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RESEARCH REPORT

Differential Expression of Matrix Metalloproteinases in the Serum of Patients with Mucopolysaccharidoses

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Abstract Mucopolysaccharidoses (MPS) represent a heterogeneous group of hereditary disorders, characterized by accumulation of glycosaminoglycans within the lysosomes. The objective of this study was to elucidate the expression and activity of matrix metalloproteinases (MMPs) in the serum of pediatric patients with MPS. Serum gelatinase activity was assessed by gelatin zymography and the concentration of circulating MMP-2, MMP-9, and of tissue inhibitors of MMPs (TIMP)-1 and TIMP-2 was measured by ELISA in the serum of seven patients with MPS (five with MPS III, 1 with MPS II and 1 with MPS VI), and healthy age- and sex-matched participants. Serum activity and protein levels of MMP-9 were significantly reduced whereas of MMP-2 were significantly increased in patients with MPS III, as compared to controls. There were no significant alterations in serum protein levels of TIMP-1 and TIMP-2 in patients with MPS III, as compared to controls. In MPS II, proMMP-2 activity and protein levels of MMP-2 were significantly increased, as compared to control. In MPS VI, enzyme replacement therapy reduced the activity and protein levels of MMP-9 up to 4 months after the initiation of treatment. The reported alterations in the expression of MMPs in the serum of patients with MPS suggest that these molecules may be used as potential biomarkers for the diagnosis, follow-up and response to therapy in patients with MPS.

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Introduction

Mucopolysaccharidoses (MPS) represent a heterogeneous group of hereditary disorders characterized by the accumulation of glycosaminoglycans (GAGs) within the lysosomes (Neufeld and Muenzer 2001). To date, 11 distinct types of MPS have been described, each one resulting from the deficient activity of a specific lysosomal hydrolase (Clarke 2008). In each disease, the primary enzyme deficiency leads to the accumulation of different types of GAGs resulting in a wide spectrum of clinical features that progress with age. Short stature and skeletal abnormalities, hepatosplenomegaly, hernias, and coarse facial features are prominent in most types of MPS with different involvement of cardiovascular, respiratory, and central nervous system in each syndrome (Muenzer 2004).

Although crucial steps have been made toward understanding the full etiopathogenetic repertoire of MPS, the exact mechanisms by which deficiencies of lysosomal hydrolases ultimately lead to disease manifestations are not clear. Recent findings indicate that the primary accumulation of GAGs within the lysosomes may trigger a cascade of events which influence various biochemical and physiological processes of the cell (Clarke 2008). The introduction of enzyme replacement therapy (ERT) increased the scientific interest in identifying molecular biomarkers of the disease and underlined the need for establishing new methods for rapid and early diagnosis of these disorders. Currently, there are no specific biomarkers for the diagnosis and treatment follow-up, apart from qualitative and quantitative measurement of urinary GAG

excretion (Gallegos-Arreola et al. 2000). Both techniques indicate the likely presence of an MPS disorder, rather than providing a definitive diagnosis or reflecting total body burden of disease.

GAGs accumulate within the lysosomes of various types of cells, including the cells of the immune system, and therefore it is not surprising that in many lysosomal storage disorders, altered immune responses are observed (Castaneda et al. 2008). Furthermore, it is widely accepted that these macromolecules have both pro- and anti-inflammatory properties, play a role as co-receptors for some cytokines (Mulloy and Rider 2006), whereas chemokines exert their biological functions through interactions with proteoglycans (Proudfoot 2006). Thus, there is emerging evidence for the involvement of inflammation in the pathophysiology of MPS. Accordingly, several mediators of the inflammatory response have been tested as possible molecular biomarkers for these disorders (Ohmi et al. 2003; Richard et al. 2008; Villani et al. 2007; Simonaro et al. 2001).

