

Original article

Effects of low doses of inhaled fluticasone propionate on inflammation and remodelling in persistent-mild asthma

In asthma a dysregulation of eosinophil apoptosis and an imbalance of metalloproteinase-9 (MMP-9) and tissue inhibitor metalloproteinase-1 (TIMP-1) play an important role in airway inflammation and remodelling. We evaluated the effects of a low dose of inhaled fluticasone propionate (FP) (100 µg bid by Diskus) for 4 weeks in 24 steroid naive patients with mild persistent asthma, symptomatic and with a sputum eosinophilia $\geq 3\%$ on clinical outcomes and inflammatory markers such as the induced sputum eosinophils, the induced sputum apoptotic eosinophils, the levels of MMP-9 and TIMP-1 and their molar ratio in the induced sputum supernatants. After FP treatment forced expiratory volume (FEV1) and FEV1/forced vital capacity values, PEF (L/min), sputum apoptotic eosinophils, and MMP-9/TIMP-1 molar ratio in sputum supernatants of asthmatic subjects were significantly increased in comparison with baseline, while sputum eosinophils significantly decreased. Change (Δ) in FEV1 after treatment with FP negatively correlated with the Δ in sputum eosinophils, while the Δ in MMP-9 values positively correlated with Δ in TIMP-1 values. This study shows that the clinical improvement achieved by the use of low doses of FP in asthmatics is related, at least in part, to the resolution of eosinophilic inflammation and the downregulation of remodelling markers.

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Corticosteroids represent the first line treatment in asthma and the most powerful anti-inflammatory drugs available for the treatment of this disease. As indicated by GINA guidelines (1), increasing doses of corticosteroids should be administered according to the clinical severity of the disease. Although previous studies in mild to moderate asthmatics failed to show a clear dose–response effect for improvements in lung function with inhaled glucocorticosteroids in low doses (2, 3), it has been demonstrated that low doses of inhaled corticosteroids can prevent asthma death (4) and that, in adults and adolescents, the fluticasone propionate (FP) dose–response curve begins at a dose around 100–200 µg/day and tends to reach a plateau at a dose around 500 µg/day (5, 6). This evidence suggests

that in mild moderate asthma a treatment with low doses of inhaled corticosteroids may be effective to control asthma symptoms, but it is still not known whether they can equally control inflammatory and remodelling processes.

The chronicity of airway inflammation is associated with the infiltration of the airways by activated eosinophils and the accumulation of eosinophils in the inflamed airways of asthmatic subjects is the result not only of an increased recruitment from the bloodstream but also of a reduced apoptosis of these cells (7, 8). Furthermore the chronicity of airway inflammation involves structural changes of the airway wall occurring at the level of the extracellular matrix proteins (ECM) proteins, and in this context, a key role is played by the imbalance between metalloproteinase-9 (MMP-9) and tissue inhibitor metalloproteinase-1 (TIMP-1) in the pathogenesis of remodelling (8, 9).

The effect of inhaled corticosteroids in reducing eosinophilic inflammation in asthma is well known. While several studies demonstrated the effects of high doses of

Abbreviations: FP, fluticasone propionate; MMP-9, metalloproteinase-9; TIMP-1, tissue inhibitor metalloproteinase-1; ECM, extracellular matrix proteins; FEV1, forced expiratory volume; FVC, forced vital capacity; PD₂₀, provocative dose of Mch producing a reduction of 20% FEV1.

corticosteroids on eosinophil apoptosis (9, 10) and their ability to modulate the plasmatic levels of MMP-9/TIMP-1 molar ratio in severe asthma, and although a few studies showed that low doses of steroids are able to decrease the eosinophilic airway infiltration (11, 12), it is still not known whether low doses of inhaled corticosteroids are able to increase eosinophil apoptosis and to downregulate the release of MMP-9 and TIMP-1 in mild persistent asthma.

