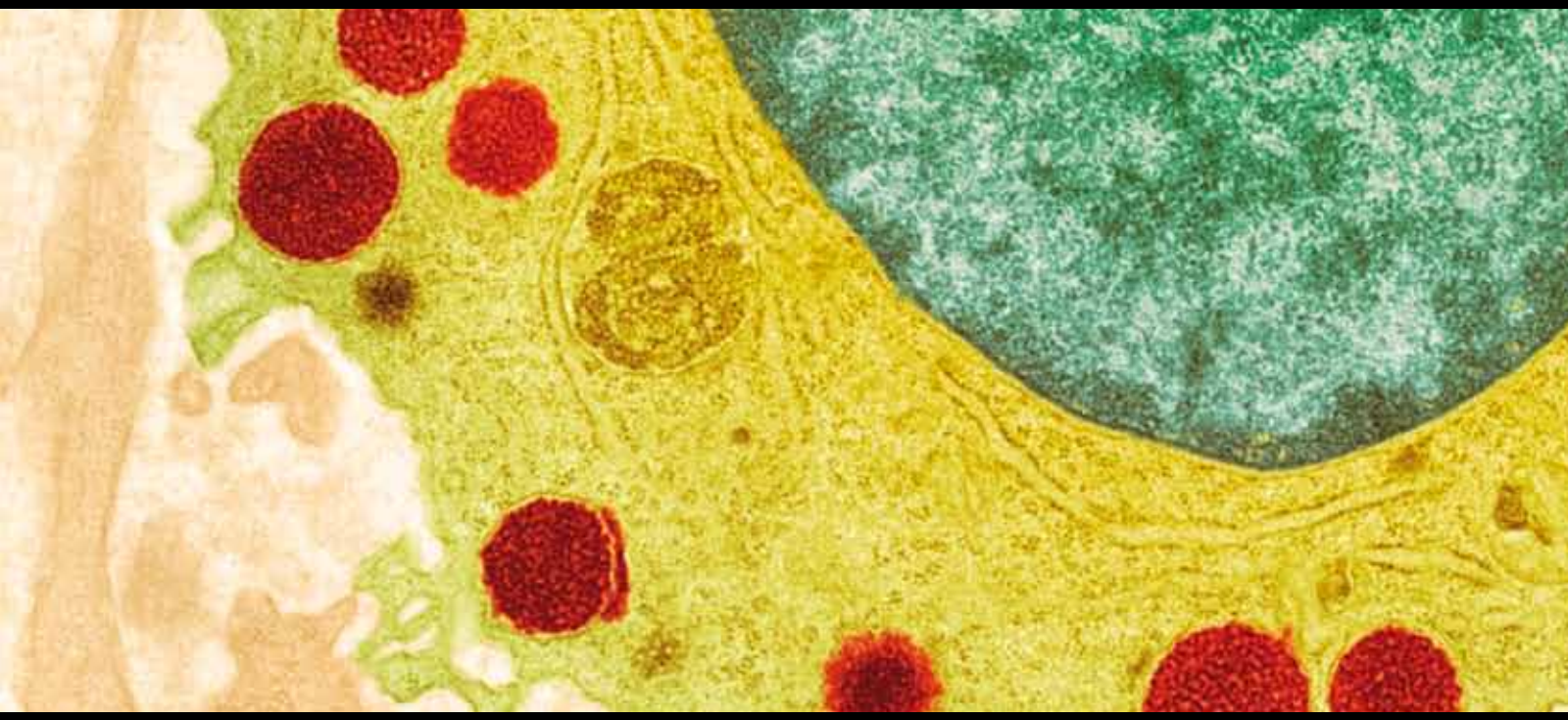
Journal of Allergy

Aspirin Exacerbated Respiratory Disease

Guest Editors: Luis M. Teran, Stephen T. Holgate, Hae-Sim Park, and Anthony P. Sampson



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Editorial Board

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Editorial

Aspirin Exacerbated Respiratory Disease

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As guest editors of this special issue of the *Journal of Allergy*, we are pleased to be able to present a range of articles, both reviews and original research papers, on the important topic of aspirin-exacerbated respiratory disease (AERD). AERD is a synonym for the "aspirin triad" of asthma, nasal polyposis and sensitivity to aspirin originally described by Widal ninety years ago. AERD patients typically experience severe bronchoconstriction and/or rhinoconjunctival reactions to aspirin, and also to other non-steroidal anti-inflammatory drugs (NSAID), even those which they have not encountered previously [1]. The reactions reflect non-allergic hypersensitivity as many AERD patients are not atopic and the reactions to NSAIDs are not usually IgE-mediated. While the aspirin triad has been described as emerging typically in early middle age, aspirin-intolerance, as determined in bronchoprovocation studies, may be apparent in 21% of adult asthmatics and 5% of asthmatic children, suggesting that it is under-recognised as a factor in asthma episodes [2]. Even in the absence of NSAID ingestion, AERD patients have relatively severe chronic asthma with a high proportion requiring long-term oral corticosteroid therapy, representing an unmet need of poorly-controlled asthma [1]. Nasal polyposis in AERD can be severe and require recurrent surgery.

Many of the papers in this special issue hinge upon the key insight in the field of AERD made by the late Professor Andrzej Szczeklik of Krakow. His "cyclooxygenase theory" recognised that the ability of NSAIDs to trigger adverse reactions in AERD patients correlates with their potency as cyclooxygenase inhibitors [3]. He extended the theory with the realisation that arachidonic acid, the substrate of the cyclooxygenase (COX) pathway, is also the substrate of the 5-lipoxygenase (5-LO) pathway that generates the cysteinyl-leukotriene family of mediators. The most persistent anomaly described in AERD is that even in the absence of NSAID ingestion, the levels of cysteinyl-leukotrienes are constitutively elevated in the bronchoalveolar lavage fluid, sputum, exhaled breath condensate, nasal secretions, saliva, blood and urine of AERD patients compared to aspirin-tolerant individuals [4]. That the further triggering of cysteinyl-leukotriene synthesis is important in acute AERD was established by studies with leukotriene synthesis inhibitors and leukotriene receptor antagonists [5]. The central mystery in AERD remains why only these patients, and not others with comparably severe asthma, show acute adverse responses to NSAIDs. This special issue of *J. Allergy* includes papers from many of the leading laboratories and opinion leaders working on this question around the world.

In the review article by Dr Maria Garcia-Cruz and her colleagues in Mexico, AERD and the cyclooxygenase theory are discussed with special emphasis on rhinosinusitis, its diagnosis and its treatment. Pathophysiological mechanisms are reviewed including recent studies of cytokine and chemokine anomalies in AERD, particularly in relation to *Staphylococcus aureus* enterotoxins which may drive abnormal allergic responses. The theme of rhinosinusitis is extended by the wide-ranging review paper from the group of Prof. Meyer in Hamburg, writing with Dr Schäfer from Erlangen, who stress the life-threatening nature of AERD and survey its molecular mechanisms, treatment and future directions for research.

Genetic studies into AERD, including candidate gene approaches and genome-wide association studies, may throw light on the underlying pathological mechanisms or provide

genetic biomarkers for improved diagnosis and treatment. The area is expertly reviewed by Dr Palikhe and colleagues based in Korea, while its importance is underlined by the original research presented by Dr Falfan-Valencia and colleagues in Mexico, describing a novel association between AERD and a polymorphism in the gene encoding the inflammatory cytokine interleukin-1β.

Over-production of cysteinyl-leukotrienes by LTC_4 synthase and over-expression of the cysteinyl-leukotriene $CysLT_1$ receptor have been postulated as central components of AERD pathology in the upper and lower airways [5]. The paper by Dr Steinke and his colleagues in Virginia, USA, provides a powerful argument that the Th2 cytokine interleukin-4 not only upregulates LTC_4 synthase and $CysLT_1$ receptors, but also down-regulates key components of the COX pathways, including COX-2, PGE_2 synthase and the EP2 receptor, and that this accounts for the key features of AERD. They further outline a molecular mechanism by which IL-4 expression could itself be modulated by aspirin. Dr Jackson and colleagues from Southampton, UK, report that the allied Th2 cytokine IL-13 appears not to upregulate LTC_4 synthase in human lung macrophages, perhaps supporting this unique role for IL-4 in AERD.

An enduring problem in the field of AERD is early and accurate diagnosis of the condition. Patients may not recognise an association between their use of NSAIDs and asthma exacerbations, while the gold standard for many years has been provocation testing using various oral or inhaled aspirin protocols that are not practicable in many clinical settings. The review in this issue by Dr Schäfer and Prof. Maune surveys the wide range of *in vitro* models that have been proposed as a basis for laboratory testing for NSAID sensitivity, including tests based on platelet aggregation, lymphocyte transformation, and others based on levels of eicosanoids and other mediators in serum or exhaled breath condensate. The original paper from Dr Abuaf and colleagues in Paris describes studies on a basophil activation test which shows promise in the diagnosis of AERD, at least in the sub-group of patients with greatest hypersensitivity.

Finally, this special issue of *J. Allergy* includes a paper by Dr Mastalerz and colleagues from the group in Krakow, Poland, led by Prof Szczeklik. Based on liquid chromatography and mass spectrometry of exhaled breath condensates, this paper completes a broad survey of lipid anomalies in AERD, including prostanoids and novel products of the 5- and 15-lipoxygenase pathways. This is one of the last papers to bear the name of Andrzej Szczeklik as an author before his untimely death in February 2012, and this special issue of the *Journal of Allergy* is dedicated to his memory.

A Remembrance of Professor Andrzej Szceklik (1938–2012). This special issue of the Journal of Allergy is privileged to include a paper (Mastalerz et al.) coauthored by Professor Andrzej Szczeklik of Krakow University shortly before his death, aged 73, on February 3rd this year. This paper is therefore one of Professor Szczeklik's last publications in a career in which he achieved high national and international honours and great scientific eminence for his founding insights and enduring contributions to the field of AERD. Andrzej Szczeklik was born in 1938 and graduated with a Diploma in Medicine from Krakow in 1961. His postgraduate training included work in Sweden at the Karolinska Institute and at Uppsala University, and in the USA at the University of North Carolina, Chapel Hill. After several years in Wroclaw, Poland, he returned to Krakow in 1972 and rose to become the Chairman of the Department of Medicine at the Jagiellonian University in 1989. From 1990 to 1993, he was president of the Copernican Academy and in 1993–1996 the Vice-Rector of Jagiellonian University Medical College. Szczeklik was well known by his scientific reputation to all the contributors to this special issue for his lifelong research into aspirin-intolerance. He led the international field following his insight in 1975 that aspirin intolerance arises from the pharmacological activity of aspirin and other NSAIDs in inhibiting prostaglandin synthesis by cyclooxygenase. The "cyclooxygenase theory" is the basis of much of the research focused on lipid mediators that followed in the field of AERD. Szczeklik's achievements were recognised by a plethora of awards and honours, including the Gold Medal of the Jagiellonian University, the AAAAI Robert A Cook Lectureship, memberships of the Polish Academy of Sciences, the Royal College of Physicians in London, and the Pontifical Academy of Sciences in the Vatican, and a number of visiting professorships and honorary doctorates at universities in the UK, Europe, and Japan. During the 1980s, he was active in the Solidarity Movement and remained a major contributor to the broader cultural life of his native Poland. Those who knew Andrzej as a friend will remember him not only as a gentleman and a scholar, but as an accomplished pianist and the author of three thoughtful and well-received books, "*Catharsis: The Art of Medicine*", "*Kore*", *and* "*Immortality: Promethean Dream of Medicine*" published posthumously, reflecting on a long life in medicine and the values that link the humanities and sciences. He will be sadly missed by his many scientific and clinical friends across the world.

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Review Article

Aspirin Sensitivity and Chronic Rhinosinusitis with Polyps: A Fatal Combination

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Aspirin-exacerbated respiratory disease (AERD) refers to aspirin sensitivity, chronic rhinosinusitis (CRS), nasal polyposis, asthma, eosinophil inflammation in the upper and lower airways, urticaria, angioedema, and anaphylaxis following the ingestion of NSAIDs. Epidemiologic and pathophysiological links between these diseases are established. The precise pathogenesis remains less defined, even though there is some progress in the understanding of several molecular mechanisms. Nevertheless, these combinations of diseases in patients classified by AERD constitute a fatal combination and may be difficult to treat with standard medical and surgical interventions. This paper reviews in brief the epidemiology, clinical features, diagnosis, molecular pathogenesis, and specific therapies of patients classified by AERD and postulates future attempts to gain new insights into this disease.

1. Introduction

Patients suffering from nasal polyps remain one of the more challenging groups of patients to manage. Unfortunately, the precise pathogenesis of nasal polyp formation remains poorly defined. However, inflammation of the upper and lower airways is well documented, and epidemiologic and pathophysiological links between chronic rhinosinusitis (CRS) without or with nasal polyps, asthma, and/or eosinophilic inflammation have been established by recent investigation [1–3]. The association of nasal polyps, asthma, and hypersensitivity to aspirin was first described by Widal et.al in 1922 [4] and thereafter popularized by Samter and Beers in 1968 thoroughly characterizing the clinical picture [5]. This syndrome has been termed "Syndrome de Widal" or "Samter's Triad." Severe cutaneous and systemic adverse reactions upon ingestion of "aspirin" were first documented in 1902 by Hirschberg [6], shortly after the market launch of aspirin. The diverse terms used in medical literature describing the adverse reactions upon ingestion of nonsteroidal anti-inflammatory drugs (NSAIDs) had been recently reviewed and summarized [7].

This subset of patients with recurrent nasal polyps, asthma, and NSAIDs remains one of the more challenging groups of patients. The term aspirin-exacerbated respiratory disease (AERD) refers to the clinical syndromes of chronic rhinosinusitis (CRS), nasal polyps, bronchoconstriction in asthmatics, and/or eosinophil inflammation in the upper and lower airways, urticaria, angioedema, and anaphyalxis following the ingestion of NSAIDs blocking the COX-1 enzyme. In this concern, NSAIDs are an exacerbating factor rather than an underlying disease. This classification system was proposed by Stevenson et al. in 2001 [8] allowing a better understanding, which type or clinical reactions constitute the subject of the publication. AERD comprises the description of physical reactions, underlying airway-related diseases, and inhibitors of cyclooxygenase (COX). AERD is subdivided, based on physical reactions, to (1) NSAID-induced rhinitis and asthma, (2) NSAID-induced urticaria/angioedema,

(3) multiple-drug-induced urticaria/angioedema, (4) single-drug-induce anaphylaxis, and (5) single-drug- or NSAID-induced blended reaction; by definition, there are none underlying diseases concerning the subclassification (3) to (5).

The appearance of diseases mentioned above in combination with the intake of NSAIDs constitutes a fatal combination for some patients. Therefore, current epidemiology, clinical features, diagnostic approaches, molecular pathogenesis, and AERD specific therapies will be elaborated and postulates of future attempts to gain new insights into this disease will be presented.

2. Epidemiology

AERD has been estimated to affect 0.3 to 2.5% of the general population [2, 8, 9]. The frequency of symptoms associated with AERD published in literature is 5–10% with rhinitis, 5–30% with nasal polyps, 10% with bronchial asthma, 25–30% with nasal polyps and bronchial asthma, and 5–10% with urticaria/Quincke's edema [1, 2, 9–13]. The estimation of prevalence of AERD varies depending on the determination through questionnaire (11–20%), medical record (~3%), or oral provocation test (21%) [2]. Therefore, AERD might be over- as well as underdiagnosed depending on the diagnostic tool used. The onset of AERD is typically during the third decade and is more commonly reported in females (~3 : 2) [14, 15]. Ethnic preferences are not described and only rare familial associations were mentioned [2, 9–12].

CRS is estimated as the most frequent chronic diseases worldwide with an intense impact on healthcare system and on the quality of life of patients [15]. More than 30 million Americans are involved [16] causing over 6 billion US $ burden for the health care system worldwide [17]. The prevalence of CRS is difficult to estimate due to different diagnostic criteria, heterogenous group of patients, treatment by different medical professions, and inconsistent definitions but is assumed to reveal 5% ranging from 1 to 19%. Up to 70% of patients with CRS also suffer from asthma and aspirin sensitivity [14, 18–23]. The incidence and prevalence of *CRS with nasal polyps(CRSwNP)* is estimated with 2–4% [14, 18–24] and 31–60% [13, 25, 26], respectively. CRSwNP was observed in 7% of asthmatics but rises to 15% of patients with CRSwNP and aspirin sensitivity [13, 27, 28].

These tremendous discrepancies of aspirin-sensitive patients who suffer from nasal polyps have most likely been caused by the diagnostic techniques which have been used.

3. Clinical Features

In patients classified by AERD rhinitis appears first during the third decade with concomitant onset of nasal congestion, hyposmia, chronic rhinorrhea, and progress to chronic pansinusitis followed by nasal polyps which frequently relapses after surgery. Finally, the disease results in NSAID-triggered hypersensitivity of the lower airways with the symptoms of asthma. About fifty percent of the patients demonstrate chronic, severe, corticoid-dependent asthma, often accompanied by systemic anaphylactoid reactions. Based on our recent knowledge, once appeared, AERD remains throughout life, even though sporadic disappearance of intolerance has been reported [1–3, 29–31].

Although there are typical clinical features, AERD is most likely underdiagnosed as exemplified above. Atopy is present in approximately 30% of patients classified by AERD, which was significantly higher in patients with positive rather than negative skin tests [32].

The formation of nasal polyps in patients suffering from AERD follows an aggressive course filling the nasal cavity, often protruding anteriorly in the face or posteriorly into the nasopharynx. Facial deformation is common, due to midfacial expansion, which occurs as a consequence of the increased pressure on the bones from nasal polyps [32, 33]. A strong positive correlation has also been found between the number of polypectomies and the peripheral blood eosinophil count [34, 35].

4. Diagnostic Approaches

The diagnosis of AERD is a major challenge not only in patients suffering from CRS with/without nasal polyps and/or bronchial asthma, but also in individuals without known underlying airway-related diseases [1–3]. The diagnostic approach of AERD is based on the clinical picture as outlined above. This will be supported in preposition by imaging techniques, including computed tomography or endoscopy. Some patients have a definitive history of adverse reactions to NSAIDs. However, many patients also had not experienced AERD, suggesting that aspirin challenge tests are critical for diagnosis [36]. The confirmative diagnosis for AERD can definitely be established by aspirin challenge [1–3, 12, 32, 36–39]. Patients receive increasing doses of aspirin during the challenges. There are four routes of provocation challenge: (1) oral, (2) bronchial inhalation, (3) nasal inhalation, (4) and intravenous [36–41]. In Europe, inhalation, nasal, or oral challenge are used; in the United States oral aspirin challenge is performed. The challenge test should be performed when asthma is stable (i.e., forced expiratory volume in the first second of expiration (FEV1) is >70% of expected value, with a variability of <10%). Increasing challenge doses of aspirin are administered (oral: ~20 to 500 mg, inhalation: ~0.2 to 182 mg cumulative dose, or lower, depending on the route of administration and severity of reported symptoms), spaced by 1.5 to 2 hours (or shorter). Nasal obstruction is measured by rhinomanometry, acoustic rhinomanometry, and/or peak nasal inspiratory flow; bronchial obstruction by FEV1. Challenges are interrupted, if a decrease of FEV1 ≥20% of baseline is measured [28, 42–45].

The challenge procedure bears the risk of severe asthma exacerbations and should be done only by trained specialists with availability of equipment and medication for the treatment of acute asthma attacks if they develop. Hypersensitivity emerged in general within 1 to 4 hours after intake of NSAIDs but may occur within minutes or takes up to 24 hours. Life-threatening reactions may occur in some patients, especially those with AERD

[1–3, 28, 39–42, 44–47]. Therefore, NSAID challenge must be performed in a specialized hospital under supervision of the patient by skilled health professionals. These require proper emergency equipment, observation, and follow-up care. A brief algorithm of the diagnostic procedure is given in Table 2.

Challenge tests will fail if AERD is still not thoroughly distinctive or provocation is precluded on ethical grounds (e.g., pregnancy, children of young age), unstable asthma, asthma nonresponsive to corticosteroids, patients on β-blocker, anatomical alterations (e.g., massive nasal polyps), missing compliance of the patient (e.g., asthmatic experiences and therefore fear of life threatening symptoms), unavailability of specific technical and/or medical equipment (e.g., measurement of respiratory function, appropriate emergency unit), or inadequately trained staff [1, 38]. Furthermore, long-term developments such as CRS with and without polyps or gastrointestinal complications cannot be adequately followed [1] or if a prognostic goal has to be considered in patients without typical aspirin-exacerbated respiratory symptoms. In addition, oral challenge tests ruled out hypersensitivity in 50% of the patients otherwise characterised by NSAID hypersensitivity [48]. Skin test responses are typically negative. Medical history revealed best positive and negative predictive values in comparison to oral challenges, but high false-positive and negative rates were also reported [49]. In such cases *in vitro* tests might be an useful option. During the last decades several *in vitro* tests had been developed. The most promising *in vitro* tests are analysing peripheral blood leukocytes (PBLs) focusing on genes, receptors, and metabolites of the eicosanoid cascade. The characteristics and relevance for supporting the diagnosis of AERD are very recently reviewed by Schäfer and Maune [7].

The diagnosis of AERD is crucial for the onset of an appropriate therapy, but aspirin sensitivity usually is diagnosed very late during the chronological sequence of the disease. As outlined above, aspirin sensitivity might become obvious in several organs until all symptoms of AERD will have been developed (for more details, see [1]). Therefore, a full medical history placing special attention to the existence of respiratory symptoms associated to AERD is essential for an early diagnosis (see Table 1).

Table 1: Organ manifestation of symptoms in AERD. The classification of AERD is based on the clinical picture and becomes obvious in diverse organs at different times in the course until all symptoms of AERD will have been developed. Accurately timed diagnosis of AERD is a major challenge in patients suffering from CRS with as well as without nasal polyps and/or bronchial asthma. But also individuals without known underlying airway-related diseases have to be considered as aspirin sensitive. Thoroughly taken medical history and scrutinising the patient's organ manifestation remain a fundamental challenge. Some of the most prominent symptoms associated with AERD are summarised (without the claim of being complete) for the identification of early indicators of AERD.

Organ manifestation	Symptoms
Airways	(i) Rhinosinusitis without nasal polyps
	(ii) Rhinosinusitis with nasal polyps
	(iii) Dyspnoea
	(iv) Bronchial asthma
	(v) Laryngeal oedema
Skin	(i) Urticaria
	(ii) Angioedema
Gastrointestinal tract	(i) Vomiting
	(ii) Diarrhoea
	(iii) Dyspepsia
	(iv) Gastric bleeding
	(v) Peptic ulcer disease
	(vi) Intestinal ulcer
Other organs	(i) Cardiovascular diseases
	(ii) Anaphylaxis
	(iii) Sepsis
	(iv) Tinnitus

5. Molecular Pathogenesis

The molecular pathogenesis of AERD has only been partially elucidated. There are several theories that try to explain AERD. The theories trying to explain AERD include (i) alteration of the arachidonic metabolism and its receptors/enzymes, (ii) release of inflammatory mediators and cytokines, and (iii) microorganisms such as virus and bacteria.

Driven by the known inhibitory action of NSAIDs on a subset of the eicosanoid pathway, that is, the cyclooxygenase pathway, Szczeklik and colleagues speculated in an early paper in 1975 that the cyclooxygenases and their metabolic products, mainly prostaglandin E (PGE), reflect a central modulating role in patients classified by AERD [50]. A decade later he presented his theory, that a viral respiratory infection may be an inciting event that starts the inflammatory processes that lead to respiratory inflammation and AERD in genetically susceptible individuals [51]. The bronchoprotective effects of PGE2 were confirmed by Pavord and Tattersfield in 1992 [52], and elevated peptido leukotrienes (pLT) in nasal polyps were documented in 1996 by Baenkler and colleagues [53]. The interdependence of the pathways of cyclooxygenase and 5-lipoxygenase elucidated and exemplified first in 1999 by Schäfer and colleagues. They demonstrated that a profile of eicosanoids (i.e., PGE2 and pLT) is specific for AERD and might play a role in the pathogenesis of aspirin exacerbated asthma. Furthermore, an altered profile of expression, synthesis, and metabolic activity of receptors and enzymes was hypothesised [42]. Subsequent studies confirmed this concept of an AERD-specific eicosanoid pattern investigating the synthesis of eicosanoid mediators as well as the expression of receptors and enzymes in nasal secretion, urinary excretion, polypous, mucosal, and bronchial tissues in respect to the cyclooxygenase and lipoxygenase pathways [54–57]. A concept of pathogenic

Table 2: Proposed algorithm of diagnosis of AERD. The diagnosis of AERD is a major challenge in patients suffering from CRS with/without nasal polyps, bronchial asthma, and/or unknown underlying diseases. The diagnostic approach of AERD is based on the clinical picture. This might be supported by imaging as well as in vitro techniques. The confirmative diagnosis for AERD is definitely established by aspirin challenge following increasing doses of aspirin. The routes of administration are (1) oral, (2) bronchial inhalation, (3) nasal inhalation, and (4) intravenous. Nasal or bronchial obstruction has to be monitored adequately. Provocation must be performed only when asthma is stable and is precluded on ethical grounds, unstable asthma, asthma nonresponsive to corticoids, or patients on β-blockers. Aspirin challenge tests should be performed by trained specialist in centres with the availability of adequate equipment and medication for emergency.

Diagnostic procedure of AERD	
Prior to aspirin challenge	
(1) Medical history	(i) Individual history (ii) Family history
(2) Severity of symptoms (suspected from historical reactions)	(i) No (ii) Mild (iii) Moderate (iv) Severe
(3) Class of NSAID	(i) Strong COX-1 inhibitors (ii) Poor COX-1 inhibitors (iii) Preferentially COX-2 inhibitors (iv) Selective COX inhibitors
(4) Physical examination (a) Localisation of symptoms (b) Stable asthma	(i) Airways (ii) Skin (iii) Gastrointestinal tract (iv) Other organs (v) FEV1 >70% and with 10% of best prior value
(5) Medication (a) Drug responsiveness (b) Actual medication	(i) Asthma responsiveness to corticoids (ii) Systemic/topic corticoids (iii) β-blockers (iv) Antihistamines (v) Others
Patient selection for aspirin challenge	
(1) Suspected reactions (2) Responsiveness to drugs (3) Anatomical alterations (4) Compliance of patient (5) Pretreatment	(i) Mild-to-moderate prior historical reactions (ii) Responsiveness to corticoids, leukotriene modifiers, β-blockers (iii) No aggressive polyp formation (iv) In need of daily aspirin (v) Continuing of all medications for upper and lower airways, including inhaled an intranasal corticosteroids (vi) Leukotriene modifier drug 2–4 weeks prior to (in case of safety reasons)
Aspirin challenge	
In vivo provocation according to an appropriate protocol	(i) Determination of airway stability (FEV1 >70%, 10% variability, every 1–3 h) (ii) Discontinue antihistamines 48 h before challenge
In vitro challenge, in case of	(i) Unstable asthma (ii) Unresponsiveness to corticoids (iii) Anatomical alterations (iv) Ethical grounds (v) Unavailability of technical and/or medical equipment (vi) In cases of non-airway-related symptoms and those not becoming obvious upon *in vivo* aspirin challenge
Treatment of aspirin-induced reactions	
Ocular Nasal Laryngeal Bronchial Gastrointestinal Urticaria/angioedema Hypotension	(i) Topical antihistamines (ii) (Oral) antihistamines or diphenhydramine, topical decongestant (iii) Racemic epinephrine nebulization (iv) Inhalation of β-agonist every 5 minutes until confortable (v) Empting (vi) Intravenous ranitidine (vii) Intravenous diphenhydramine (viii) Epinephrine administered intramuscularly

mechanisms, focusing on the imbalance of eicosanoid pathway was outlined recently [7]. To adumbrate this complex concept in brief, PGE as well as the corresponding receptors and enzymes are diminished even before NSAID challenge, whereas the pLT and the corresponding receptors and selected enzymes are elevated. This imbalance is potentiated upon intake of NSAIDs (for further details, see [7] and references therein).

In addition, it was shown that T cells, cocultured with parainfluenza, repiratory syncytial virus, or rhinovirus produced proliferation of CD3+ and CDR4+ cells and released cytokines that attract eosinophils [58], supporting the theory of Szczeklik [51]. Recent studies also have implicated interleukin-10 and tumor growth factor-β1 polymorphism through gene interaction in AERD and rhinosinusitis [59], and cyclooxygenases are modulated by cytokines [7]. Patients suffering from asthma and aspirin sensitivity as well as expressing the HLA A1/B8 phenotype have a higher incidence of AERD [60]. Elevated release of inflammatory mediators, for example, tryptase, histamine, ECP, IL-5, GM-CSF, RANTES, and eotaxin predominantly by mast cells and eosinophils, were described in patients suffering from CRSwNP and aspirin sensitivity [61–71]. The overproduction of IL-5 might intensify eosinophilic inflammation in aspirin-sensitive patients [72] and is correlated to increased levels of IgE and *Staphylococcus aureus* enterotoxins(SAE) present in nasal polyp tissue but is not specific for patients suffering from CRSwNP and aspirin sensitivity [73].

In summary, there is a complex network of molecular pathomechanisms and transcellular metabolism of eicosanoids which are implicated in the pathogenesis of CRSwNP and aspirin sensitivity [7, 66, 67, 73, 74].

6. Therapies

The treatment of CRS depends on the stage and extent of the disease. Several reviews and meta-analyses during the last decade point to slight differences in the pathogenesis of CRSwNP in association with aspirin intolerance compared to CRS. Therefore, we will review therapeutic approaches, which will specifically address this issue. For more details, please refer to a recent review by Alobid and Mullol [75].

6.1. Aspirin Desensitization. Aspirin desensitization might be beneficial for some patients classified with AERD demonstrating upper and lower airway inflammation. Aspirin desensitization is recommended for those AERD individuals with corticoid-dependent asthma or those requiring daily NSAID therapy for other medical reasons, for example, coronary artery diseases or chronic arthritis. Oral administration may reveal definitive improvements in both lower and upper airways in most patients with aspirin sensitivity [76]. The precise mechanism by desensitization in aspirin therapy is unclear. However, the synthesis of pLT by PBLs and nasal mucosa was reduced after desensitization [42, 77]. Another study demonstrated decreased bronchial responsiveness to inhaled leukotriene E4 on the day of desensitization therapy [78]. Modulation of further intracellular biochemical parameters might be another molecular mechanism (for more details on suggested mechanisms, see [7, 42]).

Focusing on the clinical symptoms, long-term aspirin desensitization (1 to 6 years) reduced significantly the use of oral corticoid treatment for asthma, the dosage of nasal corticosteroids, the numbers of sinus infections, sinus surgery per year, and hospitalization and also improved olfaction [3]. Furthermore, the rate of recurrence of nasal polyp in patients undergoing desensitization after one and six years was 6.9% and 65%, whereas without aspirin desensitization the rate was 51.3% and 93.5%, respectively [79]. Intranasal aspirin treatment in patients with bilateral CRSwNP resulted in delayed polyps recurrence, and 8 of 16 patients remained without symptoms for 15 months. The clinical outcome was significantly better than that from those treated with corticosteroids for recurrence prevention. Furthermore, endoscopy and acoustic rhinomanometry indicated a lower polyp size on the aspirin-treated nostril [80]. A double-blind, randomized, placebo-controlled trial revealed no effect on nasal airway using 16 mg of intranasal aspirin every 48 h after 6 months of treatment, but significantly decreased expression of the cys-LT1 receptor in the turbinate mucosa of aspirin treated patients [81]. Intranasal lysine-aspirin (up to 50 mg/d) in addition to routine therapy reduced polyp size without adverse effects in the lower airways [82]. Therefore, currently there is level Ib of evidence with recommendation A to use aspirin desensitization in aspirin-sensitive patients, although the evidence and recommendation to treat CRSwNP patients is still low [75].

6.2. Leukotriene Modifier Drugs. Leukotriene modifier drugs interrupt the leukotriene pathway and have an established impact in the therapy of asthma and allergic rhinitis. Two classes of leukotriene modifier drugs have been approved for asthma treatment: the cysteinyl leukotriene 1 receptor antagonist (montelukast, pranlukast, zafilrukast) and the 5-lipoxagenase (5LO-) inhibitor (zileuton). Both have been widely prescribed for symptom control of the upper and lower airways of patients classified by AERD [83–85].

If added to standard medication (including steroids) of patients suffering from CRS with or without nasal polyps, leukotriene modifier drugs will result in an overall improvement in nasal symptoms scores by 72%, thereby producing side effects in 11% [86]. Patients suffering from CRSwNP and treated with montelukast revealed an improvement only in some symptom scores and health-related quality-of-life parameters, whereas the nasal polyp scores and the ECP levels were not significantly altered [87, 88]. A subjective improvement in nasal symptoms was documented in 64% of aspirin-tolerant and in 50% of aspirin-sensitive patients. As significant improvements were observed only in aspirin-tolerant patients, the selective role of anti-leukotrienes in patients suffering from CRS with aspirin sensitivity was questioned [89]. Sinus symptoms improved in 60% of the patients treated with antileukotrienes, and an overall benefit was seen in 80% of patients suffering from Samter's triad [90]. Montelukast in addition to steroids significantly reduced headache, facial pain, and sneezing after eight weeks

of treatment. However, no significant effect were observed on the overall symptom score, nasal blockage, hyposmia, or nasal discharge [88]. Reduced eosinophilic inflammation, viability, and cytokine production in nasal polyps following montelukast therapy was described [91].

Postoperative treatment schemes with leukotriene inhibitor revealed similar results, and no significant differences were found one year after surgery [92]. Another study investigating the postoperative effects of montelukast and intranasal mometasone medication in patients suffering from CRSwNP revealed most likely complementary results. Both treatments caused a significant reduction in the SNOT-22 scores and in the rate of nasal polyps, but only a marginal advantage of montelukast [93].

These findings point to a possible role of leukotriene modifier drugs in the treatment of specific subpopulations, but significant scientific evidence is still lacking. Therefore, leukotriene modifier drugs reveal a limited level of efficacy (III) and have a low degree of recommendation (C) in patients suffering from CRSwNP [75].

6.3. Surgery. Sinus surgery is recommended when medical treatment fails. Therefore, surgical procedures are often viewed as adjunctive to medical therapy [64, 94]. Additionally, medical treatment should be continued after surgery.

The greatest review on sinus surgery with 11,147 patients has been published by Dalziel et al. who screened 444 articles and evaluated 33 articles published between 1978 and 2001 [95]. In 75–95% of the cases patients consistently evaluated their symptoms to be "improved" or "greatly improved." Additionally, surgical procedures with 1.4% overall complications for functional endoscopic sinus surgery (FESS) compared with 0.8% for conventional procedures proved to be safe.

Approximately two-thirds of the 3,128 patients participating in the National Comparative Audit suffered from CRSwNP but had no differences in clinical parameters, drug use, or general health when compared to CRSw/oNP [96]. Irrespective of the extent of surgery in the whole group of patients, a clinically significant improvement in SNOT-22 scores was demonstrated up to 36 months postoperatively. Interestingly, patients with CRSwNP benefited more from surgery than those without polyps.

Sinus surgery also significantly improved lung function and reduced systemic steroid use in patients with CRSwNP and concomitant asthma, whereas this was not the case in aspirin-sensitive asthma patients [97]. However, nasal breathing and quality of life improved in most patients.

The most recent prospective study investigated the effects of sinus surgery as well as fluticasone propionate nasal drops 400 μg twice daily on nasal and lower airway parameters in asthmatics with CRSwNP. FESS significantly improved mean asthma symptom scores and daily peak expiratory flow rate (PEFR) and all nasal parameters, including subjective and objective olfaction tests. FESS improved nasal and asthma symptoms in patients with NP [98].

Taken together, sinus surgery has been proven to be an effective tool to treat CRSwNP after first-line medical therapies.

7. Future Attempts

7.1. Antibodies

7.1.1. Anti-IgE/Anti-IL-5. Patients with CRSwNP had higher total IL-5 and IgE levels in nasal secretions, nasal polyp homogenisates, and blood serum than in controls. Anti-IgE or anti-IL-5 antibody only showed minimal beneficial effects on symptoms, eosinophilia and peak nasal inspiratory flow (PNIF) [99–104]. Based on current data, the evidence for efficacy of available anti-IgE and anti-IL-5 antibodies on the market is very low, and more studies are needed in order to recommend their use in the treatment of CRSwNP patients [75].

7.2. Staphylococcus aureus Enterotoxins. Nasal colonization by *Staphylococcus aureus* is a frequent event in CRSwNP patients. Accordingly, when specific IgE directed against *Staphylococcus aureus* enterotoxins (SAE) was found in nasal polyp tissue homogenates for the first time [23], a new paradigm was proposed, which indicated that these superantigens may be involved in the pathogenesis of eosinophilic CRSwNP [105]. By their superantigenic activity, enterotoxins may activate inflammatory cells in an antigen-nonspecific way. Indeed, nasal application of *Staphylococcus aureus* enterotoxin B is capable of aggravating experimental allergic asthma [86].

Interestingly, an increased colonization rate of *Staphylococcus aureus* and IgE to SAEs was reported in nasal polyps, specifically in subjects with asthma and AERD [87]. Thereby, IgE to SAEs was also coincident with higher levels of IL-5, eotaxin and eosinophil cationic protein which are known to potentiate and prolong the eosinophilic inflammation leading to polyps' development. Additionally, the presence of IgE against SAEs in CRSwNP correlated with an increased number of T cells expressing the TCR β-chain variable region known to be induced by microbial superantigens giving a link to the clinical importance of SAE IgE in polyps [88].

Moreover, new insights were gained into the modulatory effects of *Staphylococcus aureus* enterotoxin B (SAEB) on nasal polyp tissue [89]. SAE directed the mucosal inflammation to a Th2-driven pattern contributing to persistent inflammation by suppression of Treg lymphocytes [89]. Interestingly, SAE might only locally activate B cells, because a significant increase of local IgE antibodies can be observed in polyp patients, while independent of serum IgE levels of the same patients [23].

