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Short Report

Increased release and activity of matrix metalloproteinase-9 in patients with mandibuloacral dysplasia type A, a rare premature ageing syndrome

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Mandibuloacral dysplasia type A (MADA; OMIM 248370), a rare disorder caused by mutation in the *LMNA* gene, is characterized by post-natal growth retardation, craniofacial and skeletal anomalies (mandibular and clavicular hypoplasia, acroosteolysis, delayed closure of cranial sutures, low bone mass and joint contractures), cutaneous changes and partial lipodystrophy. Little is known about the molecular mechanisms by which *LMNA* mutations produce bone alterations. An altered bone extracellular matrix (ECM) remodelling could play a pivotal role in this disorder and influence part of the typical bone phenotype observed in patients. Therefore, we have focused our investigation on matrix metalloproteinases (MMPs), which are degradative enzymes involved in ECM degradation and ECM remodelling, thus likely contributing to the altered bone mineral density and bone metabolism values seen in five MADA patients. We evaluated the serum levels of several MMPs involved in bone development, remodelling and homeostasis, such as MMP-9, -2, -3, -8 and -13, and found that only the 82 kDa active enzyme forms of MMP-9 are significantly higher in MADA sera compared with healthy controls ($n = 16$). The serum level of MMP-3 was instead lower in all patients. No significant differences were observed between controls and MADA patients for the serum levels of MMP-2, -8 and -13 and of tissue inhibitor of metalloproteinase 2, a natural inhibitor of MMP-9. Similarly, normal serum levels of tumour necrosis factor alpha (TNF-α), interleukin (IL)-6 and IL-1β were detected. These data suggest a possible involvement of MMP-9 in MADA disease, underlying the potential use in diagnosis and therapy.

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Key words: bone remodelling – laminopathies – mandibuloacral dysplasia – MMPs

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Mandibuloacral dysplasia type A (MADA; OMIM 248370) is a very rare multisystemic autosomal recessive disorder belonging to the heterogeneous group of diseases collectively called primary laminopathies. MADA patients are characterized by several features including post-natal growth retardation along with typical skeletal abnormalities such as hypoplasia of the mandible and clavicles, shortening of distal phalanges, delayed closure of the cranial sutures and joint contractures; osteoporosis; cutaneous change such as atrophic skin; partial alopecia; mottled hyperpigmentation; partial lipodystrophy and, finally, progeroid appearance (1, 2). Some of these patients are hyperinsulinemic and insulin resistant with impaired glucose tolerance or overt diabetes mellitus. This disorder is caused by homozygosity or compound heterozygosity for different mutations

in the *LMNA* gene encoding for A-type lamins, lamin A/C (3–5). A-type lamins, expressed in most differentiated tissues, are intermediate filament proteins (6), which are the major components of the nuclear lamina, together with B-type lamins. They form a meshwork located between the inner nuclear membrane and the chromatin, so they play a fundamental role in the maintenance of the size and shape of the nucleus (7) and in several nuclear processes such as transcription, chromatin organization and DNA replication. Mutations in the *LMNA* gene can bring about cardiac and skeletal muscle diseases, lipodystrophy and premature ageing phenotypes (8–10). Mandibuloacral dysplasia (MAD) is also due to a mutation in another gene (i.e. *ZMPSTE24*) that encodes for a zinc metalloproteinase involved in post-translational proteolytic cleavage of carboxy-terminal residues of farnesylated prelamin A to form mature lamin A (11). This mutation determines MAD with type B lipodystrophy (OMIM 608612). The patients show generalized loss of subcutaneous fat involving the face, trunk, and extremities and metabolic complications. Moreover, they have several facial and skeletal anomalies very similar to the MADA patients.

