

High urinary ferritin reflects myoglobin iron evacuation in DMD patients

Jérémy Rouillon^{a,*}, Thibaud Lefebvre^{b,c}, Jérôme Denard^a, Vincent Puy^{d,e}, Raed Daher^{b,c}, Jérôme Ausseil^{d,e}, Aleksandar Zocevic^a, Paul Fogel^f, Katell Peoc'h^b, Brenda Wong^g, Laurent Servais^h, Thomas Voitⁱ, Herve Puy^{b,c}, Zoubida Karim^{b,c}, Fedor Svinartchouk^{a,**}

^a Généthon, Evry, France

^b INSERM U1149 CNRS ERL 8252, Centre de Recherche sur l'inflammation, Université Paris Diderot, site Bichat, Sorbonne Paris Cité, France, 16 rue Henri Huchard, 75018 Paris, France

^c Laboratory of excellence, GR-Ex, Paris, France

^d Unité INSERM U1088, CURS-Université de Picardie Jules Verne, Amiens, France

^e Laboratoire de Biochimie, CHU Amiens, F-80054 Amiens, France

^f Statistical consulting, Paris, France

^g Division of Pediatric Neurology, Cincinnati Children's Hospital Medical Center, USA

^h Service of Clinical Trials and Databases, Institut de Myologie, Paris, France

ⁱ University College London, NIHR Biomedical Research Centre, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK

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Abstract

Duchenne muscular dystrophy (DMD) is an X-linked disease caused by mutations in the dystrophin gene leading to the absence of the normal dystrophin protein. The efforts of many laboratories brought new treatments of DMD to the reality, but ongoing and forthcoming clinical trials suffer from absence of valuable biomarkers permitting to follow the outcome of the treatment day by day and to adjust the treatment if needed. In the present study the levels of 128 urinary proteins including growth factors, cytokines and chemokines were compared in urine of DMD patients and age related control subjects by antibody array approach. Surprisingly, statistically significant difference was observed only for urinary ferritin whose level was 50 times higher in young DMD patients. To explain the observed high urinary ferritin content we analysed the levels of iron, iron containing proteins and proteins involved in regulation of iron metabolism in serum and urine of DMD patients and their age-matched healthy controls. Obtained data strongly suggest that elevated level of urinary ferritin is functionally linked to the renal management of myoglobin iron derived from leaky muscles of DMD patients. This first observation of the high level of ferritin in urine of DMD patients permits to consider this protein as a new urinary biomarker in muscular dystrophies and sheds light on the mechanisms of iron metabolism and kidney functioning in DMD.

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1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked disease caused by mutations in the dystrophin gene leading to

the absence of the normal dystrophin protein. The disease has an incidence of 1/4000–1/5000 male newborns and is caused by a strong destabilisation of the dystrophin associated protein complex at the sarcolemma [1] leading to chronic inflammation, progressive increase of muscle fibrosis and degeneration [2]. DMD patients become wheelchair bound around the age of 12–14 [3] and die in their third or fourth decade due to cardiorespiratory failure [4,5]. The efforts of many laboratories brought new treatments of DMD to the reality, but ongoing and forthcoming clinical trials suffer from absence of valuable biomarkers permitting to follow the treatment results

* Corresponding author. Généthon, 1 bis rue de l'Internationale, 91002, Evry, France.

** Corresponding author. SQY Therapeutics, UVSQ, UFR des sciences de la santé, 2 Avenue de la source de la Bièvre, 78180 Montigny-le-Bretonneux, France.

E-mail addresses: jrouillon@genethon.fr (J. Rouillon), fedor.svinartchouk@uvsq.fr (F. Svinartchouk).

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day by day and to correct the treatment if needed. Human urine is one of the major body fluids with relatively stable protein composition, which can be obtained in large quantities using non-invasive procedures [6]. It is considered as an attractive source of biomarkers [7,8] and several approaches such as 2-DE, MALDI, LC-MS and SELDI have been used to characterise the human urinary proteome [9–11]. Recently, by using a mass spectrometry approach, we identified fragments of titin as urinary biomarkers offering the possibility to develop a simple, non-invasive and easy-to-use test for the pre-screening of muscular dystrophies including DMD [12].

In the present study, we used an antibody array approach to prospectively evaluate urine levels of chemokines, cytokines and growth factors in urine from DMD patients and healthy controls. From 274 proteins available for the analysis on the arrays, 128 urinary proteins passed the threshold of assay sensitivity. Surprisingly, statistically significant difference was observed in the level of only one protein, ferritin, which was highly increased in DMD patients compared to healthy controls.

