

BIOMARKERS

Fibroblast growth factor-2 is a sputum remodeling biomarker of severe asthma

Élyse Y. Bissonnette, PhD^{1*}, Anne-Marie Madore, PhD^{2*}, Jamila Chakir, PhD¹, Michel Laviolette, MD¹, Louis-Philippe Boulet, MD¹, Qutayba Hamid, MD³, Céline Bergeron, MD⁴, Karim Maghni, PhD⁵, and Catherine Laprise, PhD²

¹Centre de recherche de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec, Université Laval, Quebec City, QC, Canada,

²Département des sciences fondamentales, Université du Québec à Chicoutimi, Chicoutimi, QC, Canada, ³Meakins-Christie Laboratories, McGill University, Montreal, QC, Canada, ⁴Centre de Recherche du CHUM, Université de Montréal, Montreal, QC, Canada, and ⁵Département de Médecine, Centre de recherche de l'Hôpital du Sacré-Coeur de Montréal, Université de Montréal, Montreal, QC, Canada

Abstract

Objective: Given the large phenotypic diversity of asthma, our aim was to characterize molecular profiles related to asthma severity using selected remodeling biomarkers in induced sputum. **Methods:** Induced sputum from healthy controls, patients with mild to moderate asthma and severe asthma were collected. Twelve selected biomarkers previously associated to airway remodeling such as connective tissue growth factor (CTGF), fibroblast growth factor (FGF)-2, matrix metalloproteinase (MMP)-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, MMP-13, procollagen type 1 and tissue inhibitor of metalloproteinase (TIMP)-1 were measured in sputum samples using ELISA or Luminex technology. FGF-2 level was also evaluated in bronchial biopsies using immunohistochemistry. **Results:** Sputum of severe asthma was characterized by reduced percentage of macrophages and increased percentage of neutrophils and eosinophils. FGF-2, MMP-1 and TIMP-1 levels increased with asthma severity. Interestingly, only FGF-2 level inversely correlated with FEV₁/FVC ratio. Although percentage of eosinophils correlated with asthma severity, it did not correlate with FGF-2 levels. Increased levels of FGF-2 with asthma severity were confirmed in bronchial biopsies by immunohistochemistry. **Conclusions:** Level of FGF-2 in induced sputum represents a relevant remodeling biomarker of asthma severity and significantly correlates with pulmonary function. FGF-2 sputum biomarker is proposed to reveal the phenotype of asthma characterized by fixed airflow obstruction.

Keywords

FEV₁/FVC ratio, FGF-2, MMP-1, remodeling, severity

History

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Introduction

Asthma is a chronic lung disease characterized by respiratory symptoms, airway inflammation, airway hyper-responsiveness and usually, reversible airflow obstruction [1]. Its prevalence increased considerably over the last two decades reaching 14.1% in Canada and 10.9% in United States [2]. The heterogeneity of asthma clinical presentation, disease progression and treatment response suggests that asthma is a collection of phenotypes or pathologies rather than a single disease [1,3]. This led to different classifications of asthma sub-phenotypes [3], but the most widely used is still based on asthma severity, given the lack of detailed characterization of these phenotypes.

Many studies focused on the discovery of novel biomarkers for asthma and its severity to refine asthma phenotypes,

treatment and prevention strategies. Many biomarkers were identified in urine, blood, bronchoalveolar lavage, induced sputum (IS), exhaled breath condensate and bronchial biopsy of asthmatic patients [4,5], but the translation of their clinical applicability is rare. Some of these sampling methods are too invasive and cannot be performed repeatedly, reducing the interest of such biomarkers in clinics. However, IS represents a potentially promising method to quantify both inflammatory cells and biomarkers of asthma severity. Indeed, the level of eosinophils in IS is recognized as a validated, useful, non-invasive method for evaluating airway inflammation in asthma before and after intervention [5]. Other mediators correlating with asthma phenotypes or severity were also identified in IS including tumor necrosis factor (TNF), interleukin-8 (IL-8), matrix metalloproteinase (MMP)-9, MMP-9/tissue inhibitor of metalloproteinase (TIMP)-1 ratio, cysteinyl-leukotrienes and 8-isoprostane [6]. However, the majority of identified asthma biomarkers using all sampling methods mainly reflect airway inflammation [4], few of them refer to airway remodeling which is also an important pathological component of asthma.

*These authors contributed equally to this study.

Correspondence: Catherine Laprise, PhD, Département des sciences fondamentales, Université du Québec à Chicoutimi, 555 boulevard de l'Université, Chicoutimi, Québec, Canada, G7H 2B1. Tel: 418-545-5011 (#5659). Fax: 418-615-1203. E-mail: catherine.laprise@uqac.ca

Airway remodeling refers to cellular and structural alterations in the airways, including epithelial changes, increased in smooth muscle cells, activation and proliferation of myofibroblasts and deposition of new matrix in the airway walls. Several biological pathways and markers are involved in these changes as fibroblast growth factor (FGF)-2, which contributes principally to smooth muscle cells hyperplasia and MMPs and TIMPs, which regulates the balance between synthesis and degradation of extracellular matrix [7,8]. Remodeling is not just a progressive long-term process due to persistent inflammation as thought in the beginning. In fact, airway remodeling occurs early in the disease in parallel with inflammation and was held responsible for fixed airflow obstruction observed in a sub-group of severe asthma patients [9]. Although airway remodeling is recognized to play an important role in asthma pathophysiology, its evaluation is rarely performed because of the requirement of invasive methods (e.g. biopsy). Thus, there is a major clinical need to identify biomarkers associated with or that are predictive of this process using non-invasive technique.