Deficiency of Zmpste24 in mice ($Zmpste24^{-/-}$) phenocopies features MAD like such as accumulation of prelamin A. The most striking pathological phenotype is multiple spontaneous bone fractures, akin to those occurring in mouse models of osteogenesis imperfecta; moreover, they show a significant reduction in cortical and trabecular bone volumes (12). The osteogenesis requires the carefully coordinated interplay of many factors, and in this regard, proteolytic remodelling of extracellular matrix (ECM) is a condition *sine qua non* (13). Bone also continuously undergoes remodelling through a dynamical balance between resorption and deposition achieved by osteoclasts and osteoblasts, respectively. In this framework, a very important role in bone development, remodelling and homeostasis (14) is played by matrix metalloproteinases (MMPs), which are a family of zinc-dependent neutral endopeptidases characterized by their ability to degrade ECM components and break down the basement membrane. This influences bone development, remodelling and homeostasis (14). In general, MMPs' proteolytic activity is regulated at three main levels: transcription, proenzyme activation, and inhibition, which must be balanced in a concerted fashion to avoid undesired degenerative processes. MMP expression is cell and tissue specific and can be induced by various signals, such as inflammatory cytokines, growth factors and the extracellular MMP inducers (15, 16). Furthermore, the expression of various MMPs can be upregulated or downregulated by integrin-derived signals, ECM proteins, physical stress, and changes in cell shape (17).

MMPs are synthesized as inactive zymogens, pro-MMPs, which are usually activated in the pericellular space by other MMPs or serine proteinases through a proteolytic cleavage within MMP pro-domains. Their production and activity are usually very low in normal adult resting tissues, and with some exceptions, they are maintained at virtually undetectable levels by tissue inhibitors of metalloproteinase (TIMPs) and non-specific proteinase inhibitors (18, 19). The balance between MMPs and their inhibitors is essential in many physiological conditions where rapid remodelling of ECM is required, and any alteration could lead to pathological conditions, such as cancer (20). However, their expression may become elevated during specific physiological processes (such as tissue remodelling during growth phases, pregnancy and parturition and in post-menopausal osteoporosis) and/or in many pathological conditions (e.g. chronic inflammatory processes and malignancy) (21). In fact, elevated serum levels of MMPs have been reported in polycystic kidney disease (22), multiple sclerosis (23), rheumatoid arthritis (24), and many different human tumours including carcinomas of the lung, colon, breast and prostate (25–28).

In this study, we have analysed, by quantitative and qualitative enzyme assays, the behaviour of several circulating MMPs in MADA patient's serum. We measured the serum concentrations of gelatinase A (MMP-2) and gelatinase B (MMP-9), stromelysin 1 (MMP-3), neutrophil collagenase (MMP-8), collagenase-3 (MMP-13) and finally TIMP-2. We also determined the serum level of several inflammatory cytokines. As a whole, it emerges that MMP-9 is likely to be involved in the pathophysiology observed in MADA patients and that inhibition of MMP-9 function might protect against the progressive development of osteoporosis observed in MADA patients. As a corollary, MMP-9 might be a suitable biomarker for validating new drugs in MADA and possibly in other laminopathies.

Materials and methods

Patients and serum samples

The study was performed on MADA patients previously characterized (3). We collected five patients (two males and three females) recruited in Italy between 2000 and 2007 with different age, ranging from 5 to 40 years (the median age was

22.5 years). Four patients, homozygous for the R527H mutation, showed acroosteolysis, craniofacial abnormalities, hypoplasia of clavicles, and type A lipodystrophy (3). There is no significant difference concerning the phenotypic severity between all examined patients, indicating the independence of this disorder by age and sex. One patient was compound heterozygous with the classical R527H mutation and another missense mutation in exon 7 (V440M) (29). She showed an MADA-like phenotype with the absence of clavicular dysplasia and normal metabolic profiles associated with muscle hyposthenia and generalized hypotonia. Bone mineral density (BMD) was determined by dual-energy X-ray absorptiometry (DEXA) at the lumbar spine and femoral neck sites. All BMD results were expressed in kg/m^2 and as standard deviation from the mean of an age-matched and sex-matched population (*Z*-score).

Parental or patient's informed consent was obtained. Blood samples were collected by a single venipuncture. After clotting, serum was separated by centrifugation at 1300 ***g*** for 15 min at 4°C and prepared in tubes without additive. Serum was divided into aliquots and stored at −80°C until use. The control group was represented by 16 healthy volunteers, 8 females and 8 males with age ranging from 10 to 50 years. Furthermore, a group of eight post-menopausal osteoporotic women (age range 45–52 years) was used as a positive control. No therapy known to interfere with bone metabolism was administered at the time of the study. Serum samples were collected from all the study subjects in the morning after an overnight fasting.

