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Physiological matrix metalloproteinase concentrations in serum during childhood and adolescence, using Luminex® Multiplex technology

Kathryn M. Thrailkill[1,*], **Cindy S. Moreau**[1], **Gael Cockrell**[1], **Pippa Simpson**[1], **Rajiv Goel**[1], **Paula North**[2], **John L. Fowlkes**[1], and **Robert C. Bunn**[1]

1 *Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR, USA*

2 *Department of Pathology, University of Arkansas for Medical Sciences, Little Rock, AR, USA*

Abstract

Matrix metalloproteinases are a family of zinc-dependent proteinases which are involved in the breakdown and remodeling of extracellular matrix. As children grow and adolescents reach pubescence, their bodies undergo changes that require age-related morphogenesis of the extracellular matrix, possibly requiring unique patterns of matrix metalloproteinase (MMP) expression during periods of rapid tissue growth (i.e., childhood) or accelerated tissue remodeling and expansion (i.e., adolescence). Therefore, we have characterized age-specific and gender-specific differences in circulating concentrations of MMPs (specifically MMP-1, -2, -3, -8 and -9) in 189 serum samples obtained from healthy subjects, aged 2−18 years. MMP concentrations were measured using Fluorokine® MultiAnalyte Profiling kits and a Luminex® Bioanalyzer, as well as by commercial ELISA. Serum levels of MMP-1, -2, -3, -8, and -9 in healthy pediatric subjects represent log-normal distributions. MMP-2 was significantly negatively correlated with age ($r=-0.29$; $p<0.001$), while MMP-3 was significantly positively correlated with age ($r=0.38$; $p<0.001$). Although plasma, not serum, is considered the appropriate blood sample for measurement of MMP-8 and -9, serum levels of MMP-8 and -9 were also found to be highly positively correlated with each other ($r=0.76$; $p<0.01$). MMP results obtained by Fluorokine® MultiAnalyte Profiling methods correlated well with conventional ELISA methods and use of this technology provided several advantages over ELISA.

Keywords

collagenase; endopeptidases; gelatinase; particle-based flow cytometry; puberty; stromelysin

Introduction

Matrix metalloproteinases (MMPs) are an ever-expanding family of zinc-dependent endopeptidases involved in the breakdown, remodeling and physiological homeostasis of extracellular matrix (ECM) (1,2). Currently, there are over 20 human MMP-related enzymes, grouped into several subtypes, including collagenases, gelatinases, stromelysins, membrane-type MMPs (MT-MMPs) and other, less well-characterized, distinct enzymes (3). Targets of these powerful degradative enzymes include structural ECM molecules (i.e., interstitial collagens, laminins, fibronectins, and heparan sulfate proteoglycans), cell-cell adhesion molecules, cell surface receptors, growth factors, cytokines and chemoattractant proteins, as well as other proteinases (2). Because of their potent degradative capacity, the activity of these

*Corresponding author: Kathryn M. Thrailkill, MD, Arkansas Children's Hospital, 800 Marshall St., Slot 512−6, Little Rock, AR 72202, USA Phone: +1−501−364−1430, Fax: +1−501−364−6299, E-mail: thrailkillkathrynm@uams.edu.

enzymes is tightly regulated at all levels (i.e., transcription, post-transcription, secretion, protein processing and tissue localization). For example, proinflammatory cytokines such as interleukin-1β and tissue necrosis factor-α stimulate MMP production, while other cytokines, hormones, and growth factors such as transforming growth factor-β, interleukin-4, corticoid hormones and insulin-like growth factors (IGFs) down-regulate MMP synthesis (4).

To date, MMPs have been implicated in many pathologic processes characterized by either degradation of connective tissue matrices or pro-inflammatory states, such as rheumatoid arthritis and osteoarthritis, cancer growth and metastases, metabolic bone disease, emphysema and atherosclerotic heart disease (5–7). Serum or plasma levels of MMPs are altered in various diseases, and have been considered as potential clinical markers of disease activity. For example, in adult clinical studies, serum MMP-2 levels have been reported to be increased in patients with liver cirrhosis (8), malignant thyroid cancer (9), and endometriosis (10). Serum or plasma MMP-9 levels appear to be increased in patients with a diversity of cardiovascular related inflammatory conditions, including congestive heart failure (11), stroke (12,13), obstructive sleep apnea syndrome (14), and a history of myocardial infarction (15), as well as patients with amyotrophic lateral sclerosis (16), and acute allograft rejection following liver transplantation (17). Plasma levels of MMP-3 are increased in several connective tissue disorders, including rheumatoid arthritis (18) and systemic lupus erythematosus (18).