Urinary level of ferritin is extremely low in healthy individuals [13,14]. High level of urinary ferritin was previously described in patients with chronic haemolytic anaemia as a consequence of glomerular filtration of haem-iron from free haemoglobin [14,15]. In these patients, the free haemoglobin is actively filtered and catabolised by the kidney thus releasing iron. Liberated iron is then incorporated into the ferritin storage compartment and removed from the body when tubular epithelium is shed into the urine [15]. In contrast to urinary ferritin, the serum ferritin level is widely used as an indicator of iron status and its presence in the blood is mainly attributed to a leakage from damaged cells [16].

To explain the observed high urinary ferritin content we analysed the levels of iron, iron containing proteins and proteins involved in regulation of iron metabolism in serum and urine of DMD patients and their age-matched healthy controls. Obtained data strongly suggest that elevated level of urinary ferritin is functionally linked to the renal management of myoglobin iron derived from leaky muscles of DMD patients. This first observation of the high level of ferritin in urine of DMD patients sheds light on the mechanisms of iron metabolism and kidney functioning in DMD.

2. Materials and methods

2.1. Human sample collection

The study was conducted as part of ADNA (Avancées Diagnostiques pour de Nouvelles Approches thérapeutiques) project according to the principles of the declaration of Helsinki “Ethical Principles for Medical Research Involving Human Subjects”. The study protocol and informed consent were approved by the Institutional Review Board (IRB) and informed consent was obtained from all subjects prior to the study.

Samples from DMD patients and from age- and sex-matched healthy control subjects were collected at the Cincin-

nati Children’s Hospital Medical Center, USA. For all patients, DMD was diagnosed on the basis of genetic testing (Supplementary Table S1). Subjects with a known medical disorder, mental retardation or autistic/pervasive developmental disorders as well as intercurrent illness (infections, cold or fever) or elevated CRPs were not included in the study.

Blood of DMD patients and healthy controls was collected in dry tubes (BD Vacutainer, #367815) and samples were allowed to clot at room temperature for 20 min. After 10 min of centrifugation at 2000 g, serum was collected and stored at -80°C . Urine samples were centrifuged immediately after collection (1800 g, 10 min, 4°C) and supernatants were stored at -80°C . Before use, urine samples were concentrated 10 times using Amicon Ultra-15 Centrifugal Filter Units (cut-off 3 kDa, Millipore) following the manufacturer’s instructions.

2.2. Protein quantification

Protein concentration was determined using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad) according to the manufacturer’s instructions with bovine serum albumin as a standard.

2.3. Antibody arrays

RayBio® Human Cytokine Antibody Arrays 6, 7, 8, 9 and 10 (AAH-CYT-G4000-6, 7, 8, 9 and 10, RayBiotech) permit the detection of 274 proteins using capture antibodies spotted in duplicate on a glass slide (<https://www.raybiotech.com/g-series-array-list-of-targets>). Each slide contains 8 wells allowing the detection from 50 to 60 proteins in 8 samples. For each urine sample, 30 μg of proteins were incubated with arrayed antibody support followed by protein detection with a cocktail of biotinylated-antibodies and revelation with Cy3-labelled streptavidin. The fluorescent signal was measured using the laser scanner GenePix 4000B (Axon Instruments) at excitation/emission wavelengths of 532/605 nm. Intensity of fluorescent signals was quantified using GenePix pro V6.0 software. Median intensity values for each protein were normalized to positive control present on each well. To reduce variation between glass slides within urine samples, series of slides were processed simultaneously (all urine samples were analysed the same day on a given slide). Only proteins with mean fluorescence intensity ≥ 200 were taken for the analysis. The threshold of 200 increases the probability to detect the respective proteins by the ELISA kits provided by RayBio Company (our unpublished observations).

2.4. ELISA

Urine and serum levels of ferritin in DMD patients and healthy controls were determined by commercial ELISA kits (ELH-Ferritin, RayBiotech). After determination of optimal sample dilutions, all assays were performed according to the manufacturer’s instructions.

2.5. Biochemical and clinical parameters

Levels of serum ferritin, C-reactive protein (CRP), transferrin, soluble transferrin receptor (sTfr), transferrin saturation rate (satTfrR), creatine kinase (CK), hemopexin, haptoglobin, serum myoglobin, urine ferritin, and urine protein concentration, were determined in a dedicated clinical laboratory (Department of Clinical Biochemistry, Bichat University Hospital, Paris, France) using Dimension Vista, Immulite and BN ProSpec® automates following manufacturer instructions (Siemens Saint Denis Cedex, France). Urine myoglobin was analysed by a chemiluminescent microparticle immunoassay (CMIA) iStat Myoglobin (Abbott) after an initial screening using dip-and-read test strips for haemoproteins.