Materials and methods

Subjects

Induced sputum and bronchial biopsy samples were obtained from three sites of the Respiratory Health Network (RHN) Tissue Bank of the Fonds de recherche du Québec en santé (FRQS) (www.tissuebank.ca): Institut Universitaire de Cardiologie et de Pneumologie de Québec, Université Laval (93 samples), Hôpital Sacré-Cœur de Montréal, Université de Montréal (20 samples) and McGill University Health Centre (50 samples). Each of these individuals was seen at a specialized clinic to confirm the diagnosis and determine the medication needed. Subjects were classified according to the Global Initiative for Asthma reports [10]. A total of 163 subjects were classified into three different groups: non-asthmatic (NA) healthy control group (without asthma and allergy, $n = 25$), mild to moderate asthma (MMA) including individuals with or without inhaled corticosteroid (ICS) treatment ($n = 73$) and severe asthma (SA) ($n = 65$). Severe asthmatic subjects were differentiated from mild to moderate asthmatic subjects according to their medication: severe asthma group took ≥ 500 $\mu\text{g/day}$ of ICS (fluticasone equivalent) conjugated with long-acting β_2 -agonists or anti-leukotrienes [10]. Description of subjects is summarized in Table 1 and a detailed description is available in the Supplementary Material. The study was approved by local ethic committees and all subjects gave informed consent.

Induced sputum and biomarker measurements

Induction and processing of IS, and total and differential cell counts were done as previously described [11] at the different tissue bank sites. Samples were then stored at -80°C . Biomarkers studied were selected according to their known functions and possible involvement in the airway remodeling process observed in asthma [7,12]. Connective tissue growth factor (CTGF) and FGF-2 were assessed by ELISA (R & D Systems, Minneapolis, MN). Levels of procollagen type 1 were measured using PINP RIA kit (Immunodiagnostic systems Inc., Phoenix, AZ). MMPs (MMP-1, MMP-2,

Table 1. Clinical characterization of the subjects.

		Asthma	
	NA <i>n</i> = 25	MMA <i>n</i> = 73	SA <i>n</i> = 65
General descriptive data			
M: F ratio	1:2.13 ³	1:1.43	1:0.63 ¹
Mean age, Yr (range)	24 (18–40) ^{2,3}	34 (18–68) ^{1,3}	50 (20–67) ^{1,2}
Smoking status, <i>n</i> (%)			
Non smoker	23 (92)	55 (77)	45 (69)
Ex-smoker	2 (8)	12 (17)	20 (31)
Smoker	0 (0)	4 (6)	0 (0)
Clinical descriptive data			
Age of onset		15 (11) ³	25 (17) ²
FEV ₁ , % pred (SD) ^a	97 (39) ³	88 (32) ³	68 (22) ^{1,2}
FEV ₁ post-BD, % pred (SD) ^a	103 (53) ³	94 (48) ³	70 (26) ^{1,2}
FVC, % pred (SD) ^b	107 (45) ³	102 (41) ³	82 (24) ^{1,2}
FVC post-BD, % pred (SD) ^b	106 (55) ³	105 (53) ³	83 (31) ^{1,2}
PC ₂₀ , mg/ml (SD) ^c	81.7 (4.4) ²	1.2 (3.5) ¹	6.6 (4.1)
FEV ₁ /FVC ratio (SD)	0.82 (0.07) ^{2,3}	0.75 (0.18) ^{1,3}	0.67 (0.10) ^{1,2}
Allergy, <i>n</i> (%)	0 (0)	63 (97)	54 (93)
Medication			
ICS, <i>n</i>	0 (0)	32 ³ (244 ³)	65 ² (1700 ²)
(average dose, µg/day) ^d			
LABA, <i>n</i> (%) ^e	0 (0)	5 (7) ³	63 (97) ²
Others, <i>n</i> (%) ^f	0 (0)	61 (84) ³	65 (100) ²

Significant differences between two groups are as follows: ¹indicates a difference with the NA, ²the MMA or ³the SA group (Kruskal–Wallis followed by pairwise comparison tests or Chi-square with correction for multiple testing, $p < 0.05$).

Abbreviations: NA, non-asthmatic healthy control subjects; MMA, mild to moderate asthma; SA, Severe asthma.

^aMean and standard deviation (SD) of the forced expiratory volume in one second (FEV₁) in percentage of the predicted value (available for 23 NA, 65 MMA and 62 SA), FEV₁ post-bronchodilator treatment (post-BD) (available for 14 NA, 45 MMA and 59 SA).

^bMean and SD of the forced vital capacity (FVC) in percentage of the predicted value (available for 21 NA, 41 MMA and 61 SA), FVC post-BD (available for 14 NA, 41 MMA and 57 SA).

^cGeometric means and SD of the provocative concentration of methacholine that induces a 20% fall in FEV₁ (available for 20 NA, 37 MMA and 2 SA).

^dInhaled corticosteroids (ICS) treatment in average fluticasone equivalent doses in $\mu\text{g/day}$ (number of subjects that take ICS considering all subjects and doses available for 30 MMA and 64 SA).

