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Original article

Circulating MMP-9 and TIMP-1 in acute exacerbations and after remission induced by oral corticosteroids in asthmatic children.

Background *Matrix metalloproteinase-9 (MMP-9) is a collagenase implicated in extracellular matrix (ECM) turnover. Tissue inhibitor of metalloproteinase (TIMP-1) binds MMP-9 to inhibit its proteolytic activity; it also modulates cell proliferation, apoptosis, angiogenesis and the development of irreversible airflow obstruction and remodeling.*

Objective: *To investigate the serum levels of MMP-9, TIMP-1 and IL-8 in asthma exacerbations and convalescence, and the possible effect of oral corticosteroids on them.*

Methods: *Circulating levels of MMP-9 and TIMP-1 were measured using ELISA in 33 children with mean age (7.35 ±2.4 years) experiencing an asthma exacerbation before and after oral corticosteroid therapy and 30 age-matched children with stable asthma. Children were recruited during emergency or routine visits to Pediatric Chest Clinic, Ain Shams University. Circulating MMP-9 and TIMP-1 were correlated to degree of airway obstruction as measured by % decrease in FEV1.*

Results: *In the present study plasma levels of MMP-9 correlated to MMP-9 specific enzyme activity .Significantly higher circulating levels of MMP-9 and TIMP-1 were detected in patients with asthma exacerbations compared to patients with stable asthma. The degree of airway obstruction measured by % decrease in FEV1 correlated positively to both MMP-9 and TIMP-1 levels and correlated negatively to MMP-9/TIMP-1 ratio during acute exacerbations. MMP-9 correlated to blood neutrophil, eosinophil and lymphocyte counts during acute exacerbations. After oral steroids, there was a significant decline in circulating MMP-9. However TIMP-1 did not show significant change. Decrease in MMP-9/TIMP-1 ratio correlated to FEV1 changes.*

Conclusion: *MMP-9 is a serum marker of asthma exacerbations that is correlated to degree of airway obstruction and blood inflammatory cells. Imbalance between MMP-9 and TIMP-1 after asthma exacerbation might contribute significantly to airway remodeling and TIMP-1 production in acute asthma might not be suppressed by corticosteroids. MMP-9 and TIMP-1 might be important targets for therapeutic applications in patients with asthma.*

Keywords: Asthma, MMP-9, TIMP-1, Asthma exacerbations, Airway remodeling.

Laila M. A. G. Hegazy
and
Salwa Abu El Hana*

From the Departments of Pediatrics and Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt.*

Correspondence:
Laila M. A. G. Hegazy, MD, MRCP
Assistant Professor of Pediatrics
7 Labib El Shahid St., Heliopolis, Cairo, PO 11351.
E-mail: *lailahegazy @gmail.com*

INTRODUCTION

Remodeling of the lung architecture is a hallmark of many lung diseases, for example, loss of alveolar walls in emphysema, subepithelial fibrosis in asthmatic airways, intralveolar fibrosis in idiopathic pulmonary fibrosis (IPF), cavity formation in tuberculosis and bronchiectasis in cystic fibrosis. All of these pathologic changes involve extensive alterations of lung extracellular matrix (ECM). Matrix metalloproteinases (MMPs) have been proposed to be crucial in causing these changes because of their capacity to cleave structural proteins such as collagens and elastin[1]. The inflammatory process underlying asthma involves a complex interaction of cell communication that results in increased airway wall thickness; these include increase in vascularity, edema, smooth muscle hypertrophy and hyperplasia of mucus glands. Increase in thickness of the basement membrane and subepithelial fibrosis are currently recognized as hallmarks of airway remodeling[2]. These changes are believed to be a part of repair process designed to restore tissue

integrity in response to inflammatory insult[3]. Investigations suggest that repeated antigen challenges, which provoke episodes of acute inflammation, promote airway remodeling in asthma, by altering the homeostasis of extracellular matrix[4]. Matrix metalloproteinases (MMPs) have been proposed to be key factors in causing those changes because of their capacity to cleave structural proteins such as collagens and elastin[5].

In common with all MMPs; the gelatinases are produced in a latent form and can be inactivated when combined with specific inhibitors of metalloproteinase. The MMPs digest collagen, mediate vascular leakage and induce migration of inflammatory cells through basement membranes[6]. MMPs may also be involved in invasion of malignant cells or tumor angiogenesis[7].

In normal lungs, MMP-9 is not produced by resident cells, but under various forms of stimulation, Bronchial epithelial cells, Clara cells, alveolar type 2 cells, fibroblasts, smooth muscle cells, macrophages, NK cells, eosinophils, mast cells and neutrophils, all produce MMP-9[1]. Neutrophils are the richest source of MMP-9, upon stimulation, neutrophils release MMP-9, and IL-8 has been shown to induce MMP-9 release from neutrophils[3].