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases classified according to domain structure into collagenases, gelatinases, stromelysins, matrilysines, membrane-types, and others (Nagase and Woessner 1999). They represent key enzymes involved in the dissolution of extracellular matrix (Woessner 1991) and have been implicated in various processes, both normal and pathological, usually related to inflammation and cell apoptosis (Borkakoti 1998; Rydlova et al. 2008). Most MMPs are secreted as zymogens and require proteolytic activation, whereas their transcription, translation and proenzyme activity are regulated by growth factors, cytokines, and tissue inhibitors of metalloproteinases (TIMPs) (Brew et al. 2000; Clark et al. 2008).

In the present prospective case–control study, we examined the enzyme activity and expression of gelatinases, MMP-2 and MMP-9 as well as the expression of TIMP-1 and TIMP-2 in the serum of patients with MPS. The goal of this study was to elucidate the etiopathological mechanisms involved in this group of disorders aiming to provide new insights into the molecular mechanisms of these syndromes and unravel new potential biomarkers for the diagnosis, follow-up and response to therapy in patients with MPS. We demonstrate that MPS are associated with alterations in gelatinase activity and circulating levels of both MMP-2 and MMP-9.

Methods

Participants

Seven patients with MPS, followed up at the outpatient clinic of the 1st Department of Pediatrics of the Aristotle University of Thessaloniki at Hippokration General Hospital formed the study group. Patient's age was between 7 and 26 years old (14.21 ± 2.81). Five out of seven patients were male. Concerning the type of MPS, five out of seven patients suffered from MPS III (Sanfilippo syndrome), one from MPS II (Hunter syndrome) and one from MPS VI (Maroteaux–Lamy syndrome). The last patient is under ERT for the last 9 months. The control group consisted of healthy age- and sex-matched participants, as follows: 5 controls for each patient with MPS III (25 in total) and 10 controls for each patient with MPS II and MPS VI. Subjects' characteristics are presented in Table 1.

Table 1 Characteristics of patients

Patient number	Sex	Age (years)	MPS type
1	Male	26	VI
2	Male	7	III B
3	Female	7	III B
4	Male	16.5	III B
5	Female	21	III B
6	Male	14	III C
7	Male	8	II

Diagnosis of MPS was based in measurement of urine GAG excretion and was confirmed with enzyme activity assays and genetic testing for the identification of the causative mutation. Exclusion criteria in patients diagnosed with MPS were the coexistence of acute or chronic inflammatory disease and the use of drugs known to suppress inflammatory response.

Guardians and parents gave written consent for the participation of their children in the study. Whole blood samples were collected after single venipuncture and centrifuged at 1,750 g, for 10 min, at 4°C (High Speed Refrigerated Bench-top Centrifuge, Shanghai Lishen Scientific Equipment Co., Ltd, China). Serum samples were stored at 80°C until analysis. The study conformed fully with the Declaration of Helsinki for biomedical research on human subjects.

Gelatin Zymography

The gelatinolytic activity of MMPs was determined by gelatin zymography analysis using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under denaturing but nonreducing conditions (Papakonstantinou et al. 2005). In brief, 10 μl of serum samples from patients and healthy subjects were diluted 1:100 and 6 μl of the diluted samples were applied on an 8% SDS–PAGE containing 0.1% gelatin (25 mA, 2 h, at room temperature). Gels were then equilibrated in 2.5% Triton X-100 buffer

for 1 h and subsequently incubated in 50 mM Tris-HCl, pH 7.3 buffer containing 200 mM NaCI, 5 mM $CaCl_2$ and 0.1% Triton X-100 for 18 h, at 37°C. Bands of enzymatic activity were visualized by negative staining with standard Coomassie brilliant blue R-250 dye solution. Molecular size of bands displaying enzymatic activity were estimated by comparison to purified proMMP-2 (72 kDa), active MMP-2 (64 kDa), proMMP-9 (92 kDa), and active MMP-9 (78 kDa) (Anawa Trading, Wangen). Prestained standard protein molecular weight markers used were: myosin (250 kDa), phosphorylase (148 kDa), bovine serum albumin (98 kDa), L-glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (6 kDa) and insulin, B chain (4 kDa) (SeeBlue Plus2 Prestained, Invitrogen, USA). Gelatinolytic activity was quantified using a computer-assisted image analysis program (1D Image Analysis Software, Kodak Digital Science v.3.0, Eastman Kodak, Rochester, NY, USA). All experiments were performed in triplicate.