^eLong acting beta agonist (LABA) data available for all subjects.

^fOthers comprise long acting and short acting anticholinergics, short acting β_2 -agonists, anti-leukotrienes and anti-histamines (data available for all subjects).

MMP-3, MMP-7, MMP-8, MMP-9, MMP-12 and MMP-13) were quantified with the MultiAnalyte Profiling Fluorokine MAP assay (R&D Systems) and the BioPlex system (Biorad, Mississauga, Canada) based on the Luminex technology. TIMP-1 was quantified using an ELISA kit (Calbiochem EMD Chem. Inc. San Diego, CA).

Immunohistochemistry

Bronchial biopsies and sputum were obtained from the same subjects when it was possible. The inclusion criteria were exactly the same than for measurement in IS to avoid bias in sample selection. After embedding in paraffin blocs, samples were stored at room temperature. Sections (5 μm) for 8 non-asthmatic subjects, 17 patients with mild to moderate asthma and 7 with severe asthma (Table 1) were cut from paraffin embedded tissues using a microtome (Surgipath Medical Industries, Richmond, IL). Biopsy specimens were processed

for immunohistochemistry as previously described [13] with a mouse anti-human FGF-2 (# NBP1-47359, Novus Biological, Littleton, CO). For negative control preparations, the primary antibody was replaced with the relevant isotype and species-matched primary antibody. Quantification of the staining was performed using Image Pro-plus software (MediaCybernetics, Rockville, MD). All slides were labeled by randomly assigned numeric code to achieve blinding as to group designation. Stained slides were analyzed at a magnification of 20 $\times$, using a SPOT RT slider camera (diagnostic Instruments, Sterling Heights, MI). Quantification of the staining at the submucosa was expressed as a ratio of intensity staining/ μm^2 using color segmentation as we previously reported [14].

Statistical analyses

All statistical analyses were done using SPSS Statistics version 20 (IBM, Markham, Ontario, Canada). Kruskal–Wallis analyses were performed between all biomarkers measured and asthma sub-phenotypes, followed by pairwise comparisons to find significant differences between sub-phenotype groups. Spearman's correlation tests were performed between biomarkers and cell type distribution, forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) ratio, post-bronchodilator (BD) treatment FEV_1 /FVC ratio, FEV_1 and post-BD FEV_1 . As it is known that FEV_1 /FVC declines as the age of individuals increases [15], significant correlations between biomarkers and FEV_1 or FEV_1 /FVC ratio were also performed using non-parametric partial correlations with age as covariate. Multiple logistic regressions were performed for significant biomarkers and asthma severity to assess possible dependence between these biomarkers.

Samples of the mild to moderate asthma group came from different sites of the RHN Tissue Bank and thus were prepared by different individuals. To verify whether this could cause discordance in data, we performed analyses to compare phenotypic and dosage data between sites for the mild to moderate asthma group. Comparisons were performed using Mann–Whitney test or Chi-square test with correction for multiple testing when necessary.

Results

Percentage of eosinophils as marker of asthma severity

Given that eosinophils level is often used as a marker of asthma severity, differential cell count was evaluated in IS of non-asthmatic subjects and patients with mild to moderate asthma and severe asthma to verify the reliability of this marker. Percentage of eosinophils was significantly higher in both asthmatic groups compared with control (Figure 1, $p < 0.005$) and the level of eosinophils increased with asthma severity (NA = $0.04 \pm 0.02\%$, MMA = $4.50 \pm 0.94\%$ and SA = $7.47 \pm 1.34\%$). The percentage of macrophages, neutrophils and lymphocytes was similar in mild to moderate asthma and control. However, there were striking differences in cell population in severe asthma with a significant reduction ($p < 0.005$) in percentage of macrophages ($22.52 \pm 3.58\%$ in SA versus $58.32 \pm 8.02\%$ in NA) and an

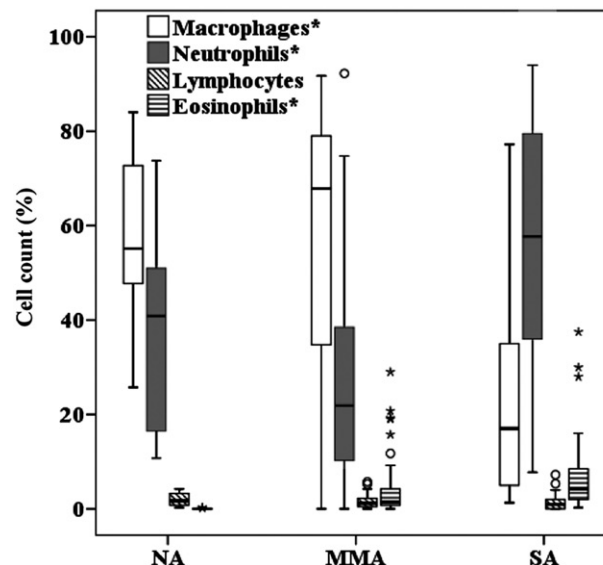


Figure 1. Differential cell counts related to asthma severity. Severe asthma is characterized by reduced alveolar macrophage and increased neutrophil and eosinophil percentages. Significant differences for Kruskal–Wallis analyses (p values are all < 0.005) are indicated by *. NA: non-asthmatic healthy control subjects, MMA: mild to moderate asthma, SA: severe asthma. Phenotypic groups include 14–35 subjects.

increase in percentage of neutrophils ($56.50 \pm 6.06\%$ in SA versus $36.66 \pm 6.26\%$ in NA). There was no significant difference in percentage of lymphocytes between control and severe asthma.

