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Pattern of airway inflammation and remodelling in mild persistent atopic asthma and in mild persistent asthma related to Gastroesophageal Reflux

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Key words

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Summary

Background: *The increase of basement membrane thickness (BMT) represents a structural feature described as commonly characterizing airway remodelling in asthma, even if the non-atopic condition had been investigated only episodically from this point of view. Gastroesophageal-reflux is a pathological condition which can frequently cause and/or sustain asthma in non-atopic individuals.* ***Objectives:*** *The aim of the study was to measure BMT; some inflammatory mediators in BAL; cys-leucotrienes (LTE_4) in urine; e-NO, and BHR to Methacholine (MCh) in mild atopic and in mild non-atopic, GER-related asthma.* ***Methods:*** *After their informed consent, 25 mild atopic (40.9 years ± 13.1 sd, FEV_1=95.9% pred. ± 12.9 sd) and 39 non-atopic, GER-related asthmatics (57.3 years ± 14.2 ds, FEV_1=101.3% pred. ± 12.2 sd), non-smoker and of a comparable asthma duration, underwent measurements of basal lung function and bronchial response to MCh (PD_{20} FEV1); endobronchial biopsies and BAL (in the right middle lobe), and a 24-h gastroesophageal pHmetry.* ***Results:*** *Atopic and GER-related asthma showed two distinct patterns of airway inflammation. The eosinophilic contribution to airway inflammation was systematically prevailing in the former group, such as: EOS=10.7% ± 13.4 sd vs 2.0% ± 2.8 sd, p=0.001; ECP=344.9 mcg/l ± 635.9 sd vs 59.2 mcg/l ± 75.1 sd, p=0.001.* **Conclusions:** *Data from the present study are suggesting that persistent mild atopic and mild GER-related asthma seem to represent two distinct phenotypes of asthma in terms of airway remodelling, and in particular of BMT involvement.*

Introduction

Asthma is a disease condition characterized by airway inflammation; variable airflow obstruction; bronchial hyperresponsiveness (BHR), and respiratory symptoms, such as cough, wheeze and attacks of breathlessness (1). It has been generally recognized that asthma is also characterized by variable degrees of chronic inflammation and structural changes within the airways (2).

These structural alterations, globally called "*airway remodelling*", encompass complex changes in composition, content, and organization of various cellular and molecular constituents of airway walls (3). Epithelial thickening; increased airway smooth muscle mass; bronchial gland enlargement; angiogenesis, and changes in the extracellular matrix components represent the most striking abnormalities which involve large and small airways, together with the surrounding peri-bronchiolar areas (4).

If the accumulation of eosinophils, mast cells and TH_2 lymphocytes in the bronchial mucosa is prevailing in the allergic forms of asthma, a substantial accumulation of neutrophils in addition to eosinophils and mast cells have been described as characterizing non-allergic asthma (5).

Because of the constant accessibility in bronchial biopsies, basement membrane (BM) has been studied extensively in asthma, and its thickness increase is reported as the "remodelling marker" in several studies (6). It consists of the thickening of the subepithelial lamina reticularis that lies urderneath the true basal lamina of bronchial epithelium.
Actually, the debate on whether or not atopic and non-atopic asthmatics show distinct patterns of airway remodelling is still open, particularly in the early stages of the disease. From this point of view, increasing attention has been paid to bronchial asthma when related to the presence of acid gastro-esophageal reflux (GER) in the last decade, and the causative (or triggering) role of GER has been progressively emphasised particularly in non-atopic subjects (7-10).
GER-related asthma can thus represent a challenging and a suitable model for investigating and comparing the role of allergic and non-allergic (acid in the case) stimuli in affecting the patho-physiological picture of asthma.
Aim of the present study was to measure and compare some morphological, cellular, and biological indices, together to lung function and BHR in mild atopic and non-atopic, GER-related asthma.

