

Correlation of MMP-3 and MMP-9 with Crohn's Disease Activity in Children

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Abstract

Background Recently published data indicate that the inflammation in Crohn's disease (CD) may be accompanied by elevated levels of matrix metalloproteinases.

Aims The goals of the present study were the estimation of MMP-3 and -9 concentrations in sera of children with Crohn's disease, the examination of correlation between the concentrations of MMP-3 and -9 and clinical activity of the disease in the relation to the control group and the evaluation of the utility of MMP-3 and -9 concentration measurements as markers of disease activity.

Methods Serum concentrations of MMP-3 and -9 were estimated in 82 children (45 CD patients divided into severe, moderate and mild subgroups; 37 controls) and correlated with disease activity estimated by the Pediatric Crohn's Disease Activity Index (PCDAI), CRP, seromucoid and ESR.

Results Mean MMP-3 concentrations were: 2.49 ng/ml (95% CI: 1.76–3.52) for mild, 16.44 ng/ml (95% CI: 10.34–26.15) for moderate, 5.25 ng/ml (95% CI: 2.73–10.11) for severe CD and 1.95 ng/ml (95% CI: 1.53–2.48) for the control group (differences between all three groups were statistically significant; $P < 0.001$). Median MMP-9 concentrations were: 2.14 ng/ml (95% CI: 0–8.9) for mild, 14.21 ng/ml (95% CI: 4.53–21.48) for moderate, 42.2 ng/ml (95% CI: 5.74–61.27) for severe CD and 1.3 ng/ml (95% CI: 0.7–2.18) for the control group. MMP-9 concentrations in

moderate and severe CD differed from the concentrations in mild CD ($P = 0.002$) and control group ($P = 0.0001$). MMP-3 concentration significantly correlated with MMP-9, PCDAI and ESR, while MMP-9 concentration significantly positively correlated with MMP-3, PCDAI, and CRP. Diagnostic utilities of the tests were: MMP-3 accuracy 75%, positive likelihood ratio (LR+) = 4.11 and negative likelihood ratio (LR-) = 0.51, sensitivity 56%, specificity 87%, Youden index 0.43; for MMP-9, accuracy 73%, LR+ = 5.14 and LR- = 0.50, sensitivity 56%, specificity 89%, Youden index 0.45; and for CRP, accuracy 74%, LR+ = 8.56 and LR- = 0.54, sensitivity 49%, specificity 94%, Youden index 0.43.

Conclusions MMP-9 serum concentration increasing along with the activity of the disease, exhibiting high specificity and correlating well with the indices of inflammation might be of better usefulness in the prediction of CD activity status in children than MMP-3.

Keywords Inflammatory bowel disease · Crohn's disease · Children · Metalloproteinases · PCDAI

Introduction

Matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases participating in the rearrangement and degradation of extracellular elements of connective tissue [1, 2]. The activity of metalloproteinases is under multilevel regulation, and their physiological equilibrium is tightly balanced by the interactions between activating factors and inhibitors, among which are tissue inhibitors of metalloproteinases (TIMPs) [2, 3]. A disturbance in this balance as well as in the formation and degradation of MMPs results in the enhancement of

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synthesis or destruction of extracellular matrix components. The changes in MMPs concentration reflecting the state of their activity have been demonstrated in many pathological processes such as cardiovascular disease [4], rheumatoid arthritis [5], initiation and progression of various cancers [6–11], and initiation and maintenance of inflammatory processes including inflammatory bowel disease (IBD) [12–14]. The destruction and rearrangement of intestinal tract wall observed in IBD raises questions as to the role which MMPs could play in the etiopathogenesis of IBD. Experimental models of induced bowel inflammation have demonstrated the elevation of MMPs in the tissues affected by pathological processes [15, 16]. A growing body of evidence, coming from both animal and human studies, points towards MMP-3 and -9 as the key players in the loss of intestinal epithelium integrity observed in the development of IBD [2, 12, 15, 17–20]. Therefore, in the face of the increasing incidence of IBD [21–24], a possibility of modulation and monitoring the disease process by an influence on the activity of these zinc endopeptidases is gaining increasing attention [2, 16, 19, 25–27].