The present study was carried out to verify whether low doses of inhaled FP, in association with clinical improvements, were able to increase sputum eosinophil apoptosis and to reduce the MMP-9 and TIMP-1 levels and their molar ratio in sputum supernatants of mild persistent asthmatics.

Materials and methods

Patients

After the screening of 60 patients, 24 patients with persistent mild asthma and a percentage of eosinophils in the induced sputum $\geq 3\%$ were selected in the study (Table 1).

Bronchial asthma was diagnosed according to the GINA guidelines (1). The inclusion criteria were the following: a history of asthma of at least 6 months, a forced expiratory volume (FEV1) $\geq 80\%$ of predicted value, and a percentage of eosinophils in the induced sputum $\geq 3\%$. In this study, three patients with FEV1 lower 80% were also included because they reported nocturnal and diurnal symptoms that could be registered, according to the GINA guidelines, such persistent mild asthma. All patients were not treated with inhaled corticosteroids in the previous 3 months.

Protocol

This was a multicentre study that aimed to assess the efficacy of low doses of FP on markers of airway inflammation. The run-in phase was as follows: all patients performed a baseline evaluation consisting in the measurement of pulmonary function, bronchial hyperresponsiveness to methacholine (Mch), 2-weeks PEF moni-

oring and recording of symptoms and rescue medication use. Patients were then treated with inhaled FP (dry powder Diskus, 100 μg bid) for 4 weeks, after that pulmonary function test, bronchial hyperresponsiveness to Mch measured as provocative dose of Mch causing a 20% decrease in FEV1 with respect provocative dose of Mch producing a reduction of 20% FEV1 (PD₂₀) and sputum induction and collection were repeated. This study was approved by the local ethic committees.

Sputum induction and processing

Sputum induction and processing were performed as previously described (8). Induced sputum supernatants and induced sputum cells were stored at -80°C until analysed. All biological samples were coded and stored in one centre (National Research Council, Palermo, Italy).

Apoptosis evaluation

Apoptotic eosinophils were detected in induced sputum cell fraction of asthmatic patients before and after FP treatment, by using the TUNEL technique (13).

MMP-9 and TIMP-1 evaluation

The levels of MMP-9 and TIMP-1 were measured in induced sputum supernatant by using a quantitative sandwich enzyme immunoassay technique (13).

Statistical analysis

Results are expressed as medians and 25–75 percentiles. Statistical analysis comparing changes before and after FP treatment was performed using Wilcoxon signed rank test. Spearman's rank correlation was performed to assess the correlation between data.

Results

Clinical characteristics of patients

The majority of patients displayed clinical and functional improvement after FP treatment (Table 1). The differences of FEV1 and PD₂₀ before and after treatment were statistically significant ($P < 0.0008$ and $P < 0.003$ respectively) (Table 1; Fig. 1).

Total and differential sputum cell counts

The total cell count, the viability and the percentage of the sputum cells before and after FP treatment are reported in the Table 2. Interestingly, the percentage of eosinophils was significantly decreased after treatment with FP ($P < 0.0001$) (Fig. 2A).

Eosinophil apoptosis

The reduction of sputum eosinophils after FP treatment was associated with an increased eosinophil apoptosis (Fig. 2B) (Table 2).

Table 1. Demographic characteristics of patients

Characteristics	Treatment with inhaled FP		<i>P</i> value (before/after)
	Before	After	
Subject (<i>n</i>)	24		
Male/female	10/14		
Mean age (years)	38 (31–45)		
FEV1 (% predicted)	96.0 (90.0–102.0)	101.5 (95.0–109.0)	<0.0008
PEF (l/min)	449.7 (368.9–531.8)	494.2 (406.0–580.3)	<0.0035
FEV1/FVC (%)	73.1 (70.0–81.5)	77.3 (73.6–81.4)	<0.0030
PD ₂₀ (mg of Mch)*	70.0 (32.3–274.5)	318.0 (115.5–856.5)	<0.0026

Data are expressed as median and 25–75 percentiles.

P-value Wilcoxon signed rank test.