2.5.1. Liquid chromatography-tandem mass spectrometry assay of hepcidin levels

Hepcidin levels were measured by a liquid chromatography-tandem mass spectrometry method [17,18] after solid phase extraction of serum samples.

2.6. Determination of serum and urinary iron by inductively coupled plasma mass spectrometry (ICP-MS)

In serum total iron was determined by an assay developed by Smith et al [19] adopted for automatic measurement on Dimension Vista analyser system (Siemens).

Urinary iron was quantified by inductively coupled plasma mass spectrometry (ICP-MS). 200 µL of urine stored at –80 °C were used for quantification. Elemental analyses were performed using inductively coupled plasma mass spectrometry (ICP-MS; Varian® 820-MS) with a collision/reaction cell system autosampler (SPS3, Varian®). The system was operated at a radio frequency power of 1550 W with an argon plasma gas flow rate of 15 L/min. Standard solutions for the external calibrations (VWR International, Leuven, Belgium) and internal standards (Yttrium ICP Standard; Merck, Darmstadt, Germany) were prepared freshly before every run in 20% nitric acid. Iron was measured in reaction cell mode using H₂ gas (3.5 mL/min). Data were quantified using a 4-point calibration curve (0; 0.4454; 0.8864; 1.755 µmol/L). Each sample measurement series was established from a dosed addition range with standard controls (ClinCheck 2 urine, Precision Instruments, Munich, Germany) in 1% HNO₃. Data are expressed in µmol/L.

2.7. Evaluation of kidney function

Estimation of glomerular filtration rate (eGFR) was performed based on the serum level of cystatin-C because it is less affected by muscle mass compare to the conventional serum creatinine [20,21]. The eGFR was calculated based on the cystatin-C Filler equation [20], which is the closest estimation of the measured GFR in DMD patients [22]:

$$eGFR = 10^{1.962 + \left(1.123 \times \log\left(\frac{1}{c_{\text{cysC}}}\right)\right)}$$

2.8. Gel filtration chromatography

A pool of three concentrated urines (450 µl) was applied onto a HiPrep 16/60 Sephacryl S-200 HR (GE Healthcare) column equilibrated with PBS. Elution was carried out with PBS at 0.3 mL/min, the collection of 600 µl fractions started 24 mL after the loading of the sample. The protein peaks were monitored by absorption at 280 nm.

2.9. Western blot

Protein samples were separated by SDS-PAGE electrophoresis (4%–12% gradient, NuPAGE Novex Bis-Tris Gel 1.0 mm, Life Technologies) and transferred onto Protran Premium membrane (nitrocellulose, GE Healthcare). Antibodies against Ferritin (1:1000, Genetex: GTX-62019) were used as primary antibodies followed by incubation with the corresponding IRDye-800CW-conjugated antibodies (1:10 000, LI-COR Biosciences) according to the manufacturer's instructions. Infrared fluorescence of the secondary antibodies was read on an Odyssey Imaging System (LI-COR Biosciences). Band intensities were measured by the Odyssey application software (LI-COR Biosciences, Image Studio Lite 4.0 Version).

2.10. Statistical analysis

Statistical analyses were performed using the GraphPad Prism version 6.04. Data are expressed as mean ± SEM if not otherwise specified. For comparisons between means, homogeneity of variances was assessed by Fisher–Snedecor's test and the Student's t-test (two-tailed) was applied. Pearson's correlation was used for correlation studies and data were analysed with a 95% confidence interval and p < 0.05 was considered significant.

3. Results

3.1. Detection of differentially present proteins in urine by antibody array analysis

For this analysis, urine samples from DMD patients and age- and sex-matched healthy control subjects were organized in a total of 12 pools according to the patients' age (Table 1). Each pool included urine from at least 4 individuals where each individual was represented by equal quantity of proteins. Pools were analysed by multiplexed antibody arrays permitting to determine the relative levels of 274 proteins including cytokines, chemokines, growth factor and soluble receptors. Only proteins with mean fluorescence intensity of ≥200 on the array were taken for the analysis. The threshold of 200 increases the probability to detect the respective proteins by the ELISA kits provided by RayBio Company (our unpublished observations). With this criterion 128 from 274 proteins on the antibody arrays were eligible for the analysis (Supplementary Table S2). To find differentially present proteins, obtained

Table 1
Schema of samples assembling into groups and subgroups for the analysis by antibody array approach.