In counterbalance, tissue inhibitor of metalloproteinase (TIMP) binds covalently in 1:1 portion MMPs and inhibits their enzyme activity. TIMP-1 induces proliferation of fibroblasts, and an imbalance between TIMP and MMP might favor airway remodeling in asthma[8].

In the present study, we aimed at measuring the serum level of MMP-9 and its inhibitor TIMP-1 in asthma exacerbations before and after oral corticosteroids as well as patients with stable asthma to determine the effect of exacerbations on the derangement of MMP-9/TIMP-1 ratio which might have an effect on the long term complications of asthma such as the development of airway remodeling.

METHODS

Study population:

The study was conducted on 63 well known asthmatic children, 6 years of age and older, recruited from the emergency department and the Pediatric Chest Clinic of Ain Shams University Children's Hospital. The diagnosis of asthma was established according to the American Thoracic Society criteria [9]. Classification of asthma severity was based on GINA guidelines[10]. They were divided into 2 groups:

1-Asthma exacerbation group

Thirty-three children were selected during moderate to severe exacerbations, either on their scheduled visit or during emergency visit, their ages ranged from 6.2 to 9.7 years with a mean age of 7.35±2.4 years. Minimum criteria for diagnosis of asthma exacerbations included intense subjective breathlessness, audible wheeze on auscultation and a PEF < 70% of personal best value in the previous 3 months.

2-Stable asthma group: Thirty age matched children with stable asthma, their ages ranged from 6.4-8.5 years with mean age of 7.38±2.5 years. They were recruited on their scheduled visit if symptoms and PEF had been stable with no change in treatment for one month at least.

Both groups were on low dose maintenance inhaled steroids and inhaled bronchodilators on demand before start of study.

Study Design:

All patients underwent full detailed questionnaire, complete clinical evaluation, pulmonary functions including FEV_1, FVC, PEF measurement (using Med Graphics TM spirometry, pulmonary function system, 350 Oak groves Parkway St Paul, Minnesota, USA Tel: 651 484 4874), routine laboratory investigations including complete blood count using coulter counter and chest radiography to exclude concomitant pneumonia. Serum samples were collected on the visits and stored at -80ºC until time of assay. Serum MMP-9, TIMP-1, MMP-9/TIMP-1 and IL-8 were determined in patients with acute asthma exacerbations and compared to patients with stable asthma. Patients with acute asthma were treated using oral steroids (prednisone 1mg/kg/d) for 10 days and were monitored for subsequent convalescence which was defined by continuous resolution of subjective symptoms and physical findings for at least 2 weeks with a morning PEF>80% personal best value.

In acute exacerbations, the plasma level of MMP-9, TIMP-1, MMP-9/TIMP-1, and IL-8 were re-determined at convalescence and correlated to the FEV1 change after oral steroids. Three patients failed to attend for convalescence sampling and were excluded from the results.

Study measurements:

Plasma MMP-9 and TIMP-1 Assay:

Using quantitative sandwich immunoassay (ELISA), supplied by Oncogene research products 10394 Pacific court CA92121.

IL-8 assay:
Using sandwich ELISA technique supplied by Diaclone research Bd a FLEMING Bp 1985-25020 BESANCON Cedex, France.

Measurement of MMP-9-Specific Enzyme Activity
To confirm that changes in pro-MMP-9 concentrations at the protein level correlate with actual changes in MMP-9-specific proteolytic function, plasma and culture supernatant samples were concurrently evaluated using a MMP-9 bioassay (Biotrak; Amersham Pharmacia Biotech LTD). According to the protocol of the manufacturer, 100 µL of test sample was incubated in 96-well plates in duplicates with a modified pro-urokinase and chromogenic peptide substrate. When incubated at 37°C, the pro-urokinase was cleaved in a specific manner by biologically active MMP-9 contained in the samples. Enzyme activity was then measured by the amount of cleaved indicator peptide detected at a 405-nm wavelength and transformed into relative enzyme concentration by comparison to a standard curve.

Statistical methods
The results were statistically analyzed via a standard computer program SPSS for Windows 98. Spearman's (rank correlation coefficient) test was used for correlation between two nonparametric variables; Pearson's correlation was used to reflect the degree of linear relationship between two normally distributed variables. Student t test was used for comparing means of two parametric samples. For all tests p values of <0.05 were considered significant.

RESULTS

Clinical characteristics of the studied groups are summarized in table 1.

