performed proteome analysis on the RBC lysate of a patient suffering from congenital HA due to a homozygous frameshift mutation in the *NT5C3A* gene. Stable isotope metabolic labeling of the sample peptides was used to relatively quantify the RBC lysate proteome against different controls, and Orbitrap MS was applied to analyze the samples. The results showed that 12 proteins were strongly differentially regulated for this P5N-deficient patient while not for the diverse controls, among which, as expected, P5N was downregulated.

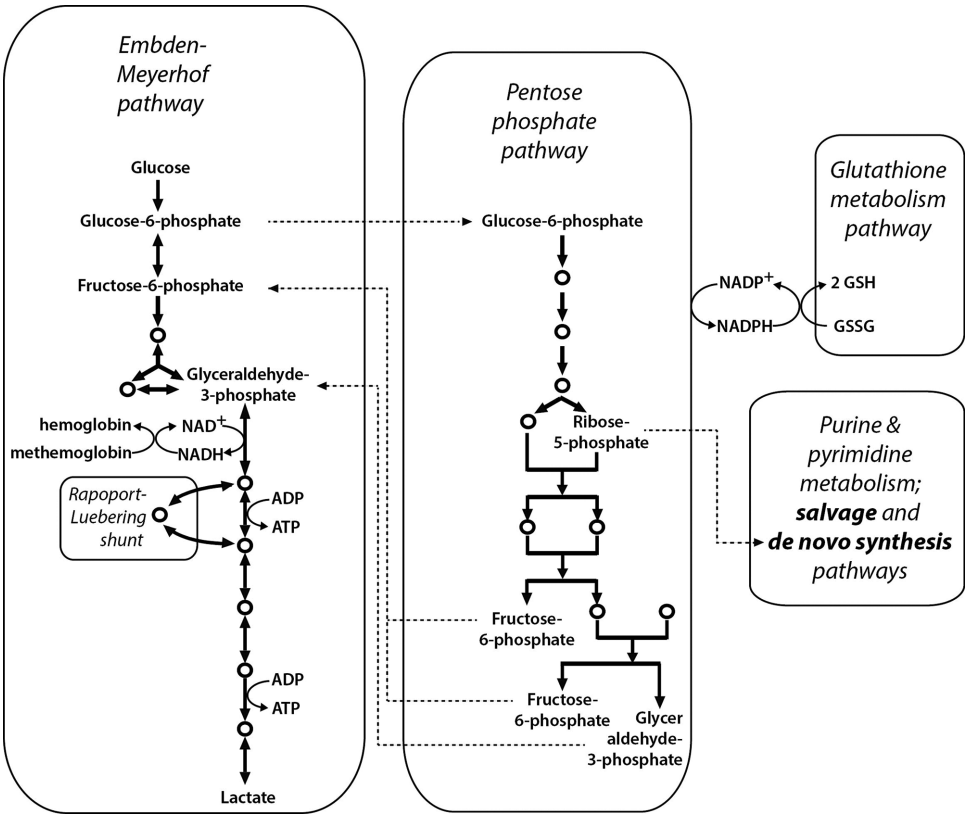

Figure 1. Interdependent connectivity between key metabolic pathways in RBCs and progenitor cells. The single source of metabolic energy for RBCs is glucose metabolism through the Embden–Meyerhof pathway, where multistep anaerobic conversion of glucose generates ATP, with lactate as an end product. The RBC-specific Rapoport–Luebering shunt regulates 2,3-bisphosphoglycerate (2,3-BPG) production relative to energy requirements in the cell. RBCs need NAD+, which is formed in the step catalyzed by glyceraldehyde-3-phosphate dehydrogenase. These NAD+ molecules are converted into NADH, which is used to reduce methemoglobin to hemoglobin. Other reducing power is created by the reduction of NADP+ into NADPH in the pentose phosphate pathway (PPP). The PPP is connected with the Embden–Meyerhof pathway via glucose-6-phosphate, fructose-6-phosphate, and glyceraldehyde-3-phosphate. NADPH functions primarily as input for reduction of oxidized glutathione in the glutathione metabolism pathway. Glutathione operates by detoxifying cellular peroxides or by reducing protein sulfhydryl groups. In RBC progenitor cells, purines and pyrimidines are being produced in the salvage pathway or the de novo pathway. Ribose-5-phosphate, a product of the PPP, is used as input for the de novo pathway of purine and pyrimidine synthesis, and eventually of RNA synthesis. Only metabolites important for the described research have been indicated, others have been indicated with a circle.

Interestingly, most of the other regulated proteins were not previously linked to HA or deemed essential for activity of the RBC. This suggests that their regulation occurs as secondary effect due to P5N mutation. Our analysis also revealed remarkable downregulation of the PPP-enzyme transketolase (TKT). Immunoblotting on RBC samples from a cohort of patients, who were each affected by different *NT5C3A* gene mutations, suggests that downregulation of TKT is a general characteristic of P5N deficiency.

