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Proteomic analysis of plasma profiles in children with recurrent bone fractures

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The aim of the study is proteomic analysis of the plasma profile in children with recurrent bone fractures. The study involved 16 children: 6 patients with recurrent low-energy fractures and normal bone mass and 10 with osteogenesis imperfecta. In the analysis of the protein profile, the two-dimensional protein electrophoresis was used (Ettan DALT II, Amersham Bioscience). The images of protein gels were compared with controls. The protein spots with changed expression were cut from the gel and the amino acid sequence was analyzed with the mass spectrometry method (Q-Tof Premier™ API MASS SPECTROMETR, Waters) for protein identification. The most prevalent protein with changed expression, with respect to controls, was haptoglobin observed in 6 patients with a severe form of osteogenesis imperfecta. Increased haptoglobin concentration in these patients was confirmed by the ELISA method. Peptides corresponding to alpha-1 acid glycoprotein and serum amyloid P-component, apolipoprotein A-I, and transthyretin were detected in one, two and three children, respectively. Conclusions: 1) The results show increased haptoglobin which may be suggestive of an inflammatory component taking part in the course of osteogenesis imperfecta. 2) Further studies to explain the possible relationship of this protein with increased bone fragility are necessary.

Keywords: bone fractures, proteomic plasma profile

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INTRODUCTION

Recurrent bone fractures are a serious paediatric problem (Khosla e*t al.,* 2003). The results of own studies conducted to date as well as observations of other authors show that most frequently they are not only the effect of increased incidence of injuries in the developmental age, but also a symptom of bone mineralization or structure disturbances (Manias *et al.,* 2006). Many fractures in children may be a consequence of decreased bone mineral density or abnormalities in calcium and phosphate balance and bone metabolism. In some cases, however, bone mass corresponding to skeletal mineralization is normal, and still repeated fractures occur in these patients. In such circumstances abnormal bone tissue composition or structure is suspected and diagnostics as well as treatment of these disturbances are particularly difficult (Clark *et al.,* 2006). The main component of the organic bone part are proteins — 90% collagen, and the other 10% — non-collagen proteins. Direct evaluation of proteins in the bone tissue is difficult due to the necessity of invasive sampling of this tissue for histomorphometric analysis (Pernow *et al.,* 2009). Bone is however a metabolically active tissue and in the process of bone remodelling both the propeptides subsequently forming its structure and the bone protein degradation products enter the blood. To date, only quantitative assessment of some proteins — commonly accepted bone formation and bone resorption markers — in the serum was performed (Cundy *et al.,* 2007). In the recent years, owing to the dynamic development of molecular biology, a possibility of qualitative assessment of proteins with proteomic methods emerged and first attempts have already been made to use this technique for studies on the pathogenesis of some motor system conditions (Mayer-Kuckuk *et al.,* 2006; de Ceunick, 2008; Zheng *et al*., 2009; Zhang *et al*., 2010). However no papers concerning qualitative assessment of proteins circulating in the blood in patients with recurrent fractures have been published to date, although such publications might contribute to an indirect, deepened analysis of the composition and structure of the organic component of the bone tissue. Only few authors dealt with protein expression in *in vivo* studies of osteogenesis imperfecta in a mouse model, however no such analyses were done in humans (Forlino *et al.,* 2007). Therefore research was undertaken whose purpose is to try to explain the aetiopathogenesis of repeated bone fractures based on a complex qualitative plasma protein profile analysis in children with recurrent fractures of unknown aetiology (RF) and in patients with osteogenesis imperfecta (OI).

MATERIALS AND METHODS

The study involved 16 children, including 6 patients with recurrent low-energy bone fractures (RF) not related to a decrease in bone mass (aged 12–16 years, number of fractures ranging from 4 to 7) and 10 patients with diagnosed osteogenesis imperfecta (OI type I in 4 children — OIt1, type III in 6 children — OIt3;

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Abbreviations: RF, recurrent fractures; OI, osteogenesis imperfecta; OIt1, osteogenesis imperfecta type I; OIt3, osteogenesis imperfecta type III.

age: 4 months — 15 years). The tests were done at least 2 months after the occurrence of the last fracture. Active inflammatory conditions were excluded in all children: no clinical symptoms of inflammation were present, C-reactive protein levels assessed by a turbidimetric method and quantitative serum electrophoresis were normal. The study enrolled patients in whom chronic diseases promoting secondary skeletal mineralisation disturbances were excluded (diseases requiring long-term systemic glucocorticoid therapy, diseases of endocrine glands, autoimmune diseases, systemic diseases, intestinal malabsorption syndromes, among others). The results were compared with proteomic plasma profile of 5 healthy children without previous fractures (controls — C). Clinical, densitometric and biochemical characteristics of the studied patients are shown in Table 1 and Table 2. Bone turnover markers were assessed by commercially available ELISA tests, liver metabolite of vitamin D was determined by a radiocompetitive method and parathormone by chemiluminescence.