In the present study plasma levels of MMP-9 correlated to MMP-9 specific enzyme activity (r=0.9) (p=0.009).

MMP-9 correlated to the counts of blood neutrophils (p<0.005) eosinophils (p<0.05) and lymphocytes (p<0.005). (Table 2).

Plasma levels of MMP-9, TIMP-1, and IL-8 were significantly elevated in acute asthma exacerbations in comparison to stable asthmatics. There was no significant difference between asthma exacerbations and stable asthma regarding mean MMP-9/TIMP-1 ratio. (Table 3).

During asthma exacerbations, the mean MMP-9 correlated positively with that of TIMP-1 (r = 0.651) and that of IL-8(r = 0.411). There was no difference in the mean MMP-9 and TIMP-1 between moderate and severe asthma. However, the degree of airway obstruction (measured by % decrease in baseline FEV_1 compared to the predicted value for age and height) correlated positively with MMP-9 (r = 0.514), TIMP-1(r = 0.779), and IL-8 (r = 0.367) and correlated negatively with MMP-9/TIMP-1 ratio (r = -0.355). (Table 4).

After oral corticosteroids, there had been a significant decline in mean MMP- 9 in patients who passed into remission (72.8ng/dl) (p<0.05), however TIMP-1 showed no significant decrease (p>0.05). The MMP-9/TIMP-1 ratio was significantly lower in remission (0.172) compared to exacerbation (0.53) (p<0.005). (Table 5).

The change in FEV_1 closely correlated with the MMP-9/TIMP-1 ratios (p = 0.0005).

Table 1. Descriptive clinical characteristics of studied patients

	Exacerbations	**Stable**
Number	33	30
Gender M/F	24/9	20/10
Age (years) mean (SD)	7.35(2.4)	7.38(2.5)
Asthma duration (years) mean (SD)	4.1(0.48)	4 (0.35)
Atopic /non atopic	10/23	9/21
Asthma severity (moderate/severe)	16/17	15/15
PEF % of personal best	68.3 %	89.4%

Table 2. Spearman's correlation between MMP-9 level and inflammatory cell counts during exacerbation.

Correlation	**rho**	**P value**
Neutrophils	0.72	<0.005
Eosinophils	0.46	<0.05
Lymphocytes	0.70	<0.005

rho= rank order correlation coefficient.

Table 3. Mean values (SD) of MMP-9, TIMP-1, MMP-9/TIMP-1 and IL-8 in plasma of asthmatic children during exacerbations (Ex) compared to stable asthmatics (St).

	Ex (n=33) Mean (SD)	**St (n=30) Mean (SD)**	**t**	**p**	
MMP-9 (ng/ml)	165 (41.6)	68.8 (17.2)	7.1	<0.001	S
TIMP-1 (ng/ml)	382.7(76.7)	133 (40)	9.84	<0.001	S
MMP-9/TIMP-1	0.46 (0.17)	0.55 (0.17)	-1.43	>0.05	NS
IL-8 (ng/ml)	242(22.2)	67.4 (21.7)	2.7	<0.05	S

NB: values are given as mean (SD). S= significant NS= non-significant

Table 4. Correlation between MMP-9, TIMP-1, MMP-9/TIMP-1 and IL-8 and severity of airway obstruction as measured by % decrease in baseline FEV1 in acute exacerbation.

		MMP9	**TIMP-1**	**MMP-9/TIMP-1**	**IL-8**
Percent decrease in baseline FEV_1	r	0.514	0.779	-0.355	0.367
	p	HS	HS	S	S

S= significant HS= highly significant

Table 5. Mean values of MMP-9, TIMP-1, MMP-9/TIMP-1 and IL-8 in asthmatic children during exacerbation compared to remission after oral corticosteroids

	Exacerbations Mean (SD)	**Remission Mean (SD)**	**t**	**p**	
MMP-9 (ng/ml)	165.2(40)	72.8(22.3)	2.5	0.02	S
TIMP-1 (ng/ml)	290.2(66)	278.6(60)	0.268	0.339	NS
MMP-9/TIMP-1	0.531(0.27)	0.172(0.12)	2.4	0.022	S
IL-8 (ng/ml)	229.25(80)	73.3(24)	6.16	0.00048	S