2 Methods

2.1 Patient characteristics

Patient A is a 49-year-old female of Italian descent, who was diagnosed with congenital HA associated with P5N deficiency due to a homozygous frameshift mutation in the *NT5C3A* gene (c.576del) [12]. She stems from a consanguineous marriage (her parents are cousins). The patient's brother and sister are both asymptomatic. Laparoscopic splenectomy and cholecystectomy was performed at age 28 due to cholelithiasis. To assess the effect of P5N deficiency on the proteome of the RBCs of patient A, we performed proteomic analysis in which we compared the patient's RBC proteome with that of four healthy controls (HCs). Also, two other HA patients (HA not due to P5N deficiency) were analyzed upon differences in RBC protein levels due to a high reticulocyte count. Blood samples were collected from all patients and controls after obtaining informed consent in accordance with the Declaration of Helsinki (2008). Details of the patient and control cohorts are provided in the Supporting information.

2.2 RBC sample preparation and quantitative MS

RBC samples were prepared as described before [13–16]. Briefly, packed RBCs were isolated using α-cellulose columns according to the protocol by Beutler and Gelbart [13], followed by further preparation of the lysates as described by Dodge et al. [14]. RBC lysates were subsequently depleted for both hemoglobin and carbonic anhydrase 1 as described by Ringrose et al. [15].

Doubly depleted protein samples of patient A, patient controls (PCs) PC1, PC2, and HCs (HC1–HC4) were prepared for quantitative analysis by MS using stable isotope dimethyl labeling according to the protocol by Boersema et al. [17]. Strong cation exchange (SCX) chromatography [18] was performed to fractionate the samples prior to quantitative RP-LC-MS/MS analysis. This approach using double depletion, followed by SCX and RP-LC-MS/MS is comparable to the method used by Roux-Dalvai et al. [19], since it provided us with a similar number of identified proteins. The MS proteomics data have been deposited to the ProteomeXchange Consortium [20] via the PRIDE partner repository with the dataset identifier PXD001837, title is "Erythrocyte soluble proteome analysis in hemolytic anaemia patients." Detailed information about the experiments is provided in the Supporting information.

2.3 Data processing and data analysis

Raw data files were processed for identification and quantification using MaxQuant [21] (version 1.1.1.36). Further details about the raw data analysis are provided in the Supporting information. We tested whether specific proteins in the samples from patient A, or from PC1, or PC2, were significantly differentially regulated compared to the HC samples. A one-sample two-tailed Student's *t*-test was performed on the 2log-transformed ratios of each of the patient samples separately, each against the HC samples. For patient A, a significance cutoff was set at $p \leq 0.05$. For these *t*-tests, only proteins that were quantified in a minimum of three HCs were taken into account. The regulated proteins were subsequently assigned to their respective metabolic pathways.

2.4 Validation of results by Western Blot

Western blot analysis was used to validate nano-LC-MS data from proteomic analysis. Where applicable, the analysis was performed on both nondepleted RBC soluble protein fraction and doubly depleted sample. Further details about the Western blot validation experiments are provided in the Supporting information.

3 Results

3.1 Proteome analysis

An overview of the experimental setup of the proteomic analysis is shown in Fig. 2. RBC samples were collected from patient A, from two patients who were used as HA controls (PC1 and PC2), and from four HCs (HC1–HC4). After double depletion of the very high abundance proteins, hemoglobin and carbonic anhydrase 1, proteins were digested and labeled with stable isotope dimethyl labels [17]. HC peptide mixtures were pooled (control pool; HC1:HC2:HC3:HC4 = 1:1:1:1) and labeled with a "light" dimethyl label (CH_3), which was used as internal standard. Seven separate peptide samples from patients (patient A, PC1, and PC2) or HCs (HC1–HC4) were labeled with an "intermediate" dimethyl label (CD_3). Each of the seven samples were mixed with the internal standard (sample:internal standard = 1:1), and peptides of each mixture were separated using SCX chromatography. The SCX fractions that contained the bulk of the peptides were analyzed by LC-MS/MS, and peptides were quantified relative to the internal standard. In total, 1146 protein groups were identified in all RBC samples.

We considered that patient A shows elevated reticulocyte counts compared to the HCs, which may additionally cause changes in protein levels. Therefore, we also selected PCs showing elevated reticulocyte counts, that is, patients PC1 and PC2, who are both affected by HA of different origin, that is, by hereditary xerocytosis (dehydrated stomatocytosis; further details are provided in Supporting information Table 1). These two PCs account for any observed variation in protein expression level biased by the elevated reticulocyte count. Further, sample variability was kept to a minimum due to the various sample preparation and handling steps within the workflow [22]. Indeed, the elution patterns of the depletion of hemoglobin (Supporting information Fig. 1A) and the second depletion of the carbonic anhydrase 1 (Supporting information Fig. 1B) were highly similar for all seven samples, apart from variations in peak intensities due to sample protein concentration differences. The patterns of the proteins after SDS-PAGE were also very similar (Supporting information Fig. 1C). The observed variations in protein band intensities are likely due to differences in genetic background of all seven individuals and/or the presence or absence of congenital HA. Also, after peptide stable isotope dimethyl labeling, the SCX chromatography patterns (Supporting information Fig. 1D) and overall proteome content (Supporting information Fig. 2A and B) are comparable, indicating that our sample preparation approach does not introduce bias in subsequent analyses.