Substrate zymography

Gelatin substrate zymography was used for the evaluation and characterization of serum proteinases. Serum sample (1 μg of proteins/lane; protein determination following Bradford [1976]) was mixed with a fivefold excess of sample buffer (0.25 M Tris, 0.8% sodium dodecyl sulphate (SDS), 10% glycerol and 0.05% bromophenol blue), and electrophoresis was run on 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels containing 1 mg/ml of gelatin, as described (30). Electrophoresis was performed at 200 V/40 mA for each gel in 25 mM Tris, 0.2 M glycine, and 1% (w/v) SDS running buffer, pH 8.2, until the tracking dye reached the bottom of the gel. After electrophoresis, SDS from gels was removed by four 15-min washings in 2% Triton X-100. The gelatin gels were then incubated at 37°C for 18 h in incubation buffer (50 mM Tris–HCl buffer pH 7.6, 0.15 M NaCl, 10 mM $CaCl_2$, 2% Triton X-100). The gels were then stained with 0.5% Coomassie blue and destained in 10% acetic acid and 40% methanol until proteinase bands were clearly visible. Proteinase bands were further characterized by adding 20 mM ethylenediaminetetraacetic acid (EDTA) or 0.3 mM 1,10-phenanthroline (MMP inhibitors) or 1 mM phenylmethylsulphonyl fluoride (PMSF) (serine proteinase inhibitor) to the incubation buffer. Protein markers (Bio-Rad, Hercules, CA) were used as molecular weight standards. The intensities and area of gelatinolytic activity have been measured and quantified by an image analysis software (IMAGEQUANT TL; Amersham Biosciences, Piscataway, NJ). An arbitrary unit (AU) scale was used, and results were statistically analysed.

Western immunoblots

Western blotting was carried out using the same serum sample to identify proteinases and their inhibitors (1 μg of proteins/lane; protein determination following Bradford [1976]). Each sample was mixed with a fivefold excess of sample buffer (0.25 mol/l Tris, 0.8% SDS, 10% glycerol and 0.05% bromophenol blue) and run on 12% SDS-polyacrylamide gel either after 2 min of boiling in presence of 2-mercaptoethanol. Gels were then transferred to nitrocellulose membrane (Amersham, Buckinghamshire, UK) in TRANS-BLOT (Bio-Rad) using Towbin buffer (25 mM Tris, 192 mM glycine, and 20% methanol). Unspecific binding sites were blocked with 5% dry low-fat milk in phosphate buffered saline (PBS) for 1 h at 37°C; membranes were then washed once with PBS and further incubated overnight at 4°C with the right concentration of mouse monoclonal (1 mg/ml; R&D Systems, Minneapolis, MN) antibodies directed against human gelatinase A (MMP-2), human gelatinase B (MMP-9), human neutrophil collagenase (MMP-8), human collagenase-3 (MMP-13) and TIMP-2. Thereafter, three washes (5 min each) with PBS/Tween-20 (0.2%) were performed, and membranes were then incubated with a 1/1000 dilution of horseradish peroxidase-labelled goat anti-mouse antibodies (Bio-Rad) in PBS for 2 h, followed by further washings (three washes, 5 min each) with PBS/Tween-20 (0.2%). The bands were visualized by incubation of the membrane (4-chloronaphtol 0.6 mg/ml and 30% H_2O_2 0.6 μl/ml) or by using ECL detection systems (Amersham). Results were analysed by an image analysis software (IMAGEQUANT TL; Amersham Biosciences). An AU scale was used, and results were statistically analysed.