FP, fluticasone propionate; FEV1, forced expiratory volume; FVC, forced vital capacity; PD₂₀, provocative dose of Mch producing a reduction of 20% FEV1; Mch, methacholine.

*PD₂₀ was evaluated on 23 patients.

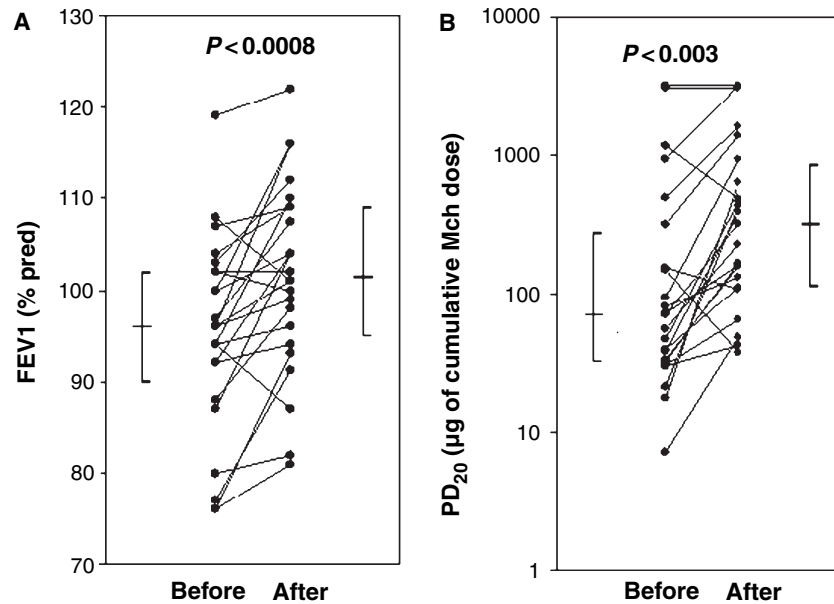


Figure 1. Effects of inhaled low doses of fluticasone propionate (before and after treatment) on forced expiratory volume (FEV1) measurements (A) and provocative dose of methacholine producing a reduction of 20% FEV1 (B) in patients ($n = 23$) with mild persistent asthma. Results are expressed as individual data points and the lateral bars represent median values and 25–75 percentiles. Statistical analysis was performed by Wilcoxon *U*-test.

Table 2. Cell analysis of sputum cells

	Treatment with inhaled FP		<i>P</i> -value (before/after)
	Before	After	
Viability (%)	71.9 (67.9–75.0)	75.0 (69.8–80.0)	ns
Volume (ml)	3.8 (1.8–7.3)	4.4 (2.8–7.3)	ns
Squamous cells (%)	28.5 (20.9–40.5)	39.4 (19.0–54.0)	ns
Corrected cell counts (million cells/ml)	0.92 (0.65–1.28)	0.92 (0.38–1.28)	ns
Differential cell counts (%)			
Macrophages	38.8 (29.1–52.8)	31.8 (19.8–64.3)	ns
Neutrophils	43.4 (27.3–52.6)	62.6 (30.4–74.0)	<0.0207
Lymphocytes	0.6 (0.2–1.3)	0.7 (0.0–1.2)	ns
Eosinophils	12.2 (6.0–24.1)	4.6 (1.6–12.2)	<0.0001
Epithelial cells	0.0 (0.0–1.6)	0.0 (0.0–0.9)	ns
Apoptotic eosinophils (%)*	12.1 (0.0–20.0)	29.9 (16.2–50.0)	<0.0005

Data are expressed as median and 25–75 percentiles.

P-value Wilcoxon signed rank test.

FP, fluticasone propionate; ns, not significant.

*Percentage of apoptotic eosinophils was evaluated on 22 patients.