Immunoassays

Concentration of MMP-2, MMP-9, TIMP-1, and TIMP-2 (ng/ml) was quantified in duplicate in the serum of patients with MPS and healthy participants, using an ELISA kit (R&D Systems Europe, Abingdon, UK), performed according to manufacturer's instructions. The MMP-2 and MMP-9 assays measure total MMP-2 and MMP-9 (proenzymes and activated forms). Sensitivity of the method employed was: MMP-2: 0.16 ng/ml, MMP-9: 0.156 ng/ml, TIMP-1: 0.08 ng/ml, and TIMP-2: 0.011 ng/ml. Preliminary investigation established the appropriate serum dilution for each MMP and TIMP as follows: MMP-2, 1:10; MMP-9, 1:40; TIMP-1, 1:100; TIMP-2, 1:50.

Statistical Analysis

Normal distribution of data was checked using Shapiro–Wilk analysis for all dependent variables. Student's t test was used for variables following normal distribution, whereas Mann–Whitney U test was used to compare differences between the mean values of continuous variables violating the assumption of normality. Two-tailed levels of significance were used in all statistical calculations. All data are expressed as mean ± SEM. Difference was considered to be statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$. The relationship between continuous variables was investigated using Pearson product-moment correlation coefficient and Spearman's Rank Order Correlation (rho). The computer software SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical calculations and analyses.

Results

Differential Expression of Gelatinase Activity in Serum of Patients with MPS III and MPS II

Gelatin zymography analysis revealed that serum from patients with MPS III, as well as from control subjects express gelatinase activity of variable molecular mass (Fig. 1a). The two gelatin lysis bands with the lower molecular mass migrated as purified proMMP-2 (72 kDa) and MMP-2 (64 kDa) with proMMP-2 being the most prominent (Fig. 1a). The other two lysis bands of higher molecular mass, which exhibited the same intensity, correspond to the proform of MMP-9 (92 kDa) and the activated MMP-9 (78 kDa) (Fig. 1a). Quantitation of the lysis bands, using a computer-assisted image analysis program revealed that the activity of proMMP-9 and MMP-9, was significantly reduced in Sanfilippo patients, as compared to controls ($p = 0.016$ and 0.028, respectively, for proMMP-9 and MMP-9) (Fig. 1b). In the same group of patients, enzyme activity of MMP-2 was significantly increased compared to healthy controls ($p = 0.046$), whereas no difference was observed concerning proMMP-2 ($p = 0.917$) (Fig. 1b).

Furthermore, we observed that the ratio of latent (proMMP-2) to activated MMP-2 in the serum of Sanfilippo patients was 5.5:1, whereas in healthy subjects the same ratio was 11:1, indicating a higher degree of MMP-2 activation in the serum of Sanfilippo patients. We found no differences in the ratio of proMMP-9 to MMP-9 between Sanfilippo patients and controls (1:1).

Finally, we found a strong negative correlation between activated MMP-9 and age ($r = -0.926$, $p = 0.024$) in MPS III patients, as opposed to controls, where no correlation was observed ($r = -0.414$, $p = 0.441$) (Table 2). No significant correlation was found between the activity of proMMP-9, proMMP-2, and MMP-2 and the age of patients with MPS III (Table 2).