Enzyme-linked immunosorbent assay quantification

The bone alkaline phosphatase (BALP) and serum cross-linked C-terminal telopeptide fragments of degraded type I collagen (CTX-I) considered bone remodelling markers were analysed in sera together with levels of MMP-2, -3, and -9 and of physiological inhibitors, TIMP-2. We also quantified the levels of cytokines, tumour necrosis factor alpha (TNF-α), interleukin (IL)-6 and IL-1β. Measurements were performed using a commercial enzyme-linked immunosorbent assay (ELISA) kit (BAP EIA Kit; Metra Quidel, San Diego, CA; Serum CTX-I EIA Kit, CrossLaps®; Quidel, San Diego, CA; Quantikine Human/Mouse/Rat MMP-2 total; R&D Systems; Immunoassay Kit Human MMP-3; Invitrogen, Carlsbad, CA; Quantikine Human MMP-9 total; R&D Systems; Human TIMP-2 ELISA Kit; RayBiotech, Inc., Norcross, GA; Immunoassay Kit Human TNF-α Ultra Sensitive; Invitrogen; IL-6 Kit ELISA, IL-1β ELISA; eBioscience, San Diego, CA) according to the manufacturer's instructions. All measurements were performed in duplicate. Both the MMP-2 and the MMP-9 assays are specific for pro- and active MMP-2 and -9. Serum samples were diluted in assay buffer standards and incubated in a microwell plate pre-coated with specific antibodies at room temperature. Any antigen present in the samples was bound to the wells, and the excess was removed by extensive washing. These antigens were then detected by a peroxidase-labelled antibody, and the amount of peroxidase was determined by the addition of tetramethyl benzidine substrate. Reactions were stopped by adding acid solution, and the absorbance was read at 450 nm in a microtitre plate spectrophotometer. Serum concentrations were determined from the corresponding standard curves run for each plate separately. A standard curve was obtained by using recombinant proteins.

Statistical analysis

A computer database containing all data measured from patients was created. These data were analysed using the STAT VIEW 512+ program from Brain Power, Inc. (Calabasas, CA). Regression analysis, correlation test and Student's *t*-test have been used. All results are expressed as mean ± SD.

Results

Bone metabolism

MADA serum levels of C-telopeptides of type I collagen (CTX-I), a marker of bone resorption, were well above the pre-menopausal range (0.68 ± 0.07 ng/ml). Nevertheless, patients showed normal serum levels of BALP (32.3 ± 5.6 IU/l), considered a marker of bone formation. On the other side, our set of patients showed a very low BMD at lumbar spine (*Z*-score −1.77 ± 0.14) in comparison with the control group, as measured by DEXA, and the same was true for the femoral neck BMD (*Z*-score −3.4 ± 0.23) (Table 1).

Table 1. Clinical and biochemical parameters of patients and controls

				p		
	Control	MADA	Osteoporotic	Control *vs* MADA	Control *vs* osteoporotic	MADA *vs* osteoporotic
Sex (M/F)	7/9	2/3	0/8	—	—	—
Age (years, range)	10–50	5–40	45–52	—	—	—
BMI (kg/m^2)	24.38 ± 0.7	17.2 ± 0.51	22.9 ± 0.49	<0.01	NS	0.05
BMD lumbar spine (*Z*-score)	0.27 ± 0.12	−1.77 ± 0.14	−2.3 ± 0.12	<0.01	<0.0005	NS
BMD femoral neck (*Z*-score)	0.33 ± 0.07	−3.4 ± 0.23	−1.9 ± 0.12	<0.005	<0.03	0.05
BALP (IU/l)	29.4 ± 9.3	32.3 ± 5.6	21.6 ± 1.6	NS	<0.03	<0.02
CTX-I (ng/ml)	0.12 ± 0.09	0.68 ± 0.07	0.55 ± 0.11	<0.03	<0.05	NS
MMP-9 (ng/ml)	0.6925 ± 0.1335	4.586 ± 0.5296	3.041 ± 0.3415	<0.0001	<0.0001	NS
MMP-2 (ng/ml)	24.74 ± 1.104	27.82 ± 3.993	NA	NS	—	—
MMP-3 (ng/ml)	5.154 ± 0.7575	1.818 ± 0.9151	7.495 ± 0.9365	0.0341	0.07	0.002
TIMP-2 (ng/ml)	4.605 ± 0.1281	4.643 ± 0.1198	NA	NS	—	—
TNF-α (pg/ml)	0.018 ± 0.043	0.0034 ± 0.006	NA	NS	—	—
IL-1β (pg/ml)	0.5365 ± 0.9979	0.3344 ± 0.6561	NA	NS	—	—
IL-6 (pg/ml)	1.855 ± 0.4824	3.683 ± 4.028	NA	NS	—	—

BALP, bone alkaline phosphatase; BMD, bone mineral density; BMI, body mass index; CTX-I, C-terminal telopeptide fragments of degraded type I collagen; F, female; M, male; MADA, mandibuloacral dysplasia type A; MMP, matrix metalloproteinase; NA, not available; NS, not significant; IL, interleukin; TIMP-2, tissue inhibitor of metalloproteinase 2; TNF-α, tumour necrosis factor alpha.