Nasal polyps typically show upregulation of proinflammatory pLTs and downregulation of PGE2 (see prior section for more details). In tissue of CRSwNP patients with an immune response to SAE, the production of pLTs, LTB4 and LXA4 is further upregulated [90], while SAEB significantly downregulated PGE2, COX2, and prostanoid receptor EP2 mRNA expression in fibroblasts, pointing to a direct role of SAE in regulating eicosanoids as a possible mechanism of SAE inflammatory reaction [88].

8. Conclusions

The latest nomenclature of AERD considers the obvious pathogenic association of chronic rhinosinusitis with/without polyps and asthma following the exposure to NSAID, which is usually last diagnosed. AERD often reveal moderate-to-severe phenotypes. Especially, the coexistence of CRSwNP elicits a more severe clinical appearance characterized by a prolonged and fatal course leading to a higher prevalence of recurrences and a coincidence with other atopic diseases. However, diagnosis in these patients is challenging despite the availability of various techniques.

AERDs are a distinct clinical entity characterized by acute NSAID triggered respiratory reactions like chronic hyperplastic rhinosinusitis with/without eosinophilia, formation of nasal polyps, or asthma. Significant morbidity and even mortality, particularly if higher doses of NSAIDs are ingested, may occur, if AERD is not recognized and appropriately treated. Concerned patients should be educated regarding NSAIDs and their avoidance preventing life-threatening asthma attacks.

The standard procedure confirming the diagnosis of AERD is still *in vivo* aspirin challenge, completed by latest blood testing, for example, in patients where *in vivo* provocation tests are contraindicated, including patients with unstable asthma, asthma nonresponsive to corticosteroid therapy, patients with β-blockers, and patients with potential compliance problems. Future areas of investigation should focus on the identification of further biomarkers improving early diagnosis using various diagnostic techniques.

The treatment of CRS and nasal polyps is essential to effectively control asthma and to prevent secondary infections. An adjusted medication, including aspirin desensitization, will have a positive impact on course of the disease and the patients' quality of life.

Moreover, children of parents with AERD suffer from CRSwNP and rhinosinusitis more often than children of healthy parents. This might point to a genetic background in terms of polymorphisms of COX-1, COX-2, 5-LO, and/or 15-LO pathways and/or receptors which need to be elucidated.

Our current knowledge on AERD focuses on a pathogenic concept based on decreased PGE2 and increased levels of pLT associated with overexpression of LTC4-synthase, which is accompanied by an altered transcellular metabolism of mediators of the eicosanoid cascade. This also involves local production of IgE directed to *Staphylococcus aureus* enterotoxins and overproduction of IL-5. There is a clear need to understand the implication of the metabolites of the eicosanoid cascade, their receptors, enzymes, and genes, the physiological and pathological impact of microbes, as well as the local and systemic function of dendritic cells, mast cells, and Treg cells.

The therapeutic interventions concerning the treatment of patients classified by AERD should include aspirin desensitization and medication with leukotriene modifier drugs being the most promising drugs at the moment. After failure of conservative treatment and/or in case of a severe phenotype, functional sinus surgery is an important option completed by further conservative therapies. Finally, therapeutic approaches treating *Staphylococcus aureus* and SAE effects by antibiotics or appropriate vaccination are promising.

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Review Article

Rhinosinusitis and Aspirin-Exacerbated Respiratory Disease

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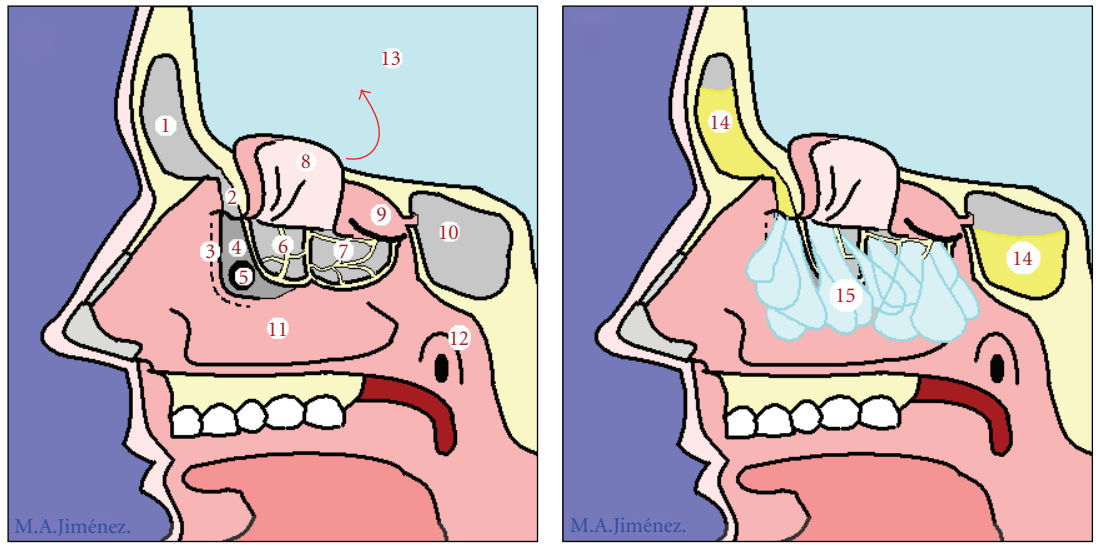
Rhinosinusitis is a feature of aspirin-exacerbated respiratory disease (AERD), which in the initial phase is manifested as nasal congestion, mostly affecting females at the age of around 30 years on average. Subsequently, nasal inflammation progresses to chronic eosinophilic rhinosinusitis, asthma, nasal polyposis, and intolerance to aspirin and to other NSAIDs. While it has been long established that NSAIDs cause inhibition of cyclooxygenase-1 (COX-1), leading to excessive metabolism of arachidonic acid (AA) to cysteinyl-leukotrienes (cys-LTs), there is now evidence that both cytokines and staphylococcus superantigens amplify the inflammatory process exacerbating the disease. This paper gives a brief overview of the development of chronic rhinosinusitis (CRS) in sensitive patients, and we share our experience in the diagnosis and management of CRS in AERD.

1. Introduction

The acute reaction to aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) in aspirin-exacerbated respiratory disease (AERD) patients is a life-threatening condition characterized by upper airway symptoms (nasal obstruction and rhinorrhea) and lower airway symptoms (shortness of breath and respiratory distress). AERD patients, however, suffer from chronic manifestations of the disease including chronic rhinosinusitis, nasal polyps, and asthma—usually resistant to any treatment. The AIANE study showed that up to 80% of AERD patients required intermediate to high doses of inhaled steroids, and up to 50% of them had to take oral steroids in order to obtain asthma control. Similarly, the TENOR study showed that asthma was severe in 66% of AERD subjects, 34% received high systemic steroidal doses, 67% needed antileukotrienes, and 20% of them required orotracheal intubation during acute reactions. It must be noted that inflammation is usually present in both upper and lower airways and some AERD patients can present with a disease limited to the upper respiratory tract and no lower airways symptoms at all [1, 2]. Here, we review some of the most relevant aspects of rhinosinusitis in AERD.

2. Rhinosinusitis in AERD

Chronic rhinosinusitis is a major condition in AERD, which in its initial phases is manifested only as nasal congestion, with asthma starting about two years after the initial nasal symptoms. Chronic rhinosinusitis (CRS) is defined as an inflammatory condition involving the mucosa underlying the nasal cavity and the paranasal sinuses that can also affect the underlying bone. It usually lasts more than twelve consecutive weeks. In the case of AERD, CRS becomes a life-time condition, which is usually difficult to control. The clinical symptoms and signs to evaluate CRS are divided into major and minor criteria. The major criteria are nasal obstruction, facial pressure, nasal discharge, and/or postnasal drip. The minor criteria are the presence of purulence, anosmia and/or hyposmia, chronic cough, headache, dental pain, ear pressure, fatigue, and halitosis. The clinical evaluation is based on anterior rhinoscopy and nasal endoscopy that usually reveals mucosal oedema and hyperemia, with or without polyps, and frequently purulent secretions [3]. CRS can be divided into two mutually exclusive histological subtypes based on the presence of polyps or glandular hypertrophy. CRS with nasal polyps (CRSwNP) affects the full thickness of the nasal



(1) Frontal sinus.
(2) Frontal recess.
(3) Uncinate process.
(4) Hiatus semilunaris.
(5) Maxillary sinus ostium.
(6) Anterior ethmoidal cells.
(7) Posterior ethmoidal cells.
(8) Middle turbinate (folded upwards).
(9) Superior turbinate.
(10) Sphenoid sinus.
(11) Inferior turbinate.
(12) Eustachian tube.
(13) Anterior cranial fossa.
(14) Retained secretions within frontal and sphenoid sinuses.
(15) Nasal polyps.

(a) (b)

Figure 1: Schematic representation of (a) normal nasosinusal anatomy and (b) nasosinusal polyposis.

mucosa, which is replaced with an oedematous, generally eosinophilic, epithelium-coated "bag" of interstitial matrix "ground substance." In contrast, CRS without nasal polyps (CRSsNP) or "hyperplastic rhinosinusitis" is characterized by glandular hypertrophy as demonstrated by Malekzadeh and colleagues [4, 5].

Eosinophils play an important role in the pathogenesis of CRS. Indeed, a greater number of these cells have been reported in both the upper and lower airways of patients of aspirin-sensitive patients as compared with aspirin-tolerant patients [6–8]. On activation, eosinophils release a vast array of mediators including leukotrienes, basic proteins (major basic protein and eosinophil cationic protein), cytokines, and oxygen-free radicals that cause local tissue damage. Saitoh et al. [9] found a correlation between the number of infiltrated eosinophils and both epithelial damage and BM thickening. CRSwNP is the most frequent form observed in aspirin-sensitive patients. We have found increased levels of eosinophil cationic protein in nasal secretions of aspirin-sensitive patients [10].

2.1. Nasal Polyps. A majority of patients with aspirin intolerance will develop nasal polyps during the course of the disease. Nasal polyps are inflammatory pseudotumoral masses that most frequently start to grow from the ostiomeatal complex and the cells of the anterior ethmoidal sinus. They can affect the totality of the remaining sinusal cavities including the posterior ethmoidal cells, the maxillary, and the frontal or the sphenoidal sinuses, and they also can extend to the olfactory cleft, the sphenoethmoidal recess, and the nasal cavities (Figure 1). Nasal polyposis in AERD patients is present in up to 80 to 90% of patients and tends to be more aggressive and difficult to treat medically, also presenting with higher recurrence rates after surgery. In the AIANE study that included 500 ASA-intolerant patients from 14 different centres, nasosinusal polyposis was diagnosed on nasal endoscopy in 60% of the patients, but the prevalence rose to 90% when CT-scans of the nose and sinuses were performed [11].

Nasal polyps are formed by lax connective tissue stroma with oedema, inflammatory cells with a predominant eosinophil infiltration, mucosal glands, and newly formed vascular structures. The mucosal covering of the nasal polyps is a columnar glandular pseudostratified epithelium that also plays a major role in cytokine and inflammatory mediator release. There is no doubt that eosinophils are the main inflammatory cells found in the tissue specimens of nasal polyps, but neutrophils can also be present in large amounts and can even be predominant, notably in Asiatic patients. Other inflammatory cells can also be found in nasal polyps, such as lymphocytes, monocytes, plasma cells, and fibroblasts. Chronic sinusitis with nasal polyposis is also characterized by an intense oedematous stroma with albumin deposition, formation of pseudocysts, and subepithelial and perivascular inflammatory cell infiltration. Remodelling is a dynamic process regulated by diverse mediators among which TGFβ is the most important. In addition to being a key factor in the generation or the deficit of T-reg cells, TGFβ

is also a critical factor implicated in the remodelling process in the airways through the attraction and proliferation of fibroblasts and upregulation of extracellular matrix synthesis.

3. Epidemiology

The full prevalence of aspirin-induced rhinosinusitis can be difficult to assess, as it primarily relies on the clinical history. We believe that this condition is underdiagnosed worldwide and is only confirmed after an ASA challenge testing under ideal conditions. False negative results can occur when the patient is receiving systemic steroid or antileukotriene treatment. Systematic reviews report an incidence of 21.1% of ASA intolerance in asthmatic patients after ASA challenge testing. The incidence rises to 24% in patients with severe asthma and up to 40% when the association of asthma and chronic rhinosinusitis with polyposis is present [12–15].

Usually ASA intolerance appears in 30–40-year-old patients with a previous history of chronic rhinosinusitis and/or asthma. Some patients refer an acute viral-like nasal episode that never resolved fully afterwards. Nasosinusal symptoms usually progress to chronic sinusitis and nasal polyposis appears years later. Intrinsic asthma tends to appear after the rhinitis, but before the onset of nasal polyposis (Figure 2).

Skin testing for allergy can be positive in up to 30–60% of AERD patients, but no association between ASA intolerance and IgE-mediated mechanisms has been identified. Nevertheless, a high prevalence of food intolerance or antibiotic allergies has been found [16–18].

4. Physiopathology

4.1. Arachidonic Acid. The physiopathologic mechanisms of AERD are still not fully understood. However, it is now well established that an alteration of arachidonic acid metabolism takes place, and it is characterized by an imbalance between cyclooxygenase (COX) and lipoxygenase pathways that results in an overactive lipoxygenase pathway. Thus, COX-1 inhibition after ASA or NSAIDs intake finally results in an overproduction of leukotrienes that leads to airway inflammation. Increased levels of leukotriene C_4 synthase with elevated levels of LTC_4, LTD_4, and LTE_4 with an overexpression of leukotriene producing enzymes such as 5-lipoxygenase (5-LO) and LTC_4 synthase in nasal polyp tissue have been documented. Increased levels of urinary leukotriene metabolites have also been associated with hyperplasic CRSsNP and CRSwNP in both ASA-tolerant (ATA) and ASA-intolerant (AIA) patients. These urinary leukotriene metabolite levels tend to drop significantly after sinus surgery, probably as a consequence of the elimination of a great amount of leukotriene producing cells in the ethmoidal sinus. Indeed increased LTE_4 levels and $CysLT_1$ receptor overexpression have been identified in nasosinusal mucosa of aspirin-sensitive patients [19]. In an *in-vitro* study, Kowalski et al. [20] found that after incubation with 200 μg of acetylsalicylic acid, 15-hydroxyeicosatetraenoic (15-HETE) was overproduced by nasal polyp epithelial cells and peripheral blood leucocytes of ASA-intolerant patients. Specificity and sensitivity of this test were both over 80%. 15-HETE has several proinflammatory effects such as inflammatory mediator release from the mast cells, mucosal glycoprotein release and bronchial smooth muscle contraction and thus could be a major trigger of airway inflammatory reactions. Supporting these observations, Mastalerz and colleagues show significant upregulation of some arachidonate lipoxygenation products in asthmatic subjects with aspirin hypersensitivity, as manifested by high baseline levels of 5-, 15-HETE in exhaled breath condensate [21].

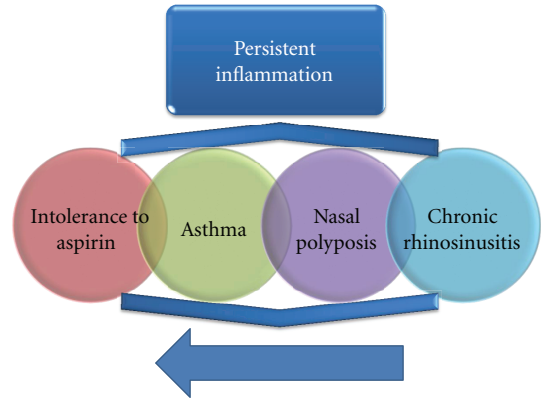


Figure 2: Natural history of aspirin-exacerbated respiratory disease.

The products of arachidonic acid also include lipoxins (LXs) and eoxins. The LXs modulate leukocyte trafficking and vascular tone, and in contrast to cys-LTs they have potent anti-inflammatory effects. A reduction in their levels may be a feature of AERD.

4.2. Cytokines. There has been a significant number of studies indicating that cytokines, in particular TH2 cytokines such as IL-4, regulate inflammatory processes in CRS. Increased IL-4 gene expression and IL-4 protein have been found in the upper airways in subjects with chronic sinusitis found as compared with controls. IL-4 promoter polymorphisms are also associated with nasal polyps in aspirin-sensitive patients. We have demonstrated that IL-4 is a major stimulus for eotaxin-2/CCL24 production. IL-13 and IFN-gamma also induce CCL24 production but they are less potent stimuli compared with IL-4 [22]. Pods et al. have reported increased expression of both eotaxin (CCL11) and eotaxin-2 in nasal polyps of patients suffering AERD, further supporting our findings in nasal polyposis [23]. The role of eotaxins in nasal polyps has been reviewed previously [24]. In a separate study, we have also shown that AERD patients with CRS release the eosinophil attractant CCL5 (RANTES) in nasal secretions, and lysine aspirin nasal challenge further increases the release of this chemokine. Consistent with this finding, Kupczyk et al. showed that these patients release increased levels of CCL5 and extended our observation by demonstrating the release of the eosinophil activating chemokine MCP-3. However, they did not show increased levels of CCL5 following

lysine-aspirin challenge. We performed nasal challenge using a nitrocellulose filter, while they applied the nasal challenge by aerosol. These methodological differences may explain the differences observed in these two studies using the aspirin challenge. The release of CCL5 by nasal polyps has also been documented [25, 26].

IL-5 plays a prominent role in eosinophilic-driven processes, and it has been documented in CRSwNP in both aspirin-sensitive and aspirin-tolerant patients. A modest increase of IL-5 has been found in bronchial biopsies from AIA [27]. Interestingly, this cytokine is decisive regarding the impact of staphylococcus-aureus-(SE-) derived enterotoxins, which function as superantigens. S. aureus enterotoxin B (SEB) shifts the cytokine pattern further in nasal polyps toward T-helper-2 cytokines (increases interleukin-2, interleukin-4, and interleukin-5 greater than twofold), but it reduces the T-regulatory cytokines interleukin-10 and TGF-beta1. S. aureus-derived enterotoxins also influence local immunoglobulin synthesis and induce polyclonal immunoglobulin E production, which may contribute to severe inflammation via activation of mast cells [28]. Increased specific IgE to both S. aureus enterotoxin A (SEA) and SEB has been detected in nasal polyps from both subject groups, but median levels were markedly higher in AIA subjects than in ATA subjects [29].

Using a microarray cDNA technique, Sekigawa et al. [30] reported allelic associations of single nucleotide polymorphisms (SNPs) of INDO and IL1R2, in nasal polyps derived from aspirin-sensitive, but not aspirin-tolerant, patients. In contrast, Stankovic et al. identified periostin as a distinctive marker in nasal polyps derived from aspirin-sensitive patients [31].

Protein profiling is another approach, which has been used to investigate the pathogenesis of aspirin sensitivity in AERD patients. Zander et al. showed upregulation of β-adaptin and heat shock protein 70 (HSP70) in pooled nasal polyps samples from AIA using protein microarray [32]. β-adaptin has been reported to be one of many proteins that form complexes in clathrin-coated pits and vesicles during receptor-mediated endocytosis, while HSP70 seem to be involved in environmental stress as it occurs during the inflammation process indicating a greater degree of tissue damage. The increased expression of HSP70 may represent a cytoprotective adaptive response and result in altered cell regulation. This, in turn, may contribute to cellular proliferation and ultimately a more aggressive form of nasal polyposis refractory to treatment characteristically seen in aspirin-sensitive patients.

4.3. Other Mechanisms. It has been proposed that autoimmunity may play a crucial role in AERD patients. However, it is possible that studies supporting this observation may reflect differences between ethnic groups as no association between AERD and autoimmunity markers has been proved [33].

Infection of the upper airways of AERD patients by anaerobic bacteria, Gram-negative organisms, staphylococcus aureus, and other bacteria is a frequent a complication. These bacteria are sources of antigens, which can form biofilms which in turn may amplify the inflammatory process. For instance, Bachert et al. demonstrated the presence of staphylococcus enterotoxins A and B in 50% of patients with polyposis in the homogenized polyp tissue. It is important to note that these same antigens also have the ability to stimulate the production of specific IgE antibodies [28].

Chronic viral respiratory infections have been suggested to play an important role in AERD via regulation of cytotoxic lymphocytes [34]. To date, however, there is not convincing evidence that this could be the case. Because the ethmoidal osteomeatal complex (OMC) in the nasal middle meatus is a site of deposition of toxic inhalants, viral particles, and airborne allergens, it is tempting to hypothesize that chronic infection of the ethmoidal sinus could play a role in the genesis of the inflammatory process in the upper airways of AERD patients. However, it does not explain the severity of asthma in these patients.

5. Diagnosis

The diagnosis of chronic sinusitis in AERD can occasionally be confusing in its early stages, since it usually precedes the onset of the AERD triad (asthma, ASA intolerance, and nasal polyposis) [35]. It can be easily confused with an allergic rhinitis although the skin tests for allergy will often be negative. However, either a nasal smear or blood count with high eosinophil numbers may give an initial diagnosis of nonallergic rhinitis with eosinophilia (NARES), an entity described in 1980 by Mullarkey et al. [36] and Jacobs et al. [37]. NARES has been suggested to be the precursor of nasal polyposis in both aspirin-tolerant and intolerant patients [38]. As the disease progresses, bronchial asthma and intolerance to aspirin will appear making the diagnosis of AERD easier (Figure 2).

In contrast to the early diagnosis of rhinitis, the diagnosis of rhinosinusitis with polyps is much easier to perform. Symptoms such as hyposmia, nasal obstruction, anterior rhinorrhea, postnasal discharge, and occasionally cephalea are suggestive of nasal polyposis. Moreover, either anterior rhinoscopy or nasal endoscopy enables the examiner to see the protrusion of gray or pink translucent, multilobulated, nonfriable, and usually clustered tissue, which characterizes the polyps. A CT scan of the paranasal sinuses may be useful to determine the extension of the polyps in the sinusal cavities.

Aspirin challenge has been widely used to confirm the adverse reaction to NSAIDs. It is indicated in patients suffering asthma, rhinosinusitis, and nasal polyposis and in those with a history of near fatal reactions, but with negative history of ASA intolerance (15% of asthmatic patients with negative history may be intolerant to ASA). In patients with asthma and negative history of ASA intolerance, but with risk factors (rhinosinusitis, nasal, polyposis, a history of near fatal reactions) the risk increases and the test is totally required. Currently, there are four ASA challenges: oral, bronchial, nasal and intravenous. The oral challenge is considered the "gold standard", and it is practiced mainly in the USA. In contrast, the aspirin bronchial inhalation challenge is used mainly in Europe. The aspirin nasal challenge is the safest

since it induces bronchospasm rarely [39]. We use this last method as a diagnostic tool in our clinic on a routine basis.

Once AERD has been diagnosed, it is essential to assess whether it is associated with respiratory allergy. It has been proved that most patients with nasal polyps are sensitized against common allergens [40]. Therefore, all patients with AERD should undergo skin or serological tests as part of their initial evaluation and, if necessary, specific immunotherapy should be instituted.

6. Treatment

Undoubtedly, corticosteroids remain the cornerstone in the treatment of chronic hypereosinophilic sinusitis, whether accompanied or not by nasal polyposis [41–44]. Nasal topical steroids such as mometasone furoate, triamcinolone acetonide, or budesonide are used almost systematically as they have demonstrated a clear benefit in the control of mucosal oedema and of nasal polyposis. The prescription of topical nasal steroids is also very important for patients after endoscopic sinus surgery for nasal polyposis, as they can decrease the recurrence and growth of polyps [45]. Nasal lavages with saline solution or other commercial seawater sprays are also recommended to all patients. These should be performed at least twice a day in order to remove nasal secretions and crusts as much as possible before the application of the nasal steroid, thus facilitating its better absorption.

Systemic steroids are also commonly used, notably in cases of moderate-to-severe nasal polyposis or poorly controlled asthma, since it has been demonstrated that after their administration there is, in addition to an improvement in FEV_1, a decrease in polyp size and a reduction in both obstructive symptoms and rhinorrhea [46]. Some patients also show a partial or complete recovery of the sense of smell. It is generally recommended to administer short courses of prednisone calculated up to 1 mg/kg/day, with a maximum of 80 mg a day, which should always be taken in a daily single dose, early in the morning, in order to strengthen the circadian rhythm of endogenous cortisol. We do not recommend steroids in low doses for prolonged periods because of the long-term deleterious effects on the hypothalamic-pituitary-adrenal axis.

The prescription of antibiotics should be reserved only for cases of bacterial infection with the presence of purulent discharge and symptoms such as cephalea, rhinorrhea, posterior dripping, and fever. In such cases, the same antibiotics recommended for any acute bacterial nonpolypoid sinusitis are used empirically, specifically directed against haemophilus influenzae, streptococcus pneumonia, and moraxella catarrhalis. The recommended first choice antibiotic is amoxicillin in combination with clavulanic acid; some other options are second or third generation cephalosporins, macrolides, or fluoroquinolones. Some studies have demonstrated a beneficial effect with the administration of low doses of macrolides for prolonged periods in chronic sinusitis with or without nasal polyposis. In fact, it has been observed in some patients, especially of oriental races, that prolonged administration of low doses of these drugs may have an immunomodulatory effect leading to a reduction in the size of nasal polyps [47]. This immunomodulatory effect of macrolides is perhaps less pronounced in Caucasian patients, whose nasal polyps have a predominantly eosinophilic tissue infiltration, characteristic of an inflammation mediated by a TH2 response.

The use of antileukotrienes has been considered an important adjunctive treatment of both bronchial asthma and chronic hypereosinophilic rhinosinusitis with or without polyps in the AERD [48]. However, both our experience and different reports in the literature show that their effect varies greatly from patient to patient. Their use can be justified by the overproduction of leukotrienes present in AERD. However, because of their cost and relative effectiveness, we do not consider them as a part of the primary treatment, and we prefer to reserve them for cases in which conventional treatment with nasal lavages, topical nasal steroids, and one to two short courses of systemic steroid per year are insufficient.

Aspirin desensitization may be beneficial in selected patients, but it must always be performed under supervised conditions. Several protocols of desensitization have been proposed allowing the completion of the procedure, usually within three to five days. The standard protocol for desensitization is an extension of the oral aspirin challenge protocol and all the safety precautions recommended for the challenge should be employed. Increasing aspirin doses (100–300 mg) are generally administered orally, although the intranasal administration is also a good alternative. Once desensitized, the patient must take a full dose of the prescribed amount of aspirin daily in order to maintain the desensitization effect. Up to 30% of patients will not tolerate the side effects of daily aspirin intake, and other COX-1 inhibitors such as ibuprofen can trigger bronchospasm in these individuals. It has been demonstrated that desensitization can improve asthma control and prevent the progressive growth of nasal polyps [49].

Although several biochemical events occur directly after achieving aspirin desensitization, such as downregulation of arachidonic acid metabolism, decreased inflammatory cell activation, downregulation in cysteinyl-leukotriene $CysLT_1$ receptor expression, the real mechanism of aspirin desensitization remains unknown [50, 51]. Sweet et al. studied 107 AERD patients in a retrospective survey after 6 years of followup. Data from this study clearly demonstrated the clinical benefit of desensitization therapy for aspirin-sensitive patients, including a reduction in the number of hospitalization and emergency room visits, upper airway tract infections, sinus surgeries, and also, for many, an improvement in the sense of smell. However, twenty percent of the 65 patients of the therapy group reported upper digestive symptoms [52]. ASA desensitization is the only treatment that has shown clear impact on the natural course of AERD. Evidence suggests that desensitization reduces the growth and recurrence rate of nasal polyps in aspirin-sensitive patients in the long term. This indeed reduces the need for sinus surgery and allows a decrease in intranasal corticosteroid use. However, the impact of ASA desensitization on asthma has not been as consistent or reliable as the effects observed in the upper airway.

Surgical treatment must also be considered when nasal polyposis fails to respond to medical treatment. Nowadays endoscopic sinus surgical techniques are preferred because of their proven safety and reduced morbidity. Several studies confirm that endoscopic sinus surgery in AERD patients not only improves nasal symptoms but also enables a better control of asthma [53]. Therefore, surgical treatment should be considered in all cases of nasosinusal polyposis when medical treatment fails to improve nasosinusal symptoms, when more than two short courses of systemic steroids per year are needed, or when asthma is not controlled. Another special indication for surgery is anosmia or hyposmia, and even more so in patients with "professional noses" such as chefs, winemakers, sommeliers, and perfume creators. However, the impact of surgery on olfaction is never guaranteed and surgery by itself could increase olfactory losses.

Currently, there are three types of surgery indicated for nasal polyposis. In simple polypectomy, only the visible polyps in the nasal cavities are removed, without penetrating into the ethmoidal cells. Functional ethmoidectomy includes the opening of all the affected ethmoidal cells, with the resection of all polyps, but with minimal removal of the ethmoidal mucosa. Finally, the nasalisation technique consists of a complete bilateral ethmoidectomy with eradication of all the ethmoidal mucosa, except the one covering the frontal recess boundaries. Wide antrostomy and sphenoidotomy are also performed, and when needed, the middle turbinate can also be included in the resection [54]. The choice of surgical technique will certainly depend on the preference and personal training of each surgeon; however, increasing reports in the literature favor a more radical removal of the ethmoidal mucosa. In fact, it has been demonstrated that limited ethmoidectomy without mucosal removal offers long-term results comparable to those of a simple polypectomy; therefore, we do not consider that the risk of a partial ethmoidectomy is justified if a more limited and more secure procedure can achieve similar results [55]. Nasalisation appears to offer better long-term results with a lower rate of recurrence of polyposis, less nasal obstruction and an improvement of olfaction that is more stable over time. It is also noteworthy that a radical surgery has a more positive and prolonged impact on the stabilization of bronchial asthma both in ASA-tolerant and intolerant patients [56]. Jankowski et al. compared nasalisation with conventional ethmoidectomy, proving that a more radical surgery on the ethmoid mucosa does offer better functional results, both on olfaction and general nasal function [56–58]. Some authors actually advocate the systematic resection of the middle turbinate, since this provides a more complete removal of the ethmoidal mucosa, resulting in a lower incidence of polyposis recurrence in the long term. However, based on our experience, we believe that the middle turbinate is an important anatomical landmark that is helpful in cases of revision surgery, and also, with its preservation, frontal recess stenosis is less likely to occur. Thus, we prefer to resect the middle turbinate only when it is affected severely by polypoid disease or when it has lost its structural support after the ethmoidectomy. In this latter situation, the resultant middle turbinate lateralization could occlude the surgical cavity, thereby impairing sinus lavages or topical steroid penetration and causing polyp relapse and chronic sinusitis.

The surgical goals of nasalisation are the eradication of the ethmoidal mucosa and the creation of a cavity as wide as possible, in order to facilitate the subsequent cleaning and the proper dissemination of topical steroid nasal spray [59]. It is also important to promote good mucosal healing through immediate postoperative application of a parenteral depot systemic steroid and the early reinstitution of topical nasal steroid use. Clinically, the goals of surgery are first, improvement of nasal obstructive symptoms with, reduced rhinorrhea and postnasal discharge; second, a stable and sustained improvement of smell; finally, a better control of bronchial asthma. Despite all the medical and surgical treatments described above, there are cases in which stabilizing a patient with AERD is extremely difficult. Some studies have reported cases in which methotrexate proved to be useful [60].

7. Conclusion

Despite all the major advances in research, understanding fully the mechanism of AERD still remains a challenge to the modern medical world. It is now well established that aspirin causes inhibition of cyclooxygenase-1 (COX-1) associated with excessive metabolism of arachidonic acid (AA) to cysteinyl-leukotrienes (cys-LTs), while there is a rapid decrease in the synthesis of COX-1 products including prostaglandin E_2, which is known to have bronchodilator and anti-inflammatory properties. Moreover, cytokines and staphylococcus superantigens further amplify the inflammatory process in the airways of AERD patients. Chronic treatment with moderate-to-high doses of inhaled steroids is indicated. However, it is important to keep in mind that a multidisciplinary approach is the cornerstone for a successful treatment of these patients.

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Review Article

Pathogenic Mechanisms and *In Vitro* Diagnosis of AERD

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Aspirin-exacerbated respiratory disease (AERD) refers to chronic rhinosinusitis, nasal polyposis, bronchoconstriction, and/or eosinophilic inflammation in asthmatics following the exposure to nonsteroidal anti-inflammatory drugs (NSAIDs). A key pathogenic mechanism associated with AERD is the imbalance of eicosanoid metabolism focusing on prostanoid and leukotriene pathways in airway mucosa as well as blood cells. Genetic and functional metabolic studies on vital and non-vital cells pointed to the variability and the crucial role of lipid mediators in disease susceptibility and their response to medication. Eicosanoids, exemplified by prostaglandin E_2 (PGE_2) and peptidoleukotrienes (pLT), are potential metabolic biomarkers contributing to the AERD phenotype. Also other mediators are implicated in the progress of AERD. Considering the various pathogenic mechanisms of AERD, a multitude of metabolic and genetic markers is suggested to be implicated and were introduced as potential biomarkers for *in vitro* diagnosis during the past decades. Deduced from an eicosanoid-related pathogenic mechanism, functional tests balancing PGE_2 and pLT as well as other eicosanoids from preferentially vital leukocytes demonstrated their applicability for *in vitro* diagnosis of AERD.

1. Introduction

Diagnostic tests assist the physician in assuring an appropriate treatment of the symptoms and as also the disease from which a patient is suffering. *In vitro* diagnostic tests are widely used in the practice of modern medicine. Nonsteroidal anti-inflammatory drugs (NSAIDs) are amongst the most frequently used drugs for the treatment of a variety of symptoms and diseases. Therefore, it is unsurprising that adverse reactions to NSAIDs arise in some patients.

The diagnosis of NSAID-triggered, or exacerbated symptoms and diseases, is usually based on medical history or provocative challenge testing [1–8]. In some cases the latter is precluded on ethical grounds (e.g., pregnancy, children of young age), anatomical alterations (e.g., massive nasal polyposis), missing compliance of the patient (e.g., asthmatic experiences and therefore fear of life threatening symptoms), unavailability of specific technical and/or medical equipment (e.g., measurement of respiratory function, appropriate emergency unit), or inadequately trained staff [7, 8].

Several approaches attempted to diagnose and confirm NSAID-triggered symptoms and related diseases by *in vitro* diagnostic tools during the last 110 years. Some of them were discarded, others are under investigation. *In vitro* tests, and the results derived when they are used, frequently play a vital role in the overall diagnostic process. To ensure that each reader has the same basic knowledge, we will describe some rudimentary background information on terminology, suggested pathomechanism, test theory and test performance before discussing the *in vitro* test for diagnosis of NSAID-triggered symptoms and underlying diseases in more detail.

To some extent there is a known discrepancy of medical history and clinical symptoms upon exposure to NSAIDs, that is, that the provocation test shows negative outcome, whereas patients' history documented positive reaction. This may require an additional (*in vitro*) diagnosis to support the physician's decision for an appropriate treatment of the patient. Unfortunately, any diagnostic procedure, clinically and *in vitro*, is hampered by one or more inherent as well

Table 1: Terms used for reactions of NSAID-triggered hypersensitivity. NSAID: nonsteroidal anti-inflammatory drugs; COX: cyclooxygenase.

Terms used	Predominant manifestation/location of symptoms	Supposed underlying pathomechanism
Syndrome de Widal	Airways	Pathomechanism unknown, hyperreactivity/-sensitivity to aspirin and aspirin-like drugs
Samter's triad	Airways	Pathomechanism suspected to altered sensitivity of chemoreceptor, hyperreactivity of airway mucosa to aspirin and aspirin-like drugs
Aspirin idiosyncrasy	Anywhere, ubiquitous	"Peculiarity" of hypersensitive reaction to aspirin and aspirin-like drugs which is not elicit by immunoglobulin-mediated/immunologic reactions, but by dysfunction or loss of function of enzymes
Aspirin allergy	Anywhere, Ubiquitous	Involvement of immunoglobulin-mediated/immunological reactions directed to aspirin and aspirin-like drugs
Pseudoallergic reaction to aspirin	Anywhere, ubiquitous	Reaction to aspirin and aspirin-like drugs, causing symptoms as seen by allergic, reactions (i.e., immunoglobulin-mediated/immunologic), but without involvement of immunological reactions
Aspirin intolerance	Anywhere, ubiquitous	Pathomechanism unknown/not defined, but aspirin and aspirin-like drugs are not tolerated by an individual
Aspirin sensitivity	Anywhere, ubiquitous	Pathomechanism unknown, but hyperreactivity/-sensitivity to aspirin and aspirin-like drugs, symptomatic description
Aspirin-sensitive asthma	Lower airways	Hyper-reactivity to aspirin and aspirin-like drugs causing airway obstruction
Aspirin-induced asthma	Lower airways	Pathomechanism unknown, but initiated/induced by aspirin and aspirin-like drugs
Aspirin-exacerbated respiratory disease (AERD)	Airways, systemic	Exacerbated by NSAIDs blocking COX-1 pathway
NSAID-induced rhinitis and asthma (NIRA)	Airways	Exacerbated by NSAIDs blocking COX-1 pathway
NSAID-induced urticaria/angioedema (NIUA)	Skin, systemic	Exacerbated by NSAIDs blocking COX-1 pathway
Single drug-induced urticaria/angioedema (SDUA)	Skin	Exacerbated by a single NSAID blocking COX-1 pathway
Multi-drug-induced urticaria/angioedema (MDUA)	Skin	Exacerbated by multiple NSAIDs blocking COX-1 pathway
Single drug-induced anaphylaxis (SDA)	Systemic	Sensitisation to a single NSAID blocking COX pathway, suggested immunoglobulin-mediated/immunologic pathomechanism
NSAID-blended reaction (NBR)	Airways, skin	Pathomechanism unknown; not AERD, not NIRA, presumably not immunoglobulin-mediated/immunologic

as exogenous factors. While some of them are known, most remain unknown, leading to some uncertainty of the test outcome.

