

URINARY LTE_4 IS HIGHER AFTER NASAL PROVOCATION TEST WITH L-ASA IN BRONCHIAL THAN IN ONLY NASAL RESPONDERS

C. Micheletto (1), M. Visconti (1), S. Tognella (1), F. Trevisan (1), R.W. Dal Negro (1)

Aspirin induced asthma (AIA) is a syndrome characterised by intolerance to acetylsalicylic acid (ASA), nasal polyps and bronchial asthma, being the metabolic shift of arachidonic acid toward the lipoxigenase pathway and hyper-production of cysteinyl-leukotrienes (cys-LTs) the current pathogenetic hypothesis. The research for both sensitive indicators and safe diagnostic tests is still attracting.

Aim of the study was to compare the levels of urinary LTE_4 in baseline and after Nasal Provocation Test (NPT) with L-ASA from subjects affected by aspirin-intolerance and characterized by only a nasal response to ASA to those from subjects with both a nasal and a bronchial response to the same challenge.

Methods: After their written consent, 74 subjects with mild to moderate AIA (16 male, mean age 45.3 years $\pm$ 12.3 sd, mean basal FEV_1 = 78.1 % pred. $\pm$ 6.2.4sd, FEV_1 reversibility = 14.3%bsln $\pm$ 2.1ds after salbutamol 200 mcg) performed a NPT with L-ASA (total maximal dose 25 mg). Spirometry (FEV_1), acoustic rinometry (nasal volume – VOL; nasal Resistance – Req; AR; TM Hood Lab., USA), and urinary LTE_4 (Cayman Chemical, MI, USA, via Triturus System, Grifols, Spain) were checked in all subjects in basal conditions and 90' after NPT.

Statistics: t test between means $\pm$ sd, assuming $p < 0.05$, and linear regression between all variables considered.

Results: In 69 ASA-intolerant-asthmatics, mean FEV_1 did not change significantly following NPT (78.7 % pred. $\pm$ 5.1 sd in baseline; 78.5 % pred. $\pm$ 4.1 sd after NPT, $p = \text{ns}$) even though in the presence of a significant decrease of VOL. (12.6 $\text{cm}^3 \pm 4.1$ sd in baseline; 6.2 $\text{cm}^3 \pm 4.6$ sd after NPT, $p = 0.003$); of a substantial increase in Req (0.9 $\text{cmH}_2\text{O/l/min} \pm 0.1$ ds in baseline; 2.4 $\text{cmH}_2\text{O/l/min} \pm 0.2$ after NPT, $p = 0.04$), and of urinary LTE_4 excretion (333.0 $\text{pg/mg} \pm 161.7$ in bsln; 558.0 $\text{pg/mg} \pm 171.6$ 90' after NPT with L-SA, $p = 0.02$).

In only 5 subjects, the nasal response occurred concomitantly to a significant broncho-constriction after the NPT: mean FEV_1 changed from 77.9 % pred. ± 3.9 in bsln to 46.6 % pred. ± 4.3 after NPT ($p < 0.001$); mean VOL from 13.9 $\text{cm}^3 \pm 4.7$ sd to 5.6 $\text{cm}^3 \pm 2.8$ sd ($p < 0.001$); mean Req from 1.1 $\text{cmH}_2\text{O/l/min} \pm 0.2$ in bsln to 2.5 $\text{cmH}_2\text{O/l/min} \pm 0.4$ after NPT ($p = 0.02$) in these subjects. In ASA-intolerant bronchial responders, the severity of respiratory reactions proved related to the extent of urinary LTE_4 response, which on the other hand, proved significantly higher than that observed in ASA-intolerant subjects with only nasal response and in ASA-tolerant subjects (LTE_4 from 333.0 $\text{pg/mg} \pm 161.7$ in baseline up to 558.0 $\text{pg/mg} \pm 171.6$ 90 min. following the NPT with L-ASA the nasal-responders, $p = 0.04$, but from 412.0 $\text{pg/mg} \pm 102.8$ in baseline up to 978.0 $\text{pg/mg} \pm 108.7$ after NPT in bronchial responders, $p < 0.001$ from baseline).

Conclusions: Nasal challenge with ASA affects significantly both nasal VOL and Req, and LTE_4 excretion in all ASA-intolerant subjects. During the nasal challenge, severity of respiratory reactions proves associated with the highest basal LTE_4 synthesis. This feature reflects a spectrum of respiratory tract reactions where cysteinyl-LTs can play a specific diagnostic role.

Aspirin induced asthma - Nasal provocation test - L-ASA - LTE_4 excretion.