The study obtained approval of the Bioethics Committee of the Medical University of Łódź (No. RNN/24/07/KE). Parents provided written consent for participation of their children in the study.

Fasting blood samples for tests were taken in the morning, to a tube with EDTA, and they were subsequently centrifuged at 1500 rpm for 15 minutes. Plasma samples obtained were stored at the temperature –70 °C to the time of analysis.

Two-dimensional polyacrylamide gel electrophoresis. Proteins were separated by two-dimensional electrophoresis, using ready-made gels with immobilized pH gradients (Amersham-Biosciences). Albumin and IgG were removed from samples of human plasma using ProteoPreb Blue Albumin and IgG Depletion Kit (Sigma). For the first dimension, samples containing approximately 70 μg of soluble protein in lysis buffer were mixed with IPG Resewelling Solution (8 M urea, 1% Chaps, 0.4% DTT, 0.5% Pharmalyte) to obtain a final volume of 450 μl. They were then loaded onto 24 cm immobilized pH linear gradient strip gels (IPG, pH 4–7). IEF strips were allowed to rehydrate for 5 h, and isoelectric focusing was performed according to a protocol by gradual increase of

Table 1. Clinical characteristic of studied children

Diagnosis (Patient initials)	Age (month or year[b])	Gender	Localisation/number of previous bone fractures	Time from the last fracture (min)	Other skeletal symptoms
Recurrent fractures and normal bone mass (RF)					
RF(DM)	12 years	F	forearm, phalanges / 7	2	none
RF(KP)	13 years	M	forearm, phalanges / 4	36	none
RF(TŁ)	14 years	M	forearm, phalanges, crural, metatarsal / 5	5	none
RF(CD)	14 years	M	humeral, clavicle, scapula, vertebrae, femoral / 6	3	back and legs pain
RF(BM)	15 years	M	forearm, metatarsal, phalanges / 6	8	back pain
RF(SP)	16 years	M	phalanges / 7	36	back pain
Osteogenesis imperfecta type I (Olt1)					
Olt1(ŚN)	3 years	M	forearm, phalanges, femoral, iliac / 5	8	arms and legs pain
Olt1(GW)	7 years	M	forearm, humeral / 6	12	legs pain
Olt1(KK)	8 years	M	forearm, vertebrae, ribs / 7	12	legs pain
Olt1(TA)	15 years	M	forearm, humeral, clavicle, crural / 8	8	legs pain
Osteogenesis imperfecta type III (Olt3)					
Olt3(MW)	4 months	F	forearm, humeral, femoral, crural, ribs / many fractures in fetal period	4	bone deformation and pain
Olt3(KZ)	9 months	F	forearm, humeral, femoral, ribs / 7 + many fractures in fetal period	6	bone deformation and pain
Olt3(OM)	2 years	F	cranial, femoral, crural / 6 + many fractures in fetal period	6	bone deformation and pain
Olt3(PP)	4 years	F	forearm, humeral, femoral, crural, ribs, vertebrae / 40 + many fractures in fetal period	2	bone deformation and pain
Olt3(MA)	1 year	M	forearm, humeral, femoral, crural / 1+ many fractures in fetal period	11	bone deformation and pain
Olt3(NB)	2 years	M	humeral, clavicle, femoral / 9 + many fractures in fetal period	3	bone deformation and pain
Controls without fractures(C)					
C(JA)	6 years	F	none	-	none
C(SA)	1 month	M	none	-	none
C(DP)	2 years	M	none	-	none
C(RJ)	11 years	M	none	-	none
C(NM)	17 years	M	none	-	none