The first description of MMP-like activity was related to the metamorphosis of the tadpole (19). This and many other subsequent discoveries strongly suggest that this well-characterized family of metalloproteinases is also intimately involved in normal tissue growth and ECM remodeling (20). Morphogenesis and tissue growth, remodeling, and repair are sentinel features of childhood and adolescence. However, little is known about the production, secretion, and clearance of these important proteinases throughout normal growth and development in humans. At present, normal values for serum concentrations of commercially assayable MMPs are unavailable for the pediatric age range, limiting our ability to quantitatively compare pediatric disease-specific MMP abnormalities with MMP concentrations in healthy children. Furthermore, without such data, we have little or no understanding of which MMPs may be important in mediating events associated with somatic growth and development. Recognizing that tissue-specific production and/or localization of MMPs is potentially a dynamic process, with unique patterns of expression of these proteinases during periods of rapid tissue growth (i.e., childhood) or accelerated tissue remodeling and expansion (i.e., adolescence), the present study was developed to characterize potential age-specific and/or gender-specific differences in circulating concentrations of MMPs (specifically MMP-1, -2, -3, -8 and -9) across the pediatric and adolescence age range. Moreover, the intent of this study was to demonstrate the utility of a multiplexed, particle-based flow cytometric assay for the efficient, cost-effective simultaneous analysis of a panel of MMPs using a small sample volume.

Materials and methods

Residual serum samples (0.5−2.0 mL) were obtained from otherwise healthy children who were being tested (through the Arkansas Children's Hospital, Division of Pediatric Allergy and Immunology clinic) to exclude the possibility of a specific food allergy because of a past anecdotal coincidence of minor illness or urticaria following a particular food exposure. Residual serum samples were maintained only from those patients who were found to have a *negative* food allergy evaluation. Specifically, a total of 189 samples from children fulfilling the following inclusion criteria were obtained: 1) age 2−18 years; 2) normal serum total IgE level; and 3) negative radioallergoimmunosorbent (RAST) testing to one or more food categories (results from all tested categories had to be negative for inclusion). At the time of collection, patient age, sex, and race were recorded from the hospital database system by clinical laboratory personnel and samples were coded with a unique identifying number. Coded

serum samples, along with the coded demographic parameters, were then transported to the research laboratory, such that research personnel remained blinded to patient identifying information. Serum samples were stored at −20°C until batch analysis was performed. The experimental protocol was approved by the institutional Review Board of the University of Arkansas for Medical Sciences. The requirement for informed consent of subjects was waived for this protocol, recognizing that an informed consent document, as a means of identifying subjects, would provide an exclusive, unnecessary risk to subject confidentiality.

MMP-1, MMP-2, MMP-3 and MMP-8 were measured simultaneously, while MMP-9 was measured separately in individual human serum samples using Fluorokine® MultiAnalyte Profiling (F-MAP) kits from R&D Systems (Minneapolis, MN, USA). Kits were run on a Luminex® 100™ Bioanalyzer (Luminex Corp., Austin, TX, USA) according to the kit manufacturer's instructions. Fluorokine® MultiAnalyte Profiling kits contained distinct groups of microspheres (each group bearing unique fluorescence intensity and a specific MMP antibody), biotinylated MMP antibodies, and phycoerythrin-conjugated streptavidin. Serum samples were incubated with antibody-coated microspheres, which bind to specific MMPs present in the serum. Next, microsphere-MMP complexes were washed and incubated with biotinylated MMP antibodies, which bind to MMPs present on the microspheres. A final incubation was performed in which phycoerythrin-labeled streptavidin was allowed to bind to biotinylated MMP antibodies present on microspheres. Microspheres were then loaded into a Luminex® 100™ Bioanalyzer, which quantifies the amount of phycoerythrin fluorescence present on each of the distinct microsphere groups. At least 50 individual microspheres were counted for each MMP, and the median fluorescence intensity was used for subsequent calculations. Intra-assay (precision within an assay; one sample replicated eight times) and inter-assay precision (precision between different assays; four samples each replicated on 4 different days) were: 5.2% and 7.5% for MMP-1; 4.1% and 9.9% for MMP-2; 3.6% and 10.3% for MMP-3; 8.4% and 16.4% for MMP-8; and 3.1% and 8.7% for MMP-9, respectively. Each of the microsphere sets was reported by the manufacturer to exhibit less than 0.5% cross-reactivity and interference with the other MMP family members studied.