Identification of remodeling biomarkers for asthma severity

Twelve selected biomarkers were measured in IS of the three groups studied. Among them, levels of CTGF, MMP-2, MMP-3, MMP-7, MMP-9, MMP-13 and procollagen type I were not significantly different between the three groups. However, levels of FGF-2, MMP-1, MMP-8, MMP-12 and TIMP-1 significantly differed between groups. Multiple regression analyses were performed and revealed no dependency between these biomarkers (data not shown).

FGF-2

Level of FGF-2 was similar in control and mild to moderate asthma (Figure 2A). However, there was a significant increase of FGF-2 level ($p = 0.006$) in IS of severe asthmatic patients compared with controls and patients with mild to moderate asthma (77.3 ± 17.9 , 26.1 ± 4.7 and 28.4 ± 3.1 pg/ml, respectively). To verify whether corticosteroid treatment affect FGF-2 level, a t -test was performed in subjects with mild to moderate asthma with and without ICS treatment. There was no significant effect of treatment on FGF-2 level ($p = 0.216$). A negative correlation was found between FGF-2 levels and FEV_1 /FVC ratios ($\text{Rho} = -0.299$, $p = 0.037$) and post-BD FEV_1 ($\text{Rho} = -0.384$, $p = 0.009$), suggesting a relation with asthma severity and fixed airflow obstruction. The analyses considering age as covariate gave similar results as a tendency was observed for the correlation between FGF-2 and