The *nomenclature* for NSAID-triggered hypersensitivity reaction in medical literature might be confusing because of the diverse terms employed over last decades and the multiple clinical manifestations in humans. A list of terms used is given in Table 1, making no claim to be complete. Supporting the communication we consider the proposed terminology of "Report of the Nomenclature Review Committee of the World Allergy Organisation", dating from 2003 [7]. This nomenclature is independent of the target organ or patient age group, but is based on the mechanisms that initiate and mediate reactions on our current knowledge, assuming that as knowledge about basic causes and mechanisms improves, the nomenclature will need further review. In this context "*hypersensitivity*" describes objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons. The terminology "aspirin-exacerbated respiratory disease" (*AERD*) characterises physical reactions, underlying respiratory diseases, and inhibitors of cyclooxygenase (COX) and refers to the clinical syndrome of chronic rhinosinusitis (CRS), nasal polyposis, bronchoconstriction in asthmatics, and/or eosinophil inflammation in the upper and lower airways following the ingestion of NSAIDs blocking the COX-1 enzyme [9]. An assignment of AERD in the context of adverse drug reactions (ADR) and drug hypersensitivity is given in Figure 1.

NSAIDs are colloquially named "aspirin" or "aspirin-like drugs". Aspirin, the trade name of acetylsalicylic acid (ASA),

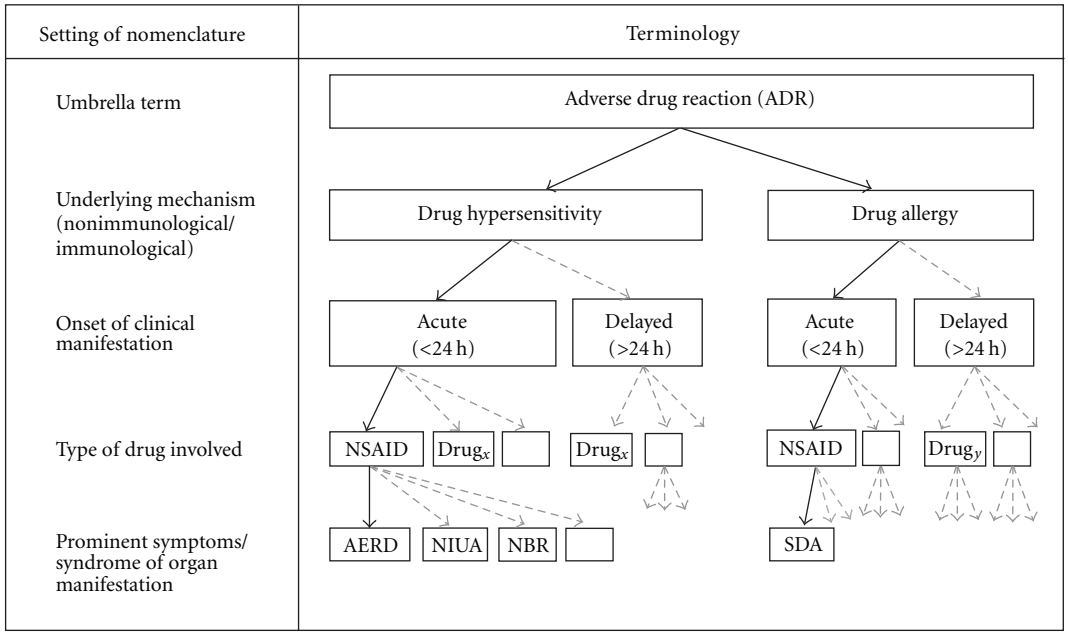


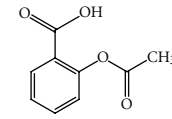
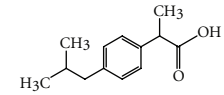
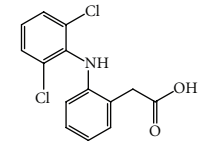
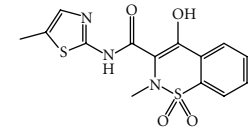
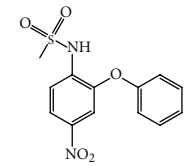
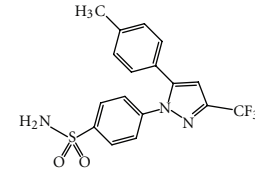
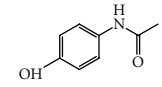
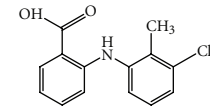
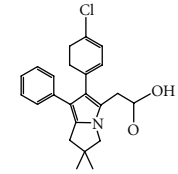
Figure 1: Allocation of terms used for adverse reactions to drugs. The diagram files the term AERD in the context of ADR, drug hypersensitivity, and drug allergy. The terms were gathered from "Report of the Nomenclature Review Committee of the World Allergy Organization" [7], and the proposed classification of allergic and pseudoallergic reactions to drugs that inhibit cyclooxygenase enzymes [9]; AERD: aspirin-exacerbated respiratory diseases, NSAID: nonsteroidal anti-inflammatory drugs, NIUA: NSAID-induced urticaria/angioedema, NBR: NSAID-blended reaction, SDA: single drug-induced anaphylaxis. Definition of ADR according to the World Health Organization [10]: any noxious, unintended, and undesired effect of a drug, which occurs at doses used in humans for prophylaxis, diagnosis, or therapy. This definition excludes therapeutic failures, intentional and accidental poisoning (i.e., overdose), and drug abuse.

patented in 1899 by Bayer AG in Germany and in 1900 in the USA, was thereafter successfully marketed all over the world and still remains one of the world's safest, least expensive, and most frequently used drug [12]. *In vivo* absorption of salicylate and acetylsalicylic acid varies greatly from one individual to another but is reasonably constant within the same individual. Bound and unbound salicylate shows no differences in aspirin-tolerant and aspirin-intolerant patients, and the rate of deacetylation in serum is the same for aspirin-intolerant patients and normal controls [3, 13]. The pharmacological hallmark of acetylsalicylic acid and other NSAIDs is the blocking of COX-enzymes causing reduction and/or loss of prostaglandin (PG) production as demonstrated in 1971 by Ferreira and colleagues [14], Smith and Willis [15], and Vane [16]. Meanwhile there are several other NSAIDs known to inhibit the three known COX-isoenzymes, depending on their selectivity (an overview is given in Table 2, for review see [17]).

The characterisation of NSAID-triggered airway diseases, AERD, was first published by Widal et al. in 1922 [2] describing the symptoms, and was annotated by the eponym "*Syndrome de Widal.*" As it was written in French it was not until Samter and Beers popularised this syndrome 35 years later and the syndrome was annotated "*Samter's triad*" [3]. Severe cutaneous and systemic adverse reactions upon ingestion of "aspirin" was first documented in 1902 by Hirschberg [1], shortly after the market launch of aspirin. Nearly 90 years ago it was proposed that aspirin activates rather than inhibits peripheral chemoreceptors causing bronchoreactivity [18], increases blood flow, vascular permeability in skin and permeability of various membranes, bronchoconstriction, secretion of mucous glands, and alters in aspirin-intolerant patients [19]. Although NSAIDs, and aspirin in particular, are beneficial for their indicated use for most patients, these drugs account for 21–25% of all adverse drug reactions [20]. NSAIDs are well-known elicitors of upper and lower airway diseases and symptoms of other organs of adults as well as of children [5, 6, 8, 21].

Symptom-based diagnosis of AERD is usually performed by medical history, which is confirmed by *in vivo* provocation tests. For this purpose, oral, nasal, bronchial, or intravenous challenges with NSAIDs blocking the COX-1 enzyme are performed followed by measuring of nasal or pulmonary function [4–9, 12, 22]. The most common causes of adverse drug reactions are acetylsalicylic acid (~80%), ibuprofen (41%), and pyrazolones (~9%), but also nonselective COX-2 inhibitors are implicated. Medication, usage, and availability are most likely to be responsible for regional differences concerning published prevalence of adverse reactions to single NSAIDs. Therefore, the prevalence of aspirin hypersensitivity in the general population ranges from 0.6 to 2.5% and is up to ~30% in asthmatics also suffering from chronic nasal polyposis. The risk of severe adverse effects caused by challenge tests, ethical reasons, and/or other contraindications (see above) make an *in vitro* diagnostic test for AERD desirable [7, 8].

Table 2: NSAIDS: classification, mechanism of action, representative structures. NSAIDs can be classified based on their chemical structure or mechanism of action; older NSAIDs were classified by chemical structure or origin, newer ones more often by their mechanism of action; COX: cyclooxygenase, 5-LO: 5-lipoxygenase.

Chemical class	Example	Inhibitory action	Representative example
Salicylates	Acetylsalicylic acid (Aspirin), diflunisal, mesalamine, salsalate	Nonselective; COX-1, COX-2,	 Acetylsalicylic acid
Propionic acid derivatives	Fenoprofen, flurbiprofen, ibuprofen, ketoprofen, naproxen, oxaprozin	Nonselective, COX-1, COX-2	 Ibuprofen
Acetic acid derivatives	Diclofenac, etodolac, indomethacin, ketorolac, nabumetone sulindac	Nonselective, COX-1, COX-2	 Diclofenac
Enolic acid (oxicam) derivatives	Droxicam, isoxicam meloxicam, piroxicam, tenoxicam	Nonselective, preferential COX-2	 Meloxicam
Sulphonanilides	Nimesulide	Nonselective, preferential COX-2	 Nimesuleid
Selective COX-2 inhibitors (coxibs)	Celecoxib, parecoxib, etoricoxib	COX-2	 Celecoxib
p-amino phenol derivatives	Paracetamol, phenacetin	COX	 Paracetamol
Fenamic acid derivatives (fenamates)	Acid, flufenamic acid, meclofenamic, mefenamic acid	COX	 Flufenamic acid
Others	Licofelone	COX, 5-LO	 Licofelone

In vitro diagnosis of AERD is discussed in literature with some controversy, most likely based on insufficient and in part contradicting data of earlier and recent publications, as well as by former papers mentioning the unavailability of or inability to establish *in vitro* tests [4, 9]. Most clinicians have some acquaintance of their use. However, the underlying concepts pertaining to diagnostic tests in general, and to their use for diagnosis of a diseases in particular, are often less familiar, and perhaps less well understood. The current concepts point to the pathways of lipids (exemplified by eicosanoids) and other molecules related to them (e.g., cytokines, growth factors, cell surface markers, second messengers of cell signalling, enzymes and receptors). These will be summarised in brief and completed by some basic theoretical aspects.

Eicosanoids (notation introduced in 1980 by Corey et al. [23], a shorthand nomenclature of eicosanoids was given in 1987 by Smith and Willis [24]) are oxygenated metabolites of the (5*Z*, 8*Z*, 11*Z*, 14*Z*)-5,8,11,14-eicosatetraenoic acid, widely known as arachidonic acid (AA). Arachidonic acid is the main source of the eicosanoid cascade in humans involving more than 50 enzymes generating a multiplicity of eicosanoids [25, 26]. Concerning NSAID-triggered hypersensitivity and AERD, we selected and focused on the COX- and 5-lipoxygenase (5LO-) pathway. Both pathways are intimately linked to AERD and their implication is well documented (see subsequent literature). Beside these pathways and their metabolites, others such as those of cytokines, growth factors, or second messengers of signal transduction are also known to be implicated in AERD and related diseases. However, it is beyond the scope of this paper to cover all of them in known detail.

Via the COX-pathway prostanoids (i.e., prostaglandins (PG), thromboxane (TX)) are generated. The COX-pathway is blocked by NASIDs [14–16, 27] by acetylating the COX enzyme [28] and by causing inhibition of the conversion of arachidonic acid to PG [16]. COX-1 is constitutively expressed in most tissues and cells and is involved in cellular housekeeping functions. COX-2 is induced by inflammatory stimuli such as cytokines, growth factors, immunoglobulins, or bacterial toxins. Putative COX-3 mRNA is present in several tissues, including that from humans, but functional protein was still not found in humans. COX-3 is switched on later in inflammation and is suggested for biosynthesis of endogenous anti-inflammatory mediators. Its clinical relevance to COX-3 remains unproven. All COX isoenzymes are modified by NSAIDs with different efficacy (for review see [17, 27, 29]). The resulting metabolite PGH_2 is further metabolised by PGE-synthase forming PGE_2. The complexity of COX expression was demonstrated for human airways. There were no differences in the total number of cells stained for COX-1 and COX-2 irrespective of whether tolerant or intolerant to NSAIDs. The number and percentage of mast cells, however, that express COX-2 was significantly increased in patients intolerant to NSAIDs. Furthermore, the expression of COX-2 in epithelial and submucosal cellular was increased in asthmatics [30]. Additionally, the expression of COX-2 was downregulated in polypous tissue as well as in bronchial muscular cells from patients with AERD [31, 32]. PGE_2 acts on at least four different seven-transmembrane-domain G-protein-coupled receptor subtypes, nominated EP_1 to EP_4. Binding on the EP_2 or EP_4 causes bronchodilatative effects, whereas binding to EP_1/EP_3 causes opposite effects [24].

The lipoxygenase pathway comprises several enzymes, generating several leukotrienes (LT). Focusing on the 5LO-pathway, LTA_4 is generated from AA, which is further metabolised by the LTC_4-synthase forming LTC_4, containing three amino acid groups, which is actively exported in the extracellular space. An overexpression of the promotor of the LTC_4-synthase gene was observed in some patients with AERD [33]. The amino acids are degraded by subsequent enzymatic processes forming LTD_4 and LTE_4. These metabolites have been named in 1960 by Brocklehurst as slow-reacting substances of anaphylaxis (SRS-A) [34] and were identified in 1982 by Hammarstrom and Samuelsson introducing the term leukotrienes for their occurrence in leukocytes and the characteristic chemical structure of conserved three conjugated double bonds (see Figure 2). These LT are characterised by a short half-life compared to other lipid mediators and are collectively named peptidoleukotrienes (pLT) based on their integral part of amino acids [35, 36].

The discovery of the 5-LO pathway caused an enormous interest in this area, largely displacing the "classic" prostaglandins. pLT are potent vaso- and bronchoconstrictors and have several other biological activities, including an ability to increase vascular permeability or to produce negative ionotropic effects in cardiac contractions [37, 38]. The pLT unfold their potential by currently three known seven-transmembrane-domain G-protein-coupled receptor types, named $cysLT_1$ and $cysLT_2$. A third dual orphan receptor GPR17 binds uracil nucleotides and pLT [39, 40]. Increased expression of $cysLT_1$ and $cysLT_2$ receptors is correlated to AERD [41–43].

The chemotactic metabolite LTB_4, also generated from LTA_4 but formed by a separate enzymatic pathway, is 100-fold less potent concerning bronchoconstriction and acts on a separate LTB_4 receptor [38, 44]. Other lipid mediators are lipoxins (LX). LXA_4 is known to inhibit LTC_4 response and is decreased in patients with AERD [45, 46]. Further pathogenetic aspects in AERD are extensively reviewed by Palikhe et al. in this journal [47].

Attempting to condense the findings outlined above, a complex eicosanoid-protein interaction network has been discovered over the past decades, comprising lipid-derived mediators, second messengers, cytokines, receptors, enzymes, and activation of genes. Eicosanoids have a crucial role as mediators in inflammatory diseases like AERD. The enzymes and receptors of the eicosanoid cascade are found to be quite ubiquitous but also feature differences regarding distribution and expression in tissue and cells in normal circumstances as well as in patients with AERD. The COX-pathway can be attributed to the control of proliferative states, the 5LO-pathway to wound healing and tissue repair. Both pathways are embedded in other metabolic pathways, for example, the network of cytokines and neuropeptides, which in turn are also interconnected [48]. Gene expression and variability differs between AERD and NSAID-tolerant

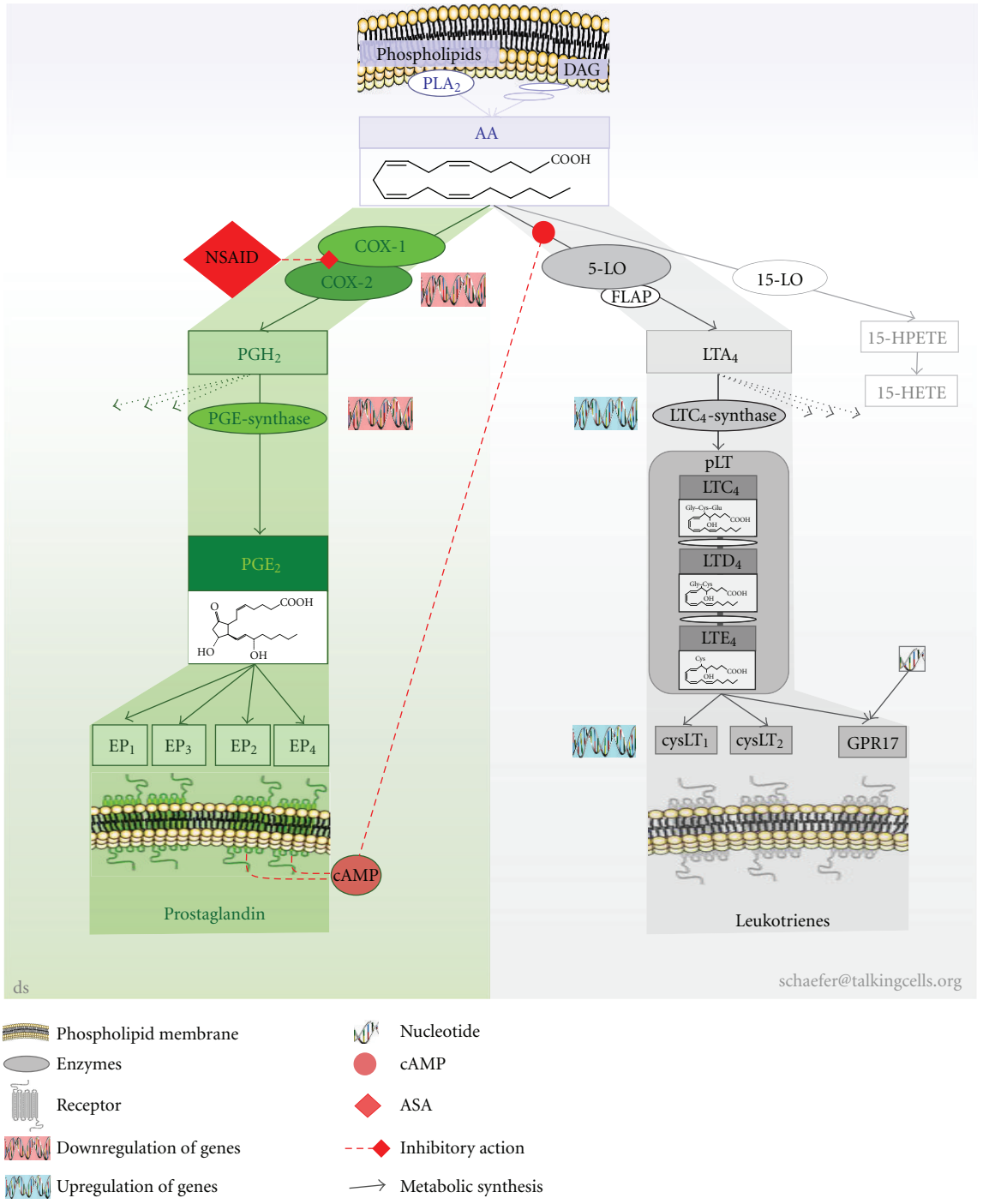


Figure 2: COX and 5-LO pathway in the metabolism of PGE_2 and pLT for *in vitro* diagnosis of NSAID-triggered hypersensitivity. Simplified pictogram of eicosanoid pathways in the metabolism PGE_2 and leukotrienes implicated for *in vitro* diagnosis of NSAID-triggered hypersensitivity. AA is enzymatically cleaved by calcium-dependent PLA_2 from phospholipids (predominantly) or from DAG (minor amounts). AA is metabolised by the COX-pathway or 5-LO pathway (but also by several other pathways not figured out here). COXs generate PGH_2, which is further processed by PGE-synthase forming PGE_2 (other PGH_2 metabolising pathways not mentioned here). PGE_2 binds to EP subtypes of which EP_2 and EP_4 generate cAMP for signalling cascade. cAMP in turn causes negative feedback on the 5-LO pathway. AA is also metabolised by the 5-LO pathway (in part assisted by FLAP) generating LTA_4. LTA_4 is further processed by calcium-dependent LTA_4-synthase forming amino acids bearing LTC_4, which is exported and extracellularly metabolised by enzymes forming LTD_4 and LTE_4, collectively named pLTs All three LTs bind to cysLTs or GPR17 with differential selectivity. 5-LO: 5-lipoxygenase, AA: arachidonic acid, ASA: acetylsalicylic acid, cAMP: cyclic-adenosine monophosphate; cysLT: receptor of pLT, DAG: diacylglycerole, COX: cyclooxygenase, EP: PGE-receptor,GPR17: orphan receptor, binding pLT and nucleotides, HPETE: hydroxyperoxy-eicosatetraenoic acid, HETE: hydroxy-eicosatetraenoic acid, NSAID: nonsteroidal anti-inflammatory drugs, PLA_2: phospholipase A_2, PG: prostaglandin, pLT: peptidoleukotrienes.

individuals with peculiarities with respect to ethnic background.

Some of these elements may directly interact with intracellular effectors to trigger multiple signalling cascades, while others act extracellularly. These components control and modulate cell migration, growth, proliferation, and activity of tissues and organs, which will result in differentiated reactions, unveiling symptoms like CRS, nasal polyposis, or asthma. A schematic overview is pictured in Figure 2.

2. Concept of Pathogenic Mechanisms

We will mention some of the known pathogenic mechanisms, elaborated in respect to AERD and to their supposed relevance to AERD, but limited to *in vitro* diagnosis of AERD and NSAID-triggered hypersensitivity.

Since the first description of adverse reactions to aspirin in airways [2], it is common knowledge that AERD is triggered by NSAIDs [3–9, 11–13, 21, 22, 30, 43, 46, 47, 49–52]. NSAIDS are known to modify the metabolism of unsaturated lipids, pinpointing eicosanoids [16]. Eicosanoids comprises a complex network of lipids essentially involved in the pathomechanisms of NSAID-triggered hypersensitivity or AERD.

NSAID hypersensitivity is characterised by an imbalance of eicosanoid synthesis (i.e., PGE_2 and pLT) prior to as well as after exposure to aspirin. This was initially documented in 1999 as a result of analysing cultured peripheral blood cells [49] as well as nasal mucosa of the same patients [50]. The concept of the imbalance of eicosanoid synthesis [49] was taken up and approved recently by a theoretical study [51] and supported by former studies [52]. The genetic as well as functional modifications may be reasonable [33, 41–43, 46–51] but details are not fully understood, as expression of COX-2 is enhanced in macrophages [48] but no differences of COX-1 or COX-2 expression in patients with AERD and NSAID-tolerant individuals is found [30].

The reduced levels of PGE_2 in AERD might be one initial factor for a diminished endogenous inhibition of the housekeeping function of PGE_2, when activating the EP_2 or EP_4 receptor. These receptor types initiate the production of cyclic adenosine monophosphate (cAMP), a second messenger, after binding of PGE_2 [48]. The synthesis of pLT is reduced by a cAMP-dependent intracellular signal transduction mechanism [11, 48, 52].

The reduced basal synthesis of housekeeping and induced PGE_2 [11, 49–52], as well as the postulated [49] and validated overexpression of LTC_4-synthase [33] and cysLT receptors [41–43] give rational arguments to explain at least in part the shift toward an elevated basal synthesis of pLT. This PGE_2-pLT shift will be further elevated upon exposure to NSAIDs, but also by other agents initiating the eicosanoid cascade (e.g., cytokines like interleukine-1, or bacterial antigens). Thus, the reduced housekeeping/induced PGE_2 most likely accounts for reduced production of cAMP, which is induced upon coupling of PGE_2 on EP_2 or EP_4 receptors, but can be induced by other signal transduction pathways [48].

Thus, the diminished availability of the housekeeping (basal) and induced PGE_2 will cause a reduced generation of suppressive acting endogenous cAMP upon exposure to COX-inhibiting agents.

In this context, PGE_2, pLT, NSAIDs, cAMP, and other factors (e.g., bacterial toxins, availability of arachidonic acid, cytokines, and others) will most likely contribute in a highly complex manner to the multifactorial exacerbation of NSAID-triggered symptoms and diseases.

3. Theoretical Consideration of *In Vitro* Diagnosis

Since the latter half of the 1980s enzyme immunoassay (EIA) tests are widely used to screen and diagnose a multitude of diseases. Results are mostly classified by a binary outcome as "positive" ("reactive") or negative ("nonreactive"), based on the protocols provided by the test manufacture and evaluation in the laboratory. The classification is the result of an ordered sequence of several steps, which had been initiated via the testing procedure.

Measurement repeatability and reproducibility are investigated during the approval process. For convenience, we will assume that the laboratory performing the test will maintain the complex process of the measurement system, and that the distribution of the results of "disease-free" and "diseased" individuals are normally distributed (see Figure 4).

In an "ideal" world these two normal distributions will not overlap. Regrettably the world of diagnostic testing is rarely unequivocally ordered. Many (currently and probably in perpetuity) unknown factors alter these distributions causing overlap to some extent. Regardless of where the test outcome threshold is situated on the measurement scale, some disease-free individuals and diseased (i.e., AERD) will be incorrectly classified as "negative" (known as "false-negative," dark shaded area left-hand side of Figure 4) or "positive" (known as "false-positive," grey area right-hand side of Figure 4), respectively. This represents one type of diagnostic test error.

Because any diagnostic test procedure has a single outcome threshold, moving the threshold to the right will reduce the false-positive results of disease-free individuals, but automatically will increase the false-negative error rate of the diseased individuals. Similarly, adjusting the threshold to the left will reduce the false-negative error rate, but automatically increases the false-positive error rate (i.e., classifying disease-free individuals as patients with AERD).

Only changing the distribution of test results in one or both groups would simultaneously reduce the rates of both types of diagnostic test errors (i.e., false-positive and false-negative results). Unfortunately, in realty this will not be practicable, due to the complex pathomechanisms underlying AERD, and the composition of the groups investigated like age, sex, medication, mentioned symptoms, interindividual variability of symptoms and syndromes, and our limited knowledge and understanding of the "plus-minus" clearly defined disease [53].

The terms *sensitivity (SE), specificity (SE),* and posttest probabilities in this concern refer to probability of an (*in vitro*) diagnostic test outcome, not to the equality of reagent or chemicals. Tests with a high sensitivity will correctly

identify virtually all patients with NSAID-triggered hypersensitivity with a high probability; tests with high specificity identify all disease-free individuals correctly with a high probability. This becomes obvious when referring to Figure 4: sensitivity and specificity correspond to the area under the probability curve (i.e., the distribution) of patients with NSAID-triggered hypersensitivity (sensitivity of the test, on the right) and disease-free individuals (specificity of the test, on the left). Unfortunately, inadequacies in the pathological and clinical symptoms or comorbid components and symptom stage, including age and sex distribution of disease-free individuals as well as patients with disease were described ~30 years ago [54] and continued to hamper any diagnostic test [53].

What physicians are really interested in knowing is the extent to which a positive or negative test result accurately predicts the true status of the patient, that is, disease-free or patient with, for example, AERD. This is commonly referred to as the posttest probability of a disease (e.g., AERD), or predictive value of a positive test result (PPV). In case of a negative test result the posttest probability of being disease-free, that is, the predictive value of a negative test result (NPV) is of interest. These values depend on not only the sensitivity and specificity, but also on the pretest probability (or prevalence) of the disease (e.g., AERD). The mathematical algorithm connecting the three probabilities sensitivity, specificity, and prevalence is known as Bayes' theorem (originally published 1763 by R. Price [55] after the death of the English clergyman Thomas Bayes). It might be easier to grasp the sense of this relationship more directly than looking on the mathematical algorithm: the prevalence of AERD is arguable in respect to the supposed prevalence of 1.2 to 2.8% of a population [5, 8, 20]. However, as outlined before, there is some uncertainty concerning the *prevalence* (i.e., the pretest probability) of AERD due to the impossibility of diagnosing this syndrome by an absolute unfailing method. This marks a further limitation for "precisely" defining the outcome of an *in vitro* test by mathematical characteristics.

The probability term *likelihood ratio*, introduced in 1968 by Lustedt and popularised in the 1980s by Sacket et al. is a ratio of the two probabilities sensitivity and 1-specificity, describing the relative probability of a positive diagnostic test result in diseased individuals compared to disease-free individuals which can be calculated [56, 57]. For *ruling-in a disease* the likelihood ratio should be at least 1, preferably much higher (graphically this represents the area on the right site of the test threshold of Figure 4). In case of *ruling out a disease*, the likelihood ratio of a negative test result is chosen. These values should ideally be much smaller than one.

As easily deduced from the above-mentioned aspects, the definition of an optimal threshold is not only a question of statistics but rather depends on how the test result will be used. For screening purpose the threshold will be relatively low, resulting in higher false-positive outcomes. This requires additional diagnostic testing to ensure a therapeutic regime. In case of AERD a low threshold line will capture all patients, even those without obvious symptoms. The low threshold also covers the risk that a patient with a potential NSAID-triggered hypersensitivity but without obvious symptoms would undergo life-threatening reactions upon exposure to NSAIDs, would not be detected. Thus, the low threshold uncovers those patients with currently mild NSAID-triggered hypersensitivity for appropriate treatment before the disease worsens in the future. The latter is visualised, in part, by Figure 5, sketching schematically the course of NSAID-triggered hypersensitivity: The symptoms and underlying disease(s) do not relate in a uniform fashion, rather a pattern of exacerbation and remission is more like an exponentially growing sinus line. This pattern will be superimposed on the residual changes of the underlying disease and is a further challenge of *in vivo* and *in vitro* diagnosis of AERD.

4. *In Vitro* Diagnosis of AERD

The change in knowledge and concepts concerning the pathogenic mechanisms of AERD reflects the diversity of *in vitro* diagnostic approaches developed during the last century.

4.1. Serum-Specific IgE against NSAIDs (SIgNT). The SIgNT examines serum or plasma collected from patients suffering from AERD and other manifestations of NSAID-triggered symptoms. The samples are filled into tubes coated with NSAIDs, including derivatives, or with NSAIDs/derivatives coupled to a carrier. After an incubation and washing step an anti-IgE or anti-IgG antibody labelled with a tracer (e.g., fluorochrome or chromogen finally converted by an enzyme) is added. Resulting values of the measurement will identify diseased patients if the value exceeds a predefined threshold (cutoff).

Underlying this approach was the observation, that adverse reactions to NASIDs displayed symptoms such as allergic reactions (the term "allergy" was introduced in1906 by von Pique as immunoglobulin mediated type of reaction [58]). Therefore, an immunologic reaction was assumed. Numerous attempts at detecting an antibody directed against Aspirin, derivatives thereof (e.g., anti-aspiryl antibodies), or to any other supposed NSAIDs failed to demonstrate an unequivocal antibody [3]. Even though antibodies were detected in 1940 by Butler et al. [59] and Zhu and colleagues [60], or propyphenazone-specific antibodies by the group of Ferreira [59], or were suspected by the group of Settipane [61]. These results have not been confirmed in the following decades [62, 63]. Also serum level of IgE in aspirin-intolerant patients did not differ from non-atopic population [61].

Nevertheless, these investigations contributed some substantial insights to our current understanding of AERD and to other NSAID-triggered symptoms as nonimmunologically mediated diseases. Thus, a SIgNT for the detection of antibodies directed to any NSAID could not be established and is not available for *in vitro* diagnosis of AERD.

4.2. Histamine Release Test (HRT). The HRT examines urine samples from patients exposed to NSAIDs or supernatants of cell culture medium of peripheral blood cells (PBLs) incubated *in vitro* with varying concentrations of different NSAIDs.

The first approach (analysing urinary samples) would not be classified as an *in vitro* test as it affords an *in vivo* provocation/exposure of the patient. There are some essential drawbacks, arguing why this procedure (*in vivo* challenge) might not be suitable in some cases (because of, for example, ethical reasons, age, compliance, technical; see Section 1). Using PBLs for measurement of histamine release has to be designated as an *in vitro* diagnostic test.

The known bronchoconstrictive effect of histamine stimulated the attempt to look for an altered histamine release in patients with AERD [64]. Early investigations demonstrated elevated urinary excretion of a histamine metabolite [65] and elevated plasma histamine levels [66]. These measurements were, however, not confirmed in nasal lavage upon provocation [67, 68]. Preincubation of leucocytes with Aspirin failed to alter spontaneous or calcium ionophore-induced histamine release in patients with AERD [69, 70]. This was confirmed for bronchial lavage [71] and for leucocytes by our study performed *in vivo* as well as *in vitro* exposure [49, 50]. There are also some inconsistent results in former studies. Okuda and colleagues reported elevated histamine release induced by platelet-activating factor from leukocytes of patients with AERD [72], Hosemann and colleagues measured lower histamine content in polypous tissue of patients with AERD than in analgesic-tolerant patients [73], and the group of Stevensson reported elevated plasma histamine levels in only three of seventeen patients [74]. The low efficiency of histamine release by *in vitro* stimulation according to the CAST-protocol (see CAST) was also affirmed by a more recent study [75].

Even though the HRT was promising, as it depicts a pathomechanistic element of AERD, and it might be suitable to confirm AERD/NSAID sensitivity in specifically selected patients (e.g., with an underlying allergic comorbidity), it is not suggested for routine *in vitro* diagnosis of patients with AERD taking into consideration all data currently available.

4.3. Lymphocyte Transformation Test (LTT). The LTT (synonyms are lymphocyte proliferation test or lymphocyte stimulation test) examines the activity of lymphocytes, notably of T-lymphocytes selected from PBLs upon exposure to varying NSAIDS at different concentrations. Most widely used for quantifying the proliferation is the measurement of ^{3}H-thymidin uptake by dividing cells from samples of anticoagulated blood.

The relevance of the LTT as model system for analysing patients with hypersensitivity to Aspirin was discussed more than 40 decades ago [76–80]. Some NSAIDs do inhibit others from enhancing the proliferation, but this was not seen consistently [81–85].

A later study demonstrated an enhanced proliferation of normal lymphocytes, but a diminished ^{3}H-thymidin uptake by lymphocytes from patients with AERD [86]. NSAIDs are considered suitable for LTT investigation [87]. But the inconsistency of results, and the more indirect relation of detecting lymphocyte proliferation to our current pathomechanistic understanding of AERD, often implicated unclear results. These findings questioned the clinical relevance of the LTT for the detection of adverse reaction to NSAIDs. Therefore, the LTT is actually not referred to be a suitable tool for *in vitro* diagnosis for patients with AERD.

4.4. Platelet Aggregation Testing (PAT). The PAT examines survival and aggregation of platelets separated from venous PBLs. The platelets are exposed to varying concentrations of those NSAIDs which are of interest, for a defined time as validated by the performing laboratory.

Around 25 years ago it was suggested that platelets might have a pivotal role in AERD [88–93]. In a subsequent study, a group led by Picado, detected no differences in any indices of platelet function studied between aspirin-tolerant and patients with AERD despite a slightly elevated aspirin-triggered $PGF_{2\alpha}$ release [94]. These results are somehow unexpected, as platelets are known to be potent producer of eicosanoids. Despite this approach and the implication of the platelet behaviour in NSAID-triggered symptoms, the PAT has not been approved for *in vitro* diagnosis of AERD.

4.5. Serum-$PGF_{2\alpha}$ Test (SPT). The SPT examines serum selected from peripheral blood. Upon addition of a predefined concentration of ASA *in vitro*, samples are analysed using a radio-immunosorbent assay. Samples exceeding a predefined serum level of $PGF_{2\alpha}$ indicate patients with AERD.

This approach was introduced in 1991 by Willilams and colleagues and demonstrated no changes in PGE_2 or PGD_2 but lower plasma level of $PGF_{2\alpha}$ in patients with AERD before addition of aspirin, and elevated levels of $PGF_{2\alpha}$ after addition of aspirin, when compared to aspirin-tolerant asthmatics [95]. Small concentrations of aspirin given to platelet suspensions generated $PGF_{2\alpha}$ [96]. This confirmed the hypothesis of an NSIAD-triggered alteration of prostanoid metabolism and altered serum protein binding capacities in patients with AERD. Regrettably, there are no further publications documenting the routine use of this promising approach.

4.6. Mediators in Nasal Lavage (MNLT). The MNLT examines nasal lavage collected from patients exposed *in vivo* to lysine aspirin. The nasal lavage is stored appropriately. After thawing and centrifugation the supernatant is analysed using specific enzyme immunoassays for two cytokines, MCP-3 and RANTES [97].

It was proposed that patients with AERD are characterised more likely by a chronic rather than an acute overproduction of MCP-3 and RANTES. The MNLT increased our pathomechanistic understanding of AERD, but an *in vivo* provocation step is presupposed. Hence, this approach does not meet the criteria of an *in vitro* test. Even though, the MNLT would be suitable to confirm AERD.

4.7. Exhaled Breath Condensate Eicosanoid Testing (EBCET). The EBCET examines exhaled breath condensate of unexposed patients with AERD. The condensate is stored until analysis using an enzyme immunoassays specific for 8-isoprostanes, LTB_4, and PGE_2. Eicosanoid values exceeding a predefined threshold characterise patients with a positive test outcome.