Materials and methods

Patients

The study was conducted in accordance with the declaration of Helsinki and was approved by the local Ethics Committee.
After their informed consent, 64 non-smoker, mild persistent asthmatics were investigated: 25 mild atopic asthmatic (AA; 13 females; 40.9 years ± 13.1 sd, FEV_1= 96.8% pred. ± 13.4 sd, $PD_{20}FEV_1$=309.1 mcg ± 432.7 sd), and 39 non-atopic, GER-related asthmatics (GER-A; 23 females; 57.3 years ± 14.2 ds, FEV_1 = 101.0% pred. ± 11.8 sd, $PD_{20}FEV_1$=1268.4 mcg ± 1099.4 sd).
Asthma was of a comparable mean duration in the two groups (15.2 and 13.6 years, respectively), even if with a later onset in the GER-A group. All subjects had been free from respiratory infections for at least 8 weeks, and from any cardiovascular disease.
Allergic asthmatics: all the 25 patients had a positive skin prick test (>3 mm) for at least perennial allergen tested (DPH, cat, dog, horse, house dust mite, clodosporium and alternaria), and had never complained any significant digestive symptom in their past. All subjects were regularly treated with low dosed ICS (daily dose equivalent to Bechlomethasone dipropionate 200mcg bid) and salbutamol prn.
Non-allergic, GER-related asthmatics: all the 39 subjects had a negative skin prick test, and were complaining significant digestive symptoms (such as the daily occurrence of acid regurgitations and heartburns, or at least ≥2 times/week), since before their asthma onset (11). oreover, the presence of a pathological GER had been instrumentally documented; 34/39 subjects were used to assume digestive treatments periodically and salbutamol prn, while 21/39 were also assuming low dosed ICS.
In all subjects, other causes or triggers of asthma (such as; occupational; hormonal; food and aspirin related; psychological, etc.) were preliminarily and carefully excluded.

Diagnosis of gastroesophageal reflux

The presence of a pathologic acid GER were detected (such as, confirmed or excluded) in the previous 12 weeks in all subjects by means of a 24h-gastro-oesophageal pH-monitoring: a combined monocristant antimony catheter was used with a pressure sensor to locate the LES (Digitrapper MKIII; Synectics Med., Stockolm, Sweden), and the diagnostic DeMeester's criteria were assumed (12).

Lung function and bronchial hyperrectivity

Lung function was basally assessed by a spirometric test (Masterscreen, Vyasis-Jaeger; Hoechberg; Germany). FEV1 was expressed in absolute value (l) and in % predicted (CECA 1993, in the range 18-70 years) (13).
The bronchial response to MCh was also assessed and results were expressed in mcg MCh causing a 20% FEV1 fall from baseline (PD_{20} FEV1). The bronchial challenge was performed by doubling MCh doses (ranging 50-3150mcg) via the APS System (Vyasis-Jaeger; Hoechberg; Germany) up to the PD_{20} FEV1 (14).

Exaled nitric oxide (e-NO)

Measurements of exhaled nitric oxide concentration were performed according to the ATS/ERS recommendations (15). Subjects were invited to exhale against a resistance to determine the closure of the soft palate and thereby to avoid the contamination with the NO of the nasal cavities. The level of e-NO was assessed via a chemoluminescence analyzer (Sievers 280, GE analytical Instruments, Boulder, CO, USA), and values were expressed in ppb (normal values ≤ 15 ppb).