DMD	D1-1	D1-2	D1-3	D1-4	D2-1	D2-2
Age (number)	3–4 (7)	4–6 (11)	6–7 (4)	7–10 (4)	12–16 (6)	16–20 (7)
Healthy controls	C3-1	C3-2	C3-3	C3-4	C4-1	C4-2
Age (number)	3–4 (5)	4–6 (6)	6–7 (5)	7–10 (5)	12–16 (10)	16–20 (7)

DMD D1-1 to D1-4: urine from young DMD patients of 3 to 10 years old; **DMD D2-1 to D2-2:** urine from DMD patients of 12 to 20 years old. Healthy controls **C3-1 to C3-4** and **C4-1 to C4-2:** age-matched healthy controls to the young and older DMD patients respectively. Numbers below each pool indicate the interval of age and the number of patients (in parentheses).

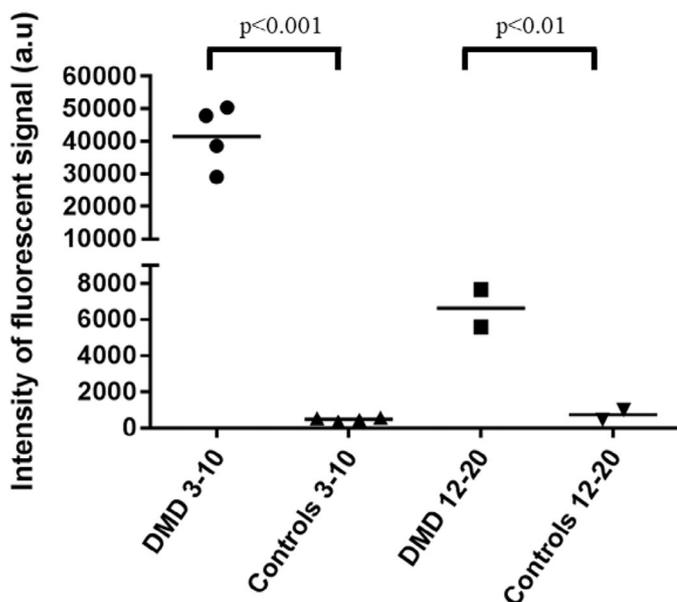


Fig. 1. Analysis of urinary ferritin levels by antibody arrays. The fluorescent signal was measured using the laser scanner GenePix 4000B and intensity of fluorescent signal (a.u.: arbitrary units) was quantified using GenePix pro V6.0 software.

data were analysed with the Bioconductor – DESeq package, version 1.16 [23], a tool routinely used to find differentially expressed genes in high throughput sequencing data. This package is particularly useful to analyse count data from a small number of biological samples. Two sets of analyses were performed: pools corresponding to 3–10 years old DMD patients were compared to their respective age-matched healthy controls and 12–20 years old DMD patients were compared to their respective age-matched healthy controls. The results of differential analyses were filtered by using adjusted p-value (False Discovery Rate) ≤ 0.05 . The two differential analyses resulted in the identification of only one differentially present protein: ferritin. The level of this protein was highly increased in DMD patients (3–10 years: $41,487 \pm 4856$ IFS (intensity of fluorescent signals) for DMD patients versus 477 ± 52 for healthy controls, p-value < 0.001 ; 12–20 years: 6621 ± 1035 IFS for DMD patients versus 731 ± 269 IFS for healthy controls, p-value < 0.01) (Fig. 1).

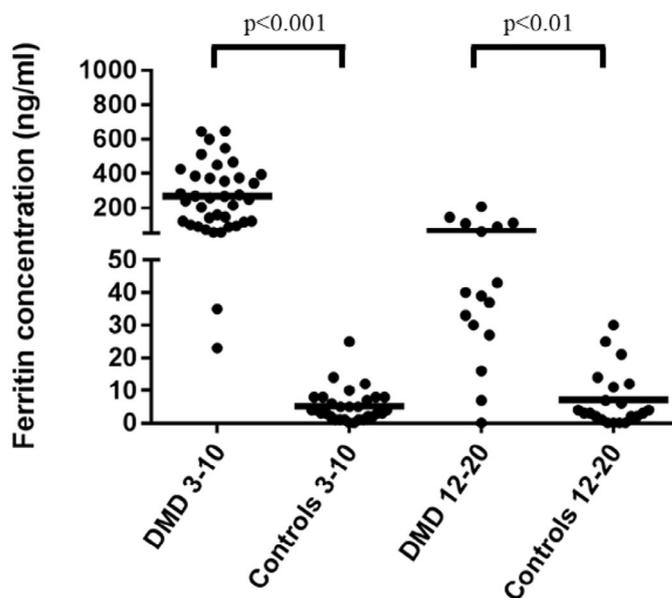


Fig. 2. Individual analysis of urinary ferritin in DMD patients and healthy controls by the ELISA assay. Clinical and biochemical data of DMD patients and respective healthy controls are presented in Table 2.