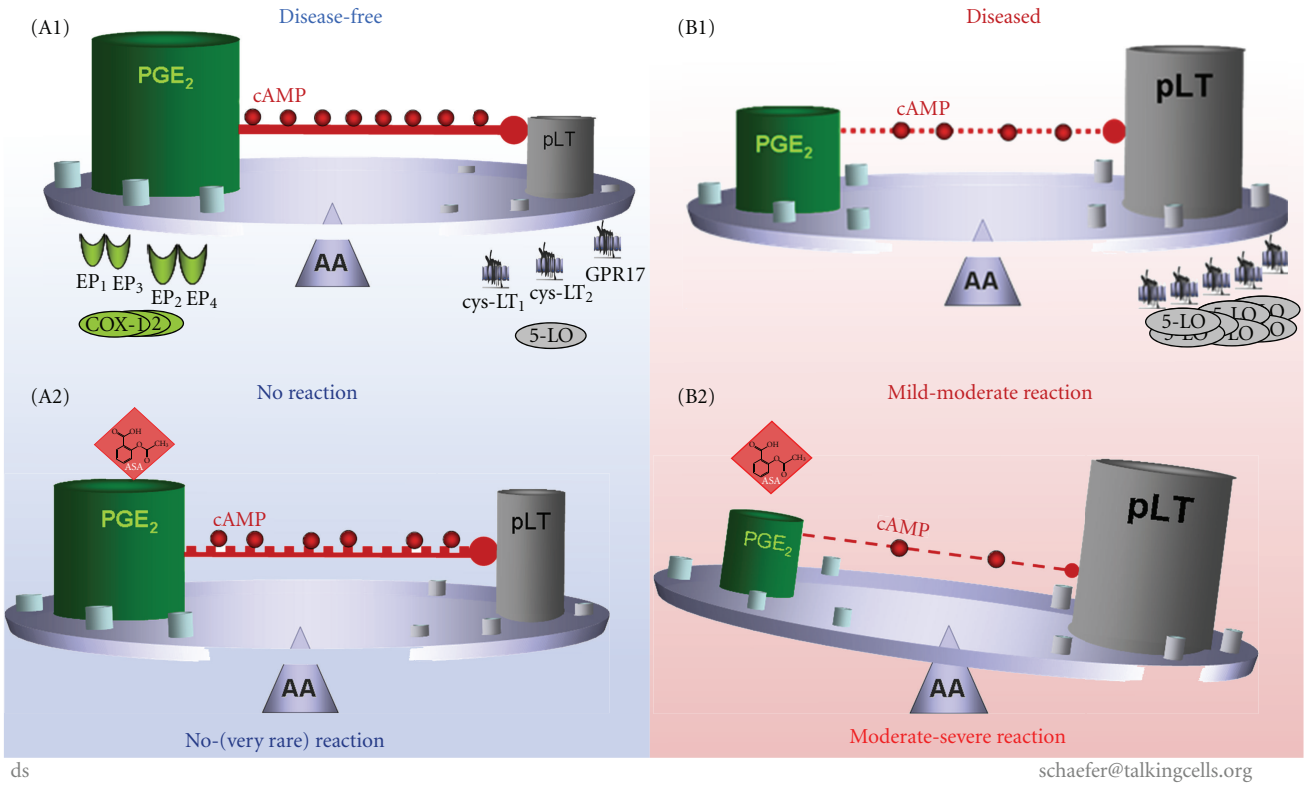


Figure 3: Causal concept of NSAID-triggered eicosanoid imbalance for *in vitro* diagnosis of AERD. The causal concept of NSAID-trigger eicosanoid imbalance for *in vitro* diagnosis of AERD is best allegorised as a tray balancing all parameters (which might be relevant for the pathway) on a needle. *Disease-free individuals*: housekeeping PGE_2 balances synthesis of pLT (e.g., by induction of endogenous cAMP, which inhibits synthesis of pLT); expression of enzymes or receptors are unremarkable (A1). Upon exposure to NSAIDs the PGE_2 level is diminished but remains high enough ensuring "uncritical" levels of pLT (even though cAMP might by diminished); expression of enzymes and/or receptors are not modified (A2). *Patients with AERD*: synthesis of housekeeping PGE_2 is diminished, but still balances synthesis of pLT (e.g., by reduced endogenous cAMP); expression of enzymes (up regulation of LTC_4-synthase) or receptors (up regulation of cysLT) can be mutated in some cases (B1). Exposure to NSAIDs/aspirin blocks the COX-pathway causing reduced synthesis of PGE_2 (and consequently further reduced cAMP level), and consequently the metabolism of arachidonic acid is shifted to the 5LO-pathway provoking elevated synthesis of pLT; expression of enzymes and/or receptors may by altered, but not modified by NSAIDs (B2).

This study was presented in 2002 by the group of Barnes, demonstrating elevated 8-isoprostanes and pLT (in ~50% of aspirin-intolerant asthmatics), no reduced PGE_2 and unchanged LTB_4 in exhaled breath condensate of patients with AERD exposed to NSAIDs [98]. These outcomes are in line with other studies also highlighting the implication of leukotrienes and prostanoids regarding diseases of the upper and lower airways [33, 49, 52, 99–103]. The EBCET was confirmed by the group of Szczeklik [104]. A very recent study extended the analysis of eicosanoids in exhaled breath condensate using gas chromatography/mass spectrometry and high-performance liquid chromatography/mass spectrometry. Before lysine aspirin challenge the amount of 5- and 15-HETE was higher in aspirin-intolerant asthmatics than in aspirin-tolerant asthmatics [105].

The approach of the EBCET depicts some of our recent knowledge on pathomechanisms concerning patients with AERD. This approach affords special equipment, mostly located in specialised centres. The EBCET, however, might become of some diagnostic value and would confirm AERD.

4.8. Cellular Allergen Stimulation Test (CAST). The CAST examines cytokine-primed enriched basophilic granulocytes separated by density-gradient sedimentation from EDTA-anticoagulated venous PBLs. Cells are incubated for 15 up to 40 minutes with varying concentrations of variable NSAIDs in combination with complement factor 5a, or anti-IgE as positive control or vehicle. Reaction is stopped by freezing; supernatants are analysed by an enzyme immunoassay specified for cysteinyl-leukotrienes. Values from NSAID-stimulated samples have to exceed a predefined threshold (cutoff) of cysteinyl-leukotrienes (=pLT) released from a control sample to reveal a positive test outcome.

The CAST, introduced in 1993 by de Weck detects a biomarker with high relevance in AERD [106]. Different protocols were published. A sensitivity of 41 to 82% and a specificity of 82 to 100% were published. These variances are a consequence of method, as well as other details (e.g., sample preparation, selection of NSAID, duration of exposure, inclusion/exclusion criteria, age, sex, and number of patients/controls) [8, 73, 107–110]. Costimulation with complement

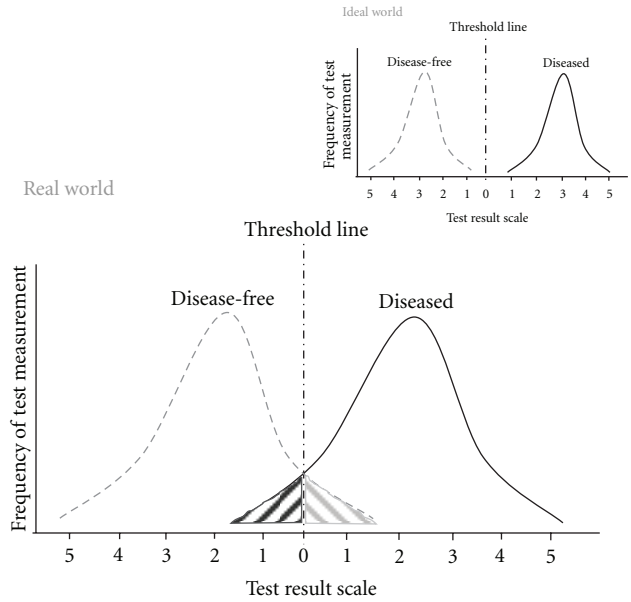


Figure 4: Framework for diagnostic test outcomes. Schema of "real" world diagnostic test outcomes; test measurement: clinical parameters like age, sex, ethnic group, height, weight, and so forth or analytical parameters like temperature, IgE, histamine, inter-leukins, lipid mediators; shaded areas exemplify the false-positive (false-negative) measurement of disease-free (diseased) individuals, respectively; insert: pictured "ideal" world.

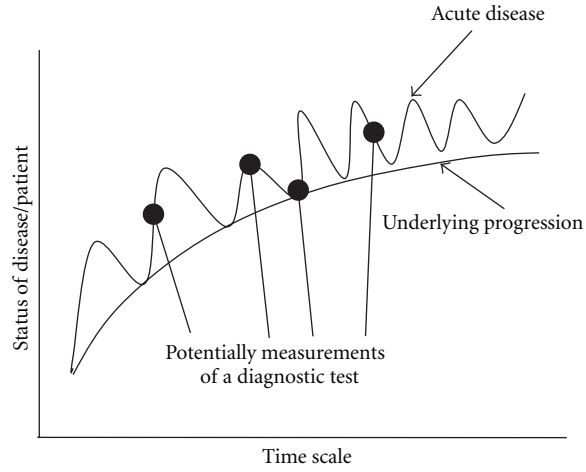


Figure 5: Hypothetical progress of AERD over time.

factor 5a was claimed by the group of Weber to improve sensitivity [107]; they investigated patients with various underlying diseases. Low efficiency was reported with no diagnostic utility and superiority to the HRT [75]. Nevertheless, the CAST was successfully established for diagnosis of allergies [109].

According to a more recent study, the CAST uncovers a pathway which was different from the classical IgE-mediated pathway. CAST uses doses of ASA for *in vitro* stimulation causing nonspecific basophile activation, and thereby eliminates the usefulness of a cell based diagnostic test for AERD. Therefore, it was suggested that the CAST would have low value in diagnosing AERD and other diseases [108, 110].

4.9. Basophile Activation Test (BAT). The BAT, also named FAST (Flow-cytometric Allergen Stimulation Test), examines basophilic granulocytes separated from EDTA-anticoagulated venous PBLs. Cells are incubated with varying concentrations of different NSAIDs for up to 40 minutes. Thereafter, basophilic granulocytes are double-marked with antibodies directed to IgE and CD63 (or CD203). The number of positively stained basophiles is measured using a fluorescence

activated flow cytometer combined with appropriate software. A positive test outcome is defined by a laboratory-defined threshold (cutoff) of positively stained basophiles.

The BAT was introduced in 2000 by the group of de Weck [111]. CD63 is a cell surface glycoprotein that mediates signal transduction events that play a role in the regulation of cell development, (platelet) activation, growth and motility. CD203 represents a transmembrane ecto-nucleotide pyrophosphatase/phospho-diesterase-I enzyme (E-NPP), which cleaves phosphodiesters and phosphosulfate bonds. Both proteins are expressed on activated basophils. During the last decade follow-up studies were initiated to improve and ensure the technical procedures, thereby using the term BAT [112–115].

The BAT depicts an altered appearance of granulocytes, which are known to be implicated in AERD. Variable values of sensitivity (~10–64%) and specificity (~75–100%) were published depending on the protocols used (e.g., sample preparation, selection of NSAID, duration of exposure, inclusion/exclusion criteria, age, sex, and number of patients/controls). The clinical use of the BAT is controversially discussed [112–115], pointing to inherent factors influencing the opportunities and limitations of an *in vitro* diagnostic test.

4.10. Flow Cytometric Assay and CAST (Flow-CAST). The Flow-CAST uses two techniques, the CAST (enzyme immunoassay) and BAT (flow cytometric assays). The outcomes of both tests are combined.

As reviewed in 2005 by the group of de Weck, the sensitivity and specificity varied depending on the NSAID tested [116]. The global sensitivity was annotated ~67%, the specificity 93%. Combination of BAT with CAST elevated sensitivity (to ~73%) but reduced specificity (to 71%). The Flow-CAST was proved for diagnosis of beta-lactam allergy [117]. It was proposed that in case of a negative result, a NSAID hypersensitivity cannot be excluded and a provocation challenge remains necessary if clinically indicated.

This approach demonstrates the usefulness of combining diagnostic procedures as mentioned in the introduction part, but demonstrates also the drawbacks as explained. From a practical point of view, performing both tests makes great demands on laboratory equipment as well as manpower, and therefore impacts on cost-effectiveness. The advantages of this procedure compared to others remain to be established.

4.11. Aspirin-Sensitive Patients Identification Test (ASPI Test). The ASPITest examines PBLs exposed *in vitro* to varying concentrations of NSAIDs. The release of 15-hydroxyeicosatetraenoic acid (15-HETE) is analysed using an enzyme immunoassay specific for 15-HETE. Values exceeding a predefined amount threshold line (cutoff, ~6% exceeding basal release) identify patients with AERD [118].

The report by Kowalski and colleagues in 2005 concluded that the aspirin-triggered release of 15-HETE from PBLs does, to some extent, mimic the reactions observed *in vivo*. 15-HETE was detected in epithelial cells of nasal polypous tissue as well as in PBLs from patients with AERD, but not in asthmatics without NSAID hypersensitivity [31, 119, 120]. Already in 1991 the group of Picado demonstrated the *in vivo* evidence of elevated release of 15-HETE in nasal secretions of allergic patients [121]. It was demonstrated, that a PGE_1 analogue (misoprostol) inhibited the aspirin-triggered 15-HETE release. A recent study investigating eight ASA-intolerant patients confirmed the elevated level of 15-HETE [120]. Variable values of sensitivity (~63–83%) and specificity (~50–82%) were published.

The ASPITest depicts a pathomechanistic link to AERD and obviously confirms the clinical finding in patients with AERD. Hitherto, there are only few promising publications and future studies will have to prove to which extent the ASPITest will be applicable for routine use for *in vitro* diagnosis of AERD and related diseases.

4.12. Functional Eicosanoid Testing and Typing (FET). The FET examines PBLs of heparinised venous blood. PBLs are diluted in an appropriate buffer before exposure to ASA, neuropeptides, and arachidonic acid. The reaction is stopped by freezing. Upon thawing and centrifugation the samples are analysed using specific enzyme immunoassays for PGE_2 and pLT. Measured data are calculated using appropriate software. The resulting individualised dynamic eicosanoid pattern is classified in values ranging from 0.0 to 3.0. This outcome is then more roughly classified as normal (0.0 to 0.5), mild (<0.5), moderate (<1.5), and severe (<2.5 to 3.0); these values also represent a probability of severity of the symptoms.

This approach was introduced in 1999 by Schäfer and colleagues and thereafter improved by integrating the growing knowledge of pathomechanistic concepts [11, 49–52, 122]. The FET depicts two biomarkers which are intimately involved in AERD and NSAID-triggered symptoms/diseases. First studies demonstrated the confirmation of clinically diagnosed AERD prior to, during provocation, and after successful treatment [123, 124]. Subsequent studies demonstrated the differentiation of non-airway-related but NSAID-triggered diseases [11, 125–127]. Others applied the FET for monitoring medical treatment in patients with AERD [128, 129] or characterisation of pathophysiological aspects [130]. Values for sensitivity and specificity varied depending on the underlying disease (airways: 96 and 89%, skin: 96 and 97%, gastrointestinal tract: 64–98 and 82–89%, resp.) [8].

The FET provides context-dependent cell-based confirmative as well as prospective information. This approach confirms AERD, but also differentiates and/or characterises underlying diseases of closely related symptoms; in addition, depending on the intended diagnostic challenge (as exemplified in Figures 6(a) and 6(b)). The FET differentiates obviously different symptoms of NSAID-triggered hypersensitivity of varying underlying disease. Future studies will have to demonstrate whether the FET, in addition to confirming or differentiating AERD, might provide some prognostic value in NSAID-triggered diseases.

5. Conclusions

During the last decades our knowledge concerning the pathogenic mechanisms, the terminology of NSAID-triggered

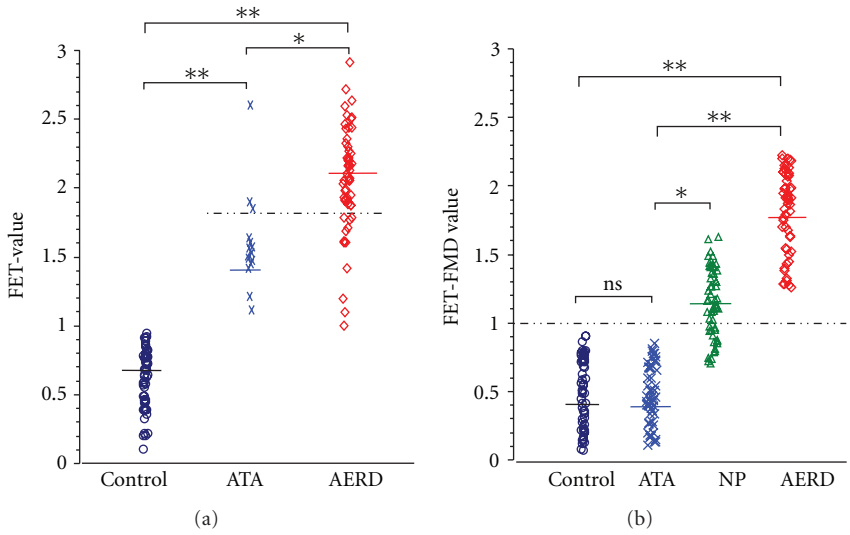


Figure 6: (a): FET and NSAID-triggered eicosanoid imbalance of individuals suffering from diseases with lower airway symptoms. The FET was performed and the FET values were calculated according to the total eicosanoid pattern score of [11] using PBLs. Patients suffering from NSAID-triggered bronchoconstrictive symptoms were confirmed and characterised by clinical and *in vitro* diagnosis. Allergy was ruled out by medical history, skin test, and *in vitro* test for total and specific immunoglobulin. The mean FET value (solid line) of controls, ATA, and AERD was 0.7, 1.4, and 2.1, respectively. FET values > 1.0 characterise patients with lower airway symptoms. FET values ≥ 1, 8 (dashed line, potential threshold) differentiate NSAID-tolerant asthmatics and patients with AERD; ATA: patients suffering from aspirin-tolerant asthma, AERD: patients suffering from aspirin exacerbated respiratory disease; ($n = 53$ for each group, $^*P < 0.05$, $^{**}P < 0.01$). (b): FET and functional metabolic differentiation of patients with and without NSAID-triggered eicosanoid of lower and upper airway symptoms. The functional metabolic differentiation (FMD) of subgroups of patient was achieved by *in vitro* provocation of PBLs and calculation of the FET value according to the total eicosanoid pattern score of [11], but by amending the FET value by subtracting the difference of the sum of the enzymatic capacity (EC) of PG- and LT-synthesis as well as the difference of the ASA- and neuropeptide-induced eicosanoid balances (EB) from the primary FET value (EC and EB were calculated according to [11]). The FET-FMD value takes into account two metabolites of the eicosanoid pathway and their *in vitro* modification by ASA and neuropeptide. The latter had been shown to be intimately implicated in hyperresponsiveness of airway ([11] and ref. therein). The FET-FMD value reveals the differentiation of ATA, NP, and AERD, but without discrimination of ATA and healthy controls. The mean value of FET-FMD (solid line) was 0.4, 0.4, 1.1, and 1.7, for controls, ATA, NP, and AERD, respectively. The threshold of FET-FMD was ≥1.0 (dashed line) for NSAID-triggered lower and upper symptoms of the airways. In conclusion, this approach confirmed and characterised NSAID-triggered symptoms by clinical and *in vitro* diagnosis. ATA: patients suffering from bronchial asthma, but tolerant to NSAIDs, NP: patients suffering from nasal polyposis, AERD: patients suffering from aspirin exacerbated respiratory disease with asthmatic symptoms; $n = 53$ for each group, ns: not significant, $^*P < 0.05$, $^{**}P < 0.01$. Allergy was ruled out by medical history, skin test and *in vitro* test of total an specific immunoglobulin.

symptoms and NSAID-exacerbated diseases (e.g., AERD) and the technical possibilities have continuously improved. This facilitated the development of new approaches for *in vitro* diagnosis, starting from no *in vitro* tests available 110 years ago to twelve *in vitro* tests developed during the last decades. Some characteristics and suggestions for intended use of the *in vitro* tests discussed are summarised in Table 3.

Our understanding of AERD and NSAID hypersensitivity moved form an immunoglobulin-triggered pathomechanism, diagnosed in the serum, to a multiplexed highly interconnected (eicosanoid) imbalance based on pathogenic understanding, diagnosing parameters from cell cultures, for example, genes, enzymes, mediators (lipids, cytokines, pH, and others), receptors, and others. A multitude of parameters were suggested. Surface marker of basophiles and lipid mediators remained to be the most promising biomarkers. Dynamic multiparametric approaches were favoured as compared to static single parametric approaches. A schematically simplified pictogram of the COX- and 5-LO pathway referred to for *in vitro* diagnosis is given in Figure 2.

The complexity of interacting parameters accounts for the initial situation where NSAIDs (see Table 2) start to act. If there is an imbalance of several metabolic and/or genetic parameters, the block of the COX pathway by NSAIDs will cause an exacerbation of one or more of prestage(s) of symptoms of a disease. Diagnosing the balance and imbalance of the eicosanoid cascade might be fundamental for diagnosing and treating NSAID-triggered diseases (see Figures 1 and 3). These approaches might be hampered by high individual variability of underlying diseases, genetics, enzymatic/cellular function/activity, and by inclusion and exclusion criteria during sample collection for *in vitro* diagnosis. The (*in vitro*) test outcome has to be carefully interpreted by an appropriately trained physician and researcher concerning terminology, inclusion, and exclusion criteria, test theory,

Table 3: Selected characteristics and suggestion for use of tests described *in vitro* diagnosis. ASPI Test: aspirin-sensitive patients identification test, BAT: basophile activation test, CAST: cellular antigen stimulation test, EBCET: exhaled breast condensate eicosanoid testing, Flow-CAST: flowcytometric assay and CAST, HRT: histamine release test, FET functional eicosanoid testing and typing, LTT: lymphocyte transformation test, MNLT: mediators of nasal lavage test, PAT: platelet aggregation test, SIgNT: serum-specific immunoglobulin E against NSAIDs test, SPT: serum-$PGF_{2\alpha}$ test; LT: leukotrienes, PG: prostaglandin, CD: cluster of differentiation, HETE: hydroy-eicosatetraenoic acid; SE: sensitivity, SP: specificity; PPV: positive predictive value, NPV: negative predictive value; n.v.d.: no values described — not suggested, (—) suggested, actually not in use, ? suggested upon further validation, (+) suggested with restrictions, + suggested.

In vitro test	Test parameter	Test sample	SE(%)	SP (%)	PPV (%)	NPV (%)	Suggestion for *in vitro* diagnosis
SIgNT	IgE, IgG	serum	n.v.d.	n.v.d.	n.v.d.	n.v.d.	—
HRT	histamine	culture medium, PBLs	n.v.d.	n.v.d.	n.v.d.	n.v.d.	—
LTT	proliferation	lymphocytes	n.v.d.	n.v.d.	n.v.d.	n.v.d.	—
PAT	aggregation	platelets	n.v.d.	n.v.d.	n.v.d.	n.v.d.	(—)
SPT	$PGF_{2\alpha}$	serum	n.v.d.	n.v.d.	n.v.d.	n.v.d.	?
MNLT	MCP-3, RANTES	nasal lavage	n.v.d.	n.v.d.	n.v.d.	n.v.d.	—
EBCET	8-isoprostane	exhaled breast condensate	n.v.d.	n.v.d.	n.v.d.	n.v.d.	?
CAST	cysLT	culture medium, basophiles	41–82	82–100	~96	~78	(—)
BAT	CD63, CD203	culture medium, basophiles	60–70	<90	~95	~56	(—)
Flow-CAST	cysLT, CD63	basophiles	~10–67	~75–100	n.v.d.	n.v.d.	(+)
ASPI Test	15-HETE	culture medium, PBLs	63–83	>50–82	79	86	?
FET	PGE_2, pLT	culture medium, PBLs	96 (64–98)	83 (82–89)	90 (70–96)	93 (69–98)	+

and last but not least, the most recent hypothesis and models of pathogenic mechanisms.

All *in vitro* tests, currently available, consider our current pathogenic and clinical understanding of AERD. But the intended use by the clinician or researcher will also account for the selection of the most appropriate *in vitro* diagnostic procedure (e.g., screening purpose, confirmation of a clinical diagnosis, individual risk assessment, proof of, prognostic probability, and/or differentiation of symptomatic appearance, monitoring of treatment, effect of single drugs, and many more). Considering the limitations of clinical diagnosis of AERD (see above), the "provocation" test is yet designated as "gold standard" in clinical diagnosis, but is usually restricted to confirm acute physical reactions of hyper reactive lower airways and requires the necessity for patients' provocation. But this "gold standard" will fail if AERD is still not thoroughly distinctive, a prognostic goal has to be considered, or provocation is precluded.

The relevance of the diagnostic test outcome and its interpretation will improve if the users of an *in vitro* diagnostic procedure consider all information provided. In this concern, functional cellular *in vitro* approaches mimic some of the complex *in vivo* processes seen in patients with AERD. The imbalance of eicosanoids might be a rational decision-making model for *in vitro* diagnosis of AERD as well as NSAID-triggered hypersensitivity. Future research will demonstrate whether and which functional *in vitro* approach will prove to be the "gold standard" of *in vitro* diagnosis of AERD to support treatment of patients with AERD and related diseases.

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Research Article

Exhaled Eicosanoids following Bronchial Aspirin Challenge in Asthma Patients with and without Aspirin Hypersensitivity: The Pilot Study

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Background. Special regulatory role of eicosanoids has been postulated in aspirin-induced asthma. *Objective*. To investigate effects of aspirin on exhaled breath condensate (EBC) levels of eicosanoids in patients with asthma. *Methods*. We determined EBC eicosanoid concentrations using gas chromatography/mass spectrometry (GC-MS) and high-performance liquid chromatography/mass spectrometry (HPLC-MS^2) or both. Determinations were performed at baseline and following bronchial aspirin challenge, in two well-defined phenotypes of asthma: aspirin-sensitive and aspirin-tolerant patients. *Results*. Aspirin precipitated bronchial reactions in all aspirin-sensitive, but in none of aspirin-tolerant patients (ATAs). At baseline, eicosanoids profile did not differ between both asthma groups except for lipoxygenation products: 5- and 15-hydroxyeicosatetraenoic acid (5-, 15-HETE) which were higher in aspirin-induced asthma (AIA) than inaspirin-tolerant subjects. Following aspirin challenge the total levels of cysteinyl-leukotrienes (cys-LTs) remained unchanged in both groups. The dose of aspirin had an effect on magnitude of the response of the exhaled cys-LTs and prostanoids levels only in AIA subjects. *Conclusion*. The high baseline eicosanoid profiling of lipoxygenation products 5- and 15-HETE in EBC makes it possible to detect alterations in aspirin-sensitive asthma. Cysteinyl-leukotrienes, and eoxins levels in EBC after bronchial aspirin administration in stable asthma patients cannot be used as a reliable diagnostic index for aspirin hypersensitivity.

1. Introduction

Exhaled breath condensate (EBC) is a simple, noninvasive technique for monitoring airway inflammation. The measurement of eicosanoids in the expired breath condensate has proven to be a useful noninvasive method for the assessment and monitoring of airway inflammation in inflammatory diseases such as asthma and other pulmonary diseases [1, 2].

Eicosanoids, including prostaglandins (PGs), thromboxane A_2 (TXA_2), and leukotrienes (LTs), are lipid mediators involved in the pathogenesis of asthma. TXA_2 is rapidly converted to thromboxane B_2 (TXB_2), a chemically stable metabolite. Thus, thromboxane synthesis in biological tissues has been monitored by measuring TXB_2 [1, 2]. Eoxins C_4, D_4, and E_4 (EXC_4, EXD_4, EXE_4) are 15-lipoxygenase (15-LO) analogues of cysteinyl leukotrienes (cys-LTs). Eoxins were metabolized to eoxin E_4 and detectable in EBC [3]. Another group of lipoxygenation products is 5-, 12- and 15-hydroxyeicosatetraenoic acid (5-HETE, 12-HETE, 15-HETE). Isoeicosanoids or isoprostanes are prostaglandin-like compounds produced by nonenzymatic lipid peroxidation of arachidonic acid. The 8-isoprostane is the best biomarker of oxidative stress and lipid peroxidation [1–3].

A special regulatory role of eicosanoids was postulated in aspirin-induced asthma (AIA) [4–6]. AIA is characterized by nasal polyps, persistent asthma, and aspirin hypersensitivity [4]. These nasal/sinus and bronchial syndromes with aspirin hypersensitivity have been named aspirin-exacerbated respiratory disease (AERD). Indeed, in this distinct asthma phenotype [7], observations have accumulated pointing to (1) overproduction of cysteinyl-leukotrienes (cys-LTs), which are potent proinflamatory mediators and bronchoconstrictors [4, 6, 8], (2) upon ingestion of aspirin, cys-LTs are further released in rising amounts probably from eosinophils

Table 1: Clinical characteristics of the patients.

	AIA ($n = 21$)	ATA ($n = 23$)	P
Age (y)	44.9 ± 13.96 39 (34 ÷ 58)	39.8 ± 8.71 39 (32 ÷ 45)	N.S. (0.33)
Female/Male	10/11	15/8	N.S. (0.36)
Duration of asthma (y)	12.36 ± 8.07 13 (6÷18)	10.26 ± 11.33 7 (1 ÷ 17)	N.S. (0.19)
Inhaled steroids yes/no	18/3	22/1	N.S. (0.34)
Inhaled steroids (μg/d) flutikason	616.57 ± 425.83 500 (500 ÷ 1000)	726.09 ± 667.06 500 (250 ÷ 1000)	N.S. (0.93)
FEV_1 baseline (% predicted) placebo/aspirin day	89.87 ± 10.83 90.01 (83.45 ÷ 95.29)	91.45 ± 11.47 91.3 (84.81 ÷ 102.1)	N.S. (0.57)
Total IgE (IU/mL)	115.06 ± 111.41 76.3 (42.8 ÷ 158)	162.86 ± 177.82 66.8 (24.1 ÷ 179)	N.S. (0.78)
Skin prick test (n) positive/negative	10/9	15/8	N.S. (0.53)
Blood eosinophil count	472.67 ± 295.39* 424 (324 ÷ 513)	248.22 ± 173.32* 232 (119 ÷ 309)	0.003

Values are expressed as mean ± SD, and median (25% and 75% percentiles).
AIA: aspirin-induced asthma.
ATA: aspirin-tolerant asthma.

and mast cells and this is accompanied by worsening of asthmatics symptoms [9, 10], (3) depressed prostaglandin E_2 (PGE_2) production by peripheral blood cells [11], nasal polyps [12, 13], bronchial fibroblasts [14], diminished EP_2 receptor on the inflammatory cells, and association with EP_2 gene polymorphism [15, 16], (4) aspirin-precipitated asthmatic attacks are not associated with changes in the systemic prostaglandin E_2 production, it might stem from the release of PGE_2 from inflammatory cells during the clinical reactions to aspirin [17], (5) both prostaglandin D_2 (PGD_2) and its metabolite increase after aspirin-induced bronchoconstriction suggesting that this reaction is associated with mast cell activation [18–20].

Aspirin-induced changes in the levels of eicosanoids, such as prostaglandins (PGs) and cys-LTs, have been examined in various biologic samples, such as plasma [19, 20], saliva [19], induced sputum [19, 21], nasal washing fluid [22, 23], bronchoalveolar lavage [24], and urine [9, 10, 17, 25]. So far, there are few published articles on eicosanoids in EBC in aspirin-induced-asthma patients [26–28]. These studies concentrated on baseline levels of eicosanoids in EBC independent of any steroid therapy used. Interestingly, one of these studies reported prostaglandins (PGE_2, $PGF_2\alpha$, $9\alpha 11\beta PGF_2$) and cys-LTs levels in breath condensates in asthmatic patients after oral aspirin challenge [28]. In the present study, we focus on exhaled breath condensate concentrations of eicosanoids following bronchial aspirin challenge, local administration of aspirin in aspirin-induced asthma patients.

The aim of this study was to evaluate the changes in wide eicosanoid spectrum concentrations in EBC during asthmatic response following aspirin inhalation. We hypothesized that the profile of eicosanoids in EBC after local aspirin administrated is markedly different in aspirin-induced asthma patients as compared to asthmatics who tolerate aspirin well. These results were validated by specific analytical techniques, such as gas chromatography/mass spectrometry (GC-MS) or by high-performance liquid chromatography/mass spectrometry (HPLC-MS).

2. Material and Methods

2.1. Subjects. The study population consisted of 21 asthmatic patients sensitive to aspirin (AIA) and 23 asthmatics who tolerated aspirin well (ATA). The diagnosis of asthma was established according to GINA 2008 update. The patients' characteristics are presented in Table 1.

The diagnosis of aspirin intolerance was confirmed by oral aspirin provocation tests, performed during 36 months preceding the study. All ATA patients occasionally used aspirin without any adverse reactions. The patients had stable asthma and their baseline FEV_1 was >70% of the predicted value on the study day. None had experienced an exacerbation or a respiratory tract infection in the 6 weeks preceding the study. Nearly 70% of ATA patients had intermittent asthma, 13% mild persistent asthma, and 17% moderate persistent. In AIA group, 67% of patients had intermittent asthma, 19% mild persistent asthma, and 14% moderate persistent asthma. According to Asthma Control Test 33% of AIA patients and 26% of ATA patients had controlled asthma, and 33% and 48% had partly controlled asthma, 33% and 26% had uncontrolled asthma, respectively. In ATA group, 2 patients were current smokers and 3 exsmokers. There were 7 exsmokers and no current smokers in AIA group. The average level of FEV_1 and FEV_1/FVC in AIA patients was 89.9% and 73.2%, in ATA patients was 91.5% and 78.5%, respectively.

The subjects were instructed to withhold medications that decrease bronchial responsiveness prior to aspirin challenge. Short-acting β_2-agonists were not used 8 hours before the challenge. Long-acting β_2-agonists and theophylline were

Table 2: Eicosanoids values at baseline and following aspirin challenge in AIA and ATA patients. Results of eicosanoids were recalculated as parts per million (ppm) of palmitic acid (PA).

	AIA ($n = 21$)		ATA ($n = 23$)		P (ANOVA)
	Baseline	Challenge	Baseline	Challenge	After the challenge
PGD_2	5.22 ± 5.93	4.85 ± 3.92	4.71 ± 3.38	4.36 ± 2.83	
(parts/million of PA)	3.18	3.69	4.27	3.85	0.54
GC/MS	(1.35 ÷ 5.85)	(1.56 ÷ 6.99)	(2.02 ÷ 6.37)	(1.78 ÷ 6.31)	
PGD_2	4.65 ± 3.14	3.76 ± 2.91	3.85 ± 2.06	3.72 ± 1.58	
(parts/million of PA)	3.56	3.00	2.99	3.33	Unable
HPLC/MS/MS	(1.93 ÷ 7.02)	(1.81 ÷ 5.66)	(2.11 ÷ 5.38)	(2.47 ÷ 4.83)	
$9\alpha 11\beta PGF_2$	2.20 ± 2.01	2.21 ± 2.02	0.73 ± 0.39	0.79 ± 0.44	
(parts/million of PA)	1.24	1.30	0.61	0.75	Unable
GC/MS	(0.27 ÷ 4.34)	(0.28 ÷ 3.90)	(0.45 ÷ 1.05)	(0.45 ÷ 1.02)	
PGF2α	1.67 ± 1.59	1.41 ± 1.38	1.14 ± 1.26	0.99 ± 1.18	
(parts/million of PA)	0.97	0.86	0.64	0.49	0.82
GC/MS	(0.26 ÷ 2.71)	(0.42 ÷ 2.16)	(0.43 ÷ 1.16)	(0.38 ÷ 1.38)	
6-keto-PGF1α	30.23 ± 18.50	29.27 ± 16.26	27.98 ± 24.71	29.64 ± 27.20	
(parts/million of PA)	32.15	32.96	16.80	16.12	0.74
GC/MS	(11.76 ÷ 45.67)	(17.09 ÷ 37.44)	(12.20 ÷ 39.60)	(13.82 ÷ 41.81)	
6-keto-PGF1α	30.51 ± 19.45	28.92 ± 16.65	26.79 ± 23.71	28.77 ± 25.48	
(parts/million of PA)	32.08	32.51	15.60	18.15	0.11
HPLC/MS/MS	(11.13 ÷ 44.05)	(15.78 ÷ 38.12)	(11.91 ÷ 35.68)	(13.20 ÷ 28.65)	
11-dehydro TXB2	19.34 ± 10.77	20.16 ± 11.23	16.43 ± 8.14	17.72 ± 9.79	
(parts/million of PA)	21.67	21.40	15.10	15.55	0.82
GC/MS	(12.18 ÷ 28.14)	(12.25 ÷ 26.23)	(9.77 ÷ 21.96)	(11.74 ÷ 17.17)	
11-dehydro TXB2	18.86 ± 10.82	20.50 ± 11.95	16.15 ± 8.20	16.87 ± 8.81	
(parts/million of PA)	19.69	21.48	14.49	14.65	0.66
HPLC/MS/MS	(9.18 ÷ 27.50)	(11.26 ÷ 25.83)	(9.37 ÷ 22.04)	(10.97 ÷ 19.78)	
LTC_4	14.51 ± 15.87	15.57 ± 36.94*	9.76 ± 14.06	4.16 ± 3.08*	
(parts/million of PA)	9.22	6.61	5.40	3.24	0.003*
HPLC/MS/MS	(2.18 ÷ 22.31)	(2.08 ÷ 11.50)	(2.79 ÷ 10.84)	(1.65 ÷ 5.84)	
LTD_4	3.11 ± 2.80	2.68 ± 2.21	3.31 ± 2.59	2.57 ± 1.55	
(parts/million of PA)	2.18	2.25	2.68	2.16	0.67
HPLC/MS/MS	(0.83 ÷ 4.41)	(1.22 ÷ 3.53)	(1.60 ÷ 4.41)	(1.42 ÷ 3.58)	
LTE_4	6.14 ± 3.79	11.96 ± 19.51*	5.45 ± 2.83	6.47 ± 2.57*	
(parts/million of PA)	5.19	6.38	4.88	6.54	0.03*
HPLC/MS/MS	(3.23 ÷ 8.50)	(4.95 ÷ 14.57)	(3.10 ÷ 7.76)	(4.42 ÷ 7.59)	
Total cysLTs	23.40 ± 19.22	30.54 ± 56.90	18.51 ± 15.42	13.20 ± 5.41	
(parts/million of PA)	19.74	15.70	14.14	12.94	0.33
HPLC/MS/MS	(6.67 ÷ 31.22)	(6.68 ÷ 29.20)	(10.95 ÷ 21.25)	(8.17 ÷ 18.75)	
LTB_4	154.84 ± 187.68	73.88 ± 77.38*	101.37 ± 163.65	70.69 ± 73.37	
(parts/million of PA)	70.28	50.49	49.72	32.29	0.03*
HPLC/MS/MS	(24.57 ÷ 168.26)	(28.71 ÷ 86.75)	(19.05 ÷ 126.82)	(19.01 ÷ 91.91)	
5 HETE	23.95 ± 39.08*	11.21 ± 11.26	6.08 ± 3.83*	5.41 ± 4.41	
(parts/million of PA)	9.98	6.78	4.53	4.22	Unable
HPLC/MS/MS	(4.71 ÷ 17.95)	(2.92 ÷ 16.24)	(3.82 ÷ 8.32)	(2.63 ÷ 6.68)	
12 HETE	23.58 ± 25.35	14.58 ± 17.15	9.22 ± 6.48	7.17 ± 9.85*	
(parts/million of PA)	12.49	6.62	7.93	5.23	Unable
HPLC/MS/MS	(4.27 ÷ 32.00)	(4.31 ÷ 15.53)	(4.11 ÷ 12.84)	(3.87 ÷ 6.26)	

Table 2: Continued.