Gelatin zymography analysis revealed that the serum of the patient with MPS II exhibited no lysis bands corresponding to proMMP-9, MMP-9, and MMP-2, but only one band, migrating as proMMP-2 (Fig. 2a). Quantification of the gelatinolytic activity revealed a 1.6-fold increase in proMMP-2 activity in MPS II, as compared to healthy controls (Fig. 2b).

Enzyme Replacement Treatment Alters Gelatinase Activity in Serum of Patients with MPS VI

The patient with MPS VI is under ERT and thus, we obtained consecutive blood samples before the initiation of treatment, as well as, 1, 2, 3, 4 and 6 months following the administration of the enzyme. Gelatin zymography analysis

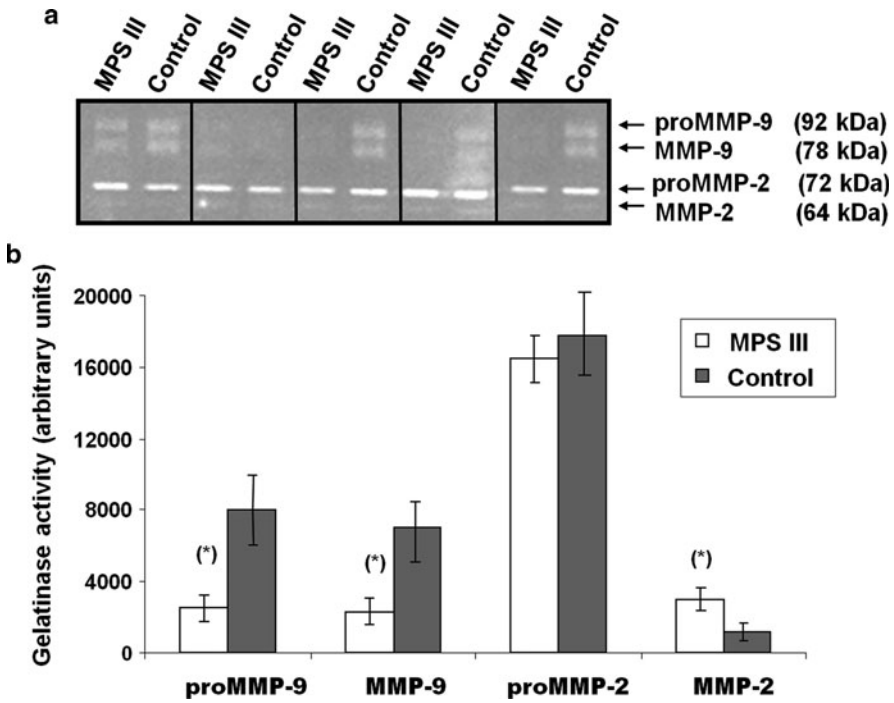

Fig. 1 Gelatinase activity is altered in patients with Sanfilippo disease. (**a**) Representative gelatine zymography in the serum of patients with MPS III ($n = 5$) and age- and sex-matched controls ($n = 5$, for each MPS III patient). (**b**) Quantitative analysis of gelatinolytic activity in serum of patients with MPS III and healthy controls using a computer-supported image analysis program. Each *bar* represents the mean ± SED from zymographies which were performed in triplicate for each patient. Statistical significance: (*) $p < 0.05$

Table 2 Correlation analysis between age and gelatin zymography values

Variable	Patients with MPS III ($n = 5$)	Controls ($n = 25$)
proMMP-9	$r = -0.876$, $p = 0.052$	$r = -0.149$, $p = 0.791$
MMP-9	$r = -0.926$, $p = 0.024$*	$r = -0.414$, $p = 0.441$
proMMP-2	$r = 0.154$, $p = 0.805$	$r = 0.723$, $p = 0.189$
MMP-2	$r = -0.333$, $p = 0.584$	$r = -0.678$, $p = 0.199$

*Statistically significant

revealed that the serum of the patient with MPS VI exhibited no lysis bands corresponding to MMP-9 and MMP-2, but only for proMMP-9 and proMMP-2 (Fig. 3a). Quantification of zymography lysis bands revealed that before treatment, proMMP-9 activity was decreased by 30% (Fig. 3b), whereas proMMP-2 activity was increased by 146%, (Fig. 3c), as compared to healthy controls.