3.2. Individual analysis of ferritin

In order to confirm the differences in ferritin levels and to estimate its individual variability, urinary ferritin was analysed by ELISA in each subject of the entire cohorts (34 DMD patients from 3 to 10 years old versus 25 age-matched controls; 12 DMD patients from 12 to 20 years old versus 20 age-matched controls). The results of the ELISA assay completely corroborate the data obtained by antibody arrays: ferritin level in young DMD patients was significantly higher than in healthy controls ($251 \text{ ng/mL} \pm 30$ versus $4.5 \text{ ng/mL} \pm 0.7$, p-value < 0.001). Level of urinary ferritin decreased with age of DMD patients remaining 10 times higher than in the healthy controls ($70 \text{ ng/mL} \pm 19$ versus $7.2 \text{ ng/mL} \pm 2$, p-value < 0.01) (Fig. 2).

In order to confirm high ferritin content in urine of DMD patients, we performed urinary ferritin analysis in an independent cohort of 47 DMD patients and 72 healthy control subjects collected in several specialised centres located in France, Belgium, and Romania. Data obtained on this independent cohort (Supplementary Fig. S1) confirmed the high urinary ferritin content observed in patients collected in Cincinnati Children's Hospital Medical Center, USA.

3.3. Urinary ferritin and iron metabolism

Next we asked the question if urinary protein was excreted in the form of ferritin (bound with iron) or in the form of apo-ferritin (not combined with iron). Concentration of urinary iron was significantly higher in young DMD patients compared to age-matched healthy controls ($0.62 \mu\text{mol/L} \pm 0.09$ versus $0.15 \mu\text{mol/L} \pm 0.03$ respectively, p-value < 0.001) and furthermore, urinary iron tightly correlated with urinary fer-