	AIA ($n = 21$)		ATA ($n = 23$)		P (ANOVA)
	Baseline	Challenge	Baseline	Challenge	After the challenge
15 HETE	22.65 ± 24.08*	14.84 ± 9.22	7.73 ± 5.52*	10.47 ± 17.07	
(parts/million of PA)	15.48	12.80	6.72	6.13	0.23
HPLC/MS/MS	(7.80 ÷ 24.57)	(8.35 ÷ 15.39)	(3.17 ÷ 11.75)	(4.62 ÷ 11.25)	
EXC_4	2.72 ± 2.45	2.99 ± 3.08	2.00 ± 1.67	1.99 ± 1.69	
(parts/million of PA)	2.09	2.59	1.41	1.61	0.79
HPLC/MS/MS	(1.17 ÷ 3.42)	(0.65 ÷ 4.32)	(1.09 ÷ 2.38)	(0.68 ÷ 2.38)	
EXD_4	2.98 ± 2.21	2.77 ± 2.52	3.03 ± 2.50	2.87 ± 2.73	
(parts/million of PA)	2.12	1.97	2.57	2.22	0.69
HPLC/MS/MS	(0.82 ÷ 4.57)	(1.12 ÷ 4.13)	(0.90 ÷ 4.09)	(0.97 ÷ 4.46)	
EXE_4	7.86 ± 5.11	8.48 ± 16.15	8.09 ± 9.65	5.65 ± 5.28	
(parts/million of PA)	7.06	3.82	3.79	3.65	0.95
HPLC/MS/MS	(4.00 ÷ 11.43)	(2.89 ÷ 7.03)	(2.67 ÷ 12.23)	(2.33 ÷ 8.97)	
8-iso-PGF2α	0.73 ± 0.40	0.79 ± 0.38	0.72 ± 0.26	0.82 ± 0.32	
(parts/million of PA)	0.68	0.76	0.67	0.73	0.09
GC/MS	(0.40 ÷ 1.04)	(0.54 ÷ 1.11)	(0.58 ÷ 0.83)	(0.55 ÷ 1.08)	

Median (25% and 75% percentiles).
AIA: aspirin-induced asthma. ATA: aspirin-tolerant asthma. PA: palmitic acid.
* P-values < 0.05; AIA versus ATA at baseline or after the challenge.
P-values:
* AIA versus ATA at baseline.
*baseline versus challenge in AIA.
*baseline versus challenge in ATA.

withdrawn for 24 hours. Short-acting antihistamines and cromones were stopped 5 days before the challenge. Inhaled steroids were allowed at a dose ≤2000 μg budesonide per day. None of the patients were treated with systemic corticosteroids or leukotriene modifying drugs.

Baseline and following bronchial aspirin challenge, exhaled breath condensate eicosanoids (see Table 2) levels were measured in all subjects. The patients gave informed consent and the study was approved by the University Ethics Committee.

2.2. Study Design. The single-blind, placebo-controlled bronchial challenge test with aspirin was carried out during one day in all study patients [29]. The test began with the inhalation of 7 breaths of placebo (saline). FEV_1 was measured at 10 and 20 minutes after placebo inhalation. The postsaline FEV_1 obtained at 20 minutes was used as "postsaline baseline" value.

The consecutive doses of lysine-aspirin were inhaled every 30 minutes by increasing the concentration of lysine-aspirin and by changing the number of breaths (increasing doses of 0.18, 0.36, 0.90, 2.34, 7.20, 16.2, 39.60, 115.20 mg, at 0.5 hour intervals, up to the cumulative dose of 181.98 mg). FEV_1 was measured at 10, 20, and 30 minutes after each dose. The challenge procedure with aspirin was interrupted, if a bronchospastic reaction occurred (FEV_1 dropped ≥ 20%), or if the maximum cumulative dose of aspirin was reached. The cumulative dose of aspirin causing a 20% fall in FEV_1 was calculated and recorded as PD_{20} (provocation dose of aspirin). FEV_1 and extrabronchial symptoms were recorded at baseline, before the challenge tests, and then every 30 minutes until 6 hours after the last dose of aspirin.

In patients with positive bronchial aspirin challenge (AIA), exhaled breath condensate samples were collected for wide eicosanoid spectrum (see Table 2) estimations at baseline and at the time of appearance of the bronchial symptoms (time 0). In ATA patients, whose aspirin challenge was negative, exhaled breath condensate samples were collected at baseline and 0.5 hours after the last aspirin dose, that is, when the cumulative doses of 181.98 mg was reached (time 0).

2.3. Lung Function. Pulmonary function tests were performed on a flow-integrating computerized pneumotachograph (Pneumoscreen, E. Jaeger, Germany).

2.4. Exhaled Breath Condensate (EBC). EBC was collected according to ATS/ERS [1] using ECO Screen instrument of Jaeger (GmbH Hoechberg, Germany). Following tidal breathing for 15–20 min, 1-2 mL of clear fluid was collected and immediately deeply frozen.

2.5. Biochemical Assays. Exhaled breath condensate concentration of eicosanoids was measured by gas chromatography/mass spectrometry (GC-MS) and by high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS^2) or both; see Table 2. Results of eicosanoids were recalculated as parts per million (ppm) of palmitic acid (PA)

or expressed as picograms per milliliter (pg/mL). Detection limits for eicosanoids measurements were between 0.17 pg/mL for 12-HETE and 0.89 pg/mL for PGD_2. Intraassay coefficients of variance were less than 10% and interassay coefficients of variance were less than 15%. Accuracy of measurements were better than 98.7%. Detailed analytical procedure and deuterated standards used were described elsewhere [3, 27].

2.6. Statistical Analysis. Summary statistics were expressed as mean (M), standard deviation (SD), median (Me), and 25% and 75% percentiles. General linear model (GLM) including repeated measures analysis of variance, which takes into account the fact that the outcome measurements are repeated over time within subject was used for multiple comparisons. Logarithmic transformation was used when needed as variance stabilizing transformation. Correlation between variables was estimated with the Spearman rank order correlations. A P-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Clinical Reactions. There was no statistical difference in the clinical characteristics between patients with aspirin-induced asthma (positive aspirin challenge test), and those who tolerated aspirin well (negative aspirin challenge test) except for blood eosinophil count, Table 1. None of the patients developed symptoms after administration of placebo. In aspirin-sensitive asthmatics, bronchial reactions developed after 0.18 mg in 1 subject, after 0.36 mg in 2 subjects, after 0.9 mg in 3 subjects, following 2.34 mg in 2, after 7.2 mg in 5 subjects, after 16.2 mg in 4 subjects, following 39.6 mg in 2 subjects, and after 115.2 mg in 2 subjects. The mean cumulative dose of aspirin was 32.108 mg. All the symptoms were relieved by short-acting β_2-agonists. None of ATA patients developed any clinical symptoms following aspirin challenges.

3.1.1. Cyclooxygenase Products

Exhaled Breath Condensate Concentration of Prostanoids. At baseline (Table 2), exhaled breath condensate levels of PGD_2, such as its metabolite $9\alpha 11\beta PGF_2$, and $PGF_2\alpha$, 6-keto-$PGF_1\alpha$ and nonenzymatic isomer (8-iso-PGF_2), did not differ significantly between the study groups. Statistically, results did not differ dependently on the methods used (HPLC/MS/MS or GC/MS) for marking eicosanoids and results shown (ppm of PA or pg/mL).

Following aspirin administration, no significant differences in EBC levels of PGD_2 measured by HPLC/MS/MS (results were recalculated as ppm of PA) and $9\alpha 11\beta PGF_2$ measured by GC/MS (results were expressed as ppm of PA) were found in AIA ($P = 0.13$ and $P = 0.82$, resp.) and in ATA subjects ($P = 0.69$ and $P = 0.43$, resp.). The same marked result (no differences) was observed when PGD_2 was measured by GC-MS.

In cases when 11-dehydro TXB_2 resulted as ppm of PA, at baseline (Table 2) and following aspirin challenge (ANOVA, $P = 0.66$) exhaled breath condensate level of 11-dehydro TXB_2 did not differ significantly between the study groups.

Negative correlation was founded between provocation doses of aspirin and exhaled PGD_2 and its $9\alpha 11\beta PGF_2$, $PGF_2\alpha$, 6-keto-$PGF_1\alpha$, and 11-dehydro TXB_2 levels only in AIA patients.

3.1.2. Lipoxygenation Products

Exhaled Breath Condensate Concentration of Cysteinyl-Leukotriene (Cys-LTs). At baseline (Table 2), exhaled breath condensate levels of leukotrienes C_4, D_4, and E_4 did not differ significantly between the AIA and ATA groups ($P = 0.43$, $P = 0.22$, $P = 0.79$, resp.).

In both study groups, following aspirin challenge, EBC level of LTC_4 decreased significantly (ANOVA, $P = 0.003$). No significant differences in EBC levels of LTD_4 was found in AIA and in ATA group patients (ANOVA, $P = 0.67$), where the level remained unchanged and at a constant. Exhaled LTE_4 after aspirin challenge increased significantly in both study groups (ANOVA, $P = 0.03$). However, total level of cys-LTs (the sum of LTC4, LTD4, LTE4) showed no changes in either group studied. Statistically the results of cys-LTs in EBC were identical independent of units (measurement) used (ppm of PA or pg/mL).

The dose of inhaled steroid used by study patients and FEV_1 values had no effect on magnitude of the response of the cys-LTs and its duration.

Negative correlation was founded between provocation doses of aspirin and exhaled LTC_4 ($r = -0.47$, $P = 0.04$), LTD_4 ($r = -0.46$, $P = 0.04$) and LTE_4 ($r = -0.43$, $P = 0.05$) levels only in AIA patients.

Exhaled Breath Condensate Concentration of Leukotriene B_4. At baseline (Table 2), exhaled breath condensate level of leukotriene B_4 did not differ significantly between the AIA and ATA groups ($P = 0.36$).

Following aspirin administration, EBC levels of LTB_4 decreased significantly only in aspirin-sensitive patients (ANOVA, $P = 0.03$). No differences were observed when LTB_4 was expressed as pg/mL.

Exhaled Breath Condensate Concentration of HETE. At baseline (Table 2), exhaled breath condensate levels of 5-HETE and 15-HETE were significantly higher in AIA as compared to ATA groups ($P = 0.03$, $P = 0.001$, resp.). Figure 1. Following bronchial aspirin administration, EBC levels of 5- and 15-HETE (ANOVA, $P = 0.23$) remained unchanged in AIA ($P = 0.37$ and $P = 0.23$, resp.) and ATA patients.

At baseline (Table 2), exhaled breath condensate levels of 12-HETE did not differ significantly between the AIA and ATA groups ($P = 0.13$). Following aspirin administration, EBC levels of 12-HETE decreased significantly only in ATA group ($P = 0.03$, due to outliers Wilcoxon Matched pairs test was used).

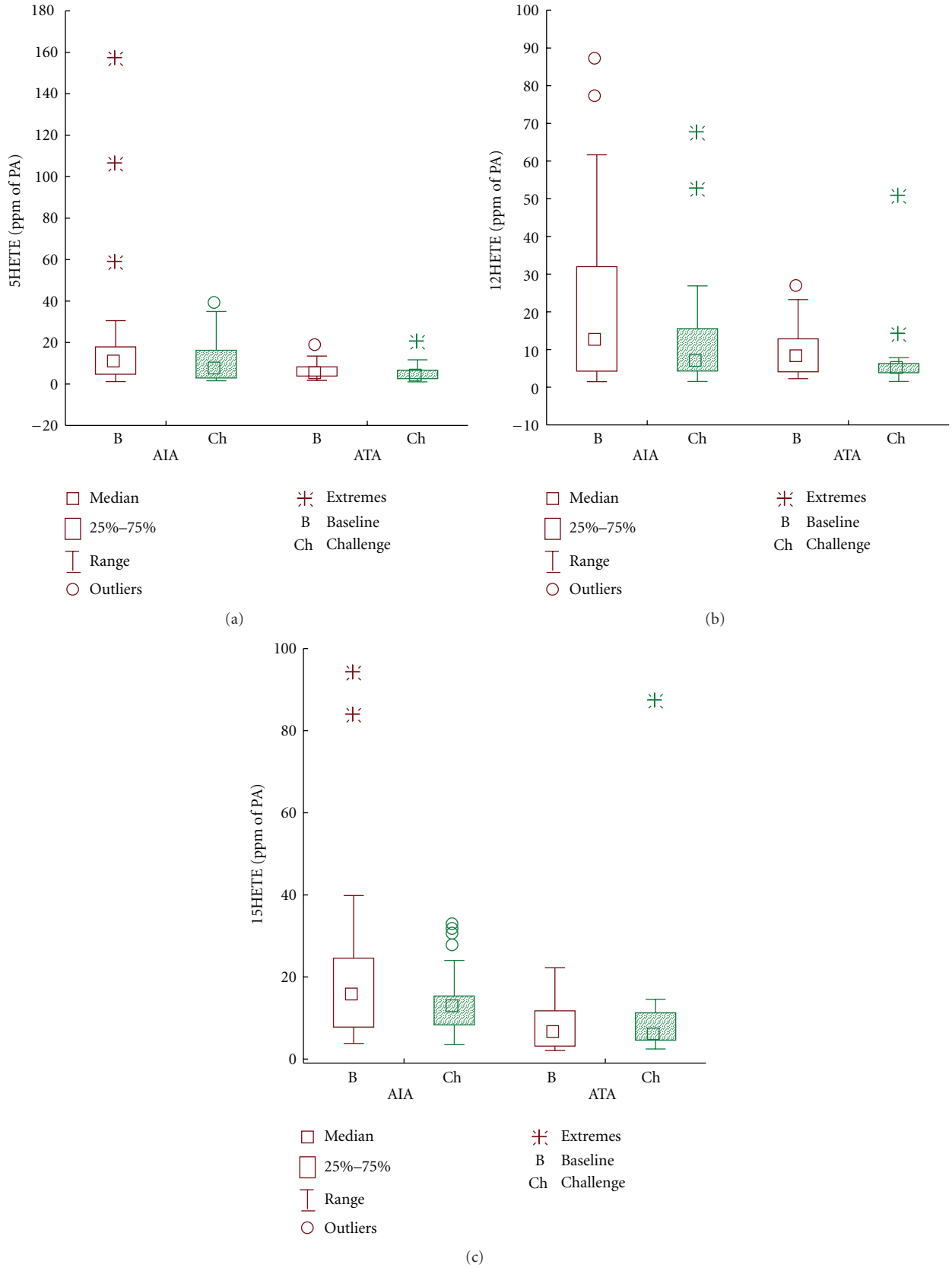

Figure 1: Eicosanoids' levels in exhaled breath condensate before and after bronchial aspirin challenge. (a) 5-HETE, (b) 15-HETE, and (c) 12-HETA. AIA: aspirin-induced asthma. ATA: aspirin-tolerant asthma. B: baseline. Ch: challenge.

Table 3: Eicosanoids values at baseline and following aspirin challenge in AIA and ATA patients. Results of eicosanoids were recalculated as picograms per milliliter (pg/mL).

	AIA ($n = 21$)		ATA ($n = 23$)		P (ANOVA)
	Baseline	After the challenge	Baseline	After the challenge	After the challenge
PGD_2	1.49 ± 1.17	1.54 ± 1.02	2.35 ± 2.06	1.80 ± 1.22	
(pg/mL)	1.01	1.21	1.64	1.53	0.35
GC/MS	(0.82 ÷ 1, 68)	(0.79 ÷ 2.11)	(1.09 ÷ 2.70)	(0.85 ÷ 3.20)	
PGD_2	1.53 ± 0.81	1.14 ± 0.58	1.76 ± 1.14	1.63 ± 0.93	
(pg/mL)	1.39	1.09	1.48	1.45	0.05
HPLC	(0.93 ÷ 2.02)	(0.67 ÷ 1.36)	(0.99 ÷ 2.19)	(0.80 ÷ 2.07)	
$9\alpha 11\beta PGF_2$	0.48 ± 0.33	0.50 ± 0.34	0.32 ± 0.18	0.31 ± 0.17	
(pg/mL)	0.36	0.34	0.29	0.28	0.59
GC/MS	(0.16 ÷ 0.69)	(0.25 ÷ 0.75)	(0.20 ÷ 0.35)	(0.21 ÷ 0.38)	
PGF2α	0.42 ± 0.34	0.40 ± 0.35	0.43 ± 0.33	0.39 ± 0.45	
(pg/mL)	0.28	0.27	0.30	0.23	0.82
GC/MS	(0.19 ÷ 0.56)	(0.15 ÷ 0.59)	(0.23 ÷ 0.44)	(0.16 ÷ 0.35)	
6-keto-PGF1α	8.94 ± 4.30	8.70 ± 3.72	9.95 ± 4.54	9.55 ± 4.19	
(pg/mL)	7.17	7.22	7.48	7.46	0.53
GC/MS	(6.75 ÷ 7.79)	(7.02 ÷ 7.62)	(7.24 ÷ 15.02)	(7.24 ÷ 11.49)	
6-keto-PGF1α	8.99 ± 4.41	8.59 ± 3.93	9.69 ± 4.37	9.46 ± 3.95	
(pg/mL)	7.26	6.96	7.40	7.65	0.60
HPLC/MS/MS	(6.32 ÷ 8.41)	(6.59 ÷ 8.54)	(7.08 ÷ 13.54)	(7.13 ÷ 10.27)	
11-dehydro TXB2	5.74 ± 0.87*	5.94 ± 0.89	6.52 ± 0.92*	6.39 ± 0.94	
(pg/mL)	5.74	6.17	6.81	6.62	0.18
GC/MS	(5.14 ÷ 6.34)	(5.16 ÷ 6.49)	(6.35 ÷ 7.00)	(5.70 ÷ 7.16)	
11-dehydro TXB2	5.51 ± 0.90*	5.96 ± 1.11	6.40 ± 1.17*	6.18 ± 1.01	
(pg/mL)	5.49	6.03	6.63	6.18	0.03*
HPLC/MS/MS	(4.77 ÷ 6.16)	(5.24 ÷ 6.55)	(6.01 ÷ 7.03)	(5.55 ÷ 7.09)	
LTC_4	4.17 ± 4.56	3.69 ± 7.01	4.35 ± 4.57	1.87 ± 1.73*	
(pg/mL)	2.30	2.02	2.46	1.06	0.01*
HPLC/MS/MS	(1.23 ÷ 6.42)	(1.13 ÷ 3.36)	(1.05 ÷ 6.18)	(0.59 ÷ 2.74)	
LTD_4	0.88 ± 0.60	0.85 ± 0.59	1.58 ± 1.28	1.18 ± 0.88	
(pg/mL)	0.69	0.65	1.34	0.95	0.16
HPLC/MS/MS	(0.43 ÷ 1.19)	(0.50 ÷ 1.03)	(0.55 ÷ 2.04)	(0.41 ÷ 1.77)	
LTE_4	2.03 ± 0.92	3.20 ± 3.63*	2.46 ± 1.43	2.78 ± 1.44*	
(pg/mL)	1.83	2.33	1.93	2.45	0.04*
HPLC/MS/MS	(1.28 ÷ 2.71)	(1.57 ÷ 3.44)	(1.30 ÷ 3.45)	(1.54 ÷ 3.69)	
Total cysLTs	7.30 ± 4.98	8.02 ± 10.75	8.39 ± 5.62	5.83 ± 3.40	
(pg/mL)	5.97	6.01	7.24	4.90	0.24
HPLC/MS/MS	(4.14 ÷ 9.66)	(3.91 ÷ 6.75)	(3.06 ÷ 12.75)	(2.87 ÷ 8.87)	
LTB_4	69.09 ± 102.42	26.15 ± 25.60*	54.23 ± 99.10	34.81 ± 37.83	
(pg/mL)	29.66	16.99	27.73	15.35	0.02*
HPLC/MS/MS	(10.26 ÷ 50.00)	(10.41 ÷ 30.38)	(6.40 ÷ 57.18)	(6.37 ÷ 53.27)	
5 HETE	7.15 ± 9.80	3.60 ± 2.69	2.75 ± 1.94	2.61 ± 2.99	
(pg/mL)	2.94	3.41	2.29	1.67	0.79
HPLC/MS/MS	(1.96 ÷ 7.41)	(1.67 ÷ 5.29)	(1.43 ÷ 3.12)	(0.78 ÷ 3.00)	
12 HETE	6.64 ± 6.16	5.85 ± 10.45	4.03 ± 2.68	3.45 ± 6.75*	
(pg/mL)	4.05	2.82	3.10	1.93	0.02*
HPLC/MS/MS	(2.91 ÷ 7.76)	(1.52 ÷ 4.22)	(1.53 ÷ 5.84)	(1.25 ÷ 2.85)	

Table 3: Continued.

	AIA ($n = 21$)		ATA ($n = 23$)		P (ANOVA)
	Baseline	After the challenge	Baseline	After the challenge	After the challenge
15 HETE	6.99 ± 4.87*	5.81 ± 5.14	3.48 ± 2.78*	5.29 ± 11.76	
(pg/mL)	5.96	4.77	2.95	2.39	0.30
HPLC/MS/MS	(4.04 ÷ 8.27)	(2.53 ÷ 6.52)	(1.75 ÷ 3.98)	(1.92 ÷ 2.97)	
EXC_4	1.05 ± 0.82	0.92 ± 0.89	0.89 ± 0.57	0.83 ± 0.73	
(pg/mL)	0.79	0.59	0.80	0.61	0.74
HPLC/MS/MS	(0.45 ÷ 1.61)	(1.18 ÷ 0.58)	(0.36 ÷ 1.40)	(0.25 ÷ 1.07)	
EXD_4	1.71 ± 2.29	1.78 ± 2.82	1.70 ± 1.76	1.29 ± 1.54	
(pg/mL)	0.73	0.62	1.06	0.74	0.909
HPLC/MS/MS	(0.30 ÷ 1.51)	(0.24 ÷ 1.93)	(0.27 ÷ 2.31)	(0.39 ÷ 1.96)	
EXE_4	5.47 ± 8.61	3.68 ± 6.01	4.01 ± 4.97	2.58 ± 2.63	
(pg/mL)	2.15	1.05	1.59	1.35	0.07
HPLC/MS/MS	(0.97 ÷ 4.60)	(0.71 ÷ 2.66)	(0.89 ÷ 5.66)	(0.72 ÷ 3.89)	
8-iso-PGF2α	0.25 ± 0.12*	0.28 ± 0.21	0.33 ± 0.15*	0.32 ± 0.10	
(pg/mL)	0.20	0.21	0.35	0.34	0.92
GC/MS	(0.17 ÷ 0.28)	(0.19 ÷ 0.26)	(0.20 ÷ 0.42)	(0.25 ÷ 0.39)	

Median (25% and 75% percentiles).
AIA: aspirin-induced asthma. ATA: aspirin-tolerant asthma.
* P-values < 0.05; AIA versus ATA at baseline or after the challenge.
P-values:
* AIA versus ATA at baseline.
*baseline versus challenge in AIA.
*baseline versus challenge in ATA.

The dose of inhaled steroid used by patients and FEV_1 values had no effect on magnitude of the response of the 5- and 15-HETE. At baseline, negative correlation was found between the doses of steroids and EBC levels of 12-HETE only in aspirin-sensitive subjects ($r = -0.45$, $P = 0.04$).

The dose of aspirin, had no effect on the magnitude of response of 5- and 15-HETE.

Exhaled Breath Condensate Concentration of Eoxins. At baseline and following aspirin challenge, exhaled breath condensate levels of eoxins C_4, D_4, and E_4 did not differ significantly between the AIA and ATA groups (see Table 2).

At baseline and following aspirin challenge no correlation was found between provocation doses of aspirin, inhaled steroid therapy and FEV_1 values, and eoxins EBC levels in aspirin-sensitive and aspirin-intolerant patients.

4. Discussion

In this study, we used a validated analytic platform [27, 30, 31] to analyze eicosanoids in EBC of asthmatic patients. A highly sensitive method of gas chromatography/mass spectrometry or high-performance liquid chromatography/mass spectrometry or both were used to measure spectrum of eicosanoids-nonvolatile compounds present in EBC [3, 27, 31]. Novel concepts for the standardization of EBC material measurements have been introduced to obtain characteristics of eicosanoid patterns produced by asthmatic lungs [27]. The assessment of palmitic acid content in EBC among many other methods [32] seems to be a convenient solution for compensating the "dilution factor" [3]. It has recently been demonstrated that dilution of nonvolatile compounds in water differs between the subjects by more than 1 order of magnitude and depends on ventilation mechanics [33, 34]. For that reason, in this paper, data are recalculated as parts per million of palmitic acid. For better understanding, EBC eicosanoid levels are given in both applicable units: ppm of PA (Table 2) and in pg/mL (Table 3) independently of performed assay.

The aim of this study was to compare a wide profile of eicosanoids released to the epithelial surface of the asthmatic lung in subjects with and without aspirin hypersensitivity at baseline and following bronchial aspirin challenge, most of them on chronic inhaled steroid therapy. We demonstrated for the first time exhaled eicosanoids following local administration of aspirin in aspirin-induced asthma patients.

Comparing subjects with AIA and ATA no significant differences were observed in EBC levels of cyclooxygenase pathway prostanoid products (PGD_2 and its metabolite 9α11$\beta$$PGF_2$, PGF2$\alpha$, 6-keto-PGF1$\alpha$, and 11-dehydro TXB_2) and nonenzymatic isomer (8-iso-PGF_2) in neither baseline nor after aspirin inhalation. Opposing data has been earlier reported as lower [28] and other times higher [27] baseline PGD_2 metabolite, namely, 9α,11$\beta$$PGF_2$, levels in EBC in AIA patients. Differences observed in this previous study between both asthma phenotypes [27] can possibly be explained by lower FEV_1 values and more severe of disease in patients with aspirin hypersensitivity compared to subjects who tolerated aspirin well. Moreover, a significant predictor of decreased FEV_1 was increased 9α,11β-PGF_2 only in AIA which did

not correlate in ATA subjects [27]. In our study, all patients presented comparable FEV_1 values, and most likely, similar bronchoconstricting eicosanoid levels such as PGD_2 and its metabolite. Mast cells are probably the main source of PGD_2 overproduction. Higher global production of PGD_2 metabolite $9\alpha,11\beta PGF_2$ was also present in the blood and urine at baseline in AIA subjects [18, 20]. Concentration of $9\alpha,11\beta$-PGF_2 was not changed by the oral-systemic [28] and how indicated our data bronchial-local administration of aspirin.

Our results revealed a significant upregulation of some arachidonate lipoxygenation products in asthmatic subjects with aspirin hypersensitivity, as manifested by high baseline levels of 5-, 15-HETE in EBC. This data is consistent with the latest observations [27]. These findings are related to an overexpression of lipoxygenases enzymes, particularly 5- and 15-LO in the asthmatic lung with aspirin hypersensitivity. These enzymes are expressed in eosinophils, activated macrophages, and also in lymphocytes and mast cells. Kowalski and colleagues have demonstrated that aspirin triggers specific generation of 15-HETE from nasal polyp epithelial cells [12] and peripheral blood leukocytes [35, 36] from aspirin-sensitive but not aspirin-tolerant subjects with asthma/rhinosinusitis. Also, they have demonstrated that two alternatively spliced variants of COX-1 mRNA present in human leucocytes may be differently expressed in patients with asthma. The relative expression of those variants has been correlated to aspirin-triggered 15-HETE generation suggesting association of this phenomenon with the pathogenesis of aspirin-induced asthma [37].

No elevation of baseline 12-HETE in EBC comparing AIA and ATA subjects was observed. Whereas, after bronchial aspirin administration, there was a statistically significant decrease in 12-HETE concentration noted only in ATA subjects. On that basis, we assume blood platelets, the main source of 12-LO, may possibly play some role in pathogenesis of aspirin hypersensitivity. However, 12-LO was originally cloned from respiratory epithelia, where 15-LO activity was also found [38].

Our study did not demonstrated baseline local overproduction of cys-LTs in the airways in AIA and is consistent with an earlier study [28]. Baseline EBC levels of LTC_4, LTD_4, and LTE_4 did not differ between aspirin tolerant and hypersensitive subjects. Contrary to this finding in subjects with AIA, a higher baseline level of cys-LTs in EBC was also reported [26, 27]. This might have been a result of low FEV_1 values and minor control of asthma (severity index) [27] or steroid-naive [26] in aspirin hypersensitivities compared to aspirin tolerant subjects. The levels of exhaled cys-LTs were lower in those AIA subjects who received inhaled steroid therapy [26]. Following bronchial aspirin challenge levels of particular cys-LTs showed some variations but the total concentration of cys-LTs remained unchanged in both study groups. Up till now, cys-LTs level in EBC has not been measured after local (inhaled) aspirin administration. However, it has been shown [28] that cys-LTs levels in EBC after oral (systemic administration) aspirin challenge increased significantly in subjects with AIA. Varying results of different studies implicate that levels of cys-LTs in EBC cannot be a convenient indicator of asthma phenotype as their level after challenge test possibly depends on manner of aspirin administration and probably the doses of steroids inhaled. Results can be surprising because, a key enzyme—LTC4 synthase, is overexpressed in bronchial mucosa of patient with AIA [39]. Furthermore, circulating steroid blood eosinophils—main source of LTE_4—carry more mRNA transcripts for this enzyme [40].

As was reported in childhood asthma [41] and adult asthmatics [27], 15-LO analogues of cys-LTs, eoxins C_4, D_4, and E_4, showed no increase at baseline in asthmatic subjects independent of aspirin hypersensitivity. Their concentrations were not changed by the bronchial aspirin challenge. Role of those eicosanoids has been recently investigated in aspirin hypersensitive patients.

The high-sensitivity eicosanoid profiling of lipoxygenation products (5HETE, 15HETE) in EBC makes it possible to detect alterations in asthma, especially in its distinct phenotype characterized by hypersensitivity to aspirin and other nonsteroidal anti-inflammatory drugs. Cysteinyl-leukotriene levels in EBC after aspirin challenge in stable asthma patients, not steroid naive, most probably cannot be used as a reliable and sensitive index for aspirin hypersensitivity. In stable AIA patients on chronic inhaled steroid therapy of global (urinary) rather than in local (breath condensate) production of postchallenge cys-LTs is of greater and more sensitive value for aspirin hypersensitivity. We believe that quantitate cell analysis and measurements of released eicosanoides in induced-sputum will be more applicable for that purpose.

List of Abbreviations

AIA:	Aspirin-intolerant asthma
ATA:	Aspirin-tolerant asthma
EBC:	Exhaled breath condensate
GC-MS:	Gas chromatography-mass spectrometry
HPLC-MS/MS:	High-performance liquid chromatography-tandem mass spectrometry
COX:	Cyclooxygenase
PG:	Prostaglandin
$9\alpha,11\beta$-PGF_2:	PGD_2 semistable metabolite
6-keto-$PGF_1\alpha$:	Prostacyclin metabolite
11-dehydro-TXB2:	Thromboxane A2 metabolite
EP2:	Prostaglandin receptor 2
LO:	Lipoxygenase
HETE:	Hydroxyeicosatetraenoic acid
LT:	Leukotriene
Cys-LTs:	Cysteinyl leukotrienes
8-iso-$PGF_2\alpha$:	Isoprostane $F_2\alpha$
NSAIDs:	Nonsteroidal anti-inflammatory drugs.

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Review Article

Interleukin-4 in the Generation of the AERD Phenotype: Implications for Molecular Mechanisms Driving Therapeutic Benefit of Aspirin Desensitization

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Aspirin-exacerbated respiratory disease (AERD) is explained in part by over-expression of 5-lipoxygenase, leukotriene C4 synthase (LTC_4S) and the cysteinyl leukotriene (CysLT) receptors (CysLT1 and 2), resulting in constitutive over-production of CysLTs and the hyperresponsiveness to CysLTs that occurs with aspirin ingestion. Increased levels of IL-4 have been found in the sinus mucosa and nasal polyps of AERD subjects. Previous studies demonstrated that IL-4 is primarily responsible for the upregulation of LTC4S by mast cells and the upregulation of CysLT1 and 2 receptors on many immune cell types. Prostaglandin E_2 (PGE_2) acts to prevent CysLT secretion by inhibiting mast cell and eosinophil activation. PGE_2 concentrations are reduced in AERD reflecting diminished expression of cyclooxygenase (COX)-2. IL-4 can inhibit basal and stimulated expression of COX-2 and microsomal PGE synthase 1 leading to decreased capacity for PGE_2 secretion. Thus, IL-4 plays an important pathogenic role in generating the phenotype of AERD. This review will examine the evidence supporting this hypothesis and describe a model of how aspirin desensitization provides therapeutic benefit for AERD patients.

1. Introduction

Aspirin-exacerbated respiratory disease (AERD) is a syndrome characterized by asthma that, when present, is often severe and can be associated with aggressive airway remodeling [1], the presence of extensive hyperplastic eosinophilic sinusitis with nasal polyp (NP) formation [2], and intolerance to aspirin and other nonselective cyclooxygenase (COX) inhibitors [3–5]. A central feature of AERD is its association with profound overproduction and overresponsiveness to cysteinyl leukotrienes (CysLT) [6, 7]. These CysLTs have important proinflammatory and profibrotic effects that contribute both to the extensive hyperplastic sinusitis and nasal polyposis that characterize AERD and to the severity of these patients' asthma [1, 8, 9]. Various cytokines have been shown to modulate CysLT expression and responsiveness. This review will focus on the role of IL-4 in the induction and maintenance of the AERD phenotype and consider implications of aspirin desensitization in altering the leukotriene synthesis and responsiveness pathways.

2. Dysregulation of Cysteinyl Leukotriene Production in AERD

The overproduction of CysLTs in part reflects the increased expression of its primary synthesis enzymes 5-lipoxygenase (5-LO) and leukotriene C_4 synthase (LTC_4S). Upregulation of these enzymes is readily observed in the lungs and nasal polyps of AERD subjects [8, 10–12]. The overexpression of these enzymes results in constitutive excess production of the CysLTs as can be demonstrated in bronchoalveolar lavage samples or through quantification of urinary LTE_4 [6, 7]. It is this upregulation of CysLT synthesis pathways

that underlies the observed life-threatening surge in CysLT secretion following ingestion of aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) in AERD [13–16].

3. IL-4 Dysregulation of LTC_4S

Several cell types including mononuclear phagocytes, basophils, mast cells, and eosinophils express LTC_4S and are thereby capable of CysLT production and secretion. Mast cells typically express modest levels of LTC_4S and its upregulation can be mediated by IL-4 (but not by IL-5 or IL-13) [17]. However, studies investigating the source of CysLTs in AERD have suggested that eosinophils might be the most important cell type driving the observed overexpression of LTC_4S [11]. In our studies, we were not able to demonstrate a cytokine mechanism for increasing LTC_4S expression in eosinophils. In part, this may reflect the short-lived nature of circulating eosinophils and their limited capacity for gene transcription and phenotypic modulation. Arguably, the "aspirin sensitive" phenotype of eosinophils in AERD reflects the impact of influences acting upon eosinophil progenitors in the bone marrow or, as increasingly recognized, long-lived transcriptionally active progenitors in the airway tissue itself [18].

4. IL-4 in AERD

Relatively little is known regarding the expression of IL-4 in sinus disease. The best study examined subjects with chronic hyperplastic eosinophilic sinusitis and nasal polyps, separating them on the basis of being allergic or nonallergic. IL-4 was prominently expressed in the tissue of the allergic subgroup when compared to either healthy controls or the nonallergic subgroup [19]. Examination of nasal secretions from subjects with chronic sinusitis found higher levels of IL-4 protein when compared with controls [20]. In another study that looked at allergic subjects with chronic sinusitis, IL-4 transcripts were found to be high in the ethmoid sinus mucosa and nasal turbinate tissue [21]. To our knowledge, however, specific expression of IL-4 in AERD has not been delineated. Our studies have demonstrated elevated levels of IL-4 expression at the mRNA and protein levels in AERD in comparison to control sinus tissue (unpublished results).

5. CysLT Receptor Dysregulation in AERD

AERD subjects also demonstrate markedly increased sensitivity to CysLTs [22], reflecting in part their upregulation of CysLT receptors [23]. The two well-characterized CysLT receptors can be distinguished by their relative potency for the CysLTs: CysLT1 receptor $LTD_4 > LTC_4 \gg LTE_4$ and CysLT2 receptor $LTD_4 = LTC_4 \gg LTE_4$. The relative insensitivity of either of these receptors to LTE_4 is in contrast to the unique sensitivity of AERD subjects to this lipid mediator and has led to the suggestion that additional CysLT receptors must exist. This is more extensively reviewed elsewhere [24] and in the absence of definitive characterization, these will not be further addressed here. CysLT type 1 receptors are prominently expressed on airway smooth muscle [25] and these receptors primarily mediate the CysLT-induced bronchospasm associated with allergen exposure [26, 27]. The role of these receptors in bronchospasm following aspirin ingestion, however, is not clear. Our studies, and those of others, have shown varied distribution of the CysLT receptors on peripheral blood leukocytes [28–31]. While both receptors are widely expressed on eosinophils and mast cells only CysLT1 receptors can be found on neutrophils. Further, very few circulating T lymphocytes normally express either class of receptor (~4–8%) [28, 29, 31]. Interestingly, while the CysLT1 receptor has been found on lung fibroblasts as well, nasal polyp-derived fibroblasts express neither the CysLT1 nor 2 receptors [32].

Table 1: IL-4 modulation of CysLT receptor mRNA expression on leukocyte.

	CysLT1 receptor	CysLT2 receptor
Monocytes	1.6 ± 0.4	$2.5 \pm 0.7^{\dagger}$
T lymphocytes	$4.3 \pm 2.1^{\dagger}$	$18.9 \pm 10.1^{\S}$
B lymphocytes	$3.5 \pm 0.6^{\dagger}$	$11.1 \pm 2.7^{\dagger}$
Eosinophils	2.4 ± 0.9	4.2 ± 1.5

$^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\S}P < 0.001$.