A unique subset of 40 samples per MMP analyte was also assayed for MMP-2, -3, -8 and -9 by commercial ELISA to establish the reliability of the Fluorokine® MultiAnalyte Profiling (F-MAP) method compared with the more standard and popular method of measuring MMPs. Specifically, the R&D Systems Quantikine colorimetric sandwich ELISAs that use this manufacturer's specific MMP polyclonal antibodies were employed. (The R&D Systems MMP-1 ELISA measures pro-MMP-1, rather than total MMP-1, making a similar F-MAP vs. ELISA comparison for MMP-1 inappropriate.) These 40-sample subsets were randomly generated and unique for each analyte, representing a distribution of four samples from each decile across the assay range for each MMP (MMP-2, -3, -8 and -9), as obtained by F-MAP.

Statistical analysis

Limited demographic data obtained from the medical record (age, sex, and race) were recorded into an SPSS (SPSS 12.0 for Windows; SPSS, Chicago, IL, USA) data set. Exploratory data analysis, including summary statistics (mean, median, range, frequency tables) and plots (scatter plots, boxplots) were used to examine the distribution of and relationship between variables. Subjects were grouped according to patient age into the following cohorts: 2 to ≤8 years (pre-pubertal); 8 to ≤10 years (representing ~Tanner stage 2 puberty); 10 to ≤12 years (~Tanner stage 2−3 puberty); 12 to ≤15 years (~Tanner stage 4 puberty); and 15 to ≤18 years (~Tanner stage 5 puberty). Data were compared for each gender and age cohort separately. In addition, data were analyzed using age as an independent variable. A priori, it could be expected that MMP measurements would be skewed. The size of the standard deviation values also suggested that this was the case. Therefore, logarithms of the MMPs were used for analysis.

The distribution of each MMP was examined using histograms. We compared MMP results between age groups, gender and race using ANOVA with a post hoc Tukey test. Type III sums of squares were used. Correlations were examined using Pearson correlations and scatter diagrams. To further aid interpretation, smooth splines were fit showing variation over age groups (Figure 3).

Results

A total of 189 samples were obtained. Study subjects ranged in age from 2 to 18 years, with a mean±standard error (SE) age of 9.27±0.34 years. The study population included 88 females (46.6%) with a mean age of 9.94±0.50 years and 101 males (53.4%) with a mean age of 8.69 ±0.45 years. Samples provided a racial distribution for subjects of 80.4% Caucasian, 16.9% African-American, 1.6% Hispanic and 1.1% other/unspecified. Subjects were sub-classified according to age, with the sample size of each age cohort as follows: 2 to ≤8 years, n=79; 8 to ≤10 years, n=29; 10 to ≤12 years, n=22; 12 to ≤15 years, n=34; and 15 to ≤18 years, n=25.

A summary of MMP results for the entire subject group is shown in Table 1. The manufacturer's expected values (for an adult population) for each analyte are also shown. Analysis of histograms of individual MMP results (Figure 1) suggests that serum levels of MMPs in healthy children and adolescents represent log-normal distributions.

MMP-1 (collagenase-1), MMP-8 (collagenase-2) and MMP-9 (gelatinase B) results were not significantly different from normal ranges reported for these analytes in adult subjects (Table 1). In addition, there was no significant difference among MMP-1, MMP-8 or MMP-9 results for different age groups ($p>0.20$) when the population was examined as a whole. Analysis of MMP-9 results by gender-race subgroups yielded no statistically significant differences (data not shown). However, mean serum levels of MMP-8 in African-American males were generally higher than MMP-8 levels in other sex-race combinations, and statistically significantly higher than MMP-8 results for Caucasian females (data not shown; $p\leq0.001$). MMP-1 results for Caucasian males in the 8−10-year age group were statistically significantly higher than MMP-1 results for Caucasian males in the 12−15-year age group (data not shown; $p<0.05$).

Two MMPs, MMP-2 (gelatinase A) and MMP-3 (stromelysin-1), were correlated with age. Specifically, MMP-2 concentrations were significantly negatively correlated with age (Figure 2; $R^2=0.086$, $p<0.001$) and when analyzed across the age subsets, MMP-2 results in the 2−8-year age group were statistically higher than MMP-2 results in the 15−18-year group ($p<0.05$). Overall, the mean MMP-2 result for all pediatric subjects was ~47% higher than the normative mean value reported for adults (Table 1). When analyzed across specific age groups and stratified according to race and gender, serum MMP-2 results suggest a slight increase in MMP-2 concentration coincident with the expected age of early puberty, occurring earlier in African-American females than in other gender-race combinations (Figure 3) (21).