Measurements of MMP-9 and TIMP-1 levels in sputum supernatant

The MMP-9 to TIMP-1 molar ratio significantly increased after the treatment with FP ($P < 0.0007$) (Fig. 3A) (Table 3). The levels of MMP-9 and TIMP-1 in the sputum supernatants are not significantly different. Although the levels of MMP-9 as well as of TIMP-1 in the sputum supernatants were both affected by the treatment with FP, these differences did not reach the statistical significance (Fig. 3B and C). Furthermore, we

expressed the change of MMP-9 and of its natural inhibitor TIMP-1, after treatment with FP, as absolute difference (Δ) and we found that the Δ in TIMP-1 was significantly correlated with the Δ in MMP-9 ($\text{Rho} = 0.63$; $P < 0.005$) (Fig. 4).

Correlation between inflammatory and functional parameters

In order to identify an association between inflammatory and functional parameters, we plotted the changes in FEV1 values with the changes in the number of induced sputum eosinophils after FP treatment and we found that the Δ of FEV1 levels was inversely correlated with Δ of the number of eosinophils ($\text{Rho} = -0.48$, $P < 0.03$) (Fig. 5).

Discussion

The main result of this study is the observation that a low dose of FP is able to increase eosinophil apoptosis and to modulate markers of airway remodelling also in mild persistent asthmatics and in a short period of time, in association with the well known positive effects on functional findings and sputum eosinophilia. This fact underscores other possible mechanisms of the efficacy of low doses of inhaled corticosteroids in asthma, such as the improvement of eosinophil apoptosis and the improvement of airway remodelling.

Mild-moderate asthma is characterized by a high clinical heterogeneity. Many of these patients may

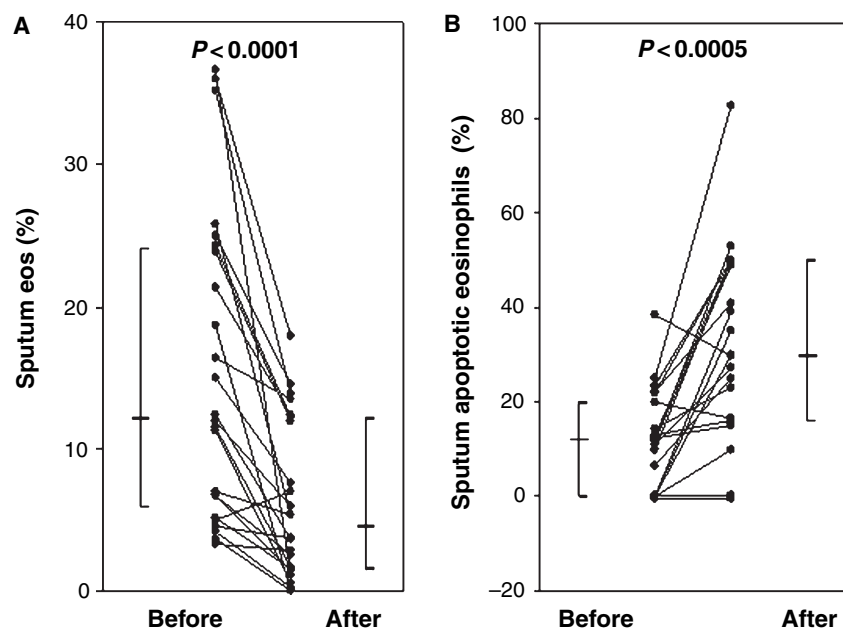


Figure 2. Effects of inhaled low doses of fluticasone propionate (before and after treatment) on the percentage of induced sputum eosinophils (A) ($n = 23$) and on percentage of induced sputum apoptotic eosinophils (B) ($n = 22$) in patients with mild persistent asthma. Results are expressed as individual data points and the lateral bars represent median values and 25–75 percentiles. Statistical analysis was performed by Wilcoxon *U*-test. Mch, methacholine.