Quantitative polymerase chain reaction data, presented as fold change in comparison to unstimulated cells, which was set at 1. See legend for Table 2 for details.

6. IL-4 Dysregulation of CysLT1 and 2 Receptors

As with LTC_4S expression, the expression of the CysLT receptors is tightly regulated by cytokines, including, most prominently, IL-4. IL-4 upregulates cell surface expression of both CysLT1 and CysLT2 receptors on mast cells [33, 34]. Similarly, IL-4 stimulates cell surface expression of the CysLT1 receptor on monocytes and CysLT2 receptors on endothelial cells [35]. We investigated modulation by IL-4 of CysLT receptor expression on peripheral blood mononuclear cells and eosinophils [31]. The most impressive results were observed for IL-4 stimulation of the CysLT2 receptor. Significant increases in expression of CysLT2 receptor transcripts were seen on T and B lymphocytes, monocytes, and eosinophils. Additionally, IL-4 significantly upregulated CysLT1 receptor transcript and protein expression on T and B cells (Table 1 and Figure 1). This increased expression in secondary to IL-4 stimulation is mechanistically explained by the identification of a STAT6 response element in the CysLT1 receptor promoter region [36].

7. Prostaglandin (PG) E_2 and PGE_2 Receptor Dysregulation in AERD

In addition to modulation of CysLTs, the pathophysiology of AERD also involves downregulation of the prostaglandin synthesis pathway. PGE_2 displays both pro- and anti-inflammatory functions reflecting its ability to interact with 4 distinct receptors (EP1–4) each having various activating or

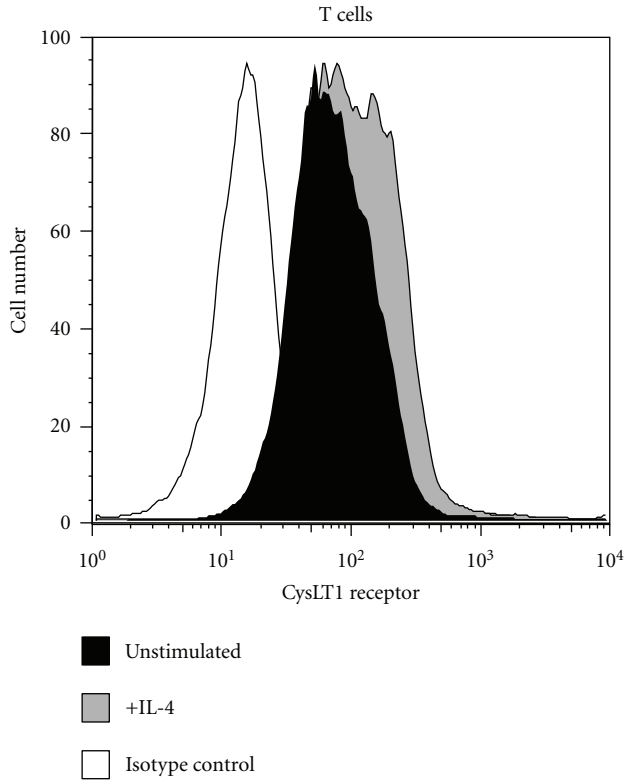

Figure 1: Cytokine modulation of cysteinyl leukotriene receptor protein expression on T cells. T cells were separated from blood using magnetic bead affinity chromatography and stimulated with 20 ng/mL IL-4 for 16 hrs before cells were collected for analysis. Cell surface expression of the CysLTR1 receptor was evaluated using rabbit polyclonal anti-CysLTR1 followed by labeling with FITC-conjugated goat anti-rabbit IgG. Isotype control is shown in white, unstimulated in black, and IL-4 stimulated in gray. Reprinted with permission of the American Thoracic Society [31].

inhibitory functions. The ability of PGE_2 acting through EP2 receptors to block eosinophil and mast cell degranulation is central to the pathogenesis of AERD and it has been shown that patients with AERD constitutively display low levels of PGE_2 [12, 37]. The further reduction of tissue PGE_2 concentrations by aspirin and other NSAIDs through COX inhibition precipitates the activation of these cells in AERD, and infusion of PGE_2 protects against these non-IgE-mediated reactions [38, 39]. The sensitivity of AERD patients to low tissue PGE_2 concentrations is amplified by the reduced expression of the anti-inflammatory EP2 receptors also observed in this condition [40].

Several studies have investigated the mechanism behind the reduced levels of PGE2 in these patient and have indicated a correlation with a decrease in the responsible upstream enzymes. The production of PGE_2 from arachidonic acid involves the sequential synthesis of PGG_2/PGH_2 by the two cyclooxygenase enzymes (COX-1 and COX-2) followed by the synthesis of PGE_2 by the microsomal PGE_2 synthases (mPGES-1, mPGES-2) and cytosolic PGE_2 synthase (cPGES). COX-2 mRNA and protein expression are diminished in NPs of subjects with AERD [12, 41, 42].

Table 2: PgE_2 metabolic pathway gene expression in control and AERD tissue.

Gene	Control ΔC_T[1]	AERD ΔC_T	$\Delta\Delta$CT ($2^{\Delta\Delta CT}$)[2]
COX-1	10.5 ± 0.4[1]	11.3 ± 2.1	-0.8 (.57)
COX-2	6.0 ± 0.6	7.9 ± 2.8*	-1.9 (.27)
mPGES-1	4.0 ± 0.7	5.0 ± 0.5	-1 (.5)
mPGES-2	2.8 ± 0.8	4.5 ± 0.4*	-1.7 (.31)
cPGES	-0.1 ± 0.6	0.0 ± 0.5	+0.1 (1.07)

* $P < 0.05$.
[1]Quantitiatve polymerase chain reaction data are presented as ΔC_T, which is the difference in threshold cycle of expression of each gene compared to housekeeping gene (each cycle corresponds to ~1 $\log_2$ difference in mRNA concentration; a higher ΔC_T represents less mRNA).
[2]$\Delta\Delta C_T$ is the difference in ΔC_T of gene expressed in control compared to AERD tissue.
$2^{\Delta\Delta CT}$ is the relative expression of gene in AERD compared to control tissue.

Our studies have confirmed this diminished expression of COX-2 (Table 2 and [43]). We found no significant change in COX-1 or cPGES transcript expression but, along with COX-2, did show diminished expression of mPGES-2 and a trend towards diminished mPGES-1 expression (Table 2). It is mPGES-1 that is most relevant to PGE_2 production in inflammatory disorders such as AERD as it is functionally coupled with COX-2 [44]. In general, mPGES-2 is thought to be primarily expressed by the heart and brain and the relevance of this dysregulation in AERD is unclear and may merely reflect the different histologies of control sinus epithelium and AERD NPs.

Diminished COX-2 expression and the reduced capacity to synthesize PGE_2 contributes to the severity of inflammation observed in AERD and accentuates the sensitivity of these individuals to the inhibition of PGE_2 synthesis associated with aspirin and other NSAIDs. This may also explain the paradoxical absence of symptoms in AERD patients that are typical in other forms of chronic sinusitis, such as pain and pressure. With this relative absence of COX-2, AERD subjects become dependent upon COX-1 for the PGE_2 that is necessary to restrain mast cell and eosinophil activation. Most AERD patients tolerate selective COX-2 inhibitors supporting this concept regarding the unique importance of COX-1-derived PGE_2.

8. IL-4 Dysregulation of PGE_2 Synthesis Pathways

We investigated the molecular mechanism underlying inhibition of PGE_2 synthesis pathways in AERD, focusing on influences of IL-4, reflecting again, its prominent expression in AERD, its previously described influences on the prostaglandin metabolic pathways [45, 46], and its involvement in the other facets of arachidonate dysregulation that have been previously discussed. For example, in contrast to IL-4 and despite being highly expressed in AERD, IL-5 did not influence PGE_2 production or responsiveness in our studies (unpublished data). Our studies were performed on nasal polyp-derived fibroblasts, mononuclear phagocytic

Table 3: IL-4 modulation of PgE_2 metabolic pathway gene expression.

	COX-1	COX-2	mPGES-1
Monocytes	1.1 ± 0.7	0.3 ± 0.2†	0.2 ± 0.1†
Fibroblasts	0.6 ± 0.7	0.3 ± 0.1†	0.5 ± 0.1
Eosinophils	0.6 ± 0.6	0.7 ± 0.7	1.0 ± 0.5

Quantitative polymerase chain reaction data, presented as fold change in comparison to unstimulated cells, which was set at 1. †$P < 0.01$.

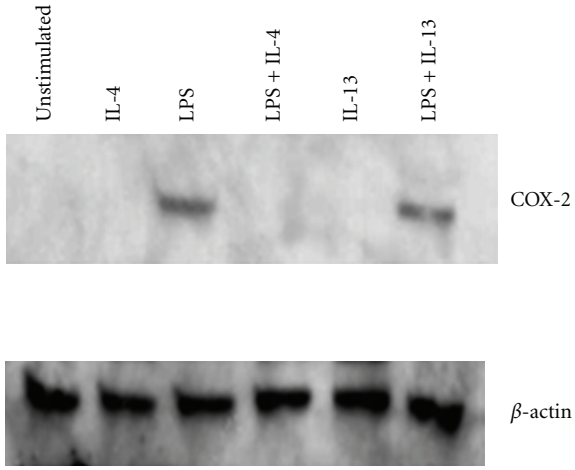

Figure 2: COX-2 protein expression in resting and LPS-stimulated monocytes. Monocytes were isolated from blood by magnetic bead purification. Cells were treated with IL-4 (10 ng/mL), IL-13 (10 ng/mL), or LPS (1 μg/mL) for 24 hrs and whole cell lysates collected. Proteins were separated on a 10% SDS acrylamide gel and transferred to nitrocellulose. The membrane was probed with anti-COX-2 and then stripped and reprobed with anti-β-actin.

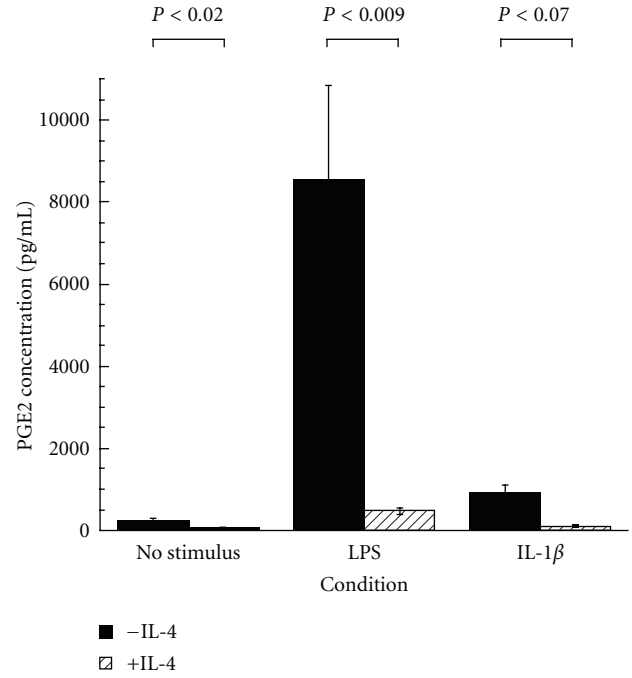

Figure 3: IL-4 inhibits monocyte PGE_2 secretion. IL-4 was added to the cells (10 ng/mL) alone or either with LPS (1 μg/mL) or IL-1β (10 ng/mL). Cells were incubated for 24 hrs before supernatants were collected. PGE_2 levels were measured by ELISA and reported as pg/mL [43].

cells, and eosinophils. Monocytes were utilized both as representative inflammatory cells, but also because PGE_2 is their dominant prostaglandin product. Significant inhibition of COX-2 and mPGES-1 (but not COX-1) mRNA expression was observed in response to IL-4 (Table 3). This appears to be a generalized effect of IL-4 insofar as similar inhibition was also observed in fibroblasts. Similar to IL-5, IL-13 also had no biological effect (not shown). No influence of IL-4 was observed in eosinophils. This may be a function of the low levels of COX-2 expressed in these cells, whose primary arachidonate product are the CysLTs, but again, may also reflect our use of terminally differentiated cells. The IL-4 effect on monocyte mRNA was further extended to expression of COX-2 protein as evaluated by Western blot. While low basal protein levels of COX-2 were observed, IL-4 (and not IL-13) inhibited LPS-stimulated COX-2 protein expression (Figure 2).

Inhibition of COX-2 and mPGES-1 synergize to result in dramatically less stimulated PGE_2 secretion by monocytes (Figure 3). Thus, in addition to upregulating CysLT pathways, IL-4 contributes to the sensitivity of AERD patients by the inhibition of PGE_2 production by aspirin/NSAIDs and, in particular, to nonselective (COX-1 and COX-2) inhibitors. However, it is necessary to remark that more than just loss of the tempering influences of PGE_2 underlies these reactions, otherwise all asthmatics and, indeed, even healthy subjects would react to aspirin/NSAID ingestion with activation of their mast cells and eosinophils. Clearly, additional currently uncharacterized biochemical mechanisms, in addition to the relatively low levels of EP2 previously mentioned, must be uniquely driving the tendency of these compounds to trigger the mast cell, eosinophil, and perhaps other inflammatory cells in AERD.

9. Aspirin Desensitization for AERD—Implications for IL-4

Aspirin desensitization is an effective treatment for AERD and has been associated with diminished need for nasal endoscopic surgery, improved sense of smell, fewer bouts of acute sinusitis, reduced need for oral corticosteroids, and less severe asthma [3, 47, 48]. The molecular mechanism of the beneficial effects of aspirin has not been determined, but we believe this could be related to the ability of this compound to inhibit the biological activities of IL-4. Consistent with this concept are the observations that successful aspirin desensitization is associated with reversal of many of the IL-4-modulated features of AERD discussed above, including the ability of desensitization to downregulate both CysLT1 receptor expression [22, 23] and leukotriene synthesis [49] and reverse the inhibition of the PGE_2 synthesis pathway; presumably by blocking the IL-4

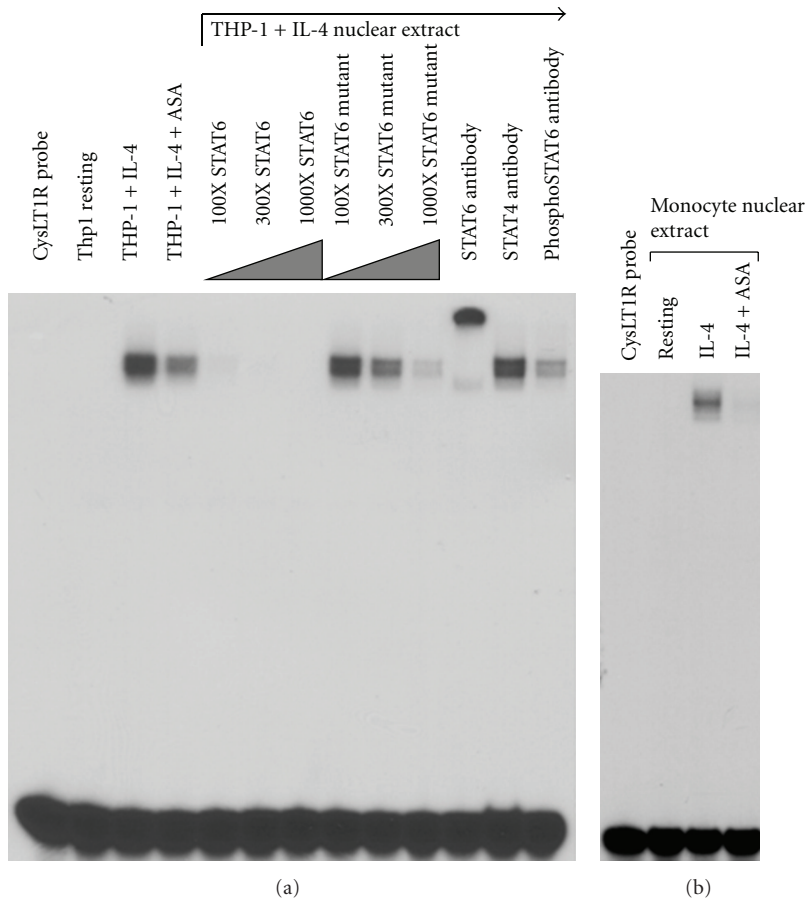

Figure 4: EMSA for STAT6. (a) EMSAs were performed using ^{32}P-labeled oligomers comprising the STAT6 site within the CysLT1R promoter. Nuclear extracts were purified from THP-1 mononuclear cell lines in the resting state, IL-4 stimulated (10 ng/mL), and IL-4 stimulated in the additional presence of aspirin (10 mM). STAT6 binding was evaluated by performing EMSAs in the presence of 100–300-fold molar excess unlabeled STAT6 consensus sequence (comprising the ε heavy chain promoter) or a mutated STAT6 consensus sequence. EMSAs were also performed using STAT6, phosphoSTAT6, and, as a control, STAT4 antibodies. (b) Relevance to normal tissue was evaluated using nuclear extracts prepared as above, derived from enriched peripheral blood-derived mononuclear phagocytes [43].

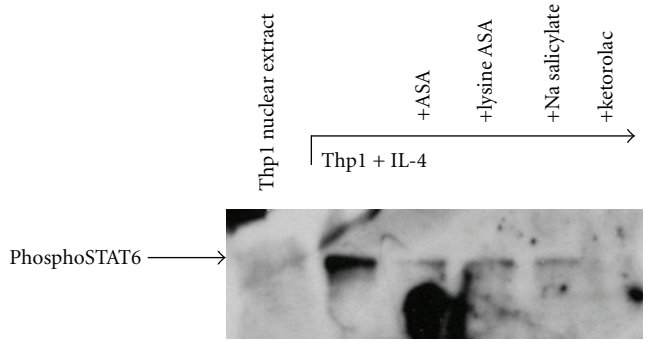

Figure 5: Western hybridization of nuclear extracts. Nuclear extracts obtained as described for Figure 4 were electrophoresed on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Presence of phosphoSTAT6 was determined via probing with anti-phosphoSTAT6 antibodies and a secondary peroxidase-labeled antibody [43].

induced-inhibition of mPGES-1 synthesis (Tanya Laidlaw, personal communication). The mechanism by which aspirin might block these effects is not immediately obvious. Aspirin (and other NSAIDs) are known to have off-target effects (effects not related to cyclooxygenase inhibition) including modulation of nuclear trafficking of numerous transcription factors such as NFAT, NF-κB, and STAT6 [50–52]. Many of these off-target effects of aspirin only occur at significantly

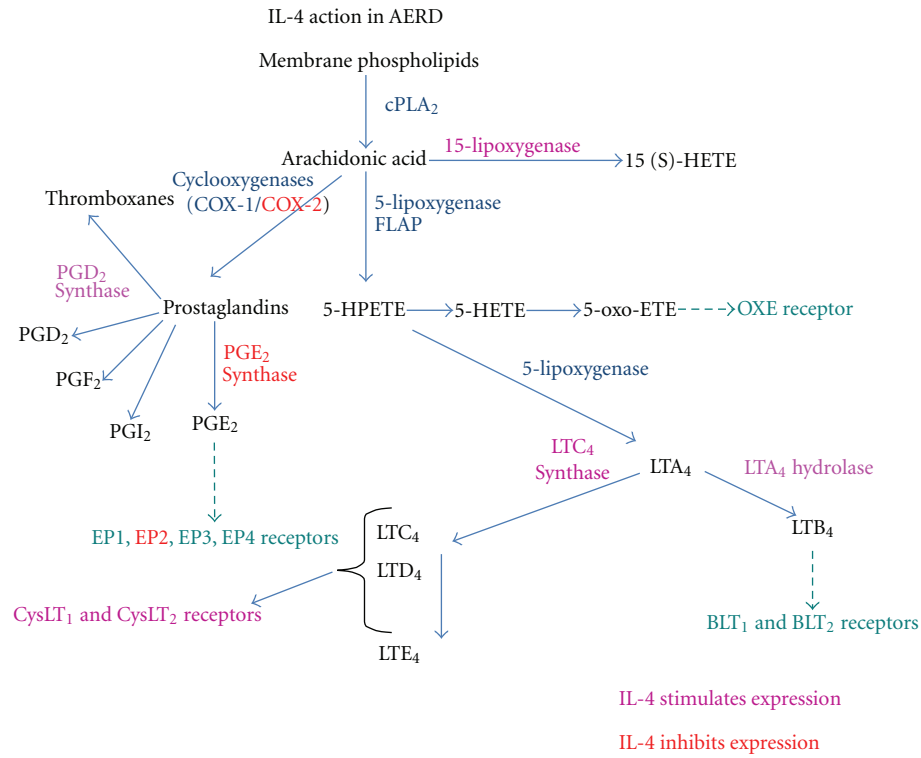

Figure 6: Summary of IL-4 activity on the leukotriene and prostaglandin synthesis pathways. Activation of gene synthesis by IL-4 is shown in pink while inhibition of gene synthesis by IL-4 is shown in red.

higher concentrations than those required for COX-1 or COX-2 inhibition [51]. The concentration of aspirin known to be effective after aspirin desensitization (up to 1300 mg/d) is somewhat higher than that required to inhibit COX-1 or COX-2, consistent with the concept that aspirin could be acting through one of these COX-independent pathways. Of relevance to our work, aspirin is known to directly inhibit T cell IL-4 expression [53]. Recently, continuous ingestion of aspirin for 6 months following desensitization has been shown to reduce sputum IL-4 levels [54]. Alternatively, we focused on recognition that engagement of the IL-4 receptor by IL-4 induces the activation of STAT6 via the Janus kinases. This STAT6 activation is critical for many of the biological activities of IL-4. As mentioned, a STAT6 site in the CysLT1 receptor promoter has been identified and was shown to be involved in IL-4-mediated transcription regulation [36]. Similarly, a putative STAT6 site has been identified in the LTC_4S gene (Bing Lam, unpublished). Previous studies demonstrate that aspirin inhibits the activation of STAT6 [55]. These observations suggested to us that aspirin may produce its clinical utility in AERD through direct inhibition of the IL-4-activated STAT6 pathway. Aspirin hypersensitivity in AERD reflects inhibition of cyclooxygenase and as such also occurs with other COX inhibitors [56]. Although *desensitization* can be induced to other COX inhibitors, it is not established whether the therapeutic benefit that follows desensitization reflects COX inhibition or some of these other off-target anti-inflammatory effects of NSAIDs and salicylates.

10. Aspirin Modulation of STAT6 Nuclear Trafficking

Our studies investigated the inhibition by aspirin and other NSAIDs of the STAT6-mediated regulation of the CysLT1 receptor and LTC_4S genes [43]. In a dose-dependent fashion, aspirin inhibited transcription of IL-4-induced CysLT1 receptor expression (not shown). Subsequently, via electrophoretic mobility shift assays (EMSA) we confirmed the presence of STAT6 binding-sites within both the CysLT1R (Figure 4) and LTC_4S promoters (not shown).

The presence of a mobility shift specifically mediated by STAT6 was confirmed by the ability of molar excess unlabeled DNA probes, comprising the STAT6 site within the ε heavy chain promoter, to block binding—but not a mutated version of this STAT6 probe. That this shift was produced by pSTAT6 was further confirmed using anti-STAT6 and anti-pSTAT6 antibodies. These results were extended to other NSAIDs including ketorolac, but not sodium salicylate (not shown). Ketorolac has been successfully utilized to diagnosis aspirin intolerance [56] and these observations suggest a plausible basis for it to have clinical efficacy in AERD. The mechanism by which aspirin blocks STAT6 expression is not known, but has been suggested to involve nuclear trafficking and recycling of transcription factors [51]. Our studies do not distinguish whether aspirin acts to either block induction of pSTAT6 or trafficking of newly activated pSTAT6 into the nucleus. However, our Western hybridization data do confirm the absence of functional pSTAT6 protein within the

nuclei of aspirin-treated cells (Figure 5). Even more impressive, was the inhibition provided by ketorolac, supportive of recent findings regarding the efficacy of this compound in therapeutic desensitization of AERD [57]. These data thereby provide evidence that the CysLT1 receptor and LTC_4S promoters have STAT6 binding sites that are occupied following IL-4 induction and inhibited by aspirin. As such, aspirin desensitization may provide effective therapy for AERD, at least in part, through mitigation of STAT6 activation, thereby downregulating the leukotriene pathways—as is observed clinically in successfully desensitized subjects.

Countering the argument that aspirin may function in AERD as an IL-4-STAT6 antagonist is the lack of an obvious recognition of its utility in aspirin-tolerant asthma or even allergic rhinitis; disorders that arguably also involve IL-4 and STAT6 expression. It could be disputed that if aspirin is an effective anti-IL-4 agent it should be effective in all asthmatics. A subset of asthmatics is recognized who have aspirin-responsive asthma [58] and aspirin may have modest efficacy in patients with chronic sinusitis without aspirin intolerance (Donald Stevenson, unpublished). However, AERD is a distinct disorder from aspirin-tolerant asthma and these subjects are unique in their production of and sensitivity to leukotrienes. Efficacy of aspirin desensitization in AERD may therefore reflect the heightened importance of these leukotriene-dependent and, by extension, aspirin-responsive mechanisms in AERD. For example, leukotriene modifiers, in particular leukotriene synthesis inhibitors, seem uniquely efficacious in AERD in comparison to aspirin tolerant asthmatics [59]. Furthermore, the relative lack of efficacy of aspirin in aspirin-tolerant asthmatics, despite its putative ability to block IL-4, parallels the failure of IL-4-targeting biotherapeutics in these subjects (although suggests that perhaps these agents would have greater efficacy if used in AERD).

11. Summary

While the exact mechanisms driving AERD are not fully understood, part of the explanation is the marked overexpression of the 5-LO and LTC_4S genes, resulting in constitutive overproduction of CysLTs, and the decrease in PGE_2 production that prevents mast cell and eosinophil activation. These studies strongly suggest that AERD is derived, at least in part, from either the increased production or hyperresponsiveness to IL-4, although no obvious mechanism underlying this dysregulation has been identified. This increased expression of an IL-4 signature, as summarized in Figure 6, can have activating and inhibitory effects on gene expression in the LT and PG pathways. The net result of enhanced IL-4 levels is to increase synthesis of and responsiveness to LTs, while blocking production of protective PGE_2. Compounds that target these molecules may lead to new therapeutic options for the treatment of AERD.

Abbreviations

5-LO: 5-lipoxygenase
AERD: Aspirin-exacerbated respiratory disease
COX: Cyclooxygenase
CysLT: Cysteinyl leukotriene
LTC_4S: Leukotriene C_4 synthase
NF-κB: Nuclear factor κB
NFAT: Nuclear factor of activated T cells
NP: Nasal polyposis
NSAID: Nonsteroidal anti-inflammatory drugs
PG: Prostaglandin
PGES: Prostaglandin E_2 synthase
STAT: Signal transducer and activator of transcription.

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Research Article

Comparison of CD63 Upregulation Induced by NSAIDs on Basophils and Monocytes in Patients with NSAID Hypersensitivity

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Background. An in vitro basophil activation test, based on the detection of CD63 upregulation induced by NSAIDs, has been described. Its clinical significance remains controversial. *Objectives*. In patients with a history of nonallergic NSAID hypersensitivity, stratified according to the severity of the symptoms, to assess with NSAIDs the predictive value of basophil (BAT) and monocyte (MAT) activation tests. *Patients/Methods*. Sixty patients who had NSAIDs-induced or exacerbated urticaria/angiooedema and 20 controls was included. After incubation with NSAIDs or acetaminophen, leukocytes were analysed for CD63 upregulation. *Results*. With aspirin, the sensitivity (37%) and specificity (90%) of BAT agree with already published results. In contrast, when patients had had cutaneous and visceral reactions, the frequency of positive BAT 14/22 (64%, $P < 0.001$) or MAT 10/22 (46%, $P < 0.01$) were increased. *Conclusions*. Positive tests were more frequent among patients having a severe hypersensitivity contrasting with the other patients who had results similar to controls.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are after antibiotics, the second most frequently suspected agents causing drug hypersensitivity. The prevalence of acetyl salicylic acid (ASA, aspirin) hypersensitivity ranges from 0.6% to 2.5% in the general population, from 4.3% to 11% in asthmatic patients [1], and from 20 to 40% in chronic idiopathic urticaria (CIU) [2]. It also occurs in subjects with no known underlying disease, otherwise normal when they abstain from taking NSAID.

Hypersensitivity may occur shortly, within 15 minutes or longer, up to 24 hours after NSAID intake. In general it develops within 1 to 4 hours [3]. Some patients might have life-threatening reactions, especially those with aspirin-exacerbated respiratory diseases (AERDs, Widal syndrome), which associate aspirin sensitivity, asthma, nasal polyposis, and airway remodelling [1].

In most patients the adverse reaction is nonallergic. Those with eicosanoid metabolism dysfunction or other alterations are prone to hypersensitivity when NSAIDs inhibit the enzyme cylooxygenase-1 (Cox-1) [3–14]. Selective NSAIDs strongly inhibit COX-2, but they are weak inhibitors of COX-1, so they are well tolerated in patients with NSAID-sensitive asthma or CIU [4, 5]. The concentration inhibiting efficiently COX-1 or COX-2 may differ as much as 3 logs between the strongest and weakest inhibitors (Table 1) [15–17]. Pharmacological profiles as well as hypersensitivity depend on their inhibitory activities.

The diagnosis of NSAID hypersensitivity is based on clinical histories and provocation challenges with aspirin or NSAIDs [19–21]. Skin test (ST) responses are typically negative except when there is a true allergy. Oral challenge tests rule out hypersensitivity in 50% of the patients [20] which suggests that clinical histories are not sufficient to diagnose true NSAID hypersensitivity. Due to the severe

Table 1: Comparison of NSAIDs and acetaminophen concentrations incubated with leukocytes to serum concentrations at usual therapeutic dosage and to 50% inhibitory concentrations (IC50) of cyclooxygenase-1 and -2.

Drug	Concentrations				
	Tested*		In serum**	IC50***	
	mg/mL	μM	μM	COX-1	COX-2
ASA	0.01–0.1–1	31–310–3100	111	4.45	13.88
Diclofenac	0.0013–0.013–0.13	4.2–42.2–422	6.1	0.26	0.01
Ketoprofen	0.025–0.25–2.5	98.3–983–9830	9.4	0.11	0.88
Celecoxib	0.005–0.05–0.5	13.1–131–1310	1.6	82	6.8
Acetaminophen	0.01–0.1–1	66.1–661–6610	117	113.7	25.8

*Each NSAID and and acetaminophen (APAP) were tested at three ten-fold serial dilutions.
**Serum concentrations at usual therapeutic dosage.
***Concentration of drug that inhibited 50% of COX-1 in platelets or COX-2 in monocytes [15–17].

reactions that might occur in some patients, it was not desirable to use oral challenge systematically. There is a need for laboratory tests; hence flow cytometric determination of CD63 upregulation on basophils incubated with aspirin and other NSAIDs has been described. Sensitivity has been shown to be 43% with aspirin or diclofenac [7, 22, 23]. However, conflicting results have also been published about the specificity or the sensitivity of the test and the clinical significance [21, 24–26].

In ASA-induced urticaria or asthma, in addition to basophils, neutrophils or other leukocytes are also activated [8, 27]. The aim of the present study is in patients suffering from nonallergic NSAID hypersensitivity, stratified according to the severity of clinical symptoms, to compare the clinical performance of the basophil activation test (BAT) with the monocyte (MAT) activation test.

2. Patients and Methods

2.1. Patients. Sixty-five patients referred by the patients' physician or by an emergency unit to the Dermatology and Allergy Center of Hôpital Tenon, Paris, between 2006 and 2009 for evaluation of a history of NSAID and/or APAP (acetaminophen, paracetamol) hypersensitivity were included in the study. Among them, 5 patients had APAP hypersensitivity alone. Half of patients according to the physician report or the patient's declaration had a history of hypersensitivity to one of these drugs during the last year, for the other half it was older.

NSAID-induced or exacerbated urticaria or angiooedema and associated symptoms were clinically classified [21, 28, 29], and severity of clinical symptoms was graded according to the published works [18]. Hypotension was defined as a systolic blood pressure below 100 mm. Patients with recurrent angiooedema were investigated for complement fractions C3 and C4 and C1 inhibitor, but their results were in normal ranges.

Patients suspected of immediate or delayed allergy to an NSAID were discarded. Those with asthma or Widal syndrome were not included because they were followed up in the department of respiratory system diseases.

The study protocol was in accordance with the local ethical committee guidelines, and all subjects gave their consent before being included. Tests were done only for a diagnostic purpose.

2.2. Controls. Leukocytes from 12 normal subjects, members of the hospital's staff, and 8 patients with allergic reactions induced by drugs other than NSAIDs were used as controls for BAT, MAT, and NAT. All the controls had never experienced NSAID hypersensitivity and had taken at least once 1 g aspirin within the last 12 months.

2.3. Skin Tests. Skin tests (STs), prick ST and intradermal ST, were done as previously described [30, 31].

2.4. Oral Challenge Test. In this study no oral challenge test with NSAIDs was done for a diagnostic purpose. In patients needing an analgesic antipyretic drug, double blind, placebo-controlled, orally given APAP tests were carried out when the STs were negative. Protocol for patients needing NSAID therapy was similar, they received selective NSAIDs celecoxib or nimesulide. All the oral challenges were done by the same practitioner.

2.5. NSAIDs for Flow Cytometry. They were purchased in solution for intravenous or intramuscular use. Acetyl salicylate lysine (Aspegic, Sanofi-Aventis), acetaminophen (APAP, paracetamol, perfalgan, Bristol-Myers Squibb), ketoprofen (profenid, Sanofi-Aventis), diclofenac (voltaren, Novartis Pharma) and celecoxib (Celebrex, Pfizer Inc.) were diluted in the dilution buffer (2 mM $MgCl_2$, 1.2 mM $CaCl_2$, and 2 g/L bovine serum albumin in phosphate-buffered saline, PBS). The dilution buffer was used, instead of a NSAID, as the negative control in two tubes, and calcium ionophore 2.5 μg/mL (Sigma) was used as the positive control. Calcium ionophore induced CD63 upregulation in at least 50% of basophils and monocytes and 25% of neutrophils.

2.6. Antibodies. R-phycoerythrin-conjugated anti-CD33 monoclonal antibody (MAB), fluorescein-isothiocya nate-(FITC-) conjugated anti-CD63 MAB, R-phycoerythrin-conjugated anti-CD203c MAB, tandem dye R-phycoerythrin-cyanin-5.1-conjugated anti-CD45 MAB, and ECD-conjugated (tandem dye phycoerythrin-Texas red) strep- tavidin were purchased from Beckman Coulter- Immunotech, Marseille,

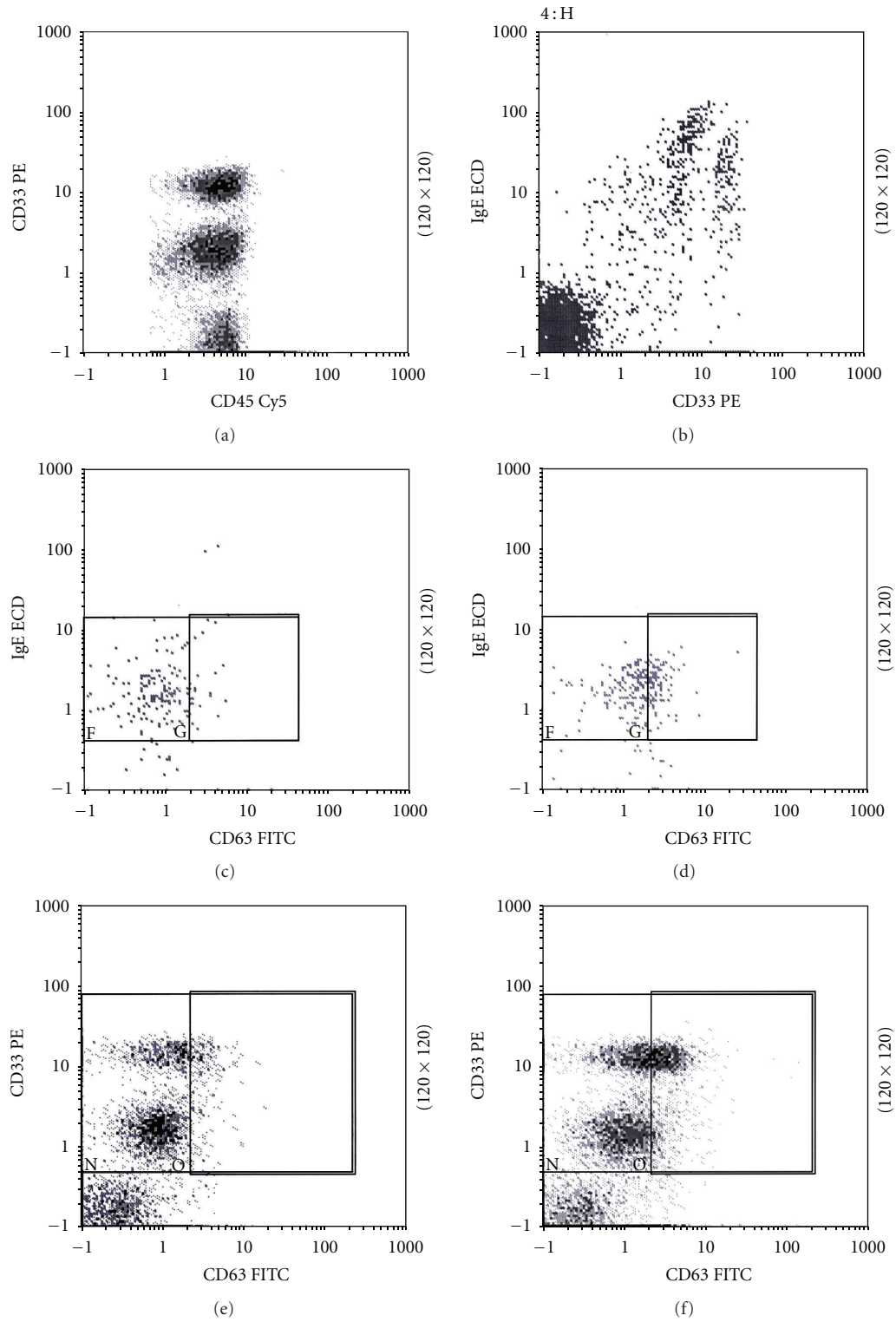

Figure 1: Detection by flow cytometry of CD63 upregulation on leukocytes activated by NSAIDs. (a) and (b) Targeting of leukocytes: (a) shows at the top CD33 bright cells, the monocytes, CD33 dim cells, polymorphonuclears, and CD33-negative cells, lymphocytes. (b) Shows among anti-IgE-labelled cells, CD33dim cells, the basophils, and C33 bright, the monocytes. The percentage of monocytes among IgE-labelled cells varies from 0 to 50%, depending on the patient. (c) and (d) Activation of basophils: (c) basophils incubated with buffer. (d) Basophils incubated with 1 mg/mL of ASA and upregulation of CD63 on 30% of basophils. (e) and (f) Activation of monocytes and neutrophils. (e) Monocytes at the top, and neutrophils under, incubated with buffer. (f) After incubation with 1 mg/mL of ASA, upregulation of CD63 on 25% of monocytes and 5% of neutrophils.