Bronchoscopy

Fibreoptic bronchoscopies were performed on an out-patient basis in accordance with established guidelines (16). All bronchoscopies were performed in the morning after a premedication with 200-400mcg nebulised salbutamol. Immediately before bronchoscopy, midazolam was administered *i.v. via* a cannula, which remained *in situ* until the patient was fully recovered. During the procedure, subjects had continuous monitoring of the pulse oximetry and received oxygen *via* nasal cannulas as required to maintain oxygen saturation >93%. The nose and the oropharynx were anaesthetized with lignocaine spray, the vocal cords with 4% lignocaine delivered *via* the bronchoscope. The bronchoscope (Pentax FB19TX, Tokyo, Japan) was inserted nasally where possible and the oral route was used only as a second choice. At least six endobronchial biopsies (EBB) were then obtained from the proximal airways, usually from subsegmental and segmental carinae of the right or left lower lobes, or right middle lobe. BAL was performed under flexible bronchoscopy at the same time as EBB. After inspection of the bronchial tree, 60-180ml of pre-warmed 0.9% saline were instilled into the right middle lobe and then gently aspirated.

EBB preparation

After fixation in neutral buffer formaldehyde 4% and embedding in paraffin, each EBB was sliced and then stained with haematoxylin-eosin; 4-to-6 sections were fixed into a slide for subsequent analysis. For each patient the best slide was selected, with respect to the presence of mucosal and sub-mucosal layers.

Eosinophils (Eos) and Eosinophilic Cationic Proteine (ECP)

ECP measurements on BAL were carried out according to the standardized instructions provided by the manufacturer (CAP System ECP FEIA, Thermofisher Diagnostics, Upsala, Sweden)

IL-8, and TNFα assay

Concentrations of IL-8 and TNFα in pooled secretion supernatant were measured on BAL by means of an automated immuno-analyzer (Immulite®; Diagnostic Product Corp., Los Angeles, CA, USA). The technique is based on a solid phase (bead) with two sites for the chemiluminescent enzyme immunometric assay (17).

Urinary LTE_4

Urine samples were obtained in the morning before bronchoscopy. Urinary LTE_4 were measured by an enzyme immunoassay (ACE™ Competitive Enzyme Immunoassay, Cayman Chemical, Ann Arbor, Mich, USA). LTE_4 concentrations were measured by enzyme immunoassay according to the manufacturers' instructions; values were expressed in pg/mg creatinine (pg/ml/mg creatinine) and reported to normal values (18).

Statistics

Quantitative variables were expressed as mean and standard deviation; categoriacal variables as absolute and percentage frequencies. The Mann-Whitney test and the Wilcoxon test were used for comparing means between the two groups, and $p<0.05$ was assumed as statistically significant. The statistical analysis was performed using the STATA software vers.12 (STATACORP, 4905 Lakeway Drive, Texas, USA).

Results

Demographics of patients and means ± sd of basal lung function and of the pH-metry score measured in the two groups are reported in table 1, together with the corresponding results of statistical comparisons.

The two groups had their lung function in the normal range, and were comparable in terms of both gender and basal FEV_1 ($p=0.80$), but not of age ($p<0.001$) and bronchial response to MCh (Tab. 1).

Mild AA and mild non-atopic GER-A resulted clearly different in terms of their biological pattern of airway inflammation (Tab. 1). In particular, despite their indistinguishable neutrophilic component of inflammation (Tab. 2), the eosinophilic contribution was absolutely prevailing in mild AA subjects: EOS=10.7% ± 13.4 sd vs 2.0% ± 2.8 sd, p=0.001; ECP=344.9 mcg/l ± 635.9sd vs 59.2 mcg/l ±

Table 1 - Demographics and lung function compared in the two groups of patients

	AA	A-GER	p
m/f	12/13	16/23	
Age (years)	40,9±13.1 sd	57.3±14.2 sd	0.01
FEV_1 (%pred.)	95.9±12.9 sd	101.3±12.2 sd	0.08
PD_{20} MCh (mcg)	309.1±432.7 sd	1268.4±1099.4 sd	0.001

Table 2 - Comparability of Neutrophil count; IL-8, and TNFα concentrations in the two groups of patients

	AA	A-GER	p
Neutrophils (%)	2.64±1.1	3.04±1.0	0.18
IL-8 (pg/ml)	982.13±1587.98	1034.9±1939.8	0.28
TNFα (pg/ml)	24.83±12.5	25.37±7.8	0.29

75.1 sd, p=0.001. Even though much higher in this group, concentrations of u-LTE_4 (394.7pg/ml ± 291.6 sd vs 251.1 pg/ml ± 199.1 sd) and of e-NO (38.1 ppb ± 29.5 sd vs 25.2 ppb ± 27.1sd) were not significantly different from those of GER related subjects (p=0.09 and p=0.09, respectively (Fig.1).