Identification of proteinases in the serum samples

In all sera, different gelatin-degrading activities were observed, corresponding to molecular weights of 207, 116, 94, 92, 82, and 62 kDa. In all MADA sera, we detected four forms (i.e. corresponding to 207, 116, 92 and 82 kDa, respectively) that could be related to gelatinase B (MMP-9) corresponding to the molecular weights of the disulphide-linked homodimer, the complex with neutrophil gelatinase B-associated lipocalin (NGAL), pro-MMP-9 and active MMP-9, respectively. Conversely, in control serum, we detected (Fig. 1a) only the active forms at 92 and 82 kDa (31). In the serum from MADA patients, the levels of the active form of MMP-9 were markedly enhanced, showing a considerable activity of this gelatinase with respect to the control serum (Fig. 1b). Proteinase activities corresponding to bands weighing about 207 and 116 kDa were detected only in four MADA patients with a mean value of 63,332 ± 13,382 and 82,442 ± 51,690 AU, respectively (Fig. 1b). The band corresponding to the molecular weight of 62 kDa, corresponding to the active MMP-2, was present in all samples (control and MADA patients) with a similar or slightly higher activity in MADA sera with respect to the control ones (Fig. 1a,b). Most bands (207, 116, 92, 82, and 62 kDa) were completely abolished when gels have been incubated in a buffer containing 10 mM EDTA or 0.3 mM 1,10-phenanthroline (MMP inhibitors), confirming that the activity should be confidently attributed to a metalloproteinase (data not shown). A proteolytic activity related to a 94 kDa enzyme was present in 100% (97,883 ± 8.645 AU) of control serum but never in samples from MADA patients (Fig. 1b). This activity was completely inhibited by 0.1 mM PMSF in the incubation buffer, whereas no effect was observed upon addition of 1,10-phenanthroline or EDTA (data not shown), indicating that this activity should be instead related to a serine proteinase, probably the plasmin. Western blot experiments have been performed, employing antibodies against gelatinase A (MMP-2, molecular weight 72–62 kDa) and gelatinase B (MMP-9, molecular weight 92–82 kDa), to characterize the band seen by gelatin zymography analysis. Results obtained show the presence of multiple forms of MMP-9 enzyme in the serum from MADA patients, whereas in serum control, only the high-molecular weight form was found (Fig. 2a). The pattern of MMP-2 (both forms) was similar in all samples tested (data not shown), showing no correlation with the pathology.

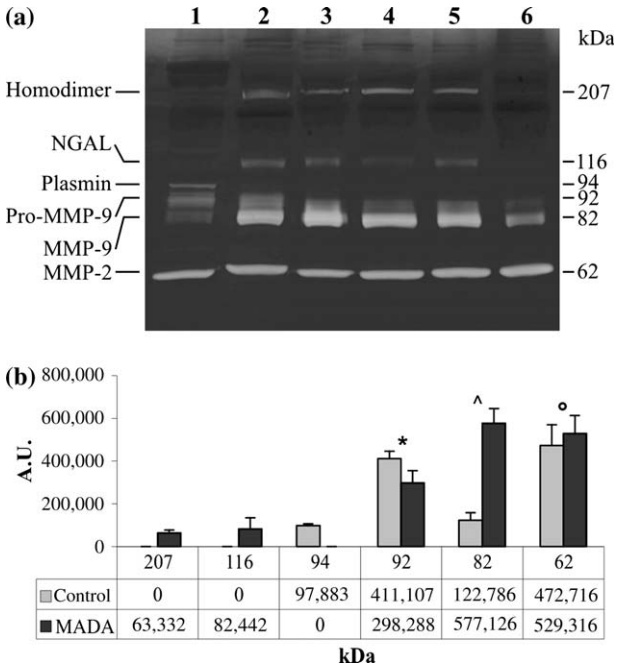

Fig. 1. (**a**) Gelatinolytic activity in serum samples. Gelatin substrate zymography of sera collected from MADA patients with R527H/R527H (lanes 2, 3, 4, and 6) or R527H/V440M genotypes (lane 5) matched with a healthy control (lane 1). Zymographic profile of lane 1 reflects that one identified in all 16 different sera collected from normal subjects. The latent, pro, and active forms of MMP-9 and the active form of MMP-2 are indicated on the left; molecular masses (kDa) are indicated on the right. (**b**) Densitometric analysis of MMPs. The intensities of zymographic bands have been measured and quantified by an image analysis software (IMAGEQUANT TL; Amersham Biosciences). The mean of each band ± SD is reported in a bar graph. The mean value of control is derived from normalized values of all 16 healthy controls. An arbitrary unit scale was used. *$p < 0.05$ control *vs* patients; ^$p < 0.005$ control *vs* patients; °$p = 0.1$ control *vs* patients. MADA, mandibuloacral dysplasia type A; MMP, matrix metalloproteinase; NGAL, neutrophil gelatinase B-associated lipocalin.