In contrast, MMP-3 concentrations were significantly positively correlated with age (Figure 4; $R^2=0.148$, $p<0.001$). When examined by individual age groups, mean MMP-3 results in the 15−18-year age group were statistically significantly higher than mean MMP-3 results in other age groups ($p<0.05$). Moreover, the mean MMP-3 result for all pediatric subjects was ~48% lower than the normative mean value reported for adults (Table 1). In contrast to results reported for adults (18), when evaluated by gender, there was no significant difference among serum MMP-3 results for males vs. females at any age.

MMP-8 (collagenase-2) and MMP-9 (gelatinase B) are both synthesized by differentiating granulocytes, stored in circulating neutrophils, and released following neutrophil activation

(3). Therefore, serum levels of these MMPs are thought to be influenced by release of MMPs by degranulation of neutrophils during ex vivo blood clotting in the test tube (22). It is significant, therefore, that serum levels of MMP-8 and MMP-9 were highly positively correlated (Figure 5; R^2=0.488; p<0.001).

Assay characteristics

MMP results generated by F-MAP and by ELISA were highly positively correlated for MMP-3 (R^2=0.97; p<0.001), MMP-8 (R^2=0.96; p<0.001) and MMP-9 (R^2=0.87; p<0.001; Figure 6). MMP-2 results generated by F-MAP and by ELISA were less tightly correlated (R^2=0.45; Figure 6).

Discussion

We have previously identified and characterized *urinary* MMP excretion in pediatric patients and demonstrated that *urinary* excretion of MMPs is age-specific, with unique alterations detected during puberty (23). These data, as well as the work of others, suggest that regulation of MMPs during puberty is very likely distinctive and reflective of exuberant ECM expansion and tissue remodeling during pubertal growth and sexual maturation. For example, high concentrations of type I collagen breakdown products are detected in the urine of adolescents, and these concentrations correlate positively with growth rate during the pubertal growth spurt and with gender, being higher in females than in males (24). Because MMPs are the principal mediators of collagen turnover, it is important to study the temporal changes in MMP concentrations throughout childhood and adolescent development, both as a reference source for existing and future pediatric studies of disease-related MMP dysregulation, and to aid in our understanding of the role of MMP activity in normal ECM physiology.

To the best of our knowledge, this report is the first demonstration of the clinical utility of the F-MAP method for simultaneously measuring multiple MMP values in a single, specifically pediatric, serum specimen. Using this technology, we have demonstrated that serum values of MMP-2 and MMP-3 are age-dependent, and differ significantly from commercial normative ranges for these analytes for adult populations. In addition, similar to our previous study demonstrating an increase in MMP-2 concentration in urine during the pubertal years (23), MMP-2 data were suggestive of an early to mid-pubertal increase in serum MMP-2 values. In contrast, we did not identify an age correlation for either MMP-8 or MMP-9. However, recognizing that serum levels of these MMPs reflect release of MMPs following neutrophil degranulation, it is possible that any age correlation was obscured by this effect, and would require analysis of plasma specimens. In addition, caution should be exercised in interpreting serum values of MMP-8 and MMP-9, as plasma is considered the preferred blood sample for measurement of these proteases.

While this subject population represented a presumed healthy pediatric and adolescent cohort, the limited demographic information obtained at the time of specimen collection, as well as the blinded nature of these specimens, makes it possible that this assumption was not uniformly accurate. In addition, it is possible that other variables inherent in a pediatric population beyond age, race and sex (i.e., changing body mass index, timing of puberty, minor concurrent illness) would influence MMP results, but could not be factored into this analysis due to the nature of the study design. Nevertheless, the large number of samples available for analysis should provide a representative assessment of normative data and dampen any confounding variables.

Because MMPs are produced by many tissues, it is likely that the serum concentrations measured reflect a variety of sources. For example, the balanced regulation of MMPs and their naturally occurring inhibitors (tissue inhibitors of metalloproteinases, TIMPs) has been shown to be a critical component of many normal physiologic processes, including cartilage

development and endochondral bone formation (25–27), normal bone turnover and repair (28,29), reproductive tissue ECM remodeling during puberty and pregnancy (30–32), menstruation (33), growth of mammary tissues and lactation (34), testicular development (35) and normal spermatogenesis (35). Notably, many of these physiologic processes become active during puberty or are augmented during the pubertal growth spurt. Moreover, pubertal hormones, specifically growth hormone and IGFs (36–39), androgens (40) and estrogen (41, 42), have all been shown to regulate MMP expression and/or secretion.