Finally, also the thickness of reticular basement membrane was significantly more pronounced (near twofold) in AA than in GER-A mild asthmatics (5.3 μm ±1.6 sd vs 3.0 μm ± 1.3sd; p=0.001) (Fig. 2).

Figure 2 - BMT compared in the two groups

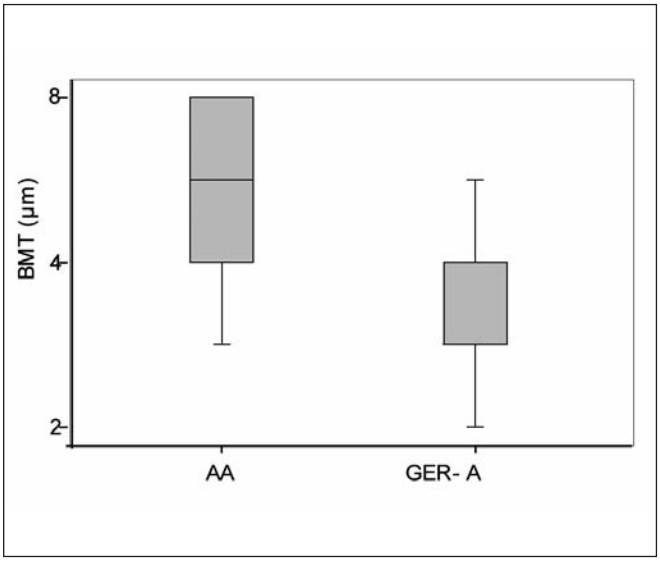


Figure 1 -EOS % (panel a); ECP concentration (panel b); urinary LTE4 (panel c), and e-NO (panel d) compared in the two groups of subjects