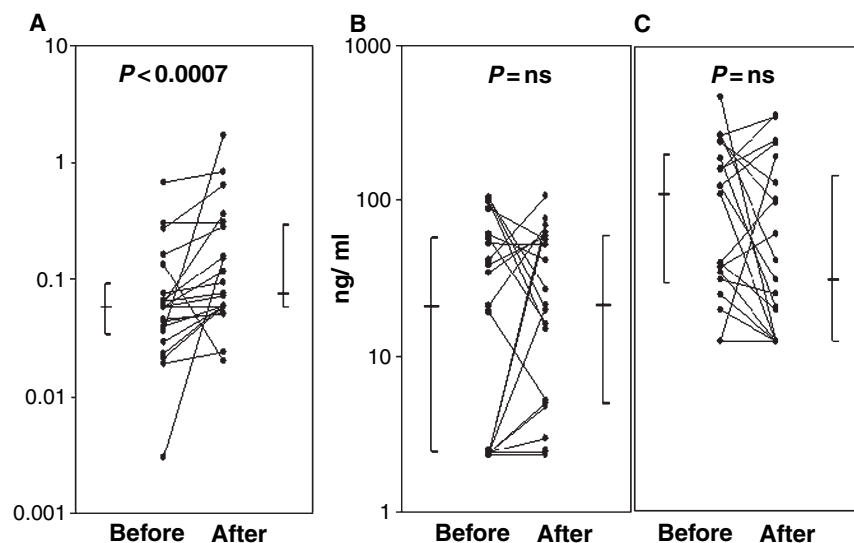


Figure 3. Effects of inhaled low doses of fluticasone propionate (before and after treatment) on the measurements of metalloproteinase-9 (MMP-9)/tissue inhibitor metalloproteinase-1 (TIMP-1) molar (A) and of MMP-9 (B) and TIMP-1 (C) levels in induced sputum from patients with mild persistent asthma ($n = 21$). Results are expressed as individual data points and the lateral bars represent median values and 25–75 percentiles. Statistical analysis was performed by Wilcoxon *U*-test.

experience severe exacerbations as well as daytime and nocturnal symptoms, confirming that appreciable morbidity is associated with the label of 'mild asthma'. Although such patients are treated, mainly in the primary care setting, often without inhaled corticosteroids, several evidences show that corticosteroid-free mild asthmatics can clinically be controlled by low doses of inhaled

steroids (3, 14). Despite these pieces of evidence, it is still unclear whether such a treatment is also able to play a role in the control of airway inflammation and remodelling (15, 16). So far, discordant results have been obtained on the relationship between dose of inhaled corticosteroids and control of airway inflammation and remodelling. Some studies have evaluated the efficacy of

Table 3. Analysis of induced sputum cells

	Treatment with inhaled FP		P-value (before/after)
	Before	After	
MMP-9 (ng/ml)*	20.8 (2.4–58.4)	21.3 (5.0–60.0)	Ns
TIMP-1 (ng/ml)*	112.0 (29.7–201.5)	31.1 (12.5–147.7)	Ns
MMP-9/TIMP-1 (molar ratio)	0.06 (0.03–0.09)	0.08 (0.06–0.29)	<0.0007

Data are expressed as median and 25–75 percentiles.

P-value Wilcoxon signed rank test.

FP, fluticasone propionate; MMP-9, metalloproteinase-9; TIMP-1, tissue inhibitor metalloproteinase-1; ns, not significant.