For these studies, we investigated the use of a new methodology to measure multiple MMPs in a single sample of serum. We utilized a Luminex system-based assay that uses a 10×10 bead matrix, in which each bead set is made up of a specific proportion of red/orange fluorescent dye. The choice of this assay has several advantages over conventional ELISA techniques to measure MMPs. First, it is capable of simultaneously measuring multiple MMPs in a single biological sample, thereby eliminating significant operator error in sampling (e.g., pipetting) which is possible with multiple MMP analyses. Second, smaller sample volumes are required for multiplexing; thus, this technology is more amenable to the pediatric population, in which blood volumes may be restricted. Third, our data suggest that when serum is analyzed there is a high degree of correlation between conventional ELISAs and the multiplex assay for MMP-3, MMP-8, and MMP-9; however, comparison of absolute values between assay techniques may not be valid. Finally, the assays are robust and capable of measuring each MMP over a wide range of values. Other considerations not directly addressed by our study, but which might also make this approach more attractive than ELISA, are that the fluorescent readout is more direct, stable, and sensitive than colorimetric methods used for ELISAs, and typically, if measuring more than six analytes, the multiplex approach can be less expensive (43). Thus, the overall evaluation of this new technology to assay MMP concentrations in human serum shows it to be reproducible, specific, sensitive, and robust.

In conclusion, particle-based flow cytometry, specifically, F-MAP techniques for measurement of serum concentrations of MMP-1, -2, -3, -8, and -9, has effectively demonstrated age-specific differences in the serum concentrations of specific MMPs. These findings suggest that care should be exercised in interpreting the role of MMPs in the pathophysiology of various childhood diseases unless critical pediatric normative data are available and utilized for comparison.

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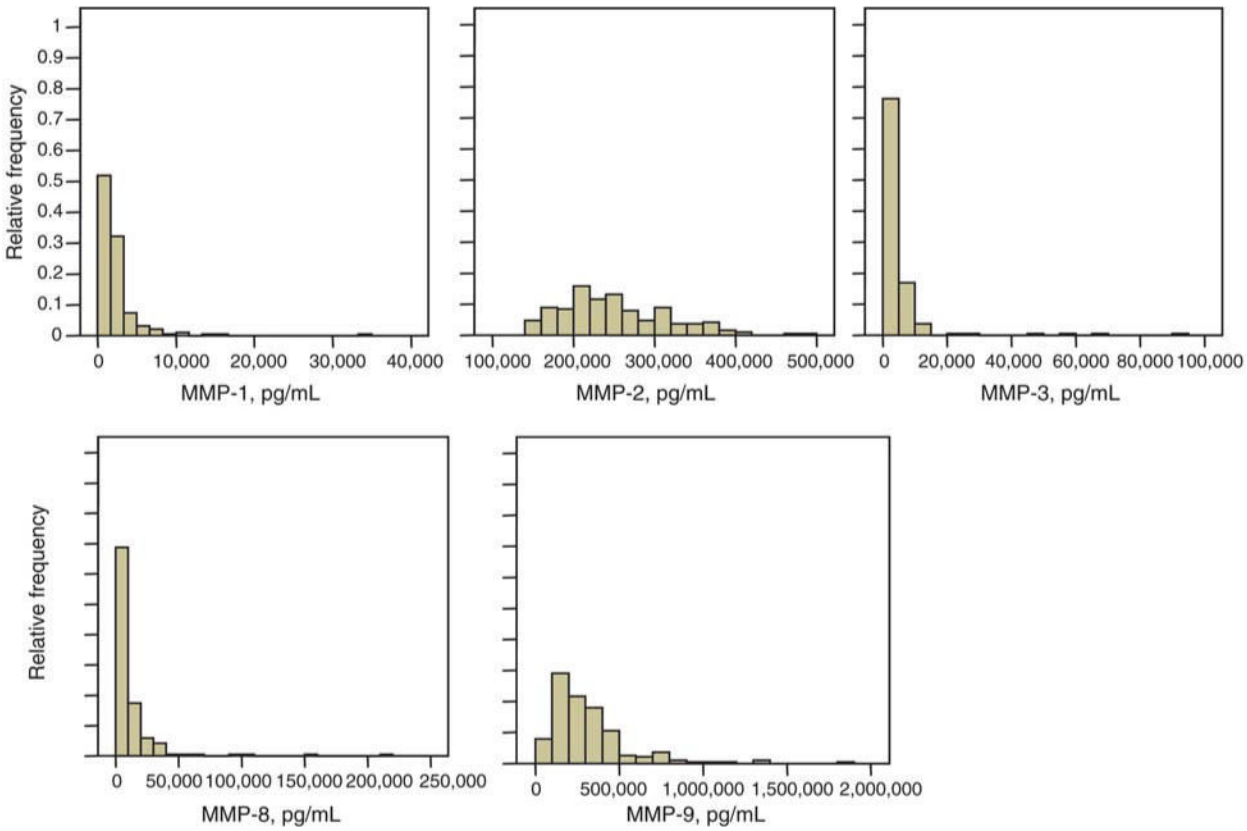

Figure 1.
Serum levels of MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9 in healthy pediatric subjects. MMP results for each analyte are presented as a histogram of relative frequency vs. MMP concentration in pg/mL.