ERT altered gelatinase activity in the serum of the patient with MPS VI. As shown in Fig. 3b, proMMP-9 was significantly reduced 1 month after the initiation of ERT. Thereafter, proMMP-9 activity was increased to reach, 6 months after treatment, the same level as observed in the age- and sex-matched controls.

The activity of proMMP-2 was initially decreased to control levels, 1 month after the initiation of ERT (Fig. 3c). However, 2 months after treatment, proMMP-2 activity was increased to the same level as before ERT and remained at this increased level up to 6 months following ERT (Fig 3c).

Immunoassays of MMPs and TIMPs

The concentration of MMP-2, MMP-9, TIMP-1, and TIMP-2 in serum of patients with MPS was assessed by ELISA. In Sanfilippo patients, circulating levels of MMP-2 were significantly increased ($p = 0.028$) (Fig. 4a), whereas serum concentration of MMP-9 was significantly decreased ($p = 0.047$) (Fig. 4b), as compared to sex- and age-matched controls. Regarding TIMP-1 and TIMP-2, no significant differences were found in circulating levels of both TIMPs between Sanfilippo patients and controls ($p = 0.095$ and 1.0, respectively, for TIMP-1 and TIMP-2) (Fig. 4c, d).

No significant correlations were found in patients with MPS III between all measured parameters and age, as revealed by statistical analysis (Table 3).

Regarding the MPS II patient, we observed a 2.6-fold increase in serum concentration of MMP-2, a 0.25-fold

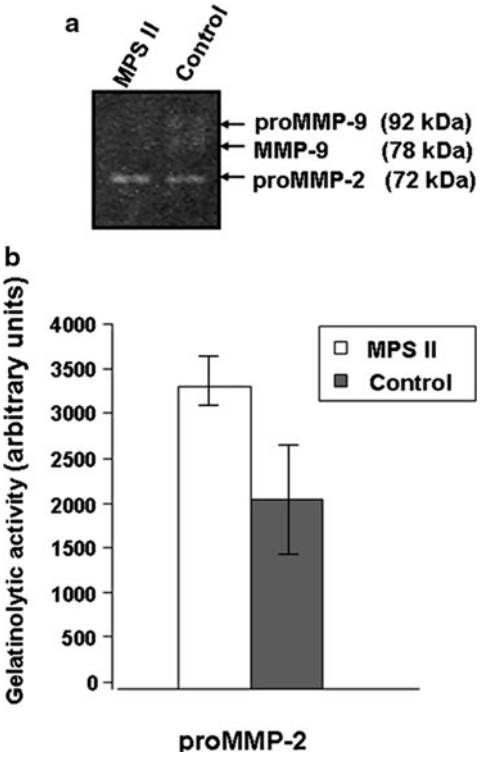

Fig. 2 Gelatinase activity is increased in MPS II. (**a**) Representative gelatine zymography in the serum of a patient with MPS III and age- and sex-matched controls ($n = 10$). (**b**) Quantitative analysis of gelatinolytic activity in serum of a patient with MPS II and healthy controls using a computer-supported image analysis program. Each *bar* represents the mean ± SED from zymographies, which were performed in triplicate for each patient