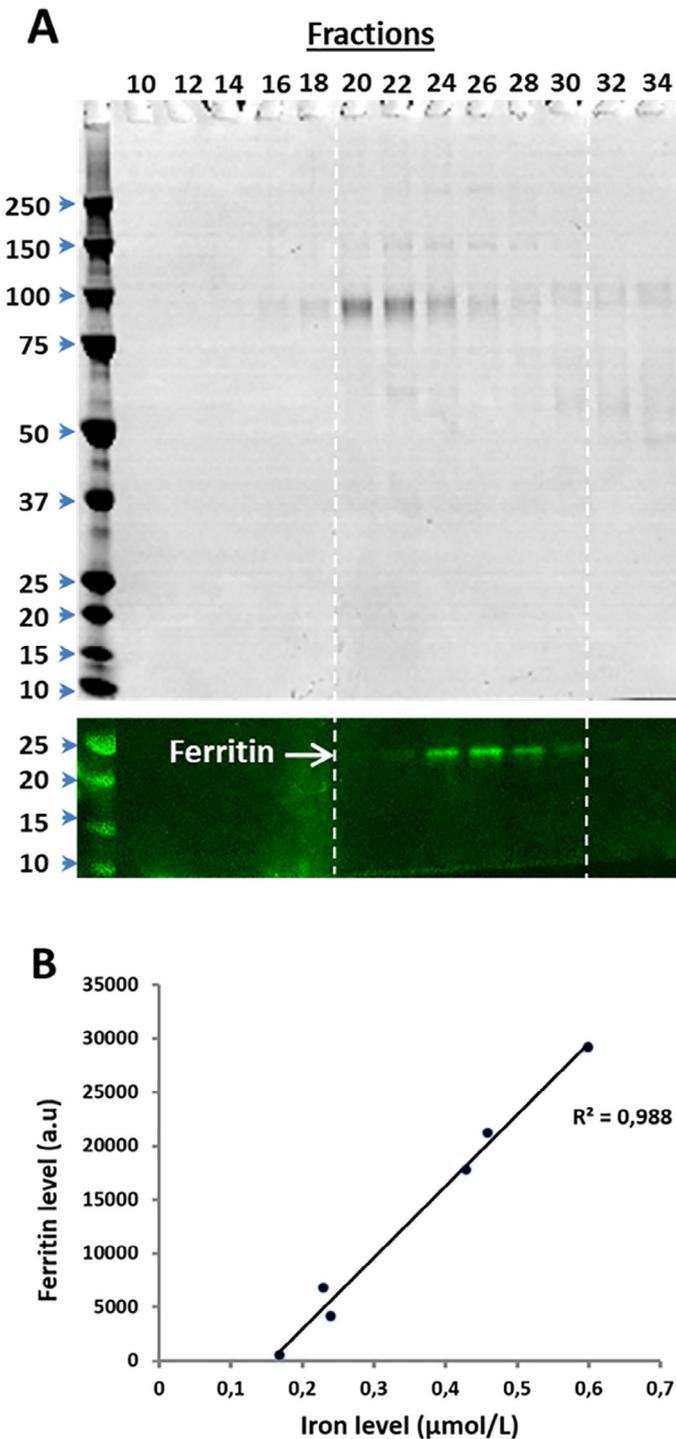


Fig. 3. Co-elution of ferritin and iron after urine fractionation by gel filtration chromatography. A pool of three concentrated urines was analysed by gel filtration chromatography and ferritin and iron levels were quantified by Western blot and Inductively Coupled Plasma Mass Spectrometry respectively in total of 46 fractions (every second fraction obtained after chromatography). Results for the first fractions (fractions 10–34; every second fraction was taken for the analyses) are presented on the figure, while in lower molecular mass fractions no ferritin and only traces of iron were detected. **A.** Upper panel: Coomassie staining of proteins in the first fractions; Lower panel: Western blot analysis of ferritin in the same fractions. **B.** Correlation between iron level and ferritin quantities in fractions 20–30. First line on the left: Molecular mass markers.

ritin ($R=0.76$). To confirm that urinary iron is bound to ferritin, co-elution of ferritin and iron was monitored after urine fractionation by gel filtration chromatography (Fig. 3). All ferritin were eluted in the first fractions corresponding to the high molecular weight proteins thus demonstrating that urinary ferritin is present in a multi-subunit form. The levels of iron in each ferritin containing fraction perfectly correlated with the quantities of ferritin ($R=0.99$, p -value <0.001) indicating that practically all urinary iron were bound to ferritin. Only traces of iron were detected in ferritin-free fractions (mean level: $0.04 \mu\text{mol/L} \pm 0.01$) while in the ferritin containing fractions the mean level of iron was $0.36 \mu\text{mol/L} \pm 0.07$.

To find the origin of urinary ferritin, we quantified its levels in serum. Surprisingly, in young DMD patients, urinary ferritin ($251 \text{ ng/mL} \pm 30$) exceeded nearly 10 times its serum level ($28 \text{ ng/mL} \pm 2.8$) while in healthy individuals concentration of ferritin was higher in serum compared to urine ($28.4 \text{ ng/mL} \pm 2.3$ versus $4.5 \text{ ng/mL} \pm 0.7$). Moreover, despite the significant increase of urinary ferritin in DMD patients, serum parameters reflecting iron status: serum ferritin, serum iron, serum transferrin, transferrin saturation, soluble transferrin receptor, serum hepcidin and total iron binding capacity were within the normal reference ranges (Table 2).

3.4. Origin of urinary ferritin and iron in DMD patients

Increased urinary iron and ferritin were already described in haemolytic anaemia and in animal models of haem-induced injury as a consequence of glomerular filtration of haem-iron from free haemoglobin. In such cases, haemolytic syndromes were associated with a dysregulation of iron metabolism [15,24]. However, no significant difference was observed in parameters reflecting either iron metabolism or haemolysis between DMD patients and healthy controls in the study cohort (Table 2). Serum haptoglobin and hemopexin were within the normal range. These results prompted us to hypothesise that another haem-iron carrier protein, myoglobin, is the source of increased urinary iron and ferritin levels in DMD patients. Indeed, in accordance with previously published data [25,26], serum level of myoglobin was significantly higher in young DMD patients compared to respective healthy controls ($2097 \mu\text{g/L} \pm 236$ versus $27 \mu\text{g/L} \pm 2$ respectively, p -value <0.001). In DMD patients myoglobin level decreased with age but remained higher than in respective healthy individuals ($456 \mu\text{g/L} \pm 136$ and $61 \mu\text{g/L} \pm 4$ respectively, p -value = 0.01). Importantly, myoglobin was detected neither in urine of DMD patients nor in healthy controls suggesting a mechanism of myoglobin re-absorption. Because serum myoglobin significantly correlated with urinary ferritin ($R=0.43$, p -value <0.01 ; Supplementary Fig. S2A) and urinary iron ($R=0.37$, p -value <0.05 ; Supplementary Fig. S2B) in all studied DMD patients, we suggest that metabolism of serum myoglobin plays a crucial role in increased urinary iron and ferritin levels in DMD patients.