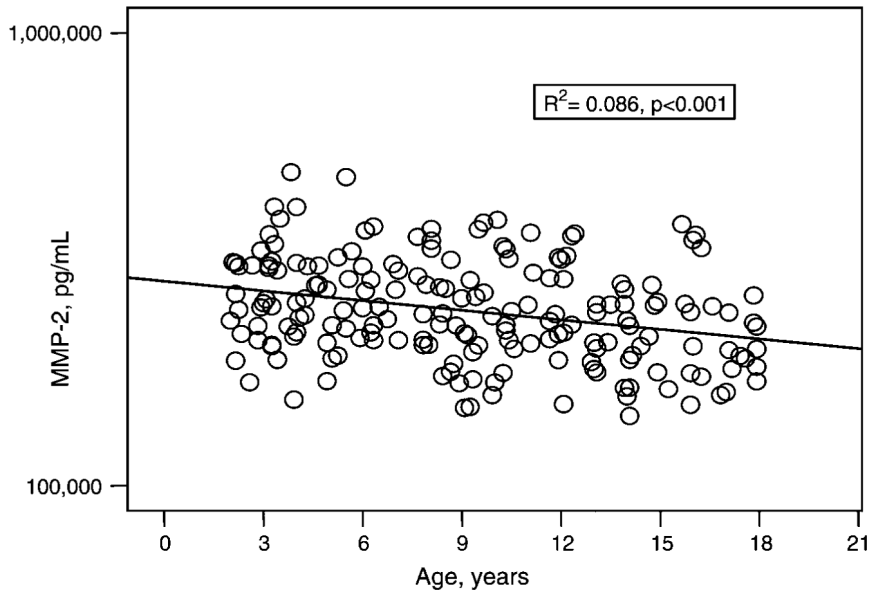

Figure 2.
Serum concentrations of MMP-2 (pg/mL) for 189 healthy pediatric subjects, aged 2−18 years. MMP-2 concentrations were significantly negatively correlated with age (R^2=0.086, p<0.001).

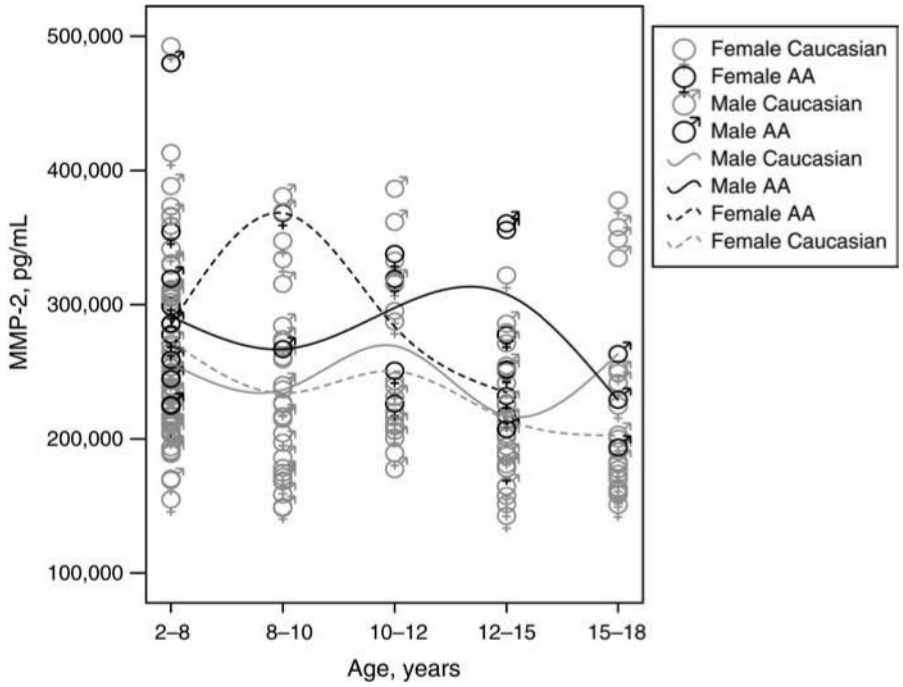

Figure 3.
Serum concentrations of MMP-2 (pg/mL) for Caucasian and African-American (AA) females and males by age group. For each sex-race combination, a slight increase in MMP-2 concentration coincident with early puberty was noted.