Table 2. Densitometric and biochemical characteristic of studied children

Diagnosis (Patient initials)	Total body BMD g/cm² (Z–score)	Spine L2L4 BMD g/cm² (Z–score)	25OHD ng/ml	PTH pg/ml	OC ng/ml	PINP ng/ml	CTX ng/ml	NTX mMBCE/mM creatinine	OPG pmol/l	sRANKL pmol/l
Recurrent fractures and normal bone mass (RF)										
RF(DM)	0.969 (0.0)	0.898 (–0.3)	40.3	34.0	160.4	364.3	1.8	399	4.0	0.0
RF(KP)	0.648 (–0.2)	0.853 (–0.4)	29.8	24.7	139.3	375.7	1.5	1080	4.5	0.0
RF(TŁ)	0.980 (0.5)	0.953 (–0.4)	46.7	18.8	99.1	244.9	1.8	1604	3.9	0.0
RF(CD)	1.011 (1.3)	0.925 (–0.1)	33.1	48.4	135.9	327.1	1.8	162	2.6	0.3
RF(BM)	0.970 (0.1)	0.923 (–0.8)	38.0	27.0	93.8	178.1	1.2	444	2.5	0.1
RF(SP)	1.077 (–0.8)	1.116 (–0.7)	23.2	33.1	111.0	213.7	2.2	290	2.5	0.0
Osteogenesis imperfecta type I (Olt1)										
Olt1(ŚN)	0,437 (–1,8)	–	75.8	8.6	107.3	290.2	0.4	630	2.5	0.0
Olt1(GW)	0.748 (–1.5)	0.428 (–3.1)	22.6	27.5	124.8	247.4	0.5	869	2.1	0.1
Olt1(KK)	0.835 (–1,5)	–	44.8	51.4	142.9	266.8	0.8	599	4.0	0.3
Olt1(TA)	0.704 (–2.8)	0.596 (–3.5)	9.5	21.6	113.6	384.5	1.7	509	4.5	0.0
Osteogenesis imperfecta type III (Olt3)										
Olt3(MW)	0.327 (–1.4)	–	34.3	9.8	26.5	287.2	0.3	312	6.3	0.0
Olt3(KZ)	0.419 (–1.6)	–	70.5	15.3	41.7	193.1	0.4	306	5.5	0.0
Olt3(OM)	–	–	41.5	19.2	126.1	259.1	0.2	330	4.5	0.0
Olt3(PP)	0.454 (–3.8)	–	73.2	29.2	43.0	73.2	0.3	390	5.5	0.0
Olt3(MA)	0.492 (–1,9)	–	42.2	25.7	36.1	184.3	0.4	1314	3.4	0.1
Olt3(NB)	0.726 (3.1)	–	40.3	30.3	38.6	147.9	0.3	396	4.8	0.0

Abbreviations: BMD, bone mineral density by dual energy X–ray absorptiometry; 25OHD, serum liver metabolite of vitamin D; PTH, serum parathormon, OC, serum osteocalcin; PINP, serum N–terminal propeptide of procollagen type I; CTX, serum C–terminal telopeptide of collagen type I; NTX, urine N–terminal telopeptide of collagen type I; OPG, serum osteoprotegerin; sRANKL, serum soluble receptor activator of nuclear factor kappa B ligand.

voltage (30 V for 5 h, 500 V for 1 h, 1000 V for 1 h, followed by 60 kVh at 8000 V), using an Ettan IPGphor system (Amersham Bioscience). Before the second dimension of electrophoresis the strips were equilibrated for 15 minutes in a solution containing 65 mmol DTT 6 M urea 30% glycerol 2% SDS and 50 mmol Tris/HCL (pH 6.8). Thereafter, they were subjected to a new equilibration solution for 15 minutes where DTT was replaced by 243 mmol iodoacetamide. Second-dimension SDS electrophoresis was performed on 12.5% polyacrylamide gels using the Ettan Dalt II vertical system (Amersham Bioscience). Protein spots were visualized by silver-staining according to the method compatible with the analysis of protein mass spectrometry (Shevchenko *et al.,* 1996). Silver-stained gels were digitized using a laser Image Scanner (Amersham-Bioscience). The images of protein gels were compared with controls. Computerized 2D gel analysis was performed with the Image Master 2D software package Version 3.0. Protein spots with changed abundance as well as those that appeared only in patients were excised from the gel subjected to in-gel digestion with trypsin.

Mass spectrometry, data base search, and data processing. Proteins in each gel slice were subjected to reduction with 10 mM dithiothreitol, alkylation with 50 mM iodoacetamide, and tryptic digestion with sequencing grade modified trypsin (10 μg/mL; Promega) at 37° C for 14 h. After in-gel digestion, the product peptides were extracted stepwise with two portions of 35 μL of 0.1% trifluoroacetic acid in 2% acetonitrile and loaded on a RP-C18 nanoAcquity UPLC pre-column (Waters). Peptides were eluted to a C18 nanoAcquity UPLC column (75 μm × 25 cm; Waters) by acetonitrile gradient in the presence of formic acid and then directly applied into an electrospray spectrometer (QUADRUPOLE TIME-OF-FLIGHT TANDEM Q-Tof Premier™ API MASS SPECTROMETR, Waters). Linear acetonitrile gradient and 0.1% formic acid were used for peptide elution. In spectra acquisition the reflectron status was on (kV: 2.04;