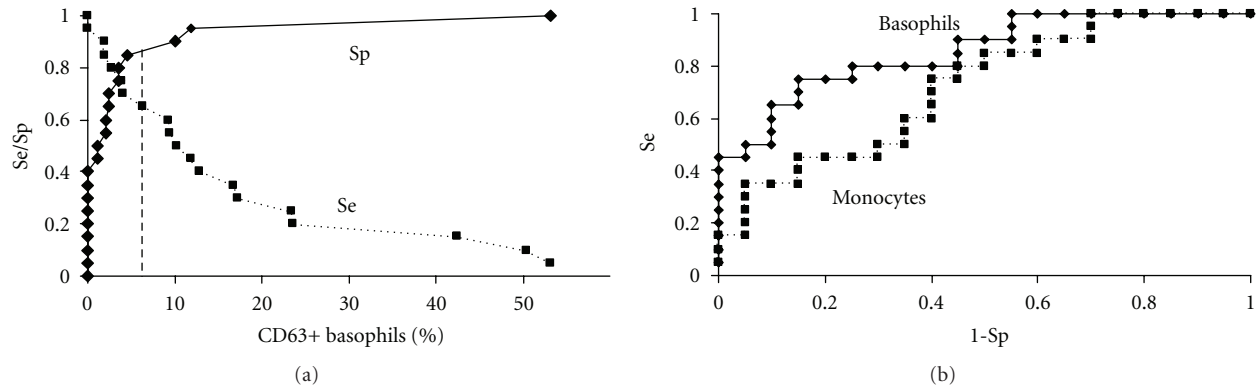

Figure 2: ROC curves for basophils and monocytes incubated with 1 mg/mL of ASA. (a) shows the sensitivity (Se) and the specificity (Sp) of BAT. The cut-off value was determined at 6% of activated basophils (vertical dashed line). (b) shows ROC curves for the activation of basophils and monocytes. The diagnostic performance of the activation of basophils was better (area under the curve for activated basophils 0.855) than that of the activation of monocytes (area under the curve 0.718), $P < 0.001$.

France. Biotinylated goat anti-human IgE polyclonal antibody was purchased from Vector Laboratories, Burlingame.

2.7. Leukocyte Activation Tests. The BASIC (basophils isolated from blood and analysed by flow cytometry) assay was done as previously described [30, 31]. Lithium-heparin anticoagulated peripheral blood was centrifuged at 500 g for 20 minutes at 20°C on a layer of ficoll ($d = 1.077$). The lymphocyte layer and leukocytes suspended in ficoll between lymphocytes and red cells were harvested. After lymphocytes the largest population among these cells were neutrophils, then monocytes, and basophils were the smallest one.

100 μL aliquots of the leukocyte suspension containing 10^6 leukocytes were mixed with 100 μL of antigen or dilution buffer and then incubated for 30 min at 37°C in a CO_2 (7%) incubator, after which the cells suspensions were fixed by the addition of 50 μL of 1% paraformaldehyde in PBS.

The leukocytes were then quadruple labelled by adding 10 μL FITC-conjugated anti-CD63 antibody, 20 μL R-phycoerythrin-conjugated anti-CD33 antibody 10 μL tandem dye R-phycoerythrin-cyanin-5.1-conjugated anti-CD45 antibody, and 1/50 diluted 50 μL biotinylated anti-IgE antibody. For the analysis of the CD203c upregulation, basophils were quadruple labelled using the same antibodies, except that the anti-CD33 antibody was replaced by 20 μL R-phycoerythrin-conjugated anti-CD203c. After incubation and washing biotinylated anti-IgE antibodies were labelled by adding 10 μL ECD-conjugated (tandem dye phycoerythrin-Texas red) streptavidin.

Analysis of leukocytes surface markers was performed on an Epics XL flow cytometer (Beckman Coulter, Marseille, France). On the histogram defined by forward scatter and side scatter, the first gating was done by a bit map around lymphocytes and monocytes, basophils were found in this gate. Another gate was done around polymorphonuclear leukocytes, for neutrophils. The next gatings were done for basophils around IgE+ CD45+ CD33dim cells (Figure 1). These cells previously had been identified as basophils [31–33]. Monocytes were gated around CD45 bright and CD33 bright cells [33], a small proportion of them being IgE+ [31]. The second gating for neutrophils was done around CD45+ CD33dim IgE− cells (Figure 1). When CD203c, a specific marker of basophils [34, 35] was used, basophils were gated around CD203c+ IgE+ CD45+ cells.

In each assay, upregulation of CD63 was measured on at least 400 cells for basophils, monocytes, or neutrophils.

2.8. Cut-Off for Positive Results. The tube-to-tube reproducibility was determined by labelling leukocytes of 10 patients and counting 12 tubes per patient. A cut-off value for positive results was chosen at 2 standard deviations (6%) exceeding the value of the nonstimulated control tube.

The best cut-off value for activated basophils and monocytes was established by analysing the receiver operating characteristic (ROC) curves of results observed in a selected group of patients with severe hypersensitivity versus controls (Figure 2). The optimal cut-off point deduced from ROC curves was approximately 6% of activated cells. It was similar to that calculated in tube-to-tube reproducibility (see above).

2.9. Statistical Analysis. Results of BAT and MAT were blind-analysed, the operator was not informed of the patients' diagnosis. Conversely, the diagnosis of allergy was done before the results of BAT and MAT were available. Statistical analysis was done with χ^2, paired χ^2, a two-tailed Fisher's test, Wilcoxon's nonparametric test, and Spearman's rank correlation.

3. Results

3.1. Clinical History and Classification of Patients with NSAID Hypersensitivity. Among 60 patients with NSAID hypersensitivity, 30 had no underlying disease and had recovered when they abstained from taking NSAIDs. These patients were diagnosed as having NSAIDs-induced urticaria/angioedema according to [21]. The most frequent and

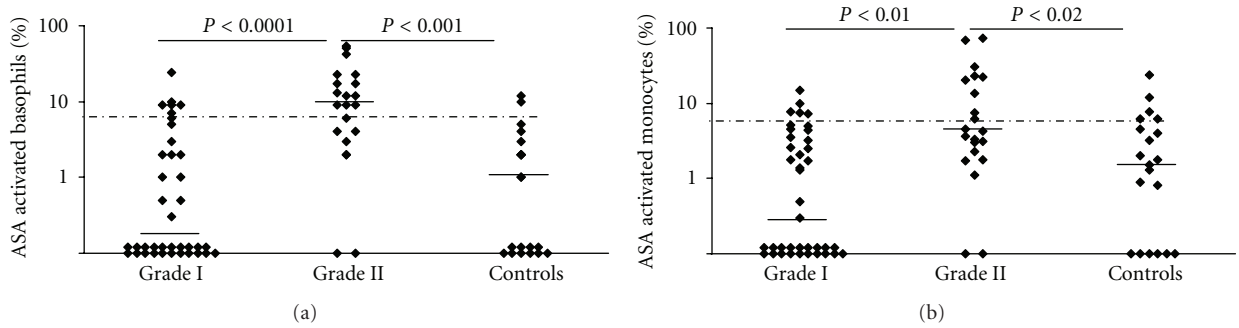

Figure 3: Activation of patients' and controls' basophils and monocytes incubated with 1 mg/mL of ASA. (a) Basophils, (b) monocytes. Grading of reactions in the NSAID hypersensitivity was done according to severity of clinical symptoms [18] (Table 3). The median value is shown by the horizontal bold lines. The cut-off for a positive BAT or MAT was determined at 6% of activated cells (dashed line). Statistical analysis was performed by Wilcoxon's nonparametric test.

Table 2: Description of patients suffering from NSAID hypersensitivity.

Group of patients*		Age	Sex ratio	Atopy**	C/E IU***	HS ≥ 2**** NSAIDs
Grade I	$n = 38$	44	29F/9M	9 (24%)	18 (47%)	25 (66%)
Grade II	$n = 22$	43	16F/6M	7 (32%)	12 (55%)	17 (77%)
Total	$n = 60$	44	45F/15M	16 (27%)	30 (50%)	42 (70%)

* Patients with a history of urticaria or angiooedema and no visceral disorder were classified as having had a hypersensitivity of grade I and those with at least one visceral disorder were classified as grade II according to published works [18].
** Patients had history of atopic dermatitis, allergic rhinitis, or asthma, but they had healed at the time of NSAID hypersensitivity.
*** The patients with "C/E IU" had chronic or episodic idiopathic urticaria or angiooedema; NSAID hypersensitivity was diagnosed when symptoms worsen relapsed, or were unusual and recovered when they stopped NSAID intake.
**** Patients who had histories of hypersensitivity induced by at least two chemically distinct NSAIDs.

characteristic clinical manifestation of the NSAID hypersensitivity was facial angiooedema without flares, observed in 20 patients (67%) ($P > 0.01$).

The remaining 30 patients had chronic and/or episodic (intermittent) idiopathic urticaria and/or angiooedema (C/E IU). NSAID-induced angiooedema was as frequent as urticaria. These patients were diagnosed as having NSAIDs-exacerbated urticaria/angioedema according to [21]. The demography of patients is described in Tables 2 and 3.

Forty-two among 60 patients (70%) had histories of hypersensitivity induced by at least two chemically distinct NSAIDs (Table 2). For the remaining 18 patients we cannot settle if they were true single- or multiple-NSAID reactors because they abstained from taking NSAIDs after the first hypersensitivity reaction. Twenty patients (33%) had also a clinical history of APAP hypersensitivity associated with NSAID hypersensitivity (not shown). All patients with APAP hypersensitivity had a negative ST to APAP. Out of 20 patients with clinical history of APAP and NSAID hypersensitivity and 5 patients with only APAP hypersensitivity, respectively, 5 (25%) and 1 (20%) had APAP oral challenge test positive.

Twenty-two patients with a history of urticaria or angiooedema and with at least one visceral disorder hypotension, laryngeal oedema, dyspnoea, abdominal pain, vomiting or diarrhoea after NSAID intake, were classified as having had a hypersensitivity of grade II (Table 4). After NSAID intake, 21 reacted before 6 h (21/22 = 95%, median 1 h). In contrast, in patients with a history of urticaria or angiooedema with no visceral involvement (grade I), hypersensitivity occurred later (median 8 h) ($P < 0.0001$) (Table 3).

Among 18 patients with only one known NSAID hypersensitivity, 7 had at least one visceral disorder, frequency (7/18) was similar to patients with hypersensitivity induced by at least two chemically distinct NSAIDs (15/42). No clinical or biological data could discriminate one group from the other.

3.2. Tests for the Detection of In Vitro Activated Leukocytes by NSAIDs. Targeting of leukocytes and detection of CD63 upregulation on the membrane of activated cells were done as indicated in Figure 1. A cut-off value for positive results was chosen at 2 standard deviations (in tube-to-tube reproducibility), 6% exceeding the value of the nonstimulated control tube (see Section 2 for more details). This value was similar to the optimal cut-off point deduced from ROC curves for the basophil activation test (BAT) or the monocyte activation test (MAT) (Figure 2).

3.3. CD63 Upregulation Induced by ASA

3.3.1. On Basophils. Twenty-two out of 60 patients with a history of NSAID hypersensitivity had a positive BAT to ASA at 1 mg/mL; therefore the sensitivity was 37%. Two controls among 20 (10%) had positive BAT to ASA, the specificity of the test could be estimated close to 90%.

Table 3: Skin symptoms and time to onset of the NSAID-induced hypersensitivity.

Group of patients		Time to onset*		NSAID-induced skin symptoms		
		Median	<6 H	AO	AO + Urticaria	Urticaria
Grade I	$n = 38$	8 H	14 (37%)	18 (47%)	9 (24%)	11 (29%)
Grade II	$n = 22$	1 H	21 (95%)	12 (55%)	7 (32%)	3 (14%)
Total	$n = 60$	4.5 H	35 (58%)	30 (50%)	16 (27%)	14 (23%)

*The time to onset of symptoms after NSAID intake was shorter in patients with grade II hypersensitivity than in patients with grade I ($P < 0.0001$).

Table 4: Description of severe reactions (grade II) observed in patients with NSAID hypersensitivity.

Grade II reactions/time to onset*		Visceral disorders (VD)				
		Laryngeal oedema	Dyspnoea**	Hypotension	G-intestinal disorders	At least one VD***
<6 H	$n = 21$	9 (43%)	8 (38%)	6 (29%)	3 (14%)	21 (100%)
8 H	$n = 1$	1	0	0	0	1
Total	$n = 22$	10 (45%)	8 (37%)	6 (27%)	3 (14%)	22 (100%)

*Time to onset of symptoms after NSAID intake. **Dyspnoea observed in patients with no laryngeal oedema. ***Patients with a history of urticaria or angiooedema and with at least one visceral disorder were classified as having had a hypersensitivity of grade II according to published works [18].

In patients suffering from NSAID hypersensitivity restricted to cutaneous reaction (grade I), positive BAT to ASA was not more frequent than in control group (Figure 3 and Table 5).

In contrast, in patients who had had a grade II hypersensitivity, basophils were more strongly activated and BAT was more frequently positive (14/22 = 64%) than in patients with grade I (8/38 = 21%) or controls (Figure 2 and Table 5) ($P < 0.001$, Wilcoxon's nonparametric test). Therefore, a positive BAT in a patient with NSAID hypersensitivity had for a grade II hypersensitivity a positive predictive value of 64% (14/22). The negative predictive value was 79%; only 8 out of 38 patients with a negative BAT had had a grade II hypersensitivity.

A positive BAT to ASA correlates with the precocity of the hypersensitivity. The patients with a positive BAT had reported an interval of 2.5 hours (median value) between NSAID intake and symptoms. On the other hand among patients with negative test the interval was 8 hours ($P < 0.001$).

3.3.2. On Monocytes and Neutrophils. Though less sensitive (46%), results of MAT correlate quite well with BAT (Spearman's rank correlation, $r = 0.71$ for ASA, $r = 0.49$ for diclofenac, $P < 0.001$). The mean percentage of activated monocytes with ASA was greater in patients with grade II hypersensitivity than in those with grade I ($P < 0.01$) or controls ($P < 0.02$, Wilcoxon's nonparametric test) (Figure 2). In control group, in comparison with BAT, the number of results exceeding the cut-off was increased and not significantly different from that of patients. Therefore, the specificity of MAT was relatively low, 75% (Table 5).

Activation of neutrophils with ASA was rather insignificant (Table 4).

3.4. Results Observed with Other NSAIDs and APAP. Activation by diclofenac of basophils and monocytes was of the same magnitude of ASA and results correlated rather well (Spearman rank correlation, $r = 0.59$, $P < 0.01$). In contrast, it activated neutrophils better than ASA (Table 5, $P < 0.01$). The mean percentage of activated basophils was greater in grade II patients (median 4.3, range 28–0%) than in grade I patients (median 0.7, range 19–0%) ($P < 0.03$, Wilcoxon's nonparametric test). However, the number of values exceeding the cut-off was not so great to be significant between grade II, grade I or controls (P = NS) (Table 4). Ketoprofen, celecoxib, and APAP activated basophils, monocytes, or neutrophils, with values exceeding the cut-off in 10 to 16% of patients and in 0 to 5% of controls (results not shown). APAP did not activate significantly cells of patients with NSAID and APAP hypersensitivity ($n = 20$) or APAP hypersensitivity alone ($n = 5$) (results not shown).

3.5. Optimal NSAID Concentrations Activating Leukocytes. Each NSAID and APAP were tested at three ten-fold serial dilutions (Table 1). For each drug the highest concentration was calculated in relation with usual pharmacological doses and tested on leukocytes in order to check the absence of toxic effects. For ASA and diclofenac, available solutions for IV and IM use were at least diluted 1/200 to obtain a final concentration of 1 mg/mL for ASA and 0.125 mg/mL for diclofenac. We avoided 1/20 diluted solutions because they seemed toxic. The percentage of positive tests decreased with increasing dilution; at 1/2000 they were two times less frequent than at 1/200 (results not shown). Results shown are those observed at the dilution 1/200 (Table 4). For ketoprofen and APAP, as they seldom activate leukocytes, available solutions were less diluted: 1/20, 1/200, and 1/2000 (final concentration in Table 1). No toxic effect had been observed with these dilutions.

3.6. Comparison of Two Basophil Activation Markers, CD63 and CD203c. In 23 patients and 8 controls, basophils were double labelled with the two activation markers. Activation

Table 5: Activation of basophils (BAT), monocytes (MAT), and neutrophils (NAT) induced in vitro by ASA (1 mg/mL) or diclofenac (0.125 mg/mL).

Group of patients		Patients with positive tests*					
		BAT+		MAT+		NAT+	
		ASA	Diclofenac	ASA	Diclofenac	ASA	Diclofenac
NSAID HS	$n = 60$	22 (37%)	20 (33%)	14 (23%)	15 (25%)	8 (13%)	16 (27%)
Grade I**	$n = 38$	8 (21%)	10 (26%)	4 (11%)	9 (24%)	6 (16%)	12 (31%)
Grade II	$n = 22$	14 (64%)	10 (46%)	10 (46%)	6 (27%)	2 (9%)	4 (19%)
Controls	$n = 20$	2 (10%)	5 (25%)	5 (25%)	3 (15%)	0 (0%)	2 (10%)

*The optimal cut-off point for a positive test deduced from ROC curves was 6% of activated cells (see Section 3). Activation was detected by CD63 upregulation. **Grading of reactions according to severity of clinical symptoms (Tables 2 and 4).

of patients' basophils with ASA, diclofenac, or ketoprofen was detected 32 times with CD63, 6 times with CD203c, and 4 times with both CD63 and CD203c (results not shown). CD63 was at least 5 times more sensitive than CD203c ($P < 0.0001$). In the control group, basophils activation was detected 5 times with CD63 and 10 times with CD203c. NSAIDs-induced upregulation of CD203c was not significantly different between patients and control group.

4. Discussion

The sensitivity (37%) and specificity (90%) of BAT for the diagnosis of NSAID induced hypersensitivity were low. These values rather agree with published results. The sensitivity of BAT was assessed between 33 and 77% with ASA and between 17 and 52% with diclofenac [7, 22, 23, 26, 36–38]. Our cut-off for positive results, at 6% of activated basophils, was slightly higher than the published values. In order to improve the sensitivity of the test, instead of the results observed with a single NSAID, those observed with ASA, diclofenac, and naproxen were combined in an index (ADN index) [26]. As a consequence the sensitivity increased from 43% to 65%.

Our study further shows that, though less frequent than on the basophils, ASA upregulates CD63 on the monocytes of some patients with NSAID hypersensitivity and of some control subjects who tolerate ASA well. Because BAT and MAT have a low sensitivity and specificity in the diagnosis of NSAID hypersensitivity, this raises the question of the clinical significance of a positive test.

Our results suggest a linkage between positive BAT or MAT with ASA and a history of a severe NSAID hypersensitivity. Indeed positive tests were more frequent in patients with severe hypersensitivity than in patients with only cutaneous symptoms or controls ($P < 0.001$). Conversely, in patients with only cutaneous symptoms after NSAID intake the frequency of positive BAT or MAT was quite similar to that in controls. Because the patients with NSAID hypersensitivity are heterogeneous, the conflicting results could be explained by bias of selection [7, 22, 23, 26, 36–38]. Stratification of the patients according to the severity of the clinical symptoms has not been used in the appraisal of the clinical significance of a positive test.

The patients included in this study had been suspected of a nonallergic hypersensitivity to NSAIDs. For most of them this was confirmed because 70% had histories of hypersensitivity with at least two chemically distinct NSAIDs; moreover, in addition to basophils, monocytes were activated in vitro by NSAIDs. Because activation of monocytes is not IgE dependent, a positive MAT might contribute to identify a nonallergic hypersensitivity. Among 18 patients with only one known NSAID hypersensitivity, 7 patients were MAT positive with ASA or diclofenac.

Results observed in BAT and MAT agree with clinical studies about harmlessness of celecoxib, a selective COX-2 inhibitor [4, 5]. It is a poor activator when compared to ASA or to diclofenac. However, results observed with ketoprofen are contradictory: it is one of the strongest COX-1 inhibitors [15, 16] (Table 1) but was a poor activator in BAT and MAT even tested at higher concentrations than ASA or diclofenac. This suggests that inhibition of COX-1 is not enough to activate leukocytes. It remains to determine the signification of cellular responses to NSAIDs and the correlation with the disease evolution. Though nonimmune, NSAID hypersensitivity in most patients is acquired and occurs around the age of forty years (Table 2). In a follow up for 4 years, a third of the patients recovered [3]. Intolerance to NSAID might precede by years the onset of CIU [39].

Twenty out of 60 (33%) patients reported in addition to NSAID APAP hypersensitivity, but, when tested with APAP, skin tests, BAT, and MAT were negative. The mechanism by which APAP affects fever and pain is still debated. It remains a weak COX inhibitor, but it is more potent in inhibiting COX-2 than COX-1, like selective COX-2 inhibitors [17].

One team published contradictory results about the sensitivity of the BAT, it reported that diclofenac induces basophil degranulation without increasing CD63 expression in sensitive patients [25]. However, the highest concentration of diclofenac they used was 10 μg/mL, which is 8 times lower than the concentration used in the other published works [22, 23, 26]. We observed, in agreement with published works for basophils and also for monocytes, that they were better activated by increasing the concentration of NSAIDs. But the too high concentrations ($\geq$5 mg/mL for ASA, $\geq$1.25 mg/mL for diclofenac) were toxic or activated nonspecifically the basophils of the controls [36, 37, 40].

CD203c upregulation, compared to CD63, poorly detected basophil activation by NSAIDs, but there is a controversy about CD203c upregulation by NSAIDs [37, 38].

Discrepancies about the sensitivity of CD203c compared to that of CD63 might have different explanations [31, 34, 41].

In summary, in patients suffering from NSAID hypersensitivity restricted to cutaneous reaction, in vitro activation of basophils or monocytes by NSAIDs was similar to that of control subjects. In contrast, a group of patients who had had early and quite severe reactions (grade II) had with ASA significantly stronger activation of basophils and monocytes.

Conflict of Interests

The authors state no conflict of interests.

Acknowledgments

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Research Article

The *IL1B-511* Polymorphism (rs16944 AA Genotype) Is Increased in Aspirin-Exacerbated Respiratory Disease in Mexican Population

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Aspirin exacerbated respiratory disease (AERD) is characterized by chronic hyperplastic rhinosinusitis, nasal polyposis, asthma, and aspirin sensitivity. The mechanisms which produce these manifestations of intolerance are not fully defined, current research focuses on cyclooxygenase 1 (COX-1) inhibition, metabolism of arachidonic acid, and the COX pathway to the lipoxygenase (LO) route, inducing increased synthesis of leukotrienes (LT). The biological plausibility of this model has led to the search for polymorphisms in genes responsible for proinflammatory cytokines synthesis, such as *IL1B* and *IL8*. We performed a genetic association study between *IL8-251* (rs4073) and *IL1B-511* (rs16944) polymorphisms in AERD, aspirin-tolerant asthma (ATA), and healthy control subjects. Using allelic discrimination by real-time PCR, we found statistically nonsignificant associations between AERD, ATA, and healthy control subjects for the GG and GA genotypes of *IL1B* (rs16944). Interestingly, the AA genotype showed an increased frequency in the AERD patients versus the ATA group (GF = 0.19 versus 0.07, $p = 0.018$, OR 2.98, and 95% CI 1.17–7.82). This is the first observation that *IL1B* polymorphisms are involved in AERD. Thus, future studies must investigate whether interleukin-1β is released in the airways of AERD patients and whether it relates to genetic polymorphisms in the *IL1B* gene.

1. Introduction

Aspirin-exacerbated respiratory disease (AERD) is a syndrome characterized by chronic hyperplastic rhinosinusitis, nasal polyposis, asthma, and aspirin sensitivity, as described in 1922 by SzcZeklik et al. [1, 2]. The prevalence of AERD is variable; Stevenson and Szczeklik reported in 2006 that AERD occurs in 3% of adult patients with asthma in the United States, with the onset of symptoms during the third decade of life, and that it is more common in women than in men, with approximately 70% versus 30% in Europe [2] and 57% versus 43% in the USA [3]. The mechanisms underlying aspirin intolerance are not fully defined, with current research focusing on cyclooxygenase 1 (COX-1) inhibition by aspirin and other NSAIDs diverting arachidonic acid metabolism from COX pathways to the lipoxygenase (LO) pathway. This leads to increased synthesis of the cysteinyl-leukotrienes (LT), LTC_4, LTD_4, and LTE_4, resulting in bronchoconstriction, mucus hypersecretion, and possibly the development of polyps and urticaria [1]. The biological plausibility of this hypothesis fact has led to the search for polymorphisms in genes responsible for LT synthesis, to explore associations between these polymorphisms and local tissue levels of the proteins.

Other factors such as polymorphisms in the genes for proinflammatory cytokines including *TNF*, *IL1B*, *IL6,* and *IL8* are involved in chronic inflammatory and autoimmune diseases. Interleukin 1 (IL-1) is a cytokine associated with inflammatory responses and found in two forms, IL-1α (produced by the *IL1A* gene) and IL-1β (*IL1B*), with both

Table 1: Genetic data on SNPs investigated in study.

SNP	Chr	Gene		Alleles		
		Symbol	Position	Change	Ancestral	MAF
rs4073	4	*IL8*	−251	A/T	A	$T = 0.492$
rs16944	2	*IL1B*	−511	G/A	A	$A = 0.462$

Chr: Chromosome, MAF: Minor allele frequency, MAF source: 1000 genomes phase 1 from dbSNP.

genes located on chromosome 2. IL-1 is expressed in nasal polyps, nasal epithelium, macrophages, activated T lymphocytes, and monocytes; its expression is regulated by adhesion molecules, and others inflammatory cytokines [4]. *IL1B* polymorphisms have been associated with inflammatory bowel disease and gastric cancer among other diseases [5]. Recently, genetic polymorphisms in proinflammatory cytokines such as IL-1β have been recognized as key players in the pathogenesis of asthma [6]. Similarly, IL-8 has been implicated in the asthmatic inflammatory process, and genetic variation in this cytokine has been associated with both the susceptibility and the severity of this disease [7]. In the present study, we have investigated the frequencies of polymorphisms in the genes encoding these two cytokines in AERD patients.

2. Materials and Methods

2.1. Subjects. Patients with aspirin-exacerbated respiratory disease (AERD) ($n = 78$) and aspirin-tolerant asthma (ATA) ($n = 135$) were recruited from the allergy and otolaryngology departments at the Instituto Nacional de Enfermedades Respiratorias (INER). Healthy control subjects (HCS) ($n = 134$) were invited to participate through AERD-screening campaigns. All participants underwent simple spirometry, inhaled methacholine challenge, and nasal challenge with lysine-aspirin (L-ASA) according to international guidelines to determine the degree of bronchial hyperresponsiveness and confirm AERD diagnosis. The AERD group had positive L-ASA and methacholine challenges, the asthmatic group had positive methacholine challenge, but negative L-ASA challenge, and the healthy control subjects were volunteers with negative L-ASA and methacholine challenges. A positive L-ASA challenge was defined as a decrease of at least 40% in total nasal airflow after L-ASA application compared with baseline measures; methacholine challenge was considered positive with a decrease of at least 20% in forced expiratory volume in one second (FEV_1) compared with baseline FEV_1 after the administration of different concentrations of methacholine (beginning with 0.03 mg/mL, increasing gradually the concentration twice every 2 minutes until the concentration of 32 mg/mL); in case it does not have it, it is considered negative. Blood samples were collected for genotyping studies in the HLA laboratory research. The study was approved by the science and bioethics committees of INER, and all participants gave their informed consent [8, 9]. Patients and healthy control subjects had ancestry of at least two generations born in Mexico and were thus considered to be Mexican Mestizo in descent [10, 11].

2.2. DNA Extraction. Peripheral blood was drawn by venipuncture, and genomic DNA was obtained using the commercial BDtract DNA isolation kit (Maxim Biotech, San Francisco, Calif, USA). The DNA was quantified by absorption of ultraviolet light at 260 nm wavelength using an ACTGene spectrophotometer (ACTGene, Inc., NJ, USA).

2.3. SNP Selection. We selected two polymorphisms in two genes related to chronic inflammation: rs16944 in *IL1B* and rs4073 in *IL8*. Genetic data from each of the polymorphisms are described in Table 1.

2.4. Genotyping. Allelic discrimination of SNPs rs16944 (*IL1B*) and rs4073 (*IL8*) was performed by real-time PCR (RT-PCR) on a 7300 Real Time PCR System (Applied Biosystems, Calif, USA) using Taqman commercial probes (Applied Biosystems, USA) for each of the polymorphisms mentioned above and followed the cycling program: preread 50°C, 1 minute; absolute quantitation: 50°C, 2 minutes, 1 cycle; 95°C, 10 minutes, 1 cycle; 95°C, 15 seconds, 60°C 1 min, 40 cycles; postread 50°C, 1 minute. The results were assessed taking into account the allelic discrimination and absolute quantitation in all samples; additionally, we included four contamination controls per plate (nontemplate controls). The interpretation was performed with Sequence Detection Software (v. 1.4). The fluorescence signal detectors used were VIC which was assigned to the B allele and FAM assigned to the A allele for both SNPs.

2.5. Statistical Analysis. Statistical analysis was performed between groups of cases (AERD and ATA) versus the healthy control subjects by χ^2 test with 3×2 tables, using SPSS (v. 15.0) software for Windows, to identify the difference between the allele and genotype frequencies of each polymorphism evaluated. A p-value <0.05 was considered significant. In addition, odds ratios and 95% confidence intervals were calculated with Epi-info (v. 6.04) software.

3. Results

Clinical data for the three groups were compared and are described in Table 2. We performed a genetic association study of the *IL8* (rs4073) and *IL1B* (rs16944) gene polymorphisms in the three groups. Genetic data for the SNPs included in this study are shown in Table 1; minor allele frequency (MAF) of the polymorphisms tested in healthy control subjects had a similar distribution to that reported in international databases (Table 1). Gene frequencies for each genotype within the three subject groups are shown in

Table 2: Summary of clinical characteristics of AERD, ATA, and HCS.

	AERD	ATA	HCS
Subjects	78	135	134
Gender (male/female)	31/47	49/86	79/55
Mean age (years, SD)	42.0 (14.4)	36.7 (17)	24 (8.9)
Premethacholine challenge FEV_1 (%)	99.6 (17.3)	101.4 (12.9)	98.2 (13.3)
Postmethacholine challenge FEV_1 (%)	75.1 (13.8)	76.9 (14.4)	95.4 (11.6)
Pre-L-ASA challenge nasal flow (mL/sec)	640.75 (162.2)	689 (178.2)	670.2 (167.5)
Post-L-ASA challenge nasal flow (mL/sec)	488 (142.8)	658.5 (186.3)	682.4 (165.7)

AERD: Aspirin-exacerbated respiratory disease, ATA: Aspirin-tolerant asthma, HCS: Healthy control subjects, SD: Standard deviation, FEV_1: Forced expiratory volume in one second, L-ASA: Lysine aspirin.

Table 3: Genotype frequencies of *IL8* and *IL1B* genes in AERD, ATA, and HCS.

Gene/SNP Genotype	AERD		ATA		HCS	
	$n = 78$	GF (%)	$n = 135$	GF (%)	$n = 134$	GF (%)
IL8						
rs4073						
AA	35	0.449 (44.87)	54	0.400 (40.00)	53	0.396 (39.55)
AT	33	0.423 (42.31)	57	0.422 (42.22)	63	0.470 (47.01)
TT	10	0.128 (12.82)	24	0.178 (17.78)	18	0.134 (13.43)
IL1B						
rs16944						
GG	23	0.295 (29.49)	57	0.422 (42.22)	47	0.351 (35.07)
GA	40	0.513 (51.28)	68	0.504 (50.37)	72	0.537 (53.73)
AA	15	0.192 (19.23)	10	0.074 (7.41)	15	0.112 (11.19)

AERD: Aspirin-exacerbated respiratory disease, ATA: aspirin-tolerant asthma, HCS: healthy control subjects, GF: genotype frequency.

Table 3. The frequency of genotypes AA, AT, and TT of the *IL8* rs4073 SNP was not statistically significant and different between the three studied groups.

Analysis of GG and GA genotypes of the *IL1B* (rs16944) SNP for AERD and ATA patients versus the healthy control subjects showed nonstatistically significant associations. Interestingly, the AA genotype showed increased frequency in the AERD patients when compared to the ATA group (GF = 0.19 versus 0.07); this association was statistically significant ($p = 0.018$, OR 2.98, and CI 1.17–7.82) (Tables 3 and 4) and was not found when AERD or ATA groups were compared to healthy control subjects. There is no difference in AA versus AG + GG using contrast of healthy control subjects versus AERD patients (data not shown).

4. Discussion

The airways of aspirin-sensitive patients are characterized by chronic inflammation with cell infiltration even when they are not exposed to aspirin or other NSAIDs [12]. In addition to alterations in the metabolism of arachidonic acid, several proinflammatory cytokines have been associated with AERD. The role of IL-1, however, has not been investigated previously. Here, we report that AERD patients show an increased frequency of the *IL1B*-511 polymorphism (rs16944 AA genotype) compared to aspirin-tolerant asthmatics.

Interleukin-1β has been reported to be involved in the genesis of both asthma [13, 14] and chronic rhinosinusitis with nasal polyposis in a Turkish population [15]. Most studies have attempted to establish the association of polymorphisms in the *IL1B* promoter gene, mainly at positions-511 G/A (rs16944) and -31 C/T. For example, Park et al., in 2004, did not find any association between these polymorphisms and either asthma or atopy in a Korean population [16]. In 2007, Erbek et al. described a susceptibility for developing nasal polyps associated with the *IL1B-511* polymorphism (rs16944) [15], but Mfuna Endam et al., in 2010, failed to reproduce this finding in Canadian patients with chronic rhinosinusitis [17]. In 2003, Allen et al. did not find any association with *IL1* gene polymorphisms in asthmatic families ($n = 244$), but they reported an association with the DNA microsatellite D2S308 in these asthma families ($p = 0.00001$) [18]. In parallel, Karajalein et al. evaluated 245 patients with asthma and nasal polyposis and did not find any association between the polymorphism *IL1B-511*

Table 4: Statistical association of *IL1B* rs16944 AA in AERD and ATA patients.

Gen/SNP	AERD		ATA		*P*	OR	CI (95%)
	$n = 78$	GF (%)	$n = 135$	GF (%)			
IL1B/rs16944							
AA	15	0.192 (19.23)	10	0.074 (7.41)	0.018	2.98	1.17–7.82

AERD: Aspirin-exacerbated respiratory disease, ATA: aspirin-tolerant asthma, GF: genotype frequency, *p* value Yates correction's, OR: odds ratio, 95% IC: 95% Confidences Interval.

C/T and nasal polyps [19]. Evidence for the role of IL-1 in pulmonary immune responses has been gathered in murine models of allergic asthma using IL-1R1-deficient [IL-1R1 (−/−)] mice; changes observed in these mice include significant reduction of pulmonary eosinophilic inflammation, diminished goblet cell hyperplasia, and reduction of cell recruitment to the lungs, as compared to control BALB/c mice [20]. However, there are no studies linking gene promoter polymorphisms and levels of expression of the cytokine in the lung microenvironment.

In the present study, we have demonstrated that the frequency of the *IL1B-511* polymorphism (rs16944 AA genotype) is three-fold higher in AERD (19.2%) than in ATA patients (7.4%), suggesting that patients carrying this polymorphism may exhibit genetic susceptibility to develop AERD.

Findings on the biological functionality of the rs16944 polymorphism have not been consistent across studies. The AA genotype has been associated with higher gastric mucosal levels of IL-1β in bacterial infections [21], while mononuclear cells from subjects with the GG genotype showed an increased release of IL-1β after stimulation with lipopolysaccharide [22]. Recent studies suggest that the functional role of rs16944 may depend on *IL1B* promoter region haplotypes including rs16944 [23–26]. Although the findings are inconsistent, these previous studies suggest that rs16944 could affect the expression levels of IL-1β. Our report is the first demonstration of the involvement of *IL1B* polymorphism in AERD. The sample size is relatively small, particularly in the AERD group, which may limit the statistical power, so it would be desirable to replicate our findings in an independent population.

Future studies must investigate whether this cytokine is released in the airways of AERD patients and whether its levels relate to genetic polymorphisms in the *IL1B* gene.

Interleukin-8 has been implicated in asthma and found in high concentrations in bronchoalveolar lavage fluid of patients with acute asthma exacerbations [26]. In fact, polymorphic *IL8* alleles (−251T and 781C) have been associated with asthma in a European population [7, 27], but not in asthmatics of Korean origin [7]; the differences could be explained by the different ethnic populations studied. In contrast, Korean asthmatics were found to show four nonsynonymous amino acid substitutions in the IL-8 receptor A (IL8RA) and an association of one synonymous variation in IL8RB [28]. In the present study, we did not find a significant difference in the rs4073 A/T between the AERD group and ATA patients.