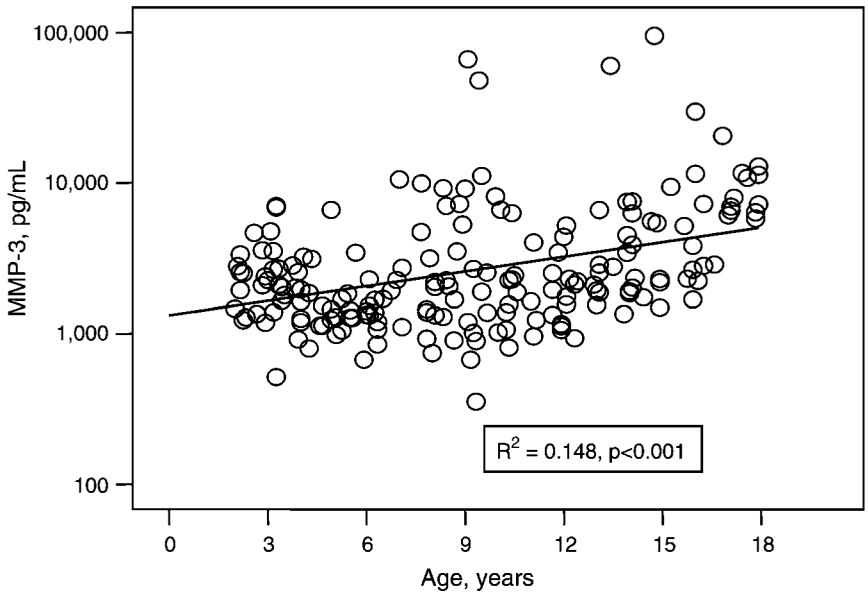

Figure 4.

Serum concentrations of MMP-3 (pg/mL) for 189 healthy pediatric subjects, aged 2−18 years. MMP-3 concentrations were significantly positively correlated with age (R^2=0.148, p<0.001).

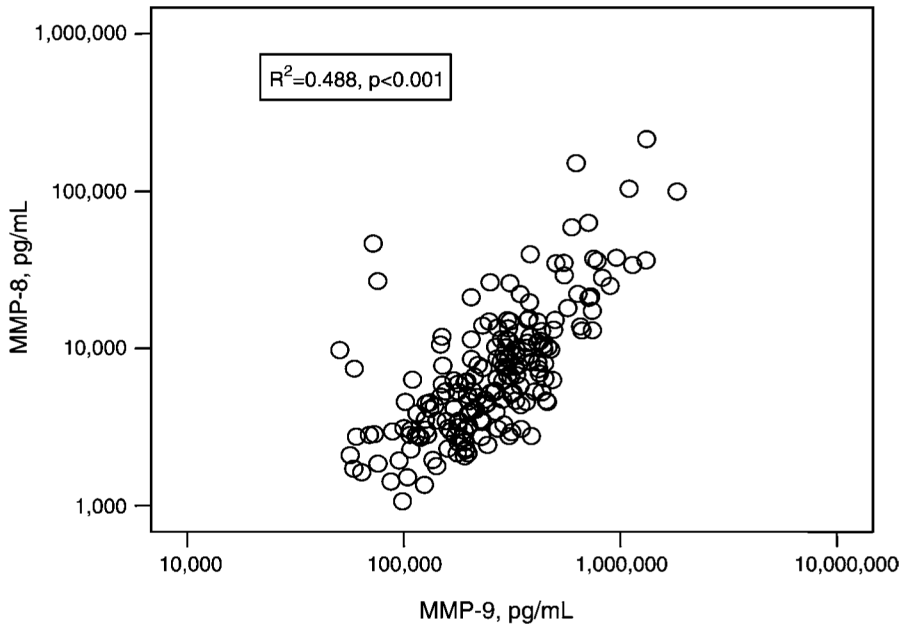

Figure 5.
Correlation between serum concentrations of MMP-8 and MMP-9 (pg/mL) in 189 healthy pediatric subjects, aged 2−18 years. Serum levels of MMP-8 and MMP-9 are highly positively correlated (R^2=0.488, p<0.001).