3.2 Differentially regulated proteins in P5N-deficient patient A

Patient A has a homozygous frameshift mutation in the *NT5C3A* gene (c.577delG), resulting in a shorter P5N protein of 206 amino acids, while the wild-type protein consists of 297 amino acids. From the differential proteome analysis of patient A, it was shown that P5N was downregulated up to 27.9 times ($p = 1.28 \times 10^{-4}$) as compared to the HCs (Supporting information Table 2). Proteome analysis further

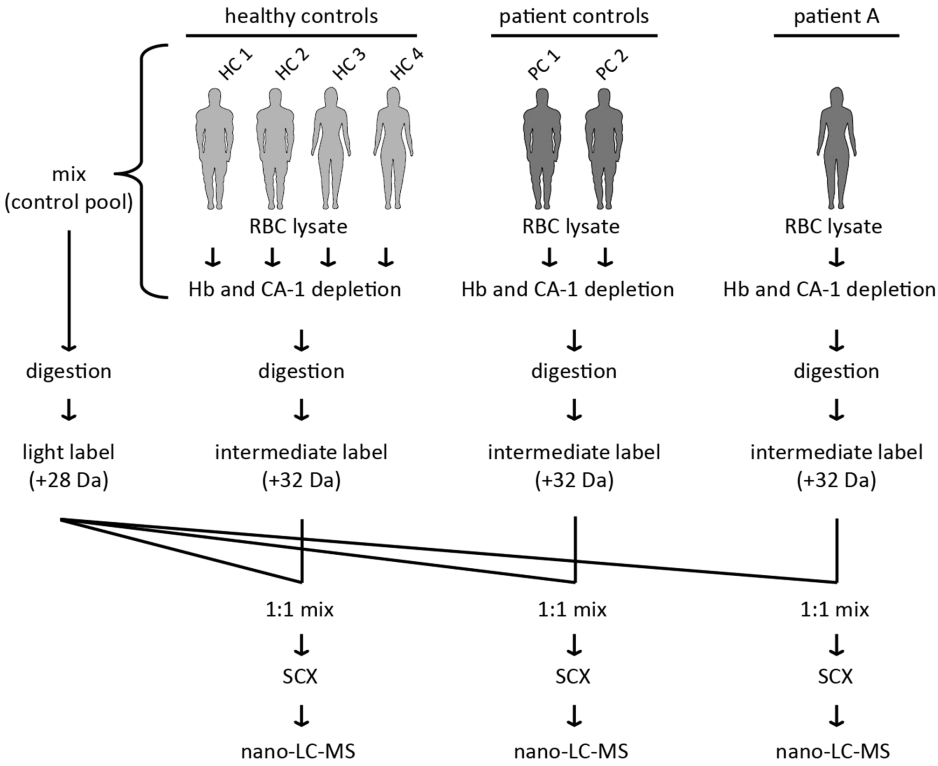

Figure 2. An overview of experimental design. RBC cytosolic samples were prepared from a set consisting of one pyrimidine 5′-nucleotidase (P5N) deficient patient (hemolytic anemia (HA) patient A), two patient controls (PCs; HA patients PC1 and PC2; these patients are *not* P5N-deficient), and four healthy controls (HCs; HC1–HC4). These seven samples were doubly depleted for hemoglobin (Hb) and carbonic anhydrase 1 (CA-1), protease digested, and thereafter chemically labeled with dimethyl groups that were labeled with stable isotopes (CD_3). As internal standard, a mixture of the four HCs was prepared (1:1:1:1 mix of HC1, HC2, HC3, and HC4), and labeled with a different isotope (CH_3). Patient or control samples were subsequently combined 1:1 with the internal standard, based on the peptide content. These seven mixtures were each subjected to SCX separation, followed by quantitative nano-LC-MS/MS analysis.

confirmed that patient A P5N was indeed shorter than the wild-type protein. Namely, the four peptides in the region of amino acid 206–297, which were detected in the control samples, were not detected in patient A samples.