INTRODUCTION

Patients with Aspirin-induced asthma (AIA) characteristically exhibit the clinical signs of aspirin (ASA) or of other non-steroidal anti-inflammatory drugs sensitivity, such as bronchial asthma and chronic polypoid rhinosinusitis (1). After ingestion of aspirin, asthmatic patients with AIA have attacks of bronchospasm that are usually accompanied by symptoms of nasal congestion, mucous secretion and/or skin reactions, such as urticaria.

Watery rhinorrhea and nasal obstruction, leading to frequent development of nasal polyps, usually precede the occurrence of asthma and aspirin intolerance for months or years (2). Aggressive nasal polyps and asthma run a protracted course, despite the avoidance of ASA and cross-reacting drugs. AIA is difficult to treat and frequently these patients require therapy with systemic corticosteroids to control symptoms (3). The precise mechanism underlying this non-immunological hypersensitivity to ASA and to other non-steroidal anti-inflammatory drugs still remains to be clarified; however, it has been assumed that the power of these pharmacological agents in provoking asthma is related to their activity as inhibitors of cyclooxygenase (COX) and the induction of elevated local and

(1) Lung Dept., Orlandi General Hospital, Bussolengo – Verona (Italy).

systemic leukotriene release (4-6). The most recent hypothesis is suggesting that the genetic predisposition to the up-regulation of cysteinyl-leukotrienes (cys-LTs) pathway could be related to an overactive expression of LTC₄ synthase (7).

When compared to ASA tolerant subjects, patients with AIA are also characterized by a lower production of prostaglandin E₂ (PGE₂) (8-9), being this alteration in PGE₂ production probably due to an inadequate COX-2 regulation (10). As PGE₂ exerts an inhibitory action on inflammatory leukocytes, its reduced expression in aspirin-induced rhinosinusitis was suggested as partly responsible for the increased inflammatory infiltrate and production of cys-LTs that characterise AIA (11).

Even in stable clinical conditions, AIA patients have an increased basal production of cys-LTs (such as urinary LTE₄) (12-14). They are also characterised by a significant increase in urinary LTE₄ excretion following aspirin ingestion (15-17), ASA inhalation (18) and nasal instillation with L-ASA (19). Other peculiar features of these patients are their higher levels of urinary LTE₄ and LTB₄ glucuronide (a chemotactic factor for eosinophils and neutrophils) following the intravenous aspirin challenge (20).

The research for both sensitive indicators and safe diagnostic tests is still attracting. Nasal provocation test (NPT) with Lysine-aspirin (L-ASA) has been introduced a few years ago for assessing AIA also in patients with instable asthma (21): the test has been described as a highly specific and sensitive one, but negative results do not exclude possible intolerance to aspirin. More recently, the acoustic rhinomanometry has been suggested as the most helpful diagnostic procedure for NPT with L-ASA, being its use less limited than the anterior rhinomanometry by the presence of nasal polyps and nasal obstructions (22-23). As previously demonstrated (19), measures of urinary LTE₄ excretion contributed significantly to magnify the discriminant and the diagnostic value of NPT.

The aim of the present study was to measure both the basal and the post-NPT urinary LTE₄ levels in subjects only a nasal responder to ASA and to compare with those in subjects with both nasal and bronchial response to the same challenge.

MATERIALS AND METHODS

Subjects

After their written consent, 74 subjects with mild-to-moderate AIA (16 male, mean age 45.3 years $\pm$ 12.3 sd; mean basal FEV₁ = 78.1 % pred. $\pm$ 6.2.4sd, FEV₁ reversibility = 14.3%bsln $\pm$ 2.1ds after salbutamol 200

mcg) performed a NPT L-ASA (total maximal dose 25 mg). In all subjects, spirometrical FEV₁; acoustic rhinomanometry, and urinary LTE₄ were measured in basal conditions and 90' after NPT.

All patients were non-smoker and performed the NPT with L-ASA after having stopped their medications (oral antihistamines and nasal corticosteroids for 7 days, and nasal inhaled anti-H₁ agents for at least 48 hours before NPT, Inhaled Corticosteroids for at least 7 days, short acting β_2 -agonist for 6 hours, long-acting β_2 -adrenergic for 12 hours, anti-LTs agents for 7 days).