Table 3. Detailed criteria of protein identification by peptide mass fingerprints and MS/MS sequencing

Attribute	Value
Search Engine Type	PLGS
Databanks	Ident_Swiss_Human Ident_Trembl_Human
Species	Human
Molecular Weight Range	0 to 200000 Da
pI Range	0 to 14
Maximum Hits to Return	50
Primary Digest Reagent	Trypsin
Secondary Digest Reagent	None
Monoisotopic or Average	Monoisotopic
Mass Values	MH+
Peptide Charge	2+

μA: 58), the ionization source was electrospray and the following settings were used: capillary (kV) 3.0; source temperature (°C) 80; desolvation temperature (°C) 180. Proteins were identified by peptide mass fingerprinting, adding confidence to the protein identification. Detailed criteria of protein identification both by peptide mass fingerprints and MS/MS sequencing were summarized in Table 3. The Swiss-Prot protein database was searched with the PLGS 2.2.5 program. The list of top candidates for each sample was verified by inspection of the quality of sequencing data. We examined their automatic ordering manually in terms of their reliability scores and MS spectrum profiles to pick up only highly reliable peptide data (sorted data). Parameters used for database searching were the following: precursor-ion mass tolerance — 15 ppm, fragment mass tolerance — 0.2 Da, number of missed cleavages — 1, variable modifications — carbamidomethyl C, fixed modifications — oxidation M.

Quantitative haptoglobin evaluation. Serum concentration of haptoglobin in children with elevated abundance of this protein in proteomic analysis (OIt3) and in controls was determined by the ELISA method (enzyme-linked immunosorbent assay) with the Haptoglobin Human ELISA kit (ABCAM, UK). The sensitivity threshold of this method was <20 ng/ml, intra-assay coefficient of variability (CV) was 5.1%, inter-assay was 8.0%. Reference values for healty subjects were 0.3–2 g/l. The statistical analysis was made by means of the Statistica 6.0 software. The results were accepted as statistically significant at $p < 0.05$.

RESULTS

In the studied group of patients proteins were detected whose expression was changed with respect to controls as well as proteins that appeared only in the individuals affected by recurrent fractures. Among the spots tested to date, peptides corresponding to the alpha and beta chains of human haptoglobin protein occurred most frequently — in all six children (100%) with type III osteogenesis imperfecta (Table 4, Fig. 1). On the contrary, the spots corresponding to the above mentioned proteins were found neither in children with recurrent fractures of unknown aetiology and with type I osteogenesis imperfecta nor in the control group. Additionally, in three patients, including two with multiple recurrent fractures and one with type III osteogenesis imperfecta, peptides corresponding to the human transthyretin protein were found (Table 4, Fig. 2). Another repeatedly found protein was human apolipoprotein A-I in two children (Table 4), including one with recurrent fractures (Fig. 2) and one with type III osteogenesis imperfecta. Additionally, in one of the patients with type III osteogenesis imperfecta peptides corresponding to human serum amyloid P-component were found.

Table 4. Proteins identified in plasma from children with recurrent fractures of unknown aetiology and with osteogenesis imperfecta type III

Diagnosis (patient initials)	Gel slice No	Protein name	Protein accesion No	Protein mass (kDa)	Peptides matches	Probability (%)	Sequence coverage (%)	Peptide sequences
Reccurent fractures and normal bone mass (RF)								
RF (DM)	1	human apolipoprotein A I	P02647	30,778	13	100	50.2	DLATVYVDVLK DYVSQFEGSALGK LLDNWDSVTSTFSK ETEGLR VQPYLDDFQK WQEEMELYR AELQEGAR LSPLGEEMR THLAPYSDELR ATEHLSTLSEK AKPALEDLR QGLLPVLESFK VSFLSALEEYTK
	2	human transthyretin	P02766	15,887	3	100	27.9	AADDTWEPFASGK VEIDTK YTIAALLSPYSYSTTAVVTNPK
	3	human transthyretin	P02766	15,887	6	100	52.4	GSPAINVAVHVFR KAADDTWEPFASGK AADDTWEPFASGK TSESGELHGLTTEEEFVEGIYK VEIDTK YTIAALLSPYSYSTTAVVTNPK
RF (BM)	4	human transthyretin	P02766	15,887	2	100	18.8	KAADDTWEPFASGK AADDTWEPFASGK

