

# Increased glycosaminoglycans in the urine of asthmatic children on inhaled corticosteroids

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Increased extracellular matrix (ECM) deposition in the airway wall contributes to the airway remodeling in asthmatics. Glycosaminoglycans (GAGs) are polysaccharides attached to a protein core in order to form proteoglycans, a component of the ECM. In this study, we investigated the possible influence of long-term treatment with inhaled corticosteroids (ICS) on urinary GAGs levels of asthmatic children. Seventy asthmatic children (41 boys), aged 6.8–12.5 yr, participated in the study. About 44 were treated with inhaled budesonide via turbuhaler for 2–35 months (median 12 months) and 26 were on relief medications. About 30 healthy controls were also studied. GAGs were precipitated from early morning urine samples, collected, isolated and quantified using uronic acid-carbazole reaction and expressed as uronic acid (UA) in  $\mu\text{g/g/Cr}_u/\text{m}^2$ . Urinary GAGs values did not differ significantly between controls and asthmatics but significant differences were found between children on ICS and asthmatics on relief medications ( $p < 0.001$ ). There was a positive correlation between the daily dose of inhaled budesonide and the urinary GAGs values ( $r = 0.32$ ,  $p = 0.037$ ) whereas a threshold distinguishing 'low' vs. 'high' doses of ICS was found to be at  $300 \mu\text{g}/\text{m}^2$  per day with a significant difference in urinary GAGs secretion ( $p = 0.006$ ). Our data show that urinary GAGs secretion is reduced in asthmatic children that used only relief medication but it is increased in those on long-term treatment with ICS. A dose dependent effect of ICS was also detected.

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The term 'remodeling' describes the structural alterations that occur in the lung of asthmatic patients, even before a clear clinical diagnosis, due to prolonged chronic inflammation within the airways (1–3). It involves two distinct processes; a reversible process of regeneration 'ad integrum' leaving no trace of previous injury and a non-reversible replacement of damaged tissue by connective tissue (4). Remodeling of the subepithelial matrix of the bronchial wall is a well-known feature of moderate and severe asthma (5). During remodeling extracellular matrix (ECM) deposition in the airway wall is increased, resulting in excess interstitial collagen and fibronectin deposition (6).

Glycosaminoglycans (GAGs) are long, straight chain polysaccharides composed of repeated disaccharide units which occur either in free form or attached to a protein core to form proteoglycans (PGs) (7). Proteoglycans are important structural and functional components of the ECM, pericellular and basement membranes (8). These macromolecules influence cell adhesion, migration and proliferation, properties which largely depend on the nature of GAGs side chain (9).

GAGs are involved in the inflammatory mechanisms of asthma by interacting with cytokines (10–12). Endobronchial biopsies obtained from patients with mild asthma showed that proteoglycans' deposition was significantly increased in

asthmatics as compared with that in control subjects (13). It has been also shown in a rat model of pulmonary emphysema, that the disappearance of heparin sulphate proteoglycan in the lung was accompanied by an increase of GAGs in the urine (14). There have been few studies demonstrating an increase of GAGs secretion in the urine of asthmatic subjects during acute asthma attacks (15).

Use of prophylactic agents with topical anti-inflammatory activity, aims to arrest the remodeling process by preventing any non-reversible airway damage, leading to airway wall reconstruction. Inhaled corticosteroids (ICS) are unquestionably considered the cornerstone of chronic asthma management, as they reduce symptoms and frequency of asthma exacerbation, improve lung function and reverse remodeling process (1–4, 16–18).

Considering the inhibitory role of ICS on airway remodeling, we hypothesized that long-term anti-asthmatic treatment with ICS is accompanied by decreased proteoglycans' deposition on the basement membrane of asthmatic children and therefore by increased GAGs in the urine. To test this hypothesis we investigated a cohort of asthmatic children in a cross sectional study looking at the effect of long-term treatment with inhaled budesonide in urinary GAGs levels.

## Materials and methods

### Patients

Seventy asthmatic children (41 boys), aged 6.8–12.9 yr that were followed at the Department of Allergology–Pulmonology at Penteli Children's Hospital in Athens were studied. The patients were clinically stable for the last 2 months and their FEV<sub>1</sub> at the time of testing was >80%. Patients were excluded from the study if they had used skin or intranasal or systemic corticosteroids during the last 3 months. Children with a history of other chronic illness were also excluded.