Acoustic rhinomanometry for NPT with L-ASA

Each patient performed the NPT according to the method described by Casadevall and co-workers by means of the acoustic rhinometry (19). This method consists in the measurement of acoustic reflections from the nasal cavity of a sound pulse created by a spark in a sound tube connected to the nasal cavity via a nosepiece. Unlike conventional rhinomanometry, acoustic rhinomanometry does not require generation of nasal flow, and therefore its use is less limited by the presence of nasal polyps and nasal obstruction. The response was evaluated by the Eccovision Acoustic Rhinomanometry System (TM Hood Laboratories, USA) with the measurement of (24):

- calculated resistance, based on a tube with the same area and laminar flow (REQ, mmH₂O/l/min);
- the total volume of the nostrils (VOL, cm³) represents the nasal cavity volume in the analysis segment;
- the minimal cross sectional area (cm²);
- its distance from the nosepiece (cm).

Rhinomanometric measurements were performed while the subject was in apnoea after a non-forced expiration. The rhinomanometer was calibrated daily with a calibration tube provided by the manufacturer. The analysis of data was performed using the Kwikstat program (TM Texasoft).

Baseline nasal function was measured with Acoustic Rhinomanometry, while the subjects were in a sitting position, then 80 μ l of L-ASA solution (180 mg/ml L-ASA) was applied locally through nose droplets on the inferior nasal concha in both nostrils. The total deposited dose of L-ASA was equivalent to 25 mg of acetylsalicylic acid. Acoustic Rhinomanometry was then performed bilaterally every 10 minutes for the next 2 hours.

L-ASA was prepared freshly each day by dissolving crystalline L-ASA in 0.9 % sodium chloride to produce a solution containing 180 mg/ml.

NPT was considered positive when: nasal resistance increased more than 40 % in at least one nostril as

compared with the corresponding baseline value; when the volume of one nostril decreased more than 10% from baseline; both parameters sustained for at least two consecutive measurements, and were accompanied by clinical symptoms persisting at least 30 minutes. The dose of aspirin, the duration of the observation period, and criteria for positivity of the test were established on the basis of previous experiments (17-19).

Simultaneously, pulmonary function (FEV_1 ; forced expiratory volume in 1 sec) was measured by using a computerized pneumotachograph (Masterlab, Jaeger). We considered the maximal fall for REQ, VOL and FEV_1 observed in the 2 hours following the NPT.

Urinary LTE_4

Urine samples were obtained before NPT with L-ASA and 2 hours after the nasal instillation of L-ASA. The time for urine collection was based on a previous measure of urinary LTE_4 after inhalation challenge (17).

Urinary LTE_4 were measured by enzyme immunoassay (ACE™ Competitive Enzyme Immunoassay, Cayman Chemical, Ann Arbor, Mich, USA), as reported by Pradelles *et al* (25), via the Triturus System (Grifols, Spain).

This assay is based on the competition between LTE_4 and an LTE_4 -acetylcholinesterase (AChE) conjugate (LTE_4 tracer) for a limited amount of LTE_4 antiserum. Because the concentration of the LTE_4 tracer is held constant while the concentration of LTE_4 varies, the amount of LTE_4 tracer that is able to bind to the LTE_4 antiserum will be inversely proportional to the concentration of LTE_4 in the well. This antibody- LTE_4 complex binds to a mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate to AChE) is added to the well. This reagent consists of acetylthiocholine and 5,5'-dithio-bis-(2-nitrobenzoic

acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine. The non-enzymatic reaction of thiocholine with 5,5'-dithio-bis-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm. AChE has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. In addition, the enzyme is highly stable under the assay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts and preservatives. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412 nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of LTE_4 tracer bound to the well, which is inversely proportional to the amount of free LTE_4 present in the well. LTE_4 was measured by enzyme immunoassay on all samples according to the manufacturers' instructions and expressed in pg/mg creatinine (pg/mg).

■ **Statistical analysis:** Mean values $\pm$ sd obtained before and after NPT for each variable were compared by t test, and $p < 0.05$ was assumed as the lowest limit for the statistical significance.

Linear regression was calculated for all variables considered.

■ **Results:** In 69 ASA-intolerant-asthmatics, FEV_1 did not change significantly following NPT (78.7 % pred. $\pm$ 5.1 sd in baseline and 78.5 % pred. $\pm$ 4.1 sd after NPT, $p = ns$) (FIG. 1) even though in the presence of a significant decrease of nasal VOL. (from $12.6 \text{ cm}^3 \pm 4.1$ sd in baseline to $6.2 \text{ cm}^3 \pm 4.6$ sd after NPT, $p = 0.003$) (FIG. 2), of a substantial increase of Req (from $0.9 \text{ cmH}_2\text{O/l/min} \pm 0.1$ ds in baseline to $2.4 \text{ cmH}_2\text{O/l/min} \pm 0.2$ after NPT, $p = 0.04$), and of urinary LTE_4 excretion (from $333.0 \text{ pg/mg} \pm 161.7$ in bsln to $558.0 \text{ pg/mg} \pm 171.6$ 90' after NPT with L-SA, $p = 0.02$) (FIG. 3). Only in 5 subjects a significant broncho-constriction after the NPT also occurred: in these subjects, mean