To characterize the presence of other MMPs in the serum, we performed Western blots employing antibodies against neutrophil collagenase (MMP-8) and collagenase-3 (MMP-13). Also in this case, we observed the two collagenases in all samples, and no significant differences were detected between MADA sera and control sera (Fig. 2b,c).

As shown in Fig. 3a, the values of total MMP-9 in sera from MADA patients range between 3.45 and 6.34 ng/ml, with a mean concentration of 4.586 ± 0.5296 ng/ml, and they are significantly higher ($p < 0.0001$) than the value of control serum (mean concentration 0.6925 ± 0.1335 ng/ml) (Fig. 3a). However, the values of total MMP-2 in sera from MADA patients were similar to the control ones (mean concentration 27.82 ± 3.993 *vs* 24.74 ± 1.104 ng/ml, $p = 0.3031$) (Fig. 3b). Interestingly, plasma level of

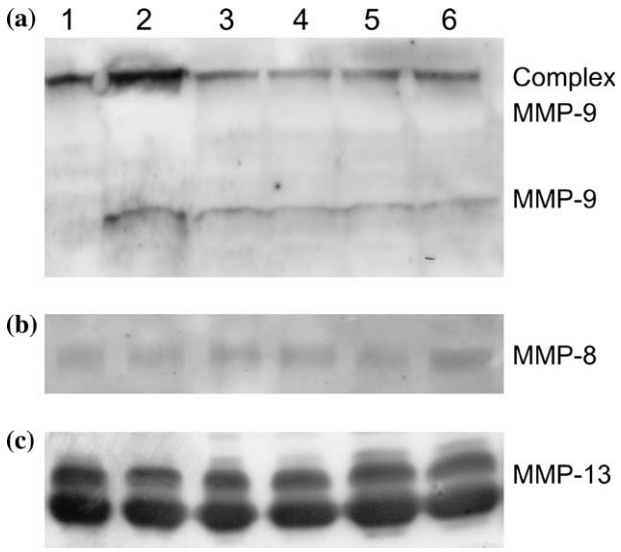

Fig. 2. (**a**) Western blot analysis of MMP-9 in total serum. MMP-9 was found primarily in its complex form in control sample (lane 1); complex and active MMP-9 were detected in mandibuloacral dysplasia type A (MADA) subject sample (lanes 2–5). (**b** and **c**) Representative Western blot of MMP-8 and -13 revealing the same pattern in all MADA and control serum samples. MMP, matrix metalloproteinase.