Asthma severity was graded according to the Guidelines for the Diagnosis and Management of Asthma, Expert Panel Report II, in four stages: intermittent, mild persistent, moderate persistent and severe persistent (19). Children included in the study suffered from intermittent or mild/moderate persistent asthma. Mild asthmatics were receiving usual relief medications ( $\beta_2$ -agonists) on an 'as required' basis, whereas persistent asthmatics were treated with inhaled budesonide via turbuhaler (with or without the addition of an inhaled long-acting  $\beta_2$ -agonist) for at least 2 months and as required inhaled short-acting

$\beta_2$ -agonists. No other controller medication were allowed. Short and long-acting  $\beta_2$ -agonists were withheld for 48 h before testing.

Atopic status was assessed by skin prick testing and/or RAST. A battery of 20 common inhaled allergens was tested.

About 30 healthy non-asthmatic children matched for age and sex served as controls.

### Methods

The testing procedure was based on the Di Ferrante method modified by Pennock (20, 21). Early morning urine samples were collected. A 100 ml sample of urine from each patient was preserved by the immediate addition of methanol (1 ml methanol/100 ml urine) and refrigerated at  $-30^\circ\text{C}$ . Urinary creatinine and specific gravity were measured and the analysis was only continued if the creatinine content was in agreement with that expected from the child's body surface area. A 25 ml urine sample was centrifuged at 3000 rpm for 15 min and 5 ml of supernatant were precipitated with 10% CPC (cetylpyridinium chloride). The precipitate was collected by centrifugation, washed twice with 95% ethanol containing 10% potassium acetate and dissolved in 1 ml of 0.05 M NaOH. Uronic acid was measured using uronic acid-carbazole reaction (22). GAGs were expressed as uronic acid (UA) in mg UA/g urine creatinine/m<sup>2</sup> body surface area ( $\mu\text{g/g Cr/m}^2$ ).

The study was approved by the ethical committee of Penteli Children's Hospital and parental informed consent was obtained for all children.

Results were expressed as mean  $\pm$  s.e.m. One way ANOVA, independent two-side Student's *t*-test and bivariate correlation by Pearson coefficient were employed. Statistical analysis was performed with SPSS 10.0 for Windows.

## Results

About 44 children (25 boys) with a mean age ( $\pm$  s.d.) 9.6 ( $\pm$  2.3) years were treated with inhaled budesonide for their persistent asthma. The median duration of inhaled budesonide treatment was 12 months, (range 2–35 months), and the median daily dose was 400  $\mu\text{g}$  or 467  $\mu\text{g}/\text{m}^2$ , ranging from 200 to 800  $\mu\text{g}$  or from 156 to 750  $\mu\text{g}/\text{m}^2$ . The mean age of the 30 children with intermittent asthma was 9.8 ( $\pm$  2.1) years and the mean age of the control group was 9.4 ( $\pm$  1.8) years.

There were no significant differences in the urinary GAGs measured values in between

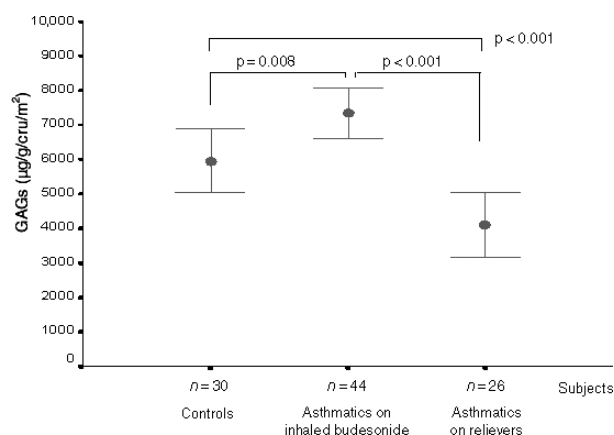


Fig. 1. Urinary GAGs levels of healthy controls, asthmatic children on inhaled budesonide and asthmatic children on relief medication. There is a significant difference between children on inhaled budesonide and controls, between children on inhaled budesonide and those receiving relief medication as well as between controls and asthmatics on relief medication.

controls and asthmatic children as a whole (difference 225.4; 95% CI: -602.5-1052.7;  $p = 0.59$ ). However, values differed significantly between children on inhaled budesonide and children receiving usual relief medication on an 'as required' basis (difference 3339.5; 95% CI: 2253.9 to 4424.2;  $p < 0.001$ ), between children on inhaled budesonide and controls (difference 1465.6; 95% CI: 401.5-2529.7;  $p = 0.008$ ) as well as between asthmatics on relief medication and controls (difference 1873.5; 95% CI: 1318.0-2428.9;  $p < 0.001$ ) (Fig. 1). There were no gender or age differences in the urinary GAGs measured values; there were no differences between atopic and non-atopic asthmatics.