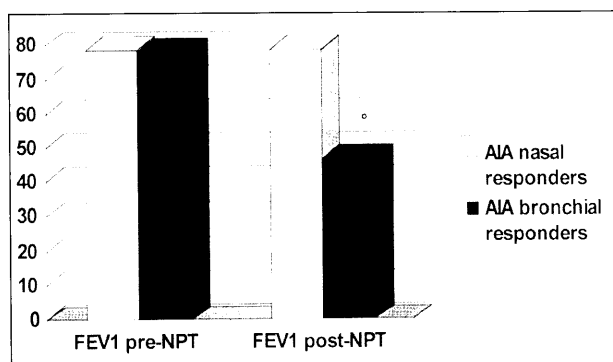


Fig. 1: FEV_1 (% predicted) before and after Nasal Provocation Test in nasal and in bronchial responders.
° $p < 0.001$ vs baseline.

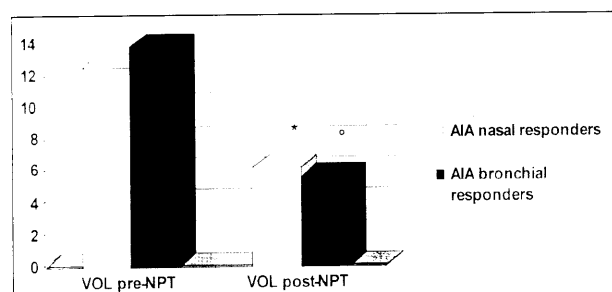


Fig. 2: Nasal Volume (cm^3) before and after Nasal Provocation Test in nasal and in bronchial responders.
* $p = 0.003$ vs baseline.
° $p = 0.002$ vs baseline

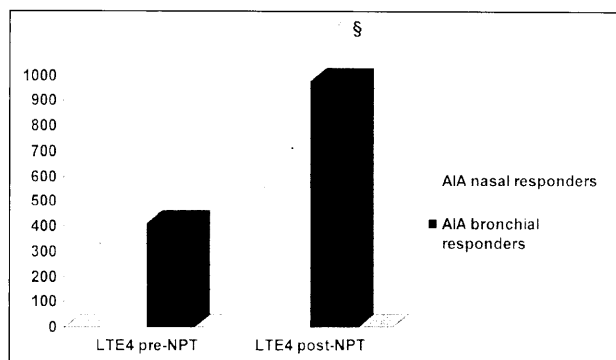


Fig. 3: LTE₄ (pg/mg) before and after Nasal Provocation Test in nasal and in bronchial responders.

* $p = 0.02$ vs baseline ° $p < 0.001$ vs baseline § $p = 0.02$ vs LTE₄ post-NPT of nasal responders.

FEV₁ dropped from 77.9 % pred. $\pm$ 3.9 in bsln to 46.6 % pred. $\pm$ 4.3 after NPT ($p < 0.001$) (FIG.1); Vol. from 13.9 cm³ $\pm$ 4.7 sd to 5.6 cm³ $\pm$ 2.8 sd, $p < 0.001$ (FIG.2), and Req from 1.1 cmH₂O/l/min $\pm$ 0.2 in bsln to 2.5 cmH₂O/l/min $\pm$ 0.4 after NPT, $p = 0.02$. Severity of respiratory reactions proved related to the extent of urinary LTE₄ response (412.0 pg/mg $\pm$ 102.8 in bsln, 978.0 pg/mg $\pm$ 108.7 after NPT, $p < 0.001$ from baseline, $p = 0.02$ vs LTE₄ post-ASA of AIA-nasal group) (FIG.3). When compared to the corresponding basal values, Req and VOL changes were discriminant for ASA intolerance accordingly to the criteria described by Milewski *et al.* (17).

The diagnostic value of nasal changes following NPT was further emphasized by the changes observed in urinary LTE₄ (from 333.0 pg/mg $\pm$ 161.7 in baseline up to 558.0 pg/mg $\pm$ 171.6 90 min. following the NPT with L-ASA the nasal-responders, $p = 0.04$, but from 412.0 pg/mg $\pm$ 102.8 in baseline up to 978.0 pg/mg $\pm$ 108.7 after NPT in bronchial responders, $p < 0.001$ from baseline).