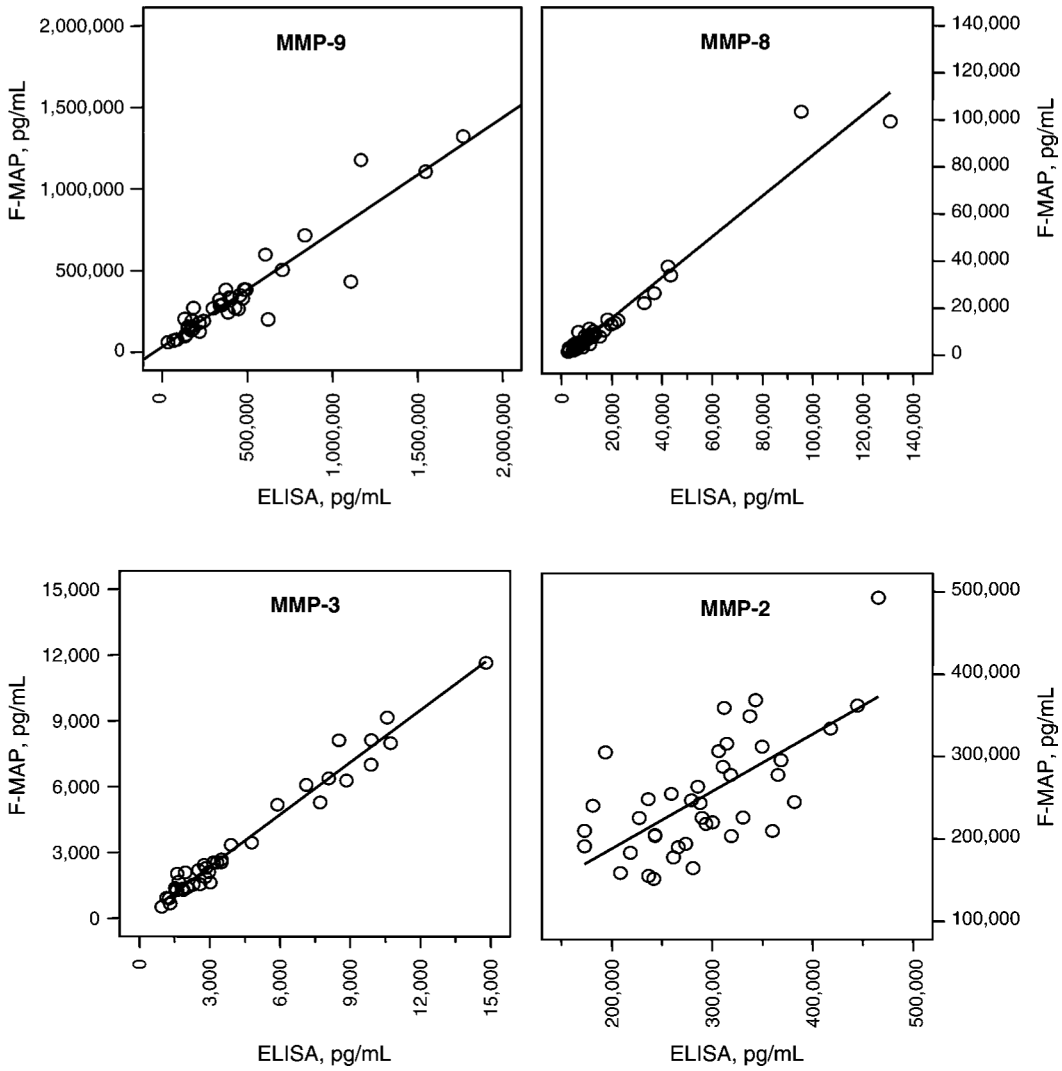

Figure 6.

Correlations between MMP results generated by Fluorokine® MultiAnalyte Profiling and MMP results generated by ELISA: MMP-3, R^2=0.97, p<0.001; MMP-8, R^2=0.96, p<0.001; MMP-9, R^2=0.87, p<0.001; MMP-2, R^2=0.45.

Table 1

Descriptive statistics.

Analyte	Concentration, pg/mL					
	Pediatric data				Expected values*	
	Minimum	Maximum	Mean	SD	Range	Mean
MMP-1	109.49	34,335.60	2421.74	3199.40	439−6934	2702
MMP-2	142,555.95	492,568.83	251,389.36	67,149.97	36,462−264,400	169,963
MMP-3	353.93	94,744.06	4788.66	10,175.62	2792−26,170	9132
MMP-8	1065.54	214,190.82	12,381.61	22,622.96	1391−79,021	16,611
MMP-9	50,591.69	1,829,283.54	318,816.19	248,005.96	85,406−1,638,927	441,732

*Adult normal range and mean according to the manufacturer.