However, most of the studies conducted so far are dealing with the changes of the metalloproteinases on the tissue level and there are only scarce reports indicating whether these changes are reflected on the systemic level, and on the possibility of diagnostic usefulness of metalloproteinases in IBD management.

The goals of the present study were: the estimation of MMP-3 and -9 concentrations in sera of children with Crohn's disease, the examination of correlation between the concentrations of MMP-3 and -9 and clinical activity of the disease in the relation to the control group and the evaluation of the utility of MMP-3 and -9 concentration measurements as markers of disease activity.

Materials and Methods

The study was conducted prospectively and comprised 45 patients with recognized CD, aged from 2 to 18 years (mean age 12.69) treated in the Department and Clinic of Paediatrics, Gastroenterology and Nutrition of Wrocław Medical University in the years 2007–2008. The disease was diagnosed on the basis of the Porto criteria [28], which included physical examination, ultrasound studies of the abdomen, endoscopic studies of the alimentary tract, serial radiologic studies, and histopathological studies of mucosal specimens of large intestine and biochemical blood tests. Based on Montreal criteria [29] in a majority of patients ileocaecal and large intestine types of CD were recognized. The patients presented type B₁ (nonstricturing, nonpenetrating) with the exception of one child with type B₂

(stricturing). For disease activity assessment, the Pediatric Crohn's Disease Activity Index (PCDAI) proposed by Hyams et al. [30, 31] was applied. The PCDAI includes subjective patient reporting of symptoms, physical examination, nutritional parameters and several common laboratory tests results (hematocrit, erythrocyte sedimentation rate, albumin). On the basis of calculated index the patients were classified into three groups: severe (≥ 51), moderate (26–50) and mild (≤ 25). The children were treated with 5'-aminosalicylate (5-ASA), azathioprine or prednisone. None of the children included in the study received biological treatment (infliximab, adalimumab), which inhibits TNF- α and therefore might influence the level of MMPs. A control group consisted of 37 children aged from 1 to 18 years (mean age 10.1), hospitalized in the Clinic, in whom organic diseases as well as metabolic disorders were excluded on the basis of diagnostic procedures. The children included in the control group were diagnosed with growth failure and functional disorders such as functional dyspepsia, irritable bowel syndrome and functional constipation. Characteristics of the study population are presented in Table 1.

Blood samples were drawn by venous puncture in a fasting state in the morning of admission to the hospital before a treatment was started or intensified (i.e. corticosteroids administration). Sera were obtained from clotted (30 min., room temperature) and centrifuged (15 min, 1,500 \times g) blood. Serum samples were stored at -80°C until analysis.

MMP-3 and -9 concentrations were estimated by enzyme double-antibody indirect immunoassays with Quantikine Human Total MMP-3 Immunoassay and Duo-Set Human MMP-9/TIMP-1 Complex provided by R&D Systems (Minneapolis, MN, USA) in accordance with manufacturer protocol.

Data on C-reactive protein (CRP), seromucoid and erythrocyte sedimentation rate (ESR) were retrieved from patients' medical records.

Statistical Analysis

Serum concentrations of MMP-3 had normal distribution after log-transformation, while these of MMP-9 had the skewed distribution as analyzed with D'Agostino–Pearson test for normality. Therefore, the average concentrations of MMP-3 are presented as means and MMP-9 as median values, both accompanied by 95% confidence interval (95% CI). Homogeneity of variances was tested with Levene's test. The significance of differences between groups was examined with *t* test for independent samples with Welch correction if necessary or Mann–Whitney *U* test with respect to data distribution. Correlation analysis was conducted with Spearman or Pearson's test with respect to data

Table 1 Characteristics of the study group

Group	Disease activity	PCDAI	Number of children	Gender		Age (years)	
				Females	Males	Range	Mean
CD group	Severe	55 (52.5–60) ^a	9	9	0	2–16	12.6
	Moderate	30 (27.5–32.3) ^b	13	7	6	7–18	13.8
	Mild	7.5 (5–11.6) ^c	23	10	13	2–18	10.6
	Total		45	26	19	2–18	11.9
Control group			37	23	14	1–18	10.1