In total, 303 proteins were found to be significantly regulated in patient A ($p \leq 0.05$) as compared to the HCs, of which 29 are involved in one or two key metabolic and functional pathways of the RBC (Supporting information Table 2). These pathways are the PPP, Embden–Meyerhof pathway, and three metabolism pathways for purine/pyrimidine, pyruvate, and glutathione. Some of the regulated proteins in patient A were found to belong to pathways such as ubiquitin-mediated proteolysis, or other proteasome-related pathways (Supporting information Table 2). Importantly, it can be concluded that many of the 303 significantly regulated proteins were regulated to the same extent for patient A as for the control patients; PC1 and PC2 (Supporting information Table 2). This indicates that regulation is due to the high reticulocyte count of patient A, rather than the P5N deficiency. For example, upregulation of proteins such as pyruvate kinase, a highly age-dependent RBC enzyme, occurs in patients A, PC1, and PC2 to similar extent (2.3-fold up, 2.6-fold up, and 2.7-fold up, respectively). This confirms a direct correlation to an elevated reticulocyte count and the relatively young age of RBCs in HA patients.

Twelve RBC proteins of patient A showed markedly differentially expressed levels compared to both HC samples (HC1–HC4) and PC samples (PC1, PC2) (Supporting information Table 2; Fig. 3). Among these, as expected, P5N (5NT3) was regulated. For ten of the other regulated proteins, the relation to P5N deficiency or altered functioning of the RBC is not directly clear. These are the strongly upregulated proteins, 14-3-3 protein eta (1433F), which act as an adapter protein and have been implicated in the regulation of a large spectrum of both general and specialized signaling pathways [23, 24], and peptidyl-prolyl cis-trans isomerase (FKBP3) that enhances the folding of proteins [25, 26]. The rest of the strongly regulated proteins in the RBCs of patient A is downregulated (Fig. 3). Aldo-keto reductase (AK1C3) catalyzes the conversion of aldehydes and ketones to alcohols. The GTP cyclohydrolase 1 (GFRP) feedback regulatory protein mediates its inhibition by tetrahydrobiopterin, which is reversed by L-phenylal [27, 28]. N-acetylneuraminate lyase (NPL) is a protein that catalyzes the cleavage of sialic acid to form

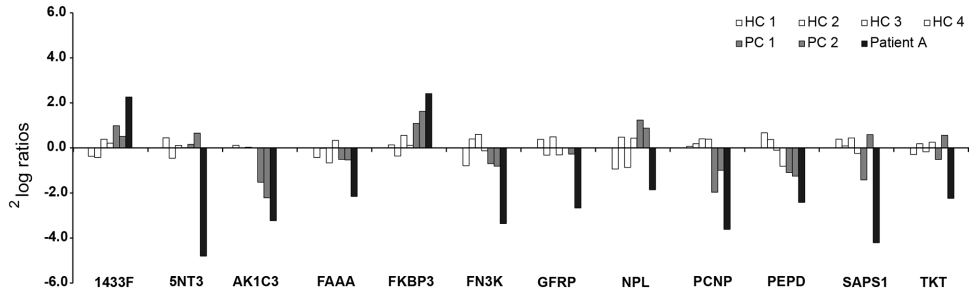

Figure 3. Proteins found to be differentially regulated in the cytosolic 5′-deficient patient A compared to both healthy controls (HCs; HC1–HC4) and patient controls (PCs; PC1 and PC2). A total of 12 proteins were found to be regulated, among which pyrimidine 5′-nucleotidase (5NT3) and transketolase (TKT) were both downregulated. Only two proteins were upregulated, 14-3-3 protein eta (1433F) and peptidyl-prolyl cis-trans isomerase (FKBP3). The other downregulated proteins are aldo-keto reductase (AK1C3), fumarylacetoacetase (FAAA), fructosamine-3-kinase (FN3K), GTP cyclohydrolase 1 (GFRP), N-acetylneuraminate lyase (NPL), PEST proteolytic signal-containing nuclear protein (PCNP), Xaa-Pro dipeptidase (PEPD), serine/threonine-protein phosphatase 6 regulatory subunit 1 (SAPS1). For further details, see Supporting information Table 2.

pyruvate and N-acetylmannosamine via a Schiff base intermediate. It prevents sialic acids from being recycled and returning to the cell surface. The PEST proteolytic signal-containing nuclear protein (PCNP) is possibly involved in cell-cycle regulation [29]. Serine/threonine protein phosphatase 6 regulatory subunit 1 (SAPS1) is the regulatory subunit of protein phosphatase 6. Fumarylacetoacetase (FAAA) is an enzyme involved in the catabolism of amino acids, it catalyzes the reaction of 4-fumarylacetoacetate to acetoacetate and fumarate. Fructosamine-3-kinase (FN3K) is a protein that is known to be present in RBCs, although its function within these cells is not well understood. The protein may have a role in deglycation of fructoselysine and other glycated proteins, and also in the phosphorylation of glycated lysozyme, fructoselysine, fructoseglycine, 1-deoxy-1-morpholinofructose, and fructose [30, 31]. Xaa-Pro dipeptidase (PEPD) is a protein that splits dipeptides with a prolyl or hydroxyprolyl residue at the C-terminal position, which plays an important role in collagen metabolism.