Osteogenesis imperfecta type III (Olt3)								
Olt3 (MW)	5	human apolipoprotein A I	P02647	30,778	19	100	67.4	VKDLATVYVDVLK DLATVYVDVLK DYVSQFEGSALGK LLDNWDSVTSTFSK EQLGPVTQEFWDNLEK DLEEVK VQPYLDDFQK WQEEMELYR QKVEPLR AELQEGAR LHELQEK LSPLGEEMR AHVDALR THLAPYSDELR LAEYHAK ATEHLSTLSEK AKPALEDLR QGLLPVLESFK VSFLSALEEYTK
	6	human serum amyloid P-component	P02743	25,387	8	100	34.5	VFVFPR AYSDLSR AYSLFSYNTQGR DNELLVYK VGEYSLYIGR QGYFVEAQPK IVLGQEQDSYGGK GYVIIKPLVWV
		human apolipoprotein A I	P02647	30,778	8	100	32.6	EQLGPVTQEFWDNLEK DYVSQFEGSALGK VQPYLDDFQK THLAPYSDELR LLDNWDSVTSTFSK VSFLSALEEYTK WQEEMELYR ATEHLSTLSEK
	7	human haptoglobin alpha beta chains	P00738	45,205	2	100	3.7	LRTEGDGVYTLNNEK TEGDGVYTLNNEK
		human transthyretin	P02766	15,887	2	100	18.8	KAADDTWEPFASGK AADDTWEPFASGK
Olt3 (KZ)	8	human haptoglobin alpha beta chains	P00738	45,205	4	100	8.4	TEGDGVYTLNDK TEGDGVYTLNDKK TEGDGVYTLNNEK NPANPVQR
	9	human haptoglobin alpha beta chains	P00738	45,205	6	100	9.4	LRTEGDGVYTLNDK TEGDGVYTLNDK TEGDGVYTLNDKK LRTEGDGVYTLNNEK TEGDGVYTLNNEK NPANPVQR
	10	human haptoglobin alpha beta chains	P00738	45,205	4	100	8.4	TEGDGVYTLNDK TEGDGVYTLNDKK TEGDGVYTLNNEK NPANPVQR
Olt3 (OM)	11	human haptoglobin alpha beta chains	P00738	45,205	2	100	6.2	TEGDGVYTLNDK TEGDGVYTLNNEK
Olt3 (PP)	12	human haptoglobin alpha beta chains	P00738	45,205	6	100	8.6	LRTEGDGVYTLNDK TEGDGVYTLNDK TEGDGVYTLNDKK KQWINK LRTEGDGVYTLNNEK TEGDGVYTLNNEK
Olt3 (MA)	13	human haptoglobin alpha beta chains	P00738	45,205	3	98.5	6.7	TEGDGVYTLNDK LRTEGDGVYTLNNEK TEGDGVYTLNNEK
	14	human haptoglobin alpha beta chains	P00738	45,205	2	100	3.7	LRTEGDGVYTLNNEK TEGDGVYTLNNEK
Olt3 (NB)	15	human haptoglobin alpha beta chain	P00738	45,207	7	100	7.9	STMQELNSR LASYLDK MTLDDFR QGVDADINGLR EVTQLR TEGDGVYTLNDKK KQWINK