CD Crohn's disease, PCDAI pediatric Crohn's disease activity index, PCDAI expressed as median values accompanied by 95% CI. Values with the same superscripts do not differ statistically

type and distribution. In case of association between MMP-3 and PCDAI, due to nonlinear distribution of data, a regression analysis was applied [$y = -1.1652 + 0.9324x + (-0.01285x^2)$, where $y = \text{MMP-3}$, $x = \text{PCDAI}$] and a coefficient of determination (R^2) calculated. Tukey test was used for the detection of outliers. Differences in incidence rates were analyzed with Fisher's exact test. All tests were two-sided and P values ≤ 0.05 were considered significant.

The diagnostic values of MMP-3, -9 and CRP were evaluated using receiver operating characteristic (ROC) curves analysis. The overall performance was expressed as the area under ROC curve (AUC) with 95% CI and P statistics for the difference between calculated AUC and AUC = 0.5 (marker without discriminative power). Cut-off values corresponding to the highest accuracy were determined and the related sensitivities and specificities together with likelihood ratios for positive and negative results (+LR and -LR) were calculated. Youden index (sensitivity + specificity - 1), a summary measure, was calculated as well. Statistical analysis was conducted with MedCalc[®] version 11.4.4.0 statistical software.

Ethical Considerations

The study protocol was approved by the Medical Ethics Committee of Wroclaw Medical University, Wroclaw, Poland and the study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Results

Metalloproteinase Concentrations and Disease Activity Stage

Both MMP-3 (4.99 ng/ml, 95% CI: 3.54–7.01) and MMP-9 (7.15 ng/ml, 95% CI: 3.09–14.07) concentrations in the sera of patients suffering from Crohn's disease were higher than those in the control group (1.95 ng/ml, 95% CI:

1.53–2.48, $P < 0.0001$ and 1.30 ng/ml, 95% CI: 0.70–2.18, $P < 0.01$, respectively). However, our study showed different profiles of MMP-3 and -9 concentrations in sera of children suffering from CD in different clinical activity stages of CD. MMP-3 concentration was increasing up to the moderate stage of the disease with a subsequent decline in the severe stage (Fig. 1). The concentrations of MMP-3 in the severe and moderate groups were statistically higher than in the mild and control groups ($P < 0.001$). There were no statistical differences between mild and control groups.

In contrast to MMP-3, MMP-9 concentrations were increasing along with the disease activity (Fig. 2). There were statistically significant differences between both the moderate and severe groups and a mild group ($P = 0.002$). Concentrations in the severe group exhibited the highest values; however the differences between severe and moderate groups did not reach statistical significance. Concentration of MMP-9 in the moderate and severe groups differed from those in the control group ($P = 0.0001$).

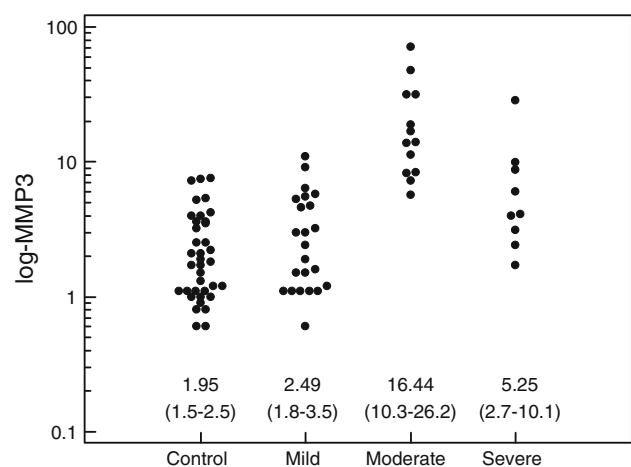


Fig. 1 MMP-3 concentration in serum with respect to the status of Crohn's disease activity in children. Closed circles represent MMP-3 concentration (ng/ml) in individual serum samples. Geometric mean values accompanied by 95% CI are given for each group