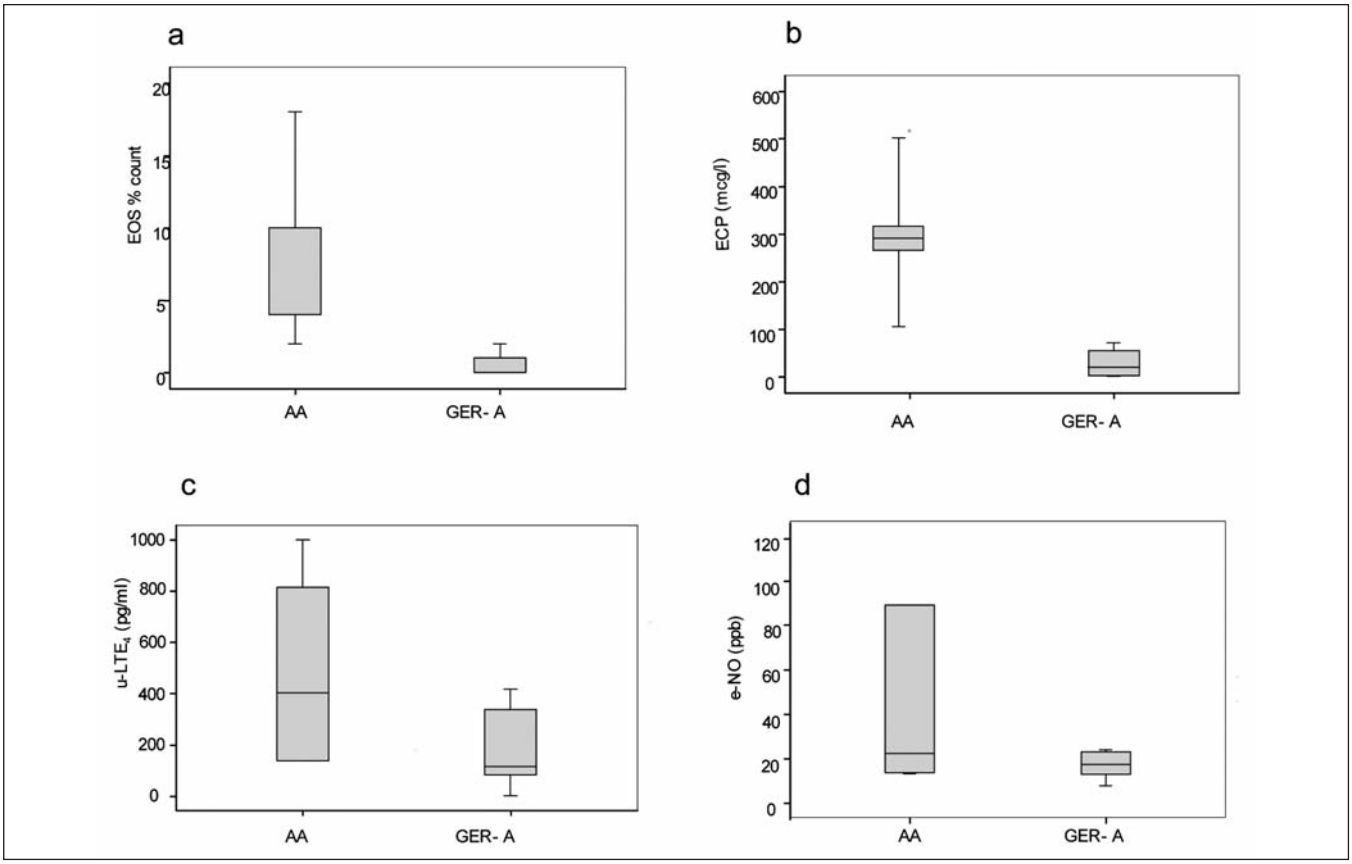
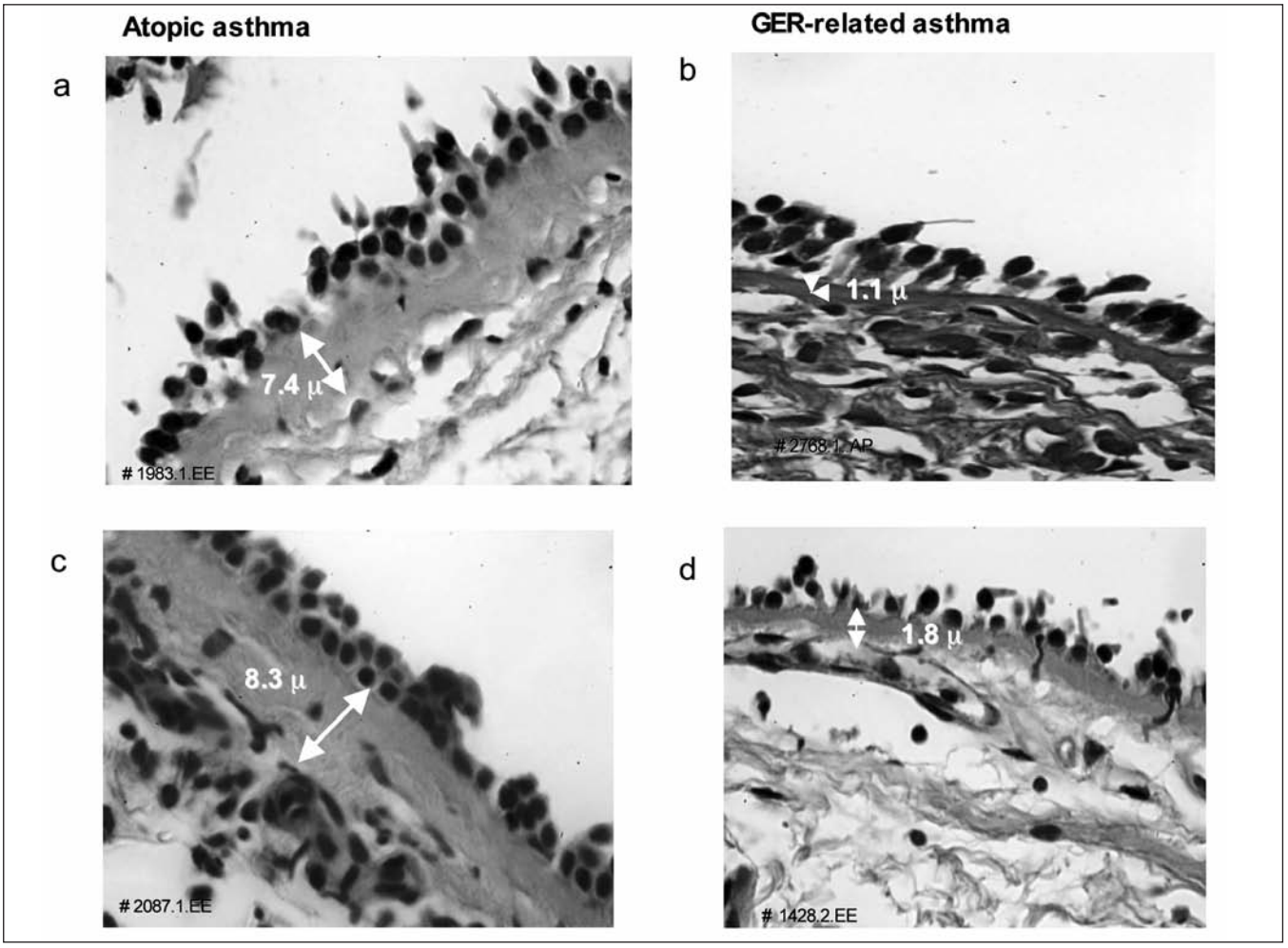