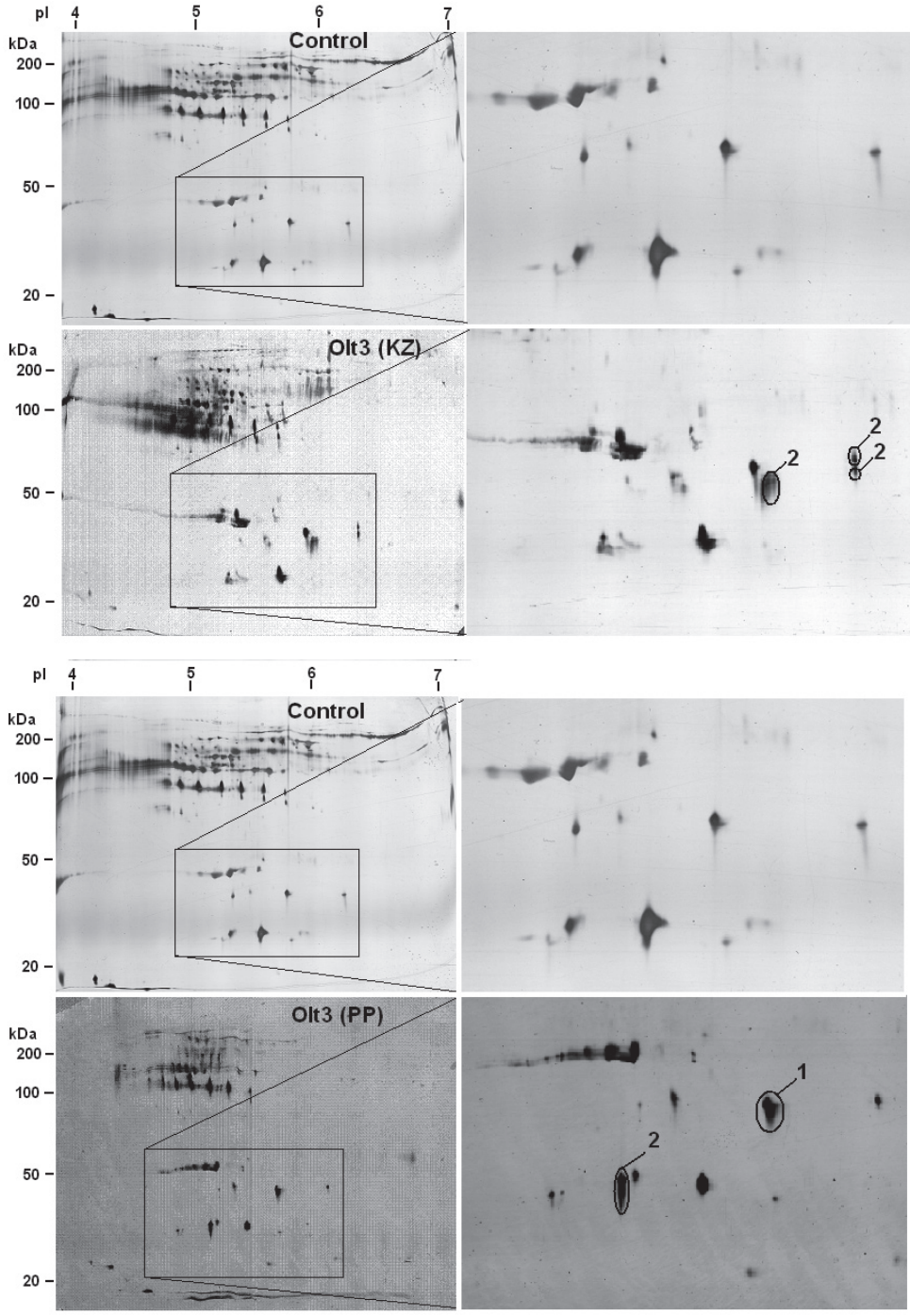

Figure 1A, B. Silver-stained 2D gel electrophoregram of plasma from two children (KZ, PP) with osteogenesis imperfecta type III (Olt3) in comparison with controls.
Panels on the right side show a magnified region of the gels (control, Olt3). The protein spots with significant changes in intensity were labeled with Arabic numbers: 1, human alpha-1 acid glycoprotein; 2, human haptoglobin alpha beta chains.

Quantitative analysis revealed that serum concentration of haptoglobin was significantly higher in children with OIt3 than in controls; mean values were 4.28 ± 2.41 *v.* 1.17 ± 1.25 g/l, respectively, $p=0.003$ (Fig. 3).

DISCUSSION

Search for the causes of recurrent bone fractures have been going on for a long time and for the time being these causes are still unclear (Manias *et al.,* 2006). At the beginning, decreased bone mineral density was considered the main factor, but after years of observation it appeared not to be the only and sufficient cause of fractures, as fractures frequently occur at normal or only slightly decreased mineral density (Clark *et al.,* 2006). Qualitative disturbances in bone structure, in particular in the collagen matrix, were then suspected. Our study included children with recurrent clinically significant fractures, in whom no disturbances in skeletal mineralization were found with available diagnostic methods, thus raising a suspicion of disturbances in the bone protein