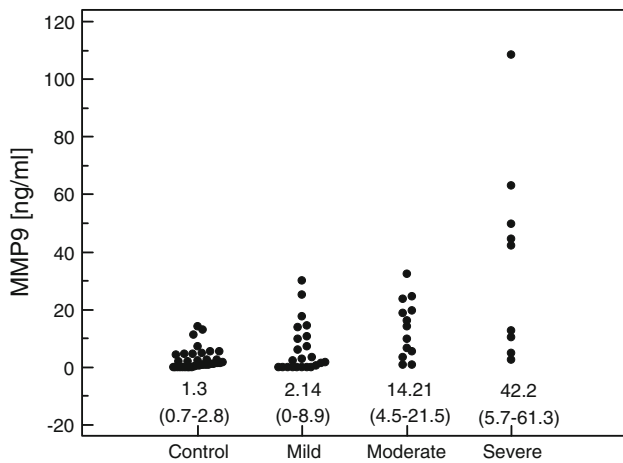


Fig. 2 MMP-9 concentration (ng/ml) in serum with respect to the status of Crohn's disease activity in children. Closed circles represent MMP-9 concentration in individual serum samples. Median values accompanied by 95% CI are given for each group

Correlations Between MMP-3, -9 and the indices of Crohn's Disease

Concentrations of both metalloproteinases correlated with each other (Table 2). There was a significant correlation between the concentrations of MMP-3 and disease activity score (PCDAI) and ESR. MMP-3 correlated also with seromuroid ($r = 0.39, P = 0.009$); however after the implementation of Tukey test for outliers the correlation lost its significance ($r = 0.23, P = 0.125$). A weak, but not statistically significant correlation between MMP-3 concentrations and CRP was observed ($\rho = 0.19, P = 0.183$).

MMP-9 on the other hand correlated fairly well with PCDAI, CRP and seromuroid (Table 2). There was also a weak, statistically not significant correlation between MMP-9 and ESR ($\rho = 0.26, P = 0.088$).

There was a strong positive correlation between PCDAI and CRP, ESR and seromuroid (Table 2).

Performance of MMP-3 and -9 as Disease Markers

ROC analysis revealed that MMP-3 concentration indicated CD presence with 75% accuracy using the concentration of 4.2 ng/ml as an optimal cut-off value for discrimination between patients with CD and controls (95% CI: 0.642–0.839, $P < 0.0001$). The likelihood ratios of positive and negative results obtained on the basis of optimal threshold values specific for CD were as follows: +LR = 4.11 and -LR = 0.51 with sensitivity and specificity equal to 56 and 87%, respectively. Youden index was 0.43.

In case of MMP-9 the calculated cut-off value for discrimination between patients with CD and controls was 5.58 ng/ml and using this value the diagnostic accuracy of MMP-9 was 73% (CI: 0.819–0.821, $P < 0.001$). The likelihood ratios of positive and negative results obtained on the basis of optimal threshold values specific for CD were as follows: +LR = 5.14 and -LR = 0.50 with sensitivity and specificity equal to 56 and 89%, respectively. Youden index was 0.45.

We also evaluated diagnostic utility tests for CRP in the cohort of our patients. Cut-off value was estimated as 4.3 mg/l. Using this value, diagnostic accuracy was 74% (95% CI: 0.63–0.83, $P < 0.0001$). The likelihood ratios of positive and negative results obtained on the basis of optimal threshold values specific for CD were as follows: +LR = 8.56 and -LR = 0.54 with sensitivity and specificity equal to 49 and 94%, respectively. Youden index was 0.43.

Discussion

To the best of our knowledge our study demonstrates for the first time a correlation between Crohn's disease activity in children and MMP-3 and -9 concentrations in serum.