*MMP-9 and TIMP-1 were evaluated on 21 patients

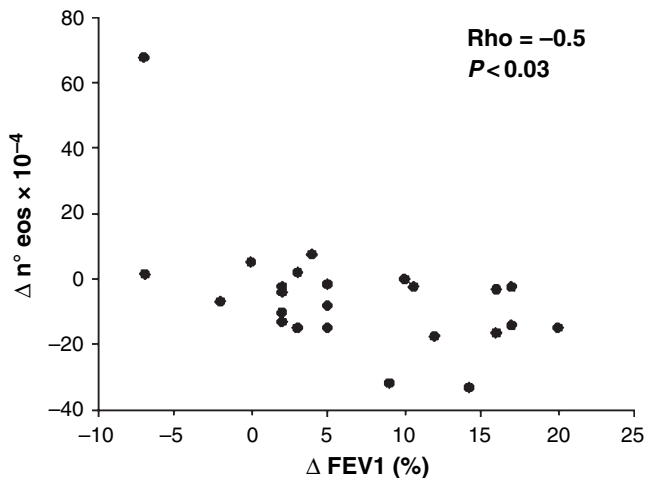


Figure 4. Correlation between the change of metalloproteinase-9 (MMP-9) values (ng/ml) (Δ MMP-9) in induced sputum cells and the absolute differences of tissue inhibitor metalloproteinase-1 (TIMP-1) values (ng/ml) (Δ TIMP-1) before and after treatment with low doses of inhaled FP in patients with mild persistent asthma ($n = 21$). Statistical analysis was performed by Spearman's rank test.

different doses of inhaled corticosteroids on sputum eosinophilia but a clear dose-dependent effect was not observed, with low doses showing similar effects to high doses (2, 14, 16). Olivieri et al. (17) showed that a treatment with 250 μ g of FP bid determined a significant reduction of eosinophilia and subepithelial fibrosis in bronchial biopsy of moderate asthmatics. Moreover, Gershman et al. (16) found that high doses (1000 μ g/day) of FP were associated with a larger reduction in sputum eosinophil percentages than was a low-dose (100 μ g/day) of FP, but these differences between the two groups were not statistically significant. Furthermore, another study performed in steroid naive subjects was unable to show clinical statistically significant differences between a dose of 100 and 1000 μ g/day of FP for all clinical outcome measures, bronchial response to Mch, and markers of inflammation in induced sputum (16).

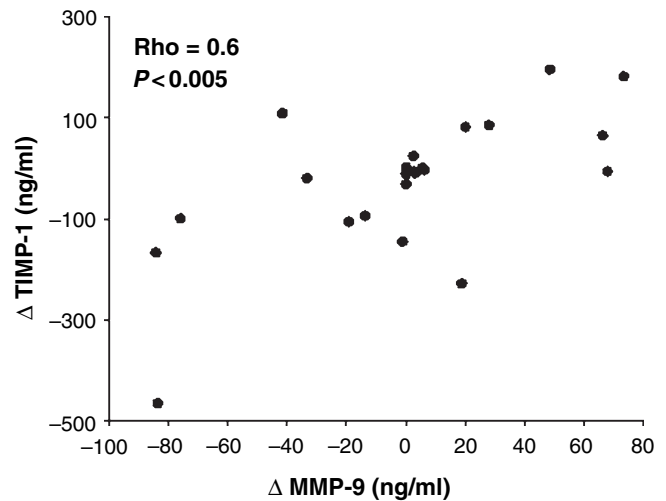


Figure 5. Correlation between the absolute differences of the induced sputum eosinophils number (Δ no EOS) and the change of forced expiratory volume (FEV1) (% pred values) (Δ FEV1) before and after treatment with low doses of inhaled fluticasone propionate in patients with persistent mild asthma. Statistical analysis was performed by Spearman's rank test.

In our study several indicators of asthma control were examined, including FEV1, PEF, FEV/forced vital capacity and Mch dose response. A 4-week treatment period with low doses of FP resulted in statistically significant improvements of all these parameters.

We found that the improvement of the functional parameters (FEV1 values) after treatment with FP was associated with the anti-inflammatory activity of FP on the airways involving a reduction of absolute eosinophil numbers in the induced sputum. One possible mechanism of this effect is through the increase of cell apoptosis (12). In the present study, the percentage of apoptotic induced sputum eosinophils was significantly increased in comparison to baseline after treatment with low doses of FP. Studies *in vivo* and *in vitro* (12, 15, 18) show that the eosinophils undergo apoptosis after corticosteroid therapy (15). We identified a positive correlation between clinical and inflammatory improvements. We found that the absolute differences (Δ) in FEV1 was inversely correlated with the absolute differences (Δ) of the eosinophils in the induced sputum after FP treatment. Taken together these findings demonstrate that low doses of FP improve the FEV1 values of mild persistent asthmatics and that this phenomenon is related to the reduction of the number of eosinophils in their airways. Moreover, the reduction of the number of these cells is likely related to the effect of low doses of FP in increasing eosinophil apoptosis.