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Research Article

Interleukin-13, but Not Indomethacin, Increases Cysteinyl-Leukotriene Synthesis in Human Lung Macrophages

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Aspirin-exacerbated respiratory disease (AERD) is associated with constitutively elevated synthesis of bronchoconstrictor cysteinyl-leukotrienes, associated with increased expression of leukotriene (LT)C_4 synthase and Th2 cytokines and airway eosinophilia. We examined whether interleukin-13 can increase LTC_4 synthase gene transcription and cysteinyl-leukotriene synthesis in macrophages isolated from resected human lung tissue and whether an NSAID (indomethacin) can trigger further cysteinyl-leukotriene synthesis in these cells. Overnight culture of human lung macrophages with IL-13 (10 ng/mL) increased spontaneous and ionophore-stimulated production of cysteinyl-leukotrienes by 42% ($P = 0.02$) and 52% ($P = 0.005$), respectively, as quantified by enzyme immunoassays, but PCR gene transcription assays did not demonstrate an effect on LTC4S mRNA. The addition of indomethacin (100 μM) did not modulate cysteinyl-leukotriene production in either IL-13-treated or untreated macrophages. We conclude that while IL-13 enhances cysteinyl-leukotriene synthesis in human lung macrophages, it does not replicate the enhanced LTC_4 synthase expression observed in the AERD lung nor confer sensitivity to NSAIDs.

1. Introduction

Aspirin-exacerbated respiratory disease (AERD) is a syndrome in which chronic asthma is accompanied by nonallergic hypersensitivity to nonsteroidal anti-inflammatory drugs (NSAIDs), leading to acute bronchoconstriction and exacerbation of other lower and upper airway symptoms [1, 2]. The ability of classical NSAIDs to inhibit prostaglandin synthesis by cyclooxygenase (COX) isozymes, particularly COX-1, is implicated in these acute exacerbations [3], but it is not known how they activate mast cells, eosinophils, macrophages, epithelium, and other cells to release a range of inflammatory mediators in susceptible subjects.

Prominent among these mediators are the cysteinyl-leukotrienes (cys-LTs), which are potent bronchoconstrictor lipids synthesised by the 5-lipoxygenase (5-LO)/LTC_4 synthase pathway [4]. Compared to NSAID-tolerant asthmatics, AERD patients have chronically elevated production of cys-LTs, as demonstrated in bronchoalveolar lavage (BAL) fluid, induced sputum, exhaled breath condensate, and urine [2]. Together with the clinical efficacy of antileukotriene drugs [5], this suggests a key role of cys-LTs in chronic AERD, even when NSAIDs are entirely avoided. Exposure to NSAIDs is postulated to shunt the shared substrate arachidonic acid from the prostaglandin (cyclooxygenase) pathway to the leukotriene (5-lipoxygenase) pathway, or to reduce production of a prostaglandin, putatively PGE_2, that normally suppresses leukotriene synthesis via an EP receptor mechanism [2], possibly via phosphorylation of LTC_4 synthase [6]. Increased cys-LT production is prominent in the acute bronchoconstriction that results, but it is not understood why NSAIDs trigger the acute surge in cys-LT levels only in AERD subjects.

In 1998, we described with our collaborators a marked overexpression of LTC_4 synthase, the terminal enzyme for the cellular biosynthesis of the first of the cys-LTs, LTC_4, in the bronchial mucosa of AERD patients [7]. LTC_4 synthase overexpression was also described in nasal polyps from aspirin-sensitive rhinitic patients [8]. A model for NSAID sensitivity was proposed in which enhanced expression of LTC_4 synthase in airway macrophages, mast cells, and eosinophils provides the enzymatic capacity for constitutive overproduction of cys-LTs in AERD [7, 9]. Indeed, total

numbers of LTC_4 synthase-positive cells in the bronchial mucosa correlate strikingly with increased cys-LT levels in BAL fluid in AERD subjects [7]. Overexpression of LTC_4 synthase may also explain why only AERD subjects respond adversely to NSAID challenge, as suggested by the unique relationship between bronchial LTC_4 synthase and airway hyperresponsiveness to aspirin challenge [7].

The view that LTC_4 synthase overexpression is a central anomaly in AERD is supported by recent work in LTC_4 synthase transgenic mice [10], although the potential mechanisms involved remain unclear. LTC_4 synthase can be upregulated by cytokines, including interleukin (IL)-3 and IL-5 in maturing eosinophils [11] and IL-4 and IL-13 in human cord-blood mast cells [12], while TNFα downregulates LTC_4 synthase in monocytes [13]. Immunoexpression of IL-5, but not IL-3 or GMCSF, is increased in AERD biopsies relative to aspirin-tolerant subjects [7], but there are no studies of IL-13 in AERD lung. Macrophages from resected human lung may represent a useful cellular model for AERD as they are primary cells that express both the 5-LO/LTC_4 synthase and COX biosynthetic pathway enzymes, and also receptors for IL-13 that mediate increased expression of the $CysLT_1$ receptor [14]. We hypothesised that culture of human lung macrophages with IL-13 would increase LTC_4 synthase gene transcription and cys-LT synthesis compared to control macrophages. We also hypothesised that the NSAID, indomethacin, would trigger a further release of cys-LTs only in the IL-13-treated cells.

2. Methods

2.1. Materials. RNALater and the DNA Mastermix were purchased from Ambion (Warrington, UK). TaqMan Universal Master Mix, β-actin and LTC4S gene expression assays, and MicroAmp optical adhesive film were purchased from Applied Biosystems (Warrington, UK). The ImProm-II reverse transcription system was purchased from Promega (Southampton, UK). The cysteinyl-leukotriene enzyme immunoassay (EIA) kit was from Cayman Chemical Europe (Tallinn, Estonia). TRIzol was purchased from Invitrogen (Paisley, UK). Recombinant human IL-13 was purchased from PeproTech (London, UK). Calcium ionophore calcimycin (A23187), indomethacin, Trypan Blue solution (0.4%), and dimethylsulphoxide (DMSO) were from Sigma Chemical Company (Poole, UK).

2.2. Isolation and Culture of Human Lung Macrophages. Samples of lung tissue (wet weight 1.8–35.2 g) were collected from male and female patients undergoing bullectomy or lobectomy for lung cancer at the Southampton General Hospital, in accordance with ethical approval (08/HO502/32) from the Southampton and South-West Hampshire Research Ethics Committee. None had a history of asthma, but all were current or exsmokers. Tissue samples were dissected into 3 mm fragments and suspended in Dulbecco's phosphate-buffered saline (PBS) containing 0.1 M NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, and 10 mM Na_2PO_4 (pH 7.4). The suspension was centrifuged (80 ×g, 20°C) for 5 min, the supernatant was discarded, and the pellet was resuspended in 30 mL of lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA; pH 7.4). After 5 min at room temperature, the suspension was filtered (70 μm filter) and centrifuged (80 ×g, 20°C, 5 min) to remove erythrocyte fragments and other debris. The pellet was resuspended in RPMI medium supplemented with antibiotics (penicillin 55 U/mL, streptomycin 5 mg/mL, and gentamycin 10 mg/mL). The resulting cell population was >90% pure macrophages by morphology and had viability >90% as determined by exclusion of Trypan Blue dye. Macrophage aliquots were cultured in RPMI (0.5×10^6 cells/mL) at 37°C for 16 h overnight with or without IL-13 (10 ng/mL) in a 5% CO_2 humidified atmosphere.

For cys-LT assays, IL-13-treated and untreated cells were incubated for a further 30 min in fresh RPMI medium (0.5×10^6 cells in 1 mL) in a 37°C water bath with no addition (control), with indomethacin (100 μM), with calcimycin (A23187, 1 μM), or with both indomethacin and A23187 at the same concentrations. Calcimycin (A23187) is a calcium ionophore that liberates arachidonic acid from membrane phospholipids and activates 5-LO to initiate leukotriene synthesis. Indomethacin is a classical NSAID which inhibits both COX-1 and COX-2 at the concentration used. These reagents were diluted from stock solutions in dimethylsulfoxide (DMSO) such that final DMSO concentration in the cell incubations was always <0.2%. At the end of the incubation, tubes were removed onto ice and centrifuged (240 ×g, 4°C) for 5 minutes to pellet the cells for RNA analyses. The supernatants were treated with two volumes of ethanol to precipitate protein, which was removed by centrifugation. Ethanolic supernatants were then evaporated to dryness *in vacuo* in a GyroVap rotary evaporator and stored at −20°C before cys-LT immunoassays.

2.3. RT-qPCR Assay for LTC4S. For LTC4S gene transcription assays, aliquots (10×10^6 cells) of IL-13-treated and untreated cell pellets were mixed with 0.5 mL RNALater and kept at 4°C for 24 h, then stored at −20°C before RNA extraction and reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) assays for LTC4S and beta-actin mRNAs. Macrophages were thawed, vortexed, and transferred into RNA-free Eppendorf tubes. PBS (1 mL) was added, and Eppendorfs were vortexed and centrifuged (2800 ×g, 20°C) for 5 min to decrease the viscosity of the RNALater and allow the macrophages to form a pellet. RNALater was then removed and the RNA extracted using TRIzol (Invitrogen, Paisley, UK) by the manufacturer's protocol. Residual genomic DNA was digested using Ambion DNA-*free* (Applied Biosystems, Warrington, UK). For each RNA sample, the A260/A280 ratio measured by spectrophotometry (Nanodrop ND1000, ThermoFisher Hemel Hempstead, UK) was >1.8, indicating an adequate level of purity, and RNA was stored at −80°C. To generate cDNA, 1 μg of RNA was reverse transcribed (Improm-II RT system, Promega, Southampton, UK) using random hexamer primers and stored at −20°C.

RT-qPCR was performed on the Lightcycler 480 (Roche Diagnostics, UK) in 384-well reaction plates using ~100 ng of template cDNA in quadruplicate 20 μL reactions using

TaqMan gene expression assays for LTC4S (Hs00168529_m1) with ACTB (beta-actin, Hs99999903_m1) as a reference gene, both using FAM-labelled hydrolysis probes and TaqMan universal mastermix II for 40 cycles, as in the manufacturer's protocol (Applied Biosystems). Gene expression relative to ACTB was calculated using the $2^{-\Delta\Delta Cq}$ method. Paired statistical comparison between IL-13-treated and untreated cells from $n = 12$ donors was performed by Wilcoxon signed rank test for nonparametric data.

2.4. Cysteinyl-Leukotriene Immunoassays. Evaporated supernatants from 30 min incubations of IL-13-treated and untreated macrophages with and without indomethacin and calcimycin were resuspended in appropriate volumes of PBS buffer and aliquots taken in duplicate for EIA quantification of released cys-LTs. The total cys-LT EIA kits (Cayman Europe) use a monoclonal primary antibody with 100% specificity for LTC_4 and LTD_4 and 79% specificity for LTE_4. Cross-reactivity to LTB_4, various HETEs, and arachidonate is less than 4% and the assay has high sensitivity (34 pg/mL) for cys-LTs. The assay is based on competition with a standard LTC_4-acetylcholinesterace tracer with Ellman's reagent as substrate. Cys-LTs were assayed in duplicate, and concentrations are expressed as nanograms of LTC_4 released per million viable macrophages. Data are presented as mean ± SEM for $n = 8$ tissue donors, and comparisons between mean values were made by two-tailed paired Student's t-tests, with $P < 0.05$ considered significant.

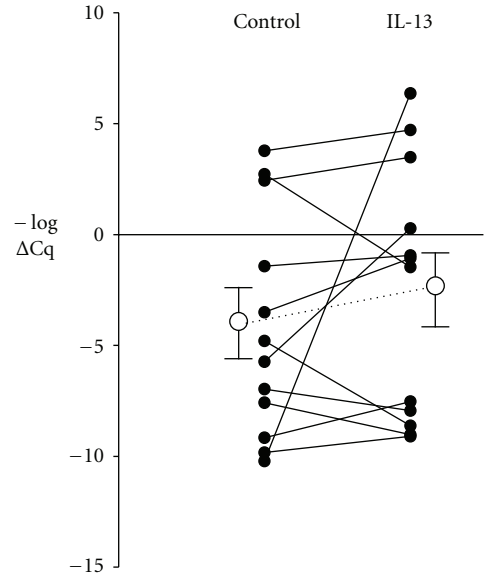

Figure 1: RT-qPCR assay data for LTC4S mRNA in macrophages from 12 lung tissue donors after 16 hours of culture in the presence and absence of 10 ng/mL IL-13. Values are normalised to the housekeeping gene (β-actin), with ΔCq values representing the number of doubling cycles taken to reach the threshold, and plotted on an inverted logarithmic y-axis (as $-\Delta Cq$) so that an increase in LTC4S mRNA is shown as a higher value on the axis. The mean log change in $-\Delta Cq$ value was 0.49, representing no significant change in LTC4S mRNA with IL-13 culture ($P = 0.3$).

3. Results

For LTC4S gene transcription assays, human lung macrophages from 12 donors (9 male, three female; mean age 64 years, range 49–78 years) were cultured overnight for 16 hours with or without IL-13 (10 ng/mL). LTC4S mRNA expression in each cell sample was detected in quadruplicate using RT-qPCR, with β-actin as the housekeeping gene. The mean expression of LTC4S mRNA at 16 hours was not significantly different in IL-13-treated macrophages compared to their untreated cells (mean log ΔΔCq value = 0.49, $P = 0.33$, $n = 12$) (Figure 1). The comparison remained nonsignificant when the single outlying value, caused by an anomalously low control (β-actin) value in the untreated cells from one donor, which was not apparent in the IL-13-treated cells from the same donor, was excluded from the analysis.

Following 16-hour cultures with or without IL-13, macrophages from representative donors (8 males, mean age 67 years, range 56–76) underwent 30-minute incubations for detection of total cys-LT release (Figure 2). IL-13 pretreatment significantly increased spontaneous cys-LT release from 544 ± 215 pg/million cells to 825 ± 292 pg/million cells ($P = 0.02$), a mean increase of 52 ± 17%. Incubation with indomethacin (100 μM) did not significantly change cys-LT release when compared with spontaneous release in either IL-13-treated or untreated cells ($P > 0.05$).

As expected, the calcium ionophore A23187 (calcimycin, 1 μM) boosted the mean release of total cys-LTs by about 10-fold compared with spontaneous release. Mean release of total cys-LTs after 30 min of A23187 stimulation was significantly greater in IL-13-treated cells (7770 ± 630 pg/million cells) than in cells not treated with IL-13 (5480 ± 670 pg/million cells) ($P = 0.005$), a mean increase of 42 ± 10% (Figure 2). Coincubation of indomethacin (100 μM) and A23187 (1 μM) however did not show different values for cys-LT release compared with A23187 alone in either IL-13 pretreated or untreated cells.

4. Discussion

Interleukin (IL)-13 is a Th2 cytokine with well-established roles in promoting airway responsiveness, mucus secretion, and chemokine production in the allergic lung, acting principally via a receptor shared with IL-4 and leading to phosphorylation of the transcription factor STAT6 [15]. IL-4 can powerfully upregulate LTC_4 synthase expression and activity in human cord-blood mast cells [12], while IL-13 can upregulate the principal receptor for cysteinyl-leukotrienes, CysLT1R, on human airway smooth muscle cells [16] and on macrophages [14]. This suggested that increased IL-4/IL-13 activity may be responsible for upregulating tissue expression of LTC_4 synthase, as observed in the upper and lower airways in AERD patients [7, 8], and for enhancing responsiveness to cys-LTs by increasing the expression of $CysLT_1$ receptors, as described in AERD nasal biopsies [17].

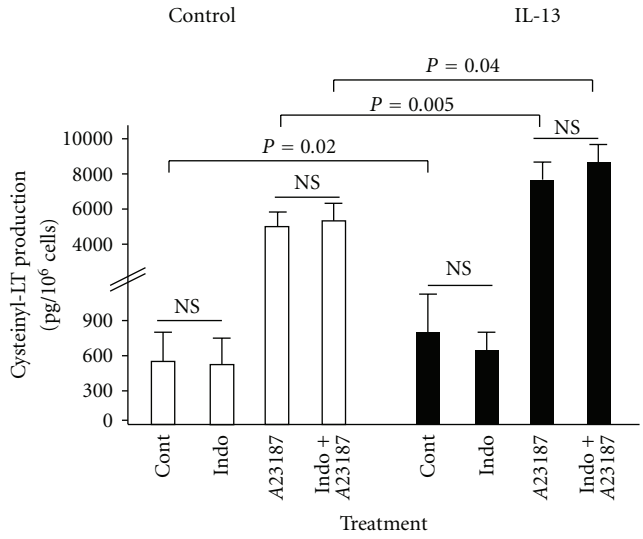

Figure 2: Enzyme immunoassay data on release of total cysteinyl-leukotrienes (cys-LT, pg/million cells) by macrophages from eight lung tissue donors after culture for 16 hours in the absence or presence of IL-13 (10 ng/mL). Macrophages were then washed and resuspended in fresh medium for a 30 min incubation with no further addition (Cont), with the NSAID indomethacin (Indo, 100 μM), with the calcium ionophore calcimycin (A23187, 1 μM), or with both indomethacin and calcimycin (Indo + A23187). Pretreatment with IL-13 significantly increased spontaneous cys-LT release ($P = 0.02$) and that induced by calcimycin ($P = 0.005$), but neither was affected by indomethacin in either IL-13 treated or untreated cells.

This study therefore explored whether IL-13 can upregulate LTC_4 synthase transcriptional expression in macrophages isolated from resected human lung and whether it leads to a higher release of cys-LTs, either spontaneously or in response to a calcium ionophore, compared with macrophages not cultured with IL-13. Taqman gene expression assays failed to show significant changes in LTC4S mRNA, standardised to the beta-actin housekeeping gene (ACTB), in IL-13 cultures of macrophages from twelve lung tissue donors (Figure 1). Increases in LTC4S mRNA are detectable after six hours of IL-4 treatment in human mast cells derived from cord blood mononuclear cells, and maximal at 24 to 120 hours [12], suggesting that the 16-hour culture with IL-13 employed in our experiments was reasonable. IL-13 may be less potent on myeloid cells than IL-4, and our cells were mature lung macrophages from mainly elderly subjects undergoing lobectomy or bullectomy; these cells may not be as responsive to IL-13 or other stimuli as cord-blood-derived mononuclear cells from healthy neonates.

Despite the lack of effect on LTC4S transcription, culture of lung macrophages with IL-13 did cause significant increases in cys-LT release, an effect that was apparent both on the low levels of spontaneous cys-LT production in unstimulated cells and on the tenfold higher levels of cys-LT release measured in cells stimulated with calcimycin A23187 (Figure 2). Calcimycin acts as a receptor-independent trigger of arachidonate release from membrane phospholipids; a high turnover of substrate through the 5-LO/LTC_4 synthase pathway was intended to simulate rate-limiting conditions, such that an increase in LTC_4 synthase enzyme expression induced by IL-13 could be revealed by a raised ceiling of cys-LT synthesis. In the event, the lack of upregulation of LTC4S mRNA suggests either a nontranscriptional effect of IL-13 or an action on other components of the pathway, possibly on 5-LO activating protein (FLAP), which is inducible by cytokines and increases markedly during human alveolar macrophage maturation [18]. The increases in cys-LT release seen in the IL-13-cultured macrophages were relatively modest (42–52%), but suggest that IL-13 could contribute to increased cys-LT releasability in macrophages in the allergic asthmatic or AERD lung.

NSAIDs such as indomethacin are proposed to cause acute AERD reactions by shunting of arachidonate from the inhibited COX pathway to the 5-LO/LTC_4 synthase pathway or by suppressing synthesis of an inhibitory prostanoid such as PGE_2, thus enhancing cys-LT production [6]. Although isolated anomalies in COX isozyme expression, prostanoid synthesis, and EP receptor signalling have been described in AERD cells and tissues [19, 20], a coherent picture of a systemic prostanoid defect in AERD has yet to emerge [2]. Human alveolar macrophages constitutively express COX-1 and synthesise PGE_2 [21]. We postulated that endogenous COX pathways in human lung macrophages may therefore provide an adequate target for NSAID action, resulting in enhanced cys-LT release if the 5-LO/LTC_4 synthase pathway has previously been induced by IL-13. No effect of indomethacin was observed however on either spontaneous or A23187-stimulated cys-LT release in either IL-13-treated or untreated cells (Figure 2). In the absence of an effect of IL-13 on LTC4S mRNA levels, it is not possible to reject our hypothesis that LTC_4 synthase overexpression in a single cell type could provide a simple cellular model of the key functional changes within the AERD lung. *In vivo*, intercellular interactions may be required, possibly with PGE_2 derived from airway epithelial cells [22] or it may depend on the recruitment of new populations of LTC_4-synthase-expressing cells, such as eosinophils. It is nevertheless intriguing that human mast cells derived in culture from the blood mononuclear cells of AERD subjects show an intrinsically raised capacity for cys-LT synthesis and that this is suppressed by PGE_2 [23], suggesting that the AERD paradigm can be detected at the level of a single cell type and that it persists in prolonged cell culture. Further comparative studies are required in primary lung cell populations from normal and AERD subjects.

The key features of AERD have recently been replicated in ovalbumin-sensitised LTC_4 synthase transgenic mice (LTC4S-Tg), including its overexpression in airway macrophages and other leukocytes, leading to increased cys-LT synthesis both before and after NSAID challenge, accompanied by dramatically increased Th2 cytokines, including IL-13 [10]. This model suggests that primary dysregulation of LTC_4 synthase in resident lung cells including macrophages may initiate overproduction of cys-LTs in patients with AERD. The cys-LTS may then promote the secondary synthesis of IL-4, IL-5, and IL-13 from lymphocytes [10] and eosinophils [24], leading to myocyte CysLT1R expression [16],

suppression of epithelial PGE_2 synthesis and EP2 expression [22], and further induction of LTC_4 synthase [11]. While the LTC4S −444A/C promoter polymorphism [25] has been discounted as an aetiological factor in most AERD populations [26], other genetic, immunological, and microbial factors that could directly dysregulate LTC_4 synthase in human lung cells merit further investigation.

Acknowledgments

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Review Article

Genetic Mechanisms in Aspirin-Exacerbated Respiratory Disease

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Aspirin-exacerbated respiratory disease (AERD) refers to the development of bronchoconstriction in asthmatics following the exposure to aspirin or other nonsteroidal anti-inflammatory drugs. The key pathogenic mechanisms associated with AERD are the overproduction of cysteinyl leukotrienes (CysLTs) and increased CysLTR1 expression in the airway mucosa and decreased lipoxin and PGE2 synthesis. Genetic studies have suggested a role for variability of genes in disease susceptibility and the response to medication. Potential genetic biomarkers contributing to the AERD phenotype include *HLA-DPB1, LTC4S, ALOX5, CYSLT, PGE2, TBXA2R, TBX21, MS4A2, IL10, ACE, IL13, KIF3A, SLC22A2, CEP68, PTGER,* and *CRTH2* and a four-locus SNP set composed of *B2ADR, CCR3, CysLTR1*, and *FCER1B*. Future areas of investigation need to focus on comprehensive approaches to identifying biomarkers for early diagnosis.

1. Introduction

Aspirin-exacerbated respiratory disease (AERD) refers to the development of bronchoconstriction in asthmatics following the ingestion of aspirin or other nonsteroidal anti-inflammatory drugs. It is defined by a clinical syndrome associated with moderate-to-severe asthma and eosinophil inflammation in the upper and lower airways, resulting in chronic rhinosinusitis and asthma [1]. Additionally, the airways of AERD show epithelial disruption, cytokine production, and the upregulation of inflammatory molecules [2]. The prevalence of aspirin hypersensitivity in the general population ranges from 0.6 to 2.5% and is higher in asthmatics [3].

The dysregulation of arachidonic acid metabolism also accounts for the susceptibility to AERD. Metabolites involved are prostaglandins (PGs), leukotrienes (LTs), and thromboxane (TBX). Inhibition of COXs by acetyl salicylic acid (ASA) in the respiratory tract alters arachidonic acid metabolism, leading to a reduction in PGE2. This may increase AERD susceptibility by overproduction of CysLTs [4, 5]. The lipoxygenase (LOX) pathway produces the leukotrienes LTA4, LTB4, and LTC4 as metabolites. 15-lipoxygenase (15-LO) is one of the LOX family members and catalyses the conversion of arachidonic acid to 15-hydroxyperoxyeicosatetranoic acid (15-HPETE). 15-hydroxyeicosatetranoic (15-HETE), a more stable derivative of 15-HPETE, is another important product, which acts as an anti-inflammatory mediator and functional antagonist of LTs [6]. Further products of 15-HPETE include eoxins (EXs) EXA4 and 15-HETE can be conjugated with glutathione, leading to the formation of EXC4, EXD4, and EXE4. AERD has also been correlated with increased CysLT receptors: CysLTR1 and CysLTR2 [7–9]. The third CysLT receptor, the G protein-coupled receptor 17 (GPR17) [9], is located at an intermediate phylogenetic position between two distinct receptor families: the purinergic receptor (P2Y) and CysLT receptor for extracellular nucleotides and CysLTs, respectively, [10]. Overexpression of CysLTR1 was detected in the nasal mucosa of patients with AERD, compared with aspirin-tolerant asthma (ATA) [11]. Considering the pathogenic mechanism of AERD, various genetic markers have been suggested in various ethnic groups and are summarized in this paper.

2. Key Results Regarding Genetic Mechanisms

2.1. Leukotriene Related Genes and Their Mechanism. Based on evidence showing a close association of leukotrienes and

AERD, initial research was performed on the association between *LTC4S* −444A > C promoter polymorphism and AERD. In the population investigated (Polish), the C allele was identified as a risk factor; however, this finding was not replicated in Japanese, American, or Korean populations [12–15]. SNPs of 5-lipoxygenase; *ALOX5* at −1708G > A, 21C > T, 270G > A, and 1728G > A and ALOX5 activating protein (*ALOX5AP*, 218A > G) were studied in a Korean population where it was discovered that the haplotype ALOX5 ht1 [G-C-G-A] was significantly higher in AERD than in ATA, suggesting a possible contribution of ALOX5 in AERD [16]. We identified three SNPs (−634C > T, −475A > C, and −336A > G) in the promoter region of CysLTR1, and mutant variants of these SNPs were associated with the AERD phenotype [17]. The mutant variants showed higher promoter activity, suggesting that these polymorphisms may modulate CysLTR1 expression increasing AERD susceptibility. In the case of *CysLTR2*, the frequencies of minor alleles for −819T > G, 2078C > T, and 2534A > G were significantly higher in the AERD group [18] when compared with ATA.

2.2. Cyclooxygenase, Prostanoid, and Human Leukocyte Antigen Markers and Related Mechanisms. It has been suggested that AERD is associated with both COX1 and COX2. Aspirin inhibits both of these proteins, with a greater effect on COX1. COX2 expression was downregulated in nasal polyps collected from AERD patients [19]. Decreased production of prostaglandin E2 (PGE2) by nasal epithelial cells of AERD has been observed [20]. PGE2 production in airway smooth muscle cells has been shown to downregulate COX2 mRNA expression [21]. Two SNPs of *TBXA2R*, −4684T > C, and +795T > C, were shown to be associated with the phenotype of AERD in a Korean population [22, 23]. The prostaglandin E2 receptor subtype 2 gene (*PTGER2*) was associated with the risk of AERD by decreasing the level of transcription, resulting in a reduction of the "PGE2 braking" mechanism of inflammation and involvement in the molecular mechanism underlying AERD in the Japanese population [24]. A further report in the Korean population showed that prostaglandin E2 receptor subtype 3 (*PTGER3*) may be an important genetic factor for aspirin intolerance in Korean asthmatics [25]. The human leukocyte antigen (HLA) allele *DPB*1*0301 was identified as a strong marker for AERD, because patients with this allele showed typical characteristics of AERD including a decreased forced expiratory volume in 1 s (FEV_1) and increased prevalence of rhinosinusitis with nasal polyps [26], as previously noted in a Polish population [27].

2.3. Eosinophil-Related Genetic Mechanisms. Eosinophil infiltration into the upper and lower airways is a key feature of AERD. Increased numbers of eosinophils and mast cells have been observed in the bronchial mucosa of AERD [28, 29]. Recent studies demonstrated that the chemoattractant receptor molecule expressed in Th2 cells, the *CRTH2* −466T > C polymorphism, could increase serum and cellular eotaxin-2 production by lowering CRTH2 expression, leading to eosinophilic infiltration in AERD patients [30]. A further study indicated that the chemokine CC motif receptor (CCR3) may be related to eosinophil migration. The *CCR3* −520T > C was significantly associated with AERD patients where mRNA expression was also significantly increased after ASA provocation [31]. IL-13 polymorphisms at −1510A > C and 1055C > T are associated with the development of rhinosinusitis in AERD patients. *IL-13* Arg110Gln may be associated with an increased eosinophil count and eotaxin-1 level, leading to an increase in eosinophilic inflammation in the upper and lower airways of patients with AERD [32] (Table 1).

2.4. AERD and Viral Infection. Szczeklik has hypothesized that AERD develops as the result of chronic viral infection [33]. Viral respiratory infections have been suggested to contribute to allergic sensitization, leading to the development of asthma and in subjects with established asthma; they are known to exacerbate allergic disease [34]. Aspirin hypersensitivity is diminished in some AERD patients during acyclovir treatment of herpes simplex infection [35]. Moreover, elevated levels of IgG4, derived from chronic antigenic stimulation of viral origin, have been noted in AERD patients [36]. A further study investigating the exacerbation of AERD with airway infection of respiratory syncytial virus was reported [37]. Recently, a study indicated that the polymorphisms in the Toll-like receptor 3 (*TLR3*) gene, *TLR3* −299698G > T and 293391G > A, were associated with the AERD phenotype. TLR3 recognizes dsRNA, activates nuclear factors, and increases interferon-gamma, which is a signal to other cells and increases antiviral defenses. As functional deterioration of TLR3 can predispose individuals to increased susceptibility to viral infections, the detection of TLR3 polymorphisms may be informative for risk assessment in AERD susceptibility [38]. The suggested mechanism is that specific cytotoxic lymphocytes are produced in response to viral infection. Activity of these lymphocytes is suppressed by PGE2, which is produced by pulmonary alveolar macrophages. If PGE2 levels are decreased, cytotoxic reactions are preceded by COX inhibitors and cytotoxic lymphocyte-mediated attacks lead to the destruction of virus affected cells in the respiratory tract. Reactive oxygen species, toxic metabolites, and mediators released then precipitate asthma attacks.

2.5. Other Suggested Mechanisms. The ubiquitin-proteasome pathway-related gene (*UBE3C)* has been recently studied in a Korean population and indicated that rs3802122 and rs6979947 is associated with AERD [39]. A further study indicated that the kinesin family number 3A (*KIF3A*) gene and its polymorphism might have an effect on AERD, because rs3756775 revealed a significant association with the percentage decline in FEV_1 after aspirin provocation [40]. Recently, the genome-wide methylation profile of nasal polyps showed that genes involved in lymphocyte proliferation, cell proliferation, leukocyte activation, cytokine biosynthesis, immune responses, inflammation, and immunoglobulin binding were hypomethylated. In the arachidonic pathways, PGDS, ALOX5AP, and LTB4R were

Table 1: Genetic mechanisms of AERD.

Gene name	SNPs	Clinical phenotype	Mechanism
Leukotriene synthesis			
LTC4S	−444A > C	C allele had high genotype frequency compared with A allele	C allele may be the risk allele due to overproduction of CysLTs
ALOX5	−1708G > A, 21C > T, 270G > A, 1728G > A	ALOX5 ht1(GCGA) had higher haplotype frequency	ALOX5 ht1(GCGA) may be the risk haplotype
CYSLTR1	**−634C > T, −475A > C, −336A > G**	ht2(TCG) showed higher frequency in AERD and higher promoter activity	Higher CysLTR1 mRNA expression may be responsible for pathogenesis
CYSLTR2	−819T > C	the frequencies of rare allele were increased in AERD and fall in FEV1 after aspirin provocation	Elevation of CysLTs production
COX/PG pathway and HLA allele			
PTGER	rs7543182 rs959	These two polymorphisms retained their susceptibility to aspirin intolerance in first and second cohorts	PTGER3 might play a significant role in aspirin hypersensitivity
TBXA2R	+795T > C	AERD patients with homozygous +795 C allele had a greater percent fall in FEV1 after aspirin exposure compared with TBXA2R+795 CT or TT genotypes.	TBXA2R+795T > C may increase bronchoconstrictive response to ASA
HLA	**DPB1*0301**	Patients with DPB1*0301 allele had higher prevalence of Rhino-sinusitis and lower FEV1 values.	HLA markers may be important for LTRA therapy
Gene name	SNPs	Clinical Phenotype	Mechanism
Eosinophil activation			
CRTH2	**−466T > C**	−466T allele had higher frequency in AERD and increased serum, cellular eotaxin-2 production and lower mRNA expression	−466T allele may be the risk allele by activation of eosinophils
CCR3	**−520T > C**	The frequencies of rare genotypes were higher in AERD and −520G allele showed higher promoter activity	Higher mRNA expression of CCR3 may cause eosinophil activation
IL 13	1510A > C, 1055C > T, Arg110Gln	Increase eotaxin-1 and peripheral eosinophil count	Eosinophil activation may occur
Mast cell activation			
FCERIG	**−237A > G −344C > T**	AA type of −237A > G showed high serum total IgE; CC/CT of −344C/T had higher SEA	Mast cells may be activated
MS4A2R	E237G	FcER1b −109T allele had higher frequency and high promoter activity	Increased mRNA expression of −109T allele may cause mast cell activation mediated by MS4A2R receptor
Other mechanisms			
IL-10 and TGF-β1	−1082 A > G and −509C > T	The frequency of rare alleles (the CT or TT genotype of TGF-β1) 509C/T and AG or GG genotype of (IL-10)1082A/G was significantly higher in AERD and −1082G had higher promoter activity	Alteration in IL-10 production caused by the −1082A/G in IL-10 may contribute to disease pathogenesis which is strengthened by a genetic interaction with TGF-β1.
ACE	−262A > T, −115T > C	The frequencies of the rare alleles were higher in AERD −262T had lower promoter activity and fall of FEV1 after aspirin provocation	Downregulation of ACE expression

Table 1: Continued.

Gene name	SNPs	Clinical phenotype	Mechanism
KIF3A	rs 3756775	Fall of FEV1 and higher mRNA expression of KIF3A in the ASA induced bronchial epithelial cells and protein expression in nasal polyp epithelia in AERD	Abnormality of cilia predisposing to AERD
SLC6A12	rs499368, rs557881	The minor allele frequencies were higher in AERD and fall of FEV1 after aspirin provocation	GABA signaling pathway in the airway epithelium may play a role
CEP68	7572857G > A	Fall of FEV1 after aspirin provocation by A allele	Change in polarity of the protein structure due to nonsynonymous SNP which replaces Gly with Ser

IL13: interleukin 13, CCR3: chemokine receptor 3, CRTH2: chemoattractant receptor, IL10: interleukin 10, TGF: transforming growth factor, MS4A2R: high affinity immunoglobulin epsilon receptor beta-subunit (FcERI) TBXA2R: thromboxane receptor, CysLTR1: cysteinyl leukotriene 1, CysLTR2: cysteinyl leukotriene 2, ALOX5: arachidonate 5 lipoxygenase, HLA: human leukocyte antigen, LTC4S: leukotriene C4, ACE: angiotensin-converting enzyme KIF3A: kinesin family number 2A, SLC22A2: solute carrier family 6, CEP68: centrosomal protein, PTGER: prostanoid gene, TEC: total eosinophilic count, TF: transcription factor, MAZ: myc-associated zinc finger protein, SEA: Staphylococcus enterotoxin A, FEV_1: forced expiratory volume in 1 s, AERD: aspirin-exacerbated respiratory disease.

hypomethylated whereas PTGES was hypermethylated [41]. The calcium channel voltage-dependent gamma subunit 6 (*CACNG6*) gene encodes a protein that stabilizes the calcium channel. *CACNG6* has been studied in AERD, which revealed that rs192808C > T may be associated with the risk of AERD in a Korean population [42].

2.6. AERD and Genome-Wide Studies. Genome-wide association studies (GWAS) have recently emerged as a technology that can predict genetic variations across the genome associated with human diseases and clinical responses to drug treatment. Recently, GWAS for asthma and related phenotypes have reported several susceptible genes. Candidate gene approaches have been used for most of the genetic association studies of AERD. GWAS suggested that the nonsynonymous *CEP68* rs 7572857G > A variant, replacing glycine with serine, showed a higher decline in FEV_1 due to aspirin provocation than other variants and could be a susceptible gene for AERD. Gly74Ser could also affect the polarity of the protein structure [43].

2.7. Gene-Gene Interactions. Gene-gene interactions have also been proposed in the pathogenesis of AERD, and a few studies indicated that the genetic effects of CysLTs and *LTC4S* −444A > C synthesis increased the lower level of FEV_1 after lysine ASA inhalation [18]. *TBXA2R* 795T > C polymorphism was associated with *HLA DPB*1*0301 in AERD patients compared with ATA [23]. Recently, a synergistic effect between the *TGF-beta1*-509C/T and *IL-10*-1082A/G polymorphisms on the phenotype of AERD was noted when stratified by the presence of rhinosinusitis [44]. Moreover, Kim et al. reported a significant epistatic effect with a four-locus genetic interaction in the susceptibility to aspirin intolerance in asthmatic patients. This model includes four SNPs: *B2ADR* −46A > G, *CCR3* −520T > G, *CysLTR1* −634C > T, and *FCER1B* −109T > C [45]. These findings should be validated further in other cohorts.

3. Conclusions

AERD often produces a moderate-to-severe phenotype; however, diagnosis in these patients is challenging despite the availability of various techniques. A hypothesis has been put forward, mostly focused on the overproduction of CysLTs and arachidonic acid pathways. Most of the genetic studies have been performed using techniques such as GWAS and the candidate gene approach. However, replication studies in different ethnic groups will be essential to validate the reported data and apply this knowledge in clinical practice. Future areas of investigation should focus on identification of biomarkers for early diagnosis with various diagnostic techniques. These genetic studies will be able to extend our understanding about the molecular genetic mechanism of AERD and to find a genetic marker for predicting drug responses or hypersensitivity reactions. Furthermore, this will be helpful for the determination of new diagnostic tools and therapeutic interventions.

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