Figure 3 - Examples of bronchial biopsies in two atopic (panel a and c) and in two GER-related (panel b and d) asthmatics. MBT was clearly different independently of the extent of the epithelial damage. The corresponding slide number at bottom left.



In figure 3 are reported four examples of bronchial biopsies from two AA (Fig. 3a and 3c) and two GER-A (Fig. 3b and 3d) subjects, clearly indicating the substantial difference between the two groups of subjects in terms of BMT.

Discussion

Asthma is main characterized by the presence of respiratory symptoms associated with a variable airflow limitation; airway hyperresponsiveness; bronchial inflammation, and structural changes of airway wall.

Pathological repair of chronic inflammation may result in airway remodelling characterized by hypertrophy and hyperplasia of airway smooth muscle; submucosal gland hyperplasia, vascular proliferation, deposition of extracellular matrix (such as collagen and fibronectin) in the subepithelial basement membrane or in the submucosa. The thickening of the *lamina reticularis* below the true basement membrane is a characteristic early feature of asthmatic airways, named "basement membrane thickening" (BMT), which is associated with deposition of collagen I, collagen III, and fibronectin (19). An approximate twofold increase in BMT has been reported in asthmatics of different severity (20-21), but its reduction following anti-inflammatory treatment still has to be definitively clarified (6).

Remodelling has been attributed to the repetitive injury to the airway wall due to several cycles of inflammatory events and subsequent repair, which can represent a perpetuating damaging process.

Although remodelling has been considered a consequence of inflammation, it has also been suggested that the remodelling process *per se* might be independent of inflammation, such as a sort of a primary event in the natural history of asthma (22).