It has been shown that apoptosis of eosinophils is usually associated with the resolution of the inflammatory process (18). This is however a complex phenomenon in which repair and healing may result in an altered

structure, referred to as a remodelling of the airways. Repair usually involves two distinct processes: regeneration, which is the replacement of injured tissue by parenchymal cells of the same type, and replacement by connective tissue and its eventual maturation into scar tissue. In many instances both processes contribute to the healing response and inflammation. In asthma, the reduced apoptosis of inflammatory cells leading to the persistence of airway inflammation and maturation, modifies the processes of cell de-differentiation, migration, differentiation and maturation as well as of connective tissue deposition causing an altered restitution of airways structure and function (19). In asthma a dynamic ECM remodelling can occur because of the presence of an increased number of fibroblasts (20) and myofibroblast (21, 22) and to an increased collagen deposition (21, 23) leading to the airway wall thickening. The ECM is indeed a dynamic structure and an equilibrium between synthesis (24) and controlled degradation of ECM components is required for the maintenance of its homeostasis. Three major elements are involved: proteases and protease inhibitors (25, 26), cell receptors recruiting intracellular and secreted proteases to the cell surface; and the integral membrane proteases activating a protease cascade. Among proteases and their inhibitors, MMP-9 and TIMP-1 are capable of playing an important role in the pathogenesis of asthma. We have previously demonstrated that, in asthmatic subjects, the levels of induced sputum MMP-9 and TIMP-1 are significantly increased as compared with those from control subjects (8). We also showed that the levels of TIMP-1 exceed those of MMP-9 and were inversely correlated with the FEV1 values (8), suggesting that in asthma the remodelling of ECM is characterized by an imbalance between metalloproteinases and their inhibitors which may generate an excessive collagen deposition in the airways. In this study, after 4 weeks of low doses of FP treatment, we found that the MMP-9/TIMP-1 molar ratio in sputum supernatants was significantly increased, bringing the subjects towards the 'normal status' (8). We did not find any statistically significant modification of the absolute levels of MMP-9 and TIMP-1 after FP treatment but the finding that there

was a positive correlation between the absolute differences (Δ) in MMP-9 and the absolute differences (Δ) in TIMP-1 values after treatment with FP demonstrates that the treatment with low doses of FP is able to affect the release of both molecules although low doses of FP, indeed, increase the MMP-9/TIMP-1 molar ratio. The increase of this ratio is extremely important because suggests that these patients, after FP treatment, may have a relative increase of MMP-9 vs TIMP-1. This relative upregulation of MMP-9 might protect the asthmatic patients from the remodelling of ECM (8). To our knowledge this is the first study showing that low doses of FP regulate the balance between MMP-9/TIMP-1 in such a way to upregulate the molar ratio between MMP-9 and TIMP-1. We did not identify any correlation between MMP-9 and TIMP-1 levels and FEV1 values while we found a negative correlation between the number of eosinophils and the FEV1 values. This might be related to the fact that although eosinophils represent an important source of metalloproteinases and their inhibitors in the airways of asthmatic subjects (27, 28) the effects of steroids on the release of MMP-9 and TIMP-1 is also related to other cells which are less targeted by low doses of FP (29) so that their levels after the FP treatment did not reach a correlation with functional improvements.

In conclusion, this study shows that in corticosteroid-free mild persistent asthmatics, the clinical improvement in asthma control after low doses of inhaled FP is associated with the resolution of the eosinophilic inflammation, mainly through an improvement of eosinophil apoptosis, and with the modification of some markers of remodelling, including MMP-9 and TIMP-1 levels. Therefore, this study contributes to the clarification of the mechanisms by which low doses of inhaled corticosteroids are effective in controlling airway inflammation in asthma.

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