Table 2 Associations between MMP-3 and -9 and Crohn's disease indices

Metalloproteinases	MMP-3	MMP-9	PCDAI	CRP	ESR	Seromuroid
MMP-3		$\rho = 0.43^*$ $P = 0.003$	$R^2 = 0.174^*$ $P = 0.018$	$\rho = 0.19$ $P = 0.183$	$r = 0.32^*$ $P = 0.035$	$r = 0.23^a$ $P = 0.1253$
MMP-9			$\rho = 0.411^*$ $P = 0.005$	$\rho = 0.45^*$ $P = 0.002$	$\rho = 0.26$ $P = 0.088$	$\rho = 0.42^*$ $P = 0.004$
PCDAI				$\rho = 0.73^*$ $P < 0.0001$	$\rho = 0.73^*$ $P < 0.0001$	$\rho = 0.68^*$ $P < 0.0001$

PCDAI pediatric Crohn's disease activity index, CRP C reactive protein, ESR erythrocyte sedimentation rate

ρ Spearman correlation coefficient, r Pearson correlation coefficient, R^2 coefficient of determination [$y = -1.1652 + 0.9324x + (-0.01285x^2)$, where $y = \text{MMP3}, x = \text{PCDAI}; F = 4.41$]

* Statistically significant correlations

^a Tukey test

Local MMPs concentrations in histological specimens from various parts of the alimentary tract affected by inflammation have been found to correlate with histological changes. This is especially true with respect to MMP-3 and -9, which are emerging as the main metalloproteinases implicated in the development of IBD. Higher expression of MMPs in inflammatory altered tissues has been demonstrated by Meijer et al. [13]. They demonstrated increased expression of MMP-1, -2, -3 and -9 in samples affected by inflammation in comparison with those without inflammation from both healthy subjects and IBD patients. These results were corroborated by Louis et al. [12] and von Lampe et al. [14] who observed significant increases in MMP-3 in inflammatory altered specimens from IBD patients in comparison with the unaltered specimens as well as with a control group. Similar results were obtained in the comprehensive study of Pedersen et al. [32], who found elevated expression of MMP-1, -3 -7, -9 and -10 in the inflamed colonic epithelial cells from human mucosa. Several studies have also indicated that inhibition of metalloproteinases was leading to the attenuation of inflammation in the experimentally induced bowel inflammation in animal models [15, 16] as well as to the prevention of intestine destruction in the ex vivo fetal human model [17, 33]. These studies unequivocally point to the disturbance in the equilibrium between the synthesis and degradation of metalloproteinases in patients suffering from IBD. However, the invasive procedures required for the measurement of local concentrations of proteases in the inflammatory altered tissues preclude the application of these determinations in the continuous monitoring of the disease course. Therefore, in our studies, we explored the reliability of metalloproteinase measurements in serum. We conducted the estimations in serum and not in plasma, which might be considered a limitation of the study. It is known that platelets are actively scavenging various factors in order to release them when necessary, for example, at the site of inflammation. However, in vitro, during clotting of blood a degranulation of platelets takes place and the factors entrapped there are released into serum. Hence, it has been suggested that serum and not plasma reflects the true potential of factors that can be stored by platelets, and metalloproteinases are one of them [34].

We demonstrated that the concentrations of the studied MMPs in serum, especially MMP-9, fairly well correlate with the disease activity estimated using PCDAI index. PCDAI index is characterized by a high degree of complexity due to the analysis of many laboratory parameters and clinical symptoms. The indices of endoscopic alterations such as Crohn's Disease Endoscopic Index of Severity (CDEIS) and Simple Endoscopic Score for Crohn's Disease (SES-CD), which are used in the evaluation of the intensity of changes in CD have a limited application

because of the invasiveness of procedure, and hence cannot be used in frequent and repeated monitoring of patients [35]. Therefore, the possibility of using a measurement of a single parameter, such as for instance MMPs, in the easily available biological material, seems to be of high importance. Our studies demonstrated statistically significant differences in the concentrations of the studied metalloproteinases in serum of patients with an active inflammatory process taking place in the alimentary tract in the course of Crohn's disease in comparison to the control group. The concentrations of MMP-9 were increasing along with the disease progression with the lowest values in patients with a mild type of disease, higher in the group with moderate type of disease and highest in severe type of disease.