In baseline, urinary LTE₄ excretion was similar in both groups of subjects ($p = ns$), so confirming that the extent of airway involvement is not predictable by the basal extent of LTE₄ excretion only.

NPT proved well tolerated in the whole population: no systemic or skin reaction were registered, and the five episodes of bronchospasm were managed by only assumption of salbutamol (200 μ g + 200 μ g after 15 min) in two cases; by administration of salbutamol plus prednisone 25 mg per os in 2 cases, and of salbutamol plus betametasone 4 mg ev in one case.

DISCUSSION

AIA develops according to a clinical pattern characterized by a sequence of symptoms (3): firstly, persistent rhinitis, then asthma, aspirin intolerance, and finally nasal polyposis appear. After the occurrence of

rhinitis, it is important to precise AIA because such a particular asthma has to be considered a "difficult" asthma which frequently requires systemic steroids to control symptoms.

At present, there is no *in vitro* test for identification of aspirin intolerance, and the diagnosis can be only confirmed by oral (26) or by inhalation (27-28) aspirin provocation test. Both these procedures have several limits: while the oral test can sometimes precipitate a severe asthmatic reaction, the inhalation of increasing concentrations of Lysine-aspirin (L-ASA) solution is considered as unsuitable for patients whose asthma is not in clinical remission and whose FEV₁ is lower than 70 % of predicted value.

Actually, NPT is regarded as the method of choice for assessing AIA in aspirin intolerant subjects by means of anterior rhinomanometry (18), or acoustic rhinomanometry (21-22), or by measuring peak nasal inspiratory flow (29). In the United States a new diagnostic test for nasal challenge using a dilute solution of ketorolac has recently completed diagnostic trials (30).

In the present study, NPT confirmed as a simple and rapid test to perform, which proves highly safe for diagnosing AIA; it should then represent the method of choice to check the hypersensitivity to aspirin, particularly when it is characterised by upper airway symptoms only. In particular, differently from the older and more usual diagnostic approach, NPT, when based on the assessment of ASA-induced acoustic rhinomanometrical changes, consented the absolute protection of lung function. For these reasons, this novel procedure should be used more widely as the first-line diagnostic approach to the diagnosis of hypersensitivity to aspirin also in patients with instability of the bronchial tree, and when bronchial or oral provocation test with aspirin cannot be carried out for safety reasons.

Several observations are supporting the hypothesis that LTs may mediate AIA (5-10); in particular it has been assessed a mean fourfold increase in urinary LTE₄ levels 3-to-6 hours following aspirin ingestion (15), and after inhalation of L-ASA (18). Moreover, the severity of respiratory reactions during oral ASA challenge was associated with the extent of basal urinary LTE₄ expression (16), thus suggesting that asthmatics with aspirin-sensitive respiratory disorders are characterised by a spectrum of reactions in which leukotrienes seem to play a crucial pathogenetic role. Despite these observations, changes in urinary LTE₄ following nasal provocation test with ASA were never previously investigated in ASA-tolerant and ASA-intolerant asthmatics.

Urinary LTE₄ excretion proves significantly enhanced after simple nasal instillation of L-ASA, thus emphasizing the great diagnostic value of LTE₄ measurements even in the absence of systemic reactions. Furthermore, as reported in previous studies (15-18),

also the basal high excretion of urinary LTE_4 was described as a discriminating feature between ASA-sensitive asthmatics and non-sensitive controls.

Moreover, despite age and lung function (FEV_1), the extent of basal LTE_4 urinary excretion seems clearly related to basal (such as pre-NPT) nasal function, in particular to nasal volume. The crucial role of LTE_4 production (which is already exalted in baseline) and of nasal structural involvement (i.e. nasal hyperresponsiveness) can be regarded as a substantial contribution to the early definition of the biological and functional pattern of ASA intolerance.

In conclusion, data of the present study further support the diagnostic relevance of basal LTE_4 measurements and of nasal investigation in patients suspected of ASA intolerance thus emphasizing their intrinsic predisposition to LTs-mediated reactivity to ASA. NPT with L-ASA, when implemented with the acoustic rhinomanometry and urinary LTE_4 measures, confirms as an absolutely safe and discriminant test for patients suspected of ASA intolerance, and represents a suitable diagnostic procedure in the absence of meaningful bronchial obstruction. Finally, the severity of respiratory reactions observed during the nasal challenge proves associated with the highest elevation of basal LTE_4 synthesis: this feature further reflects a spectrum of respiratory tract reactions where LTs can play a discriminating role.

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