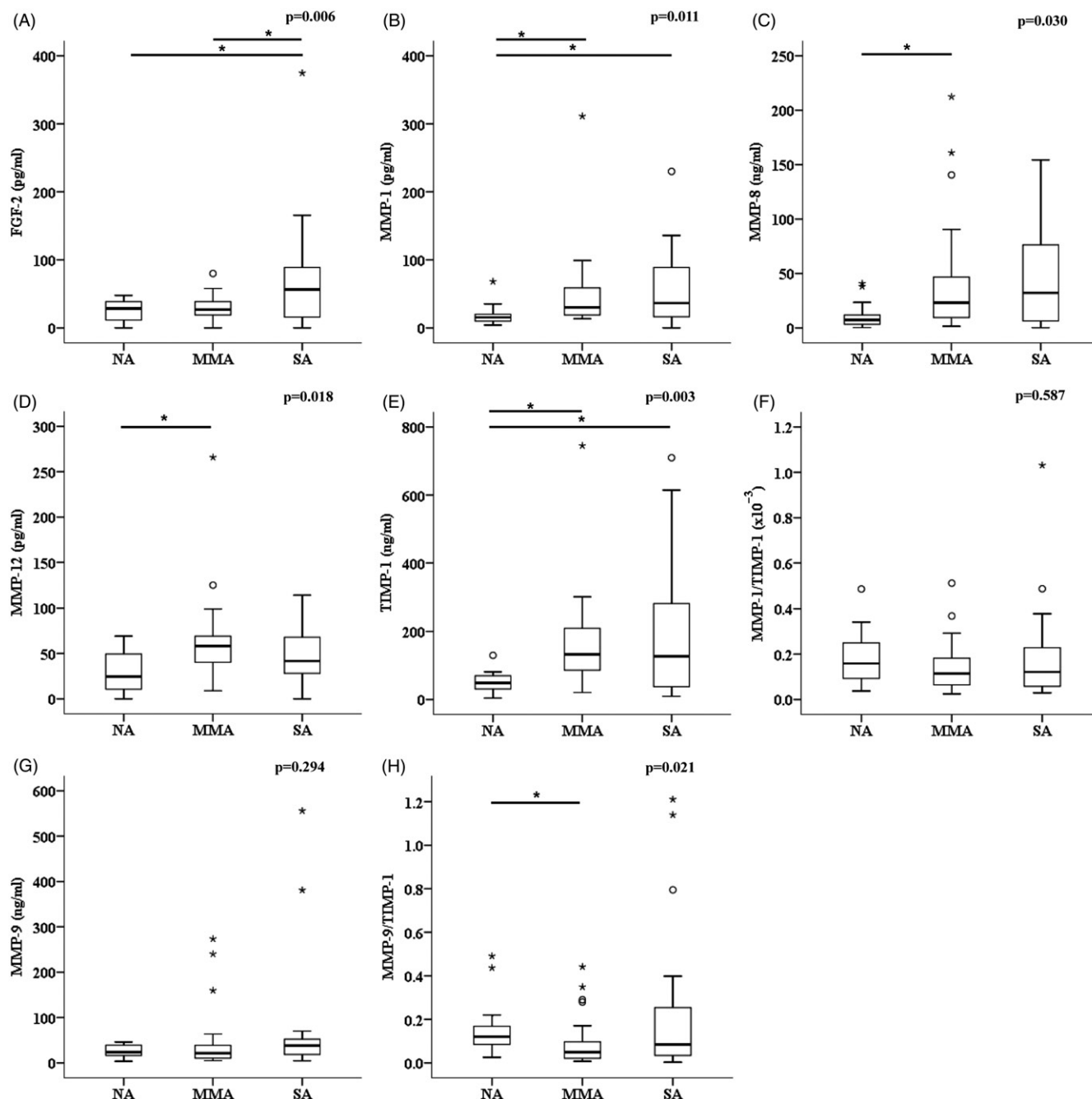


Figure 2. Biomarker levels in induced sputum. (A) Fibroblast growth factor (FGF)-2, (B) matrix metalloproteinase (MMP)-1, (C) MMP-8, (D) MMP-12, (E) tissue inhibitor of metalloproteinase (TIMP)-1, (F) MMP-1/TIMP-1 ratio, (G) MMP-9, and (H) MMP-9/TIMP-1 ratio according to asthma severity. NA: non-asthmatic healthy control subjects, MMA: mild to moderate asthma, SA: severe asthma. Significant difference (*) for pairwise comparisons and *p* values of Kruskal-Wallis analyses are indicated. Phenotypic groups include 14–31 subjects.

FEV₁/FVC ratios ($Rho = -0.256$, $p = 0.080$) and a significant correlation was observed between FGF-2 and post-BD FEV₁ ($Rho = -0.349$, $p = 0.020$). However, no significant correlation was found between FGF-2 and percentage of cell types.

MMP-1, MMP-8 and MMP-12

MMP-1 levels were significantly higher ($p = 0.011$) in IS of subjects with mild to moderate asthma and severe asthma (48.7 ± 10.7 and 58.6 ± 13.7 pg/ml) compared with controls (NA = 19.9 ± 4.9 pg/ml; Figure 2B). However, no significant difference was observed between subjects with mild to

moderate asthma and severe asthma. No correlation was found between MMP-1 level and FEV₁/FVC ratio. Interestingly, levels of both MMP-8 and MMP-12 were significantly increased in mild to moderate asthma compared with control, but the significance was lost in severe asthma (Figure 2C and 2D).

MMPs and TIMP-1

Given the importance of the balance between MMP and TIMP, levels of TIMP-1 were also measured. Both asthmatic groups showed significantly higher levels of TIMP-1

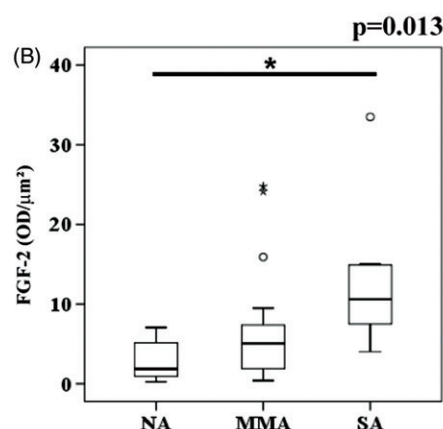
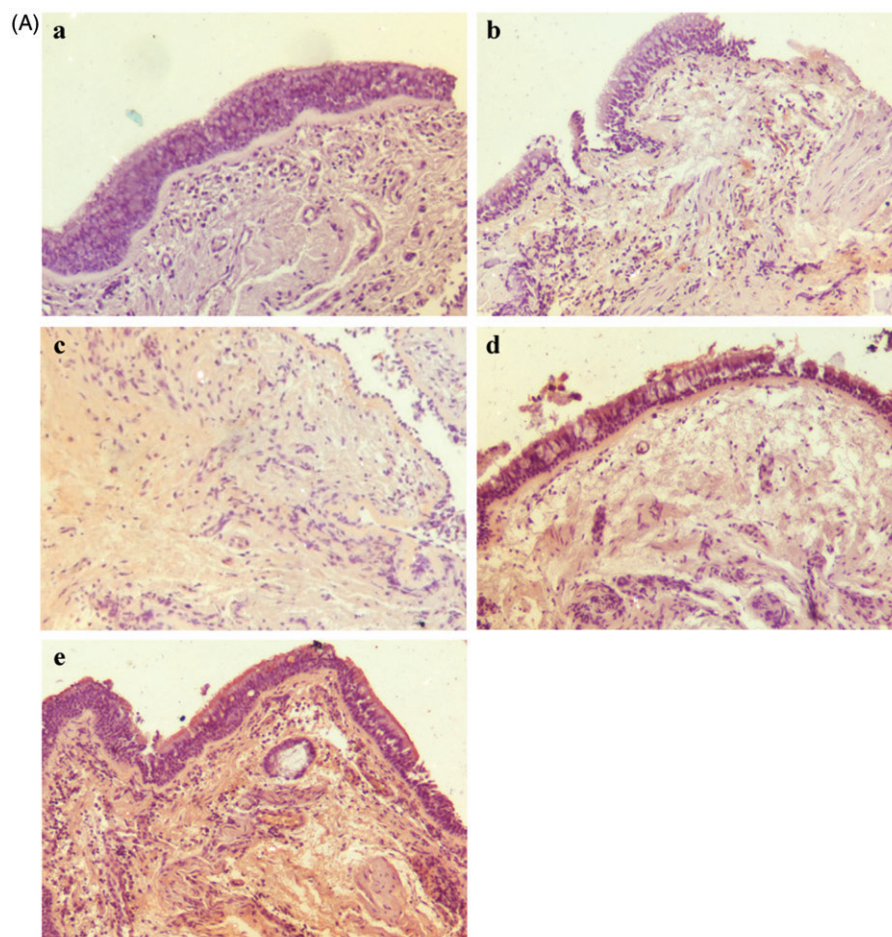
($p = 0.003$) compared with controls (NA = 51.7 ± 9.5 ng/ml, MMA = 161.8 ± 25.8 ng/ml and SA = 204.7 ± 48.0 ng/ml), but no difference was observed between mild to moderate and severe asthma (Figure 2E). No correlation was observed between TIMP-1 level and FEV₁/FVC ratio. Increase of both MMP-1 and TIMP-1 levels was observed in asthma groups compared with control, leading to similar MMP-1/TIMP-1 ratios between the three groups studied (from $3.6 \times 10^{-4} \pm 0.5 \times 10^{-4}$ to $4.7 \times 10^{-4} \pm 1.4 \times 10^{-4}$; Figure 2F) and no correlation was observed with FEV₁/FVC ratio. In contrast, while MMP-9 level did not significantly differ in the three groups studied (Figure 2G), there was a significant reduction of MMP-9/TIMP-1 ratio in mild to moderate asthma and a tendency of an increase in severe asthma compared with control subjects ($p = 0.021$ and means: NA = $6.8 \times 10^{-4} \pm 1.5 \times 10^{-4}$, MMA = $3.6 \times 10^{-4} \pm 0.9 \times 10^{-4}$, SA = 10.0×10^{-4}