The 12th protein presented in Fig. 3, TKT, was found to be downregulated about 4.7-fold ($p = 4.61 \times 10^{-4}$), and this enzyme plays an important role in the nonoxidative phase of the PPP, one of the critical pathways in the RBC (shown in Figs. 1 and 4). Three other enzymes in the oxidative phase of the PPP were present at a higher level, that is, 6-phosphogluconolactonase, 6-phosphogluconate dehydrogenase, and ribose-5-phosphate isomerase. These enzymes, however, showed a similar pattern for the PC samples, indicating that upregulation is associated with the elevated reticulocyte count in all these patients.

3.3 Validation of differential expression of TKT

Downregulation of TKT for patient A was confirmed by immunochemical detection of TKT. To establish if depletion had affected our results, Western blotting was performed on both doubly depleted and nondepleted RBC lysates. As can be concluded from Fig. 5, also this immunoblotting method revealed significant downregulation of TKT in patient A. Moreover, comparison of the extent of TKT downregulation from three instances—LC-MS/MS, doubly depleted sample, and nondepleted lysate—suggests consistency in the observed downregulation across multiple sample preparation and sample-handling procedures (Fig. 5).

To extend our findings concerning P5N deficiency, a comparison of patient A to three other patients known to be affected by P5N deficiency was performed. Each of these patients showed other, unique mutations in the *NT5C3A* gene compared to patient A (patients B, C, and D; Supporting information Table 1). Figure 6 depicts that significant downregulation of TKT to varying degrees was observed across all patients compared to HCs. These findings strongly suggest that TKT is a potential biomarker for P5N deficiency, which can be easily detected by Western blotting.

4 Discussion

Our quantitative proteome analysis was aimed at finding novel biomarkers for P5N patients. Our validation experiments showed that TKT was found to be concomitantly downregulated as a result of P5N deficiency in these patients, regardless of the origin of the mutation in the *NT5C3A* gene. Moreover, ten other regulated proteins were detected, of which the association with functioning of the RBC is not immediately clear.

The question raised was if P5N itself could act as a biomarker for P5N patients. The protein was strongly downregulated in patient A, while we could not detect the four peptides of the amino acid region 206–297 by LC-MS, suggesting detection of a truncated protein. To date, approximately 100 patients are known worldwide, who are mostly unrelated cases. Therefore, most of the known mutations of the NT5C3A gene are different, that is, homozygous versus heterozygous (some are compound heterozygous), and