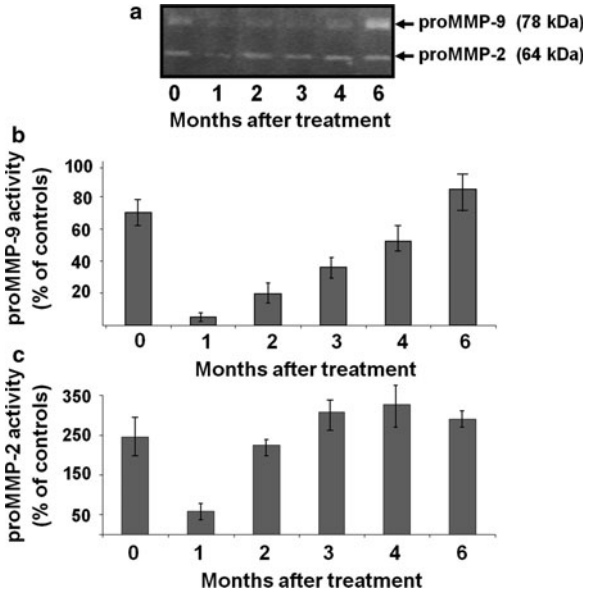

Fig. 3 Enzyme replacement therapy alters gelatinase activity in MPS VI. (**a**) Representative gelatine zymography in the serum of a patient with MPS VI before the initiation of treatment (0), as well as 1, 2, 3, 4, and 6 months after treatment. (**b**–**c**) Quantitative analysis of gelatinolytic activity using a computer-supported image analysis program. Each bar represents the mean ± SED from zymographies which were performed in triplicate and are presented as % of the gelatinolytic activity of age- and sex-matched controls ($n = 10$)

decrease in MMP-9, a 1.6-fold increase in TIMP-1 levels, and a twofold increase in circulating levels of TIMP-2, as compared to controls (Fig. 5).

Circulating levels of gelatinases and TIMPs were also assessed in consecutive blood samples taken from the patient with Maroteaux–Lamy syndrome, before treatment, as well as 1, 2, 3, 4, and 6 months following the initiation of ERT. All concentrations are expressed as % of serum levels of healthy subjects. As shown in Fig. 6, the administration of the enzyme alters serum concentration

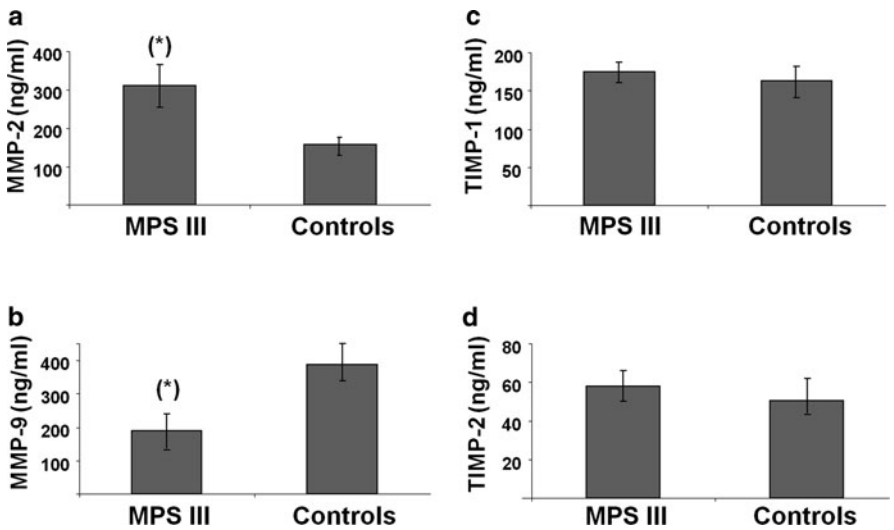

Fig. 4 Concentration of circulating MMP-2 (**a**), MMP-9 (**b**), TIMP-1 (**c**), TIMP-2 (**d**), in the serum of patients with MPS III ($n = 5$) and age- and sex-matched controls ($n = 5$, for each MPS III patient) measured by ELISA. Each bar represents mean ± SED from experiments performed in duplicate. Statistical significance: (*) $p < 0.05$