SD = Standard Deviation S= Significant NS= non-significant

DISCUSSION

In the present study, we have measured changes in the expression and activity of the circulating metalloproteinase MMP-9 and its inhibitor TIMP-1 during asthma exacerbations. MMP-9 specific enzyme activity correlated to plasma levels of MMP-9 in acute exacerbations, after remission and in stable asthma (p=0.009). This confirms that pro-MMP-9 concentrations at the protein level correlate with actual changes in MMP-9-specific proteolytic function and the wide variability of plasma activity of MMP-9 in patients with asthma .The mean plasma MMP-9 concentration was increased in patients with asthma exacerbation compared to patients with stable asthma. In acute asthma, the level of plasma MMP-9 correlated with the numbers of neutrophils, eosinophils and lymphocytes and this agrees with the results of Tanaka et al[11], Oshita et al[12] and Lee et al[6]. They suggested that airway inflammation after asthma exacerbation correlates with the overproduction of MMP-9, which then leads to airway remodeling. Gelatin zymography of sputum and BALF in these patients typically shows a prominent MMP-9–

lipocalin band, consistent with increased neutrophil counts[11].

Bossie and associates[13] concluded that MMP-9 was increased in asthma exacerbations and this correlated to steroid responsiveness. In patients with asthma, MMP-9 was increased in sputum and bronchoalveolar lavage (BAL) fluid under stable conditions[14] and after local allergen challenge[15]. Current evidence suggests that MMP-9 mediates several important pathways responsible for asthma exacerbations including airflow obstruction. Exfoliation of airway epithelial cells, induced by MMP-9, may contribute to the pathologic airway obstruction in status asthmaticus (SA)[16], increased vascular permeability and exaggerated airway hyperresponsiveness[17]. In a study by Lemjabbar et al[16] an acute 10- to 160-fold increase of 92 kDa gelatinase (MMP-9) concentration was noticed in epithelial lining fluid (ELF) from patients with SA. Concomitant elevated level of tissue inhibitor of metalloproteinase-1 (TIMP-1) was shown only in patients with SA, thus counterbalancing, at least partially, excess of activated 92 kDa gelatinase. Acutely enhanced albumin levels were only observed in patients with SA; in addition, MMP-9 and albumin levels were significantly and positively correlated ($r = 0.96$, $p < 0.0001$), suggesting that 92 kDa gelatinase may account for increased bronchial permeability in patients with SA. Several arguments support that MMP-9 during SA originates both from numerous activated chemoattracted neutrophils and from activated bronchial epithelial cells in response to in situ lung injury.

MMP-9 is over expressed in serum and/or diseased organs of patients with several disorders characterized by tissue destruction, such as rheumatoid arthritis and pulmonary emphysema[18]. MMP-9 is considered to be involved in the pathophysiology of these disorders, presumably by degrading ECM. MMP-9 has also been suggested to enhance the migration of inflammatory cells into the airways through degrading collagen[19]. Circulating MMP-9 levels may therefore reflect a "spill over" of MMP-9 produced in the airways.

We further characterized the changes in circulating MMP-9 activity in the same individuals and confirmed increased MMP-9 activity during exacerbations compared with subsequent convalescence. The levels of MMP-9 could influence the intensity and nature of the asthmatic inflammatory response. This agrees with the work of Lee and associates[6] in which they demonstrated increased MMP-9 activity in asthma exacerbations. Elevated levels of MMP-9 might be seen in patients with steroid-responsive disease characterized by eosinophilic and lymphocytic inflammation. In a study by Hoshino et al.[20] inhaled steroids reduced the amount of reticular basement membrane in association with decreased MMP-9 suggesting that enhanced MMP-9 activity promote subepithelial fibrosis in asthmatic individuals. It is therefore likely that asthma exacerbations promote airway remodeling by altering MMP-9 mediated homeostasis. In contrast, decreased levels of MMP-9 (or MMP-9 function) might be seen in patients with steroid-unresponsive, refractory disease with neutrophil-rich tissue inflammation[21].

In the present study TIMP-1, the predominant inhibitor of MMP-9, was significantly increased in acute exacerbations compared to stable asthma. Also TIMP-1 levels positively correlated to MMP-9 in asthma exacerbations. The elevated TIMP-1 might represent some control regulation of the excessive and acute release of MMP-9, however after oral steroids there was no significant decline in TIMP-1.

TIMP-1 inhibits the enzymatic activity of MMP-9 by 1:1 binding. TIMP-1 is considered to have fibrogenic properties resulting from inhibition of MMP-9 and promotion of cell growth of fibroblasts or myofibroblasts[1]. In several fibrotic diseases such as progressive systemic sclerosis[22] and idiopathic pulmonary fibrosis[23], TIMP-1 is overexpressed and considered to accelerate fibrosis and ECM deposition. In the sputum or BAL fluid of patients with stable asthma, TIMP-1 is increased compared with healthy controls and is overproduced relative to MMP-9. An excess of TIMP-1 over MMP-9 in the sputum or serum of asthmatic patients has been associated with chronic airflow obstruction[13].