$\pm 3.5 \times 10^{-4}$; Figure 2H). However, no significant correlation was found with FEV₁/FVC ratio.

Increased FGF-2 levels in biopsies of asthmatic patients

Given the increase of FGF-2 observed in IS of severe asthmatic subjects, we performed immunohistochemistry to determine whether this increase was also observed in bronchial biopsies from the three groups studied. Staining for FGF-2 in bronchial biopsies increased with asthma severity ($p = 0.013$) and a significant difference was observed in severe asthmatic subjects compared with controls (13.3 ± 3.7 and 2.9 ± 0.9 OD/ μm^2 ratio; Figure 3). Furthermore, as observed with IS, a negative correlation was found between FGF-2 level in biopsies and post-BD FEV₁ (Rho = -0.692 and $p = 0.006$) and a tendency of

Figure 3. Increased expression of fibroblast growth factor (FGF)-2 in bronchial biopsies of severe asthma subjects. (A) Representative immunostaining of FGF-2 in bronchial biopsy specimens of negative control (a), non-asthmatic healthy control subjects (b), mild asthmatic patients (c), moderate asthmatic patients (d) and severe asthmatic patients (e). (B) Relative intensity of FGF-2 levels. NA: non-asthmatic healthy control subjects, MMA: mild to moderate asthma, SA: severe asthma. Significant increase of FGF-2 level (*) was observed in SA according to pairwise comparisons results. p Value of the Kruskal–Wallis analysis is indicated. Phenotypic groups include 7–17 subjects.



negative correlation was detected between FGF-2 and FEV₁/FVC ratio (Rho = −0.451 and $p=0.060$). Both correlations are significant even considering the age as covariate (post-BD FEV₁: Rho = −0.643, $p=0.018$; post-BD FEV₁/FVC: Rho = −0.438, $p=0.079$). A negative correlation was also found between FGF-2 and post-BD FEV₁/FVC ratio for the Spearman correlation (Rho = −0.855 and $p=0.001$) and also the partial correlation considering the age as covariate (Rho = −0.867, $p=0.001$).

Analyses performed to describe the differences between MMA samples from different tissue bank sites show that the majority of phenotypic and dosage data were similar between the two sites (Supplementary Table 1). Thus, IS sampling in different research centers does not seem to alter data in our study. These comparisons were only performed for MMA subjects as in other phenotypic categories (NA and SA) there is less diversity in term of sampling sites.

Discussion

This study aims at identifying potential remodeling biomarkers of asthma severity. Our data demonstrate that the most specific biomarker is FGF-2 as we observed a significant negative correlation between FGF-2 levels, in both IS and bronchial biopsies, and pre- and post-BD FEV₁/FVC ratios and post-BD FEV₁ even when considering the age in the model, indicating the relationship between FGF-2 and asthma severity. There were significant differences in other biomarkers such as MMP-1, MMP-8, MMP-12, and TIMP-1 levels and MMP-9/TIMP-1 ratio, but no correlation was observed with lung function or asthma severity.

Asthma is characterized by both airway inflammation and remodeling. However, the majority of studies investigating biomarkers for asthma severity mainly focused on inflammatory mediators, forsaking the remodeling contribution in the pathogenesis of this disease. Strikingly, there are controversies on the relationship between lung function and inflammation [16], whereas there is more consistency with airway remodeling [7,17]. Thus, given the relationship between airway remodeling and asthma severity, the identification of remodeling biomarkers is of great interest.

We identified FGF-2 as a potential biomarker of asthma severity and fixed airflow obstruction in IS. FGF-2 level was similar in normal and mild to moderate asthma subjects, but it is specifically increased in severe asthma subjects. Previous studies indicated an increased level of FGF-2 in sputum [18] and bronchoalveolar lavage fluids of asthmatic subjects compared with controls [19]. However, their asthmatic subjects were not subdivided according to the severity of their disease, which may explain, at least in part, the difference with our data. Furthermore, our study suggests that FGF-2 level in IS may be used as a specific biomarker for severe asthma. The increase of FGF-2 was previously detected immunohistologically in bronchial tissue of mild asthmatic subjects [20]. Our study confirmed this observation and further correlated FGF-2 levels with asthma severity and fixed airflow obstruction.