Table 3 Correlation analysis between age and immunoassays values

Variable	Patients with MPS III ($n = 5$)	Controls ($n = 25$)
MMP-9	$r = -0.344$, $p = 0.517$	$r = 0.112$, $p = 0.873$
MMP-2	$r = 0.107$, $p = 0.864$	$r = 0.668$, $p = 0.291$
TIMP-1	$r = 0.813$, $p = 0.187$	$r = -0.031$, $p = 0.955$
TIMP-2	$r = 0.812$, $p = 0.188$	$r = -0.282$, $p = 0.632$

of MMP-2, MMP-9, TIMP-1 and TIMP-2, but no stable pattern was observed. MMP-2 showed a 1.5-fold increase before treatment with no significant alterations during ERT (Fig. 6a). MMP-9 concentration exhibited an almost 60% decrease, 2 and 3 months following the initiation of ERT, and thereafter its concentration increased to reach, 6 months after the initiation of treatment, the same levels as before treatment (Fig. 6b). Concerning circulating levels of TIMPs before treatment, TIMP-2 showed a 1.6-fold increase and TIMP-1 a slight decrease in comparison with healthy subjects, with those levels showing no significant alterations during the administration of ERT (Fig. 6c, d).

Discussion

Very few analytical tools are at present available to diagnose MPS disorders, as well as to predict disease

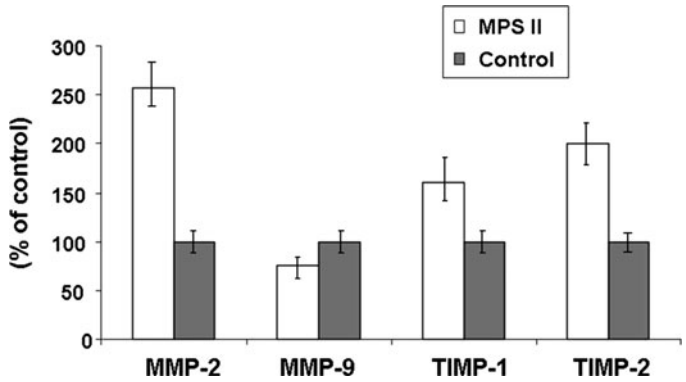

Fig. 5 Levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 measured by ELISA in the serum of one patient with MPS II. Each *bar* represents the mean ± SED from experiments performed in duplicate and are presented as % of values of age- and sex-matched controls ($n = 10$)

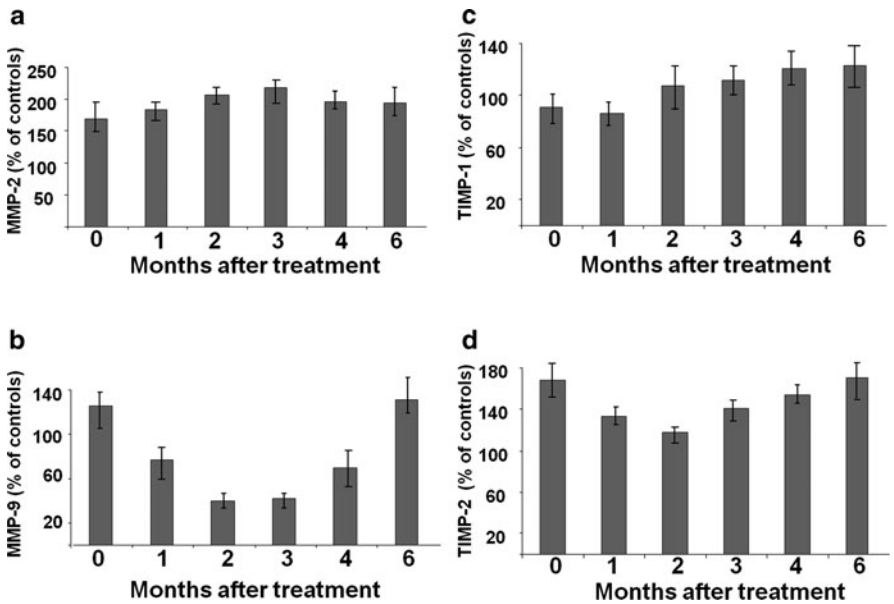

Fig. 6 Enzyme replacement therapy alters protein levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 in the serum of patients with MPS VI. The protein levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 were measured in the serum of a patient with MPS VI before the initiation of treatment (0), as well as 1, 2, 3, 4, and 6 months after treatment by ELISA. Each bar represents the mean ± SED from experiments performed in duplicate and are presented as % of values of age- and sex-matched controls ($n = 10$)