With regards to the daily dose of inhaled budesonide, there was a positive correlation between the daily dose of inhaled budesonide and the urinary GAGs measured values of children on this treatment (Pearson's correlation coefficient  $r = 0.32$ ,  $p = 0.037$ ) (Fig. 2). The threshold that distinguished 'low' vs. 'high' doses was set post hoc at  $300 \mu\text{g}/\text{m}^2$  per day; i.e urinary GAGs levels of children on a dose of ICS less than  $300 \mu\text{g}/\text{m}^2$  were significantly lower compared to those on 'high' doses (difference 2321.1; 95% CI: 692.0-3950.2;  $p = 0.006$ ) (Fig. 3).

No correlation between the duration of treatment of persistent asthma to GAGs levels in urine was observed.

## Discussion

These data show that urinary GAGs secretion is reduced in children on relief medications compared to normals, but it is increased in asthmatics

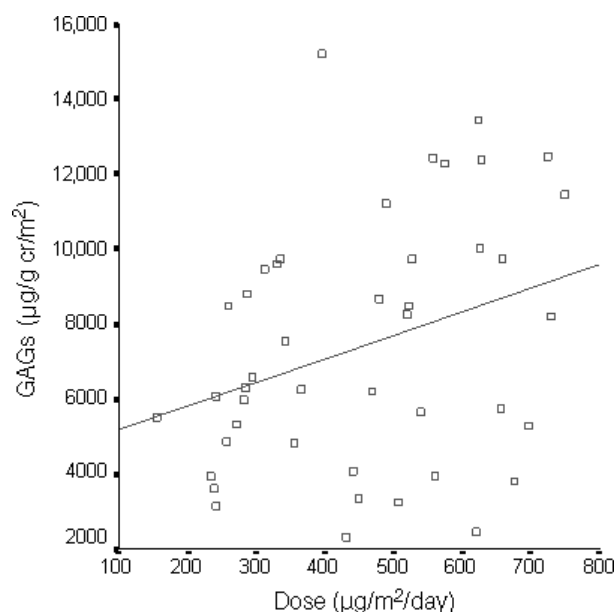


Fig. 2. A positive correlation between the daily dose of inhaled budesonide and the urinary GAGs measured values of children on this treatment was detected ( $p = 0.037$ ).

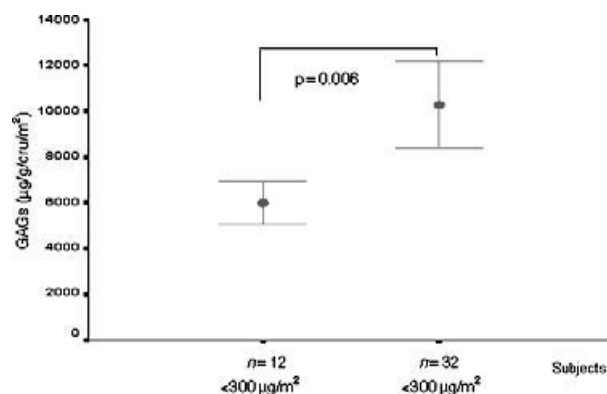


Fig. 3. Urinary GAGs levels of asthmatic children on a daily dose of inhaled budesonide  $< 300 \mu\text{g}/\text{m}^2$  and  $> 300 \mu\text{g}/\text{m}^2$ . There is a significant difference between children on 'low' and 'high' doses.

on long-term treatment with ICS. In addition, there is a dose dependent effect of ICS on urinary GAGs secretion.