Table 2

Clinical and biochemical data of young and older DMD patients and respective healthy controls.

Fluid	Group	DMD 3–10 (n = 34)	DMD 12–20 (n = 12)	Control 3–10 (n = 25)	Control 12–20 (n = 20)	Normal range
	Age (years)	5.3 ± 0.3	16.1 ± 0.7	6.7 ± 0.3	16 ± 1.4	n.a
	Height (cm)	107.8 ± 1.7	159.9 ± 3.2	n.a	n.a	n.a
	Weight (kg)	19.6 ± 0.7	47.2 ± 5.1	n.a	n.a	n.a
Serum	CK (UI/L)	25,685 ± 2129	2433 ± 1385	144. ± 16	231 ± 53	24–320
	Ferritin (ng/mL)	28 ± 2.8	102.4 ± 18.9	28.4 ± 2.3	48.4 ± 6	10–300
	Hepcidin (ng/mL)	3.8 ± 0.7	6.5 ± 1.5	6.7 ± 1.3	5.8 ± 1.9	1–20
	CRP (mg/L)	2.1 ± 0.1	3 ± 0.4	1.8 ± 0.1	2.6 ± 0.4	0–6
	Iron (μmol/L)	12 ± 0.7	13.1 ± 1.4	14.6 ± 1.4	18.9 ± 2.2	11.6–35
	Transferrin (g/L)	2.9 ± 0.1	2.8 ± 0.1	2.6 ± 0.1	2.7 ± 0.1	1.9–3.6
	TIBC (μmol/L)	71.9 ± 1.7	71.2 ± 2.2	66 ± 1.3	67.7 ± 1.9	40–85
	Transferrin saturation (%)	16.9 ± 0.9	18.9 ± 2.4	22.1 ± 2.1	28.9 ± 3.7	16–50
	sTfR (mg/L)	1.5 ± 0.1	1.2 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	0.8–1.7
	Haptoglobin (g/dL)	1.1 ± 0.04	1.1 ± 0.05	1 ± 0.04	1 ± 0.04	0.3–2
	Hemopexin (g/L)	0.8 ± 0.04	0.8 ± 0.04	0.8 ± 0.04	0.7 ± 0.05	0.4–1.2
Myoglobin (μg/L)	2097 ± 232	456 ± 136	27.1 ± 2	34.6 ± 4.1	10–100	
Urine	Ferritin (ng/mL)	251 ± 30	70 ± 19	4.5 ± 0.7	7.2 ± 2	1–16
	Protein Concentration (μg/mL)	91.3 ± 9.5	101.3 ± 10.3	77.9 ± 6.5	115.9 ± 17.8	0–200
	Urine iron (μmol/L)	0.62 ± 0.09	0.25 ± 0.08	0.15 ± 0.03	0.12 ± 0.01	n.a
	Myoglobin (μg/L)	<1	<1	<1	<1	<1

Abbreviations: CK: creatine kinase, CRP: C-reactive protein, sTfR: soluble transferrin receptor, TIBC: total iron binding capacity, n.a: not available. Values in bold are above the upper limit of the normal range values.

Table 3

Evaluation of kidney function in DMD patients and healthy controls.

Group	Mean eGFR ± SEM	p-value
DMD 3–10	136.2 mL/min/1.73 m ² ± 3.9	p-value = 0.90
Control 3–10	135.2 mL/min/1.73 m ² ± 6.7	
DMD 12–20	147.5 mL/min/1.73 m ² ± 5.4	p-value = 0.01
Control 12–20	121.2 mL/min/1.73 m ² ± 8.0	

3.5. Evaluation of renal function in DMD patients

Lifelong arrival of myoglobin to the renal cells produces chronic excess of haem-iron which could be deleterious for kidney functions. To assess renal function in DMD patients, we estimated the glomerular filtration rate (eGFR) based on the serum level of cystatin-C. Cystatin-C was chosen because its level is less affected by muscle mass compared to conventional serum creatinine [21]. The eGFR was calculated based on the cystatin-C Filler equation [20], which is the closest estimation of the measured GFR in DMD patients [22]. In young DMD patients eGFR was not significantly different from respective healthy controls (Table 3). In accordance with published data [7], eGFR decreased in older healthy controls. Intriguingly, eGFR was significantly higher in older DMD patients than in respective healthy controls (Table 3). This increase in eGFR value indicates on a moderate hyperfiltration in older DMD patients that could reflect first signs of kidney dysfunction.