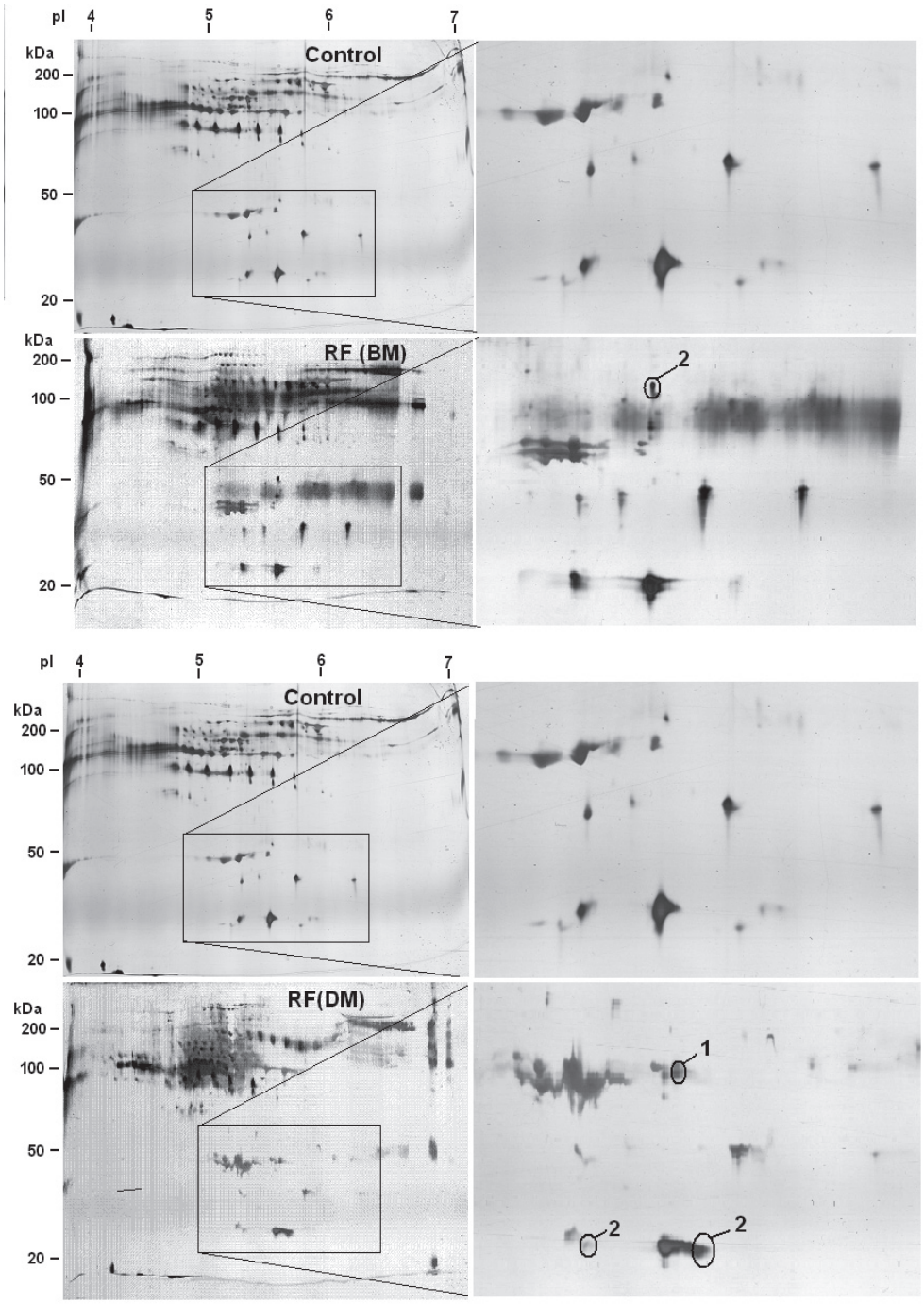

Figure 2A, B. Silver-stained 2D gel electrophoregram of plasma from two children (BM, DM) with recurrent fractures of unknown aetiology (RF) in comparison with controls.
Panels on the right side show a magnified region of the gels (control, RF). The protein spots with significant changes in intensity were labeled with Arabic numbers: 1, human apolipoprotein A-I; 2, human transthyretin.

component; it also included patients with osteogenesis imperfecta, i.e. with a genetically conditioned disease related to qualitative or quantitative collagen defects.

Analysis of the plasma protein profile with proteomic methods has shown, that haptoglobin is the most frequently occurring protein with changed abundance in children with fractures, as compared to the control group. Our proteomic results were subsequently confirmed by a quantitative ELISA method. Haptoglobin is a glycoprotein built up of alpha and beta chains, with three different phenotypes. Its main function is to bind free haemoglobin and to participate in iron recovery from the haem group; decrease of its concentration is a well-known haemolysis marker (Van Vlierberghe *et al.,* 2004). Most of the iron present in the body comes from haemoglobin metabolism, in which haptoglobin participates (D'Amelio *et al.,* 2008). Previous in vivo studies showed that iron has a significant role also in bone metabolism, including the effect of its abnormal levels on bone demineralisation. In studies done by Campos *et al.* (1998) on rats fed an iron-deficient diet, disturbances in calcium, phosphate and magnesium metabolism with a significant level of bone demineralisation were observed. Thus it is possible that increased haptoglobin found in