In a previous study (15), it was shown that urine levels of GAGs are increased in patients admitted for asthma exacerbation that returned to normal in an asymptomatic period, during a 28 days follow-up. The predominant urinary GAGs were chondroitin/dermatan sulphate that suggests the bronchi as the probable site of proteoglycan degradation (7). Abundant gelatinase B released from tissue eosinophils, which potentially contributed to the degradation of matrix proteoglycans was also found in a severe asthmatic patient (15). On the other hand,

preterm rabbit pups exposed to corticosteroids had lower lung GAGs' concentrations than control pups (23). These observations suggest that increased urinary GAGs secretion during an asthma attack could be due to either asthma exacerbation pathophysiology *per se*, or the effect of anti-asthmatic medications.

To our knowledge, there is no previously published study looking at urinary GAGs levels of asthmatic children on long-term treatment with ICS. The fact that urinary GAGs values in children on inhaled budesonide were significantly higher compared to those receiving inhaled relief medications on an 'as required' basis could be due to the efficacy of long-term ICS treatment. ICS have been shown to be efficient in reversing the remodeling process in the airways (1–2, 16–19, 24, 25). Consequently, production of abundant deconstruction material may be expected; the dose-dependent effect observed in this study strengthens this assumption. We postulate that the main source of the deconstruction material is the airway wall, as most of the currently used ICS have very limited systemic circulation (19). Indeed, systemic bioavailability of modern ICS is low and it is derived from the inhaled fraction that enters the systemic circulation after absorption from the airways mucosa. The high clearance and short half-life of budesonide in children (approximately 1.5 h) (26, 27), in association with its prolonged topical anti-inflammatory activity and high airway selectivity – possibly because of its reversible fatty acid esterification within airway tissue – offer an advantageous therapeutic index (28).

The threshold that distinguished 'low' vs. 'high' doses of inhaled budesonide in our sample was 300  $\mu\text{g}/\text{m}^2$  per day; children on 'high' doses excreted increased urinary GAGs compared to those on 'low' doses. This threshold level approximates that set by GINA and BTS/SING guidelines that have been based upon clinical effectiveness and safety criteria (29, 30). This observation supports, although it certainly does not prove, the concept that a clinically 'effective' dose of ICS may be efficient in reversing the remodeling of the airways by increasing proteoglycans degradation that results in an increase of urinary GAGs secretion. The important issue of whether quantitative determination of urinary GAGs levels can be utilized as a marker of identifying the most efficacious dose of ICS, as suggested by this study, requires confirmation.

We also detected, as compared to normals, lower urinary GAGs levels in asthmatic children that used only relief medication. This can be attributed either to asthma *per se* or to the

limited physical activity of asthmatics. It was shown recently that fibroblast growth factor-2 that is co-localized to heparan sulphate proteoglycans in epithelial and endothelial basement membranes contributes to tissue remodeling (31). Consequently, decreased urinary GAGs secretion could be associated to the marked deposition of proteoglycans in the airway wall. One can also speculate that it is the result of limited physical activity, which usually is adopted by asthmatics as a protective mechanism of exercise induced asthma; indeed decreased physical activity has been shown to reduce urinary GAGs levels in healthy subjects (32).

According to the findings of this study, there was no correlation between urinary GAGs values and atopy. This observation supports the suggestion that airway remodeling and deconstruction occur in asthmatic patients regardless of atopic status.

In addition, although asthmatic children in our study were on inhaled budesonide for a period ranging from 2 to 35 months, urinary GAGs secretion did not show any time dependence. Therefore, according to our data, the anti-inflammatory (and apparently deconstructive) effect of ICS in asthmatic airways of children on long-term corticosteroid treatment occurs early and there is no additive effect.

A limitation of the present study is the fact that no specific GAGs measurements were made; the information offered by such measurement could potentially strengthen our hypothesis. However, our observations should be viewed as a first step toward identifying an alternative indirect marker of the activity of the remodeling process in childhood asthma as well as the effect of ICS on such activity. In this respect, measurement of urinary GAGs prior to initiating treatment with ICS or after discontinuation of corticosteroids for a period of time would have been of value.

In conclusion, our results demonstrate that urinary GAGs secretion is reduced in children receiving usual relief medication on an 'as required' basis compared to normals, but it is increased in asthmatics on long-term treatment with ICS. A dose dependent effect of ICS was detected. Further investigation is needed to clarify the possible role of urinary GAGs as a marker of the effectiveness of the anti-asthmatic treatment.

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