Several studies are supporting that asthmatics as a group experience an accelerated rate of deterioration in respiratory function which is tempting to ascribe to the structural airway changes due to remodelling (23-24). BMT, with the increased deposition of matrix components, has been shown to correlate with the degree of bronchial hyperresponsiveness in atopic asthmatics, thus indicating that this structural change could be linked to important patho-physiological aspects of asthma (25). Actually, the evidence that a great proportion of asthmatics have a persistently increased bronchial responsiveness despite a long-term anti-inflammatory treatment has been suggested as likely related to the airway remodelling occurring in atopic individuals (26)

Allergic asthma has been extensively investigated from this point of view and shown to include several inflammatory changes in the airways which are mainly characterized by the involvement of eosinophils and of T helper, type 2 (Th2) lymphocytes.

Much less information is available about the pathologic characteristics of remodelling in non-allergic asthma, mainly investigated in small and scarcely selected samples of subjects (27). Some immuno-histochemical studies contributed to quantify the involvement of different inflammatory cells in bronchial biopsies from allergic and non-allergic asthmatics in stable conditions (5) and some significant differences were observed, such as: more eosinophils and lymphocytes, but fewer neutrophils in the allergic group.

Also the extent of epithelial damage was described as significantly higher in allergic asthmatics compared with control subjects and non-allergic asthmatics, even though in a study on two small samples of atopic and GER-related asthmatics of different severity the extent of epithelial damage was found quite similar in both conditions independently of the extent of eosinophilic inflammation (28).

The precise relationship between the remodelling and the various triggers or causative factors of asthma still is poorly understood, although allergen exposure of sensitised subjects is regarded as the stimulus that is most strictly implicated in remodelling events. On the other hand, the propensity for allergen challenge (and the subsequent inflammatory response) to trigger and sustain the persistence of airway injury was confirmed by experimental asthma in several animal species (29).

The role of non allergenic triggers on airway remodelling, such as GER, has been less explored and still is not exhaustively clarified. Exposures to acid GER evoke cough, bronchoconstriction (7-11), airway hyperreactivity, microvacular leakage, and heightened production of mucous, fluid, and nitric oxide (30). Moreover, the condition of airway hyperreactivity induced by acid challenges has become a specific feature characterizing the bronchial response of mild asthmatics when acid refluxer, and then of high diagnostic value in clinical terms (10).

The intimate mechanisms of acid-induced asthma are dependent on activation of capsaicin-sensitive sensory nerves; protons activate these nerves, and the subsequent release of tachykinins and other mediators modulate diverse aspects of airway dysfunction and inflammation trough the activation of NK1 and NK2 receptors on target epithelial, endothelial, mesenchymal, and inflammatory cells (31-34).

Present data tend to emphasize that the extent of airway remodelling in mild persistent asthma is quite low when GER-related. It is then presumable that, at least in its early stages, the GER-induced inflammation could affect airway structures according to a pattern (and/or timing) of damage which is different from that of repeated allergic exposure as occurring in atopic asthma of same severity.

In particular, the eosinophilic component of inflammatory process proves minimal in mild GER-related asthma. Moreover, it is also characterized by a low cys-LTs expression which, on the other hand, corresponds to a negligible involvement of BM, thus further supporting the key-role of eicosanoids in airway wall remodelling and in affecting BM thickness (35).

In conclusion, all morphological, cellular, and biological data registered in the present study are strongly suggesting the hypothesis that mild persistent GER-related asthma should be regarded as a distinct phenotype of asthma which is characterized by a negligible inflammatory component. This suggestion is supported by the low grade of aggression against airway structures (and BM in particular) which prove much more preserved than in atopic asthma of comparable severity and duration.

Authors' contribution

Roberto W Dal Negro, Claudio Micheletto designed the study
Roberto W Dal Negro, Claudio Micheletto drafted the manuscript.
Massimo Guerriero analized the statistical data
Roberto W Dal Negro, Claudio Micheletto and Massimo Guerriero revised the manuscript.
All authors have approved the final version to be published

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