FGF-2 is expressed in epithelial cells, sub-epithelial cells (airway and vascular smooth muscle cells and endothelial cells) and inflammatory cells (mast cells, eosinophils,

macrophages and T cells) of the airways [8]. FGF-2 increases the production of transforming growth factor β (TGF- β), a remodeling factor that is also involved in suppressing airway inflammation [21,22]. Furthermore, FGF-2 has mitogenic activity for airway smooth muscle cells, fibroblasts and endothelial cells [23]. The release of FGF-2 in the submucosal compartment may participate to the remodeling process in asthma through airway smooth muscle cell hyperplasia, as demonstrated *in vitro* [8]. A direct role for FGF-2 in the remodeling process may underlie its specific increase in severe asthma and its negative correlation with pre- and post-BD FEV₁/FVC ratios and post-BD FEV₁. FGF-2 may contribute to airway smooth muscle hyperplasia leading to increase airway narrowing and disease severity [24]. Strikingly, FGF-2 did not correlate with eosinophil level, suggesting that this marker may characterize a sub-phenotype of severe asthma. Furthermore, given the well-known correlation between asthma severity and remodeling [25,26], our data suggest that FGF-2 is linked to remodeling.

MMPs play a central role in inflammation, tissue remodeling and repair [27]. Their proteolytic activities are inhibited by TIMPs. Thus, an imbalance between MMPs and TIMPs may results in disease. Indeed, an excessive production of MMPs is associated with chronic lung disease including asthma [27]. Our data show a significant increase of MMP-1, MMP-8 and MMP-12 levels in IS of mild to moderate asthma, whereas only MMP-1 was also significantly higher in severe asthma compared with control. An increased MMP-1 mRNA level was previously reported in IS cells of mild asthmatic patients [28]. MMP-1 was also associated with persistent airway obstruction in asthma and induction of smooth muscle cell hyperplasia in asthmatic subjects [29]. MMP-1 may represent a potential biomarker of asthma severity. However, there was no difference in MMP-1/TIMP-1 ratio between the groups and no correlation with FEV₁/FVC ratio.

Our data suggest that levels of MMP-8 and MMP-12, and MMP-9/TIMP-1 ratio may be good markers of mild to moderate asthma, but not severe asthma. No correlation between MMP-9/TIMP-1 ratio and FEV₁/FVC ratio were observed, as well as any difference in MMP-9 levels among groups. There are some controversies on the role of MMP-9 in asthma as some studies report an increased level in bronchoalveolar lavage [30], whereas other did not find differences in IS [31]. Increased level of MMP-8 was reported in IS of asthmatic children with moderate symptoms [32], but to our knowledge, there is no report in severe asthma. The medication used by subjects with severe asthma may be responsible of the variability observed in this group.

MMP-12 is mainly associated with lung emphysema. A previous study reported no difference in MMP-12 levels in IS of asthmatic subjects [33]. However, the subjects were separated into smokers and no smokers, whereas we have very few smokers in our groups. It is worth noting that there was no difference in MMP-12/TIMP-1 ratio.

Induced sputum is a sampling technique used since 1992 [34] to assess the presence of airway inflammation and response to treatment, primarily via eosinophil and neutrophil counts [6]. Although there is some limitation especially in children [6], this sampling technique has considerable advantages. Induced sputum is a non-invasive technique and

easier to conduct than bronchoalveolar lavages or biopsies [6]. Furthermore, our data are similar in samples originating from different research centers, showing the reproducibility of this technique. In contrast to less invasive methods, such as exhaled breath condensate and exhaled nitric oxide, many biomarkers can be measured in a single sample of IS, allowing the assessment of different biological pathways to obtain a global overview of the disease and its progression. Furthermore, remodeling mediators are resistant to dithiothreitol (DTT), used in sputum processing, suggesting a great potential for remodeling biomarker discovery and clinical application using this sampling method [35].

Conclusion

In summary, we identified FGF-2 and MMP-1 as two remodeling biomarkers in IS associated with asthma severity. FGF-2 seems more specific to asthma severity as its levels inversely correlated with pre- and post-BD FEV₁/FVC ratios and post-BD FEV₁. FGF-2 could predict worse asthma outcomes through fixed airflow obstruction resulting from an excessive remodeling process. We also described for the first time the use of IS sampling to identify biomarkers of airway remodeling in asthma. To further refine the use of these biomarkers, a larger cohort of asthmatic subjects covering diverse asthma sub-phenotypes should be investigated. Functional studies will help define the clinical relevance of these biomarker level profiles to gain a better understanding of their predictive value for clinical application as prevention and diagnostic tools.