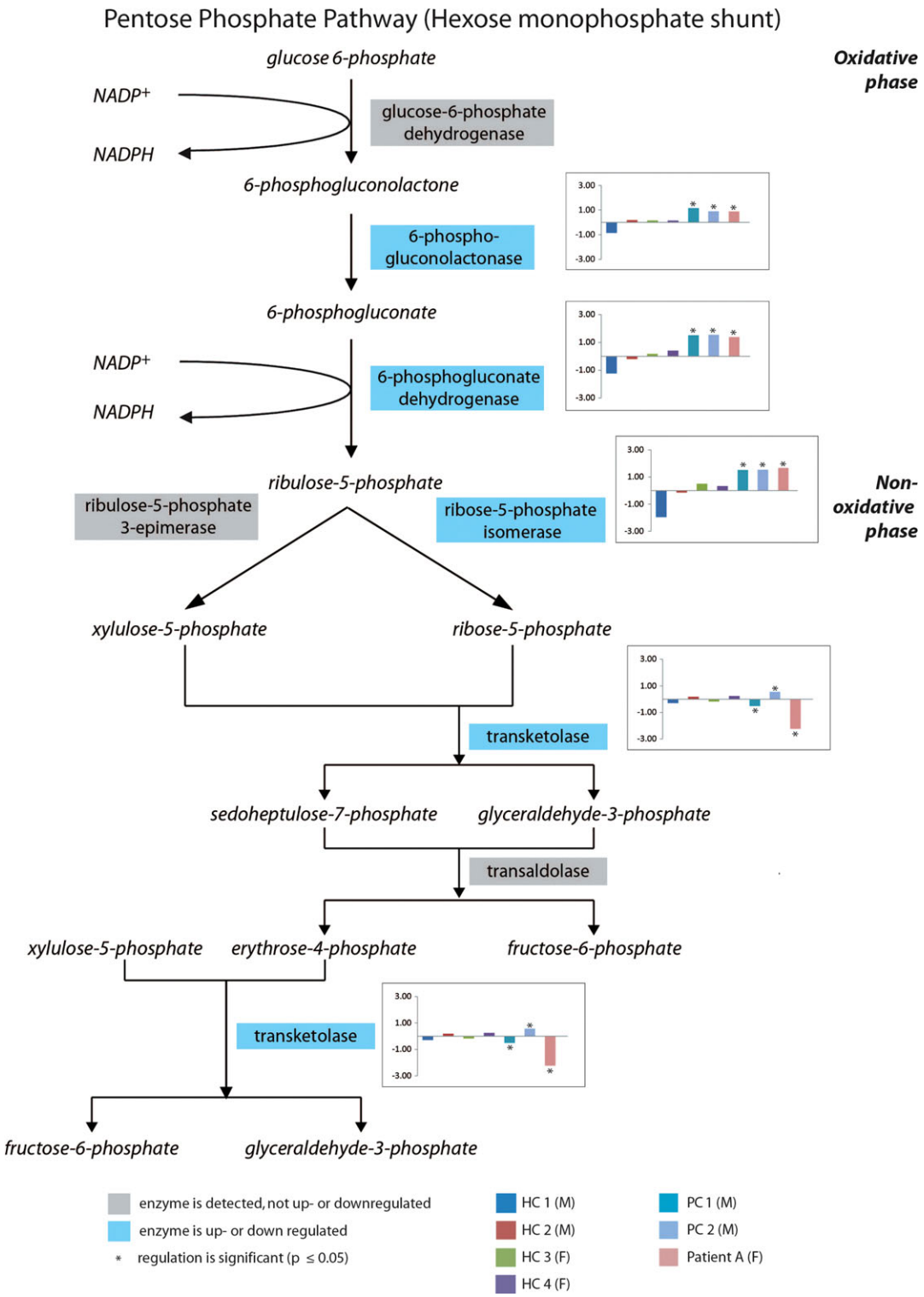

Figure 4. Schematic representation of the pentose phosphate pathway (PPP). The relative enzyme expression levels for healthy controls, patient controls (PCs), and patient A are shown. Enzyme expression levels are indicated on the *y*-axis as 2log-transformed values. PC1, PC2 and patient A expression levels, which were found to be significantly changed (one-sample, two-tailed Student's *t*-test, $p \leq 0.05$), are indicated with an asterisk on top of the bars. PPP enzymes are indicated within gray boxes if not significantly regulated, and within blue boxes if the enzyme is significantly up- or downregulated for at least one of the patient samples. Transketolase, which is involved in the nonoxidative phase of the PPP, was found to be significantly downregulated in patient A (4.7-fold, $p = 0.0005$).

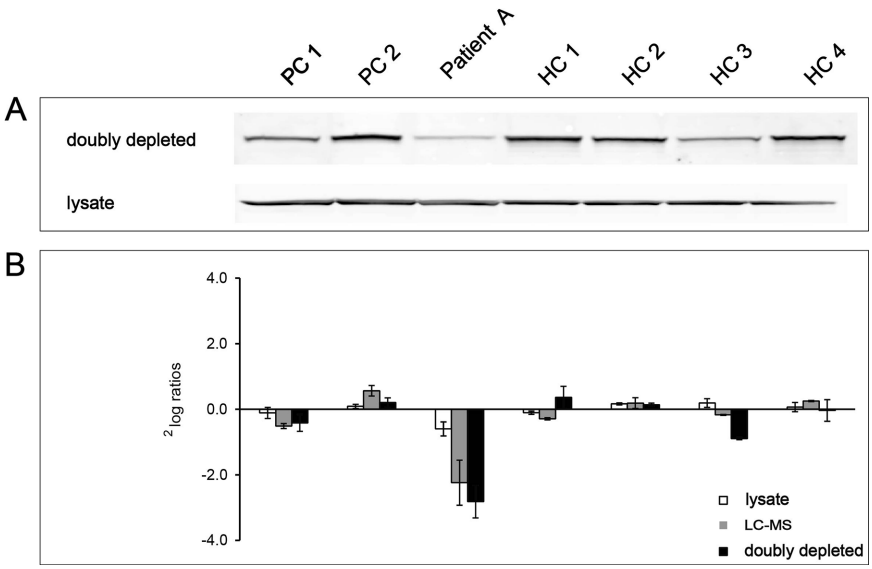

Figure 5. Validation of quantitative MS results of transketolase (TKT) downregulation in patient A using Western blot analysis. Panel A shows a typical example of Western blot of samples depleted for both hemoglobin (Hb) and carbonic anhydrase 1 (CA-1) (top; "doubly depleted"), and from analyzing RBC lysates prior to depletion (bottom, "lysate"). Panel B shows 2log-transformed ratios of TKT levels of patient A relative to the average of the HC1–HC4 levels (internal standard). Ratios are shown from RBC lysates (white bars), TKT peptides through nano-LC-MS/MS (gray bars), and from samples depleted of both Hb and CA-1 (black bars). Fluorescent band intensities of the Western blots were first normalized against total protein load band densities from temporary Ponceau-S staining, to account for differences in total protein content, prior to relative quantification as visualized in panel B. Error bars in panel B originate from multiple analyses, that is, three technical replicates for the Western blots. For the LC-MS analysis, more than one peptide was quantified for each of the samples (Supporting information Table 2B). The average ratio was calculated, and the error bars indicate the SD. In all three instances (panel B), TKT levels were found to be downregulated in patient A relative to both healthy controls and patient controls.