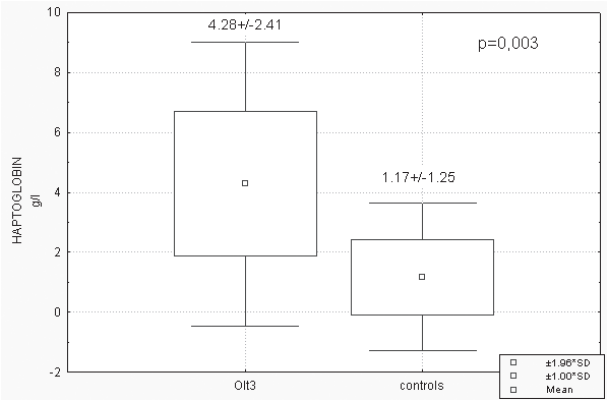

Figure 3. Serum haptoglobin concentration in children with OIt3 and controls ($p < 0.05$).

the studied subjects is associated with disturbances in the iron balance unfavourably affecting bone metabolism. The results obtained in our work also correspond with a recently published report of the team of D'Amelio *et al.* (2008) who studied the relationship between iron metabolism, including haptoglobin polymorphisms, and postmenopausal osteoporosis. The study revealed that the occurrence of fractures in this group was closely related to haptoglobin phenotype 2.2, i.e. to haptoglobin of the highest molecular weight. These authors also pointed to the fact that haptoglobin 2.2 favours deficiency of iron that is indispensable as a cofactor of enzymes synthesising collagen and bone matrix as well as a cofactor of 25-hydroxylase — an enzyme engaged in activation of vitamin D, and thus in the intestinal calcium absorption. This way they showed a close relationship between haptoglobin, iron metabolism, and the occurrence of fractures in postmenopausal women. Similar relationships may be present in the group of children studied by us and this issue seems to be worth further deepened analysis.

Haptoglobin has also immunomodulatory, antioxidative and angiogenic activity (Delanghe *et al.,* 1999). It is an acute-phase protein, its production in the liver increases in inflammatory conditions and is directly stimulated by proinflammatory proteins, such as interleukin-1, interleukin-6, and tumor necrosis factor alpha (Van Vlierberghe *et al.,* 2004). It is possible that increased expression of this protein in children with particularly numerous fractures, i.e. in children with type III osteogenesis imperfecta, is related with an underlying subclinical inflammatory process. Despite the fact that these children showed no clinical symptoms of inflammation and their C-reactive protein concentration was normal, haptoglobin was the most frequently detected protein with changed expression in their plasma, as compared to the reference group.

Proinflammatory cytokines, in particular interleukin-6, are known to be able to stimulate bone resorption (Carstanjen *et al.*, 2001; Fonseca *et al.*, 2009). This fact is supported by their increased concentration in adults with osteoporosis shown in the literature (Mundy, 2007), as well as by the results of our own previous studies in children with idiopathic osteoporosis (Rusińska & Chlebna-Sokół, 2005). It is possible that increased expression of haptoglobin in the patients studied by us was accompanied by increased concentration of other proinflammatory factors which enhanced bone resorption and thus increased the risk of fracture. A direct effect of haptoglobin on stimulation of bone resorption is also probable, as this effect was shown *in vitro* by Lerner and Frohlander (1992).

However, it remains unclear whether increased expression of this acute-phase protein in children with the most numerous fractures was, through increased bone resorption, the cause or rather the effect of the fractures. Nevertheless, to exclude such bias, blood for tests was collected, at the earliest, 2 months after the last fracture, i.e. at the time when it was already healed. Additionally, clinically occult microfractures cannot be excluded that might elicit a discrete inflammatory condition and thus affect obtained results.

As the changed expression of transthyretin, apolipoprotein A-I and serum amyloid P-component was only detected in one or two subjects in the RF and/or the OIt3 group, we will not speculate in this paper about the possible involvement of these proteins in bone fragility, however this issue may need discussion in further studies.

CONCLUSIONS

The results of pilot studies show increased haptoglobin concentration in children with OIt3, which suggests contribution of an inflammatory component to the course of severe osteogenesis imperfecta.

There is a necessity of further studies to explain the possible relationship of this protein with increased bone fragility.

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Conflict of interest statement

All authors declare no financial/commercial conflicts of interest.

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