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Declaration of interest

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Supplemental materials

Fibroblast growth factor-2 is a sputum remodelling biomarker of severe asthma

Élyse Y. Bissonnette PhD^{*1}, Anne-Marie Madore PhD^{*2}, Jamila Chakir PhD¹, Michel Laviolette MD¹, Louis-Philippe Boulet MD¹, Qutayba Hamid MD³, Céline Bergeron MD⁴, Karim Maghni PhD⁵, and Catherine Laprise PhD²

Affiliations:

1: Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec, Université Laval; 2: Département des sciences fondamentales, Université du Québec à Chicoutimi; 3: Meakins-Christie Laboratories, McGill University; 4: Centre de recherche du CHUM, Université de Montréal; 5: Centre de recherche de l'Hôpital du Sacré-Coeur de Montréal, Département de médecine, Université de Montréal

* These authors contributed equally to the study

Subjects

Clinical evaluation and sampling were performed at three sites of the Respiratory Health Network (RHN) Tissue Bank of the FRQS (www.tissuebank.ca): Institut Universitaire de Cardiologie et de Pneumologie de Québec, Université Laval (93 samples), Hôpital Sacré-Cœur de Montréal, Université de Montréal (20 samples) and McGill University Health Centre (50 samples). These subjects signed the consent form of Tissue Bank and the project was approved by all site institutional Ethics Committee.

The medical status of all participants was determined by a clinical evaluation and they also completed a questionnaire about respiratory symptoms, medication, smoking status, etc. Spirometry was carried out according to the American Thoracic Society criteria. Asthma was confirmed by improvement in forced expiratory volume in 1 second (FEV_1) $\geq 12\%$ after salbutamol 200 μg , or by the demonstration of AHR to methacholine (provocative concentration of methacholine needed to cause a 20% fall in FEV_1 $< 8 \text{ mg}\cdot\text{mL}^{-1}$ corresponding to PC_{20}). Methacholine based bronchoprovocation tests were performed according to the method described by Juniper and colleagues [1]. Bronchodilator response (BD) was measured as the increase in FEV_1 at 15 minutes after a 200 mg dose of salbutamol was inhaled. Allergy was assessed using skin-prick tests for 31 allergens. Nurses of the three different sites performed all tests.

The 163 samples were classified into non-asthmatic (NA) healthy control group (without asthma and allergy, $n = 25$), mild to moderate asthma (MMA) including individuals with or without inhaled corticosteroid (ICS) treatment ($n = 73$) and severe asthma (SA) ($n = 65$). The classification of asthmatic patients into MMA or SA group was mainly based on the severity of asthma according to medication considered required by the treating physician as proposed in the GINA report [2]. Indeed, for patients already on treatment, classification into different severity groups (intermittent, mild persistent, moderate persistent or severe persistent) according to symptoms (recurrent attacks of wheezing, cough at night, etc.) is often erroneous. Furthermore, a classification based on the intensity of treatment needed by a patient to control its symptoms is now accepted by consensus [2]. So, in this study, asthma was considered to be mild-to-moderate if they needed a low to moderate dose of inhaled corticosteroid (ICS) according to the GINA report [2] with or without additional medication, and severe if they needed a high dose of ICS, defined as being equivalent to $\geq 500 \mu\text{g/day}$ of fluticasone dipropionate in combination with long-acting beta agonists.

Table 1. Comparison between data of samples from different sampling sites of subjects for the mild to moderate asthma group

Data	p	Data	p
General descriptive data		Biomarkers	
M: F ratio	0.712	CTGF	0.077
Age	0.035	FGF-2 ⁷	0.740
Smoking status, n	0.108	MMP-1 ⁷	0.890
Clinical descriptive data		MMP-2	0.378
Epithelial cells (%)	0.803	MMP-3	0.134
Lymphocytes (%)	0.010	MMP-7	0.643
Macrophages (%)	0.097	MMP-8 ⁷	0.025
Neutrophils (%)	0.896	MMP-9	0.015
Eosinophils (%)	0.298	MMP-12 ⁷	0.890
Age of onset	0.056	MMP-13	0.397
FEV ₁ , % pred ¹	0.717	MMP-1/ TIMP-1	0.111
FEV ₁ postBD, % pred ¹	0.676	MMP-8/ TIMP-1	0.042
FVC, % pred ²	0.002	MMP-9/ TIMP-1 ⁷	0.487
FVC postBD, % pred ²	0.037	MMP-12/ TIMP-1	0.066
PC ₂₀ , mg/ml ³	0.454	Procollagen type 1	0.012
FEV ₁ /FVC ratio	0.392	TIMP-1 ⁷	0.029
Allergy, n	0.538		
Medication			
ICS, n ⁴	0.164		
ICS, average dose, µg/day ⁴	0.511		
LABA, n ⁵	0.220		
Others, n ⁶	0.011		

Mann-Whitney for quantitative data and Chi-square with correction for multiple testing for qualitative data were performed. P-values <0.05 are shown in bold.

¹ Forced expiratory volume in one second (FEV₁) in percentage of the predicted value (available for 65 mild to moderate asthma (MMA) subjects, FEV₁ post bronchodilator treatment (postBD) available for 45 MMA subjects);

² Forced vital capacity (FVC) in percentage of the predicted value (available for 41 MMA, FVC postBD available for 41 MMA);

³ Provocative concentration of methacholine that induces a 20% fall in FEV₁ (available 37 MMA).

⁴ Inhaled corticosteroids (ICS) treatment in average fluticasone equivalent dose in µg/day (number of subjects that take ICS available for all subjects and dose available for 30 MMA).

⁵ Long acting beta agonist (LABA) data available for all subjects.

⁶ Others comprise long acting and short acting anticholinergics, short acting β_2 -agonists, anti-leukotrienes and anti-histamines (data available for all subjects).

⁷ Biomarkers found significantly associated with asthma severity in this study.

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