nonsense or missense mutations. While nonsense mutations often give rise to enhanced degradation of the corresponding mRNA and protein [32], it cannot always be predicted if a particular mutation of the *NT5C3A* gene will lead to upregulation or downregulation of the P5N protein. Furthermore, detection of mutations in the P5N protein by MS is not straightforward for the diagnosis of new congenital HA patients. In general, less than 100% of the peptides of any protein is being detected by MS, therefore "not detecting peptides" cannot be used as evidence for the presence of a truncated protein. Moreover, an amino acid mutation can only be identified by LC-MS if the mutated protein is added to the database that is being used for database searching. Therefore, yet unknown mutations in the P5N protein can easily be overlooked using LC-MS. On the other hand, immunochemical detection may need the development of more than one antibody for P5N, as it cannot be predicted if the epitope will be affected by a particular mutation. Genome sequencing of these HA patients seems the routine approach of the near future, however it can never be predicted if a newly detected mutation in the *NT5C3A* gene will give rise to a nonfunctional P5N protein. With our current work, we showed that TKT is a highly potential biomarker for four patients with unrelated mutations of the *NT5C3A* gene. To date, immunochemical detection is rather routine for hospital laboratories that accomplish patient diagnostics. The value of TKT as a biomarker may well be confirmed for other P5N patients, although this is a rare disease.

What is the possible effect of mutations causing P5N deficiency? Nucleoside-5′-phosphates are basically produced by two pathways, that is, the salvage pathway and de novo synthesis pathway. Synthesis of a mole of pyrimidines costs a single mole of ATP through the salvage pathway, which is very cost-efficient, as it costs five moles of ATP through the de novo synthesis pathway. In the pyrimidine salvage pathway, RNA is first degraded into nucleosides and free bases. The nucleosides are converted back to nucleotide monophosphates, which can subsequently be interconverted into the pathways of pyrimidine biosynthesis. P5N, encoded by the *NT5C3A* gene, is the first degradative enzyme of the pyrimidine salvage pathway.

Reticulocytes exhibit very high P5N activity that declines rapidly in young RBCs, while matured RBCs show very low P5N activity [33,34]. Mutations in the *NT5C3A* gene can result in the subsequent accumulation of pyrimidine nucleotides in the RBC, which has been previously described to adversely affect key metabolic pathways [35].

One of these key pathways is the PPP, which is vital for the production of (1) NADPH in its oxidative phase, and of (2) ribose-5-phosphate in its nonoxidative phase (shown in Figs. 1 and 3). Proteins in RBCs are more than in other body cells being challenged with oxidation, therefore NADPH

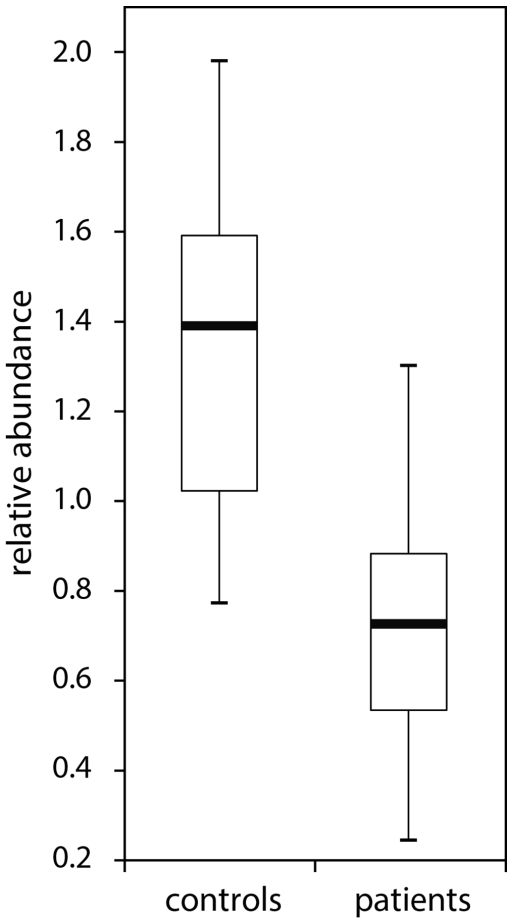

Figure 6. Box plot depicting the validation result of transketolase downregulation in pyrimidine 5′-nucleotidase (P5N) patients using Western blot analysis. Quantification values for healthy control (HC) samples ($n = 5$) and P5N-deficient patients ($n = 4$) are expressed as relative abundances calculated from intensities of fluorescent bands after normalization. Band intensities were first normalized against total protein load band densities from temporary Ponceau-S staining, before relative quantification. Transketolase was found to be significantly downregulated ($p = 1.36 \times 10^{-9}$) in all P5N-deficient patients (median abundance = 0.72) compared to the HC group (median abundance = 1.39).