severity and responsiveness to therapy. Randall et al., have shown a significant increase of heparin cofactor II-thrombin complex in the serum of patients with MPS I in relation to healthy subjects, with its levels showing responsiveness to various treatment regimens (Randall et al. 2008). In addition, with the use of SELDI-TOF mass spectrometry, it has been found that patients with MPS show an increase in the ratio of the two forms of apolipoprotein ApoCI in plasma, which was associated with an increase in the activity of dipeptidyl peptidase IV (Beesley et al. 2009). These molecules were reported to be good biomarkers both for the diagnosis and for the therapy follow-up in MPS patients.

This study includes a small population of patients, most of them suffering from subtype B of Sanfilippo disease, which is the most common type of MPS disorders in our country (Michelakakis et al. 1995). Aiming to elucidate the mechanisms underlying the etiopathogenesis of these syndromes and unravel potential biomarkers, we examined the enzyme activity and protein levels of MMP-2 and MMP-9 in the serum of MPS patients. Additionally, we studied circulating levels of TIMP-1 and TIMP-2.

Many members of the MMP family represent research targets and were examined in tissues of animal models suffering from MPS during the past years. Common characteristic of all these studies is the altered expression of enzymes studied, such as MMP-1 and MMP-13 (Simonaro et al. 2008), MMP-12 (Ma et al. 2008), as well as the two members of gelatinase family, MMP-2 and MMP-9 (Richard et al. 2008; Simonaro et al. 2005). Despite all this work, MMPs have not been sufficiently studied in human tissues, with the exception of one study, conducted by Di Natale et al., which revealed a down-regulation of MMP-9 in patients with MPS VI (Di Natale et al. 2008). Apart from the expression of MMPs, enzyme activity of these proteins is also a promising study field. Simonaro et al., have found a significant increase in enzyme activity of gelatinases in synovial membranes of animals with MPS VI and VII (Simonaro et al. 2005). Thus, this study represents the first work investigating enzyme activity and expression of MMP-2 and MMP-9 in a human population with MPS. Concerning MMP-2, we found a significant increase both in enzyme activity and serum concentration, whereas a decrease in gelatinolytic activity and circulating levels of MMP-9 was observed. Those differences were even more obvious in the Sanfilippo group of patients. Given the fact that MMPs seem to be involved in numerous neuroinflammatory diseases and an overproduction of these enzymes is coupled with a disruption of blood–brain barrier (Mun-Bryce and Rosenberg 1998), MMP-2 and MMP-9 might represent suitable biomarkers demonstrating CNS involvement. In relation to treatment responsiveness, the alteration in enzyme activity and serum expression of gelatinases following the initiation of ERT classifies these molecules as possible candidates for follow-up markers.

In conclusion, the data presented in this study provide an important baseline for the differential expression of MMPs and TIMPs in MPS. A limitation of our study is the low number of MPS patients, and especially those undergoing ERT. Thus, our results need to be reconfirmed in studies employing a larger population of MPS patients of all types, in order to define more accurately the implication of the studied molecules, in the etiopathogenesis of these disorders. However, even with the limited number of patients, our results indicate that gelatinases are potential biomarkers for the diagnosis, follow-up and response to therapy in patients with MPS and should be further examined at a genetic level.

Take-Home Message

MMPs represent potential biomarkers for the diagnosis, follow-up, and response to therapy in patients with MPS.

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