4. Discussion

In the present study the levels of 128 urinary proteins including growth factors, cytokines and chemokines were compared in urine of DMD patients and age related control subjects by antibody array approach. Surprisingly, statistically significant difference was observed only for urinary ferritin whose level was at least ten times higher in DMD patients.

Ferritin is a 24-subunit cytosolic protein composed of two chains: heavy chain (H-ferritin) and light chain (L-ferritin) which form a structure that can store up to 4500 iron ions in a nontoxic form. The H-ferritin possesses the ferroxidase activity transforming the ferrous ions Fe(II) into ferric ions Fe(III). The ferric ions are then translocated into the core of the ferritin shell where L-ferritin assists in iron mineralization thus protecting cells against free iron related production of reactive oxygen species (for review see ref. [27]).

Ferritinuria was previously described in patients suffering from chronic haemolytic anaemia [14]. Under this condition, when free haemoglobin exceeds the haptoglobin binding capacity, the excess is filtered by the kidney [8]. In renal cells, the iron liberated from haemoglobin induces ferritin production [10] which sequesters iron and prevents iron related damage. The authors proposed that the high level of urinary ferritin was the result of the shedding of tubular epithelium into the urine [14]. In DMD patients, there is no evidence that erythrocytes have survival defects [9]. In the absence of inflammation and taking into account that all tested parameters reflecting serum iron and haemolysis status in the studied cohort were within the normal reference ranges it is reasonable to suggest that the turnover of another haem-carrier protein, myoglobin, is the reason for the increased urinary iron and

ferritin in DMD patients. Indeed, increased level of myoglobin was already described in serum of DMD patients as a result of muscle leakage [11,28]. Moderate but significant correlations between serum myoglobin and urinary ferritin as well as between serum myoglobin and urinary iron supported myoglobin metabolism as a cause of increased urinary iron and ferritin. It is highly possible that correlations between serum myoglobin and urinary ferritin/iron are impacted by the long-time retention of ferritin in renal cells before being shed into the urine [29]. Because urines in the present study were obtained at the time of patients' visit (midstream urines), the retention of ferritin (and iron) in kidney could lessen the correlation between serum myoglobin and urinary ferritin/iron. It is also possible that part of serum myoglobin was cleared by the liver [30,31], but this process is difficult to quantify since hepatic clearance is highly variable [32].

In renal cells, iron released from myoglobin could provoke oxidative damage and induce kidney failure as in the case of acute rhabdomyolysis [33]. Recently, Zorova and colleagues demonstrated the importance of the activation of myoglobin and haem degradation by haem oxygenase for the reduction of renal damage and renal failure associated with rhabdomyolysis [34]. Importantly, temporary and acute increase of serum myoglobin up to 4 mg/L [35] or even 15 mg/L [36] can be tolerated by the kidney without clear renal manifestation. In our cohort, the mean level of serum myoglobin in young DMD patients was 2 mg/L, but contrary to acute rhabdomyolysis event where myoglobin is quickly eliminated from the serum [37], DMD patients suffer from chronic muscle leakage and a lifelong presence of serum myoglobin. This lifelong serum myoglobin persistence and concomitant iron renal recycling could be deleterious for patient's kidneys in the long term. The evaluation of the GFR in DMD patients and healthy subjects demonstrated no kidney function impairment in young DMD patients. However, we observed statistically significant increase of eGFR in older DMD patients indicating a moderate glomerular hyperfiltration. Hyperfiltration was suggested as an early marker of renal damage in pre-diabetes and pre-hypertension [38], and it may be the first sign of progressive loss of kidney function in diabetes, cancer or obesity (for review see ref. [39]). Importantly, hyperfiltration was already reported in DMD patients [22] and kidney failure was observed in older DMD patients [40,41]. The presence of kidney injury was also described in the *mdx* mouse [42]. Many factors including dehydration, use of high doses of diuretics or fluid intake reduction are known to accelerate renal failure in DMD patients [40,41]. Our results indicate that modified renal iron metabolism could be a component of kidney failure process. Extension of patients' life with coming of age new therapeutics [43–46] appeals for serious consideration for the role of iron in renal functions of DMD patients.

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Appendix. Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.nmd.2018.03.008.

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