production is extremely important for these cells as an input for the glutathione metabolism pathway (Fig. 1). This may be even more prominent in case of HA in general, since our results indicate that most enzymes in the oxidative phase of the PPP, that is, 6-phosphogluconolactonase, 6-phosphogluconate dehydrogenase, and ribose-5-phosphate isomerase are significantly upregulated in all HA patients (Fig. 4). Uniquely for patient A with P5N deficiency, the TKT levels were found to be strongly reduced (Fig. 3; Supporting information Table 2). TKT is an important enzyme [36], related to a number of diseases, for example, cancer [37], diabetes [38], and Alzheimer disease [39]. Kim et al. [40] studied the effect of knocking down TKT in meiotic cell cycle oocytes, and found no significant effect on transcript levels of the PPP enzymes, but found reduced transcript levels for ribokinase and the phosphoribosyl pyrophosphate synthetase 1 enzyme that convert ribose-5-phosphate into D-ribose and 5-phosphoribosyl-1-pyrophosphate, respectively. In that study, TKT was knocked down, and thus it was a primary effect [40]. In our study, P5N deficiency was the primary effect, while downregulation of TKT levels seemed a secondary effect, which may originate from distorted pathways in erythroid progenitor cells during haemopoiesis. Like all cells, also erythroid progenitor cells use ribose-5-phosphate as an input for the synthesis of purine and pyrimidine nucleotides (Fig. 1), which are subsequently being incorporated into RNA molecules. However, in case of P5N deficiency, pyrimidine nucleosides cannot be formed from pyrimidine nucleotides, and therefore synthesis of ribose-5-phosphate may be upregulated in the PPP as counterbalance. If ribose-5-phosphate is continuously being used as an input for RNA biosynthesis, this may explain that the next steps in the PPP have been downregulated, in particular the steps regulated by TKT (Fig. 4). Downregulated TKT levels in P5N patients therefore likely originate from response to P5N deficiency in erythroid progenitor cells. However, as soon as TKT is being downregulated in the progenitor cells, their level remains low in the RBCs due to lack of novel protein synthesis. Future in-depth experiments that provide deeper insight into regulation of all different steps of the PPP will allow testing this hypothesis.

Diagnosis of P5N deficiency is not always straightforward. Erythroid progenitor cells mature into reticulocytes and eventually into RBCs. Appropriate activity of P5N is highly important in this maturation process, since the nucleosides can diffuse out of the cells, while the negatively charged nucleotides cannot. Therefore, as a result of the P5N deficiency, pyrimidine nucleotides accumulate in the RBCs. This is visible by microscope as basophilic stippling, being an important morphological hallmark. However, basophilic stippling is not specific for P5N deficiency only, since it can also be observed in other congenital or acquired disorders such as lead poisoning, β-thalassemia trait, and sideroblastic anemia. For example in lead poisoning, chelation to lead ions inhibits P5N activity [41–43]. The specific diagnosis is currently made on the basis of demonstrating high concentration of pyrimidine nucleotides from UV absorption screening of deproteinized extracts at 260 and 280 nm [44], and by measuring reduced P5N enzyme activity in RBCs. As reviewed by others [45–47], several procedures exist for measuring P5N activity, however, these are arguably cumbersome and could lead to false-negative diagnosis, especially in cases where high residual enzyme activity is observed due to reticulocytosis.

An advantage of our findings is that the measurement of TKT expression levels by immunodetection or targeted MS would ease the process of diagnosing P5N deficiency in HA patients. This may serve as a preliminary screening and can be performed prior to the more laborious molecular characterization approaches [12, 46–48].

5 Conclusion

Accurate and timely diagnosis of RBC enzymopathies causing HNSHA remains a challenge. It is essential and at times crucial to determine possible therapeutic measures in managing the disease. For pyrimidine 5′-nucleotidase patients, basophilic stippling remains an important though nonspecific indicator of the deficiency. Here, we have demonstrated how quantitative proteomics is applicable in confirming divergent pyrimidine 5′-nucleotidase expression levels. We have further identified TKT as an RBC PPP enzyme of which the reduced expression levels correlate with pyrimidine 5′-nucleotidase deficiency, regardless of the specific underlying mutation in the *NT5C3A* gene. This opens new opportunities for an improved insight into the pathophysiology of P5N deficiencies, and possibly into the pathogenesis of hemolysis.

Additional research, such as metabolomics experiments to study changes in RBC metabolite levels and metabolic fluxes, would be required to fully unravel the mechanisms and biology behind disruptions in the PPP as a result of P5N deficiency. This may eventually lead to novel therapies for this group of patients.

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