An increase in the molar ratio of MMP-9/TIMP-1 may favor tissue injury, while an excess of TIMP-1 over MMP-9 has been proposed to favor airway remodeling in chronic asthma[8]. Tonnel et al[5] demonstrated that patients with decompensated asthma have a molar ratio of MMP-9 to TIMP-1 in BALF greater than that seen in patients with stable asthma, low MMP-9/TIMP-1 ratios can result in subepithelial collagen deposition, chronic MMP-9/TIMP-1 imbalance could lead to thickened airways and restricted airflow. In a study by Doherty and associates[24] MMP-9/TIMP-1 was found to be reduced even in stable asthmatic children, this imbalance might indicate early derangement of the metabolism of ECM.

In the present study, we have demonstrated decline in serum MMP-9 after oral steroids which marks the remitting inflammatory process. However, the continuing elevation of TIMP-1 after

remission indicates ongoing subepithelial fibrosis and remodeling that was initiated by asthma exacerbation, but failed to subside after remission. Interestingly, corticosteroids downregulated MMP-9 but enhanced TIMP-1. This finding is in agreement with those of Lee et al[6] who concluded that intravenous corticosteroid treatment decreased the amount of MMP-9 but increased the amount of TIMP-1. On the contrary Mattos et al[25] did not show any significant decline in sputum MMP-9 after inhaled steroids. Vignola and colleagues[26] have detected that MMP-9/TIMP-1 molar ratio in sputum supernatants of asthmatic subjects were significantly increased in comparison with baseline levels after inhaled steroids, indicating downregulation of TIMP-1. The severity of asthma, mode of steroid administration, the dose and the duration of therapy were variable in those studies. Even though it is clear that enhanced airway inflammation in asthma is associated with increased expression of MMPs, whether specific inhibitors of MMP could reduce airway injury and facilitate orderly healing in asthma is still unknown.

In the present study, serum MMP-9 correlated with degree of airway obstruction during exacerbations, moreover the percent improvement in FEV1 after corticosteroid treatment correlated to MMP-9/TIMP-1. Vignola and colleagues[14] have shown that the molar ratio between MMP-9 and TIMP-1 in the sputum of asthmatic patients is positively correlated with FEV1. The authors indicated that the excess of TIMP-1 might lead to airflow obstruction, possibly by a role in the pathogenesis of ECM remodeling. Bossé and colleagues[13] have evaluated the relationship between the responsiveness of FEV1 to oral corticosteroid treatment (methylprednisolone 40 mg/day for 2 weeks) and the pretreatment serum molar ratio of MMP-9/TIMP-1 in severe asthmatic patients requiring high doses of inhaled corticosteroids. Serum MMP-9/TIMP-1 ratio strongly correlated with the degree of increase in FEV1 after oral corticosteroid treatment. This comes in agreement with our results.

In the present study, elevated levels of IL-8 in asthma exacerbations were detected compared to stable asthmatics and after remission. IL-8 chemoattracts and activates neutrophils and eosinophils. IL-8 was detected in bronchial tissues of subjects with symptomatic asthma, and increased IL-8 concentrations have been found in induced sputum of subjects with severe asthma. IL-8 levels correlate with increased nasal aspirate neutrophil myeloperoxidase levels and there was also a correlation between myeloperoxidase levels and upper respiratory symptom severity[27]. Recently, it was shown that peripheral blood mononuclear cells from patients with severe asthma release high concentrations of IL-8 compared with controlled and uncontrolled patients with asthma[28]. Van den Steen et al[29] concluded that the activation of neutrophils by CXC chemokines such as IL-8 leads to the secretion of proteases including MMP-9. MMP-9 in neutrophils, also contributes to enhanced IL-8 activity by a positive feedback loop. Consequently, neutrophils and IL-8 are putative markers of severe asthma. In this context, inhibitors to IL-8 and MMP-9 might have therapeutic value in asthma exacerbations

In conclusion, the increase in MMP-9 production and activity observed in the present study suggests a process of extracellular matrix degradation in asthma exacerbations and proposes MMP-9 as a non-invasive systemic marker of inflammation in asthma. TIMP-1 was increased in acute asthma and correlated to MMP-9. Circulating MMP-9 level decreased after remission using oral corticosteroids causing imbalance between MMP-9 and TIMP-1. The imbalance during and after asthma exacerbation might contribute significantly to airway tissue remodeling. TIMP-1 production in acute asthma might not be suppressed by corticosteroids. MMP-9 and TIMP-1 might be important targets for therapeutic applications in patients with asthma. This study highlights the effect of asthma exacerbations on the long term outcome of asthma.

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