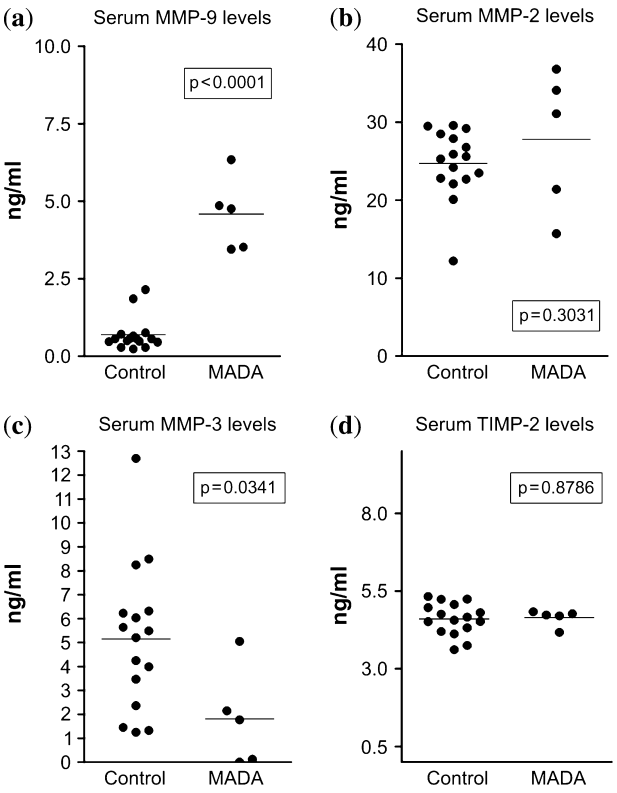

Fig. 3. Statistical analysis of the distribution of total serum level of MMP-9, -2, and -3 and TIMP-2 in MADA patients ($n = 5$) and healthy controls ($n = 16$) by enzyme-linked immunosorbent assay. The horizontal bars indicate the means. MADA, mandibuloacral dysplasia type A; MMP, matrix metalloproteinase; TIMP-2, tissue inhibitor of metalloproteinase 2.

MMP-3 in MADA patients was significantly lower compared with the normal control (mean concentration 1.818 ± 0.9151 *vs* 5.154 ± 0.7575 ng/ml, $p = 0.0341$) (Fig. 3c, Table 1). There were no statistically significant differences between age and gender.

Moreover, we compared circulating values of MMP-9 and -3 in MADA patients with levels detected in a consecutive series of patients with osteoporosis analysed according to the same analytical protocol. Interestingly, the latter group showed higher MMP-9 levels with respect to healthy control, as expected, but much lower levels (2.041 ± 0.3415 ng/ml) with respect to MADA patients. Furthermore, in osteoporotic patients, elevated MMP-3 (7.495 ± 0.9365 ng/ml) serum levels have been detected compared with the healthy control and MADA patients (data not shown).

The variation of enzymatic activity of MMP-9 in MADA patients outlined above was also compared with the amounts of tissue inhibitor of MMPs (i.e. TIMPs) and in particular TIMP-2. The total amount of TIMP-2 was found similar in all patients and controls, clearly indicating that the higher activity of MMP-9 was not related to a reduced level of natural inhibitors. The values, found by ELISA, ranged from 4.643 ± 0.1198 *vs* 4.605 ± 0.1281 ng/ml in all groups ($p = 0.8786$) (Fig. 3d). Normal levels of cytokines such as TNF-α, IL-6 and IL-1β have been detected in the serum of controls and MADA patients (TNF-α: 0.00934 ± 0.004 *vs* 0.015 ± 0.0113 pg/ml, $p = 0.7308$; IL-6: 3.683 ± 4.028 *vs* 1.855 ± 0.4824 pg/ml, $p = 0.2198$ and IL-1β: 0.3344 ± 0.6561 *vs* 0.5365 ± 0.9979 pg/ml, $p = 0.6$).

Discussion

Through a combination of biochemical assays, this report provides the first evidence that abnormalities in the maintenance of bone ECM homeostasis in MADA patients is associated to high levels of MMP-9 active form in patient's serum compared with controls. Even though this is not a proof of a prominent role played by MMP-9 in this process, it must be reminded that there is strong evidence that MMPs play a crucial role during osteogenesis and bone remodelling (32). Their synthesis by osteoblasts has been demonstrated during osteoid degradation prior to resorption of mineralized matrix by osteoclasts, and their activities are regulated by TIMPs (33).

The process of bone remodelling is complex and requires 'coupling' between osteoclastic and osteoblastic activities. Disruption of the balance between osteoclast and osteoblast activities is observed in various disease states such as osteoporosis and bone destruction by metastatic tumours. The central step of bone resorption is the removal of bone matrix by osteoclasts. In this context, MMP-9 may act not only as a solubilizer of bone matrix but also as a regulator of initiation of bone resorption and coupling to bone formation (34–40). This possibility is supported by several observations under different physiological/developmental situations and employing various experimental approaches, including proteinase inhibitor studies and transgenic mice. As a matter of fact, mice lacking MMP-9 accumulate hypertrophic cartilage within a growing region of their long bones and ultimately exhibit subtle bone shortening (41). Furthermore, upregulation of MMPs, in particular MMP-2 and -9, has been reported in many human bone-related disorders, as for instance several types of cancer-related osteolysis (42–47), osteoarthritis (48, 49), loosening of prosthesis (50), fracture healing (51), and periodontitis (52, 53). In particular, MMP-9 level was found elevated in arthritis, degrading non-collagen matrix components of the joints. MMP-9 is also active in the control of hypertrophic cartilage vascularization and skeletal development as well as in bone resorption (39, 54, 55). Thus, in recent years, both enzymes have received a great deal of attention as putative markers for clinical applications, that being mainly true for MMP-9, which is rarely expressed in adult human tissues, and it is almost always associated to inflammatory condition.