MMP-9 better correlated with the indices of disease than MMP-3. This is partially due to the fact that MMP-3 did not exhibit linear correlation with the disease activity, declining in the severe phase of CD, while the other studied parameters had linear correlations. MMP-9 significantly correlated with PCDAI, CRP and seromuroid. The correlation between MMP-9 and PCDAI was not very high; however it has been previously noted that correlations between laboratory indices of inflammation and clinical disease activity scores are not always strong, since the latter contain several components, some of which are subjective in nature [31, 35]. Nevertheless, when evaluating diagnostic utility of MMP-3, -9 and CRP, their performances in terms of both diagnostic accuracy and Youden index are comparable with the notion that MMP-9 had higher sensitivity and CRP higher specificity. It has to be noted, however, that diagnostic accuracy of CRP was based on our cohort of pediatric patients and it was better than reported elsewhere, for example, by Langhorst et al. [36] (74% vs. 64%) for adult patients.

Despite the fact that the possibility of metalloproteinase measurement in tissues other than locally collected tissue specimens from the alimentary tract significantly increases the availability of such determinations in everyday medical practice, the number of papers dedicated to this topic is limited. Manfredi et al. [37], in their studies conducted on the population of children and young adults with active course of IBD, found that the concentrations of MMP-2 and -9 in the urine of patients with CD and UC differed significantly from a control group, and concluded that the determinations of these metalloproteinases could be useful as biomarkers in the evaluation of clinical activity of IBD. Recently, the diagnostic utility of MMP-3 and -9 has been evaluated in the sera of patients with colorectal cancer [38, 39]. Mroczko et al. [38], found MMP-9 determinations in serum to be potentially useful in the differentiation between colorectal cancer patients and healthy subjects with a diagnostic accuracy of 55.9% and sensitivity of

55%. Conversely, Yeh et al. [39], who studied MMP-3, -7 and -9 in the sera of *H. pylori*-infected gastric cancer patients, did not find differences in MMP-9 concentrations between cancer patients and those suffering from gastritis or duodenal or gastric ulcers; nevertheless they observed that concomitant estimations of MMP-3 and -7 could serve as indicators of lymph node invasion and poor survival. It is worth mentioning that overall performance of both MMP-3 and -9 in our studies was a much better indicator that these metalloproteinases might be more useful in the management of IBD than cancers.

The evidence of the elevated levels of metalloproteinases in various kinds of biological materials broadens the spectrum of the possible measurements, and what is most important indicates that the studied metalloproteinases can be used as biomarkers of disease activity in the course of IBD. Especially the determinations of MMP-9, the concentrations of which were in a direct, linear relation to the disease activity computed on the basis of clinical scales of disease activity seem to create such a possibility. The fact that MMP-3 is at first increasing along with the disease activity up to the moderate level with the subsequent drop in its concentration in the severe form of CD seems to indicate that this metalloproteinase is less useful as a single indicator in the differentiation between moderate and severe forms of the disease. This phenomenon observed in our studies has not been described previously. However, in other studies the patients were classified according to the presence or absence of the inflammatory changes in the histological mucosa specimens collected from a given segment of intestinal tract. In our work the classification was based on the evaluation of clinical status of patients, and from the entire group of patients distinct subgroups of patients with mild, moderate and severe disease forms were separated. With respect to our results it is, however, worth mentioning that Kirkegaard et al. [40] studying the expression of metalloproteinases in fistulas in IBD patients demonstrated elevated expression of MMP-9 transcripts in an acute inflammation in the comparison with a chronic one, whereas at the same time the expression of MMP-3 has been elevated in tissues affected by a chronic process. Despite the fact that a direct comparison between a severe form of disease estimated on the basis of clinical evaluation and histologically confirmed acute inflammation as well as moderate disease and chronic inflammation is not possible, a trend that in acute states MMP-9 is more elevated than MMP-3 is noticeable.

The results of our studies indicate that the intensity of the disease process taking place in the intestinal tract is reflected by metalloproteinase concentrations in the blood. These zinc proteases are one of the elements of inflammatory cascade causing a systemic reaction of the organism. However, the position of metalloproteinases in

inflammatory cascade limits their specificity with respect to IBD. Yet, demonstrated in our study, relatively high, and comparable with that of others, commonly used indices, specificity of the studied metalloproteinases with respect to Crohn's disease severity creates the grounds for their potential application, possibly in combination with other factors, as indicators of this disease activity that might prove useful in the monitoring of the disease course.

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