In our study, MADA serum levels of MMP-9 were markedly enhanced (4.7-fold over control level) ($p < 0.005$), showing a considerable activity of this gelatinase with respect to the control serum. We revealed, by zymography, the presence of one band corresponding to high-molecular weight form of MMP-9, disulphide-bonded homodimeric form (207 kDa), only in four of five (80%) MADA patients. This form has been identified in a variety of pro-MMP-9-producing cells including normal (56–58) and tumour (59, 60) cells and in various biological fluids (61, 62) and tissues (63), indicating that this is a physiological form of the enzyme. In addition, we were also able to detect the gelatinolytic band corresponding to the 116 kDa form representing the complex of MMP-9 with lipocalin (NGAL), which is usually present in neutrophil granules (56, 57). Probably their apparition could be linked to high serum levels of MMP-9 itself, as both these activity bands were undetectable in serum control. Accordingly, both bands were absent in one MADA patient, showing the minor level of total MMP-9 (Fig. 1a, line 6). However, the serum from MADA patients showed a slightly lower activity of the latent form of MMP-9 (i.e. pro-MMP-9) compared with control sera, suggesting that the activation process is more active in MADA patients than in healthy subjects.

We propose that such increase of MMP-9 in the serum of MADA patients indicates a participation of this enzyme to specific imbalance of the homeostasis process involving an altered bone ECM remodelling, increased bone resorption, and cartilage damage. This hypothesis is reinforced by the absence of alterations in MADA patients for serum levels of both other MMPs (i.e. MMP-2, -8, and -13) and several other inflammatory markers (i.e. TNF-α, IL-6, and IL-1β). Notably, well-known activators of MMP-9 (such as MMP-2 and -13) were in the normal concentration range, suggesting that the induced activity and expression of MMP-9 are not a consequence of the post-secretion activation mediated by these proteases (64). Similarly, no evidence for an incomplete inhibition through TIMPs was evident because the levels of TIMP-2 were detected at physiological levels. In addition, we have surprisingly found that the serum levels of stromelysin-1 (MMP-3) were significantly lower in MADA patients than in normal subjects. This is a very important observation because MMP-3 is one of the MMPs responsible for the activation of MMP-9, and the lower serum level of MMP-3 rules out the possibility that the increased activation of MMP-9 is a simple consequence of an enhanced activity of MMP-3. In this respect, this information strengthens the idea of a direct correlation between the enhanced activity of MMP-9 and the altered bone homeostasis, eliminating other indirect influences by the activity of other MMPs. This is a relevant assessment because it was previously suggested (65–68) that a high concentration and localization of MMP-3 in the joint tissues, such as cartilage and synovium, may contribute to cartilage degradation in patients with osteoarthritis. However, the inverse correlation between MMP-9 and -3 levels detected in MADA patients could reflect the presence in these patients of an inducible factor that regulates diametrically the expression of these enzymes. The identification of osteoclast-specific markers in these diseases is crucial in establishing clinical protocols using anti-resorptive drugs and to understand the genetic regulation of osteoclast development and function (69). Bone resorption in culture-derived osteoclasts is inhibited by three resorption

inhibitors: calcitonin, alendronate and an integrin antagonist (70). Bisphosphonates, for example form the mainstay of current therapy for a wide range of human bone diseases, including osteoporosis, rheumatoid arthritis, metastatic bone cancer and hypercalcaemia (71, 72). In this context, the availability of a disease-specific osteoclastic marker is considered to be required for a proper clinical trial using this class of drugs. Further development of *in vitro* assays for the direct assessment of osteoclast function in MADA cells will lead to improved clinical treatments for the bone disease observed in MADA patients, which constitute a major target of this complex disorder. To explore this further, it will be important to better define MMP-9 activation, inhibition, and deactivation processes in MADA cells. Such studies should shed additional light on the molecular mechanisms of laminopathies and enable to develop patient-appropriate therapies.

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